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Purification and characterization of a prolyl endopeptidase isolated from *Aspergillus oryzae*

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Abstract A new fungal strain that was isolated from our library was identified as an *Aspergillus oryzae* and noted to produce a novel proly endopeptidase. The enzyme was isolated, purified, and characterized. The molecular mass of the prolyl endopeptidase was estimated to be 60 kDa by using SDS-PAGE. Further biochemical characterization assays revealed that the enzyme attained optimal activity at pH 4.0 with acid pH stability from 3.0 to 5.0. Its optimum temperature was 30 °C and residual activity after 30 min incubation at 55 °C was higher than 80 %. The enzyme was activated and stabilized by Ca^{2+} but inhibited by EDTA (10 mM) and Cu^{2+}. The K_m and k_{cat} values of the purified enzyme for different length substrates were also evaluated, and the results imply that the enzyme from *A. oryzae* possesses higher affinity for the larger substrates. Furthermore, this paper demonstrates for the first time that a prolyl endopeptidase purified from *A. oryzae* is able to hydrolyze intact casein.

Keywords Prolyl endopeptidase · *Aspergillus oryzae* · Purification

Introduction

Prolyl endopeptidases (EC 3.4.21.26) are the enzymes that hydrolyze substrates on the carboxyl site of proline residues located internally in a peptide (or ester) and they are widely distributed in plants [12, 13], mammals [1, 17, 18], bacteria [4, 8–10, 19, 24, 25, 28, 29], and fungi [6, 7, 11, 14, 21, 23, 28]. The roles of these enzymes among various organisms are diverse including the activation of cell-mediated immunity, autoimmune and inflammatory responses [2, 16, 20], and nutrient digestion. Prolyl endopeptidases are also commercially important enzymes which are used in several food processing and brewing industries particularly for the production of beer and bread-making processes [6, 14, 24].

The first microbial prolyl endopeptidase was purified from *Flavobacterium meningosepticum* and then classified as a serine protease on the basis of its inhibition by diisopropylfluorophosphate (DFP) [28]. Since then, prolyl endopeptidases were also found in many other microorganisms. From an application point of view, *F. meningosepticum* [29], *Xanthomonas* sp. [25], *Aeromonas hydrophila* [10], and *Pseudomonas* sp. KU-22 [19], which are pathogenic bacteria, are obviously not good choices for the food processing industry.

Therefore, the isolation and screening of new non-pathogenic fungi with higher prolyl endopeptidase activity could open new opportunities for the discovery and use of novel, efficient, and cost-effective prolyl endopeptidases. In this regard, Edens et al. [14] observed that low levels of an acid proline-specific endoprotease from *Aspergillus niger* in bottled beer effectively prevented chill-haze formation and left the beer form almost unaffected. Ever since, this has been widely applied in the industrial development of an enzymatic beer stabilization method. However, few works report the production of an *A. niger* proline-specific endoprotease; moreover, to our knowledge, its yield and activity were not high [6, 7, 11, 14]. Interestingly, *A. oryzae* species are often food-grade, non-toxigenic, and non-pathogenic microorganisms, which are widely used in traditional fermentation industries, e.g., soy sauce, sake, miso, shochu,

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bean curd seasoning, and vinegar production [5, 15]. In this paper, we focused our research on filamentous fungi as a source of new prolyl endopeptidases. During the screening of prolyl endopeptidase-producing organisms, we found a filamentous fungus, *A. oryzae* S1, which produced a highly specific prolyl endopeptidase with Z-Gly-Pro-pNA as the substrate. The aim of this study was to purify this unique prolyl endopeptidase produced by *A. oryzae* S1 and to study the effect of physical and chemical parameters on its activity. To the best of our knowledge, this is the first report on the production, purification, and characterization (pH, temperature, specificity, and application) of the prolyl endopeptidase from *A. oryzae*.

Materials and methods

Materials

The substrates Z-Gly-Pro-pNA, Ala-Ala-Pro-pNA, and Z-Ala-Ala-Ala-Pro-pNA were obtained from Bachem Americas, Inc. (CA, USA). The collagen was purchased from Sigma. DNA sequencing was performed using an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA). The Amicon Ultra 10,000 MWCO membrane was from Millipore (Billerica, MA, USA), and the columns including HiTrap DEAE FF and HiTrap Q FF were from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals used in this research were of the highest purity and commercially available.

Microorganism

The *A. oryzae* strains used in the present study were isolated from our library using the Czapek plating method. The fungal strain of *Aspergillus niger* 2.169 was used as a reference for comparisons in terms of proline-specific endopeptidase production. The strains were propagated onto PDA (Potato Dextrose Agar) medium plates at 28 °C

Media

The Czapek medium comprised (g/l) 2.0 g of $NaNO_3$, 1.0 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of $FeSO_4{\cdot}7H_2O$, 30 g of sucrose, and 20 g of agar. This medium generally allows for the isolation and differentiation of *Aspergillus* genus.

The selected medium comprised (g/l) Czapek medium, 2 ml 0.25 mM Z-Gly-Pro-pNA and 6 ml fast garnet (3 g/l).

The salts medium comprised (g/l) 1.0 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of $FeSO_4{\cdot}7H_2O$, 5.0 g of glucose, and 15 g of collagen (Sangon, Shanghai, China), as described by Edens et al. [6]. The salts medium was used for culture inoculation and collagen was used as the sole carbon source to induce the production of prolyl endopeptidase. The initial pH was adjusted to 5.0.

Fungal isolation and screening for prolyl endopeptidase activities

The *A. oryzae* strains stored in our library were first cultured in Czapek–agar plates containing 1.5 % (w/v) of collagen for 3–5 days at 28 °C. Then, 4 ml of 0.5 % melted agar and 2 ml of 0.25 mM Z-Gly-Pro-pNA were mixed and poured onto the agar plate. After incubation for 30 min at 28 °C, 6 ml of fast garnet was spread over the agar plate. The red color suggested the presence of activity toward the selected substrate.

Culture growth

The 3-day-old *A. oryzae* in the Czapek medium was transferred to the salts medium. The inoculum was incubated under at an agitation rate of 250 rpm for 5 day at 28 °C. Then the prolyl endopeptidase production was carried out by inoculating 50 ml of the culture medium in a 250-ml flask. Fungal mycelium was filtered through Whatman filter paper (24 mm) and centrifuged at 12,000*g* for 30 min. The supernatant was collected as a source of extracellular enzyme for further analysis.

DNA and sequence manipulation

The microorganism was identified using molecular biology techniques as described elsewhere [22]. Genomic DNA extraction was first performed by liquid nitrogen grinding and then carried out using the E.Z.NA fungal DNA kit.

18S DNA and the ITS region were submitted to PCR amplification using fungus-specific primers, namely 18F:5-CCTGGTTGATCCTGCCAGTA-3 and 18R: 5-GCTTGATCCTTCTGCAGGTT-3 and ITS4: 5-TCCTCCGCTTATTGATATGG-3 and ITS5: 5-GGAAGGTAAAAGTCAAGG-3. 18S DNA and the ITS region were amplified as described elsewhere, respectively [26, 27].

Enzyme activity

Prolyl endopeptidase activity was determined using the methodology reported by Edens [6, 14]. Firstly, the substrate (benzyloxycarbonyl-glycine-proline-*p*-nitroanilide, Z-Gly-Pro-pNA), was dissolved in 1,4-dioxane (40 %, v/v in water) at 60 °C to prepare a 0.25 mM solution. The *A. oryzae* prolyl endopeptidase activity was determined by using Z-Gly-Pro-pNA as the substrate at 35 °C (with a modification) in a citrate/disodium phosphate buffer (pH

5.0). The reaction products were monitored spectrophotometrically at 410 nm.

A unit of prolyl endopeptidase was defined as the amount of enzyme that releases 1 μmol of *p*-nitroanilide per minute under the assay conditions described.

Purification of prolyl endopeptidase from *A. oryzae*

The proteins in the crude extract were concentrated by ultrafiltration using an Amicon Ultra 10, 000 MWCO membrane. The concentrate was brought to 10, 20, 40, 60, 80, and 100 % saturation with $(NH_4)_2SO_4$, left for 4 h, respectively, and then the precipitate formed in each step was removed by centrifugation. The supernatant was further saturated with $(NH_4)_2SO_4$ overnight, while the precipitate was dissolved in a small volume of 20 mM phosphate buffer (pH 5.0), and dialyzed overnight. The steps were carried out at 4 °C unless otherwise described. The dialyzed enzyme solution was applied to HiTrap DEAE FF and HiTrap Q FF columns (Amersham Biosciences) pre-equilibrated with 20 mM phosphate buffer (pH 5.0). The column was washed with the same buffer with a linear gradient from 0 to 0.5 M NaCl, at a flow rate of 1 ml/min. The active fractions were tested according to the enzyme test method and the eluted proteins were also analyzed by SDS-PAGE. The molecular weight of the enzyme was also determined by SDS-PAGE.

Determination of pH and thermal Optima

The optimal pH for the enzyme activity was determined using citrate–phosphate buffers (pH 2.2–8.0). Enzyme stability against pH was estimated after incubating for 30 min in different pH reaction mixtures at 35 °C. The thermal dependence of prolyl endopeptidase activity was determined by measuring the enzyme activity in 0.1 M citrate–phosphate buffer (optimal pH) over a temperatures range of 15–80 °C. The thermal stability of the enzyme was detected by incubating the enzyme for 30 min at different temperatures (15–80 °C) in citrate–phosphate buffer (optimal pH).

Effect of metal ions and reagents on prolyl endopeptidase activity

Various metal ions (Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Al^{3+}) and reagents like EDTA (1, 10 mM) and phenylmethanesulfonylfluoride (PMSF, a typical serine protease inhibitor, 1 mM) were added to the enzyme and incubated for 30 min at 30 °C followed by enzyme assay under standard conditions at pH and thermal optima. Enzyme activities were expressed as relative values (%) and the sample without any metal ion or reagent was used as the control (100 %).

Enzyme kinetics

The Michaelis–Menten constant (K_m), maximal velocity (V_{max}), and k_{cat} of the purified enzyme were determined using Z-Gly-Pro-pNA, Ala-Pro-pNA, Ala-Ala-Pro-pNA, and Z-Ala-Ala-Ala-Pro-pNA as substrates in the range of 2, 4, 6, 8, and 10 mM under the optimal assay conditions in the standard method. The kinetic data were calculated from Lineweaver–Burk plots using the Michaelis–Menten equation using the software SkanIt RE for MS 2.4.2.

Hydrolysis of β-casein

One microgram of purified enzyme and β-casein dissolved in acetate buffer pH 3.5 to a concentration of 1 mg/ml were incubated for 6 h at 40 °C. Then the mixture was filtered through a 0.22-μm membrane and analyzed by RP-HPLC. The chromatographic conditions were as follows: ZORBAX SB-Aq C18 column (4.6 × 250 nm, Agilent, 5 μm); UV detector, detection wavelength 220 and 280 nm; flow rate, 1.0 ml/min; mobile phase, 0.1 % trifluoroacetic in 100 % acetonitrile, 0.1 % trifluoroacetic in 100 % water; injection volume, 10 μl.

Results and discussion

Fungal isolation and identification

Twenty new fungi were isolated from our library and were screened for prolyl endopeptidase production. Among them, the *A. oryzae* S1 strain was selected for its ability to release *p*-nitroanline from Z-Gly-Pro-pNA.

The 18S and the ITS regions of the isolated S1 strain were amplified by PCR as described in the "Materials and methods". The PCR amplified 18S DNA sequence (1,800 bp) showed the highest nucleotide identity of 99 % with the *A. oryzae* strain RIB40 (GenBank accession no. AP007173.1). Furthermore, *A. oryzae* was also the closest phylogenetic neighbor, according to the data from the ITS region sequence (600 bp). Therefore, strain S1 was identified as belonging to *A. oryzae*.

Production and purification of prolyl endopeptidase

The enzyme was purified from the crude extract using a combination of ammonium sulfate precipitation, ultrafiltration, and ion exchange chromatography as summarized in Table 1. The prolyl endopeptidase was purified by 73-fold in 18.25 % yield from the crude enzyme extract. First, the proteins in the crude extract were concentrated twofold by ultrafiltration using an Amicon system with a 10-kDa-cutoff membrane. Then, the retentates were

Table 1 Purification of the prolyl endopeptidase from *A. oryzae* S1

Purification steps	Total protein (ml)	Total activity (U)	Specific activity (U/l)	Purification (-fold)	Yield (%)
Crude extract	2,000	100	50	1	100
Ultrafiltration (10 kDa)	800	80	100	2	80
$(NH_4)_2SO_4$, dialyzed	85	29.8	350	7	29.8
HiTrap Q-FF	20	25	1,250	25	25
HiTrap DEAE-FF	5	18.25	3,650	73	18.25

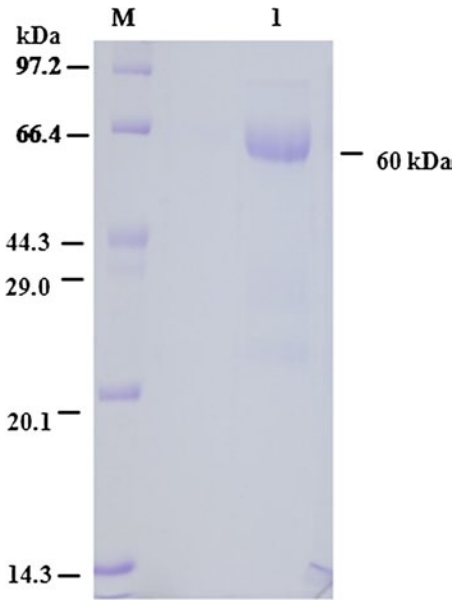

Fig. 1 SDS-PAGE of the purified prolyl endopeptidase from *A. oryzae*. *M* marker, *1* purified prolyl endopeptidase from *A. oryzae* S1

precipitated with 30–70 % $(NH_4)_2SO_4$ which achieved sevenfold enzyme purification. The precipitated proteins were dialyzed against 20 mM phosphate buffer (pH 5.0) and purified by using an anionic exchange chromatography Hitrap Q-FF column and Hitrap DEAE-FF column. The bound proteins were eluted by increasing linear gradient of 0–0.5 M NaCl in phosphate buffer (pH 5.0). Fractions exhibiting prolyl endopeptidase activity were pooled and concentrated by using a 30-kDa-cutoff membrane, and then examined further for purity by SDS-PAGE (Fig. 1). The SDS-PAGE suggested that the enzyme existed as a monomer with a molecular weight of about 60 kDa which is similar to the prolyl endopeptidase from *A. niger* [11].

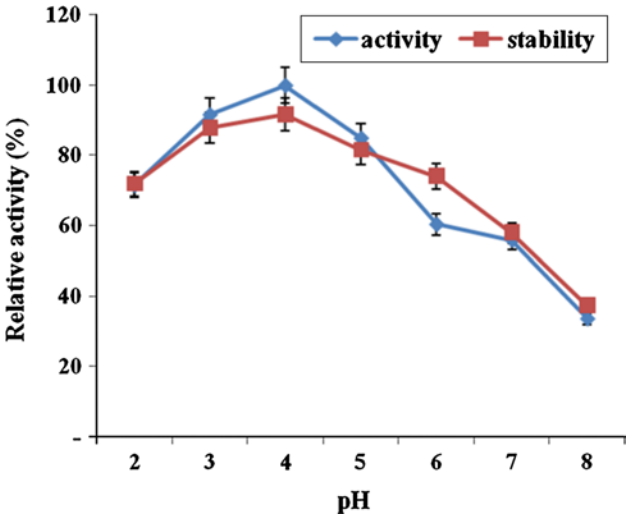

Fig. 2 Effect of pH on purified prolyl endopeptidase stability and activity. The relative activity of the prolyl endopeptidase from *A. oryzae* was determined at pH 2.2–8.0 and optimum temperature. All the experiments were conducted in triplicate

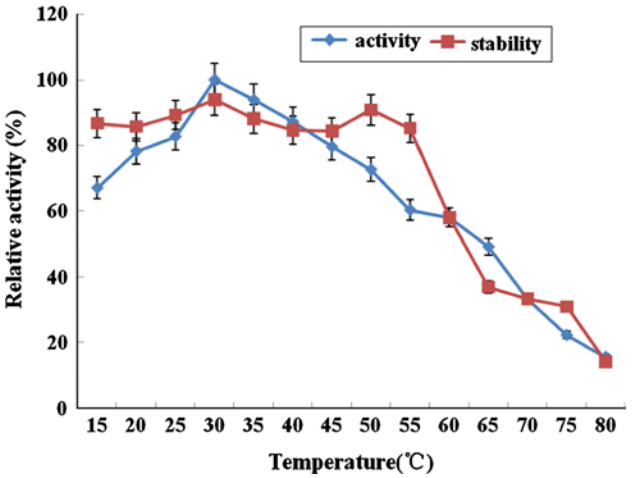

Fig. 3 Effect of temperature on the purified prolyl endopeptidase stability and activity. The relative activity of the prolyl endopeptidase from *A. oryzae* was determined at 15–80 °C and optimum pH. All the experiments were conducted in triplicate

Enzymatic and physicochemical properties

As shown in Fig. 2, the optimum pH for the prolyl endopeptidase from *A. oryzae* S1 was 4.0 with Z-Gly-Pro-pNA as the substrate. More than 70 % of the initial activity remained after incubation of the enzyme for 30 min at 35 °C between pH 3.0 and 5.0.

The optimum temperature for the prolyl endopeptidase was 40 °C (Fig. 3). The optimal temperature of the prolyl endopeptidase from *A. niger* and *Pseudomonas* sp. KU-22 was reported to be 42 and 45 °C, respectively

Table 2 Comparison of enzymatic and physiochemical properties of several prolyl endopeptidases

Origin	*Aspergillus oryzae*[a]	*Aspergillus niger* [14]	*Halobacterium halobium* S9 [4]	*Sphingomonas capsulata* [9]	*Pseudomonas* sp. KU-22 [19]	*Aeromonas hydrophila* [10]	*Xanthomonas* sp. [25]	*Flavobacterium meningosepticum* [29]	Human brain [1]
Optimum pH	4.0	4.2	8.7	8.5	8	8	7.5	7	6.8
pH stability	3.0–5.0		Wide variations of pH		8.0–11.0	7.0–8.5	6–8.5	5.0–9.0	5.5–9.5
Optimum temperature (°C)	30	50	40	43	45	30	ND	40	37
Thermal stability	55	ND	Wide variations of temperature	ND	45	35	ND	42	37
Isoelectric point	ND	Around pH 4.2	ND	7.6	4.9	5.5	6.2	9.6	4.75
Molecular weight	60,000	66,000	71,000	75,000	76,000	76,383	75,000	76,000	79,600
Inhibition with	EDTA	DFP	DFP	DFP	DFP	DFP	DFP	DFP, Z-Gly-Pro-CH_2Cl	DFP

ND not determined, *DFP* diisopropylfluorophosphate

[a] This study

[6, 19]. However, *A. hydrophila* and human brain enzyme showed optimal temperatures of 30 and 37 °C, respectively (Table 2) [1, 10]. The thermal stability of the purified prolyl endopeptidase from *A. oryzae* is also depicted in Fig. 3. The prolyl endopeptidase was quite stable up to 50 °C with a residual activity of above 70 % incubating at pH 4.0 for 30 min. A decrease in activity was observed when the enzyme was heated at temperatures above 60 °C. At higher temperatures, the prolyl endopeptidase probably underwent denaturation and lost its activity.

Effect of various metal ions and reagents on enzyme activity

The sensitivity of the purified prolyl endopeptidase from *A. oryzae* S1 to various metal ions and inhibitors was tested, and the results are shown in Table 3. Ca^{2+} ions significantly enhanced prolyl endopeptidase activity. A similar result was obtained for the proly endopeptidase from *A. niger* [11]. Zn^{2+} and Mg^{2+} did not show any considerable effect on the enzyme activity, whereas metal ions such as Fe^{2+}, Mn^{2+}, Cu^{2+}, and Al^{3+} exhibited different inhibitory effects on the enzyme activity.

The influence of some reagents on the activity of the prolyl endopeptidase from *A. oryzae* is also displayed in Table 2. The activity of the prolyl endopeptidase was slightly affected by PMSF or EDTA (1 mM), whereas the activity was moderately inhibited by 10 mM metal chelator EDTA indicating that it does require some metal ions for its activity. On the other hand, aprotinin and leupeptin hemisulfate slightly inhibited the enzyme activity.

Table 3 Effect of various metal ions on the activity of the prolyl endopeptidase from *A. oryzae* S1

Metal ions	Concentration (mM)	Relative enzyme activity (%)
None		100.0
Ca^{2+}	10	100 ± 2.8
Fe^{2+}	10	55 ± 3.1
Mg^{2+}	10	97 ± 2.9
Zn^{2+}	10	91 ± 2.3
Mn^{2+}	10	30 ± 1.8
Cu^{2+}	1	12 ± 1.5
Al^{3+}	10	65 ± 2.8
EDTA	1	95.2 ± 0.5
EDTA	10	37.3 ± 0.5
PMSF	1	98 ± 2.5
Aprotinin	1	89 ± 3.3
Leupeptin hemisulfate	1	86 ± 3.1

PMSF phenylmethanesulfonylfluoride

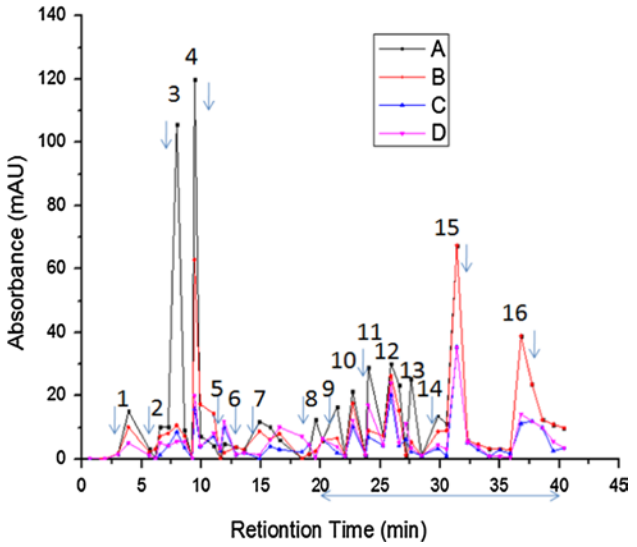

Fig. 4 Chromatogram of the casein hydrolysates analyzed by RP-HPLC using a C-18 resin. **a** Intact caseinate. **b** Hydrolysate produced by incubation with subtilisin (judged to be very bitter). **c** Hydrolysate produced by incubation with subtilisin plus *A. niger* endoprotease EPR purified in our library (judged to be the most strongly debittered) [6]. **d** Hydrolysate produced by incubation with subtilisin plus prolyl endopeptidase from *A. oryzae* purified in this study (judged to be strongly debittered)

Kinetic parameters

Kinetic data for the purified prolyl endopeptidase from *A. oryzae* S1 were tested. The effect of varying the substrate concentration on the reaction rate was investigated by using Z-Gly-Pro-pNA, Ala-Pro-pNA, Ala-Ala-Pro-pNA, and Z-Ala-Ala-Ala-Pro-pNA as the substrates. The results showed that the prolyl endopeptidase from *A. oryzae* did not hydrolyze the Ala-Pro-pNA, whereas it exhibited higher affinity for Ala-Ala-Pro-pNA ($K_m = 0.18$ mM, $k_{cat} = 107.4\ s^{-1}$, $k_{cat}/K_m = 596.67\ s^{-1}\ mM^{-1}$), in comparison to Z-Gly-Pro-pNA ($K_m = 0.26$ mM, $k_{cat} = 240\ s^{-1}$, $k_{cat}/K_m = 24\ s^{-1}\ mM^{-1}$), and Z-Ala-Ala-Ala-Pro-pNA ($K_m = 0.37$ mM, $k_{cat} = 75\ s^{-1}$, $k_{cat}/K_m = 202.7\ s^{-1}\ mM^{-1}$).

Hydrolysis of β-casein by purified prolyl endopeptidase from *A. oryzae*

During the enzymatic hydrolysis of proteins, bitter-tasting peptides such as casein are released thereby limiting their application in food processing. Because of its unusual structure, proline plays a key physiological role by protecting peptides from enzymatic degradation. Therefore, special attention was given to prolyl-specific protease hydolyzing bitter peptides and liberating aromatic amino acids. In this research, the effect of endopeptidase on the prolyl endopeptidase from *A. oryzae* was analyzed by RP-HPLC and compared to that of the recombinant endoprotease EPR from *A. niger* [11]. Figure 4 shows the chromatograms of intact caseinate, caseinate incubated with subtilisin, caseinate incubated with subtilisin plus the *A. niger* endoprotease EPR [11], and caseinate incubated with subtilisin plus the prolyl endopeptidase from *A. oryzae* purified in this study. Evidently several peptide fractions of the β-casein digested by the prolyl endopeptidase disappeared and new peaks occurred (Fig. 4), compared to the intact β-casein. On the other hand, hydrophobic peptides have longer retention times than the less hydrophobic peptides [3]. This finding strongly suggests that the prolyl endopeptidase from *A. oryzae* is able to cleave most of the hydrophobic peptidase and that the removal of these hydrophobic peptides is indeed responsible for the debittering effect that was observed, although the prolyl endopeptidase from *A. niger* may have more a significant effect on the caseinate. Enzymes hydrolyzing the Pro residues in bitter peptides are of particular interest. Although the use of proline-specific peptidase together with aminopeptidases of broad specificity has been successful in the food industry, the prolyl endopeptidase purified from *A. oryzae* has not been reported. The prolyl endopeptidase from *A. oryzae* was first screened and characterized with a relatively broad range of acid pH values. It can also cleave the β-casein into shorter fractions for the debittering effect. All of these results indicated the potential application of this enzyme in the food industry.

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