ENZYMATIC HYDROLYSIS OF PLANT PROTEINS USING FOOD-GRADE PEPTIDASES

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ZUSAMMENFASSUNG

Peptidasen werden bei der Herstellung und Verarbeitung von vielen Lebensmitteln, wie Käse, Backwaren und Getränken, eingesetzt. Eine besondere Bedeutung besitzen Proteinhydrolysate die unter anderem in hypoallergener Säuglingsnahrung, medizinischer Ernährung, Sportgetränken, und Nahrungsergänzungsmitteln Verwendung finden. Diese Hydrolysate können je nach Zusammensetzung bestimmte biologische, und technologische Funktionalitäten oder verbesserte Geschmackseigenschaften aufweisen. Pflanzenproteine sind von besonderem Interesse, da sie aus der Stärke- oder Ölproduktion als Nebenprodukte in großen Mengen anfallen. Die zur Hydrolyse verwendeten lebensmittelzugelassenen Peptidasepräparate werden meist aus Mikroorganismen gewonnen und in konzentrierter Form eingesetzt.

In der ersten Studie wurde eine Methodik entwickelt, um die Zusammensetzung kommerzieller Peptidasepräparate zu untersuchen. Zunächst, wurden die in den Präparaten enthaltenen Peptidasen mittels Massenspektrometrie identifiziert. Anschließend wurden mit Enzympräparaten Lupinproteinhydrolysate erzeugt und die Hydrolysate charakterisiert. Im Verlauf der Lupinproteinhydrolyse wurde die Aminosäurefreisetzung kontinuierlich mittels eines neu entwickelten automatisierten Hochleistungsflüssigkeitschromatographiesystems (HPLC) erfasst. Die kommerziellen Enzympräparate Flavourzyme und Protease AN zeigten Potential zur Erzeugung bioaktiver und geschmacksaktiver Hydrolysate (Freisetzung vieler Aminosäuren). Bioprase, Collupulin, Corolase, Proteinase T, Promod, und Protin hingegen erzeugten Hydrolysate mit niedrigem Hydrolysegrad und es konnten kaum freie Aminosäuren festgestellt werden.

In der zweiten Studie wurde die Komplexität des kommerziellen Peptidasepräparats Flavourzyme gezeigt. Flavourzyme ist ein Aminopeptidasepräparat aus *Aspergillus oryzae* und wird mit dem synthetischen Substrat H-Leu-*p*NA standardisiert. In der Studie wurden acht Schlüsselenzyme durch Massenspektrometrie und durch Aktivitätsfärbung von nativen Polyacrylamidgelen nachgewiesen. Es konnten zwei Aminopeptidasen, zwei Dipeptidylpeptidasen, drei Endopeptidasen und eine α-Amylase zweifelsfrei identifiziert werden. Dabei wurden fünf spezifische Markersubstrate für die *A. oryzae* Enzyme ermittelt und ein automatisiertes, neunstufiges Reinigungsprotokoll für die Isolierung der

Enzyme innerhalb von 7 h entwickelt. Abschließend wurden die gereinigten Enzyme biochemisch charakterisiert.

In der dritten Studie wurde die Produkthemmung der sieben isolierten Flavourzymepeptidasen untersucht und, darüber hinaus, der Einfluss der Peptidasen auf die Weizenglutenhydrolyse mittels statistischer Versuchsplanung (Response Surface Methode) ermittelt. Sechs der sieben Peptidasen zeigten einen signifikanten ($p < 0,05$) Beitrag bei der Weizenglutenhydrolyse auf und müssen daher bei der Optimierung eines solchen Prozesses berücksichtigt werden.

In der vierten Studie wurden Flavourzymechargen mit unterschiedlichen Herstellungsdaten auf die Lagerstabilität und die Chargenvariation der identifizierten Peptidasen untersucht. Die geringste Variation wurde für die vom Hersteller als Hauptbestandteil angegebene Aminopeptidase ermittelt. Dem gegenüber konnten erhebliche Schwankungen von anderen für die Hydrolyse wichtigen Peptidasen festgestellt werden. Die Schwankung der Endopeptidaseaktivität fiel dabei am deutlichsten aus und ist für die Reproduzierbarkeit einer Casein Batchhydrolyse unabdingbar. Es wurde deutlich, dass die Berücksichtigung aller relevanten Peptidasen erforderlich ist, um einen reproduzierbaren Hydrolyseprozess zu gewährleisten.

Die fünfte Studie untersuchte die Einflüsse verschiedener Prozessparameter einer enzymatischen Hydrolyse (Art der eingesetzten Peptidase, Substratkonzentration) sowie die Auswirkung von verfahrenstechnischen Grundoperationen (Zentrifugation, Ultrafiltration) auf die Antigenität von Weizenglutenhydrolysaten. Durch enzymatische Hydrolyse (Flavourzyme) von 100 g/L Weizengluten und anschließende 1 kDa Ultrafiltration konnten glutenfreie Hydrolysate (kompetitiver ELISA) gewonnen werden. Wurde die Substratkonzentration auf 25 g /L reduziert, war lediglich eine Zentrifugation notwendig, um ein glutenfreies Hydrolysat zu erhalten.

In der sechsten Studie wurde ein kontinuierlicher Hydrolyseprozess von Weizengluten mit dem Peptidasepräparat Flavourzyme entwickelt. Dabei wurde Ethanol als lebensmitteltaugliche mikrobielle Hürde identifiziert. Temperaturabhängige Inaktivierung und der Verlust der Aktivität durch Scherbeanspruchung und Membranpermeabilität wurden als kritische Faktoren ermittelt. Mit dem Prozess wurde über einen Zeitraum von 96 h eine konstante Produktqualität und Raum-Zeit-Ausbeute erreicht. Die Enzymproduktivität konnte im Vergleich zu einer Referenz (Batchprozess) um 450% gesteigert werden.

ABSTRACT

Peptidases are applied in the production and processing of various food products, such as cheese, pastries, beverages, meat and fish. Protein hydrolysates are, furthermore, used in hypoallergenic infant formula, clinical nutrition and sports drinks or as food supplements. Enzymatic hydrolysis is a powerful tool to improve the nutritional value of proteins and to enhance various favorable properties, such as biological activity, technological functionality and/or flavor characteristics. Plant proteins are of particular interest because they can be recovered as by-products from industrial processes, such as starch or oil production in large quantities. The food industry employs the food-grade peptidases in a concentrated form derived from plant, animal and microbial materials.

In the first study, a methodology was developed to characterize commercial peptidase preparations using mass spectrometry analysis and a novel high-performance liquid chromatography (HPLC)-based system. With this automated system, the liberation of amino acids during a lupine protein hydrolysis was monitored. Suitable industrial peptidases were identified for the production of hydrolysates resulting in high degrees of hydrolysis and the liberation of high amounts of free amino acids (Flavourzyme, Protease AN). In contrast, hydrolysates with low degrees of hydrolysis and a low release of amino acids were achieved with Bioprase, Collupulin, Corolase, Proteinase T, Promod, Protin.

In the second study, the complex composition of the commercial peptidase preparation Flavourzyme has been shown. Flavourzyme is derived from *Aspergillus oryzae* and sold as an aminopeptidase preparation and standardized applying the artificial substrate H-Leu-*p*NA. Eight key enzymes were identified by mass spectrometry, whereas some showed distinct activity on native polyacrylamide gels after activity staining. The eight enzymes identified were two aminopeptidases, two dipeptidyl peptidases, three endopeptidases and one α-amylase. Five specific marker substrates for these *A. oryzae* derived enzymes were ascertained and a fast and automated nine-step purification protocol was designed to isolate the enzymes within 7 h. Finally, the purified *A. oryzae* derived enzymes were biochemically characterized with regard to pH and temperature profiles and molecular sizes.

In the third study, the product inhibition for the seven Flavourzyme peptidases isolated was determined, since severe product inhibition of the peptidases results in a protein hydrolysate with a low or lower degree of hydrolysis. Furthermore, the impact of each

peptidase on the wheat gluten hydrolysis was analyzed using response surface methodology. One of the endopeptidases (alkaline protease 1) was least affected by product inhibition and showed the highest effect on the wheat gluten hydrolysis. The leucine aminopeptidase 2 showed a higher impact on the hydrolysis compared to the leucine aminopeptidase A, but exhibited the highest product inhibition sensitivity. It was shown that six out of the seven peptidases contributed significantly ($p < 0.05$) to the wheat gluten hydrolysis. Thus, the proposed marker substrates should be considered to achieve an improved hydrolysis process control.

In the fourth study, the activity of the seven peptidases identified was tested in different Flavourzyme batches. The impact of the storage time on the peptidase activity and the magnitude of the batch-to-batch variation were investigated applying the marker substrates identified previously. The variation was least for the aminopeptidase, which is stated as main component in the enzyme preparation. However, the variation of the other important peptidase activities was noticeable and was most distinct for the endopeptidase activity, which also decreased during the storage time of the preparation. The variation of the Flavourzyme composition affected the reproducibility of a casein batch hydrolysis process, which should be considered for any future research and industrial application.

The fifth study tested the influence of various process parameters (type of peptidase, substrate concentration) and downstream processing operations (centrifugation, ultrafiltration) on the antigenicity of wheat gluten hydrolysates. Gluten free hydrolysates (tested with a competitive ELISA) were obtained by enzymatic hydrolysis (Flavourzyme) of 100 g/L wheat gluten and subsequent 1 kDa ultrafiltration. However, when the substrate concentration was reduced to 25 g/L, centrifugation alone was sufficient to produce gluten-free hydrolysates.

In the sixth study, a principally food-grade continuous long-term hydrolysis process of wheat gluten with Flavourzyme was designed using ethanol as a food-grade microbial hurdle. Critical factors for the enzyme stability were the temperature, pump stress and enzyme leakage through the membrane. The hydrolysis and process conditions were optimized to increase the space–time yield. A stable product quality (degree of hydrolysis) and space–time yield was achieved by the continuous process over a runtime of 96 h. Due to the fact that the enzymes were reused, the enzyme productivity could be increased by 450 % compared to a reference batch process.

CHAPTER ONE

GENERAL INTRODUCTION AND THESIS OUTLINE

ABSTRACT

Proteolytic enzymes/peptidases have been used for dairy processing, baking, brewing, and in the processing of meat and fish for centuries. Enzymatic hydrolysis is a powerful tool to improve the nutritional value of proteins and to enhance various favorable properties, such as biological activity, technological functionality and/or flavor characteristics. The production of enzymatic protein hydrolysates in combination with filtration technology is considered the most effective way to obtain protein hydrolysates with defined characteristics. The peptidase preparations used is a pivotal determinant for the production of these defined hydrolysates. The bulk production of foods normally utilizes food-grade peptidase preparations of microbial origin (e.g. *Bacillus sp.*, *Aspergillus sp*.), which are produced as rather crude preparations (without the application of sophisticated and expensive purification techniques) on a large scale. Therefore, knowledge about the composition and the biochemical characteristics, such as the specificities, is essential. Various options are available for the industrial production of protein hydrolysates, such as the type of reactor used and whether the process is conducted in batch or continuous mode, using either free (homogeneous catalysis) or immobilized enzymes (heterogeneous catalysis). Proteins from agricultural commodities are the most important nutritional protein source worldwide. Several plant protein fractions can be recovered as by-products from industrial processes, such as starch or oil production in large quantities. Therefore, these isolates are economical and an alternative to animal-derived proteins.

This general introduction summarizes the basics about food-grade peptidases and their application in the food industry. Further insights about the industrial production of plant protein hydrolysates, including their potential functionalities, are highlighted. Lastly, an outline is given to link the outcomes of this thesis with the current state of research.

PROTEOLYTIC ENZYMES

Proteolytic enzymes (EC 3.4) catalyze the hydrolysis of peptide bonds as shown in Figure 1.1. They are commonly termed proteases, proteinases and peptidases [1]. However, the term peptidase is recommend by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) for all of the enzymes that hydrolyze peptide bonds [1]. Peptidases have a crucial position in many biological processes and are necessary for all living organisms [1]. In general, extracellular peptidases break down large proteins for subsequent absorption whereas intracellular peptidases play a pivotal role in metabolism regulation [2]. Peptidases play an important role in nutrition and the regulation of the turnover of intracellular proteins [2]. They also take part in the regulation of cell growth, differentiation, morphology [3–5] and the blood coagulation cascade [6, 7]. Furthermore, peptidases are involved in blood pressure regulation [8, 9] and can influence the development of cancer [10, 11] or diabetes [12]. Lastly, peptidases are responsible for the activation of zymogens (inactive enzyme precursors) by partial proteolysis.

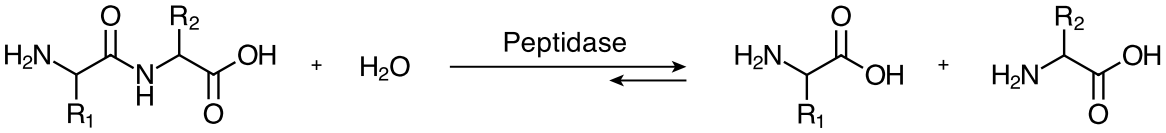

Figure 1.1: Proteolytic hydrolysis of a representative dipeptide (R1 and R2 are individual side chains).

Classification of peptidases

Even though all peptidases catalyze the same reaction - the hydrolysis of a peptide bond - many different sub-types of peptidases exist [13]. Three fundamental methods are used for the classification of peptidases. They can be grouped based on the chemical mechanism of catalysis (1), by the kind of reaction they catalyze (2) and by their evolutionary relationship and homology (3).

The classification by the chemical mechanism of catalysis (1) relates to the chemical nature of the catalytic site and the chemical groups responsible for proteolysis [13, 14]. Peptidases can be categorized by the nucleophile involved (reactive group of the corresponding amino acid side chain in the active site) into serine, threonine, cysteine, aspartic, and glutamic peptidases, and metallopeptidases. The catalytic nucleophile is either a hydroxyl group (serine and threonine peptidases), a sulfhydryl group (cysteine

peptidases) or commonly an activated water group (aspartic peptidases and metallopeptidases) [13]. In metallopeptidases the metal ion is usually zinc, cobalt, manganese or copper [13].

Peptidases are selective regarding the position of the peptide bond in the substrate. Therefore, peptidases can be grouped according to the kind of reaction they catalyze (2) into endopeptidases and exopeptidases. Endopeptidases hydrolyze internal, alpha-peptide bonds in a polypeptide chain that are usually distant from the N-terminus or C-terminus [13]. Endopeptidases can be further grouped according to their catalytic type (1) into serine endopeptidases (EC 3.4.21), cysteine endopeptidases (EC 3.4.22) aspartic endopeptidases (EC 3.4.23), metalloendopeptidases (EC 3.4.24) and threonine endopeptidases (EC 3.4.25). By contrast, exopeptidases liberate single amino acids, dipeptides or tripeptides from polypeptide chains [2]. Exopeptidases, which act on the free N-terminus of the polypeptide chain, are aminopeptidases (EC 3.4.11), dipetidases (EC 3.4.13), and dipeptidyl-peptidases and tripeptidyl-peptidases (EC 3.4.14). Exopeptidases, which act on the free C-terminus of the polypeptide chain, are called peptidyl-dipeptidases and carboxypeptidases (EC 3.4.15). Carboxypeptidases can also be further grouped into serine-type carboxypeptidases (EC 3.4.16), metallo-carboxypeptidases (EC 3.4.17) and cysteine-type carboxypeptidases (EC 3.4.18) according to their catalytic type (1). The classification of peptidases according to the type of reaction is illustrated in Figure 1.2.

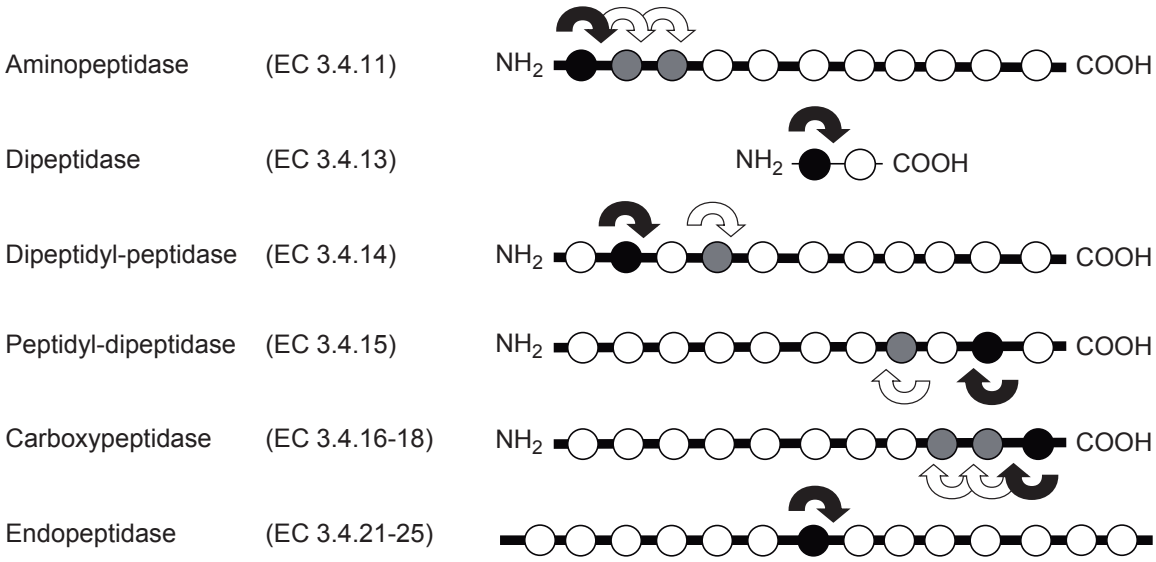

Figure 1.2: Classification of peptidases by the kind of catalyzed reaction. Amino acids are represented as beads. Black arrows show the first cleavage and white arrows show subsequent cleavages (modified after Rawlings *et al.* [13]).

In addition, a third small group of peptidases, called omega peptidases (EC 3.4.19), catalyzes the hydrolysis of terminal peptide residues that are substituted, cyclized or linked by isopeptide bonds.

Finally, peptidases are grouped according to their evolutionary relationship [15] into families and clans. Peptidases that share certain sequence homologies (especially in the part of the sequence responsible for the catalytic activity) are grouped into families [15]. A clan comprises a group of families that have arisen from a single evolutionary origin. This is the most recent classification method, since it depends on the availability of data for amino acid sequences and three-dimensional structures [13].

Terminology of peptidase specificity

In addition to the classification mentioned above, peptidases can be categorized according to their specificity/preference towards a certain amino acid sequence or amino acid residue [16]. As an example, trypsin (EC 3.4.21.4) specifically cleaves after arginine or lysine and is, therefore applied, in mass spectroscopic (MS) analysis [17]. A terminology to describe these preferences was introduced by Schechter and Berger [18]. This declaration is based on the theory that the catalytic site is considered to be flanked on one or both sides by specific subsites, each able to accommodate the side chain of a single amino acid residue from the substrate [1]. The sites are numbered, starting from the catalytic site, as S1, S2, S3, etc. towards the N-terminus of the substrate and S1', S2', S3', etc. towards the C-terminus. Accordingly, the amino acid residues in the substrate, which are accommodated, are numbered (from the scissile bond) P1, P2, P3, etc. (N-terminus) and P1', P2', P3', etc. (C-terminus) [1, 2, 13]. A schematic representation of an enzyme-substrate complex, which is labeled according to the Schechter and Berger nomenclature, is shown in Figure 1.3.

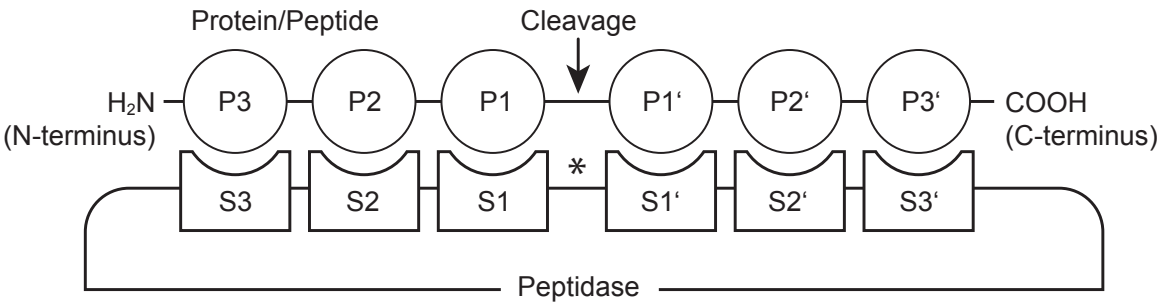

Figure 1.3: Schematic representation of an enzyme-substrate complex of a peptidase with a protein or peptide. Labeling according to the nomenclature of Schechter and Berger [18]. In this representation the catalytic site of the enzyme is marked (∗).

Synergism of endopeptidases and exopeptidases

Various applications, such as the production of flavoring hydrolysates or hypoallergenic infant formulas (see below), require protein hydrolysates with a high degree of hydrolysis (DH). A protein hydrolysis is always limited if a single peptidase is used, since every peptidase shows certain substrate specificities/preferences. In order to obtain a high DH, the combination of peptidases is essential. An exemplary proteolysis of a hypothetical protein with only one endopeptidase (1), an exopeptidase (2) and the combination of both (3) is illustrated in Figure 1.4. Beads represent amino acids from the polypeptide chain. Red beads indicate preferred cleavage sites for the endopeptidase, whereas, brown beads indicate preferred cleavage sites for the exopeptidase (aminopeptidase). White beads represent amino acid residues that cannot be cleaved when in the P1 position (see above). These synergistic effects are necessary for an efficient breakdown of proteins.

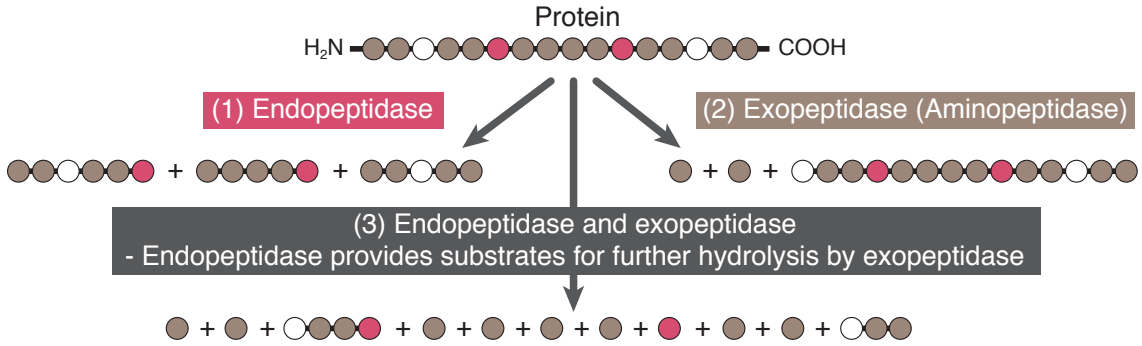

Figure 1.4: Proteolysis of a hypothetical protein by (1) an endopeptidase, (2) an exopeptidase (aminopeptidase) and (3) a combination of both. Preferred cleavage sites are indicated by pink (endopeptidase) and brown beads (exopeptidase). Amino acids represented by white beads cannot be cleaved in the P1 position.

Measurement of peptidase activity

Katal (kat) is the SI unit of catalytic activity and is defined as the amount of a catalyst that catalyzes a reaction rate of 1 mole (substrate consumed or product formed) per second. Information about the assay conditions (e.g. substrate concentration, temperature, pH, buffer systems and additives) is requisite to assess a stated enzyme activity. When using an enzyme as a process catalyst, the substrate (e.g. plant protein) can be different from that employed in its assay. Model substrates can be chromogenic (e.g. *p*NA: *p*-nitroanilide) or fluorogenic (e.g. AFC: 7-Amino-4-trifluoromethylcoumarin) protein/peptide/amino acid derivatives. Furthermore, labeled proteins, such as azocasein

(azo compound labeled casein), can also be used. The choice of substrate is crucial if peptidase activity has to be determined/detected. An endopeptidase activity, for example, cannot be measured with an amino acid derivative, since certain amino acid residues (P_i, P_i') might be missing. Moreover, since the *p*NA group is located at the C-terminus of an amino acid derivative it cannot be cleaved by a carboxypeptidase.

However, the activity of peptidases or peptidase preparations can also be determined using the original substrate of the process. The substrate (e.g. plant protein) is hydrolyzed and the amino groups released are subsequently detected after derivatization (e.g. OPA: *o*-phthalaldehyde). Furthermore, substrate/product can also be quantified, for instance, using high-performance liquid chromatography (HPLC).

Industrial applications of peptidases

The global enzymes market size was estimated at approximately USD 4.4 billion in 2013. A growing food and beverage industry coupled with an increasing use in biofuel manufacturing is expected to drive market demand in the forecast period to over USD 7.6 billion in 2020 [19]. Peptidases make around USD 1.2 billion (28 % market revenue share). They have a large variety of applications, mainly in the detergent, food, feed, leather and pharmaceutical industries [2]. By category, enzymes for food and beverage hold the dominant part, having a market share of 38 % with the highest sales in the milk and dairy segment [19]. Peptidases have been used for dairy processing, baking, brewing, and in the processing of meat and fish for centuries, although not in an isolated form [20]. A selection of peptidases, which are utilized in the food industry with the corresponding application area is listed in Table 1.1. Approximately 260 enzymes are available in the European Union, which are derived from plant (3 %) and animal (6 %) materials, but the majority are produced by fungi (filamentous ascomycetes and basidiomycetes: 58 %, yeasts: 5 %) and bacteria (28 %) [20]. More than a third of the enzymes offered originate from genetically modified organisms [20].

Table 1.1: Selection of peptidases, corresponding production hosts and industrial application areas; Adapted from Fraatz *et al.* [20].

Peptidase	EC number	Production strains	Application areas
Leucyl aminopeptidase	3.4.11.1	*Aspergillus oryzae*	Beverages, savory
Serine-type carboxypeptidase	3.4.16.x	*Aspergillus niger*	Meat, fish, dairy, savory
Trypsin	3.4.21.4	*Fusarium venenatum*	Dairy
Subtilisin	3.4.21.62	*Bacillus licheniformis*	Protein hydrolysates
Oryzin	3.4.21.63	*Aspergillus oryzae*	Bakery
Papain	3.4.22.2	*Carica papaya*	Beverages, meat
Aspartic endopeptidases	3.4.23.x	Animal origin, *A. niger, A. oryzae, B. licheniformis*	Bakery, beverages, dairy, etc.
Pepsin	3.4.23.1/2	Cattle, pig	Protein hydrolysates
Chymosin	3.4.23.4	*A. niger, Kluyveromyces lactis*	Dairy
Aspergillopepsin I	3.4.23.18	*A. oryzae, A. wentii*	Dairy
Mucorpepsin	3.4.23.23	*A. oryzae, Mucor pusillus, Rhizomucor miehei*	Meat, fish, protein hydrolysates
Thermolysin	3.4.24.27	*Geobacillus caldoproteolyticus*	Protein hydrolysates
Bacillolysin	3.4.24.28	*Bacillus amyloliquefaciens*	Bakery, beverages, meat, fish, etc.
Deuterolysin	3.4.24.39	*A. oryzae, A. wentii*	Beverages, protein hydrolysates

Peptidases in dairy technology

The application of rennet in cheese manufacture was among the earliest (from 6000 BC) food processes where exogenous enzymes were utilized [21]. Calf rennet, which is traditionally used for cheese making, is an enzyme complex and consists mainly of chymosin (EC 3.4.23.4) [22]. Milk clotting is caused by a specific cleavage of κ-casein between Phe105 and Met106 by chymosin. This causes a destabilization and, therefore, coagulation of the casein micelles due to the removal of the hydrophilic glycopeptide (caseinomacropeptide). Mostly recombinant chymosin (produced in *Kluyveromyces lactis* or *Aspergillus niger*) or microbial peptidases (e.g. aspartyl proteinases, EC 3.4.23.23, from *Rhizomucor miehei*) are used for cheese production. Peptidases are also utilized to accelerate the cheese ripening procedure. Enzyme-modified cheese (EMC) is an alternative to natural cheese in processed consumer foods [23]. Peptidases from *Aspergillus oryzae* and lactic acid bacteria are used, besides other enzyme classes, to

produce a typical cheese flavor. A high DH and, therefore, a combination of endopeptidases and exopeptidases, is generally desirable in the production of EMC [23]. Milk protein hydrolysates, which are widely used as substitutes or supplements to human breast milk, are also used in infant formula [24]. These hypoallergenic protein hydrolysates are usually obtained by combining enzymatic hydrolysis of milk protein (e.g. whey) and ultrafiltration (UF) [25]. Furthermore, milk protein hydrolysates are also used in clinical nutrition [26], sports drinks [27] or as food supplements [26].

Peptidases in cereal technology

Gluten is the most important functional component in wheat flour and anything that modifies the gluten network will have a strong influence on the dough and final bread quality [28]. Peptidases from *A. oryzae* and bacterial peptidases have been applied in bread-making technology [2]. In bread making, peptidases are used to improve dough-handling properties, dough machinability and to reduce the mixing time [29, 30]. Furthermore, peptidases can improve color, flavor, water absorption, dough extensibility, and grain and crumb texture [31, 32]. In addition, the use of peptidases improves the flavor in cakes and has a positive influence on the dough extensibility and texture of biscuits [33] and cookies [34]. Finally, peptidases are added to wafer batter in order to liquefy the gluten, which results in a uniform mixture with optimum flow properties [33].

Processing of beverages using peptidases

Protein plays a key role in the foam formation and foam stabilization of beer and is necessary for adequate yeast nutrition in the fermentation process [35]. It is common to add exogenous peptidases to supplement the limited amount present in the mashing process, especially when large amounts of unmalted cereals/adjuncts (e.g. corn, rice, rye, oats, barley and wheat) are used for the beer production [35]. Another application of peptidases is the stabilization of beer. Beer haze is usually caused by the cross-linkage of the high molecular weight protein of malt (hordein) [35]. Commercial peptidases, such as papain (EC 3.4.22.2) [36] and other plant-derived peptidases, are applied to prevent beer haze. Nowadays, gluten-free products represents an important socioeconomic issue and the market for gluten-free products is growing fast [37]. Peptidases, such as the prolyl endopeptidase (EC 3.4.21.26) from *A. niger*, can be used to produce gluten-free beer [37]. In wine processing, the peptidases Aspergillopepsin I (EC 3.4.23.18) and II (EC 3.4.23.19) from *A. niger* are used for wine haze prevention, even though it is not common

practice [38]. It is also possible to employ exogenous peptidases in the clarification of juice [39].

Peptidases in meat and fish processing

Peptidases have an important role in meat processing. Papain, bromelain (EC 3.4.22.32) and ficain (EC 3.4.22.3) are currently used for commercial scale meat tenderization [36, 40]. Zingibain (EC 3.4.22.67), a peptidase from ginger (*Zingiber officinale*), has also gained a lot of interest recently due to its collagenolytic activity [41, 42]. Exogenous peptidases are also added to improve flavor formation in fermented meat products [41].

Traditional and commercial preparations of fish protein hydrolysates from fish waste (e.g. skin, head, visceral, liver, bones) are currently used as food supplements/nutraceuticals in many countries [43, 44]. These preparations are produced, for example, by *A. oryzae* peptidases, subtilisin (EC 3.4.21.62), trypsin or papain. Although traditional fish sauce production is carried out only by endogenous peptidases, the modern commercial production uses exogenous peptidases (e.g. papain; bromelain, ficain) in the fermentation to accelerate the process [45]. Furthermore, peptidases are utilized to recover carotenoids from crustacean waste [45], to extract fish collagen from fish waste [46] or to remove the skin from fish fillets [45].

Synthesis of artificial sweeteners (aspartame)

Aspartame is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. Maintenance of the stereospecificity is crucial and, therefore, enzymatic synthesis is preferred [2]. Peptidases are able to catalyze condensation reactions under certain kinetically controlled conditions. An immobilized preparation of thermolysin (EC 3.4.24.27) from *Bacillus thermoprotyolyticus* is used for the enzymatic synthesis (condensation reaction) of aspartame, which is mainly produced by Toya Soda (Japan) and DSM (The Netherlands) [2].

Peptidases for the production of flavoring hydrolysates from agricultural crops

Peptidases have been used from ancient times to prepare soy sauce and other soy products [2]. Even though only endogenous peptidases (from *A. oryzae*) are used in the traditional production of soy sauce [47], exogenous peptidases are applied in some industrial applications [48, 49]. Aspergillopepsin I, produced by *A. saitoi*, is widely used for hydrolysis of soybean protein in the manufacture of soy sauce [49].

The food industry in Europe began using peptidases as processing aids for wheat gluten hydrolysis in the 1980s [50]. Hydrolysates of wheat gluten are used for delivering a savory taste (umami) in a wide range of culinary products [51, 52]. The desirable savory flavor of vegetable protein hydrolysates is mainly caused by free amino acids and smaller peptides [53]. Glutamic acid is especially important because of its umami taste profile [53]. Therefore, the product should have a high DH. A combination of endopeptidases and exopeptidases is essential for an efficient breakdown of proteins. The commercial peptidase preparation Flavourzyme (Novozymes, Denmark) is a common preparation for the production of flavoring hydrolysates and contains several exopeptidases and endopeptidases [54].

Perspectives on the application of protein hydrolysates

Protein hydrolysates from various edible proteins are currently used as food supplements in a broad range of applications. However, the prospective demand for protein hydrolysates goes beyond the sole nutritional support [55]. Diet has become a core component of public health and personal well-being over the last two decades [55]. As a result, there has been growing interest in food protein hydrolysates with bioactive properties [56]. A lot of effort has been put into the research to develop novel foods with enhanced functional properties. This includes the research about novel bread-making technologies [57], meat products [58], functional milk protein hydrolysates [12, 59–61] and the peptide functionalities obtained from different agricultural crops [56, 62–66] and vegetables [67]. Protein hydrolysates, for example, showed antihypertensive properties caused by the inhibitory angiotensin-converting enzyme (ACE; EC 3.4.15.1) activity of some peptides. Inhibitory dipeptidyl-peptidase 4 (DPP4; EC 3.4.14.5) peptides against type 2 diabetes have also been found. Moreover, protein hydrolysates with antioxidant, anti-inflammatory and immunomodulatory properties are known [61]. In addition to the biofunctional properties of hydrolysates, several techno-functional properties are of industrial interest. These include foam stabilization and emulsifying properties as well as radical scavenging activity. The functional properties of protein hydrolysates will be discussed in detail in a later section.

INDUSTRIAL ENZYMES

Christian Hansen started the industrial production of enzymes for use in food processing in 1874 with the extraction of chymosin from dried calves' stomachs for use in cheese manufacturing [68]. A variety of commercial enzymes from plant, animal and microbial enzymes are available nowadays. However, around 90 % of the commercial enzymes are produced in microorganism [69]. The type and field of application define the kind of process for enzyme production [69]. Enzymes for medicine and health-care products are usually required in high levels of purity, whereas enzymes used in the bulk production of, for example, food or feed are usually produced as rather crude preparations on a large scale [70]. Furthermore, the production process depends on the source and localization of the enzyme [69]. A general scheme for the production of enzymes is shown in Figure 1.5. Four general stages come into operation for all enzyme production procedures, regardless of the origin and the location of the enzyme. The first stage is enzyme synthesis (cultivation of microorganisms, plants or animals), followed by the second stage, which is the enzyme recovery (separation of the enzyme from the producing cell system) [69]. The next stage is enzyme purification (to remove impurities), followed by the enzyme formulation to obtain a stable enzyme preparation (solid or liquid) [69].

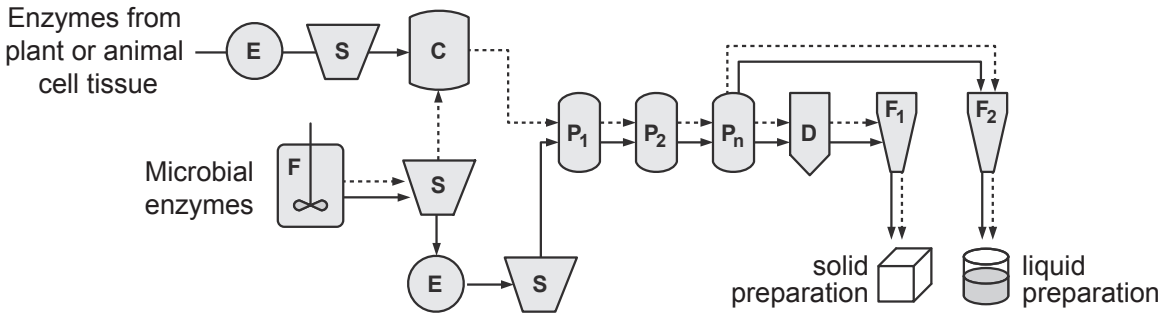

Figure 1.5: Scheme for the production of enzymes from plant/animal tissue or microbial fermentation modified after Illanes [69]. E: cell extraction; S: solid–liquid separation; C: concentration; F: fermentation; P_i: operations of purification; D: drying; F_i: formulation (solid lines: intracellular enzyme; dashed lines: extracellular enzyme).

Industrial peptidase preparations applied in the food industry

Novozymes, Dupont (Danisco) and DSM dominate the enzyme market with respective market shares of 47 %, 21 % and 6 %, respectively, even though a variety of enzyme manufacturers exist. Lists of commercial enzymes used in food processing can be

obtained from the Enzyme Technical Association (http://www.enzymetechnicalassoc.org) and the Association of Manufacturers and Formulators of Enzyme Products (http://www.amfep.org). A selection of relevant commercial peptidase preparations is listed in Table 1.2.

Table 1.2: Selection of commercial peptidase preparations applied in the food industry; Adapted from Ward *et al*. [71].

Supplier	Product trade name	Production host
Novozymes (Denmark)	Alcalase	*Bacillus licheniformis*
	Esperase	*B. lentus*
	Flavorzyme	*Aspergillus oryzae*
	Neutrase	*B. subtilis*
	Protamex	*Bacillus sp.* peptidase complex
Chr. Hansen (Denmark)	Hannilase	*Rhizomucor miehei*
	Thermolase	*Cryphonectria parasitica*
	Naturen	*Bos taurus*
	Chy-Max	*A. niger* (recombinant chymosin)
	YieldMAX	*A. oryzae*
Dupont/Danisco (USA)	Accelase	*Lactococcus lactis*
	Savorase	Enzyme complex (various sources)
	Debitrase	Lactic acid bacteria
	Marzyme	*R. miehei*
DSM (Netherlands)	BakeZyme B500BG	*Bacillus sp., Aspergillus sp.*
	Brewers clarex	*A. niger*
	Fromase	*R. miehei*
	Maxiren	*Kluyveromyces lactis* (recomb. chymosin)
	Suparen	*C. parasitica*
Amano (Japan)	Newlase F	*Rhizopus niveus*
	Protease M	*A. oryzae*
	Protease N	*B. subtilis*
	Protease S	*B. stearothermophilus*
	Peptidase R	*R. oryzae*
	Protin	*Bacillus sp.*
	Thermoase	*Bacillus sp*
AB Enzymes (Germany)	Corolase	*Bacillus sp*
Enzyme Development (USA)	ENZECO Bromelain	*Ananas comosus*
	ENZECO Papain	*Carica papaya*

Sources of industrial peptidases

Peptidases from plant origin

The use of plants as a source of peptidases is affected by the availability of land for cultivation and the climatic conditions for growth and is a time-consuming process [2]. Nevertheless, the plant peptidases papain, bromelain and ficain are applied in food processing (see above). All three peptidases belong to the cysteine peptidases and are categorized in family C1 [72]. In this clan, two additional amino acid residues (glutamine and asparagine) are involved in the catalytic mechanism besides the catalytic dyad (cysteine, histidine) [72]. Papain (EC 3.4.22.2) is extracted from the latex of green papaya (*Carica papaya*) fruit [2]. The term "papain" is also applied to the commercial crude dried latex used in industrial enzyme preparations [72]. These preparations often include papain, glycyl endopeptidase (EC 3.4.22.25), chymopapain (EC 3.4.22.6) and caricain (EC 3.4.22.30) [73]. It should be noted that papain is only a minor constituent (5 – 8 %) among the papaya endopeptidases [73]. Bromelain is either extracted from the stem (stem bromelain, EC 3.4.22.32) or the fruit (fruit bromelain, EC 3.4.22.33) of the pineapple (*Ananas comosus*). Again, commercial bromelain preparations (usually from the pineapple stem) often include other peptidases, such as ananain (EC 3.4.22.31) or comosain [74]. Ficain (EC 3.4.22.3), which is extracted from the latex of fig (*Ficus glabrata*), and actinidain (EC 3.4.22.14), which is obtained from the kiwi fruit (*Actinidia deliciosa*), are also commercially available.

Peptidases from animal origin

The most familiar peptidases of animal origin for industrial use are pancreatic trypsin, chymotrypsin, pepsin and chymosin [75]. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) belong to the serine peptidases, are categorized in family S1 and can be, for example, isolated from the bovine or porcine pancreas. Trypsin is specific for arginine and lysine at the P1 position and occurs in two isoforms (cationic and anionic trypsin). Chymotrypsin has a high specificity for the aromatic amino acids phenylalanine, tyrosine and tryptophan at the P1 position. Pepsin (EC 3.4.23.1) and chymosin (EC 3.4.23.4) belong to the aspartic acid peptidases and are categorized in family A1. Pepsin is found in the stomach of almost all vertebrates. Commercial pepsin is usually isolated from acidified pig stomach. Chymosin can be extracted from the gastric mucosa of young animals, such as calves. It specifically cleaves a single peptide bond in κ-casein to

generate insoluble *para-κ*-casein and C-terminal glycopeptide [2]. Current demands for chymosin cannot be met from animal sources [76] and, therefore, the recombinant production of chymosin and microbial milk coagulants are of industrial importance.

Peptidases from microbial origin

Microorganisms are excellent cell systems for enzyme production: they are metabolically vigorous, quite versatile, easy to propagate on a large scale by submerged or solid-state fermentation, and are simple to manipulate both environmentally and genetically, their nutritional requirements are simple and their supply is not conditioned by seasonal fluctuations [69].

Most of the industrial peptidases originate either from *Bacillus* or *Aspergillus* species, as seen in Table 1.1 and 1.2. Subtilisin (EC 3.4.21.62; S8 family) belongs to the alkaline serine peptidases and is the most dominant enzyme on the market (production of 500 tons of pure enzyme protein per year) [2]. It is produced by multiple *Bacillus sp*. (e.g. *B. licheniformis, B. amyloliquefaciens, B. subtilis*) and is specific for aromatic and hydrophobic amino acid residues at the P1 position [77]. Furthermore, thermolysin (EC 3.4.24.27) from *Geobacillus stearothermophilus* and *B. thermoproteolyticus*, and bacillolysin (EC 3.4.24.28) from *B. amyloliquefaciens*, *B. cereus* and *B. subtilis* are two metalloendopeptidases from the M4 family, which preferably cleave hydrophobic amino acids in the P1' position [78]. Aminopeptidase 1 (EC 3.4.11.22) from *G. stearothermophilus* is one of the few exopeptidases described in the *Bacillus* species.

Aspergillus species are able to produce a whole set of peptidases, which are contained in various commercial peptidase preparations. *Aspergillus niger* produces two proline-specific (at the P1 position) serine endopeptidases: prolyl endopeptidase I (EC 3.4.21.26; S9 family) and acid prolyl endopeptidase (EC 3.4.21.-, S28 family). The serine endopeptidase oryzin (EC 3.4.21.63, S8 family) and the two pepsin-like aspartic acid peptidases aspergillopepsin I (EC 3.4.23.18, A1 family) and aspergillopepsin II (EC 3.4.23.19, A1 family) are produced by *A. saitoi* and other *Aspergillus* species [38, 79, 80]. Furthermore, several metalloendopeptidases from *Aspergillus* species are known. These include deuterolysin (peptidase EC 3.4.24.39, M35 family), fungalysin (EC 3.4.24.-, M36 family) and penicillolysin (EC 3.4.24.-, M35 family) [81–83]. In addition to the multiple endopeptidases, *Aspergillus* species produce various exopeptidases, such as aminopeptidases (leucine aminopeptidase 1 and leucine aminopeptidase 2, EC 3.4.11.1, M1 family), dipeptidyl peptidases (dipeptidyl peptidase 4, EC 3.4.14.5, S9 family;

dipeptidyl peptidase 5, EC 3.4.14.-, S9 family) and serine-type carboxypeptidases (EC 3.4.16.6, S10 family) [84–86].

Besides *Bacillus* and *Aspergillus*, some peptidases from other species are of industrial importance, especially in the dairy sector. These include various peptidases from lactic acid bacteria [87, 88] and chymosin-like milk-clotting enzymes (microbial chymosin alternatives), such as endothiapepsin (EC 3.4.23.22, A1 family) from *Cryphonectria parasitica* [89] or mucorpepsin from *Rhizomucor miehei* (EC 3.4.23.23, A1 family) [90].

Industrial production of peptidases

Extraction of peptidases from animal and plant tissue

Enzymes from plant tissues/exudates or animal organs/fluids are usually by-products and are synthesized as a part of the agricultural processes (plant growth, animal breeding) [69]. These peptidases have to be extracted from the tissues/organs prior to the downstream processing, which is discussed in this section. The choice of extraction medium (pH, buffer salts, detergent and/or chaotropic agents) is, therefore, important.

Peptidases from papaya, fig and pineapple can be purified from the latex or juice directly without the need for a sophisticated extraction protocol [91–93]. The tissues containing enzymes from animal material are, typically, preserved at the slaughterhouse by salting, freezing or drying [49]. Since all gastric peptidases are generated from their inactive forms (zymogens), a combined extraction and activation is targeted. Commercial trypsin and chymotrypsin are prepared by acidic extraction from the pancreas in the presence of $CaCl_2$. Commercial pepsin is prepared from acidified pig stomach homogenate [94]. Chymosin is extracted from gastric mucosa (young animals) at neutral or weakly alkaline pH [95] or using a 50 – 100 g/L aqueous NaCl solution [70].

Microbial production of peptidases

Organisms which are used for the production of enzymes for the food industry must be “generally recognized as safe” (GRAS) or must comply with the “qualified presumption of safety” (QPS) concept. The Food and Drug Administration (FDA) and the European Food Safety Authority (efsa) approve microorganisms when these are nontoxic, nonpathogenic and generally do not produce antibiotics [70].

The first important part of the fermentation process is the appropriate selection of the medium. Cells need a source of essential elements (e.g. carbon, nitrogen, oxygen, sulfur, phosphorus, magnesium) and a variety of trace elements (e.g. metals, vitamins) to

produce enzymes [96]. A defined/simple medium should be favored over an undefined/complex medium. This is only possible in the case of well-researched fermentations [96]. The fermenters utilized for an industrial production of enzymes have typical volumes of 50 – 200 m^3, which are designed to be sterilized at 121 °C prior to inoculation with the production strains [71]. Fermenter design is important to supply sterile air and to regulate the temperature/pH. If necessary, the feeding of carbon, nitrogen or any other supplements (e.g. inducer) is carried out during the fermentation [71]. In general, the fermentation can be operated in batch, fed-batch and continuous mode [97]. As stated before, *Bacillus* and *Aspergillus* are the predominant species utilized in the production of food-grade peptidases. Both species exhibit high growth rates and this contributes to short fermentation durations [98, 99]. Furthermore, several species of *Bacillus* and *Aspergillus* have GRAS status, including *B. subtilis*, *B. licheniformis*, *A. niger* and *A. oryzae*, and the genomes of various species have been sequenced. The majority of the microbial peptidases are secreted as extracellular enzymes into the culture medium with typical durations of the fermentation processes of about 2 – 4 days (bacteria) and 3 – 5 days (fungi) [49]. Extracellular enzyme product concentrations of up to 30 g/L are possible [49]. Enzymes from *Bacillus* species are typically produced constitutively during the exponential and post-exponential phases of growth [71]. The production of peptidases in *Aspergillus* species is carried out predominantly in submerged fermentation, however, solid-state systems are also in use [71]. It should be mentioned that the fermentation conditions (e.g. pH, medium) and the manufacturing process (e.g. solid-state, submerged) affect the expression of the peptidase sets highly [86, 100, 101].
After the fermentation, the enzymes are usually recovered in the supernatant followed by a continuous centrifugation process to remove cells and other particulates (bacteria), or the fungal mycelium and other particulates are removed on a rotary vacuum filter (fungi) [71]. The resulting supernatants or filtrates are usually further concentrated and/or purified. Most commercial peptidases are marketed as liquids, because powders might cause allergies and are dangerous if inhaled [71].

Recombinant production of peptidases for the food industry

The demand for the recombinant production of enzymes is caused by the increasing sophistication of food processing [102] and to increase the production of enzymes for various commercial applications [2]. The recombinant production of chymosin is one of the most important examples of how recombinant technology helps to overcome the poor

availability of animal chymosin for cheese manufacture. Recombinant chymosin is currently commercially available using *A. niger* and *Klyveromyces lactis* as microbial hosts (Table 1.2). These strains were chosen because of their well-characterized fermentation properties and their ability to secrete high levels of prochymosin (pH shift converts prochymosin to chymosin) into the fermentation medium [103]. Besides chymosin, other peptidases are also produced recombinantly (e.g. in *Bacillus* hosts). An enzyme has to be labeled as recombinant if the native enzyme is produced in a non-native organism, or if the enzyme and/or the organism has been genetically modified [104]. The FDA and efsa specify the enzyme labeling in the US and Europe, respectively.

Downstream processing

The term "downstream processing" describes the entirety of unit operations used to refine an enzyme from a complex natural source (e.g. plant extract, fermentation broth). The choice of unit operations (listed in Table 1.3) depends the properties of the enzyme (biochemical, biophysical), the nature of the crude extract (fermentation broth or plant/animal extract) and economic considerations [104].

Table 1.3: List of unit operation and respective functions, which are used in downstream processing of industrial enzymes.

Unit operation	Functions
Homogenization	Enzyme recovery by cell disruption (intracellular enzymes)
Bead Milling	Enzyme recovery by cell disruption (intracellular enzymes)
Cell lysis	Enzyme recovery through cell lysis (intracellular enzymes)
Microfiltration	Separation of enzymes and/or cells/cell debris
Centrifugation	Centrifugal separation and/of enzymes or cells/cell debris
Ultrafiltration	Concentration of target enzyme and/or desalting
Vacuum evaporation	Concentration of target enzyme
Freeze concentration	Concentration of target enzyme
Precipitation	Purification and/or concentration of target enzyme
Chromatography	Purification and/or concentration of target enzyme
Spray drying	Drying of enzyme for formulation
Lyophilization	Drying of enzyme for formulation

During downstream processing, a compromise between purity, yield and the level of concentration has to be made [96]. The product purity is measured by specific activity ($kat/g_{protein}$). The ratio of the specific activity to the initial specific activity in the crude

extract is called the purification factor [96]. Yield is expressed as a percentage of the enzyme activity (kat) at the processing step in question compared to the initial enzyme activity contained in the crude extract [96]. The level of concentration is important because the complexity and cost of various downstream processing steps depend on the processed volume and/or the concentration of the target enzyme.

Enzyme recovery

After the production/extraction process, the target enzyme has to be recovered from the crude extract (fermentation broth, plant/animal extract). Depending on the location of the enzyme, it is separated from the cells (extracellular) or cell debris after cell disruption (intracellular). A set of unit operations (see Table 1.3) can be used to isolate the enzyme from the cells. The latter can be achieved either by mechanical forces (e.g. homogenization, bead milling) or cell lysis (chemically, enzymatically). Afterwards, the enzyme has to be separated from the cells/cell debris by centrifugation or microfiltration. Filtration technology is a powerful tool for several downstream processing steps and can be divided into microfiltration, UF, nanofiltration and reverse osmosis, depending on the pore size/molecular weight cut-off [105]. An overview of the different filtration technologies is given in Figure 1.6.

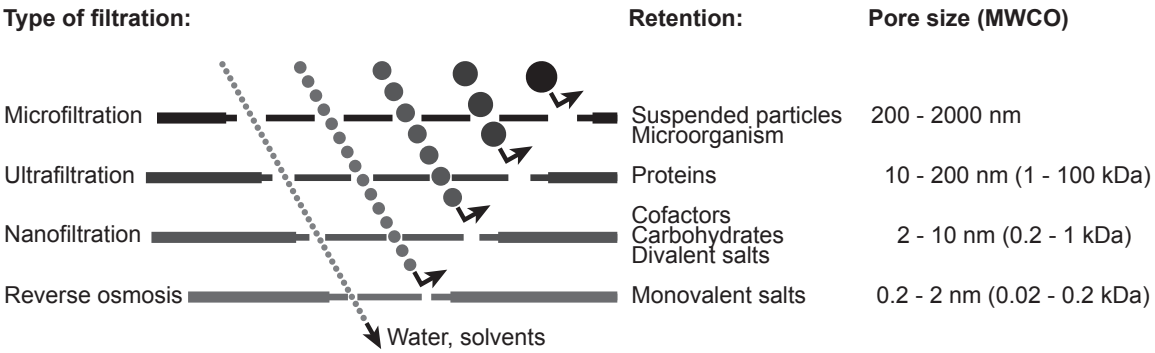

Figure 1.6: Types of filtration with retained molecules and the corresponding pore sizes and the molecular weight cutoffs; adapted from Rao *et al.* [105].

A concentration of extracellular enzyme crude extracts is often appropriate prior to the purification of the enzyme. This is usually achieved in industrial scale by UF, vacuum evaporation or freeze concentration [69]. The enzyme concentration of the starting material, prior to the purification and further refinement, has a profound impact on the final production cost [106].

Enzyme purification

If the specifications of the enzyme product require a lower concentration of components (enzyme and non-enzyme) other than the target enzyme, purification is required [104]. In industrial scale, purification can be accomplished by filtration, precipitation, extraction and chromatography [96]. Filtration can be used to separate particular molecules (microfiltration) or molecules with low molecular weights (LMWs; UF) from the target enzyme. Precipitation of a protein in an extract may be achieved by adding salts (e.g. ammonium sulfate), organic solvents (e.g. ethanol) or organic polymers (e.g. polyethylene glycol), or by varying the pH or temperature of the solution [107]. The aim is to precipitate and, therefore, separate the target enzyme or impurities due to different chemical and biophysical properties. Aqueous two-phase extraction is useful to remove biomolecules due to different solubilization behaviors.

The most sophisticated (also the most expensive) method to purify an enzyme from a complex crude extract is fast protein liquid chromatography. Separation by chromatography depends on the differential reversible interaction of proteins between a stationary phase (the chromatographic medium or the adsorbent is usually packed in a vertical column) and a mobile phase (the buffer solution) [107]. Chromatographic matrices can be classified as inorganic materials, synthetic organic polymers or polysaccharides, are bead-shaped (diameter: 10 – 100 μm) and should not contain groups that unspecifically/spontaneously bind protein molecules [107]. Depending on the functional groups attached to the matrix and, therefore, the separation principle, chromatography (Figure 1.7) can be categorized into (1) size exclusion chromatography, (2) ion exchange chromatography, (3) hydrophobic interaction chromatography and (4) affinity chromatography. Cation exchangers are acids (e.g. sulfonate anions) and anion exchangers are bases (e.g. ammonium cations) [107]. Functional groups in affinity chromatography of peptidases can be substrate/inhibitor analogs or metals.

Since the chromatographic separation is expensive, the first choice of bulk purification methods, as used for the production of food-grade enzymes, is precipitation and filtration [104]. Therefore, it should be pointed out that the peptidase preparations for the food industry are rather crude preparations and probably contain more than a single enzyme activity. After enzyme purification, a protein solution can be prepared for storage either by drying (solid formulation) or by stabilization in solution (liquid formulation) with an additive [96].

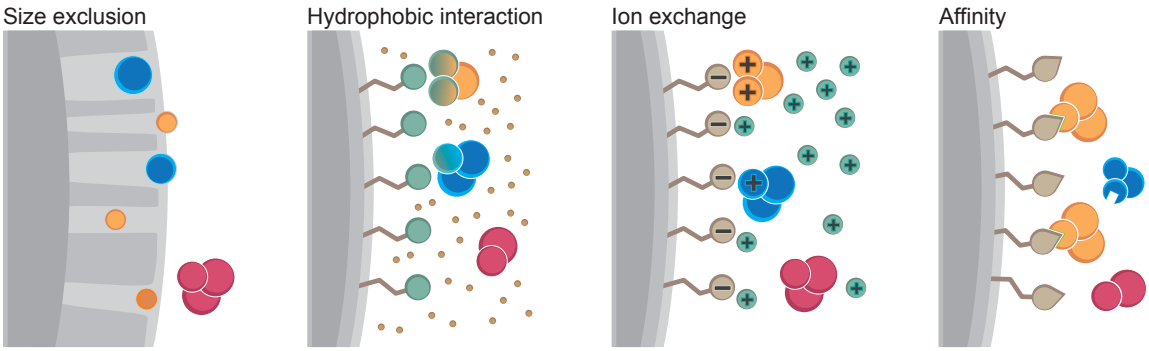

Figure 1.7: Principle chromatographic separation techniques for the purification of enzymes. In this representation, proteins elute in the order: purple, blue and orange (for details, see text).

Enzyme product formulation

With a notable exception (enzymes in solid washing detergents), most enzymes are marketed as liquid concentrates, because powdered enzyme products can cause allergies and peptidases are especially dangerous if inhaled [71]. Formulation is required to supply the desired shelf life during storage of the enzyme preparation prior to its application [104]. It is important that the additives utilized for product formulation are also acceptable in the food product [104].

One advantage of liquid formulations is that the last manufacturing step (drying) is avoided [69]. Critical issues of liquid formulations are the enzyme and microbial stability, because these systems are more dynamic compared to solid formulations [104]. Glycerol stabilizes protein structures [108] and is often used in liquid formulations in concentrations of around 50 % (v/v) [96]. Sugars or sugar alcohols can also be used as stabilizers in liquid formulations [109]. The most common preservatives utilized for food applications are sodium benzoate and potassium sorbate [104].

Solid preparations have the benefit of easy handling and transportation (less water) and a comparatively higher shelf-life (higher enzyme and microbial stability) [69]. Solid preparations can usually be stored at room temperature (liquid preparations have to be chilled or refrigerated). Vacuum drying and spray drying are the operations used most if the enzyme is robust [110]. By contrast, freeze-drying, a more expensive operation, is used for more labile enzymes [111]. The last step in enzyme production is standardization, since the producer must ensure an enzyme product of uniform quality.

INDUSTRIAL REALIZATION OF ENZYMATIC PROTEIN HYDROLYSES PROCESSES

The industrial production of protein hydrolysates is mainly realized by chemical or enzymatic hydrolysis [112]. Chemical hydrolysis can be conducted using acid or alkaline treatment of the respective protein source, which both come along with disadvantages. Using the acid hydrolysis, essential amino acids, such as tryptophan, methionine and cysteine, are destroyed [60]. Furthermore, glutamine and asparagine are converted to glutamic acid and aspartic acid, respectively [60]. Alkaline treatments can destroy cystine, arginine, threonine, serine and lysine and form toxic lysinoalanine [113]. Therefore, enzymatic hydrolysis is preferred over chemical hydrolysis because the hydrolysates obtained exhibit the same biological value as the source protein. Furthermore, hydrolysis can be carried out under mild conditions (temperature, pH) and enzymes are more specific, enabling the manufacturers to control the DH precisely and, thus, the composition/characteristics of the hydrolysates [114]. Several variables should be taken into account when designing a protein hydrolysis process. These variables include the mode of operation (continuous or discontinuous) and the choice of process parameters, such as temperature, pH and substrate concentration. Furthermore, enzyme kinetics, enzyme inhibition (due to substrate or product), thermodynamics and the physical and operational stability of the enzyme should be considered. Some of the issues mentioned can be handled by an appropriate bioreactor design.

Bioreactor design

Three types of ideal reactors are known in biochemical engineering. These reactors are (1) the batch reactor, (2) the plug-flow reactor and (3) the continuous stirred tank reactor (CSTR). Based on these three ideal reactors, six basic reactor setups (Figure 1.8) are commonly used. The industrial biotransformation of proteins, nowadays, is mainly performed in discontinuous batch processes [115]. If the biocatalyst is inexpensive or exhibits insufficient operational stability, a batch reactor (Figure 1.8A) might be the best option [96]. Furthermore, batch processing provides a certain degree of flexibility that is useful in some applications. A batch reactor with subsequent UF is the easiest way to increase the enzyme productivity (recover the enzyme) by keeping a batch reactor design (Figure 1.8B). Membrane reactors are applied to retain large components (i.e. the enzyme and the substrate), while allowing small molecules to pass through (i.e. the product) [116]. Membrane reactors can be operated as CSTRs with dead-end filtration (Figure

1.8C) or as loop or recycle reactors (Figure 1.8D) with tangential (crossflow) filtration [117]. An overview of filtration classifications and the corresponding membrane pore sizes is shown in Figure 1.6. Immobilization (see below) of the enzymes on particular carrier beads allows the packing of a fixed-bed reactor (Figure 1.8E). The immobilized enzymes can, furthermore, be operated as a fluidized-bed reactor (Figure 1.8E) to avoid axial gradients of pH, substrate or product [117]. It should be mentioned that the immobilized enzymes can also be applied in reactors A – D (Figure 1.8). The advantages of the membrane and bed reactors (Figure 1.8 C-D) are that both can be operated continuously, which means that the residence times of enzyme and reactants are uncoupled [117]. Additionally, they possess the capability for *in situ* separation of enzyme and products/substrates [117].

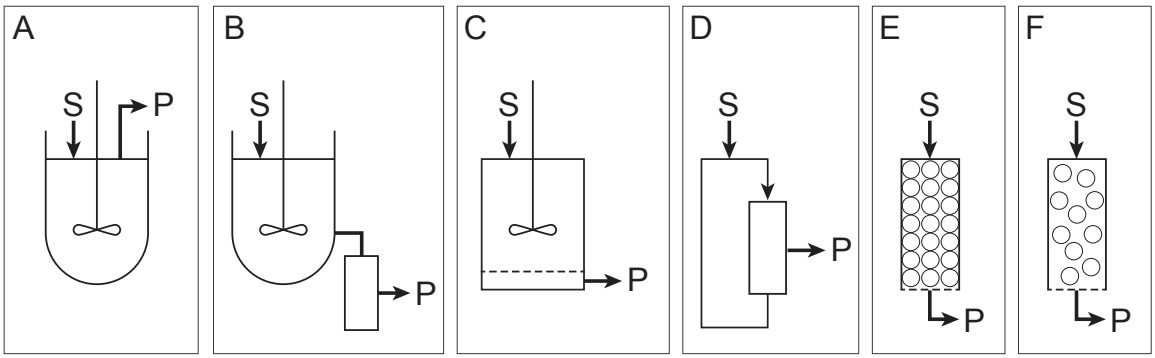

Figure 1.8: Enzyme reactor designs adapted from Bommarius [117] (A, batch reactor; B, batch-ultrafiltration reactor; C, continuous stirred-tank membrane reactor; D, recycle reactor; E, fixed-bed reactor; F, fluidized-bed reactor).

If the operational stability of the enzyme is sufficient or if an immobilization is feasible, the recovery or retention of the enzyme should be targeted to increase enzyme productivity. This cannot be realized with a batch reactor (Figure 1.8A), but can with the other reactor designs (Figure 1.8B-F). A batch reactor setup should be avoided for a reaction exhibiting substrate surplus inhibition [105]. The high substrate concentration at the beginning inhibits the enzyme activity and, therefore, a CSTR is preferred (low substrate concentration in steady state) [105]. By contrast, if an enzyme is product-inhibited, a batch reactor or a bed reactor should be chosen. The choice of reactor is also important for the hydrolysis of proteins with poor solubility characteristics, such as many plant-derived proteins. Here, a batch and fluidized-bed reactor should be preferred over membrane and fixed-bed reactors, since insoluble substrate might block the membrane or

bed. The advantages and disadvantages of the reactor setups described are summarized in Table 1.4.

Table 1.4: Advantages and disadvantages of different reactor setups for the industrial biotransformation (modified after Bommarius and Riebel [96]).

	Type of Reactor	Advantages	Disadvantages
A/B	Batch/Batch-UF	- Easy setup	- No enzyme reuse (Batch)
		- High flexibility	- Batch-to-batch variations
		- Suitable for all substrates	
C/D	Membrane	- Retention/Reuse of enzymes	- Only for soluble substrates
		- No mass transfer limitations	- Polymeric products difficult
		- Simple scale up	- Only suitable for stable enzymes
		- Continuous operation	
		- *In situ* separation of enzyme and product	
E/F	Bed	- Retention/Reuse of enzymes	- Substrate limitations (fixed-bed)
		- Potentially more stable enzymes	- Mass transfer limitations
		- Continuous operation	- Immobilization increases costs
		- *In situ* separation of enzyme and product	- Difficult to sterilize

Enzyme immobilization

Immobilization facilitates the processing of the product, increases the turnover number of the enzyme (enzyme productivity) and often enhances enzyme stability [105]. Immobilization can be achieved chemically, either by covalent bonding of the enzyme to a carrier/surface, adsorption or ionic interactions between enzyme and carrier/surface, or crosslinking [96]. Furthermore, immobilization can be realized physically by encapsulation in matrices or by embedding in a membrane (membrane reactors) [96]. The chemical immobilization and the encapsulation in matrices result in a heterogeneous catalysis. By contrast, entrapment in membranes results in a homogeneous catalysis (soluble enzyme). The shape and morphology (e.g. porosity) is an important characteristic of the immobilization carrier. Carriers can be shaped and configured as films, fibers, planar surfaces or spheres and can be made of ceramics, glass, synthetic polymers (e.g. polysulfone, polystyrene) or polysaccharides (e.g. cellulose, agarose, dextran) [96]. Membrane reactors can further be classified by driving force, which leads to a separation of enzyme, product and substrate. If separation is based on a concentration gradient, the membrane acts as a barrier only to diffusive membrane flux (dialysis) [96]. However, the

driving force can also be a charge gradient (electrodialysis) or, in most cases, a pressure gradient across the membrane (forced convection) [96].

The main advantages of immobilization are: (1) easy separation/recycling of the biocatalyst, (2) easier product processing, (3) increased enzyme stability for chemical immobilization, (4) application in bed reactors, and (5) the realization of continuous processes [105, 118]. However, the disadvantages are (1) the loss of enzyme activity and (2) mass transfer limitations in heterogeneous catalysis, and (3) additional immobilization costs [105].

Enzyme membrane reactor technology

Flaschel and Wandrey covered enzyme membrane reactor groundwork in the late-1970s [119, 120]. The enzyme membrane reactor (EMR) is a reactor system in which an appropriate membrane separation is used to keep larger compounds (i.e. enzymes and/or macromolecular substrates) in the reactor vessel, while low molecular mass molecules (i.e. products and/or inhibitors) are allowed to pass through the membrane as permeate [121]. Enzyme membrane reactors can be operated as dead-end or crossflow filtration devices such as CSTRs or porous fiber plug-flow reactors [115]. The membrane in an EMR can either be used as a separation unit with combined immobilization support or as separation unit only [121]. An overview of the general EMR configurations, operated as a crossflow CSTR system, are given in Figure 1.9.

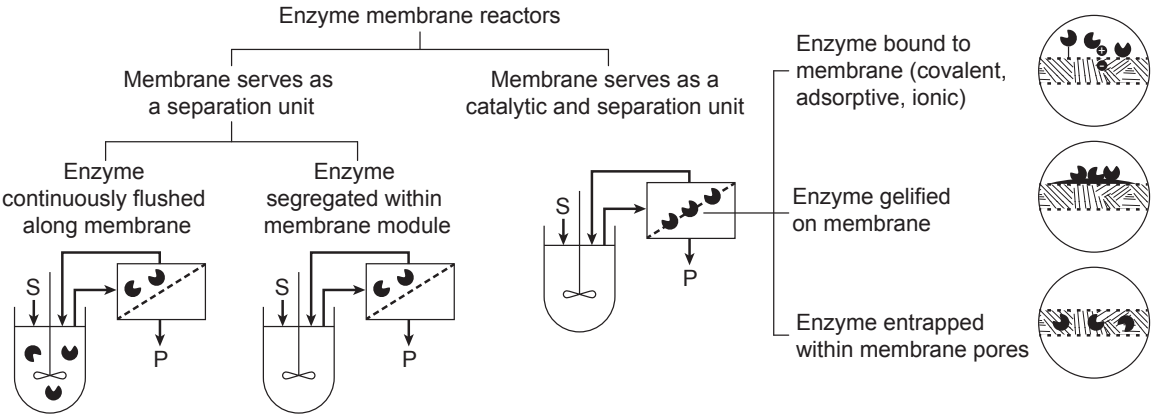

Figure 1.9: Enzyme membrane reactor configuration where the membrane either serves as separation unit or as separation unit and immobilization support. Modified after Giorno and Drioli [116].

The enzyme can be flushed along a membrane module, segregated within a membrane module, immobilized chemically (covalent, adsorptive, ionic) and by gelification, or entrapped within the membrane pores [116].

Membranes can have symmetric and asymmetric structure and can have various types of shapes and materials. Depending on the application, one can choose between polymeric (e.g. polysulfone, polypropylene, cellulose acetate, polylactic acid) or inorganic (e.g. aluminum, zirconium or titanium oxide) materials [122]. Membranes can be arranged in two shapes: tubular forms (e.g. tubes, capillaries and hollow fibers) and flat sheets (e.g. plate-and-frame, and spiral wound module) [123]. UF membranes (see Figure 1.6) with molecular weight cut-offs from 1 – 100 kDa (10 – 200 nm) are normally used to retain the enzymes inside the reactor. The retention of the enzyme activity inside the reactor is a prerequisite for a continuous economically competitive operation of an EMR [96]. The decline of enzyme activity inside the reactor can be due to (1) thermal inactivation, (2) mechanical shearing forces, (3) adsorption or "poisoning" of the enzyme through contact with the membrane, (4) enzyme leakage and/or (5) enzyme inhibition due to product accumulation [124, 125]. The utilization of enzyme membrane reactors for the production of protein hydrolysates has been reviewed on several occasions [50, 124–134].

PLANT PROTEINS

Industrial relevance

Agricultural commodities are the most important nutritional protein source and account for a daily protein supply of 48.3 g per capita [135]. By contrast, a daily protein intake of 31.8 g per capita is provided by animal products (e.g. meat, milk, egg) [135]. The Guideline Daily Amounts recommends a daily protein intake of 50 – 60 g. However, according to the Statistics Division of the Food and Agriculture Organization of the United Nations, the worldwide average daily protein intake was 80.5 g per capita in 2011 [135]. In the context of human nutrition, cereals, legumes and oil seeds are the most important groups [136]. The world production (2013) of selected important agricultural crops (cereals, legumes, oil seeds and root vegetables) is illustrated in Figure 1.10. More than 1,000 million tons of maize is produced per year followed by rice (740 million/a), wheat (715 million/a), potatoes (380 million/a) and soybeans (280 million/a) [137]. By comparison, approximately 750 million tons of milk and 310 million tons of meat (total) were produced annually (worldwide) [137]. Besides the consumption of the agricultural commodities themselves, there is an increasing industrial interest in plant protein isolates. Plant protein fractions can be recovered as by-products from various industrial processes, such as starch (e.g. cereal proteins) or oil production (e.g. oil seed proteins). These plant protein isolates have a high potential for utilization in various food applications, since they are inexpensive and highly available [66]. The modification (e.g. enzymatic hydrolysis) of these protein isolates is one way to further increase their nutritional or technological value [138], as described later.

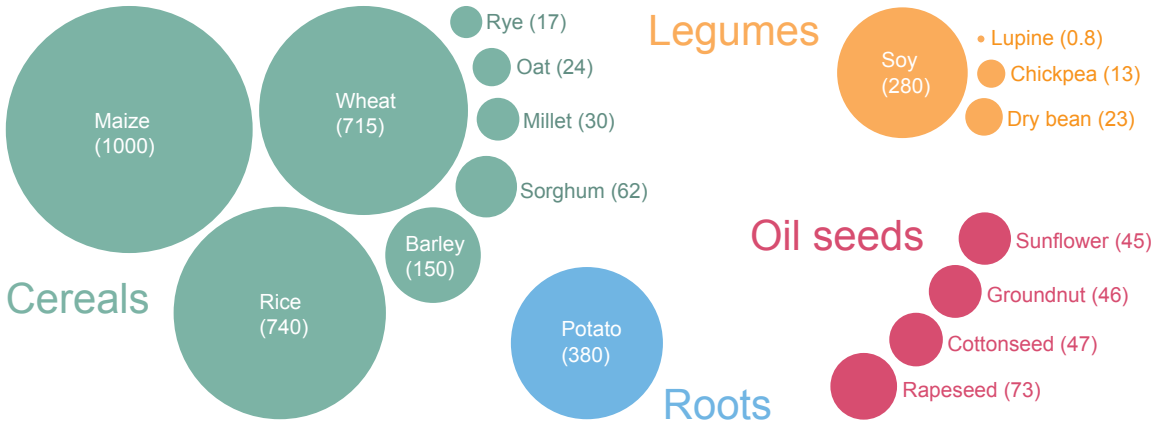

Figure 1.10: World production (Mio tons/a) of agricultural crops in 2013 reported by the Statistics Division of the Food and Agriculture Organization of the United Nations [137].

Classification and functional properties of plant proteins

In general, four levels of hierarchical organization are used to describe protein structure. The primary structure of a protein refers to its sequence of amino acids, described from the N-terminus to the C-terminus [138]. Over 300 naturally occurring amino acids have been reported [139], however, only 20 amino acids (L-stereoisomers) are commonly found in most proteins. The secondary structure refers to regular local substructures within the polypeptide chain (alpha helix, beta sheet). The resulting three-dimensional structure is the tertiary and quaternary structure for monomers and multimers, respectively. The structure of a protein affects its physicochemical and functional attributes highly. Depending on the biological function, plant proteins are classified into biocatalytic (enzymes), regulatory (hormones), transport, structural and storage proteins [140]. Plant proteins can also be grouped according to their structural and evolutionary relationship into families and superfamilies or clans [141].

Another categorization is based on their functional role in food systems. Proteins can differ in their viscosity, water-binding, gelation, elasticity, emulsification, foaming, and fat or flavor binding properties, as well as their solubility [142]. The Osborne classification, which is based on the solubility characteristics of the proteins, is an important methodology to distinguish protein fractions. According to Osborne [143], proteins can be grouped into (1) albumins, soluble in water; (2) globulins, require salt solutions for solubilization; (3) prolamins, soluble in aqueous alcohol [e.g. 60 – 80 % (v/v) ethanol]; and (4) glutelins, soluble in dilute acids or base [138, 140]. In simplified terms, the albumins correspond to metabolic proteins, whereas other fractions from seeds consist mainly of storage proteins [140].

The detailed protein distribution in wheat, for example, is 14.7 % for the albumins, 7 % for the globulins, 32.6 % for the prolamins and 45.7 % for the glutelins. Wheat gluten is composed of prolamins and glutelins, which are also called gliadins and glutenins [144]. Hence, gluten makes about 78 % of the total wheat protein. Gluten is an important plant protein composite and the main functional ingredient in bread making [145]. The structure of gluten proteins can be formed into three main groups depending on their molecular weight. A high molecular group with the high molecular weight (HMW) subunits x-type glutenins (M = 83 – 88 kDa) and y-type (M = 67 – 74 kDa) glutenins, a group of intermediate molecular weight subunits ω5-gliadins (M = 44 – 55 kDa) and ω1,2-gliadins (M = 34 – 44 kDa), and a group of LMW subunits α- and γ-gliadins (M =

28 – 35 kDa) as well as the LMW subunits of the glutenins (M = 32 – 39) [144]. α-Gliadin and γ-gliadin are mainly monomeric proteins, which, consequently, contain only intra molecular disulfide bonds. By comparison, the glutenins are protein aggregates of HMW and LMW subunits with molar masses from about 200 to a few thousand kDa, which are stabilized by intermolecular disulfide bonds, hydrophobic interactions and other forces [144].

Prolamins from other cereal origins are called hordein (barley), secalin (rye), zein (maize), oryzin (rice), kafirin (sorghum), pennisetin (millet) and avenin (oats). Some prolamins (especially gliadin) are known to be responsible for the autoimmune disorder coeliac disease [146, 147].

Composition and nutritional aspects of plant proteins

Proteins are needed in food to supply the essential amino acids and as a nitrogen source [140]. An essential/indispensable amino acid cannot be synthesized *de novo* by the organism being considered and, therefore, must be supplied in its diet [148]. These amino acids (in humans) are phenylalanine (Phe), valine (Val), threonine (Thr), tryptophan (Trp), methionine (Met), leucine (Leu), isoleucine (Ile), lysine (Lys) and histidine (His) [148].

Furthermore, six amino acids are considered conditionally essential in the human diet, meaning their synthesis is more complex and can be limited under special pathophysiological conditions (e.g. prematurity, severe catabolic distress) [148]. The conditionally essential amino acids are arginine (Arg), cysteine (Cys), glutamine (Gln), glycine (Gly), proline (Pro) and tyrosine (Tyr). Five amino acids, alanine (Ala), aspartic acid (Asp), asparagine (Asn), glutamic acid (Glu) and serine (Ser), are non-essential/dispensable and can easily be synthesized in the human body [136, 148, 149]. The amino acid composition of various selected cereal, legume and oil seed proteins is listed in Table 1.5 (cereals) and Table 1.6 (legumes, oil seeds, roots).

Table 1.5: Protein content [% (w/w)] and amino acid composition [mole %] of selected cereal proteins of industrial relevance. Data collected from [140, 150–153].

	Maize	Rice	Wheat	Barley	Sorghum	Millet	Oat	Rye
Protein	9	8	12	12	10	12	13	11
Ala	10.4	6.1	5.5	5.1	12.2	11.2	6.7	6.0
Arg [2]	3.0	6.3	2.9	3.3	2.8	3.1	5.4	3.7
Asn	5.9	5.5	3.7	4.9*	5.7	7.7*	8.1*	6.9*
Asp	1.6	3.7	1.4		2.8			
Cys [2]	2.1	2.4	2.7	1.5	2.2	1.2	2.6	1.6
Gln [2]	12.0	10.0	19.2	24.8*	11.3	17.1*	19.5*	23.6*
Glu	3.2	6.7	7.1		5.5			
Gly [2]	7.1	8.0	7.1	6.0	5.1	5.7	8.2	7.0
His [1]	2.7	2.3	2.0	1.8	1.8	2.1	2.0	1.9
Ile [1]	3.0	3.9	4.4	3.7	3.5	3.9	4.0	3.6
Leu [1]	11.8	8.3	6.9	6.8	13.8	9.6	7.6	6.6
Lys [1]	2.3	3.8	2.6	2.6	1.8	2.5	3.3	3.1
Met [1]	1.7	2.0	1.2	1.6	1.2	2.9	1.7	1.3
Phe [1]	3.7	5.0	3.8	4.3	3.7	4.0	4.4	3.9
Pro [2]	10.3	4.9	12.3	14.3	8.7	7.5	6.2	12.2
Ser	6.8	5.4	6.0	6.0	5.2	6.6	6.6	6.4
Thr [1]	4.2	4.3	3.2	3.8	3.3	4.5	3.9	4.0
Trp [1]	0.6	0.6	0.5	0.7	0.7	1.0	0.8	0.5
Tyr [2]	2.6	2.5	2.5	2.7	2.5	2.7	2.8	2.2
Val [1]	5.1	8.3	4.8	6.1	6.3	6.7	6.2	5.5

[1] essential; [2] conditionally essential; * Particular sums of Gln/Glu and Asn/Asp

Most plant proteins exhibit a relatively high amount of glutamic acid/glutamine, aspartic acid/asparagine and proline (Table 1.5 and Table 1.6). Even though cereal seeds are deficient in lysine, threonine and tryptophan, and most legume seed proteins are deficient in the sulfur-containing amino acids (methionine, cysteine, threonine) and tryptophan, plant proteins can, if combined, supply the amount and nutritional quality adequate for all ages [136, 149]. As described above, the modification of plant proteins (e.g. enzymatic hydrolysis) is one way to further increase their nutritional value.

Table 1.6: Protein content [% (w/w)] and amino acid composition [mole %] of selected plant proteins of industrial relevance. Data collected from [140, 150–160].

	Legumes				Oil seeds				Roots
	Soy	Dry bean	Chickpea	Lupine	Rapeseed	Cottonseed	Groundnut	Sunflower	Potato
Protein	38	22	20	45	20	20	26	20	2
Ala	6.4	5.7	7.9	4.9	6.4	5.6	5.4	6.1	6.4
Arg [2]	5.3	4.9	7.7	8.7	4.5	9.2	10.8	7.1	3.6
Asn	4.6	3.3*	10.7*	11.0*	7.2*	4.2	11.5*	10.3*	19.1
Asp	6.9					5.2			1.0
Cys [2]	1.7	1.4	0.7	2.7	2.8	2.0	1.4	1.5	1.2
Gln [2]	7.6	4.3*	16.2*	22.6*	18.2*	9.2	18.2*	21.5*	15.3
Glu	8.3					10.6			0.9
Gly [2]	8.9	6.6	7.2	7.4	9.0	10.0	9.8	10.2	6.0
His [1]	2.1	2.7	2.6	2.6	2.6	2.5	1.8	2.1	1.9
Ile [1]	4.5	4.7	4.9	4.0	4.5	3.2	3.1	5.3	4.2
Leu [1]	7.6	8.2	8.1	7.8	8.2	6.1	6.1	6.3	5.6
Lys [1]	5.6	6.3	6.2	4.6	5.9	4.0	3.1	2.8	4.5
Met [1]	1.2	1.2	1.5	0.6	2.0	1.6	1.1	1.6	1.1
Phe [1]	3.9	4.8	4.5	3.2	3.3	4.3	4.0	3.7	3.6
Pro [2]	7.7	4.9	5.3	4.2	8.2	5.8	6.0	5.5	4.0
Ser	5.9	7.2	4.3	6.2	4.0	5.8	6.6	4.2	5.6
Thr [1]	1.5	4.3	3.3	3.7	4.2	1.3	3.1	3.5	6.0
Trp [1]	2.5	0.9	1.0	0.4	0.4	2.2	0.8	0.9	0.8
Tyr [2]	2.7	2.2	2.3	1.2	2.0	2.1	2.7	1.8	1.9
Val [1]	5.2	5.6	5.6	4.1	6.5	5.1	4.2	5.6	7.4

[1] essential; [2] conditionally essential; * Particular sums of Gln/Glu and Asn/Asp

CHARACTERIZATION OF PLANT PROTEIN HYDROLYSATES

Chemical composition

Degree of hydrolysis

The DH is one of the most important characteristics of protein hydrolysates. It is expressed as the percentage of free amino groups (released during the enzymatic hydrolysis) compared to the maximum concentration of free amino acids at complete hydrolysis [161]. The average molecular mass of the amino acids of the respective hydrolyzed protein (calculated from the amino acid composition) is used to calculate the theoretical maximum concentration of free amino acids. With the DH, the theoretical average peptide length of a hydrolysate can be estimated. A DH of 20 %, for example, results in a theoretical average peptide length of five amino acids. There are various methods to measure the amount of free amino groups. The free amino groups are usually quantified spectrophotometrically after derivatization with, for example, OPA or 2,4,6-trinitrobenzenesulfonic acid [161, 162].

Amino acid profile

The amino acid composition/profile of protein hydrolysates can be determined after derivatization using either HPLC [163] or gas chromatography [164]. In the HPLC, these derivatives are either formed before (pre-column derivatization) or after (post-column derivatization) the chromatographic separation and are quantified by fluorometric or spectrophotometric detection. Common reagents are OPA, phenyl isothiocyanate, fluorescamine, dansyl chloride or dabsyl chloride [165–167]. In gas chromatography, alkylation, silylation, acylation and condensation reactions can be performed using a wide range of reagents to form volatile derivatives [168].

Detection of peptides and proteins by mass spectrometry

Mass spectrometry is a sensitive chemical method used to detect, identify and quantify proteins/peptides in complex samples (hydrolysates) based on their mass-to-charge ratio (m/z) down to femtomole (10^{-15} mol) amounts [169]. A sample (solid, liquid or gas) is ionized, which causes some of the sample molecules to break into charged fragments. These ions are then accelerated (i.e. electric or magnetic field), separated according to their mass-to-charge ratio, and detected [170]. Typical ionization methods are electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) or atmospheric-pressure chemical ionization (APCI). Mass-to-charge can be analyzed by,

for example, the time of flight (TOF), a quadrupole mass filter or ion traps (e.g. Orbitrap) [169]. Many mass spectrometers use two or more mass analyzers (fragmentation in between) for tandem MS (MS/MS) [169].

Gel electrophoresis

Gel electrophoresis describes a technique to separate biological macromolecules (proteins/peptides) according to their electrophoretic mobility [171]. Polyacrylamide gels are normally used for protein/peptide analysis. It is possible using this technique to separate a sample (e.g. hydrolysate or protein substrate composite) into its components.

Functional properties

The enzymatic hydrolysis of proteins can increase certain functional properties. Some of these functionalities, which are important for food-related products, are introduced in this section. An overview of the characteristic properties and the desirable functional properties of protein hydrolysates is given in Figure 1.11.

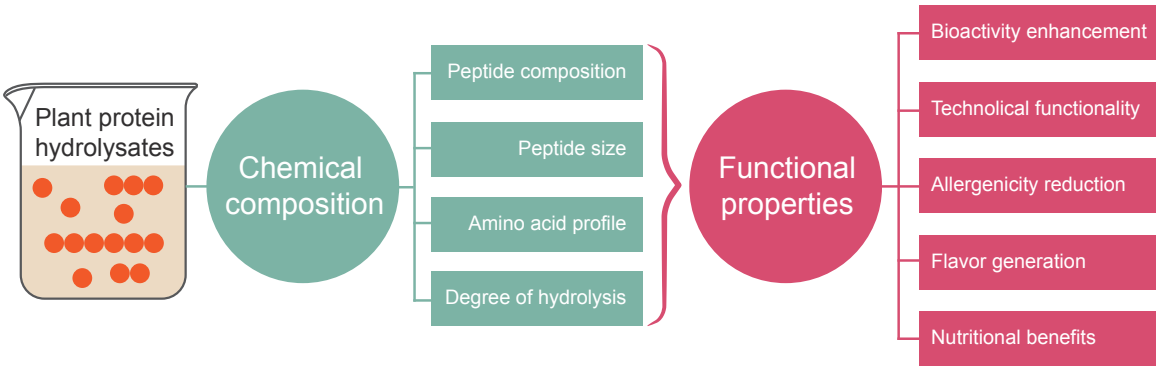

Figure 1.11: Characteristic properties and functional properties of protein hydrolysates.

Bioactive properties

Many peptides with relevant bioactive potential have been discovered in recent years. The term “bioactive peptides” refers to the ability of these peptides to have regulatory functions in the human system beyond normal and adequate nutrition [172].

Peptides liberated by the enzymatic hydrolysis of plant proteins are able to exhibit various bioactive properties. Hypotensive/antihypertensive (ACE-inhibitory) peptides have been identified in multiple enzymatic hydrolysates, such as rice, soy, chickpeas, peanuts, potatoes, maize, rapeseed or wheat by *in vitro* and *in vivo* studies [172, 173]. The inhibition of ACE is tested using the respective inhibitor/hydrolysate to determine the ACE-inhibitory activity (*in vitro*). The half maximal inhibitory concentration (IC_{50})

indicates the effectiveness in inhibiting the ACE activity. Immunomodulatory peptides from soy, rice and wheat have also been described (*in vitro* and *in vivo*) [172, 173]. The hypocholesterolemic properties of soy, lupine and potato proteins have been shown in several *in vivo* studies. The FDA even approved the health claim "25 g of soy bean protein per day may reduce the risk of heart disease" [173]. Moreover, increased hypocholesterolemic properties have been verified for soy protein hydrolysates [174]. Antithrombic peptides, which originate from soy proteins, have also been identified [173]. Furthermore, peptides originating from soy and wheat exhibited opioid agonist activities. Lastly, several plant-derived antioxidant peptides were identified in enzymatic wheat, soy, potato, rapeseed, sunflower, oat, barley, rice, and maize hydrolysates [172, 173].

Technological properties

A sufficient solubility is one requirement for the application of proteins as an ingredient in food products. Generally, plant storage proteins, such as prolamins and glutenins, exhibit, by definition, poor solubility characteristics in aqueous systems. Enzymatic hydrolysis is one way to increase the solubility as described for cereal, oilseed and legume proteins [66, 175–177]. The reduction of the secondary structure of a protein and the release of smaller peptides increases the overall nitrogen solubility.

Stable emulsions and foams are essential for the stabilization of various food products. Foaming, for example, provides a desirable and unique texture to a range of aerated foods and beverages (e.g. ice-cream, bread, cakes, meringues, beer) [175]. As a result of their amphiphilic character, proteins/peptides can adsorb to the oil/water or air/water interface and, therefore, stabilize emulsions or foams, respectively [178]. A limited hydrolysis with low DH generally increases the emulsifying and foam-stabilizing ability of various plant proteins [175, 178]. Increased emulsion and foam stabilities has been shown for several plant protein hydrolysates [175]. Finally, it should be mentioned that, the gel-forming ability of protein hydrolysates is reduced compared to the native proteins [175].

Allergenic properties

Certain plant proteins are not available to everyone, since some humans suffer from plant protein allergies. Most allergens derived from plant proteins belong to the structural and evolutionary superfamilies cupin (e.g. allergens from soybeans, peanuts, tree nuts) and prolamin (e.g. allergens from cereals, sesame, fruit, tree nuts) [179]. Besides plant protein allergies, coeliac disease is another prominent plant protein-derived disorder. Coeliac

disease is an autoimmune disorder and is caused by abnormal immune response induced by prolamins from wheat, barley and rye that result in tissue damage of the small intestine [180, 181]. The enzyme tissue transglutaminase modifies the prolamins, and the immune system cross-reacts with the small bowel tissue, causing an inflammatory reaction [180, 181]. Epitopes are the part of an antigen that is recognized by the immune system. Since the immune system is involved in both disorders (allergy and coeliac disease) and since epitopes are commonly 5 – 20 amino acids in length, the allergenic potential of plant proteins can be drastically reduced by enzymatic hydrolysis. The reduction of the antigenic activity was described, for example, for enzymatic hydrolysates from wheat, lupine, soy, chickpea and lentil proteins [182, 183]. The competitive ELISA is a common *in vitro* method to evaluate the allergenic potential of protein hydrolysates and is shown schematically in Figure 1.12. Gluten-free foods may not contain more than 20 mg/kg gluten.

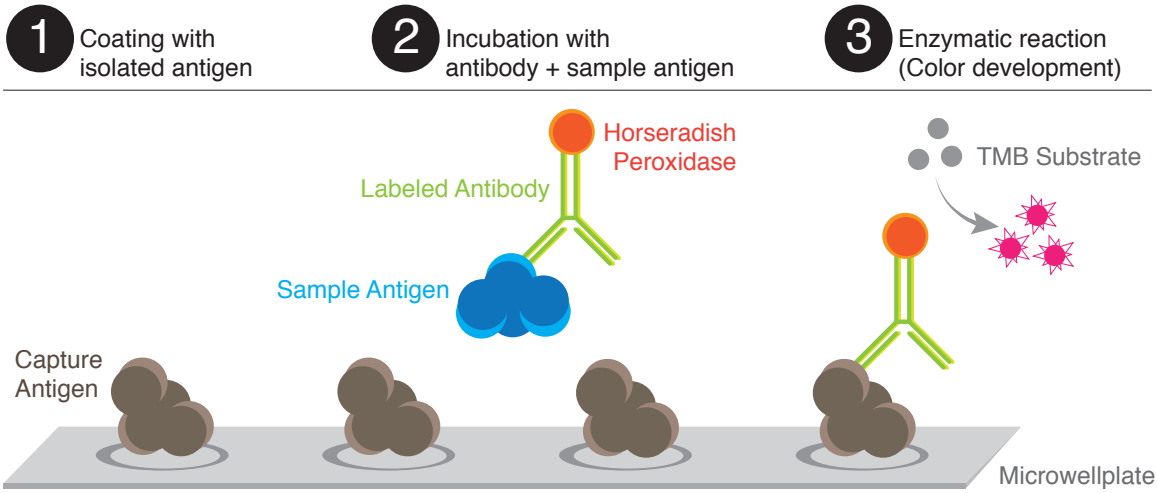

Figure 1.12: Schematic representation of a competitive ELISA including the stages coating (1), sample incubation (2) and enzymatic reaction (3).

Flavoring properties

Proteolysis plays an important role in the development of flavor in foods such as cheese, meat, sausage and fermented soy products (e.g. soy sauce) [51]. The typical flavor of vegetable protein hydrolysates enables its application as a savory ingredient in a variety of foods. This savory flavor (umami) of protein hydrolysates is complex and caused by the combination of various components [184, 185]. Key components which contribute to the desired umami flavor are assumed to be a high content of free amino acids (i.e. glutamic acid, aspartic acid), LMW peptides, salt and organic acids [53]. Therefore, a

maximum conversion of the protein (high DH) is targeted for the production of flavoring hydrolysates. Monosodium L-glutamate, for example, is one of the key drivers for the umami taste in traditional Japanese soy sauce food seasonings [184]. Even though amino acids with acidic side chains (glutamic acid, aspartic acid) and LMW peptides containing the latter exhibit salty and umami tastes [186], the influence of salts, organic acids and the food matrix on the formation and perception of the flavor is still not completely understood [51, 187, 188].

As mentioned above, various taste properties (e.g. sour, salty, sweet, bitter) also contribute to a complete and rich flavor in protein hydrolysates. Although a bitter taste is desirable in limited amounts, some protein hydrolysates exhibit intense/unwanted bitterness. Bitterness in protein hydrolysates is associated with the release of peptides (< 6 kDa) containing a high proportion of hydrophobic amino acid residues (Leu, Pro, Phe, Tyr, Ile, Trp) [186, 189]. Internally sited Pro residues are a major and distinct contributor to peptide bitterness due to the unique conformation associated with this imino acid [190]. The utilization of specific exopeptidases is one way to further increase the DH and additionally reduce bitterness [190].

Nutritional properties

It is generally accepted that protein hydrolysates containing mostly di- and tripeptides are absorbed faster than intact proteins [27]. Therefore, protein hydrolysates are used as protein supplementation in energy drinks, geriatrics products, sport nutrition and weight-control diets [26]. Furthermore, protein hydrolysates are used as medical diets to provide complete or supplemental nutritional support to individuals who are unable to ingest adequate amounts of food proteins in a conventional form [26]. Hydrolysates are also applied to provide specialized nutritional support to patients with particular physiological and nutritional needs [26]. In clinical use, protein hydrolysates are used for patients suffering from phenylketonuria, acute and chronic liver disease, Crohn's disease, pancreatitis, ulcerative colitis or the short bowel syndrome [26]. Protein hydrolysates are also used as hypoallergenic infant formula. The production of enzymatic protein hydrolysates in combination with downstream processing procedures (e.g. filtration) is considered the most effective way to obtain protein hydrolysates with defined characteristics [25, 26]. Although most protein hydrolysates for a medical application are derived from milk proteins, plant proteins have become increasingly important as a source of edible proteins with interesting nutritional properties [26].

THESIS OUTLINE

The research reported in this thesis was intended to expand the knowledge about food-grade peptidases and their application for the production of plant proteins hydrolysates with defined composition and characteristics.

Chapter one gives a general introduction about proteolytic enzymes, their application in the food industry and the production of plant protein hydrolysates employing food-grade peptidases. The enzymatic hydrolysis of proteins can increase certain favored properties, which might be useful in food-related products. A controlled and defined hydrolysis process is a prerequisite, since a desired property or functionality is dependent on the composition (amino acid and peptide profile) of the hydrolysate. In order to control the hydrolysis process, the peptidase preparation must be adequately characterized, because the latter is a key determinant for a controlled and defined process. Peptidase preparations for the food industry are produced as rather crude preparations. A three-step methodology was developed which facilitates the selection of an appropriate commercial peptidase preparation for the production of defined hydrolysates. This methodology is described in **Chapter two**. The complexity of the composition of commercial peptidase preparations has been shown for Flavourzyme, which is widely used in industrial and research applications. **Chapter three** highlights the identification, subsequent automated nine-step purification and characterization of eight key Flavourzyme enzymes. The contribution of the seven isolated Flavourzyme peptidases on a wheat gluten hydrolysis was evaluated using response surface methodology and is discussed in **Chapter four**. The latter also includes product inhibition studies of the Flavourzyme peptidases, which is relevant information for an optimal process design. Batch-to-batch variations of the Flavourzyme preparation and the influence of the variation on process reproducibility were assessed in **Chapter five**. Wheat gluten, for example, is widely used in various food applications. However, wheat gluten is not available to everyone, because some humans suffer from the autoimmune disorder coeliac disease. Therefore, information about the effect of enzymatic hydrolysis and downstream processing on the antigenicity of wheat gluten hydrolysates is valuable and is discussed in **Chapter six**. The application of Flavourzyme in a principally food-grade enzyme membrane reactor system is described in **Chapter seven** The continuous hydrolysis of the wheat gluten process was conducted for 96 h under industrially relevant process conditions using ethanol as a food-grade microbial hurdle.

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CHAPTER TWO

HOW TO CHOOSE THE APPROPRIATE PEPTIDASE PREPARATION? – A METHODOLOGY TO SELECT COMMERCIAL PEPTIDASES FOR THE PRODUCTION OF PROTEIN HYDROLYSATES WITH DEFINED COMPOSITIONS

This chapter was submitted

Merz, M., Eisele, T., Claaßen, W., Appel, D., Berends, P., Rabe, S., Blank, I. Stressler, T., Fischer, L. (2015). How to choose the appropriate peptidase preparation? – A methodology to select commercial peptidases for the production of protein hydrolysates with defined compositions. *Journal of Agricultural and Food Chemistry*. Submitted Manuscript.

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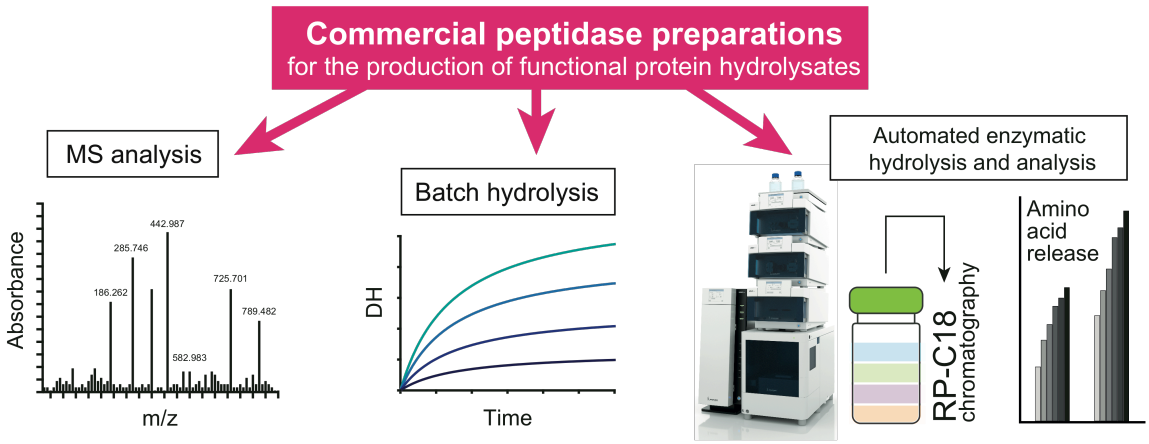
Graphical Abstract



ABSTRACT

Enzymatic hydrolysis of food protein can increase its value by improving techno functional properties such as emulsifying and foam-stabilizing capabilities or by increasing angiotensin I-converting enzyme inhibitory and radical scavenging activity. Furthermore, these hydrolysates can be used as flavoring additives in various food applications. Since these functionalities are highly dependent on the degree of hydrolysis (DH) of the hydrolysates, the enzymatic hydrolysis process must be carefully controlled. Low DH improves techno functional properties, whereas high DH targets biological activity and taste-enhancing properties. Thus, hydrolysates with both free amino acids and long-chain peptides are usually not desirable. These contrary dependencies lead to strict requirements for the commercial peptidases used. Therefore, we developed a methodology to investigate the composition of commercial peptidase preparations using MS analysis and a novel HPLC-based system for automated small-scale enzymatic reaction and analysis. We identified two suitable preparations for the production of hydrolysates with high DH and high amounts of free amino acids, and six suitable preparations for the production of hydrolysates with low DH and negligible release of amino acids.

KEYWORDS

Commercial peptidases, peptidase composition, methodology, amino acid release, automated enzymatic hydrolysis, functional hydrolysates

INTRODUCTION

The enzymatic modification of food proteins is one way to increase their value by enhancing a desired functionality. One option to achieve the latter is the hydrolysis of the proteins. By this means, for example, technological functionalities, such as emulsification and foam stabilization, can be improved [1, 2]. Additionally, angiotensin-I converting enzyme (ACE; EC 3.4.15.1) inhibitory and radical scavenging activity can be enhanced in protein hydrolysates [1]. The usage of protein hydrolysates as a flavoring ingredient is also of industrial interest [3]. The enzymatic hydrolysis of proteins is mainly performed in batch processes [4] with defined process parameters. Each of the functionalities desired is highly dependent on the degree of hydrolysis (DH) and the peptidase preparation utilized [1, 2, 5–8]. Therefore, the hydrolysis process must be carefully controlled. In general, the emulsifying and foam-stabilizing capabilities of protein hydrolysates are enhanced at lower DH [1, 2, 9–11]. By contrast, the ACE inhibitory and radical scavenging activity is improved with increased DH [1, 6]. Furthermore, a high DH is preferred for flavoring hydrolysates, since free amino acids and smaller peptides contribute to the formation of the flavors desired [3].

Due to the different dependencies of the functional property, the production of hydrolysates with both free amino acids and long-chain peptides is usually not desirable. The choice of the peptidases and the knowledge about them are crucial elements for the production of defined hydrolysates with certain functionality. The type of peptidase and the combination of exopeptidases and endopeptidases in the peptidase preparation mainly influences the DH and, therefore, the peptide/amino acid composition of the resulting hydrolysates. The complexity of the commercial peptidase preparation Flavourzyme was recently demonstrated [12]. Eight key enzymes were identified in Flavourzyme and the significant impact of six peptidases on a wheat gluten hydrolysis was shown [13].

With the methodology presented, appropriate peptidase preparations for the production of protein hydrolysates with a desired composition can be identified. Therefore, the desired property of the hydrolysate can be increased, because the mechanisms of action of the chosen industrial peptidases are better understood. We investigated the composition of ten commercial peptidase preparations by mass spectroscopic analysis. Next, classical batch hydrolyses of a model food protein (lupine) were conducted and the release of free amino acids during the protein hydrolysis was monitored using a novel HPLC-based system for automated small-scale enzymatic reaction and analysis. Thereby, the overall

exopeptidase activity of the preparations was quantified and correlated to the outcome of the classical batch hydrolysis. With this study, we revealed the potential of the peptidases tested for the production of defined hydrolysates. Furthermore, the HPLC-based system described enables the automated enzymatic hydrolysis, sampling and subsequent derivatization in mL-scale and is a versatile modification of the methods for the automated precolumn derivatization using *ortho*-phthalaldehyde (OPA) described already [14–17].

EXPERIMENTAL

Materials and Chemicals

Lupine protein isolate (Prolupin, Prolupin GmbH, Grimmen, Germany) was a gift from Nestlé Product Technology Centre (Singen, Germany). All chemicals were of analytical grade and were obtained from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany).

Commercial Peptidase Preparations

The commercial enzyme preparations were a gift from Nestlé Product Technology Center (Singen, Germany). The commercial enzymes tested were Alcalase 2.4L (Novozymes, Bagsværd, Denmark), Bioprase SP-20FG (Nagase ChemteX, Kyoto, Japan), Collupulin 200L (DSM, Delft, Netherlands), Corolase 2TS (AB Enzymes, Darmstadt, Germany), Flavourzyme 1000L (Novozymes, Bagsværd, Denmark), Maxazyme NNP DS (DSM, Delft, Netherlands), Promod 439L (Biocatalysts Ltd, Nantgarw, Wales), Proteinase T (DuPont, Aarhus, Denmark), Protease AN "Amano" SD-K (Amano Enzyme Inc., Nagoya, Japan) and Protin SD-AY10 (Amano Enzyme Inc., Nagoya, Japan). The preparations are hereinafter referred to as Alcalase, Bioprase, Collupulin, Corolase, Flavourzyme, Maxazyme, Promod, Proteinase T, Protease AN, and Protin, respectively.

Determination of Amino Groups with o*rtho*-Phthalaldehyde

Primary amino groups were determined after derivatization with OPA, according to the method of Nielsen *et al*.[18], with some modifications [19]. A volume of 25 µL of the sample was transferred into a microtiter plate and 175 µL OPA reagent was added. For the OPA reagent, 11 mM OPA, 20 mM Dithiothreitol and 11.25 % (v/v) methanol were dissolved in 120 mM sodium tetraborate decahydrate (adjusted to pH 9.8 with NaOH). The plate was incubated at 37 °C for 1 min and the absorbance was measured at 340 nm

using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using L-serine as a reference.

Degree of Hydrolysis

The DH was calculated with Eq. 2.1 according to Adler-Nissen [20], with modifications [19].

(Eq. 2.1) $$\mathbf{DH} = \frac{h}{h_{tot}} \times \mathbf{100}\ [\%]$$

where h is the concentration of free amino groups [mol/L; see above] and h_{tot} is the maximum concentration of free amino groups at complete hydrolysis [mol/L] calculated according to Eq. 2.2.

(Eq. 2.2) $$\boldsymbol{h_{tot}} = \frac{\mathbf{c_{Protein}}}{\boldsymbol{M^{*} - M_{H_2O}}}\ \mathbf{[mol/L]}$$

where $c_{Protein}$ is the concentration of protein which is hydrolyzed (g/L) and M^* is the average molecular mass of the amino acids in lupine protein (137.9 g/mol). This lupine-specific average molecular mass was calculated by considering the lupine protein amino acid composition **[21]**. The molecular mass of water (M_{H_2O} = 18 g/mol) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

Determination of the Proteolytic Activity

The enzyme activity of the commercial enzyme preparations was tested using lupine protein as a substrate. Therefore, 150 µL of lupine protein isolate (2 g/L) and 100 µL of the corresponding buffer (150 mM) were incubated at 37 °C for 5 min. The reaction was started with the addition of 50 µL diluted enzyme solution, was stopped by the addition of trichloroacetic acid (TCA, 1.5 M, 50 µL) and was centrifuged (20,000 × g, 4 °C, 5 min) afterwards. The supernatant (25 µL) was transferred into a microtiter plate and the OPA assay was performed (see above). One katal (kat) of enzyme activity was defined as the release of 1 mol L-serine equivalent amino groups per s.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Respective samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5 %) according to the method of Laemmli [22]. The protein bands of the SDS gel were located by staining with Coomassie Brilliant Blue R-250 [23]. Every lane was analyzed by mass spectrometric investigation. The protein load was 5 µg per lane and was determined by using the method of Bradford [24] and bovine serum albumin as a reference.

Mass Spectrometry Analysis

The Life Science Center at the University of Hohenheim carried out the mass spectrometric investigations. Proteins were in-gel digested (Coomassie stained SDS gel; see above) using trypsin (Roche, Germany), according to Shevchenko et al.[25]. Nano-LC-ESI-MS/MS experiments were performed on an ACQUITY nano-UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Germany). Tryptic digests were concentrated and desalted on a precolumn (2 cm x 180 µm, Symmetry C18, 5 µm particle size, Waters, USA) and separated on a 25 cm x 75 µm BEH 130 C18 reversed phase column (1.7 µm particle size, Waters, USA). Gradient elution was performed from 1 % ACN to 40 % ACN in 0.1 % FA within 30 min. The LTQ-Orbitrap was operated under the control of XCalibur 2.1.0 software. Survey spectra (m/z = 250 – 1800) were detected in the Orbitrap at a resolution of 60,000 at m/z = 400. Data-dependent tandem mass spectra were generated for the seven most abundant peptide precursors in the linear ion trap. Internal calibration was performed for all measurements using the Orbitrap detector with lock-mass ions from ambient air, as described in Olsen et al.[26].

All MS^2 samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02). Mascot was set up to search the NCBI database No. 20150430, assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 5 ppm. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification. Scaffold (version Scaffold 4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS^2-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95 % probability by the Peptide Prophet algorithm[27], with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99 % probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [28]. Proteins that contained similar peptides and could not be differentiated based on MS^2 analysis alone were grouped to satisfy the principles of parsimony.

Lupine Protein Batch Hydrolysis

All hydrolyses were carried out similarly, as described by Merz et al.[13] in deep-well plates in 400 µL scale at 37 °C and at the respective optimum pH of the commercial

peptidases. Therefore, 100 µL buffer stock solution (200 mM) was transferred into the well of the deep-well plate. A volume of 100 µL of the enzyme solution was added and incubated on a platform shaker (1000 rpm; Titramax 100, Heidolph, Schwabach, Germany) at 37 °C for 10 min. The addition of the substrate solution (200 µL, 2 g/L lupine) started the enzymatic hydrolysis. Final concentrations in the reaction volume were 1 g/L lupine protein isolate and 50 mM of the respective buffer. Final enzyme activity was 5 nkat/mL. Samples (25 µL) were taken after various times and transferred in 100 µL TCA (0.25 M) in microtiter plates to stop the reaction. Samples were further diluted if necessary, and free amino groups and the DH were determined (see above).

HPLC-based System for Automated Small-Scale Enzymatic Hydrolysis and Analysis

HPLC Setup

Automated small-scale enzymatic hydrolyses were realized with a PLATINblue UHPLC system (Knauer, Berlin, Germany). The system was equipped with a pump (P-1), a diode array detector (PDA-1), a solvent gradient manager (M-1), a column thermostat (Jetstream 2 plus) and an autosampler (AS-1). The PLATINblue chromatography system was controlled by ChromGate Data System V.3.3.1 (Build 3.3.1.902).

A homemade aluminum tray replaced one of the two sample vial trays in the autosampler. Holes were drilled into a solid aluminum block fitting the dimensions of the original tray. Additionally, a heating pad was fixed beneath the aluminum tray (Figure 2.1, tray 2) which allowed the heating of 30 vials (1.5 mL, 12x32 mm) from 20 to 70 °C independently of the standard vial tray (Figure 2.1, tray 1, 48 vials). The heating pad was regulated using a temperature switch (TSM 125, H-Tronic, Hirschau, Germany).

Four different categories of vials were defined in the autosampler instrument configuration. The sample vials contained the reaction volume of the enzymatic hydrolysis and were located in tray 2 (Figure 2.1). The precolumn derivatization of the samples was carried out in the destination vials (Figure 2.1, tray 1). A new destination vial was used automatically for each sampling. The enzyme solution (reagent A) and the neutralization buffer (reagent B) were located in tray 1 (Figure 2.1). The injection needle of the autosampler was used for the transferring and mixing steps. Aspirating and dispensing was accomplished in the mixing using the injection needle and syringe.

Figure 2.1: Modified HPLC system for an automated small-scale enzymatic hydrolysis and simultaneous analysis of 18 amino acids. The standard HPLC system was additionally equipped with an aluminum tray (tray 2) that allowed the heating of 30 vials from 20 to 70 °C independently of the standard vial tray (tray 1). Vial colors are in accordance with Figure 2.2 (red: enzyme; brown: neutralization buffer; green: destination vial; blue: sample vial).

Automated Lupine Protein Hydrolysis with Commercial Peptidases

The enzymatic hydrolysis was automated by the PLATINblue autosampler AS-1. The release of single amino acids during hydrolysis was monitored by this means and, therefore, the overall exopeptidase activity was assessed. The enzymatic hydrolysis was carried out at the respective optimum pH of the commercial peptidases in the sample vials located in tray 2 (Figure 2.1) at 37 ± 1 °C. The schematic representation of the enzymatic hydrolysis is shown in Figure 2.2A. The substrate solution (1.44 mL) consisted of 1.11 g/L lupine protein isolate (0.45 µm filtered), 22.2 mM of the respective buffer and 111 µM ethanolamine as an internal standard (IS). A volume of 160 µL of the enzyme solution (reagent A) was transferred into the sample vial and started the enzymatic hydrolysis. After the addition of the enzyme solution, the final concentrations in the reaction volume were 1 g/L lupine, 20 mM buffer and 100 µM IS. The final enzyme activity was 5 nkat/mL. A sequence was programmed for the enzymatic hydrolysis that

contained three different method modules. These modules, which are shown in Figure 2.3, were combined to achieve certain sampling times. Module A (Figure 2.3) was used at the beginning of the enzymatic hydrolysis to transfer the enzyme solution into the sample vial (substrate). After the transfer, the reaction volume was mixed thoroughly and incubated for 15 min. Subsequently, the first sample (125 µL) was taken and analyzed. The next module was used to mix the reaction volume (5 min) and to take a sample (B, Figure 2.3). Module C (Figure 2.3) was used to mix the reaction volume and to bridge the time. The chronological order of the modules was ABCBCCBCCBCCB for each enzymatic hydrolysis. Consequently, samples (125 µL) were taken after 15, 45, 105, 195, 285 and 375 min and transferred into an unused destination vial (tray 1, Figure 2.1) to conduct the precolumn derivatization (Figure 2.2B), as described below.

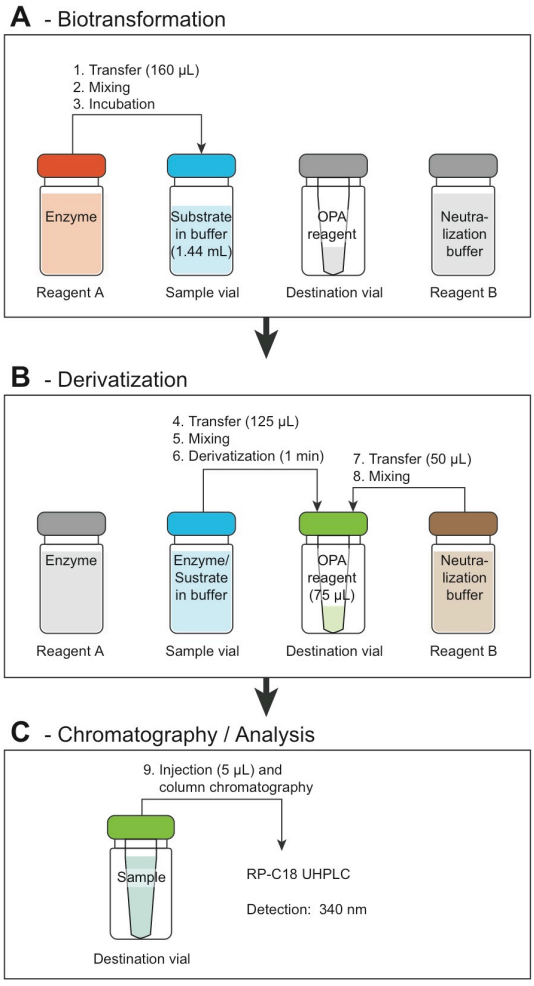


Figure 2.2: Schematic representation of the procedure for the lupine protein enzymatic hydrolysis (A; conducted in the sample vial), the subsequent precolumn derivatization (B) and the chromatographic separation (C) of the amino acid derivatives. The autosampler syringe accomplished the mixing and transferring of the respective reaction volumes.

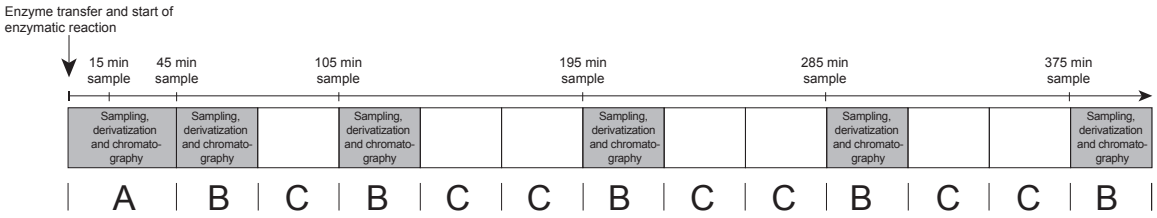


Figure 2.3: Method modules (A – C) that were used in the programmed sequence for the automated enzymatic hydrolysis of the lupine protein. Method A was used to start the enzymatic hydrolysis. Method B was used to mix the reaction volume (5 min) and to take a sample. Method C was used to mix the reaction volume and to bridge the time.

Precolumn Derivatization of Amino Acids with ortho-Phthalaldehyde

The OPA derivatization was automated by the PLATINblue autosampler AS-1. The temperature in the autosampler for the derivatization was set to 22 °C. The derivatization scheme is shown in Figure 2.2B. A sample volume of 125 µL was transferred into the destination vial (mentioned above), using a 300 µL insert that contained 75 µL of OPA reagent and mixed thoroughly. The OPA reagent consisted of 25 mM OPA, 43 mM 2-mercaptoethanol and 30 % (v/v) methanol in 85 mM sodium tetraborate buffer (pH 9.6, adjusted with NaOH). The derivatization time was 1 min, and afterwards, 50 µL of neutralization buffer (1 M sodium acetate pH 5) was added and, again, the mixture was mixed thoroughly. Subsequently, the amino acid derivatives were separated and quantified by column chromatography (Figure 2.2C; see below). A standard amino acid mixture (20 amino acids encoded by the nuclear genes) ranging from 10 to 500 µM for each amino acid, including ethanolamine as IS (100 µM), was used for the calibration. Example chromatographs of the calibration are shown in Figure S1 (Supporting information). The limit of quantification (LOQ) and limit of detection (LOD) was determined for each amino acid based on the standard deviation of response (y-intercept of the regression line) and slope of the calibration curve (LOQ = 10 σ/s; LOD = 3.3 σ/s) [29].

Chromatographic Separation

The chromatographic separation (Figure 2.2C) of the amino acid derivatives was conducted with a reversed Phase C18 column (Bluespher C18, 2.0 µm, 100 x 2 mm ID, Knauer, Germany). A sample volume of 5 µL was injected into the column. Solvent A comprised 70 mM disodium hydrogen phosphate which was adjusted with HCl to pH 6.5 and 5 % (v/v) acetonitrile was added. Solvent B consisted of H_2O_{dd}/methanol/acetonitrile

[40:30:30 (v/v/v)]. The conditions of gradient elution were: 0 – 2 min (A: 100 %; B: 0 %), 2 – 6 min (A: 90 %; B: 10 %), 6 – 20 min (A: 15 %; B: 85 %), 20 – 21 min (A: 0 %; B: 100 %), 21 – 23.5 min (A: 0 %; B: 100 %), 23.5 – 24 min (A: 95 %; B: 5 %) and 24 – 25 min (A: 100 %; B: 0 %). The flow rate increased from 0.1 to 0.5 mL/min from 0 – 1 min. Afterwards, derivatives were separated with a constant flow rate of 0.5 mL/min and detected by absorbance at 340 nm. The column temperature was set at 40 °C.

Statistical Analysis

The batch hydrolyses were conducted at least in duplicate, with two independent measurements of the free amino groups. The automated lupine protein enzymatic hydrolyses were conducted in duplicate. The standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA).

RESULTS AND DISCUSSION

The detailed composition of commercial peptidases is usually unknown. In order to gain new insights, we characterized ten commercial peptidases in respect of the hydrolysis kinetics and the overall exopeptidase activity using lupine protein as a substrate. These preparations may exhibit peptidase activities different from those stated by the supplier as a consequence of the applied purification technology. Recently, for example, multiple relevant Flavourzyme peptidases were identified and characterized **[12, 13]**.

Firstly, relevant peptidases were identified by mass spectrometry. Secondly, batch hydrolyses were carried out to check the kinetics of the release of free amino groups (tested with OPA). Thirdly, the release of 18 amino acids (L-stereoisomers) was monitored during lupine hydrolyses using a novel HPLC-based system for automated enzymatic hydrolysis and analysis. Since the retention time for valine and methionine were almost the same, the sum of both amino acids is given hereafter. Proline and cysteine could not be quantified because the absorbance of these two derivatives is very low **[30]**.

Identification of Peptidases from Commercial Peptidase Preparations by Mass Spectrometry

All peptidases in the different commercial preparations which were identified by mass spectrometry are listed in Table 2.1.

Table 2.1: Peptidases in commercial peptidases found by mass spectrometric investigations. The proteins are sorted in descending order according to the proportion of the peptidase in the preparation (percentage of the total spectra). Peptidases with the highest proportion are listed on top within the preparation.

Preparation	Peptidase	UniProtKB ID	Peptidase type	EC number
Alcalase 2.4L	Subtilisin	P00780	Endo	3.4.21.62
	Glutamyl endopeptidase	Q5K2M2	Endo	3.4.21.19
	Extracellular neutral metalloprotease	E1UT71	Endo	3.4.24.-
	Aminopeptidase	Q65DH7	Exo	na
Bioprase SP-20FG	Subtilisin	P00780	Endo	3.4.21.62
	Extracellular metalloprotease	R9TP85	Endo	3.4.21.19
	Extracellular neutral metalloprotease	E1UT71	Endo	3.4.24.-
	Aminopeptidase	Q65DH7	Exo	na
	Bacillopeptidase F	R9TU45	Endo	3.4.21.-
Corolase 2TS	Thermolysin	P00800	Endo	3.4.24.27
	Extracellular neutral metalloprotease	E1UT71	Endo	3.4.24.-
Collupulin 200L	Chymopapain	P14080	Endo	3.4.22.6
	Caricain	P10056	Endo	3.4.22.30
	Papaya proteinase 4	P05994	Endo	3.4.22.25
Flavourzyme 1000L	Leucine aminopeptidase A	Q2U1F3	Exo	3.4.11.-
	Leucine aminopeptidase 2	Q2ULM2	Exo	3.4.11.-
	Dipeptidyl-peptidase 5	Q9Y8E3	Exo	3.4.14.-
	Neutral protease 1	Q2U1G7	Endo	3.4.24.-
	Neutral protease 2	P46076	Endo	3.4.24.39
	Alkaline Protease 1	P12547	Endo	3.4.21.63
	Carboxypeptidase	Q2TWJ3	Exo	3.4.16.-
	Carboxypeptidase S1	Q96VZ9	Exo	3.4.16.6
	Aspartic protease	Q06902	Endo	3.4.23.18
	Dipeptidyl-peptidase 4	Q2UH35	Exo	3.4.14.5
Maxazyme NNP DS	Extracellular neutral metalloprotease	E1UT71	Endo	3.4.24.-
	Aminopeptidase	A7Z7S9	Exo	na
Promod 439L-P439L	Subtilisin	P00780	Endo	3.4.21.62
Protease AN	Dipeptidyl-peptidase 5	Q9Y8E3	Exo	3.4.14.-
	Alkaline Protease 1	P12547	Endo	3.4.21.63
	Neutral protease 1	Q2U1G7	Endo	3.4.24.-
	Leucine aminopeptidase A	Q2U1F3	Exo	3.4.11.-
	Aspartic protease	Q06902	Endo	3.4.23.18
	Neutral protease 2	P46076	Endo	3.4.24.39
	Leucine aminopeptidase 2	Q2ULM2	Exo	3.4.11.-
	Carboxypeptidase S1	Q96VZ9	Exo	3.4.16.6
	Dipeptidyl-peptidase 4	Q2UH35	Exo	3.4.14.5
	Carboxypeptidase	Q2TWJ3	Exo	3.4.16.-
Proteinase T	Thermolysin	P00800	Endo	3.4.24.27
Protin SD-AY10	Subtilisin	P00780	Endo	3.4.21.62

na, not applicable (no clear assignment possible)

In addition to proteolytic enzymes, other proteins which were found in the preparations were neglected and are not listed in Table 2.1. The proteins are sorted in descending order according to the proportion of the peptidase in the preparation (percentage of the total spectra). According to the mass spectrometric data obtained, the preparations are derived from *Aspergillus oryzae* (Flavourzyme, Protease AN), *Bacillus amyloliquefaciens* (Maxazyme), *Bacillus licheniformis* (Alcalase, Bioprase, Promod, Protin), *Bacillus thermoproteolyticus* (Corolase, Proteinase T) and *Carica papaya* (Collupulin). All preparations consisted of at least one endopeptidase. Subtilisin was found in Alcalase, Bioprase, Promod and Protin. Thermolysin was found in Corolase and Proteinase T. Four endopeptidases (alkaline protease 1, neutral protease 1, neutral protease 2, aspartic protease) were found in Flavourzyme and Protease AN. Three endopeptidases (chymopapain, caricain, papaya proteinase 4) were detected in Collupulin. An extracellular metalloprotease was found in Maxazyme. The mentioned findings are in accordance with the supplier specifications (if stated).

Exopeptidases were found in five of the preparations investigated. An aminopeptidase was ascertained in Alcalase, Bioprase and Maxazyme. Several exopeptidases (leucine aminopeptidase A, leucine aminopeptidase 2, carboxypeptidase, carboxypeptidase S1, dipeptidyl-peptidase 4 and dipeptidyl-peptidase 5) were found in Flavourzyme and Protease AN. This is in accordance with previous mass spectrometric analyses of *A. oryzae* peptidases **[12, 31–33]**.

Lupine Protein Batch Hydrolyses

Batch hydrolyses were carried out in the presence of 1 g/L lupine protein isolate at the respective optimum pH at 37 °C. The optimum pH for the lupine hydrolysis was determined previously (data not shown) and is listed in Table 2.2. The respective buffer system for the hydrolysis was citrate phosphate (pH 5 – 7) and Bis-tris-propane (pH 7.5 – 8). Optimum pH ranged from pH 6 to 8 (Table 2.2). The subtilisin preparations exhibited optimum pH values of pH 7 (Alcalase, Bioprase, Promod) and pH 6 (Protin). Hydrolyses of lupine protein using Alcalase were conducted at pH 8 in previous studies **[34, 35]**. An optimum pH range from pH 5.5 to 7.5 was described for commercial subtilisin and bacillolysin preparations **[36]**. The thermolysin preparations exhibited the highest activity at pH 7.5 (Corolase) and pH 8 (Proteinase T). The optimum was pH 7.5 for both preparations derived from *A. oryzae* and is in accordance with a previous study on a wheat gluten hydrolysis **[19]**. The optimum pH for Collupulin was determined at pH 6,

however, the enzyme showed insufficient stability in a buffer system above pH 5 (data not shown). The low pH-dependent stability was not observed in the literature for the three individual papaya-derived peptidases (listed in Table 2.1). However, the combination of the peptidases may lead to an increased autoproteolysis, especially in buffer systems without a substrate. The subsequent enzymatic hydrolyses with Collupulin were carried out at pH 5 to assure sufficient enzyme stability.

A standardized enzyme activity of 5 nkat/mL was applied for all batch hydrolyses. The enzyme activity of the ten commercial enzymes preparations on lupine protein are also listed in Table 2.2 and are expressed either as nkat/mL for liquid or as nkat/g for solid preparations.

Table 2.2: Commercial peptidase preparations investigated and conditions for the lupine protein hydrolysis. The enzyme activity was determined with 1 g/L lupine protein isolate at the respective optimum pH/buffer system at 37 °C.

Commercial peptidase	Protein	Optimum pH[+] [-]	Enzyme activity	Rel. activity [%]
Alcalase 2.4L	38 [a]	7	5106 ± 196 [c]	62
Bioprase SP-20FG	42 [b]	7	6940 ± 319 [d]	84
Corolase 2TS	20 [a]	7.5	549 ± 61 [c]	7
Collupulin 200L	27 [a]	6*	492 ± 46 [c]	6
Flavourzyme 1000L	71 [a]	7.5	2897 ± 211 [c]	35
Maxazyme NNP DS	11 [a]	7	343 ± 20 [c]	4
Promod 439L-P439L	25 [a]	7	3173 ± 114 [c]	39
Proteinase AN	132 [b]	7.5	8224 ± 236 [d]	100
Proteinase T	8 [a]	8	430 ± 21 [c]	5
Protin SD-AY10	46 [b]	6	4607 ± 94 [d]	56

[+] Buffer system, Citrate phosphate (pH 5 - 7) and Bis-tris-propane (pH 7.5 - 8)

[a] g/L (Liquid preparation), [b] g/kg (Solid preparation)

[c] nkat/mL (Liquid preparation), [d] nkat/g (Solid preparation)

* Hydrolyses were carried out at pH 5, due to insufficient pH stability at pH 6

The progression of the DH during lupine protein hydrolysis for each preparation is shown in Figure 2.4. Preparations which only contained endopeptidases (see above) are shown in Figure 2.4A, whereas preparations with at least one identified exopeptidase are shown in Figure 2.4B. The kinetics of the endopeptidase preparations (without exopeptidases) was

comparable and the DH ranged from 15.8 ± 0.1 (Proteinase T) to 23.2 ± 0.3 % (Promod) after 450 min of hydrolysis (Figure 2.4A).

For the two preparations with one exopeptidase identified, a DH of 23.2 ± 0.2 % (Bioprase) and 24.2 ± 0.7 % (Alcalase) was measured after 450 min (Figure 2.4B). Maxazyme, Flavourzyme and Protease AN contained multiple exopeptidases (see above) and, therefore, their hydrolysates exhibited the highest DH (34.5 ± 1.7, 56.8 ± 0.1 and 62.2 ± 0.9 %, respectively).

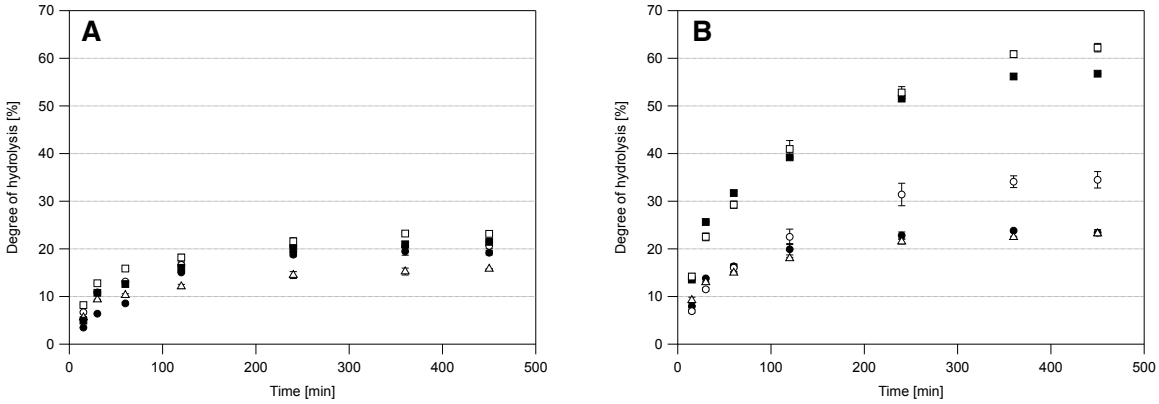


Figure 2.4: Batch hydrolyses of lupine protein isolate (1 g/L) with commercial peptidases. An enzyme activity of 5 nkat/mL was applied at 37 °C at the respective optimum pH; A: Endopeptidase preparations without exopeptidases (Open triangle: Proteinase T; filled circle: Collupulin; open circle: Corolase; filled square: Protin; open square: Promod); B: Peptidase preparations with at least one identified exopeptidase (Open triangle: Bioprase; filled circle: Alcalase; open circle: Maxazyme; filled square: Flavourzyme; open square: Protease AN).

A DH of 26 % was obtained for a lupine protein hydrolysate using Alcalase in a comparable study **[37]**. Similar results were obtained by various studies with DH ranging from 16 – 25 % depending on the substrate and method for the determination of the DH **[38–42]**. Furthermore, an average peptide length of 4.3 amino acid residues was determined by mass spectrometry in a whey protein hydrolysate obtained with Alcalase **[43]**, which corresponds to a DH of 23.3 %. Since Alcalase, Bioprase, Promod and Protin are subtilisin preparations, a comparable DH for the preparations seems reasonable. Apparently, the two aminopeptidases identified in Alcalase and Bioprase did not have an impact on the lupine protein hydrolysis. The two hydrolysates of the thermolysin preparations exhibited a DH of 15.8 ± 0.1 % (Proteinase T) and 20.6 ± 0.6 % (Corolase).

Ovotransferrin was hydrolyzed by thermolysin with a resulting DH ranging from approximately 18 to 22 % in a previous study **[44]**.

The high DH of the Flavourzyme and Protease AN hydrolysates can be explained by the synergistic effects of the endo- and exopeptidases, which was shown for the Flavourzyme peptidases previously **[13]**.

Automated Lupine Protein Hydrolysis with Commercial Peptidases

After the batch hydrolysis, the enzymatic hydrolysis of lupine protein was also performed in the autosampler of the PLATINblue HPLC system. Here, the amino acid release was monitored over 375 min in order to determine the overall exopeptidase activity of the respective commercial peptidase preparation. This knowledge is essential for the characterization of hydrolysates, since a defined peptide length and composition is important for certain functional properties.

The automated enzymatic hydrolysis and subsequent precolumn derivatization in mL scale is helpful to minimize the expenditure of manpower, reduce the consumption of chemicals and reduce the accumulated error of the different methods. The automated precolumn derivatization of amino acids with OPA is a well described and established method for the analysis of amino acids and peptides [14–17]. However, a system that combines and automates the enzymatic hydrolysis of proteins with the precolumn derivatization, including the chromatographic analysis, has not yet been described.

Regarding the amino acid monitoring, the LOQ ranged from 0.87 to 23.5 µM in the enzymatic hydrolysis vial depending on the amino acid derivative (LOD: 0.29 – 7.83 µM). Lupine protein hydrolyses were carried out under the same conditions compared to the batch hydrolyses (see above) in the sample vials of the autosampler of the HPLC system described (see above).

Example chromatographs of the automated Flavourzyme enzymatic hydrolysis after precolumn derivatization with OPA are shown in Figure 2.5. The figure includes the graphs of the substrate solution (1 g/L lupine protein in the respective buffer) and the Flavourzyme solution (in H_2O_{dd}; the same concentration as in the enzymatic hydrolysis) for referencing, as well as the 15 and 375 min enzymatic hydrolysis samples. The released amino acids were corrected for the free amino acids added initially through the enzyme preparation. The amino acid concentrations of the preparations that contained free amino acids are listed in Table 2.3. The concentrations are expressed as ppm (mg/L for liquid preparations; mg/kg for solid preparations). A total amino acid concentration of

0.5, 5.4, 32.2, 1.3 and 50.1 ppm was determined for Alcalase, Collupulin, Flavourzyme, Maxazyme and Protease AN, respectively. Amino acids were not detected in the other commercial peptidase preparations. Analogue chromatographs for the other preparations are displayed in Figure S2 – S11 (Supporting information).

The release of the amino acids during the lupine protein enzymatic hydrolysis is shown in Figure 2.6. A release of single amino acids during the hydrolysis was observed for Alcalase, Flavourzyme, Maxazyme and Protease AN. No release of single amino acids was determined for the other six peptidase preparations, even though an exopeptidase was found in Bioprase. However, the relative exopeptidase activity in Bioprase was negligible low as described previously [45].

Table 2.3: Amino acid composition of the original commercial peptidase preparations. Concentrations are stated in ppm, which corresponds to mg/L for liquid and mg/kg for solid peptidase preparations.

Amino acid	Amino acid concentration in the peptidase preparation [ppm]				
	Alcalase 2.4L [a]	Collupulin 200L [a]	Flavourzyme 1000L [a]	Maxazyme NNP DS [a]	Protease AN [b]
Aspartic acid	nd	947	2746	38	3007
Glutamic acid	508	691	2124	29	644
Asparagine	nd	299	2585	26	2746
Serine	nd	9	3322	66	53
Histidine	nd	822	668	nd	741
Glutamine	nd	5	1432	nd	3638
Arginine	nd	12	911	33	1651
Glycine	nd	1383	2894	50	3543
Threonine	nd	265	2392	68	4636
Alanine	nd	nd	3306	204	4586
Tyrosine	nd	nd	956	71	3832
Valine/Methionine	nd	86	3101	66	4275
Tryptophan	nd	nd	46	312	176
Phenylalanine	nd	149	1261	43	2826
Isoleucine	nd	103	2135	83	3065
Leucine	nd	287	2824	174	5748
Lysine	nd	309	2510	78	4894

[a] mg/L; [b] mg/kg; nd, not detectable

A total amino acid concentration of 0.42 mM was determined after 375 min of hydrolysis with Alcalase (Fig 6A). Thus, 5.6 % of the amino acids were released as single amino acids from the substrate. Mainly glutamic acid, serine, histidine, glycine and leucine were released (Figure 2.6A). To the best of our knowledge, an exopeptidase activity has not yet been described for Alcalase.

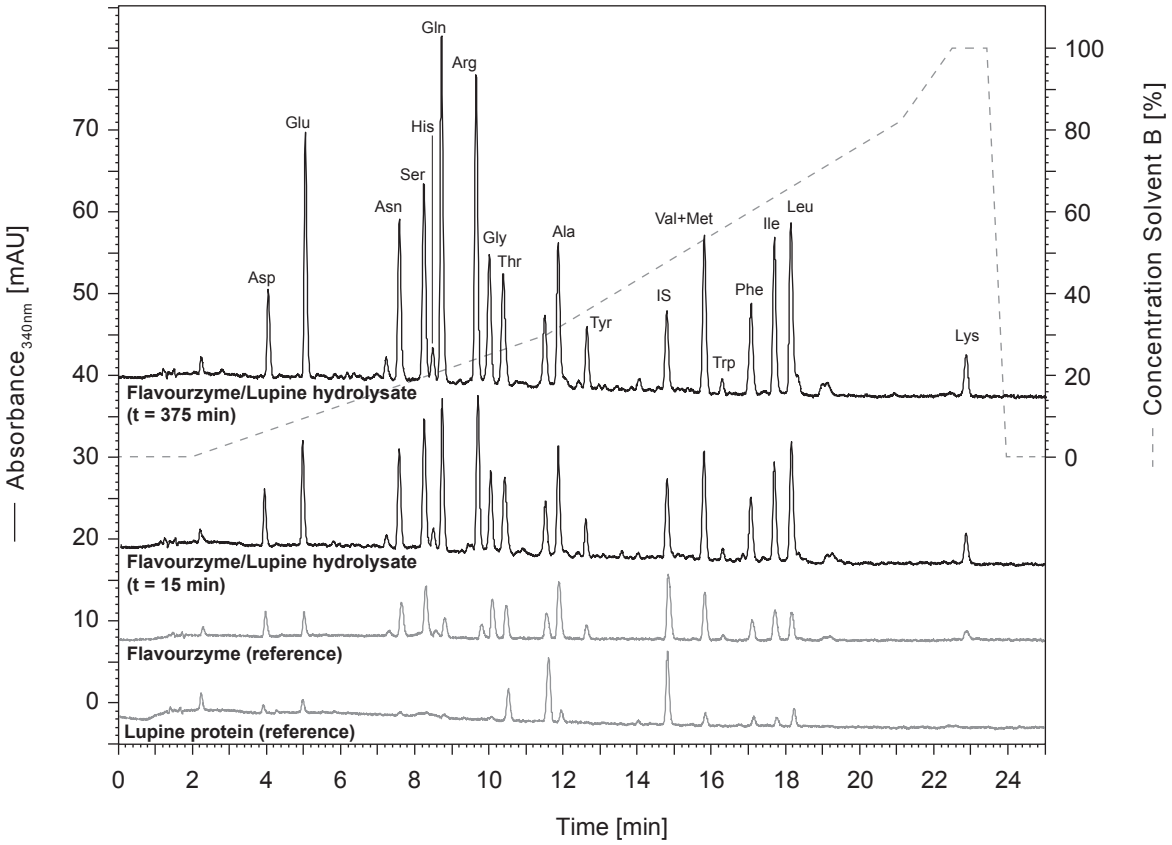


Figure 2.5: Exemplary chromatographs of the simultaneous monitoring of 18 amino acids (three-letter code) including an internal standard (IS; 100 µM ethanolamine) after precolumn derivatization with OPA. Four samples are shown from an automated enzymatic hydrolysis using the HPLC system described. Lupine protein (reference) and Flavourzyme (reference) as well as samples after 15 and 375 min of lupine protein (1 g/L) hydrolysis with Flavourzyme (5 nkat/mL) are displayed.

By comparison, a total amino acid concentration of 2.1 mM was determined for the Flavourzyme hydrolysate, which means that 28 % of the amino acids were released (Figure 2.6B). Most amino acids were released equally (between 20 and 35 % corresponding to their share in the lupine protein) during the hydrolysis with Flavourzyme. The release of serine, glutamine, glycine and tyrosine was noticeably over

average (28 %) with 58, 40, 45 and 41 %, respectively. Due to the presence of various endopeptidases and unspecific exopeptidases [46], a fairly equal distribution of all amino acids seems convincing.

The total amino acid concentration was 0.77 mM for the Maxazyme hydrolysate (375 min), resulting in an amino acid release of 10.3 % (Figure 2.6C). As seen from Figure 2.6C, mainly hydrophobic amino acids (alanine, tyrosine, valine/methionine, phenylalanine, isoleucine and leucine) were released. The release of the latter amino acids was 21, 31, 29, 16, 37 and 27%, respectively, which was distinctly over average (10.3 %). In total, 1.83 mM amino acids were determined after 375 min for the Protease AN hydrolysate. This corresponds to an amino acid release of 24.4 %. Since the same set of enzymes was determined by mass spectrometric analysis for Protease AN and Flavourzyme, the kinetics of the amino acid release was comparable.

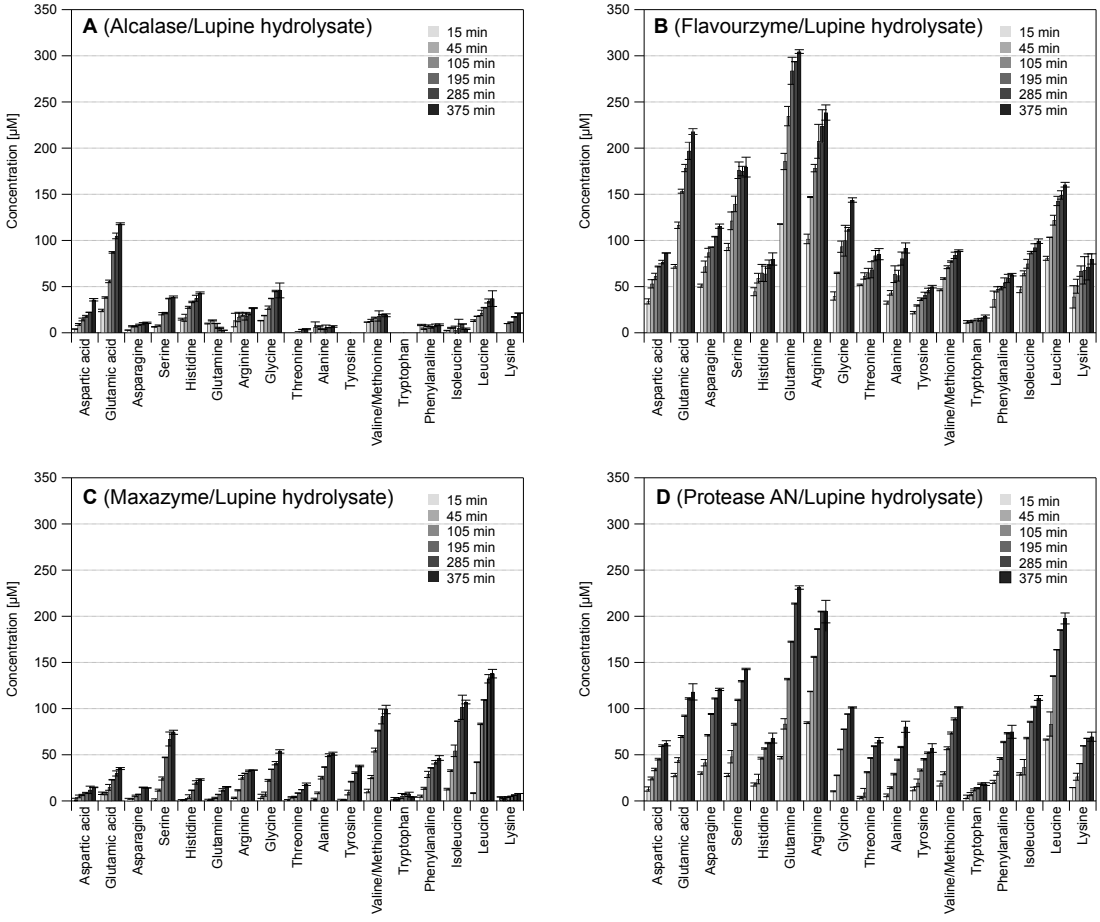


Figure 2.6: Amino acid release during automated lupine protein enzymatic hydrolysis with four different peptidase preparations. Sampling after 15, 45, 105, 195, 285 and 375 min of enzymatic hydrolysis. Peptidase preparations that are not shown here exhibited no exopeptidase activity.

In summary, the hydrolysis kinetics of lupine protein was evaluated for ten commercial peptidase preparations. A novel HPLC system for automated small-scale (mL) enzymatic hydrolysis and analysis was developed to monitor the release of 18 amino acids simultaneously during a lupine protein hydrolysis. The described system has proven to be a versatile tool for the monitoring of proteolysis reactions. Furthermore, the system was also applied for the characterization (e.g enzyme kinetics) of peptidases (data not shown). Exopeptidases were found by mass spectroscopy in three endopeptidase preparations (Alcalase, Bioprase, Maxazyme) and exopeptidase activity was confirmed in Alcalase and Maxazyme using the HPLC system described. Due to the different dependencies of the functional properties, the production of hydrolysates with both free amino acids and long-chain peptides is not desirable. Using the methodology described, we identified suitable commercial peptidases for the production of hydrolysates with high DH and high amounts of free amino acids (Flavourzyme, Protease AN) and suitable endopeptidase preparations for the production of hydrolysates with low DH and minimal release of amino acids (Bioprase, Collupulin, Corolase, Proteinase T, Promod, Protin). We suggest that this approach can be used to evaluate and select appropriate commercial peptidases to produce protein hydrolysates with desired compositions.

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ABBREVIATIONS USED

ACE, angiotensin-I converting enzyme; DH, degree of hydrolysis; h, concentration of free amino groups; h_{tot}, maximum concentration of free amino groups at complete hydrolysis; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; M^*, average amino acid molecular mass of lupine protein; OPA, *ortho*-phthalaldehyde; SDS-Page, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

SUPPORTING INFORMATION AVAILABLE

Figure S1 – S11. This material is available free of charge via the Internet at http://pubs.acs.org.

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CHAPTER THREE

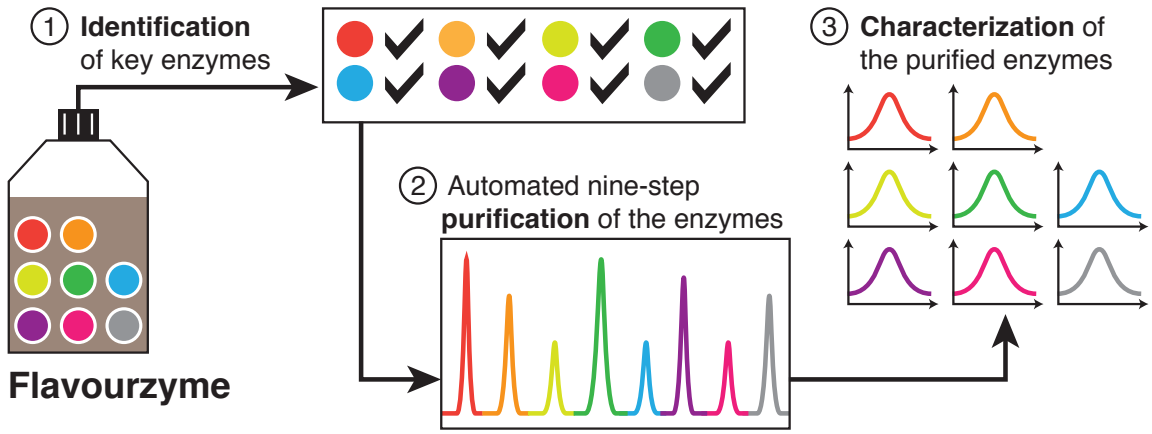
FLAVOURZYME, AN ENZYME PREPARATION WITH INDUSTRIAL RELEVANCE – AUTOMATED NINE-STEP PURIFICATION AND PARTIAL CHARACTERIZATION OF EIGHT ENZYMES

This chapter was published

Merz, M., Eisele, T., Claaßen, W., Appel, D., Rabe, S., Stressler, T., Fischer, L. (2015). Flavourzyme, an Enzyme Preparation with Industrial Relevance – Automated Nine-Step Purification and Partial Characterization of Eight Enzymes. *Journal of Agricultural and Food Chemistry*, 63(23), 5682–5693.

Time of investigation: 07/2012 - 12/2014

Graphical Abstract



ABSTRACT

Flavourzyme is sold as a peptidase preparation from *Aspergillus oryzae.* The enzyme preparation is widely and diversely used for protein hydrolysis in industrial and research applications. However, detailed information about the composition of this mixture is still missing due to the complexity. The present study identified eight key enzymes by mass spectrometry and partially by activity staining on native polyacrylamide gels or gel zymography. The eight enzymes identified were two aminopeptidases, two dipeptidyl peptidases, three endopeptidases and one α-amylase from the *A. oryzae* strain ATCC 42149/RIB 40 (yellow koji mold). Various specific marker substrates for these Flavourzyme enzymes were ascertained. An automated, time-saving nine-step protocol for the purification of all eight enzymes within 7h was designed. Finally, the purified Flavourzyme enzymes were biochemically characterized with regard to pH and temperature profiles and molecular sizes.

KEYWORDS

Flavourzyme, key enzymes, characterization, *Aspergillus oryzae* peptidases, *Aspergillus oryzae* amylase, automated purification, protein hydrolysis

INTRODUCTION

Protein hydrolysates are versatile and play a major role in a wide range of applications in various industries. Applications of protein hydrolysis processes can be found in, among others, the detergent [1], leather [2] and food industries. In the latter, protein hydrolysates are used, for example, to improve specific functionalities [3], to provide nutritional support to patients with different needs [4] or in hypoallergenic infant foods [5]. Protein hydrolysates can also be utilized to increase the emulsification or frothing capabilities in food matrices [3]. In contrast to chemical hydrolyses, the enzymatic hydrolysis processes usually avoid side reactions and do not decrease the nutritional value of the protein source [5]. A defined degree of hydrolysis is desirable for certain applications [3] and, therefore, the hydrolysis process must be carefully controlled. The comprehensive characterization of the enzymes used is, therefore, essential.

Commercial peptidases are generally derived from lactic acid bacteria [6], *Aspergillus* sp.[6], *Bacillus* sp. or from plants. Flavourzyme, for example, is sold as an industrial peptidase preparation, derived from *Aspergillus. oryzae* and used for protein hydrolysis in various industrial and research applications. As the name “Flavourzyme” suggests, the production of flavor-active compounds from various protein sources is a major application of this preparation. Investigations about Flavourzyme and the hydrolysis of vegetable proteins, such as wheat gluten or soy proteins, are described [7–10]. Animal protein sources were also used for the production of flavoring hydrolysates [11]. Furthermore, Flavourzyme was used to produce angiotensin converting enzyme inhibitory peptides from corn germ [12], lupin proteins [13] or bovine plasma [14]. Another application for Flavourzyme is the production of antioxidative or radical scavenging peptides from rapeseed [15] or tilapia proteins [16]. Protein hydrolysates from brewers’ spent grain with Flavouryzme also showed improved emulsifying and foam-forming properties [17].

Some of the extracellular proteins from various *Aspergillus* strains were identified in previous studies [18–20]. Some enzyme activities were also determined with the API-ZYM test system [6]. However, a comprehensive characterization of the proteins in the Flavourzyme preparation is not published yet. Obtaining highly purified proteins is essential when functional and structural characterization studies are targeted [21]. A possibility to gain these proteins is a fully automated purification protocol. It will allow

the purification of proteins with minimal operator intervention [21]. The development and application of such automated purification protocols has already been described [22, 23]. In the present study, we identified key enzymes in the preparation of Flavourzyme 1000L. These enzymes were purified using an automated purification protocol and partially characterized afterward.

EXPERIMENTAL

Materials and Chemicals

Flavourzyme 1000L (Novozymes, Bagsværd, Denmark) was a gift from Nestlé Product Technology Centre (Singen, Germany). All *p*-nitroanilide (*p*NA) substrates were obtained from Bachem (Bubendorf, Switzerland). Azocasein was bought from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals were of analytical grade and were obtained from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), or Sigma-Aldrich (Taufkirchen, Germany). Protein purification columns and resins were purchased from GE (Munich, Germany) or were a friendly gift from Knauer Wissenschaftliche Geräte GmbH (Berlin, Germany).

Endopeptidase Activity Assay

The azocasein assay was performed according to the method of Iversen and Jørgensen [24], with some modifications. The substrate azocasein (5 g/L) was dissolved in double-distilled water (H_2O_{dd}), and the assay was performed as follows: The substrate solution (125 µL) was preincubated with 125 µL of the respective buffer solution at the respective temperature for 5 min. The reaction was started with the addition of enzyme solution (50 µL), stopped by the addition of trichloroacetic acid (1.5 M, 50 µL) and subsequently centrifuged (20000 *g*, 4 °C, 5 min). A volume of 195 µL of the supernatant was transferred into a microtiter plate, 65 µL of NaOH (1 M) was added, and the absorbance at 450 nm was measured using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). One azocasein unit (ACU) was defined as the increase of 1 absorbance unit per minute at 450 nm in 0.25 M NaOH.

Aminopeptidase and Dipeptidyl Peptidase Activity Assay

Aminopeptidase and dipeptidyl peptidase activities were measured according to the method of Chrispeels and Boulter [25], with some modifications [26]. The assay contained 177 µL of the respective buffer solution and 50 µL enzyme solution. After 5

min of preincubation at the respective temperature the reaction was started by the addition of 12.5 µL of the corresponding substrate stock solution [*p*NA derivative dissolved in dimethylformamide (DMF)]. The substrate concentration in the final reaction volume was 3.7 mM except for Lys-Ala-*p*NA (1 mM). The reaction was stopped by the addition of 50 % (v/v) acetic acid (50 µL) and was centrifuged afterward (20000 *g*, 4 °C, 5 min). The supernatant (240 µL) was transferred into a microtiter plate and the absorbance at 405 nm was measured using a microtiter plate reader (Multiskan FC). One katal (kat) of aminopeptidase or dipeptidyl peptidase activity was defined as the release of 1 mol *p*NA per second.

Amylase Activity Assay

The amylase activity was measured according to the Somogyi-Nelson method [27, 28], with some modifications. The following two reagents were prepared: Reagent A consisted of $Na_2HPO_4 \cdot 2H_2O$ (35.2 g/L), $KNaC_4H_4O_6 \cdot 4H_2O$ (40 g/L), $CuSO_4 \cdot 5H_2O$ (8 g/L) and NaOH (4 g/L) solved in H_2O_{dd}. Reagent B consisted of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50 g/L), H_2SO_4 (42 g/L) and $Na_2HAsO_4 \cdot 7H_2O$ (6 g/L) dissolved in H_2O_{dd}.

The substrate starch (10 g/L) was dissolved at 70 °C for 60 min in H_2O_{dd} and the assay was performed as follows. The substrate solution (125 µL) was preincubated with 75 µL of citrate phosphate buffer at the respective temperature for 5 min. The reaction was started with the addition of enzyme solution (50 µL). After the reaction, a volume of 125 µL was transferred into 125 µL citrate phosphate buffer (100 mM, pH 7.5). Afterward, reagent A (100 µL) was added and the reaction mixture was subsequently incubated at 98 °C for 10 min (water bath). Finally, a volume of 100 µL of reagent B and 800 µL of H_2O_{dd} were added. The mixture was incubated at 20 °C for 30 min and then centrifuged (13000 *g*, 20 °C, 1 min). The supernatant (200 µL) was transferred into a microtiter plate and the absorbance at 450 nm was measured using a microtiter plate reader (Multiskan FC). The calibration was performed using glucose as a reference. One katal (kat) of amylase activity was defined as the release of 1 mol of glucose-equivalents per second.

Mass Spectrometric Investigations

The respective bands from the Coomassie stained SDS gel (see below) were eluted from the gel and hydrolyzed tryptically by the Life Science Center (University of Hohenheim) using a modified method according to Shevchenko et al.[29]. The protein spots were excised from the gel and incubated in 200 µL of H_2O_{dd} for 5 min at 1000 rpm. Afterward, the supernatant was replaced by 150 µL of acetonitrile and shaken at 1000 rpm. After 10

min, the supernatant was discarded and 100 µL of 100 mM NH_4HCO_3 containing 10 mM DTT was added and incubated for 30 min at 56 °C at 1000 rpm. The supernatant was subsequently replaced by 150 µL acetonitrile and 85 µL of 100 mM NH_4HCO_3 containing 55 mM iodoacetamide. After incubation for 20 min in the dark, the gel piece was washed in 150 µL of 100 mM NH_4HCO_3 for 10 min at 1000 rpm and dehydrated by the addition of 150 µL acetonitrile for 10 min at 1000 rpm. Afterward, the supernatant was discarded and 25 µL trypsin solution (10 ng/µL in 40 mM NH_4HCO_3) was added. After 30 min on ice, a further 40 mM NH_4HCO_3 was added if needed. The digestion was carried out overnight at 37 °C. The sample was centrifuged after the addition of 2 µL of 50 % (v/v) formic acid and the supernatant was used for the mass spectrometric analysis (MALDI-TOF-MS). The sample was analyzed by liquid chromatography electrospray ionization-mass spectrometry (LC-ESI-MS^2) using a nano-HPLC system (Aquity Waters, Milford, MA, USA) connected to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A gradient from 1 to 50 % (v/v) acetonitrile in 0.1 % (v/v) formic acid over 30 min was used for separation (Life Science Center, University of Hohenheim). The data were treated by XCalibur software version 2.0.7, and m/z spectral data were transformed to spectra representing mass values. All MS^2 samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02). Mascot was set up to search the NCBI database no. 20131117 (selected for Other Fungi, unknown version, 2111786 entries), assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 15 ppm. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification. Scaffold (version Scaffold 4.2.1, Proteome Software Inc., Portland, OR, USA) was used to validate MS^2-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95 % probability by the Peptide Prophet algorithm [30], with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at >99 % probability and contained at least five identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [31]. Proteins that contained similar peptides and could not be differentiated on the basis of MS^2 analysis alone were grouped to satisfy the principles of parsimony.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Native PAGE

Respective samples were analyzed by SDS-PAGE (12.5 %) according to the method of Laemmli [32]. The molecular weight protein standard mixture was obtained from New England Biolabs (NEB, broad range, 2-212 kDa; Frankfurt, Germany) and was used as a reference. The protein bands of the SDS gel were located by staining with Coomassie Brilliant Blue R-250 [33]. The protein load was 5 µg per lane for the Flavourzyme mixture and 1 µg per lane for the purified enzyme samples. The protein content was determined by using the method of Bradford [34] and bovine serum albumin as a standard.

A native molecular weight protein standard mixture (SERVA native marker, liquid mix; SERVA, Heidelberg, Germany) was used as the reference for the native PAGE (8 %). The native gel was activity stained (see below) to visualize aminopeptidase and dipeptidyl peptidase activity and to determine specific marker substrates for the automated purification protocol. Samples were diluted depending on the activity toward their particular substrate for further activity staining. An enzyme activity of approximately 50 pkat was applied per lane.

Aminopeptidase and Dipeptidyl Peptidase Activity Staining of Polyacrylamide Gels

After electrophoresis, the native PAGE gels were washed with Tris-HCl buffer (50 mM, pH 7.5). An amount of 5 mg of the corresponding *p*NA derivative was solved in 25 µL of DMF and filled to 5 mL with Tris-HCl buffer (50 mM, pH 7.5) for the activity staining. In total, 13 X-*p*NA derivatives were tested (X = Ala, Ala-Pro, Arg, Glu, Gly, His, Ile, Leu, Lys, Lys-Ala, Phe, Pro and Val). The gels were incubated in the buffer containing the respective *p*NA substrate until a yellow band appeared and were then washed with H_2O_{dd}. The bands were redyed, according to the method of Božić and Vujčić [35], for improved visibility. Therefore, the gels were incubated in 1 g/L $NaNO_2$ (dissolved in 1 M HCl) for 120 s and washed in urea buffer (10 g/L) for 30 s. The gels were incubated in 0.25 g/L *N*-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in 22 % (v/v) EtOH for 10 min for staining.

Zymography

Native gels were supplemented with casein to a final concentration of 0.1 % (w/v). An

enzyme activity of 5×10-4 ACU was applied per lane. The separation of the proteins was performed in an ice bath to prevent a hydrolysis of the casein during the enzyme migration. After electrophoresis, the gel was incubated in 0.1 % (w/v) Triton X-100 at 7 °C for 1 h and subsequently incubated in Tris-HCl buffer (50 mM, pH 7.5) at 20 °C for 16 h. Afterward, the gel was Coomassie stained [33] to visualize endopeptidase activity.

Table 3.1: Buffer Compositions Used during the Automated Nine-Step Purification Protocol.

buffer	composition	pH
A	10 mM sodium acetate, 0.1 mM $ZnCl_2$, 0.1 mM $CaCl_2$	4.85
B	10 mM sodium acetate, 0.1 mM $ZnCl_2$, 0.1 mM $CaCl_2$, 100 mM NaCl	4.9
C	1 M NaCl	-
D	20 mM Tris-HCl	7.5
E	20 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$	7.5
F	35 mM sodium acetate, 0.1 mM $ZnCl_2$, 1 mM $CaCl_2$	4.5

Automated Nine-Step Purification of Flavourzyme Peptidases

Flavourzyme enzymes were purified using a Bioline chromatography system (Knauer, Berlin, Germany). The system was equipped with a pump (S 1000), a UV detector (S 2520), a conductivity meter (S 2900), a manual injection six-port/three-channel injection valve, two seven-port/one-channel switching valves, and two six-port/three-channel injection valves. The eluting purified enzymes were detected at 280 nm and were collected using a fraction collector (Frac 3050). The fraction collector was cooled at 10 °C. The Bioline chromatography system was controlled by ChromGate Data System V.3.3.1. The fast protein liquid chromatography system and valve position setup were the same as previously reported [36]. Modifications to the system described [36] were the columns used and that a 5 mL sample loop was used for protein parking instead of a Superloop™ (GE Healthcare, Munich, Germany). Valves 1 and 2 were responsible for directing the flow to the columns. Three different modes could be operated with the positions of valves 3 and 4. The first mode (V3: inject; V4: load) was used for the standard chromatography. In the second mode (V3: load; V4: load), a protein fraction was parked in the sample loop. In the third mode (V3: inject; V4: inject), the parked protein fraction was applied to the next column. The three modes and the valve configuration are shown in the Supporting Information (Figure S1). The buffers and columns used for the

automated purification protocol are listed in Tables 1 and 2. The column temperatures were 20 °C. A schematic representation of the automated purification protocol is shown in Figure 3.1. Prior to the purification, Flavourzyme was diluted in H_2O_{dd} and, subsequently, buffer was exchanged for the first buffer in the purification protocol (buffer A, Table 3.1) using a PD10-column (GE Healthcare). A final Flavourzyme solution of 10 % (v/v) was obtained, resulting in a protein content of 7.3 g/L.

Table 3.2: Purification Stages and Columns That Were Used during the Automated Nine-Step Purification Protocol.

stage	column	chromatographic principle
I	MonoS 5/50 GL (CV = 1 mL; GE Healthcare, Munich, Germany)	ion exchange
II, IV, VI	HiTrap desalting column (3 x 5 mL ; GE Healthcare, Munich, Germany)	size exclusion
III, VIII	BioFox 17/1200 Q (CV = 4.3 mL; Knauer; Berlin, Germany)	ion exchange
V	RESOURCE PHE (CV = 1 mL; GE Healthcare, Munich, Germany)	hydrophobic interaction
VII	MonoQ 5/50 GL (CV = 1 mL; GE Healthcare, Munich, Germany)	ion exchange
IX	Superdex 200 10/300 GL (CV = 24 mL; GE; Munich, Germany)	size exclusion

A volume of 2 mL of the Flavourzyme solution was injected at a flow rate of 0.5 mL/min into a strong cation exchange chromatography **(CEX)** column (stage **I,** Table 3.2) with 100 % buffer A. The unbound protein was washed out using 10 column volumes (CV) at a flow rate of 0.5 mL/min. The flow-through volume (4.25 mL) was collected and automatically parked in the 5 mL sample loop. The column-bound protein was eluted using a one-step gradient and two linear gradients at a flowrate of 0.75 mL/min. The protein eluted from the step gradient (80 % buffer A, 20 % buffer B) was discarded. The proteins eluted from the first linear gradient (10 CV; 65 % buffer A, 35 % buffer B) and the second linear gradient (16 CV; 80 % buffer A, 20 % buffer C) were collected individually from 27.5 to 32.5 min (3.75 mL) and from 45.3 to 48.3 min (2.25 mL), respectively. Afterward, the parked protein was injected into three lined-up size exclusion chromatography (**SEC**) columns (stage **II,** Table 3.2) for a buffer exchange to 100 % buffer D at a flow rate of 2 mL/min and, subsequently, parked (5 mL) again. Then, the desalted protein solution was injected into a strong anion exchange chromatography

(**AEX)** column (stage **III,** Table 3.2) with 100 % buffer D at 0.5 mL/min. No flow-through was collected here. One bound protein fraction was eluted using a step gradient (21 % buffer C, 79 % buffer D) at a flow rate of 1 mL/min. The protein eluted (3.8 mL) was automatically parked. Additional bound protein remained on the column until further notice. Again, the parked protein was injected into three lined-up SEC columns (stage **IV,** Table 3.2) for a buffer exchange to 100 % buffer E at a flow rate of 2 mL/min and, subsequently, parked (5 mL) again. The parked protein was then injected into a hydrophobic interaction chromatography (**HIC**) column (stage **V,** Table 3.2) with 100 % buffer E at 0.5 mL/min. The unbound protein was washed out using 10 CV, and no flow-through was collected at this point. The elution was performed at a flow rate of 1 mL/min. The first bound protein fraction was eluted using a linear gradient (3.75 CV; 12 % buffer D, 88 % buffer E) and was discarded. The second bound protein fraction (3.5 mL) was eluted with a step gradient (55 % buffer D, 45 % buffer E) and was automatically parked. The third bound protein fraction was eluted by linear gradient (8 CV; 100 % buffer D) and collected from 194 to 197 min (3 mL). Next, the parked protein fraction from stage **V** (Table 3.2) was injected into three lined-up SEC columns (stage **VI,** Table 3.2) for a buffer exchange to 100 % buffer D at a flow rate of 2 mL/min and, subsequently, parked (5 mL) again. The parked protein fraction was injected into a strong AEX column (stage **VII,** Table 3.2) with 100 % buffer D at 0.5 mL/min. The unbound protein was washed out using 20 CV and no flow-through was collected here. The elution was performed at a flow rate of 1 mL/min. The first bound protein fraction was eluted using a linear gradient (4 CV; 35 % buffer D, 65 % buffer F), and the fraction was collected from 273.5 to 277.5 min (4 mL). The second bound protein fraction was eluted with a step gradient (30 % buffer C, 70 % buffer D), and the fraction was collected from 281.3 to 283.3 min (2 mL). Afterward, the remaining protein fractions on the column from stage **III** (Table 3.2) were eluted at a flow rate of 1 mL/min (stage **VIII,** Table 3.2). The first bound fraction was obtained after linear gradient elution (1.2 CV; 20 % buffer C, 80 % buffer D) from 312 to 315 min (3 mL). The gradient was kept for 2.4 CV, and then 100 % buffer D was used for 0.7 CV. A second fraction was obtained by a subsequent linear gradient elution (1.9 CV; 50 % buffer D, 50 % buffer F) from 325.5 to 327.5 min (2 mL). A third fraction (1.15 mL) was automatically collected and parked after a step gradient (35 % buffer C, 65 % buffer D). The parked protein fraction was then injected directly into a SEC column (stage **IX,** Table 3.2) with 20 % buffer C and 80 %

buffer D at a flow rate of 0.75 mL/min. A last protein fraction was obtained by isocratic elution for 1 CV from 384.8 to 387.8 min (2.25 mL). The total run time for the automated nine-step purification protocol of the Flavourzyme enzymes was 420 min (7 h). A detailed overview of the particular purification steps, including valve positions, is summarized in Table S2 (Supporting Information).

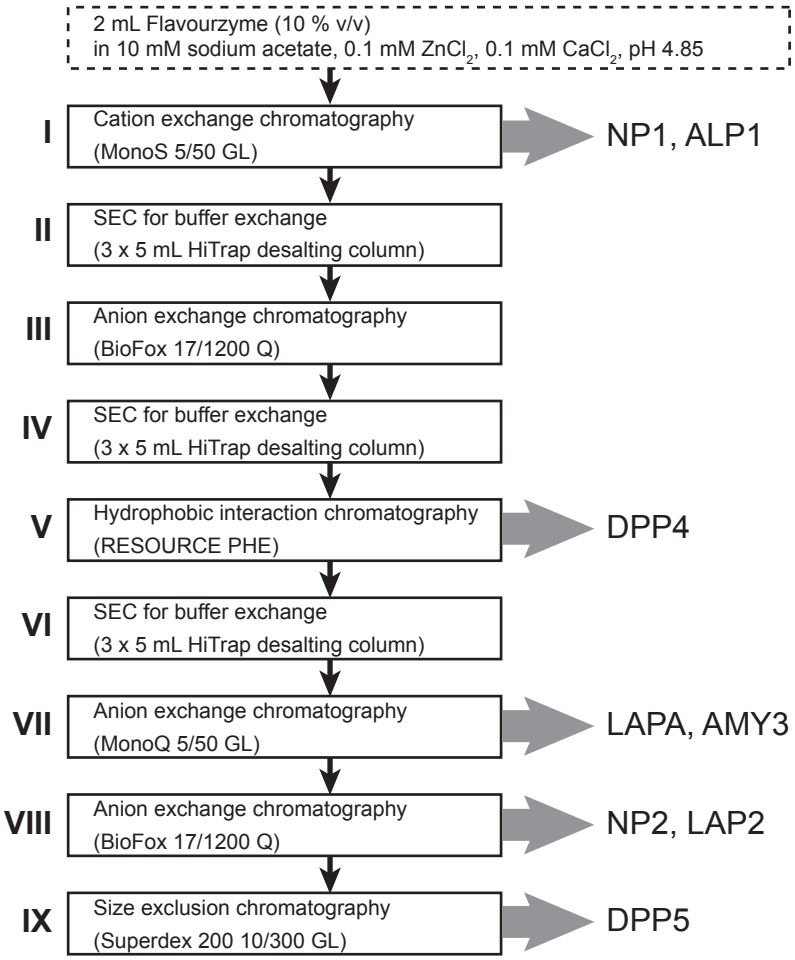


Figure 3.1: Schematic representation of the automated purification protocol for the Flavourzyme enzymes. Stages **I - IX** are in accordance with Table 3.2. Protein fractions, which were injected into the next purification stage, were always parked in a 5 mL sample loop. ALP1, alkaline protease 1; AMY3, α-amylase A type 3; DPP4, dipeptidyl peptidase 4; DPP5, dipeptidyl peptidase 5; LAPA, leucine aminopeptidase A; LAP2, leucine aminopeptidase 2; NP1, neutral protease 1; NP2, neutral protease 2.

Characterization of the Purified Flavourzyme Enzymes

Protein fractions from the automated purification protocol were taken and the buffer was exchanged using PD10 desalting columns (GE Healthcare). The exchange buffer was 10

mM Tris-HCl (pH 7.5) containing 0.1 mM $ZnCl_2$ and 0.1 mM $CaCl_2$. Formulated purified enzymes were stored at -20 °C. The enzyme samples were conveniently diluted in corresponding assay buffers for the following characterization.

Effect of pH on the Enzyme Activity

The pH profiles of the purified enzymes were determined with citrate phosphate buffer (50 mM; pH 3.0 – 7.5), Bis-Tris-propane buffer (50 mM; pH 7.5 – 10.0), CHES buffer (50 mM; pH 9.0 – 10.0) or glycine-NaOH buffer (50 mM; pH 10.0 – 12.0) at 37 °C. As an exception, the pH profile of the AMY3 was measured in the presence of 5 mM of the corresponding buffer.

Effect of Temperature on the Enzyme Activity

The temperature profiles of the purified enzymes were determined at the optimum pH in the corresponding buffer system (determined before). The assay temperatures ranged from 20 to 75 °C.

Determination of the Native Molecular Size by HPLC-SEC

The native molecular size of the purified enzymes was evaluated by SEC on an UHPLC system (PLATINblue; Knauer, Berlin, Germany). Therefore, 5 µL of the purified enzyme solution was loaded into a Yarra SEC-2000 column (Phenomenex, Aschaffenburg, Germany). The flow rate was 1 mL/min, and the mobile phase was 50 mM sodium phosphate buffer, pH 6.5, supplemented with 300 mM NaCl. Eluted proteins were detected at 280 nm. The sizes were calculated, on the basis of the retention time obtained, using a calibration curve determined with standard proteins (gel filtration LMW/HMW calibration kits; GE Healthcare). All samples were taken from the fractions of the automated purification protocol without any further preparation. Only the DPP4 fraction was concentrated 60-fold, using a vacuum centrifuge (RVC 2-33 IR, Christ, Osterode, Germany; 1 mbar, 40 °C, 4 h), due to the low protein content of the fraction.

Statistical Analysis

The standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, WA, USA). All trials were conducted at least in duplicate, with two independent measurements. The automated purification protocol was carried out five times to check the reproducibility of the system.

RESULTS AND DISCUSSION

Identification of Key Flavourzyme Enzymes

A comprehensive and comparative study was carried out to identify the key enzyme activities in the Flavourzyme preparation. The approach was as follows: First, relevant proteins from Flavourzyme were identified by mass spectrometric investigations (LC-ESI-MS^2). Second, a substrate screening using various aminopeptidase and dipeptidyl peptidase substrates was carried out. Third, if possible, the activities of the substrate screening were assigned to particular proteins by activity staining of native polyacrylamide gels or by zymography.

In total, eight enzymes were identified in the Flavourzyme mixture (Table 3.3). The table also includes the UniProtKB identifier of the particular enzyme and the molecular mass calculated from the respective UniProtKB entry (mature monomer). All enzymes identified originated from *A. oryzae* (strain ATCC 42149 / RIB 40), also known as yellow koji mold. In summary, three endopeptidases (neutral protease 1, **NP1**; neutral protease 2, **NP2**; alkaline protease 1, **ALP1**), two aminopeptidases (leucine aminopeptidase A, **LAPA**; leucine aminopeptidase 2, **LAP2**), two dipeptidyl peptidases (dipeptidyl peptidase 4, **DPP4**; dipeptidyl peptidase 5, **DPP5**), and one amylase (α-amylase A type-3, **AMY3**) were identified. The ALP1, NP1, and NP2 are also known as oryzin, fungalysin, and deuterolysin, respectively. LAPA is also known as leucine aminopaptidase 1. The sequence coverages (MS analysis) were 28, 30, 46, 60, 73, 7, 52 and 45 % for NP1, NP2, ALP1, LAPA, LAP2, DPP4, DPP5, and AMY3, respectively.

All peptidase activities could be verified either by activity staining of native polyacrylamide gels or by zymography. Three endopeptidase activities were determined by zymography (data not shown). Four specific marker substrates were revealed for the aminopeptidases and dipeptidyl peptidases after the *p*NA substrate screening and by activity staining of native gels (Figure 3.2). Here, the Flavourzyme preparation was applied on a native PAGE and activity stained with different *p*NA substrates. It can be concluded that four different aminopeptidases and dipeptidyl peptidases are present in Flavourzyme due to the different activity staining patterns (Figure 3.2). Later, it was confirmed for the purified peptidases that the substrate Ile-*p*NA was hydrolyzed only by LAPA, Pro-*p*NA was hydrolyzed only by LAP2, Ala-Pro-*p*NA was hydrolyzed only by DPP4, and Lys-Ala-*p*NA was hydrolyzed only by DPP5. With these marker substrates, a

distinction between these four enzymes was possible and advantageous for a later assessment of the purification of the Flavourzyme enzymes.

Table 3.3: Identification of Flavourzyme Enzymes by Mass Spectrometric Analysis and Assignment to the Corresponding UniProtKB Entry.

#	Enzyme	abbrev	UniProtKB ID	size* (kDa)	EC number
1	leucine aminopeptidase A	LAPA	Q2U1F3	32.5	3.4.11.
2	leucine aminopeptidase 2	LAP2	Q2ULM2	51.9	3.4.11.
3	dipeptidyl peptidase 4	DPP4	Q2UH35	85.1	3.4.14.5
4	dipeptidyl peptidase 5	DPP5	Q9Y8E3	78.6	3.4.14.
5	neutral protease 1	NP1	Q2U1G7	42.4	3.4.24.
6	neutral protease 2	NP2	P46076	19.0	3.4.24.39
7	alkaline protease 1	ALP1	P12547	29.0	3.4.21.63
8	α-amylase A type-3	AMY3	P0C1B4	52.5	3.2.1.1

* Molecular masses calculated from the respective UniProtKB entry (mature monomer).

All marker substrates, which were used for the indication of the enzymes during the automated purification, are summarized in Table 3.4. Because specific marker substrates could not be determined for the endopeptidases (NP1, NP2, ALP1), the same substrate (azocasein) was used for the evaluation of the purification. Starch was used as the substrate for AMY3.

Because Flavouryzme is an industrial enzyme preparation from *A. oryzae*, the proteomic analysis of extracellular proteins from *A. oryzae* gives an indication of the enzymes present in the preparation. Oda et al.[18] evaluated the production of extracellular proteins from *A. oryzae* RIB40 under submerged and solid-state culture conditions. The cultures were cultivated on wheat bran (solid-state and submerged). The proteins were identified by MALDI-TOF-MS. A functionality (enzyme activity) of a specific protein was not determined. The authors found that mainly glucosidases were secreted during the cultivation [18]. Oryzin (ALP1) and DPP5 were expressed under solid-state conditions. Oryzin (ALP1) was also expressed under submerged conditions [18]. Because wheat bran contains around 80 % carbohydrates [37], a preferred expression of glucosidases over peptidases seems convincing. Liang et al.[19] analyzed the extracellular proteins of *A. oryzae* AS 3.951 and RIB40 grown on soy sauce koji (soybeans) by MALDI-TOF-MS. Again, functionality (enzyme activity) was not determined. Here, an ALP1, an NP1, an

NP2, a DPP5, an X-prolyl aminopeptidase, a leucine aminopeptidase, and an *α*-amylase were found [19]. In conclusion, an impact of the cultivation medium (wheat bran or soy sauce koji) on the expression of the enzyme set can be assumed. Nakadai and Nasuno [38] also showed that the production of peptidases from *A. oryzae* ATCC 20386 varied, depending on the culture conditions. The conclusion was that submerged cultivation was preferable to solid-state cultivation for the production of peptidases [38]. In this study [38], casein and the aminopeptidase substrate (Leu-*β*NA) were used to determine the particular peptidase activity. On the other hand, solid-state cultivation was preferable for the production of glucosidases [38]. The study of Sriranganadane et al.[20] showed that different sets of peptidases are expressed and secreted depending on the pH of the culture medium. The authors detected peptidases from *Aspergillus fumigatus* by MALDI-TOF-MS. The fungus was grown on a malt agar including collagen at pH 3.5 and 7.0. The authors concluded that neutral pH favors the secretion of neutral and alkaline endopeptidases and leucine aminopeptidases (LAP2, DPP4, DPP5), whereas acidic pH promotes the secretion of endopeptidases of the pepsin family and carboxypeptidases [20]. Taking these results together, *A. oryzae* ATCC 42149/RIB 40, which is used for the production of Flavourzyme, was most probably cultivated under neutral culture conditions.

Table 3.4: Purified Enzymes from Flavourzyme That Were Obtained from the Automated Nine-Step Purification Protocol [a].

#	enzyme [b]	Marker substrate	Apparent Purity [c] [%]	Total enzyme activity	Specific enzyme activity	Recovery [%]	Purification fold [-]
1	LAPA	Ile-*p*NA	90	9.5 nkat	10.7 nkat/mg	42	7.5
2	LAP2	Pro-*p*NA	88	1.5 nkat	6.6 nkat/mg	42	29.5
3	DPP4	Ala-Pro-*p*NA	40	1.8 nkat	603 nkat/mg	36	1898
4	DPP5	Lys-Ala-*p*NA	94	30.1 nkat	133 nkat/mg	475	328
5	NP1	Azocasein	> 95	450 ACU	775 ACU/mg		na [d]
6	NP2	Azocasein	89	9.0 ACU	6.3 ACU/mg	23 [e]	na
7	ALP1	Azocasein	> 95	151 ACU	495 ACU/mg		na
8	AMY3	Starch	> 95	2950 nkat	2190 nkat/mg	54	6.3

[a] The total enzyme activity is calculated per purification batch (200 µL Flavourzyme) at 37 °C and pH 7.5. Specific enzyme activity is calculated per mg of protein, determined with Bradford; [b] ALP1, alkaline protease 1; AMY3, *α*-amylase A type-3; DPP4, dipeptidyl peptidase 4; DPP5, dipeptidyl peptidase 5; LAPA, leucine aminopeptidase A; LAP2, leucine aminopeptidase 2; NP1, neutral protease 1; NP2, neutral protease 2; [c] Apparent purity was estimated by integration of the SEC chromatograms (See Supporting Information Figures S3 – S10); [d] na = not applicable; [e] Total recovery of NP1, NP2, ALP1.

Various enzyme activities in Flavourzyme were determined using an API-ZYM test system in the study of Kilcawley et al.[6]. The authors used a solid preparation (Flavourzyme MG/A) and did not correlate a certain enzyme activity to a specific protein. They identified various esterase, peptidase, lipase, and glycosidase activities with the API-ZYM test [6]. In addition, various aminopeptidase activities were detected, whereas endopeptidase activity and carboxypeptidase activity could not be ascertained [6].

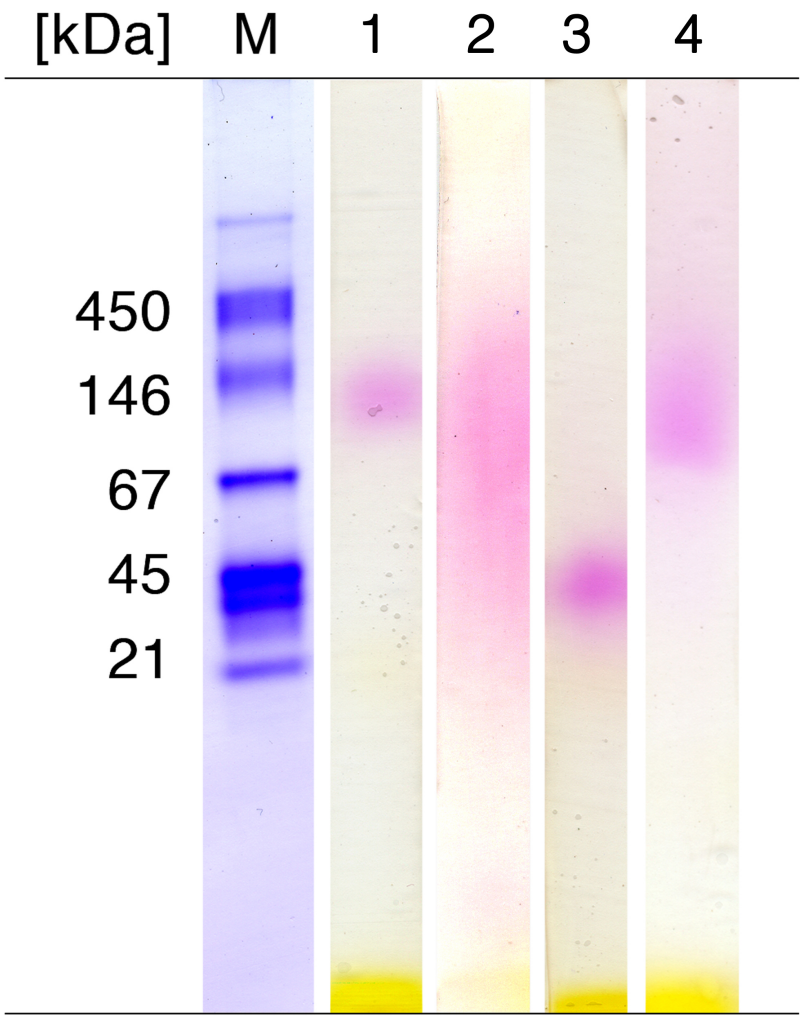


Figure 3.2: Native PAGE with activity staining of Flavourzyme preparation with different *p*NA substrates (Lanes: 1, Ala-Pro-*p*NA; 2, Lys-Ala-*p*NA; 3, Ile-*p*NA; 4, Pro-*p*NA). M indicates the protein marker (SERVA native marker, liquid mix; SERVA, Heidelberg, Germany). An enzyme activity of 50 pkat on the corresponding substrate was loaded per lane.

Automated Nine-Step Purification of Flavourzyme Enzymes

An automated purification protocol was developed to purify the eight enzymes of Flavourzyme simultaneously in a semipreparative scale. All fractions collected were tested for endopeptidase, aminopeptidase, dipeptidyl peptidase and α-amylase activity. In addition, all active fractions were investigated by MALDI-TOF-MS. The final chromatogram of the automated nine-step purification protocol is displayed in Figure 3.3. The different stages (**I-IX**) of the purification protocol correspond to Table 3.2. Encircled numbers from Figure 3.3 represent the fractions of interest (gray areas indicate the purified enzymes) and are in accordance with the consecutive numbering in Tables 3 and 4. The purified enzymes collected were immediately chilled on ice and desalted to 10 mM Tris-HCl, pH 7.5, containing 0.1 mM $ZnCl_2$ and 0.1 mM $CaCl_2$ before the enzyme activity was determined. The hatched areas in Figure 3.3 indicate fractions that were parked and, subsequently, injected into the next chromatography step.

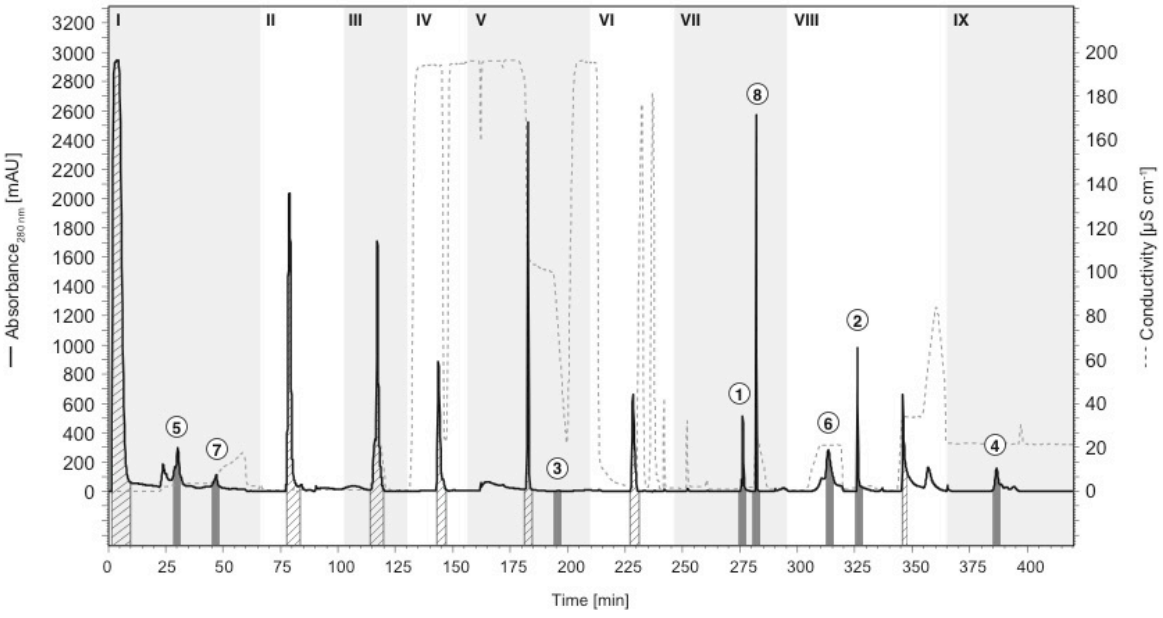


Figure 3.3: Automated nine-step purification of eight Flavourzyme key enzymes. The purification stages comply with Table 3.2 and Figure 3.1. **I, CEX** (Mono S 5/50 GL, 1 mL); **II, IV, VI, buffer exchange** (HiTrap desalting column, 3 x 5 mL), **III, AEX** (Biofox 17Q, 4.3 mL); **V, HIC** (ResourcePhe, 1 mL); **VII, AEX** (Mono Q 5/50 GL, 1 mL); **VIII, AEX** (Biofox 17Q, 4.3 mL); **IX, SEC** (Superdex 200 10/300 GL, 24 mL). Fractions of interest (isolated enzymes) are indicated with gray bars and the encircled numbers comply with the consecutive numbering in Tables 3 and 4. Fractions that were automatically injected to the next chromatography step are indicated by hatched areas.

We observed a loss of the LAPA activity after the CEX in preliminary experiments (data not shown). The LAPA is a zinc-dependent metallopeptidase (UniProtKB annotation) and, thus, the removal of the cation from the enzyme by the chromatography resin could be an explanation. Because most other enzymes identified are also metal-dependent (zinc, NP1, NP2, LAP2; calcium, AMY3), buffers A, B and F (Table 3.1) were supplemented with zinc and calcium chloride. With these additions, the recovery of the LAPA activity was about 5-fold higher (data not shown). The other enzymes were not affected by the supplementation.

As mentioned before, a schematic representation of the automated purification protocol is shown in Figure 3.1. Two protein fractions were eluted from 27.5 to 32.5 min (3.75 mL) and from 45.3 to 48.3 min (2.25 mL), respectively. The proteins eluted were identified by LC-ESI-MS^2 as NP1 (⑤; Figure 3.3) and ALP1 (⑦; Figure 3.3). A total activity of 450 ACU with a specific activity of 775 ACU/mg was obtained for NP1. A total activity of 151 ACU with a specific activity of 495 ACU/mg was obtained for ALP1 (see Table 3.4). The next fraction of interest was eluted in stage **V** (Figure 3.3) from 194 to 197 min (3 mL) and was identified by LC-ESI-MS^2 as DPP4 (③; Figure 3.3). A total enzyme activity of 1.8 $nkat_{H\text{-}Ala\text{-}Pro\text{-}pNA}$ was obtained. In comparison to the Flavourzyme preparation, the specific activity increased 1898-fold to 603 $nkat_{H\text{-}Ala\text{-}Pro\text{-}pNA}$/mg for the purified peptidase. The recovery was 36 % (Table 3.4). The next two fractions of interest were eluted in stage **VII** (Figure 3.3) from 273.5 to 277.5 min (4 mL) and 281.3 to 283.3 min (2 mL) and were identified by LC-ESI-MS^2 as LAPA (①; Figure 3.3) and AMY3 (⑧; Figure 3.3), respectively. A total enzyme activity of 9.5 $nkat_{H\text{-}Ile\text{-}pNA}$ was obtained for LAPA. The specific activity increased 7.5-fold to 10.7 $nkat_{H\text{-}Ile\text{-}pNA}$/mg for the purified peptidase. The recovery was 42 % (Table 3.4). A total enzyme activity of 2950 $nkat_{Starch}$ was obtained for AMY3. The specific activity increased 6.3-fold to 2190 $nkat_{Starch}$/mg for the purified amylase (recovery, 54 %; Table 3.4). Another two protein fractions were obtained in stage **VIII** (Figure 3.3) from 312 to 315 min (3 mL) and from 325.5 to 327.5 min (2 mL) and were identified by LC-ESI-MS^2 as NP2 (⑥; Figure 3.3) and LAP2 (② ; Figure 3.3), respectively. A total activity of 9 ACU with a specific activity of 6.3 ACU/mg was obtained for NP2 (Table 3.4). Because all endopeptidases investigated use the same substrate (azocasein), it was not possible to assign a specific recovery and a purification factor for a particular endopeptidase. In total, a recovery of 23 % of the three endopeptidases was achieved. A total enzyme activity of 1.5 $nkat_{H\text{-}Pro\text{-}pNA}$ was obtained

for the aminopeptidase LAP2. The specific activity of LAP2 increased 29.5-fold to 6.6 $nkat_{H\text{-}Pro\text{-}pNA}$/mg for the purified peptidase. The recovery was 42 % (Table 3.4). The last protein fraction of interest was obtained in stage **IX** (Figure 3.3) from 384.8 to 387.8 min (2.25 mL) and was identified by LC-ESI-MS^2 as DPP5 (④; Figure 3.3). A total enzyme activity of 30.1 $nkat_{H\text{-}Lys\text{-}Ala\text{-}pNA}$ was obtained for DPP5. The specific activity increased 328-fold to 133 $nkat_{H\text{-}Lys\text{-}Ala\text{-}pNA}$/mg for the purified peptidase. The recovery was 475 % (Table 3.4).

The reproducibility of the automated purification was tested with five independent trials. The deviation of the five purification trials was < 5 % concerning purification factors, yields of the enzymes, and alteration of the retention times.

The automated purification of proteins with a comparable setup was shown by Eisele et al.[36] and Stressler et al.[39]. Eisele et al.[36] purified angiotensin-converting-enzyme from a mammalian tissue (pig lung) with a yield of 23.6 % and a 308-fold increase of the specific activity. The automated six-step purification protocol took 8 h. Stressler et al.[39] purified a prolyl-specific aminopeptidase (PepX) from *Lactobacillus helveticus* from a crude cell-extract. They achieved a yield of 56 % with a purification factor of 162-fold. The automated four-step purification protocol took 6 h. In contrast to the literature presented, eight proteins of interests were simultaneously purified here from a complex industrial fungal enzyme preparation.

Characterization of Purified Flavourzyme Enzymes

Purified and formulated enzymes were conveniently diluted in the appropriate buffer system. The effect of the pH and the temperature on the enzyme activity was tested using the purified enzymes and is described below. Additionally, the purified enzymes were analyzed by HPLC-SEC and SDS-PAGE to determine the native sizes and the sizes under denaturing conditions, respectively. The apparent purity (Table 3.4) of the particular enzyme was estimated by integration of the HPLC-SEC chromatogram (Figures S3 – S10, Supporting Information). The extinction coefficients of the respective proteins were not considered because some areas from the HPLC-SEC chromatogram could not be assigned to a specific protein from the LC-ESI-MS^2 analysis. The major impurities in the purified fractions, found by LC-ESI-MS^2 analysis, were a *β*-glucosidase A, a N-acetylglucosaminidase, a IgE-binding protein and a glucoamylase.

All purified enzyme fractions exhibited < 5 % activity toward the other marker substrates (data not shown).

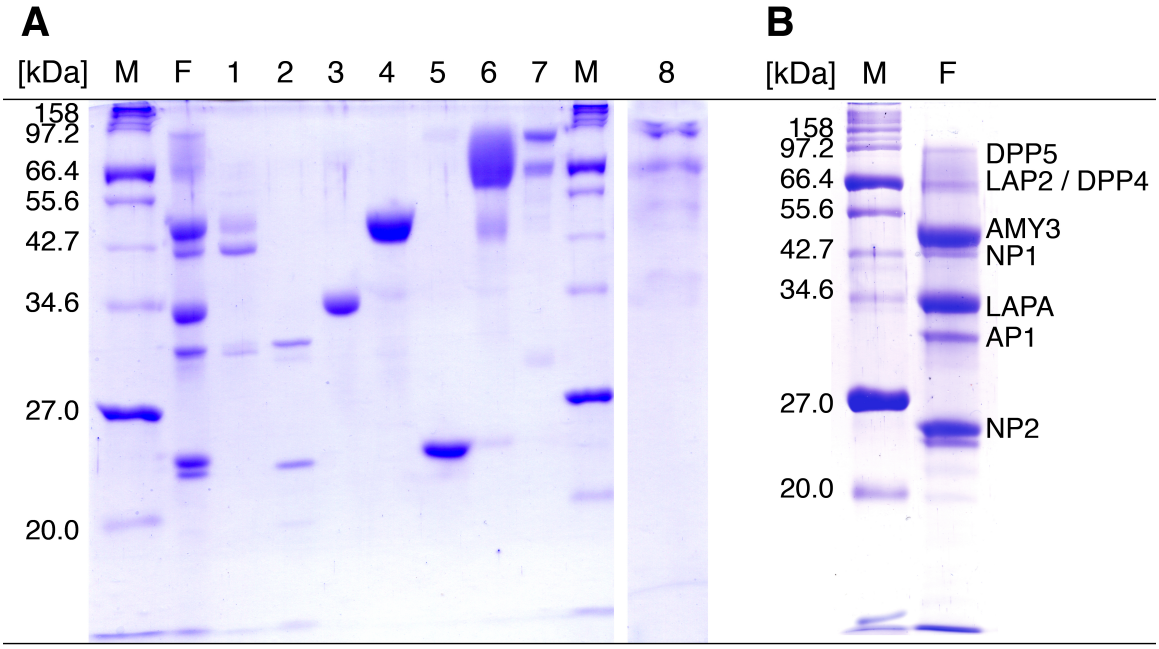


Figure 3.4: SDS-PAGE with Coomassie staining of protein fractions from the automated purification protocol (A) and Flavourzyme (B). M indicates the protein marker (Broad Range, 2-212 kDa; NEB, Frankfurt, Germany). Protein load was 1 µg per lane for the purified protein fractions (lanes 1 - 8) and 5 µg for the Flavourzyme samples (F). Lanes: 1, neutral protease 1; 2, alkaline protease 1; 3, leucine aminopeptidase A; 4, *α*-Amylase A type-3; 5, neutral protease 2; 6, leucine aminopeptidase 2; 7, Dipeptidyl peptidase 5; 8, dipeptidyl peptidase 4.

Leucine Aminopeptidase A (LAPA)

The calculated size of LAPA is 32.5 kDa (UniProtKB). Under denaturing conditions (SDS-PAGE; Figure 3.4A, lane 3), a protein band at approximately 34 kDa was observed. A native size of 36.2 kDa was determined by HPLC-SEC. These results indicate a monomeric structure for the active enzyme. The apparent purity of the isolated enzyme was 90% (Table 3.4). The pH profile of the purified LAPA is displayed in Figure 3.5-**1** and shows an optimum pH of 9.0 with reasonable activity between pH 6.5 and 10.0. The effect of the temperature on the activity is shown in Figure 3.6-**1**. The highest activity was measured at 65 °C. An extracellular leucine aminopeptidase from *A. oryzae* RIB40 was described previously by Matsushita et al.[40]. Here, the aminopeptidase exhibited the highest activity at pH 8.5 and 60 °C and showed a molecular weight of 33 kDa by SDS-PAGE analysis [40]. A leucine aminopeptidase 1 from *A. oryzae* ATCC 20386 was

purified and characterized by Nakadai et al.[41]. Again, the enzyme exhibited the highest enzyme activity at pH 8.5 and 60 °C. However, in contrast to our observations, the native size determined was 26.5 kDa by SEC analysis. In the current study, the enzyme activity at optimum pH and temperature was 75.1 $nkat_{H\text{-}Ile\text{-}pNA}$/mg.

Leucine aminopeptidase 2 (LAP2)

A monomeric molecular weight of 51.9 kDa was calculated (UniProtKB) for LAP2. No discrete protein band could be detected under denaturing conditions (SDS-PAGE; Figure 3.4A, lane 6). A broad protein band from approximately 60 to 100 kDa was observed, and a native size of 105.7 kDa was determined by HPLC-SEC. These results indicate a seemingly dimeric structure for the active enzyme. The smeared band observed on the SDS-PAGE and the native size of 105.7 kDa can be explained by the high glycosylation of the enzyme (computed glycosylation at amino acid positions 224, 307, 341, 402, 424, and 458; UniProtKB). This observation was also described by Blinkovsky et al.[42] for aminopeptidase 2 from *A. oryzae* ATCC 20386. After deglycosylation of the N-linked sugars, the protein band was sharp at 56 kDa [42]. Thus, a heterogeneous glycosylation pattern could lead to higher and variable molecular masses for a monomer. The apparent purity of the isolated enzyme was 88 % (Table 3.4). The pH profile of the purified LAP2 is displayed in Figure 3.5-**2** and shows an optimum pH of 9.5 with about 50 % relative activity at pH 8.5 and 10.5. An optimum pH of 8.0 was determined by Nakadai et al.[43], and an optimum pH of 9.5 was determined by Blinkovsky et al.[42] for leucine aminopeptidase 2 from *A. oryzae* ATCC 20386, respectively. The effect of the temperature on the activity of LAP2 is shown in Figure 3.6-**2**. The highest activity was measured at 50 °C. This is in accordance with the literature [43]. The enzyme activity of LAP2 at optimum pH and temperature was 54.2 $nkat_{H\text{-}Pro\text{-}pNA}$/mg.

Dipeptidyl Peptidase 4 (DPP4)

The size of DPP4 calculated was 85.1 kDa (UniProtKB). A single protein band could not be obtained on a SDS-PAGE (Figure 3.4A, lane 8). The presence of DPP in this fraction was ascertained by LC-ESI-MS^2 analysis. The apparent purity of DPP4 was approximately 40 % (Table 3.4) by HPLC-SEC. The native size (HPLC-SEC) of DPP4 was 206.6 kDa, which most probably indicates a dimeric form of the enzyme. The results are in accordance with the literature. The existence of DPP4, which was expressed as a 95 kDa glycoprotein, from *A. oryzae* has been shown [44]. Tachi et al.[45] also showed that DPP4 from *A. oryzae* existed as a homodimer. The pH profile of the purified DPP4 is

shown in Figure 3.5-**3**. The optimum pH was 8.5 with about 50 % relative activity at pH 7.5 and 9.5. Tachi et al.[45] determined an optimum pH of 7.0 – 7.5 with the substrate H-Gly-Pro-*p*NA. The effect of the temperature on the activity is shown in Figure 3.6-**3**. The highest activity was measured at 60 °C. The enzyme activity at optimum pH and temperature was 1471 $nkat_{H\text{-}Ala\text{-}Pro\text{-}pNA}$/mg.

Dipeptidyl Peptidase 5 (DPP5)

To the best of our knowledge, there has been no detailed biochemical characterization of a DPP5 from *A. oryzae* so far. In contrast to other dipeptidyl peptidases, DPP5 is not able to cleave X-prolyl bonds (tested with Ala-Pro-*p*NA, Gly-Pro-*p*NA, and Arg-Pro-*p*NA). This difference makes a distinction between DPP5 and DPP4 possible. Beauvais et al.[46] described a fungal DPP5 from *A. fumigatus*. They observed a monomeric size (SDS PAGE analysis) of 79 kDa after deglycosylation (88 kDa as glycosylated protein). In our study, we determined a native size of 220.3 kDa (HPLC-SEC), which indicates a dimeric or trimeric form when considering the size of 78.6 kDa calculated (UniProtKB). In addition, the HPLC-SEC analysis showed a single and distinct peak (94 % apparent purity, Table 3.4). In contrast to the latter, three distinct protein bands were obtained on a SDS-PAGE (Figure 3.4A, lane 7). All three bands were independently identified as DPP5 by LC-ESI-MS^2. The protein bands at approximately 66 and 90 kDa showed sequence coverages of 45 and 46 %, respectively. The protein band at approximately 30 kDa showed a sequence coverage of 19 %. The reason for the three bands on the SDS PAGE is not clear. The presence of fractions after electrophoresis can probably explain the broad staining of the native PAGE (Figure 3.2, lane 2). The pH profile of the purified DPP5 is shown in Figure 3.5-**4.** The optimum pH was determined to be 6.0 with about 50 % relative activity at pH 5.0 and about 80 % relative activity at pH 7.0, respectively. The highest activity was measured in Bis-Tris-propane buffer at pH 7.5. A strong inactivation (buffer effect) was observed using citrate phosphate buffer. The effect of the temperature on the activity is shown in Figure 3.6-**4**. The highest activity was measured at 65 °C. The enzyme activity at optimum pH and temperature was 555 $nkat_{H\text{-}Lys\text{-}Ala\text{-}pNA}$/mg.

Neutral Protease 1 (NP1)

The purified NP1 showed two distinct protein bands at approximately 29 and 42 kDa (SDS-PAGE analysis; Figure 3.4A, lane 1). The LC-ESI-MS^2 measurements identified both bands as NP1. Only two distinct proteins with 41.3 and 58.5 kDa were detected for the native size of the NP1 determined by HPLC-SEC. The mass calculated (UniProtKB)

for the NP1 was 42.4 kDa (Table 3.3). The two proteins were obtained in different fractions during the automated purification. Therefore, the 41.3 and 58.5 kDa proteins were separately characterized. We observed the same properties regarding pH and temperature profiles and specific enzyme activity. Thus, the respective protein fractions of the NP1 were pooled. A proteolysis during the storage of the Flavourzyme preparation seems to be a plausible explanation for two NP1 fragments. The optimum pH was determined at pH 7.0 in citrate phosphate buffer, as shown in Figure 3.5-**5**. However, the highest activity was measured in Bis-Tris-propane buffer at pH 7.5, due to a strong inactivation (buffer effect) caused by the citrate phosphate buffer. This result complies with a characterization of a neutral protease 1 from *A. oryzae* ATCC 20386 previously described [47]. The authors determined a native size of 41 kDa and an optimum pH of 7.0 with about 50 % relative enzyme activity in phosphate buffer compared to Tris-HCl buffer [47]. The effect of the temperature on the activity is shown in Figure 3.6-**5**. The highest activity was measured at 50 °C. The enzyme activity at optimum pH and temperature was 892 ACU/mg.

Neutral Protease 2 (NP2)

The NP2 showed a distinct band at approximately 25 kDa (SDS-PAGE analysis; Figure 3.4A, lane 5). A distinct peak was also obtained by HPLC-SEC analysis, which corresponds to a native size of 24.4 kDa (89 % apparent purity, Table 3.4). The size calculated was 19.0 kDa (UniProtKB). Therefore, a monomeric state of the peptidase can be assumed. The highest activity was determined at pH 5 – 5.5 (Figure 3.5-**6**) and 60 °C (Figure 3.6-**6**). A moderate influence of the buffer system was also observed. The NP2 activity was lower in the citrate phosphate buffer. The enzyme activity at optimum pH and temperature was 62 ACU/mg. Tatsumi et al.[48] expressed a neutral protease 2 from *A. oryzae* ATCC 20386 and determined a molecular size of 26 kDa (SDS). Nakadai et al.[49] also described a neutral protease 2 from *A. oryzae* ATCC 20386 and determined a native size of 19 kDa (SEC) with the highest activity at pH 5.5 – 6.

Alkaline Protease 1 (ALP1)

As described for NP1, the purified ALP1 fraction also showed two distinct proteins, with approximately 25 and 30 kDa (SDS-PAGE analysis; Figure 3.4A, lane 2). Both proteins were identified as ALP1 by LC-ESI-MS^2. The native size (HPLC-SEC) was determined at 19.5 and 41.1 kDa, respectively. The theoretical mass for the ALP1 was 29.0 kDa (UniProtKB, Table 3.3). Again, the two proteins were obtained in different fractions

during purification. The 19.5 and 41.1 kDa proteins were separately characterized. As described for NP1, we observed the same properties (pH profile, temperature profile, and specific enzyme activity) for both protein fractions. Therefore, we pooled the respective protein fractions of the ALP1 for further investigation. The pH profile of the ALP1 is shown in Figure 3.5-**7**. The optimum pH was measured at pH 7.0 with reasonable activities from pH 6.0 to 10.0. The highest activity was measured in Bis-Tris-Propane buffer at pH 7.5 due to a slight inactivation caused by the citrate phosphate buffer. The optimum temperature (Figure 3.6-**7)** was 50 °C. The enzyme activity at optimum pH and temperature was 880 ACU/mg. In contrast to our result, Guo and Ma [50] characterized an alkaline protease from *A. oryzae* Y2942 and determined a molecular size of 34 kDa (SDS-PAGE) and the highest activity at 40 °C and pH 9.0. Nakadai and Nasuno [51] described an alkaline protease 1 from *A. oryzae* ATCC 20386 and determined a native size of 23 kDa (SEC) and the highest activity at pH 10.5.

α-Amylase A Type-3 (AMY3)

The AMY3 fraction from the purification protocol showed a protein band at approximately 48 kDa (SDS-PAGE: Figure 3.4A, lane 4). A native size of 54.9 kDa was determined by HPLC-SEC and is in accordance with the molecular mass of 52.5 kDa calculated (UniProtKB). The pH profile of the purified AMY3 is shown in Figure 3.5-**8.** An optimum pH of 5.0 – 5.5 with about 50 % relative activity at pH 3.0 and 7.0 was determined. The effect of the temperature on the activity is shown in Figure 3.6-**8**. The highest activity was measured at 50 °C. The enzyme activity at optimum pH and temperature was 6850 $nkat_{Starch}$/mg. The optimum conditions determined (50 °C, pH 5.0) were also described for *α*-amylases from *A. oryzae* ATCC 76080 [52] and *A. oryzae* IFO-30103 [53]. The activity of the purified amylase of the current study was compared with a commercial amylase preparation from *A. oryzae* (Fungamyl 2500G, Novozymes, Bagsværd, Denmark). Almost the same specific enzyme activity was determined for both enzymes. The purified Flavourzyme amylase exhibited 2190 $nkat_{Starch}$/mg, whereas Fungamyl 2500G exhibited 2240 $nkat_{Starch}$/mg. The same molecular mass was also determined for both samples on SDS-PAGE (data not shown).

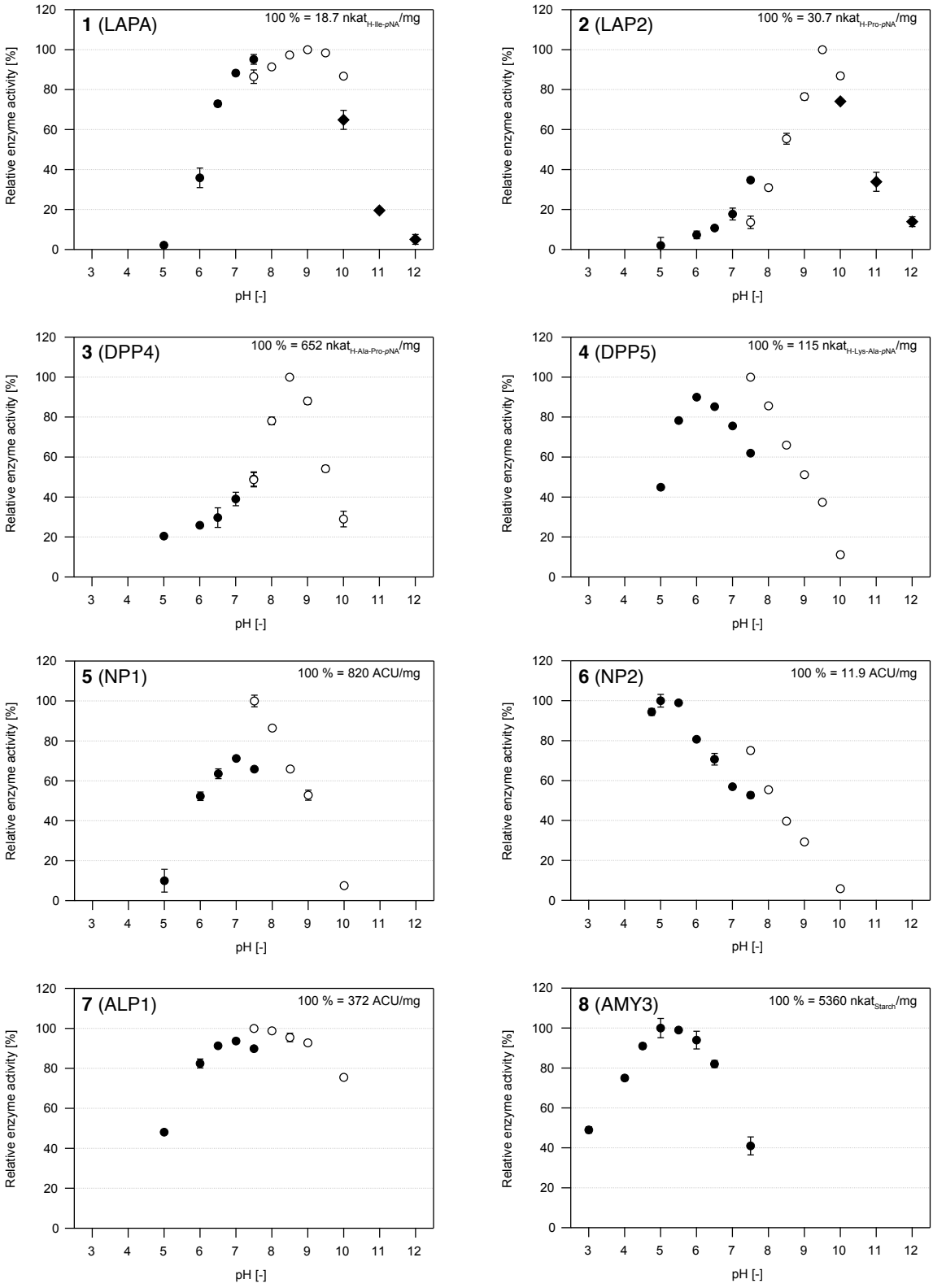


Figure 3.5: Influence of pH value on enzyme activity of purified Flavourzyme enzymes at 37 °C. Buffers: solid circles, 50 mM citrate phosphate; open circles, 50 mM Bis-Tris-propane; solid diamonds, 50 mM glycine-NaOH. AMY3 was incubated in 5 mM corresponding buffer.

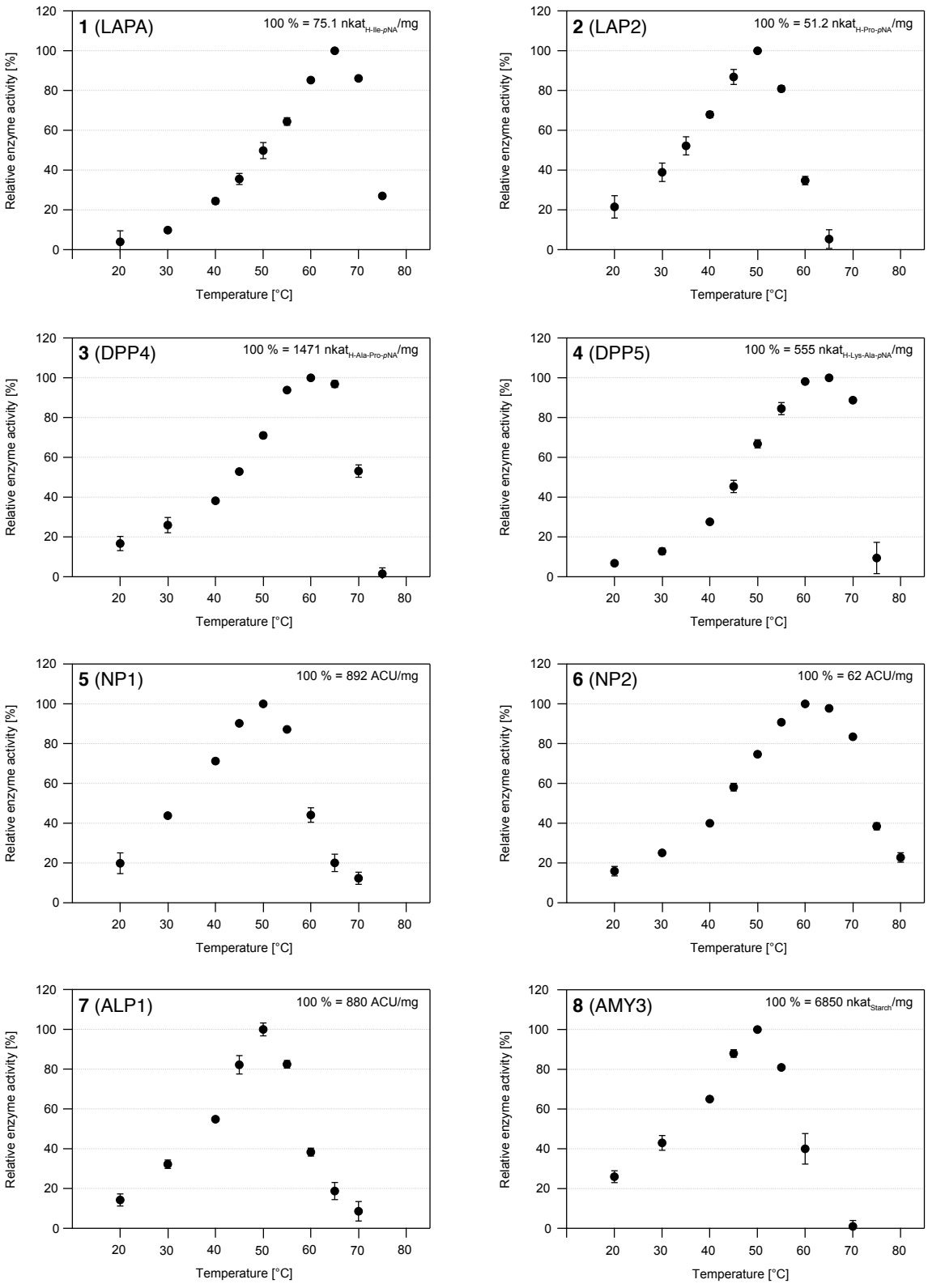


Figure 3.6: Influence of temperature on enzyme activity of purified Flavourzyme enzymes at the respective optimum pH (50 mM of the corresponding buffer). AMY3 was incubated in 5 mM corresponding buffer.

In summary, we could identify eight active key enzymes in Flavourzyme 1000L. Due to the results presented, the enzymes identified can be assigned to the different protein bands of a SDS-PAGE of Flavourzyme (Figure 3.4B). Flavourzyme 1000L is sold as an aminopeptidase preparation with at least 1000 LAPU/g (leucine aminopeptidase units/g determined with Leu-*p*NA). However, it has to be mentioned that the substrate Leu-*p*NA is only converted by the LAPA and LAP2 in the Flavourzyme complex, of which the LAPA makes around 90% of the Leu-*p*NA activity (data not shown). We showed that six proposed marker substrates [azocasein (endopeptidases: NP1, NP2, ALP1), H-Ile-*p*NA (LAPA), H-Pro-*p*NA (LAP2), H-Ala-Pro-*p*NA (DPP4), H-Lys-Ala-*p*NA (DPP5), and starch (AMY3)] could be used for the characterization of the Flavourzyme enzymes. It will now be possible to distinguish between respective enzyme activities in any Flavourzyme protein hydrolysis-processes. This is helpful to provide a better understanding of the enzymatic complex and to allow its improved use on different substrates. Furthermore, we realized a time-saving fully automated nine-step purification of the eight Flavourzyme enzymes.

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ABBREVIATIONS USED

ACU, azocasein unit; AEX, anion exchange chromatography; ALP1, alkaline protease 1; AMY3, α-amylase A type-3; CEX, cation exchange chromatography; CV, column volume, DPP4, dipeptidyl peptidase 4; DPP5, dipeptidyl peptidase 5; double distilled water, H2Odd; dimethylformamide, DMF; HIC, hydrophobic interaction chromatography; LAPA, leucine aminopeptidase A; LAP2, leucine aminopeptidase 2; NP1, neutral protease 1; NP2, neutral protease 2; pNA, p-nitroanilide; SEC, size-exclusion chromatography.

SUPPORTING INFORMATION AVAILABLE

Table S2 and Figures S1 and S3 – S10. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01665

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CHAPTER FOUR

WHEAT GLUTEN HYDROLYSIS USING ISOLATED FLAVOURZYME PEPTIDASES: PRODUCT INHIBITION AND DETERMINATION OF SYNERGISTIC EFFECTS USING RESPONSE SURFACE METHODOLOGY

This chapter was published

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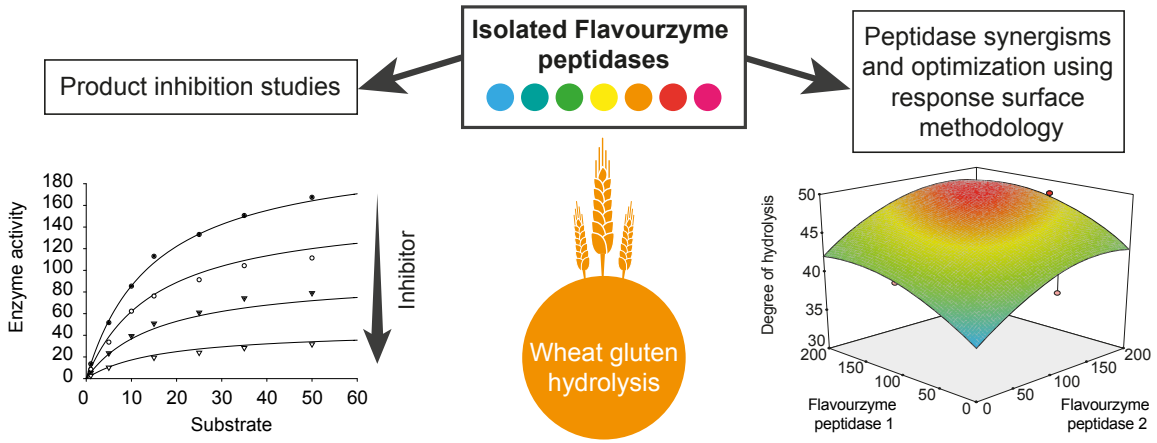
Time of investigation: 07/2014 - 07/2015

Graphical Abstract



ABSTRACT

The commercial peptidase preparation Flavourzyme is derived from the *Aspergillus oryzae* strain ATCC 42149/RIB 40 (Yellow koji mold) and is widely used for protein hydrolysis in various industrial and research applications. However, a biochemical characterization of the Flavourzyme peptidases is difficult, because obtaining purified proteins is essential when functional and structural characterization studies are targeted. Key enzyme activities (three endopeptidases, two aminopeptidases, two dipeptidyl peptidases) have recently been identified and isolated from this commercially available enzyme preparation. The impact and the synergism of theses peptidases on the complex wheat gluten hydrolysis are yet unclear. However, the knowledge about the latter is crucial for an efficient protein hydrolysis. In the present study, we determined the product inhibition for the seven isolated peptidases and analyzed the impact of each peptidase on the wheat gluten hydrolysis using response surface methodology. In general, both aminopeptidases and the three endopeptidases were of major importance. One of the endopeptidases (alkaline protease 1) was least affected by product inhibition and showed the highest effect on the wheat gluten hydrolysis. In the case of the aminopeptidases, the leucine aminopeptidase 2 showed a higher impact on the hydrolysis compared to the leucine aminopeptidase A, but exhibited the highest product inhibition sensitivity. The dipeptidyl peptidases were of only minor impact on the wheat gluten hydrolysis. To conclude: six out of the seven peptidases contributed significantly ($p < 0.05$) to the wheat gluten hydrolysis and should be considered when designing such a process.

KEYWORDS

Flavourzyme, Product inhibition, Response surface methodology, Wheat gluten hydrolysis, Synergistic effects

INTRODUCTION

Protein hydrolysates are versatile food commodities and can be used for emulsification, gelatinization or as seasoning [1]. The industrial biotransformation of proteins is currently mainly performed in discontinuous batch processes [2]. In some cases the application of the enzyme membrane reactor technology can be advantageous [3]. However the knowledge about the product and substrate inhibition of the used enzymes is important information for an appropriate bioreaction engineering [4].

Flavourzyme is a commercial peptidase preparation that is used for protein hydrolysis in various industrial and research applications [5–12]. Exemplarily, the production of flavoring hydrolysates [8], angiotensin converting enzyme inhibitory peptides [9, 10], antioxidative or radical scavenging peptides [11, 13] and emulsifying peptides [12] have been described. Additionally, Flavourzyme peptidases have already been used for protein hydrolysis in continuous membrane reactor systems [5, 6, 14].

Obtaining purified proteins is essential when functional and structural characterization studies are targeted [15]. Recently, enzymes of Flavourzyme have been identified and isolated with an automated purification protocol [16]. The impact of the seven identified and purified peptidases on the hydrolysis of proteins has not been known until now.

In this study, the isolated Flavourzyme peptidases were used to undertake product inhibition studies and to determine their influence and the synergisms on a wheat gluten hydrolysis using response surface methodology. This will provide a better understanding of the complex wheat gluten hydrolysis process using Flavourzyme peptidases and allows an improved process control. Response surface methodology is a collection of mathematical and statistical techniques that are useful for modeling and analyzing problems in which a response of interest is influenced by several variables and the objective is to determine the influence of various factors [17]. It is an established method for the description and optimization of enzymatic biotransformations [18–22].

MATERIALS AND METHODS

Materials and chemicals

Flavourzyme 1000L (Novozymes, Bagsværd, Denmark) and protein-glutaminase "Amano" 50 (Amano, Oxfordshire, United Kingdom) were a gift from the Nestlé Product Technology Center (Singen, Germany). All *para*-nitroanilide (*p*NA) substrates were obtained from Bachem (Bubendorf, Switzerland). Azocasein was bought from Sigma

Aldrich (Taufkirchen, Germany). All other chemicals were of analytical grade and were obtained from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany).

Analytical methods

Determination of amino groups with ortho-phthalaldehyde

Primary amino groups were determined after derivatization with *ortho*-phthalaldehyde (OPA), according to the method of Nielsen et al. [23], with some modifications. A sample volume of 25 µL was transferred into a microtiter plate and 175 µL OPA reagent was added. For the OPA reagent, 1.5 g L^{-1} OPA, 3 g L^{-1} DTT (dithiothreitol) and 11.25 % (v/v) methanol were solved in 120 mM sodium tetraborate decahydrate buffer (adjusted to pH 9.6 with NaOH). The plate was incubated at 37 °C for 1 min and the absorbance was measured at 340 nm using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using L-serine as a reference.

Degree of hydrolysis

The degree of hydrolysis (DH) was calculated according to Adler-Nissen [24], with modifications (Eq. 4.1).

(Eq. 4.1) $$\mathbf{DH} = \frac{h}{h_{tot}} \times \mathbf{100}\ [\%]$$

where h is the concentration of free amino groups [mol L^{-1}; see Section 2.2.1] and h_{tot} is the maximum concentration of free amino groups at complete hydrolysis [mol L^{-1}] calculated according to Eq. 4.2.

(Eq. 4.2) $$\boldsymbol{h_{tot}} = \frac{\mathbf{c_{Protein}}}{\boldsymbol{M^{*}-M_{H_2O}}}\ [\mathbf{mol\ L^{-1}}]$$

where $c_{Protein}$ is the concentration of protein which is hydrolyzed (g L^{-1}) and M^* is the average molecular mass of the amino acids in wheat gluten (133.5 g mol^{-1}). This gluten-specific average molecular mass was calculated by considering the wheat gluten amino acid composition [25]. The molecular mass of water (M_{H20} = 18 g mol^{-1}) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

Endopeptidase activity assay

The azocasein assay was performed according to the method of Iversen and Jørgensen [26], with some modifications. Azocasein (5 g L^{-1}, substrate solution) was dissolved in H_2O_{dd} and the assay was performed as follows: The substrate solution (125 μL) was

preincubated with 125 μL of the respective buffer solution at 37 °C for 5 min. The reaction was started with the addition of enzyme solution (50 μL) and stopped by the addition of trichloroacetic acid (1.5 M, 50 μL). After subsequent centrifugation (20000 × *g*, 4 °C, 5 min), a volume of 195 μL of the supernatant was transferred into a microtiter plate, 65 μL NaOH (1 M) was added and the absorbance at 450 nm was measured using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). One azocasein unit (ACU) was defined as the increase of 1 absorbance unit per min at 450 nm in 0.25 M NaOH.

Aminopeptidase and dipeptidyl peptidase activity assay

Aminopeptidase and dipeptidyl peptidase activities were measured according to the method of Chrispeels and Boulter [27], with some modifications [28]. The assay contained 177 μL of the respective buffer solution and 50 μL enzyme solution. Buffer and enzyme were preincubated at 37 °C for 5 min. The reaction was started by the addition of 12.5 μL of the corresponding *p*NA derivative solved in dimethylformamide. The final substrate concentration in the assay was 3.7 mM (Ile-, Pro-, Ala-Pro-*p*NA) or 1 mM (Lys-Ala-*p*NA), respectively. The reaction was stopped by the addition of 50 % (v/v) acetic acid (50 μL). After centrifugation (20000 × *g*, 4 °C, 5 min), the supernatant (240 μL) was transferred into a microtiter plate and the absorbance at 405 nm was measured using a microtiter plate reader Multiskan FC, Thermo Scientific, Braunschweig, Germany). One katal (kat) of aminopeptidase or dipeptidyl peptidase activity was defined as the release of 1 mol *p*NA per s.

Flavourzyme peptidases

The purification of the Flavourzyme peptidases from *Aspergillus oryzae* strain ATCC 42149/RIB 40 (Yellow koji mold) was the same as reported previously [16]. The peptidases were a leucine aminopeptidase A (LAPA), a leucine aminopeptidase 2 (LAP2), a dipeptidyl peptidase 4 (DPP4), a dipeptidyl peptidase 5 (DPP5), a neutral protease 1 (NP1), a neutral protease 2 (NP2) and an alkaline protease 1 (ALP1).

2.4. Determination of enzyme kinetics and product inhibition of Flavourzyme peptidases

A Flavourzyme/wheat gluten hydrolysate was used to determine the product inhibition of the seven isolated Flavourzyme peptidases. Therefore, a batch hydrolysis of 100 g L^{-1} wheat gluten was carried out in Tris-HCl buffer (20 mM, pH 7.5) at 37 °C with 1 % (v/v) Flavourzyme. The DH was monitored to check when the equilibrium (stable DH) was reached. This was realized after 10 h and the hydrolysis was stopped by heating the

solution to 90 °C for 15 min using a water bath. The hydrolysate obtained was freeze-dried (1 mbar; Alpha 1-2 freeze dryer, Christ GmbH, Osterode am Harz, Germany) to produce a powder. The powder was pestled and used as an inhibitor mixture for product inhibition studies.

Enzyme kinetics were determined at 37 °C at the respective optimum pH of the peptidases [16]. The substrates Ile-*p*NA, Pro-*p*NA and Ala-Pro-*p*NA in 50 mM Bis-Tris-propane buffer at pH 9, pH 9.5 and pH 8.5 was used for the LAPA, LAP2 and DPP4, respectively. Enzyme kinetics of the DPP5 was determined in 50 mM citrate phosphate buffer, pH 6, with Lys-Ala-*p*NA as a substrate. Enzyme kinetics of the NP1, NP2 and ALP1 were determined with azocasein as a substrate in citrate phosphate buffer at pH 7, pH 5 and pH 7, respectively.

A quantity of 50 µL of the corresponding assay buffer was replaced by different inhibitor mixture stock solutions for the product inhibition studies. The final inhibitor mixture concentration ranged from 0.25 g L^{-1} to 25 g L^{-1}. The final substrate concentration for the aminopeptidases and dipeptidyl peptidases varied from 0.3 to 50 mM, depending on the particular *p*NA derivative. The final substrate concentration in the enzyme activity assay for the endopeptidases ranged from 0.2 to 5 g L^{-1} azocasein. The initial reaction rate (v_{max}), the Michaelis constant (K_M), the inhibition constant (K_I) and the type of inhibition were then calculated by nonlinear regression using the Enzyme Kinetics Module (SigmaPlot 12.5; Systat Software GmbH, Erkrath, Germany).

Surface-based modeling to optimize a Flavourzyme/wheat gluten hydrolysis

A surface-based modeling was chosen to evaluate the synergistic effects of seven Flavourzyme peptidases on a wheat gluten hydrolysis. Response surface methodology is a collection of mathematical and statistical techniques that are useful for modeling and analyzing problems in which a response of interest is influenced by several variables and the objective is to determine the influence of various factors and to optimize the response [17].

The experimental design and statistical analysis were performed using Design Expert Software Version 8 (Stat-Ease Inc., Minneapolis, USA). The level of significance was evaluated by variance analysis (ANOVA). In this study, we employed a central composite design (CCD) to determine the influence of seven Flavourzyme peptidases (k = 7) on the wheat gluten hydrolysis.

The CCD consisted of 2^k factorial points (-1; 1), 2k axial points (-α; +α), and 3 center points (0). The axial points provide additional levels of the factor for purposes of estimation of the quadratic terms [29]. Furthermore, nine additional vertices points were included. In total, 154 experiments were conducted for one CCD. All experiments were conducted with 2 and 10 g L^{-1} substrate (see below) in random order resulting in 308 batch hydrolyses. Samples were taken after 6, 12 and 24 h. The two categorical factors (time and substrate concentration) were employed in the model.

The central point (0) corresponded to the proportion of the peptidases in the original Flavourzyme preparation and was defined as 100 %. For each numerical factor (k), the low level (-1) was defined as 50 % and the high level (+1) was defined as 150 %. The axial points were 0 % (-α) and 200 % (+α). The CCD of the 308 batch hydrolyses is listed in Table A.1 (Supplementary data). The axial points are listed from standard order 1 – 14, factorial points are listed from standard order 15 – 142, the additional vertices points are listed from standard order 143 – 151 and the center points are listed from standard order 152 – 154 (Table A.1).

A quadratic model was used (ANOVA) to assess the dependency for each factor on the DH (response), as described by Thompson [30]. A quadratic polynomial equation (Eq. 4.3; modified after Thompson [30]) was used to calculate the response (DH).

(Eq. 4.3) $$\mathbf{DH}\ [\%] = \boldsymbol{\beta_0} + \sum_{i=1}^{k} \boldsymbol{\beta_i X_i} + \sum_{i \le j}^{k} \boldsymbol{\beta_{ij} X_i X_j} + \sum_{i=1}^{k} \boldsymbol{\beta_{ii} X_i^2}$$

where β_0 is the constant, β_i the linear coefficients, β_{ij} the cross product coefficients, β_{ii} the quadratic coefficients, and X_i and X_j are the levels of the numerical and categorical factors (X_1: LAPA; X_2: LAP2; X_3: DPP4; X_4: DPP5; X_5: ALP1; X_6: NP1; X_7: NP2; X_8: Substrate concentration; X_9: Time). The fit of the model was evaluated by the determination of the R-squared coefficient, the predicted R-squared and the adjusted R-squared coefficient. The evaluation of the optimum values of the model was obtained by solving the regression equation using Design Expert.

Wheat gluten hydrolysis using a small-scale biotransformation screening system

A method for small-scale biotransformations was established to realize 308 parallel batch hydrolyses. A schematic representation of the system is shown in Figure 4.1. All hydrolyses were carried out in deep-well plates in 250 µL scale in 40 mM Tris-HCl buffer (pH 7.5). The 308 batch hydrolyses were carried out in parallel in four deep-well plates.

Plates were incubated at 37 °C on a platform shaker (1000 rpm; Titramax 100, Heidolph, Schwabach, Germany).

The isolated Flavourzyme peptidases were transferred into the wells of the deep-well plate according to Table A.1 (Supplementary data). The enzyme activities for the center point (100 % of each peptidase) were calculated accordingly to comply with the peptidase proportions of the original Flavourzyme preparation. Based on previous experiments a final Flavourzyme concentration of 0.1 % (v/v) was appropriate for the hydrolysis of 1 g L^{-1} wheat gluten. Therefore 100 % enzyme activity corresponded to 11 $pkat_{Ile\text{-}pNA}$ mL^{-1}, 1.8 $pkat_{Pro\text{-}pNA}$ mL^{-1}, 2.4 $pkat_{Ala\text{-}Pro\text{-}pNA}$ mL^{-1}, 3.2 $pkat_{Lys\text{-}Ala\text{-}pNA}$ mL^{-1}, 6.5 ACU mL^{-1}, 0.12 ACU mL^{-1} and 1.6 ACU mL^{-1} for the isolated LAPA, LAP2, DPP4, DPP5, NP1, NP2 and ALP1, respectively. A corresponding volume of double distilled water (H_2O_{dd}) was added to the peptidase fractions to a final volume of 200 µL.

A wheat gluten suspension treated with protein glutaminase (PG) was used as a substrate solution due to the insufficient solubility of the untreated wheat gluten. We observed in previous studies that the hydrolysis kinetics were similar for the native wheat gluten and PG-treated wheat gluten with Flavourzyme (data not shown). Therefore, 50 g L^{-1} wheat gluten was deamined with 5 g L^{-1} protein glutaminase (Protein-glutaminase "Amano" 50) in Tris-HCl buffer (200 mM, pH 7.5) at 37 °C for 8 h. After deamination, the suspension was heated to 90 °C for 15 min to inactivate the enzymes.

The addition of the substrate solution (50 µL) started the biotransformation. Final substrate concentrations of 2 and 10 g L^{-1} were targeted for the biotransformations. Samples (25 µL) were taken after 6, 12 and 24 h and transferred in 100 µL trichloroacetic acid (0.25 M) in microtiter plates to stop the reaction (Figure 4.1). Samples were further diluted if necessary, and the DH was determined (see above).

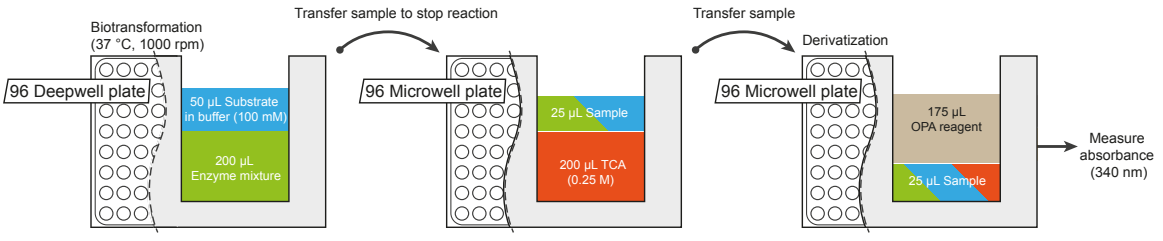


Figure 4.1: Schematic representation of the small-scale biotransformations system for the wheat gluten hydrolysis with Flavourzyme peptidases. Hydrolyses were carried out in deep-well plates in 250 µL scale in 40 mM Tris-HCl buffer (pH 7.5). Plates were incubated at 37 °C on a platform shaker (1000 rpm; Titramax 100, Heidolph).

Statistical analysis

The trials for the determination of enzyme kinetics and product inhibition of Flavourzyme peptidases were conducted at least in duplicate, with two independent measurements. The standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA). Hydrolyses for the modeling of the synergistic effects of the Flavourzyme peptidases and optimization of the wheat gluten hydrolysis were performed in single determination with two independent measurements of the free amino groups formed.

RESULTS AND DISCUSSION

Flavourzyme is widely used in research and industrial applications. As known from previous studies, Flavourzyme is a complex mixture of at least eight enzymes, including seven peptidases [16]. Product inhibition has been observed for the crude Flavourzyme [5, 14], but reliable information about enzyme kinetics is only obtained if isolated enzymes are used for characterization. Therefore, we carried out product inhibition studies with seven isolated Flavourzyme peptidases. Furthermore, the impact of each Flavourzyme peptidase on the hydrolysis of wheat gluten hydrolysis was determined using response surface methodology.

Product inhibition studies of Flavourzyme peptidases

Product inhibition was tested for the isolated Flavourzyme peptidases with a Flavourzyme/wheat gluten hydrolysate as an inhibitor mixture (see above). The DH of this hydrolysate (inhibitor mixture) was 44.7 % and results in an theoretical average gluten fragment size of 2.24 amino acids. Additionally, a total free amino acid concentration of 26.3 % was determined by HPLC analysis after precolumn derivatization with OPA (data not shown). Therefore, the theoretical average gluten fragment size is approximately four amino acids when the amount of free amino acids are subtracted.

The product inhibition studies were carried out with 5 – 25 g L^{-1} inhibitor mixture for the LAPA and DPP5, 0.25 – 5 g L^{-1} inhibitor mixture for the LAP2, 1 – 15 g L^{-1} inhibitor mixture for the DPP4 and 4.2 – 21 g L^{-1} inhibitor mixture for the NP1, NP2 and ALP1. The Michaelis-Menten kinetics of the Flavourzyme peptidases are shown on Figure 4.2, including the regression of the respective enzyme kinetic which was fitted to the apparent inhibition type. The initial reaction rates, the kinetic parameters and the apparent types of inhibition of the Flavourzyme peptidases are summarized in Table 4.1.

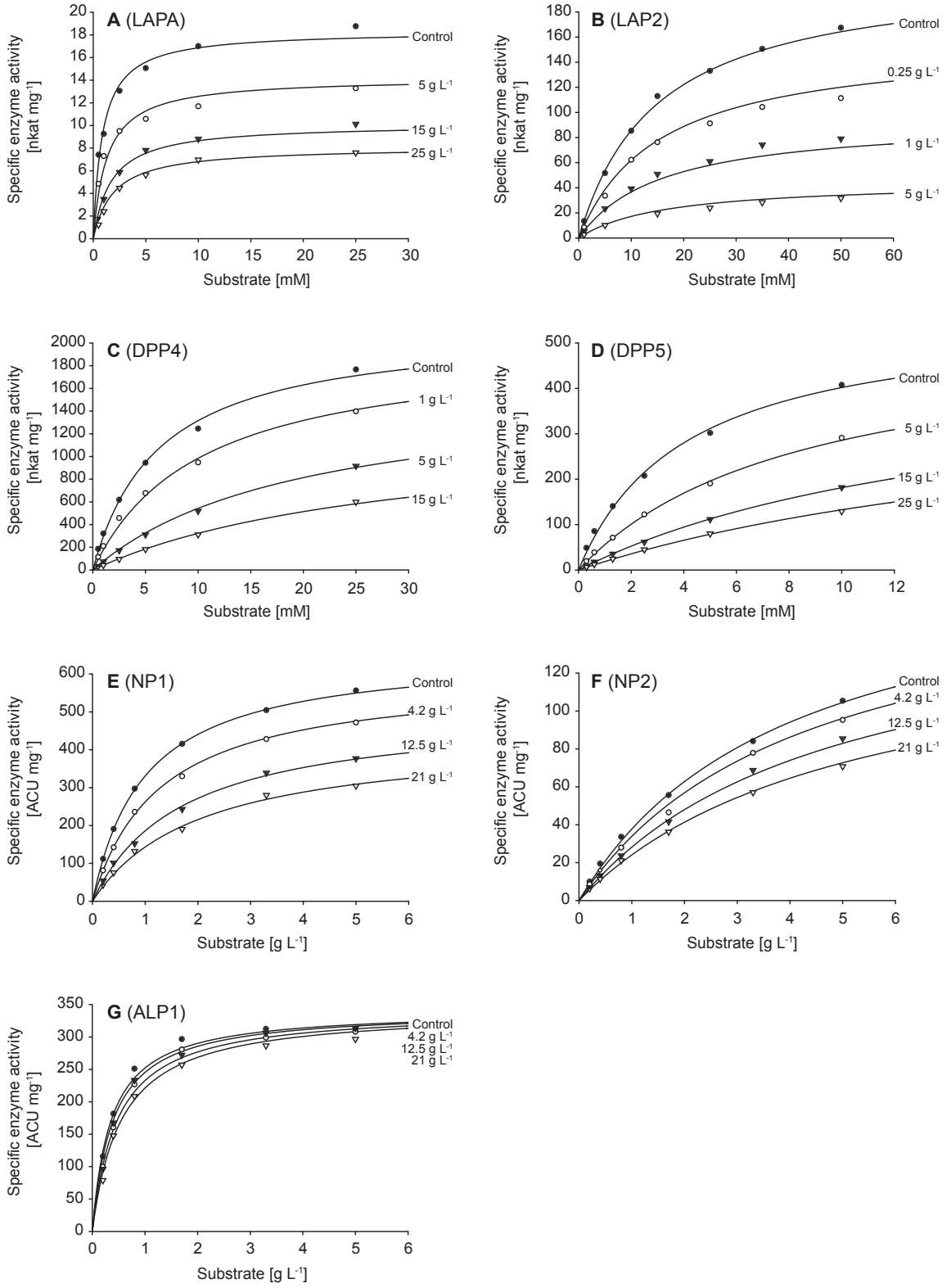


Figure 4.2: Michaelis–Menten plots of the isolated Flavourzyme peptidases with varying inhibitor mixture (Flavourzyme/wheat gluten hydrolysate) concentrations. Product inhibition studies were carried out in duplicate at 37 °C at the respective optimum pH of the peptidases. Standard deviation was less than 5 %.

Table 4.1: Apparent product inhibition type and enzyme kinetic parameters of isolated Flavourzyme peptidases after nonlinear regression using a Flavourzyme/wheat gluten hydrolysate as inhibitor mixture.

Exopeptidases	**Apparent Inhibition type**	v_{max} **[nkat mg^{-1}]**	K_M **[mM]**	$K_{I,\ apparent}$ **[g L^{-1}]**
LAPA	Mixed	18.2 ± 0.4	0.9 ± 0.1	5.1 ± 1.2
LAP2	Mixed	214 ± 8	15.1 ± 1.5	0.6 ± 0.1
DPP4	Mixed	2142 ± 57	6.3 ± 0.4	1.2 ± 0.1
DPP5	Competitive	566 ± 14	4.1 ± 0.2	4.1 ± 0.3
Endopeptidases	**Apparent Inhibition type**	v_{max} **[ACU mg^{-1}]**	K_M **[g L^{-1}]**	$K_{I,\ apparent}$ **[g L^{-1}]**
NP1	Mixed	660 ± 8	1.0 ± 0.03	8.9 ± 0.9
NP2	Competitive	176 ± 6	3.5 ± 0.2	22.8 ± 1.6
ALP1	Competitive	342 ± 5	0.4 ± 0.02	39.3 ± 9.2

Since a complex inhibitor mixture is used, the inhibition constant is expressed in g L^{-1}. Other mechanisms, such as substrate inhibition, might be of influence, due to the presence of free amino acids and gluten fragments. Therefore, the inhibition constant is hereafter expressed as apparent inhibition constant ($K_{I,\ apparent}$).

The aminopeptidases and dipeptidyl peptidases showed increased product inhibition compared to the endopeptidases. The LAP2 showed the highest product inhibition with a $K_{I,\ apparent}$ of 0.6 g L^{-1} inhibitor mixture. The ALP1 showed the least product inhibition resulting in a $K_{I,\ apparent}$ of 39.3 g L^{-1}. An apparent mixed type inhibition was observed for the LAPA, LAP2, DPP4 and NP1, whereas, an apparent competitive inhibition type was observed for the DPP5, NP2 and ALP1.

Product inhibition of the peptidases has been described for the Flavourzyme preparation previously. Berends et al. [5] tested the influence of different amino acids on the enzyme kinetic of one aminopeptidase in Flavourzyme. They concluded that mainly L-cysteine inhibited the Flavourzyme activity when H-Leu-*p*NA was used as a substrate [5]. An apparent mixed type inhibition for L-cysteine was observed [5]. Giesler et al. [14] also described product inhibition for the Flavourzyme peptidases. However, the results indicated an apparent competitive product inhibition of the Flavourzyme complex. Since the overall proteolytic activity was determined and the hydrolysis was carried out for 20 h, the initial reaction rate and the kinetic parameters could not be determined in the study [14] and are, therefore, not comparable to the current study

Modeling of the synergistic effects of the Flavourzyme peptidases on the wheat gluten hydrolysis

In order to determine the influence of each Flavourzyme peptidase on the wheat gluten hydrolysis, 308 batch hydrolyses were carried out in parallel using a small-scale biotransformation screening system (see above). Flavourzyme peptidase activities were varied from 0 – 200 % according to Table A.1 (see Supplemental data). In particular, the enzyme activities applied were 0 – 22 pkat mL^{-1} (LAPA), 0 – 3.6 pkat mL^{-1} (LAP2), 0 – 4.8 pkat mL^{-1} (DPP4), 0 – 6.4 pkat mL^{-1} (DPP5), 0 – 13 ACU mL^{-1} (NP1), 0 – 0.24 ACU mL^{-1} (NP2) and 0 – 3.2 ACU mL^{-1} (ALP1), respectively. The responses (DH) of the experiments depending on the peptidase mixture, the hydrolysis time and the substrate concentration with the corresponding standard order are listed in Table A.2 (see Supplemental data).

Table 4.2: ANOVA table for the adjusted model of response (DH) from enzymatic hydrolysis of wheat gluten with isolated Flavourzyme peptidases.

Source	Sum of squares	Degree of freedom	Mean square	F value	p-value (Prob > F)
Model	98479	61	1614	162	< 0.0001
Residual	8580	862	9.95		
Total	107059				

R^2 = 0.9199; Adj. R^2 = 0.9142; Pred. R^2 = 0.9039

The responses of the 924 sample points (308 hydrolysis; three sampling times) were used and applied to a quadratic polynomial model (see Eq. 4.3). The results of the response surface model in the form of ANOVA are given in Table 4.2. The F-value (162) and the low p-value (< 0.0001) demonstrate a high significance for the regression model [31, 32]. A lack of fit F-value of 0.99 implies that the lack of fit is not significant relative to the pure error. The model fit was evaluated by the coefficient of determination (R^2). In this case, the value of R^2 = 0.9199, which indicates a good fit. A value of R-squared > 0.75 indicates the suitability of the model [33]. The value of the adjusted coefficient of determination (Adj. R^2 = 0.9142) is also in reasonable agreement with the coefficient of determination predicted (Pred. R^2 = 0.9039) and stands for a high significance of the model [31, 32]. The correlation of the responses predicted (DH) from the model and the actual responses, which were measured, are shown in Figure 4.3. The coefficient of

variation for this model is 7.64 %. Significant model terms (p value < 0.05) of the polynomial equation (see Eq. 4.3) with the corresponding results of the response surface model in the form of ANOVA are given in Table 4.3. The results indicate that linear terms of the substrate concentration and the time have the highest impact on the DH. Regarding the peptidases, endopeptidases and aminopeptidases have a higher impact compared to the dipeptidyl peptidases. The effect of the DPP5 is not significant and is, therefore, not listed in Table 4.3. Linear effects are higher for the LAP2 and the ALP1, demonstrating that these are important factors/peptidases for the wheat gluten hydrolysis. All coefficients for the polynomial equation (see Eq. 4.3) are listed in Table 4.4.

Table 4.3: ANOVA table for the factors that showed significant (p-value < 0.05) model terms from the enzymatic hydrolysis of wheat gluten with isolated Flavourzyme peptidases.

Source	Sum of squares	Degree of freedom	F value	p-value (Prob > F)
X_1 : LAPA	657.47	1	66.05	< 0.0001
X_2 : LAP2	1445.15	1	145.18	< 0.0001
X_3 : DPP4	113.06	1	11.36	0.0008
X_5 : ALP1	2651.25	1	266.35	< 0.0001
X_6 : NP1	1097.95	1	110.3	< 0.0001
X_7 : NP2	1150.14	1	115.54	< 0.0001
X_8 : Substrate	66367	1	6667.30	< 0.0001
X_9 : Time	17867	2	897.47	< 0.0001
X_{12}	202	1	20.19	< 0.0001
X_{14}	89	1	8.92	0.0034
X_{15}	105	1	10.47	0.0013
X_{38}	259	1	25.94	< 0.0001
X_{57}	101	1	10.13	0.0015
X_{78}	45	1	4.52	0.0338
X_{89}	79	2	3.97	0.0192
X_{22}	59	1	5.90	0.0153
X_{55}	107	1	10.70	0.0011
X_{66}	64	1	6.35	0.0119

Influence of the peptidase activities on the degree of hydrolysis

The impact of the aminopeptidases (LAPA, LAP2) on the wheat gluten hydrolysis was modeled (see above) and is shown in Figure 4.4 A and B for 2 and 10 g L^{-1} substrate

(wheat gluten treated with PG), respectively. The DH in the example contour graph is plotted over the LAPA and LAP2 activity for the 6 h samples. The enzyme activity of the aminopeptidases ranges from 0 to 200 %, whereas the activity of the other enzymes remain constant at 100 %. The LAP2 seemed to have a slightly higher impact on the DH compared to the LAPA. The DH increases from 39 % to over 47 % when the LAP2 activity increases, whereas the DH increases slightly lower, from 39 % to over 45 %, when the LAPA activity increases (substrate: 2 g L^{-1}). This effect was not observed for a higher substrate concentration (10 g L^{-1}). Here, the impact of the LAPA and LAP2 activity on the DH is comparable. This can be explained by the higher product inhibition of the LAP2 compared to the LAPA, as described previously (see above). The overall higher impact of the LAP2 can also be derived from the model coefficients β_1 and β_2 (Table 4.4). The LAP2 coefficient (β_2) is +2.460 and is higher compared to the LAPA coefficient (β_1), which is +1.720. Thus, the response is influenced to a greater extent by the LAP2 activity compared to the LAPA activity. The importance of aminopeptidases from *Aspergillus oryzae* 460 on the liberation of glutamic acid from soybean protein has been shown previously [34]. With experimental design, they also found out that the LAP2 is of particular importance [34].

The impact of the dipeptidyl peptidases (DPP4, DPP5) on the wheat gluten hydrolysis is shown in Figure 4.4 C and D for 2 and 10 g L^{-1} substrate (wheat gluten treated with PG), respectively. The DH in the exemplary contour graph is plotted over the DPP4 and DPP5 activity for the 6 h samples. The enzyme activity of the dipeptidyl peptidases ranges from 0 to 200 %, whereas the activity of the other enzymes remains constant at 100 %. The impact of the DPP4 is moderate and lower compared to the aminopeptidases. The DH increases from around 45 % to around 47 % when the DPP4 activity increases from 0 to 200 % in the presence of 2 g L^{-1} substrate (Figure 4.4 C). The DPP5 has no significant impact on the DH and is, therefore, not listed in Table 4.3. The low impact of the dipeptidyl peptidases can be derived from the low model coefficients β_3 (+0.670) and β_4 (+0.120) for the DPP4 and DPP5, respectively (Table 4.4).

The impact of the endopeptidases (ALP1, NP1, NP2) on the wheat gluten hydrolysis was modeled (see above) and is shown in Figure 4.5 A – D. The DH in the exemplary contour graphs is plotted over the ALP1 and NP1 activity (Figure 4.5 A and B) and over the ALP1 and NP2 activity (Figure 4.5 C and D) for the 6 h samples with different substrate concentrations (Figure 4.5 A and C: 2 g L^{-1}; Figure 4.5 B and D: 10 g L^{-1}). The enzyme

activity of the respective endopeptidases ranges from 0 to 200 %, whereas activity of the other enzymes remain constant at 100 %.

Table 4.4: Model coefficients of the quadratic polynomial equation (Eq. 4.3) calculated by quadratic regression.

Factor	Coefficient	Factor	Coefficient
Intercept	+44.220 (β_0)	X_{37}	+0.410 (β_{37})
X_1 : LAPA	+1.720 (β_1)	X_{38}	-1.020 (β_{38})
X_2 : LAP2	+2.460 (β_2)	$X_{39[1]}$	-0.057 ($\beta_{39[1]}$)
X_3 : DPP4	+0.670 (β_3)	$X_{39[2]}$	+0.210 ($\beta_{39[2]}$)
X_4 : DPP5	+0.120 (β_4)	X_{45}	-0.840 (β_{45})
X_5 : ALP1	+3.310 (β_5)	X_{46}	-0.280 (β_{46})
X_6 : NP1	+2.110 (β_6)	X_{47}	-0.810 (β_{47})
X_7 : NP2	+2.150 (β_7)	X_{48}	+0.170 (β_{48})
X_8 : Substrate	-8.480 (β_8)	$X_{49[1]}$	-0.130 ($\beta_{49[1]}$)
$X_{9[1]}$: Time	-5.310 ($\beta_{9[1]}$)	$X_{49[2]}$	-0.220 ($\beta_{49[2]}$)
$X_{9[2]}$: Time	-0.170 ($\beta_{9[2]}$)	X_{56}	-0.150 (β_{56})
X_{12}	-1.850 (β_{12})	X_{57}	-1.390 (β_{57})
X_{13}	-0.830 (β_{13})	X_{58}	-0.280 (β_{58})
X_{14}	-1.240 (β_{14})	$X_{59[1]}$	-0.460 ($\beta_{59[1]}$)
X_{15}	+1.380 (β_{15})	$X_{59[2]}$	+0.330 ($\beta_{59[2]}$)
X_{16}	-0.370 (β_{16})	X_{67}	-0.280 (β_{67})
X_{17}	+0.230 (β_{17})	X_{68}	-0.630 (β_{68})
X_{18}	-0.016 (β_{18})	$X_{69[1]}$	-0.025 ($\beta_{69[1]}$)
$X_{19[1]}$	+0.130 ($\beta_{19[1]}$)	$X_{69[2]}$	+0.086 ($\beta_{69[2]}$)
$X_{19[2]}$	+0.067 ($\beta_{19[2]}$)	X_{78}	-0.420 (β_{78})
X_{23}	+0.290 (β_{23})	$X_{79[1]}$	-0.310 ($\beta_{79[1]}$)
X_{24}	-0.320 (β_{24})	$X_{79[2]}$	+0.050 ($\beta_{79[2]}$)
X_{25}	+0.210 (β_{25})	$X_{89[1]}$	+0.015 ($\beta_{89[1]}$)
X_{26}	+0.180 (β_{26})	$X_{89[2]}$	+0.350 ($\beta_{89[2]}$)
X_{27}	+0.550 (β_{27})	X_{11}	-0.760 (β_{11})
X_{28}	-0.280 (β_{28})	X_{22}	-2.040 (β_{22})
$X_{29[1]}$	-0.200 ($\beta_{29[1]}$)	X_{33}	-0.540 (β_{33})
$X_{29[2]}$	-0.100 ($\beta_{29[2]}$)	X_{44}	-0.680 (β_{44})
X_{34}	+0.630 (β_{34})	X_{55}	-2.710 (β_{55})
X_{35}	-0.750 (β_{35})	X_{66}	-2.090 (β_{66})
X_{36}	+0.110 (β_{36})	X_{77}	-0.960 (β_{77})

All endopeptidases have a high impact on the wheat gluten hydrolysis, as can be seen from the contour graphs (Figure 4.5) and the model terms (Table 4.3). The significant impact of the endopeptidases can also be derived from the model coefficients β_5, β_6 and β_7 (Table 4.4). The highest linear coefficient of the seven peptidases is the one for the ALP1 (3.310). The model coefficients for the NP1 and NP2 are +2.110 and +2.150, respectively. The cross product coefficient of the endopeptidases and the substrate concentration is -0.280 (β_{58}) for the ALP1, -0.620 (β_{68}) for the NP1 and -0.460 (β_{78}) for the NP2, indicating that the ALP1 is least affected by the substrate concentration, followed by the NP2 and NP1. The coefficients of the endopeptidases and the substrate concentration are in accordance with the results, which were obtained from the product inhibition studies. Nakadai et al. [34] also tested the influence of ALP1, NP1 and NP2 on soy protein hydrolysis. In contrast to our study, they only detected a significant effect of the NP1 on the soluble nitrogen after soy protein hydrolysis [34].

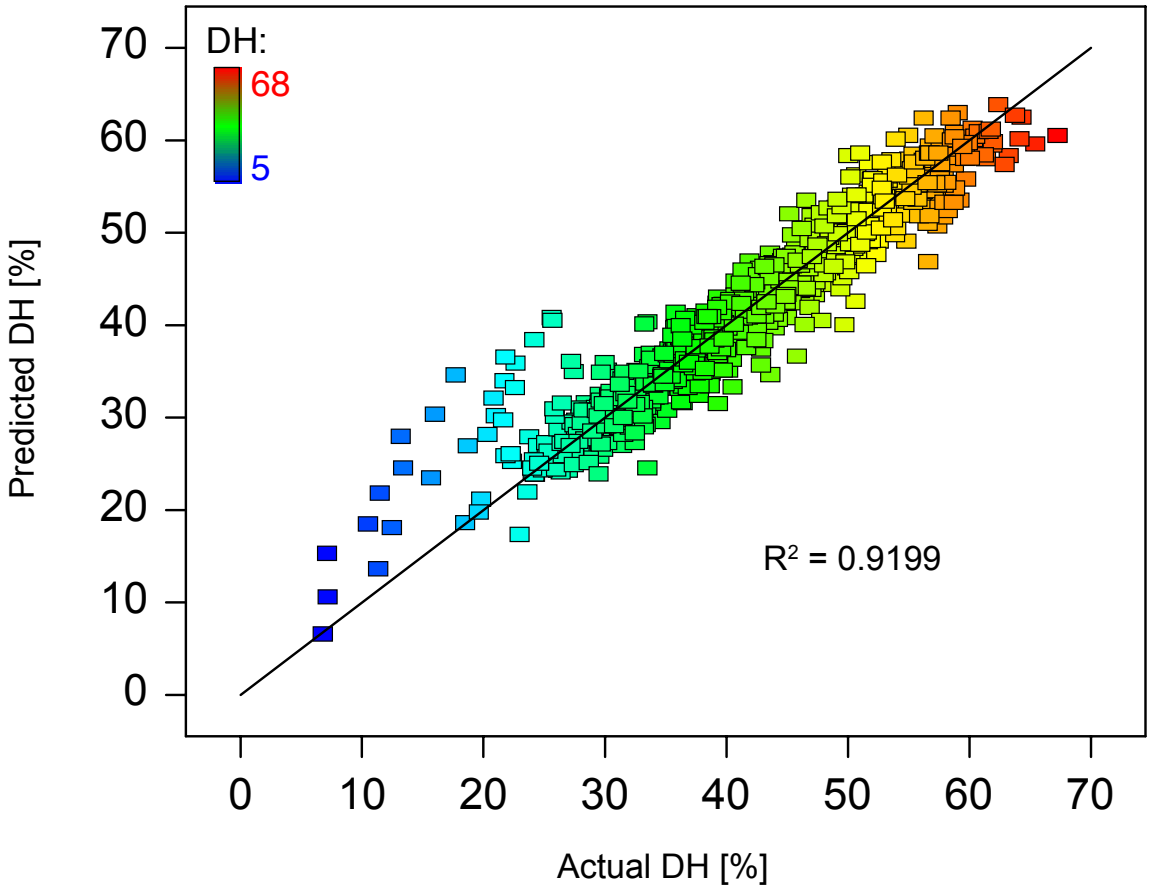


Figure 4.3: Predicted response versus actual response of the quadratic model used for wheat gluten hydrolysis by Flavourzyme peptidases.

A significant synergistic effect was observed for the LAPA and the ALP1 (β_{15} = +1.380), as shown in Tables 3 and 4. Significant antagonistic effects were observed for the LAPA and LAP2 (β_{12} = -1.850), for the LAPA and DPP5 (β_{14} = -1.240) and for the ALP1 and NP2 (β_{57} = -1.390).

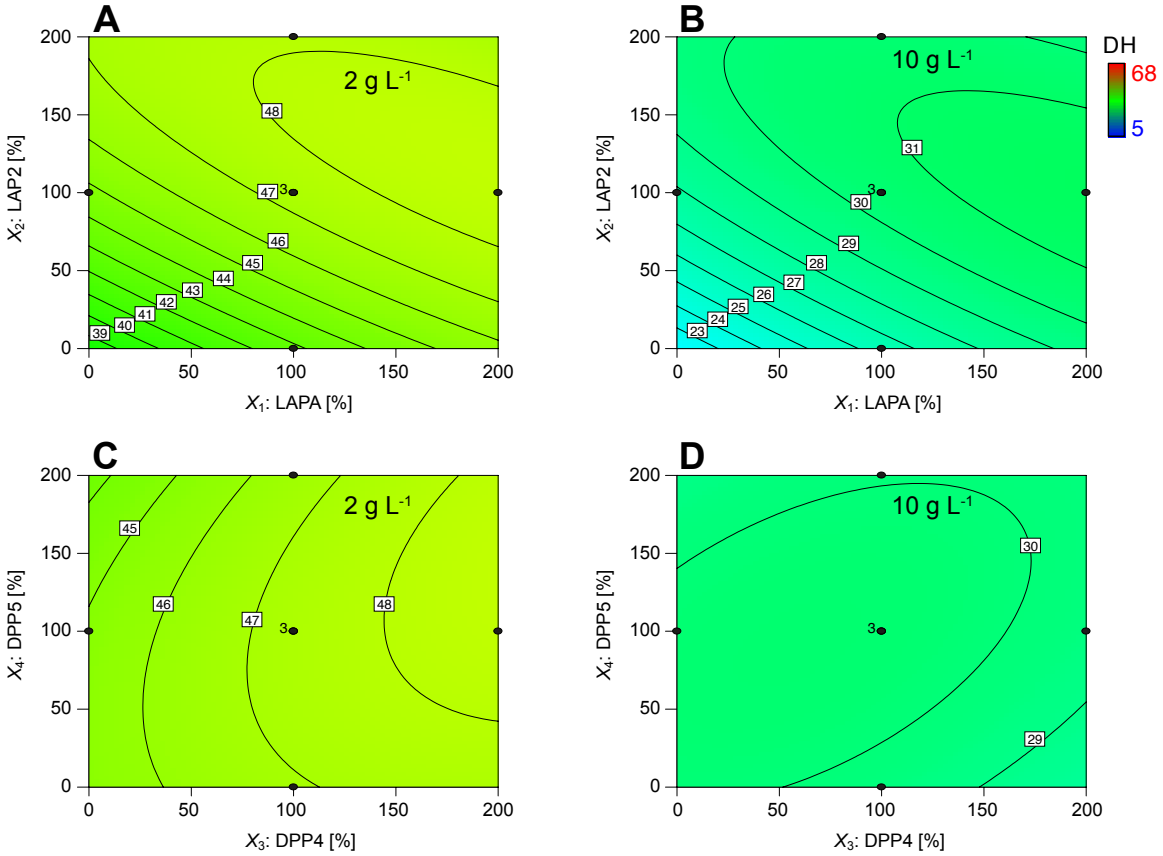


Figure 4.4: Response surface plots displaying the relative effect of two variables (aminopeptidases and dipeptidyl peptidases) on DH of PG-treated wheat gluten hydrolysis while other factors remained at center level (100 %). The effect of the variables is shown for 2 g L^{-1} (A and C) and 10 g L^{-1} substrate (B and D). Plots display the relative effect at a fixed reaction time of 6 h. The unit of the response (DH) was percent (%). Filled circles indicate actual design points of the CCD.

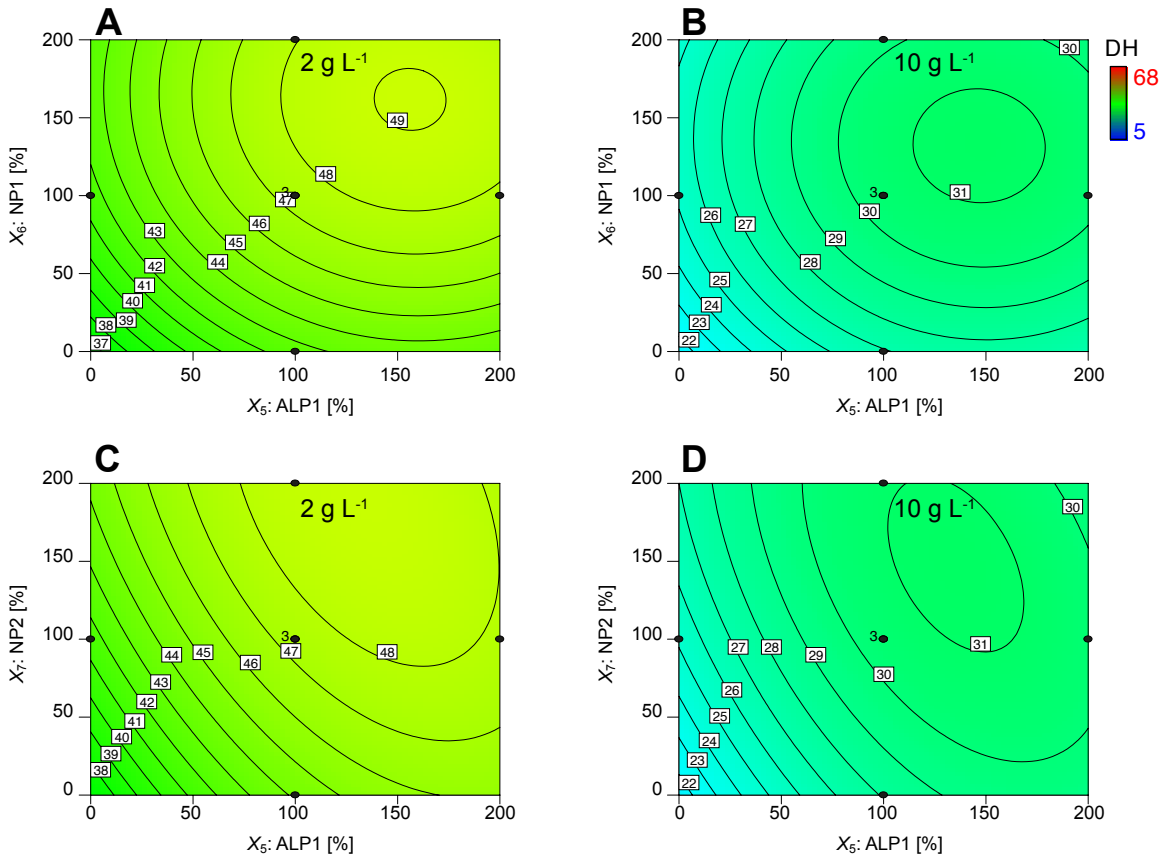


Figure 4.5: Response surface plots displaying the relative effect of two variables (endopeptidases) on DH of PG-treated wheat gluten hydrolysis while other factors remained at center level (100 %). The effect of the variables is shown for 2 g L^{-1} (A and C) and 10 g L^{-1} substrate (B and D). Plots display the relative effect at a fixed reaction time of 6 h. The unit of the response (DH) was percent (%). Filled circles indicate actual design points of the CCD.

Influence of the substrate concentration and hydrolysis time on the degree of hydrolysis

The influence of the substrate concentration and the reaction time is shown in Figure 4.6. The substrate concentration affects the response (DH) significantly, as is also seen from the corresponding model terms and coefficients (Tables 4.3 and 4.4). The DH decreases with higher substrate concentration, which can be explained by the product inhibition of the peptidases described above. Furthermore, and as expected, the DH is higher when the reaction time is prolonged. However, due to the characteristic saturation curve of the hydrolysis, the DH does not increase linearly with increased reaction time. Thus, the time-dependent coefficients ($\beta_{9[1]}$ and $\beta_{9[2]}$) are negative.

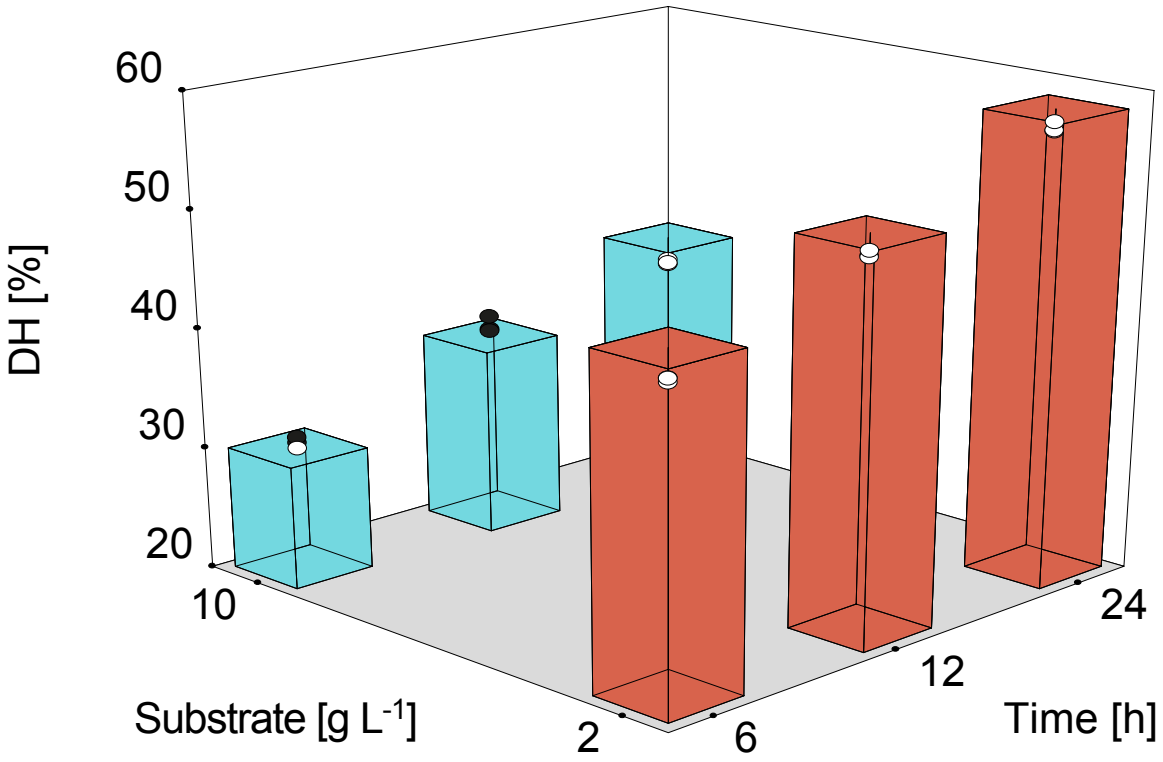


Figure 4.6: Response surface plot displaying the relative effect reaction time and substrate concentration on DH of PG-treated wheat gluten hydrolysis while other factors remained at center level (100 %). Circles indicate actual design points of the CCD; Filled circles: Actual value above predicted value; Open circles: Actual value below predicted value.

Optimization of the wheat gluten hydrolysis

The optimum DH was obtained by solving the regression equation (see Eq. 4.3). The confirmation experiments at the optimum were carried out for 2 and 10 g L^{-1} substrate concentrations and 24 h hydrolysis time. The optimal values of the test variables with 2 g L^{-1} substrate are as follows: X_1 (LAPA): 200 %; X_2 (LAP2): 158 %; X_3 (DPP4): 99 %; X_4 (DPP5): 0 %; X_5 (ALP1): 182 %; X_6 (NP1): 152 %; X_7 (NP2): 200 %.

The optimal values of the test variables with 10 g L^{-1} substrate are as follows: X_1 (LAPA): 200 %; X_2 (LAP2): 136 %; X_3 (DPP4): 0 %; X_4 (DPP5): 0 %; X_5 (ALP1): 200 %; X_6 (NP1): 116 %; X_7 (NP2): 179 %.

The model predicts that the maximum DH that can be obtained using the optimum conditions of variables above is 65.66 % and 47.09 % for 2 and 10 g L^{-1} substrate, respectively. The verification of the results using the optimized conditions was accomplished by carrying out the experiments in quintuplicate. The DH obtained with the

optimized parameters was 64.56 ± 1.4 % for 2 g L^{-1} substrate and 44.57 ± 0.7 % for 10 g L^{-1} substrate. The result showed that the experimental values were in agreement with the predicted values of the model within a 95 % confidence interval (2 g L^{-1}: 62.33 – 68.97 %; 10 g L^{-1}: 43.25 – 50.91 %).

The results showed the applicability of the response surface methodology for the hydrolysis of wheat gluten with seven Flavourzyme peptidases. This methodology was also used previously to optimize the hydrolysis of duck blood corpuscle [35] and fish soluble concentrate [36] with Flavourzyme.

CONCLUSIONS

The type and the magnitude of product inhibition were evaluated for seven isolated Flavourzyme peptidases. Product inhibition was observed for all seven peptidases and was highest for the LAP2 and DPP4 with $K_{I,\ apparent}$ values of 0.6 and 1.2 g L^{-1}, respectively. The ALP1 showed only minor product inhibition ($K_{I,\ apparent}$ = 39.3 g L^{-1}). The continuous and selective removal of released amino acids (e.g. nanofiltration, electrodialysis) would be one way to improve the hydrolysis process, since, exopeptidases exhibited the most distinct product inhibition. Furthermore, the impact of each isolated Flavourzyme peptidase on the wheat gluten hydrolysis was evaluated by response surface methodology. The highest impacts were observed for the endopeptidases and aminopeptidases. The ALP1 particularly showed the highest impact on the wheat gluten hydrolysis. Additionally, the LAP2 exhibited a higher influence on the wheat gluten hydrolysis compared to the LAPA. In conclusion, six out of the seven peptidases contributed significantly to the wheat gluten hydrolysis. The complexity of a Flavourzyme/wheat gluten hydrolysis process was demonstrated by the results presented and protein hydrolysis studies with Flavourzyme should be designed and evaluated accordingly.

ACKNOWLEDGEMENTS

We gratefully acknowledge the cooperation with the Nestlé Product Technology Centre at Singen, Germany.

ABBREVIATIONS

ACU, azocasein unit; ALP1, alkaline protease 1; CCD, central composite design; DH, degree of hydrolysis; DPP4, dipeptidyl peptidase 4; DPP5, dipeptidyl peptidase 5; DTT, dithiothreitol; h, concentration of free amino groups; h_{tot}, maximum concentration of free amino groups at complete hydrolysis; LAPA, leucine aminopeptidase A; LAP2, leucine aminopeptidase 2; NP1, neutral protease 1; NP2, neutral protease 2; OPA, *ortho*-phthalaldehyde; *p*NA, *para*-nitroanilide; PG, protein glutaminase.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version.

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CHAPTER FIVE

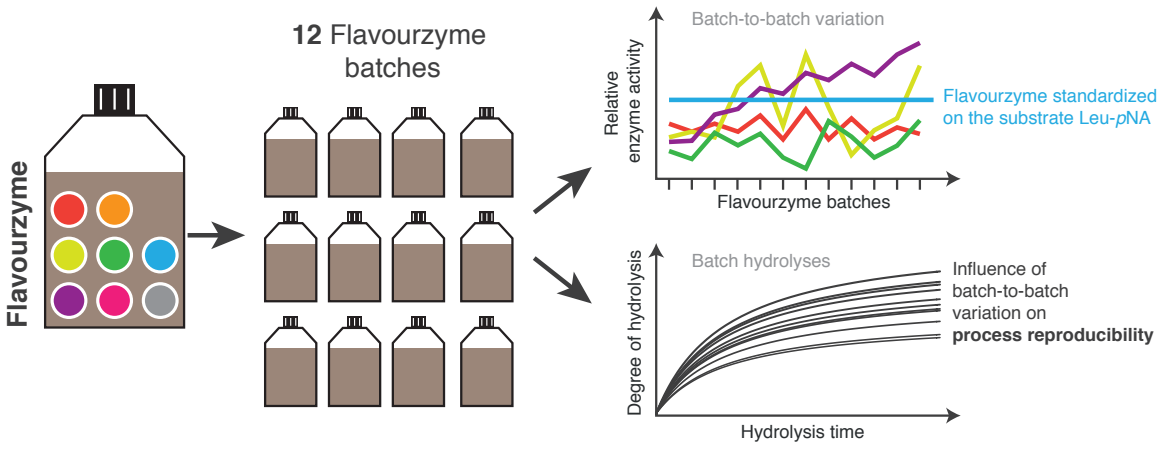
BATCH-TO-BATCH VARIATION OF THE COMMERCIAL PEPTIDASE PREPARATION FLAVOURZYME IN RESPECT OF KEY ENZYME ACTIVITIES AND ITS INFLUENCE ON PROCESS REPRODUCIBILITY

This chapter was submitted

Merz, M., Appel, D., Berends, P., Rabe, S., Blank, I., Stressler, T., Fischer, L. (2015). Batch-to-batch variation of the commercial peptidase preparation Flavourzyme in respect of key enzyme activities and its influence on process reproducibility. *European Research and Technology*. Submitted manuscript.

Time of investigation: 01/2014 - 07/2015

Graphical Abstract



ABSTRACT

The synergy of endopeptidases and exopeptidases is the key for an efficient hydrolysis of proteins. Flavourzyme is sold as a commercial peptidase preparation from *Aspergillus oryzae* that exhibits various endo- and exopeptidase activities and, therefore, generates protein hydrolysates with high degrees of hydrolysis. The manufacturer (Novozymes) standardizes the enzyme preparation for one peptidase activity, determined with the marker substrate H-Leu-*p*NA. However, seven peptidases of Flavourzyme were recently identified and purified, and the significant contribution of six of them to wheat gluten hydrolysis was demonstrated. The knowledge about the batch-to-batch variation and storage stability of the Flavourzyme preparation regarding the other peptidase activities are still unclear, and this is important information for the usage of the enzyme preparation to gain reproducible protein hydrolysis processes. In the present study, we tested twelve Flavourzyme batches for the activity of the seven peptidases. The impact of the storage time on the peptidase activities and the magnitude of the batch-to-batch variation were investigated. In contrast to the activity determined with H-Leu-*p*NA as a substrate, the variations of the other peptidase activities were noticeable. The variation of the endopeptidase activity was most distinct and the activity decreased during the storage time of the preparation. The variation of the Flavourzyme composition also affected the reproducibility of a casein batch hydrolysis process, which should be taken into account for any future research and industrial application.

KEYWORDS

Aspergillus oryzae peptidases, Flavourzyme, Commercial enzyme preparation, Protein hydrolysis, Batch-to-batch variation, Process reproducibility

INTRODUCTION

Flavourzyme is an industrial peptidase preparation obtained from *Aspergillus oryzae* and is of importance for both industrial and research applications [1–8]. The preparation is versatile and can be used to produce hydrolysates with different favorable properties for various industries. The production of flavoring hydrolysates [1, 2, 4], angiotensin-converting enzyme inhibitory peptides [5, 6], antioxidative or radical scavenging peptides [7, 9] and emulsifying peptides [8] have already been described.

Eight enzymes of the Flavourzyme preparation were recently identified and purified by an automated purification protocol [10]. These enzymes were a leucine aminopeptidase A (LAPA), a leucine aminopeptidase 2 (LAP2), a dipeptidyl peptidase 4 (DPP4), a dipeptidyl peptidase 5 (DPP5), a neutral protease 1 (NP1), a neutral protease 2 (NP2), an alkaline protease 1 (ALP1) and an alpha amylase A type-3 (AMY3) [10]. However, the manufacturer of Flavourzyme 1000L (Novozymes) standardizes the enzyme preparation on the marker substrate H-Leucine-*para*-nitroanilide (Leu-*p*NA) and, thus, Flavourzyme contains at least 1000 leucine aminopeptidase units (LAPU) per gam preparation. Since this substrate mainly covers one aminopeptidase [10] and since other enzymes are not quantified nor specified, the batch-to-batch variation of the other enzymes is, as yet, unclear. Actually, the significant contribution of six isolated Flavourzyme peptidases on wheat gluten hydrolysis was shown [11]. Thus, the proportion of all peptidases in the Flavourzyme preparation is a deciding factor, due to the synergy of endopeptidases and exopeptidases, for an efficient breakdown of proteins. Therefore, batch-to-batch variations might also affect the reproducibility of protein hydrolysis processes.

As mentioned above, Flavourzyme is obtained from the *Aspergillus oryzae* strain ATCC 42149/RIB 40 (Yellow koji mold). It is produced by submerged fermentation of the fungi and purified afterwards [12]. However, the submerged fermentation of filamentous fungi is challenging [13]. Even small variations of the broth viscosity, fungal morphology or culture pH can affect the cultivation [13] and, therefore, lead to batch-to-batch variations. In the case of *Aspergillus fumigatus*, for instance, it was shown that the expression of different peptidase sets depends on the culture pH [14].

In the present study, we tested the composition and enzyme distribution of twelve different Flavourzyme batches. The respective batches were tested for the protein content and the enzyme activity of eight different substrates. Some of the substrates used were recommended as marker substrates to characterize the key enzymes in Flavourzyme [10].

The different Flavourzyme batches were used for a casein hydrolysis and the influence of the batch-to-batch variation on the process reproducibility was evaluated.

MATERIALS AND METHODS

Substrates and chemicals

All *p*NA substrates were obtained from Bachem (Bubendorf, Switzerland). Azocasein was bought from Megazyme (Bray, Ireland). All other chemicals were of analytical grade and were obtained from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany).

Flavourzyme batches

Flavourzyme 1000L (Novozymes, Bagsværd, Denmark) batches were gifts from Nestlé Product Technology Centre (Singen, Germany). A total of twelve batches were tested. The storage time of the different batches at 7 °C varied between 1 and 22 months. Batches were labeled alphabetically (Batch A – L). A list of the Flavouzyme batches used with the corresponding storage time and the LOT number is listed in Table 5.1. All batches were within the recommended time of utilization (24 months) given by Novozymes at the time of the investigation [12].

Analytical methods

Determination of amino groups with ortho-phthalaldehyde

Primary amino groups were determined after derivatization with *ortho*-phthalaldehyde (OPA), as described previously [2]. A sample volume of 25 µL was transferred into a microtiter plate and 175 µL OPA reagent was added. For the OPA reagent, 1.5 g/L OPA, 3 g/L DTT (dithiothreitol) and 11.25 % (v/v) methanol were solved in sodium tetraborate decahydrate buffer (120 mM; adjusted to pH 9.6 with NaOH). The plate was incubated at 37 °C for 1 min and the absorbance was measured at 340 nm using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using L-serine as a standard.

Degree of hydrolysis

The degree of hydrolysis (DH) was calculated according to Adler-Nissen [15], with modifications described previously [11] (Eq. 5.1).

(Eq. 5.1) $$\mathbf{DH} = \frac{h}{h_{tot}} \times \mathbf{100}\ [\%]$$

where h is the concentration of free amino groups [mol/L; see above] and h_{tot} is the maximum concentration of free amino groups at complete hydrolysis [mol/L] calculated according to Eq. 5.2.

(Eq. 5.2) $$\boldsymbol{h_{tot} = \frac{c_{Protein}}{M^* - M_{H_2O}}\ [mol/L]}$$

where $c_{Protein}$ is the concentration of protein which is hydrolyzed (g/L) and M^* is the average molecular mass of the amino acids in casein (136.9 g/mol). This casein-specific average molecular mass was calculated by considering the casein amino acid composition [16]. The molecular mass of water (M_{H20} = 18 g/mol) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

Table 5.1: Flavourzyme LOT numbers with the corresponding storage time at 7 °C at the time of investigation. Batches were labeled alphabetically according to the LOT number in ascending order.

Internal label	LOT number	Storage time (7 °C) [month]
Batch A	HPN 00479	22
Batch B	HPN 00482	20
Batch C	HPN 00495	17
Batch D	HPN 00497	13
Batch E	HPN 00498	11
Batch F	HPN 00500	10
Batch G	HPN 00512	6
Batch H	HPN 00514	4
Batch I	HPN 00515	3
Batch J	HPN 00518	3
Batch K	HPN 01001	1
Batch L	HPN 01002	1

Endopeptidase activity assay

The azocasein assay was performed according to the method of Iversen and Jørgensen [17], with some modifications [10] at 37 °C. Azocasein (5 g/L, substrate solution) was dissolved in H_2O_{dd} and the assay contained 125 µL substrate solution and 125 µL of Tris-HCl buffer (100 mM, pH 7.5). The reaction was started with the addition of enzyme solution (50 µL) and stopped by the addition of trichloroacetic acid (TCA, 1.5 M, 50 µL).

After subsequent centrifugation (20,000 × *g*, 4 °C, 5 min), a volume of 195 µL of the supernatant was transferred into a microtiter plate, 65 µL NaOH (1 M) was added and the absorbance at 450 nm was measured using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). One azocasein unit (ACU) was defined as the increase of 1 absorbance unit per min at 450 nm in 0.25 M NaOH.

Aminopeptidase and dipeptidylpeptidase activity assay

Aminopeptidase and dipeptidyl peptidase activities were measured according to the method of Chrispeels and Boulter [18], with some modifications [10, 19]. The assay contained 177 µL of Tris-HCl buffer (50 mM, pH 7.5) and 50 µL enzyme solution. The reaction was started by the addition of 12.5 µL of the corresponding *p*NA derivative solved in dimethylformamide (DMF). The final substrate concentration in the assay was 3.7 mM (Ile-, Leu-, Pro-, Ala-Pro-*p*NA) or 1 mM (Lys-Ala-*p*NA), respectively. The reaction was stopped by the addition of 50 % (v/v) acetic acid (50 µL). After centrifugation (20,000 × *g*, 4 °C, 5 min), the supernatant (240 µL) was transferred into a microtiter plate and the absorbance at 405 nm was measured using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). One katal (kat) of aminopeptidase or dipeptidyl peptidase activity was defined as the release of 1 mol *p*NA per s.

Amylase activity assay

The amylase activity was measured according to the Somogyi-Nelson method [20, 21], with some modifications [10]. The following two reagents were prepared: Reagent A consisted of $Na_2HPO_4 \cdot 2\ H_2O$ (35.2 g/L), $KNaC_4H_4O_6 \cdot 4\ H_2O$ (40 g/L), $CuSO_4 \cdot 5\ H_2O$ (8 g/L) and NaOH (4 g/L) solved in H_2O_{dd}. Reagent B consisted of $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ (50 g/L), H_2SO_4 (42 g/L) and $Na_2HAsO4 \cdot 7\ H_2O$ (6 g/L) solved in H_2O_{dd}.

The substrate starch (10 g/L) was solved at 70 °C for 60 min in H_2O_{dd} and the assay was performed as follows. The substrate solution (125 µL) was preincubated with 75 µL of citrate phosphate buffer (16.6 mM, pH 5) at 37 °C for 5 min. The reaction was started with the addition of enzyme solution (50 µL). After the reaction, a volume of 125 µL was transferred into 125 µL citrate phosphate buffer (100 mM, pH 7.5). Afterwards, reagent A (100 µL) was added and the reaction mixture was subsequently incubated at 98 °C for 10 min (water bath). Finally, a volume of 100 µL of reagent B and 800 µL of H_2O_{dd} were added. The mixture was incubated at 20 °C for 30 min and then centrifuged (13,000 *g*, 20 °C, 1 min). The supernatant (200 µL) was transferred into a microtiter plate and the

absorbance at 450 nm was measured using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using glucose as a reference. One kat of amylase activity was defined as the release of 1 mol glucose-equivalents per s.

Overall proteolytic activity of Flavourzyme on casein

The standard assay contained 125 µL of sodium caseinate (20 g/L) and 125 µL Bis-tris-propane-HCl buffer (100 mM, pH 7.5). The reaction was started with the addition of 50 µL diluted Flavourzyme, stopped by the addition of TCA (1.5 M, 50 µL) and centrifuged (20,000 × g, 4 °C, 5 min) afterwards. The supernatant (25 µL) was transferred into a microtiter plate and the OPA assay was performed (see above). One kat of overall proteolytic activity was defined as the release of 1 mol serine equivalent amino groups per s.

SDS-PAGE and native PAGE

Respective samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 %), according to the method of Laemmli [22]. The molecular weight protein standard mixture was obtained from New England Biolabs (NEB, Broad Range, 2-212 kDa; Frankfurt, Germany) and was used as a reference. The protein bands of the SDS gel were located by staining with Coomassie Brilliant Blue R-250 [23]. The protein load was 5 µg per lane for the Flavourzyme samples. The protein content was determined using the method of Bradford [24] and bovine serum albumin as a standard.

Casein batch hydrolyses

Batch hydrolyses of casein with different Flavourzyme batches were conducted similarly, as described previously for wheat gluten hydrolyses [11].

The corresponding Flavourzyme batch was diluted in Tris-HCl buffer (200 mM, pH 7.5) and a volume of 200 µL was transferred into a deep well plate. The addition of 200 µL substrate solution (50 g/L sodium caseinate in water) started the enzymatic hydrolysis. Thus, hydrolyses were carried out in 400 µL scale with Tris-HCl buffer (100 mM, pH 7.5) with 25 g/L sodium caseinate. An enzyme activity of 300 $nkat_{H\text{-}Leu\text{-}pNA}$/mL was targeted in the final reaction volume. Plates were incubated at 37 °C on a platform shaker (1000 rpm; Titramax 100, Heidolph, Schwabach, Germany). Samples (30 µL) were taken after various times and transferred into microtiter plates containing 120 µL TCA (0.25 M)

to stop the reaction. Samples were further diluted to determine the DH (see above) or to check the amino acid/peptide pattern with RP-HPLC (see below).

RP-HPLC analysis of casein hydrolysates

Automated small-scale hydrolyses were realized with a PLATINblue UHPLC system (Knauer, Berlin, Germany). The system was equipped with a pump (P-1), a diode array detector (PDA-1), a solvent gradient manager (M-1), a column thermostat (Jetstream 2 plus) and an autosampler (AS-1). The PLATINblue chromatography system was controlled by ChromGate Data System V.3.3.1 (Build 3.3.1.902).

The OPA derivatization was automated by the PLATINblue autosampler AS-1. The temperature in the autosampler for the derivatization was set to 22 °C. A volume of 75 mL OPA reagent was added to the sample from the batch hydrolysis (125 µL; diluted 20-fold in H_2O_{dd}) and mixed thoroughly. The OPA reagent consisted of 25 mM *ortho*-phthalaldehyde, 43 mM 2-mercaptoethanol and 30% (v/v) methanol in 85 mM sodium tetraborate buffer (pH 9.6, adjusted with NaOH). The derivatization time was 1 min and afterwards 50 µL of neutralization buffer (1 M sodium acetate pH 5) was added and, again, the mixture was mixed thoroughly.

The chromatography was conducted with a reversed Phase C18 column (BlueOrchid C8, 1.8 µm, 100 x 2 mm ID, Knauer, Germany). A sample volume of 5 µL was injected into the column. For solvent A, 70 mM disodium hydrogen phosphate was adjusted with HCl to pH 6.5 and 5% (v/v) acetonitrile was added. Solvent B consisted of H_2O_{dd}/methanol/acetonitrile [40:30:30 (v/v/v)]. The conditions of gradient elution were: 0-4 min (A: 100%; B:0%), 4-13.5 min (A: 0%; B:100%), 13.5-14 min (A:0%; B:100%), 14-14.1 min (A:100%; B:0%), 14.1-15 min (A:0%; B:100%). The flow rate was 0.7 mL/min and derivatives were detected by absorbance at 340 nm. The column temperature was set at 45 °C.

Statistical analysis

The standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA). All trials were conducted at least in duplicate, with two independent measurements.

RESULTS AND DISCUSSION

Protein content and distribution

The batches investigated are listed in Table 5.1 and are labeled alphabetically according to the LOT number in ascending order. The protein concentration (Bradford with bovine serum albumin as a standard) of the Flavourzyme batches tested is shown in Table 5.2. The protein content ranged from 37.0 ± 1.8 to 78.5 ± 1.4 g/L, resulting in an average protein content of 61.9 g/L. The variations of the protein content indicated batch-to-batch variations of the Flavourzyme preparations tested. The variation of the protein content was 23 % (60.4 and 78.5 g/L) for the consecutive batches K and L (consecutive LOT numbers). The corresponding distribution of proteins contained in the particular Flavourzyme batches was analyzed by SDS gel electrophoresis and is shown in Figure 5.1. The protein bands on the gel were assigned to particular enzymes according to a previous study of our group [10]. Protein band intensities at approximately 50, 34 and 25 kDa (AMY3, LAPA and NP2, respectively) were comparable and indicated that the expression and presence of these enzymes was quite constant. By contrast, the protein bands at approximately 42 and 30 kDa (NP1 and ALP1) seemed to vary and tended to be less intense with increased storage time. The NP1 was not detected in batches A and B and was hardly detectable in the batches C, D, E and F. The NP1 protein band was clearly visible in batches G – L. The ALP1 was not detectable in batch B and is hardly detectable in batches A and C. The ALP1 protein band was clearly visible in batches D to L and was most intense for the batches K and L. Since the enzyme DPP5 fragmented on a SDS gel [10], the protein band at approximately 66 kDa was evaluated representatively for this enzyme. The band intensity of the DPP5 was similar for all batches tested. A distinct band was not observed for LAP2, because this enzyme protein is heterogeneously glycosylated [10]. The DPP4 was not detectable on the gel due to its low proportion in the Flavourzyme preparation [10].

Enzyme activity on different substrates

The manufacturer uses the substrate H-Leu-*p*NA as the marker substrate for the specification of the Flavourzyme activity. According to Novozymes (Bagsværd, Denmark), Flavourzyme 1000L exhibits at least 1000 LAPU per gram of liquid preparation. One LAPU is defined as the release of 1 mM *p*NA from the substrate H-Leu-*p*NA (1.53 mM) at 37 °C, pH 8 (100 mM Tris-HCl, 0.2 mM $ZnCl_2$) per min.

The activities of the different enzymes in the current study were tested with the substrates H-Leu-*p*NA, H-Ile-*p*NA, H-Pro-*p*NA, H-Ala-Pro-*p*NA, H-Lys-Ala-*p*NA, azocasein, casein and starch. This was realized for twelve Flavourzyme batches and the particular enzyme activities on these substrates are listed in Table 5.2. The different enzyme activities were standardized on the batch with the least storage time (batch L). The enzyme activity with H-Leu-*p*NA as a substrate was similar for all batches tested and ranged from 87 to 102 %. The activity of batch L (100 %) was 20.3 ± 0.4 µkat/mL (37 °C, pH 7.5), which accounts for 1102 ± 9 LAPU/g (37 °C, pH 8.0, 0.2 mM $ZnCl_2$). The activity of the batches with H-Ile-*p*NA as a substrate was comparable and ranged from 89 to 104 % (100 % equals to 120 ± 4.8 nkat/mL). The tendencies were the same for both substrates mentioned above, thus, both substrates were converted by the same peptidase (LAPA) as previously described by Merz et al. [10]. In conclusion, the LAPA activity meets the manufacturer's specification of 1000 LAPU/g even after a storage-time of 22 months.

In contrast to the activity with H-Leu-*p*NA and H-Ile-*p*NA as a substrate, the activity of the batches with H-Pro-*p*NA as a substrate, the marker substrate for the LAP2, varied in the range between 47 and 100 % (100 % = 28.0 ± 1.4 nkat/mL). In this case, a slight trend of a decreased activity was observed for longer storage times. However, the batch-to-batch variation should be taken into account. The consecutive batches K and L (consecutive LOT numbers), for example, showed a difference of 24 % when H-Pro-*p*NA was used as a substrate, although the storage time was comparable.

A batch-to-batch variation for the batches K and L was also observed in the case of the DPP4 activity (H-Ala-Pro-*p*NA as a substrate; 45 % difference; 100 % = 43.8 ± 0.4 nkat/mL). A correlation of the DPP4 activity with the storage time was not clearly observable.

The DPP5 activity (H-Lys-Ala-*p*NA as a substrate) was similar for the Flavouryzme batches tested and ranged from 87 to 103 % (100 % equals to 37.7 ± 0.4 nkat/mL), although a batch-to-batch variation of 13 % was observed for batches K and L.

In general, the variation of the endopeptidase activity (NP1, NP2, ALP1), tested with azocasein as a substrate, was the most noticeable. The endopeptidase activity of the Flavourzyme batches tested ranged from 9 to 100 % (29.600 ± 110 ACU/mL). The activity decreased with prolonged storage times indicating that the endopeptidase activity is the least stable compared to the other peptidases in the Flavourzyme preparation.

Nevertheless, a 16 % batch-to-batch variation was also observed for batches K and L. This fact can be an explanation why Kilcawley et al. [25] did not measure any endopeptidase activity in Flavourzyme whereas others [2, 10, 26] described the latter.

Table 5.2: Protein content and relative enzyme activity on eight different substrates of twelve Flavourzyme batches. Enzyme activities were standardized (100 %) on the activity of the most recent batch (batch L).

		Relative enzyme activity [%]							
Internal label	Protein [g/L]	H-Leu-*p*NA	H-Ile-*p*NA	H-Pro-*p*NA	H-Ala-Pro-*p*NA	H-Lys-Ala-*p*NA	Azo-casein	Casein/ OPA	Starch
Batch A	39.6 ± 2.3	98 ± 1	101 ± 0	47 ± 0	63 ± 1	97 ± 2	18 ± 1	16 ± 2	71 ± 8
Batch B	37.0 ± 1.8	102 ± 0	104 ± 0	64 ± 2	44 ± 2	97 ± 0	9 ± 0	12 ± 0	74 ± 8
Batch C	46.5 ± 3.1	95 ± 3	97 ± 3	61 ± 0	42 ± 0	92 ± 4	33 ± 0	37 ± 2	85 ± 1
Batch D	56.8 ± 1.9	94 ± 1	97 ± 4	57 ± 1	46 ± 0	103 ± 7	49 ± 1	42 ± 0	92 ± 1
Batch E	50.4 ± 1.2	95 ± 2	95 ± 4	51 ± 0	51 ± 1	102 ± 1	47 ± 0	49 ± 1	82 ± 1
Batch F	42.8 ± 2.4	91 ± 3	93 ± 2	48 ± 1	36 ± 0	93 ± 0	44 ± 1	44 ± 2	79 ± 1
Batch G	68.0 ± 0.9	97 ± 2	94 ± 1	61 ± 3	64 ± 0	107 ± 1	83 ± 0	76 ± 0	87 ± 2
Batch H	65.7 ± 1.7	102 ± 0	99 ± 2	89 ± 2	71 ± 1	103 ± 3	82 ± 1	62 ± 1	93 ± 2
Batch I	63.9 ±1.1	101 ± 0	97 ± 3	86 ± 2	62 ± 2	98 ± 3	81 ± 2	66 ± 4	92 ± 0
Batch J	67.3 ± 1.8	95 ± 1	89 ± 1	81 ± 2	52 ± 1	99 ± 1	78 ± 1	64 ± 5	86 ± 6
Batch K	60.4 ±2.5	101 ± 3	93 ± 0	76 ± 0	55 ± 1	87 ± 0	84 ± 1	88 ± 1	92 ± 3
Batch L	78.5 ± 1.4	100 ± 2	100 ± 4	100 ± 5	100 ± 1	100 ± 1	100 ± 0	100 ± 0	100 ± 1

* 100 %: H-Leu-*p*NA, 20.3 µkat/mL; H-Ile-*p*NA, 120 nkat/mL; H-Pro-*p*NA, 28.0 nkat/mL; H-Ala-Pro-*p*NA, 43.8 nkat/mL; H-Lys-Ala-*p*NA, 37.7 nkat/mL; Azocasein, 29.600 ACU/mL; Casein, 27.8 µkat/mL; Starch, 16.1 µkat/mL

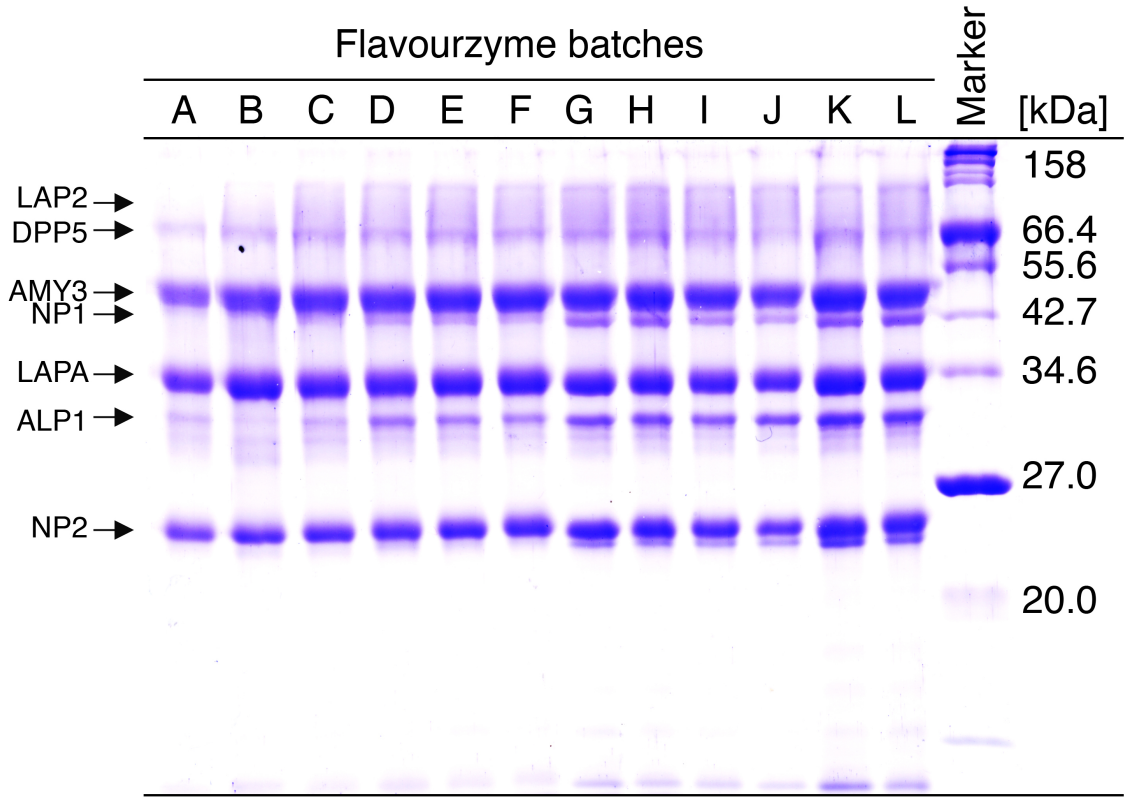


Figure 5.1: SDS-PAGE with Coomassie staining of Flavourzyme batches A – L. A protein marker was used as a reference (Broad Range, 2-212 kDa; NEB, Frankfurt, Germany). Protein load was 5 µg per lane. Protein bands were assigned according to Merz et al. [10]; ALP1, Alkaline protease 1; AMY3, Alpha-Amylase A type-3; DPP5, Dipeptidyl peptidase 5; LAPA, Leucine aminopeptidase A; LAP2, Leucine aminopeptidase 2; NP1, Neutral protease 1; NP2, Neutral protease 2.

The overall proteolytic activity of the Flavourzyme batches was tested by determining the amino groups released after casein hydrolysis. The activity of the batches tested ranged from 12 to 100 % (27.8 ± 0.1 µkat/mL) and was comparable to the activity determined with azocasein as a substrate. This is in accordance with the results of Merz et al. [11], that the endopeptidases have a crucial impact on the overall proteolytic activity.
Finally, the activity of the Flavourzyme batches on starch was tested to determine the variation of the AMY3 activity. The activity of the batches tested ranged from 71 to 100 % (16.1 ± 0.2 µkat/mL). A slight trend towards a decreased activity was observed for longer storage times. The highest batch-to-batch variation of 10 % was observed for batches D and E.

Batch hydrolyses of casein

Enzymatic hydrolyses of casein (25 g/L) were carried out with 300 $nkat_{H\text{-}Leu\text{-}pNA}$/mL at 37 °C, pH 7.5 (100 mM Tris-HCl), for 480 min. The progression of the DH during the hydrolysis is shown in Figure 5.2A for the twelve Flavourzyme batches tested.

As seen in Figure 5.2A, different equilibriums were obtained for the Flavourzyme batches. However, the influence of the enzyme composition of the batches on the DH was more distinct at the beginning of the enzymatic hydrolysis (20 – 120 min).

After 480 min (Fig 2A), the DH varied from 55.5 ± 2.3 to 64.1 ± 2.9 % and was least for batches A, B and C with 56.3 ± 1.4, 55.5 ± 2.3 and 57.8 ± 2.3 %, respectively. These three batches also exhibited the least endopeptidase activity and overall proteolytic activity (see above). The DH at equilibrium was comparable for batches D – L. The final DH of the hydrolysis was plotted over the measured endopeptidase activity (Table 5.2) to check the impact of the latter on the hydrolysis process. As seen in Figure 5.2 B the DH was clearly dependent on the endopeptidase activity. The significant impact of the Flavourzyme endopeptidase activity on the hydrolysis was also reported recently [11]. It seems that the endopeptidase activity and the overall composition of the Flavourzyme preparation affects the initial progression of the DH to a greater extent compared to the final DH at equilibrium. The final DH of the batch hydrolyses after 480 min are summarized in Table 5.3. It should be highlighted that comparative studies on the casein hydrolysis using Flavourzyme were not mentioned due to the significant differences of the experimental setups, substrates and DH determination methods.

Additionally, the hydrolysate samples (460 min) of the enzymatic batch hydrolysis (see above) were tested by RP-HPLC chromatography to see if different peptide/amino acid patterns were obtained by the Flavourzyme batches. Exemplarily, chromatographs of the hydrolysates obtained with batch B and batch L are shown in Figure 5.3. Even though detailed information about the composition was not obtained with this investigation, the results indicated that, to some extend, the chromatographs, and therefore the composition of the hydrolysates, were different.

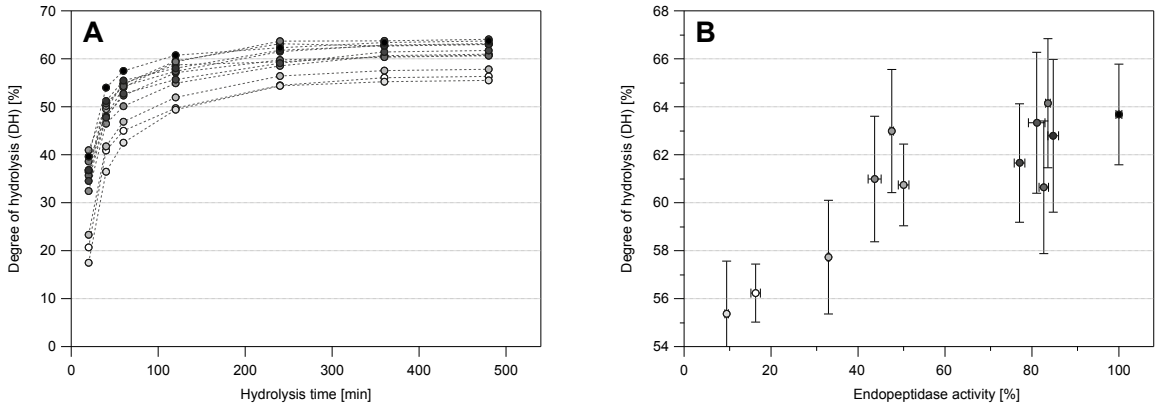


Figure 5.2: A: Batch hydrolyses of sodium caseinate (25 g/L) with Flavourzyme batches A – L (300 $nkat_{H\text{-}Leu\text{-}pNA}$/mL) at 37 °C, pH 7.5 (100 mM Tris-HCl). Twelve shades of grey represent the batches tested, with increased saturation from batch A to batch L (white: batch A; black: batch L). Values represent the mean values of two independent measurements with a standard deviation of less than 5 %. B: Dependence of the DH from the batch hydrolyses (Figure 5.2A) and the endopeptidase activity (Table 5.2). The endopeptidase activity of the respective Flavourzyme batch was plotted over the obtained DH from the batch hydrolyses using the respective batch.

Table 5.3: Degrees of hydrolysis after 480 min of hydrolysis (see Figure 5.2) of 25 g/L sodium caseinate with 300 $nkat_{H\text{-}Leu\text{-}pNA}$/mL of Flavourzyme batches A – L.

Internal label	DH [%]
Batch A	56.3 ± 1.4
Batch B	55.5 ± 2.3
Batch C	57.8 ± 2.3
Batch D	60.8 ± 1.6
Batch E	63.0 ± 2.6
Batch F	61.0 ± 2.7
Batch G	64.1 ± 2.9
Batch H	60.7 ± 2.8
Batch I	63.2 ± 3.1
Batch J	61.7 ± 2.4
Batch K	62.9 ± 3.1
Batch L	63.7 ± 2.1

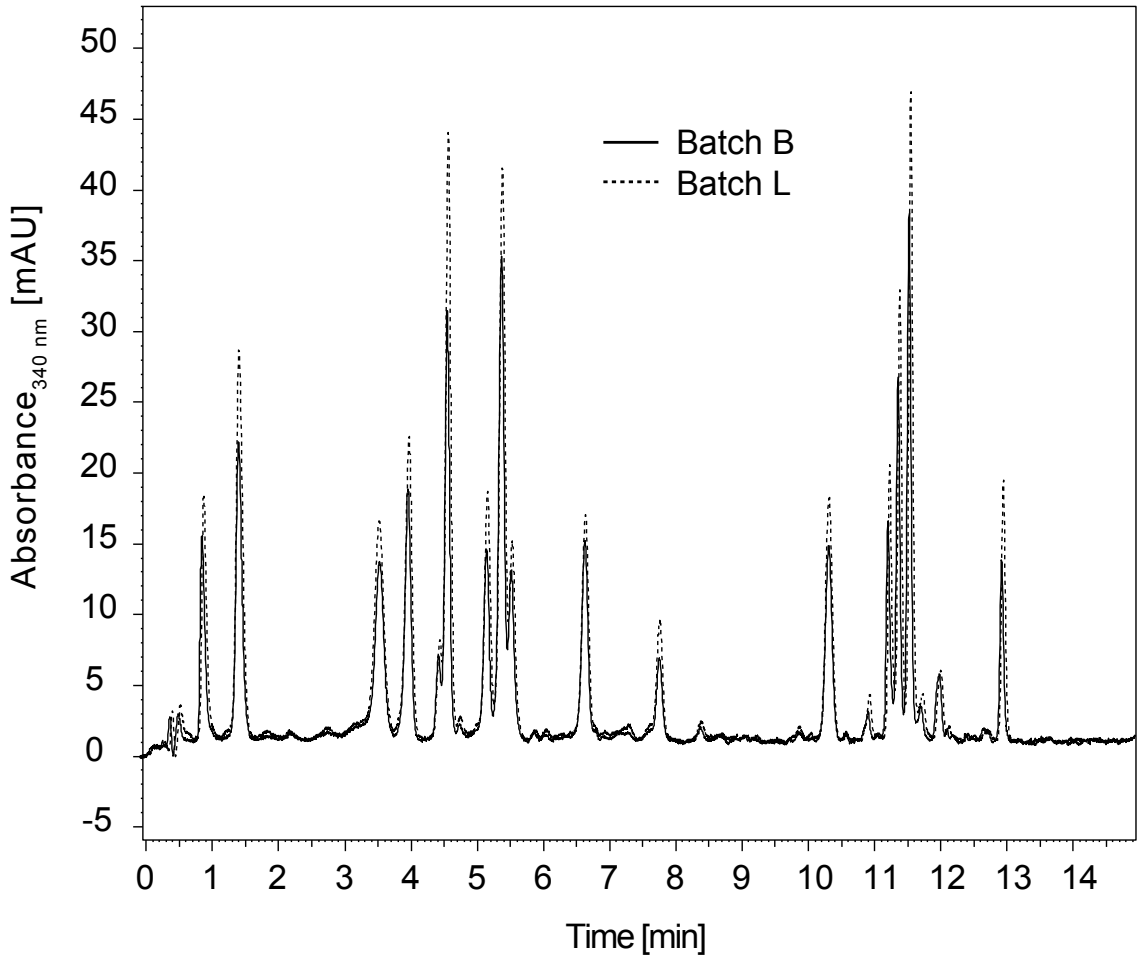


Figure 5.3: RP-HPLC chromatographs of casein hydrolysates obtained with Flavourzyme batches B and L after derivatization with OPA. Respective samples of the enzymatic hydrolysis after 460 min were diluted 20-fold prior to the derivatization and analysis.

CONCLUSIONS

The enzyme activity of twelve Flavourzyme batches on eight different substrates was evaluated. Coherences of the storage time and the endopeptidase activity were observed. Even though the enzyme preparation meets the specifications (LAPU), other enzyme activities decreased over time.

However, specific statements about different enzyme stabilities are difficult due to the fact that batch-to-batch variations were also observed. These variations were distinctly determined for the consecutive batches K and L (consecutive LOT numbers HPN 01001 and HPN 01002). Variations of the enzyme activity of 7, 24, 45, 13, 16, 12 and 8 % were ascertained with the substrates H-Ile-*p*NA, H-Pro-*p*NA, H-Ala-Pro-*p*NA, H-Lys-Ala-*p*NA, azocasein, casein and starch, respectively. The batch-to-batch variations were also

confirmed by variations of the protein content of the preparations. The variation of the enzyme activity also affected a casein hydrolysis, which resulted in different DH and peptide/amino acid pattern for the respective Flavourzyme batches. The results from the batch hydrolyses were in accordance with the enzyme activity screening performed.

The synergy of the different peptidases is the reason for an efficient breakdown of proteins. In our study, we figured out that batch-to-batch variations of Flavourzyme were noticeable and that the composition of the preparation influenced the outcome of a casein batch hydrolysis. Furthermore, the endopeptidase activity especially exhibited insufficient stability in the preparation. The knowledge about the batch-to-batch variation and the storage stability of the peptidases has to be kept in mind when using an enzyme preparation such as Flavourzyme. Consequently, the efficacy and the reproducibility of protein hydrolysis processes can be improved if all relevant peptidase activities are considered.

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ABBREVIATIONS

ACU, azocasein unit; ALP1, alkaline protease 1; AMY3, Alpha-Amylase A type-3; DH, degree of hydrolysis; DMF, dimethylformamide; DPP4, dipeptidyl peptidase 4; DPP5, dipeptidyl peptidase 5; DTT, dithiothreitol; H_2O_{dd}, double distilled water; h, concentration of free amino groups; h_{tot}, maximum concentration of free amino groups at complete hydrolysis; kat, katal; LAPA, leucine aminopeptidase A; LAP2, leucine aminopeptidase 2; LAPU, leucine aminopeptidase units; NP1, neutral protease 1; NP2, neutral protease 2; OPA, *ortho*-phthalaldehyde; *p*NA, *para*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Compliance with Ethics requirements

This article does not contain any studies with human or animal subjects.

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CHAPTER SIX

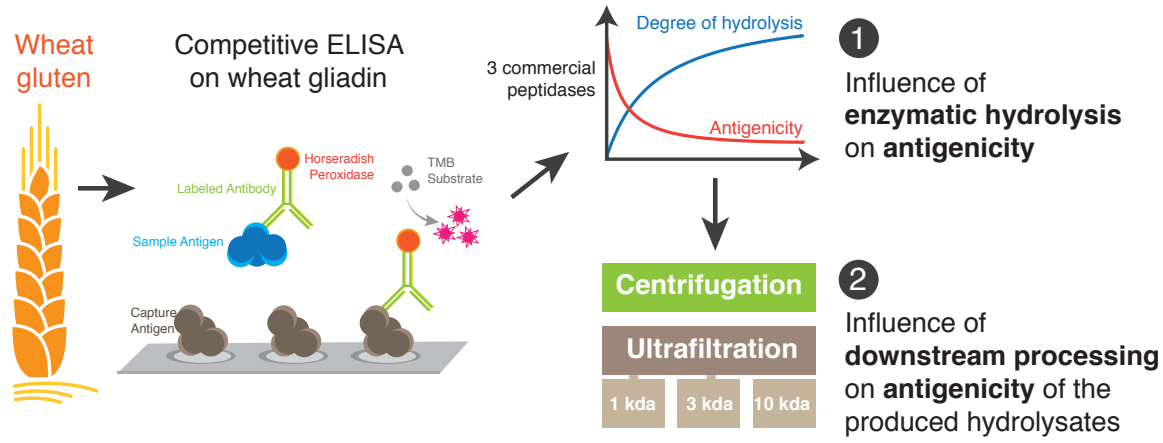
PRODUCTION OF WHEAT GLUTEN HYDROLYSATES WITH REDUCED ANTIGENICITY EMPLOYING ENZYMATIC HYDROLYSIS COMBINED WITH DOWNSTREAM UNIT OPERATIONS

This chapter was submitted

Merz, M., Kettner, L., Langolf, E., Appel, D., Blank, I., Stressler, T., Fischer, L. (2015). Production of wheat gluten hydrolysates with reduced antigenicity employing enzymatic hydrolysis combined with downstream unit operations. *Journal of the Science of Food and Agriculture*. Submitted manuscript.

Time of investigation: 01/2013 - 09/2015

Graphical Abstract



ABSTRACT

Background

A certain segment of the population is not able to consume some of the plant proteins, which are the main protein support in human nutrition. Celiac disease is a prominent autoimmune disorder and the only therapy is a strict adherence to a gluten-free diet. The aim of this study was to identify suitable combinations of enzymatic hydrolysis and common unit operations in food processing (centrifugation, ultrafiltration) to produce gluten-free wheat gluten hydrolysates for food application. To analyze the hydrolysates, a simple and cheap competitive ELISA protocol was designed and validated in this study as well.

Results

The competitive ELISA was validated using gliadin spiked skim milk protein hydrolysates, due to the latter application of the assay. A recovery of approximately 90 % was achieved under the described extraction and assay conditions. The limit of quantification was 4.19 mg kg^{-1}, which allowed the identification of gluten-free (<20 mg kg^{-1}) hydrolysates. Enzymatic hydrolysis, including the type of peptidase, and the downstream processing highly affected the antigenicity of the hydrolysates.

Conclusion

Enzymatic hydrolysis and downstream processing operations, such as centrifugation and ultrafiltration, reduced the antigenicity of wheat gluten hydrolysates. Gluten-free hydrolysates were obtained with Flavourzyme after centrifugation (25 g L^{-1} substrate) and after 1 kDa ultrafiltration (100 g L^{-1} substrate). A multiple peptidase complex, such as Flavourzyme, seems to be required for the production of gluten-free hydrolysates.

KEYWORDS

Antigenicity, ELISA, wheat gluten hydrolysate, enzymatic hydrolysis, centrifugation, ultrafiltration

INTRODUCTION

Agricultural crops derive the majority of proteins for human nutrition [1]. Unfortunately, allergies or other health disorders lead to the fact that a certain segment of the population is not able to consume some of the plant proteins. Coeliac disease (CD), for example, is a prominent autoimmune disorder and is caused by abnormal immune response induced by prolamins from wheat, barley and rye that result in tissue damage of the small intestine [2, 3].

The only therapy of CD is a strict adherence to a gluten-free diet [4]. These dietetic foods underlie the regulations of the "Codex Alimentarius Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten". The current draft regulates that gluten-free food may exhibit a maximum gluten concentration of 20 mg kg^{-1} of the original product [5]. Commonly, for the gluten quantification, the alcohol-soluble proteins, called prolamins, are extracted with aqueous ethanol and quantified by means of an immunochemical method, such as an enzyme-linked immunosorbent assay (ELISA). Competitive-ELISA is a common and reliable method for the *in vitro* determination of the antigenicity of wheat gluten hydrolysates [6, 7], because only one epitope is needed for binding. Thus, a competitive ELISA is preferred over a sandwich ELISA for the analysis of partially hydrolyzed gluten (gluten peptides) [4]. Due to the fact that gluten fraction contains approximately equal amounts of prolamins and glutelins the gluten to prolamin conversion ratio is generally taken as 2 [4].

Since the immune system is involved in both disorders (allergy and coeliac disease) and since epitopes are commonly 5 – 20 amino acids in length [8], the allergenic potential of wheat gluten can be drastically reduced by enzymatic hydrolysis. The enzymatic hydrolysis in combination with downstream processing operations is an affective way to reduce the allergenicity of protein hydrolysates [9]. This is important, because, in general, plant protein hydrolysates can be used to enhance certain functional properties or as food supplements. In Europe, the food industry began using peptidases as processing aids for wheat gluten hydrolysis in the 1980s [10]. Hydrolysates of wheat gluten is used for delivering savory taste (umami) in a wide range of culinary products [11, 12].

The aim of this study was to investigate the influence of enzymatic hydrolysis employing three different commercial peptidases in combination with common unit operations in

food processing (centrifugation, ultrafiltration). To analyze the hydrolysates, a simple and cheap competitive ELISA protocol was designed and validated.

EXPERIMENTAL

Materials and chemicals

Anti-gliadin (wheat)–peroxidase conjugate antibody produced in rabbit (polyclonal) and skim milk powder were obtained from Sigma (Taufenkirchen, Germany). Flavourzyme 1000L (Novozymes, Bagsværd, Denmark), Bioprase SP-20FG (Nagase ChemteX, Kyoto, Japan), Thermoase PC10F (Amano Enzyme Inc., Nagoya, Japan) and wheat gluten (>830 g kg^{-1} protein) were a gift from the Nestlé Product Technology Center (Singen, Germany). All other chemicals were of analytical grade and were obtained from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany). The preparations are hereinafter referred to as Bioprase, Flavourzyme and Thermoase, respectively.

Analytical methods

Determination of amino groups with ortho-phthalaldehyde

Primary amino groups were determined after derivatization with *ortho*-phthalaldehyde (OPA) according to the method of Nielsen et al. [13] with some modifications [14]. A volume of 25 µL of the sample was transferred into a microtiter plate and 175 µL OPA reagent was added. For the OPA reagent 11 mM OPA, 20 mM dithiothreitol (DTT) and 112.5 mL L^{-1} methanol were dissolved in 120 mM sodium tetraborate decahydrate (adjusted to pH 9.8 with NaOH). The plate was incubated at 37 °C for 1 min and the absorbance was measured at 340 nm using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using L-serine as a reference.

Degree of hydrolysis

The degree of hydrolysis (DH) was calculated with Eq. 6.1 according to Adler-Nissen [15] with modifications [14].

(Eq. 6.1) $$\mathbf{DH} = \frac{h}{h_{tot}} \times \mathbf{100}\ [\%]$$

where h is the concentration of free amino groups [mol L^{-1}; see above] and h_{tot} is the maximum concentration of free amino groups at complete hydrolysis [mol L^{-1}] calculated according to Eq. 6.2.

(Eq. 6.2) $$h_{tot} = \frac{c_{Protein}}{M^* - M_{H_2O}} \; [\mathbf{mol\ L^{-1}}]$$

where $c_{Protein}$ is the concentration of protein which is hydrolyzed (g L^{-1}) and M^* is the average molecular mass of the amino acids in wheat gluten (137.9 g mol^{-1}). This gluten-specific average molecular mass was calculated by considering the amino acid composition of wheat gluten [16]. The molecular mass of water (M_{H2O} = 18 g mol^{-1}) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

Determination of the proteolytic activity

The enzyme activity of the commercial peptidases was tested using wheat gluten as a substrate. Therefore, 150 µL of a wheat gluten suspension (10 g L^{-1}) and 100 µL of Bis-Tris propane buffer (150 mM) at the respective optimum pH were incubated at 37 °C for 5 min. The reaction was started with the addition of 50 µL diluted enzyme solution, was stopped by the addition of **trichloroacetic acid** (TCA; 1.5 M, 50 µL) and was centrifuged (20,000 × g, 4 °C, 5 min) afterwards. The supernatant (25 µL) was transferred into a microtiter plate and the OPA assay was performed (see above). One katal (kat) of enzyme activity was defined as the release of 1 mol L-serine equivalent amino groups per s.

Production of wheat gluten hydrolysates

Wheat gluten batch hydrolyses

Wheat gluten hydrolyses were carried out in 250 mL scale with 25 or 100 g L^{-1} wheat gluten at the respective optimum pH of the peptidases (Flavourzyme, pH 7.5; Bioprase, pH 7.0; Thermoase, pH 7.5) at 37 °C. The biotransformation was started by the addition of 25 nkat mL^{-1} of the respective commercial preparation (Bioprase, Flavourzyme, Thermoase). Samples (5 mL) were taken regularly, heated to 90 °C for 15 min immediately to stop the hydrolysis and subsequently the DH was determined (see above). The samples were freeze-dried (Alpha 1–2; Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) and the antigenicity of the hydrolysate powder was determined (see below). After 465 min of batch hydrolysis a last sampling was taken and, subsequently, the total reaction volume was transferred into 50 mL Falcon tubes and heated to 90 °C for 30 min to inactivate the enzymes.

The same procedure was repeated with Flavourzyme and 25 g L^{-1} wheat gluten to additionally check the influence of the substrate concentration on the antigenicity.

Downstream processing of wheat gluten hydrolysates

The inactivated hydrolysate after 465 min of batch hydrolysis (see above) was centrifuged for 15 min (10,000 g, 5 °C) to remove insoluble and non-hydrolyzed compounds. The centrifuged hydrolysate (supernatant) was further filtrated using different ultrafiltration molecular weight cutoffs (MWCO). Minimate™ tangential flow filtration capsules (Pall, Dreieich, Germany) were used for the cross flow filtration of the hydrolysates. Each hydrolysate, prepared with Flavourzyme, Bioprase and Thermoase, was filtered using 1, 3 and 10 kDa capsules. The hydrolysate was filtered at a constant trans-membrane pressure of 1 bar using a tube pump (TB, Medorex, Nörten-Hardenberg, Germany). A volume of 5 mL of each filtrate (1, 3 and 10 kDa) was collected. The samples were freeze-dried and the antigenicity of the hydrolysate powder was determined (see below).

Determination of the antigenicity of wheat gluten hydrolysates

Gliadin extraction from wheat gluten for antigen coating

The gliadin from wheat gluten was isolated by means of a modified preparative Osborne fractionation [17]. Therefore, 150 g wheat gluten powder was defatted under stirring in 300 mL n-hexane for 3 h at 20 °C. Afterwards, the defatted wheat gluten was filtered and dried at 40 °C over night. To remove the albumin and globulin impurities the defatted wheat gluten (50 g L^{-1}) was incubated in Tris-HCl buffer (20 mM; pH 7,5; 0,4 M NaCl) at 20 °C for 30 min under stirring (1300 rpm). The supernatant was discarded after centrifugation (10000 g, 10 min, 15 °C). The pellet was suspended in aqueous ethanol (700 mL L^{-1}), mixed using a Ultraturrax (9500 rpm, 30 s) and then incubated at 20 °C for 30 min under stirring (1300 rpm). The gliadin fraction was obtained in the supernatant after centrifugation (10,000 g, 10 min, 15 °C). The supernatant was dialyzed (10 kDa MWCO) against 100 mM acetic acid and freeze-dried. The obtained powder was used as antigen for coating and as reference for the calibration.

Competitive enzyme-linked immunosorbent assay (ELISA)

MaxiSorp ELISA plates (F96 wells; Nunc, Thermo Scientific) were coated with 2 µg mL^{-1} purified gliadin (see above) for 4 h at 20 °C under moderate stirring (300 rpm, ThermoMixer comfort with MTP thermoblock, Eppendorf, Hamburg, Germany). Afterwards the plate was washed three times with phosphate buffered saline (PBS) using an ELISA washer (3D-IW8 Inteliwasher, Biosan, Riga, Latvia).

To extract the gliadin from the respective samples the sample preparation was as follows: An amount of 25 mg or 50 mg of sample was dissolved in 1 mL aqueous ethanol (575 mL L^{-1}), vortexed for 1 min, and sonicated for 15 min. Afterwards, the suspension was incubated at 50 °C under shaking (1000 rpm, ThermoMixer comfort) for 40 min. The extracted gliadin was obtained in the supernatant after centrifugation (8,000 g, 1 min, 25 °C).

The gliadin containing extract (100 µL) was then incubated in 875 µL blocking buffer (23 g L^{-1} skim milk powder dissolved in PBS) for 30 min. Afterwards, a volume of 125 µL antibody solution (conjugated anti-gliadin) was added and the reaction volume to a final antibody concentration of 12.5 µg mL^{-1}. The sample antigen/antibody solution was further incubated for 30 min at 20 °C (300 rpm, ThermoMixer comfort). The final concentrations after the addition of antibody were 100 mL L^{-1} extracted sample, 20 g L^{-1} skim milk powder and 12.5 µg mL^{-1} antibody. A volume of 50 µL of the obtained solution was transferred into the well of the coated ELISA plate and incubated at 20 °C for 4 h (300 rpm, ThermoMixer comfort with MTP thermoblock). Next, the plate was washed three times with PBS and, afterwards, 100 µL 3,3',5,5' tetramethylbenzidine (TMB, OptEIA, BD Biosciences, San Jose, USA) was transferred into each well of the ELISA plate and incubated at 20 °C in the dark for 40 min. The reaction was stopped by the addition of 0.32 M sulfuric acid and the absorbance was measured at 450 nm using a microtiter plate reader (Multiskan FC, Thermo Scientific, Braunschweig, Germany). The calibration was performed using the isolated gliadin (see above) as a reference in a final concentration range from 10 - 250 ng mL^{-1} gliadin. A calibration was performed for every ELISA plate.

Validation of the competitive enzyme-linked immunosorbent assay (ELISA)

The ELISA was validated using skim milk protein hydrolysates, which were supplemented with different amounts of gliadin. The skim milk protein hydrolysates were prepared as follows: Skim milk protein (100 g L^{-1}) was hydrolyzed with 10 mL L^{-1} Flavourzyme for 7 h until a steady DH was reached. The hydrolysates were then heated to 90 °C for 30 min to inactivate the enzymes. Afterwards, the purified gliadin (see above) was added and the hydrolysates were freeze-dried. Three samples were produced with final gliadin concentrations in the hydrolysate powder of 50, 100 and 500 mg kg^{-1}, respectively. The extraction procedure and the extraction medium were optimized using these spiked hydrolysate samples. The gliadin recovery is expressed as the percentage of

gliadin, which could be extracted from the skim milk hydrolysate powder and determined by the ELISA.

Statistical analysis

A single batch hydrolysis in 250 mL scale was carried out for every substrate/peptidase combination. All ELISA samples were tested in triplicate, at least. The calibration with gliadin (10 - 250 ng mL^{-1}) was evaluated after logarithmic transformation of the gliadin concentration followed normalization of the absorbance values (A/A_0). Due to the normalization the absorbance ranged from 0 to 1 for each calibration. These values were taken for linear regression. The standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA). The limit of detection (LOD) was calculated as the compound concentration causing 5% inhibition of color development (A/A_0 = 0.95 of the normalized plot of the calibration) [18, 19]. The limit of quantification (LOQ) was calculated as the compound concentration causing 20% inhibition of color development.

RESULT AND DISCUSSION

The present study evaluated the influence of the enzymatic hydrolysis on the antigenicity of wheat gluten hydrolysates using peptidases from three different origins. The peptidases were Flavourzyme, Bioprase and Thermoase (Table 6.1). Furthermore, the effect of two standard unit operations in food processing (centrifugation, ultrafiltration) on the antigenicity was tested. An overview about the operations and analyses from this study is given in Fig 6.1.

Table 6.1: Characteristics of the peptidase preparations and enzymatic hydrolysis conditions to produce wheat gluten hydrolysates with reduced antigenicity.

Peptidase Preparation	Supplier	Type of peptidase (Origin)	Optimum pH [-]	Enzyme activity
Bioprase	Nagase	Subtilisin *(Bacillus licheniformis)*	7.0	5714 nkat g^{-1}
Flavourzyme	Novozymes	Endo- and exopeptidase mixture *(Aspergillus oryzae*)	7.5	1142 nkat mL^{-1}
Thermoase	Amano	Thermolysin (*Bacillus thermoproteolyticus)*	7.5	1714 nkat g^{-1}

Preparation of wheat gluten hydrolysates

Three different commercial peptidases (see Table 6.1) were used for the production of the wheat gluten hydrolysates. The commercial peptidases were a subtilisin, a thermolysin (from *Bacillus sp.*) and a peptidases mixture from *Aspergillus oryzae*. Hydrolyses were carried out with 25 or 100 g L^{-1} wheat gluten in 25 mM Bis-Tris propane at the respective optimum pH of the peptidases (Flavourzyme, pH 7.5; Bioprase, pH 7.0; Thermoase, pH 7.5) at 37 °C. According to the optimum temperature of the peptidases (Flavourzyme, 55 °C; Bioprase, 70 °C; Thermoase, 65 °C) a sufficient thermal stability was assumed for 465 min of hydrolysis. A final enzyme activity of 25 nkat mL^{-1} was applied in the final reaction volume.

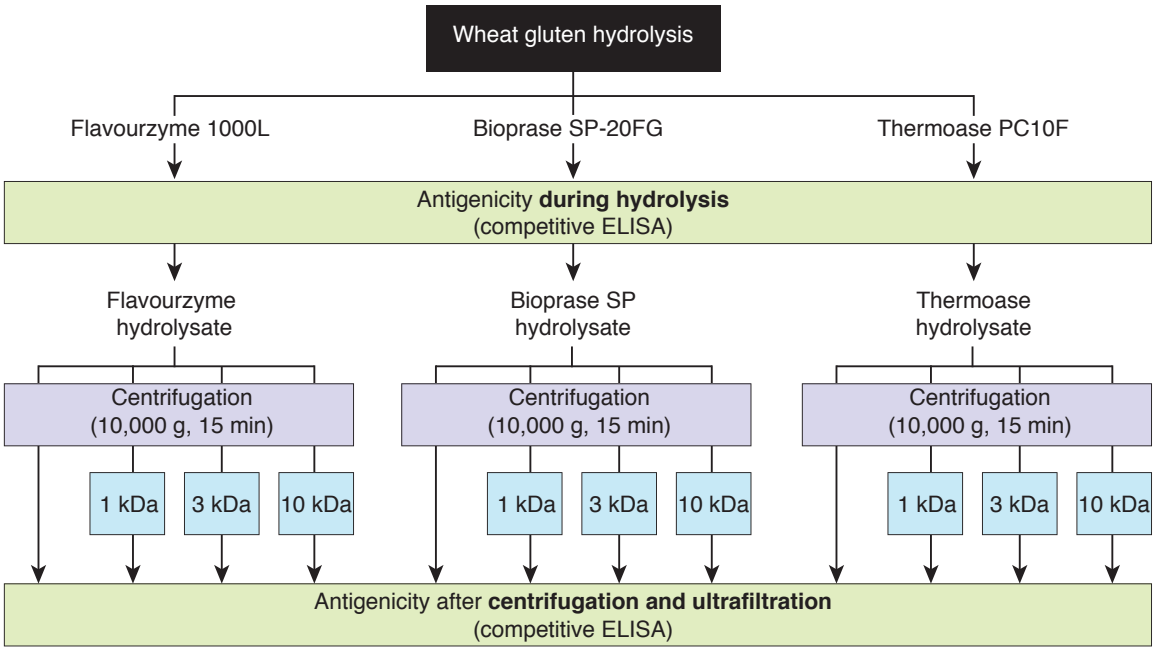


Figure 6.1: Production flowchart of enzymatic protein hydrolysates. Antigenicity was monitored during the enzymatic hydrolysis and after various downstream processing steps (centrifugation, ultrafiltration). Three commercial peptidases (Flavouryzme, Bioprase, Thermoase) were used for the enzymatic hydrolysis of wheat gluten.

The results of the batch hydrolyses with 100 g L^{-1} wheat gluten are shown in Figure 6.2. Different trends of the DH were observed for the different peptidases used. The conversion of the wheat gluten was highest for Flavourzyme followed by Bioprase and Thermoase. The final DH after 465 min of hydrolysis was 42.5 ± 1.1, 14.4 ± 0.8 and 8.85 ± 0.10 % for Flavourzyme, Bioprase and Thermoase, respectively. Because Flavourzyme

consists of several peptidases [20] a higher DH was obtained for the Flavourzyme hydrolysate compared to the endopeptidase preparations. After the enzymatic hydrolysis the hydrolysates were heated for enzyme inactivation and further processed using two common unit operations. Hydrolysates were centrifuged and ultra filtrated using either 1, 3 or 10 kDa membrane cutoffs. In general the DH of the hydrolysates decreased after the filtration depending on the MWCO. The tested DH of the hydrolysates before and after the respective unit operation step are summarized in Table 6.2. The DH of the Flavourzyme hydrolysate, for example, decreased from 44.6 ± 0.3 % after centrifugation to 39.7 ± 0.6, 38.9 ± 0.6 and 37.8 ± 0.1 % after 10, 3 and 1 kDa filtration, respectively. Additionally hydrolyses were carried out with Flavourzyme in presence of 25 g L^{-1} substrate (wheat gluten). A higher DH of 61.0 ± 1.9 % was obtained after 465 min of hydrolysis (Table 6.2), due to the fact that Flavourzyme peptidases are affected by product inhibition [21].

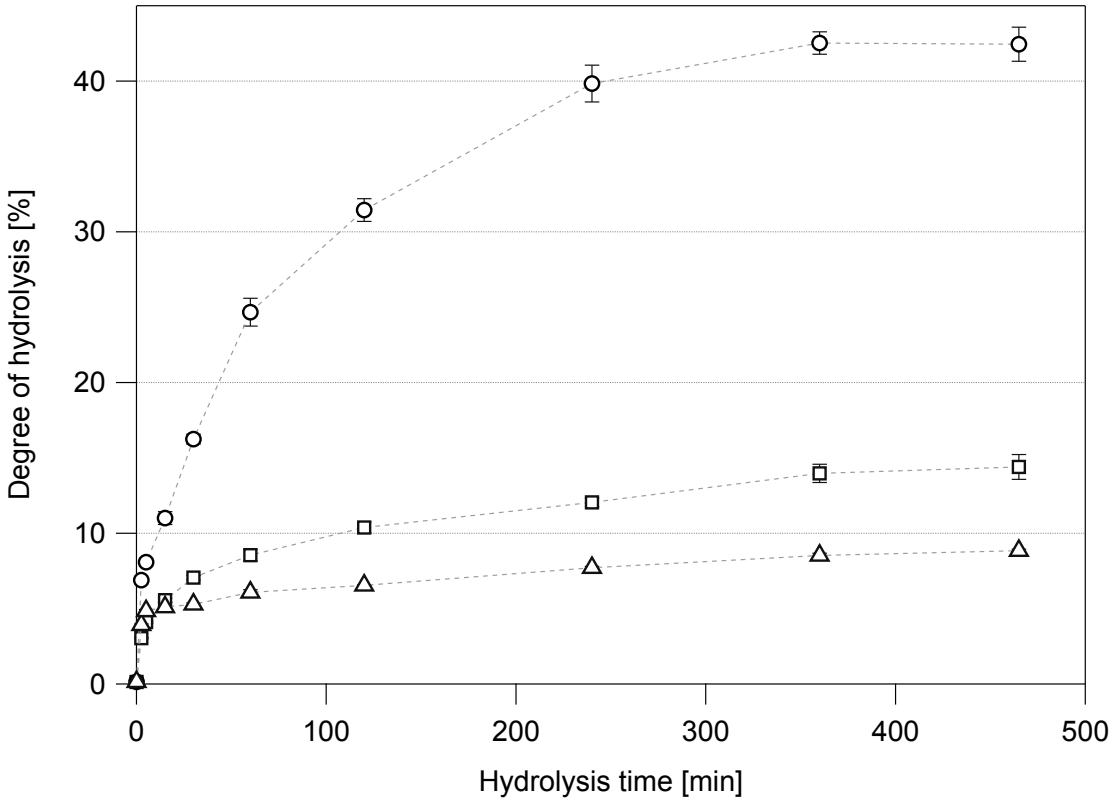


Figure 6.2: Batch hydrolyses of 100 g L^{-1} wheat gluten employing the commercial peptidases Flavourzyme (circles), Bioprase (squares) and Thermoase (triangles). Enzymatic hydrolysis was carried out for 465 min at 37 °C and the respective optimum of the peptidase.

Table 6.2: Degree of hydrolysis (DH) of wheat gluten hydrolysates measured after enzymatic hydrolysis (with different commercial peptidases) and after various downstream unit operation steps.

	Degree of hydrolysis of hydrolysate [%]			
	100 g L^{-1} wheat gluten			25 g L^{-1} wheat gluten
	Flavourzyme	Bioprase	Thermoase	Flavourzyme
Hydrolysate (t=465 min)	42.5 ± 1.1	14.4 ± 0.8	8.85 ± 0.10	61.0 ± 1.9
Centrifuged	44.6 ± 0.3	16.0 ± 0.4	8.66 ± 0.20	61.9 ± 0.3
10 kDa filtered	39.7 ± 0.6	15.3 ± 0.3	8.52 ± 0.15	61.2 ± 0.3
3 kDa filtered	38.9 ± 0.6	14.1 ± 0.6	8.51 ± 0.05	58.9 ± 0.5
1 kDa filtered	37.8 ± 0.1	14.1 ± 0.2	8.49 ± 0.06	54.3 ± 0.4

Antigenicity of wheat gluten hydrolysates evaluated by competitive enzyme-linked immunosorbent assay (ELISA)

A competitive ELISA system was established and validated to check the influence of the enzymatic hydrolysis and of two common unit operations in the food industry (centrifugation and ultrafiltration) on the antigenicity of wheat gluten hydrolysates. The successful utilization of polyclonal antibodies for the quantification of prolamins was previously described [22].

Validation of the competitive ELISA

The ELISA test system was optimized in respect of the capture antigen (purified gliadin; see above) and the antibody titer, and the respective incubation times and temperatures (data not shown). With regard of the analysis of protein hydrolysates the developed ELISA assay was validated using skim milk protein hydrolysate, which was spiked with various amounts of purified gliadin. After 7 h of skim milk protein hydrolysis a DH of approximately 55 % was achieved. After lyophilization of the spiked skim milk hydrolysates final gliadin concentrations were 50, 100 and 500 mg kg^{-1}. The recovery was dependent on the ethanol concentration and was highest for 575 mL L^{-1} aqueous ethanol (concentration was varied from 500 to 700 mL L^{-1}; data not shown). The gliadin recovery with the described extraction method (see above) was 89.9 ± 9.6 % (n=14) for the extraction of 25 mg sample and 89.8 ± 10.9 % (n=26) for the extraction of 50 mg sample. The LOD of the competitive ELISA was 8.7 ng mL^{-1}. Considering the sample extraction and preparation the LOD of gliadin in the hydrolysate sample was 3.48 mg kg^{-1} and 1.74 mg kg^{-1} for the extraction of 25 and 50 mg sample, respectively. The LOQ of the

competitive ELISA was 20.9 ng mL^{-1} resulting in a LOQ in the hydrolysate sample of 8.39 mg kg^{-1} and 4.19 mg kg^{-1} for the extraction of 25 and 50 mg sample, respectively. This allowed the analysis of hydrolysate samples with an amount of gluten as low as 8.38 mg kg^{-1} (assuming a gluten to gliadin ratio of 2) [4] when working with a minimum sample dilution of 1:10 (see above) of the hydrolysate extracted at a concentration of 50 mg mL^{-1} of 575 mL L^{-1} aqueous ethanol. It has to be mentioned that the sensitivity of the standard (according to the Codex Alimentarius) competitive ELISA method is 2.44 mg kg^{-1} gluten [6, 23]. Nevertheless, it is possible to identify gluten free hydrolysates, which may not contain more than 20 mg kg^{-1} gluten [24, 25].

Influence of the enzymatic hydrolysis on the antigenicity of wheat gluten hydrolysates

The influence of the type of peptidase and the DH on the antigenicity of wheat gluten hydrolysates is illustrated in Figure 6.3 (Flavourzyme), Figure 6.4 (Bioprase) and Figure 6.5 (Thermoase). The gliadin concentration was reduced from 3.64×10^5, 3.77×10^5 and 3.94×10^5 mg kg^{-1} to 8.58×10^2, 1.13×10^4 and 1.05×10^4 mg kg^{-1} gliadin during the 465 min of hydrolysis with Flavourzyme, Bioprase and Thermoase, respectively. The initial gluten concentration (gluten/gliadin = 2) was, therefore, 7.28×10^5, 7.54×10^5 and 7.87×10^5 mg kg^{-1}. These values for the reference (t = 0) seem convincing, taking into account that the protein content of the wheat gluten powder is approximately 830 g kg^{-1} and that the recovery of the gliadin extraction is 89 %. For all peptidase preparations the antigenicity drastically decreased with increased DH. The DH dependent reduction of the antigenicity is higher for the two endopeptidases compared to the peptidase mixture (exopeptidases and endopeptidases). As seen in Figure 6.3 to 6.5, for example, the gliadin concentration was halved with a DH of approximately 6.9, 3.5 and 3.9 % for Flavourzyme, Bioprase and Thermoase, respectively. It might be more likely to inactivate an epitope when a peptide bond is cleaved inside the polypeptide chain compared to a cleavage of a C- or N-terminal amino acid. Because Flavourzyme showed the highest detoxification potential the hydrolysis with Flavourzyme was also carried out in presence of 25 g L^{-1} wheat gluten to reduce the influence of product inhibition. For this trial, a concentration of 2.15×10^2 mg kg^{-1} gliadin could be detected after 465 min of hydrolysis, which is around 25% of the gliadin concentration with 100 g L^{-1} substrate (see above). Nevertheless, no gluten-free hydrolysates were obtained by sole enzymatic hydrolysis.

Therefore, food processing unit operations (centrifugation, ultrafiltration) were employed to further reduce the antigenicity.

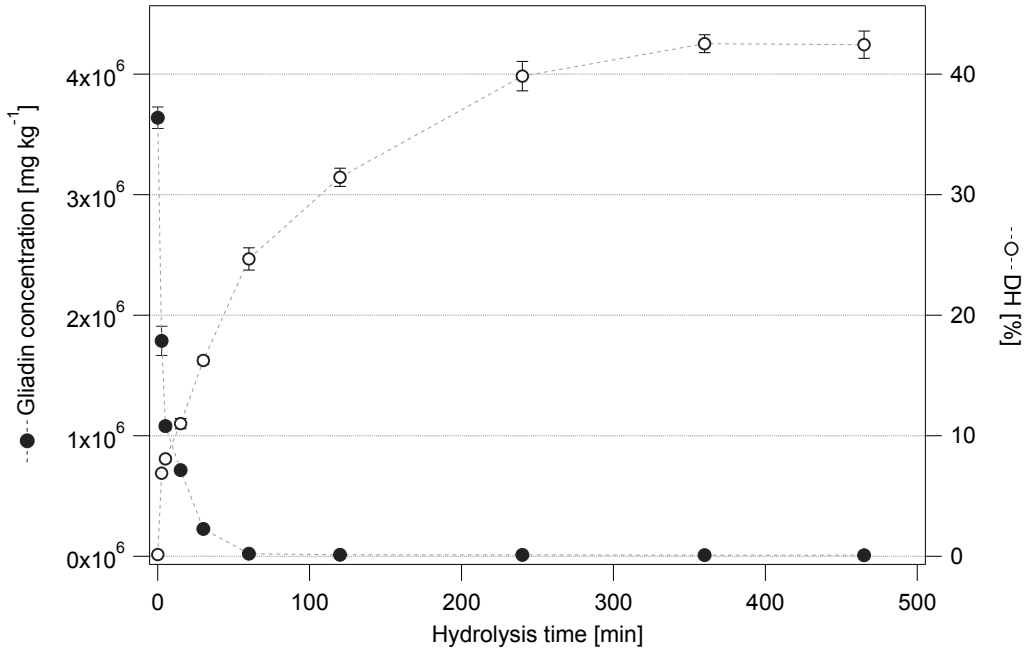


Figure 6.3: Gliadin concentration (filled circles), as a parameter for the antigenicity, and the degree of hydrolysis (DH) during the batch hydrolysis of 100 g L^{-1} wheat gluten using 25 nkat mL^{-1} Flavourzyme (37 °C, pH 7.5).

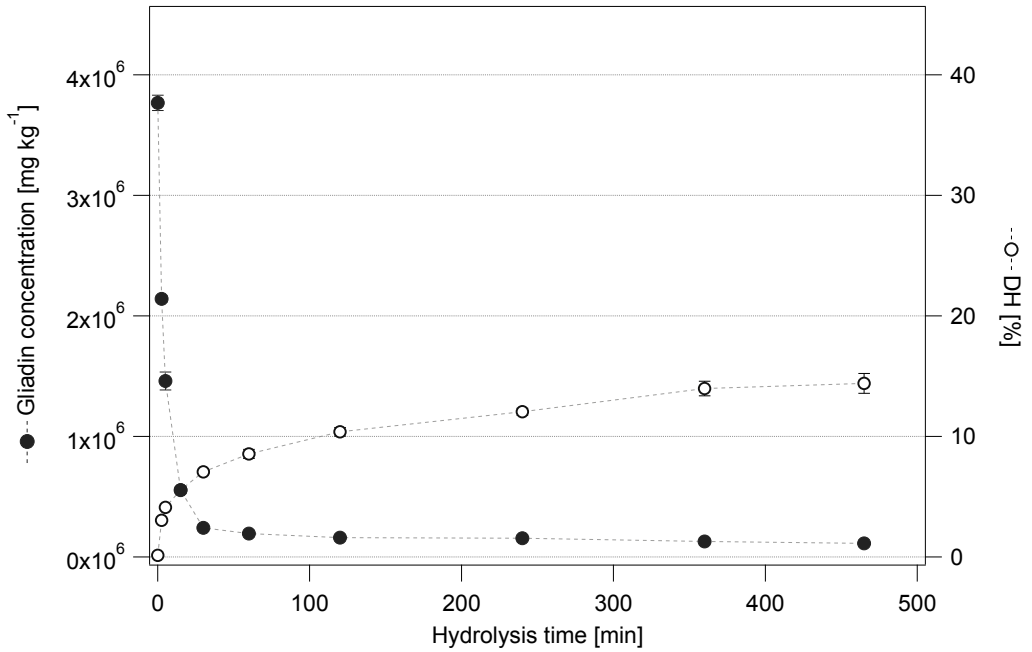


Figure 6.4: Gliadin concentration (filled circles), as a parameter for the antigenicity, and the degree of hydrolysis (DH) during the batch hydrolysis of 100 g L^{-1} wheat gluten using 25 nkat mL^{-1} Bioprase (37 °C, pH 7.0).

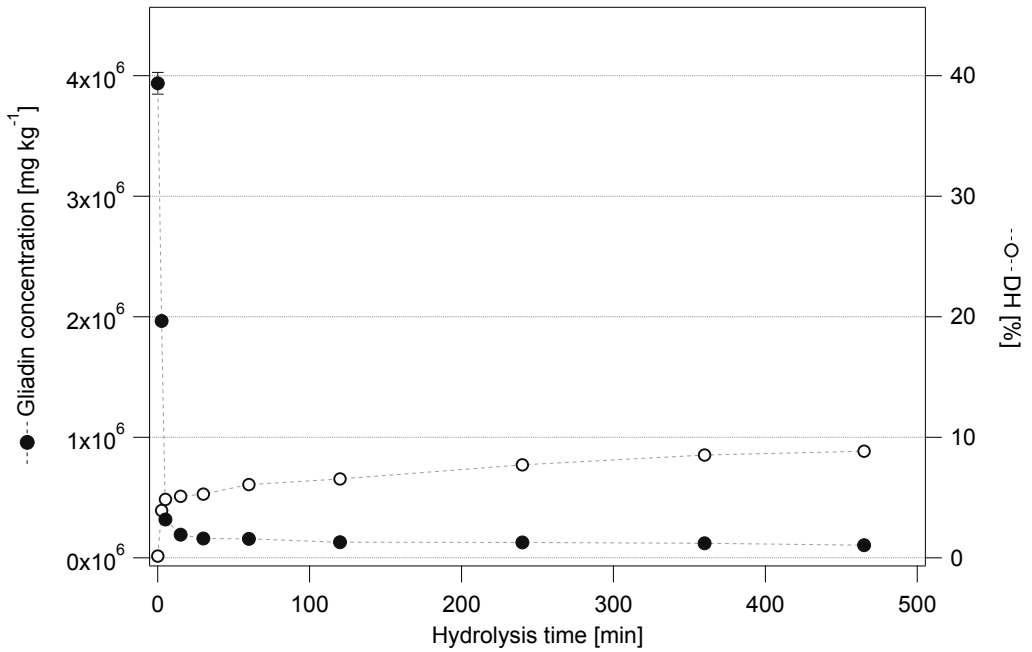


Figure 6.5: Gliadin concentration (filled circles), as a parameter for the antigenicity, and the degree of hydrolysis (DH) during the batch hydrolysis of 100 g L^{-1} wheat gluten using 25 nkat mL^{-1} Thermoase (37 °C, pH 7.5).

Influence of centrifugation and ultrafiltration on the antigenicity of wheat gluten hydrolysates

After the batch hydrolysis the enzymatic wheat gluten hydrolysates (see above) were centrifuged and, subsequently, either 1, 3 or 10 kDa filtered (Figure 6.1). The influence of the downstream unit operations (centrifugation, ultrafiltration) on the antigenicity of the hydrolysates is shown in Table 6.3. The antigenicity drastically decreased after centrifugation of the hydrolysates (t = 465 min). Using 100 g L^{-1} wheat gluten as substrate, the antigenicity was reduced from 858 ± 37, 11296 ± 614 and 10520 ± 437 mg kg^{-1} to 23.7 ± 0.8, 771 ± 24 and 1635 ± 24 mg kg^{-1} gliadin for Flavourzyme, Bioprase and Thermoase, respectively. A sole centrifugation was sufficient to obtain a gluten-free hydrolysate powder from the Flavourzyme/wheat gluten hydrolysis with 25 g L^{-1} substrate. The final gliadin concentration in the hydrolysate was 9.51 ± 0.30 mg kg^{-1}, corresponding to a gluten concentration of 19.02 mg kg^{-1} (gluten/gliadin ration = 2). A gluten-free hydrolysate was obtained for the Flavourzyme hydrolysate with 100 g L^{-1} substrate after centrifugation and subsequent 1 kDa ultrafiltration. The gliadin concentration was 8.23 ± 0.4 mg kg^{-1} (16.46 mg kg^{-1} gluten). The antigenicity decreased with lower MWCO. This trend was observed for all three hydrolysates (Flavourzyme,

Bioprase, Thermoase). A gluten free hydrolysate (100 g L^{-1}) was obtained after 1 kDa ultrafiltration of the Flavourzyme/wheat gluten hydrolysate. In general the antigenicity of the Flavourzyme/wheat gluten ultrafiltrates were lower compared to the Bioprase/wheat gluten and Thermoase/wheat gluten ultrafiltrates. Numerous previous studies described the enzymatic detoxification properties of fungal and bacterial peptidases during the production of sourdough [26]. However, the enzymatic hydrolysis and subsequent downstream processing of wheat gluten hydrolysates to produce gluten-free hydrolysates for the application in food products was only minorly discussed yet.

Table 6.3: Gliadin concentration determined of wheat gluten hydrolysates quantified by the competitive ELISA after enzymatic hydrolysis (with different commercial peptidases) and after various downstream unit operation steps.

	Gliadin concentration in dried hydrolysate [mg kg^{-1}]			
	100 g L^{-1} wheat gluten			25 g L^{-1} wheat gluten
	Flavourzyme	Bioprase	Thermoase	Flavourzyme
Hydrolysate (t=465 min)	858 ± 37	11296 ± 614	10520 ± 437	215 ± 11
Centrifuged	23.7 ± 0.8	771 ± 24	1635 ± 24	9.51 ± 0.30
10 kDa filtered	11.9 ± 0.1	126 ± 5.9	211 ± 4.2	2.22 ± 0.10
3 kDa filtered	11.2 ± 0.4	110 ± 5.4	156 ± 1.7	2.01 ± 0.23
1 kDa filtered	8.23 ± 0.40	59.0 ± 1.9	44.1 ± 1.9	1.87 ± 0.07

CONCLUSION

The influence of the enzymatic hydrolysis of wheat gluten on the antigenicity was investigated. Furthermore, common unit operations in food processing (centrifugation, ultrafiltration) were used to further decrease the allergenic potential of the hydrolysates. Gluten-free wheat gluten hydrolysates could be produced by enzymatic hydrolysis of wheat gluten using the complex commercial peptidase mixture Flavourzyme. A sole centrifugation was sufficient to obtain gluten-free hydrolysates when 25 g L^{-1} wheat gluten was applied as a substrate. Ultrafiltration with 1 kDa MWCO was necessary to obtain a gluten-free hydrolysate when the substrate concentration was increased to 100 g L^{-1}. In general, the antigenicity of the wheat gluten hydrolysates was highly dependent on the DH and drastically decreased along with the enzymatic hydrolysis. Gluten-free

hydrolysates were not obtained with the peptidases Bioprase and Thermoase, because the DH was moderately low. Also centrifugation and ultrafiltration was not sufficient to obtain gluten-free products with these peptidases. However, the work presented provides important information when these hydrolysates are applied, for example, as an ingredient in a food application. Depending on the proportion of the hydrolysate, an overall gluten-free food product might be obtained.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

A/A_0, normalized absorbance values; PBS, phosphate buffered saline; DH, degree of hydrolysis; ELISA, enzyme-linked immunosorbent assay; h, concentration of free amino groups; h_{tot}, maximum concentration of free amino groups at complete hydrolysis; kat, katal; LOD, limit of detection; LOQ, limit of quantification; OPA, *ortho*-phthalaldehyde; TCA, trichloroacetic acid.

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CHAPTER SEVEN

CONTINUOUS LONG-TERM HYDROLYSIS OF WHEAT GLUTEN USING A PRINCIPALLY FOOD-GRADE ENZYME MEMBRANE REACTOR SYSTEM

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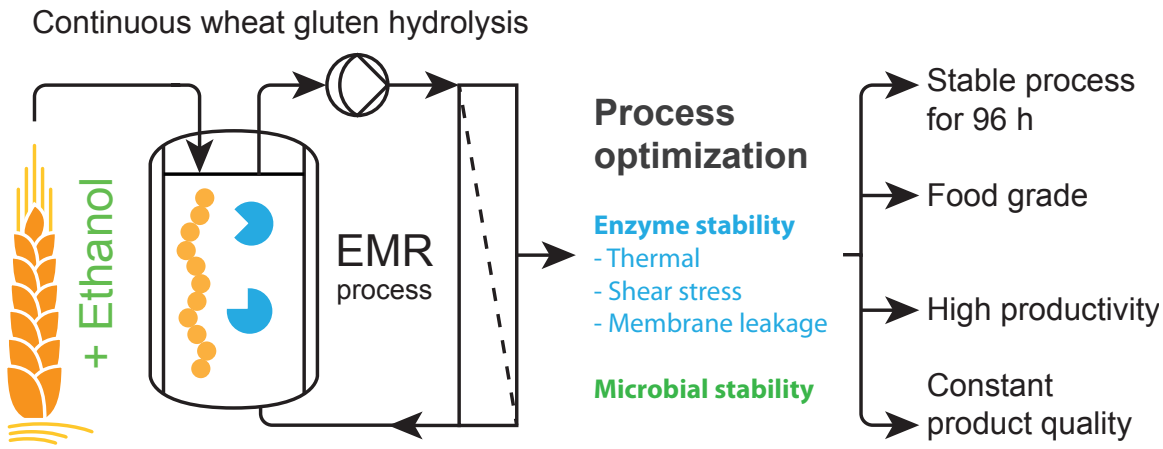
Graphical Abstract



ABSTRACT

The potential of the enzyme membrane reactor technology has been shown in several studies. In our study, we designed a principally food-grade continuous long-term hydrolysis process of wheat gluten in an enzyme membrane reactor with the proteolytic enzyme preparation Flavourzyme. Among others, ethanol was the most suitable food-grade processing aid for avoiding contamination and showed high potential for application. The Flavourzyme /wheat gluten process was comprehensively characterized in the enzyme membrane reactor. Critical factors for the enzyme stability were the temperature, pump stress and enzyme leakage through the membrane. The hydrolysis and process conditions were optimized to increase the space–time yield. Respective process parameters were chosen to obtain sufficient microbial, enzyme and process stability for the whole process time. The long-term hydrolysis was carried out in the presence of 8% (v/v) ethanol with a substrate concentration of 100 g L^{-1} at 37 °C and pH 7.5 for 96 h. The continuous process resulted in stable product quality (degree of hydrolysis) and space–time yield (6.33 g h^{-1} L^{-1}) over time. However, a discontinuous removal of accumulated dry matter was inevitable for the present enzyme membrane reactor process and was performed for 30 min every 24 h. Due to the fact that the enzymes were reused, the enzyme productivity could be increased by 450% compared to a reference batch process.

KEYWORDS

Wheat gluten hydrolysis, Enzyme membrane reactor, Continuous, Seasoning, Ethanol, *Aspergillus oryzae* peptidase, Protein hydrolysates

INTRODUCTION

Cereal grain protein hydrolysates are versatile food commodities and play a major role in the food additive industry. Depending on the degree of hydrolysis (DH), these hydrolysates can be used for emulsification, gelatinization or as seasoning [1]. Wheat gluten powder contains up to 85% protein, is highly available [2] and its hydrolysates are already used in a wide range of culinary products [3–6]. For these applications, the desirable product should have a high DH [1]. The industrial biotransformation of proteins, nowadays, is mainly performed in discontinuous batch processes [7]. However, the use of an enzyme membrane reactor (EMR) for a continuous biotransformation is promising [8] and features various advantages. The enzymes are located in a reaction space, entrapped by a membrane and, thus, can be reused, which is a significant economical benefit [9]. Additionally, hydrolysates standardized with a molecular weight cut-off (MWCO) can be produced at high and constant space–time yields (STYs) [10]. The selective removal of products from the reaction site can also increase the conversion of product-inhibited or thermodynamically unfavorable reactions [8]. As a result, process efficiency and enzyme productivity can be increased compared to the classical batch processes in the case enzyme stability is sufficient and the lifecycle time satisfactory.

Membrane technology has been successfully used in the production of protein fractions with specific functional properties [2, 11–13] and for the continuous production of protein hydrolysates in laboratory scale in an EMR [14–24]. However, microbial stability is not often mentioned or discussed in literature during long-term (>10 h) continuous hydrolysis [15, 20] or non-food-grade additives, such as sodium azide, were used [14]. The microbial stability of a protein biotransformation is crucial, especially at moderate process conditions, and cannot be taken for granted.

In this study, an approach for a long-term hydrolysis with a principally food-grade EMR system was carried out to achieve a stable process with a high enzyme productivity and a constant STY over 96 h. The industrial peptidase preparation Flavourzyme, which contains endo- and exopeptidase activities [18], was used for the wheat gluten hydrolysis. Factors affecting the operating stability of the wheat gluten enzymatic hydrolysis process, including microbial stability and enzyme stability inside the EMR, were identified and considered for the continuous process design.

MATERIALS AND METHODS

Materials and chemicals

Wheat gluten and Flavourzyme 1000 L (Novozymes) were obtained from Nestlé Product Technology Centre (Singen, Germany). The 2,4,6-trinitrobenzene sulfonic acid (TNBS) and the azocasein were bought from Sigma (Taufkirchen, Germany). The exopeptidase substrate H-Leu-*p*NA was obtained from Bachem (Bubendorf, Switzerland). Ethanol was obtained from the department of Yeast Genetics and Fermentation Technology (150f) at the University of Hohenheim (Stuttgart, Germany).

Analytical methods

Determination of amino groups with 2,4,6-trinitrobenzene sulfonic acid

Primary amino groups were determined after derivatization with TNBS according to the method of [25] with some modifications. A volume of 7.5 µL of the sample was transferred into a microtiter plate and sodium phosphate buffer (0.2125 M, 60 µL) and TNBS reagent [0.1% (w/v) in H2O, 60 µL] were added. The plate was incubated at 60 °C for 10 min, HCl (0.1 M, 120 µL) was added afterwards and the absorbance was measured at 340 nm using a microtiter plate reader (Multiskan FC). The calibration was performed using l-leucine as a reference.

Degree of hydrolysis

The DH was calculated according to Adler-Nissen [25] with modifications (Eq. 7.1).

(Eq. 7.1) $$\mathbf{DH} = \frac{h}{h_{tot}} \times \mathbf{100}\ [\%]$$

where h is the concentration of free amino groups [mol L^{-1}; see above] and h_{tot} is the maximum concentration of free amino groups at complete hydrolysis [mol L^{-1}] calculated

(Eq. 7.2) $$\boldsymbol{h_{tot}} = \frac{\mathbf{c_{Protein}}}{M^{*} - M_{H_2O}}\ [\mathbf{mol\ L^{-1}}]$$

where $c_{Protein}$ is the concentration of protein which is hydrolyzed (g L^{-1}) and M^* is the average molecular mass of the amino acids in wheat gluten (133.5 g mol^{-1}). This gluten-specific average molecular mass was calculated by considering the wheat gluten amino acid composition [26]. The molecular mass of water (M_{H2O}= 18 g mol^{-1}) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

Total proteolytic activity of Flavourzyme on wheat gluten

Wheat gluten was used as the substrate. For the substrate solution, 100 g L^{-1} wheat gluten was suspended in the corresponding buffer and heated to 60 °C under stirring for at least 2 h and a sedimentation was carried out at 20 °C for 1 h. This supernatant was used for activity measurement and contained a soluble protein content of 2 g L^{-1}. The protein content was determined with the method of Bradford [27] and bovine serum albumin fraction V (BSA) as the reference. The standard assay contained 250 µL of substrate solution and was preincubated at 37 °C for 5 min. Then, the reaction was started with the addition of 50 µL appropriately diluted Flavourzyme sample and was stopped by the addition of TCA (2 mol L^{-1}, 50 µL) and was centrifuged (20,000 × g, 4 °C, 5 min) afterwards. The supernatant (7.5 µL) was transferred into a microtiter plate and the TNBS assay was performed (see above). One katal (kat) of total proteolytic activity was defined as the release of 1 mol l-leucine-equivalent amino groups per second at the defined assay conditions. Since, Flavourzyme is known to contain exo- and endopeptidase activities both were also separately quantified (see below).

Endopeptidase activity assay

The assay was performed according to the method of Iversen and Jørgensen [28] with modifications. The reaction was carried out in 1.5 mL reaction tubes using a thermomixer (ThermoMixer comfort, Eppendorf, Hamburg, Germany). The substrate azocasein (2.5 g L^{-1}) was dissolved in Tris-HCl buffer (50 mmol L^{-1}, pH 7.5). The standard assay was performed as follows: the substrate solution (250 µL) was preincubated at 37 °C for 5 min. The reaction was started with the addition of an appropriately diluted Flavourzyme sample (50 µL), stopped by the addition of trichloroacetic acid (TCA, 2 mol L^{-1}, 50 µL) and centrifuged (20,000 × g, 4 °C, 5 min, 5417 R, Eppendorf) afterwards. The supernatant (195 µL) was transferred into a microtiter plate, NaOH (1.5 mol L^{-1}, 50 µL) was added and the absorbance was measured at 450 nm using a microtiter plate reader (Multiskan FC, Thermo, Schwerte, Germany). One azocasein unit (ACU) was defined as the increase of one optical density unit per minute at 450 nm in 0.25 mol L^{-1} NaOH.

Exopeptidase activity assay

Exopeptidase activity was measured according to the method of Chrispeels and Boulter [29] with modifications [30]. The standard assay contained 177 µL of Tris-HCl buffer (50 mmol L^{-1}, pH 7.5) and 50 µL appropriately diluted Flavourzyme sample. Buffer and enzyme were preincubated at 37 °C for 5 min. The reaction was started with the addition of 12.5 µL (3.7 mmol L^{-1}) L-leucine-*para*-nitroanilide (Leu-*p*NA) solved in

dimethylformamide, stopped by the addition of 50% (v/v) acetic acid (50 µL) and was centrifuged (20,000 × g, 4 °C, 5 min) afterwards. The supernatant (240 µL) was transferred into a microtiter plate and the absorbance was measured at 405 nm using a microtiter plate reader (Multiskan FC). One katal (kat) of exopeptidase activity was defined as the release of 1 mol *p*-nitroanilide per second.

Determination of dry matter content

The dry matter content (DM) was determined by weighing the dried hydrolysate samples. Respective samples (1 mL) were dried in a vacuum centrifuge concentrator (RVC 2-33 IR, Christ, Osterode, Germany) at 100 Pa and 50 °C for 4–6 h until a constant weight was reached.

Flavourzyme /wheat gluten preparation

Different wheat gluten concentrations (25, 50, 100, 200 g L^{-1}) were suspended and hydrolyzed with Flavourzyme under standardized conditions in order to obtain a liquid and to allow investigations in an EMR setup. For the preparation wheat gluten (25–200 g L^{-1}) was suspended in Tris-HCl buffer (20 mmol L^{-1}, pH 7.5) containing 8% (v/v) ethanol. The suspension was stirred with a magnetic stirrer (Rheo Basic, IKA, Staufen, Germany) at 500 rpm and the reaction was started by the addition of appropriately diluted Flavourzyme (20 $nkat_{glutenTNBS}$ mL^{-1} if not mentioned otherwise). The temperature was kept at 37 °C. After 16 h of hydrolysis the equilibrium of the DH was reached for all enzyme activity/wheat gluten combinations. The liquid was used for further trials without inactivation of the Flavourzyme peptidases after the mentioned 16 h.

The effect of temperature and pH on the total proteolytic activity of Flavourzyme

Temperature and pH profiles of the total proteolytic activity were determined under standard assay conditions with the addition of 8% (v/v) ethanol. The temperature profile was determined in Tris-HCl buffer (50 mmol L^{-1}, pH 7.5) in the range from 20 to 70 °C. The pH profile was determined at 37 °C from pH 5 to pH 11 in the corresponding buffer (MES-NaOH, Tris-HCl, CHES-NaOH; 50 mmol L^{-1}). Relative activities were plotted over temperature and pH, respectively.

EMR setup

The EMR flow scheme and setup is shown in Figure 7.1. Unless otherwise mentioned, a diaphragm pump (P1; Hydracell G03X, Verder, Haan, Germany) was used to circulate

the reaction volume of 1 L. The flow rate was 3.3 L min^{-1} resulting in 3.3 volume replacements per min. Hollow fiber Al2O3 ceramic membranes (l = 45 mm, Ø 6 mm, A = 0.0085 m^2, Atech innovations, Gladbeck, Germany) were used for crossflow ultrafiltration (F1) of the hydrolysates with 1, 5 or 10 kDa membrane MWCO. With this setup, a Reynolds (Re) number of 4900 could be achieved. Physical properties of a 10% (w/v) whey protein solution [31] were used for the Reynolds number estimation. With a calculated Reynolds number of Re= 4900, which is above the critical Reynolds number calculated of $Re_{critical}$ = 2300 [32], a turbulent tube flow was assumed. Regeneration and cleaning of the membranes was carried out with NaOH (0.5 mol L^{-1}) and NaOCl (10 mmol L^{-1}) at 75 °C for 10 min after each trial. Afterwards, the membranes were cleaned with water for 30 min. The pump and filtration unit were connected using pressure-resistant flex tubes (Riegler, Bad Urach, Germany) made of stainless steel. A safety valve (V2) was used to limit the maximum pressure over the membrane to 6 bar. The temperature of the reaction volume was regulated using a water bath (W1; Julabo ED-5, Seelbach, Germany) connected to the double-vessel reaction chamber (B2; 1 L, Ochs Gerätebau, Bovenden, Germany). The hydrolysate inside the reactor was stirred using a magnetic stirrer (R2; Ika Rheo Basic). A constant transmembrane pressure of 2 bar was regulated using a ball valve (V1) and was read from barometers (PI1, PI2). The permeate was collected and weighed immediately (Precisa 800M, Precisa, Switzerland) for the determination of the membrane flux. New substrate for the long-term continuous hydrolysis was fed continuously using a tube pump (P2; TB, Medorex, Nörten-Hardenberg, Germany). A level sensor (LIC) inside the reactor controlled the current supply for the tube pump and, therefore, ensured a constant reaction volume of 1 L. The hysteresis of the level control was set to 50 mL. The feed and reaction volumes were stirred moderately to ensure homogeneity. The feed vessel was located in an ice bath for the whole trial.

Operating stability of the EMR process

Microbial stability of wheat gluten suspensions

The trials were carried out in 50 mL scale in Falcon tubes. Three food-grade additives were tested for their ability to prevent microbial growth in wheat gluten suspensions. Wheat gluten (100 g L^{-1}) was incubated in Tris-HCl buffer (20 mmol L^{-1}, pH 7.5) at 37 °C for 36 days. Ethanol [C_2H_5OH; 2–10% (v/v)], sodium chloride [NaCl; 2–15% (w/v)] and hydrogen peroxide [H2O2; 0.005, 0.05 and 0.5% (v/v)] were tested individually with

and without the addition of Flavourzyme (20 $nkat_{glutenTNBS}$ mL^{-1}). Samples were taken daily and examined microscopically for microbial contamination. The microbial stability was defined as the absence of microorganisms in the visual range at 400-fold magnification during microscopy (Axio Scope.A1, Zeiss, Jena, Germany).

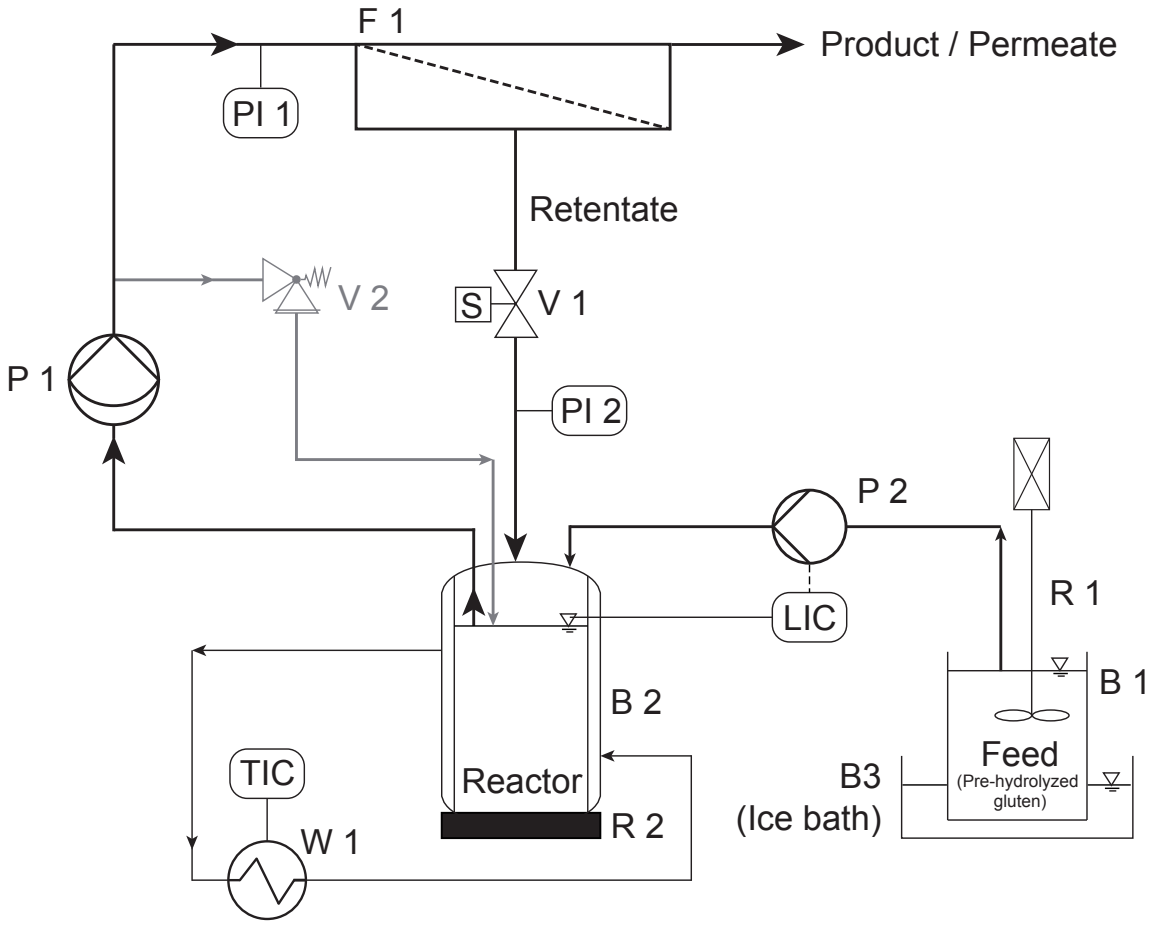


Figure 7.1: Schematic flow diagram of the experimental EMR setting including two stir tank reactors (B1, B2), a water bath (W1) with a thermostat (TIC), a diaphragm pump (P1), a feed pump (P2), a crossflow filtration unit (F1), two barometers (PI1, PI2), a level indicator (LIC) and valves (V1, V2); grey line indicates the membrane bypass which is activated if the pressure exceeds 6 bar.

Thermal enzyme stability

Flavourzyme was incubated at 50 °C in 2 mL reaction [20 mmol L^{-1}, pH 7.5; 8% (v/v) ethanol] tubes in a thermomixer to determine the thermal stability. Flavourzyme was incubated in Tris-HCl buffer solution (20 mmol L^{-1}, pH 7.5) containing 8% (v/v) ethanol for 192 h to check the operating thermal stability of the peptidases at 37 °C. The trials at

37 °C were carried out in 50 mL scale in Falcon tubes. All trials were carried out in the presence of 100 g L^{-1} wheat gluten to consider the stabilizing effect of the substrate. Samples were taken after various incubation times and chilled on ice immediately. Residual activities were measured using corresponding enzyme assays (see above). The initial activity prior to incubation was set to 100% and relative enzyme activity was plotted against incubation times.

Influence of the pump system on the enzyme stability

The influence of shear stress on the operational stability of the Flavourzyme peptidases was tested using two types of pumps. The trials were carried out in the EMR without filtration (0 bar of transmembrane pressure). A gear type pump (Gather, Mettmann, Germany) and a diaphragm pump (Hydracell G03X) were used as circulation pumps. A Tris buffer solution (1 L, 20 mmol L^{-1}, pH 7.5) containing 8% ethanol (v/v) with 20 nkatglutenTNBS mL^{-1} Flavourzyme was circulated at 20 °C. Samples were taken after 400, 800, 1,200, 4,800, 6500 and 10,000 volume replacements and were compared to a blank sample also stored at 20 °C without pumping. The samples were chilled on ice and residual activity was measured using the corresponding assay (see above). The residual activity was plotted over volume replacements.

Membrane leakage of enzyme activity

Flavourzyme/wheat gluten preparations (see above) were used for this trial. The gluten concentrations in the preparation were 25, 50, 100 and 200 g L^{-1}, respectively. Ceramic membranes with MWCO of 1, 5 and 10 kDa were used and tested with each gluten concentration. For trials without gluten (control) 20 $nkat_{glutenTNBS}$ mL^{-1} Flavourzyme was dissolved in buffer and 8% (v/v) ethanol. Each trial was carried out in the EMR with a transmembrane pressure of 2 bar. Permeate samples of 1 mL were taken when a steady state of the membrane flux was reached and were chilled on ice immediately. The permeate was tested for endopeptidase, exopeptidase and total proteolytic activity (see above).

Characteristic variables to evaluate a membrane separation process

In order to evaluate the membrane separation process, the STY and the product quality (DH) were determined for different gluten concentrations and with membranes with either a 1, 5 or 10 kDa MWCO. Again, Flavourzyme /wheat gluten preparations (see above) were used for this trial (25, 50, 100 and 200 g L^{-1}). Hydrolysates were filtered in

the EMR (1, 5 and 10 kDa MWCO; 2 bar transmembrane pressure) and permeates were analyzed for DH (see above) and DM (see above). The membrane flux was measured every minute by weighing the permeate until a steady state was reached. Eq. 7.3 was used to calculate the STY for every substrate/MWCO combination.

(Eq. 7.3) $$\mathbf{STY} = \boldsymbol{J} \times \mathbf{DM} \times \boldsymbol{V}\ [\mathbf{g\ h^{-1}\ L^{-1}}]$$

where J is the flux through the membrane [L h^{-1}], DM is the dry matter in the permeate [g L^{-1}] and V is the reactor volume [L]. To evaluate the applicability of a combination of substrate concentration and MWCO also the product quality has to be taken into account. Thus, Eq. 7.4 was used to estimate the performance of a certain combination.

(Eq. 7.4) $$\mathbf{Performance} = \mathbf{STY} \times \frac{\mathbf{DH}}{\mathbf{100}}\ [\mathbf{g\ h^{-1}\ L^{-1}}]$$

With the performance, both the STY and the product quality (DH; see above) were considered. The enzyme productivity (Eq. 7.5) was defined as the amount of product ($m_{Product}$) which can be produced with a certain enzyme activity (EA_{tot}). The initial activity in the EMR ($EA_{Initial}$), the enzyme activity in the feed stream (EA_{Feed}) and the flow rate of the feed stream (v_{Feed}; equivalent to the membrane flux) were considered for the total activity.

(Eq. 7.5) $$\mathbf{Enzyme\ productivity} = \frac{\mathbf{m_{Product}}}{\mathbf{EA_{tot}}} = \frac{\mathbf{m_{Product}}}{\mathbf{EA_{Initial}} + \int_0^t \mathbf{EA_{Feed}} \times \boldsymbol{v}_{\mathbf{Feed}}\, \boldsymbol{dt}}\ [\mathbf{mg\ nkat^{-1}}]$$

Continuous wheat gluten hydrolysis in the EMR

The loss of enzyme activity through the permeate during operation of the EMR has to be added in the feed stream to maintain a certain enzyme activity during the long-term EMR process. The total loss of enzyme activity (EA_{Total}) due to enzyme leakage through membrane ($EA_{Leakage}$), thermal inactivation ($EA_{Thermal}$) and pump stress (EA_{Pump}) was calculated with Eq. 7.6. These variables were considered as main reasons for the activity loss.

(Eq. 7.6) $$\mathbf{EA_{tot}} = \mathbf{EA_{Leakage}} + \mathbf{EA_{Thermal}} + \mathbf{EA_{Pump}}\ [\mathbf{nkat\ mL^{-1}}]$$

Particular losses can be calculated as follows:

(Eq. 7.7) $$\mathbf{EA_{Thermal}} = \frac{\boldsymbol{k}_{\mathbf{T}} \times \mathbf{EA_{Initial}} \times \boldsymbol{V}}{\boldsymbol{J}}\ [\mathbf{nkat\ mL^{-1}}]$$

(Eq. 7.8) $$\mathbf{EA_{Pump}} = \frac{\boldsymbol{k}_{\mathbf{P}} \times \mathbf{EA_{Initial}} \times \boldsymbol{V}}{\boldsymbol{J}}\ [\mathbf{nkat\ mL^{-1}}]$$

where $EA_{Thermal}$ and EA_{Pump} are the losses due to thermal inactivation and pump stress, respectively. The temperature-dependent inactivation rate k_T [h^{-1}] and the pump stress-dependent inactivation rate k_P [h^{-1}] are multiplied with the initial enzyme activity in the

EMR ($EA_{Initial}$) and the reactor volume (V). The inactivation rate constants were calculated by first order expression according to Eq. 7.9, where EA_t is the enzyme activity after a particular incubation time and EA_0 is the initial enzyme activity.

(Eq. 7.9) $$\ln\left[\frac{EA_t}{EA_0}\right] = -k_{T/P} \times t$$

The time-dependent activity loss (k * $EA_{Initial}$) is divided by the membrane flux of the EMR to obtain the volumetric activity, which has to be added to the feed. The loss of enzyme activity due to membrane leakage ($EA_{Leakage}$) equals the activity measured in the permeate.

Based on the calculations above the feed solution (100 g L^{-1} wheat gluten) was pre-hydrolyzed with 2 $nkat_{glutenTNBS}$ mL^{-1} Flavourzyme at 37 °C in Tris-HCl buffer (20 mmol L^{-1}, pH 7.5) and in the presence of 8% (v/v) ethanol to increase homogeneity and to compensate activity losses. When a DH of 15% was reached, the Flavourzyme/wheat gluten suspension was pumpable and was transferred into the feed vessel (B1, Figure 7.1). The feed vessel was stored on ice to prevent an inactivation of Flavourzyme during the long-term operation.

For the continuous process a wheat gluten concentration of 100 g L^{-1} was hydrolyzed in the EMR (reactor B2, Figure 7.1) with 20 $nkat_{glutenTNBS}$ mL^{-1} Flavourzyme as initial enzyme activity. The hydrolysis was carried out at 37 °C and pH 7.5 in the presence of 8% (v/v) ethanol. After 16 h of hydrolysis (DH equilibrium) the transmembrane pressure was set to 2 bar and the EMR process started (t = 0). A 10 kDa ceramic membrane was used for cross-flow filtration. The flux was measured regularly by scaling the permeate. The permeate, the retentate and the feed samples (1.5 mL each) were taken at various times to determine the DM (see above), the DH (see above) and the enzyme activity (see above). The retentate samples were also checked for ethanol concentration (Enzymatic test kit, R-Biopharm, Darmstadt, Germany) to ensure a constant ethanol concentration of 8% (v/v). If the concentration was decreased, ethanol was added accordingly to regain 8% (v/v). Since, an accumulation of insoluble dry matter, such as starch, was inevitable the latter was removed regularly by centrifugation. The total reaction volume of 1 L was removed from the reactor at 24, 48, 72 and 94 h and was centrifuged (10,000 * g, 4 °C, 15 min). After centrifugation, the supernatant was returned to the reactor and the EMR process continued. In the present lab scale EMR a continuous solution for the removal of the insoluble dry matter could not be found.

Statistical analysis

All experiments were carried out in duplicate with two independent measurements. The continuous hydrolysis approach for 96 h was performed once. Standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA).

RESULTS AND DISCUSSION

Microbial stability of wheat gluten suspensions

Later experiments showed that temperature could not be used as microbial hurdle since Flavourzyme was not sufficiently stable (see below). Therefore the following experiments were carried out at an appropriate temperature of 37 °C. The main bacterial spoilage in wheat is caused by *Bacillus* species [33]. The germination of Bacillus spores must be avoided to obtain a safe and stable hydrolysis process. Therefore, wheat gluten suspensions (100 g L^{-1}) were incubated at 37 °C for 36 days. Investigations on ethanol (C_2H_5OH), sodium chloride (NaCl) and hydrogen peroxide (H_2O_2) have been carried out to find a reliable principally food-grade microbial hurdle. Ethanol could be removed from the product and recycled in the drying process when producing powders. Sodium chloride is already used as additional hurdle in concentrations of 15–20% in the industrial production of soy sauce [34]. Incubated suspensions were analyzed daily for microbial growth (Table 7.1).

Table 7.1: Investigated microbial hurdles at 37 ◦C for wheat gluten suspensions (100 g L^{-1}). Microbial growth was monitored for a maximum of 36 days.

Microbial hurdle	Concentration [%]	Time of microbial stability
na (control)	na	12–16 h
Ethanol (C_2H_5OH)	4	2 d
	6	5 d
	7	6 d
	7.5	36 d
	8	36 d
Hydrogen peroxide (H_2O_2)	0.05	12–16 h
	0.5	36 d
Sodium chloride (NaCl)	10	12–16 h
	15	24 h

na, not applicable

Microbial growth in control suspension without ethanol, sodium chloride or hydrogen peroxide was observed after 12–16 h of incubation. The microbial stability could be increased to 24 h in the presence of 15% (w/v) NaCl. A concentration of 0.5% (v/v) H_2O_2 was necessary to keep the wheat gluten suspension stable for 36 days. The suspension was microbially stable for 2 d with 4% ethanol, 5 d with 6% ethanol, 6 d with 7% ethanol and 36 days with 7.5% ethanol (v/v), respectively. The ethanol sensitivity of *B. subtilis* spores was previously described by Bohin et al. [35]. These authors reported a 77% growth rate reduction at 37 °C in the presence of 5.8% (v/v) (1 mol L^{-1}) ethanol. The growth rate reduction with 1.74% (v/v) and 2.9% (v/v) ethanol was 22% and 54%, respectively. Hence, the prevention of germination in the presence of 8% (v/v) ethanol (1.38 mol L^{-1}) seems forceful. Thus, ethanol was chosen as microbial hurdle, due to the uncertainty of amino acid oxidations by H_2O_2 [36]. Ethanol was applied in a concentration of 8% (v/v) for all further investigations.

The effect of temperature and pH on the total proteolytic activity of Flavourzyme

The effect of temperature and pH on the total initial proteolytic activity is shown in Figure 7.2. The highest initial activity was determined at 50 °C (2,688 $nkat_{glutenTNBS}$ mL^{-1}). Concerning the pH value, the highest initial activity was determined in Tris-HCl buffer (20 mmol L^{-1}) at pH 7.5 (2,016 $nkat_{glutenTNBS}$ mL^{-1}; Figure 7.2b). In previous studies, with a Design of Experiments (DoE) of soy protein isolates, a pH value of 7.12 was determined as the optimum pH for hydrolysis with Flavourzyme [37]. In addition, the optimum pH for hydrolysis of fish proteins was reported at pH 7.5 for Flavourzyme [38]. Cheison et al. [18] determined the optimum conditions for a whey protein hydrolysis at pH 7.0 and 50 °C. The results obtained in the current study are in accordance with the literature as described above. Although 8% (v/v) ethanol was present in the buffers used, no shifting effect on the optimum temperature and pH of the total proteolytic activity of Flavourzyme was determined in a control sample (data not shown). However, if 8% (v/v) ethanol was present in the substrate solution a decrease of the total proteolytic activity of 30% was measured. This could probably be explained due to the denaturation of the enzyme molecules by ethanol [39].

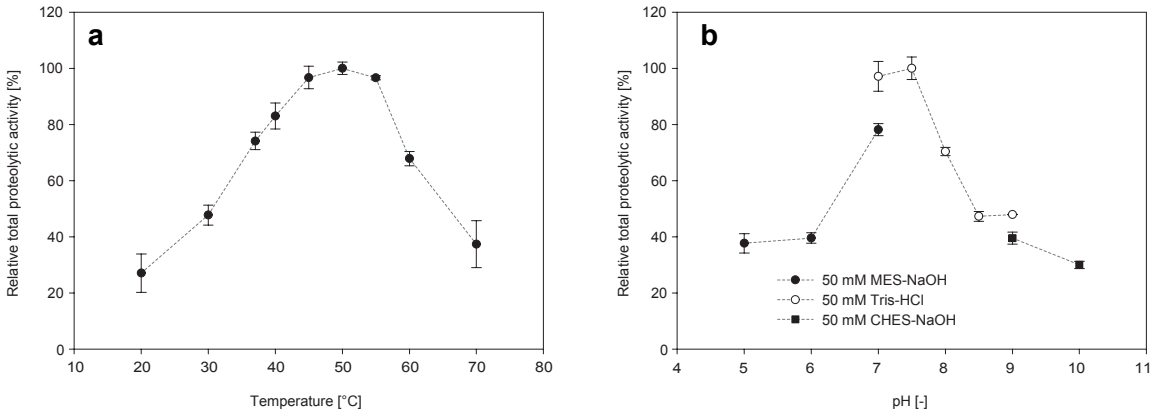


Figure 7.2: Effect of temperature (a) and pH (b) on the Flavourzyme total proteolytic activity in presence of 8% (v/v) ethanol (a: 100% ≡ 2,688 $nkat_{glutenTNBS}$ mL^{-1}; b: 100% ≡ 2,016 $nkat_{glutenTNBS}$ mL^{-1}. Results represent the mean ± standard deviation of two independent measurements).

Investigations about the operating stability of Flavourzyme

Another important parameter for a stable and economical EMR process is the retention of the enzyme inside the EMR [10]. This was checked by enzyme activity measurement of permeate and retentate. According to [21], the decline of enzyme activity inside the reactor can be due to (a) thermal inactivation; (b) mechanical shearing forces; (c) adsorption or "poisoning" of the enzyme through contact with the membrane; (d) enzyme leakage and/or (e) enzyme inhibition due to product accumulation. Except (c), these factors were investigated in order to determine their effect on the long-term operational stability of Flavourzyme in the EMR. Since an inert ceramic membrane was used the membrane poisoning was assumed to be of minor influence for this process after saturation of the membrane with substrate.

Thermal enzyme stability

All experiments were carried out in the presence of 100 g L^{-1} wheat gluten, which was the target concentration for the later EMR process. In preliminary studies, the thermal stability of the Flavourzyme peptidases (separately endoand exopeptidase activity) was firstly tested at the optimum temperature of its total initial activity (50 °C, see above). The half-life of endopeptidase activity at 50 °C was 1.93 h, which would not allow a long-term hydrolysis process. Thus, the operational stability of the endopeptidase and exopeptidase activity was then checked at lower temperatures (data not shown). The temperature of 37 °C revealed the best compromise of stability and activity of

Flavourzyme . The operational stability of the endo- and exopeptidase activity at 37 °C for 196 h is shown in Figure 7.3a. The resulting residual endopeptidase activity was 67%. The corresponding extrapolated half-life at 37 °C was 422 h. The residual activity for the exopeptidase activity was even higher (82%) after 196 h. Based on the results presented, all subsequent batch experiments and the continuous hydrolysis were carried out at 37 °C. The endopeptidase stability seemed to be the crucial parameter regarding thermal inactivation. An inactivation kinetic was determined and the resulting thermal inactivation rate (k_T) was 0.0017 h^{-1}.

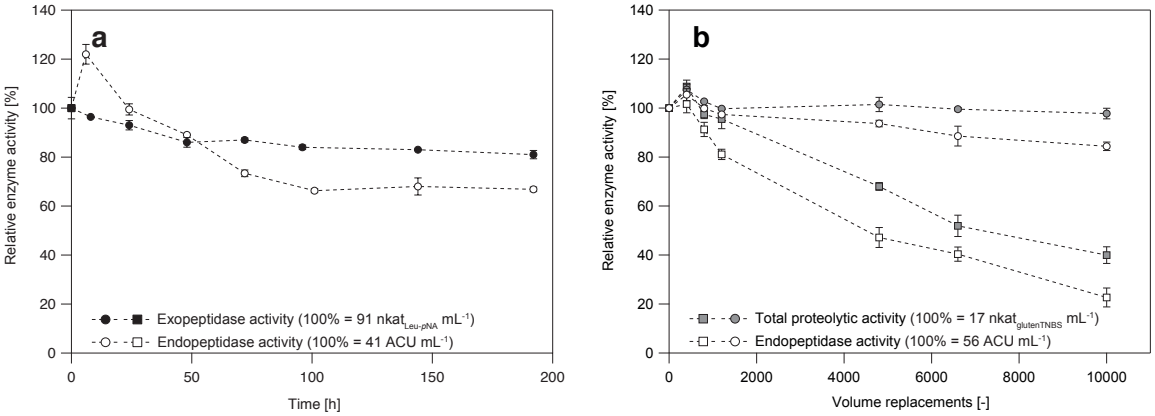


Figure 7.3: Stability of Flavourzyme. (a) Thermal stability demonstrated with its endo- and exopeptidase activity at 37 °C and (b) mechanical stress on total proteolytic activity and endopeptidase activity using two different pumps at 20 °C. Diaphragm pump (circles) and gear type pump (squares). Results represent the mean ± standard deviation of two independent measurements.

Influence of the pump system on Flavourzyme stability

Two different pump systems were tested separately to check whether this affected the enzymatic activity loss during operation (see Figure 7.3b). The following experiments were carried out in buffer system at 20 °C in order to focus on the impact of the pump systems on the enzyme activity.

The gear type pump had a much higher impact on enzyme inactivation compared to the diaphragm pump, as shown in Figure 7.3b. After 10,000 volume replacements (i.e. after 50 h of pumping) the residual proteolytic activity was only 40% with the gear pump compared to 98% with the diaphragm pump. The remaining endopeptidase activity after 10,000 volume replacements was 23% with the gear type pump and 84% with the diaphragm pump. The exopeptidase stability was comparable to the total proteolytic

stability and is omitted in Figure 7.3b for more clearness. Sannier et al. [40] also showed a significant inactivation of pepsin after 24 h of pumping with a gear type pump, whereas Guadix et al. [41] could not measure any mechanical inactivation of subtilisin with a rotary pump within 250 min of pumping. On the basis of these results, the diaphragm pump was chosen for all subsequent EMR trials. The endopeptidase stability again seemed to be the crucial activity regarding inactivation due to mechanical stress. An inactivation kinetic was determined and the resulting pump stress-dependent inactivation rate was $k_P = 0.0032\ h^{-1}$ for the endopeptidase activity.

Membrane flux and membrane leakage of Flavourzyme activity

Flavourzyme/wheat gluten preparations (see above) were used for this trial. The gluten concentrations in the preparation were 25, 50, 100 and 200 g L^{-1}, respectively. The use of a hydrolyzed wheat gluten preparation was appropriate because this solution is in contact with the membranes during the final process (see Figure 7.1). Investigations were carried out in the EMR using 1, 5 and 10 kDa membrane MWCOs (see Figure 7.4). Preliminary studies showed that a steady state concerning the flux was reached after 30 min of filtration for all tested membranes and hydrolysate concentrations (data not shown).

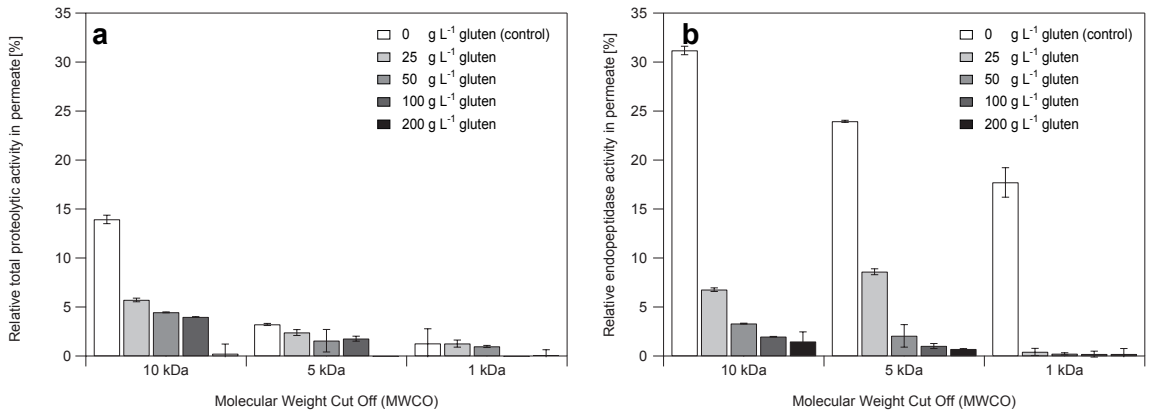


Figure 7.4: Effect of MWCO and gluten concentration in the Flavourzyme/wheat gluten preparation on the membrane leakage of the total proteolytic activity (a) and the endopeptidase activity (b). Activity in permeate was determined at steady state of the flux. In case of exopeptidase no activity was found in any Flavourzyme /wheat gluten permeate (100% total proteolytic activity was 21 $nkat_{glutenTNBS}$ mL^{-1}; 100% endopeptidase activity was 53 ACU mL^{-1}). Results represent the mean ± standard deviation of two independent measurements.

No exopeptidase activity could be measured in permeate in presence of any Flavourzyme/wheat gluten preparation. In case of buffer (control) a slight leakage of 3.28% was observed. The leakage of total proteolytic and endopeptidase activity was clearly dependent on the wheat gluten concentration in the Flavourzyme/wheat gluten preparation. The total proteolytic activity in the permeate (10 kDa MWCO), for example, was 13.9% and 0.23% for the buffer solution and 200 g L^{-1} gluten, respectively (10 kDa MWCO). The endopeptidase activity in the permeate decreased from 31.2% to 1.46% for the buffer solution and 200 g L^{-1} gluten, respectively. Thus, the leakage of enzyme activity decreased with higher gluten concentrations in the preparation.

Gluten concentrations and membrane MWCO for the EMR process

Flavourzyme/wheat gluten preparations (see above) with varying gluten concentrations were used for this trial and transferred into reactor B2 (Figure 7.1) in the EMR. The liquid was filtered at 37 °C using different MWCOs (1, 5 and 10 kDa) with a constant trans-membrane pressure of 2 bar. The results of the experiments are presented in Table 7.2.

Table 7.2: The membrane flux and subsequent permeate analysis after the filtration of Flavourzyme/wheat gluten preparations (see above). Permeate was analyzed for DM and DH after 30 min of filtration (steady state). Results represent the mean ± standard deviation of two independent measurements.

	Wheat gluten concentration [g L^{-1}]			
	25	50	100	200
J_N [L^{-1} h^{-1} m^{-2}]				
1 kDa MWCO	13.1 ± 0.4	13.4 ± 1.8	11.3 ± 0.2	9.0 ± 0.7
5 kDa MWCO	35.9 ± 1.6	40.1 ± 3.8	32.5 ± 1.2	23.7 ± 1.4
10 kDa MWCO	39.3 ± 4.4	38.7 ± 2.1	33.1 ± 2.7	23.0 ± 1.8
DM [g L^{-1}]				
1 kDa MWCO	16.0 ± 0.3	32.4 ± 0.4	62.0 ± 3.4	106.6 ± 0.6
5 kDa MWCO	19.1 ± 0.3	38.3 ± 0.7	74.0 ± 1.9	125.6 ± 5.0
10 kDa MWCO	26.2 ± 6.3	35.8 ± 1.4	83.3 ± 9.6	128.0 ± 7.4
DH [%]				
1 kDa MWCO	72.3 ± 2.0	65.4 ± 2.2	51.1 ± 2.6	21.8 ± 0.4
5 kDa MWCO	82.3 ± 0.9	73.1 ± 2.9	53.5 ± 1.8	28.8 ± 2.0
10 kDa MWCO	75.9 ± 5.1	66.6 ± 1.1	52.2 ± 3.7	27.1 ± 0.5

The flux was dependent on the substrate concentration. The membrane flux, for example, decreased from 39.3 L h^{-1} m^{-2} (25 g L^{-1}) to 23.0 L h^{-1} m^{-2} (200 g L^{-1}) for the 10 kDa MWCO. This can be explained by a higher content of non-hydrolyzed substrate and insoluble dry matter in the reactor and the formation of a thicker coating layer on the membrane. In addition, the DM of the permeate increased from 16.0, 19.1 and 26.2 g L^{-1} (25 g L^{-1}) to 106, 125.6 and 128 g L^{-1} (200 g L^{-1}) in the permeate with 1, 5 and 10 kDa MWCO, respectively. The dry matter recovery (DM in the permeate related to the initial substrate concentration) decreased with increasing substrate concentrations. A further important point in protein hydrolysis is the product inhibition of the peptidases used. An indication for the product inhibition of the Flavourzyme peptidases is that the DH of the permeates is also lower for higher substrate concentrations. The DH decreased from 72.3, 82.3 and 75.9% (25 g L^{-1}) to 21.8, 28.8 and 27.1% (200 g L^{-1}) in the permeate with 1, 5 and 10 kDa MWCO, respectively. These results are in accordance with the literature. The product inhibition of Flavourzyme peptidases has already been reported [14, 42].

With the results listed in Table 7.2, the STY and the performance could be determined according to Eqs. 7.3 and 7.4. The STY and the DH are shown in Figure 7.5a. The STY increased with higher substrate concentrations whereas the DH decreased. The highest STY was calculated for 200 g L^{-1} and was 25.2 and 24.8 g h^{-1} L^{-1} for 5 and 10 kDa, respectively. Previous studies [17], [19] and [23] also showed similar relationships between the STY and the DH, depending on the substrate concentration. For an efficient EMR process the STY and the DH should be as high as possible. Thus, the performance was calculated because of the contrary tendencies of the STY and the DH.

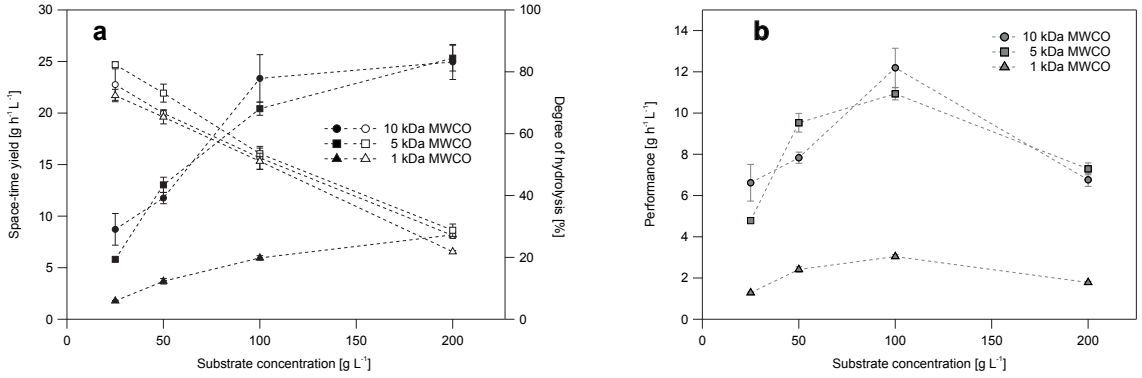


Figure 7.5: Effect of substrate concentration and MWCO on the STY and DH (a) and on the performance (b) of the EMR (a: STY is represented by filled symbols, DH is represented by open symbols. Results represent the mean ± standard deviation of two measurements).

The performance (STY*DH; Eq. 7.4) of the experiments is shown in Figure 7.5b. The highest performance was 12.2 g h^{-1} L^{-1} with a substrate concentration of 100 g L^{-1} and a MWCO of 10 kDa. This experimental setup was used for further experiments.

Continuous wheat gluten hydrolysis in the EMR

The highest performance for the EMR was determined with 100 g L^{-1} gluten, a MWCO of 10 kDa at 37 °C and a pH of 7.5. Ethanol [8% (v/v)] provided sufficient microbial stability. These parameters were chosen for the continuous wheat gluten hydrolysis in the EMR. A diaphragm pump was chosen due to its moderate impact on enzyme inactivation and trans-membrane pressure was kept constant at 2 bar.

In order to estimate the best suitable enzyme amount for 100 g L^{-1} gluten batch experiments with three different enzyme concentrations were performed and judged by the DH (data not shown). An enzyme activity of 20 $nkat_{glutenTNBS}$ mL^{-1} Flavourzyme seemed to be sufficient to achieve an acceptable DH within the predicted residence time of 3.57 h.

Since, a loss of enzyme activity during operation was observed and monitored (see above) the compensation for this loss could be calculated. This calculation was based on a presumption of a maximum flux of 33.1 L h^{-1} m−2 (0.28 L h^{-1} for A = 0.0085 m^2) and an initial enzyme activity of 20 $nkat_{glutenTNBS}$ mL^{-1} Flavourzyme. The total enzyme activity, which had to be added into the feed calculated according to Eq. 7.6 was 1.15 $nkat_{glutenTNBS}$ mL^{-1}. In practice the feed was substituted with 2 $nkat_{glutenTNBS}$ mL^{-1} to compensate for the theoretical estimated enzyme activity loss. Thermal and mechanical inactivation as well as leakage through the membrane were considered as the main reasons for activity loss.

The continuous EMR process was started after a 16 h batch hydrolysis in reactor B2 (see Figure 7.1) and was carried out for 96 h (Table 7.3). During the EMR process the permeate output was constantly compensated by the feed (pre-hydrolyzed gluten; B1; see Figure 7.1). Due to the added Flavourzyme activity of 2 $nkat_{glutenTNBS}$ mL^{-1} in the feed the endo- and exopeptidase activity during the EMR process remained between 100 and 121%. This confirmed the predicted calculations (see above). A slight leakage of endopeptidase activity was observed as expected.

The accumulation of insoluble dry matter was inevitable and caused a steady decrease in the membrane flux. A continuous solution for the removal of the insoluble dry matter was not possible for the present lab scale EMR. Discontinuous centrifugation steps of the EMR reaction volume were performed. In an industrial application, the usage of disc

stack separator technology would be a conceivable solution to overcome this problem. The DM was measured in the retentate to monitor the accumulation of these insolubles. The DM accumulated in the EMR to 131, 135, 131 and 159 g L^{-1} prior to the centrifugation steps and dropped to 116, 115, 119 and 115 g L^{-1} after centrifugation, respectively.

Table 7.3: Analysis of the retentate and permeate during the continuous EMR process. Results represent the mean ± standard deviation of two independent measurements.

	EMR runtime [h]								
	0	23	26	47	50	71	74	93	96
Retentate									
Endopeptidase activity[a] [%]	100±0.1	104±1.7	106±1.0	106±0.6	105±1.0	108±0.1	106±3.6	104±0.7	104±0.3
Exopeptidase activity[b] [%]	100±0.8	101±0.6	100±0.8	107±1.3	104±0.6	117±0.3	116±0.1	121±1.5	121±1.6
DM [g L^{-1}]	81.3±1.5	131±4.6	116±4.2	135±5.9	115±2.2	131±3.6	119±3.0	159±6.9	115±4.1
DH [%]	51.0±1.3	59.9±0.6	58.7±2.1	59.6±0.2	57.5±2.1	60.7±0.8	60.3±2.7	58.4±3.2	58.8±0.8
Permeate									
Endopeptidase activity[a] [%]	na	1.19±0.05	1.28±0.12	1.49±0.06	0.92±0.25	0.59±0.01	0.85±0.04	0.58±0.09	0.53±0.03
Exopeptidase activity[b] [%]	na	nd	nd	nd	nd	nd	nd	nd	nd
DH [%]	na	53.0±1.8	51.3±4.9	49.6±0.8	48.6±4.8	50.9±4.5	51.4±3.7	52.0±4.1	51.9±0.7
STY [g h^{-1} L^{-1}]	na	6.08±0.36	6.53±0.15	6.19±0.18	6.65±0.64	6.01±0.03	6.63±0.02	5.97±0.35	6.39±0.13

na, not applicable; nd, not detectable

[a] 100%, 35.8 ACU mL^{-1}

[b] 100%, 81.7 $nkat_{Leu\text{-}pNA}$ mL^{-1}

The DH inside the EMR remained nearly constant after 23 h and ranged between 57.5 and 60.7%. As a consequence, also the DH of the permeate was stable at around 50%. A total permeate volume of 8 L (eight volume replacements) was obtained after 96 h. The STY was quite stable during the whole process (6.33 ± 0.32 g h^{-1} L^{-1}). The average flux was around 10.9 L h^{-1} m^{-2}.

The feed was also tested during the continuous hydrolysis. The DH of the feed increased only slightly from 17.5 to 19.6 and 20.2% after 13, 61 and 96 h of continuous hydrolysis. The enzyme activity in the feed solution remained constant at 2 $nkat_{glutenTNBS}$ mL^{-1} for 96 h.

The enzyme productivity of the 96 h continuous EMR process was calculated according to Eq. 7.5 and is shown in Figure 7.6. An enzyme productivity of 18.7 mg $nkat^{-1}$ could be achieved after 96 h (eight volume replacements). Without enzyme inactivation during the process, the enzyme productivity would increase linearly, as described by [16], [17] or [19]. In order to compare the efficiency of the EMR process with a batch process the

latter was performed under the same conditions as the EMR process. A wheat gluten suspension was hydrolyzed with 20 $nkat_{glutenTNBS}$ mL^{-1} Flavourzyme at 37 °C and pH 7.5. The batch process hydrolysis time was about 16 h to achieve a DH of 50%, which is comparable to a DH of 51.1% in the permeate of the EMR process. Thus, a reasonable batch cycle time of 24 h was assumed considering a runtime of 16 h and a subsequent downtime of 8 h for cleaning and recharging.

The enzyme productivity of the batch process was 4.13 mg $nkat^{-1}$ and cannot increase with volume replacements (see Figure 7.6) due to the batch process characteristic. As a result, the enzyme productivity was 450% higher for the EMR process compared to the batch process under the same conditions. The STY of the EMR process after 96 h (6.33 g h^{-1} L^{-1}; 0.0085 m^2 filtration area) was also 85% higher compared to the batch process (3.44 g h^{-1} L^{-1}). Compared to the literature [14] the STY was also around 40% higher. They could achieve an average STY of around 4.5 g h^{-1} L^{-1} with 200 $nkat_{Leu\ pNA}$ mL^{-1} Flavourzyme and 10 g L^{-1} wheat gluten at 50 °C and pH 5.0.

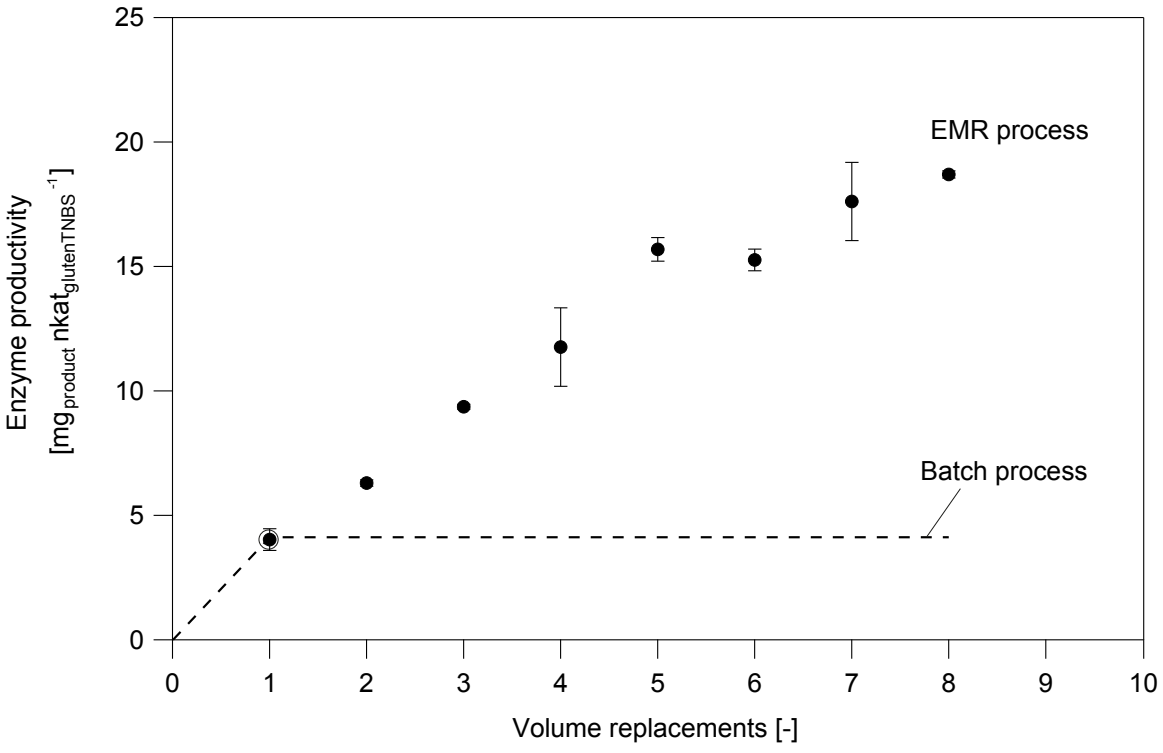


Figure 7.6: Enzyme productivity of the EMR process (96 h= eight volume replacements) compared to a batch process. Results represent the mean ± standard deviation of two independent permeate samples.

CONCLUSION

The proof of principle of a stable and productive food-grade EMR process has been shown. A long-term EMR hydrolysis process of wheat gluten and the enzyme preparation Flavourzyme was realized and operated for 96 h. A concentration of 8% (v/v) ethanol was identified as a suitable process aid to maintain microbial stability. Thermal inactivation, pump stress and membrane leakage were identified as the main reasons for enzyme activity loss inside the EMR. The enzyme productivity of the EMR process could be increased by 450% compared to a batch process under the same conditions. The results above indicated that the EMR process designed can be used for a cost-effective industrial production of hydrolysates from cereal proteins. Ethanol showed high potential for the application as preservative for the processing of food components. However, a solution for the continuous removal of the insoluble dry matter during the EMR process and an adequate process design for the handling of ethanol in an industrial application have to be considered.

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ABBREVIATIONS

ACU, azocasein unit (endopeptidase activity); DH, degree of hydrolysis; DM, dry matter; EA, enzyme activity; EMR, enzyme membrane reactor; g, constant of gravitation; h, concentration of free amino groups; h_{tot}, theoretical concentration of free amino groups at complete hydrolysis; J, membrane flux; J_N, normalized flux; k_T, temperature-dependent inactivation rate; k_P, pump stress-dependent inactivation rate; M, molecular weight; MWCO, molecular weight cut-off; $nkat_{glutenTNBS}$, total proteolytic activity; $nkat_{H\text{-}Leu\text{-}}pNA$, exopeptidase activity; pNA, para-nitroanilide; STY, space–time yield; TNBS, 2,4,6-trinitrobenzene sulfonic acid; V, reactor volume

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Co-authored scientific publications

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Glück, C., Rentschler, E. Krewinkel, M., **Merz, M.**, von Neubeck, M., Wenning, M., Scherer, S., Stoeckel, M., Hinrichs, J., Stressler, T. & Fischer, L. (2015). Thermostability of peptidases secreted by raw milk associated microorganisms. Manuscript intendet to be submitted to the *International Dairy Journal*.

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Conferences with oral presentation

Merz, M., Eisele, T., Fischer, L. (2013). Effiziente Proteinhydrolyse mittels kontinuierlicher Enzym-Membran-Reaktortechnologie für den Einsatz im Lebensmittelbereich. Dechema Himmelfahrtstagung, Bad Wildungen (Germany).

Conferences with poster presentation

Merz, M., Fischer, L. (2014). Application of a novel HPLC-based system for automated biotransformation and analysis. 7th International Congress on Biocatalysis, Hamburg (Germany).

Merz, M., Ewert, J., Stressler, T., Fischer, L. (2014). Enzyme membrane reactor technology as a promising tool to increase enzyme efficiency. 1st Bioeconomy Congress, Stuttgart (Germany).

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07/2012 – 09/2015 - Ph.D (Dr. rer. nat), University of Hohenheim (Stuttgart, Germany), Institute of Food Science and Biotechnology, Department of Biotechnology and Enzyme Science, Prof. Lutz Fischer. Dissertation title: „Enzymatic hydrolysis of plant proteins using food-grade peptidases“

10/2006 – 06/2012 - Engineer's degree, Food Technology (Dipl.-LM-Ing.); Grade: 1.1, University of Hohenheim (Stuttgart, Germany), Diploma thesis (Department of Biotechnology and Enzyme Science): „Continuous wheat gluten hydrolysis using an enzyme membrane reactor“.

10/2005 – 08/2006 - Food Technology, Technical University (Berlin, Germany).

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Work experience

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