Appl Microbiol Biotechnol (2015) 99:7837–7846
DOI 10.1007/s00253-015-6838-0

MINI-REVIEW

Prolyl-specific peptidases for applications in food protein hydrolysis

Nicole Mika[1] · **Holger Zorn**[1] · **Martin Rühl**[1]

Received: 7 April 2015 / Revised: 8 July 2015 / Accepted: 11 July 2015 / Published online: 4 August 2015

Abstract Various food proteins including, e.g. gluten, collagen and casein are rich in L-proline residues. Due to the cyclic structure of proline, these proteins are well protected from enzymatic degradation by typical digestive enzymes. Proline-specific peptidases (PsP) belong to different families of hydrolases acting on peptide bonds (EC 3.4.x.x). They occur in various organisms including bacteria, fungi, plants and insects. Based on their biochemical characteristics, PsP type enzymes are further grouped into different subclasses of which prolyl aminopeptidases (EC 3.4.11.5, PAP), prolyl carboxypeptidases (EC 3.4.17.16, PCP) and prolyl oligopeptidases/prolyl endopeptidases (EC 3.4.21.26, POP/PEP) are of major interest for applications in food biotechnology. This mini review summarises the biochemical assays employed for these subclasses of PsP and their structural properties and the reaction mechanisms. A special focus was set on PsP derived from fungi and insects and important industrial applications in the field of food biotechnology. The degradation of gluten and collagen as well as the hydrolysis of bitter peptides are discussed.

Keywords Prolyl aminopeptidase · Prolyl carboxypeptidase · Gluten · Collagen · Debittering

✉ Holger Zorn
Holger.Zorn@lcb.chemie.uni-giessen.de

[1] Institute of Food Chemistry and Food Biotechnology, Justus Liebig University Giessen, Heinrich Buff-Ring 58, 35392 Giessen, Germany

Introduction

L-Proline is a unique proteinogenic amino acid in which the α-carbon and the nitrogen are combined in a pyrrolidine ring. The ring disables the mobility at the α-carbon and, thus, conformational changes of peptides and proteins harbouring proline residues. As a consequence thereof, proline-rich proteins, such as gluten or collagen are well protected from enzymatic degradation (Gass and Khosla 2007). Nevertheless, specific peptidases are able to cleave the peptide bond between proline and any other amino acid. These peptidases are known as prolyl-specific peptidases (PsP).

The activity of PsP is often restricted to substrates shorter than 30 amino acids, and hydrolysis at a central position (endo) or at the C- or N-terminal side (exo) is possible. A protein conformation consisting of a seven-bladed β-propeller domain, which is present in most PsP, and α/β-hydrolase domains prevent larger peptides and proteins to enter the active site of the enzyme (Fülöp et al. 1998). However, PsP from, e.g. *Flavobacterium meningosepticum* and *Pyrococcus furiosus*, may also hydrolyse larger peptides (Shan et al. 2004, Harris et al. 2001). Apart from their applications in biotechnology, PsP play an important role in biomedical research. Diseases like Parkinson, type 2 diabetes, depression or coeliac disease are associated with proline-rich proteins. As a consequence, PsP might provide access to new therapeutic treatments. These fields of application have been well reviewed, e.g. by Gass and Khosla (2007) and by Rosenblum and Kozarich (2003).

Some years ago, PsP were known from animals, plants and bacterial sources only. In the last few years, substantial research was dedicated to fungi and especially to insects as potential sources for novel PsP. This mini review focuses

mainly on recent studies on PsP from fungi and insects and furthermore on applications of PsP in the field of protein hydrolysis in food biotechnology.

Structure and reaction mechanism

PsP belong to several subclasses. Based on their preferred hydrolysis pattern, they are classified into the following: prolyl aminopeptidases (PAP; EC 3.4.11.5) releasing an N-terminal proline from a peptide, Xaa-Pro aminopeptidases (EC 3.4.11.9) releasing any N-terminal amino acid linked to proline and Xaa-Pro dipeptidases (EC 3.4.13.9) which hydrolyse dipeptides. Additionally, dipeptidyl-peptidases II (EC 3.4.14.2), dipeptidyl-peptidase IV (EC. 3.4.14.5), Xaa-Pro dipeptidyl-peptidase (PDP; EC 3.4.14.11) and Xaa-Xaa-Pro tripeptidyl-peptidase (PTP; EC 3.4.14.12) releasing di- or tripeptides from the N-terminus have been described. Lysosomal Pro-Xaa carboxypeptidase (EC 3.4.16.2) and membrane Pro-Xaa carboxypeptidases (PCP; EC 3.4.17.16) release a C-terminal amino acid from proline-containing proteins. Endo-acting prolyl oligopeptidases (POP; EC 3.4.21.26) which are also addressed as prolyl endopeptidases (PEP) are able to hydrolyse oligopeptides at a central position.

The catalytic reaction for the latter PsP type is based on an induced-fit mechanism, which has been proposed by Li et al. (2010a) on the basis of different native and closed POP structures of the bacterium *Aeromonas punctate*. Crystallographic studies of a eukaryotic POP from the parasite *Trypanosoma brucei* and comparison to the POP of *A. punctate* verified this mechanism (Canning et al. 2013). In the open state the POP forms a large cavity between the α/β-hydrolase and the β-propeller domains. Upon substrate binding, a conformational change is induced leading to the closed state where the catalytic reaction is performed. The active site is composed of a catalytic triad which consists of a serine, an aspartic acid and a histidine residue (Fig. 1).

The typical mechanism of serine peptidases has been well described for various enzymes, including, e.g. chymotrypsin (Berg et al. 2012). In short, the hydroxyl group of the catalytic serine is initially acylated by the carbonyl carbon atom of the substrate, thus forming a tetrahedral intermediate. The proton of the hydroxyl group is intermediately transferred to the imidazole ring of the histidine, while the aspartic acid residue stabilises the transition state. In the next step, the peptide bond is cleaved under transfer of the hydrogen from the imidazole ring to the amino group of the hydrolysed substrate. Finally, the serine residue is deacylated by adding a water molecule to the substrate (Polgár 2005).

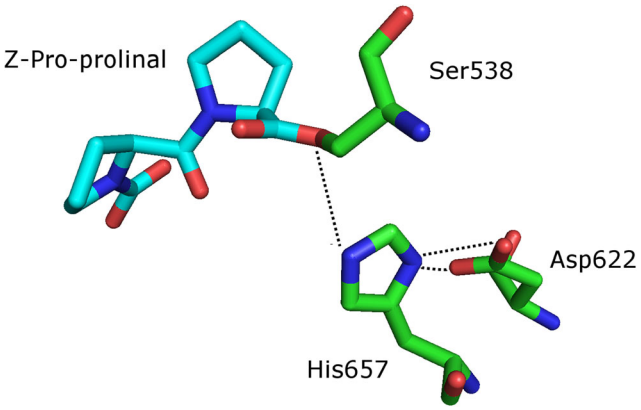

Fig. 1 Detailed view of the *Aeromonas punctate* POP active site of the closed state with the PsP inhibitor Z-Pro-prolinal (3IVM.pdb, Li et al. 2010a). *Dotted lines* show hydrogen bonds between the amino acids of the catalytic triad

Biochemical assays

PsP activities are determined by various substrates of which most contain the chromophore *p*-nitroaniline. This chromophore is bound via its amino group to the carboxy group of proline forming an amide bond as in, e.g. benzyloxycarbonyl-glycine-proline-*p*-nitroanilide (Z-Gly-Pro-pNA). The peptide bond at the carboxy side of proline is hydrolysed by PsP resulting in a benzyloxycarbonyl-dipeptide and *p*-nitroaniline. The release of the latter is determined photometrically at a wavelength of 410 nm (Fig. 2).

Several further synthetic substrates have been used for the biochemical classification of PsP, such as, e.g. Z-Gly-D-Pro-pNA, Z-Gly-Pro-Ala, Z-Gly-Pro-D-Ala and Z-Ala-Ala-Ala-Pro-pNA (Yoshimoto et al. 1988, Sattar et al. 1990, Chen et al. 2012). To differentiate prolyl oligopeptidases from amino- and carboxypeptidases, the substrates listed in Table 1 have been suggested.

To characterise the PCP activity of larvae of the mealworm *Tenebrio molitor*, Goptar et al. (2013) used the dipeptide Z-Pro-Phe as a substrate. Based on the amino acid sequence motif of the active serine site GGSYGG (S9c), the enzyme was supposed to be a POP, but significant activity was only observed with the PCP substrate.

Li et al. (2010b) identified a recombinant peptidase from *Phanerochaete chrysosporium* expressed in *Escherichia coli* to be a PAP as it only showed activity against Pro-pNA and not against the proline-containing substrate Ala-Ala-Pro-Leu-pNA. A similar approach was used for a purified PAP from the thermophilic fungus *Talaromyces emersonii* (Mahon et al. 2009). In this study, the authors used the proline-containing substrates succinyl-Ala-Ala-Pro-Phe-pNA, succinyl-Ala-Ala-Pro-pNA, Gly-Pro-pNA as well as Pro-pNA, from which only the latter was hydrolysed by the purified peptidase. Kitazono et al. (1994) used oligopeptides (8-mer and 12-mer) with an N-terminal proline residue to show

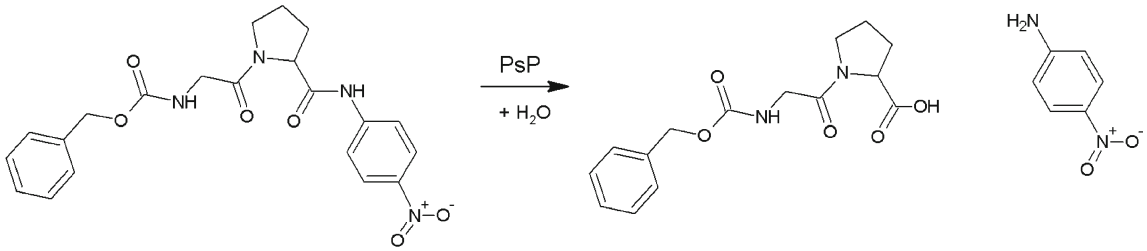

Fig. 2 Hydrolysis of Z-Gly-Pro-pNA by a prolyl-specific peptidase (PsP) into Z-Gly-Pro and *p*-nitroaniline

PAP activity of an *Aeromonas sobria* peptidase heterologously produced in *E. coli*.

PEP activity is usually used synonymous to POP activity as both show endo activity. As substrates, oligopeptides of 3 to 33 amino acids containing a central proline have been employed (reviewed by Cunningham and O'Connor 1997, Shan et al. 2004). The hydrolysis products may be detected by means of MALDI-TOF-MS (Stepniak et al. 2006), ESI-MS (Mika et al. 2015) or HPLC-UV (Shan et al. 2004, 2005). Additionally, immunological tests, such as Western blots or enzyme-linked immunosorbent assays (ELISA), have been used (Stepniak et al. 2006, Walter et al. 2014, 2015). Immunological tests are especially important in cases where PsP are used for hydrolysis of gluten which can cause celiac disease (cf. below).

Sources for PsP

PsP have been found in all domains of life: in archaea (Harwood et al. 1997, Lee et al. 2007, Harris et al. 2001), bacteria (Shan et al. 2004, Banbula et al. 1999) and eukaryotes (Yoshimoto et al. 1988, Goptar et al. 2013). Well-characterised bacterial endo-acting PsP are derived, e.g. from *F. meningosepticum* (FM-PEP), *Sphingomonas capsulate* (SC-PEP) and *Myxococcus xanthus* (MX-PEP). They typically show maximum activity at pH 6–7 (Shan et al. 2004, 2005) and are easily inactivated at low pH values (Edens et al. 2005). An efficient degradation of immune-sensitive peptides requires high PEP concentrations and long incubation times, which has been shown for FM-PEP. Here, 9 μg were necessary to hydrolyse 200 μg of gliadin peptides at 37 °C for 3 h to achieve a non-immune-sensitive peptide solution (Matysiak-Budnik et al. 2005). Thus, the biotechnological potential of bacterial PEP is limited.

Fungi

A number of filamentous fungi are known to secrete extracellular peptidases belonging to the PsP family. Early studies reported on PsP activities in the fruiting bodies of different basidiomycetous mushrooms, of which the two representatives *Lyophyllum cinerascens* and *Agaricus bisporus* showed the highest activities (Yoshimoto et al. 1988, Sattar et al. 1990). In both studies, purified enzymes were tested for the hydrolysis of up to 20 different proline-containing substrates, and a post-proline cleaving activity was detected. Thus, the so defined POP had a relative molecular weight of about 76 kDa and showed the highest activities at 37 °C at pH 6.8 and 7.5 for the enzymes of *A. bisporus* and *L. cinerascens*, respectively. The pattern of hydrolysis of all substrates was similar for both POP having the highest catalytic efficiency for the substrate Z-Ala-Pro-2-β-naphthylamide. Substrates having a D-amino ac-

Table 1 Substrates used for the characterisation of prolyl-specific peptidases

Substrates	PAP EC 3.4.11.5	PCP EC 3.4.16.2	POP (PEP) EC 3.4.21.26	Ref.
Pro-Phe-Gly-Lys	x			Fuke and Matsuoka 1993; Bolumar et al. 2003
Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, Pro-2-naphthylamine	x			Kitazono et al. 1994
Z-Pro-Phe		x		Goptar et al. 2013
Z-Gly-Pro-pNA			x	Kang et al. 2014
Z-Gly-Pro-7-amido-4-methylcoumarin			x	Birney and O'Connor 2001
Z-Gly-Pro-Leu-Ala			x	Yoshimoto et al. 1988;
Z-Gly-Pro-Leu-Gly-Pro			x	Sattar et al. 1990

PAP prolyl aminopeptidase, *PCP* prolyl carboxypeptidase, *POP* prolyl oligopepetidase, *PEP* prolyl endopeptidase

id before or after proline were not hydrolysed (Yoshimoto et al. 1988, Sattar et al. 1990). Likewise, at least one amino acid residue N-terminal of proline was required for the hydrolysis of proline-containing peptides with an enzyme derived from the inky cap mushroom *Coprinopsis clastophylla* (Chen et al. 2012). Comparable to the POP from *A. bisporus* and *L. cinerascens*, the POP of *C. clastophylla* had a pH optimum of 8.0 and showed the highest activity at 37 °C. Additionally, some non-edible toadstools have a high proline-specific activity. *Amanita bisporigera* and *Amanita phalloides*, which are called destroying angel or death angel, respectively, do harbour a toxic combination of a POP and a propeptide which is hydrolysed into a cyclic toxic octa- or heptapeptide. These toxins, known as amatoxin or phallotoxin, are only produced by some of the *Amanita* and related species and are absent in ascomycetes (Luo et al. 2009, 2010; Hallen et al. 2007). The propeptides of the toxins consist of about 35 amino acids with a conserved N-terminal leader peptide and a conserved C-terminal sequence flanking a variable core peptide of 7–8 amino acids (Oman and van der Donk 2010). In the basidiomycete *Galerina marginata*, the POP first cleaves the leader peptide after its proline residue and then the C-terminal sequence leading to a peptide of 7–8 amino acids with a C-terminal proline. Afterwards, cyclisation of the linear peptide occurs (Luo et al. 2014). In the genomes of basidiomycetous and ascomycetous fungi available at the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/programs/fungi/index.jsf, Walnut Creek, CA), 47 hits were found in the genomes of basidiomycetes and none in the genomes of ascomycetes when conducting a tblastn search (11.02.2015, E-value 10^{-150}) with the POP from *A. bisporigera* (accession number ADN19205). Only in *G. marginata*, a hit against the protoxin of *A. bisporigera* (accession number A8W7M4) was detected. Interestingly, in *G. marginata*, the coding sequence for the POP and the gene coding for the fungal protoxin are only 3 kbp away from each other (Riley et al. 2014).

In dermatophytic ascomycetous fungi, POP are essential for the hydrolysis of keratin. These fungi secrete peptidases cleaving after proline (Ala-Ala-Pro-pNA, Ala-Pro-pNA) only at neutral pH (Sriranganadane et al. 2011). On the other hand, acidophilic POP have been found in various *Aspergillus* species (Kubota et al. 2005, Edens et al. 2005, Kang et al. 2014). The POP detected in *Aspergillus niger* differ slightly from each other in their molecular weight and in their optimum reaction conditions. While the POP described by Kubota et al. (2005) was characterised by a molecular weight of 54.5 kDa and a temperature optimum of 60 °C at pH 3.7, the enzyme detected by Edens et al. (2005) showed a molecular weight of 66 kDa and was most active at 50 °C and at pH 5.0. A POP of *Aspergillus oryzae* with a molecular weight of about 60 kDa showed highest activity at 30 °C and at pH 4.0 (Kang et al. 2014). These POP have become of special interest in recent years due to their wide spread applications in the food industry (cf. below, Table 2). Besides POP-type enzymes, also PAP are present in the fungal kingdom. PAP have been identified and characterised, e.g. in the ascomycetes *A. oryzae* and *A. niger* (Matsushita-Morita et al. 2010, Basten et al. 2005). Both PAP have similar properties with a native molecular weight of ~315 kDa and seem to be composed of six monomeric units. A similar PAP was found in *Debaryomyces hansenii* (MW ~370 kDa) which showed maximum activity at 45 °C and pH 7.5 (Bolumar et al. 2003). In *Penicillium camemberti*, an ascomycete well-known from the production of cheese, a PAP with a native relative molecular weight of 270 kDa is supposed to be composed of four subunits. The optimal reaction conditions were similar with a pH optimum of 7.5 and a temperature optimum of 45 °C (Fuke and Matsuoka 1993). In addition, PAP have also been reported for basidiomycetes, such as *P. chrysosporium* (Li et al. 2010b). The recombinant PAP produced in *E. coli* showed an optimal activity at pH 8.0 and between 45 and 50 °C.

Insects

Insects represent a rich and highly promising source for new PsP enzymes. Especially cereal pests (Fig. 3) are equipped with a wide range of peptidolytic enzymes to regulate their feed intake. As a consequence, investigations in the field of insect (or "yellow") biotechnology are mostly focused on the characterisation of the beetles' digestive systems, especially on the gut of the beetles. Even though the protein digestion in humans and beetles differs significantly, the beetles' peptidases might find use in industrial applications.

Probably due to its size and its good availability, the yellow mealworm *Tenebrio molitor* represents the best examined beetle. Vinokurov et al. (2006) analysed the diversity of digestive peptidases in the gut of *T. molitor* larvae derived from the insect itself or the microbiome present in the gut. A complex enzymatic system of protein digestion was found within the gut of the larvae. Diverse sets of peptidases were localised in different areas of the gut, depending on a sharp pH gradient. For example, serine peptidases were mainly found in the posterior midgut at pH 5.2–5.6 (Vinokurov et al. 2006). In 2008, Goptar et al. identified two PsP (PPCP1 and PPCP2) in *T. molitor*. The maximum activity of PPCP1 was determined to be at pH 5.6 for the substrate Z-Ala-Ala-Pro-pNA and had a molecular weight of 101 kDa. PPCP2 showed maximum activity against Z-Ala-Pro-pNA at a pH of 7.9 and had a molecular weight of 63 kDa. Based on the preferred activity against Z-Ala-Pro-pNA, both enzymes were defined as POP. In a more recent study, Goptar et al. (2013) isolated and characterised a PCP from larvae of *T. molitor*. The authors used degenerated primers, derived from a predicted PCP nucleotide sequence from the genome of the related beetle *Tribolium castaneum*, for cDNA amplification of *T. molitor*.

Table 2 Different classes of prolyl-specific peptidases and their characteristics

Organism		PsP type	pH optimum	Temperature optimum (°C)	Molecular weight (kDa)	References
Bacteria	*Aneurinibacillus* sp.	PAP	8.5–10.5	55	35	Murai et al. 2004
	Aeromonas caviae	PAP	8.5	50	29.5	Izawa et al. 1997
	Lactobacillus casei ssp. *casei LLG*	PDP	7.0	50	79	Habibi-Najafi and Lee 1994
	Flavobacterium meningosepticum	PEP	6.0–7.0	n.d.	n.d.	Shan et al. 2004
	Myxococcus xanthus	PEP	6.0–7.0	n.d.	n.d.	Shan et al. 2004
	Sphingomonas capsulate	PEP	6.0–7.0	n.d.	n.d.	Shan et al. 2004
	Porphyromonas gingivalis	PTP	6.0–8.0	n.d.	82	Banbula et al. 1999
Fungi	*Aspergillus oryzae*	PAP	7.5	60	50	Ding et al. 2014
	Debaryomyces hansenii	PAP	7.5	45	~370[a]	Bolumar et al. 2003
	Penicillium camemberti	PAP	7.5	45	270[b]	Fuke and Matsuoka 1993
	Phanerochaete chrysosporium	PAP	8.0	45	38	Li et al. 2010b
	Agaricus bisporus	POP	7.5	37	76	Sattar et al. 1990
	Aspergillus niger	POP	3.7	60	54.5	Kubota et al. (2005)
		POP	5.0	50	66	Edens et al. 2005
	Aspergillus oryzae	POP	4.0	30	60	Kang et al. 2014
	Coprinopsis clastophylla	POP	8.0	37	84	Chen et al. 2012
	Lyophyllum cinerascens	POP	6.8	37	76	Yoshimoto et al. 1988
Insects	*Tenebrio molitor*	PCP	5.6	n.d.	105	Goptar et al. 2013
		POP	7.9	n.d.	63	Goptar et al. 2008
		POP	5.6	n.d.	101	
	Sarcophaga peregrine	POP	n.d.	n.d.	84	Ohtsuki et al. 1997

PAP prolyl aminopeptidase, *PCP* membrane Pro-Xaa carboxypeptidases, *PDP* Xaa-Pro dipeptidyl peptidase, *POP* prolyl oligopepetidase, *PEP* prolyl endopeptidase, *PTP* Xaa-Xaa-Pro tripeptidyl-peptidase

n.d. not determined

[a] Consists of seven monomers of approximately 53.5 kDa each

[b] Consists of four monomers of approximately 66 kDa each

Fig. 3 Cereal pests *Sitophilus granarius* (**a**), *Rhizopertha dominica* (**b**), *Tenebrio molitor* adult beetle (**c**), *Tenebrio molitor* larva (**d**)

The authors supposed the enzyme to be a dimer with a molecular weight of 105 kDa and determined a pH optimum of 5.6, which correlates well with its localisation in the gut of the larvae.

According to Mika et al. (2015), insect-associated peptidases may efficiently hydrolyse various food proteins. An extensive degradation of proline-rich proteins like casein, rice protein and gluten was shown for the cereal pests *Rhizopertha dominica*, *Oryzaephilus surinamensis*, *T. molitor* and *Alphitobius diaperinus*. In the beetle extracts, PsP activities of 4.6×10^{-6} to 1.2×10^{-3} U mg^{-1} ground beetle were determined. The highest PsP activity was determined in *R. dominica* which showed a preferred cleavage after proline during the degradation of proline-rich peptides from wheat and barley.

While digestive enzymes are predominantly localised within the gastrointestinal system of the beetles, further digestive organs, like salivary glands, produce peptidolytic enzymes as well. Bezdi et al. (2012) analysed the protein patterns secreted by the salivary glands of the sunn pest *Eurygaster integriceps*. They detected 31 digestive enzymes, whereof four enzymes were identified as serine peptidases. Darkoh et al. (2010) characterised a PEP from *E. integriceps*. In this study, the enzyme was not isolated from the beetle itself, but from infested wheat grains. The optimum activity was determined between pH 8 and 10 at temperatures between 20 and 35 °C. Surprisingly, the enzyme was able to hydrolyse gluten whereby a polymerisation of the treated gluten with SDS was reduced. Another PsP gene was identified in nymphs of the brown planthopper *Nilaparvata lugens*, which is highly homologous to a PsP gene of *Drosophila melanogaster* (Yang et al. 2006). Already in 1997, Ohtsuki et al. (1997) cloned the cDNA encoding a PsP of another Brachycera, the flesh fly *Sarcophaga peregrine*. The deduced amino acid sequence of the PsP showed a 53 % homology to a mammalian PsP. The 84 kDa peptidase was produced in *E. coli* and the specific activity of the enzyme against succinyl-Gly-Pro-7-amido-4-methylcoumarin was 1.19 U mg^{-1}.

Applications

As extensive reviews on pharmaceutical and medicinal applications of PsP are available (cf. above), this mini review focuses on applications of PsP in the field of food biotechnology. PsP are inter alia needed for an efficient degradation of gluten or collagen and for debittering of peptides. To mention one commercial example, a PsP is produced by the Dutch company DSM Food Specialties: EndoPro™ is a food-grade enzyme which is secreted by an *Aspergillus* species and may be used in different fields of food biotechnology.

Degradation of gluten

The degradation of proline-rich proteins like gluten or collagen represents a big challenge in biotechnological applications, especially for the production of allergen-free products. Protein hydrolysates that are produced from proline-rich proteins may cause serious problems for patients suffering from celiac disease. Celiac disease describes an intestinal disorder due to an uncontrolled immune response, caused by gluten proteins that are resistant to proteolytic degradation within the gastrointestinal tract (Stepniak et al. 2006). About 1 % of the world population is suffering from celiac disease. It occurs in people of all ages and is currently only treated by a strict gluten-free diet (Green and Cellier 2007). Typical symptoms of celiac disease include, for example, diarrhoea, malnutrition and growth disturbances. According to the codex alimentarius, codex stan 118–1979 ('codex standard for foods for special dietary use for persons intolerant to gluten'), gluten is defined as a 'protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant'. Gluten-free foods are described as dietary foods with a maximum gluten amount of 20 mg kg^{-1} in total, based on the food as sold or distributed to the consumer. To eliminate traces of gluten or to produce gluten-free foodstuffs, an enzymatic degradation of immune sensitive peptides by means of PsP represents a promising approach. Even an oral administration of PsP is mentioned in literature to ease the patients' lives (Sollid and Khosla 2005, Cerf-Bensussan et al. 2007).

Since many fungi and insects efficiently hydrolyse gluten, new PsP are constantly being found in these organisms. Edens et al. (2005) described a PEP from *A. niger*, AN-PEP. This peptidase seems to belong to the serine peptidase family S28 (PCP) rather than to the family S9 (POP) and showed degradation of immune-sensitive gluten peptides as well as of intact gluten proteins (Edens et al. 2005, Stepniak et al. 2006). The peptidase showed maximum activity at pH 4.0–5.0 and was highly thermostable. Additionally, the comparison of AN-PEP with bacterial FM-PEP at their optimal pH showed in average a 60 times faster degradation of immune-sensitive gluten peptides with the fungal enzyme (Stepniak et al. 2006). Edens et al. (2005) did not detect any homology of AN-PEP to bacterial PEP, but low homologies to a human PCP and a dipeptidyl aminopeptidase of rat. Mitea et al. (2008) further characterised AN-PEP against the background of celiac disease. In an adapted human gastrointestinal tract, AN-PEP improved the digestion of gluten. As a result, immune-sensitive gluten peptides did not reach the duodenum compartment, where they trigger inflammatory reactions. Walter et al. (2014) tested AN-PEP also for the degradation of celiac disease relevant peptides and compared the activity to PsP containing bran extracts from germinated cereals. Different from the extracts of germinated bran, AN-PEP was able to reduce

the gluten content of three types of wheat starch below the critical level of 20 mg kg ^{1} which was determined by means of competitive ELISA using the R5 antibody (RIDASCREEN® Gliadin competitive, R-Biopharm, Darmstadt, Germany). AN-PEP has also been used in a clinical study and was well tolerated by the celiac disease patients (Tack et al. 2013). A key advantage of the use of AN-PEP in food biotechnological applications is the easy and low-cost production of the enzyme and, of course, the adherence to food-grade quality standards. Apart from the hydrolysis of peptides causing celiac disease, AN-PEP was successfully applied to prevent chill-haze in beer. The enzyme treatment showed comparable results to the control stabiliser polyvinylpolypyrrolidone (Lopez and Edens 2005).

Toft-Hansen et al. (2014) reported on a further PEP from *A. niger*, AnP2. AnP2 showed a maximum activity at pH 3.5–5.0 and was stable at pH 2.5–7.5. The degradation of gluten was shown at 37 °C by SDS-PAGE analysis. Furthermore, the ability of AnP2 to detoxify native or deamidated gluten peptides was tested with various gluten-reactive T-cell lines from children and adults. Here, AnP2 blocked the response against the toxic peptides and the enzyme, therefore, represents an auspicious candidate for biotechnological applications as well as for medical treatments of celiac disease.

In insects, an efficient degradation of gluten was shown by Mika et al. (2015). In gluten-containing zymograms, peptidolytic activity was observed with various cereal pests. The responsible peptidases were localised within in the gastrointestinal system of the beetles. After purification of an enzyme extract derived from *R. dominica*, a trypsin-like serine peptidase was identified. Based on the specific hydrolysis of gliadin and hordein fractions from wheat and barley after proline, a prolyl specificity of the peptidase was assumed. The PEP from *E. integriceps* (cf. above) was also tested for its ability to degrade gluten (Darkoh et al. 2010). The enzyme activity was shown by use of an SDS-gluten assay, in which hydrolytic activity led to a degradation of the gluten-containing gel. The gel height corresponded inverse linearly to the enzyme activity. Konarev et al. (2011) extracted a peptidase from salivary glands of *E. integriceps* which was able to hydrolyse glutenin. The protein extracts showed glutenin hydrolysis bands in an IEF zymogram. The purified peptidase had a molecular weight of 28 kDa and the deduced amino acid sequence showed homologies to already known insect serine peptidases. Furthermore, a preferred cleavage position between the glutamine-glycine bond was determined by means of MALDI-TOF-MS.

Debittering

Several types of peptidases play an important role in the production of various foodstuffs. For the production of, e.g. beverages or cheese, peptidases are indispensable. Unfortunately, the peptidolytic hydrolysis of food proteins like soy protein, rice protein, gluten or casein often leads to the release of bitter peptides and thus negatively influences the products' taste. Depending on the respective parameters of a food manufacturing process, the intensity of the formation of bitter peptides may vary. Brühl et al. (2007) detected bitter off-taste compounds in cold-pressed linseed oil. A negative influence of high temperatures and extended storage times was observed. Amongst others, the cyclic octapeptide cyclolinopeptide E, consisting of Pro-Leu-Phe-Ile-MetO-Leu-Val-Phe, was identified to impart a bitter taste. Toelstede and Hofmann (2008) screened for bitter metabolites in Gouda cheese and identified proline-rich bitter peptides, which had their origin in α- and β-casein. Especially, the length and the amino acid composition of the released peptide fragments play a crucial role (Guigoz and Solms 1976). Ishibashi et al. (1988) assumed a correlation of the bitterness of peptides with the number of hydrophobic amino acids like proline, valine, leucine, phenylalanine or alanine. In 1983, a bitter heptapeptide (Arg-Gly-Pro-Pro-Phe-Ile-Val) was identified by Fukui et al. (1983) in a casein hydrolysate. Here, the proline residues in position three and four were crucial for the development of bitter taste. Furthermore, an intense bitterness was observed when arginine is adjacent to proline residues (Otagiri et al. 1985). Thus, PsP play a key role in debittering processes (FitzGerald and O'Cuinn 2006).

A PsP from the bacterium *Aeromonas caviae* was purified and characterised (Izawa et al. 1997). The aminopeptidase had a molecular weight of 29.5 kDa and showed debittering activity. PsP activity was confirmed by the hydrolysis of the substrates Pro-pNA and Pro-Phe, and the enzyme hydrolysed the bitter peptides Val-Tyr-Pro-Phe and Arg-Arg-Pro-Phe-Phe. The degradation of bitter peptides due to bacterial organisms often focusses on lactic acid bacteria. In 1995, Habibi-Najafi and Lee purified and characterised a PAP from *Lactobacillus casei* ssp. *casei* LLG by showing its highest specificity against the synthetic substrates Pro-β-naphthylamide and Pro-7-amino-4-methylcoumarin (Pro-AMC) as well as against the dipeptide Pro-Leu. Interestingly, the activity against Pro-Gly was 20 times lower. The enzyme had a molecular weight of 46 kDa, and optimum reaction conditions were tested with the substrate Pro-AMC with a maximum activity at pH 7.5 and 40 °C. Besides, a Xaa-Pro dipeptidyl peptidase (PDP) was isolated from the same organism (Habibi-Najafi and Lee 1994). In a further study, Habibi-Najafi and Lee (2007) tested also debittering effects of this PDP. After previous digestion of β-casein with trypsin, the fractionated hydrolysates were treated with the PDP. A complete degradation of two proline-rich bitter peptides was observed, and the bitterness of other peptides containing proline decreased as well. Bouchier et al. (2001) purified different aminopeptidases from *Lactococcus lactis* ssp. *cremoris* AM2. A general

aminopeptidase, a PDP and a PAP were verified via activity against the substrates Lys-pNA, Gly-Pro-AMC and Arg-Pro-Pro, respectively. After incubation of a tryptic hydrolysate of β-casein with the enzymes, best results were obtained with a combination of these enzymes. The omission of the PAP and the PDP resulted in a limited hydrolysis of the bitter peptides, whereas sequential application of the general aminopeptidase and the PAP/PDP led to an increase in peptide degradation and a decrease in bitterness.

For fungal enzymes, debittering properties have also been reported. The AN-PEP from *A. Niger* (cf. above) has an acidic pH optimum and showed strong debittering features after incubation with casein hydrolysates. The debittering was demonstrated in a sensory test as well as by RP-HPLC analysis (Edens et al. 2005). Li et al. (2010b) characterised a PAP from the white-rot basidiomycete *P. chrysosporium*. The enzyme showed a maximum peptidolytic activity against Pro-pNA at pH 8.0 and 45 °C. The removal of free amino acids was shown by the hydrolysis of natural peptides. Peptide solutions from milk, collagen and BSA were degraded by the PAP. Therefore, a degradation of bitter compounds may be presumed. In 2008, Li et al. tested peptidases from supernatants of *Actinomucor elegans* in combination with the serine endopeptidase Alcalase 2.4 L™ (EC 3.4.21.62, Novozymes, Denmark) for their debittering qualities in soybean protein hydrolysates. The supernatants from *A. elegans* showed the highest activity against Gly-Pro-pNA and *N*-carbobenzoxy-Ile-Leu, indicating the presence of a PsP. In a two-step reaction, the Alcalase first cleaves at hydrophobic amino acid residues whereby bitter peptides may be formed. Afterwards, the *A. elegans* extract acts as an exopeptidase and releases the terminal amino acid. As a result, a high degree of hydrolysis as well as a low bitterness may be achieved. The degradation of bitter peptides was verified by SDS-PAGE, ESI-MS and in a sensory evaluation. The bitterness was reduced with increasing amounts of the *A. elegans* supernatant.

Hydrolysis of collagen

As a by-product of the meat industry, collagen often occurs in wastewater of butcheries. Like gluten, collagen and the gelatin derived thereof are rich in proline and 4-hydroxyproline. Thus, an efficient hydrolysis of collagen by PsP represents an interesting option for the biotechnological production of low molecular weight peptides and free amino acids.

Fungal collagen hydrolysing peptidases have been described only rarely. Nevertheless, several peptidases have been detected in fungal human pathogens. Ibrahim-Granet et al. (1994, 1996) purified and characterised peptidases from *Aspergillus fumigatus* and *Trichophyton schoenleinii*. The peptidases showed activity against the substrate phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg (PZ-Pro-Leu-Gly-Pro-Arg) and, due to characterisation experiments with various peptidase inhibitors, were defined as metallopeptidases. Thus, both peptidases potentially categorise into the PCP class. In application tests, the *T. schoenleinii* peptidase cleaved native rat type I collagen. Besides *T. schoenleinii*, further fungi such as *Conidiobolus coronatus*, *Fonsecaea pedrosoi* and *Wangeiella dermatidis* showed an activity against PZ-Pro-Leu-Gly-Pro-Arg and may have the potential to hydrolyse collagen (Ibrahim-Granet et al. 1996). A non-pathogenic fungi as a source for a collagen-degrading enzyme has been published only recently. Ding et al. (2014) isolated a PAP from *A. oryzae* and combined it successfully with a neutral peptidase for the degradation of collagen. The PAP had a molecular weight of 50 kDa and showed the highest activity at 60 °C and pH 7.5. Compared to fungal peptidases, bacterial peptidases are described more comprehensively and several PsP have been characterised for their ability to degrade collagen. Murai et al. (2004) described a PAP from the thermophilic bacterium *Aneurinibacillus* sp. Strain AM-1 after induction with gelatin. Maximum activity was observed at pH 8–10 and at 55 °C. Similar to the results of Ding et al. (2014), the exclusive usage of the PAP from *Aneurinibacillus* sp. resulted in a low hydrolytic activity against collagen. After adding a collagenolytic peptidase from *Geobacillus collagenovorans* MO-1 (Okamoto et al. 2001), the hydrolysis of collagen increased significantly. On the other hand, the enzymatic degradation of collagen by single enzymes has been described as well. A PTP was isolated from the pathogenic bacterium *Porphyromonas gingivalis* by Banbula et al. (1999). The bacterium occurs in the human oral cavity and triggers periodontal disease. The molecular weight of the peptidase was calculated to be 82 kDa. A pH optimum of 6.0–8.0 was determined, and the enzyme was stable at 37 °C for at least 12 h. It was shown that the enzyme cleaves specific peptides with a proline residue on the third position from the amino terminal end of partially degraded collagen fragments. As mentioned before, collagen is also rich in 4-hydroxyproline. Compared to proline, peptidase specificity for 4-hydroxyproline is only rarely discussed in the literature. Nevertheless, a specific hydrolysis after 4-hydroxyproline (Hyp) in Ala-Hyp-pNA was described by another type of PsP, dipeptidyl peptidase IV, which was isolated from pig kidney (Heins et al. 1988).

Conflict of interest The authors declare that they have no competing interests.

Funding N. Mika is grateful for the financial support by the excellence initiative of the Hessian Ministry of Science and Art which encompasses a generous grant for the LOEWE Center for Insect Biotechnology & Bioresources.

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