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ORIGINAL ARTICLE

Characterization of recombinant prolyl aminopeptidase from *Aspergillus oryzae*

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Abstract

Aims: Prolyl aminopeptidase (PAP) degrades only amino-terminal proline from peptides. The food-grade fungus *Aspergillus oryzae* produces this enzyme only in small amounts. In this paper, we present efficient production of recombinant PAP with an overexpression system of *A. oryzae* and characterization of its biochemical properties.

Methods and Results: The gene encoding PAP was overexpressed as a His-tag fusion protein under a taka-amylase gene (*amyB*) promoter with a limited expressing condition in *A. oryzae*. The PAP activity in the mycelia grown in rich medium containing glucose (repressing condition) was twice that in starch (inducing condition). The enzyme prepared as cell-free extract was partially purified through two-step column chromatography. The PAP was estimated to be a hexameric protein and exhibited salt tolerance against NaCl of up to 4 mol l^{-1}.

Conclusions: *Aspergillus oryzae* PAP was produced under the repressing condition of *amyB* promoter in a PAP-overexpressing strain and purified 1800-folds. Overproduction of PAP under promoter-inducing conditions led to an increase in inactive PAP, possibly because of irregular folding.

Significance and Impact of the Study: PAP with a high specific activity and salt tolerance may be used effectively in the manufacturing processes of fermented foods.

Introduction

Studies of physiologically functional peptides obtained from food have greatly progressed recently. For instance, lactotripeptide (Nakamura *et al.* 1995; Seppo *et al.* 2003; Mizuno *et al.* 2004; Sano *et al.* 2005) is the main focus of these investigations. These peptides are composed of several amino acids, and they may be produced by the combined reaction of several proteases. Proteases with high substrate specificity are especially important in extracting these peptides from proteins. Therefore, we searched for such aminopeptidases in the genome information for *Aspergillus oryzae* (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao), which was described by Machida *et al.* (2005). The utilization of *A. oryzae* as a source of enzymes is particularly important because *A. oryzae* has been widely used in the manufacture of fermented foods in East Asia, and also in the production of food and pharmaceutical grade enzymes. Moreover, considering its long history of use in food production and fermentation, *A. oryzae* is designated 'generally recognized as safe' by the US Food and Drug Administration. The production of large amounts of enzymes with a self-cloning system of *A. oryzae* is being established. Social acceptance of

self-cloning enzyme production may be higher than recombinant enzyme production. In our previous study, we produced aspartyl aminopeptidase (DAP), specific for acidic amino acids with an *A. oryzae* overproducing strain (Kusumoto *et al.* 2008). In the study of DAP, we used the promoter of the taka-amylase gene *amyB* to induce overproduction of the enzyme with His-tag at the C terminus. As DAP is a metalloprotease, the specific activity of this enzyme is greatly increased by the addition of cobalt ions to the culture medium.

In the present study, we focused on an *A. oryzae* ortholog of prolyl aminopeptidase (PAP) in the genome database. As the enzyme clarified in *Aspergillus niger* only removes the amino-terminal proline from peptides (Basten *et al.* 2005), the gene product of the *A. oryzae* ortholog was also expected to have the same activity. The PAP of *A. niger* was characterized as the first eukaryotic PAP. The enzyme was consisted of a hexameric protein and showed homology against the PAP family functioning as a multimeric enzyme. PAP orthologs have also been identified in several fungal genome databases (Basten *et al.* 2005). However, only *A. niger* PAP has been enzymatically characterized.

PAPs have been found in various organisms, and several bacterial PAPs have been purified and characterized. Recently, these enzymes have been classified into two groups according to their molecular masses and substrate specificities. One group consists of monomeric enzymes obtained from *Bacillus coagulans* (Kitazono *et al.* 1992), *Lactobacillus delbrueckii* (Atlan *et al.* 1994; Gilbert *et al.* 1994) and *Serratia marcescens* (Yoshimoto *et al.* 1999; Ito *et al.* 2000), for instance. The other group consists of a multimeric protein that is either a tetramer or a hexamer obtained from *Aeromonas sobria* (Kitazono *et al.* 1994a), *A. niger* (Basten *et al.* 2005) and *Streptomyces aureofaciens* (Uraji *et al.* 2007), for instance.

In the present study, we report the overproduction, purification and enzymatic characterization of *A. oryzae* PAP, including new features not observed in *A. niger* PAP. We also examined the condition of PAP production for a high degree of specific activity of the enzyme in the mycelia of *A. oryzae*.

Materials and methods

Chemicals

Pro-*p*NA, Met-*p*NA, Gly-*p*NA, Arg-*p*NA, Val-*p*NA, Pro-*β*NA, hydroxyl-Pro-*β*NA and angiotensin II were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Ala-*p*NA, Leu-*p*NA, Lys-*p*NA and Glu-*p*NA were obtained from the Peptide Institute (Osaka, Japan). Asp-*p*NA, Phe-*p*NA, Ile-*p*NA, His-*p*NA and the peptides of AP, PA, PLG(NH_2), PPGFSPFR and PLSRTLSVAAKK were obtained from Bachem (Bubendorf, Switzerland). Other chemicals used in this study were of certified reagent grade.

Strains and media

Aspergillus oryzae RIB40 was used in this experiment. To isolate the mRNA pool, this strain was grown in yeast extract-polypeptone-dextrose (YPD) liquid medium, which was a rich medium containing glucose (1·0% (w/v) yeast extract, 2·0% (w/v) polypeptone, 1·0% (w/v) D-glucose; pH 5·5), at 30°C for 20 h with shaking. YPD liquid medium and Czapek-Dox (CD) medium, a minimum medium containing glucose (0·3% (w/v) $NaNO_3$, 0·052% (w/v) KCl, 0·152% (w/v) KH_2PO_4, 2 mmol l^{-1} $MgSO_4$, 1·0% (w/v) D-glucose, and 0·1% (v/v) trace elements solution: 0·1% (w/v) $FeSO_4{\cdot}7H_2O$, 0·88% (w/v) $ZnSO_4{\cdot}7H_2O$, 0·04% (w/v) $CuSO_4{\cdot}5H_2O$, 0·01% (w/v) $Na_2B_4O_7{\cdot}10H_2O$ and 0·005% (w/v) $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$), were used to grow transformants. To investigate the induction conditions of the *amyB* promoter for PAP with higher specific activity, the carbon source of the CD medium was changed; CDS: 1·0% (w/v) starch instead of 1·0% (w/v) D-glucose or CDgly: 1·0% (w/v) glycerol instead of 1·0% (w/v) D-glucose. The YPS consisted of 1·0% (w/v) starch instead of 1·0% (w/v) D-glucose of YPD liquid medium.

Transformation experiment

Escherichia coli transformation was performed as previously described (Hanahan 1983). The transformation of *A. oryzae* was performed as described by Gomi *et al.* (1987), except that the recipient strain was cultivated with Czapek-Dox (CD) medium, and the regeneration medium was CD containing 0·1 mg l^{-1} pyrithiamine associated with 0·8 mol l^{-1} NaCl as an osmotic stabilizer.

Construction of vector plasmid for overexpression of *pamA*

A MultiSite Gateway® system (Invitrogen, Carlsbad, CA, USA) was utilized to construct a vector plasmid for overexpressing *pamA* (a gene encoding the PAP of *A. oryzae*) in *A. oryzae*. The procedure followed was that of Kusumoto *et al.* (2008), except that the primers PAPF1GW (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAAATGGCTGCCAAACTAGTAGACAAGAAGC-3′, underline: attB1 sequence) and PAPR1GW (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAATCAATAGAGTCGTCCCTCAAGGC-3′, underline: attB2 sequence) were used. The plasmid pGAPH contained the *amyB* promoter of *A. oryzae* RIB40, *pamA* coding sequence for the Met

initiation codon through the carboxy-terminal amino acid, 11 amino acids followed by HA- and His-tags (YPA FLYKVEPGYDVPDYASHHHHHH), the *amyB* terminator of RIB40 and *ptrA* as a marker for pyrithiamine resistance, in that order.

RT-PCR

The total RNAs of *A. oryzae* RIB40 were extracted from mycelia grown at 30°C for 20 h in YPD liquid medium culture. mRNAs were purified from total RNAs using Oligotex^{TM}–dT30 <Super> mRNA Purification Kit (Takara Bio, Shiga, Japan). cDNA of *pamA* was generated using a RT-PCR Kit ReverTraAce-$\alpha^{®}$ (Toyobo, Osaka, Japan). PAPF1GW and PAPR1GW primers were used.

Nucleotide sequence determination

The DNA sequence was determined using a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI 310 automatic sequencer according to the manufacturers' instructions (Applied Biosystems Japan, Tokyo, Japan). The sequence data were analyzed using GENETYX-MAC sequence analysis software (Genetyx Corp., Tokyo, Japan).

Purification of PAP active fraction from *A. oryzae*

The *A. oryzae* transformed strain PAP12 was grown in 1·5 l of YPD medium at 30°C for 20 h with shaking at 120 rev min^{-1}. Conidia (1×10^8) of the PAP12 strain were inoculated into 150 ml of YPD medium in each 500-ml flask. A mycelial pellet was obtained by filtration through MiraCloth (Calbiochem, La Jolla, CA, USA). Mycelia were frozen in liquid nitrogen and then powdered with a mortar and pestle. Cell-free extract (CE) was prepared by adding 200 ml of buffer A (20 mmol l^{-1} imidazole, 20 mmol l^{-1} Tris–HCl (pH 7·5), 300 mmol l^{-1} NaCl) to the powdered mycelia (30 g) and was centrifuged twice at 22 400 ***g*** and 4°C for 20 min. The supernatant was collected and used as CE for purification of PAP protein.

The CE solution was then loaded onto 15 ml of Ni-IMAC gel (Ni-IMAC Profinity; Bio-Rad Japan, Tokyo, Japan) packed in an Econo-column (Bio-Rad Japan), pre-equilibrated with buffer A; the column was maintained at 4°C (cold room). After the column was rinsed with 10 times the bed volume using the same buffer (150 ml) as the gel, about three bed volumes (50 ml) of buffer B (100 mmol l^{-1} imidazole contained in buffer A) was applied to the column to elute the bound proteins.

The eluted fraction (50 ml) was added to an Amicon 100 tube (Millipore, Billerica, MA, USA) to concentrate the protein solution to 0·5 ml by centrifugation at 4000 ***g*** at 4°C. The concentrated eluted fraction was desalted with PD-10 (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with buffer C (20 mmol l^{-1} Tris–HCl (pH 7·5)).

The concentrated and desalted sample was then loaded onto an anion-exchange column MonoQ 5/50 GL (GE Healthcare) pre-equilibrated with buffer C at room temperature, and bound proteins were eluted using a 45-ml linear gradient from 0·15 to 0·4 mol l^{-1} NaCl in 20 mmol l^{-1} Tris–HCl (pH 7·5) with an Acta Prime Plus protein purification system (GE healthcare). Each fraction tube contained 0·5 ml of eluted protein. The PAP activity in each fraction was assayed with prolyl-*p*NA. The protein concentration of each fraction was estimated with the absorbance at 280 nm measured by Acta Prime Plus (GE Healthcare) in real time. The molecular mass of the PAP was determined using a Superose 12 gel filtration column (GE Healthcare) pre-equilibrated with buffer D [50 mmol l^{-1} Tris–HCl (pH 7·5), 150 mmol l^{-1} NaCl].

A gel filtration calibration kit (GE Healthcare) was used to determine the molecular masses of the separated proteins. As reference proteins, hexokinase (100 kDa), aldolase (158 kDa), catalase (240 kDa) and ferritin (440 kDa) were used. Retention times were plotted against molecular masses and compared with the retention time of the enzyme.

General protein techniques

The protein content in the enzyme samples was measured by the Bradford method (Bradford 1976) using a protein assay kit (Bio-Rad Japan). Aliquots of 10 μl of the purified proteins were mixed with the same volume of Laemmli sample buffer (Bio-Rad Japan) containing 5% (v/v) β-mercaptoethanol. They were then heated at 95°C for 10 min to denature the protein. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses were performed as previously described (Laemmli 1970) using 7·5% (w/v) polyacrylamide gels and the Mini-Protean 3 system (Bio-Rad Japan). Protein bands were stained with Coomassie brilliant blue R-250. An XL-Ladder (APRO Science, Tokushima, Japan) was used as a protein molecular mass maker. Internal amino acid sequence of a 48-kDa protein was determined by APRO Science using Procise 494 HT protein sequencing system (Applied Biosystems) by the method of the Edman degradation process following fraction of the digestion with lysyl endopeptidase.

Western blot analysis

After SDS–PAGE, proteins were transferred onto the PVDF membrane (Clear Blot Membrane-P; ATTO,

Tokyo, Japan) using semi-dry type blotting apparatus (Horiz-Blot, AE-6677; ATTO). His-tag® monoclonal antibody (Takara Bio) and ECL Plus Western blotting detection reagents including a secondary antibody (GE Healthcare) were used for detection, according to the manufacturers' instructions. MW maker was used for the XL-Ladder (APRO Science).

Aminopeptidase assay

Aminopeptidase activity was determined with artificial substrates. Activity was measured using 13 kinds of amino acids coupled to *para*-nitroanilide (*p*NA) as substrates (Atlan *et al.* 1994). The standard reaction mixture contained 2 mmol l^{-1} amino acid-*p*NA as substrate in 20 mmol l^{-1} Tris–HCl (pH 7·5). They were then incubated at 30°C, and the reaction was terminated by adding a quarter volume of 40% (v/v) acetic acid at predetermined times. The absorbance at 415 nm was measured using a Mini 1240 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) to determine the amount of liberated *p*NA.

Prolyl aminopeptidase activity was also examined using Pro-*β*NA (beta-naphtylamide), hydroxyl-Pro-*β*NA as the substrate following Kitazono *et al.* (1994b) and Ito *et al.* (2000). The reaction mixture consisted of 0·8 ml of 20 mmol l^{-1} Tris–HCl buffer (pH 7·0), 0·1 ml of enzyme solution and 0·1 ml of a 1 mmol l^{-1} solution of substrates. After 30-min incubation at 30°C, the reaction was stopped by adding 1 ml of Fast Garnet GBC (1 mg ml^{-1}) solution containing 10% (v/v) Triton X-100 in 1 mol l^{-1} sodium acetate (pH 4·0). The absorbance at 550 nm was measured after 20 min.

When peptide substrates were used for aminopeptidase activity, the proline-releasing activity was determined using the discontinuous ninhydrin assay described by Troll and Lindsley (1955). An aliquot of 10 *μ*l of enzyme was mixed with 1 mmol l^{-1} peptide in 20 mmol l^{-1} Tris–HCl (pH 7·5) and incubated at 30°C (total 1 ml). Thereafter, samples were taken at various times, and the reaction was terminated by the addition of 450 *μ*l of 1 mol l^{-1} sodium acetate (pH 2·8). The final pH of the total reaction mixture was 3·5. Next, 50 *μ*l of 10% (w/v) ninhydrin in ethanol was added, and the mixtures were incubated for 10 min at 80°C. Colour development was measured spectrophotometrically at 440 nm with a Mini 1240 UV-Vis spectrophotometer (Shimadzu), and the amount of proline liberated was calculated from a proline calibration curve generated in a similar way. The reaction rate was determined from the slope of the line through the linear part of the activity plot. In the case of angiotensin II and AP used for substrate, we performed the modified method described by Ichishima (1972) and Takeuchi and Ichishima (1986). The detection of the reaction of the amino group and ninhydrin was measured by the colour development at an absorbance of 570 nm.

The enzyme activity was defined in a term of unit (U), where 1 U was the amount of enzyme that produced 1 *μ*mol of *p*NA, *β*NA or proline per min. The K_m value for PAP against Pro-*p*NA or PA was calculated from Lineweaver–Burk plots. The result represented the mean of three independent experiments.

Biochemical characterization of PamA

The optimal pH for PamA enzymatic activity was determined using McIlvaine buffers ranging from pH 3 to 8 and 200 mmol l^{-1} HEPES buffers in the range from pH 7 to 12. Pro-*p*NA was used as the substrate. The pH stability of PAP was determined by pre-incubation of the purified enzyme in McIlvaine buffers ranging from pH 3 to 8 at 30°C for 30 min, followed by the standard enzyme reaction. A sample pre-incubated at pH 7·5 was used as a reference.

To determine its thermal stability, the purified enzyme was pre-incubated at 0, 30, 37, 50 and 60°C for 30 min followed by the standard enzyme reaction. A sample pre-incubated at 0°C was used as the reference.

The effects of chemicals about protease inhibitors and metal ions were investigated. The protease inhibitors, tosyl lysyl chloromethyl ketone (TLCK), tosyl phenylalanyl chloromethyl ketone (TPCK), leupeptin, bestatin, EDTA and *p*-chloromercuribenzenesulfonate (PCMB) on enzyme activity were measured in 200 mmol l^{-1} HEPES buffer (pH 7·5). The effects of metal ion salts, $CoCl_2$, $MnCl_2$, $CaCl_2$, $MgCl_2$, and $ZnCl_2$ and *β*-mercaptoethanol on enzyme activity were measured in 20 mmol l^{-1} Tris–HCl (pH 7·5). The purified enzyme was incubated with the respective compound for 30 min at 30°C, and its activity was then measured for 30 min at 30°C using a standard enzyme assay containing each compound.

The effect of NaCl on the enzyme activity was investigated by the measurement of the activity in 20 mmol l^{-1} Tris–HCl (pH 7·5) containing 0–4·8 mol l^{-1} NaCl. The purified enzyme was incubated in 20 mmol l^{-1} Tris–HCl (pH 7·5) containing 0–4·8 mol l^{-1} NaCl for 30 min at 30°C, and its activity was then measured for 30 min at 30°C at a final concentration of 2 mmol l^{-1} Pro-*p*NA.

Results

Isolation and overexpression of *pamA* gene

An ortholog of the prolyl aminopeptidase gene (*papA*) of *A. niger* was found in the genome database of *A. oryzae*, DOGAN (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao). This gene was designated as *pamA*.

There was no data for this gene in the EST (Expressed sequence tag) database of *A. oryzae* (http://nribf2.nrib.go.jp/EST2/), suggesting that the expression level of this gene was considerably low. To examine whether *pamA* was expressed, RT-PCR was performed using mRNA prepared from the mycelia of RIB40 grown in YPD liquid medium for 20 h at 30°C. Next, the PCR product was obtained and sequenced. Sequence analysis showed that this DNA was the cDNA of *pamA*, which did not have an intron, compared with the *pamA* genomic sequence. The presence of the cDNA suggested that *pamA* mRNA was expressed in liquid culture (YPD medium) and that this *pamA* gene contained eight introns. The amino acid sequence of *pamA* gene product (PamA) deduced from its cDNA sequence contained 447 amino acids and was seven amino acids longer than the sequence registered in the genome database of *A. oryzae* (*A. oryzae* genome database; database number AO090003000302) (440 amino acids). Then, the sequence of cDNA of PamA we obtained was submitted in GenBank/EMBL/DDBJ, accession no. AB512522. The seven amino acids were GGLDSIH starting from the 259th amino acid of PamA (translation starting codon Met as +1). The coding sequence of PamA showed 78% identity to the *papA* of *A. niger* (GenBank/EMBL/DDBJ, accession no. AJ315565). The PamA amino acid sequence contained a GXSXG motif highly conserved in serine protease (GQSFG, amino acids number 160-165).

To overexpress the *pamA* gene, its genomic DNA was obtained by PCR with the genomic DNA of *A. oryzae* RIB40 as a template. A plasmid vector pGAPH for overexpressing *pamA* was introduced to *A. oryzae* RIB40 by transformation. The isolated 15 transformants were analyzed for the specific activity of PAP (Pro-*p*NA hydrolyzing activity per mg protein) in the cell-free extract (CE) of the mycelia grown in CDS medium (induction medium for *amyB* promoter). Compared with the host strain RIB40 (0·5 mU mg^{-1}, Table 1), the transformants showed 2–18 times the specific activity. Among those strains, the PAP12 strain (9·4 mU mg^{-1}, Table 1) was selected as the enzyme producer. The CE of the PAP12 strain was subjected to Western blot analysis with anti-His-tag monoclonal antibody, and the signal was detected at 51 kDa. This result suggested that the introduced *pamA* was expressed as a His-tag fusion protein in *A. oryzae* RIB40, and the PamA showed PAP activity.

Table 1 Medium composition and Pro-*p*NA hydrolyzing activity

Strain	Medium	mU mg^{-1} (CE)
PAP12	YPD (1% glucose)	12·0
PAP12	YPS (1% starch)	6·5
PAP12	CD (1% glucose)	9·0
PAP12	CDS (1% starch)	9·4
PAP12	CDgly (1% glycerol)	10·0
RIB40	YPD (1% glucose)	1·5
RIB40	CDS (1% starch)	0·5

Examination of medium composition suitable for the production of PamA

We examined the culture conditions suitable for the production of PamA (estimated as Pro-*p*NA hydrolysis activity) in the PAP12 strain. PAP12 was grown in media containing starch as a carbon source for the induction of *amyB* promoter (YPS (yeast extract-polypeptone-starch) and CDS (Czapek-Dox medium containing starch)), and in media containing glucose (YPD and CD) or glycerol (CDgly) for repression of the *amyB* promoter. The CE of the mycelia grown in each medium was prepared. The specific activity for the PAP of CE showed the highest value using YPD medium (12·0 mU mg^{-1}, Table 1) (repressing condition). Unexpectedly, specific activity for the PAP of CE in YPS (inducing condition) was approximately half of that of CE in YPD (repressing condition), although the signal of recombinant PamA of CE of PAP12 in YPS medium was detected much stronger than that of CE of PAP12 in YPD medium by Western blot analysis (Fig. 1a,b). In CD media, the activity at inducing condition (CDS) (9·0 mU mg^{-1}, Table 1) was also the similar value of that at repressing condition (CD) (9·4

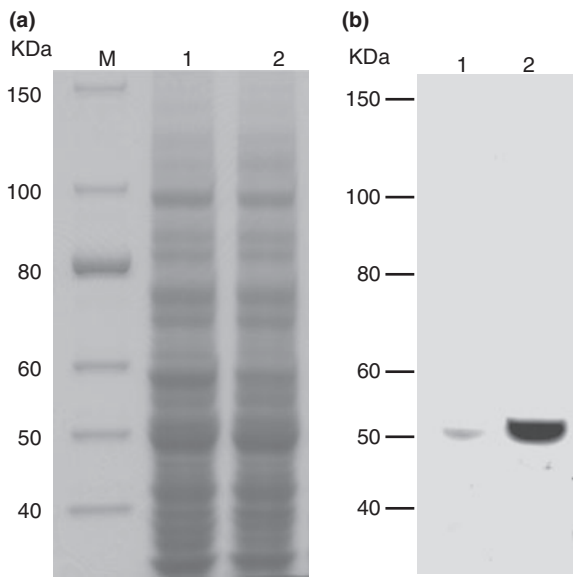

Figure 1 SDS–PAGE (a) and Western blot analysis (b) of cell-free extract (CE) of PAP12 strain in YPD or YPS medium. M: molecular mass marker, lane 1: CE of PAP12 in YPD medium, lane 2: CE of PAP12 in YPS medium. 20 μg of the total protein was loaded to each lane.

mU mg^{-1}, Table 1). Mycelia grown in CD media containing starch, glucose or glycerol also showed lower activity than in YPD. Moreover, the specific activity of the CE of PAP12 with YPD (12·0 mU mg^{-1}, Table 1) was eight times higher than the CE of the RIB40 host strain (1·5 mU mg^{-1}, Table 1) with the same medium. Therefore, PamA production was performed using PAP12 mycelia grown in YPD medium.

Purification of PamA from *A. oryzae*

CE of PAP12 was prepared from the mycelia (30 g wet weight) grown in 1·5 l of YPD medium. Purification of PamA was performed using a Ni-IMAC column with one-step elution and a MonoQ column with a salt gradient. The fraction obtained from the Ni-IMAC column showed hydrolysis of only Pro-*p*NA among 13 aminoacyl-*p*NA substrates (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Val), even after reaction for 20 h. PAP activity in the fraction obtained from the Ni-IMAC column was further purified with MonoQ ion-exchange column chromatography (Fig. 2). A broad protein peak for low salt concentration and a sharp protein peak were observed, but PAP activity only coincided with the latter protein peak. The active fraction (MonoQ-Frac.40) showed 1800 times more specific activity of CE (Table 2).

SDS–PAGE analysis revealed that two protein bands (51 kDa and 48 kDa) were observed in the MonoQ-Frac.40 sample (Fig. 3a). The His-tag signal in the Western blot analysis was detected at 51 kDa (Fig. 3b). However, the amino acid sequence analysis revealed that the sequence of one of the lysyl endopeptidase-digested peptides of the 48-kDa protein matched exactly with QFITNTMYHNAL (416–427 amino acid residues) of PamA. Therefore, we confirmed that the 51-kDa and 48-kDa proteins in the MonoQ-Frac.40 sample were gene

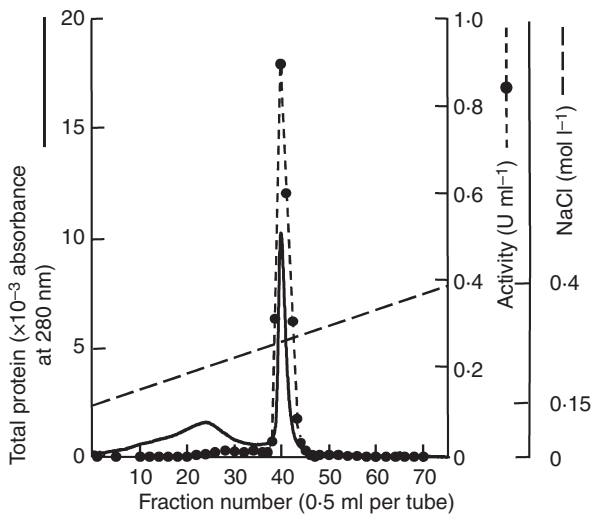

Figure 2 Fractionation of the fraction obtained from the Ni-IMAC column by the MonoQ chromatography. Dotted line with (●): prolyl aminopeptidase activity, solid line: absorbance at 280 nm, dashed line: NaCl concentration.

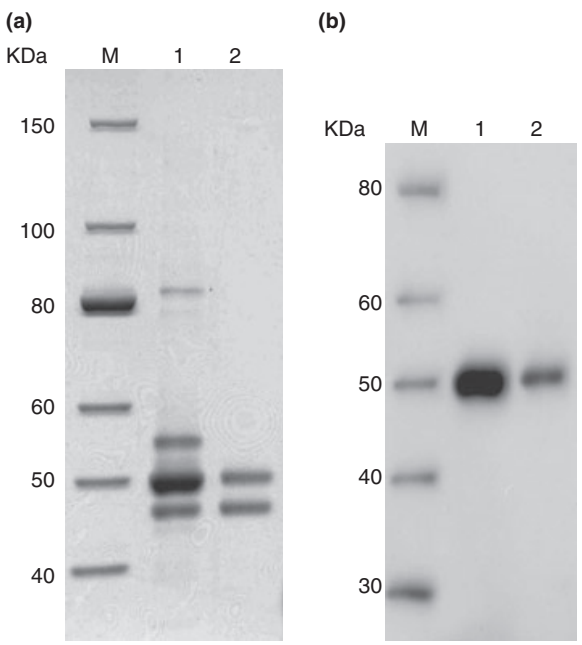

Figure 3 SDS–PAGE (a) and Western blot analysis (b) of partially purified prolyl aminopeptidase (PamA). M: molecular mass marker, lane 1: the fraction obtained from the Ni-IMAC column, lane 2: MonoQ-frac.40.

Table 2 Purification of recombinant prolyl aminopeptidase from *Aspergillus oryzae* PAP12 strain in YPD medium

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	Yield (%)	Volume (ml)
Cell-free extract	7·6	78×10^{2}	$9{\cdot}8 \times 10^{-3}$	1	100	200
Ni-IMAC column	1·6	0·6	2·6	$2{\cdot}7 \times 10^{2}$	21	50
MonoQ (MonoQ-Frac.40)	1·1	6×10^{-2}	$1{\cdot}8 \times 10^{1}$	$1{\cdot}8 \times 10^{3}$	15	2

Activity was measured using the substrate Pro-*p*NA.

products of *pamA*. Gel filtration chromatography of the MonoQ-Frac.40 sample showed a single protein peak coinciding with the activity peak. The molecular mass of the PamA enzyme was calculated as 313 kDa. So, the purified PAP was estimated to constitute a hexameric protein. The MonoQ-Frac.40 sample was considered to be a purified PamA sample and was subjected to biochemical characterization of PamA. The MonoQ-Frac.40 sample showed hydrolysis of only Pro-*p*NA among 13 aminoacyl-*p*NA substrates.

Biochemical characterization of PamA

PamA purified from *A. oryzae* was examined for hydrolysis activity on several peptides of chain lengths of two, three, eight and 12 amino acids (Table 3). PamA released Pro from amino terminus of a dipeptide PA, and not from AP. The hydrolysis rate on a dipeptide PA and a tripeptid PLG(NH_2) was identical. That on long-chain peptides, PPFG (8) and PLSR (12), was about 0·1 times that of PA. When a dipeptide AP or an octapeptide angiotensin II, in which the N-terminal amino acid is Asp, was reacted with PamA, no reaction was detected.

The K_m of PamA for Pro-*p*NA was estimated to be 0·095 ± 0·014 mmol l^{-1} at 30°C, and that for PA was 0·31 ± 0·024 mmol l^{-1} at 30°C, based on Lineweaver–Burk plots. PamA also hydrolyzed synthetic substrates Pro-*β*NA and hydroxy-Pro-*β*NA, in which the hydrolyzing ratio of hydroxy-Pro-*β*NA was about 2·1 times that of Pro-*β*NA (Table 3). These results again confirmed that PamA is an aminopeptidase, specific for amino-terminal proline.

The optimal pH of PamA for hydrolyzing Pro-*p*NA was between 7 and 11, with the highest activity at 7·5 (Fig. 4a). PamA was stable around pH 7 (Fig. 4b) up to a temperature of 37°C and was inactivated over 50°C (Fig. 4c). Next, the influence of several protease inhibitors and metal ions on the activity of PamA was examined (Table 4). Serine protease inhibitors TLCK and TPCK inhibited the PamA activity, and 4 mmol l^{-1} TLCK

Table 3 Hydrolysis of various substrates by PamA

Substrate	Rate of hydrolysis (U mg^{-1})
PA	25·7 ± 1·0
AP	ND
PLG(NH_2)	22·2 ± 1·4
PPFG (8)	4·5 ± 0·7*
PLSR (12)	2·4 ± 0·4
Pro-*p*NA	18·3 ± 1·2
Pro-*β*NA	20·0 ± 1·7
Hydroxy-Pro-*β*NA	42·4 ± 3·3

Activity was measured using the substrate peptides, Pro-*p*NA, Pro-*β*NA and hydroxy-Pro-*β*NA. PLSR (12), PLSRTLSVAAKK; PPGF (8), PPGFSPFR; ND, no activity detected.

*Showed overall rates of hydrolysis for both proline residues. Results are the mean of three independent experiments.

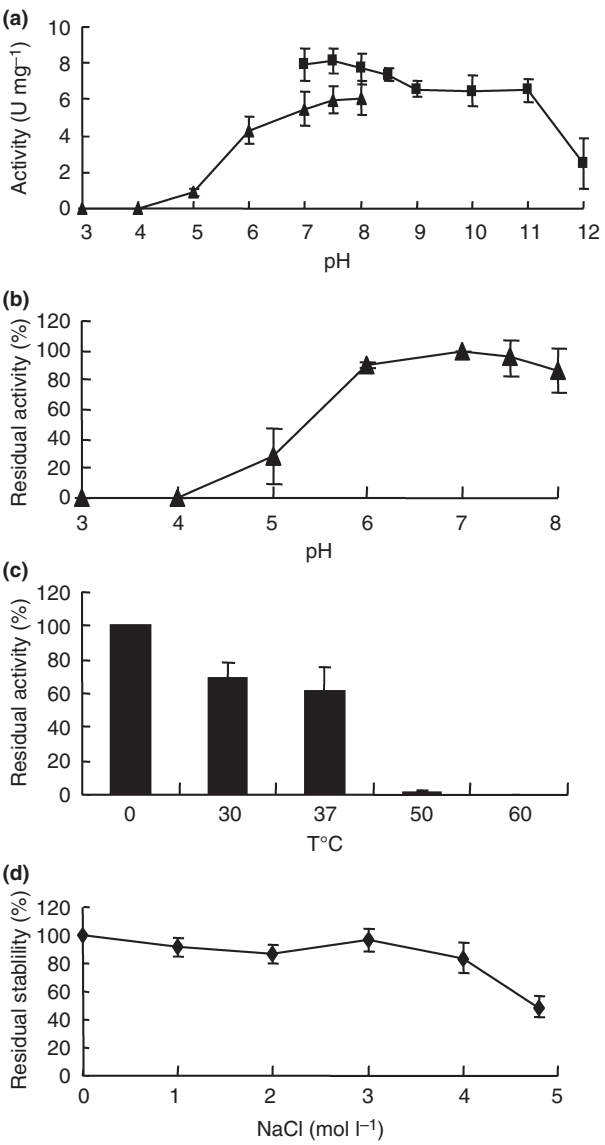

Figure 4 Biochemical characterization of PamA. (a) pH optimum of PamA. The following buffers were used: McIlvaine (–▲–) and HEPES (–■–). (b) pH stability of PamA. Residual activity was calculated relative to a sample kept at pH 7·0 at 30°C. (c) Thermal stability of PamA. Residual activity after a 30-min incubation at the indicated temperatures is expressed relative to a sample kept at 0°C. (d) Effects of NaCl concentrations on stabilities of PamA. The activities of these enzymes were determined at 30°C for 30 min after mixing PamA with various concentrations of NaCl for 30 min. Residual stability is expressed relative to a sample at 0 mol l^{-1} NaCl. Results are the mean of three independent experiments.

Table 4 Effects of several potential inhibitors and metal ions on the activity of PamA

Chemical	Concentration (mmol l^{-1})	Relative activity (%)
None		100
TLCK	0·02	66 ± 10
TLCK	0·5	51 ± 5
TLCK	4	37 ± 6
TPCK	0·02	69 ± 15
Leupeptin	0·04	68 ± 17
Bestatin	0·002	65 ± 13
EDTA	1	68 ± 10
PCMB	0·1	0
$CoCl_2$	1	78 ± 0
$MnCl_2$	1	95 ± 6
$CaCl_2$	40	94 ± 4
$MgCl_2$	1	98 ± 2
$ZnCl_2$	1	0
$ZnCl_2$ + β-Mercaptoethanol	1 + 5	61 ± 2
β-Mercaptoethanol	5	101 ± 2

Activity was expressed as the percentage of activity remaining after a 30-min incubation of the enzyme in the presence of the potential inhibitor or metal ion at 30°C. Activity was measured using the substrate Pro-*p*NA. Results are the mean of three independent experiments.
TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone.

achieved 37% remaining activity. A serine and cysteine protease inhibitor, leupeptin, also inhibited the activity to 68% remaining activity at 0·04 mmol l^{-1}. An aminopeptidase B inhibitor bestatin and metal ion chelating reagent EDTA also inhibited the activity to 65 and 68% remaining activity, respectively. The sulfhydryl reagent PCMB inhibited the activity completely. Metal ion salts of $MnCl_2$, $CaCl_2$ and $MgCl_2$ did not show any effect on PamA activity. The addition of $CoCl_2$ showed the 22% inhibition of the activity. Then, $ZnCl_2$ at a concentration of 1 mmol l^{-1} inhibited the PamA activity completely; however, 5 mmol l^{-1} β-mercaptoethanol repressed the inhibition of $ZnCl_2$. Next, the effect of salt concentration on PamA activity was examined (Fig. 4d). PamA activity was identical at concentrations of zero to 4 mmol l^{-1} NaCl. The activity at 4·8 mmol l^{-1} NaCl was half of that with no NaCl.

Discussion

PamA was efficiently produced in the mycelia of a recombinant *A. oryzae* strain under a limited expressing condition of a strong *amyB* promoter. Moreover, some newly characterized features of fungal PAP were described.

In the production of *A. niger* PAP (Basten *et al.* 2005), approximately 30 copies of the *papA* gene with an authentic promoter were integrated into the *A. niger* host strain. The transformant then showed a six times increase in prolyl aminopeptidase activity compared with the host strain. In this study, the *pamA* transformant PAP12 strain showed 18 times PAP-specific activity of cell-free extract (CE) in comparison with the host strain, grown in the inducing medium of the *amyB* promoter (CDS medium). However, from the results shown in Table 1, a suitable condition for PamA production was the use of a repressing condition medium (YPD) rather than an inducing condition medium (YPS).

In our preliminary experiment, CE was prepared from the mycelia grown in YPS (inducing condition) medium. Then, the PAP active fraction was purified using a Ni-IMAC column. The active fraction was applied to MonoQ column chromatography. The elution pattern showed two protein peaks, a broad peak and a sharp peak. The broad protein peak did not show any PAP activity. The sharp protein peak showed the PAP activity, which corresponded to the sharp peak of MonoQ column chromatography in the case of YPD medium (Fig. 2). The broad protein peak was estimated to be approximately 100 kDa (dimer) by Superose 12 gel filtration chromatography. SDS–PAGE and Western blot analysis of the broad peak indicated a 51-kDa protein only (data not shown). From these results, it was speculated that the overexpression of the *pamA* gene with amyB promoter under the inducing condition causes an irregular folding of recombinant PamA. Such protein would form homodimer complex and accumulate as inactive dimer in the mycelia of PAP12. In the present study in the case of YPD medium, a small broad protein peak was also resolved at the low salt concentration of the fraction obtained from the Ni-IMAC column with MonoQ column chromatography (Fig. 2, fractions 10–30).

The similar results were observed when the recombinant aspartyl aminopeptidase was overproduced in *A. oryzae* (Kusumoto *et al.* 2008). In this case, the specific activity was elevated by the addition of cobalt ion in the culture medium as this enzyme is a member of metalloprotease. An irregular folding of this protein was observed in the case of no addition of cobalt ions. Likewise, the accumulation of the inactive PAP may be attributable to the irregular folding of PamA even under the repressing condition of *amyB* promoter, and the amount of the inactive PamA may increase with strong transcription under the inducing condition of the *amyB* promoter. The problem of accumulation of the inactive protein should be resolved in future to produce useful enzymes using a self-cloning technique.

In this study, we were able to separate the inactive PamA from an active fraction by MonoQ column chromatography. The purified PamA was a hexameric protein,

which was consisted of recombinant 51-kDa PamA with His-tag in C-terminal and 48-kDa PamA (Fig. 3a, lane 2). We anticipated that the 48-kDa PamA was not only native PamA but also recombinant PamA without His-tag. This 48-kDa PamA would be partly generated from the properly folded recombinant PamA by deletion of C-terminal His-tag in cytosol. This MonoQ column chromatography process reached to 1800-folds purification of PamA finally. This led to the further characterization of the PamA enzyme.

PamA was active on synthetic substrates, Pro-βNA and hydroxy-Pro-βNA, in addition to Pro-*p*NA. The substrate spectra of Pro-containing synthetic compounds may depend on each member of the PAP family derived from different biological sources. PAP from *Aeromonas sobria*, which is a tetrameric enzyme, hydrolyzes Pro-βNA and hydroxyl-Pro-βNA (Kitazono *et al.* 1994a); PAP from *Bacillus coagulans*, which is a monomeric enzyme, hydrolyzes Pro-βNA but not hydroxyl-Pro-βNA; and PapA from *A. niger* hydrolyzes hydroxyl-Pro-βNA and Pro-*p*NA but not Pro-βNA (Basten *et al.* 2005). The difference between the preference for different synthetic substrates of the PAP of *A. oryzae* (PamA) and *A. niger* (PapA) may be attributable to the 22% difference in amino acid sequence.

The effects of several inhibitors, including serine protease inhibitors, on PamA tend to those of PapA of *A. niger*. The exceptions were the effects of leupeptin (a serine and cysteine protease inhibitor), bestatin (an aminopeptidase B inhibitor) and the metal ion chelating reagent EDTA. These reagents at the tested concentration inhibited PamA activity to 65–68% of the remaining activity (Table 4), whereas no influence on these compounds was observed for the PAP activity of *A. niger* (Basten *et al.* 2005). We have no information on the reason for these differences. We also observed that $ZnCl_2$ completely inhibited the PamA activity, and that β-mercaptoethanol repressed the extent of $ZnCl_2$ inhibition (Table 4). Basten *et al.* (2001) reported the same result for lysine aminopeptidase of *A. niger*, where $ZnCl_2$ may be involved in the oxidation of four cysteine residues and β-mercaptoethanol repressed the oxidation. Therefore, it is suggested that the four conserved cysteine residues (amino acid numbers 138, 144, 167 and 185 of PamA) are involved in supporting the active site Ser residue.

We found the salt tolerance of PamA, where the activity was identical in zero to 4 mol l^{-1} NaCl. The activity was half of the maximum in 4·8 mol l^{-1} NaCl. The salt tolerance of PamA is the first finding among fungal PAP. Uraji *et al.* (2007) described the salt tolerance of the PAP of *Streptomyces aureofaciens* (TH-3PAP), where the PAP activity in 1–3 mol l^{-1} NaCl increased 1·5–2 times without NaCl. NaCl concentrations of 1–4 mol l^{-1} are equivalent to 5–20% NaCl. Therefore, *A. oryzae* PamA may be applied in salty fermented foods such as soy sauce (12–16% NaCl) and soy bean paste (6–13% NaCl) as a food-grade enzyme additive.

Our preliminary data suggest that authentic PAP activity in *A. oryzae* RIB40 may be small, about 1/18 of the recombinant *A. oryzae* PAP12. There is no information about the PAP activity of other industrial *A. oryzae* strains. Oka and Nagata (1974a,b) described acidic and neutral peptides detected in soy sauce. There is no detectable amount of peptide containing the amino-terminal Pro. Therefore, it is possible that small amounts of PAP activity may be enough to hydrolyze such amino-terminal Pro-containing peptides released during the fermentation of soy sauce. However, it is possible that the maturation time of fermented foods may be shortened by using an active PAP-containing enzyme mix. Moreover, some food sources containing a high proportion of Pro residue, such as materials containing collagen, may be efficiently hydrolyzed by the addition of an active enzyme mix of PamA and other suitable endo-type proteases. These hydrolytes may be good sources for supplying Pro. The fact that PamA purified with one-step elution of Ni-IMAC was specific enough to hydrolyze only amino-terminal Pro-containing peptides may lead to an efficient method for preparing such a PAP-containing enzyme mix. Further research is necessary to investigate these speculations.

Our findings may be important in producing new types of food-grade enzymes, and for the food industry in the future, such as in the development of new type of foods, for instance, by increasing functional peptides. We intend to investigate the regulation mechanism of PamA expression to develop a strain of *A. oryzae* that produces high levels of PamA, for instance, by traditional mutagenesis or by a self-cloning technique.

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