J Ind Microbiol Biotechnol (2013) 40:855–864
DOI 10.1007/s10295-013-1284-4

BIOTECHNOLOGY METHODS

Gene cloning and enzymatic characterization of an endoprotease Endo-Pro-*Aspergillus niger*

Chao Kang · Xiao-Wei Yu · Yan Xu

Received: 5 March 2013 / Accepted: 29 April 2013 / Published online: 18 May 2013

Abstract A novel endoprotease Endo-Pro-*Aspergillus niger* (endoprotease EPR) was first successfully expressed at high level in the methylotrophic yeast *Pichia pastoris* and the purification procedure was established. The endoprotease EPR is 95 % identity with proline specific endopeptidase from *A. niger* CBS513.88 (EMBL; AX458699), while sharing low identity with those from other microorganisms. The purified endoprotease EPR was a monomer of 60 kDa. Furthermore, the peptide mass fingerprinting (PMF) analysis confirmed that the purified protein was an endoprotease Endo-Pro-*Aspergillus niger*. A three-dimensional model revealed that the active site of the enzyme was located in $Ser_{(179)}$-$Asp_{(458)}$-$His_{(491)}$, based on template 3n2zB with sequence identity of 17.6 %. The optimum pH and temperature of the endoprotease EPR were pH 4–5 and 35 °C, and the stabilities were pH 3–7 and 15–60 °C, respectively. Furthermore, the endoprotease EPR had the ability to digest peptides with the C-terminal of proline as well as alanine, and was also capable of hydrolyzing larger peptides. The properties of the endoprotease EPR made it a highly promising candidate for future application in the field of brewing and food process.

C. Kang · X.-W. Yu (✉) · Y. Xu (✉)
State Key Laboratory of Food Science and Technology, The Key Laboratory of Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Ave, Wuxi 214122 Jiangsu, People's Republic of China
e-mail: bioyuxw@yahoo.com.cn

Y. Xu
e-mail: yxu@jiangnan.edu.cn

C. Kang
e-mail: kangchao61@hotmail.com

Keywords *Aspergillus niger* · Proline-specific endoprotease · Endo-Pro-*Aspergillus niger* · Expression · *Pichia pastoris*

Introduction

Prolyl endopeptidase (EC 3. 4. 21. 26), also called proline-specific endoprotease, belongs to the serine protease family and has the ability to cleave peptides at internal proline residues [30]. As is known, the cyclic amino acid proline, due to its unique structural properties, plays a key physiological role by protecting peptides from enzymatic degradation, and links to a wide range of diseases [2, 13, 17, 18], including depression, Parkinson's disease, and celiac sprue, as well as other diseases (blood pressure regulation, anorexia, bulimia nervosa, et al.). In contrast, the currently available proteolytic enzymes cannot efficiently cleave the peptide bond involving proline residues of proline-rich proteins, such as casein, gluten, collagen, and gelatin. However, many researchers found that the prolyl endopeptidase can overcome these problems. Prolyl endopeptidase also attracted numerous medical researchers and was proposed as a potential therapeutic approach because of its highly efficient degradation in gluten [14, 27].

The microbial prolyl endopeptidase was first purified from *Flavobacterium meningosepticum* and then classified as a serine protease on the basic of its inhibition by DFP [31]. Since then, prolyl endopeptidase activity was also found in *Xanthomonas* sp. [28], *Aeromonas hydrophilic* [11], *Pseudomonas* sp. KU-22 [15], *Sphingomonas capsulate* [10], *Lactobacillus helveticus* [25], *Halobacterium halobium* S9 [3], *Myxococcus xanthus* [6], *Aspergillus niger* [4, 12, 32], *A. oryzae* [19]. However, from an application point of view, *F. meningosepticum*,

Xanthomonas sp., *A. hydrophila*, *Pseudomonas* sp. KU-22, *Myxococcus xanthus*, which belong to pathogenic bacteria, are obviously not good choices for food processing industry, while *H. halobium* S9 also showed numerous serious drawbacks, such as culture condition with an extremely high salt concentration and preference for cleaving Pro in the penultimate position combination with hydrophobic amino acid in the C-terminal side of peptides. On the contrary, *Aspergillus* sp., known as the food-grade microorganisms, may be considered as safe and attractive microorganisms for producing prolyl endopeptidase [14, 27]. In addition, it was observed that using low levels of an acid proline-specific endoprotease from *A. niger* in bottled beer could effectively prevent chill-haze formation, but leaving the beer form almost unaffected [12]. Since then, this enzyme has been widely applied in brewing industry for beer stabilization. At the same time, the first proline-specific protease gene from *A. niger* was cloned and expressed in *A. niger* CBS513.88. The overexpressed proline-specific protease was of good debittering effect to the peptides responsible for the bitter taste of casein hydrolysate containing several proline residues [4]. Furthermore, genome of *A. niger* CBS518.13 was sequenced and analyzed, which revealed that the endoprotease Endo-Pro-*Aspergillus niger* (endoprotease EPR) was highly homologous to the proline-specific endoprotease with the same particular conserved catalytic triad Ser-Asp-His of serine protease. At the same time, the proline-specific endoprotease was also confirmed to be the S28 family of clan SC of serine proteases [16]. Endoprotease EPR was also considered to be proline-specific endoprotease. However, to our knowledge, few researchers have reported the production of *A. niger* proline-specific endoprotease with low yield and activity [4, 5, 16].

In recent years, the yeast *Pichia pastoris*, known as a powerful expression system for the production of high levels of various recombinant heterologous proteins, together with its economic use, has applied for both basic laboratory research and industrial manufacture [9]. Therefore, in this study, we constructed recombinant *P. pastoris* strains capable of producing endoprotease Endo-Pro-*Aspergillus niger* at high levels and the purified endoprotease EPR from the culture supernatant was characterized.

Materials and methods

Strains, vectors, reagents, and enzymes

A. niger 2.169 strains, *Escherichia coli* JM 109 strains, the plasmid expression vector pPIC9 K, and strain for protein expression *P. pastoris* GS115, were stored in our library. The plasmid vector pMD19-T, restriction endonucleases, *Taq* DNA polymerase and T4 DNA ligase, were purchased from TaKaRa Biotechnology. The Z-Gly-Pro-pNA substrate was obtained from Bachem (King of Prussia, PA, USA). The standard mini Plasmid Prep Kit and the DNA gel extraction kit were purchased from Omega (OMEGA bio-tek, USA). DNA sequencing was performed using an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA). The Amicon Ultra 30, 000 MWCO membrane was from Millipore (Billerica, MA, USA), and the HiTrap DEAE FF column was from Amersham Biosciences (Piscataway, NJ, USA).

Cloning of endoprotease EPR gene and construction of the expression plasmid

A. niger 2.169 was grown in a medium containing 1.0 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $FeSO_4.7H_2O$, 5 g of glucose, and 15 g of collagen (Sigma), as described by Edens et al. The collagen was used as the sole carbon source to induce the expression of the gene encoding for endoprotease EPR. Young mycelia were harvested after 48 h grown at 30 °C. Total RNA from *A. niger* was isolated using the TRIzol reagent exactly as described by the supplier (Sangon Biotech. Shanghai, China) and its purity was evaluated by electrophoresis on 2 % agarose gel.

Reverse transcription was performed by using 2 μg totals RNA, 1X Prime Script Buffer, 25 pmol Oligo dT Primer (50 μM) and 50 pmol Random 6 mers (100 μM). Reactions were carried out at 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 10 min. The resulting cDNA from the *A. niger* was then used for PCR. PCR mixture contained 0.5 μM final concentrations of sense (P1: 5′-CCG*GA ATTC*GCTCGCCCCCGTCTTGT) and antisense (P2: 5′-C C*GCGGCCGC*TCAAGCATAATACTCCTCCACCC) primers, which contained added sites for the restriction enzymes *Eco*RI and *Not*I (underline), respectively, TaKaRa Taq DNA polymerase 1.25 U, 10* PCR Buffer (Mg^{2+} plus) 5 μl, dNTP Mixture (each 2.5 mM) 4 μl, primer 1 (20 μM) 1 μl, primer 2 (20 μM) 1 μl, and cDNA 0.5 μg, in a 50-μl volume PCR amplification was performed by incubating the samples at 94 °C for 3 min of preheating, followed by 30 cycles at 94 °C for 30 s, at 63 °C for 60 s, and at 72 °C for 60 s, with a final extension at 72 °C for 10 min. At the end of amplification, samples were submitted to electrophoresis on 1.5 % agarose gel with a 2,000-bp DNA ladder as a size marker. The amplified bands of about 1,530 bp were visualized by Golden View I staining.

The PCR-amplified fragment encoding endoprotease EPR was cloned into the pMD19-T cloning vector and subjected to double-stranded DNA sequencing. After *Eco*RI-*Not*I digestion, the endoprotease EPR gene was cloned in the pPIC9 K vector between the *Eco*RI (5′ end) and *Not*I (3′ end) restriction sites to generate the plasmid

pPIC9 K-EPR. The resulting plasmid (pPIC9 K-EPR) was transformed into *E. coli* DH5α, and then the recombinant *E. coli* cells were selected on ampicillin containing LB plates and screened by PCR using the AOX1, P1, P2 primers. Plasmid DNA was purified from the recombinant *E. coli* DH5α and subjected to DNA sequence analysis to confirm the endoprotease EPR cDNA fragment.

Transformation and expression of endoprotease EPR in *P. pastoris*

Pichia pastoris wild-type strain GS115 was used as a host for the expression of the gene encoding endoprotease EPR. The expression vector pPIC9 K-EPR described above was linearized by digestion with restriction enzyme *Bgl*II and introduced into *P. pastoris* wild-type strain GS115 by electroporation using a Micropulser (Bio-Rad, Hercules, CA, USA). According to the manufacturer's recommendations (Invitrogen), the following culture media: minimal dextrose medium (MD), buffered glycerol-complex medium (BMGY), and buffered minimal methanol (BMMY), were prepared for the transformation of *P. pastoris*, selection of recombinant clones, and expression of endoprotease EPR. For cultures in liquid BMMY, which contain methanol as an inducer and carbon source, methanol was added every 24 h to a final concentration of 1 % (v/v). All cultures were carried out at 28 °C with shaking 250 rpm. Every day (0, 24, 48, 72, and 96 h), just before the addition of 1 % methanol, a 1-ml sample of the expression medium was collected to measure endoprotease EPR activity and for expression analysis by SDS-PAGE.

Enzymes activity

Endoprotease EPR activity was determined using the methodology reported by Edens et al. 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 250-μM solution. The expression endoprotease EPR activity was determined by using Z-Gly-Pro-pNA as substrate at 37 °C in a citrate/disodium phosphate buffer (pH 5.0). The reaction products were monitored spectrophotometrically at 410 nm. One unit of the endoprotease EPR activity was defined as the quantity of enzyme that releases 1 μmol of *p*-nitroanilide per minute under the conditions specified.

Purification of the endoprotease EPR

After 96 h of culture, the entire medium was harvested by centrifugation at 10,000 × *g* for 20 min. The endoprotease EPR secreted in the supernatant was purified by a four-step procedure consisting of ammonium sulfate precipitation, dialysis, Amicon Ultra 30, 000 MWCO membrane (Millipore), and ion-exchange chromatography. The following steps were carried out at 4 °C unless otherwise described. Briefly, the supernatant was fractionated with ammonium sulfate (60–80 % saturation) over night, and after centrifugation the precipitate was dialyzed in water, then concentrated with Amicon Ultra 30,000 MWCO membrane (Millipore), and applied to a HiTrap DEAE FF column (Amersham Biosciences) pre-equilibrated with 20 mM Tris–HCl (pH 5.0). The column was washed with the same buffer with a linear gradient from 0 to 0.5 M NaCl, and the enzyme eluted at around 0.3 M NaCl; the fractions containing activity were pooled and kept at 4 °C. The eluted proteins were also analyzed by SDS-PAGE.

Peptide mass fingerprinting analysis of the endoprotease EPR

PMF analysis of the endoprotease EPR was analyzed using MALDI-TOF–MS as follows. The sample was first analyzed by 12 % SDS-PAGE and then stained with silver nitrate. The recombinant endoprotease EPR band was cut out of the gel to be a 1-mm^3 rubber block by homemade cut-off device, decolorized in 200–400 μl 100 mM NH_4HCO_3/30 % acetonitrile solutions for about 1 h, and then the supernatant was removed. Then, the rubber block was incubated in 90 μl 100 mM NH_4HCO_3, 10 μl 100 mM DTT at 56 °C for 30 min, and stored in 70 μl 100 mM NH_4HCO_3, 30 μl 200 mM IAM3 at dark for 20 min; and then washed with 100 μl 100 mM NH_4HCO_3 for 15 min, 100 μl 100 % ACN for 5 min, respectively. The above sample without any liquid was reacted with 5 μl 2.5–10 ng/μl trypsin at 4 °C for 1 h. After removal of the supernatant, the sample (without any trypsin) was incubated in 20–30 μl 25 mM NH_4HCO_3 at 37 °C for about 20 h, and then the enzymatic hydrolysates that resulted from digestion of the protein with trypsin were introduced into a mass spectrometer. The positive-ion mode was employed and the mass spectrometer with the application of a spray voltage was set at 3.2 kV. MASCOT search tool (URL, http://www.matrixscience.com) was used for identification of tryptic maps.

Determination of pH and thermal optima

To establish the pH activity profiles of the endoprotease EPR, the citrate/disodium phosphate buffers with different pH values were prepared. A synthetic chromogenic peptide Z-Gly-Pro-pNA was used as the substrate for the enzyme.

Endoprotease EPR activity dependence in terms of pH was determined using 0.1 M citrate–phosphate buffers (pH of 2, 3, 4, 5, 6, 7, 8, and 9). Enzyme stability against pH was determined after incubating the enzyme for 30 min at 37 °C. Thermal dependence of endoprotease EPR activity

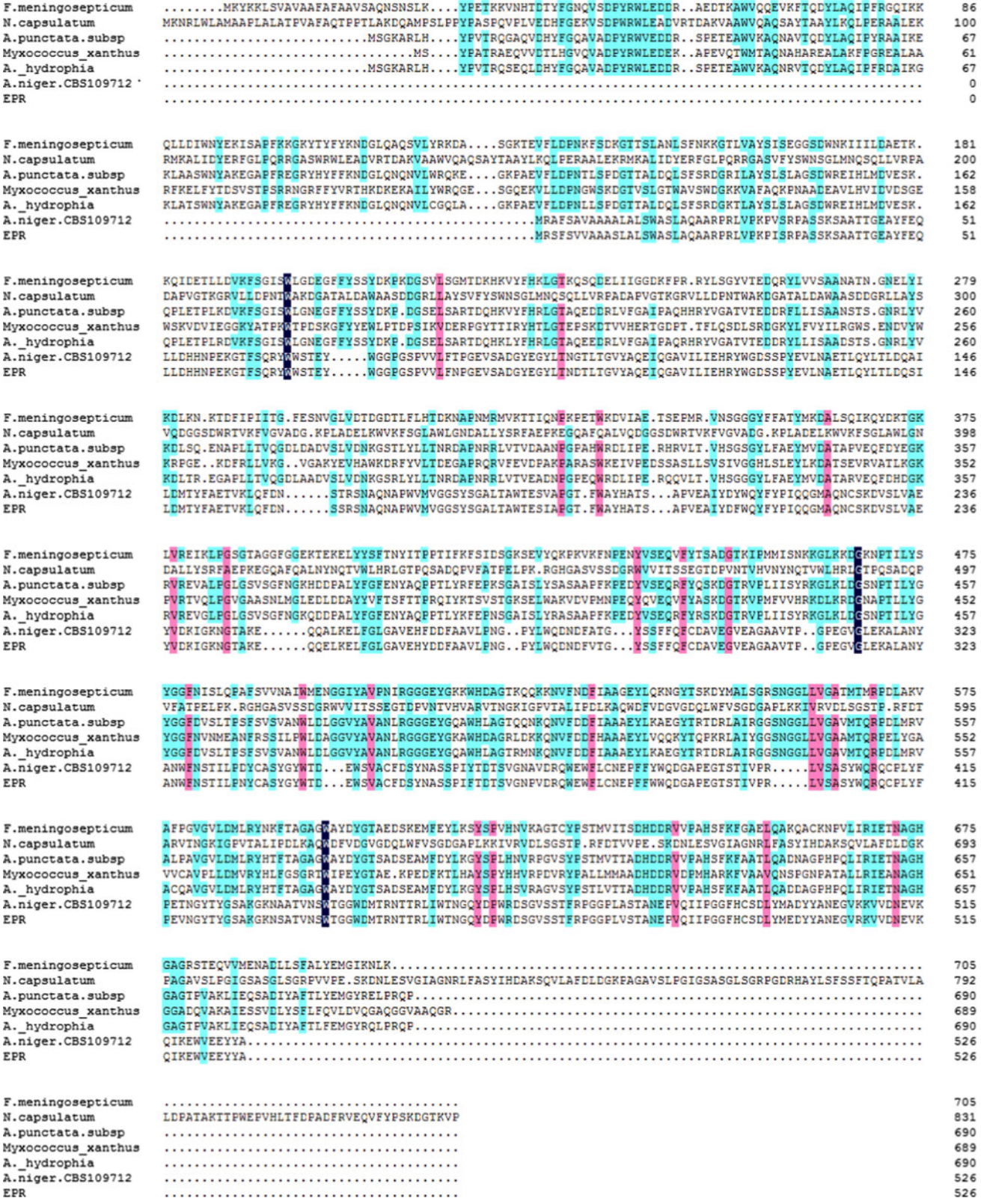

Fig. 1 Comparison of the amino acid sequence identity of endoprotease EPR with other prolyl endopeptidases from *F. meningosepticum*, *N. capsulatum*, *Aeromonas punctata* spp. *punctata*, *Myxococcus xanthus*, *A. hydrophia*, and *A. niger* CBS109712

was determined incubating the reaction mixture (enzyme in 0.1 M citrate–phosphate buffer pH 5 and the substrate) at temperatures between 15 and 80 °C. To evaluate thermal stability, 50 µl of the enzyme solution and 500 µl of 0.1 M citrate–phosphate buffer pH 5 were incubated for 30 min at different temperatures (15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80 °C). Then the activity was determined according to the standard enzyme activity test.

Inhibitors

Various inhibitors such as phenylmethylsulfonyl fluoride (PMSF, typical serine protease inhibitor, 0.1 M), EDTA (metal ions, 0.1 M), aprotinin (0.1 M), and leupeptin hemisulfate (0.1 M) were added to the enzyme and incubated for 30 min at 37 °C followed by enzyme assay under standard condition at 37 °C and pH 4.0. The sample without any inhibitor was taken as control (100 %).

Degradation of peptides and hydrolysis of intact protein by endoprotease EPR

The potential of endoprotease EPR in degrading various peptides and whole protein was given below. Two peptides with the sequences N-SKETTMPLW-OH (400 mg/ml) and N-SKETTMALW-OH (240 mg/ml) were incubated with the purified endoprotease EPR, at 35 °C for 30 min, respectively. The resulting peptide sequences were confirmed by LC/MS/MS. The ability of endoprotease EPR to hydrolyze the whole protein, such as β-casein, bovine serum albumin (BSA) and collagen, was studied. A total of 1 U of endoprotease EPR was incubated with 1 ml different proteins (1 g/l) in 50 mM sodium phosphate buffer (pH 5.0) at 35 °C for 24 h. Their hydrolysates were analyzed by RP-HPLC.

Results and discussion

Analysis of transformed clones and expression of the endoprotease EPR

Total RNA from *A. niger* was isolated and submitted to the reverse transcription. The resultant cDNA was used for PCR and yielded a 1-530-bp DNA fragment containing the whole coding region with the expected signal endoprotease EPR sequence. The homology alignment is shown in Fig. 1. Sequences of several reported prolyl endopeptidases from *F. meningosepticum*, *N. capsulatum*, *Aeromonas punctata* spp. *punctata*, *Myxococcus xanthus*, *A. hydrophia*, and *A. niger* were aligned with endoprotease EPR, which showed 10.19, 11.86, 10.71, 12.46, 11.27, and 95.82 % identity to the endoprotease Endo-Pro *Aspergillus niger*, respectively. Although the homology from different sources was very low, the sequence Gly-X-Ser-X-Gly was conserved among them.

The recombinant plasmid pPIC9 K-EPR containing the endoprotease EPR gene was transformed into the *P. pastoris* wild-type strain GS115. Some of the colonies selected on MD plates (ten of 34 colonies) were tested, and transformants were selected with G418 (2.0 mg/ml) by PCR using primers specific for endoprotease EPR to confirm the integration of the endoprotease EPR coding region into the *P. pastoris* genome and six positive clones were selected. The clone with the highest activity and one negative control, pPIC9 K (without the insert), was initially inoculated

Table 1 Purification of the recombinant protease endoprotease EPR

Purification step	Volume (ml)	Total activity (mU)	Specific activity (U/l)	Purification (-fold)	Yield (%)
Supernatant	300	150,000	500	1	100
Ammonium sulfate precipitation, dialysis	80	120,000	1,500	3	80
Amicon Ultra 30,000 MWCO membrane	60	108,000	1,800	6	72
HiTrap DEAE- FF	15	94,500	6,300	12.6	63

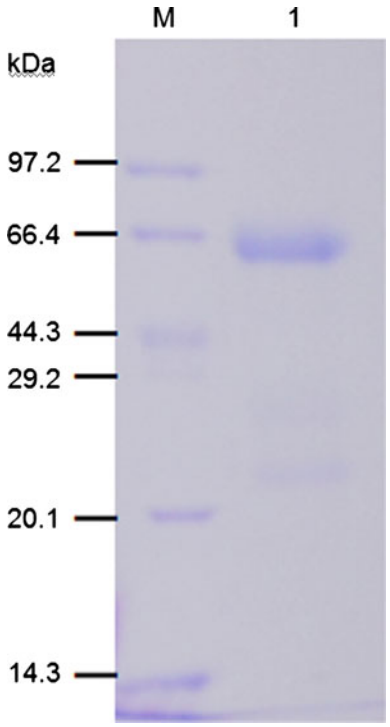

Fig. 2 SDS-PAGE of the purified recombinant endoprotease EPR protein. *M* protein marker; *1* endoprotease EPR

into BMGY and later into BMMY. The cells and the supernatant of these clones were then collected by centrifugation.

Purification and peptide mass fingerprinting (PMF) analysis of the endoprotease EPR

To confirm the expression efficiency of the target protein, the concentration of induction methanol (0.5 and 1 %) and the inducing period were optimized. The supernatants were taken at various time points (0, 12, 24, 48, 72, 80, 96 h). The results showed that the endoprotease EPR activity increased in a time-dependent manner within 80 h, but then slowed down at 96 h, which implied that the optimized inducing period of the transformant was around 80 h. Also, the final methanol concentration of 1 % was better than 0.5 %. After 80 h of methanol induction, cultures were harvested and the supernatants were collected for purification of endoprotease EPR.

The prolyl endopeptidase was purified 12.6-fold with 63 % yield from the crude enzyme extract (specific activity 500 U/l). A summary for this purification step is given in Table 1. Firstly, the proteins in the crude extract precipitated with 30–70 % $(NH_4)_2SO_4$ achieved threefold enzyme purification. Fractions exhibiting prolyl endopeptidase activity purified 12.6-fold (6,300 U/l) were pooled. The SDS-PAGE result suggested that the enzyme appeared as a monomer with molecular weight of about 60 kDa (Fig. 2).

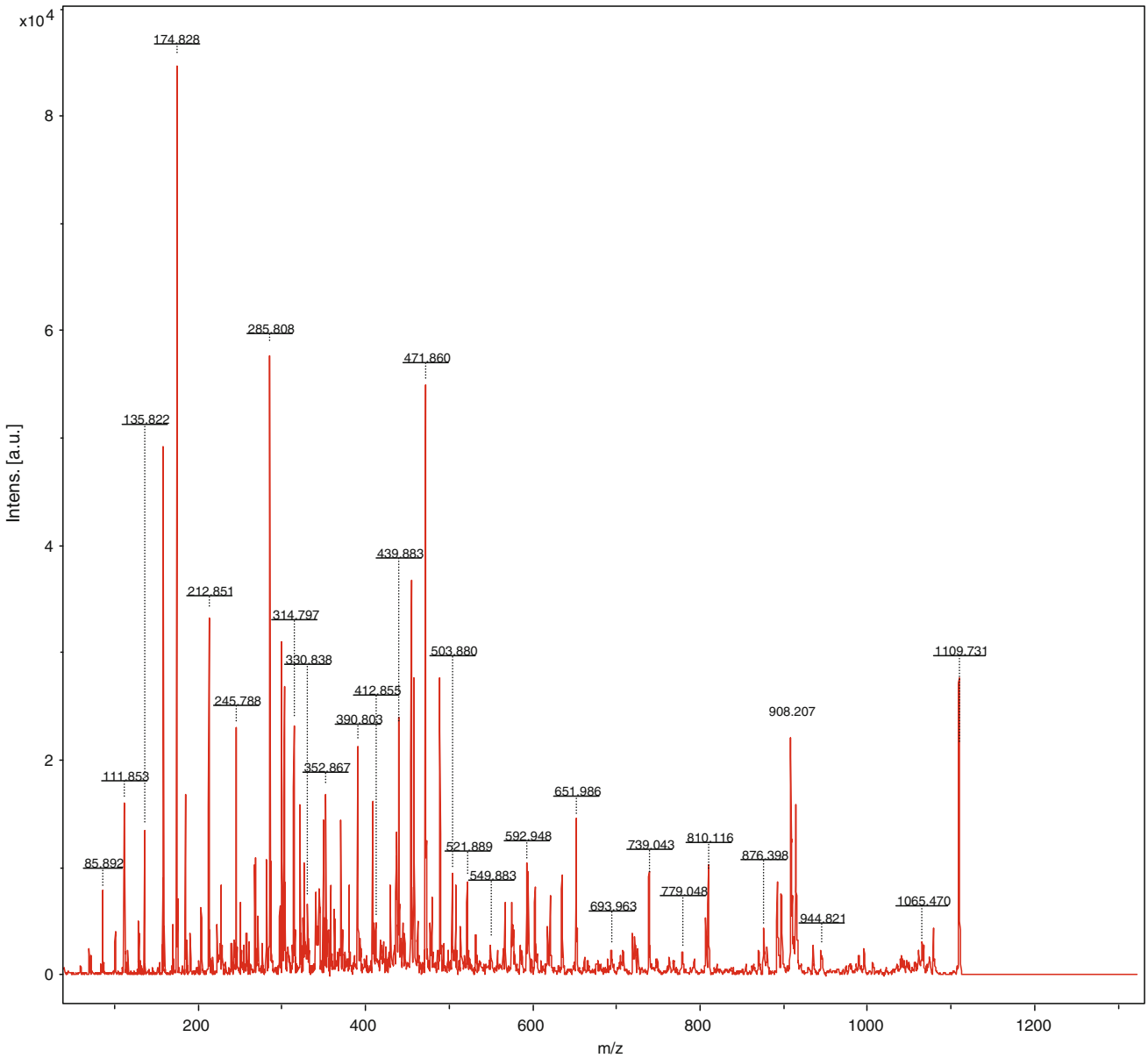

Fig. 3 Spectra of the peptide mass fingerprinting analysis of the endoprotease EPR

Peptide mass fingerprinting (PMF) is known to be an excellent, fast, and powerful search engine to differentiate peptidase even with highly similar properties. Therefore, the identification of the recombinant endoprotease EPR was analyzed by PMF analysis. As shown in Fig. 3, mass values of the peptides resulted from the endoprotease EPR digestion ranged from 877 to 3,600. The peptide mass fingerprinting data were analyzed using the MASCOT search tool (http://ww.matrixscience.com) and showed that a unique MS/MS fragmentation of LVSASYWQR matches with the recombinant endoprotease EPR, which further confirmed that the purified recombinant protein is what we want.

Proline-specific endopeptidases (PEPs) are a unique class of serine proteases, and most of their structures are unknown, excluding the structures from pig PEP, *S. capsulate* PEP, and *Myxococcus xanthus* PEP [21]. A web-based tool for protein structure homology modeling-Swiss Model and the software Discovery Studio 3.1 were used for predicting the simulated structure of the endoprotease EPR, which revealed that the active site was located in $Ser_{(179)}$-$Asp_{(458)}$-$His_{(491)}$, based on the template 3n2zB with sequence identity of 17.6 %. The result indicated that the endoprotease EPR shared the same active site Ser-Asp-His with human prolylcarboxypeptidase belonging to S28 protease family [20, 23].

Properties of endoprotease EPR produced by *P. pastoris*

As shown in Fig. 4a, the highest recombinant endoprotease EPR activity was observed at pH 4-5, measured in various citrate/disodium phosphate buffers and the residue enzyme activities were above 82 % of the maximum activity between pH 3 and 7. The optimum pH (pH 4) of the recombinant enzyme was similar to the prolyl specific endopeptidase (AN-PEP) reported by Edens et al. [12] (around pH 4.2), which was different from those in the mammalian and bacterial. The enzyme was stable over a range of acid pH 3–7, which indicated that the recombinant endoprotease EPR belonged to a low-pH form and the general base/acid-catalyzed acylation is the rate limiting. After incubation below pH 3 and above pH 8 for 30 min, the enzyme retained no more than 50 % of the maximum activity, and the activity was almost completely abolished at pH 2 and pH 9.

The endoprotease EPR expressed in *P. pastoris* presented its maximum activity at 35 °C (Fig. 4b), which was similar to proline endoprotease enzymes from *A. oryzae* FS1-32 (37 °C) [1], and its thermal stability was between 15 and 60 °C. The stability test revealed that the residual enzyme activity was 80-100 % after incubation at 15–60 °C for 30 min (Fig. 4b). Unlike these, optimal temperatures for AN-PEP [6, 12] and proline endoprotease

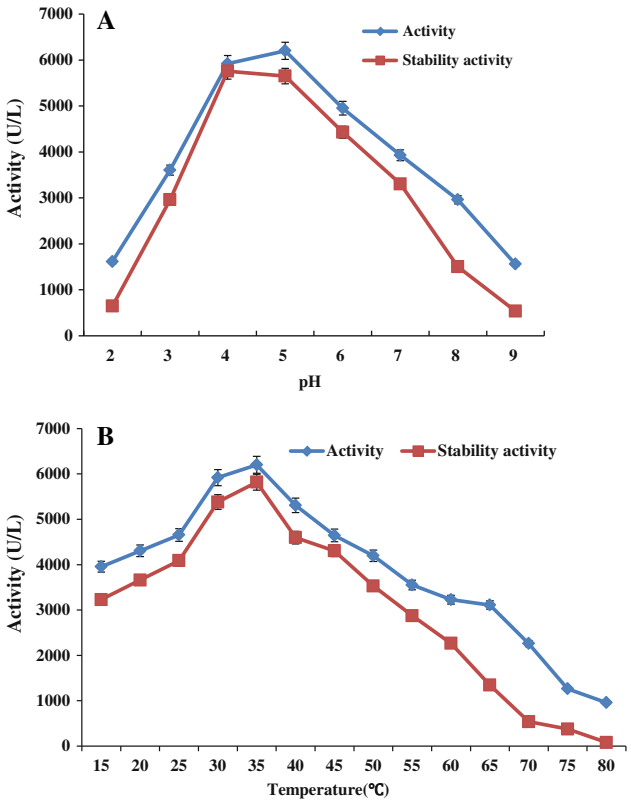

Fig. 4 **a** The effect of pH on the recombinant endoprotease EPR activity and stability. The activity was determined under conditions at pH 2.0–9.0 and 37 °C. The pH stability was determined by incubating enzyme at various pH at room temperature for 2 h and assay at optimum temperature (35 °C). **b** The effect of temperature on recombinant endoprotease EPR activity and stability. The activity for optimum temperature was determined under conditions at 15–80 °C and pH 5.0. The effect of temperature on enzyme stability was determined by incubating the enzyme for 30 min at temperatures in the range of 15–80 °C. All the experiments were conducted in triplicate

enzymes from *Pyrococcus furiosus* were both 50 °C and between 85 and 90 °C [8], respectively. Therefore, the recombinant endoprotease EPR may have an advantage over the enzyme denature in the latter processing stages. The incubation at 65–75 °C for 30 min reduced the activity of the enzyme by 30 % and after incubation at 80 °C for the same period almost total activity was lost.

The effects of PMSF, EDTA, aprotinin, and leupeptin hemisulfate on the recombinant endoprotease EPR activities were studied. Among the inhibitors tested, the aprotinin and leupeptin hemisulfate showed middle effects on both enzyme activities (87.3 and 82.5 %, respectively), while PMSF had only a slightly inhibiting effect on enzyme activity under the same reaction condition. However, EDTA showed a significant negative effect on the activity of the enzyme. It was worth mentioning that the addition of $CaCl_2$ can partially relieve the inhibition by EDTA, which hinted that the enzyme maybe associated

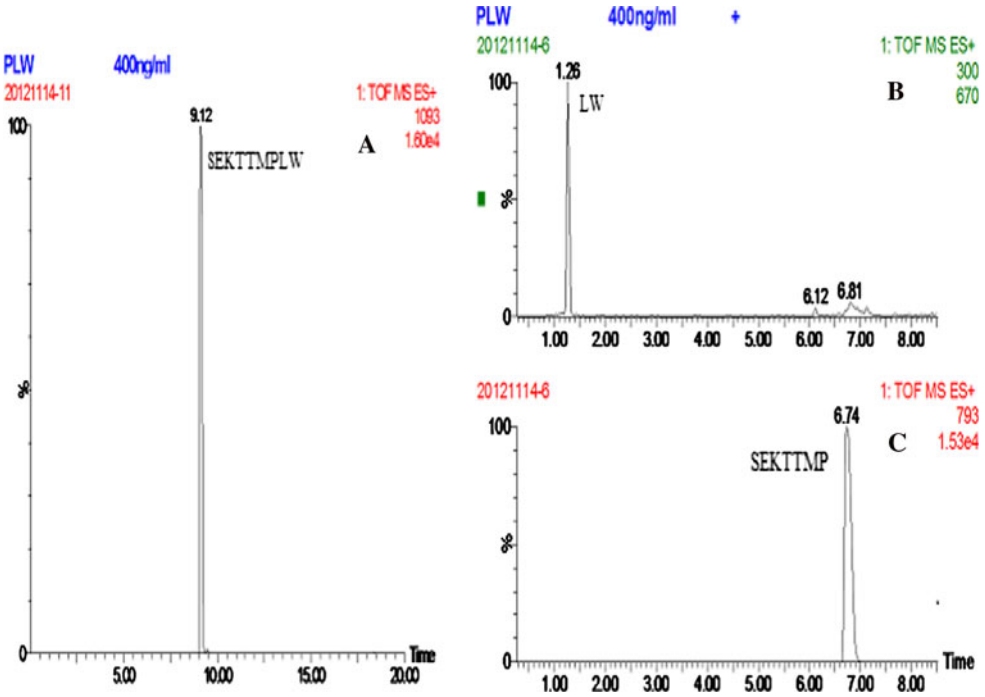

Fig. 5 The reaction products of a standard substance N-SEKTTMPLW-OH hydrolyzed by the recombinant endoprotease EPR were identified by LC–MS/MS upon identical retention time and mass fragmentation patent. **a** Total ion chromatograms of the stander peptide and MS/MS spectrum of the stander peptide peak at retention time 9.12 min. **b**, **c** Total ion chromatograms of enzymatic conversion of stander peptide, and MS/MS spectrum of the stander peptide peak at retention time 1.26 min (LW), 6.74 min (SEKTT MP), respectively

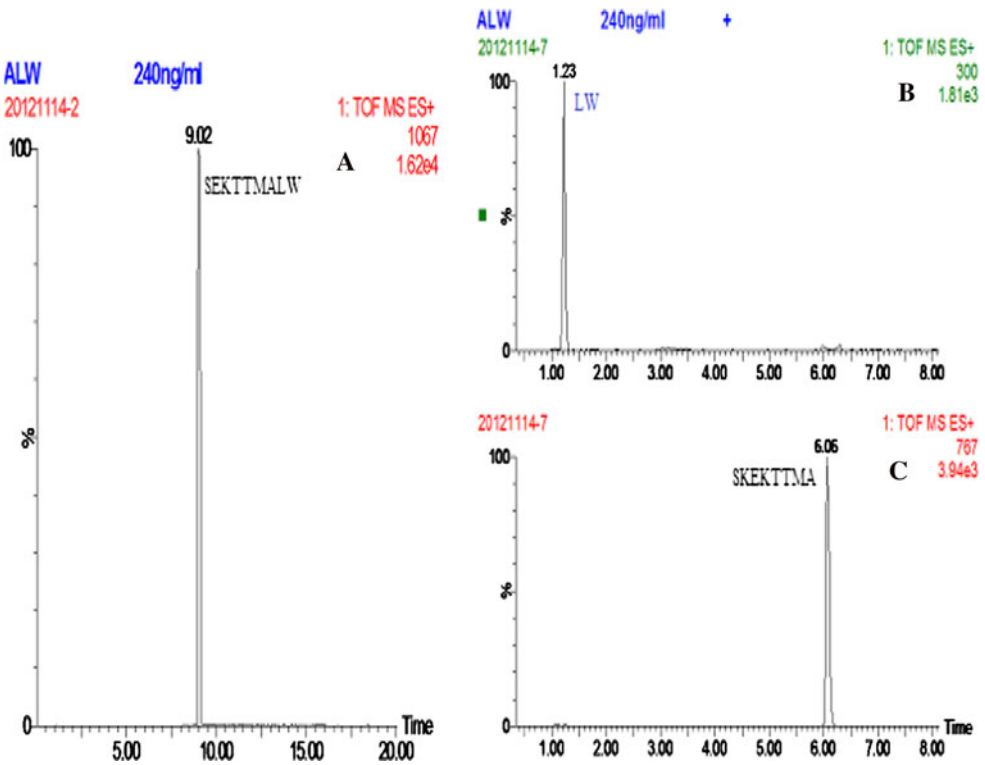

Fig. 6 The reaction products of a standard substance N-SEKTTMALW-OH hydrolyzed by recombinant endoprotease EPR were identified by LC–MS/MS upon identical retention time and mass fragmentation patent. **a** Total ion chromatograms of the stander peptide, and MS/MS spectrum of the stander peptide peak at retention time 9.02 min. **b**, **c** Total ion chromatograms of enzymatic conversion of stander peptide, and MS/MS spectrum of the stander peptide peak at retention time 1.23 min (LW), 6.06 min (SEKTT MA), respectively

with metal ions and required calcium ions for its optimal activity. This phenomena might be attributed to calcium ions involved in the stabilization of the enzyme molecular structure. In fact, calcium ions are known to be inducers and stabilizers of many enzymes [26, 29] and also protect them from conformational changes [24].

In comparison with chromogenic substrates Ala-Pro-pNA, Ala–Ala-Pro-pNA, Z-Gly-Pro-pNA, and Z-Ala-Ala–Ala-Pro-pNA, according to the kinetic assay, the recombinant endoprotease EPR toward the dipeptide Ala-Pro-pNA showed almost no activity, while Ala–Ala-Pro-pNA and Z-Ala-Ala–Ala-Pro-pNA were better substrates for the

endoprotease EPR. The research implied that the enzyme preferred larger substrates. Two peptides with the sequences N-SKETTMPLW-OH (Mw 1092.29) and N-SKETTMALW-OH (Mw 1066.25) were incubated with the purified recombinant endoprotease EPR, respectively. The resulting peptide sequences N-SKETTMP-OH (Mw 792) (Fig. 5) and N-SKETTMA-OH (Mw 766.7) were confirmed by LC/MS/MS (Fig. 6). Until recently, researchers found that certain proline-specific endoprotease could degrade a 33-mer gluten-derived and intact protein [22]. What limits the enzyme specificity is the substrate accessibility to the proline-specific endoprotease activity site instead of the chain length specificity [7]. In addition, HPLC analysis in our study showed that the pure recombinant endoprotease EPR also could digest the β-casein and bovine serum albumin.

Acknowledgments Financial support from the National High Technology Research and Development Program of China (863 Program) (No. 2012AA022207 and 2008AA10Z304), the Fundamental Research Funds for the Central Universities (JUSRP11014), and the Ministry of Education, R.P. China, and from NSFC (20802027) is greatly appreciated.

References

1. Araki H, Ouchi H, Uesugi S, Hashimoto Y, Shimoda T (1993) Prolyl endopeptidase and production thereof. EPO patent EP19920111124
2. Arentz-Hansen H, McAdam SN, Molberg Ø, Fleckenstein B, Lundin KE, Jørgensen TJ, Jung G, Roepstorff P, Sollid LM (2002) Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. Gastroenterology 123:803–809
3. Capiralla H, Hiroi T, Hirokawa T, Maeda S (2002) Purification and characterization of a hydrophobic amino acid specific endopeptidase from *Halobacterium halobium* S9 with potential. Process Biochem 38:571–579
4. Edens L, Dekker P, van der Hoeven R, Deen F, de Roos A, Floris R (2005) Extracellular prolyl endoprotease from *Aspergillus niger* and its use in the debittering of protein hydrolysates. J Agric Food Chem 53:7950–7957
5. Esparza Y, Huaiquil A, Neira L, Leyton A, Rubilar M, Salazar L, Shene C (2011) Optimization of process conditions for the production of a prolylendopeptidase by *Aspergillus niger* ATCC 11414 in solid-state fermentation. Food Sci Biotechnol 20:1323–1330
6. Gass J, Ehren J, Strohmeier G, Isaacs I, Khosla C (2005) Fermentation, purification, formulation, and pharmacological evaluation of a prolyl endopeptidase from *Myxococcus xanthus*: implications for celiac sprue therapy. Biotechnol Bioeng 92:674–684
7. Gass J, Khosla C (2007) Prolyl endopeptidases. Cell Mol Life Sci 64:345–355
8. Harwood VJ, Denson JD, Robinson-Bidle KA, Schreier HJ (1997) Overexpression and characterization of a prolyl endopeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol 179:3613–3618
9. Higgins DR, Cregg JM (1998) Methods in molecular biology: *Pichia* protocols. Totowa, NJ
10. Kabashima T, Fujii M, Meng Y, Ito K, Yoshimoto T (1998) Prolyl endopeptidase from *Sphingomonas capsulata*: isolation and characterization of the enzyme and nucleotide sequence of the gene. Arch Biochem Biophys 358:141–148
11. Kanatani A, Yoshimoto T, Kitazono A, Kokubo T, Tsuru D (1993) Prolyl endopeptidase from *Aeromonas hydrophila*: cloning, sequencing, and expression of the enzyme gene, and characterization of the expressed enzyme. J Biochem 113: 790–796
12. Lopez M, Edens L (2005) Effective prevention of chill-haze in beer using an acid proline-specific endoprotease from *Aspergillus niger*. J Agric Food Chem 53:7944–7949
13. Mentlein R (1988) Proline residues in the maturation and degradation of peptide hormones and neuropeptides. FEBS Lett 234:251–256
14. Mitea C, Havenaar R, Drijfhout JW, Edens L, Dekking L, Koning F (2008) Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. Gut 57:25–32
15. Oyama H, Aoki H, Amano M, Mizuki E, Yoshimoto T, Tsuru D, Murao S (1997) Purification and characterization of a prolyl endopeptidase from *Pseudomonas* sp. KU-22. J Ferment Bioeng 84:538–542
16. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K et al (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25:221–231
17. Polgar L (2002) The prolyl oligopeptidase family. Cell Mol Life Sci 59:349–362
18. Polgar L (2002) Structure-function of prolyl oligopeptidase and its role in neurological disorders. Curr Med Chem 2:251–257
19. Riggle HM, Fisher MA (2009) Purification of a prolyl endopeptidase from *Aspergillus oryzae* and evaluation of its ability to digest gluten. http://oasys2.confex.com/acs/237nm/techprogram/P1239602.HTM
20. Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385
21. Shan L, Mathew II, Khosla C (2005) Structure and mechanistic analysis of two prolyl endopeptidase: role of interdomain dynamics in catalysis and specificity. PNAS 102:3599–3604
22. Shan L, Molberg Ø, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C (2002) Structural basis for gluten intolerance in celiac sprue. Science 297:2275–2279
23. Soisson SM, Patel SB, Abeywickrema PD, Byrne NJ, Diehl RE, Hall DL, Forf RE, Reid JC, Rickert KW, Shipman JM, Sharma S, Lumb KJ (2010) Structural definition and substrate specificity of the S28 protease family: the crystal structure of human prolylcarboxypeptidase. BMC Struct Biol 10:16
24. Sousa F, Ju S, Erbel A, Kokol V, Cavaco-Paulo A, Gubitz GM (2007) A novel metalloprotease from *Bacillus cereus* for protein fibre processing. Enzyme Microb Technol 40:772–781
25. Sridhar VR, Hughes JE, Welke DL, Broadbent JR, Steele JL (2005) Identification of endopeptidase genes from the genomic sequence of *Lactobacillus helveticus* CNRZ32 and the role of these genes in hydrolysis of model bitter peptides. Appl Environ Microbiol 71:3025–3032
26. Subba C, Sathish T, Ravichandra P, Prakasham RS (2009) Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. Process Biochem 44:262–269
27. Stepniak D, Spaenij-Dekking L, Mitea C, Moester M, de RuA, Baak-Pablo R, van VP, Edens L, Koning F (2006) Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. Am J Physiol Gastrointest Liver Physiol 291:621–629

28. Szwajcer-Dey E, Rasmussen J, Meldal M, Breddam K (1992) Proline-specific endopeptidases from microbial sources: isolation of an enzyme from a *Xanthomonas* sp. J Bacteriol 174:2454–2459
29. Takii Y, Kurlyama N, Suzuki Y (1990) Alkaline serine protease produced-from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP 1239. Appl Microbial Biotechnol 34:57–62
30. Walter R, Simmons WH, Yoshimoto T (1980) Proline-specific endo- and exopeptidases. Mol Cell Biochem 30:111–127
31. Yoshimoto T, Walter R, Tsuru D (1980) Proline-specific endopeptidase from *Flavobacterium*: purification and properties. J Biol Chem 255:4786–4792
32. Xianchang X, Di Y, Fuping L, Yanlin G, Yu L (2009) Cloning of proline-specific endoprotease gene of *Aspergillus niger* and expression in *Pichia pastoris*. Ind Microbiol (in Chinese) 39:7–12