ENZYMATIC HYDROLYSIS OF VEGETABLE AND INSECT PROTEINS USING TECHNICAL ENZYME PREPARATIONS

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~ Ein stiller Gedanke geht auch an die, die heute leider nicht mehr bei uns sein können ~

PUBLICATIONS AND CONFERENCE TALKS

Peer reviewed original publications

Kora Kassandra Grossmann, Michael Merz, Daniel Appel, Lutz Fischer

A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis.

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Impact of peptidase activities on plant protein hydrolysates regarding bitter and umami taste.

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New insights into the flavoring potential of cricket (Acheta domesticus) and mealworm (Tenebrio molitor) protein hydrolysates and their Maillard products.

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Conference talks

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High throughput determination of substrate specificity and peptidase side activities from selected technical enzyme preparations approved for food.

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Place, Date Signature of supervisor

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ABBREVIATIONS AND SYMBOLS

$c_{protein}$	concentration of protein
DH	degree of hydrolysis
DNA	deoxyribonucleic acid
EC	Enzyme Commission (number)
e.g.	lat. exempli gratia (for example)
Eq.	equation
h	hydrolyzed bonds
h_{tot}	total number of peptide bonds in the protein
i.e.	lat. id est (that is)
IU	international units
kat	katal
M	molecular mass
n.d.	no data
OPA	*o*-phthaldialdehyde
*p*NA	*p*-nitroanilide
R&D	research & development
SI units	international system of units
TEP/-s	technical enzyme preparation/-s

The abbreviations used in the publications are listed in the respective studies.

ABSTRACT

The present dissertation covers the usage of technical enzyme preparations (TEPs) for vegetable and insect protein hydrolysis, due to the mounting interest in alternative protein sources to cover the increasing demand for food from a growing world population. The TEPs, as defined in this study, are enzyme preparations that include side activities and are used in food processing. Today, TEPs are used by food manufacturers based on the supplier's information that usually states the main enzyme activity and includes information on side activities only in some cases. However, knowledge about the activity profile is crucial as side activities can contribute to the properties of the protein hydrolysates produced (e.g. degree of hydrolysis [DH], liberation of amino acids) and the final food product quality.

In the **first study**, an automated photometric analyzer (GalleryTM Plus, Thermo Fisher Scientific) was introduced for the comprehensive activity determination of TEPs. The new setup of the analyzer covered 32 synthetic and natural substrates in order to determine aminopeptidase, carboxypeptidase, dipeptidylpeptidase and endopeptidase activities distinguishably. Accordingly, the overall proteolytic activity of TEPs was quantified and detailed information about the substrate spectra and peptidase side activities was generated. Furthermore, several batches of the industrial TEP Flavourzyme1000L were measured. By determining 32 peptidase activities, batch variations were shown. As Flavourzyme1000L is standardized by its supplier Novozymes on only one activity (leucine aminopeptidase), the additional 31 new peptidase activities determined showed differences of the side activities of the batches. In addition, the study showed that the detailed information of the peptidase activities of the TEPs could explain the properties of the resulting lupine protein hydrolysates (DH and liberation of amino acids). Due to the determination of 32 peptidase activities (so-called "activity fingerprint"), TEPs were selected specifically to increase, for example, the DH. The two TEPs P278 and DZM were selected due to their complementary peptidase activities, as an example of this study. The combination of these two TEPs resulted in an increase of the DH of 47%. Now, TEPs can be selected targeted more on the basis of their peptidase activities to, for example, increase the hydrolysis efficiency of lupine protein by combining complementary peptidase activities.

In the **second study**, six food-grade TEPs (Flavourzyme1000L, ProteaseP “Amano”6SD, DeltazymAPS-M-FG, Promod278, ProteAX-K and PeptidaseR) were investigated regarding their influence on the hydrolysis of soy, pea and canola protein. The hydrolysates were investigated analytically concerning their DH and free amino acid profiles and sensorically concerning the taste attributes umami and bitter. By using a random forest model, the taste attributes bitter and umami were connected to specific peptidase activities (exo- and endopeptidase activities). Furthermore, out of the six selected TEPs, the usage of ProteAX-K showed high umami and low bitter taste of the vegetable protein hydrolysates (soy, pea and canola). In line with the first study, the second study showed that the detailed information of the peptidase activities of the TEPs could explain the properties of the resulting vegetable protein hydrolysates. Based on these new insights, TEPs can be selected more specifically based on their peptidase activity profiles to direct the formation of desired taste attributes of the protein hydrolysates.

In the **third study**, two TEPs with various peptidase activities (Flavourzyme1000L, ProteaseA “Amano”2SD) were applied for the hydrolysis of insect proteins. This study investigated the potential of cricket and mealworm protein and their hydrolysates regarding their sensory potential. The sensory profiles of the insect proteins were altered by, firstly, applying proteolytic hydrolysis and then a Maillard reaction (30 min, T = 98°C, 1% (w/v) xylose) to the hydrolysates. The initially earthy-like flavor of the insect proteins resulted in modified taste profiles described by e.g., savory-like attributes, due to both processing steps. Furthermore, 38 odor-active molecules (1 alcohol, 5 acids, 11 aldehydes, 5 ketones and 16 heterocyclic compounds) were identified by gas chromatography-olfactometry (GC-O). The identified molecules are also found in meat and edible seafood products. The third study showed that the flavoring profile of insect proteins was modified and can be developed further by the respective processing.

ZUSAMMENFASSUNG

Die vorliegende Dissertation befasst sich mit der Verwendung von technischen Enzympräparaten (TEP) zur Hydrolyse von Pflanzen- und Insektenproteinen. Alternative Proteinquellen sind von zunehmendem Interesse, um die steigende Lebensmittelnachfrage der wachsenden Weltbevölkerung zu decken. In dieser Studie werden TEP als Enzympräparate definiert, die zur Herstellung von Lebensmitteln verwendet werden und meist Nebenaktivitäten aufweisen. In der Regel werden TEP von Lebensmittelherstellern basierend auf den Informationen der Produzenten verwendet. Diese nennen vorwiegend die Hauptenzymaktivität und selten weitere Nebenaktivitäten der Präparate. Detaillierte Informationen über Nebenaktivitäten sind allerdings wesentlich, da diese die Eigenschaften der erzeugten Proteinhydrolyse (z. B. Hydrolysegrad, Freisetzung von Aminosäuren) und die finale Lebensmittelqualität beeinflussen können.

In der **ersten Studie** wurde ein automatisiertes photometrisches Analysengerät (Gallery™ Plus, Thermo Fisher Scientific) vorgestellt, um TEP umfangreich hinsichtlich ihrer Peptidaseaktivitäten zu bestimmen. Die neue Installierung des Analysengerätes beinhaltete 32 synthetische und natürliche Substrate, um Aminopeptidase-, Carboxypeptidase-, Dipeptidylpeptidase- und Endopeptidaseaktivitäten von TEP differenzierbar zu messen. Dabei wurden die allgemeine proteolytische Aktivität der TEP quantifiziert, sowie detaillierte Informationen über das Substratspektrum und über proteolytische Nebenaktivitäten generiert. Zudem wurden mehrere Chargen des industriellen TEP Flavourzyme1000L gemessen. Durch die Bestimmung der 32 Peptidaseaktivitäten wurden Batchvariationen aufgezeigt. Da Flavourzyme1000L vom Hersteller Novozymes auf nur eine Aktivität (Leucin Aminopeptidase) standardisiert wird, haben die weiteren 31 neu bestimmten Peptidaseaktivitäten die Unterschiede der Nebenaktivitäten aufgezeigt. Ferner hat diese Studie gezeigt, dass die detaillierten Informationen über die Peptidaseaktivitäten der TEP Auswirkungen auf den resultierenden Hydrolysegrad und die Aminosäurefreisetzung von Lupinprotein-hydrolysaten haben. Durch die Bestimmung der 32 Peptidaseaktivitäten (egl. „*activity fingerprint*“) wurden TEP gezielt ausgewählt, um beispielsweise den Hydrolysegrad zu erhöhen. Unter anderem wurden die zwei TEP Promod278 und DeltazymAPS-M-FG auf Grund ihrer komplementären Peptidaseaktivitäten ausgewählt und kombiniert, was zu

einem Anstieg des Hydrolysegrades um 47% führte. Von nun an können TEP gezielter aufgrund ihrer Peptidaseaktivitäten ausgewählt werden, um durch die Kombination von komplementären Aktivitäten z. B. eine effizientere Hydrolyse zu erzielen.

In der **zweiten Studie** wurde der Einfluss von sechs lebensmitteltauchglichen TEP (Flavourzyme1000L, ProteaseP “Amano”6SD, DeltazymAPS-M-FG, Promod278, ProteAX-K, PeptidaseR) auf die Hydrolyse von Soja-, Erbsen- und Canolaprotein untersucht. Die Hydrolysate wurden hinsichtlich des Hydrolysegrads, der Aminosäurefreisetzung und der Geschmacksattribute umami und bitter sensorisch untersucht. Mit Hilfe eines Random Forest Algorithmus wurden diese beiden Geschmacksattribute bestimmten Peptidaseaktivitäten (Exo- und Endopeptidase-aktivitäten) zugeordnet. Ferner zeigte, von den sechs untersuchten TEP, der Einsatz von ProteAX-K einen stark umami und schwach bitteren Geschmack der Pflanzenproteinhydrolysate (Soja, Erbse und Canola). Die Ergebnisse der ersten Studie konnten somit untermauert werden, auch hier konnten die detaillierten Informationen über die Peptidaseaktivitäten der TEPs die Eigenschaften der resultierenden Pflanzenproteinhydrolysate erklären. Durch diese neuen Erkenntnisse können TEP auf Grund ihrer Peptidaseaktivitäten künftig gezielter ausgewählt werden, um die Entwicklung von Geschmacksattributen der Hydrolysate zu beeinflussen.

In der **dritten Studie** wurden zwei TEP mit verschiedenen Peptidaseaktivitäten (Flavourzyme1000L, ProteaseA “Amano”2SD) zur Hydrolyse von Insektenprotein eingesetzt. Diese Studie hat das sensorische Potential von Grillenprotein (*Acheta domesticus*) und Mehlwurmprotein (*Tenebrio molitor*) und deren Hydrolysate untersucht. Mittels proteolytischer Hydrolyse und anschließender Maillard Reaktion (30 min, T = 98°C, 1% (w/v) Xylose) wurden die Geschmacksprofile der Insektenproteine verändert. Die zunächst erdig schmeckenden Insektenproteine zeigten nach der technologischen Verarbeitung veränderte Geschmacksprofile durch z. B. herzhafte Attribute auf. Ferner wurden 38 geruchsaktive Moleküle (1 Alkohol, 5 Säuren, 11 Aldehyde, 5 Ketone and 16 hetero-zyklische Verbindungen) mittels olfaktorischer Gaschromatographie (GC-O) bestimmt. Die identifizierten Moleküle kommen auch in Fleisch- und essbaren Meerestieren vor. Die dritte Studie hat gezeigt, dass das Geschmacksprofil der Insektenproteine durch technologische Verarbeitung verändert und weiterentwickelt werden kann.

1 CHAPTER ONE: INTRODUCTION, AIM AND OUTLINE

1.1 PROTEOLYTIC ENZYMES

Proteolytic enzymes are enzymes that catalyze the hydrolysis of a peptide bond, as the reaction principle shows in Figure 1. According to their catalytic mechanism, proteolytic enzymes are classified with the EC number 3.4, referring to hydrolases, utilizing water to break a peptide bond. Several terms, such as proteases, proteinases and peptidases, are used in literature for proteolytic enzymes. The term protease was well established before 1928, but then Grassmann and Dyckerhoff found out that there are two types of proteolytic enzymes: some acting best on intact proteins and others showing a preference for small peptides. These first thoughts then led to the establishment of endo- and exopeptidases by Bergmann and Ross in 1936. Summarizing, it was proposed to use the term peptidase, being equal to the old term protease, for referring generally to peptide bond hydrolases. Endopeptidases replaced, more or less, the term proteinase, referring to endo-acting peptide bond hydrolases, whereas the term exopeptidases was established for exo-acting peptide bond hydrolases [1]. Thus, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology recommends the term peptidases for enzymes hydrolyzing peptide bonds [2].

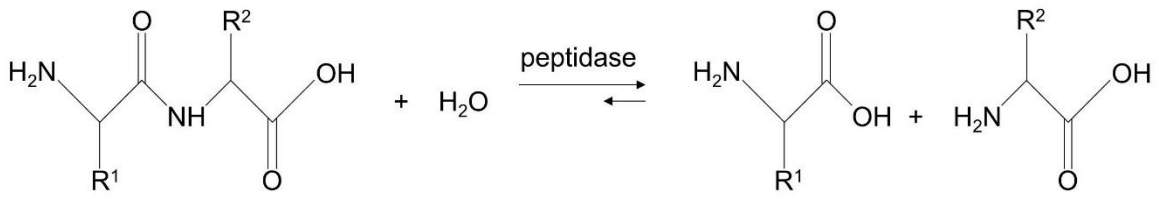

Figure 1: Reaction principle of the proteolytic hydrolysis of a peptide bond (R^1 and R^2 represent two individual side chains).

1.1.1 Classification of peptidases

Further classifications of proteolytic enzymes have been established since the 1980s, when the fundamentals were laid out. Although catalyzing the same reaction (e.g. hydrolysis of a peptide bond), proteolytic enzymes can be classified based on I) their catalytic type, II) their active site or III) their molecular structure and homology.

Catalytic types of proteolytic enzymes (I) refer to specific chemical groups. The latter are reactive groups of amino acid side chains and can be either hydroxyl groups, sulfhydryl groups or activated water molecules. There are seven catalytic types: serine, threonine, cysteine, aspartic, metallo, glutamic peptidases and asparagine peptide lyases [3]. Hydroxyl groups act as reactive groups for serine and threonine peptidases, and sulfhydryl groups for cysteine peptidases. Regarding aspartic and metallo peptidases, the water molecule is either directly bound by the side chains of the aspartic residues or is held in place by metal ions, enabling charged amino acid side chains to be ligands for the metal ions. Zinc serves most commonly as the metal ion, but cobalt, manganese and copper can also act accordingly [4]. Glutamic peptidases have a catalytic dyad of Gln35 and Glu136 that activate a bound water molecule for a nucleophile attack on the carbonyl carbon atom of the scissile peptide bond [3, 5]. The seventh catalytic type of proteolytic enzymes does not cover hydrolases similar to the first six cases, but lyases, namely, asparagine peptide lyases. These proteolytic enzymes are characterized by the usage of asparagine as the proteolytic nucleophile in order to break down peptide bonds [6].

The classification by the active site (II) is based on catalyzing the hydrolysis of a peptide bond in a particular position in the polypeptide chain of the substrate molecule. Accordingly, peptidases are classified generally into exo- or endopeptidases. Exopeptidases hydrolyze one to three residues from the terminus, requiring either a free N-terminal amino group, a C-terminal carboxyl group or both. Due to this characteristic, exopeptidases acting on the free N-terminus of the polypeptide chain are classified as aminopeptidases (EC 3.4.11), dipeptidases (EC 3.4.13), dipeptidyl-peptidases and tripeptidyl-peptidases (EC 3.4.14). Acting on the C-terminus, further exopeptidases are grouped into peptidyl-dipeptidases (EC 3.4.15) and carboxypeptidases (EC 3.4.16-18). The latter include three further divisions due to their catalytic type: serine-type carboxypeptidases (EC 3.4.16), metallo-carboxypeptidases (EC 3.4.17) and cysteine-type carboxypeptidases (EC 3.4.18). Endopeptidases internally hydrolyze the alpha-

peptide bond in a polypeptide chain, acting away from the N-terminus or C-terminus. Today, endopeptidases are subdivided into six further groups of serine endopeptidases (EC 3.4.21), cysteine endopeptidases (EC 3.4.22), aspartic endopeptidases (EC 3.4.23), metallo-endopeptidases (EC 3.4.24), threonine endopeptidases (EC 3.4.25) and endopeptidases of unknown mechanism (EC 3.4.99) [4]. According to the Comprehensive Enzyme Information System BRENDA, the group of serine endopeptidases compromises 789 organisms and encompasses the largest sub-group of endopeptidases [7]. Figure 2 illustrates the classification of peptidases by the active site of their catalyzed reaction.

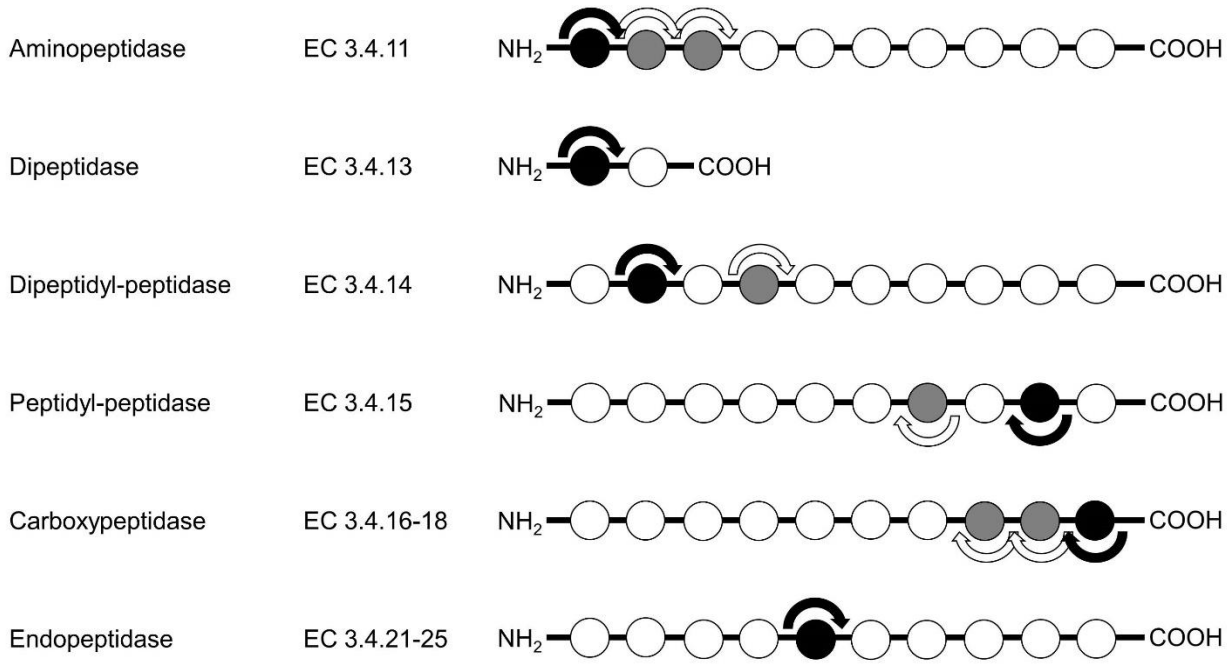

Figure 2: Classification of peptidases according to the active site of their catalyzed reaction. The amino acids are presented as beads. The arrows illustrate the cleavages, the first in black and further cleavages in white (modified after Rawlings et al. [4]).

A further group of peptidases that do not require a free N-terminus or C-terminus in the substrate encompasses the omega peptidases. Although they have this feature in common with the endopeptidases, they are distinguished by commonly acting close to one of the terminus. Omega peptidases also hydrolyze non-alpha bonds and remove substituted, cyclized or linked (by isopeptide bonds) terminal residues. Currently covering a group of 106 organisms, omega peptidases are placed in the sub-class EC 3.4.19 by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology [4, 7-8]. The third classification (III) is based on grouping proteolytic enzymes by

their molecular structure and homology. Among the three approaches, this classification of peptidases is the newest, as it is based on data of amino acid sequences and structures in three dimensions. This information started to develop according to new methods and data availability in the early 1990s when peptidases were assigned to families, based on the comparison of their amino acid sequences. Members are assigned to families based on them displaying a statistically significant relationship to at least one other member of the respective family and requiring that the relationship exists in the peptidase unit (e.g. the part of the molecule responsible for the catalytic activity). Members of families whose proteins have diverged so far that their primary structure no longer proves their significant relationship are grouped into clans. Based on homologies in their three dimensional structure, members of clans furthermore consider both the arrangement of catalytic residues in the polypeptide chain and limited sequence similarities around the catalytic amino acid [4, 9].

1.1.2 Terminology of peptidases specificity

Schechter and Berger invented a conceptual model in 1967 to describe the specificity of peptidases [10]. In this concept, each specific subsite of a peptidase is allocated to the side chain of an amino acid residue. Starting from the catalytic site, the active sites of the enzyme are numbered towards the N-terminus of the substrate by the abbreviations S1, S2, Sn and by additional apostrophes S1´, S2´… Sn´ towards the C-terminus. The residues of the substrate allocated are then numbered from the scissile bond of the enzyme with the positions P1, P2…Pn and P1´, P2´…Pn´, respectively [4, 10]. Figure 3 shows a scheme of an enzyme-substrate complex, based on the model that was invented by Schechter and Berger [10].

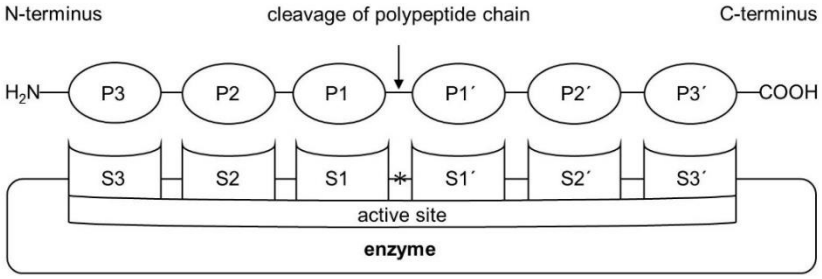

Figure 3: Scheme of an enzyme-substrate complex between a peptidase and a polypeptide chain, according to the nomenclature of Schechter and Berger [10]. The scissile bond in the active site of the enzyme is indicated by an asterisk (*), “S” represents the subsites of the enzyme and “P” the positions of the substrate.

1.1.3 Synergism of peptidases – impact on the degree of hydrolysis

Protein hydrolysis is limited to a certain extent because every peptidase shows substrate specificities towards either single amino acids or specific amino acid sequences. However, high degrees of hydrolysis (DH) are required for several reasons, such as the improvement of techno-functional features [11] or the reduction of allergenicity proteins [12]. The DH is defined as the proportion of the total number of peptide bonds that are cleaved during hydrolysis [13]. As shown in Equation 1 below, it is calculated by dividing the number of hydrolyzed bonds (h) by the total number of peptide bonds in the respective protein (h_{tot}), multiplied by 100 and presented in percent:

$$DH = \frac{h}{h_{tot}} \times 100\ [\%] \qquad \text{Eq. 1}$$

The total number of peptide bonds in the protein (h_{tot}) is calculated from the amino acid composition of the respective protein, as shown in Equation 2: the protein concentration that is hydrolyzed ($c_{protein}$) is divided by the difference of the average molecular mass of the amino acids in the respective protein (M*) and the molecular mass of water (M_{H_2O}). The molecular mass of water is subtracted, due to the fact that water is added during the hydrolysis of a peptide bond:

$$h_{tot} = \frac{c_{protein}}{\left(M^{*} - M_{H_2O}\right)} \qquad \text{Eq. 2}$$

Figure 4 illustrates the synergism of different catalytic types of peptidases. The first part of the illustration [A] shows how two endopeptidases act together. The combination of two different endopeptidases enlarges the DH because endopeptidases can have different preferred cleavage residues. The second part of the illustration [B] shows how two exopeptidases (aminopeptidases) work together. The first aminopeptidase cleaves a specific amino acid residue from the polypeptide chain, enabling a second aminopeptidase to cleave its specific residue of the remaining protein. The third part [C] illustrates the synergism from endo- and exopeptidases in a combined manner that has just been described. These synergistic effects enlarge the breakdown of the polypeptide chain and, therefore, increase the DH within the protein.

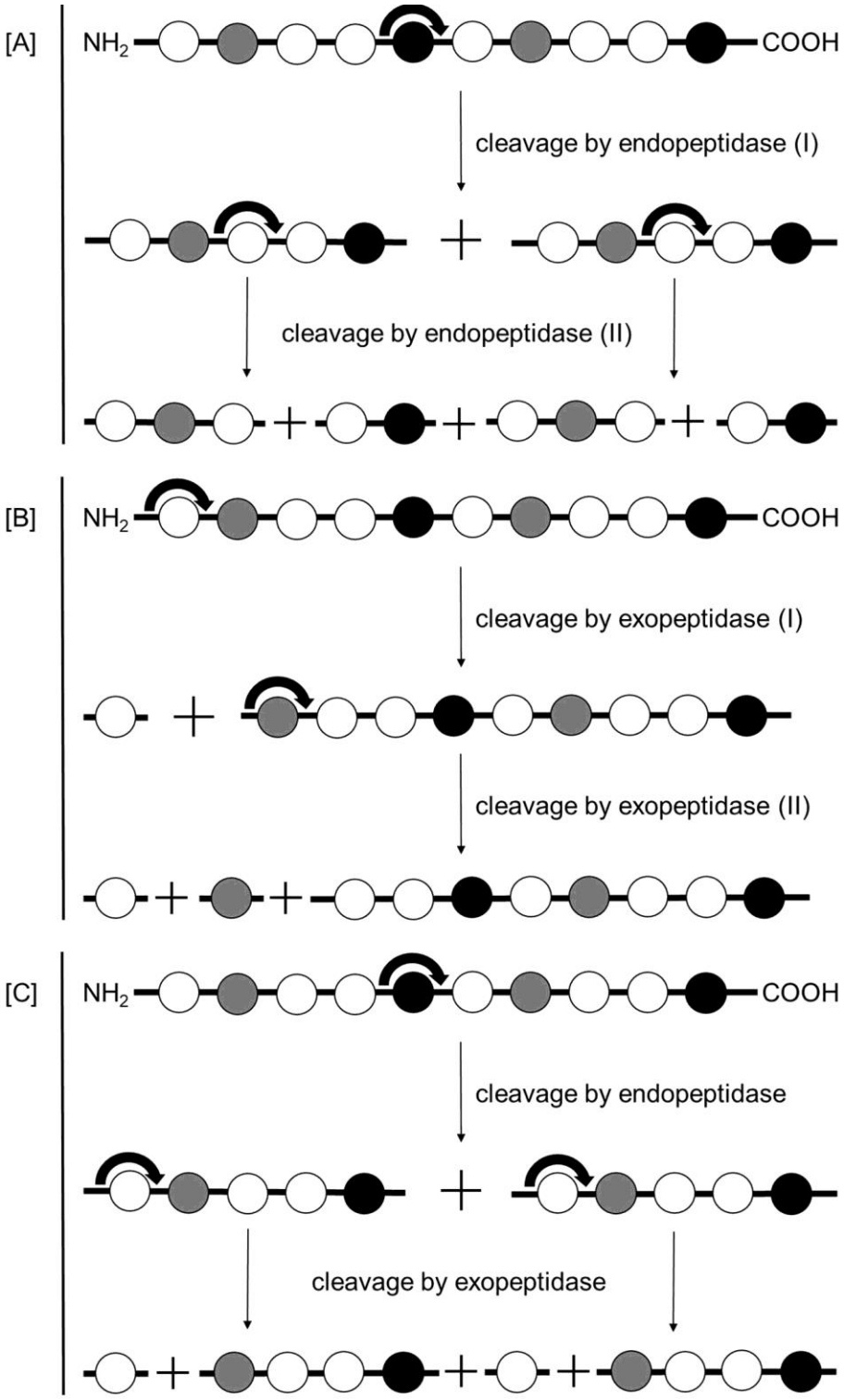

Figure 4: Scheme of the synergism of different catalytic types of peptidases. [A] Synergism of two individual endopeptidases, [B] two individual exopeptidases (aminopeptidases) and [C] one endo- and one exopeptidase. The colored beads represent the different amino acids and the bent arrows illustrate the cleavage position due to the specific residue.

1.1.4 Measurement of peptidases activities

Before 1972, the Enzyme Commission of the International Union of Biochemistry recommended expressing the enzyme activity in international units (IU). One IU was defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute under standard conditions (e.g. temperature, optimal pH and optimal substrate concentration). In order to adhere to the International System of Units (SI units) the Commission on Biochemical Nomenclature recommended in 1972 expressing the reaction rate in moles per second, proposing katal as the new unit for enzyme activity. One katal has therefore been defined as the catalytic activity that will raise the rate of reaction by one mole per second under specified assay conditions [14]. Table 1 summarizes the two common units including their dimensions and conversions.

Table 1: Overview of the two most common units for enzyme activities, their dimensions and conversions (adapted from Bisswanger) [15].

Unit	Dimension	Conversion
1 katal (kat)	= 1 mol s^{-1}	= 60,000,000 IU
1 international unit (IU)	= 1 µmol min^{-1}	= 0.0000000167 kat = 16.7 nkat

The assay conditions are crucial for the activity determination of enzymes. The main factors to be considered are temperature, pH, ionic strength and appropriate substrate and enzyme concentrations [15]. Since enzyme activity is considered as the maximum catalytic potential of the enzyme, its activity is often referred to as optimal reaction conditions. The optimal substrate concentration is considered to be the concentration at which the initial rate of reaction is at its maximum. The optimal value for pH lies within the range of maximum stability [14]. A general standard temperature for all assays cannot be defined because enzyme kinetics are highly dependent on temperature. Nevertheless, three temperatures have been commonly established: 37, 25 and 30°C referring to the human physical temperature, an average room temperature and a compromise of the two, respectively. The conditions at 30°C are close to the physical temperature, thermal denaturation is not feared and the enzymes are more active than at 25°C [15]. Furthermore,

assay time should be short in order to neglect both the effect of the products on the reaction and the reduction in substrate concentration.

The experimental measurement of enzyme activities is based on the determination of the initial reaction rates of the enzymes and the evaluation can be established on substrate depletion or product buildup. One or the other can be used if the stoichiometry of the reaction is well-known and defended. Otherwise, the measurement of product which has built up is recommended since product buildup determines significant differences between small magnitudes. Substrate depletion measures small differences between large magnitudes, implying more error. When neither of the approaches are easily measurable, enzyme activity can also be determined by coupling reactions, transforming the product to a final analyte that can then be determined analytically [14].

Several methods have been described to observe peptidase activities. Photometric assays are commonly applied due to easy handling and a relative low susceptibility against disturbances. Chromogenic substrates including *p*-nitroanilide (*p*NA) can serve as model substrates to determine specific peptidase activities (indicated by H-X-*p*NA for a random substrate in Figure 5).The release of *p*NA can be detected by its absorption at 405 nm and one katal of peptidase activity can be defined as the release of one mol *p*NA per second [16].

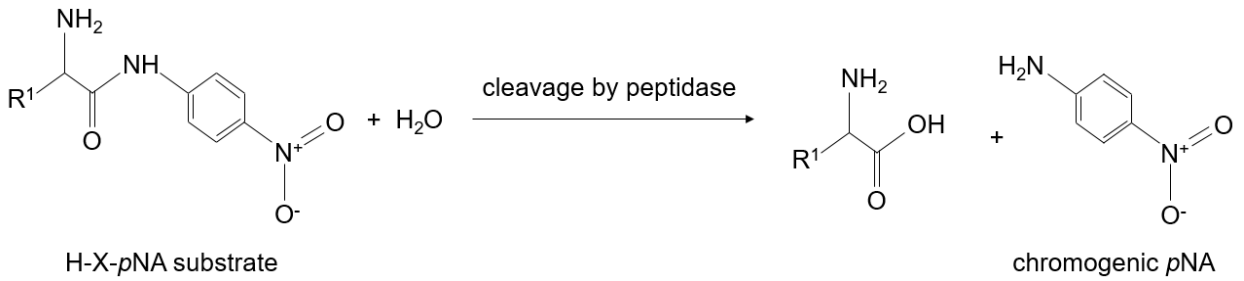

Figure 5: Reaction principle of the release of *p*NA from a random H-X-*p*NA substrate by a peptidase.

If the catalytic reaction cannot be observed photometrically or requires more sensitivity, fluorogenic substrates can be used. Being 300-500 times more sensitive than absorbance methods, fluorimetry correlates the fluorescence intensity directly proportional to the fluorophore concentration. Although fluorescence spectrometry has the advantage over absorption spectrometry to be able to measure at much higher signal-to-noise ratios, spectrofluorometers are often more complicated to handle and imply more error sources. Therefore, fluorometric assays require deeper experience and are not as usual as photometric methods. Fluorometric substrates are based on peptidyl derivatives of fluorescent amines, such as 4-methyl-7-coumarylamides, and peptides that contain a fluorescent donor being quenched by an acceptor chromophore via resonance energy transfer. When the amide bond between the peptide and the fluorophore is hydrolyzed, a highly fluorescent-free 7-amino-4-methylcoumarin is released [17].

Apart from the synthetic substrates, natural substrates, such as caseins or plant proteins, can be used to measure peptidase activity. These substrates are used to determine general proteolytic activity. The substrate is hydrolyzed, and the amino groups released can be subsequently detected after derivatization with, for example, *o*-phthaldialdehyde (OPA). Figure 6 illustrates the reaction principle of OPA reacting with primary amino groups in the presence of reduced sulfhydryl groups (e.g. dithiothreitol) to a light-absorbing compound that can be detected at 340 nm. The enzyme activity can then be expressed as one katal, referring to one L-serine equivalent per second. The selection of serine as a standard is due to the fact that serine shows a response in reactions that is very close to the average response of amino acids [18].

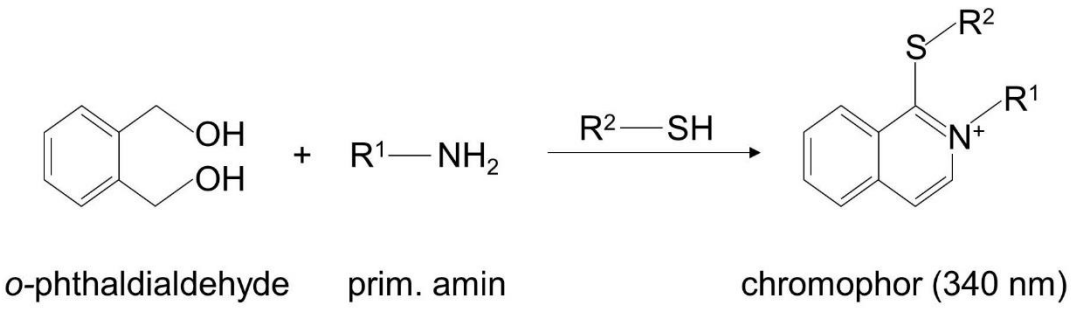

Figure 6: Reaction principle of *o*-phthaldialdehyde (OPA) with primary amines (R^1-NH_2) and in the presence of reduced sulfhydryl groups (R^2-SH) to a light-absorbing compound at 340 nm (modified after Nielsen et al.) [18].

Summarizing, different peptidase activities can be measured depending on the choice of method and substrate. Therefore, it is crucial to state the assay conditions and the substrate used when presenting proteolytic activities. In addition, particular caution must be taken when comparing proteolytic activities under different conditions. Enzymatic assays should generally be adapted to the specific features of the individual enzyme and the instrumentation available.

1.1.5 Origins of peptidases

Peptidases are produced from a variety of sources, such as animals, plants and microorganisms, including fungi, bacteria and viruses. Animal and plant sources covered only 6 and 3%, respectively, of the sources for food enzymes in 2014. The majority, approximately 60%, was produced by filamentous fungi (e.g. ascomycetes and basidiomycetes), yeasts (5%) and bacteria (28%). About one-third of the commercially available enzymes are derived from genetically modified organisms [19]. These numbers may have changed to date due to the increasing demand of food enzymes and the fast developments in the enzyme segments. However, as accurate numbers reflecting the status are difficult to access, the numbers presented in this section, nevertheless, aim to provide an indicative picture. Originating from microorganisms, a large number of enzymes derive from fungal *Aspergillus* sp. and bacterial *Bacillus* sp. [20]. Famous examples of plant- deriving enzymes are bromelain and papain. Originating from the pineapple (*Ananas comosus*) and papaya fruit (*Carica papaya*), respectively, both belong to the cysteine endopeptidases and are classified by EC 3.4.22.33 for fruit bromelain, EC 3.4.22.32 for stem bromelain and EC 3.4.22.2 for papain [21].

Animal-derived peptidases are, for example, trypsin, chymotrypsin, and pepsin. Trypsin and chymotrypsin are serine endopeptidases, classified with EC 3.4.21 and pepsins are aspartic endopeptidases classified with EC 3.4.23. Trypsin was first discovered by Wilhelm Kuehne in 1876 [22] and can derive, the same as chymotrypsin and pepsin, from mammalian intestines [23]. A highly studied system in the history of proteolytic enzymes is the human immunodeficiency virus 1 retro pepsin, commonly known as HIV-1 protease (EC 3.4.23.16), which is involved in the life cycle of the human immunodeficiency virus (HIV), the retrovirus causing the acquired immunodeficiency syndrome (AIDS) [24].

Table 2 shows a selection of peptidases, their classifying EC number together with their source of origin and production strains.

Table 2: List of a selection of peptidases, EC numbers, sources of their origin and production strain (s) (adapted from Fraatz et al.) [19]. GM: genetically modified.

Peptidase	EC number	Origin source(s)	Production strain(s)
Aminopeptidase	3.4.11.x	Microbial	*Aspergillus niger, A. oryzae, Rhizopus oryzae*
Leucyl aminopeptidase	3.4.11.1	Microbial	*Aspergillus oryzae*
Serine-type carboxypeptidase	3.4.16.x	Microbial	*Aspergillus niger* (GM)
Serine endopeptidase	3.4.21.x	Microbial	*Aspergillus oryzae, A. wentii, Bacillus amyloliquefaciens*, *B. licheniformis, Cryphonectria parasitica, Fusarium venenatum* (GM), *Rhizomucor miehei*
Chymotrypsin	3.4.21.1	Microbial Animalic	*Bacillus licheniformis* (GM), beef pancreas
Trypsin	3.4.21.4	Microbial	*Fusarium venenatum* (GM)
Thrombin	3.4.21.5	Animalic	Cattle, pig
Prolyl oligopeptidase	3.4.21.26	Microbial	*Aspergillus niger* (GM)
Subtilisin	3.4.21.62	Microbial	*Bacillus licheniformis*
Oryzin	3.4.21.63	Microbial	*Aspergillus oryzae*
Aqualysin 1	3.4.21.111	Microbial	*Bacillus amyloliquefaciens* (GM)
Papain	3.4.22.2	Plant	*Carica papaya*
Bromelain	3.4.22.32 3.4.22.33	Plant	*Ananas comosus*
Aspartic endopeptidases	3.4.23.x	Microbial Animalic	*Aspergillus niger* (GM), *A. oryzae, A. wentii, Bacillus licheniformis, Micrococcus caseolyticus,* Cattle, pig
Pepsin A	3.4.23.1	Animalic	Cattle, pig
Pepsin B	3.4.23.2	Animalic	Pig, bovine rennet
Chymosin	3.4.23.4	Microbial Animalic	*Aspergillus niger (*GM*), Escherichia coli* (GM), *Kluyveromyces lactis* (GM), Calf
Aspergillopepsin I	3.4.23.18	Microbial	*Aspergillus oryzae* (non-GM and GM), *A. wentii*
Endothiapepsin	3.4.23.22	Microbial	*Cryphonectria parasitica*
Mucorpepsin	3.4.23.23	Microbial	*Aspergillus oryzae* (GM), *Mucor pusillus, Rhizomucor miehei*
Thermolysin	3.4.24.27	Microbial	*Geobacillus caldoproteolyticus*
Bacillolysin	3.4.24.28	Microbial	*Bacillus amyloliquefaciens* (non-GM and GM)
Deuterolysin	3.4.24.39	Microbial	*Aspergillus oryzae, A. wentii*

1.2 INDUSTRIAL RELEVANCE, PRODUCTION AND APPLICATIONS OF PROTEOLYTIC ENZYMES

Enzymes are used in various applications in industry, ranging from the sectors of pharmaceuticals to food processing, from the detergent to biofuels, from the textile industry to paper pulp, and oil and grain processing. The following paragraphs introduce the major companies of the global enzyme market and then focuses on the peptidase enzyme segment and a selection of relevant applications of proteolytic enzymes in the food sector. Figure 7 shows the revenue numbers of the global enzyme market in 2016 and the projected global enzyme market in 2021, illustrating an increase in all sectors of food, animal feed, technical applications and detergents (sorted into forecasted increase by color intensities). Currently (2021), the global market for enzymes confirms these numbers with $6.4 billion and $8.7 billion by 2026 forecast [25].

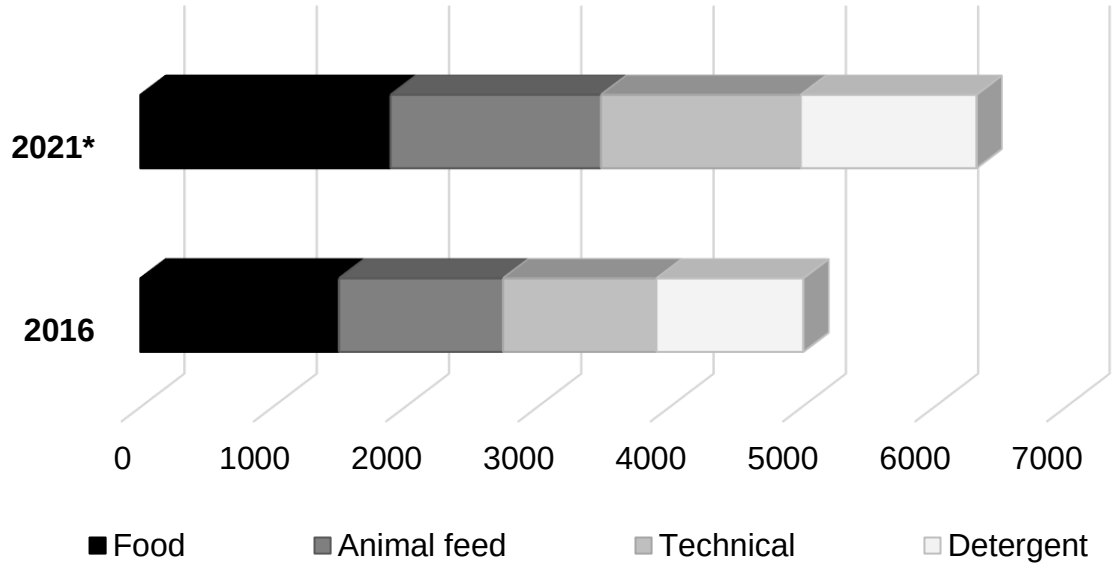

Figure 7: Revenue numbers of the global enzyme market in 2016 and the projected numbers in 2021 (indicated with an asterisk) of the four sectors of food, animal feed, technical applications and detergents. Data in millions, sorted into forecasted increase by color intensities (adapted from Chapman et al.) [26].

1.2.1 Major companies of the global enzyme market

In January 2018, the market research company Technavio listed the top ten vendors in the worldwide food enzyme market and presented the companies: DuPont Danisco, Amway, BASF, Novozymes, DSM, Amano, Nuritech, AB Enzymes, Roche and Aum Enzymes [27]. Table 3 lists these ten enzyme companies, including their countries and sectors offering products with product examples.

Table 3: Overview of the top ten enzyme companies [27] including examples of product sectors and products. The information concerning the product sectors and examples were collected from the respective company homepages.

Company	Country	Product sectors	Product examples
DuPont™ Danisco®	USA	Food and beverages, animal feed, dietary supplements	POWERBake®, FoodPro®
Amway™	Germany	Nutrition, health, skincare, detergents	Nutrilite™, Legacy of Clean®
Verenium Corporation, BASF	Germany	Animal feed, detergents, pulp and paper, grain and oil processing	Fuelzyme®, Lavergy™
Novozymes	Denmark	Food and beverages, animal feed, detergents, agriculture, textiles and leather, pulp and paper processing, biofuels	Alcalase®, Flavourzyme®
DSM	The Netherlands	Food and beverages, animal feed	Brewers Clarex, Maxilact®
Amano	Japan	Food and beverages, agriculture, dietary supplements, cosmetic, pharmaceutical	Protease A “Amano”2 SD, Protease P “Amano”6 SD
Nuritech	New Zealand	Animal feed	AcidStart™, Sil-All®
AB Enzymes	Germany	Food and beverages, animal feed, detergents, textiles, pulp and paper, grain and oil processing	BIOTOUCH®, ROHALASE®
Roche Costom Biotech	Switzerland	Industrial diagnostics and pharma	Recombinant trypsin, endopeptidases
Aum Enzymes	India	Food and beverages, animal feed, detergents, textiles and leather processing, effluent treatment	Cleanzyme, Fermentzyme

1.2.2 An overview of the peptidase segment

The industrial enzyme market has reached a value of $ 6.4 billion in 2021 and will grow to $ 8.7 billion by 2026, at a compound annual growth rate of 6.3% for 2021-2026 forecast [25]. The request for industrial enzymes is driven mainly by the Asia-Pacific countries (e.g. India, China, Japan) for biofuel, the research and development (R&D) sectors and consumer products. Peptidases belong to one of the six segments of the global food enzyme market: I) amylases, II) cellulases, III) peptidases, IV) lipases, V) phytases and VI) others. Peptidases compose the largest of those segments as they are widely applied in many areas, such as the food, beverage, detergent and pharmaceutical sectors [28].

The peptidase enzyme market is divided geographically into five key regions: North America, Latin America, Europe, Asia-Pacific and Middle East & Africa. Due to the large cleaning and detergent industry sector, North America has become a leader in the worldwide peptidase market. In Europe, Germany is the market leader. As it has already been mentioned, the market in the Asian-Pacific countries shows the highest growth rates due to a constantly developing and modernizing industry, providing extensive opportunities for the peptidase market growth [29]. Figure 8 illustrates an overview of the enzyme market segments, its sectors by main applications and geographical regions.

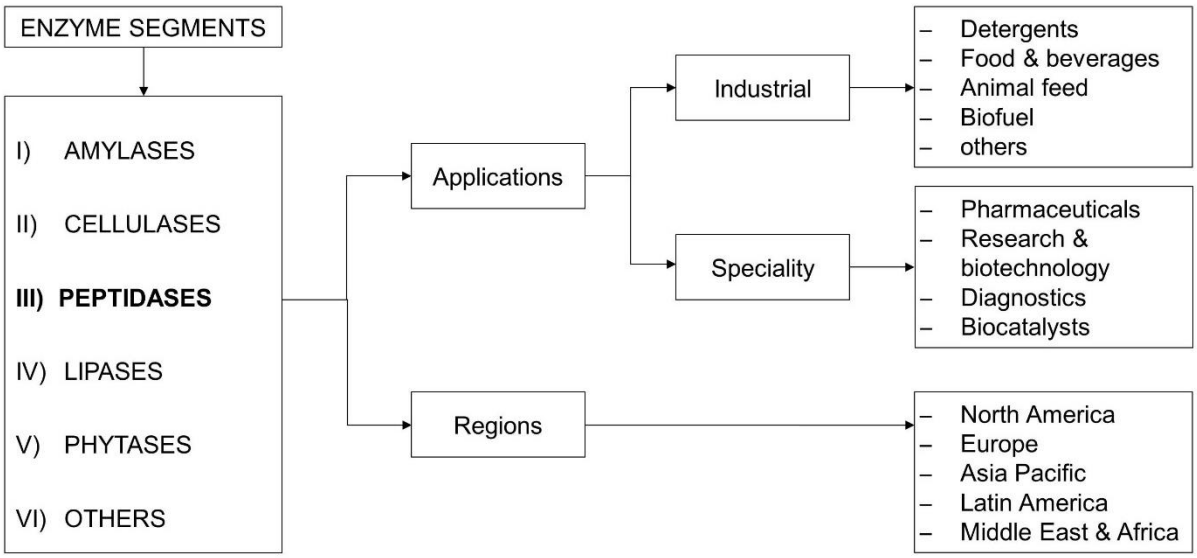

Figure 8: Overview of the enzyme market segments in industrial and specialty applications and the five key geographical regions of the peptidase enzyme market [28-29].

1.2.3 Industrial production of enzymes

Enzymes are produced at different levels of production, going from bulk high tonnage processes (commodities) to small-scale applications (research, enzyme specialties), according to their target application. High levels of purity are required for specialty enzymes that are used in medicine and health-care sectors and are, therefore, produced in rather small quantities (grams). On the opposite side of the spectrum, enzymes applied in the bulk production of food, feed, fabrics and fuel are commonly produced as crude preparations in large-scale tons.

The source and localization of the enzyme determines the production process. Enzymes from animal and plant sources are extracted or recovered mainly from the tissue or fluids. By contrast, microbial enzymes are produced mainly by fermentation and subsequent recovery from either the fermentation medium or the cell paste after extraction. Following both production methods, several operations of purification are applied, depending on the purity required for the enzyme application. Drying and formulating are the final processing steps in the production, giving the enzyme preparation its final presentation, including stabilization and standardization [30]. Figure 9 shows a scheme to produce enzymes.

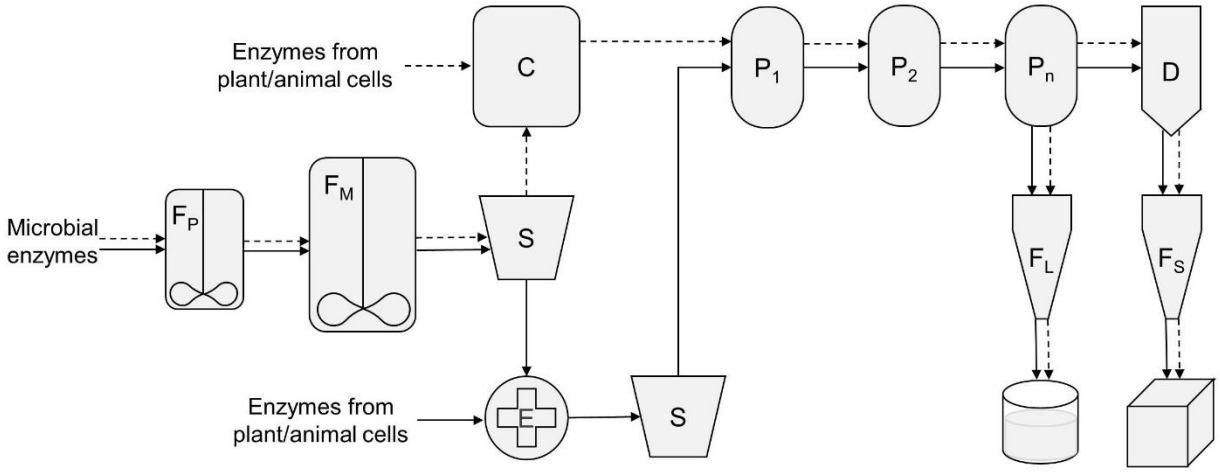

Figure 9: Scheme for the production of enzymes from microbial, plant and animal derived sources (adapted from Illanes) [30]. Dashed arrows: extracellular enzymes; continuous arrows: intracellular enzymes; F_P: pre-cultivation for F_M: main fermentation; S: separation; E: extraction; C: concentration; $P_{1\text{-}n}$: purification steps; D: drying; F_L: liquid formulation; F_S: solid formulation.

Most enzymes are derived from microbial sources and microbial enzymes can be either extra- or intracellular. Extracellular enzymes are secreted by a cell and function outside of that cell. Intracellular enzymes must be released by further processing, such as cell rupture or permeabilization. Alternatively, as they usually remain associated with the cell, they are themselves used as catalysts [31]. As shown in Figure 10, microbial enzyme production can generally be divided into five stages: I) application research and protein engineering, II) strain development, III) microbial fermentation, IV) downstream processing and V) enzyme formulation [32].

Figure 10: Flowchart of the five general stages of microbial enzyme production (adapted from Dodge) [32].

I) Application research and protein engineering

Before starting the production, a comprehensive understanding of the enzyme application is essential for selecting the appropriate enzyme. Screening systems should be robust and simulate the final application performance. Furthermore, it is advantageous if one works with different large libraries. The challenges are then to transfer the requirements of the optimal industrial conditions to biochemical screening assays, considering properties such as temperature, pH, ionic strength, inhibitors, stability, solubility, and specificity issues. Nevertheless, screening systems only simulate the enzyme performance. Therefore, it is highly recommended to always include application trails with the final candidates in the respective scale to validate the screening system selected with the final application. Although most enzymes are produced microbially, many are produced using recombinant deoxyribonucleic acid (DNA) technology (e.g. protein engineering). With this method, properties of the strains can be improved that are important for industrial application [32].

II) Strain development

The selection of an appropriate production host is the next step after having identified the target enzyme. The majority of industrial food enzymes are produced by microorganisms, mainly *Bacillus* species [33] and filamentous fungi [34]. These hosts are recognized as safe

by regulatory authorities, represent good secretors, can be genetically manipulated and reach high biomass concentrations during cultivation. Both genetically modified organisms (GMOs) and non-GMOs are used for the production of food enzymes. Although food enzymes from non-GMOs are more accepted by the consumer [35], enzymes from GMOs are engineered to overexpress the enzyme desired and are, therefore, not only expressed at higher levels but also tend to have a higher purity in comparison to enzymes from non-GMOs. If GMOs are used for the enzyme production, the Food and Drug Administration is responsible for the product labeling in the United States [36] and the European Union in Europe [37].

III) Microbial fermentation

Enzymes are produced from microbial cultures and fermentation unit operations are used to generate the enzymes [38]. In order to harvest, sufficient enzymes have to be produced during fermentation, which occurs in all microbial growth phases. The strains selected are usually pre-cultured on a small scale, before being used for the inoculation of the fermentation media. Sterile fermentation conditions are needed since only growth of the selected strain is targeted. Such conditions are typically created by heat inactivation or filtration. During fermentation, mass and heat transfers are to be considered in order to achieve optimal growth of the microbial catalyst. The regulation of sufficient oxygen supply and heat (both mechanical and metabolic) removal are essential since most industrial enzyme fermentations are aerobic processes [32].

Three basic modes are applied for fermentation: batch, fed-batch and continuous. Batch mode is the oldest and simplest, as all components are included from the beginning of the process. After fermentation, the products are harvested. The continuous mode constantly supplies the microbe with fresh nutrients and removes media. The mode most frequently used for enzyme production is fed-batch fermentation [39]. Growth-limiting nutrients are fed to the microbes in a batch system installation to extend the growth period and, consequently, the enzyme production.

Apart from the three modes, both types of fermentation, submerged and solid-state, are applied for enzyme production. A well-known process that has been carried out for centuries by solid state is Japanese *koji* - fermentation of soybeans by *Aspergillus oryzae* [40]. Most processes involved in industrial enzyme production are performed by

submerged fermentation due to challenges of foaming from, for example, oxygen supply, gas and heat removal and homogeneous mixing issues [32].

IV) Downstream processing

Fermentation broths are not ready to be sold as a final product due to a number of issues. Living microbial cells and rDNA must first be removed and the water activity needs to be reduced in order to avoid degradation and for subsequent concentration. Several operations are applied in downstream processing, such as centrifugation and filtration steps, resulting in a clarified enzyme broth. After removing residual process aids, such as antifoam oils and flocculants, together with other undesirable residuals, the crude enzyme concentrate can be further purified [32]. The extent of enzyme purification is dependent on its further application. Purification is commonly kept to a minimum because enzyme purification on an industrial scale is cumbersome and expensive. The yield of enzyme recovery is commonly more targeted than the enzyme purity, as the benefit for producing at higher purity does not compensate for the higher costs. Figure 11A illustrates a guideline for the number of purification steps considering the purification factor and yield of recovery, whereas Figure 11B shows the common steps of a purification strategy.

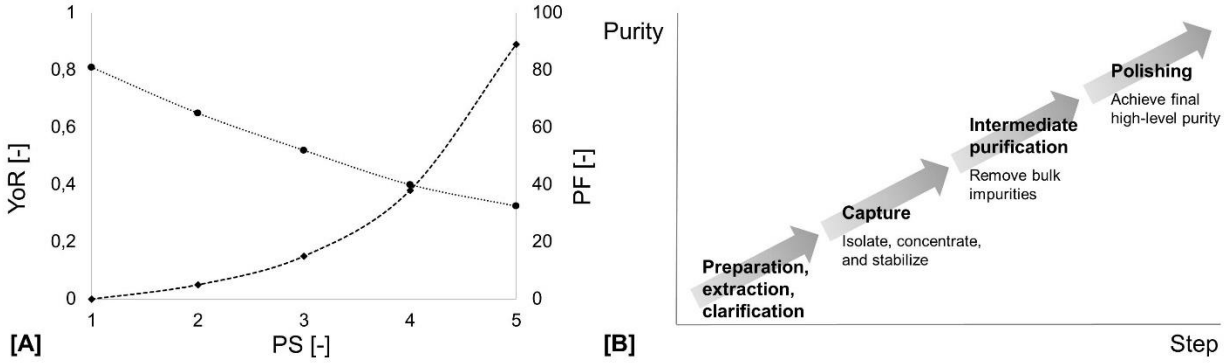

Figure 11: [A] Guideline for the number of purification steps (PS) as a function of the purification factor (PF) and yield of recovery (YoR) (adapted from Illanes) [30]. [B] A three-step purification strategy after sample preparation (adapted from GE Healthcare) [41].

Exemplarily, high purities might be required for pharmaceutical usage and highly specific research applications. A three-step purification strategy has been developed to simplify protein purification in terms of planning and execution, as shown in Figure 11B. After sample preparation, the strategy consists of three steps: capture, intermediate purification and polishing. The capture step includes the isolation, concentration and stabilization of

the target product. If possible, critical contaminants should be removed to the greatest extent. Further bulk impurities, such as other proteins, nucleic acids, endotoxins and viruses are removed during the intermediate purification stage, which is only necessary if the previous capture step has not been efficient enough. This guideline helps to combine purification methods in order to reach the set goals best, while not implying that each protocol consists of only three steps. The number of purification steps will depend on the starting material, purity requirements and indented application of the protein [41].

V) Enzyme formulation

The purified enzymes have to be stabilized in order to supply the enzymes with sufficient shelf-life. According to their intended use, formulations are generally in either solid or liquid states. In the solid product formulation, the enzyme is concentrated, mixed with the respective stabilizing agents and then converted into a dry form either by spray drying, freeze drying, encapsulation or tableting. Freeze drying can be beneficial to the enzyme products as it avoids heat generation, however, it is usually more expensive. By including the enzyme in the core, encapsulation can lead to an improved stability and specific release of the enzyme under controlled conditions, given by, for example, pH, temperature and water activity. Enzyme tableting also incorporates the enzymes in a stabilized matrix and offers easy dosing and handling. Nevertheless, spray drying is commonly applied for solid product formulation. After mixing the enzyme with all the stabilizers required, the formulation is atomized into small droplets through a nozzle. The atomized liquid is then transferred into a spray tower and exposed to temperatures of up to 170°C. Thus, the actual enzyme powder only undergoes temperatures of below 100°C due to evaporation cooling. Spray drying has the main advantage of producing very homogenous products. A disadvantage is that, due to the small particle sizes, spray dried enzyme formulations tend to be very dusty, making them difficult to handle in large quantities. In order to reduce the dustiness, the powders can be treated with low levels of oil or agglomerated to larger particles. Liquid product formulation can be challenging in their own way because liquid systems are more dynamic due to the presence of water. By maintaining the protein structure and, therefore, preventing protein denaturation, carbohydrates, for example, sucrose, dextrose, sugar alcohols and polyols, are common liquid stabilizers. Enzyme stabilizers are added after the enzyme has been concentrated to help control the water

activity. A subsequent filtration step removes undissolved particles and is sometimes also performed to reduce the microbial level in the final product [32].

Sodium benzoate and potassium sorbate are the main preservatives used to control microbial contamination for enzymes used in food applications. Controlling a broad spectrum of microorganisms, both sodium benzoate and potassium sorbate are effective at a low pH. Moisture-free products such as solid formulations when stored appropriately, are generally known to exhibit a lower risk of microbial and fungal growth/contamination compared to liquid formulations. The demand for natural preservatives, such as plant extracts or peptides, is increasing due to consumer preferences.

1.2.4 Industrial applications of peptidases in the food sector

Proteolytic enzymes have found a wide range of application areas, as indicated in the product sectors in Table 3. Although also applied in the areas of, for example, animal feed and detergents, the following paragraphs list several examples into the food sector and provides an insight to their industrial relevance. Furthermore, the following passages only focus on the applications of peptidases and not on enzymes in general.

Peptidases in dairy processing

Peptidases are involved in several sectors of the dairy industry, such as cheese production and milk processing. Concerning cheese production, peptidases are involved in the two fundamental steps of coagulation and ripening. Microbial acidic proteases (E.C.3.4.23) are used for their ability to coagulate the casein milk protein for subsequent cheese production and taste formation [42]. In the context of ripening, a protease from *Pseudomonas fluorescens* R098 has found application as a debittering agent for the hydrolysis of bitter tasting peptides in cheese [43]. Peptidases from lactic acid bacteria contribute to the flavor and aroma characteristics of cheese by hydrolyzing the different types of caseins (α-, β-, κ-casein) [44] and by debittering via exopeptidase activities (e.g., aminopeptidases PepN (EC 3.4.11.2) and proline-specific aminopeptidase PepX (EC 3.4.14.11) from *Lactobacillus helveticus)* [45].

Chymosin (also called rennin) is an aspartic endopeptidase (EC 3.4.23.4) and the main peptidase found in rennet which is produced in the stomachs of ruminant mammals. By hydrolyzing the Phe-Met-bond at 105-106 in the κ-casein, the casein micelles are disrupted, the milk coagulates and the casein proteins are released for the further formation of curds and whey for cheese production [46].

Peptidases in cereal processing

Peptidases play an important role particularly in the bakery industry. Gluten is the main storage protein of wheat grains at around 85-90% (w/w) [47] and the most important functional compound in wheat flour [48]. Gliadins and glutenins compose the main protein fractions and the gluten matrix mainly determines the dough quality of bread and of other bakery products, such as pasta and biscuits [49]. Both fractions contribute to the rheological properties of dough but with divergent functions. While the gliadins contribute mainly to the viscosity and extensibility of the dough system, the glutenins influence the strength

and elasticity [47]. Peptidases are used to hydrolyze these protein fractions and, thereby, modify dough characteristics (e.g., serine peptidase (EC 3.4.21) of the enzyme preparation TS-E-555, Danisco Cultor) [50] and to generate free amino acids to serve as precursors for flavor generation [51].

Apart from the techno-functional reasons mentioned above, peptidases are also used to produce gluten-free products. This is particularly important as an increasing section of the population suffers from wheat-related disorders, such as celiac disease, wheat allergy and non-celiac gluten sensitivity. Celiac disease is defined as an inflammatory disease of the upper small intestine (e.g. duodenum, jejunum) in genetically susceptible individuals caused by the ingestion of gluten proteins from wheat, rye, barley and possibly oats. By considering celiac disease as both a food sensitivity disorder and an autoimmune condition, celiac disease has been defined as an immune-mediated food sensitivity with an autoimmune component [52]. Wheat allergy is defined as an adverse immunologic reaction to wheat proteins, with IgE antibodies playing a key role in the pathogenesis [53]. On the other hand, non-celiac gluten sensitivity refers to individuals who develop symptoms after consuming gluten-containing food but lack any evidence of celiac disease or wheat allergy [54]. People affected by these, usually life-long diseases, suffer from bowel distress, such as cramps, bloating, gas and constipation. Moreover, depression, infertility and osteoporosis may also result from these disorders [55]. Although products have been developed to break down gluten and support patients lifestyles (e.g. Tolerase® G, an enzyme preparation with proline-specific oligopeptidase activity (EC 3.4.21.26), DSM, Delft, The Netherlands), the only certain solution is a complete avoidance of gluten in a strict diet [55].

The United Nations Food and Agriculture Organization defines in the Codex Alimentarius (CODEX STAN 118-1979) food standards for gluten-intolerant consumers with special dietary requirements. It defines *"**gluten-free foods** as dietary foods […] consisting of or made only from one or more ingredients which do not contain wheat […], rye, barley, oats (if contaminated with wheat, rye or barley in foods covered by this standard) or their crossbred varieties, and the **gluten level does not exceed 20 mg kg^{-1} in total**, based on the food as sold or distributed to the consumer […]" [56].* Moreover, several toxic gluten peptides rich in proline and glutamine residues have been identified [52], and this is where the usage of peptidases comes into play. Studies have proposed detoxifying gluten, for

example, by bacterial prolyl endopeptidases from *Flavobacterium meningosepticum*, *Sphingomonas capsulate* and *Myxococcus xanthus*, degrading the gluten proteins into inactive short peptides [57]. Further prolyl endopeptidases (EC 3.4.22.18) from fungal *Aspergillus niger* and *A. oryzae* have been investigated due to their gluten-degrading capacities [58]. Intestinal *Lactobacillus* strains (e.g., *L. ruminis*, *L. johnsonii*, *L. amylovorus* and *L. salivarius*) have been investigated for being capable of degrading immunotoxic gluten peptides. Due to their proteolytic activity, referring to prolyl endopeptidases (EC 3.4.21.26) , aminopeptidases type N (EC 3.4.11.2) and proline iminopeptidases (EC 3.4.11.5), their usage as probiotics have been suggested [59].

Peptidases in beverage processing

Proteolytic enzymes are involved in several beverage sectors, such as beer, wine and juice processing. During the brewing process, peptidases break down the protein matrix around the starch grains, improve mash extraction and foam stability and liberate free amino acids from the polypeptide chains for further reactions [60]. Efforts have been invested in producing gluten-free beer to respond to the specific requirements of the gluten-associated diseases mentioned in the previous section. Celiac-active peptides involving proline residues were cleaved by using special malt with high gluten-specific peptidase activity, resulting in a gluten-free (e.g. gluten content < 20 mg kg^{-1}) wort [61]. Another approach was to include a peptidase preparation with proline-specific endopeptidase activity from *A. niger* (Brewers Clarex, DSM, Delft, The Netherlands) in the brewing process, also achieving a gluten-free beer with no impact on the sensory profile [62]. Along with several types of enzymes, such as amylases, cellulases and pectinases [63], peptidases have proved to be advantageous for wine processing. An aspartic protease (EC 3.4.23) from the yeast *Metschnikowia pulcherrima* was able to degrade grape proteins which are responsible for undesired turbidity in wine [64]. Similar results were observed when applying commercial enzyme preparations such as Zumizyme AP protease (BioBright Japan) and Papaine protease (Novozymes SA). The wines produced from raw materials that were previously treated with the peptidase preparations resulted in significantly lower turbidity [65]. As is the case for wines, proline-specific and acid peptidases from *A. niger* are also applied in juice processing for clarification reasons [66].

Peptidases in meat and seafood processing

The need for extended meat production is increasing due to the rising demand for meat and methods are being developed to add value to lower quality meat. Peptidases can be used to tenderize tough meat parts and, therefore, upgrade meat of poorer quality [67]. Plant-derived cysteine peptidases (EC.3.4.22), such as papain (EC 3.4.22.2 from *Carica papaya*), bromelain (EC 3.4.22.32 from *Ananas comosus*) and ficin (EC 3.4.22.3 from *Ficus glabrata*), are traditionally used to break down the cross-linking of the fibrous protein which results in more tender meat. Furthermore, cysteine peptidases are also used to remove muscle residues from bones, producing a meat slurry that can subsequently be used for canned meat and soups, whereas the cleaned bones are used for producing gelatin [68]. Apart from the functional aspects, serine endopeptidases (EC 3.4.21) from *Streptomyces griseus* have also shown the potential to enhance the flavor development of meat products due to the liberation of free amino acids during hydrolysis [69]. In a similar way to meat processing, peptidases are applied in the seafood processing sector to increase the yield, facilitate processing steps and improve product quality [70]. Fish by-products can be applied as flavor formulations in the fish industry by the usage of both exo- and endopeptidases which produce amino acid-rich extracts through proteolytic activities. This was shown by using commercial enzyme preparations with leucine aminopeptidase activity (EC 3.4.11.1) and non-specific casein proteases from *Aspergillus* sp. and *Bacillus* sp. [71].

1.2.5 Technical enzyme preparations

The worldwide enzyme market offers various technical enzyme preparations (TEPs) due to the high demand for processing aids in a wide range of applications (detergent, pharmaceutical, food and beverage industry) [72]. As presented in the previous chapters 1.1.5 Origins of peptidases and 1.2.3 Industrial production of peptidases, both the origin and processing impact the final enzyme preparation. The Food and Drug Administration states that food processing enzyme preparations contain one or several active enzymes that are responsible for the intended technical purpose in food production [36]. The TEPs, as defined in this study, are enzymes with moderate purity to be used in food processing. Today, TEPs are used by food manufacturers based on the supplier's information that usually states the main enzyme activity and includes information on side activities only in

some cases. The TEP Flavourzyme1000L is standardized by its supplier Novozymes by leucine aminopeptidase units (LAPU), using Leu-*p*NA as a synthetic substrate, as an example of a specific activity. The TEP Promod278 is standardized by its supplier Biocatalysts Ltd. by casein protease units, using casein as a natural substrate, as an example of an unspecific activity. In general, some data sheets of the TEPs' state side activities, such as endopeptidase or glutaminase activities, but detailed information about the substrate specificity and specific peptidase activities are usually not available. Table 4 shows an overview of the main proteolytic TEPs used in this dissertation and presents the enzyme information available provided by the specification sheets of the respective suppliers. As shown by these examples, the suppliers declare only one specific activity or unspecific enzyme activities. Only in the case of Flavourzyme1000L, generic side activities are declared by "*protease*" side activities.

Table 4: An overview of the proteolytic technical enzyme preparations including the respective information on the supplier, origin and enzyme information declared. All information was provided by the enzyme specifications of the respective suppliers.

ID	Supplier	Origin	Declared enzyme	Declared activity	Declared assay substrate	Declared side activities
Alcalase2.4L	Novozymes	*Bacillus licheniformis*	Serine endoprotease (Subtilisin)	2.5 AU-Ag^{-1}	n.d.	n.d.
Flavourzyme1000L	Novozymes	*Aspergillus oryzae*	Leucine aminopeptidase	1000 LAPUg^{-1}	Leu-*p*NA	*"protease"*
DeltazymAPS-M-FG	WeissBio Tech GmbH	*Aspergillus niger*	n.d.	19,000 SAP Ug^{-1}	n.d.	n.d.
FoodPro51 FP	Danisco, DuPont	*Aspergillus oryzae*	Aspergillopepsin I	n.d.	n.d.	n.d.
Promod278	Biocatalysts	*Carica papaya and Bacillus subtilis*	Casein protease	700 Ug^{-1}	Casein	n.d.
ProteaseA "Amano"2SD	Amano Enzyme Inc.	*Aspergillus oryzae*	Neutral protease	20,000 Ug^{-1}	L-Tyr	n.d.
ProteaseP "Amano"6SD	Amano Enzyme Inc.	*Aspergillus melleus*	Semi alkaline protease	600,000 Ug^{-1}	L-Tyr	n.d.
ProteAX-K	Amano Enzyme Inc.	*Aspergillus oryzae*	Peptidase	1400 Ug^{-1}	n.d.	n.d.
PeptidaseR	Amano Enzyme Inc.	*Rhizopus oryzae*	Peptidase	420 Ug^{-1}	L-Leu-Gly-Gly	n.d.
Flavorpro750	Biocatalysts	*Aspergillus oryzae*	Casein protease	55 Ug^{-1}	Casein	n.d.
Flavorpro766	Biocatalysts	*"a mixed/ microbial source"*	Leucine aminopeptidase	203 LAPUg^{-1}	Leu-*p*NA	n.d.
Flavorpro839	Biocatalysts	*"a mixed/ microbial source"*	Leucine aminopeptidase	250 LAPUg^{-1}	Leu-*p*NA	n.d.

1.3 ALTERNATIVE PROTEIN SOURCES – PLANT AND INSECT PROTEINS

The worldwide population has currently (2021) reached over 7.7 billion people and, according to recent forecasts, it is expected to exceed nine billion over the next 20 years [73]. Due to this growth and strong economic transitions, meat consumption is constantly increasing. In 2014, the average meat consumption worldwide was over 40 kg per person per year, with the highest meat consumers in high-income regions, such as Europe and North America with values of over 100 kg per person per year [74]. The most common sources of meat are beef, pork and poultry [75], making meat the main protein source [76]. Due to this increasing demand, alternative protein sources are becoming increasingly required. Plant proteins are important in the food industry not only for their nutritional value. They have also been shown to bring additional value to meat products for technological reasons, such as water-binding capacities [77], or for texture-improving properties [78]. Apart from plant proteins, proteins from various insect species have registered an increasing interest in the last couple of years. Insects are consumed mainly in Asia, Africa and South America [79], however, due to their protein value, they are becoming more appealing in other parts of the world. The most commonly consumed insects are beetles (*Coleoptera*, 31%), caterpillars (*Lepidoptera*, 18%), bees, wasps and ants (*Hymenoptera*, 14%), followed by grasshoppers, locusts and crickets (*Orthoptera*, 13%), cicadas, leafhoppers, planthoppers, scale insects and true bugs (*Hemiptera*, 10%), termites (*Isoptera*, 3%), dragonflies (*Odonata*, 3%), flies (*Diptera* 2%) and other orders (5%) [80]. From these, the European Food and Safety Authority lists twelve species which are reported to have the greatest potential to be used as food and feed in the European Union, namely flies (*Musca domestica*, *Hermetia illucens*), mealworms (*Tenebrio molitor*, *Zophobas atratus*, *Alphitobus diaperinus*), moths (*Galleria mellonella*, *Achroia grisella*), silkworms (*Bombyx mori*), crickets (*Acheta domesticus*, *Gryllodes sigillatus*), locusts (*Locusta migratora migratorioides*) and grasshoppers (*Schistocerca Americana*). In the following section, a selection of plant proteins (e.g. soy, pea, canola, lupin) and two edible insect species (e.g. mealworm and cricket) are introduced and their applications and/or importance in the food industry are presented.

1.3.1 A selection of plant proteins – soy, pea, canola and lupin

Soy is the largest produced among the plant proteins, with almost 350 million metric tons in the 2017/2018 market year [81]. Although it has been a constant part of nutrition for more than 2,000 years, especially in East Asia, soybeans only became popular in other parts of the world in the 1960s. Soy is an economical and a high-quality plant protein, therefore, it has been widely used in the food industry since that time [82]. With a protein content of approximately 40% [83], soybeans form the basis of several foods, such as tofu [84], especially consumed as meat alternatives in vegetarian diets. Soy protein has become a viable alternative for others seeking non-animal sources of protein in their diet. The protein digestibility-corrected amino acid score for soy protein has been reported to be equivalent to the score of animal protein [85]. Unfortunately, soy represents a major source of food allergies, containing primary food allergens including P34/Gly m Bd 30k, a member of the cysteine peptidase family [86]. Due to allergenicity and controversial economic aspects, such as rainforest destruction for soybean cultivation [87], other plant proteins such as lupine, pea and canola have also gained increasing interest in the food industry.

Pea and canola are recognized as important protein sources and their market shares are continuously increasing due to the growing demand for alternative proteins [88]. Peas have long been important components in the food industry due to their composition of starch, proteins, fibers, vitamins, minerals and phytochemicals. Peas (*Pisum sativum L.*) contain 22-32% protein [89], depending on the genotype and environmental conditions [90]. The majority of the pea proteins consist of globulin storage proteins with around 50-60% of the total protein [91]. The amino acid profile of pea protein products has been reported to be similar to that of soybeans and lupins, containing mainly glutamine, aspartic acid, arginine and lysine. Thus, products from peas have resulted in higher amounts of arginine, valine and methionine [92] and, therefore, cover a part of the recommended human requirements for amino acids [93].

The term *canola* (***Can***adian ***o***il **l**ow ***a***cid) was introduced for specific rapeseed varieties in the 1970s [94]. Deriving from the genus *Brassica*, these varieties belong to the so-called “00-rape”. This rape is defined as being low in erucic acid (< 2%) and gluco-sinolates (30 µ mol g^{-1}) [95], both being undesirable compounds due to their bitterness [96]. The two main storage proteins of canola are cruciferin (12S globulin) and napin (1.7-2S albumin) with 60 and 20% of the total protein in mature seeds, respectively [97]. Canola

proteins are applied in the food industry not only for techno-functional reasons, such as emulsifying properties [98], but also due to their nutritive and health beneficial values. Canola products possess a high-quality protein profile due to their amino acid composition that is comparable to other protein sources, such as soy [95], and, therefore, serve as a suitable plant protein alternative.

With a protein content of approximately 40 to 50% [83], lupines serve as a valuable source of protein. Lupins belong to the leguminose family (*Fabaceae*), genus *Lupinus* [99]. The four main species include *L. albus, L. angustifolius, L. luteus* and *L. mutabilis*, grown mainly in Europe, Australia, the Mediterranean region and in South Africa, respectively [100]. Since lupines are able to grow on different soils and climate zones, they are valuable not only for their nutritional value but also for economic reasons [101]. Although they are used less than soy and pea protein, lupine flour, protein isolates and concentrates are used mainly in bakery products to add nutritional value or for functionality reasons [100]. However, lupins are known to possess an allergenic potential similar to peanuts, due predominantly to the storage proteins α-conglutin (legumin-like, 11 S), β-conglutin (vicilin-like, 7 S), γ-conglutin (7 S) and δ-conglutin (2 S-albumin) [102].

1.3.2 A selection of insect proteins – mealworm and cricket

The mealworm *Tenebrio molitor* and the house cricket *Acheta domesticus* belong to the twelve species that are listed by the European Food and Safety Authority to have the potential to be used in the food and feed industry [103]. The yellow mealworm *Tenebrio molitor* is economically the most important species used for the large-scale conversion of biomass into protein. Therefore, industrial companies, such as Ynsect, have selected this species and are developing the large-scale farming of this insect [104]. The protein content in fresh insects is approximately 25%, whereas it can reach 60 to 70% in dry matter [105]. The amino acid profile of mealworm protein includes a variety of both essential and non-essential amino acids, showing similar total values to those of soy [106]. Studies have shown that larvae of *Tenebrio molitor* possess potential for seasoning sauces [107], as a novel non-meat ingredient [108] and for functional properties [106] for food applications.

The house cricket *Acheta domesticus* exhibits a fivefold feed conversion efficiency compared to pigs [109] and possesses, therefore, potential for aiding to meet the increasing food demand to the growing population worldwide. With a protein content of 50 to 60%

(dried, defatted insects) and a well-balanced amino acid profile [110], crickets have gained attention for protein applications in the food industry. It is suggested that flour from *Acheta domesticus* could be used as an effective non-meat functional ingredient for meat products [111]. Further research has shown that cricket powder can be added to wheat flour to obtain protein-enriched bread without lacking technological and sensory properties [112]. Unfortunately, both mealworm and cricket protein possess allergenic potential. Since insects are closely related to *Crustaceans* and mites (*Acari*), edible insects are related with allergens such as tropomyosin, α-amylase or arginine kinase [113].

1.4 THE BENEFIT AND OPPORTUNITIES OF ENZYMATIC PROTEIN HYDROLYSIS

Enzymatic hydrolysis has been applied for thousands of years, ranging back to 6000 BC in references to the Sumerians and Babylonians for the brewing of beer or back to the old Egyptians for wine making and baking bread [114]. Enzymes liberate various molecules by breaking down complex structures and achieve an alternation of the matrix properties in terms of techno-functional, nutritional and sensory properties. The following paragraphs give an insight into the possibilities of using peptidases in the food industry to alternate protein properties, outline important features and highlight the benefits and further potentials of enzymatic protein hydrolysis. The examples and research results that will be discussed refer mainly to the plant and insect proteins that have been introduced in this section so far and part of the research of this dissertation.

As explained in detail at the beginning of the proteolytic enzymes' introduction section, peptidases act on the peptide bonds of polypeptide chains and cleave proteins into smaller fragments and molecules. Since proteins are composed of amino acids, breakage of the peptide bonds results in peptides of various sizes and single amino acids, depending on the specificity and kinetics of the peptidase activities. The DH gives the extent to which a protein has been hydrolyzed as a reflection of broken peptide bonds and, therefore, an average size of the peptides present. The DH can be determined through various methods, such as after derivatization with OPA. This technique has been detailed in the *Synergism of peptidases - impact on the degree of hydrolysis* section. Depending on the DH, the resulting peptides and amino acids alter the properties of the hydrolysates in many respects and serve as precursors for further reactions that are explained in more detail in the following paragraphs.

1.4.1 Impact on techno-functional properties

Enzymatic hydrolysis leads to lower molecular weight distributions due to its cleavage function and, therefore, decreases the apparent viscosity of protein systems [115]. An increased protein solubility has been determined in various proteins [116], including plant proteins, such as soy [115], rapeseed [117] and insect proteins [118]. Enzymatic hydrolysis also alternates the protein foaming properties due to the increased protein solubility and

structure modification. Although it has been suggested that low molecular weight fractions contribute to the rapid foam formation, irregularities have been observed with the foam stability [119]. Foams are two-phase systems that are composed of air bubbles surrounded by continuous liquid lamellar phases and can be stabilized by both surfactants or proteins [120]. Both foam capacity and stability depend on protein functionalities and change when proteins are hydrolyzed into smaller fragments, altering protein structures and exposing functional amino acids in the matrix [95].

Emulsification properties of canola protein [121] and cricket protein [118] have been reported to be enhanced by the usage of serine endopeptidase activities (EC 3.4.21) from *Bacillus licheniformis*. Emulsifiers are surface-active compounds that consist of a hydrophilic head group and a lipophilic tail and can therefore operate between aqueous and oil phases. Emulsifiers lower the surface or interfacial tensions by positioning themselves at the air/water or oil/water interface [122]. The improved emulsifying properties after hydrolysis, by mainly endo-peptidase activities (E.C. 3.4.21.62, E.C. 3.4.24.38) derived from *Bacillus amyloliquefaciens*), have been explained by the protein hydrophobicity. Hydrophobic sites are exposed due to hydrolysis, leading to an increased surface activity and adsorption at the interface and, finally, to a strong protein-oil interaction [119].

1.4.2 Impact on nutritional quality

The quality of proteins is highly determined by the amino acid profile because amino acids are the most important building blocks of proteins. Based on their nutritional/physiological roles, amino acids can be divided into essential and non-essential groups. Valine, leucine, isoleucine, phenylalanine, tryptophan, methionine and threonine belong to the essential amino acids. Histidine is essential for infants, and lysine and arginine have been defined as semi-essential. Amino acids that cannot be synthetized by humans and, thus have to be ingested via foods are glycine, alanine, proline, serine, cysteine, tyrosine, asparagine, glutamine, aspartic acid and glutamic acid [123]. Amino acids are highly important nutritionally and contribute to the formation of enzymes, hormones and other important substances for maintaining health [124]. Protein hydrolysates can have higher nutritional value than the protein raw material since peptidases, more precisely exopeptidases, liberate amino acids from the polypeptide chain. Proteolytic hydrolysis has been generally shown to improve digestion and the absorption of poorly digestible proteins by degrading

the complex structures [125]. Furthermore, research has shown that enzymatically hydrolyzed proteins have the potential to be used in fat replacing systems of, for example, ice cream hydrolysis by serine endopeptidase activities (EC 3.4.21) from *Bacillus licheniformis* [115], and contribute, therefore, to the invention of healthier foods.

When discussing the nutritional value of proteins, the topic of allergenicity has to be mentioned. A number of proteins cannot be consumed by humans because the former possess various allergens. Peptidases have been applied to reduce the allergenic potential of several proteins by degrading toxic-active peptides responsible for allergenic reaction. Research has shown that prolyl-specific peptidases can degrade gluten to levels below 20 mg kg^{-1}, resulting in a gluten-free labeling of food products. Several peptidases have been investigated for gluten degradation, e.g., plant cysteine endopeptidase (EC 3.4.22) from barley and wheat or fungal prolyl endopeptidase (EC 3.4.21.26) from *Aspergillus* sp.[126]. Further information is also included in the upper chapter 1.2.4 Industrial applications of peptidases in the food sector - peptidases in cereal processing.

1.5 SENSORY POTENTIAL OF ENZYMATIC PROTEIN HYDROLYSATES

During hydrolysis, peptides and amino acids are released which not only alter the techno-functionality but also the sensory attributes of the hydrolysates. Sensory refers to the interaction of taste, odor, and textural feelings when food is consumed. The term flavor describes the overall sensation of these interactions and results from components responsible once for taste and once for odors. Components responsible for the odor are often designated as aroma substances. While they are volatile, these substances are perceived by the odor reception sites of the smell organ, such as the olfactory tissue of the nasal cavity. Gas chromatography is coupled with an olfactometer in order to detect such substances. The gas chromatography separates the volatile mixtures, while the human nose can detect the odor intensity of the analytes [127]. Aroma substances reach the receptors via ortho nasal and retro nasal, which refer to the nose and the throat, respectively. Components responsible for taste are generally nonvolatile at room temperature and, therefore, interact with the taste receptors located in the taste buds of the tongue [128].

1.5.1 Taste properties of amino acids and peptides

Taste perception includes the five sectors: sour, sweet, bitter, salty and umami [129]. Bitter, salty and umami are the three most interesting taste profiles when dealing with hydrolysates. While bitterness is an issue of hydrolysates and mostly targeted to be decreased or even removed, research on salty and umami tasting peptides has been followed with great interest. During hydrolysis, taste-active amino acids and peptides are released, contribute to the flavor of the hydrolysate and/or serve as precursors for further flavor reactions [123].

Peptides consist of amino acids and, therefore, the bitter perception of peptides is determined mainly by the amino acid composition containing predominantly hydrophobic residues. Furthermore, research has shown that the arrangements of the amino acids and the spatial structure also influence the bitterness intensity. Basic residues at the N-terminal and hydrophobic or bulky residues at the C-terminus enhance the bitterness intensity. Furthermore, short distances between the N- or C-terminal group and hydrophobic groups promote bitterness intensity. Proline, valine, leucine, phenylalanine

and tryptophan have been determined as the most bitterness-influencing amino acids [130]. Therefore, depending on the cleavage of the proteolytic activity and the resulting peptides, bitterness can increase and has been observed in several studies [131]. However, specific enzymatic treatment has also been shown to be used for the debittering of hydrolysates by further hydrolysis and degradation of bitter peptides. The application especially of proline-specific exo- and endopeptidases has shown potential for debittering due to the contribution of the proline residues to peptide/hydrolysate bitterness [132].

Although salt is an important substance for food quality, an excess of salt intake is strongly linked to several health issues, such as hypertension (an increase in blood pressure), resulting in serious cardiovascular diseases [133]. Research has been performed for salt replacers as salt is not only important for food quality but also for savory flavor reasons. Although salt replacers, such as potassium, magnesium or calcium chloride, can be applied to salt-reduced foods, flavor and the overall acceptability tends to be correlated negatively [134]. Thus, arginyl dipeptides have been identified to have salt taste enhancing properties [135] and peptidases of Basidiomycota (fungi) have shown high peptidolytic activity to liberate these salt taste enhancing peptides. Therefore, peptidases can be applied to produce hydrolysates with salt tasting peptides. These can then be further applied to foods to achieve salty attributes while lacking the addition of salt [136].

Umami is considered as the fifth basic taste and umami substances are considered as flavor enhancers. Monosodium glutamate is the salt of the amino acid glutamic acid and possesses a unique umami flavor [137]. Apart from the salts of glutamic acid, the salts of aspartic acid and 5´nucleotides also contribute to the umami taste [138]. Monosodium glutamate and the salts of glutamic and aspartic acid are used as food flavorings [139], and although these umami substances occur naturally in various foods, such as meat, fish and mushrooms, research has shown that these substances are liberated due to proteolytic activity. Therefore, hydrolyzed foods possess higher levels of umami substances and, therefore, umami taste [138]. Summarizing this paragraph, the application of peptidases results in protein hydrolysates that, depending on the substrate and the peptidase/-s applied show different sensory profiles.

1.5.2 Protein hydrolysates for flavor generation in Maillard reactions

Hydrolysates can serve as basis for flavor generation since amino acids are released during hydrolysis due to enzymatic proteolytic activity. Although amino acids give flavor to food on their own, they contribute mainly during heating to the reactions of other flavor compounds. Maillard products are important flavor substances of food products and characterize important flavor notes, such as roasty notes of meat products [140].

Figure 12 shows a scheme for combining proteolytic hydrolysis for the liberation of free amino acids and smaller peptides and subsequent Maillard reactions to generate flavor-active Maillard products.

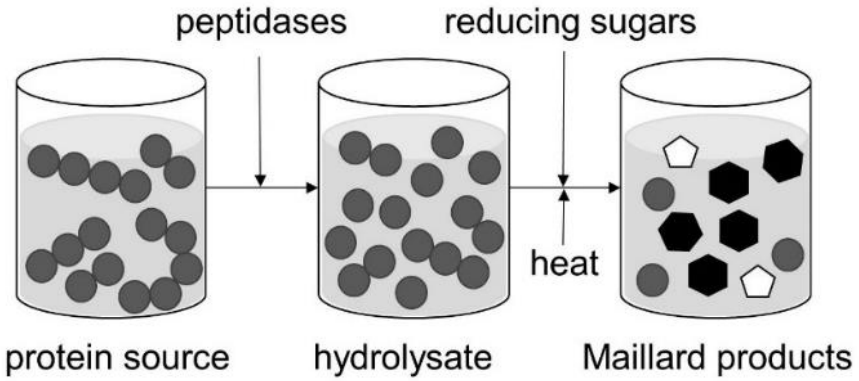

Figure 12: Scheme for combining, firstly, proteolytic hydrolysis of a protein source with subsequent reactions of the hydrolysates to produce flavor-active Maillard products.

Maillard reactions are very complex and include various reactions. The first reaction scheme was introduced by Hodge in 1953 and, since then, researchers have developed this scheme. Maillard reactions are initiated by the condensation of amino groups of proteins, peptides and/or amino acids with carbonyl groups of reducing sugars, resulting in the formation of a Schiff base and Amadori or Heyns product rearrangement [141]. As an example, Figure 13 shows a simplified scheme where the free amino group of an amino acid reacts with the carbonyl group of a reducing sugar to produce a condensation product, N-substituted glycosylamine, which rearranges further to Amadori products. The subsequent degradation of the Amadori products is dependent on the pH, forming

I) reductones, II) fission products or III) furfurals. All these compounds are highly reactive and form other reaction products [142]. Maillard reactions and their formation are complex reactions and are described in excellent studies [143]. As this dissertation focuses on the investigation of enzymatic protein hydrolysis, this section intends mainly to give a short insight into Maillard reactions.

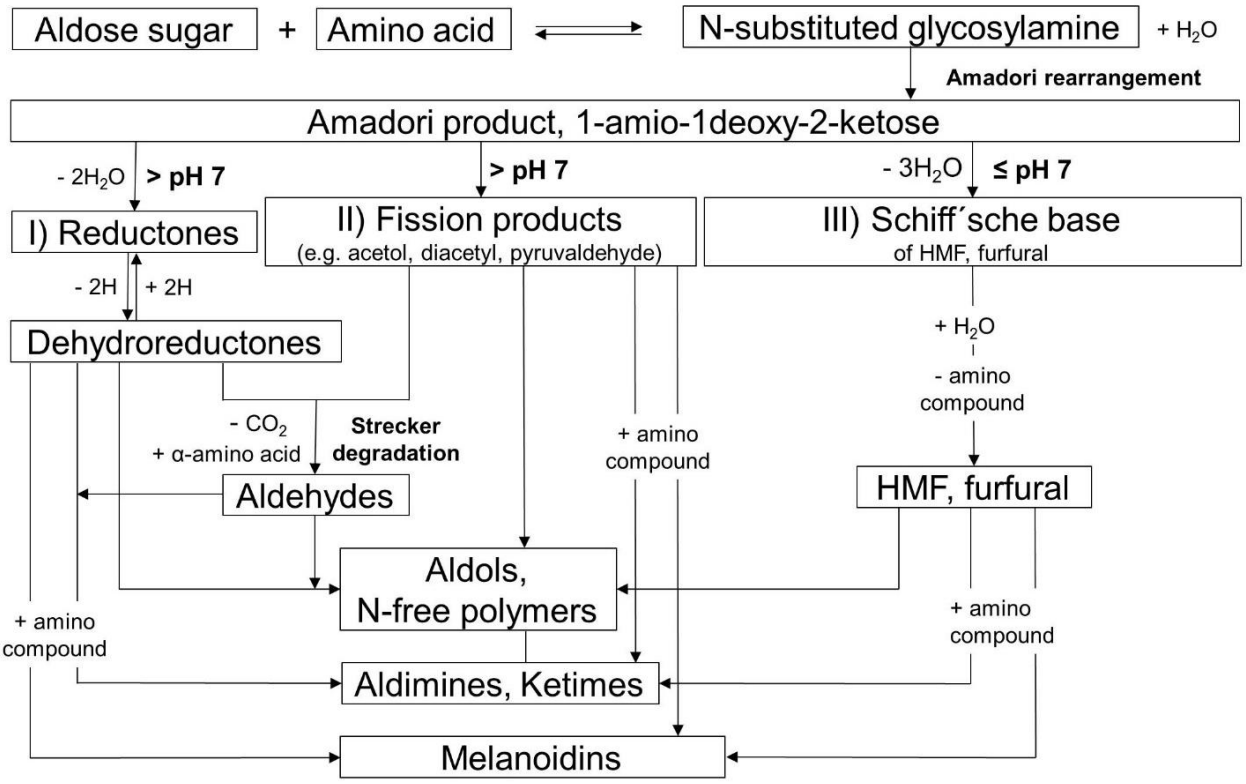

Figure 13: Basic scheme of Maillard reactions, showing an example for initiating the reaction by an aldose sugar and amino acid compound (modified after Hodge) [141].

1.6 THESIS AIM AND OUTLINE

The aim of this research was to increase the knowledge of technical enzyme preparations (TEPs) and their impact on protein hydrolysis. It was of interest to increase the process efficiency and investigate the further potential of protein hydrolysis. The TEPs are applied in various sectors in the food and beverage industry, ranging from the dairy sector to cereal, meat and seafood processing. Although the TEPs are standardized on one specific enzyme activity, little about the substrate spectra and side activities is commonly known. Therefore, this research includes three scientific studies to enlarge the knowledge of the specific activities of TEPs and their influence on the characteristics of the hydrolysates regarding the degree of hydrolysis, profiles of free amino acids and sensory properties.

This thesis is divided into five main chapters. The first chapter introduces the topic of peptidases and provides basic knowledge on their classification, terminology and other relevant sectors to achieve a scientific background for understanding the research experiments performed. It also highlights the industrial relevance and applications of peptidases by focusing on the food industry. Several protein sources and the potential of peptidases to modify protein characteristics for techno-functional, nutritional or sensory properties are introduced. Chapters two to four present three scientific research studies that were published in scientific research journals. The second chapter introduces a new measurement approach to evaluate TEPs by their peptidase activities, referring to 32 synthetical and natural substrates. This study highlights the advantage of detailed knowledge about the substrate specificity and side activities of peptidases and their influence on the performance and efficiency of lupin protein hydrolysis. The third chapter investigates six food-grade TEPs concerning their influence on soy-, pea- and canola protein. In addition to the characteristics of the hydrolysates, a special focus is on the taste attributes bitter and umami. The fourth chapter investigates the potential of cricket and mealworm protein hydrolysates regarding their flavoring potential. The earthy-like tasting protein profiles were modified into complex and savory taste profiles by applying proteolytic hydrolysis and Maillard reaction. Furthermore, odor-active molecules were identified by gas chromatography-olfactometry that are also present in meat and seafood products. The fifth chapter covers a final discussion of studies and this thesis.

2 CHAPTER TWO: THE FIRST PUBLICATION

2.1 A FAST AND NOVEL APPROACH TO EVALUATE TECHNICAL ENZYME PREPARATIONS FOR AN EFFICIENT PROTEIN HYDROLYSIS.

Kora Kassandra Großmann, Michael Merz, Daniel Appel and Lutz Fischer

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Highlights

- A fast and novel measurement approach to evaluate technical enzyme preparations by their activity profiles
- Automated measurement of 32 peptidase activities within less than one hour
- The information generated by the activity profiles were transferrable to the protein hydrolysate properties
- The measurement approach enables a specific selection of technical enzyme preparations for a targeted and efficient hydrolysis

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ORIGINAL PAPER

A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis

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Abstract

The objective of this study was to establish a fast approach (< 1 h) for the evaluation of technical enzyme preparations (TEPs). An automated photometric analyzer (Gallery™ Plus) was equipped with 32 synthetic and natural substrates to measure aminopeptidase, carboxypeptidase, dipeptidyl peptidase and endopeptidase activities distinguishably and the proteolytic activity towards lupine protein of TEPs. The established so-called "activity fingerprints" (AFPs) delivered detailed information about the substrate spectra and peptidase side activities, noticing furthermore batch variations of Flavourzyme1000L. Based on their AFPs, particular TEPs were selected for lupine protein hydrolysis and the hydrolysates were analyzed regarding the degree of hydrolysis and the free amino acids. It was demonstrated that the information of the AFPs were applicable to predict important properties of the resulting hydrolysates. Consequently, the hydrolysis efficiency was improved (increase of 47%). The system introduced enables the targeted selection of TEPs for enzymatic protein hydrolysis, resulting in specific food protein hydrolysates.

Keywords Technical enzyme preparation · Peptidase activity · Substrate screening · Protein hydrolysis · Targeted hydrolysate attribute

Abbreviations

Alc	Alcalase2.4L
AFP	Activity fingerprint
BEH	Ethylene bridged hybrid
CAGR	Compound annual growth rate
CV	Coefficients of variation
DPP	Dipeptidyl peptidase
DH	Degree of hydrolysis
DZM	DeltazymAPS-M-FG
FP51	FoodPro51FP
FVZ	Flavourzyme1000L
H_2O_{dd}	Purified water
h	Concentration of free amino groups
h_{tot}	Maximum concentration of free amino acids at complete hydrolysis
LAPU	Leucine aminopeptidase units
LOD	Limit of detection
LOQ	Limit of quantification
OPA	*ortho*-Phthalaldehyde
P278	Promod278
PepR	PeptidaseR
*p*NA	*para*-Nitroaniline
TEP	Technical enzyme preparation
UPLC	Ultra-performance liquid chromatography

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Introduction

The worldwide market offers various technical enzyme preparations (TEPs) and, due to their wide range of application (detergent, pharmaceutical, food and beverage industry),

new preparations are added continuously [1, 2]. The food and beverage industry covers the major part of the enzyme market and is expected to grow from nearly $ 1.5 billion in 2016 to $ 1.9 billion in 2021, at a compound annual growth rate (CAGR) of 4.7% [3], the Grand View Research forecasting even 10.9% from 2016 to 2024 [4]. Hydrolases (Enzyme Class 3) are the major class of enzymes which are used in industrial applications. Of them, peptidases (EC. 3.4) catalyze the hydrolysis of peptide bonds [5] and cover about one-third of the total enzyme market [4].

The TEPs currently used in the food industry are derived mainly from microorganisms, such as *Aspergillus* spp. [6–9] and *Bacillus* spp. [10–13]. The TEPs, produced either by solid state or submerged fermentation, are formulated to enable an easy application. The enzymes are usually stabilized by the addition of carbohydrates, such as sucrose, dextrose and sugar alcohols [14], and preserved by commonly adding sodium benzoate or potassium sorbate. Solid product forms are produced by spray-drying the mixtures of enzymes, stabilizers and preservatives [15]. According to the Food and Drug Administration, the enzyme preparations which are used in food processing contain one or several active enzymes that are responsible for the intended technical purpose in food production [16]. However, TEPs are standardized either on one specific or an unspecific peptidase activity. Flavourzyme1000L (Novozymes A/S, Denmark), which is standardized by leucine aminopeptidase units (LAPU) using the synthetic substrate Leu-*p*NA is exemplary for the first. For the second, Promod278 (Biocatalysts Ltd., UK), which is standardized by casein protease units using a natural substrate casein, is exemplary. The data sheets of the TEPs state side activities, such as endopeptidase or glutaminase activities, only in some cases. The TEPs generally contain several enzyme activities due to different degrees of complexity in the cultivation of microorganisms (e.g., media, process conditions), different types of formulations and to avoid high purification costs [17, 18]. Although TEPs have been described as containing several practice-relevant activities [19, 20], a reliable approach for the fast determination of important specific activities is missing. Due to this lack, TEPs are commonly chosen without detailed information about their process performance or are termed imprecisely as exopeptidases or endopeptidases [21–24].

In the present study, a fast measurement approach (< 1 h) was found to generate so-called activity fingerprints (AFPs) of TEPs. One AFP included 32 synthetic and natural substrates to determine specific peptidase activities of TEPs. Detailed and comprehensive information on the composition and proteolytic potential (endo- and exopeptidase activities) of a particular TEP was generated from this. Furthermore, batch hydrolyses of lupine protein were subsequently performed and the degree of hydrolysis (DH) and the free amino acid profiles of the hydrolysates were analyzed. Lupine protein was chosen due to its good solubility and high content of essential amino acids [25].

Materials and methods

Chemicals and substrates

All chemicals were of analytical grade and were obtained from Merck Chemicals GmbH (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen, Germany). All *para*-nitroanilide (*p*NA) substrates were obtained from Bachem (Bubendorf, Switzerland). Soluble lupine protein isolate, which is derived from the seeds of blue lupins (*Lupinus angustifolius*), was obtained from Prolupin GmbH (Grimmen, Germany). The lupine protein isolate consists of 87–95% protein, < 7% ash, < 3% fat and < 1.5% fibers, according to the supplier's information.

Technical enzyme preparations

The technical enzyme preparation (TEP) DeltazymAPS-M-FG originally derives from *A. niger* and was obtained from WeissBio Tech GmbH (Ascheberg, Germany). The TEPs Flavourzyme1000L and Alcalase2.4L were obtained from Novozymes (Bagsværd, Denmark) and derive from *A. oryzae* and *B. licheniformis*, respectively. Flavorpro750 MPD, Flavorpro766 MPD, Flavorpro839 MPD and Promod278 were obtained from Biocatalysts (Wales, UK). According to the supplier, Flavorpro750 MPD derives from *A. oryzae.* Flavorpro766 MPD and Flavorpro839 MPD are described as from "a mixed source" and "a microbial source". Promod278 derives from *B. subtilis*. The TEPs Peptidase R and Protease P "Amano" 6SD were obtained from Amano Enzyme Inc. (Nagoya, Japan) and originally derived from *R. oryzae* and *A. melleus,* respectively. FoodPro51 FP was obtained from Danisco, DuPont (Wilmington, Delaware, US) and derives from *A. oryzae*. Four batches of the TEP Flavourzyme1000L were investigated (provided by the Nestlé PTC Food, Singen). The batch numbers were HPN01005, HPN01010, HPN01011, HPN02003 with storage times (production date to the time of the investigation) of 18, 12, 12 and 8 months, respectively. All TEPs were stored at 7 °C and protected from light.

Determination of the enzyme activities by the novel measurement approach

The automated photometric analyzer Gallery™ Plus (Thermo Fischer Scientific, Waltham, MA, USA) is commonly used for routine analysis in the food and beverage industry. This system was used in this study to establish a methodology for the determination of the enzyme activities

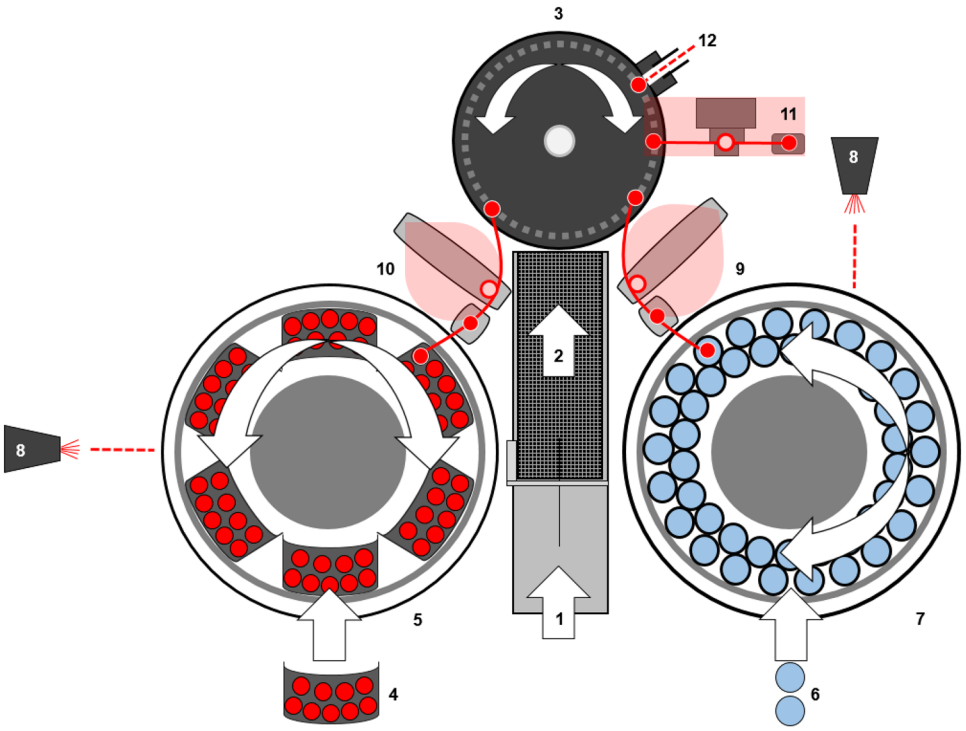


Fig. 1 The interior of the Gallery™ Plus Automated Photometric Analyzer [modified after Thermo Scientific]. (1) Cuvette entry point, (2) cuvette loader, (3) incubator, (4) sample racks, (5) sample disk, (6) reagents, (7) reagent disk, (8) barcode reader, (9) reagent dispenser, (10) sample dispenser, (11) mixer and (12) photometer unit

of TEPs. Due to the programming capacity of this system, two different photometric assays (*p*NA and *ortho*-phthalaldehyde: OPA) were implemented. As shown in Fig. 1, the system is set up with two segments, one for samples (left) and one cooled segment (10 °C) for reagents (right). The sample segment consists of six sub segments, each with nine sample positions (54 sample positions in total). The reagent segment consists of 42 reagent vials. Two independent pipettors (sample and reagent) transfer samples and reagents into the cuvettes, where the reaction takes place. The cuvettes are in an incubator for incubation and measurement to ensure a defined temperature. After finalizing the programmed assay, the absorption is measured at the wavelength defined. According to the substrate, specific calibrations were defined in the assay protocol (see Table 1 in the supporting information for more information). An algorithm was used within the assay protocol to adapt the dilution (of the TEP) for each substrate automatically depending on the respective enzyme activity. This automatic dilution ensured that the measured activity was within the linear range of the deposited calibration. The concentration of product formed during the enzymatic reaction was calculated considering the specific dilution factor. The values were then transferred into an excel data library, where the enzyme activities were calculated and are therefore presented in all AFPs in nkat mL^{-1} TEP.

A total of 32 substrates were selected, covering a wide range of different specific exo- and endopeptidase activities. Some have already been used for screening purposes and reported previously in literature [19, 20, 26]. The substrates were selected to cover four peptidase classes and the proteolytic activity towards lupine protein as a natural substrate: (1) aminopeptidases, (2) carboxy-/endopeptidases, (3) dipeptidyl peptidases, (4) endopeptidases and (5) proteolytic activity towards lupine protein. All available substrates (a total of 15) were selected to cover as many different (1) aminopeptidase activities as possible. The substrates (3) H-Ala-Pro-*p*NA and (3) H-Lys-Ala-*p*NA were selected to measure the activity of (3) dipeptidyl peptidases (DPP), for example, DPP2, DPP4, DPP5 [19, 27]. The substrates for the (2) carboxy-/endopeptidase activities included a protective group at the N- and C-terminal amino acids with different chemical properties (e.g., positively/negatively charged, aliphatic, aromatic) to distinguish the (2) carboxy-/endopeptidase activities from the (1) aminopeptidase activities. The terminology "carboxy-/endopeptidase activity" is used considering that endopeptidases might accept the carboxypeptidase substrates (although the substrates are protected from N-terminal digestion) and, thus, affect the carboxypeptidase activity results. The substrates for the (4) endopeptidase activities were selected to cover both specific activities, such as trypsin (using, e.g., Bz-Arg-*p*NA · HCl), and nonspecific activities, such as subtilisin (using, e.g., Z-Ala-Ala-Leu-*p*NA). Lupine protein was chosen to cover the (5) proteolytic activity towards a natural substrate.

Determination of the aminopeptidase, dipeptidyl peptidase and endopeptidase activities with *p*NA substrates

The exopeptidase (amino- and dipeptidyl peptidase) and endopeptidase activities were determined according to the method of Chrispeels and Boulter [28], with the following modifications. The assays were carried out using an automated photometric analyzer Gallery™ Plus (Thermo Fischer Scientific, Waltham, MA, USA) at 37 °C. A volume of 100 µL bis–tris-propane HCl (200 mM, pH 7.0) was mixed with 50 µL of *p*NA substrate and incubated for 60 s. A volume of 50 µL of diluted TEP sample (generally 10%, w/v for solid preparations, v/v for liquid preparations) was added and incubated for 600 s. The release of *p*NA was measured at 405 nm and 1 katal (kat) of peptidase activity was defined as the release of 1 mol *p*NA per second. Stock solutions of the respective *p*NA substrates were prepared in dimethylformamide with a concentration of 400 mM. The substrate solutions were dissolved in 10 mM HCl, or dimethylformamide to a final concentration of 4 mM or 40 mM, respectively, prior to the application. A detailed overview of the specific substrates and the solvent types are shown in Table 1 (supporting information). An overview of the pipetting scheme of the *p*NA assay using the programmed photometer is provided in Table 2 the supporting information section. The blank was measured using purified water (H_2O_{dd}) and performed in the same way as the pipetting scheme of the activity assay (Table 2 supporting information).

Determination of the carboxy-/endopeptidase activity and the proteolytic activity towards lupine protein after derivatization with *ortho*-phthalaldehyde

The activity of the carboxy-/endopeptidases and the proteolytic activity towards lupine protein were measured indirectly by determining the generation of amino groups after derivatization with OPA. The method of Nielsen et al. [29] was modified as follows. The assays were carried out using an automated photometric analyzer Gallery™ Plus (Thermo Fischer Scientific, Waltham, MA, USA). A volume of 20 µL bis–tris-propane HCl (200 mM, pH 7.0) was mixed with 10 µL of substrate and incubated for 60 s. A volume of 10 µL of diluted TEP (generally 10%, w/v for solid preparations, v/v for liquid preparations) was added and incubated for 600 s. A volume of 240 µL OPA Reagent (OPA Solution Complete, Sigma-Aldrich, Taufkirchen, Germany) was added, incubated for 30 s and the absorption was measured at 340 nm. An overview of the pipetting scheme of the OPA assay using the programmed photometer is provided in Table 3 (supporting information). The blank was measured using purified water (H_2O_{dd}) and in the same way as the pipetting scheme of the activity assay (Table 3 supporting information).

The carboxy-/endopeptidase activities were measured at 37 °C using the synthetic substrates Z-Ala-Gly-OH, Z-Ala-Glu-OH, Z-Gly-Phe-OH, Z-Ala-Lys-OH, Z-Ala-Ser-OH and Z-Ala-Leu-OH. Further information concerning the specific substrates and the solvent types are shown in Table 1 in the supporting information section. L-glycine, L-glutamic acid, L-phenylalanine, L-lysine, L-serine and L-leucine, were used for the calibration, respectively. One katal (kat) of peptidase activity was defined as the release of 1 mol L-amino acid equivalents per s. The proteolytic activity on the natural lupine protein was measured using L-serine as a reference. One katal (kat) of peptidase activity was defined as the release of 1 mol L-serine equivalents per second.

Batch hydrolyses of lupine protein

Setup of lupine protein batch hydrolyses

Batch hydrolyses of lupine protein were performed with several commercially available TEPs in 40-mL scale in Falcon tubes. The final substrate concentration was 10% (w/v) lupine protein isolate, suspended in bis–tris-propane HCl buffer (100 mM, pH 7.0). The final TEP concentration was 6% (v/v) or (w/v), depending on the type of formulation (liquid or solid) of the TEP in order to ensure equilibrium after 8 h of hydrolysis. The hydrolyses were performed at 37 °C and 850 rpm by using ThemoMixerC (Eppendorf, Hamburg, Germany). Samples of 800 µL were taken after various times and transferred into Eppendorf tubes containing 200 µL trichloroacetic acid (2.5 M) to terminate the reaction. The samples were shaken at 3000 min^{-1} for 3 s using a universal shaker (MS3basic, IKA®, Staufen, Germany) and centrifuged at 14,100 rcf for 3 min (MiniSpinPlus, Eppendorf, Hamburg, Germany). The samples were diluted as required to determine the DH (see "Manual determination of the degree of hydrolysis with *ortho*-phthalaldehyde in batch hydrolysates") or analyze the free amino acid profile via ultra-performance liquid chromatography (UPLC) (see "Ultra-performance liquid chromatography analysis for the free amino acid profiles of the lupine protein hydrolysates"). As a reference, the substrate suspension without TEP was treated and measured in the same way as the sample suspensions with TEP.

Both the substrate for the hydrolyses (lupine protein) and the TEPs themselves were analyzed for their DH and free amino acids and subtracted from the batch hydrolysate samples. Consequently, only the product release caused by the enzymatic liberation was captured. Total free amino acid concentration of the TEPs ranged between 0.02 and 0.2 g 100 g^{-1} and the total free amino acid concentration of the substrate suspension (lupine protein) was 0.1 g 100 g^{-1}.

Manual determination of the degree of hydrolysis with *ortho*-phthalaldehyde in batch hydrolysates

Primary amino groups were determined after derivatization with OPA, according to the method of Nielsen et al. [29], with some modifications. A sample volume of 25 µL was transferred into a microtiter plate and 175 µL OPA Reagent (OPA Solution Complete, Sigma-Aldrich, Taufkirchen, Germany) was added. The plate was incubated at 24 °C for 10 s and shaken for 10 s. The absorbance was measured at 340 nm using a UV–Vis multimode microplate reader (SpectraMaxM5, Molecular Devices, Sunnyvale CA, USA). L-serine was used as a reference for the calibration.

The DH was calculated according to the description of Adler-Nissen [30], with modifications [31]. The following equation (Eq. 1) describes the DH as the percentage of the concentration of free amino groups h (mol L^{-1}) compared to the maximum concentration of free amino acids at complete hydrolysis h_{tot} (mol L^{-1}):

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100\ (\%). \tag{1}$$

The maximum concentration of free amino acids at complete hydrolysis (Eq. 2) is described as the protein concentration that is hydrolyzed $c_{protein}$ (g L^{-1}) divided by the difference of the average molecular mass of the amino acids in lupine protein M^* (137.9 g mol^{-1}) and the molecular mass of water MH_2O (18.0 g mol^{-1}). The lupine protein-specific average molecular mass was calculated by considering the lupine protein amino acid composition [25]. The molecular mass of water was subtracted, since water is added during the hydrolysis of a peptide bond.

$$h_{\mathrm{tot}} = \frac{c_{\mathrm{protein}}}{(M^* - M_{\mathrm{H_2O}})} (\mathrm{mol\ L^{-1}}). \tag{2}$$

Ultra-performance liquid chromatography analysis for the free amino acid profiles of the lupine protein hydrolysates

The free amino acids of the lupine protein hydrolysates were determined by UPLC, as described previously [32]. An Acquity® UPLC H-Class System (Waters, Milford, USA) was used equipped with a quaternary solvent manager (QSM), a sample manager with Flow-Through Needle (FTN), a column heater (CH-A) and a photodiode array (PDA) detector. A Waters AccQ·Tag™ Ultra RP Column, Acquity® UPLC Ethylene-Bridged-Hybrid (BEH) C18 (pore diameter 130 Å, particle size 1.7 µm, inner diameter 2.1 mm, length 100 mm) column was used. Norvaline (Sigma-Aldrich, Taufkirchen, Germany) was used as an internal standard with a concentration of 25 µmol L^{-1} (1:100 stock solution 2.5 M in 0.1 M HCl). The column temperature was 43 °C and the sample temperature was 20 °C. The injection volume was 100 µL. The eluent gradient, the flow rate (0.7 mL min^{-1}) and the detection wavelength (260 nm) were applied according to the recommendations of Waters Corporation [33].

Statistical analysis

The samples of the batch hydrolyses were measured in duplicate using two independent measurements. The data were evaluated with the standard deviation calculated with Excel (Microsoft, Redmond, USA).

The measurements for the AFPs of the TEPs were performed in single determination due to economic reasons and screening intentions. The AFP measurements of Flavourzyme1000L batch HPN02003 were performed exemplarily in triplicate to show the deviation of the assays in the automated screening system. Figure 1 (supporting information) shows the coefficients of variation (CV) of the 32 substrates. The measurements using the substrates Z-Asn-*p*NA, Bz-Phe-Val-Arg-*p*NA, Z-Ala-Lys-OH, lupine and Z-Ala-Ser-OH showed CVs above 15%. The CV for the substrate Z-Asn-*p*NA above 15% was negligible since no enzyme activity was measured. The CVs regarding the substrates Z-Ala-Ser-OH, Z-Ala-Lys-OH and Bz-Phe-Val-Arg-*p*NA, and the natural lupine protein was due to poor solubility of the substrates in the assay. Furthermore, the small volumes of the pipetting scheme of the OPA assay, which were due to the setup limitations of the Gallery™, could have contributed to the CVs. The limit of detection (LOD; defined as 3 × standard deviation of the blank) and the limit of quantification (LOQ; defined as 10 × standard deviation of the blank) values for the enzyme activities in the AFPs were calculated for each assay and can be found in Table 4 in the supporting information.

Results and discussion

Technical enzyme preparations are used in a wide range of applications in the food industry and usually consist of enzyme(s), stabilizers and preservatives. The knowledge about the TEPs' substrate spectrum and potential side activities is limited but crucial for their selection for process application. Technical peptidase preparations are especially known to present more than one kind of enzyme activity. Therefore, a fast system was established to create AFPs of TEPs to provide detailed information of the substrate spectrum and activity profiles from technical peptidase preparations. The automated photometric analyzer Gallery™ Plus (Thermo Fischer Scientific, Waltham, MA, USA) is generally used for routine analysis in the food and beverage

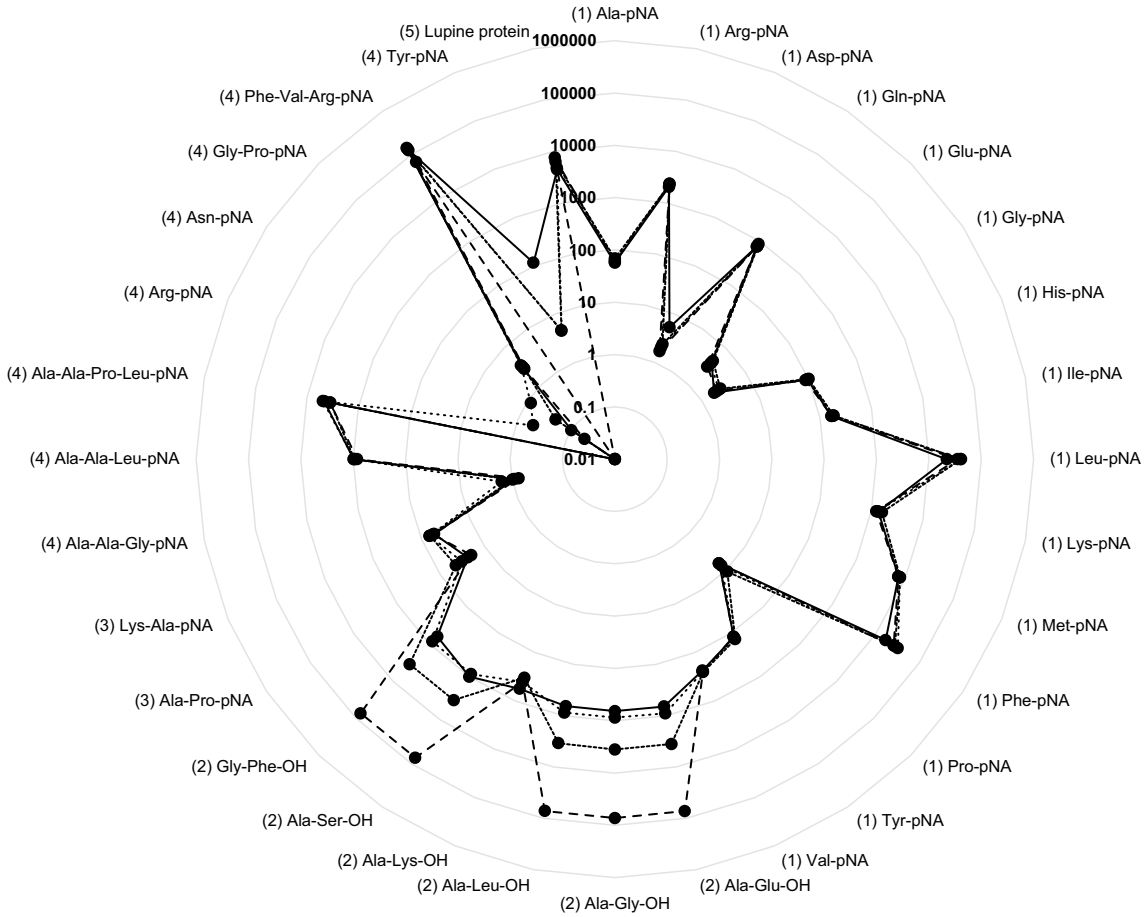


Fig. 2 Activity fingerprints (AFPs) of specific peptidase activities of the technical enzyme preparation (TEP) Flavourzyme1000L batch HPN01005 (long dashes), batch HPN01011 (short dashes), batch HPN01010 (dotted dash) and batch HPN02003 (solid line) with storage times of 18, 12, 12 and 8 months, respectively. The numbers represent clusters of (1) aminopeptidase activities, (2) carboxy-/endopeptidase activities, (3) dipeptidyl peptidase activities, (4) endopeptidase activities and (5) proteolytic activity towards lupine protein. The values represent the enzyme activity in nkat mL^{-1} TEP (logarithmic scale), measured at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM), substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein. Refer to "Statistical analysis" for statistical information

industry. In the present study, the Gallery™ Plus was used to establish a methodology for the determination of enzyme activities of TEPs. The methodology established delivers a fast approach (32 assays are performed in less than 1 h, depending on the number of dilutions) to evaluate TEPs comprehensively for their variety of activities while measuring both exo- and endopeptidase activities. According to our experiences, the automated system is approximately 6–8 times faster than the manual measurement of all activities (32 substrates). Please refer to the description of the measurement approach in "Determination of the enzyme activities by the novel measurement approach" for detailed information concerning the Gallery™ setup. By performing batch hydrolysis of lupine protein, it was further demonstrated that the information generated based on the AFPs can be used to select TEPs specifically for hydrolysis and to combine TEPs according to process targets (e.g., DH, free amino acids).

Activity fingerprints for the characterization of technical enzyme preparations

The AFPs of different batches of the TEP Flavourzyme1000L were created by implementing the novel measurement approach and the results are shown in Fig. 2. The peptidase activities are placed in five different groups referring to (1) aminopeptidase activity, (2) carboxy-/endopeptidase activity, (3) dipeptidyl peptidase activity, (4) endopeptidase activity and (5) the proteolytic activity towards lupine protein as a natural substrate. The activities are displayed in nkat mL TEP^{-1} in logarithmic scale ranging from 10 pkat mL^{-1} to 1 mkat mL^{-1}. The current values for the enzyme activities were additionally provided in Table 5 (supporting information) for the sake of completeness. Please refer to the statistical analysis Sect. 2.5 for the LOD and LOQ values. Flavourzyme1000L exhibited

Table 1 General information of three commercially available technical enzyme preparations (TEPs): Flavorpro750 MPD, Flavorpro766 MPD and Flavorpro839 MPD (Biocatalysts)

Name	Activity	Source	Format	pH_{range}	T_{range}	Description
Flavorpro750 MPD	55 CPU g^{-1}[a]	*Aspergillus* spp.	Brown powder	5.5–7.5	45–55 °C	Casein protease used for the production of superior tasting and non-bitter whey protein hydrolysates
Flavorpro766 MPD	203 LAPU g^{-1}[b]	Mixed source	Beige powder	5.0–7.5	45–55 °C	Protease designed for extensive hydrolysis of wheat gluten—high level of exopeptidase activity—smooth, non-bitter flavor
Flavorpro839 MPD	250 LAPU g^{-1}[b]	Microbial	Brown powder	6.0–7.0	45–55 °C	Protease preparation containing endopeptidase and exopeptidase activities—to hydrolyze proteins such as meat and fish, to create specific flavors

[a]Casein protease units per gram protein

[b]Leucine aminopeptidase units per gram protein

activities on all 15 (1) aminopeptidase substrates with its leading activity towards (1) H-Leu-*p*NA, as is declared by the supplier. The activities towards (1) H-Leu-*p*NA were 35 µkat mL^{-1}, 41 µkat mL^{-1}, 41 µkat mL^{-1} and 22 µkat mL^{-1} for the Flavourzyme1000L batches HPN01005, HPN01010, HPN01011 and HPN02003, respectively. Similar values of 20.3 ± 0.4 µkat mL^{-1} (37 °C, pH 7.5) have been reported and accounted to 1102 ± 9 LAPU g^{-1} (37 °C, pH 8.0, 0.2 mM $ZnCl_2$) for a comparison with the manufacturer's specification of 1000 LAPU g^{-1} [34].

Apart from this main activity (LAPU g^{-1}), the AFPs of the Flavouryme1000L batches showed variations regarding all (2) carboxy-/endopeptidase activities, for example, towards (2) Z-Ala-Glu-OH, ranging from 0.7 (HPN02003) to 72 µkat mL^{-1} (HPN01005). Further variations were measured regarding (4) endopeptidase activities, for example, towards (4) Bz-Arg-*p*NA and (4) Bz-Tyr-*p*NA, referring to trypsin-like and chymotrypsin activities as described in the literature, respectively [35, 36]. Batch-to-batch variations have been reported previously but only investigating the endopeptidase activity using azocasein as a substrate [34]. The novel measurement approach presented in this study generated activity patterns (AFPs) of TEPs with more precise information on specific peptidase activities (exo- and endopeptidase activities) and illustrates more clearly, therefore, specific peptidase side activities and batch variations (e.g., in the case of Flavourzyme1000L, several (2) carboxy-/endopeptidase and (4) endopeptidase activities).

Activity fingerprints for the specific selection of technical enzyme preparations

Due to the increasing number of enzyme preparations on the market, their appropriate selection has gained in importance [3, 4]. Table 1 shows an example of the information provided concerning three commercially available TEPs: Flavorpro750 MPD, Flavorpro766 MPD and Flavorpro839 MPD. These three TEPs show apparent differences in their specified activities, sources and pH-dependent activity and are presented differently in their general descriptions. The respective AFPs of these TEPs, investigated by the novel measurement approach, illustrated the peptidase activity patterns (Fig. 3). Despite small quantitative differences, the three TEPs displayed similar activity patterns under the conditions tested. All three TEPs contained several exopeptidase and endopeptidase activities in similar activity ranges (see Table 5, supporting information for the current values). The information generated by the measurement approach delivered a detailed and comprehensive description of the TEPs' activities. Therefore, the AFPs help to choose appropriate TEPs for application and minimize the number of experiments using only preselected TEPs. As an example, if different protein hydrolysates are targeted, it is of advantage to also choose TEPs with different AFPs.

Usage of the information generated by the activity fingerprints of technical enzyme preparations to influence the resulting lupine protein hydrolysates predictably

The AFPs of the TEPs Flavourzyme1000L and Alcalase2.4L are presented in Fig. 4a. Although Flavourzyme1000L has been investigated intensively in literature [19], further information on substrate specificity was obtained with the AFPs in this study. Alcalase2.4L exhibited negligible or low (1) exopeptidase activities, but noticeable (2) carboxy-/endopeptidase and (4) endopeptidase activities. Low aminopeptidase activity and mainly endopeptidase activity of Alcalase2.4L have been reported previously [37]. In this study, both AFPs of Flavourzyme1000L and Alcalase2.4L delivered more detailed information on their activity profiles

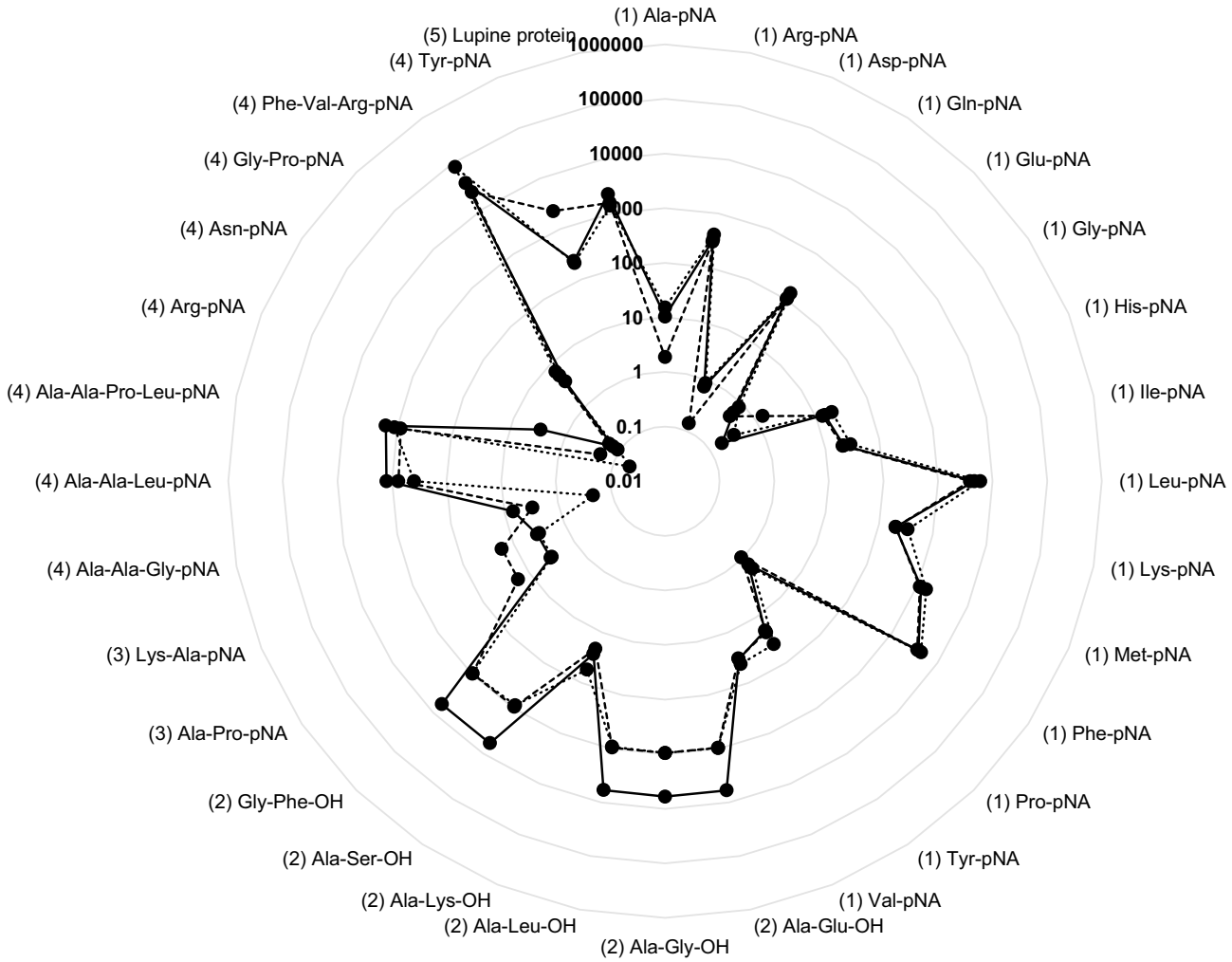


Fig. 3 The AFPs of specific peptidase activities of the three TEPs Flavorpro750 MPD (dotted dash), Flavorpro766 MPD (short dashes) and Falvorpro839 MDP (solid line). The numbers represent clusters for (1) aminopeptidase activities, (2) carboxy-/endopeptidase activities, (3) dipeptidyl peptidase activities, (4) endopeptidase activities and (5) proteolytic activity towards lupine protein. The values represent the enzyme activity in nkat mL^{-1} TEP (logarithmic scale), measured at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM), substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein. Refer to "Statistical analysis" for statistical information

than has been published previously. The current values of the enzyme activities are provided in Table 6 in the supporting information section.

Batch hydrolyses of lupine protein were conducted to establish a relation between the information of the AFPs and the performance of TEPs in protein hydrolysis. The lupine protein hydrolyses were carried out with [1] Flavourzyme1000L, [2] Alcalase2.4L and [3] a combination of Flavourzyme1000L and Alcalase2.4L in a ratio of 1:1 (Fig. 4b). The hydrolysis using Flavourzyme1000L resulted in a degree of hydrolysis of 52.62 ± 0.14% after 8 h (DH_{8h}), which could be explained by the complex mixture of exo- and endopeptidase activities of Flavourzyme1000L. The hydrolysis with Alcalase2.4L, containing fewer exopeptidase activities and mainly endopeptidase activities (see AFP of Alcalase2.4L, Fig. 4a), resulted in a DH_{8h} of 14.70 ± 0.22% (Fig. 4b). The combination of Flavourzyme1000L and Alcalase2.4L has been reported to increase the DH using potato protein and anchovy powder [21, 22, 38]. However, due to the information generated from the AFPs in this study, a combination of Flavourzyme1000L and Alcalase2.4L was not expected to increase the DH_{8h}. This was confirmed by the DH_{8h} of the TEP combination with 53.35 ± 0.73%, showing no significant increase compared to DH_{8h} = 52.62 ± 0.14% using Flavourzyme1000L only.

The lupine protein hydrolysates were analyzed for their free amino acid profiles by UPLC (Fig. 4c) to investigate the coherence of the information of the AFPs regarding the release of free amino acids during hydrolysis. Several free amino acids were generated which can be related to the exopeptidase activities shown in the AFP of Flavourzyme1000L (Fig. 4a). By contrast, the hydrolysates of Alcalase2.4L showed negligible

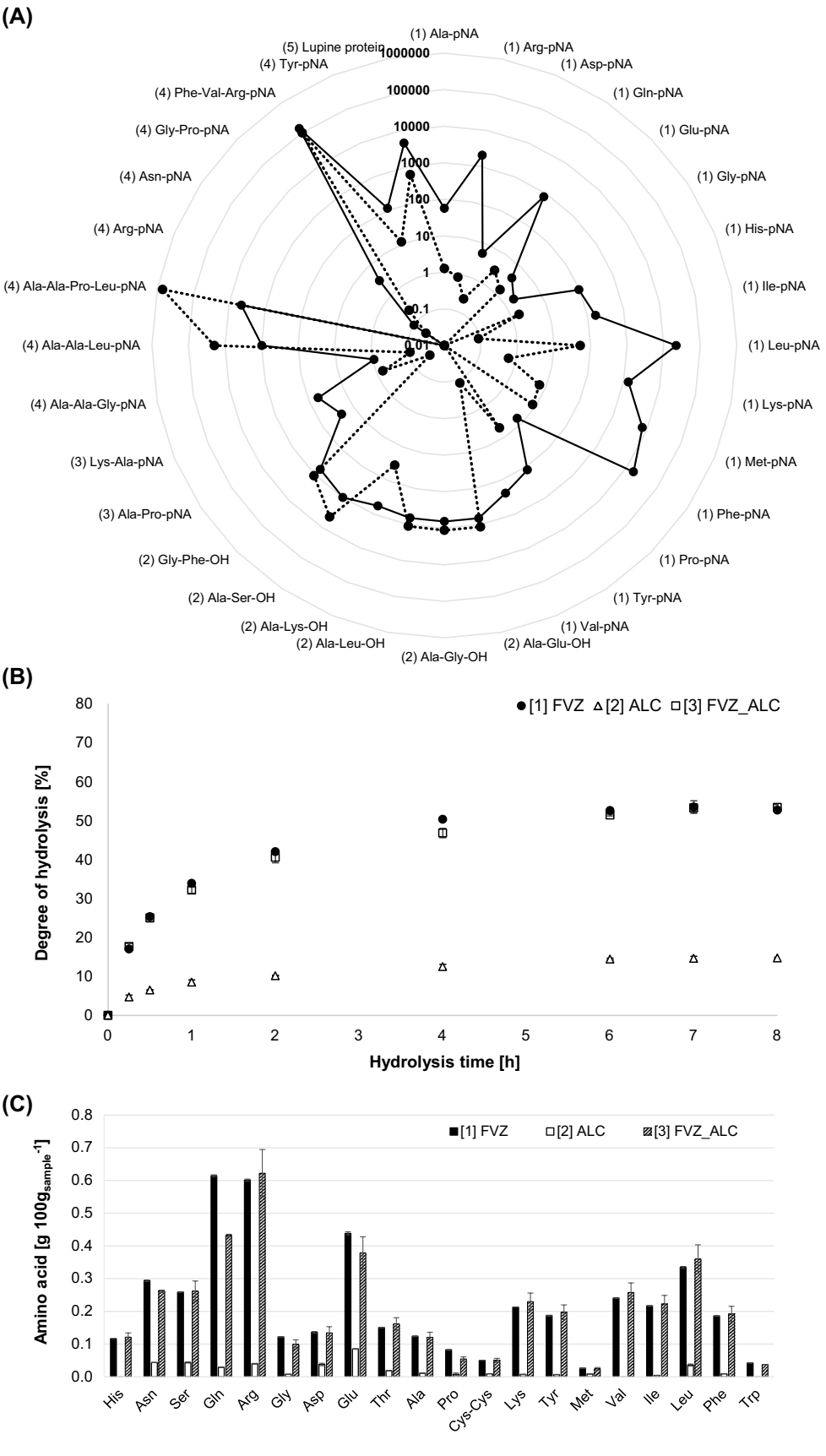
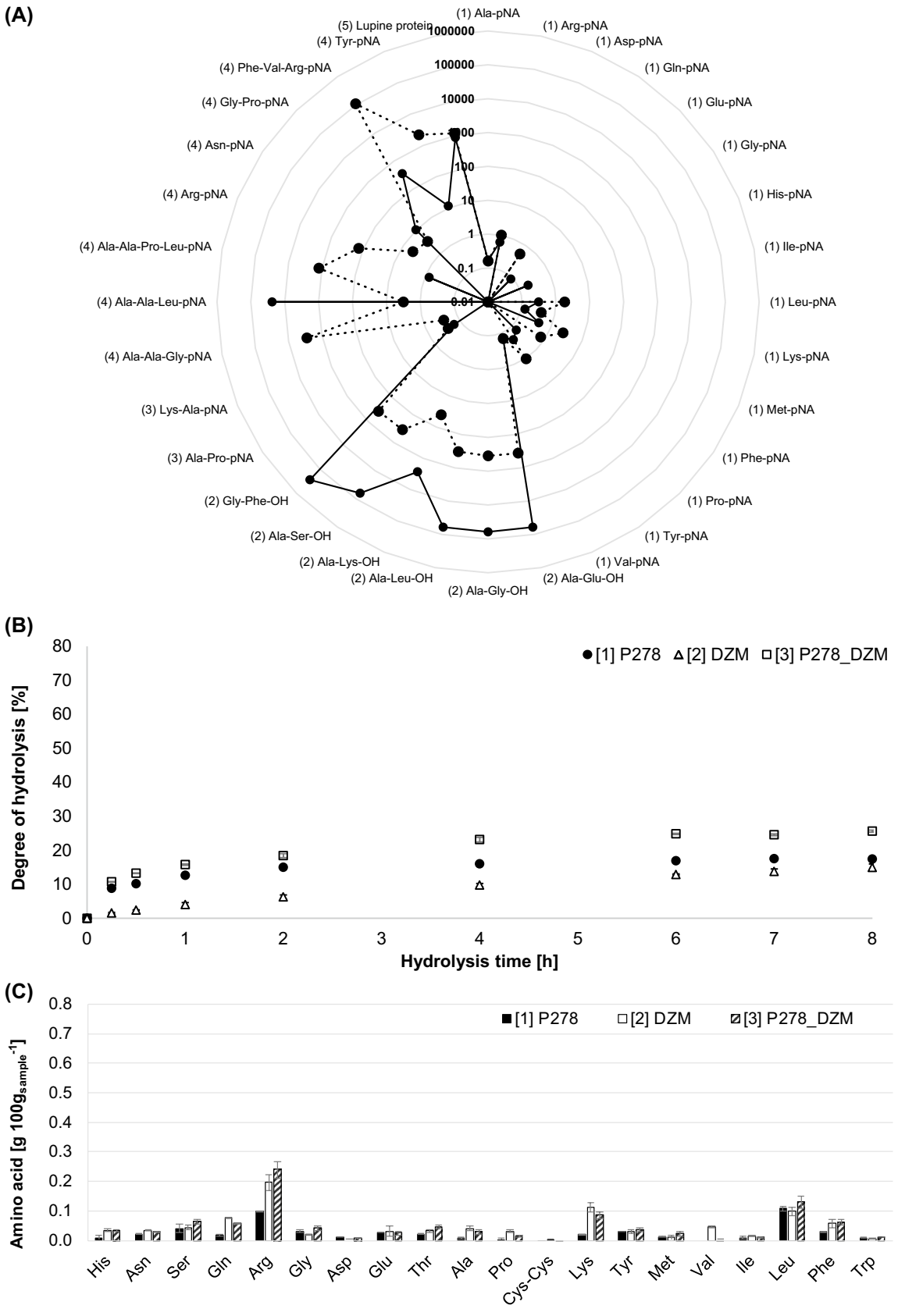


Fig. 4 **a** The AFPs of specific peptidase activities of Flavourzyme1000L (FVZ, solid line) and Alcalase2.4L (ALC, dotted dash). The values represent the enzyme activity in nkat mL^{-1} TEP (logarithmic scale), measured at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM), substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein. **b** Batch hydrolysis of 10% (w/v) lupine protein with 6% (v/v) FVZ (filled circles), 6% (v/v) ALC (unfilled triangles) and a combination of 3% (v/v) FVZ and 3% (v/v) ALC (unfilled squares) at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM). **c** Free amino acid profiles of lupine protein hydrolysates using [1] FVZ (black bars), [2] ALC (white bars) and [3] a combination of FVZ and ALC (shaded bars), measured by ultra-performance liquid chromatography (UPLC). The reference values of the substrate and the TEPs were subtracted to capture the values only due to enzymatic liberation during hydrolysis. Refer to "Statistical analysis" for statistical information


(A)
(1) Ala-pNA
(1) Arg-pNA
(1) Asp-pNA
(1) Gln-pNA
(1) Glu-pNA
(1) Gly-pNA
(1) His-pNA
(1) Ile-pNA
(1) Leu-pNA
(1) Lys-pNA
(1) Met-pNA
(1) Phe-pNA
(1) Pro-pNA
(1) Tyr-pNA
(1) Val-pNA
(2) Ala-Glu-OH
(2) Ala-Gly-OH
(2) Ala-Leu-OH
(2) Ala-Lys-OH
(2) Ala-Ser-OH
(2) Gly-Phe-OH
(3) Ala-Pro-pNA
(3) Lys-Ala-pNA
(4) Ala-Ala-Gly-pNA
(4) Ala-Ala-Leu-pNA
(4) Ala-Ala-Pro-Leu-pNA
(4) Arg-pNA
(4) Asn-pNA
(4) Gly-Pro-pNA
(4) Phe-Val-Arg-pNA
(4) Tyr-pNA
(5) Lupine protein
1000000
100000
10000
1000
100
10
1
0.1
0.01
(B)
[1] P278
[2] DZM
[3] P278_DZM
Degree of hydrolysis [%]
Hydrolysis time [h]
(C)
[1] P278
[2] DZM
[3] P278_DZM
Amino acid [g 100g$_{sample}^{-1}$]
His
Asn
Ser
Gln
Arg
Gly
Asp
Glu
Thr
Ala
Pro
Cys-Cys
Lys
Tyr
Met
Val
Ile
Leu
Phe
Trp

◂**Fig. 5** **a** The AFPs of specific peptidase activities of Promod278 (P278, dotted dash) and of DeltazymAPS-M-FG (DZM, solid dash). The values represent the enzyme activity in nkat mL^{-1} TEP (logarithmic scale), measured at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM), substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein. **b** Batch hydrolysis of 10% (w/v) lupine protein with 6% (w/v) P278 (filled circles), 6% (w/v) DZM (unfilled triangles) and a combination of 3% (w/v) P278 and 3% (w/v) DZM (unfilled squares) at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM). **c** Free amino acid profiles of lupine protein hydrolysates using [1] P278 (black bars), [2] DZM (white bars) and [3] a combination of P278 and DZM (shaded bars), measured by UPLC. The reference values of the substrate and the TEPs were subtracted to capture the values only due to enzymatic liberation during hydrolysis. Refer to "Statistical analysis" for statistical information

amounts of free amino acids, which can be explained by the lack of exopeptidase activities (AFP of Alcalase2.4L, Fig. 4a). The combination of Flavourzyme1000L and Alcalase2.4L did not increase the release of free amino acids, but showed similar values as was achieved with Flavourzyme1000L on its own.

These findings demonstrated that the AFPs delivered valuable information about the TEPs' characteristics, which could be transferred to their hydrolysis characteristics. Consequently, TEPs for protein hydrolysis could be selected more rationally by their AFPs, rather than based on the general information provided by the suppliers.

Advanced hydrolysis by combination of technical enzyme preparations based on their activity fingerprints

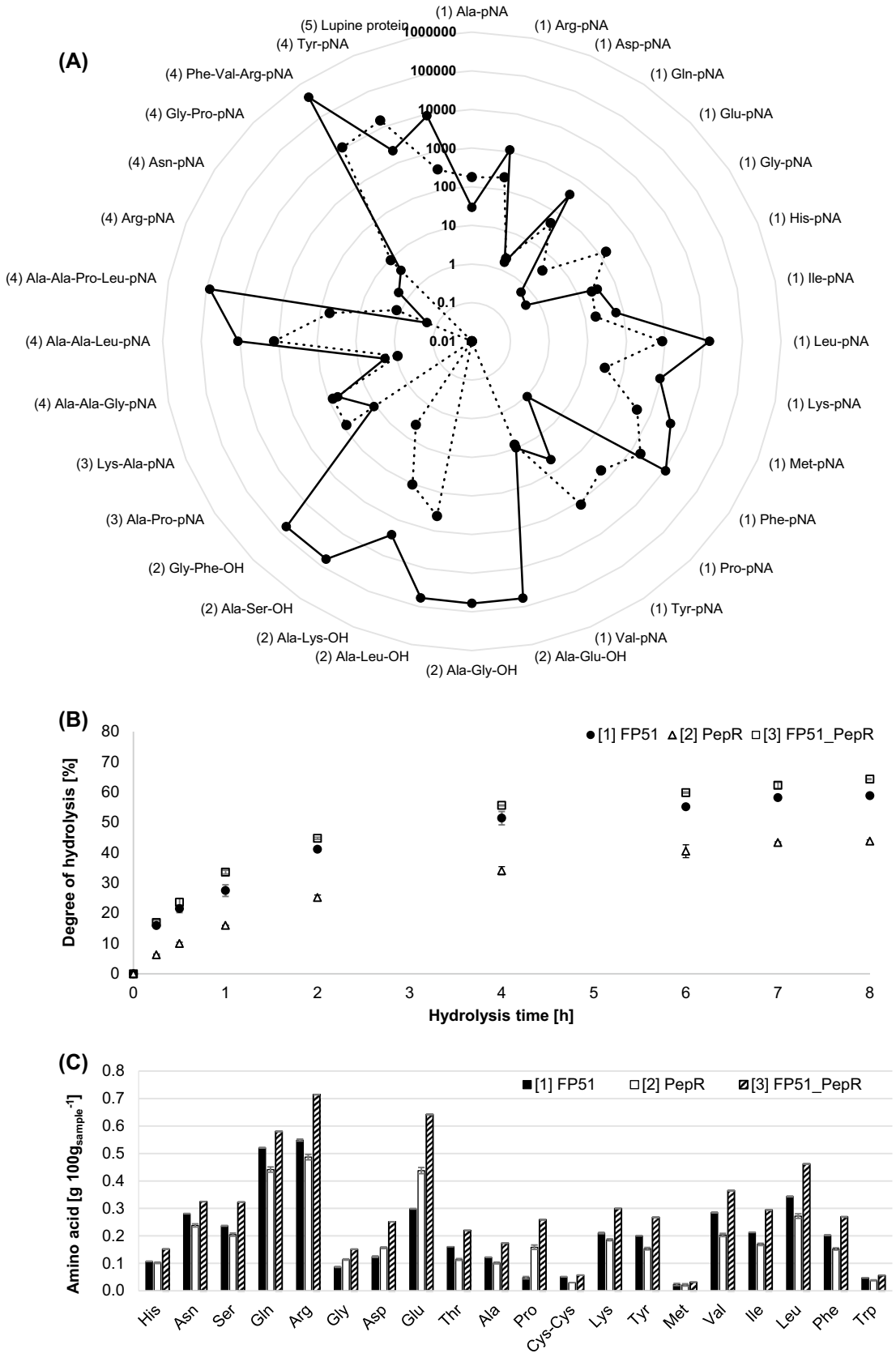
Based on the assumption that a hydrolysate composition might be predicted from the AFPs, it was, furthermore, investigated whether the DH could be improved by the selection and combination of TEPs with complementary activities. Therefore, TEPs with different peptidase activity patterns were chosen to perform batch hydrolysis of lupine protein. The TEPs were selected based on the complementary activities shown in their AFPs (see Table 6, supporting information for the current enzyme activity values). In the first example, P278 and DZM were combined based on complementary (4) endopeptidase activities. In the second example, FP51 and PepR were combined based on complementary (1) exopeptidase activities. The respective Figs. 5 and 6 were set up the same as Fig. 4, showing the AFPs of the TEPs used [A], the DH during lupine protein hydrolysis [B] and the analysis of the free amino acids of the resulting lupine protein hydrolysates after 8 h process time [C]. A random regression forest was applied to relate the single activities to the values of DH. Due to the high complexibility, no clear correlation was observed in this point. Nevertheless, a relationship between the activities of the peptidase classes and the values for DH were observed.

Combination of two technical enzyme preparations with mainly endopeptidase activities

Both AFPs of the TEPs Promod278 (P278) and DeltazymAPS-M-FG (DZM) exhibited mainly (2) carboxy-/endopeptidase and (4) endopeptidase activities and were complementary in some of the activities (see Fig. 5a). The DZM displayed higher activities for the (2) carboxy-/endopeptidase activities and higher activities for the remaining (4) endopeptidase activities towards (4) Z-Ala-Ala-Leu-*p*NA and (4) Z-Gly-Pro-*p*NA, P278. The hydrolysis of lupine protein using P278 resulted in a DH_{8h} of 17.42 ± 0.23% and a DH_{8h} of 14.94 ± 1.03% using DZM (Fig. 5b). The combination of P278 and DZM increased the DH_{8h} to 25.62 ± 0.31%, which is an overall increase of 47%. Due to the lack of exopeptidase activities for both P278 and DZM (see AFPs, Fig. 5a), the generation of free amino acids was negligible (Fig. 5c). The rational combination of two complementary TEPs containing mainly (2) carboxy-/endopeptidase and (4) endopeptidase activities has been shown to improve the hydrolysis efficiency (Fig. 5b) without generating more free amino acids (Fig. 5c). Free amino acids might not be desired in some applications (e.g., milk hydrolysates for hypoallergenic products) since they have been reported to have an impact on flavor [39–41].

Combination of two complex technical enzyme preparations containing exopeptidase and endopeptidase activities

The AFPs of FP51 and PepR both showed several exo- and endopeptidase activities (Fig. 6a) and were chosen because of their complementary exopeptidase activities. PepR displayed higher activities towards (1) aminopeptidase activities, such as (1) H-Ala-*p*NA, (1) H-Glu-*p*NA, (1) H-Gly-*p*NA, (1) H-Pro-*p*NA and (1) H-Tyr-*p*NA. By contrast, FP51 displayed higher activities towards the other ten (1) aminopeptidase substrates. The hydrolysis of lupine protein (Fig. 6b) resulted in a DH_{8h} of 43.83 ± 0.45% and 58.84 ± 0.32% for PepR and FP51, respectively. The increased DH_{8h} of FP51 could be explained by the higher absolute activities towards a wider substrate specificity in comparison to PepR. However, the combination of FP51 and PepR resulted in an increase of DH_{8h} to 64.33 ± 0.14%. This total increase of 9% might be explained by the complementary exopeptidase activities (Fig. 6a). Since both TEPs exhibited exopeptidase activities, free amino acids were generated during hydrolyses using FP51 and PepR (Fig. 6c). Tendencies for a correlation between the activities of the AFP and the generation of free amino acids could be drawn. The aminopeptidase activity indicated tendencies to correlate with the amount of its respective amino acid in the hydrolysate in 12 out of 15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(1) Ile-pNA
(1) Leu-pNA
(1) Lys-pNA
(1) Met-pNA
(1) Phe-pNA
(1) Pro-pNA
(1) Tyr-pNA
(1) Val-pNA
(2) Ala-Glu-OH
(2) Ala-Gly-OH
(2) Ala-Leu-OH
(2) Ala-Lys-OH
(2) Ala-Ser-OH
(2) Gly-Phe-OH
(3) Ala-Pro-pNA
(3) Lys-Ala-pNA
(4) Ala-Ala-Gly-pNA
(4) Ala-Ala-Leu-pNA
(4) Ala-Ala-Pro-Leu-pNA
(4) Arg-pNA
(4) Asn-pNA
(4) Gly-Pro-pNA
(4) Phe-Val-Arg-pNA
(4) Tyr-pNA
(5) Lupine protein
1000000
100000
10000
1000
100
10
1
0.1
0.01
(B)
[1] FP51 [2] PepR [3] FP51_PepR
Degree of hydrolysis [%]
Hydrolysis time [h]
(C)
[1] FP51 [2] PepR [3] FP51_PepR
Amino acid [g 100g$_{sample}^{-1}$]
His Asn Ser Gln Arg Gly Asp Glu Thr Ala Pro Cys-Cys Lys Tyr Met Val Ile Leu Phe Trp

◂**Fig. 6** **a** The AFPs of specific peptidase activities of FoodPro51FP (FP51, solid dash) and of PeptidaseR (PepR, dotted dash). The values represent the enzyme activity in nkat mL^{-1} TEP (logarithmic scale), measured at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM), substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein. **b** Batch hydrolysis of 10% (w/v) lupine protein with 6% (w/v) FP51 (filled circles), 6% (w/v) PepR (unfilled triangles) and a combination of 3% (w/v) FP51 and 3% (w/v) PepR (unfilled squares) at 37 °C, pH 7.0 (bis–tris-propane HCl, 100 mM). **c** Free amino acid profiles of lupine protein hydrolysates using FP51 (black bars), PepR (white bars) and a combination of FP51 and PepR (shaded bars), measured by UPLC. The reference values of the substrate and the TEPs were subtracted to capture the values only due to enzymatic liberation during hydrolysis. Refer to "Statistical analysis" for statistical information

cases. FP51, for example, exhibited a higher activity towards H-Leu-*p*NA (AFP, Fig. 6a) and resulted in a higher release of free leucine (Fig. 6c). PepR exhibited a higher activity towards H-Pro-*p*NA and resulted in a higher release of free proline. These findings showed that the process efficiency of the hydrolysis was increased by choosing the TEP based on its AFP. Furthermore, the hydrolysis could be guided to release specific amino acids of interest, for example, increase the amount of dietary-essential amino acids.

Conclusions

In this study, a reasonable fast approach was presented to comprehensively evaluate TEPs for an efficient protein hydrolysis at pH 7 and 37 °C. However, the assay conditions can be adapted if other process parameter are targeted, leading to respective results. By implementing the novel measurement approach, AFPs of TEPs were created delivering detailed information on substrate specificity and peptidase activities towards 32 synthetic substrates and one natural substrate: 15 aminopeptidase activities, six carboxy-/endopeptidase activities, two dipeptidyl peptidase activities, eight endopeptidase activities and one proteolytic activity towards lupine protein as a natural substrate. Depending on the TEP, the measurement of an AFP could be performed in less than 1 h and 6–8 times faster than the manual determination (according to our experience). It was shown that the novel information generated by the AFPs increased the knowledge regarding the substrate specificity and peptidase side activities (including batch variations) of the TEPs to choose predictably the most suitable TEP for a targeted protein hydrolysate. The information generated by the AFPs was used to anticipate the outcome of a lupine protein hydrolysis and the generation of free amino acids. Consequently, TEPs were applied more systematically to protein hydrolysis based on their AFP. Thus, a more efficient hydrolysis process was achieved (e.g., DH) leading to specific protein hydrolysates with desirable and predictable attributes (e.g., free amino acids) and higher yields. The novel approach might be useful for a greater efficiency of TEPs when applied in industrial processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This research does not contain any studies with human participants or animals performed by any of the authors.

References

1. Patel AK, Singhania RR, Pandey A (2016) Novel enzymatic processes applied to the food industry. Curr Opin Food Sci 7:64–72. https://doi.org/10.1016/j.cofs.2015.12.002
2. Adrio JL, Demain AL (2014) Microbial enzymes: tools for biotechnological processes. Biomolecules 4(1):117–139. https://doi.org/10.3390/biom4010117
3. Dewan SS (2017) Global market for enzymes in industrial application. BCC Research Biotechnology, BCC Research, Faridabad
4. Grand View Research (2016) Enzymes market analysis and segment forecasts to 2024. https://www.grandviewresearch.com/industry-analysis/enzymes-industry. Accessed 24 July 2018
5. Barrett AJ, McDonald JK (1986) Nomenclature protease proteinase and peptidase. Biochem J 237(3):935. https://doi.org/10.1042/bj2370935
6. Matsushita-Morita M, Tada S, Suzuki S, Hattori R, Marui J, Furukawa I, Yamagata Y, Amano H, Ishida H, Takeuchi M, Kashiwagi Y, Kusumoto K (2011) Overexpression and characterization of an extracellular leucine aminopeptidase from *Aspergillus oryzae*. Curr Microbiol 62(2):557–564. https://doi.org/10.1007/s00284-010-9744-9
7. Liang Y, Pan L, Lin Y (2009) Analysis of extracellular proteins of *Aspergillus oryzae* grown on soy sauce koji. Biosci Biotechnol Biochem 73(1):192–195. https://doi.org/10.1271/bbb.80500
8. Salamin K, Eugster PJ, Jousson O, Waridel P, Grouzmann E, Monod M (2017) AoS28D, a proline-Xaa carboxypeptidase secreted by *Aspergillus oryzae*. Appl Microbiol Biotechnol 101(10):4129–4137. https://doi.org/10.1007/s00253-017-8186-8
9. Eugster PJ, Salamin K, Grouzmann E, Monod M (2015) Production and characterization of two major *Aspergillus oryzae* secreted prolyl endopeptidases able to efficiently digest proline-rich peptides of gliadin. Microbiology 161:2277–2288. https://doi.org/10.1099/mic.0.000198
10. Graycar TP, Bott RP, Power SD, Estell DA (2013) Subtilisins. In: Rawlings ND, Salvesen G (eds) Handbook of proteolytic enzymes. Academic Press, New York, pp 3148–3155

11. Van den Burg B, Eijsink V (2013) Thermolysin and related *Bacillus* metallopeptidases. In: Rawlings ND, Salvesen G (eds) Handbook of proteolytic enzymes. Academic Press, New York, pp 540–553
12. Hageman JH (2004) Bacillopeptidase F. In: Rawlings ND, Salvesen G (eds) Handbook of proteolytic enzymes. Academic Press, New York, pp 3170–3171
13. Schallmey M, Singh A, Ward OP (2004) Developments in the use of *Bacillus* species for industrial production. Can J Microbiol 50(1):1–17. https://doi.org/10.1139/W03-076
14. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB (2011) Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development. Adv Drug Deliv Rev 63(13):1118–1159. https://doi.org/10.1016/j.addr.2011.07.006
15. Dodge T (2010) Production of industrial enzymes. In: Whitehurst RJ, van Oort M (eds) Enzymes in food technology. Wiley-Blackwell, New York, pp 44–58
16. U.S. Food and Drug Administration (2010) Guidance for industry, enzyme preparations: recommendations for submission of chemical and technical data for food additive petitions and GRAS notices. https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm217685.htm. Accessed 24 July 2018
17. Cherry JR, Fidantsef AL (2003) Directed evolution of industrial enzymes: an update. Curr Opin Biotechnol 14(4):438–443. https://doi.org/10.1016/s0958-1669(03)00099-5
18. Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13(4):345–351. https://doi.org/10.1016/s0958-1669(02)00328-2
19. Merz M, Eisele T, Berends P, Appel D, Rabe S, Blank I, Stressler T, Fischer L (2015) Flavourzyme, an enzyme preparation with industrial relevance: automated nine-step purification and partial characterization of eight enzymes. J Agric Food Chem 63(23):5682–5693. https://doi.org/10.1021/acs.jafc.5b01665
20. Kilcawley KN, Wilkinson MG, Fox PF (2002) Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. Enzyme Microb Technol 31(3):310–320
21. Kamnerdpetch C, Weiss M, Kasper C, Scheper T (2007) An improvement of potato pulp protein hydrolyzation process by the combination of protease enzyme systems. Enzyme Microb Technol 40(4):508–514. https://doi.org/10.1016/j.enzmictec.2006.05.006
22. Zhang M, Mu T-H, Wang Y-B, Sun M-J (2012) Evaluation of free radical-scavenging activities of sweet potato protein and its hydrolysates as affected by single and combination of enzyme systems. Int J Food Sci Technol 47(4):696–702. https://doi.org/10.1111/j.1365-2621.2011.02895.x
23. Jung S, Roussel-Philippe C, Briggs JL, Murphy PA, Johnson LA (2004) Limited hydrolysis of soy proteins with endo- and exoproteases. JAOCS 81(10):953–960
24. Cheung LKY, Aluko RE, Cliff MA, Li-Chan ECY (2015) Effects of exopeptidase treatment on antihypertensive activity and taste attributes of enzymatic whey protein hydrolysates. J Funct Foods 13:262–275. https://doi.org/10.1016/j.jff.2014.12.036
25. Sujak A, Kotlarz A, Strobel W (2006) Compositional and nutritional evaluation of several lupin seeds. Food Chem 98(4):711–719. https://doi.org/10.1016/j.foodchem.2005.06.036
26. Kilcawley KN, Wilkinson MG, Fox PF (2002) Properties of commercial microbial proteinase preparations. Food Biotechnol 16(1):29–55. https://doi.org/10.1081/fbt-120004200
27. Fujimura S, Hirai K, Shibata Y (2002) Dipeptidyl peptidase with strict substrate specificity of an anaerobic periodontopathogen *Porphyromonas gingivalis*. FEMS Microbiol Lett 209:127–131
28. Chrispeels MJ, Boulter D (1974) Control of storage protein metabolism in the cotyledons of germinating mung beans role of endopeptidase. J Plant Physiol 55:1031–1037
29. Nielsen PM, Petersen D, Dambmann C (2001) Improved method for determining food protein degree of hydrolysis. J Food Sci 66(5):642–646
30. Adler-Nissen J (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J Agric Food Chem 27(6):1256–1262
31. Merz M, Ewert J, Baur C, Appel D, Blank I, Stressler T, Fischer L (2015) Wheat gluten hydrolysis using isolated Flavourzyme peptidases: product inhibition and determination of synergistic effects using response surface methodology. J Mol Catal B Enzym 122:218–226. https://doi.org/10.1016/j.molcatb.2015.09.010
32. Berends P, Merz M, Kochjohann A, Philipps L, Blank I, Stressler T, Fischer L (2016) Sensory and antigenic properties of enzymatic wheat gluten hydrolysates produced in an enzyme membrane reactor in comparison with batch. Eur Food Res Technol 243(5):807–816. https://doi.org/10.1007/s00217-016-2794-x
33. Waters (2012) Acquity® UPLC H-class and H-class bio amino acid analysis system guide. Revision B. http://www.waters.com/webassets/cms/support/docs/acq_uplc_h-class_aaa_sysgd_rev_b.pdf. Accessed Apr 2019
34. Merz M, Appel D, Berends P, Rabe S, Blank I, Stressler T, Fischer L (2015) Batch-to-batch variation and storage stability of the commercial peptidase preparation Flavourzyme in respect of key enzyme activities and its influence on process reproducibility. Eur Food Res Technol 242(7):1005–1012. https://doi.org/10.1007/s00217-015-2606-8
35. Westrom BR, Ohlsson B, Karlsson BW (1987) Development of porcine pancreatic hydrolases and their isoenzymes from the fetal period to adulthood. Pancreas 2(5):589–596
36. Blocher M, Walde P, Dunn IJ (1999) Modeling of enzymatic reactions in vesicles: the case of α-chymotrypsin. Biotechnol Bioeng 62(1):36–43
37. Smyth M, FitzGerald RJ (1998) Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. Int Dairy J 8:819–827
38. Kim N, Son SH, Maeng JS, Cho YJ, Kim CT (2016) Enzymatic hydrolysis of anchovy fine powder at high and ambient pressure, and characterization of the hydrolyzates. J Sci Food Agric 96(3):970–978. https://doi.org/10.1002/jsfa.7173
39. Nishimura T, Kato H (1988) Taste of free amino acids and peptides. Food Rev Int 4(2):175–194
40. Solms J (1969) Taste of amino acids and peptides. J Agric Food Chem 17(4):686–688
41. Fuke S, Konosu S (1991) Taste-active components in some foods: a review of Japanese research. Physiol Behav 49(5):863–868

2.2 SUPPORTING INFORMATION

"A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis"

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SUPPORTING INFORMATION

Table S1: Overview of the substrates, solvents and concentrations for the measurement of the peptidase activities established in the novel measurement approach. The substrates are sorted according to the peptidase class covered and by the respective activity assay.

SUBSTRATE	STOCK SOLUTION		FINAL SUBSTRATE SOLUTION		PEPTIDASE CLASSES COVERED	CALIBRATION WITH	COMMENTS
	Concentration	**Solvent**	**Concentration**	**Solvent**			
H-Ala-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	Direct measurement of *para*-nitroaniline released (absorbance at 405 nm)
H-Arg-*p*NA · 2 HCl	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Asp-*p*NA · HCl	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Gln-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Glu-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Gly-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-His-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Ile-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Leu-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Lys-*p*NA · 2 HBr	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Met-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Phe-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Pro-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Tyr-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	
H-Val-*p*NA	400 mM	DMF	4 mM	10 mM HCl	(1) aminopeptidase	*p*NA [c)]	

Table continues on the next page

TABLE S1 CONTINUED

SUBSTRATE	STOCK SOLUTION		FINAL SUBSTRATE SOLUTION		PEPTIDASE CLASSES COVERED	CALIBRATION WITH	COMMENTS
	Concentration	**Solvent**	**Concentration**	**Solvent**			
H-Ala-Pro-*p*NA · HCl	400 mM	DMF	4 mM	10 mM HCl	(3) dieptidyl-peptidase	*p*NA [c]	Direct measurement of *para*-nitroaniline released (absorbance at 405 nm)
H-Lys-Ala-*p*NA · 2 HCl	400 mM	DMF	4 mM	10 mM HCl	(3) dieptidyl-peptidase	*p*NA [c]	
Bz-Asn-*p*NA	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Suc-Ala-Ala-Pro-Leu-*p*NA	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Z-Gly-Pro-*p*NA	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Bz-Tyr-*p*NA	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Bz-Arg-*p*NA · HCl	400 mM	DMF	4 mM	10 mM HCl	(4) endopeptidase	*p*NA [c]	
Z-Ala-Ala-Leu-*p*NA	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Boc-Ala-Ala-Gly-*p*NA	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Bz-Phe-Val-Arg-*p*NA · HCl	400 mM	DMF	40 mM	DMF	(4) endopeptidase	*p*NA [c]	
Z-Ala-Gly-OH	400 mM	DMF	4 mM	2 mM NaOH	(2) carboxy-/endopeptidase	L-glycine (5 mM in H_2O_{dd})	Indirect measurement of amino acids released after derivatization with *ortho*-phthalaldehyde (absorbance at 340 nm)
Z-Ala-Glu-OH	400 mM	DMF	4 mM	2 mM NaOH	(2) carboxy-/endopeptidase	L-glutamic acid (5 mM in H_2O_{dd})	
Z-Gly-Phe-OH	400 mM	DMF	4 mM	2 mM NaOH	(2) carboxy-/endopeptidase	L-phenylalanine (5 mM in H_2O_{dd})	
Z-Ala-Lys-OH	400 mM	H_2O	4 mM	2 mM NaOH	(2) carboxy-/endopeptidase	L-lysine (5 mM in H_2O_{dd})	
Z-Ala-Ser-OH	400 mM	DMF	4 mM	2 mM NaOH	(2) carboxy-/endopeptidase	L-serine (5 mM in H_2O_{dd})	
Z-Ala-Leu-OH	400 mM	DMF	4 mM	2 mM NaOH	(2) carboxy-/endopeptidase	L-leucine (5 mM in H_2O_{dd})	
Lupine protein	-	-	10 g L^{-1}	H_2O_{dd}	general proteolytic activity	L-serine (5 mM in H_2O_{dd})	

[c] *para*-nitroaniline [0.8 mM in H_2O_{dd}]

Table continued from the previous page

Table S2: Overview of the pipetting scheme that was implemented in the novel measurement approach concerning the pNA assay.

Volume [µL]	Blank	Activity assay
100	Buffer	Buffer
50	H_2O_{dd}	Substrate
Incubate for 60 s at 37°C		
Blank absorption value		
50	Sample (TEP [d])	Sample (TEP [d])
Incubate for 600 s at 37°C and measure absorption at 405 nm		

[d] Technical enzyme preparation

Table S3: Overview of the pipetting scheme that was implemented in the novel measurement approach concerning the OPA assay.

Volume [µL]	Blank	Activity assay
20	Buffer	Buffer
10	H_2O_{dd}	Substrate
Incubate for 60 s at 37°C		
10	Sample (TEP [e])	Sample (TEP [e])
Incubate for 600 s at 37°C		
240	OPA [f]	OPA [f]
Incubate for 30 s at 37°C and measure absorption at 340 nm		

[e] Technical enzyme preparation
[f] *ortho*-phthalaldehyde

Table S4: Overview of the limits of detection (LOD) and limits of quantification (LOQ) of the programmed enzyme activity assays, including the definitions of the blanks.

ASSAY	BLANK	LOD [nkat mL^{-1}]	LOQ [nkat mL^{-1}]
***p*NA assay**	*p*NA	0.05	0.18
OPA assay	L-serine	126.64	422.13
OPA assay [g]	L-lysine	29.78	99.27

[g] Only for the substrate (2) Z-Ala-Lys-OH

Table S5: Overview of the enzyme activities towards the 32 substrates of the activity fingerprints of Flavourzyme1000L (four batches) and of the technical enzyme preparations Flavorpro750 MPD, Flavorpro766 MPD and Flavorpro839 MPD. The enzyme activities are displayed in nkat mL^{-1} TEP, measured at 37°C, pH 7.0 [Bis-Tris-propane HCl, 100 mM], substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein.

Substrate	EA [nkat mL^{-1}]						
	Flavourzyme1000L				Flavorpro		
	HPN01005	HPN01010	HPN01011	HPN02003	750 MPD	766 MPD	839 MPD
H-Ala-*p*NA	58.77	63.12	68.59	56.95	15.11	1.87	10.35
H-Arg-*p*NA · 2 HCl	2203.97	2328.72	2339.19	2031.29	403.34	297.21	323.66
H-Asp-*p*NA · HCl	1.71	2.04	2.39	5.34	0.88	0.14	0.75
H-Gln-*p*NA	761.46	805.45	880.64	789.15	138.59	103.03	106.97
H-Glu-*p*NA	3.16	3.85	4.50	4.09	0.82	0.48	0.59
H-Gly-*p*NA	2.17	2.45	2.66	1.92	0.33	1.41	0.18
H-His-*p*NA	88.21	93.20	102.37	96.56	20.20	13.29	14.65
H-Ile-*p*NA	168.64	180.59	184.26	165.73	29.25	20.80	21.94
H-Leu-*p*NA	35430.36	40686.11	41213.62	22073.77	5935.85	3870.30	4635.65
H-Lys-*p*NA · 2 HBr	1242.78	1312.01	1606.27	1366.94	340.65	203.36	201.64
H-Met-*p*NA	7358.32	7910.71	7918.98	7320.87	1508.55	1131.07	1186.79
H-Phe-*p*NA	25469.92	31795.49	27143.94	16757.61	4355.80	3601.69	3789.53
H-Pro-*p*NA	6.53	7.59	11.01	6.55	1.85	0.94	1.44
H-Tyr-*p*NA	120.07	127.84	138.17	124.04	39.03	21.45	19.75
H-Val-*p*NA	249.46	248.11	254.95	236.77	42.15	32.64	33.83

Table continues on the next page

TABLE S5 CONTINUED

Substrate	EA [nkat mL^{-1}]						
	Flavourzyme1000L				Flavorpro		
	HPN01005	HPN01010	HPN01011	HPN02003	750 MPD	F766 MPD	839 MPD
Z-Ala-Glu-OH	72210.60	900.60	3558.58	661.00	963.82	954.02	5941.10
Z-Ala-Gly-OH	72796.73	872.54	3549.98	654.71	951.63	953.69	5998.94
Z-Ala-Leu-OH	71768.67	871.47	3425.55	649.88	911.84	948.14	5863.20
Z-Ala-Lys-OH	407.87	403.97	332.08	563.24	54.79	21.10	26.76
Z-Ala-Ser-OH	73306.00	876.80	3467.29	1004.87	864.63	939.64	5913.91
Z-Gly-Phe-OH	74372.20	847.68	3509.52	626.00	957.68	967.09	5999.19
H-Ala-Pro-*p*NA · HCl	20.01	32.96	44.92	23.99	3.10	17.18	3.33
H-Lys-Ala-*p*NA · 2 HCl	57.93	67.67	67.56	54.36	3.11	17.27	3.44
Boc-Ala-Ala-Gly-*p*NA	0.75	1.60	0.99	0.91	0.22	3.00	6.86
Z-Ala-Ala-Leu-*p*NA	841.64	964.14	945.44	980.28	395.61	765.06	1264.63
Suc-Ala-Ala-Pro-Leu-*p*NA	3546.63	4861.49	3495.36	4583.53	1130.10	862.63	1635.72
Bz-Arg-*p*NA · HCl	0.01	0.49	0.01	0.01	0.05	0.19	2.93
Bz-Asn-*p*NA	0.05	0.85	0.23	0.10	0.11	0.17	0.14
Z-Gly-Pro-*p*NA	2.77	3.45	2.91	3.20	6.80	5.44	3.84
Bz-Phe-Val-Arg-*p*NA · HCl	68185.44	126306.02	143196.55	138791.10	83073.93	23285.63	37035.36
Bz-Tyr-*p*NA	0.00	4.54	4.65	115.60	211.91	2253.52	231.14
Lupine protein	7575.57	4779.66	6245.17	4438.57	1397.20	1578.62	2279.61

Table continued from the previous page

Table S6: Overview of the enzyme activities towards the 32 substrates of the activity fingerprints of the respective TEPs (Flavourzyme1000L, Alcalase2.4L, DeltazymAPS M-FG, Promod278, FoodPro51FP, PeptidaseR). The values for Flavourzyme1000L represent the average (av) of a triplicate determination, including the standard deviation (sd). The enzyme activities are displayed in nkat mL^{-1} TEP, measured at 37°C, pH 7.0 [Bis-Tris-propane HCl, 100 mM], substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein.

Substrate	Flavourzyme1000L		Alcalase2.4L	Deltazym APS-M-FG	Promod278	FoodPro51	PeptidaseR
	EA (av) [nkat mL^{-1}]	EA (sd) [nkat mL^{-1}]	EA [nkat mL^{-1}]				
H-Ala-*p*NA	56.95	0.37	1.27	0.17	0.16	29.26	177.26
H-Arg-*p*NA · 2 HCl	2031.29	46.34	0.81	0.63	1.03	1123.28	211.84
H-Asp-*p*NA · HCl	5.34	0.02	0.24	0.01	0.01	1.63	2.06
H-Gln-*p*NA	789.15	9.75	2.99	0.01	0.50	367.22	47.25
H-Glu-*p*NA	4.09	0.03	1.44	0.09	0.01	0.63	3.84
H-Gly-*p*NA	1.92	0.03	0.01	0.01	0.01	0.48	149.66
H-His-*p*NA	96.56	0.40	1.65	0.19	0.01	33.01	22.95
H-Ile-*p*NA	165.73	2.63	0.09	0.01	0.01	63.90	18.43
H-Leu-*p*NA	22073.77	635.12	52.54	0.31	1.83	14068.40	843.65
H-Lys-*p*NA · 2 HBr	1366.94	51.66	0.62	0.13	0.40	919.40	32.36
H-Met-*p*NA	7320.87	225.30	6.57	0.42	2.47	3682.09	421.82
H-Phe-*p*NA	16757.61	1018.29	8.12	0.01	0.74	10749.20	1768.85
H-Pro-*p*NA	6.55	0.08	0.01	0.15	0.01	1.07	535.54
H-Tyr-*p*NA	124.04	0.16	5.19	0.22	1.05	48.23	1209.07
H-Val-*p*NA	236.77	4.67	0.13	0.14	0.15	9.71	7.86

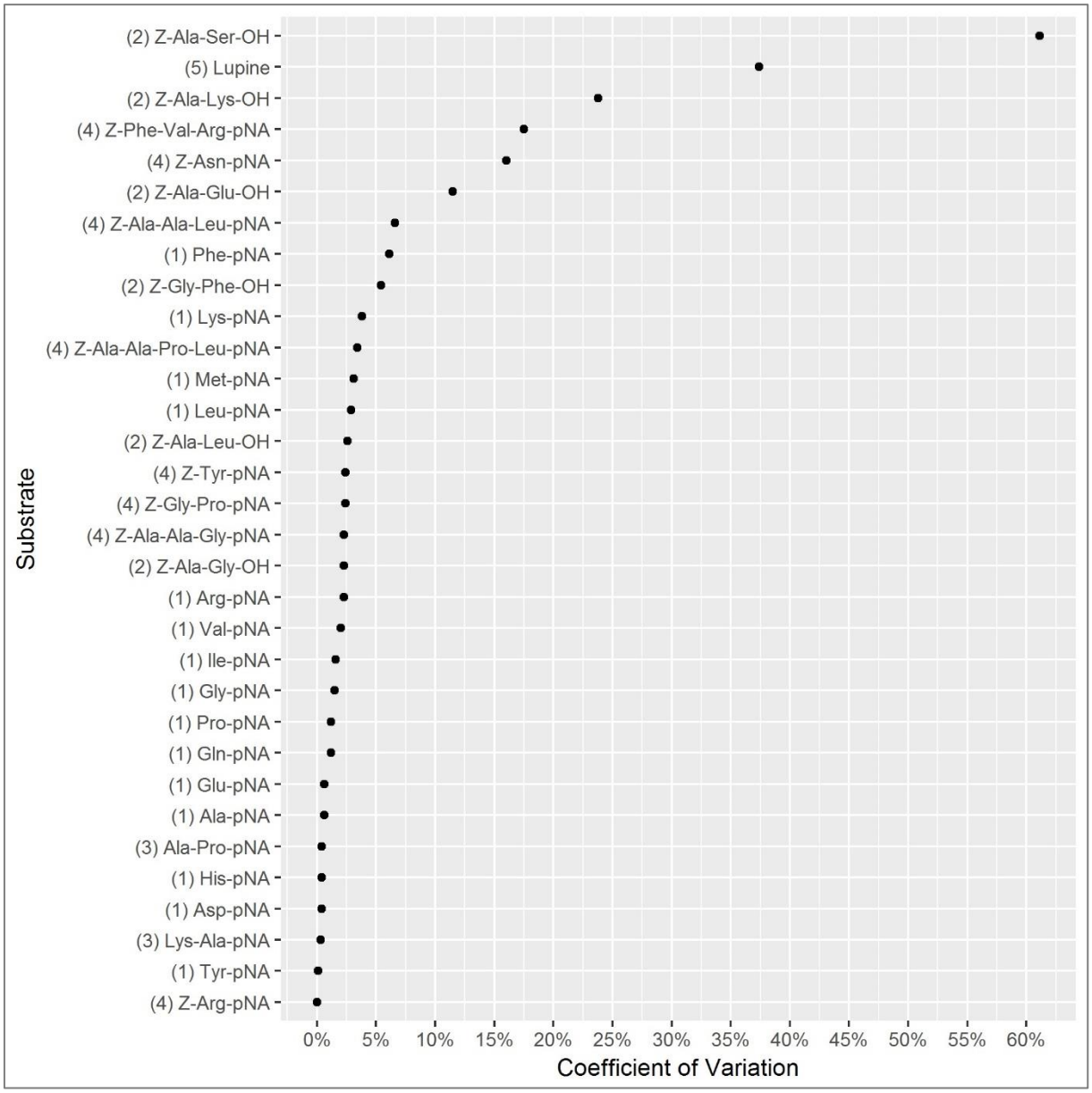
Table continues on the next page

TABLE S6 CONTINUED

Substrate	Flavourzyme1000L		Alcalase2.4L	Deltazym APS-M-FG	Promod278	Food-Pro51	PeptidaseR
	EA (av) [nkat mL^{-1}]	EA (sd) [nkat mL^{-1}]	EA [nkat mL^{-1}]				
Z-Ala-Glu-OH	661.00	75.75	1151.59	60818.99	359.70	59979.60	0.01
Z-Ala-Gly-OH	654.71	14.96	1140.62	62045.92	352.80	60662.35	0.01
Z-Ala-Leu-OH	649.88	16.69	1100.86	60674.00	322.97	59090.34	407.09
Z-Ala-Lys-OH	563.24	133.90	35.02	2711.17	40.87	2628.45	103.30
Z-Ala-Ser-OH	1004.87	613.50	4406.80	62358.00	347.98	60323.36	4.01
Z-Gly-Phe-OH	626.00	33.61	1094.87	271388.24	369.77	60783.18	0.01
H-Ala-Pro-*p*NA · HCl	23.99	0.10	0.03	0.16	0.26	10.99	80.06
H-Lys-Ala-*p*NA · 2 HCl	54.36	0.15	0.66	0.01	0.26	56.60	76.93
Boc-Ala-Ala-Gly-*p*NA	0.91	0.02	0.09	0.01	2812.00	1.95	0.90
Z-Ala-Ala-Leu-*p*NA	980.28	64.78	19576.72	23596.08	3.15	11165.27	1310.72
Suc-Ala-Ala-Pro-Leu-*p*NA	4583.53	157.34	724219.16	0.01	1234.43	81352.56	55.98
Bz-Arg-*p*NA · HCl	0.01	0.01	0.01	0.76	135.97	0.18	1.28
Bz-Asn-*p*NA	0.10	0.02	0.04	0.01	4.69	1.87	0.01
Z-Gly-Pro-*p*NA	3.20	0.08	0.23	10.21	3.31	3.92	9.26
Bz-Phe-Val-Arg-*p*NA · HCl	138791.10	24275.25	99602.49	356.82	108461.68	389621.76	10701.85
Bz-Tyr-*p*NA	115.60	2.74	11.68	11.57	2185.12	2182.03	15297.23
Lupine protein	4438.57	1661.97	588.68	883.30	1186.67	8876.23	341.27

Table continued from the previous page

Fig. S1: Coefficients of variation of the enzyme activities by the novel measurement approach of Flavourzyme1000L batch HPN02003 towards the 32 substrates of the activity fingerprints, measured in triplicate at 37°C, pH 7.0 (Bis-Tris-propane HCl, 100 mM), substrate concentration 1 mM for the synthetic substrates and 2.5 g L^{-1} for lupine protein.



3 CHAPTER THREE: THE SECOND PUBLICATION

3.1 IMPACT OF PEPTIDASE ACTIVITIES ON PLANT PROTEIN HYDROLYSATES REGARDING BITTER AND UMAMI TASTE.

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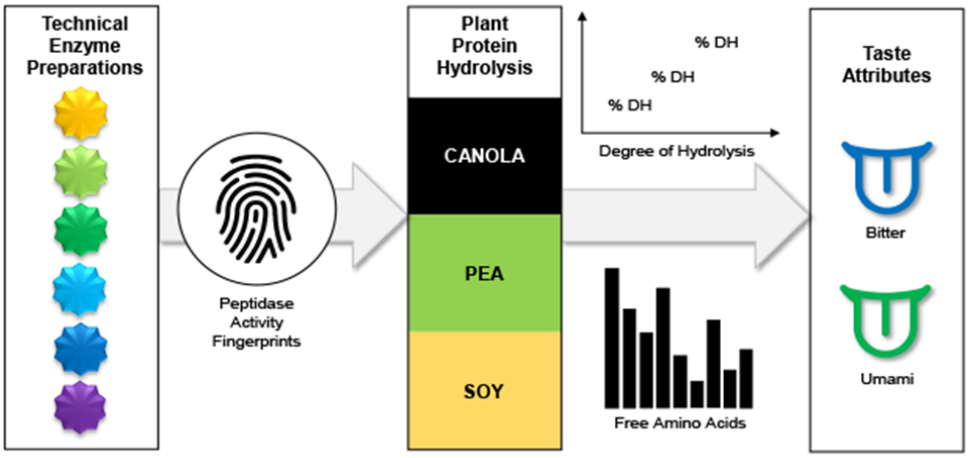
Graphical Abstract



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Impact of Peptidase Activities on Plant Protein Hydrolysates Regarding Bitter and Umami Taste

Kora Kassandra Großmann, Michael Merz, Daniel Appel, Thorn Thaler, and Lutz Fischer*

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ABSTRACT: The aim of this study was to investigate six food-grade peptidase preparations, namely, Flavourzyme 1000L, Protease P "Amano" 6SD, DeltazymAPS-M-FG, Promod278, ProteAX-K, and Peptidase R, regarding their use for the hydrolysis of soy, pea, and canola protein. The relationship between the specific peptidase activities and, first, the degree of hydrolysis, second, the free amino acid profiles of the hydrolysates, and, third, the corresponding taste of the hydrolysates was analyzed using a random forest model. The taste attributes bitter and umami were of special interest. The peptidase ProteAX-K was the biocatalyst most suited for the high umami and low bitter taste of the plant-based protein hydrolysates based on the experimental results and the random forest model.

KEYWORDS: *activity profiling of proteases, enzymatic food protein hydrolysis, plant proteins: soy, pea, canola, modulation of taste attributes bitter and umami*

INTRODUCTION

Nowadays, plant-based protein products are gaining increasing interest because the resources of animal protein are limited. Wheat protein is the main global plant protein by market volume, followed by soy. However, because of the potential allergenicity of both, other plant-derived proteins, such as pea and canola protein, are gaining increasing interest.[1] Enzymatic plant protein hydrolysates, especially soy protein hydrolysates, are commonly added to foods for either techno-functional, nutritional, flavoring, or beneficial health reasons.[2,3] Apart from the latter, hydrolysis often occurs with changes in sensory taste properties. The formation of the bitter taste of protein hydrolysates has been linked to peptides comprising hydrophobic amino acids that were previously hidden inside the protein structure but are exposed to the surface of the peptide chains after hydrolysis.[2] Furthermore, the bitterness of the protein hydrolysates has been referred to peptides with a low-molecular weight of about 0.36−2.10 kDa in size.[4] A variety of commercially available peptidase preparations (flavourzyme, alcalase, neutrase, protamex, papain, and bromelain) were investigated for plant protein hydrolysis by Seo et al., and the resulting changes in the bitter taste of the different hydrolysates were reported.[5−7] Soy protein isolates were also investigated by enzymatic hydrolysis, and the corresponding hydrolysates showed a bitter taste.[8] Apart from these studies referring to the generation of bitterness, enzymatic hydrolysis has also been reported as a possible method of reducing bitterness. The usage especially of particular exopeptidases was described as revealing a debittering effect.[9] Thus, the substrate selectivity of the particular peptidase used determined the bitterness or nonbitterness of the resulting hydrolysates.[10]

Umami is one of the five basic tastes, which is often associated with particular umami substances, such as glutamate, the nucleotide monophosphates 5′-inosinate, or 5′-guanylate. These substances can occur naturally in many foods and deliver a certain umami flavor.[11] Glutamate is the salt of free glutamic acid and often appears in the form of monosodium glutamate. However, glutamate has no particular taste when it is integrated in a peptide chain as part of a protein or peptide. On the other hand, free umami-tasting glutamate can be easily generated by proteolysis.[12] The review by Zhao et al. described the use of various peptidases for the generation of protein hydrolysates with an umami taste.[13]

A recent study showed that the activity profiles from peptidases (so-called "activity fingerprints": AFPs), obtained with many different natural and synthetic substrates, were used to predict important properties, such as the degree of hydrolysis (DH) and number of free amino acids, of the resulting hydrolysates.[14] Following these results, this new study investigated the impact of AFPs from the selected peptidases on the taste attributes of the plant protein hydrolysates generated. The taste attributes of the plant hydrolysates in focus were bitterness and umami. Finally, a mathematic model was suggested to describe and analyze the results of the taste generated.

MATERIALS AND METHODS

Chemicals and Substrates. All chemicals were of analytical grade and obtained from Merck Chemicals GmbH (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen, Germany). All *para*-nitroanilide (*p*NA) substrates were obtained from Bachem

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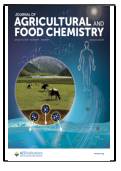

(Bubendorf, Switzerland). The soy protein ALPHA8IP was obtained from Solae LLC (St. Louis, MO, USA), which derives from soybeans (*Glycine Max*.L.). According to the supplier, the product composition is a min. 67% protein, max. 8% moisture, max. 1.5% fat, and max. 8% ash. The pea protein Nutralys was obtained from Roquette (Lestrem, France), deriving from the yellow pea (*Pisum Sativum*). The product composition is 79% protein, 1% dietary fiber, 9% total fat, 7% moisture, and 4% ash. The canola protein was CanolaPRO from DSM (Geleen, The Netherlands), obtained from a press cake of the seeds of the rape plant (*Brassica napus* and/or *Brassica juncea*). The product composition is a min. 90% protein, max. 2% fat, max. 7% carbohydrates, and max. 4% ash. The total amino acid composition of all three plant proteins from the suppliers can be looked up in the Supporting Information (Table S2).

Technical Enzyme Preparations. The technical enzyme preparation (TEP) DeltazymAPS-M-FG was obtained from WeissBio Tech GmbH (Ascheberg, Germany) and an enzyme preparation derived from *A. niger*. Flavourzyme 1000L was obtained from Novozymes (Bagsvaerd, Denmark) and derives from *A. oryzae*. The TEP Promod278 was obtained from Biocatalysts (Wales, UK), originally deriving from *Carica papaya* and *Bacillus subtilis*. Peptidase R, Protease P "Amano" 6SD, and ProteAX-K were obtained from Amano Enzyme Inc. (Nagoya, Japan) and originally derive from *R. oryzae*, *A. melleus* and *A. oryzae*, respectively. All TEPs were stored at 7 °C and protected from light.

Determination of the Enzyme Activities of the Technical Enzyme Preparations. The TEPs were investigated for their enzyme activities toward 29 substrates, as previously described in the literature.[14] An automated photometric analyzer Gallery Plus (ThermoFischerScientific, Waltham, MA, USA) was implemented to perform two photometric enzymatic assays (*p*NA assay and *ortho*-phthalaldehyde [OPA] assay) creating AFPs of the TEPs. Fifteen aminopeptidase activities (1), six carboxy-/endopeptidase activities (2), two dipeptidylpeptidase activities (3), and eight endopeptidase activities (4) were measured using several synthetic substrates. The numbers in brackets (1−4) refer to the four different groups of peptidase activities. It must be mentioned that the substrates for the carboxypeptidase activities (2) might also be accepted by endopeptidases and might, therefore, affect the carboxypeptidase activity results. The assays were done at 37 °C, 100 mmol L^{-1} Bis-Tris-propane HCl, pH 7.0, and with a substrate concentration of 1 mmol L^{-1} for the synthetical substrates. The release of *p*NA was measured at 405 nm and one katal (kat) of peptidase activity was defined as the release of 1 mol *p*NA per sec. The carboxypeptidase activities were measured after derivatization with the OPA reagent (OPA Solution Complete, Sigma-Aldrich, Taufkirchen, Germany) at 340 nm, using the synthetic substrates *Z*-Ala-Gly-OH, *Z*-Ala-Glu-OH, *Z*-Ala-Ser-OH and *Z*-Ala-Leu-OH. L-glycine, L-glutamic acid, L-serine and L-leucine were used, respectively, for the calibration. One kat of peptidase activity was defined as the release of 1 mol L^{-1} amino acid equivalents per sec. The blanks for both assays were done similarly to the pipetting scheme using $H_2O_{dd.}$ and can be looked up in the Supporting Information of the system description.[14]

Enzymatic Batch Hydrolysis of Soy, Pea, and Canola Protein. Batch hydrolyses of three plant proteins (soy, pea, and canola) were done with six different TEPs. The hydrolyses were carried out in 900 mL scale in stirring reactors (Distek, New York, USA). The final substrate concentration was adjusted to 10% (w/v) protein suspended in tap water (pH 7.3 at 21.1 °C). The final TEP concentration was 2% (v/v) or (w/v), depending on the TEPs' type of formulation (liquid or solid). The hydrolyses were done at 50 °C and 250 rpm. A hydrolysis timeframe of 2 h was selected because of internal safety regulations for the subsequent sensory characteristics after hydrolysis. The combination of 2 h and 2% enzyme dosage was chosen to reach an equilibrium of DH (data not shown). After 2 h of hydrolysis, the hydrolysates were heated to 95 °C to inactivate the enzymes for 15 min at 95 °C. Therefore, the complete process time was 2.5 h, and all samples will refer to this time. The hydrolysates were freeze-dried (Alpha 1-4 LSCplus, Christ, Osterode am Harz, Germany) and stored in an airtight container and protected from light.

Determination of the Degree of Hydrolysis of the Batch Hydrolysates. Primary amino groups were determined after derivatization with OPA, according to the method of Nielsen et al.,[15] with some modifications. After completing the hydrolysis, samples of 800 μL were taken and transferred into Eppendorf tubes containing 200 μL of trichloroacetic acid [2.5 mol L^{-1}] to terminate the reaction. The samples were shaken for 3 s at 3000 min^{-1} using a universal shaker (MS3basic, IKA, Staufen, Germany) and centrifuged for 3 min at 14,100 rcf (MiniSpinPlus, Eppendorf, Hamburg, Germany). The samples were diluted as required to determine the DH within the calibration range (L-serine was used as a reference for the calibration). A sample volume of 25 μL was transferred into a microtiter plate and 175 μL of OPA Reagent (OPA Solution Complete, Sigma-Aldrich, Taufkirchen, Germany) was added. The plate was incubated at 24 °C for 10 s and shaken for 10 s. The absorbance was measured at 340 nm using a UV−vis multimode microplate reader (SpectraMaxM5, Molecular Devices, Sunnyvale CA, USA).

The DH was calculated according to the description of Adler-Nissen,[16] with modifications.[17] Equation 1 describes the DH as the percentage of the concentration of free amino groups h [mol L^{-1}] compared to the maximum concentration of free amino acids at complete hydrolysis h_{tot} [mol L^{-1}].

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100(\%) \tag{1}$$

The maximum concentration of free amino acids at complete hydrolysis (eq 2) is described as the protein concentration that is hydrolyzed $c_{protein}$ [g L^{-1}] divided by the difference of the average molecular mass of the amino acids in soy, pea, and canola protein (136.13, 135.20, and 134.62 g mol^{-1}, respectively) and the molecular mass of water (18.0 g mol^{-1}). The soy, pea, and canola protein-specific average molecular mass was calculated by considering the amino acid compositions. The molecular mass of water was subtracted because water is added during the hydrolysis of a peptide bond.

$$h_{\mathrm{tot}} = \frac{c_{\mathrm{protein}}}{M^* - M_{\mathrm{H_2O}}} (\mathrm{mol\ L^{-1}}) \tag{2}$$

Determination of the Free Amino Acids of the Batch Hydrolysates. The free amino acids were determined by ultra-performance liquid chromatography as described previously.[18] An Acquity UPLC H-Class System (Waters, Milford, USA) which was equipped with a quaternary solvent manager, a sample manager with flow-through needle, a column heater (CH-A), and a photodiode array (PDA) detector was used. A Waters AccQ•Tag Ultra RP Column, Acquity UPLC ethylene-bridged-hybrid (BEH) C18 (pore diameter 130 Å, particle size 1.7 μm, inner diameter 2.1 mm, length 100 mm) column was used. Norvaline (Sigma-Aldrich, Taufkirchen, Germany) was used as an internal standard with a concentration of 25 μmol L^{-1} (1:100 stock solution 2.5 mmol L^{-1} in 0.1 mol L^{-1} HCl). The injection volume was 100 μL. The column temperature was 43 °C and the sample temperature was 20 °C. The eluent gradient, the flow rate (0.7 mL min^{-1}), and the detection wavelength (260 nm) were set according to the recommendations of Waters Corporation.[19] Cystein was measured as cystin because of the method constraints.

The six TEPs and the substrates (soy, pea, and canola protein) were analyzed to determine their free amino acid compositions. These values were subtracted from the results of the batch hydrolysates. Consequently, only the product release by the enzymatic liberation was captured.

Taste Investigation of the Plant Protein Hydrolysates. A sensory evaluation of the plant protein hydrolysates was performed to investigate the influence of the different TEPs during hydrolysis on the taste attributes, bitter and umami. The panel consisted of 17 participants and, therefore, covered the number of participants required according to the German standard DIN-ISO 8587. All panelists took part in a basic panelist screening including basic taste

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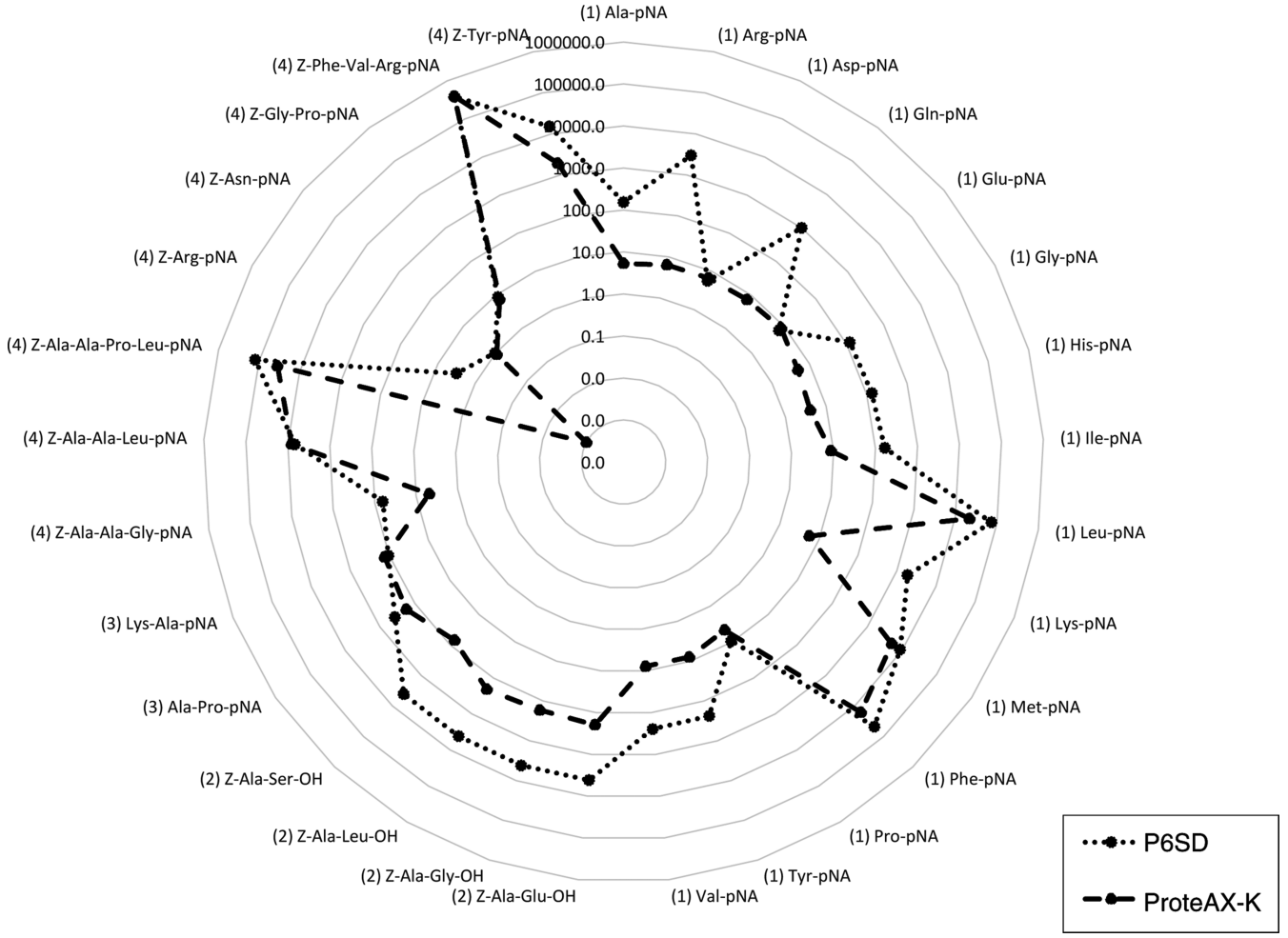

Figure 1. Activity fingerprints of the TEPs P6SD (black dotted line) and ProteAX-K (black dashed line). The enzyme activities are shown on a logarithmic scale in nkat mL^{-1} TEP, measured at 37 °C, pH 7.0 [bis-trispropane HCl, 100 mmol L^{-1}], and 1 mmol L^{-1} substrate concentration.

recognition (sweet: 10 g L^{-1} sugar, salty: 1.5 g L^{-1} NaCl, bitter: 0.5 g L^{-1} caffeine × 2, sour: 0.6 g L^{-1} citric acid, umami: 0.9 g L^{-1} MSG), umami ranking (0.5 g L^{-1}–4.5 g L^{-1} MSG) and aroma recognition test. A triangle test was carried out prior to investigating the sensory characteristics of the hydrolysates to guarantee the bitter perception of the panelists. Therefore, a caffeine solution (0.3 g L^{-1}) was used according to the German standard DIN10959. All panelists identified the caffeine solution and were, therefore, qualified for the taste investigation. The freeze-dried hydrolysates and the plant proteins as references were dissolved in tap water at 2% (w/v) concentration. The samples were scored on a scale from 0 to 3 regarding the attributes, bitter and umami, because the internal tasters were familiar with the 3-point category scale and anchor words were used to help understand the scale. The tasting was carried out at room temperature (24 °C). The samples were labeled with three-digit codes and an individual questionnaire was provided for documentation. A two-way ANOVA was done with the product as a fixed factor and the panelist as a random factor to calculate which descriptors were rated significantly ($p < 0.05$) different between samples. Analysis of the attributes with statistically significant differences was followed by Duncan's multiple range test to find out which products differed significantly ($p < 0.05$).

Modeling of the Information Obtained in the Activity Fingerprints and the Sensory Investigation of the Hydrolysates. So-called random forests were used to relate the enzyme activities to the sensory characteristics of the hydrolysates. The random forests is a tree-based approach, which constructs a set of uncorrelated decision trees (regression trees in our case) and returns the mean prediction of this set.[20] Five thousand trees with a minimal terminal node size of three were used for this study. Each tree was fitted with a bootstrapped sample (with replacement) from the original data set. The bootstrapping was done in such a way that each observation was used in about two-thirds of the trees. Observations not used for a particular permutation (out-of-bag observations) were used to measure the relative importance of each activity. This was done by permuting the out-of-bag observations 500 times and recording the increase in the mean square error (MSE) for each predictor. A large increase in the MSE indicates that the particular predictor is important. Thus, this study was particularly focused on the predictors which featured a large MSE increase (Figure 5). The random forests rely on stochastic sampling. Hence, a seed was set for the random generator to make the results reproducible. All statistical analyses were carried out using R version 3.4.2 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Statistical Analysis. The standard deviations were calculated using Excel (Microsoft, Redmond, WA, USA). The batch hydrolyses were done in single determination. The analytics were done in duplicate. Please refer to the section of the sensory investigation of the batch protein hydrolysates for the statistical information regarding the sensory characteristics.

RESULTS AND DISCUSSION

The objective of this study was to perform plant protein hydrolyses using several TEPs and to combine the latter's activity profiles with the resulting taste of the hydrolysates. Therefore, TEPs were selected based on the differences in their activity profiles AFPs and were used to hydrolyze soy, pea, and

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Table 1. Degree of Hydrolysis (%) of the Soy, Pea, and Canola Protein Hydrolysates Using Six TEPs (50 °C, 2 h)

	TEP					
protein	P6SD[a] (%)	FVZ[a] (%)	ProteAX-K[a] (%)	PepR[a] (%)	DZM[a] (%)	P278[a] (%)
soy	72	38	55	32	8	24
pea	70	52	24	29	15	22
canola	52	42	48	38	13	13

[a]Please see abbreviation section for the full names of the TEPs.

canola protein. These hydrolysates were characterized concerning the DH, the free amino acid profiles, and, most importantly, the taste. A mathematical model was created to set the AFPs of the TEPs in correlation with the taste of the hydrolysates.

Selection of Relevant Technical Enzyme Preparations. A total of 67 TEPs, both commercially available ones and prototypes, were tested regarding their substrate selectivity and corresponding activity, and the, so-called, AFPs were obtained (data not shown). Six of the commercial TEPs were then selected because their particular activities with individual substrates differed the most from each other in selectivity or total activity value. These TEPs were FVZ, DZM, P278, PepR, P6SD, and ProteAX-K (for abbreviations, see Materials and Methods). Thus, if these TEPs were used for protein hydrolysis, the resulting sensory profiles of the hydrolysates should differ as well. The activities of four TEPs (FVZ, DZM, P278, and PepR) have been reported previously[14] and are shown in the supplement (Table S1). The AFPs of the TEPs P6SD and ProteAX-K are shown in Figure 1, and the absolute activity values are given in Table S1 (Supporting Information).

Four TEPs showed both exo- and endopeptidase activities (P6SD, ProteAX-K, PepR, and FVZ), and two of them only endopeptidase activity (DZM and P278). The TEP P6SD was the most active with all substrates tested when all its activities were added up (total activities with all substrates accepted; P6SD a total of 689,270 nkat mL^{-1}; ProteAX-K a total of 482,332 nkat mL^{-1}; DZM a total of 269,875 nkat mL^{-1}; FVZ a total of 198,561 nkat mL^{-1}; P278 a total of 116,233 nkat mL^{-1}; and PepR a total of 33,398 nkat mL^{-1}). The TEP ProteAX-K showed high activities toward Leu-*p*NA (21,843 nkat mL^{-1}), Met-*p*NA (5123 nkat mL^{-1}), and Phe-*p*NA (16,197 nkat mL^{-1}). PepR was interesting because of its high activities toward Pro-*p*NA (536 nkat mL^{-1}) and Tyr-*p*NA (1209 nkat mL^{-1}). The most prominent commercial peptidase is FVZ, with seven different peptidases[21] and had a similar AFP[14] as P6SD. However, it was interesting to compare it with P6SD and the others. Both DZM and P278 showed only endopeptidase activities[14] with different AFPs regarding particular substrates (see Table S1).

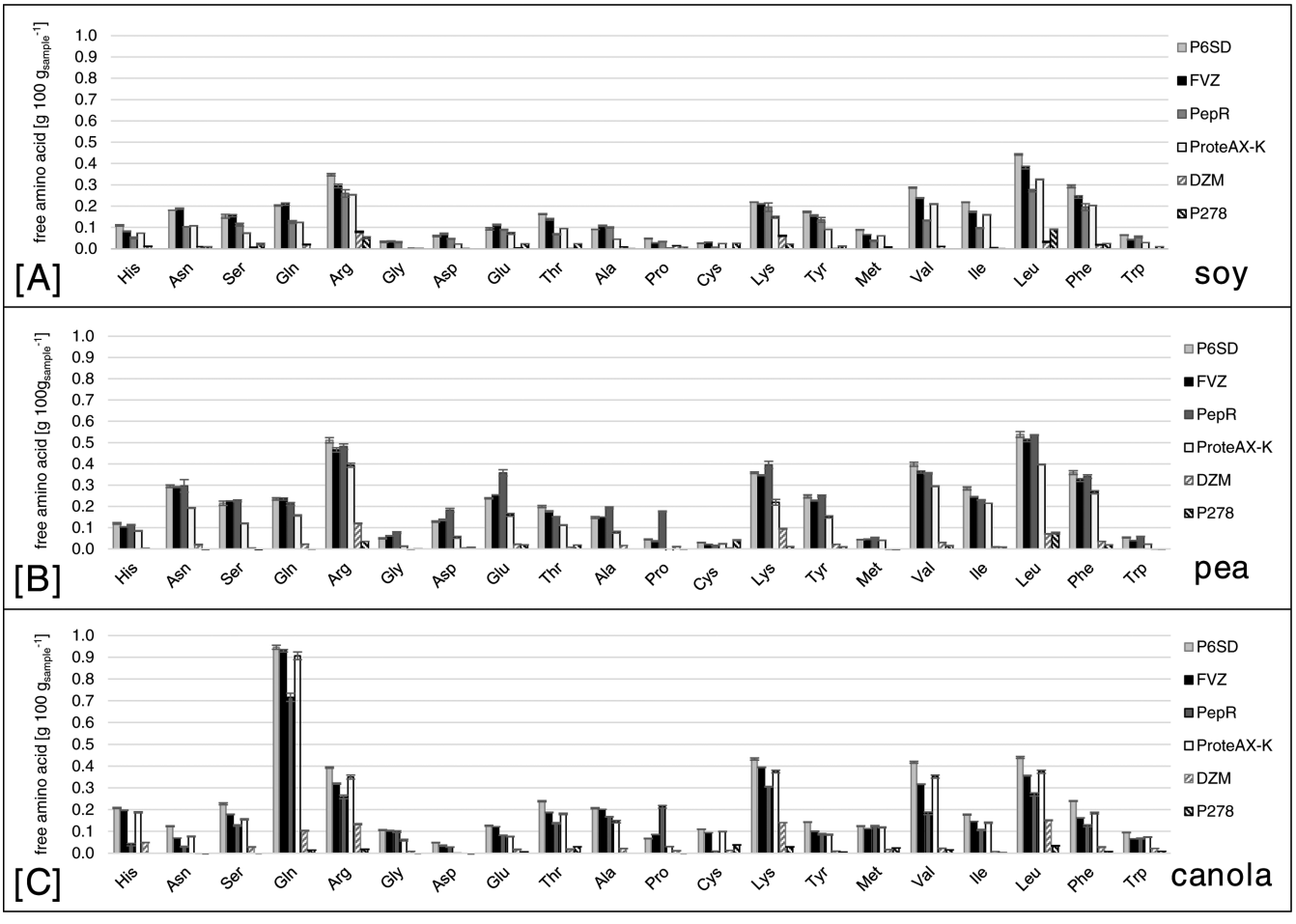

Figure 2. Free amino acid profiles of the hydrolysates of soy [A], pea [B], and canola protein [C] using the series of six TEPs for each. The values refer to the amounts that were liberated during hydrolysis.

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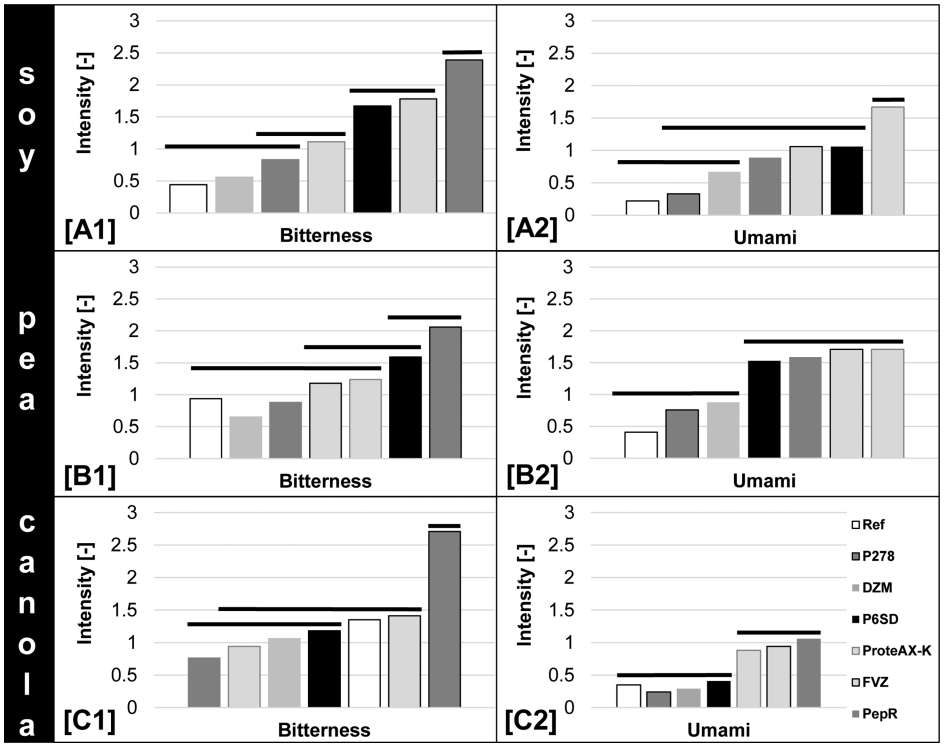

Figure 3. Bar chart with mean scores for the taste attributes bitter and umami for [A] soy, [B] pea, and [C] canola protein hydrolysates, hydrolyzed with each the series of six TEPs. Bars under the same line are not significantly different from each other (Duncan, $p < 0.05$).

Table 2. Total Amount of Free Amino Acids (g 100 g_{sample}^{-1}) of the of the Soy, Pea, and Canola Protein Hydrolysates Using Six TEPs (50 °C, 2 h)[b]

	TEP					
protein	P6SD[a]	FVZ[a]	ProteAX-K[a]	PepR[a]	DZM[a]	P278[a]
soy	4.86 (49.10%)	4.17 (44.15%)	3.97 (31.57%)	3.17 (32.03%)	0.80 (4.33%)	0.22 (4.81%)
pea	3.29 (56.89%)	2.96 (53.85%)	2.12 (37.77%)	2.15 (59.47%)	0.29 (6.24%)	0.32 (3.16%)
canola	4.49 (54.03%)	4.25 (46.36%)	2.98 (44.07%)	4.70 (35.23%)	0.49 (8.83%)	0.25 (2.40%)

[a]Please see abbreviation section for the full names of the TEPs. [b]The yield of amino acid liberation is additionally listed in brackets.

Enzymatic Batch Hydrolysis of Soy, Pea, and Canola Protein. The six TEPs described above were used for the hydrolysis of soy, pea, and canola protein in a 900 mL scale. The time frame of the hydrolysis processes needed to be short in order to avoid microbial contaminations, as the usage of additives for preservation was not permitted and the hydrolysates had to be tasted by humans afterward. Preliminary hydrolysis experiments showed that an overdosage of 2% (w/v or v/v) of the TEPs at 50 °C resulted in the maximum DH desired for each protein (10% w/v) in 2.5 h (time including the heat inactivation step). Consequently, the safety regulations for the sensory testing of the protein hydrolysates by humans were covered and their sensory investigation was possible to be performed safely.

The batch hydrolysates of soy, pea, and canola protein were investigated for their DH and are shown in Table 1. The highest DH of 72, 70, and 52% was obtained for soy, pea, and canola protein, respectively, with P6SD. These results are in line with previous observations. Commercial peptidases were characterized including Flavourzyme and DH were reported between 15 and 62%, depending on the commercial enzyme and its proteolytic set.[22] As seen above, P6SD was the most active toward the substrates tested, thus resulting in higher DH.

On the other hand, DZM resulted in the lowest DH of 8, 15, and 13% for soy, pea, and canola protein, respectively. Both FVZ and DZM resulted in their highest DH when using the pea protein as a substrate (52 and 15%, respectively). The highest DH for P6SD, P278, and ProteAX-K was measured for soy protein hydrolysis (72, 24, and 55%, respectively). In the case of PepR, the canola protein resulted in the highest value with a DH of 38%, in comparison to 32% for soy and 29% for pea protein. In conclusion, the TEPs FVZ and DZM cleaved pea protein better than canola or soy protein, P6SD and P278 cleaved soy protein better than both the other proteins, and ProteAX-K and PepR cleaved canola protein preferentially the best.

Free Amino Acid and Taste Profiles of the Protein Hydrolysates. The hydrolysates of soy, pea, and canola protein using the six TEPs selected were analyzed for their free amino acid profiles, and the results are shown in Figure 2. As mentioned in the Materials and Methods section, the protein sources and the TEPs themselves were analyzed for their content of free amino acids, and these values were subtracted from the values of the later protein hydrolysates. Thus, the values of the hydrolysates, as shown in Figure 2, show only the amino acids liberated during protein hydrolysis. The total free amino acid concentrations of the TEPs ranged between 0.06

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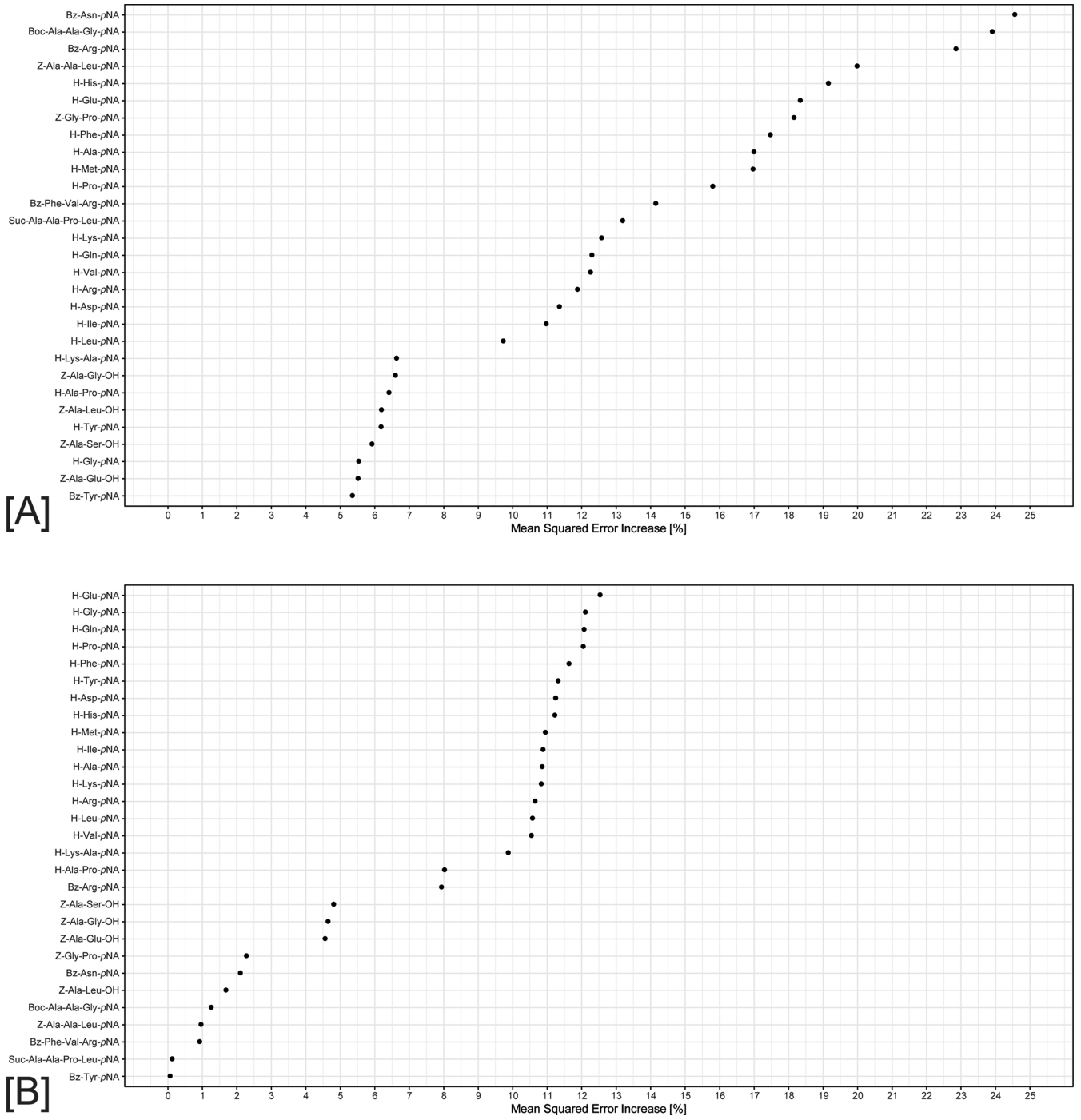

Figure 4. Variable importance of the several enzyme activities for the random forest model on the sensory attribute [A] bitter and [B] umami of the plant protein hydrolysates. Each dot represents the increase of the mean squared error when the particular term is not present in the model.

and 0.98 g 100 g^{-1} and the total free amino acid concentration of the substrate suspensions were 0.02 and 0.04 g 100 g^{-1} for the soy and pea, respectively. No free amino acids were detected in the canola protein suspension. Furthermore, the taste of the protein hydrolysates was investigated focusing on the attributes bitter and umami. The tasting results for each protein hydrolysate are shown in Figure 3A1,A2 with soy, Figure 3B1,B2 with pea, and Figure 3C1,C2 with canola protein. The nonhydrolyzed proteins were also evaluated (reference). The lines above the bars represent the significances, and the samples below the same line were not statistically different from each other (Duncan test).

As shown in Figure 2, P6SD and FVZ liberated the highest total amounts of amino acids, followed by PepR and ProteAX-K. The TEPs DZM and P278 liberated low amounts of free amino acids. For the respective values, please see Table 2. This could be explained by the exopeptidase activities of P6SD, FVZ, PepR, and ProteAX-K, as shown in Figure 1 and Table S1. The TEPs DZM and P278 contained mainly endopeptidase activities[14] and, therefore, did not liberate significant amounts of free amino acids.

The TEP PepR liberated higher amounts of free proline in comparison to the other TEPs, as shown in Figure 2B,C, whereas, free proline was described in the literature as having a

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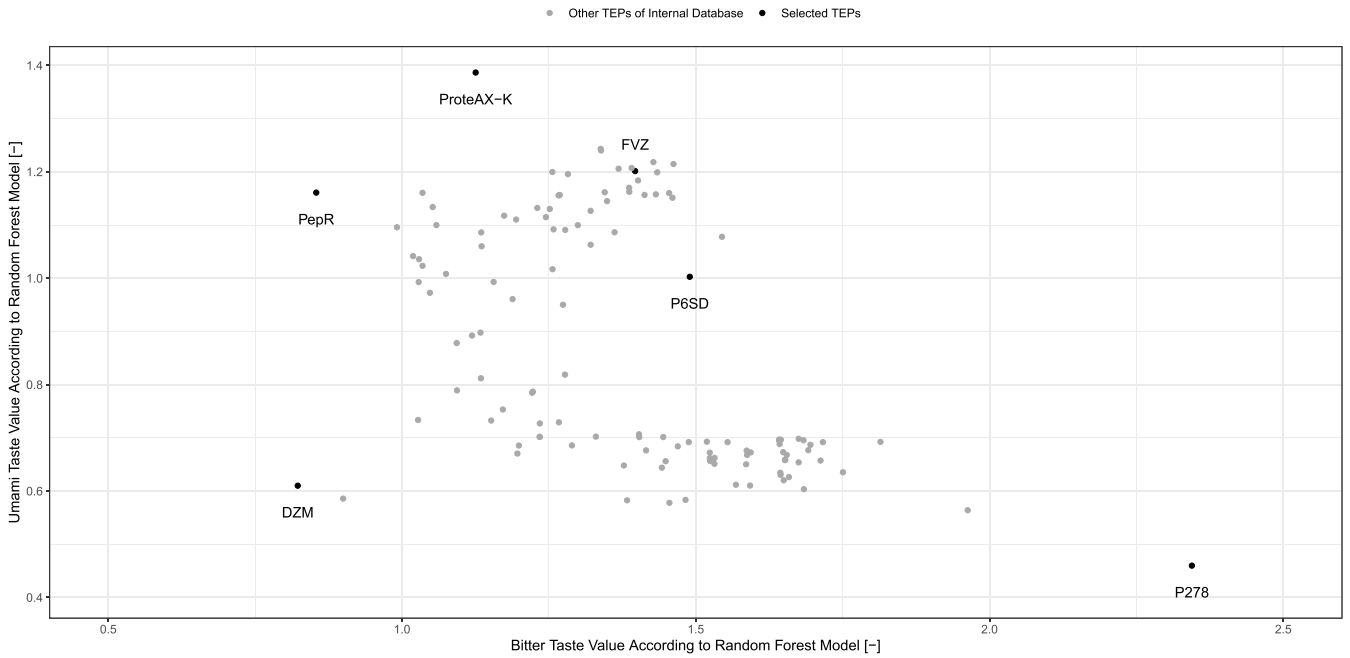

Figure 5. Predicted umami and bitter scores of an internal database of 67 TEPs by the random forest model fitted. The six TEPs discussed are highlighted in the figure.

sweeter taste,[23] proline bound in peptides exhibited a bitterer taste.[24] Therefore, the hydrolysates of pea and canola protein using PepR were expected to be low in the bitter taste. This was confirmed in the taste results, as shown in Figure 3B1,C1. In the case of canola protein in Figure 3C1, the hydrolysate using PepR was rated even lower in bitterness than the reference. According to the activity profile, PepR showed the highest activity toward H-Pro-*p*NA with 535 nkat mL^{-1}, compared to the other TEPs with activities of 9.31 nkat mL^{-1} and below (Table S1). These results were in line with previously published results, illustrating that prolyl-specific peptidases showed debittering effects.[25]

The amount of glutamine liberated was significantly higher when using canola protein than soy or pea protein, as shown in Figure 2C. The TEPs with exopeptidase activities liberated glutamine amounts of 0.95 ± 0.01, 0.93 ± 0.01, 0.72 ± 0.02, and 0.94 ± 0.01 g 100 g^{-1}_{sample} for P6SD, FVZ, PepR, and ProteAX-K, respectively. The amounts for glutamine for soy- and pea proteins were all below 0.25 g 100 g^{-1}_{sample}, hardly one-third of the values for the canola protein. This could be explained by the differences of the plant proteins in protein sequences and the cleavage specificity of the TEPs, assuming that the canola protein is more accessible for the TEPs selected to release free glutamine. This high liberation of free glutamine could be interesting for the protein hydrolysate supplemented to foods because glutamine was reported to have potential health beneficial properties, for example, maintaining the gut mucosal integrity and function.[26]

Because glutamic and aspartic acid presumably contributed to the umami taste,[27] it was interesting to investigate the potential relationships between the TEPs P6SD, FVZ, PepR, and ProteAX-K, which released these amino acids (see Figure 2), TEPs peptidase activities (Table S1), and the taste results of the corresponding hydrolysates (Figure 3). First, the release of glutamic and aspartic acid by these TEPs fitted very well to their activity values toward the corresponding substrates H-Asp-*p*NA and H-Glu-*p*NA (Table S1). Second, the corresponding hydrolysates of these TEPs were rated significantly higher in umami taste than the references (Figure 3), with the exception of the canola protein hydrolysate produced by P6SD that was rated in the same umami taste intensity as the reference.

Data Analysis of Activity Fingerprints and Sensory Pattern by Modeling. A random forest model was used to evaluate the information obtained by the AFPs of the TEPs regarding the resulting taste of their hydrolysates for further analysis of the experimental results. Random forests is a popular approach in several research fields because of its simple applicability to classification and regression challenges. They are frequently applied because they achieve a high prediction accuracy and enable to identify informative variables.[28] All activities of the AFPs of the TEPs and the results of the tastings were included in the model. Therefore, Figure 4A,B represent the variable importance of the several TEP activities for the random forest model regarding the attributes bitter and umami, respectively. The higher the MSE increase of the TEP toward the activities stated, the higher the impact on the bitter taste of the hydrolysates was. Therefore, the endopeptidase activates (4) toward the substrates *Z*-Ala-Ala-Gly-*p*NA, *Z*-Asn-*p*NA, and *Z*-Arg-*p*NA were rated as the most important ones for the bitter attribute. These results were in line with previously reported data showing that the peptides liberated by endopeptidase activities have an influence on the bitter taste perception.[29]

The hydrolysates of the TEPs ProteAX-K and FVZ were rated the most umami, followed by the hydrolysates of PepR and P6SD (see Figure 3). The hydrolysates of the TEPs P278 and DZM were rated similar to the references and were, therefore, not significantly different. Because the TEPs P278 and DZM had mainly endopeptidase activities, it can be concluded that endopeptidase activities did not contribute to the umami taste. On the contrary, the TEPs with exopeptidase activities contributed to the umami taste. This could be confirmed in the random forest model; the activities toward

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the substrates for the aminopeptidase activities showed the highest impact on the umami attribute. All 15 substrates for aminopeptidase activities are rated more important than the other activities, with the activities toward Glu-*p*NA, Pro-*p*NA, Ala-*p*NA, and Asp-*p*NA being the four most important ones, as shown in Figure 4B. Peptides with glutamic acid, alanine, and asparagine were reported previously to contribute to the umami taste.[30] Cheung et al. investigated the effects of exopeptidase treatment on the taste attributes of whey protein hydrolysates produced by ProteAX and PepR. Similar to the results of this study, they showed that the exopeptidase treatment increased the umami taste and related this result to the release of terminal amino acids.[31]

Furthermore, an internal database of AFPs from 67 TEPs was screened based on the random forest model presented. Figure 5 shows the resulting forecasted impact on the attributes umami and bitter. The six TEPs selected in this study are highlighted by their names and the other TEPs of the internal database are shown in grey. According to this, ProteAX-K was the TEP most suited for a high umami and low bitter taste of plant-based protein hydrolysates. These findings were in line with the previously reported results, where the usage of ProteAX resulted in decreased bitterness and increased umami of other hydrolysates, such as whey protein, bovine muscle, and porcine plasma.[31,32] The model showed, furthermore, that the six TEPs selected had different influences on the formation of bitter and umami tastes. This confirmed that the selection of the TEPs via the AFPs was meaningful because it was targeted to select the TEPs that differed the most in their resulting taste of the corresponding hydrolysates.

This study showed that the information obtained by AFPs from various peptidases (TEPs) could be correlated to the liberation of free amino acids of the hydrolysates. Furthermore, these particular activities of the TEPs were shown to influence the resulting bitter and umami tastes of the hydrolysates. Thus, the approach might contribute to the forecast of taste impacts on protein hydrolysates based on prior activity investigations using AFPs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c05447.

Specific values of the peptidase activities of the six TEPs selected; activity profiles of P6SD and ProteAX-K; activity values of FVZ, PepR, DZM, and P278;[14] and overview of the total amino acid composition of the three respective plant proteins (soy, pea, and canola) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AFP, activity fingerprint; BEH, ethylene-bridged hybrid; DPP, dipeptidylpeptidase; DMF, dimethylformamid; DH, degree of hydrolysis; DZM, deltazym APS-M-FG; FVZ, Flavourzyme 1000L; H_2O_{dd}, purified water; h, concentration of free amino groups; h_{tot}, maximum concentration of free amino acids at complete hydrolysis; LAPU, leucine aminopeptidase units; LOD, limit of detection; LOQ, limit of quantification; OPA, *ortho*-phthalaldehyde; P278, Promod278; P6SD, Protease P "Amano" 6SD; PepR, PeptidaseR; *p*NA, *para*-nitroaniline; TEP, technical enzyme preparation

■ REFERENCES

(1) Chandrakumar, B. J. G.; Ranjan, B.; Bahuguna, A.; Pillai, K.; Reddy, K. H.; Maakan, H. Protein Ingredients Market Size, Share & Trends Analysis Report by Product (Plant Protein, Animal/Dairy Protein), by Application Food and Beverages, Personal Care & Cosmetics), and Segment Forecast, 2020−2027 https://www.grandviewresearch.com/industry-analysis/protein-ingredients-market (accessed April 20, 2020).

(2) Sun, X. D. Enzymatic hydrolysis of soy proteins and the hydrolysates utilisation. *Int. J. Food Sci. Technol.* **2011**, *46*, 2447−2459.

(3) Liu, R.; Wang, L.; Liu, Y.; Wu, T.; Zhang, M. Fabricating soy protein hydrolysate/xanthan gum as fat replacer in ice cream by combined enzymatic and heat-shearing treatment. *Food Hydrocolloids* **2018**, *81*, 39−47.

(4) Kim, M. R.; Choi, S. Y.; Lee, C. H. Molecular characterization and bitter taste formation of tryptic hydrolysis of 11S glycinin. *J. Microbiol. Biotechnol.* **1999**, *9*, 509−513.

(5) Seo, W. H.; Lee, H. G.; Baek, H. H. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. *J. Food Sci.* **2008**, *73*, S41−S46.

(6) Humiski, L. M.; Aluko, R. E. Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. *J. Food Sci.* **2007**, *72*, S605−S611.

(7) Guo, X.; Tian, S.; Small, D. M. Generation of meat-like flavourings from enzymatic hydrolysates of proteins from *Brassica* sp. *Food Chem.* **2010**, *119*, 167−172.

(8) Meinlschmidt, P.; Sussmann, D.; Schweiggert-Weisz, U.; Eisner, P. Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality, and sensory properties. *Food Sci. Nutr.* **2016**, *4*, 11−23.

(9) Nishiwaki, T.; Yoshimizu, S.; Furuta, M.; Hayashi, K. Debittering of enzymatic hydrolysates using an aminopeptidase from the edible basidiomycete *Grifola frondosa*. *J. Biosci. Bioeng.* **2002**, *93*, 60−63.

(10) Ewert, J.; Claassen, W.; Stressler, T.; Fischer, L. An innovative two-step enzymatic membrane bioreactor approach for the continuous production of antioxidative casein hydrolysates with reduced bitterness. *Biochem. Eng. J.* **2019**, *150*, 107261.
(11) Beauchamp, G. K. Sensory and receptor responses to umami: an overview of pioneering work. *Am. J. Clin. Nutr.* **2009**, *90*, 723S−727S.
(12) Kurihara, K. Umami the fifth basic taste: history of studies on receptor mechanisms and role as a food flavor. *BioMed Res. Int.* **2015**, *2015*, 10.
(13) Zhao, Y.; Zhang, M.; Devahastin, S.; Liu, Y. Progresses on processing methods of umami substances: A review. *Trends Food Sci. Technol.* **2019**, *93*, 125−135.
(14) Großmann, K. K.; Merz, M.; Appel, D.; Fischer, L. A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis. *Eur. Food Res. Technol.* **2019**, *245*, 1695−1708.
(15) Nielsen, P. M.; Petersen, D.; Dambmann, C. Improved method for determining food protein degree of hydrolysis. *J. Food Sci. Food Chem. Toxicol.* **2001**, *66*, 642−646.
(16) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256−1262.
(17) Merz, M.; Ewert, J.; Baur, C.; Appel, D.; Blank, I.; Stressler, T.; Fischer, L. Wheat gluten hydrolysis using isolated Flavourzyme peptidases: Product inhibition and determination of synergistic effects using response surface methodology. *J. Mol. Catal. B: Enzym.* **2015**, *122*, 218−226.
(18) Berends, P.; Merz, M.; Kochjohann, A.; Philipps, L.; Blank, I.; Stressler, T.; Fischer, L. Sensory and antigenic properties of enzymatic wheat gluten hydrolysates produced in an enzyme membrane reactor in comparison with batch. *Eur. Food Res. Technol.* **2016**, *243*, 807−816.
(19) Waters, *Acquity®, UPLC H-Class and H-Class Bio Amino Acid Analysis System Guide*. Revision B. 2012, (Revision B.).
(20) Breiman, L. Random forests. *Mach. Learn.* **2001**, *45*, 5−32.
(21) Merz, M.; Eisele, T.; Berends, P.; Appel, D.; Rabe, S.; Blank, I.; Stressler, T.; Fischer, L. Flavourzyme, an enzyme preparation with industrial relevance: automated nine-step purification and partial characterization of eight enzymes. *J. Agric. Food Chem.* **2015**, *63*, 5682−5693.
(22) Merz, M.; Claassen, W.; Appel, D.; Berends, P.; Rabe, S.; Blank, I.; Stressler, T.; Fischer, L. Characterization of commercially available peptidases in respect of the production of protein hydrolysates with defined compositions using a three-step methodology. *J. Mol. Catal. B: Enzym.* **2016**, *127*, 1−10.
(23) Birch, G. G.; Kemp, S. E. Apparent specific volumes and tastes of amino acids. *Chem. Senses* **1989**, *14*, 249−258.
(24) Ishibashi, N.; Kubo, T.; Chino, M.; Fukui, H.; Shinoda, I.; Kikuchi, E.; Okai, H.; Fukui, S. Taste of Proline-containing Peptides. *Agric. Biol. Chem.* **1988**, *52*, 95−98.
(25) Mika, N.; Zorn, H.; Rühl, M. Prolyl-specific peptidases for applications in food protein hydrolysis. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 7837−7846.
(26) Lenaerts, K.; Mariman, E.; Bouwman, F.; Renes, J. Glutamine regulates the expression of proteins with a potential health-promoting effect in human intestinal Caco-2 cells. *Proteomics* **2006**, *6*, 2454−2464.
(27) Manninen, H.; Rotola-Pukkila, M.; Aisala, H.; Hopia, A.; Laaksonen, T. Free amino acids and 5'-nucleotides in Finnish forest mushrooms. *Food Chem.* **2018**, *247*, 23−28.
(28) Hapfelmeier, A.; Ulm, K. A new variable selection approach using Random Forests. *Comput Stat Data Anal* **2013**, *60*, 50−69.
(29) Saha, B. C.; Hayashi, K. Debittering of protein hydrolyzates. *Biotechnol. Adv.* **2001**, *19*, 355−370.
(30) Zhang, Y.; Venkitasamy, C.; Pan, Z.; Liu, W.; Zhao, L. Novel umami ingredients: umami peptides and their taste. *J. Food Sci.* **2017**, *82*, 16−23.
(31) Cheung, L. K. Y.; Aluko, R. E.; Cliff, M. A.; Li-Chan, E. C. Y. Effects of exopeptidase treatment on antihypertensive activity and taste attributes of enzymatic whey protein hydrolysates. *J. Funct. Foods* **2015**, *13*, 262−275.
(32) Fu, Y.; Liu, J.; Hansen, E. T.; Bredie, W. L. P.; Lametsch, R. Structural characteristics of low bitter and high umami protein hydrolysates prepared from bovine muscle and porcine plasma. *Food Chem.* **2018**, *257*, 163−171.

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3.2 SUPPORTING INFORMATION

"*Impact of peptidase activities on plant protein hydrolysates regarding bitter and umami taste*"

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TABLE S1: Overview of the enzyme activities of the technical enzyme preparations (TEPs) Flavourzyme1000L, P6SD, DeltazymAPS-M-FG, Promod278, ProteAX-K and PeptidaseR towards 29 substrates. The enzyme activities are displayed in nkat mL^{-1} TEP, measured at 37°C, pH 7.0 [Bis-Tris-propane HCl, 100 mM], substrate concentration 1 mM.

TEP[a)]	Flavourzyme 1000L[b)]	P6SD	DeltazymAPS-M-FG[b)]	Promod278[b)]	ProteAX-K	PeptidaseR[b)]
Substrate	**EA [nkat mL^{-1}]**					
H-Ala-*p*NA	56.95	154.34	0.17	0.16	5.35	177.26
H-Arg-*p*NA · 2 HCl	2031.29	2990.01	0.63	1.03	6.41	211.84
H-Asp-*p*NA · HCl	5.34	5.68	0.01	0.01	6.71	2.06
H-Gln-*p*NA	789.15	1013.79	0.01	0.50	7.16	47.25
H-Glu-*p*NA	4.09	7.04	0.09	0.01	8.33	3.84
H-Gly-*p*NA	1.92	123.68	0.01	0.01	4.93	149.66
H-His-*p*NA	96.56	134.72	0.19	0.01	4.09	22.95
H-Ile-*p*NA	165.73	167.05	0.01	0.01	8.72	18.43
H-Leu-pNA	22073.77	73580.61	0.31	1.83	21843.24	843.65
H-Lys-*p*NA · 2 HBr	1366.94	1879.24	0.13	0.40	5.73	32.36
H-Met-*p*NA	7320.87	8905.74	0.42	2.47	5123.22	421.82
H-Phe-*p*NA	16757.61	47211.83	0.01	0.74	16196.56	1768.85
H-Pro-pNA	6.55	9.31	0.15	0.01	4.64	535.54
H-Tyr-*p*NA	124.04	238.45	0.22	1.05	7.95	1209.07
H-Val-*p*NA	236.77	246.87	0.14	0.15	7.78	7.86

Table continues the next page

TABLE S1 CONTINUED

TEP[a)]	Flavourzyme 1000L[b)]	P6SD	DeltazymAPS-M-FG[b)]	Promod278[b)]	ProteAX-K	PeptidaseR[b)]
Substrate	**EA [nkat mL^{-1}]**					
Z-Ala-Glu-OH	661.00	4141.33	60818.99	359.70	196.76	0.01
Z-Ala-Gly-OH	654.71	4171.44	62045.92	352.80	169.81	0.01
Z-Ala-Leu-OH	649.88	4108.38	60674.00	322.97	206.06	407.09
Z-Ala-Ser-OH	1004.87	373.52	62358.00	347.98	70.15	4.01
H-Ala-Pro-*p*NA · HCl	23.99	154.34	0.16	0.26	175.77	80.06
H-Lys-Ala-*p*NA · 2 HCl	54.36	2990.00	0.01	0.26	131.02	76.93
Boc-Ala-Ala-Gly-*p*NA	0.91	64.04	0.01	2812.00	4.84	0.90
Z-Ala-Ala-Leu-*p*NA	980.28	6822.06	23596.08	3.15	8110.85	1310.72
Suc-Ala-Ala-Pro-Leu-*p*NA	4583.53	125623.75	0.01	1234.43	35392.40	55.98
Bz-Arg-*p*NA · HCl	0.01	3.18	0.76	135.97	0.01	1.28
Bz-Asn-*p*NA	0.10	1.04	0.01	4.69	0.91	0.01
Z-Gly-Pro-*p*NA	3.20	8.64	10.21	3.31	7.45	9.26
Bz-Phe-Val-Arg-*p*NA · HCl	138791.10	388059.08	356.82	108461.68	392707.21	10701.85
Bz-Tyr-*p*NA	115.60	15067.97	11.57	2185.12	1918.36	15297.23
Sum	**198561.12**	**689269.85**	**269874.99**	**116232.65**	**482332.42**	**33397.77**

[a)] Technical enzyme preparation

Table continued from the previous page

[b)] Reported previously in Grossmann, K. K.; Merz, M.; Appel, D.; Fischer, L. A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis. *Europ. Food Res. Technol.* **2019**, *245* (8), 1695-1708.

TABLE S2: Overview of the total amino acid composition (g $100g_{protein}^{-1}$) of soy protein ALPHA®8IP, pea protein Nutralys® and canola protein CanolaPRO™ (data obtained from the suppliers).

Amino Acid	Soy	Pea	Canola
Ala	4.3	4.3	4.4
Arg	7.1	8.7	6.7
Asx*	11.0	11.5	6.0
Cys	1.4	1.0	3.7
Glx*	19.1	16.7	23.7
Gly	4.0	4.0	5.1
His	2.3	2.5	3.2
Ile	4.3	4.7	3.7
Leu	7.6	8.2	7.2
Lys	6.1	7.1	6.4
Met	1.3	1.1	2.2
Phe	4.9	5.5	3.9
Pro	5.0	4.3	7.0
Ser	5.0	5.1	4.1
Thr	3.7	3.8	4.0
Trp	1.1	3.8	1.4
Tyr	3.4	1.0	2.1
Val	4.6	5.0	5.0

*Glx = Glu+Gln, Asx = Asp+Asn

4 CHAPTER FOUR: THE THIRD PUBLICATION

4.1 NEW INSIGHTS INTO THE FLAVORING POTENTIAL OF CRICKET (*ACHETA DOMESTICUS*) AND MEALWORM (*TENEBRIO MOLITOR*) PROTEIN HYDROLYSATES AND THEIR MAILLARD PRODUCTS.

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Highlights

- Enzymatic hydrolysis of insect proteins develops savory and diverse taste profiles
- Insect proteins hydrolysates and Maillard products show different sensory profiles
- Identified odor-active molecules are also present in meat and seafood products
- Insect proteins have potential for savory flavoring

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New insights into the flavoring potential of cricket (*Acheta domesticus*) and mealworm (*Tenebrio molitor*) protein hydrolysates and their Maillard products

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ABSTRACT

Insect proteins have an earthy-like flavor and have not shown great flavor potential for food applications so far. In this study, insect proteins of cricket *Acheta domesticus* and mealworm *Tenebrio molitor* larvae were first enzymatically hydrolyzed using two peptidase preparations (Flavourzyme1000L and ProteaseA "Amano"2SD). Xylose was then added to facilitate Maillard reactions (30 min, T = 98 °C, 1% (w/v) xylose). A comprehensive sensory evaluation showed that both the hydrolysis and the Maillard reactions changed the flavor description of the samples significantly to more complex and savory-like taste profiles (27 descriptors for cricket and 39 descriptors for mealworm protein). In addition, 38 odor-active molecules were identified using gas chromatography–olfactometry (1 alcohol, 5 acids, 11 aldehydes, 5 ketones and 16 heterocyclic compounds). The results showed impressively that the flavoring potential of insect proteins was significantly enhanced with respective processing.

1. Introduction

Insects have been part of the human diet for centuries, especially in the Asian, Latin American and African regions (Jongema, 2012). Alternative food sources have become more and more important due to the increasing food demand of the growing population. According to the European Food Safety Authority, *Acheta domesticus* and *Tenebrio molitor* belong to a proposed list of twelve insect species showing potential for use as food and feed in the European Union (EFSA Scientific Commitee, 2015). They are, furthermore, listed in the Food Composition Database for Biodiversity (BioFoodComp) and are considered to be high in protein, according to the World Health Organization and Food and Agricultural Organization of the United Nations food labeling requirements (Nowak, Persijn, Rittenschober, & Charrondiere, 2016).

Being high in protein, edible insects have gained attention for use in enzymatic processing. Various studies have described the enzymatic breakdown of the proteins into peptides and single amino acids for either functional, nutritional or sensory reasons (Leni, Soetemans, Caligiani, Sforza, & Bastiaens, 2020; Osimani et al., 2018; Purschke, Meinlschmidt, Horn, Rieder, & Jäger, 2017; Roncolini et al., 2020; Zhang et al., 2017). Enzymatic hydrolysis of *Locusta migratoria* protein flour has been reported to improve techno-functional properties which can be beneficial for insect-based ingredients for food applications (Purschke et al., 2017). Adult cricket (*Gryllodes sigillatus*), larvae of mealworm (*Tenebrio molitor*) and adult locust (*Schistocerca gregaria*) have well-balanced nutrient profiles referring to amino acids, fatty acids and trace elements (Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015).

Although research has been done investigating important aspects regarding the use of edible insects for food applications, there are few sensory data on insect hydrolysates. A small number of studies have shown that edible insects can improve the nutritional value, being high in protein and essential amino acids (Zielinska, Baraniak, Karas, Rybczynska, & Jakubczyk, 2015). Following this theme, studies have shown that insects can be integrated into foods, for example, in baked and pasta products (Cabuk & Yilmaz, 2020; Osimani et al., 2018; Roncolini et al.,

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2020). Apart from the nutritional benefits, sensory evaluations have shown that the simple addition of insect powders does not also result in sensory improvements. Regarding food applications, edible insects are used commonly as dry powder or meal (Fasolin et al., 2019; Govorushko, 2019). A recently published study investigated the fortification of pasta with grasshopper and mealworm flour. The sensory evaluation showed that the insect fortification did not contribute to higher flavor intensity and decreased the overall acceptability (Cabuk et al., 2020). Further studies have shown that the integration of insect powders decrease the global liking due to its notable, insect-like flavor (Osimani et al., 2018). Allowing that the simple fortification of insect powders faces sensory challenges, this study investigated the sensory changes if insect proteins are further processed.

The aim of this study was to investigate the effect of the enzymatic hydrolysis and subsequent Maillard reaction of cricket (*Acheta domesticus*) and mealworm (*Tenebrio molitor*) protein on the flavor characteristics of the resulting products to generate further insights into the flavor potential of insect proteins. A comprehensive sensory evaluation was done showing the flavor potential of insect proteins, including comprehensive gas chromatography–olfactometry (GC-O) analysis.

2. Materials and methods

2.1. Chemicals and substrates

All chemicals were of analytical grade and obtained from Merck Chemicals GmbH (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen, Germany). All *para*-nitroanilide (*p*NA) substrates were obtained from Bachem (Bubendorf, Switzerland). The cricket protein was obtained from Thailand Unique (Udon Thani, Thailand) in powder format and derived from the house crickets (*Acheta domesticus)*. According to the supplier, the product composition is 68% protein, 5.6% fat, 5.5% carbohydrates, 1% moisture and 0.5% fiber. The mealworm protein was obtained from Ynsect (Évry, France) in powder format and derived from mealworm larvae (*Tenebrio molitor*). The product composition, according to the supplier, is 70% protein, 13% fat, 8% fiber, 4% moisture and 3% ash.

2.2. Technical enzyme preparations

Two technical enzyme preparations (TEPs) were investigated in this study. Flavourzyme1000L (F) was obtained from Novozymes (Bagsværd, Denmark) and derives from *A. oryzae* with an aminopeptidase content of 17% (w/w), according to the supplier. ProteaseA "Amano" 2SD (A) was obtained from Amano Enzyme Inc. (Nagoya, Japan) and originally derives from *A. oryzae* with a protease content of 65%, according to the supplier. The TEPs were stored at 7 °C and protected from light.

Flavourzyme was selected because it is well-known in the food industry, and has been characterized and used in various studies (Berends, Appel, Eisele, Rabe, & Fischer, 2014; Grossmann, Merz, Appel, & Fischer, 2019; Merz, Appel, et al., 2015; Merz, Eisele, et al., 2015). ProteaseA was selected due to the pre-experimental sensory screening of twelve TEPs where the hydrolysates using ProteaseA achieved the roundest, balanced and umami-like taste profile (data not shown).

2.3. Determination of the enzyme activities of the TEPs

The TEPs were investigated for their enzyme activities towards 29 substrates as described previously (Grossmann et al., 2019). An automated photometric analyzer Gallery^{TM} Plus (ThermoFischerScientific, Waltham, MA, USA) was used to do two photometric enzymatic assays (peptide nucleic acid-based [*p*NA] and o-phthalaldehyde [OPA] assay) creating activity fingerprints of the TEPs. Fifteen aminopeptidase activities (1), six carboxy-/endopeptidase activities (2), two dipeptidylpeptidase activities (3) and eight endopeptidase activities (4) were measured by using several synthetic substrates. The numbers in brackets (1–4) refer to the four different groups of peptidase activities. It has to be mentioned that the substrates for the carboxypeptidase activities (2) might also be accepted by endopeptidase and may, therefore, affect the carboxypeptidase activity results. The assays were done at 37 °C, 100 mmol L^{-1} BIS-TRIS-propane HCl, pH 7.0, with substrate concentrations of 1 mmol L^{-1}. The release of *p*NA was measured at 405 nm and one katal (kat) of peptidase activity was defined as the release of 1 mol *p*NA per second. The carboxypeptidase activities were measured after derivatization with OPA reagent (OPA Solution Complete, Sigma-Aldrich, Taufkirchen, Germany) at 340 nm, using the synthetic substrates Z-Ala-Gly-OH, Z-Ala-Glu-OH, Z-Ala-Ser-OH and Z-Ala-Leu-OH. Regarding the calibration, L–glycine, L–glutamic acid, L-serine and L-leucine were used, respectively. One katal (kat) of peptidase activity was defined as the release of 1 mol L^{-1} amino acid equivalent per second. The blanks for both assays were done similarly to the pipetting scheme using double-distilled water and can be looked up, as can the limit of detection and limit of quantification, in the supporting information of the system description (Grossmann et al., 2019).

2.4. Batch hydrolysis and Maillard reaction of cricket and mealworm protein

Batch hydrolyses of cricket and mealworm protein were done each with Flavourzyme and ProteaseA. The hydrolyses were done in 800 mL scale in stirred reactors (Distek, New York, USA) at 50 °C and 300 rpm. The final substrate concentration was adjusted to 5% (w/v) protein, suspended in double distilled water (pH = 7.3 at T = 21.1 °C). The final TEP concentration was 2% (v/v) or (w/v), depending on the type of formulation (liquid or solid) of the TEPs. A hydrolysis timeframe of 2 h was selected for the subsequent sensory evaluation after hydrolysis due to internal safety regulations. The combination of 2 h and 2% enzyme dosage was chosen to reach an equilibrium of the degree of hydrolysis (DH; data not shown). After 2 h of hydrolysis, the hydrolysates were heated for 15 min at 90 °C to inactivate the enzymes. After inactivation, a volume of 200 mL was collected for the sensory evaluation of the hydrolysates. A volume of 300 mL was transferred into a separate reactor beaker (preheated to the same temperature of 90 °C) and the temperature was adjusted to 98 °C. Regarding the subsequent Maillard reaction, 1% (w/v) xylose was added and the reaction time was set at 30 min. Other time and temperature conditions were tested (data not shown) but the selected conditions resulted in the most interesting flavor profiles. The samples were stored on ice to terminate the reaction. The hydrolysates and samples were centrifuged for 10 min at 10,000 rpm (Sorvall RC 6+, Thermo Scientific, Waltham, MA USA) after the Maillard reaction to investigate the soluble fractions and to avoid potential disturbance of the subsequent assays (e.g. DH determination, UPLC). The supernatant was collected and stored at −20 °C and protected from light.

2.5. Determination of the degree of hydrolysis of the insect protein processed

Primary amino groups were determined after derivatization with OPA, according to the method of (Nielsen, Petersen, & Dambmann, 2001), with some modifications. Samples of 800 μL were taken at different time points during hydrolysis and were transferred into Eppendorf tubes containing 200 μL trichloroacetic acid [2.5 mol L^{-1}] to terminate the reaction. Further samples were taken after the heat inactivation and the Maillard reaction. The samples were mixed for 3 s at 3000 min^{-1} using a universal shaker (MS3basic, IKA®, Staufen, Germany) and centrifuged for 3 min at 14100 rcf (MiniSpinPlus, Eppendorf, Hamburg, Germany). The samples were diluted as required to determine the DH. A sample volume of 25 μL was transferred into a microtiter plate and 175 μL OPA Reagent (OPA Solution Complete, Sigma-Aldrich, Taufkirchen, Germany) was added. The plate was incubated at 24 °C for 10 s and mixed for 10 s. The absorbance was measured at 340 nm

using an ultraviolet–visible multimode microplate reader (SpectraMaxM5, Molecular Devices, Sunnyvale CA, USA). L-serine was used as a reference for the calibration.

The DH was calculated according to the description of Adler-Nissen (Adler-Nissen, 1979), with modifications (Merz, Ewert, et al., 2015). The following equation (Eq. (1)) describes the DH as the percentage of the concentration of free amino groups **h** [mol L^{-1}] compared to the maximum concentration of free amino acids at complete hydrolysis $\mathbf{h_{tot}}$ [mol L^{-1}].

$$DH = \frac{h}{h_{tot}} \times 100\ [\%] \quad (1)$$

The maximum concentration of free amino acids at complete hydrolysis (Eq. (2)) is described as the protein concentration that is hydrolyzed $c_{protein}$ [g L^{-1}] divided by the difference of the average molecular mass of the amino acids in cricket and mealworm protein [130.3 g $mol^{-1}$and 131.6 g mol^{-1}, respectively] and the molecular mass of water [18.0 g mol^{-1}]. The cricket and mealworm protein-specific average molecular mass was calculated by considering the amino acid compositions provided by the supplier (for the mealworm protein) and by an external lab (for the cricket protein). The molecular mass of water was subtracted because water is added during the hydrolysis of a peptide bond.

$$h_{tot} = \frac{c_{protein}}{M^* - M_{H_2O}}\ [\text{mol L}^{-1}] \quad (2)$$

2.6. Determination of the free amino acids of the insect proteins processed

The free amino acids were determined by ultra-performance liquid chromatography (UPLC) as described previously (Berends et al., 2016). Samples were taken after 2 h of hydrolysis, the heat inactivation and the Maillard reaction. A volume of 8 mL sample was inactivated with 2 mL TCA [2.5 M]. An Acquity® UPLC H-Class System (Waters, Milford, USA) was used, which was equipped with a quaternary solvent manager, a sample manager with flow-through needle, a column heater and a photodiode array detector. A Waters AccQ•Tag™ Ultra RP Column, Acquity® UPLC Ethylene-Bridged-Hybrid (BEH) C18 (pore diameter 130 Å, particle size 1.7 µm, inner diameter 2.1 mm, length 100 mm) column was used. Norvaline (Sigma-Aldrich, Taufkirchen, Germany) was used as an internal standard with a concentration of 25 µmol L^{-1} (1:100 stock solution 2.5 mmol L^{-1} in 0.1 mol L^{-1} HCl). The injection volume was 100 µL. The column temperature was 43 °C and the sample temperature was 20 °C. The eluent gradient, the flow rate (0.7 mL min^{-1}) and the detection wavelength (260 nm) were applied according to the recommendations of Waters Corporation (Waters, 2012). Cystein was measured as cystin due to the method constraints. The two TEPs and the two insect proteins were analyzed to determine their free amino acid compositions. These values were subtracted from the results of the batch hydrolysates. Consequently, only the product release by the enzymatic liberation was captured.

2.7. Gas chromatography–olfactometry (GC-O)

A volume of 5 mL of sample and 1 g of NaCl were transferred into a 20 mL headspace vial and analyzed by means of gas chromatography–mass spectrometry in combination with olfactometry detection. Regarding solid phase microextraction analysis, samples were incubated in an agitator at 65 °C for 10 min and extracted for 30 min using a 2 cm DVB/CAR/PDMS fiber (Supelco, Merck, Darmstadt, Germany). Desorption was carried out using a SSL injector in splitless mode at 2 mL min^{-1} for 3 min. A GC system 7890B (Agilent Technologies, Waldbronn, Germany) was used with a DB-FFAP (30 m × 0.32 mm × 0.25 µm) column (Agilent J & W GC, Waldbronn, Germany) with a helium flow rate of 2 mL min^{-1}. The oven temperature was programmed from 40 °C (3 min), raised at 6 °C min^{-1} to 230 °C (15 min). The transfer line was kept at 250 °C. Mass spectrometry detection was done by a mass spectrometer MSD 5977B (Agilent Technologies, Waldbronn, Germany) in scan mode from *m/z* 33–300. The split ratio between olfactory port and mass spectrometer was 1:1. The Olfactory Detection Port ODP3 (Gerstel, Muelheim an der Ruhr, Germany) was kept at 250 °C and the effluent was evaluated by two trained panelists in two different chromatographic runs. Odor intensity was evaluated on a scale from one to four. Data treatment was carried out using Mass Hunter Workstation Software Qualitative Analysis Navigator B.08.00 (Agilent Technologies, Waldbronn, Germany). Compounds were identified by their mass spectra in comparison with NIST 2017, version 2.3, and by comparing their retention index with those of reference compounds listed in an internal library. Retention indices of the compounds were calculated by using the retention data of the linear alkane series.

2.8. Sensory evaluation of the insect protein hydrolysates and Maillard products

The samples were defrosted at room temperature (24 °C) for the sensory evaluation and mixed at a ratio of 1:1 with NaCl (0.2% (w/v)) to enhance taste properties. The following samples were selected for the sensory evaluation: The unprocessed proteins as references, the hydrolysates after heat inactivation, the Maillard product with xylose and the hydrolysate, which was heated in a similar way to the Maillard product without additional xylose.

The sensory evaluation of samples was done in two separate sessions with a panel of 13 (for the cricket protein samples) and 15 (for the mealworm protein samples) panelists. All samples were described by the respective panelists ensuring the recommended number of panelists according to descriptive analysis recommendations (Lawless & Heymann, 2010). The attributes umami and bitter were evaluated on a scale of 0–3, 0 referring to not perceivable and 3 referring to strongly perceivable, since the internal tasters were familiar with the 3-point category scale and anchor words were used to help them understand the scale. Before the sensory evaluation of the samples, all panelists took part in a separate training session. Therefore, all panelists took part in a basic panelist screening including basic tastes recognition (sweet: 10 g L^{-1} sugar, salty: 1.5 g L^{-1} NaCl, bitter: 0.5 g L^{-1} caffeine × 2, sour: 0.6 g L^{-1} citric acid, umami: 0.9 g L^{-1} MSG), umami ranking (0.5 g L^{-1} – 4.5 g L^{-1} MSG) and an aroma recognition test. A triangle test was carried out prior to investigating the sensory characteristics of the hydrolysates to guarantee the bitter perception of the panelists. Therefore, a caffeine solution (0.3 g L^{-1}) was used, according to the German standard DIN10959. All panelists identified the caffeine solution and were, therefore, qualified for the taste investigation. The tasting was carried out at room temperature (24 °C). Plain white bread and still water were provided for taste neutralization between the samples. The samples were labeled with three-digit codes and an individual questionnaire was provided for documentation. A two-way ANOVA was done for the evaluation of the sensory attribute's umami and bitter with the product as a fixed factor and the panelist as a random factor to calculate which descriptors were rated significantly ($p < 0.05$) different between samples. Analysis of the attributes with statistically significant differences was followed by Duncan's multiple range test to find out which products differed significantly ($p < 0.05$).

Additionally, the panelists were asked to give a general description of the samples. To investigate the relationship between the sensory attributes of the samples, a Correspondence Analysis (CA) was performed. The CA is a generalization of the Principal Component Analysis (PCA) and allows to highlight potential relationships between categorical variables. A biplot was resorted to visualize the results. The biplot spots both the relationship between the samples among themselves as well as the relation between the categorical sensory attributes. Attributes pointing to the same direction are likely to be elicited together, while attributes pointing in to opposing directions are likely not occurring together. Moreover, the relationship between the samples and the

Table 1
Enzyme activities (EA) of the technical enzyme preparations (TEPs) ProteaseA and Flavourzyme towards 29 synthetic substrates in nkat mL^{-1} TEP, measured at 37 °C, pH 7.0 [Bis-Tris-propane HCl, 100 m*M*], substrate concentration 1 m*M*. Values on black background: > 10-fold higher activity; values on dark grey background: > 5-fold higher activity; values on light grey background: > 2-fold higher activity in comparison to the other TEP.

TEP	ProteaseA	Flavourzyme
Substrate	**$EA_{average}$**	**$EA_{average}$**
(1) Ala-*p*NA	30 ± 0	57 ± 0
(1) Arg-*p*NA	1460 ± 81	2031 ± 46
(1) Asp-*p*NA	844 ± 6	5 ± 0
(1) Gln-*p*NA	492 ± 9	789 ± 10
(1) Glu-*p*NA	0 ± 0	4 ± 0
(1) Gly-*p*NA	2 ± 0	2 ± 0
(1) His-*p*NA	47 ± 1	97 ± 0
(1) Ile-*p*NA	97 ± 1	166 ± 3
(1) Leu-*p*NA	13014 ± 145	22074 ± 635
(1) Lys-*p*NA	904 ± 8	1367 ± 52
(1) Met-*p*NA	5375 ± 235	7321 ± 225
(1) Phe-*p*NA	10522 ± 1181	16758 ± 1018
(1) Pro-*p*NA	1 ± 0	7 ± 0
(1) Tyr-*p*NA	67 ± 1	124 ± 0
(1) Val-*p*NA	178 ± 7	237 ± 5
(2) Z-Ala-Glu-OH	955 ± 133	661 ± 76
(2) Z-Ala-Gly-OH	884 ± 38	655 ± 15
(2) Z-Ala-Leu-OH	978 ± 44	650 ± 17
(2) Z-Ala-Ser-OH	874 ± 58	1005 ± 614
(3) Ala-Pro-*p*NA	345 ± 8	24 ± 0
(3) Lys-Ala-*p*NA	77 ± 0	54 ± 0
(4) Z-Ala-Ala-Gly-*p*NA	1 ± 0	1 ± 0
(4) Z-Ala-Ala-Leu-*p*NA	8162 ± 486	980 ± 65
(4) Z-Ala-Ala-Pro-Leu-*p*NA	64505 ± 10289	4584 ± 157
(4) Z-Arg-*p*NA	0 ± 0	0 ± 0
(4) Z-Asn-*p*NA	0 ± 0	0 ± 0
(4) Z-Gly-Pro-*p*NA	41 ± 3	3 ± 0
(4) Z-Phe-Val-Arg-*p*NA	363613 ± 8589	138791 ± 24275
(4) Z-Tyr-*p*NA	1085 ± 20	116 ± 3

(1) Substrates for aminopeptidases, (2) carboxypeptidases/endopeptidases, (3) dipeptidylpeptidases and (4) endopeptidases.

sensory attributes can be derived from their relative position in a sense that similar directions indicate a higher likelihood to be named for the respective sample. The statistical analyses were carried out using R version 3.6.3 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). and the library FactoMineR: An R Package for Multivariate Analysis.

3. Results & discussion

3.1. Peptidase activities of the technical enzyme preparations selected

The two TEPs, Flavourzyme and ProteaseA, both originate from *A. oryzae*. However, the TEPs are standardized on different activities because they are different from each other. Flavourzyme is standardized on 1000 LAPU g^{-1} referring to leucine amino peptidase units, whereas ProteaseA is standardized on 20,000 U g^{-1} referring to a general endopeptidase activity using Hammertstein casein as a substrate. Furthermore, Flavourzyme is supplied in a liquid formula stabilized with sucrose (30% (w/w)), potassium chloride (10% (w/w)) and potassium sorbate (0.2% (w/w)). ProteaseA is supplied in a solid formula, spray dried and contains 35% dextrin. These differences in standardization units and formulation rouse interest to investigate these two enzyme preparations further in more detail. As seen in Figure S1 in the supplementary material, ProteaseA showed a similar protein pattern but also indicated differences to the detailed characterization of Flavourzyme (Merz, Eisele, et al., 2015). Flavourzyme and ProteaseA were measured for their peptidase activity "fingerprints" to further investigate the differences between both enzyme preparations (Grossmann et al., 2019). The respective exo- and endopeptidase activities with the selected substrates are shown in Table 1. The peptidase specificity of Flavourzyme has been described previously (Grossmann et al., 2019) and these values were included in Table 1 for comparison. The conspicuous values were highlighted to visualize the differences between the two preparations: Values on a black background: > 10-fold higher activity; values on a dark grey background: > 5-fold higher activity; and values on a light grey background: > 2-fold higher activity in comparison to each other. As shown in Table 1, Flavourzyme and ProteaseA showed similar values for all exopeptidase (1) activities, apart from the activity towards Asp-*p*NA, where Flavourzyme showed 5 nkat mL^{-1} and ProteaseA 844 nkat mL^{-1}. A 10-fold higher activity was measured for ProteaseA towards Ala-Pro-*p*NA, referring to one dipeptidyl peptidase activity, and towards several substrates, referring to endopeptidase activities (Z-Ala-Ala-Pro-Leu-*p*NA, Z-Gly-Pro-*p*NA). The enzyme preparation ProteaseA showed a > 5-fold higher activity towards Z-Ala-Ala-Leu-*p*NA, Z-Tyr-*p*NA and 2-fold higher activity towards Z-Phe-Val-Arg-*p*NA. As illustrated by these results, Flavourzyme and ProteaseA showed significant differences in their peptidase specificities, which will result in differences of the composition of their hydrolysates (e.g. free amino acid profiles) and this again will influence the subsequent formation of Maillard products after further heat processing of the hydrolysates in the presence of sugar.

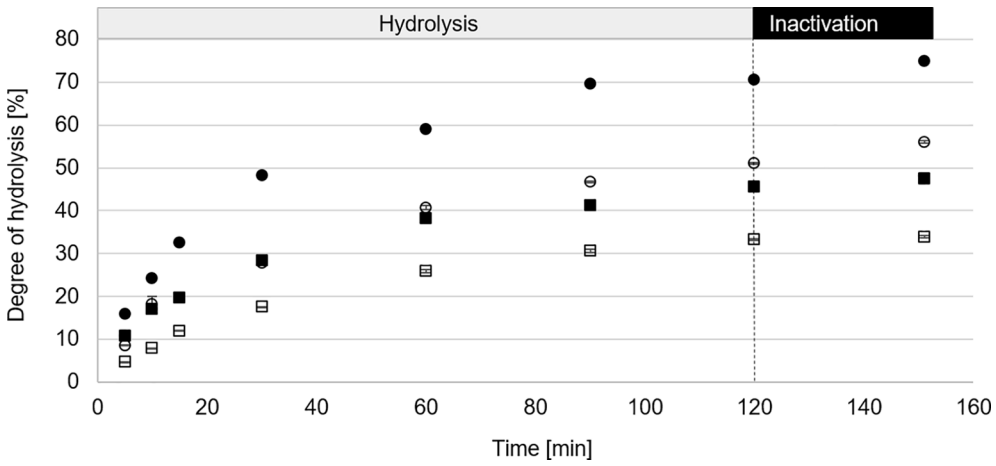

Fig. 1. Batch hydrolysis of cricket- (□ Flavourzyme, ■ ProteaseA) and mealworm protein (○ Flavourzyme, ● ProteaseA) at 50 °C for 2 h, followed by heat inactivation at 90 °C for 15 min. Substrate concentration: 5% protein (w/v), enzyme concentration: 2% (w/v).

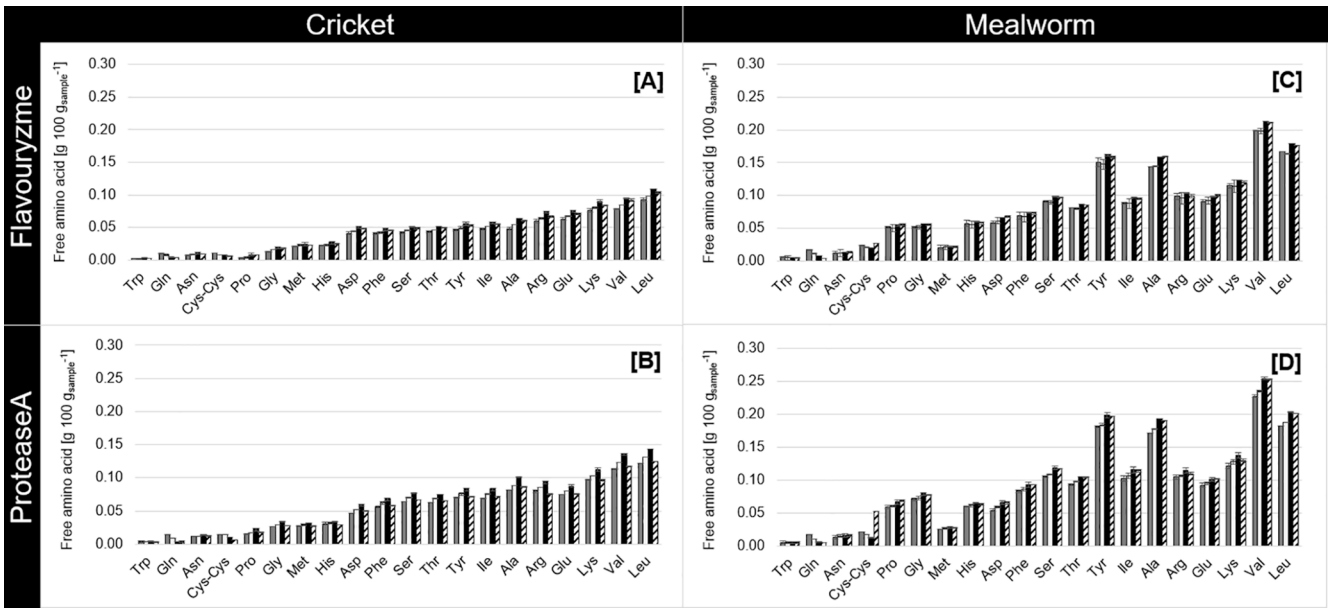

Fig. 2. Free amino acid profiles of the insect hydrolysates after 2 h of hydrolysis (grey bars), heat inactivation (white bars), heat treatment without xylose (black bars) and Maillard reaction step with 1% (w/v) xylose (bars with diagonal black lines). [A] cricket protein hydrolyzed with Flavourzyme, [B] cricket protein hydrolyzed with ProteaseA, [C] mealworm protein hydrolyzed with Flavourzyme, [D] mealworm protein hydrolyzed with ProteaseA. The values refer to the amounts that were liberated during hydrolysis.

3.2. Characteristics of the batch hydrolysates: Degree of hydrolysis and generation of free amino acids

Batch hydrolysates were done with both enzyme preparations, Flavourzyme and ProteaseA, for 2 h. The DH and the free amino acid profiles were determined to investigate the hydrolysates of the insect proteins. The time courses of the DH of the insect protein hydrolysates are shown in Fig. 1. After 2 h, the DH was 46% for ProteaseA and 33% for

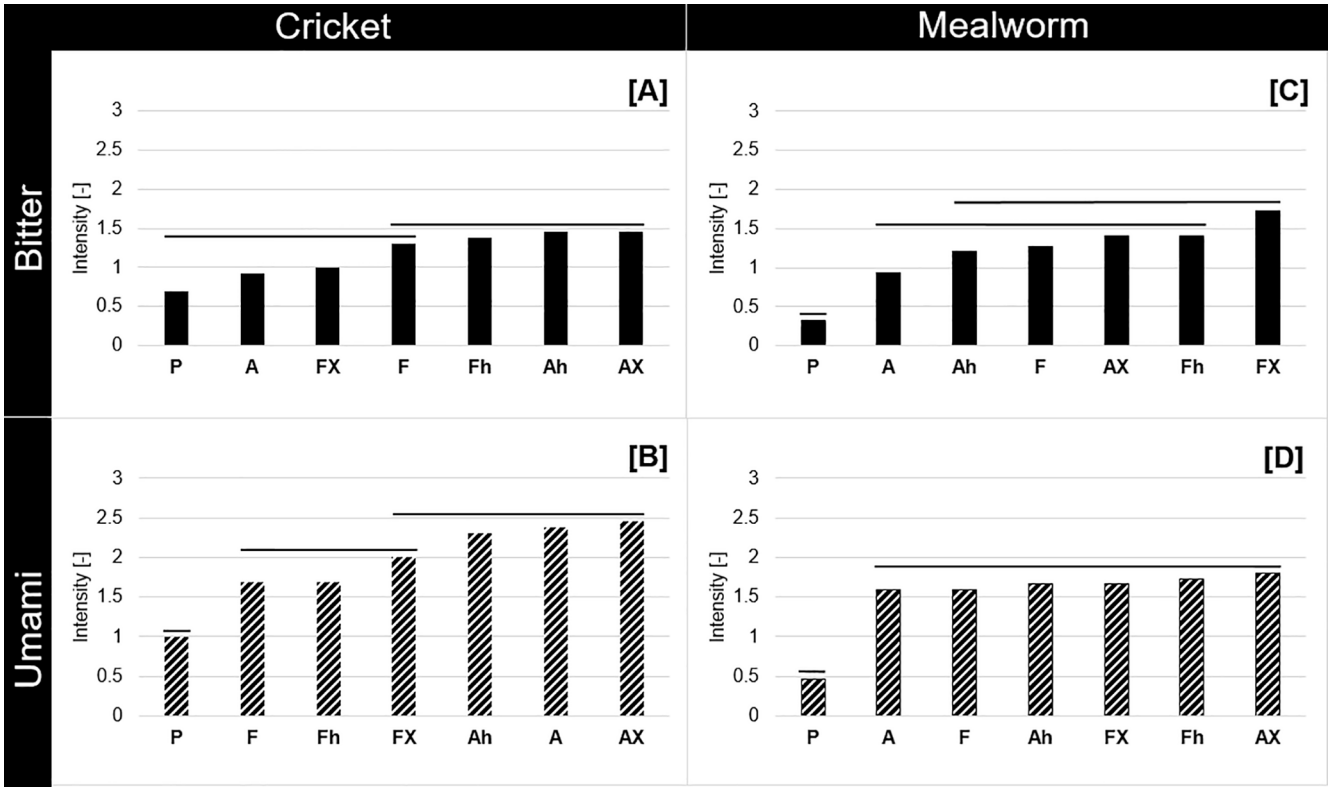

Fig. 3. Sensory evaluation of the differently processed cricket [A, B] and mealworm protein [C, D]. The black bars refer to the attribute bitterness and the black bars with diagonal stripes refer to the attribute umami. The attributes were rated from 0 (not perceivable) to 3 (strongly perceivable). Bars below the same line are not significantly different from each other (Duncan, $p < 0.05$). P, unprocessed insect protein; F, hydrolysate using Flavourzyme; A, hydrolysate using ProteaseA; FX and AX, Maillard products; Fh and Ah, hydrolysates after heat treatment without xylose.

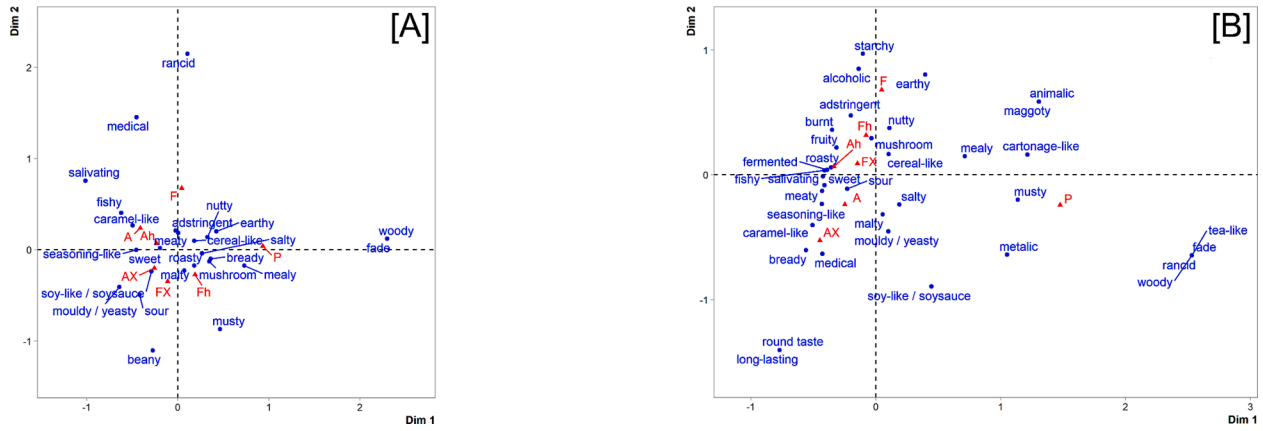

Fig. 4. Correspondence analysis factor map showing the comprehensive sensory evaluation of the differently processed cricket [A] and mealworm proteins [B]. P, unprocessed insect protein; F, hydrolysate using Flavourzyme; A, hydrolysate using ProteaseA; FX and AX, Maillard products; Fh and Ah, hydrolysates after heat treatment without xylose. The figure shows all taste descriptors mentioned by the sensory panelists when tasting the samples for the distinctive qualitative description analysis (●, mentioned descriptors; ▲, particular sample).

Flavourzyme using cricket protein. By comparison, the hydrolysates with mealworm protein resulted in a higher DH of 71% for ProteaseA and 51% for Flavourzyme. The usage of ProteaseA resulted in a higher DH in comparison to Flavourzyme for both insect proteins. One reason might be the higher endopeptidase activities of ProteaseA (Table 1), enabling further hydrolysis of the proteins due to the higher synergistic effects of exo- and endopeptidases (Grossmann et al., 2019). Both TEPs hydrolyzed the mealworm protein to a higher degree than the cricket protein. This increased hydrolysis efficiency could be explained by a more favorable accessibility of the mealworm proteins to the active site of the TEPs due their differences in protein structure, protein sequence and thus, cleavage locations compared to the cricket proteins. The same preference was the case with the liberation of the free amino acids, as shown in Fig. 2. Higher amounts of free amino acids were generally liberated with mealworm protein as the substrate. After the hydrolysis of the cricket protein, a total amount of amino acids of 0.8 g 100 $g^{-1}_{\text{cricket protein}}$ were liberated using Flavourzyme and 1.1 g 100 $g^{-1}_{\text{cricket protein}}$ using ProteaseA. Almost the double amount (1.6 g 100 $g^{-1}_{\text{mealworm protein}}$ for Flavourzyme and 1.8 100 $g^{-1}_{\text{mealworm protein}}$ for ProteaseA) were liberated in the case of the mealworm protein hydrolyses. Apart from the absolute values, both enzyme preparations produced similar profiles of liberated amino acids with each substrate. The preferred liberated amino acids were leucine, isoleucine and lysine for the cricket protein and valine, leucine, tyrosine and alanine for the mealworm protein. After heat treatment of the samples, a slight increase for almost all liberated amino acids was measured since the peptidases did not denature immediately when increasing the temperature of the samples. It was observed that the samples with xylose slightly decreased the concentrations of the amino acids compared to the sample without xylose, see Fig. 2.

3.3. Sensory evaluation of the insect protein hydrolysates and further heat processing with xylose for the generation of Maillard products

Both cricket and mealworm protein hydrolysates showed a quite flat taste in preliminary sensory evaluations (data not shown). It is well-known in food science/industry that peptides and amino acids are highly valued because they can react with reduced carbohydrates and, thereby, affect the product flavor significantly (Van Lancker, Adams, & De Kimpe, 2011). Therefore, it was of interest to investigate flavor modification of cricket and mealworm protein hydrolysates when further processing them through Maillard reactions under defined conditions using both analytical and sensory evaluations.

3.3.1. Evaluation of bitterness and umami

The sensory evaluation of the insect protein samples processed under different conditions are shown in Fig. 3. The unprocessed insect proteins served as references and were compared to the hydrolysates produced enzymatically using Flavourzyme (F) or ProteaseA (A). Those hydrolysates F or A, where xylose was added and heated up, are indicated by FX and AX. The hydrolysates F or A that were only heated without xylose are indicated by the abbreviation Fh and Ah. As seen in Fig. 3A, the bitterness of the cricket protein samples had a tendency to increase after enzymatic hydrolyses, enabling Maillard reactions caused by adding xylose and heating. This was also in the case of the umami taste (Fig. 3B), especially for the samples obtained with ProteaseA. Here, a significant increase of the umami taste was recognized (A, AX, Ah). The same experiments with mealworm protein showed a significant increase in bitterness of the processed samples (Fig. 3C), with the FX sample as the most bitter one compared to all the others. Although the umami attribute was also increased for the mealworm samples after processing (Fig. 3D), no clear link to one of the peptidase preparations, Flavourzyme or ProteaseA, or to the processing steps was recognized. The increase of bitterness after the enzymatic hydrolysis of proteins is a common observation and has been reported previously due to the formation of bitter peptides (Fu, Liu, Hansen, Bredie, & Lametsch, 2018; Temussi, 2012). The increase of umami in Fig. 3D might be explained by the liberation of amino acids, such as glutamic and aspartic acid, which contribute to the umami taste (Poojary, Orlien, Passamonti, & Olsen, 2017; Y. Zhang, Venkitasamy, Pan, & Wang, 2013).

3.3.2. Further distinctive qualitative description analysis

This comprehensive study evaluated the samples concerning their general taste description further to complete the sensory investigations. Fig. 4 shows all the taste descriptors mentioned by the sensory panelists when tasting the samples. Descriptors pointing in the same direction as the samples are likely to describe the main attributes. Samples or attributes further away from the origin (0/0) are better represented than those sitting in the center of the map. Thus, this association is more likely to hold true when the sample and the descriptor are not located around the center. The correspondence analysis for the cricket samples (Fig. 4A-cricket) explains 52% of the total variants (Dim1: 33%, Dim2: 19%). The correspondence analysis for the cricket samples (Fig. 4B-mealworm) explains 61% of the total variants (Dim1: 44%, Dim2: 17%). As shown in Fig. 4A, the cricket protein reference was associated with the attributes fade, woody, mealy and earthy-like. The sensory attributes were changed after enzymatic hydrolysis with Flavourzyme to the descriptors of rancid, medical and fishy, and further generation of Maillard reactions led to a change in the taste attributes, for example, beany, musty, sour, and malty. The sensory descriptions of the hydrolysates and the further reacted samples using ProteaseA were more similar and

Table 2
Odor-active components of cricket and mealworm protein (P), their hydrolysates using Flavourzyme (F) or ProteaseA (A) and their Maillard products (FX, AX) identified using gas chromatography–olfactometry.

No.	Compound	Odor	RI (Wax)	Cricket P	F	FX	A	AX	Mealworm P	F	FX	A	AX	Source	Literature (referring to odor, compound and source)
Alcohols															
1	2-Ethyl-1-hexanol	Green, flowery	1503	2	3		3	1	2	2	1	3	3	Seafood	(Cai et al., 2016; Laohakunjit et al., 2014)
Acids															
2	2-Methylpropanoic acid	Cheesy, fatty	1566						2	2	2	2	1	Seafood	(Lapsongphon et al., 2015)
3	Butanoic acid	Sweaty, rancid	1615	2	1	1	2		2		2			SeafoodInsectsMeat	(Lapsongphon et al., 2015; Mall et al., 2017) (Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018) (Kim et al., 2013; Straßer et al., 2014)
4	2-/3-Methylbutanoic acid	Sweaty	1657	2	1	1	1	1	4	3	4	2	1	Seafood Insects Meat	(Lapsongphon et al., 2015; Mall et al., 2017) (Kiatbenjakul et al., 2015) (Straßer et al., 2014)
5	Hexanoic acid	Sweaty, cheesy	1828	1	3				2	3	2			Seafood Insects Meat	(Laohakunjit et al., 2014) (Bou-Maroun et al., 2011; Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018) (Kim et al., 2013; Song, Zhang, Xiao, Niu, Hayat, & Eric, 2012)
6	Octanoic acid	Green, sweet	2053	2			3	3	2	2	1		1	Seafood Meat	(Laohakunjit et al., 2014) (Kim et al., 2013; Song et al., 2012)
Aldehydes															
7	2-/3-Methylbutanal	Malty	911	3	1	1	3	3	3	2	3		2	Seafood Meat	(Cai et al., 2016; Jónsdóttir, Ólafsdóttir, Chanie, & Haugen, 2008; Schlueter et al., 1999) (Kim et al., 2013; Song et al., 2012; Straßer et al., 2014; Xie et al., 2008)
8	Hexanal	Green	1079	2	2		2	2	2	3	3	2	2	Seafood Insects Meat	(Cai et al., 2016; Ganeko et al., 2007; Han et al., 2018; Jónsdóttir et al., 2008; Laohakunjit et al., 2014; Schlueter et al., 1999) (Bou-Maroun et al., 2011; Mahattanatawee et al., 2018) (Kim et al., 2013; Song et al., 2012; Straßer et al., 2014; Xie et al., 2008)
9	Octanal	Citrus	1290		4	3	2		3	2	2		3	Seafood Insects Meat	(Ganeko et al., 2007; Han et al., 2018; Schlueter et al., 1999) (Mahattanatawee et al., 2018) (Kim et al., 2013; Song et al., 2012; Xie et al., 2008)
10	(E)-2-Octenal	Fatty	1424		2		2	2	3		2	3	4	Seafood Meat	(Han et al., 2018) (Schlueter et al., 1999) (Xie et al., 2008)
11	Methional	Potato	1457	3	4	4	4	4		3	4	4	4	Seafood Insects	(Cai et al., 2016; Ganeko et al., 2007; Schlueter et al., 1999) (Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018)
12	(E)-2-Nonenal	Tallowy, cucumber-like	1533						2	2	1	3	3	Seafood Insects Meat	(Lapsongphon et al., 2015; Schlueter et al., 1999) (Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018) (Straßer et al., 2014; Xie et al., 2008)
13	Phenylacetaldehyde	Honey-like	1636	3	4	4	4	4	4	4	2	4	4	Seafood Meat	(Mall et al., 2017; Schlueter et al., 1999) (Kim et al., 2013; Straßer et al., 2014)
14	(E,E)-2,4-Nonadienal	fatty, green	1693		2	1	2		2	1	2	2		Seafood Insects Meat	(Mall & Schieberle, 2017; Schlueter & Steinhart, 1999) (Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018) (Kim et al., 2013; Song et al., 2012; Straßer et al., 2014; Xie et al., 2008)
15	(E,E)-2,4-Decadienal	Fatty	1800	3	3	3	2	2	1			3	3		(Mall & Schieberle, 2017; Schlueter & Steinhart, 1999)

(*continued on next page*)

Table 2 (*continued*)

No.	Compound	Odor	RI (Wax)	Cricket P	F	FX	A	AX	Mealworm P	F	FX	A	AX	Source	Literature (referring to odor, compound and source)
														Seafood	(Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018)
														Insects	
														Meat	(Kim et al., 2013; Song et al., 2012; Straßer et al., 2014; Xie et al., 2008)
16	Trans-4,5-Epoxy-(E)-2-decenal	Metallic	2005						2	3	4	2	3	Insects	(Kiatbenjakul et al., 2015)
														Meat	(Kim et al., 2013; Straßer et al., 2014)
17	2-Phenyl-2-butenal	Flowery, honey-like	1940		3		2	2						Malt Honey	(Alissandrakis et al., 2007; Vandecan et al., 2010)
Ketones															
18	2,3-Butanedione (diacetyl)	Buttery	982	2	1	1	3	3	3	3	3	1	3	Seafood	(Laohakunjit et al., 2014; Prost, Hallier, Cardinal, Serot, & Courcoux, 2006)
19	2,3-Pentanedione	Buttery	1061	2	1	1	1	1						Seafood	(Ganeko et al., 2007; Schlueter et al., 1999)
20	1-Octen-3-one	Mushroom-like, metallic	1298	3		1		3	1	2	3	3	2	Seafood	(Lapsongphon et al., 2015; Mall et al., 2017; Schlueter et al., 1999)
														Insects	(Bou-Maroun et al., 2011; Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018)
														Meat	(Kim et al., 2013; Straßer et al., 2014)
21	(Z)-1,5-Octadien-3-one	Green, metallic	1382		3	3	2	3	1	4	3			Seafood	(Lapsongphon et al., 2015; Mall et al., 2017; Schlueter et al., 1999)
														Insects	(Bou-Maroun et al., 2011)
														Meat	(Straßer et al., 2014)
24	β -Ionone	Violet-like	1926			1	3	2	3	3	4	1	3	Insects	(Mahattanatawee et al., 2018)
														Mate	(Kawakami & Kobayashi, 1991)
Heterocyclic compounds															
22	2-Acetyl-1-pyrroline	Roasty, sweet	1336	4	4	4	4	3	4	4	4	4	4	Seafood	(Cai et al., 2016; Lapsongphon et al., 2015; Mall et al., 2017)
														Insects	(Kiatbenjakul et al., 2015)
														Meat	(Kim et al., 2013; Straßer et al., 2014)
23	2-Acetyl-2-thiazoline	Popcorn-like	1750	4	3	4	2	3						Seafood	(Schlueter et al., 1999)
														Insects	(Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018)
														Meat	(Straßer et al., 2014; Xie et al., 2008)
25	4-Hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol)	Strawberry-like, caramel-like	2032	3	1	3	3	3	3	4	4	4	4	Seafood	(Lapsongphon et al., 2015)
														Meat	(Kim et al., 2013; Song et al., 2012; Straßer et al., 2014)
26	4-Hydroxy-5-methyl-3 (2H)-furanone (Norfuraneol)	Caramel-like	2121						2				3	MRS: Phe-xylose	(Cui et al., 2019)
Pyrazines															
27	Trimethylpyrazine	Earthy-like	1404	3	1	1	3	2	3	2	2	3	2	Seafood	(Laohakunjit et al., 2014)
														Meat	(Straßer et al., 2014)
28	2-/3-Ethyl-2(3),5-dimethylpyrazine	Popcorn-like	1429	1	2	1		3			2		3	Seafood	(Cai et al., 2016; Laohakunjit et al., 2014)
														Meat	(Kim et al., 2013; Xie et al., 2008)
29	2,3-Diethyl-5-methylpyrazine	Earthy-like	1494		4	3								Seafood	(Laohakunjit et al., 2014)
Volatile phenols															
30	2-Methoxyphenol (Guaiacol)	Smoky	1857						2	2	3	3	1	Seafood	(Jónsdóttir et al., 2008)
														Insects	(Kiatbenjakul et al., 2015; Mahattanatawee et al., 2018)
														Meat	(Kim et al., 2013)
31	4-Methylphenol (*p*-cresol)	Fecal, phenolic	2086	3	4	4	3	4	4	4	4	3	4	Insects	(Kiatbenjakul et al., 2015)
														Meat	(Kim et al., 2013; Song et al., 2012)
32	2-Methoxy-4-vinylphenol	Smoky, clove-like	2204						1	2	2		3	Seafood	(Jónsdóttir et al., 2008)
33	2-Aminoacetophenone	Foxy	2204	2	4				4	4	4	4	4	Seafood	(Lapsongphon et al., 2015; Mall et al., 2017)
														Meat	(Straßer et al., 2014)
34	5-Vinyl-2-methoxyphenol (*p*-vinylguaiacol)	Seasoning-like, smoky	2211			3	3	3						–	–
35	Indole	Fecal, mothball-like	2453		4	2								Seafood	(Lapsongphon et al., 2015)

(*continued on next page*)

Table 2 (*continued*)

No.	Compound	Odor	RI (Wax)	Cricket P	F	FX	A	AX	Mealworm P	F	FX	A	AX	Source	Literature (referring to odor, compound and source)
Lactones															
36	g-Octalactone	Coconut-like	1913							4	4	4	3	Meat	(Kim et al., 2013; Song et al., 2012)
37	g-Decalactone	Fatty, peach-like	2155	1	3	2	4				2	1	2	Seafood Meat	(Mall et al., 2017) (Kim et al., 2013)
38	g-Undecalacton	Flowery	2232	3			4	4						MRS: Cys-glucsoe	(Yang et al., 2015)
Unknown															
39	n.i.	Green	1604						2	3	2	2	3		

associated with mainly savory attributes, such as meaty, seasoning-like, sweet, malty and soy sauce-like. As shown in Fig. 4B, the mealworm protein reference was described with the attributes, for example, woody, fade, musty and metallic. The enzymatic hydrolysis with Flavourzyme changed the taste attributes to earthy, starchy and, after the generation of Maillard reactions, astringent to nutty, mushroom-like and roasty. Similar to the cricket protein samples, the mealworm protein samples hydrolyzed with ProteaseA (hydrolysate and after generating Maillard products) were associated with the attributes meaty, seasoning-like and sweet and were, furthermore, described as long lasting, salivating and with a round taste. In summary, these results showed that the sensory profiles of both insect proteins were modified significantly after processing: These taste profiles resulted in more complex and savory-like profiles. This showed that creative processing of insect proteins generated interesting new taste profiles and demonstrated their flavoring potential.

3.4. Identification of volatile odor-active compounds by GC-O

Thirty-eight odor-active compounds were identified using GC-O and are shown in Table 2. The compounds included 1 alcohol, 5 acids, 11 aldehydes, 5 ketones and 16 heterocyclic compounds, including 3 pyrazines, 6 volatile phenols and 3 lactones. The compounds 2-/3-methylbutanoic acid, phenylacetaldehyde, 2,3-butanedione, 2-acetyl-1-pyrroline, furaneol, trimethylpyrazine and *p*-cresol were identified in both cricket and mealworm protein in all samples. Further compounds that were identified in 80% of the samples were 2-ethyl-hexanol, 2-/3-methylbutanal, hexanal, methional, (*E,E*)-2,4-decadienal and 1-octen-3-one. Seven compounds (2-phenyl-2-butenal, 2,3-pentanedione, 2-acetyl-2-thiazoline, 2,3-diethyl-5-methylpyrazine, 5-vinyl-2-methoxyphenol, indole and g-undecalactone) were only identified in the cricket samples and seven compounds (2-methylpropanoic acid, (*E*)-2-nonenal, *trans*-4,5-epoxy-(*E*)-2-decenal, 4-hydroxy-5-methyl-3(2H)-furanone, 2-methoxyphenol, 2-methoxy-4-vinylphenol and *g*-octalactone) were only identified in the mealworm samples. As shown in Table 2, 16 of the compounds identified have been reported previously as odorants or odor-active compounds of the insect protein powder of *Eisenia foetida* and the edible insect *Lethocerus indicus* (Bou-Maroun & Cayot, 2011; Kiatbenjakul, Intarapichet, & Cadwallader, 2015; Mahattanatawee, Luanphaisarnnont, & Rouseff, 2018). Almost all compounds (30 out of 38) have been reported in seafood, such as fish, prawns and seaweed (Laohakunjit, Selamassakul, & Kerdchoechuen, 2014; Lapsongphon, Yongsawatdigul, & Cadwallader, 2015; Mall & Schieberle, 2017; Schlueter & Steinhart, 1999). Since insects are targeted to be an alternative source of protein to conventional meat production, it is interesting to note that most of the odor-active compounds have been reported in meat products, for example, cooked beef (Kim, Cadwallader, Kido, & Watanabe, 2013), roasted pork (Xie, Sun, Zheng, & Wang, 2008) and roasted livers of beef, duck and pork (Straßer & Schieberle, 2014). Four compounds (2-phenyl-2-butenal, norfuraneol, *p*-vinylguaiacol and *g*-undecalactone) were reported previously in other sources, such as malt, honey or coffee (Alissandrakis, Tarantilis, Harizanis, & Polissiou, 2007; Heinrich & Baltes, 1987; Vandecan, Saison, Schouppe, Delvaux, & Delvaux, 2010) or in model reaction systems of amino acids and reducing sugars (Cui, Yu, Xia, Duhoranimana, Huang, & Zhang, 2019; Yang et al., 2015). Most compounds were described as green, earthy or potato/mushroom-like. Twelve of the compounds identified were associated with fatty, sweaty, cheesy or popcorn odor description. The remainder of the odor-active compounds were described as honey-like, caramel-like, roasted or flowery. Volatile phenols were associated with smoky and fecal odors.

The variety of descriptors show how diverse the odor profile of processed cricket and mealworm proteins are. Volatile analysis supported the sensory evaluation; the latter refers to both pleasant and unpleasant notes and stresses the diversity of the sample attributes. Although no clear correlation could be made between specific odor-active compounds and the different process steps (hydrolysis, Maillard reaction), generic aroma profiles of the samples were different for the insect proteins and both process steps (see Table 2); for example, 2-Acetyl-2-thiazoline (compound 23) for cricket protein versus 2-Methoxyphenol (Guaiacol, compound 30) for mealworm protein. Changes due to processing were shown by e.g. Methional (compound 11) for mealworm protein or (Z)-1,5-Octadien-3-one (compound 21) for cricket protein. Consequently, processing of cricket and mealworm proteins enhanced the flavor profile complexity. This study showed a proof of principle to further optimize the processing steps with insect proteins in the future to achieve a savory- and meat-like flavor profile while simultaneously reducing undesirable aromas.

4. Conclusions

This study showed that the sensory properties of both cricket and mealworm protein were changed using enzymatic hydrolysis and the subsequent generation of Maillard reactions by adding xylose and incubation at 98 °C for 30 min. This was shown by both the sensory evaluation of bitter and umami intensity and the qualitative description analysis. The hydrolysates were a suitable source for the application of Maillard reactions due to the liberation of free amino acids and peptides during enzymatic hydrolysis. The sensory evaluation showed a generally slight increase of bitterness and a differently strong increase of umami taste for the cricket protein and strong increase of bitter and umami taste for the mealworm protein. The sensory evaluation generally showed that the profiles of both processed insect proteins were modified significantly by the processing steps to more complex and savory-like taste profiles. These samples delivered a mushroom-like and meaty taste and relevant odor-active compounds of processed seafood and meat were identified using GC-O. Thus, it was demonstrated that the sensory properties of cricket and mealworm protein can be modified by enzymatic hydrolysis alone or with additional Maillard reactions, using in this case xylose as reducing sugar. The use of insect proteins as, for example, a food supplement to increase the protein content and nutritional value of a food will benefit from the creative processing of the insect proteins prior to application.

CRediT authorship contribution statement

Kora Kassandra Grossmann: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization. **Michael Merz:** Methodology, Formal analysis, Writing - review & editing. **Daniel Appel:** Resources, Project administration. **Maria Monteiro De Araujo:** Methodology, Validation, Writing - review & editing. **Lutz Fischer:** Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256–1262.

Alissandrakis, E., Tarantilis, P. A., Harizanis, P. C., & Polissiou, M. (2007). Comparison of the volatile composition in thyme honey from several origins in Greece. *Journal of Agricultural and Food Chemistry, 55*(20), 8152–8157. https://doi.org/10.1021/jf071442y.

Berends, P., Appel, D., Eisele, T., Rabe, S., & Fischer, L. (2014). Performance of enzymatic wheat gluten hydrolysis in batch and continuous processes using Flavourzyme. *LWT - Food Science and Technology, 58*(2), 534–540. https://doi.org/10.1016/j.lwt.2014.03.035.

Berends, P., Merz, M., Kochjohann, A., Philipps, L., Blank, I., Stressler, T., & Fischer, L. (2016). Sensory and antigenic properties of enzymatic wheat gluten hydrolysates produced in an enzyme membrane reactor in comparison with batch. *European Food Research and Technology, 243*(5), 807–816. https://doi.org/10.1007/s00217-016-2794-x.

Bou-Maroun, E., & Cayot, N. (2011). Odour-active compounds of an *Eisenia foetida* protein powder. Identification and effect of delipidation on the odour profile. *Food Chemistry, 124*(3), 889–894. https://doi.org/10.1016/j.foodchem.2010.07.014.

Cabuk, B., & Yilmaz, B. (2020). Fortification of traditional egg pasta (erişte) with edible insects: Nutritional quality, cooking properties and sensory characteristics evaluation. *Journal of Food Science and Technology, ahead of Print.*. https://doi.org/10.1007/s13197-020-04315-7.

Cai, L., Li, D., Dong, Z., Cao, A., Lin, H., & Li, J. (2016). Change regularity of the characteristics of Maillard reaction products derived from xylose and Chinese shrimp waste hydrolysates. *LWT - Food Science and Technology, 65*, 908–916. https://doi.org/10.1016/j.lwt.2015.09.007.

Cui, H., Yu, J., Xia, S., Duhoranimana, E., Huang, Q., & Zhang, X. (2019). Improved controlled flavor formation during heat-treatment with a stable Maillard reaction intermediate derived from xylose-phenylalanine. *Food Chemistry, 271*, 47–53. https://doi.org/10.1016/j.foodchem.2018.07.161.

EFSA (European Food Safety Authority) Scientific Committee. (2015). Risk profile related to production and consumption of insects as food and feed. *EFSA Journal, 13*, 4257.

Fasolin, L. H., Pereira, R. N., Pinheiro, A. C., Martins, J. T., Andrade, C. C. P., Ramos, O. L., & Vicente, A. A. (2019). Emergent food proteins – Towards sustainability, health and innovation. *Food Research International, 125*, Article 108586. https://doi.org/10.1016/j.foodres.2019.108586.

Fu, Y., Liu, J., Hansen, E. T., Bredie, W. L. P., & Lametsch, R. (2018). Structural characteristics of low bitter and high umami protein hydrolysates prepared from bovine muscle and porcine plasma. *Food Chemistry, 257*, 163–171. https://doi.org/10.1016/j.foodchem.2018.02.159.

Ganeko, N., Shoda, M., Hirohara, I., Bhadra, A., Ishida, T., Matsuda, H., … Matoba, T. (2007). Analysis of volatile flavor compounds of sardine (*Sardinops melanostica*) by solid phase microextraction. *Journal of Food Scienc, 73*(1), S83–S88. https://doi.org/10.1111/j.1750-3841.2007.00608.x.

Govorushko, S. (2019). Global status of insects as food and feed source: A review. *Trends in Food Science & Technology, 91*, 436–445. https://doi.org/10.1016/j.tifs.2019.07.032.

Grossmann, K. K., Merz, M., Appel, D., & Fischer, L. (2019). A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis. *European Food Research and Technol, 245*(1). https://doi.org/10.1007/s00217-019-03280-6.

Han, J.-R., Yan, J.-N., Sun, S.-G., Tang, Y., Shang, W.-H., Li, A.-T., … Xiong, Y. L. (2018). Characteristic antioxidant activity and comprehensive flavor compound profile of scallop (*Chlamys farreri*) mantle hydrolysates-ribose Maillard reaction products. *Food Chemistry, 261*, 337–347. https://doi.org/10.1016/j.foodchem.2018.04.044.

Heinrich, L., & Baltes, W. (1987). Occurrence of phenols in coffee melanoidins. *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung, 185*(5), 366–370.

Jongema, Y. (2012). *List of edible insect species of the world.* Wageningen.

Jónsdóttir, R., Ólafsdóttir, G., Chanie, E., & Haugen, J.-E. (2008). Volatile compounds suitable for rapid detection as quality indicators of cold smoked salmon (*Salmo salar*). *Food Chemistry, 109*(1), 184–195. https://doi.org/10.1016/j.foodchem.2007.12.006.

Kawakami, M., & Kobayashi, A. (1991). Volatile constituents of green mate and roasted mate. *Journal of Agricultural and Food Chemistry, 39*(7), 1275–1279. https://doi.org/10.1021/jf00007a016.

Kiatbenjakul, P., Intarapichet, K.-O., & Cadwallader, K. R. (2015). Characterization of potent odorants in male giant water bug (*Lethocerus indicus* Lep. and Serv.), an important edible insect of Southeast Asia. *Food Chemistry, 168*, 639–647. https://doi.org/10.1016/j.foodchem.2014.07.108.

Kim, H., Cadwallader, K. R., Kido, H., & Watanabe, Y. (2013). Effect of addition of commercial rosemary extracts on potent odorants in cooked beef. *Meat Science, 94*(2), 170–176. https://doi.org/10.1016/j.meatsci.2013.01.005.

Laohakunjit, N., Selamassakul, O., & Kerdchoechuen, O. (2014). Seafood-like flavour obtained from the enzymatic hydrolysis of the protein by-products of seaweed (*Gracilaria* sp.). *Food Chemistry, 158*, 162–170. https://doi.org/10.1016/j.foodchem.2014.02.101.

Lapsongphon, N., Yongsawatdigul, J., & Cadwallader, K. R. (2015). Identification and characterization of the aroma-impact components of Thai fish sauce. *Journal of Agricultural and Food Chemistry, 63*(10), 2628–2638. https://doi.org/10.1021/jf5061248.

Lawless, H. T., & Heymann, H. (2010). Descriptive Analysis. In H. T. Lawless, & H. Heymann (Eds.), *Sensory Evaluation of Food – Principles and Practices* (pp. 227–258). New York Dordrecht Heidelberg London: Springer.

Leni, G., Soetemans, L., Caligiani, A., Sforza, S., & Bastiaens, L. (2020). Degree of hydrolysis affects the techno-functional properties of lesser mealworm protein hydrolysates. *Foods, 9*(4), 381. https://doi.org/10.3390/foods9040381.

Mahattanatawee, K., Luanphaisarnnont, T., & Rouseff, R. (2018). Comparison of aroma character impact volatiles of thummong leaves (*Litsea petiolata* Hook. f.), Mangdana water beetle (*Lethocerus indicus*), and a commercial product as flavoring agents in Thai traditional cooking. *Journal of Agricultural and Food Chemistry, 66*(10), 2480–2484. https://doi.org/10.1021/acs.jafc.7b01499.

Mall, V., & Schieberle, P. (2017). Evaluation of key aroma compounds in processed prawns (whiteleg shrimp) by quantitation and aroma recombination experiments. *Journal of Agricultural and Food Chemistry, 65*(13), 2776–2783. https://doi.org/10.1021/acs.jafc.7b00636.

Merz, M., Appel, D., Berends, P., Rabe, S., Blank, I., Stressler, T., & Fischer, L. (2015). Batch-to-batch variation and storage stability of the commercial peptidase preparation Flavourzyme in respect of key enzyme activities and its influence on process reproducibility. *European Food Research Technology, 242*(7), 1005–1012. https://doi.org/10.1007/s00217-015-2606-8.

Merz, M., Eisele, T., Berends, P., Appel, D., Rabe, S., Blank, I., … 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. https://doi.org/10.1021/acs.jafc.5b01665.

Merz, M., Ewert, J., Baur, C., Appel, D., Blank, I., Stressler, T., & Fischer, L. (2015). Wheat gluten hydrolysis using isolated Flavourzyme peptidases: Product inhibition and determination of synergistic effects using response surface methodology. *Journal of Molecular Catalysis B: Enzymatic, 122*, 218–226. https://doi.org/10.1016/j.molcatb.2015.09.010.

Nielsen, P. M., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science: Food Chemistry and Toxicology, 66*(5), 642–646.

Nowak, V., Persijn, D., Rittenschober, D., & Charrondiere, U. R. (2016). Review of food composition data for edible insects. *Food Chemistry, 193*, 39–46. https://doi.org/10.1016/j.foodchem.2014.10.114.

Osimani, A., Milanović, V., Cardinali, F., Roncolini, A., Garofalo, C., Clementi, F., … Aquilanti, L. (2018). Bread enriched with cricket powder (*Acheta domesticus*): A technological, microbiological and nutritional evaluation. *Innovative Food Science & Emerging Technologies, 48*, 150–163. https://doi.org/10.1016/j.ifset.2018.06.007.

Poojary, M. M., Orlien, V., Passamonti, P., & Olsen, K. (2017). Enzyme-assisted extraction enhancing the umami taste amino acids recovery from several cultivated mushrooms. *Food Chemistry, 234*, 236–244. https://doi.org/10.1016/j.foodchem.2017.04.157.

Prost, C., Hallier, A., Cardinal, M., Serot, T., & Courcoux, P. (2006). Effect of storage time on raw sardine (*Sardina pilchardus*) flavor and aroma quality. *Journal of Food Science, 69*(5), S198–S204.

Purschke, B., Meinlschmidt, P., Horn, C., Rieder, O., & Jäger, H. (2017). Improvement of techno-functional properties of edible insect protein from migratory locust by enzymatic hydrolysis. *European Food Research and Technology, 244*(6), 999–1013. https://doi.org/10.1007/s00217-017-3017-9.

Roncolini, A., Milanovic, V., Aquilanti, L., Cardinali, F., Garofalo, C., Sabbatini, R., … Osimani, A. (2020). Lesser mealworm (*Alphitobius diaperinus*) powder as a novel baking ingredient for manufacturing high-protein, mineral-dense snacks. *Food Research International, 131*, Article 109031. https://doi.org/10.1016/j.foodres.2020.109031.

Schlueter, S., & Steinhart, H. (1999). Changes in the odorants of boiled carp fillet (*Cyprinus carpio L.*) as affected by increasing methionine levels in feed. *Journal of Agricultural and Food Chemistry, 47*, 5146–5150.

Song, S., Zhang, X., Xiao, Z., Niu, Y., Hayat, K., & Eric, K. (2012). Contribution of oxidized tallow to aroma characteristics of beeflike process flavour assessed by gas chromatography–mass spectrometry and partial least squares regression. *Journal of Chromatography A, 1254*, 115–124. https://doi.org/10.1016/j.chroma.2012.07.056.

Straßer, S., & Schieberle, P. (2014). Characterization of the key aroma compounds in roasted duck liver by means of aroma extract dilution analysis: Comparison with beef and pork livers. *European Food Research and Technology, 238*(2), 307–313. https://doi.org/10.1007/s00217-013-2095-6.

Temussi, P. A. (2012). The good taste of peptides. *Journal of Peptide Science, 18*(2), 73–82. https://doi.org/10.1002/psc.1428.

Van Lancker, F., Adams, A., & De Kimpe, N. (2011). Chemical modifications of peptides and their impact on food properties. *Chemical Reviews, 111*(12), 7876–7903. https://doi.org/10.1021/cr200032j.

Vandecan, S. M., Saison, D., Schouppe, N., Delvaux, F., & Delvaux, F. R. (2010). Optimisation of specialty malt volatile analysis by headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry. *Analytica Chimica Acta, 671*(1–2), 55–60. https://doi.org/10.1016/j.aca.2010.05.009.

Waters. (2012). Acquity® UPLC H-Class and H-Class Bio Amino Acid Analysis System Guide. Revision B. (Revision B.).

Xie, J., Sun, B., Zheng, F., & Wang, S. (2008). Volatile flavor constituents in roasted pork of Mini-pig. *Food Chemistry, 109*(3), 506–514. https://doi.org/10.1016/j.foodchem.2007.12.074.

Yang, Z., Xie, J., Zhang, L., Du, R., Cao, C., Wang, M., … Sun, B. (2015). Aromatic effect of fat and oxidized fat on a meat-like model reaction system of cysteine and glucose. *Flavour and Fragrance Journal, 30*(4), 320–329. https://doi.org/10.1002/ffj.3248.

Zhang, S., Zhang, C., Qiao, Y., Xing, L., Kang, D., Khan, I. A., … Zhou, G. (2017). Effect of Flavourzyme on proteolysis, antioxidant activity and sensory qualities of Cantonese bacon. *Food Chemistry, 237*, 779–785. https://doi.org/10.1016/j.foodchem.2017.06.026.

Zhang, Y., Venkitasamy, C., Pan, Z., & Wang, W. (2013). Recent developments on umami ingredients of edible mushrooms – A review. *Trends in Food Science & Technology, 33*(2), 78–92. https://doi.org/10.1016/j.tifs.2013.08.002.

Zielińska, E., Baraniak, B., Karaś, M., Rybczyńska, K., & Jakubczyk, A. (2015). Selected species of edible insects as a source of nutrient composition. *Food Research International, 77*, 460–466. https://doi.org/10.1016/j.foodres.2015.09.008.

4.2 SUPPORTING INFORMATION

"New insights into the flavoring potential of cricket (Acheta domesticus) and mealworm (Tenebrio molitor) protein hydrolysates and their Maillard products"

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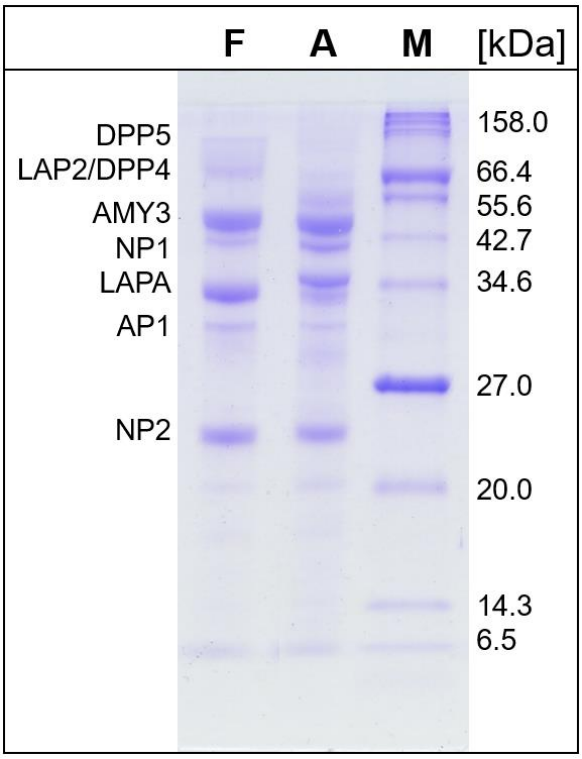

Figure.S14: SDS-PAGE with Coomassie staining of protein fractions of Flavourzyme (*F*) and ProteaseA “Amano”2SD (A). M indicates the protein marker (broad range, 2−212 kDa; NEB, Frankfurt, Germany). Protein load was 5 µg. Neutral protease 1 (NP1); neutral protease 2 (NP2), alkaline protease 1 (AP1); leucine aminopeptidase A (LAPA); α-amylase A type 3 (AMY3); leucine aminopeptidase 2 (LAP2); dipeptidyl peptidase 5 DPP5); dipeptidyl peptidase 4 (DPP4) as described for Flavourzyme by Merz et al.*.

*Merz, M., Eisele, T., Berends, P., Appel, D., Rabe, S., Blank, I., . . . 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. https://doi.org/10.1021/acs.jafc.5b01665

5 CHAPTER FIVE: FINAL DISCUSSION

Various tribes and cultures from the Middle and Near East have been applying enzymes for food processing since early civilizations have existed [116]. Consequently, enzymatic processing has been used in many areas going far beyond the food processing sector (see section 1.2 Industrial Relevance, Production and Applications of Proteolytic Enzymes). The study of enzymes has been taken up significantly by the scientific community to expand on topics such as their structure, function, regulation and kinetics [145], although enzymes were only termed as such in the 19th century.[146]. The interest in generating new insights into the usage of enzymes, more precisely, the usage of technical enzyme preparations (TEPs), was the driver of this dissertation and the foundation of the three scientific articles presented in chapters two to four. The three publications investigated TEPs not only for their substrate spectra and peptidase activities but also - and partly resulting therefrom - for the resulting impact on the hydrolysate characteristics (i.e., degree of hydrolysis, liberation of free amino acids, sensory properties). Although discussed in detail in the respective publications, this section gives a final perspective on these pieces of research, and their main conclusions, and a brief outlook into future research in this field.

As defined in this study, TEPs are enzymes with moderate purity to be used in food processing. They are processed biocatalysts that are derived, within this study, mainly from microbial sources. Apart from the differences in origin brought about by nature, the processing and potential purification steps, defined by the enzyme producers, also influence the properties and activities of the TEPs. Nowadays, TEPs are used by food manufacturers based on the supplier's information that usually states the main enzyme activity and includes information on side activities only in some cases. Defined by one peptidase activity (e.g. Flavourzyme 1000L, defined by leucine aminopeptidase units using the synthetic substrate Leu-*p*NA) or more general peptidase activities (e.g. Promod278, defined by casein protease units using a natural substrate casein), the data sheets of the TEPs provide insights only into this activity. In some cases, potential side activities are mentioned, but the lack of a measuring system to determine a range of peptidase activities was the driver of the development of "*A fast and novel approach to evaluate technical enzyme preparations for an efficient protein hydrolysis"* [147]*.*

An automated photometric analyzer (Gallery^{TM} Plus) was used to install a determination methodology for the enzyme activities of TEPs. Based on two specific photometric assays, (*p*NA and OPA), the measurement approach covered 32 synthetic and natural substrates to generate detailed and comprehensive information on the proteolytic potential, namely, endo- and exopeptidase activities. The established so-called “activity fingerprints” (AFPs) covered five different groups of peptidase activities: (1) aminopeptidase, (2) carboxy-peptidase, (3) dipeptidylpeptidase, (4) endopeptidase activities particularly, and (5) the proteolytic activity towards lupine protein as a natural substrate. As shown in the study, the AFPs did not only present detailed information about the substrate spectra and peptidase side activities but also observed batch variations of Flavourzyme1000L. These insights can be crucial, for example, for industrial applications of Flavourzyme1000L as side activities of batch variations may have an impact on the final product properties. Similar to the standardization of one activity, batch-to-batch variations have been investigated in other studies but also only on the basis of one activity, namely, using azocasein as a substrate to determine endopeptidase activity [148]. By contrast, this first study widened the determination of peptidase activities and substrate spectra by the established AFPs of the TEPs.

After setting up the system and the respective methodology, it was of interest to investigate further whether the information generated by the AFPs would impact properties of the enzymatic hydrolysis applied. Batch hydrolyses of lupine protein were done, followed by the analysis of the hydrolysates based on their DH and the liberation of free amino acids. Several TEPs were selected due their differentiating AFPs and, as shown in this first study, tendencies for a correlation between the AFPs and the hydrolysate properties could be drawn: TEPs containing a wide range of both exo- and endopeptidase activities, resulted in a high DH, such as in the case of Flavourzyme1000L with a DH_{8h} of 52.62 ± 0.14% or FP51 with a DH_{8h} of 58.84 ± 0.32% after 8 h of hydrolysis. The TEPs containing a less complex AFP and/or mainly endopeptidase activities resulted in a lower DH, for example, Alcalase2.4L with a DH_{8h} of 14.70 ± 0.22% or P278 with a DH_{8h} of 17.42 ± 0.23%. Regarding the liberation of free amino acids, tendencies were also observed indicating that the information generated in the AFPs of the TEPs could be transferred to the liberation of free amino acids. Based on the insights generated and these results, TEPs with complementary activities were selected and combined to

investigate the influence on increasing the DH and the liberation of free amino acids. As shown in the study, both combinations of complementary activities selected increased the DH by 47% and 9% for the combinations of P278-DZM and FP51-PepR, respectively.

Based on this work, the newly developed approach was successfully installed as a reliable system for the wider investigation and determination of activity patterns for TEPs. The determination and measurement of an AFP was, dependent on the TEP, completed in less than 1 h, which is (based on the authors experience) six to eight times faster than the manual determination. The new approach delivered detailed information of numerous TEPs on substrate spectra and peptidase activities and provided insights into side activities and batch-to-batch variations. As knowledge of the AFP has been demonstrated to be applicable to other hydrolysate properties, such as the DH and free amino acids, TEPs could be applied more systematically to protein hydrolysis to obtain higher TEP efficiency in industrial processes. This first study and the results presented enabled the foundation of the two following publications and was, thus, of high value. Professional experience in R&D in the food industry has confirmed that this study has generated valuable insights into TEPs by enabling various experiments to go beyond -trial and error- by systematically selecting TEPs based on their AFPs.

In terms of the outlook for future research, the AFPs could be extended beyond peptidase activities. Side activities from other enzyme sectors are seldom known, such as amylase or glutaminase side activities of proteolytic TEPs. If overlooked, this can lead to unexpected changes during the shelf life of food products. A further extension of the screening system to additional enzyme segments (e.g. amylases, lipases) could even increase the knowledge of the activity patterns and potentially also identify side activities.

Following these first findings after the system setup and method development, it was of further interest to gain insights into the "*Impact of peptidase activities on plant protein hydrolysates regarding bitter and umami taste*" [144], the second study of this dissertation. As the first study had shown that the activity patterns of TEPs could be connected to the resulting hydrolysate properties, such as the DH and free amino acid profiles, this second study aimed at additionally investigating the influence on sensory attributes. Three plant proteins, namely, soy (*Glycine Max*. L.), pea (*Pisum Sativum*) and canola (*Brassica napus* and/or *Brassica juncea*) protein, were selected as they are recognized as important

protein sources and their market shares are continuously increasing due to the growing consumer preferences for alternative proteins [88]. Six commercially available TEPs were selected after testing 67 TEPs based on their substrate selectivity and the corresponding AFPs. The six TEPs were chosen as their respective activities differed the most from each other in selectivity or total activity value. The hypothesis of the authors was, that if these TEPs were applied to protein hydrolysis, the resulting hydrolysates should also differ in their sensory profiles. The DH and free amino acid profiles were determined before investigating the sensory profiles of the hydrolysates. Although this study used other plant proteins (e.g. soy, pea, canola) than the first study (i.e. lupine protein), similar findings were revealed regarding the connection of activity patterns (AFPs) to the properties of the DH and free amino acid profiles. The TEP P6SD, for example, was the most active of all the substrates tested (689,270 nkat mL^{-1}). It also resulted in the highest DH in all batch hydrolyses with a DH of 72, 70 and 52% for soy, pea, and canola protein, respectively, and liberated the highest total amounts of free amino acids with 4.86, 3.29 and 4.49 g 100 g $sample^{-1}$ of the soy, pea and canola protein hydrolysates, respectively. These results were in line with previous studies that investigated commercial peptidases [149]. Regarding the sensory evaluation, a special focus was placed on the taste attributes umami and bitter. As these two attributes have been intensively studied within the context of protein hydrolysis [132, 150], it was of special interest to set the activity profiles of the TEPs into context with the resulting umami and bitter taste of the resulting hydrolysates. The sensory results showed that the hydrolysates were perceived as bitter and/or umami depending on the usage of TEP and, thus, on the liberation of specific free amino acids that are known to contribute to the umami or bitter taste. As an example, the TEP PepR showed the highest activity toward H-Pro-*p*NA with 535 nkat mL^{-1} and was rated low for the soy and pea protein hydrolysates and even less bitter than the reference for the canola protein hydrolysate. Therefore, these findings were in line with studies reported previously of prolyl-specific peptidases showing debittering effects [132, 151]. The activity patterns of the TEPs were analyzed using random forest techniques. The model applied connected the activity values with the resulting taste of the hydrolysates and showed, *inter alia*, that endopeptidase activities towards the substrates Z-Ala-Ala-Gly-*p*NA, Z-Asn-*p*NA, and Z-Arg-*p*NA were rated as the most important ones for the bitter attribute - a finding that was in line with data reported previously showing that

the peptides liberated by endopeptidase activities influence the bitter taste perception [152]. Additionally, the connection between the exopeptidase activities of the TEPs and the umami taste attribute was shown as the substrates for the aminopeptidase activities indicated the highest impact on the umami attribute: all 15 substrates for aminopeptidase activities were rated more important than the other activities, with the activities toward Glu-*p*NA, Pro-*p*NA, Ala-*p*NA and Asp-*p*NA being the four most important ones. These results were in line with other studies that connected peptides with glutamic acid, alanine and asparagine [153] or other exopeptidase treatment [154], resulting in the umami taste. The additional screening of the total 67 TEPs (internal database) mentioned previously according to the random forest model, positioned the six TEPs selected in the forecasted impact based on the umami and bitter attributes. The model did not only confirm the initial hypothesis, i.e. that the six TEPs selected would also differ in their sensory attributes, but also showed that ProteAX-K would deliver an high umami and low bitter taste profile, as had also been confirmed in studies reported previously [154-155]. Thus, it needs to be mentioned that the product of an enzymatic hydrolysis is dependent on a variety of factors, such as process conditions and substrate/enzyme composition. Therefore, findings need to be put in the context of the respective conditions.

In conclusion, the second study of this dissertation did not only widen the connection between the activity patterns of TEPs and the properties of the DH and free amino acid profiles to other substrates (namely soy, pea and canola protein) but also showed that the respective TEP activities influenced the umami and bitter taste of the resulting hydrolysates. As this approach could contribute further to the forecasting of taste impacts on protein hydrolysis, it could be of additional interest to investigate the formation of umami and bitter peptides as an extra connection step between the activity patterns and the taste profiles of the hydrolysates. Further peptide analytics and sensory evaluation could be useful for expanding this approach. Moreover, a forecast of the sensory attributes of protein hydrolysates from analytical data, such as from the AFPs, can be of great value for the R&D applied in the food industry considering the targeted application of TEPs and the formation of target taste and flavor development. Regarding the outlook for industrial upscaling, the enzyme dosage could be optimized. Concerning the present study, the final TEP concentration was 2% (v/v) or (w/v), depending on the TEP's type of formulation (liquid or solid). As mentioned in the materials and methods section of the publication,

2% enzyme dosage was chosen to reach an equilibrium of the DH after a duration of 2 h. The hydrolysis timeframe of 2 h was selected because of internal safety regulations for the subsequent sensory characteristics after hydrolysis. This enzyme dosage could be optimized to determine a cost-effective concentration for an industrial upscaling.

Whereas the first two studies investigated the impact of enzymatic hydrolysis on plant proteins (i.e. lupine, soy, pea and canola), the third study built on the resulting knowledge and developed "*New insights into the flavoring potential of cricket (Acheta domesticus) and mealworm (Tenebrio molitor) protein hydrolysates and their Maillard products"* [156]. Both science and the food industry are investigating alternative food sources to cover the increasing demand for food from a growing world population (July 2021: world population of over 7.7 billion people [73b]). Insects have been part of the human diet for many centuries, especially in the Asian, Latin American and African regions [157]. Although there are hundreds of edible insect species worldwide, the European Food Safety Authority lists twelve insect species which have potential to be used as food and feed in the European Union [103]. *Acheta domesticus* (crickets) and *Tenebrio molitor* (mealworms) belong to this list and were chosen for this study to investigate the sensory impact of both enzymatic hydrolysis and subsequent Maillard reaction. Following the enzymatic hydrolysis using once Flavourzyme1000L and once ProteaseA "Amano"2SD, xylose was added as a reducing sugar to promote the Maillard reaction. The samples were then investigated for their DH, free amino acid profiles, umami and bitter taste, odor-active compounds and other distinctive qualitative descriptive analyses were carried out. Also, both Flavourzyme and ProteaseA were measured for the peptidase activity patterns by the implemented method of the first study [147]. Although both enzymes applied derived from *A. oryzae*, differences in their specific substrate activities were determined. ProteaseA, for example, showed over 10-fold higher activities towards the substrates Asp-*p*NA, Ala-Pro-*p*NA, Z-Ala-Ala-Pro-Leu-*p*NA and Z-Phe-Val-Arg-*p*NA. The usage of ProteaseA also resulted in higher values for the DH of both cricket at 46% and mealworm protein at 71% compared to using Flavourzyme with 33 and 51%, respectively. Although the free amino acid profiles were similar, ProteaseA released higher total amounts of free amino acids and, therefore, goes along with the previous findings [147] that might be explained by the differences in peptidase activities (synergistic effects of both endo- and exopeptidase activities), enabling the further hydrolysis and release of amino acids. As sensory data on insect

hydrolysates are few, the focus of this study was to perform a sensory evaluation. As a first part of the sensory evaluation, all samples (reference, hydrolysates and Maillard products with and without xylose) were evaluated for the specific taste attributes bitter and umami. It was shown that both bitterness and umami generally increased due to enzymatic hydrolysis and showcased a tendency to increase even more after a subsequent Maillard reaction. Regarding the umami taste, an overall increase but no clear link to one of the processing steps was shown for the processing of mealworm protein. These results are in line with findings reported previously of increasing bitterness due to the formation of bitter peptides by enzymatic hydrolysis [155] and an increase in umami taste due to the liberation of amino acids (e.g. glutamic and aspartic acid) that contribute to the umami taste [158]. A further general taste description of all samples was done by a distinctive qualitative description analysis and showed the changes in taste descriptors due to the processing steps. Whereas the cricket protein, as such, was associated with the attributes “fade”, “woody”, “mealy” and “earthy-like” and the mealworm protein with similar ones (i.e. “woody”, “fade”, “musty” and “metallic”), the hydrolysates were described differently by attributes such as “rancid”, “medical”, and “fishy” for the cricket-protein hydrolysates with Flavourzyme and as “beany”, “musty”, “sour”, and “malty” for the other reacted Maillard products. Additional changes for the hydrolysates and Maillard products using ProteaseA were observed: the processed samples (both hydrolysis and further Maillard reaction) were associated with mainly savory attributes, such as “meaty”, “seasoning-like”, “sweet”, “malty” and “soy sauce-like” for the cricket protein and “meaty”, “seasoning-like”, “sweet” and, furthermore, described as “long lasting”, “salivating” and with a “round taste” for the mealworm protein. Complementary gas chromatography-olfactometry analysis investigated odor-active compounds of the insect proteins and as a result, 38 odor-active molecules were identified. These odor-active molecules were from different chemical groups, specifically 1 alcohol, 5 acids, 11 aldehydes, 5 ketones and 16 heterocyclic compounds. Albeit no clear link could be made between specific odor-active compounds and the processing steps of hydrolysis and subsequent Maillard reactions, generic aroma profiles of the samples differed for the insect proteins, as such, and for both processing steps. Apart from the 16 compounds that were reported previously in insect related studies [159], almost all compounds (30 out of 38) have been identified previously in seafood [160] or meat products [161].

This third study showed that the sensory attributes of cricket and mealworm protein were modified by applying enzymatic hydrolysis and subsequent Maillard reaction. With growing protein consumption stretching the existing production capacity to its limits, and the scaling of existing production methods proving to be controversial on several (particularly environmental and social) grounds, both science and the food industry need to find alternatives to meet the demands of consumers. This study generated new insights into the flavoring potential of cricket (*Acheta domesticus*) and mealworm (*Tenebrio molitor*) processed proteins. It was not only shown that both processing steps could be applied to change the taste profiles into more complex and savory-like (related to descriptors such as such as “meaty”, “seasoning-like”, “sweet”, “malty” and “soy sauce-like”) but also highlighted the link with odor-active compounds from meat and seafood products. This study showed a proof of principle to apply more specific enzymatic hydrolysis and subsequent Maillard reactions to insect proteins to generate a savory and meat-like flavor, and potentially even reduce undesirable aromas. An interesting and valuable line of further study could be to continue investigating the connection between processing steps and conditions regarding their sensory effects. The generation of additional data could be used to determine a precise link and generate targeted sensory changes due to the processing applied. This would be of great value to guide the formation of meat- and seafood-associated flavors by using alternative and sustainable proteins, such as those from insect proteins. This could be especially interesting for those regions that have an insect food history, for example, the Asian, Latin American and African regions.

REFERENCES

[1] A. J. Barrett and J. K. McDonald, *Biochemical Journal* **1986**, *237*, 935.

[2] N. D. Rawlings and A. J. Barrett in *Introduction: Aspartic and Glutamic Peptidases and Their Clans*, *Vol. 1* Academic Press, **2013**, pp. 3-19.

[3] K. Oda, *Journal of Biochemistry* **2012**, *151*, 13-25.

[4] N. D. Rawlings, F. R. Morton and A. J. Barrett, *An Introduction to Peptidases and the MEROPS Database*, Springer, The Netherlands, **2007**, p. 161-179.

[5] M. Y. Kondo, D. N. Okamoto, J. A. Santos, M. A. Juliano, K. Oda, B. Pillai, M. N. James, L. Juliano and I. E. Gouvea, *The Journal of Biological Chemistry* **2010**, *285*, 21437-21445.

[6] N. D. Rawlings, A. J. Barrett and A. Bateman, *The Journal of Biological Chemistry* **2011**, *286*, 38321-38328.

[7] D. Jahn and BrendaTeam in *BRENDA The Comprehensive Enzyme Information System*, *Vol. 4* Technische Universität Braunschweig, Germany, **2021**.

[8] J. K. McDonald, *The Enzyme List Class 3 - Hydrolyses,* pp. 1-502, http://www.enzyme-database.org/downloads/ec3.pdf, 14.10.2021.

[9] N. D. Rawlings, M. Waller, A. J. Barrett and A. Bateman, *Nucleic Acids Research* **2014**, *42*, D503-509.

[10] I. Schechter and A. Berger, *Biochemical and Biophysical Research Communications* **1967**, *27*, 157-162.

[11] B. Purschke, P. Meinlschmidt, C. Horn, O. Rieder and H. Jäger, *European Food Research and Technology* **2017**, *244*, 999-1013.

[12] F. Hall, P. E. Johnson and A. Liceaga, *Food Chemistry* **2018**, *262*, 39-47.

[13] J. Adler-Nissen in *Relationship of Structure to Taste of Peptides and Peptide Mixtures*, New York: Marcel Dekker Inc., USA, **1986**, pp. 97-112.

[14] A. Illanes in *The Concept and Determination of Enzyme Activity*, *Vol. 1* (Ed. A. Illanes), Springer, **2008**, pp. 1-56.

[15] H. Bisswanger, *Perspectives in Science* **2014**, *1*, 41-55.

[16] a) D. L. Purich in *Practical Aspects of Measuring Initial Rates and Reaction Parameters*, (Ed. D. L. Purich), Elsevier, London, Burlington, San Diego, **2010**, pp. 215-284; b) M. Merz, T. Eisele, P. Berends, D. Appel, S. Rabe, I. Blank, T. Stressler and L. Fischer, *Journal of Agricultural and Food Chemistry* **2015**, *63*, 5682-5693.

[17] W. D. Mallender, M. Bembenek, L. R. Dick, M. Kuranda, P. Li, S. Menon, E. Pardo and T. Parsons in *Biochemical Assays for High-throughput Screening*, *Vol. 35* (Ed. J. Hüser), John Wiley & Sons, Weinheim, **2006**, pp. 102-106.

[18] P. M. Nielsen, D. Petersen and C. Dambmann, *Journal of Food Science: Food Chemistry and Toxicology* **2001**, *66*, 642-646.

[19] M. A. Fraatz, N. Ruehl and H. Zorn in *Food and Feed Enzymes*, Eds.: H. Zorn and P. Czermak), Springer, Heidelberg New York Dordrecht London, **2014**, pp. 229-256.

[20] N. D. Rawlings and G. Salvesen in *Introduction*, Eds.: N. D. Rawlings and G. Salvesen), Academic Press, **2013**, pp. 3765-3932.

[21] a) A. D. Rowan in *Stem and Fruit Bromelain*, *Vol. 1* Eds.: N. D. Rawlings and G. Salvesen), Academic Press Elsevier, **2013**, pp. 1871-1875; b) I. G. Kamphuis, K. H. Kalk, M. B. A. Swarte and J. Drenth, *Journal of Molecular Biology* **1984** *179*, 233-256.

[22] E. Gross and J. Meienhofer in *The Peptide Bond*, *Vol. 1* Eds.: E. Gross and J. Meienhofer), Academic Press, New York, San Francisco, London, **1979**, pp. 1-65.

[23] J. Donlon in *Subtilisin*, Eds.: J. Polaina and A. P. MacCabe), Springer, The Netherlands, **2007**, pp. 197-206.

[24] B. M. Dunn and N. E. Goldfarb in *Human Immunodeficiency Virus 1 Retropepsin*, *Vol. 1* Eds.: N. D. Rawlings and L. M. Sagis), Elsevier, **2013**, pp. 190-199.

[25] BCCResearch, *Global Markets for Enzymes in Industrial Applications 2021-2026*, https://www.researchandmarkets.com/reports/5393441/global-markets-for-enzymes-in-industrial, 02.11.2021.

[26] J. Chapman, A. E. Ismail and C. Z. Dinu, *Catalysts* **2018**, *8*, 1-26.

[27] TechnavioBlog, *Top 10 Vendors in the Global Food Enzymes Market*, https://www.technavio.com/blog/top-10-vendors-global-food-enzymes, 16.11.2018.

[28] MarketAndMarkets, *Industrial Enzymes Market by Type (Amylases, Cellulases, Proteases, Lipases, and Phytases), Application (Food & Beverages, Cleaning Agents, and Animal Feed), Source (Microorganism, Plant, and Animal), and Region - Global Forecast to 2022* https://www.marketsandmarkets.com/Market-Reports/industrial-enzymes-market-237327836.html, 17.10.2018.

[29] FutureMarketInsights, *Protease Market: Global Industry Analysis and Opportunity Assessment 2016-2026* https://www.futuremarketinsights.com/reports/protease-market, 17.10.2018.

[30] A. Illanes in *Enzyme Production*, *Vol. 1* (Ed. A. Illanes), Springer, **2008**, pp. 57-106.
[31] M. van Oort in *Enzymes in Food Technology – Introduction*, Eds.: R. J. Whitehurst and M. van Oort), Wiley-Blackwell, **2010**, pp. 1-17.
[32] T. Dodge in *Production of Industrial Enzymes*, Eds.: R. J. Whitehurst and M. van Oort), Wiley-Blackwell, **2010**, pp. 44-58.
[33] M. Schallmey, A. Singh and O. P. Ward, *Canadian Journal of Microbiology* **2004**, *50*, 1-17.
[34] P. J. Punt, N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel, *Trends Biotechnology* **2002**, *20*, 200-206.
[35] H. A. Søndergaard, K. G. Grunert and J. Scholderer, *Trends in Food Science & Technology* **2005**, *16*, 466-474.
[36] FoodAndDrugAdministration, *Guidance for Industry Enzyme Preparations: Recommendations for Submission of Chemical and Technological Data for Food Additive Petitions and GRAS Notices,* pp. 1-12, https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-recommendations-submission-chemical-and-technological-data-food-additive-petitions, 09.11.2021.
[37] EuropeanCommission, *Guidance Document on Criteria for Categorisation of Food Enzymes*, https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_enzymes-guidance-categorisation.pdf, 23.10.2018.
[38] G. K. Chotani, T. C. Dodge, A. L. Gaertner and M. V. Arbige in *Industrial Biotechnology: Discovery to Delivery*, *Vol. 2* (Ed. J. K. Kent), Springer Science, New York, **2007**, pp. 1311-1374
[39] M. Krause, A. Neubauer and P. Neubauer, *Microbial Cell Factories* **2016**, *15*, 1-13.
[40] Y. Liang, L. Pan and Y. Lin, *Bioscience Biotechnology Biochemistry* **2009**, *73*, 192-195.
[41] GE-Healthcare, *Strategies for Protein Purification,* pp. 39-50, https://www.academia.edu/43673854/GE_Healthcare_imagination_at_work_agination_at_work_Strategies_for_Protein_Purif_ication_Handbook, 27.10.2018.
[42] V. Mandujano-González, L. Villa-Tanaca, M. A. Anducho-Reyes and Y. Mercado-Flores, *Revista Iberoamericana de Micología* **2016**, *33*, 76-82.
[43] R. Koka and B. C. Weimer, *International Dairy Journal* **2000**, *10*, 75-79.

[44] L. Ruiz-Rodríguez, J. Bleckwedel, M. E. Ortiz, M. Pescuma and F. Mozzi in *Lactic Acid Bacteria*, *Vol. 3a* Eds.: C. Wittmann, J. C. Liao, S. Y. Lee, J. Nielsen and G. Stephanopoulos), John Wiley & Sons, Germany, **2017**, pp. 395- 451.
[45] T. Stressler, T. Eisele, M. Schlayer, S. Lutz-Wahl and L. Fischer, *PLoS One* **2013**, *8*, e70055.
[46] A. Pihlanto-Leppala, E. Pahkala and V. Antila, *Agricultural Science in Finland* **1993**, *2*, 489-496.
[47] H. Wieser, *Food Microbiology* **2007**, *24*, 115-119.
[48] M. van Oort in *Enzymes in Bread Making*, Eds.: W. J.R. and M. van Oort), John Wiley & Sons, **2010**, pp. 103-143.
[49] J. R. Biesiekierski, *Journal of Gastroenterology and Hepatology* **2017**, *32*, 78-81.
[50] L. Pedersen, K. Kaack, M. N. Bergsøe and J. Adler-Nissen, *Journal of Food Science* **2005**, *70*, E152-E158.
[51] M. G. Gänzle, J. Loponen and M. Gobbetti, *Trends in Food Science & Technology* **2008**, *19*, 513-521.
[52] K. A. Scherf, P. Koehler and H. Wieser, *Journal of Cereal Science* **2016**, *67*, 2-11.
[53] A. Sapone, J. C. Bai, C. Ciacci, J. Dolinsek, P. H. Green, M. Hadjivassiliou, K. Kaukinen, K. Rostami, D. S. Sanders, M. Schumann, R. Ullrich, D. Villalta, U. Volta, C. Catassi and A. Fasano, *BMC (BioMed Central) Medicine* **2012**, *10*, 1-13.
[54] J. C. Henggeler, M. Veríssimo and F. Ramos, *Trends in Food Science & Technology* **2017**, *66*, 84-92.
[55] L. Heller in *Commercial Aspects of Gluten-Free Products*, (Ed. E. Gallagher), Wiley, **2009**, pp. 99-106.
[56] FoodAndAgricultureOrganizationOfTheUnitedNations and WorldHealthOrganization, *CODEX STAN 118-1979, Standards for Foods for Special Dietary Use For Persons Intolerant to Gluten* pp. 1-3, https://www.fao.org/fao-who-codexalimentarius/sh-proxy/es/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B118-1979%252FCXS_118e_2015.pdf, 09.11.2021.
[57] L. Shan, T. Marti, L. M. Sollid, G. M. Gray and C. Kholsa, *Biochemical Journal* **2004**, *383*, 311-318.

[58] H. M. Riggle and M. A. Fisher in *Purification of a Prolyl Endopeptidase from Aspergillus oryzae and Evaluation of its Ability to Digest Gluten Vol. 2* American Chemical Society Washington, D. C, Salt Lake City, UT, United States, **2009**.
[59] R. M. Duar, K. J. Clark, P. B. Patil, C. Hernández, S. Brüning, T. E. Burkey, N. Madayiputhiya, S. L. Taylor and J. Walter, *Journal of Applied Microbiology* **2015**, *118*, 515-527.
[60] E. Lalor and D. Goode in *Brewing with Enzymes*, *Vol. 2* Eds.: R. J. Whitehurst and M. van Oort), John Wiley & Sons, **2010**, pp. 163-194.
[61] V. Knorr, R. Kerpes, H. Wieser, M. Zarnkow, T. Becker and P. Koehler, *European Food Research and Technology* **2016**, *242*, 585-597.
[62] M. Fanari, M. Forteschi, M. Sanna, M. Zinellu, M. C. Porcu and L. Pretti, *Innovative Food Science & Emerging Technologies* **2018**, *49*, 76-81.
[63] A. Bruchmann and C. Fauveau in *Enzymes in Potable Alcohol and Wine Production*, Eds.: R. J. Whitehurst and M. van Oort), John Wiley & Sons, **2010**, pp. 195-210.
[64] L. W. Theron, M. Bely and B. Divol, *Journal of the Science of Food and Agriculture* **2017**, *97*, 3584-3593.
[65] G. W. Byarugaba-Bazirake, P. van Rensburg and W. Kyamuhangire, *American Journal of Biotechnology and Molecular Sciences* **2013**, *3*, 41-62.
[66] L. Theron and B. Divol, *Applied Microbiology and Biotechnology* **2014**, *98*, 8853-8868.
[67] R. Lantto, K. Kruus, E. Puolanne, K. Honkapää, K. Roininen and J. Buchert in *Enzymes in Meat Processing*, Eds.: R. J. Whitehurst and M. van Oort), John Wiley & Sons, **2010**, pp. 264-291.
[68] Z. Grzonka, F. Kasprzykowski and w. Wiczk in *Cysteine Proteases*, Eds.: J. Polaina and A. P. MacCabe), Springer, Dordrecht, The Netherlands., **2007**, pp. 181-196.
[69] a) J. M. Bruna, M. Fernández, J. A. Ordóñez and L. de la Hoz, *Journal of the Science of Food and Agriculture* **2002**, *82*, 526-533; b) H. He, X. Chen, J. Li, Y. Zhang and P. Gao, *Food Chemistry* **2004**, *84*, 307-311.
[70] S. Benjakul, S. Klomklao and B. K. Simpson in *Enzymes in Fish Processing*, Eds.: R. J. Whitehurst and M. van Oort), John Wiley & Sons, **2010**, pp. 211-235.
[71] I. Peinado, G. Koutsidis and J. Ames, *LWT - Food Science & Technology* **2016**, *66*, 444-452.

[72] a) A. K. Patel, R. R. Singhania and A. Pandey, *Current Opinion in Food Science* **2016**, *7*, 64-72; b) J. L. Adrio and A. L. Demain, *Biomolecules* **2014**, *4*, 117-139.
[73] a) Worldometers, *Current World Population*, http://www.worldometers.info/world-population/, 17.07.2021; b) UnistedStatesSensusBureau, *U.S. and World Population Clock*, https://www.census.gov/popclock/, 17.07.2021.
[74] H. Ritchie and M. Roser, *Meat and Seafood Production & Consumption*, https://ourworldindata.org/meat-and-seafood-production-consumption, 31.10.2018.
[75] AgricultureAndConsumerProtectionDepartment, *Sources of Meat*, http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html, 31.10.2018.
[76] K. E. Bradbury and T. Y. N. Tong, Key, T.J., *Nutrients* **2017**, *9*, 1-17.
[77] H. S. Gujral, A. Kaur, N. Singh and N. S. Sodhi, *Journal of Food Engineering* **2002**, *53*, 377-385.
[78] H. Ulu, *Food Chemistry* **2004**, *87*, 523-529.
[79] B. A. Rumpold and O. Schlueter, *Insect-based Protein Sources and their Potential for Human Consumption: Nutritional Composition and Processing,* pp. 20-24, https://academic.oup.com/af/article/5/2/20/4638722, 15.10.2021.
[80] A. van Huis, J. Van Itterbeeck, H. Klunder, E. Mertens, A. Halloran, G. Muir and P. Vantomme, *Edible Insects - Future Prospects for Food and Feed Security,* pp. 1-187, http://www.fao.org/docrep/018/i3253e/i3253e.pdf, 22.08.2018.
[81] SoybeanMealInfoCenter, *World Soybean Production*, https://www.soymeal.org/soy-meal-articles/world-soybean-production/, 31.10.2018.
[82] D. Fukushima in *Soy Proteins*, (Ed. R. Y. Yada), CRC Press, **2004**, pp. 122-154.
[83] B. Hoffmann, S. Münch, F. Schwägele, C. Neusüß and W. Jira, *Food Control* **2017**, *71*, 200-209.
[84] K. Nishinari, Y. Fang, T. Nagano, S. Guo and R. Wang in *Soy as a Food Ingredient*, (Ed. R. Y. Yada), Woodhead Publishing, **2017**, pp. 149-186.
[85] J. R. Hoffman and M. J. Falvo, *Journal of Sports Science & Medicine* **2004**, *3*, 118-130.
[86] E. M. Herman, *Abstracts of Papers, 232nd ACS National Meeting* (San Francisco, CA, United States) **2006**, pp. AGFD-052.
[87] J. M. Townsend, C. A. S. Hall, T. A. Volk, D. Murphy, G. Ofezu, B. Powers, A. Quaye and M. Serapiglia in *Energy Return on Investment (EROI), Liquid Fuel Production and*

Consequences for Wildlife, Eds.: J. E. Gates, D. L. Trauger and B. Czech), Springer, **2014**, pp. 29-62.

[88] a) GradViewResearch, *Pea Protein Market Analysis By Product (Isolates, Concentrates, Textured), By Form (Dry, Liquid), By Application (Meat Substitutes, Bakery Goods, Dietary Supplements, Beverage), And Segment Forecasts, 2018 - 2025,* pp. 1-200, https://www.grandviewresearch.com/industry-analysis/pea-protein-market, 01.11.2018; b) GrandViewResearch, *Seed Treatment Market Size, Share & Trends Analysis Report By Type (Insecticides, Fungicides, Nonchemical), By Crop Type (Maize, Soybean, Wheat, Canola, Cotton), By Region, And Segment Forecasts, 2012 - 2020,* pp. 1-85, https://www.grandviewresearch.com/industry-analysis/seed-treatment-market, 01.11.2018.

[89] W. J. Dahl, L. M. Foster and R. T. Tyler, *British Journal of Nutrition* **2012**, *108*, S3-S10.

[90] S. D. Hood-Niefer, T. D. Warkentin, R. N. Chibbar, A. Vandenberg and R. T. Tyler, *Journal of the Science of Food and Agriculture* **2012**, *92*, 141-150.

[91] J. Boye, F. Zare and A. Pletch, *Food Research International* **2010**, *43*, 414-431.

[92] S. Tömösközi, R. Lásztity, R. Haraszi and O. Baticz, *Food / Nahrung* **2001**, *45*, 399-401.

[93] WorldHealthOrganization and FoodAndAgricultureOrganizationOfTheUnitedNations, *Protein and Amino Acid Requirements in Human Nutrition - Report of a Joint FAO/WHO/UNU Expert Consultation* pp. 1-284, http://www.who.int/nutrition/publications/nutrientrequirements/WHO_TRS_935/en/, 30.10.2018.

[94] A. M. Alashi, C. L. Blanchard, R. J. Mailer and S. O. Agboola, *Food Reviews International* **2013**, *29*, 231-260.

[95] S. H. Tan, R. J. Mailer, C. L. Blanchard and S. O. Agboola, *Journal of Food Science* **2011**, *76*, R16-R28.

[96] M. Granvogl and K. Matheis in *Unraveling the Off-flavor Formation of Native Cold-pressed Rapeseed Oil Using the Molecular Sensory Science Concept*, *Vol. 2* American Chemical Society Washington, D. C, Philadelphia, PA, United States, **2016**, pp. AGFD-136.

[97] a) A.-S. Höglund, J. Rödin, E. Larsson and L. Rask, *Plant Physiology* **1992**, *98*, 509-515; b) S. P. Perera, T. C. McIntosh and J. P. D. Wanasundara, *Plants (Basel)* **2016**, *5*, 1-24.

[98] S. H. Tan, R. J. Mailer, C. L. Blanchard and S. O. Agboola, *LWT - Food Science & Technology* **2014**, *57*, 376-382.

[99] B. Uzun, C. Arslan, M. Karhan and C. Toker, *Food Chemistry* **2007**, *102*, 45-49.

[100] C. B. J. Villarino, V. Jayasena, R. Coorey, S. Chakrabarti-Bell and S. K. Johnson, *Critical Reviews in Food Science and Nutrition* **2016**, *56*, 835-857.

[101] A. Sujak, A. Kotlarz and W. Strobel, *Food Chemistry* **2006**, *98*, 711-719.

[102] E. Sirtori, D. Resta, A. Arnoldi, H. F. J. Savelkoul and H. J. Wichers, *Food Chemistry* **2011**, *126*, 902-910.

[103] EFSA-Scientific-Commitee, *EFSA Journal* **2015**, *13*, 1-60 (4257).

[104] Ynsect, https://www.ynsect.com/en/, 09.11.2021.

[105] L. Yi, M. A. J. S. Van Boekel, S. Boeren and C. M. M. Lakemond, *European Food Research and Technology* **2016**, *242*, 1285-1297.

[106] L. Yi, C. M. Lakemond, L. M. Sagis, V. Eisner-Schadler, A. van Huis and M. A. van Boekel, *Food Chemistry* **2013**, *141*, 3341-3348.

[107] J.-H. Cho, H.-L. Zhao, J.-S. Kim, S.-H. Kim and C.-H. Chung, *Innovative Food Science & Emerging Technologies* **2018**, *45*, 186-195.

[108] H.-W. Kim, D. Setyabrata, Y. J. Lee, O. G. Jones and Y. H. B. Kim, *Innovative Food Science & Emerging Technologies* **2016**, *38*, 116-123.

[109] A. v. Huis, *Annual Review of Entomology* **2013**, *58*, 563-583.

[110] M. H. Sipponen, O. E. Mäkinen, K. Rommi, R. Heiniö, U. Holopainen-Mantila, S. Hokkanen, T. K. Hakala and E. Nordlund, *European Food Research and Technology* **2017**, *244*, 19-29.

[111] H. W. Kim, D. Setyabrata, Y. Lee, O. G. Jones and Y. H. B. Kim, *Journal of Food Science* **2017**, *82*, 2787-2793.

[112] A. Osimani, V. Milanović, F. Cardinali, A. Roncolini, C. Garofalo, F. Clementi, M. Pasquini, M. Mozzon, R. Foligni, N. Raffaelli, F. Zamporlini and L. Aquilanti, *Innovative Food Science & Emerging Technologies* **2018**, *48*, 150-163.

[113] F. Francis, V. Doyen, F. Debaugnies, G. Mazzucchelli, R. Caparros, T. Alabi, C. Blecker, E. Haubruge and F. Corazza, *Food Chemistry* **2019**, *276*, 714-718.

[114] D. Vasic-Racki in *History of Industrial Biotransformations – Dreams and Realities*, Eds.: A. Liese, K. Seelbach and C. Wandrey), WILEY-VCH, Weinheim, **2006**, pp. 1-36.
[115] R. Liu, L. Wang, Y. Liu, T. Wu and M. Zhang, *Food Hydrocolloids* **2018**, *81*, 39-47.
[116] D. Panyam and A. Kilara, *Trends in Food Science & Technology* **1996**, *7*, 120-125.
[117] J. Vioque, R. Sánchez-Vioque, A. Clemente, J. Pedroche, J. Bautista and F. Millan, *Journal of the American Oil Chemists' Society* **1999**, *76*, 819-823.
[118] F. G. Hall, O. G. Jones, M. E. O'Haire and A. M. Liceaga, *Food Chemistry* **2017**, *224*, 414-422.
[119] S. Jung, P. A. Murphy and L. A. Johnson, *Journal of Food Science* **2005**, *70*, C180-C187.
[120] R. Sánchez-Vioque, C. L. Bagger, C. Rabiller and J. Guéguen, *Journal of Colloid and Interface Science* **2001**, *244*, 386-393.
[121] A. M. Alashi, C. L. Blanchard, R. J. Mailer, S. O. Agboola, J. A. Mawson and R. E. Aluko, *International Journal of Food Science & Technology* **2018**, *53*, 2316-2324.
[122] G. L. Hasenhuettl in *Overview of Food Emulsifiers*, Eds.: R. W. Hartel and G. L. Hasenhuettl), Springer US, **2013**, pp. 1-10.
[123] H.-D. Berlitz, W. Grosch and P. Schieberle in *Amino Acids, Peptides, Proteins*, Eds.: H.-D. Berlitz, W. Grosch and P. Schieberle), Springer, Berlin, Heidelberg, **2009**, pp. 8-82.
[124] C. S. Pundir, S. Lata and V. Narwal, *Biosensors and Bioelectronics* **2018**, *117*, 373-384.
[125] S. M. Rutherfurd, *Journal of AOAC International* **2010**, *93*, 1515-1522.
[126] K. A. Scherf, H. Wieser and P. Koehler, *Food Research International* **2016**.
[127] H. Song and J. Liu, *Food Research International* **2018**, *114*, 187-198.
[128] H.-D. Berlitz, W. Grosch and P. Schieberle in *Aroma Compounds*, Eds.: H.-D. Berlitz, W. Grosch and P. Schieberle), Springer, Berlin, Heidelberg, **2009**, pp. 340-402.
[129] K. Yamamoto and Y. Ishimaru, *Seminars in Cell & Developmental Biology* **2013**, *24*, 240-246.
[130] R. E. Aluko in *Structural Characteristics of Food Protein-Derived Bitter Peptides*, Eds.: M. Aliani and M. N. A. Aeskin), John Wiley & Sons, Inc. , **2017**, pp. 105-129.
[131] a) W. H. Seo, H. G. Lee and H. H. Baek, *Journal of Food Science* **2008**, *73*, S41-46; b) L. M. Humiski and R. E. Aluko, *Journal of Food Science* **2007**, *72*, S605-611.
[132] R. J. FitzGerald and G. O'Cuinn, *Biotechnology Advances* **2006**, *24*, 234-237.

[133] D. Edel, A.-G. Mercedes and G. W. Martin, *Current Nutrition & Food Science* **2008**, *4*, 290-297.
[134] S. Fellendorf, J. P. Kerry, R. M. Hamill and M. G. O'Sullivan, *LWT - Food Science & Technology* **2018**, *92*, 584-592.
[135] A. Schindler, A. Dunkel, F. Stähler, M. Backes, J. Ley, W. Meyerhof and T. Hofmann, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 12578-12588.
[136] L. Harth, U. Krah, D. Linke, A. Dunkel, T. Hofmann and R. G. Berger, *Journal of Agricultural and Food Chemistry* **2018**, *66*, 2344-2353.
[137] K. Kurihara, *BioMed Research International* **2015**, *2015*, 1-10.
[138] Y. Zhang, C. Venkitasamy, Z. Pan and W. Wang, *Trends in Food Science & Technology* **2013**, *33*, 78-92.
[139] A. Karau and I. Grayson in *Amino Acids in Human and Animal Nutrition*, *Vol. 143* Eds.: Zorn H. and C. P.), Springer, Heidelberg, Berlin, **2014**, pp. 189-228.
[140] D. S. Mottram, *Food Chemistry* **1998**, *62*, 415-424.
[141] J. E. Hodge, *Journal of Agricultural and Food Chemistry* **1953**, *1*, 928-943.
[142] S. I. F. S. Martins, W. M. F. Jongen and M. A. J. S. van Boekel, *Trends in Food Science & Technology* **2000**, *11*, 364-373.
[143] a) M. N. Lund and C. A. Ray, *Journal of Agricultural and Food Chemistry* **2017**, *65*, 4537-4552; b) F. Van Lancker, A. Adams and N. De Kimpe, *Chemical Reviews* **2011**, *111*, 7876-7903.
[144] K. K. Grossmann, M. Merz, D. Appel, T. Thaler and L. Fischer, *Journal of Agricultural and Food Chemistry* **2021**, *69*, 368-376.
[145] B. Srinivasan, *FEBS (Federation of European Biochemical Societies) Journal* **2021**, *288*, 2068-2083.
[146] C. M. Heckmann and F. Paradisi, *ChemCatChem* **2020**, *12*, 6082-6102.
[147] K. K. Grossmann, M. Merz, D. Appel and L. Fischer, *European Food Research and Technology* **2019**, *245*, 1695-1708.
[148] M. Merz, D. Appel, P. Berends, S. Rabe, I. Blank, T. Stressler and L. Fischer, *European Food Research and Technology* **2015**, *242*, 1005-1012.
[149] M. Merz, W. Claassen, D. Appel, P. Berends, S. Rabe, I. Blank, T. Stressler and L. Fischer, *Journal of Molecular Catalysis B: Enzymatic* **2016**, *127*, 1-10.

[150] Y. Zhao, M. Zhang, S. Devahastin and Y. Liu, *Trends in Food Science & Technology* **2019**, *93*, 125-135.
[151] N. Mika, H. Zorn and M. Ruhl, *Applied Microbiology and Biotechnology* **2015**, *99*, 7837-7846.
[152] B. C. Saha and K. Hayashi, *Biotechnology Advances* **2001**, *19*, 355-370.
[153] Y. Zhang, C. Venkitasamy, Z. Pan, W. Liu and L. Zhao, *Journal of Food Science* **2017**, *82*, 16-23.
[154] L. K. Y. Cheung, R. E. Aluko, M. A. Cliff and E. C. Y. Li-Chan, *Journal of Functional Foods* **2015**, *13*, 262-275.
[155] Y. Fu, J. Liu, E. T. Hansen, W. L. P. Bredie and R. Lametsch, *Food Chemistry* **2018**, *257*, 163-171.
[156] K. K. Grossmann, M. Merz, D. Appel, M. M. De Araujo and L. Fischer, *Food Chemistry* **2021**, *364*, 130336.
[157] Y. Jongema, *List of Edible Insect Species of the World*, https://www.wur.nl/en/Research-Results/Chair-groups/Plant-Sciences/Laboratory-of-Entomology/Edible-insects/Worldwide-species-list.htm, 15.10.2021.
[158] M. M. Poojary, V. Orlien, P. Passamonti and K. Olsen, *Food Chemistry* **2017**, *234*, 236-244.
[159] a) E. Bou-Maroun and N. Cayot, *Food Chemistry* **2011**, *124*, 889-894; b) K. Mahattanatawee, T. Luanphaisarnnont and R. Rouseff, *Journal of Agricultural and Food Chemistry* **2018**, *66*, 2480-2484.
[160] V. Mall and P. Schieberle, *Journal of Agricultural and Food Chemistry* **2017**, *65*, 2776-2783.
[161] S. Straßer and P. Schieberle, *European Food Research and Technology* **2014**, *238*, 307-313.