Extracellular Prolyl Endoprotease from Aspergillus niger and Its Use in the Debittering of Protein Hydrolysates

Article in Journal of Agricultural and Food Chemistry · November 2005
DOI: 10.1021/jf050652c · Source: PubMed

6 authors, including:

- **Luppo Edens**
  DSM
  28 PUBLICATIONS  1,012 CITATIONS
  See Profile

- **Peter Dekker**
  DSM Food-Specialties
  28 PUBLICATIONS  2,694 CITATIONS
  See Profile

- **Rob van der hoeven**
  Royal DSM
  41 PUBLICATIONS  710 CITATIONS
  See Profile

- **René Floris**
  NIZO food research
  43 PUBLICATIONS  1,188 CITATIONS
  See Profile

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.

Available from: Peter Dekker
Retrieved on: 23 November 2016
Extracellular Prolyl Endoprotease from *Aspergillus niger* and Its Use in the Debittering of Protein Hydrolysates

LUPPO EDENS,*† PETER DEKKER,*† ROB VAN DER HOEVEN,*† FLIP DEEN,*† ANDRE DE ROOS,*† AND RENE ÄFLORIS‡

DSM Food Specialties, Post Office Box 1, 2600 MA Delft, The Netherlands, and NIZO Food Research, Post Office Box 20, 6710 BA Ede, The Netherlands

The observation that the bitterest peptides from casein hydrolysates contain several proline residues led us to hypothesize that a proline-specific protease would be instrumental in debittering such peptides. To identify the desired proline-specific activity, a microbiological screening was carried out in which the chromogenic peptide benzoyloxycarbonyl-glycine-proline-p-nitroanilide (Z-Gly-Pro-pNA) was used as the substrate. An *Aspergillus niger* (*A. niger*) strain was identified that produces an extracellular proline-specific protease with an acidic pH optimum. On the basis of sequence similarities, we conclude that the *A. niger*-derived enzyme probably belongs to the S28 family of clan SC of serine proteases rather than the S9 family to which prolyl oligopeptidases belong. Incubating the overexpressed and purified enzyme with bitter casein hydrolysates showed a major debittering effect. Reversed phase HPLC analysis revealed that this debittering effect is accompanied by a significant reduction of the number of hydrophobic peptides present.

**KEYWORDS:** Prolyl-specific endoprotease; secretion; debittering; protein hydrolysates

INTRODUCTION

Protein hydrolysates enjoy increasing market interests as nutritional supplements for specific groups of individuals. Whey-derived products are particularly popular because of their taste profile and excellent solubility (1). However, babies naturally choose milk, which contains 4 times as much casein as whey proteins, to ensure adequate nutrition. The nutritional role of the casein fraction of milk, supplying essential amino acids, adequate calcium, and phosphate, is well recognized. Nevertheless, casein-derived hydrolysates have not gained a wide popularity. The main reason for this lack of market penetration is the very bitter off-taste, which is generated during casein hydrolysis. This bitter off-taste represents a technical problem that has not been adequately solved by the food industry (2).

A bitter off-taste is common for all protein hydrolysates, but large differences occur depending on the protein source used. According to Ney’s Q-hypothesis (3), the degree of bitterness that develops during hydrolysis is connected with the level of hydrophobic amino acids present in the protein substrate used. More recent views link bitterness to the formation of a few exceptionally bitter peptides rather than to an array of moderately bitter peptides (4). Matoba and Hata (5) observed that peptides with hydrophobic amino acids at C- or N-terminal positions appear less bitter than peptides with both ends of these hydrophobic amino acids involved in peptide bonds.

Casein is relatively rich in hydrophobic amino acids and especially in proline. Peptides 61–67, 84–89, and 193–208 of β-casein, which are isolated from cheese, have been identified as extremely bitter (6, 7). The latter peptides are comprised of several proline residues and are very hydrophobic. Recently, Capiralla et al. (8) described the identification of an endoprotease from *Halobacterium halobium* preferentially generating peptides with a proline in the penultimate and a hydrophobic amino acid residue in the C-terminal position. Upon incubating a tryptic β-casein digest with this enzyme, they noticed a significant debittering of the hydrolysate accompanied by the selective degradation of the hydrophobic peptides present. However, from an application point of view, the *Halobacterium* enzyme has a number of serious drawbacks. For example, the endopeptidase has very high salt requirements, and cleavage requires a proline in combination with a hydrophobic amino acid residue.

Peptide bonds involving proline residues are notoriously difficult to cleave as illustrated by the currently available industrial enzymes. Among the serine proteases, prolyl oligopeptidases (EC 3.4.21.26) have the unique possibility of preferentially cleaving peptides at the carboxyl side of proline residues. As suggested by their name, these enzymes can cleave only peptide substrates, while excluding large proteins. This characteristic is due to the presence of a unique domain that excludes large structured peptides from the enzyme’s active site, hereby preventing an undesired degradation of proteins present in the cytosol (9). The known prolyl oligopeptidases are typically cytosolic, nonsecreted enzymes with neutral pH optima. So far, prolyl oligopeptidases have not been found in fungi (10).
With the aim of identifying a proline-specific protease that would allow industrial application, we screened for a proline-specific protease with an acid pH optimum and secreted by a food grade microorganism.

**MATERIALS AND METHODS**

**Materials.** Sodium caseinates were obtained from DMV International (Veghel, The Netherlands) or from MD Foods (Videbaek, Denmark). Purified bovine β-casein was obtained from Sigma. Intact ovalbumine vials containing 20 mg of freeze-dried material were obtained from Pierce (Rockford, IL). Subtilisin (Alcalase AF 2.4 L, with an activity of 2.6 AU (A) as specified by the supplier) was obtained from Novozymes A/S ( Bagsvaerd, Denmark), and thermolysin (14,000 PU/ mg of Thermoase as specified by the supplier) was from Daiwa Kasei (Osaka, Japan). The proline-specific oligopeptidase from *Flavobacterium meningosepticum* was obtained from ICN Biomedicals (35 units/ mg as specified by the supplier (Aurora, OH)). The *A. niger* strain producing the proline-specific endoprotease was deposited as strain CBS 109712 at the Centraal Bureau of Schimmelcultures (CBS, Baarn, The Netherlands).

Chromogenic para-nitroanilide (pNA) peptides were obtained from Pepscan (Leiden, The Netherlands). Peptide NH₂-FRASDNDRVID-F was a gift from the Pepscan company (Lelystad, The Netherlands). Peptide NH₂-FRASDNDRVID-F. meningosepticum was cut out, washed several times with 50 mM ammonium bicarbonate, and incubated overnight at 37 °C with sequencing grade trypsin (Worthington Biochemical Corp., Lakewood, NJ). Peptides were extracted from the gel piece by washing several times with acetonitrile/ water containing formic acid (50/50/5, v/v/v). The samples were dried and stored at −20 °C until the LC/MS analysis.

**LC/MS Analysis.** Nano-HPLC equipped with a Qtof-2 (Micromass, Manchester, UK) mass spectrometer was used to separate the peptides formed during digestion of the proline-specific endoprotease with trypsin. The dried sample was solubilized in 50 μL of Milli-Q water containing 5% of formic acid. 5 μL of the peptide solution was trapped on a micro-precolumn, C₁₈, 5 x 0.3 mm (LC Packings, Amsterdam, Netherlands) using Milli-Q water containing 0.1% of formic acid at a flow-rate of 20 μL/min. The peptides were loaded onto the precolumn with 0.1% formic acid in Milli-Q at a flow-rate of 20 μL/min and were washed for 4 min to remove salts. The peptides were eluted from the precolumn onto the analytical column, PEPMAP C₁₈, 75 μm ID 100 Å (LC Packings, Amsterdam, The Netherlands), using a fast gradient elution of 0.1% formic acid in Milli-Q water (solution A) and 0.1% formic acid in acetonitrile (solution B) at a flow-rate of 200 μL/min. Partial amino acid sequences of the *A. niger* proline-specific endopeptidase could be determined by de novo sequencing of suitable peptides.

To characterize the enzymatic protein hydrolysates generated by the overproduced proline-specific endoprotease HPLC, a P4000 pump (Thermoquest, Breda, The Netherlands) equipped with an LCQ ion trap mass spectrometer (Thermoquest, Breda, The Netherlands) was used. The peptides formed were separated using a 150 x 1 mm PEPMAP C18 300 Å column in combination with a gradient of 0.1% formic acid in Milli-Q water (solution A) and 0.1% formic acid in acetonitrile (solution B) for elution. The protein concentration of the injected samples was approximately 50 μg/mL, and an injection volume of 50 μL was used. Detailed information on the individual peptides was obtained by using the “scan-dependent” MS/MS algorithm, which is a characteristic algorithm for an ion trap mass spectrometer. The partial peptide sequence information, obtained with MS/MS, could be used for database searching using the SEQUEST application from Xcalibur Bioworks (Thermoquest, Breda, The Netherlands). The databases used contained only the proteins of interest for the relevant application.

**Cloning, Overexpression, and Purification of the Proline-Specific Endoprotease.** *A. niger* CBS109712 was grown in a medium that contained collagen as sole carbon source to induce the expression of the gene encoding for proline-specific endoprotease, as described above. Young mycelia were harvested after 48 h growth at 34 °C and used for the isolation of total RNA with the Trizol method exactly as described by the supplier (Invitrogen, Breda, The Netherlands). A forward degenerate oligonucleotide primer, 5'-GCCAACGAGG- GARGCCTATYYTTYGA-3', was used for amplification and cloning of the gene encoding the proline-specific endopeptidase by 3'-RACE, as described by the supplier (Invitrogen).

The obtained cDNA fragment was cloned into pCR2.1 (Invitrogen), and the DNA sequence was determined. From this sequence, three additional primers, 5'-TTCCAGTACTCCACAGTACCCTC-3', 5'-TTGG-GAAAAGGTGCCCTTCTCC-3', and 5'-GGATTATGATGGTCCAG-GGAC-3', were designed for further amplification of the 5'-part of the gene from total RNA of *A. niger* CBS109712, using the 5'-RACE kit (Invitrogen) according to the suppliers’ instructions. The fragment was cloned into pCR2.1, and the DNA sequence was determined. The entire open reading frame of the gene encoding proline-specific endoprotease was PCR amplified from cDNA of *A. niger* CBS 109712 and cloned into the EcoRI site of expression vector pGFPIN-11 (13), where the coding region is functionally coupled to the *A. niger* gluA promoter and terminator.

*A. niger* CBS 513.88 was used as a host for the overexpression of the gene encoding the proline-specific endoprotease. The expression vector described above was linearized by digestion with restriction enzyme NotI, which removed all E. coli-derived sequences. The *A. niger* transformation was according to a standard procedure. *A. niger* transformants containing multiple copies of the expression cassette were selected on agar plates containing acetamide as nitrogen source. Transformants were cultivated for 4–5 days in baffled flask cultures in a medium at pH 6.2 containing 150 g/L maltose, 60 g/L Bacto-Soytone, 70 g/L sodiumcitrate, 1 g/L Na₂HPO₄, 15 g/L (NH₄)₂SO₄, 1 g/L MgSO₄,
0.08 g/L Tween 80, 0.2 g/L antifoam (Basildon), and 1 g/L L-arginine, after which supernatant was used for purification of the prolyl endopeptidase, as described below.

From overproducing A. niger strains, the enzyme was purified from the fermentation broth by centrifugation (11 000g) to remove the bulk of the fungal mass. The supernatant was then passed through a number of filters with decreasing pore sizes to remove all cell fragments. Finally, the ultrafiltrate obtained was diluted 10 times in 20 mM sodium acetate pH 5.1 and applied on a Q-Sepharose FF column. Proteins were eluted in a gradient from 0 to 0.4 M NaCl in 20 mM sodium acetate pH 5.1. Pooling of the active fractions followed by concentration finally yielded a preparation that showed only a single band on SDS-PAGE.

**Determination of pH Optima.** To establish the pH activity profiles of the A. niger- and F. meningosepticum-derived enzymes, buffers with different pH values were prepared. Buffers ranging from pH 2.0 to 7.0 were prepared using 0.1 M citrate, buffers ranging from pH 6.0 to 9.0 were prepared using 0.1 M tris, and buffers ranging from pH 8.0 to 12.0 were made using 0.2 M glycine. The pH was adjusted using either HCl or NaOH. The chromogenic synthetic peptide Z-Gly-Pro-AMC was used as the substrate for both enzymes. In each well of a 96-well microtiter plate, 85 µL of buffer, 10 µL of enzyme solution, and 5 µL of the substrate (4mM Z-Gly-Pro-AMC in 60% methanol) were introduced. The final concentration of the A. niger enzyme was 32 µg/mL (3.2 µM/mL), and the final concentration of the F. meningosepticum enzyme was 0.21 µg/mL (7.4 µM/mL). After mixing, the reaction was allowed to proceed for 30 min at 37°C, after which the fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm at a slit width of 40 nm in a CytoFluor multi-well plate reader (PerSeptive Biosciences, Framingham, MA).

**Specificity of A. niger-Derived Enzyme.** To establish its specificity, the chromatographically purified enzyme was tested against a collection of chromogenic peptide substrates. Stock solutions of AAX-pNA substrates (150 mM; ‘X’ representing different natural amino acid residues) were diluted 100× in 0.1 M acetate buffer pH 4.0 containing 20 mM CaCl2. The kinetic measurements were performed at 40°C in a TECAN Genios MTP Reader (Salzburg, Vienna). Increase in optical density was monitored at 405 nm during 10 min. The data generated were further processed in Excel.

**Hydrolysis of Ovalbumin and the Synthetic 27-mer.** A chromatographically purified A. niger-derived proline-specific endopeptidase with an activity of 4.5 units/mL was diluted 100-fold with 0.1 M acetate buffer pH 4.0. The ovalbumin was dissolved in acetate buffer pH 4.0 to a concentration of 1 mg/mL (22 µM). The 27-mer was dissolved in the same buffer to reach a concentration of 0.48 mg/mL (152 µM). 0.5 mL of both substrate solutions was incubated with 10 µL (0.45 mU) of the enzyme solution in an Eppendorf thermomixer at 50°C. At certain time intervals, 10 µL samples were withdrawn from the incubation mixture and kept at 20°C until SDS-PAGE. All materials used for SDS-PAGE and staining were purchased from Invitrogen. Samples were prepared using a lithium dodecyl sulfate (LDS) buffer according to manufacturers’ instructions and separated on 12% Bis-Tris gels using a 2-(N-morpholino)ethane-sulfonic acid-sodium dodecyl sulfate (MES-SDS) buffer system according to manufacturers’ instructions. Staining was performed using Simply Blue Safe Stain (Colidial Coomassie G250; Invitrogen).

**Debittering Tests.** To a solution containing 60 g/L of sodium caseinate (Miprodan 30) and water, thermolysin (Thermose; 1 g) was added and incubated for 2 h at pH 6.7 and 80°C. To a similar solution, subtilisin (Alcalase AF 2.4L; 2% (v/w) was added and incubated for 2 h at 65°C. In both cases, a clarified solution was obtained with a minor precipitate. The pH of both solutions was then adjusted to pH 5.0, after which the enzymes were inactivated through 45 min of heating at 90°C. After inactivation, 2 units of chromatographically purified A. niger prolyl endopeptidase was added per gram of casein hydrolysate, and incubation was continued for 20 h at 50°C followed by a further heat inactivation stage. Degrees of hydrolysis (DH) were established using the OPA method (14). Sensory evaluation of the protein hydrolysates formed was carried out by a panel of five people, who are trained in detecting and ranking various levels of bitterness. During these sessions (one session per hydrolysate), the taste trials were performed “blind”, and bitterness was scored on an integer-scale from 0 (no bitterness) to 4 (very bitter). At each session, a maximum of four samples and one standard as anchor point (intensity bitter being 1) were presented. The samples were offered with a software-generated code (Fizz, Biosystèmes, France) in a random sequence. Panel members were trained with quinine sulfate solutions with the following concentrations: 15 mg/L quinine sulfate > intensity bitter = 1; 20 mg/L quinine sulfate > intensity bitter = 2; 30 mg/L quinine sulfate > intensity bitter = 3; 50 mg/L quinine sulfate > intensity bitter = 4.

**RP-HPLC.** Experiments were carried out essentially as described by Visser et al. (15). However, the solvent gradient used was slightly adapted. Starting with 0% of solvent B, a gradient was generated by increasing the proportion of solvent B by 1% min−1 during 50 min, then by 4% min−1 during 5 min, and finally by 0% min−1 during 5 min, before returning to the starting conditions in 5 min.

### RESULTS

**Isolation and Characteristics of an Extracellular Proline-Specific Protease from A. niger.** A large collection of moulds capable of forming black spores was screened with the aim of identifying a secreted prolyl endopeptidase with an acid pH optimum. For this purpose, culture filtrates were incubated with the synthetic peptide Z-Ala-Pro-pNA at pH 5.0 and 50°C. The filtrate of one specific A. niger Van Tieghem strain showed a relatively high activity. From the supernatant of this strain, a minor quantity of an almost pure enzyme was obtained by chromatographic purification. According to SDS gel electrophoresis, the purified enzyme was estimated to have a molecular weight of around 66 kDa, and isoelectric focusing of the purified enzyme indicated an isoelectric point around pH 4.2. The enzyme pH optimum was determined by measuring the hydrolysis of the chromogenic peptide Z-Gly-Pro-AMC under different pH conditions. Comparison with a commercially obtained prolyl oligopeptidase from F. meningosepticum illustrated the different pH optima of the two enzymes (Figure 1). Preliminary tests at the enzyme’s pH optimum of around 4.2 indicated a temperature optimum of ca. 50°C for the A. niger-derived enzyme. To establish partial amino acid sequences, a chromatographically purified version of the enzyme was subjected to two-dimensional gel electrophoresis, after which the largest spot was cut from the gel incubated with trypsin and eluted. T/LC/MS/MS analysis was then carried out on the eluted material. From the identified peptide sequence NH2-ATTGEAYFE-COOH, a forward degenerate oligonucleotide primer was designed that enabled the cloning of the gene for the prolyl endopeptidase into an A. niger expression vector. The
Features of the A. niger Gene Encoding Prolyl Endopeptidase. From the entire coding sequence of the prolyl endopeptidase gene, its deduced protein sequence of 526 amino acids could be determined (Figure 2). At the amino terminus of the protein sequence, a putative signal sequence can be identified corresponding with the extracellular localization of the protein. Analysis of chromatographically purified mature protein revealed that the sequence NH2-ATTGEAYFE-COOH used to design oligonucleotide primer represents the amino terminus of the mature enzyme. The observation that this sequence is located approximately 20 amino acids downstream of the putative processing site of the signal sequence suggests that the prolyl endopeptidase of A. niger is synthesized as a precursor containing a pro-peptide. The novelty of the amino acid sequence of the proline-specific endopeptidase has been recorded in the EMBL database under account number AX458699.

Figure 2. Comparison of the amino acid sequences of the A. niger proline-specific protease (endoPro) with other members of the family S28 of serine proteases, using the program Clustal W (1.74). The putative signal sequence is indicated by a solid underlining, and the prosequence is indicated with a dotted underlining. The amino-terminus of the mature protein is indicated by an arrow. Putative active site residues are highlighted. Acc. nr. of sequences are DPP-II of rat (PIR:JC7668), PCP of human (PIR:A47352), and the proline-specific endoprotease of A. niger (EMBL:AX458699).

homology could be detected between the A. niger enzyme and prolyl oligopeptidases from other sources. However, closer inspection of the amino acid sequence revealed low but significant homology to Pro-X carboxypeptidases (EC3.4.16.2) and dipeptidyl aminopeptidases II (EC3.4.14.2) (Figure 2). The latter enzymes have been assigned to the family S28 of clan SC of serine peptidases (16). In the A. niger enzyme, the putative active-site configuration Ser-Asp-His can be recognized. This particular catalytic triad has been assigned to serine proteases of clan SC (17). Furthermore, the GxSYxG configuration around the active-site serine is conserved between Pro-X carboxypeptidase, dipeptidyl aminopeptidase II, and the A. niger-derived proline-specific protease. Other similarities include synthesis with a signal sequence and a pro-peptide, a preferred cleavage site carboxy-terminal of proline residues, and, finally, acidic pH optima (16). Also, the size of the A. niger enzyme is similar to the size of the other enzymes belonging to the family S28. Therefore, the secreted A. niger proline-specific protease appears to be a member of the family S28 of serine proteases rather than the clostridial proline proteases.
Overexpression and Isolation of the *A. niger* Proline-Specific Protease. The coding sequence of the proline-specific protease from *A. niger* was fused to the promoter and terminator sequences of the *A. niger* glaA gene, hereby permitting a strong expression in *A. niger* CBS513.88 (13). Transformants were cultivated in shake flasks, and their filtrates were subjected to SDS-PAGE to test for overexpression and secretion of the proline-specific protease. The overexpression of the enzyme by some transformants was confirmed by a clear increase in activity toward the synthetic peptide Z-Ala-Pro-pNA. A transformant displaying a relatively high activity was used for the production of enzyme material for subsequent tests.

Substrate Specificity. Prolyl oligopeptidases have been reported to exhibit a strong preference for cleaving C-terminal of proline residues. Peptide bonds C-terminal of alanine can also be cleaved, but with a lower efficiency (18). To test the substrate specificity of the *A. niger*-derived enzyme, the overexpressed enzyme was chromatographically purified and incubated with various Ala-Ala-X-pNa substrates at pH 4.0 and 40 °C. The results obtained confirmed the strong preference of the *A. niger*-derived enzyme for cleaving C-terminal of proline. Similar to the prolyl oligopeptidases, the *A. niger*-derived enzyme also showed some activity toward alanine-containing substrates. In comparison with peptides Ala-Ala-Pro-pNA and Z-Ala-Ala-Ala-Pro-pNA, peptide Z-Gly-Pro-pNA, a popular substrate for testing prolyl oligopeptidases, was found to form a relatively poor substrate for the *A. niger*-derived enzyme (data not shown). In combination with the finding that the enzyme exhibits almost no activity toward the dipeptide Ala-Pro-pNA, these data suggest a preference for larger substrates.

To confirm the specificity data obtained with these chromogenic tripeptides, we also incubated the enzyme with peptide 9-mers corresponding to the C-terminal amino acid sequence of alphaS1-casein. Three peptides were used, one with the natural sequence and two variants, in which the single proline residue was replaced with either an alanine or an hydroxyproline residue. The resulting peptides with the sequences N-SEKTTMPLW-OH (Mw 1091.5), N-SEKTTLALW-OH (Mw 1065.5), and N-SEKTTLMLW-OH (Mw 1107.5) were first checked for their purity in LC/MS and LC/MS/MS mode using gradient elution and then digested with a low concentration of the *A. niger*-derived prolyl endoprotease. Peptides N-SEKKTMP-OH, N-SEKKTMA-OH, and N-SEKKTMJ-OH were found to represent at least 96% of the final digestion products, showing that the *A. niger* enzyme can hydrolyze C-terminal of proline as well as alanine and hydroxyproline residues. In accordance with the results obtained from the chromogenic substrates, these LC/MS/MS results also indicate that the hydrolysis of proline-containing substrates is at least 1 order of magnitude faster than the hydrolysis of alanine-containing substrates.

Effect of Inhibitors. Because inhibitors can provide valuable information as to the catalytic type of the protease, the *A. niger*-derived enzyme was incubated with a number of different compounds. At first, the commercial protease inhibitor cocktail “Complete” was used. However, under conditions specified by the supplier, this cocktail had no significant effect on the activity of the enzyme. Also, phenylmethylsulfonyl fluoride (PMSF), a typical inhibitor of serine proteases, hardly affected the enzyme. At a concentration as high as 5 mM, only a 30% activity reduction was found. The compounds Z-Pro-Pro-aldehyde-dimethyl acetal (19) and FmocPro-PyrCN (20) are known to be potent inhibitors of prolyl oligopeptidases.
comitant with the degradation of the ovalbumine, the 27-mer peptide is also hydrolyzed as shown by the increasing fuzziness of the 3 kDa band (cf., lanes 2 and 6). On the basis of these experiments, we conclude that the *A. niger* enzyme is a true proline-specific endoprotease rather than a prolyl oligopeptidase.

**Debittering of Casein Hydrolysate with the Proline-Specific Endoprotease.** To test the postulated debittering effect by the proline-specific protease, a tasting experiment was conducted in which the bitterness of two different casein hydrolysates was compared before and after an incubation with the *A. niger*-derived proline-specific endoprotease. Casein hydrolysates were prepared by incubating sodium caseinate with a “known protease”, that is, either the metal protease thermolysin or the serine protease subtilisin. After incubation with either one of these two proteases under neutral pH conditions, the two solutions were acidified to pH 5.0 and heated to inactivate the enzymes used. The two hydrolysates obtained were clear solutions containing some precipitate. Their DH values were around 14%. One-half of each hydrolysate was then hydrolyzed further, by a subsequent incubation with the proline-specific endoprotease. The remaining hydrolysate served as a reference and was incubated under the same conditions but without any enzyme added. During this second incubation, the DH of the enzyme-containing samples was increased to ca. 19% and most of the precipitate dissolved. After another heat treatment, all four samples were freeze-dried and stored for organoleptic testing.

The taste of the four hydrolysates was evaluated after dissolution of the freeze-dried powders to reach protein concentrations equivalent to 3% caseinate. The pH of the four solutions was adjusted to 4.0 and the temperature to 14 °C. A trained panel consisting of five people scored the bitterness of each hydrolysate on a scale of 0 (not bitter) to 4 (very bitter). A reference sample of 15 mg/L quinine sulfate was given to the panelists before the taste session and was assigned a bitter intensity value of 1. The casein hydrolysates produced by incubation with just the thermolysine or the subtilisin were judged to be very bitter; that is, both solutions scored a 4 with all members of the taste panel. Upon tasting the solutions subjected to the additional incubation with the proline-specific protease, the panel unanimously gave a score of 1. Thus, during the incubation with the proline-specific protease the originally

---

**Figure 4.** Schematic representation of the possible formation of bitter and debittered hydrolysates from proline-rich substrates. (A) Peptides formed upon incubation with a conventional endoprotease and (B) peptides formed upon incubation with a conventional endoprotease plus a proline-specific endoprotease. “H” symbolizes hydrophobic amino acid residues, and “P” symbolizes proline residues. According to the hypothesis, proline-rich, hydrophobic peptides cannot be cleaved by the usual proteases and the build-up of such peptides leads to bitterness.

---

**Figure 5.** Chromatograms of various casein hydrolysates analyzed by RP-HPLC using a C-18 resin. (A) Intact caseinate. (B) Hydrolysate produced by incubation with subtilisin (judged to be very bitter). (C) Hydrolysate produced by an incubation with subtilisin followed by an incubation with the proline-specific endoprotease (judged to be strongly debittered).
very bitter hydrolysates were debittered to almost negligible levels. A schematic representation explaining these results is shown in Figure 4.

An incubation with the proline-specific protease was expected to lead to a selective degradation of the most hydrophobic peptides present, hereby debittering the hydrolysate. To confirm the disappearance of the hydrophobic peptides, both the subtilisin-treated and the subtilisin-plus-proline-specific protease-treated hydrolysates were subjected to a RP-HPLC analysis over a C-18 resin. On this resin, hydrophobic peptides have longer retention times than less hydrophobic peptides (22). Figure 5 shows the chromatograms of intact caseinate, caseinate incubated with subtilisin, and caseinate incubated with subtilisin plus the proline-specific protease. Evidently the subtilisin-treated caseinate contains many peaks with retention times that are longer than 30 min. Such hydrophobic peaks disappear upon incubation with the proline-specific protease. This finding strongly suggests that most of the hydrophobic peptides are cleaved by the proline-specific protease and that the removal of these hydrophobic peptides is indeed responsible for the debittering effect that was observed.

DISCUSSION

Although different prolyl oligopeptidases have been isolated from a large variety of organisms, it is unlikely that any of these known enzymes are suitable for use by the food industry. The main reasons are the cytosolic nature of these oligopeptidases and their unfavorable pH and temperature optima. Because the oligopeptidases are not secreted into the fermentation broth, elaborate purification steps are required, which are costly. Furthermore, their neutral pH optima in combination with relatively low temperature optima preclude efficient use under incubation conditions that are preferred for food applications. Our screening of food-grade fungi yielded a secreted proline-specific endoprotease with an acidic pH optimum that probably belongs to the S28 family of clan SC of serine proteases. The two known members of this S28 family are the exopeptidases dipeptidyl peptidase II and lysosomal Pro-X carboxypeptidase ("angiotensinase"). All three enzymes are serine proteases that can cleave peptide bonds at the carboxy-terminal end of proline residues, either within the peptide chain, or at its amino- or carboxyterminal end. Apparently, the nature (serine protease) and substrate specificity (cleavage at the C-terminal side of proline residues) is conserved in the primary amino acid sequence of these enzymes, but their endo- or exo-acting nature is not. The new proline-specific endoprotease also shows low but significant homology with thymus-specific serine peptidases (TSSP; i.e., EMBL acc. nr. AF052514). Furthermore, there is an unusual β-propeller domain regulates proteolysis. Cell 1998, 94, 161–170.

Apart from lowering the bitterness of specific hydrolysates, the removal of proline-rich protein sequences can be expected to impart other benefits on protein hydrolysates. For example, the β-casomorphine 7 (BCM-7) peptide forming the basis for the A1/A2 caseins discussion (23) is efficiently cleaved by the A. niger-derived enzyme (data not shown). Furthermore, recent scientific literature provides compelling evidence that an incomplete degradation of proline-rich peptides may contribute to the development of diseases such as celiac sprue, autism, and associated diseases (24). However, such proline-rich peptides present in wheat gluten are efficiently degraded by incubation with prolyl oligopeptidases (25). As none of the currently available industrial enzymes can cope with such problematic proline-rich protein sequences, there is little doubt that the proline-specific endoprotease from A. niger as described here will develop into an important tool for the food ingredients industry.

ACKNOWLEDGMENT

We thank Jan-Metske van der Laan for many helpful discussions and Dariusz Stepiak for his support in determining pH optima.

LITERATURE CITED


Received for review March 23, 2005. Revised manuscript received July 13, 2005. Accepted July 25, 2005.

JF050652C