Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2017

www.elsevier.com/locate/jbiosc

Enzymatic characterization of a novel Xaa-Pro aminopeptidase XpmA from *Aspergillus oryzae* expressed in *Escherichia coli*

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Received 19 January 2017; accepted 18 June 2017
Available online xxx

Xaa-Pro aminopeptidases are peptidases responsible for the cleavage of any amino acid N-terminally adjacent to a proline residue. We identified a gene encoding a putative Xaa-Pro aminopeptidase in the genome of the filamentous fungus *Aspergillus oryzae* (genome database number: AO090701000720) and named this gene *xpmA*. We produced its enzyme in a C-terminally His_6-tag-fused form in an *Escherichia coli* expression system and purified it. The purified recombinant XpmA (rXpmA) showed hydrolysis activity toward Xaa-Pro-oligopeptides, especially the two dipeptides Ala-Pro and Phe-Pro. The molecular weight of rXpmA was estimated to be 69 kDa by SDS-PAGE and 126 kDa by gel filtration, suggesting that it is a homodimer. The enzyme was activated by various divalent metal ions such as Mn^{2+}, Co^{2+}, and Mg^{2+}; in particular, the enzyme activity was increased 27.6-times relative to the no-addition control by 1 mM Mn^{2+}. Additionally, 10 mM EDTA suppressed its activity to 0.26-times of the control level. Therefore, rXpmA was a metalloprotease. Optimal hydrolytic activity of rXpmA was observed at 50°C and pH 8.5–9.0. The enzyme was stable up to 50°C and from pH 4.0 to 11.0. rXpmA showed substrate inhibition by Leu-Pro, Ser-Pro and Arg-Pro at concentrations over 4 mM, 10 mM, and 3 mM, respectively. NaCl increased the enzyme activity in the concentration range 0.5–3.0 M, suggesting that the enzyme is halophilic.

[**Key words:** Xaa-Pro aminopeptidase; Metallopeptidase; M24B family; Proline; Halophilicity; *Aspergillus oryzae*]

Aspergillus oryzae, a filamentous fungus, produces various and vast amounts of enzymes and is widely used in Japan in the production of traditional fermented foods such as soy sauce (shoyu), soybean paste (miso) and rice wine (sake). Genome analysis of *A. oryzae* strain RIB40 has been completed (1). *A. oryzae* grows and produces various enzymes in a complex fermentation process. Proteases of *A. oryzae* break down proteins into peptides and amino acids in the process. Various oligopeptides and amino acids in the fermented food are thought to contribute to tastes and flavors (2). Therefore, proteases which can increase these peptides and amino acids are considered very important enzymes for seasoning the foods. In general, proteases are classified into two groups: endopeptidases and exopeptidases (3,4). We are interested in exopeptidases, which cleave amino acid residues at the extremities of the polypeptide. Most exopeptidases show high substrate specificity. We have focused on enzymes, especially "aminopeptidases", which release free amino acid residues at the N-terminal end of polypeptides. Understanding the substrate specificities of aminopeptidases would be very valuable in understanding fermented foods and making new fermented products with novel values such as tastes, flavor or functional claims.

The genome sequence of *A. oryzae* (DOGAN: http://www.bio.nite.go.jp/dogan/project/view/AO) revealed the presence of >130 protease-like genes (5), 80% of which were uncharacterized. Based on the genome information, we have comprehensively investigated all genes annotated as aminopeptidases for use in industrial applications. So far, we have characterized several aminopeptidases of *A. oryzae*, including prolyl aminopeptidase (PamA) (6), leucyl aminopeptidase (LapA) (7), glycine-D-alanine aminopeptidase (GdaA) (8), and D-stereoselective aminopeptidase A (DamA) (9).

It was reported that soy sauce contains the peptides Gly-Pro, Ser-Pro, Thr-Pro, Ala-Pro, and Val-Pro, which all contain proline in the N-terminal penultimate position (10,11). It may be presumed that a variety of oligopeptides containing Xaa-Pro at the N-terminus are present in the fermented foods. Most of those oligopeptides, such as Tyr-Pro, Arg-Pro, Val-Pro-Phe, and Gly-Pro-Gly, were reported to have a bitter taste (2,12,13). However, other research reported that proline indicates "sweet taste; possibly complex with salty or sour components" (14). Therefore, peptidases that can hydrolyze peptide bonds between Xaa-Pro dipeptides or oligopeptides containing Xaa-Pro at the N-terminus may contribute to improved tastes of the fermented foods by increasing free prolines; this activity would occur in concert with prolyl aminopeptidases, which catalyze the cleavage of the N-terminal amino acid adjacent to a proline residue (6).

The unique structure of proline induces conformational constraints on the peptide bond, protecting it from general degradation processes (15,16). The existence of a small number of special proteases, "proline-specific peptidases", which can hydrolyze the bond

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.06.007

between proline and other amino acids has been reported (15). Some of the proline-specific peptidases belonging to the M24B family in MEROPS (the Peptide Database, Release 10.0) (17) were defined as peptidases that remove the N-terminal amino acids from peptides in which the penultimate residue is proline. The peptidases of the M24B family are classified as metallopeptidases and include two proline-specific peptidases, Xaa-Pro aminopeptidase (Aminopeptidase P, PepP) and prolidase (Xaa-Pro dipeptidase; EC 3.4.13.9) which can only hydrolyze Xaa-Pro dipeptides (15,16). We focused on the M24B family peptidases of *A. oryzae*.

In the genome of *A. oryzae*, we found six genes (genome database numbers: AO090701000720, AO090005001240, AO090011000942, AO090120000162, AO090001000597, and AO090003000847) which were annotated as belonging to the M24B family. The six genes were expressed in *Escherichia coli* and it was confirmed whether each affinity-purified product had hydrolysis activity toward Ala-Pro or Leu-Pro. Three gene products showed Leu-Pro hydrolytic activity (AO090701000720, AO090005001240, and AO090011000942; data not shown). The activities of the products of AO090005001240 and AO090011000942 have been published in patent applications by Kikkoman Corporation in the data base of Japan Platform for Patent Information (J-Plat Pat) of Japan Patent Office. AO090701000720 gene is annotated as a putative Xaa-pro aminopeptidase and its deduced amino acid sequence contains the conserved multi-domains of PepP (Xaa-pro aminopeptidase) by Conserved Domain Search of NCBI. To date, there are no reports about enzymatic characterization of Xaa-pro aminopeptidase among fungi, although prolidase of *Aspergillus nidulans* was already reported (18). Therefore, we proceeded to characterize the product of the gene, as a novel Xaa-Pro aminopeptidase from *A. oryzae*. We named the gene of interest *xpmA* and purified the recombinant Xaa-Pro aminopeptidase (rXpmA) which was expressed in *E. coli* in large quantities. We describe the substrate specificity and properties of the purified rXpmA in detail. This is the first report on characterization of a fungal Xaa-Pro aminopeptidase.

MATERIALS AND METHODS

Chemicals Ala-Pro-OH (AP), Phe-Pro-OH (FP), Ser-Pro-OH (SP), Arg-Pro-OH (RP), Gly-Pro-OH (GP), Ala-Pro-Tyr-Ala-OH, Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg-OH, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH, Leu-Pro-Phe-Phe-Asp-OH, Gly-Pro-Ala-OH, Pro-Ala-OH, Pro-Leu-Gly-NH_2, and Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-OH were obtained from Bachem (Bubendorf, Switzerland). All other chemicals used were of certified reagent grade.

Strains and media *A. oryzae* strain RIB40 (ATCC42149) was used to isolate total RNA. YPD medium (1.0% yeast extract, 2.0% polypeptone, and 2.0% D-glucose) was used for growth of *A. oryzae*. *E. coli* BL21 competent cells (Invitrogen, Thermo Fisher Science) were used for the construction of plasmid DNAs and expression of the recombinant *xpmA* gene. Luria–Bertani (LB) medium (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) was used for bacterial culture. Ampicillin (50 μg/ml) was added to cultures of plasmid-carrying *E. coli* strains.

Cloning of *xpmA* cDNA from *A. oryzae* and construction of pCold-XpmA plasmid Total RNAs of *A. oryzae* RIB40 were extracted from mycelia grown at 30°C for 20 h in YPD liquid medium culture using Isogen (Nippon Gene, Tokyo, Japan). mRNAs were purified from the total RNAs by using the Oligotex-dT30 <Super> mRNA Purification Kit (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. mRNA (100 ng) was used for cDNA pool synthesis using the ReverTraAce-α RT-PCR Kit (Toyobo, Osaka, Japan) and oligo dT primers provided in the kit. Construction of plasmid pCold-XpmA was per the instructions for the Cold Shock Expression System pCold DNA (Takara Bio). The *xpmA* cDNA, which was fused in-frame with a sequence encoding the Factor Xa recognition sequence (Ile-Glu-Gly-Arg) and a His_6-tag at the 3′-end, was amplified by PCR from the cDNA pool with three primers: xpmA F, xpmA R1, and xpmA R2 using the Phusion High-Fidelity PCR Kit (New England Biolabs, MA, USA). The amplified *xpmA* fragment was cloned into the *NdeI*-*XbaI* sites of pColdIV using an In-Fusion HD Cloning Kit (Takara Bio-Clontech). The resultant plasmid pCold-XpmA was transformed into *E. coli* BL21. Primer sequences were described in Supplementary Table S1.

Purification of recombinant C-terminal His_6-tagged XpmA Overnight preculture (2 ml) of cells of *E. coli* BL21 harboring pCold-XpmA was transferred into fresh LB medium (150 ml) containing 50 μg/ml ampicillin, and shaken at 110 strokes/min at 37°C until the optical density reached 0.4. Subsequently, the cultures were incubated at 15°C for 30 min and induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside. After allowing additional growth (110 strokes/min) at 15°C for 2 days, the cells were harvested by centrifugation, washed with buffer A (20 mM Tris–HCl, pH 7.5, containing 300 mM NaCl and 20 mM imidazole), resuspended and sonicated in 10 ml of the same buffer. The lysate was centrifuged, and the supernatant was absorbed into Ni-IMAC gel (Ni-IMAC Profinity, Bio-Rad, CA, USA) packed into an Econo-column (Bio-Rad), which was pre-equilibrated with buffer A at 4°C. The resin was washed with buffer A, followed by elution of the recombinant C-terminal His_6-tagged XpmA (rXpmA) with buffer B (20 mM Tris–HCl, pH 7.5, containing 300 mM NaCl and 100 mM imidazole). The eluted proteins were loaded onto a HiLoad 16/600 Superdex 200 pg gel filtration column (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with buffer D (50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl). Eluates were collected in fraction tubes (0.5-ml each). Peak fractions were collected and assayed for rXpmA activity using Ala-Pro as the substrate. Active fractions were pooled and dialyzed against buffer E (20 mM Tris–HCl, pH 7.5) for additional analysis. The molecular mass of purified rXpmA was determined using a Superose 12 gel filtration column (column size: 1.0 × 30 cm; flow rate: 0.5 ml/min) (GE Healthcare) pre-equilibrated with buffer D. A Gel Filtration Calibration Kit (GE Healthcare) was used for the calibration; hexokinase (100 kDa), aldolase (158 kDa), catalase (240 kDa), and ferritin (440 kDa) were used as reference proteins. Retention times were plotted against molecular mass and compared with that of rXpmA.

Protein handling and analysis Protein concentrations were determined by the Bradford method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Mini-PROTEAN TGX 10% gels (Bio-Rad). Protein bands were stained with Coomassie Brilliant Blue R-250. An XL-Ladder (APRO Science, Tokushima, Japan) was used for protein molecular mass makers. In western blot analysis, after SDS-PAGE, proteins were transferred onto a PVDF membrane (Clear Blot Membrane-P; ATTO, Tokyo, Japan) using a semidry blotting apparatus (Horiz-Blot, AE-6677; ATTO). A His-tag monoclonal antibody (Takara Bio) and ECL Plus Western Blotting Detection Reagents, including a secondary antibody (GE Healthcare), were used for detection per the manufacturer's instructions. Rainbow molecular weight markers (GE Healthcare) were used.

Deduced protein molecular masses were calculated using the ExPASy Compute pI/Mw tool (http://tw.expasy.org/tools/pi_tool.html). Protein sequence similarity searches, conserved domain analysis, obtained peptide predictions, and predicted localization of protein were performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/), MEROPS - the Peptide Database release 10.0 (http://merops.sanger.ac.uk/) (19), WoLF PSORT Advanced Protein Subcellular Localization Prediction Tool (http://www.genscript.com/wolf-psort.html), and MitoFates (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi, MITO) (19). Patent searching was performed with the Japan Platform for Patent Information (J-Plat Pat); https://www.j-platpat.inpit.go.jp/web/all/top/BTmTopPage).

Quantitative RT–PCR analysis of *xpmA* After *A. oryzae* RIB40 was cultured in YPD medium at 30°C for 20 h, it was incubated for an additional 2 h in four different culture conditions: normal, alkaline, high-salt (NaCl), and heat stress. For the control (normal) condition, *A. oryzae* was cultured at 30°C. For the alkaline condition, potassium hydroxide (KOH) was added to the medium to achieve a final pH of 10.0. For the high-NaCl condition, NaCl was added to the medium to a final concentration of 1.0 M. For the heat-stress condition, the strain was cultured at 40°C. After cultures had been grown in each condition, mycelia were collected. Total RNA was extracted from each sample and treated with DNase I (Qiagen, Hilden, Germany). 1 μg total RNA were used as templates for cDNA synthesis using ReverTraAce-α (Toyobo) with an oligo dT primer in 20 μl reaction mixtures. The reaction mixtures were diluted 500 times with distilled water. Each 5 μl aliquot of cDNA pool was applied to reaction mixtures (15 μl) of quantitative PCR analysis using an Mx3000P Real-Time QPCR System (Agilent Technologies Inc., Santa Clara, CA, USA) with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies Inc.). For amplification of *xpmA* cDNA, primers xpmA-sQrtF and xpmA-sQrtR were used. As an internal control, the primer pairs actin-F and actin-R (7) were used.

rXpmA hydrolytic activity assay A standard reaction to determine rXpmA hydrolytic activity was carried out at 30°C in 50 mM HEPES-NaOH (pH 7.5) containing 50 mM Ala-Pro (AP) with 0.05 mM $CoCl_2$ and an aliquot of 2 μl of the purified enzyme (0.438 mg/ml). The released proline was detected using the discontinuous ninhydrin assay, as described previously (6). The total reaction volume was 200 μl. The reaction was performed at 30°C for 10 min and terminated by the addition of 90 μl of 1.0 M sodium acetate (pH 2.8). The final pH of the total reaction mixture was 3.5. Next, 10 μl of 10% (w/v) ninhydrin in ethanol was added, and the mixtures were incubated for 10 min at 80°C. Color development was measured spectrophotometrically at 415 nm with a microplate reader model 680 (Bio-Rad), and the amount of liberated proline was calculated from a standard proline calibration curve generated in a similar way. The enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of proline per minute.

Please cite this article in press as: Matsushita-Morita, M., et al., Enzymatic characterization of a novel Xaa-Pro aminopeptidase XpmA from *Aspergillus oryzae* expressed in *Escherichia coli*, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.06.007