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Identification of a plant aminopeptidase with preference for aromatic amino acid residues as a novel member of the prolyl oligopeptidase family of serine proteases

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The nucleotide and amino acid sequences reported in this article for tyrosyl aminopeptidase from daikon radish (*Raphanus sativus*) have been submitted to DDBJ/EMBL/GenBank with accession number AB608259.

Genome analysis has indicated that plants, like animals, possess a variety of protease genes. However, bulk of putative proteases has not been characterized at the enzyme level. In this article, a novel enzyme that hydrolyses phenylalanyl-4-methylcoumaryl 7-amide (phenylalanyl-MCA) was purified from cotyledons of daikon radish by ammonium sulphate fractionation and successive chromatography with DEAE-cellulose, phenyl-Sepharose, Sephacryl S-200 and Mini-Q. The molecular mass of the enzyme was estimated to be 78 kDa by SDS–PAGE under reducing conditions and 74 kDa by gel filtration, indicating that the enzyme is a monomer. The deduced amino acid sequence from the cDNA nucleotide sequence indicated that the enzyme is an orthologue of *Arabidopsis* unidentified protein, acylpeptide hydrolase-like protein (AHLP; UniProt ID: Q9FG66) belonging to the prolyl oligopeptidase (POP) family of a serine-type peptidase predicted from genetic information. Good substrates identified for the enzyme include phenylalanyl-MCA, tyrosyl-MCA and enkephalin. Neither acylamino acid-releasing activity nor endopeptidase activity was detected. The enzyme cleaved enkephalin (YGGFM, YGGFL), whereas, BAM-12 P (YGGFMRRVGRPE) and dynorphin A (YGGFLRRIRPKLK) were not digested. These results suggested that the enzyme possesses strict size selectivity of substrate. We propose the name 'tyrosyl aminopeptidase' for the uncharacterized protein AHLP.

Keywords: acylpeptide hydrolase-like protein/daikon radish/POP family/S9 protease/tyrosyl aminopeptidase.

Abbreviations: AARE, acylamino acid releasing enzyme; Ac, acetyl; ACTH, adrenocorticotropic hormone; AHLP, acylpeptide hydrolase-like protein; E-64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane; MCA, 4-methylcoumaryl 7-amide; OPB, oligopeptidase B; *p*-NA, *p*-nitroanilide; PCE, Phenylalanyl-MCA cleaving enzyme; PMSF, phenylmethanesulphonyl fluoride; POP, prolyl oligopeptidase; *Suc*, succinyl; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Z, carbobenzoxy.

Genome analysis has indicated that plants and animals possess almost the same number of protease genes. The *Arabidopsis thaliana* genome has approximately 700 protease (known and putative peptidase) sequences representing all six catalytic types: aspartic, cysteine, glutamic, metallo, serine and threonine peptidases (MEROPS peptidase database; http://merops.sanger.ac.uk/). Plant proteases playing a significant role in storage protein mobilization during germination (*1*, *2*), defence systems (*3*, *4*) and senescence (*5*, *6*) have been identified. However, the enzymatic properties and physiological functions of the bulk of these proteases remain unknown.

The prolyl oligopeptidase (POP) family comprises serine proteases belonging to clan SC, family S9 (*7*, *8*). In humans, the POP family includes prolyl oligopeptidase (POP, EC 3.4.21.26), dipeptidyl-peptidase IV (DPP IV, EC 3.4.19.1), acylaminoacyl peptidase (acylamino acid-releasing enzyme, AARE, EC 3.4.19.1) (*2*, *3*) and PREPL A protein lacking catalytic activity (*9*). In plants, four members of the S9 family, POP (*10*), oligopeptidase B (OPB) (*11*), AARE (*12*) and glutamyl-peptidase (*13*) have been identified and their enzymatic properties characterized. Recent crystal structure determinations and modelling analyses showed that these enzymes share a similar three-dimensional structure containing a catalytic (peptidase) domain with an α/β hydrolase fold and a β-propeller domain (*1*, *2*, *14*, *15*). The latter domain of POP comprises a 7-fold repeat of a four-stranded anti-parallel β sheet which excludes proteins and large peptides from the active site of the catalytic domain. Most members of the family show restricted specificities and act mainly on oligopeptides, consistent with the steric hindrance associated with the catalytic site resulting from the β-propeller domain at the N-terminus. In *A. thaliana*, acylpeptide hydrolase-like protein (AHLP, UniProt ID: Q9FG66, TAIR ID: AT5G36210) was also predicted to be another POP family member by genome analysis. Blast analyses showed that mRNA encoding AHLP is widely

distributed in plants. However, AHLP has not been detected at the protein level and its enzyme activity was not characterized.

We previously purified and characterized OPB from cotyledons of daikon radish (*11*). During the course of purifying OPB, phenylalanyl 4-methylcoumaryl 7-amide (Phe-MCA) cleaving activity was found in the cotyledon extract. While EDTA-sensitive phenylalanyl aminopeptidase has been purified from Japanese cedar pollen (*16*) and chick-pea cotyledons (*17*), the Phe-MCA cleaving activity in daikon cotyledon extract was not inhibited by EDTA. In this study, a novel aminopeptidase with preference for aromatic amino acids in cotyledons of daikon radish was purified to homogeneity and its sequence determined. Our results clearly showed that the purified aminopeptidase is an orthologue of the AHLP predicted by genome analysis of *A. thaliana*.

Experimental Procedures

Materials

Peptide 4-methylcoumaryl 7-amide (MCA) substrates, glucagon, adrenocorticotropic hormone (ACTH 1–24), β-casomorphin-5, Leu-enkephalin, Met-enkephalin, [D-Ala]-Met-enkephalin, sulphated Leu-enkephalin, BAM-12P, dynorphin A, leupeptin, diprotin A, leuhistin and *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64) were purchased from the Peptide Institute (Osaka, Japan). Phenyl-Sepharose and Sephacryl S-200 were from GE Healthcare (Uppsala, Sweden). Gly–Gly–Gly, Gly–Gly–Gly–Gly and Gly–Gly–MCA were from Bachem (Bubendorf, Germany). All other chemicals used were of analytical grade.

Plant

Fresh sprouts of daikon radish (*Raphanus sativus*) grown by hydroponics were purchased from a grocery store and used for purification of the enzyme. For the analysis of enzyme expression during germination, daikon radish seeds were allowed to imbibe water on filter paper at 20°C in the dark. After 2 days, embryos were grown at 20°C under long-day conditions (16 h light, 8 h dark), until collected.

Enzyme assay

The enzyme activity was assayed using Phe-MCA, unless, otherwise indicated. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.5) and 20 μM Phe-MCA. The reaction was initiated by the addition of enzyme solution. Following incubation at 37°C for 10–20 min, the reaction was terminated by the addition of 0.1 M acetic acid and the MCA liberated was determined fluorometrically, as previously described (*18*) Acyl amino acid-releasing activity was determined using acylalanyl *p*-nitroanilide (Ac-Ala-*p*NA) (*12*). Protein was determined by the method of Bradford using BSA as a standard (*19*).

Analysis of Phe-MCA cleaving activity during germination

Twenty grams of daikon seed and cotyledons (2, 5 and 7 days after imbibition) was homogenized in 200 ml of 20 mM acetate buffer (pH 6.5) containing 0.5 mM EDTA (buffer A) using a Waring blender. The homogenate was fractionated using ammonium sulphate (60% saturation), and then subjected to Sephacryl S-200 gel filtration (column size: 2.0 × 100 cm). Enzyme activity was eluted with buffer A containing 0.1 M NaCl. The activity was detected using the synthetic substrate Phe-MCA.

Purification of Phe-MCA cleaving enzyme from cotyledons of daikon radish

All purification procedures were performed at 4°C, unless, otherwise indicated. Cotyledons of daikon radish (500g) were homogenized in 2.5 l of ice-cold buffer A using a Waring blender. The homogenate was centrifuged at 12,000*g* for 10 min at 4°C and the supernatant was fractionated with ammonium sulphate (60% saturation). The precipitate was dissolved in buffer A and then dialysed against the same buffer. Following centrifugation at 12,000*g* for 10 min at 4°C, the resultant supernatant was concentrated by ultrafiltration (Amicon YM-10 filter). The concentrate was applied to a DEAE-cellulose column (2.5 × 16 cm) equilibrated with buffer A and washed with the same buffer. Bound protein was eluted using a linear gradient of NaCl (0–0.25 M) and fractions with activity were concentrated. The concentrate was applied to a HiLoad™ 16/10 phenyl-Sepharose column equilibrated with buffer A containing 1 M ammonium sulphate. Bound proteins appeared in the first 100 ml, whereas, most of the enzyme activity was found in the following 200 ml with a slight overlap, and minor activity was eluted using a linear gradient of ammonium sulphate (1–0 M) in the same buffer. The first fractions with high specific activity were selected and concentrated by ultrafiltration. The concentrated sample was applied to a Sephacryl S-200 column (2.0 × 103 cm) and eluted with 20 mM acetate buffer (pH 6.5) containing 0.1 M NaCl. The fraction possessing activity was eluted as a single peak, concentrated by ultrafiltration, and then applied to a Mini-Q column (GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM acetate buffer (pH6.5). Bound proteins were eluted using a linear gradient of NaCl (0–0.3 M) in 20 mM acetate buffer (pH 6.5). The fraction with activity was used as the final preparation.

Sequence analysis of Phe-MCA cleaving enzyme

Purified enzyme separated by SDS–PAGE (*20*) was electroblotted onto a PVDF membrane (Immobilon™, 0.45 mm, Millipore, Bedford, MA, USA) according to the manufacturer's instructions. The protein band was detected by staining with Ponceau 3 R. For the determination of internal sequence of the enzyme, the protein band was digested with lysyl endopeptidase and the released peptides were separated by reversed-phase high performance liquid chromatography (RP-HPLC) on a μRPC C2/C18 pc3.2/3 column (GE Healthcare, Uppsala, Sweden) as previously described (*11*, *21*) Amino acid sequences were analysed using an automated protein sequencer (Shimazu PPSQ-10, Kyoto, Japan).

Cleavage specificity of Phe-MCA cleaving enzyme towards model peptides

For the cleavage specificity analysis, 2 nmol of peptide substrates (Leu-enkephalin, Met-enkephalin, sulphated Leu-enkephalin, [D-Ala]-Met-enkephalin, BAM-12P, dynorphin A, β-casomorphin-5, tyrosyl-bradykinin, angiotensin IV, glucagon and ACTH) were digested with purified enzyme (6 pmol) in 20 mM Tris–HCl (pH 7.5) at 37°C for 15 h. The mixture was then acidified with trifluoroacetic acid and separated by RP-HPLC on a μRPC C2/C18 pc3.2/3 column as previously described (*11*, *22*). The purified peptide fragments were applied to glass fibre discs that had been coated with polybrene and then analysed using an automated protein sequencer. Amino acid analysis of digests of Met-enkephalin, by the enzyme was performed using a Hitachi L-8500A amino acid analyzer (Japan).

Isolation of cDNA encoding Phe-MCA cleaving enzyme

Total RNA isolated from the cotyledons of daikon radish 8 days after imbibition was reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase using a random hexamer, as previously described (*22*). To isolate cDNA encoding the Phe-MCA cleaving enzyme (PCE), we designed PCR primers corresponding to the sequences of peptide (#11,TAPYGSWK; #14, KALSSEIVWSS) generated from lysyl endopeptidase digestion, an internal sequence (FFVTDRK^{267}) of *Arabidopsis* AHLP, and a conserved sequence (ADDITP^{670}) in the C-terminal region of plant AHLP (UniProt ID: Q9FG66). cDNA encoding the N-terminal side of PCE was amplified by PCR, using primers corresponding to TAPYGSWK (S1) and KALSSEIVWSS (AS1). cDNA encoding the C-terminal side of PCE was amplified by PCR, using primers corresponding to FFVTDRK (S2) and ADDITP (AS2). The sense (S1 and S2) and antisense (AS1 and AS2) primer sequences used were 5′-AAC CGCTCCTTATGGCTCCTGGAA -3′, 5′-TTTTTTGTTACTGAC CGAAAG-3′, 5′-CGACGACCAAACAATCTCAGAACTGAGTG CTTT-3′ and 5′-AGGAGTGATGTCATCAGC-3′, respectively. PCR was performed for 30 cycles comprising denaturation at 95°C for 1 min, annealing at 53°C for 30 s, followed by extension at 72°C for 1 min. The PCR products were then subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced. The sequence of the AS1 and S2 primer of the first PCR product was replaced with the corresponding sequence of the second PCR product in the composite sequence. cDNAs for 5′- and 3′-terminal regions

of mRNA were amplified using a FirstChoice RLM-RACE (Rapid amplification of cDNA end) Kit (Ambion, Life Technologies Co, Carlsbad, CA, USA) according to the manufacturer's protocol. The 5′-RACE inner and outer primers used were, 5′-GAAGCGGT TAAAGCGGGAAT-3′ (corresponding to nucleotide numbers 561–579) and 5′-CGACCATCTTCCCTAACAGT-3′ (583–602), respectively. The 3′-RACE inner and outer primers used were, 5′-TTGC CCTTGTCGAGTACGAA-3′ (2006–2025) and 5′-TCCCGCTATATC GACAGTCT-3′ (1828–1847), respectively. The sequences of the sense and antisense primers designed from the amino acid sequences were replaced with the corresponding internal sequence of the PCR products in the composite sequence. Amplified, full-length PCE cDNA was subcloned into pGEM-T easy vector.

Expression of PCE in Escherichia coli

The full-length PCE cDNA was ligated into the SphI and SalI sites of pQE30 vector (Quagen, Germany). Expression of PCE in *E. coli* and extraction of the enzyme were carried out as previously described (*23*).

Results

Phe-MCA cleaving activity in cotyledons of daikon radish

Phenylalanyl aminopeptidase activity, reflected by the hydrolysis of Phe-MCA at pH 7.5, was detected in all extracts of cotyledons after 2, 5 and 7 days of imbibition (Fig. 1). This activity was eluted as a single, sharp peak at a position corresponding to a molecular mass of ~74 kDa on Sephacryl S-200. The enzyme activity in the eluate was not inhibited by 10 mM EDTA. Although Phe-MCA cleaving activity was also detected in extracts of dry seed, the activity was eluted as a broad peak, suggesting the presence of other kinds of aminopeptidase. In cotyledons, Phe-MCA cleaving activity increased following germination. Compared with the enzyme activity of cotyledons after 2 days of imbibition, the activity increased 2-fold after 7 days of imbibition.

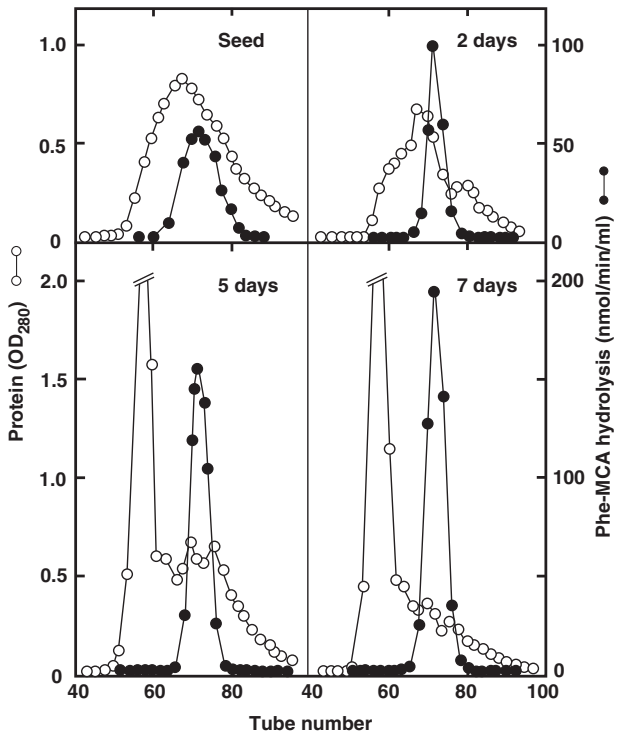

Fig. 1 Change in Phe-MCA cleaving activity during germination. The extract derived from daikon dry seeds and cotyledons (20 g) following imbibition (2, 5 and 7 days) was subjected to Sephacryl S-200 gel filtration chromatography as described in 'Experimental Procedures' section. The cleavage of Phe-MCA at pH 7.5 was assayed.

Purification of PCE

The PCE in cotyledons of daikon radish was purified by employing a series of column chromatographic steps utilizing DEAE-cellulose, phenyl-Sepharose, Sephacryl S-200 and Mini-Q (Fig. 2). Table I summarizes the results of a typical purification of PCE. The purified enzyme weighing ~32 μg, was obtained from 1 kg of cotyledons. The final preparation yielded a single protein band on SDS–PAGE under reducing condition (Fig. 3A). The molecular mass of the enzyme was estimated to be 78 kDa by SDS–PAGE and 74 kDa by gel filtration. The specific activity against Phe-MCA of the final preparation was 17.5 μmol/min/mg.

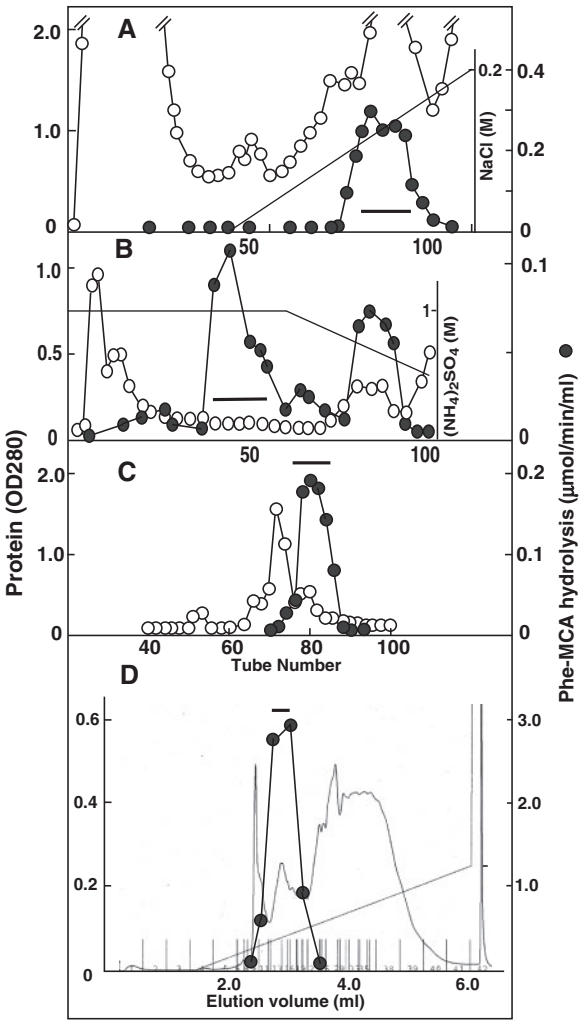

Fig. 2 Purification of PCE from cotyledons of daikon radish using a series of column chromatographic steps. (A) DEAE-cellulose column chromatography. (B) Phenyl-Sepharose chromatography, (C) Sephacryl S-200 gel filtration chromatography and (D) MiniQ column chromatography. The column chromatography was performed as described in 'Experimental Procedures' section. Fractions indicated by the horizontal bar were pooled.

Table I. Purification of PCE from cotyledons of daikon radish.

Steps	Total protein (mg)	Activity (μmol/min)	Activity (μmol/min/mg)	Yield (%)	Purification (fold)
Ammonium sulphate (0–60%)	710	67.0	0.094	100	1
DEAE-cellulose	102	26.4	0.259	39.4	2.76
Phenyl-Sepharose	19.2	7.12	0.371	10.6	3.95
Sephacryl S-200	1.14	2.50	2.19	3.73	23.3
Mini-Q	0.032	0.56	17.5	0.835	186

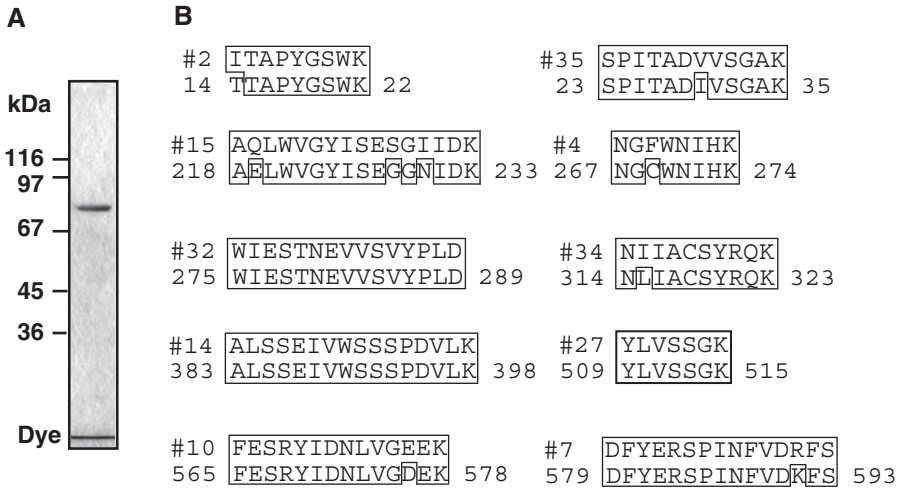

Fig. 3 SDS–PAGE and amino acid sequences of the purified enzyme. (A) SDS–PAGE (7.5% gel) of the purified enzyme (1 μg). The marker proteins were as follows: β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (67 kDa), ovalbumin (45 kDa) and glyceraldehyde-3-phosphate dehydrogenase (36 kDa). (B) Alignment of the internal sequences of the purified enzyme with AHLP (UniProt ID: Q9GF66) from *A. thaliana*. The 10 internal sequences of fragments (#2, 4, 7, 10, 14, 15, 27, 32, 34 and 35) generated by lysyl endopeptidase digestion of the purified enzyme were determined as described in 'Experimental Procedures' section. Amino acid residue numbers of AHLP are indicated on both sides of the AHLP sequence.

Sequence analysis of the purified enzyme

In an effort to characterize PCE at the protein level, the N-terminal sequence of the purified enzyme was first examined. When ~100 pmol of the enzyme was applied to a protein sequencer, no amino acid sequence was obtained, suggesting that the N-terminus of the enzyme is blocked. Consequently, the sequences of the peptides generated by lysyl endopeptidase digestion were determined. About 10 peptides were purified by HPLC and sequenced. The sequences of peptide #2, 4, 7, 10, 14, 15, 27, 32, 34 and 35 were ITAPYGSWK, NGFWNIHK, DFYERSPINFVDRFS, FESRYIDNLVGEEK, ALSSEIVWSSSPDVLK, AQLWVGYISESGIIDK, YLVSSGK, WIESTNEVVSVYPLD, NIIACSYRQK and SPITADVVSGAK, respectively. Investigations of homology using a protein database revealed that *Arabidopsis* AHLP (UniProt ID: Q9FG66), possesses highly homologous sequences to these sequences as shown in Fig. 3B. The sequences of peptide #14, 27 and 32 were identical to that of the corresponding region of *Arabidopsis* AHLP. The AHLP (sequence length: 678 amino acids, 75.4 kDa) is predicted to be a member of the POP family (S9 serine protease family) from genome analysis, however, its enzyme activity was not yet identified. These results strongly suggested that daikon PCE is an AHLP-related enzyme.

Isolation of cDNA encoding PCE

In order to deduce the primary structure of daikon PCE, cDNAs encoding the enzyme were amplified by PCR using primers designed from amino acid sequences as described in, 'Experimental Procedures' section. Finally, 5′- and 3′-RACE were performed using specific primers. By combining the nucleotide sequences of these PCR products, a nucleotide sequence comprising a total of 2,324 bp was determined. The reliability of the nucleotide sequence was confirmed by PCR using specific primers. The whole nucleotide sequence (DDBJ accession number: AB608259) contained a 5′-untranslated (nucleotide number: 1–120), open reading frame (121–2178) and 3′-untranslated (2179–2324) sequences. In the 3′-terminal region, a putative polyadenylation signal (AATAA, 2214–2218) and poly(A) tail (2314–2324) were found.

The predicted amino acid sequence (total of 685 residues) of PCE and alignment of the sequence with sequences of AHLP and acyl amino acid-releasing enzyme (AARE, UniProt ID: Q84LM4) from *A. thaliana* are

shown in Fig. 4. The amino acid sequences of all peptides generated by lysyl endopeptidase digestion match perfectly with the deduced sequence. The PCE sequence is highly homologous with the AHLP sequence (amino acid identity: 85%), unlike the case with AARE (amino acid identity: 20%) and OPB (amino acid identity: 18%, UniProt ID: Q9SX53). Active-site residues (Ser, Asp and His) of POP family serine proteases and the sequence surrounding the active Ser residue are well conserved. Alignment of the AARE sequence resulted in identification of the putative active-site residues (Ser531, Asp607, His639) of PCE (*24*). Three potential N-glycosylation sites (Asn54, Asn175 and Asn437) were also identified. Thus, amino acid sequence alignment indicated that PCE is an orthologue of *Arabidopsis* AHLP.

In order to confirm the identity of PCE, PCE cDNA was expressed in *E. coli*. Although expression level was low, EDTA-insensitive hydrolytic activity towards Phe-MCA increased markedly (Table II). Tyr–MCA cleaving activity was also increased whereas leucine aminopeptidase activity was not changed. Expression of PCE did not affect acyl amino acid releasing activity.

In plants, the deduced sequence of PCE showed high amino acid identity with sequences of predicted uncharacterized protein from grape (amino acid identity 72%, UniProt ID: E0CPB5, sequence length: 675

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PCE   ------MSSS--ESPLEEKITAPYGSWKSPITADVVSGASKRLGGTAVDSRGRLIWLESR 52
AHLP  ------MSSSSPDAAQTPLTTAPYGSWKSPITADIVSGASKRLGGTAVDSHGRLVLLESR 54
AARE  MDSSGTDSAKELHVGLDPTTEEEYATQSKLLQEFINIPSIDKAWIFNSDSGSQAMFALSQ 60

PCE   PNESGRGVLVVEGGEKEGGIDITPKEFGVRTLTQEYGGGAFRLSSSSSHDQLVVFSNYKD 112
AHLP  PNESGRGVLVLQG---ETSIDITPKDFAVRTLTQEYGGGAFQISS----DDTLVFSNYKD 116
AARE  ANLLANKKKKFMLSGHISNESNQSVNFHWAPFPIEMTGASAFVPSPSG-LKLLVIRNPEN 119

PCE   QRLYKQHFGHKDSSPKPITPDYGSPAVTYADGVFDSR-FNRFVTVREDGRQDRSNPITTI 171
AHLP  QRLYKQDITDKDSSPKPITPDYGTPAVTYADGVFDSR-FNRYVTVREDGRQDRSNPITTI 166
AARE  ESPTKFEIWNSSQLEKEFHIPQKVHGSVYVDGWFEGISWDSDETHVAYVAEEPSRPKPTF 179

PCE   VEVNLSGGDTLE-------------------------EPKVLVSGNDFYAFPRLD--PKS 204
AHLP  VEVNLSG-ETLEDP----------------------EPKVLVSGNDFYAFPRLD--PKC 200
AARE  DHLGYYKKENSLDKGIGSWKGEGDWEEEWGEAYAGKRQPALFVINVDSGEVEPIKGIPRS 239

PCE   ERLAWIEWSHPNMPWDKAQLWVGYISESGIIDKRVCVAG---------------------- 243
AHLP  ERLAWIEWSHPNMPWDKAELWVGYISEGGNIDKRVCVAG---------------------- 239
AARE  ISVGQVVWSPNSNGSAQYLVFAGWLGDKRKFGIKYCYNRPCAIYAIKFTSDEPKDDDANE 299

PCE   ----CDPDYVESPTEPKWSSRGELFFVSDRKNGFWNIHKWIESTNEVVSVYPLDGEFTKP 299
AHLP  ----CDPKYVESPTEPKWSSRGELFFVTDRKNGCWNIHKWIESTNEVVSVYPLDGEFAKP 295
AARE  FPIHNLTKSISSGFCPRFSKDGKFLVFVSAKTAVDSGAHWATESLHRID-WPSDGKLPES 358

PCE   LWVFGTNSYEIIECSEE--------KNIIACSYRQKGKSYLGILDDSKG----SCSLLD- 346
AHLP  LWIFGTNSYEIIECSEE--------KNLIACSYRQKGKSYLGIVDDSQG----SCSLLD- 342
AARE  TNIV--DVIQVVNCPKDGCFPGLYVTGLLSDPWLSDGHSLMLSTYWRSCRVILSVNLLSG 416

PCE   -IPLTDFDNITLGNQCLYVEG----ASAVLPPSVAKVTLDKHKMKALS-SEIVWSSSPDV 400
AHLP  -IPLTDFDSITLGNQCLYVEG----ASAVLPPSVARVTLDQHKTKALS-SEIVWSSSPDV 396
AARE  EVSRASPSDSDYSWNALALDGDSIVAVSSSPVSVPEIKYGKKGLDSAGKPSWLWSNIQSP 476

PCE   LKYKSFFSAP------DLIEFPTEVPGQNAYAYFYPPTNPLYNASIEEKP-----PLIVK 449
AHLP  LKYKAYFSVP------ELIEFPTEVPGQNAYAYFYPPTNPLYNASMEEKP-----PLLVK 445
AARE  IRYSEKVMAGLSSLQFKILKVPISDVSEGLAEGAKNPIEAIYVSSSKSKENGKCDPLIAV 536

PCE   SHGGPSAESRGSLNLIIQYWTSRGWAFVDVNYGGSTGYGREYRERVLRRWGIVDVDDCCG 509
AHLP  SHGGPTAESRGSLNLNIQYWTSRGWAFVDVNYGGSTGYGREYRERLLRQWGIVDVDDCCG 505
AARE  LHGGPHSVSPCSFSRTMAYLSSIGYSQLIINYRGSLGYGEDALQSLPGKVGSQDVKDCLL 596

PCE   CAKYLVSSGKADVKRLFISGGSAGGYTALTALALR-DVFKAGASLYGVADLKMLK----- 563
AHLP  CAKYLVSSGKADVKRLCISGGSAGGYTTLASLAFR-DVFKAGASLYGVADLKMLKE---- 560
AARE  AVDHAIEMGIADPSRITVLGGSHGGFLTTHLIGQAPDKFVAAAARNPVCNMASMVGITDI 656

PCE   ------EGHKFESRYIDNLVGEEKD-FYERSPINFVDRFSCPIILFQGLEDKIVNPDQTR 616
AHLP  ------EGHKFESRYIDNLVGDEKD-FYERSPINFVDKFSCPIILFQGLEDKVVTPDQSR 613
AARE  PDWCFFEAYGDQSHYTEAPSAEDLSRFHQMSPISHISKVKTPTLFLLGTKDLRVPISNGF 716

PCE   KIYQALKEKGVPVALVEYEGEEHGFRKAENIKYTLEQLMVFFARVVGGFQVADDITPLKI 676
AHLP  KIYEALKKKGLPVALVEYEGEQHGFRKAENIKYTLEQQMVFFARVVGGFKVADDITPLKI 673
AARE  QYVRALKEKGVEVKVLVFPNDNHPLDRPQTDYESFLNIAVWFNKYCKL------------ 764

PCE   DNFDTSSGV 685
AHLP  DNFDT---- 678
AARE  ---------
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Fig. 4 Alignment of the deduced amino acid sequence of PCE with AHLP (UniProt ID:Q9FG66) and AARE (UniProt ID: Q84LM4) from *A. thaliana*. Identical amino acid residues of the aligned sequences are shown in bold. The amino acid sequences identified are underlined in the sequence. The essential active-site residues are boxed. The accession number of the nucleotide sequence of daikon aminopeptidase cDNA in the DDBJ/EMBL/GenBank databases is AB608259.

Table II. Enzyme activity in the cell extract of *E. coli* carrying PCE expression vector.

	Enzyme activity (nmol/min/mg)						
	Aminopeptidase						Acylamino acid releasing enzyme
	Phe-MCA EDTA		Tyr–MCA EDTA		Leu–MCA EDTA		
Plasmid	(−)	(+)	(−)	(+)	(−)	(+)	Ac-Ala-*p*NA
pQE30	2.11	0.615 (1)	2.11	0.681 (1)	5.88	1.84 (1)	N.D.
pQE30 /PCE	33.2	27.1 (44)	32.8	29.0 (43)	6.29	2.05 (1.1)	N.D.

Hydrolytic activity towards Phe-MCA, Tyr–MCA and Leu–MCA were determined in the absence and presence of 10 mM EDTA as described in 'Experimental Procedures' section. Acylamino acid releasing activity was assayed using Ac-Ala-*p*NA as described in 'Experimental Procedures' section. Values in parentheses are activities relative to that of control (pQE30). N.D., Not detected

A.A.), poplar (71%, B9GNY4, 672 A.A.), sorghum (64%, C5Z6J1, 721 A.A.), spruce (63%, A9NWZ2, 721 A.A.), spikemoss (55%, D8TAZ4, 666 A.A.), AHLP from maize (64%, B6UHC0, 674 A.A.), putative aminopeptidase C from rice (65%, Q69Y12, 683 A.A.) and putative AARE from caster bean (70%, B9R7H7, 731 A.A.). Furthermore, sequences homologous with PCE were also found in bacteria such as peptidase S9 of *Trichodesmium erythraeum* (UniProt ID: Q115Z2, amino acid identity 48%, sequence length 644 A.A.). Among animal proteins, the PCE sequence showed highest amino acid identity with predicted protein from lancelet (40%, C3Y087, 653 A.A.), sea anemone (39%, A7RWV1, 656 A.A.) and filaria (31%, A8NDK6, 644 A.A.). These sequences showed highest homology with PCE among POP family members. On the other hand, PCE orthologue is not found in vertebrate gene products.

Enzymatic properties of the purified enzyme

The enzymatic properties of PCE were investigated using synthetic substrate. When the effect of pH on the activity towards Phe-MCA was examined, the enzyme displayed highest activity at pH 7.5 and the enzyme was stable at neutral pH (pH 6.5–7.5). When the enzyme was incubated at 37, 45 and 50°C for 20 min at pH 7.0, 80, 70 and 5% of the activity, respectively, remained. The enzyme displayed optimal activity at 37°C. The effect of various protease inhibitors on the enzyme was also examined. As shown in Table III, the enzyme activity was strongly inhibited by PMSF. However, leupeptin (inhibitor of trypsin-like serine protease and papain-like cysteine protease), E-64 (inhibitor of papain-like cysteine protease) and diprotin A (inhibitor of dipeptidyl aminopeptidase IV) had no effect. Furthermore, leuhistin (inhibitor of aminopeptidase M) and EDTA had no inhibitory effect. Bestatin, an aminopeptidase inhibitor, also had no effect (data not shown). The activity of PCE was inhibited by iodoacetamide at high concentration, although E-64 had no effect.

Cleavage specificity of the purified enzyme towards synthetic substrates

The activity of purified enzyme was examined against various synthetic substrates (Table IV). Of the substrates examined, Tyr–MCA and Phe-MCA were shown to be good substrates for PCE, like the recombinant PCE. The enzyme preferentially hydrolysed amino-terminal aromatic amino acids. Activity towards substrates for endoprotease (Z-Phe–Arg-MCA and Suc-Leu–Leu–Val–Tyr–MCA) was not found. Substrates of POP (Gly–Pro-MCA) and AARE (Ac-Ala-*p*NA) were not hydrolysed by the purified

Table III. Effects of various inhibitors on the activity of purified enzyme. Activity towards Phe-MCA was assayed at pH 7.5 in the presence of various inhibitors.

Protease inhibitors	Concentration (mM)	Inhibition (%)
PMSF	1	76
TPCK	1	26
Leupeptin	1	0
Diprotin A	0.15	0
Leuhistin	0.2	0
EDTA	20	0
E-64	1	0
Iodoacetamide	2	16
	10	100
N-Ethylmaleimide	10	30

Table IV. Cleavage specificity of the purified enzyme towards synthetic substrates.

Substrates	Relative activity (%)
Phe-MCA	100
Tyr–MC	132
Leu–MCA	10.8
Met-MCA	2.3
Arg-MCA	0
Lys-MCA	0
Z-Phe–Arg-MCA	0
Suc-Leu–Leu–Val–Tyr–MCA	0
Gly–Pro-MCA	0
Z-Leu–Arg–Gly–Gly–MCA	0
Gly–Gly–MCA	0
Ac-Ala-*p*NA	0
Ac-Phe-*p*NA	0

Enzyme activity towards MCA substrates was determined in 0.1 M Tris–HCl buffer (pH 7.5) using various substrates at a concentration of 100 μM. Acylamino acid releasing activity was determined in 50 mM Tris–HCl buffer (pH 7.5) using Ac-Ala-*p*NA and Ac-Phe-*p*NA at a concentration of 1 mM.

enzyme. Ac-Phe-*p*NA was also not hydrolysed by the enzyme. The *Km* values of Phe-MCA and Tyr–MCA were $83.1 \pm 1.4\,\mu M$ and $116 \pm 2.2\,\mu M$, respectively. These results suggested that the purified enzyme possesses aminopeptidase activity and preference for aromatic amino acid residues.

Cleavage specificity of the purified enzyme towards model peptides

The ability of PCE to hydrolyse model peptides was examined. Glucagon (amino terminus: His), adrenocorticotropic hormone (Ser) and peptides possessing a tyrosine residue at the N-terminus (β-casomorphin-5, Leu-enkephalin, Met-enkephalin, BAM-12 P, Tyr-bradykinin and dynorphin A) were digested with the purified enzyme at 37°C for 15 h. Cleavage products were separated by RP-HPLC and the amino acid sequences were determined. Although glucagon, ACTH, β-casomorphin-5, BAM-12 P, Tyr-bradykinin and dynorphin A were not cleaved at all, cleavage products of Leu-enkephalin and Met-enkephalin were detected. As shown in Fig. 5A, digestion of Leu-enkephalin resulted in the production of Gly–Gly–Phe–Leu, the peptide resulting from removal of the N-terminal Tyr residue, and the C-terminal dipeptide Phe–Leu. The tripeptide Gly–Phe–Leu was not produced. Similarly, digestion of Met-enkephalin resulted in the production of tetrapeptide (Gly–Gly–Phe–Met) and dipeptide (Phe–Met). The N-terminal amino acid of Tyr-[D-Ala]-Gly–Phe–Met was also released by the purified enzyme, whereas neither Phe–Met nor Gly–Phe–Met was generated. In contrast, O-sulphated Leu-enkephalin was not digested, suggesting that the presence of free OH group of tyrosine residue is essential for cleavage by PCE.

To confirm the cleavage specificity, Met-enkephalin was digested with the purified enzyme for 10, 30, 60, 120 and 180 min, and cleavage products were analysed using an amino acid analyzer. Elution profiles of the cleavage products on ion-exchange chromatography are shown in Fig. 5B. The peak (retention time: 17.8 min) detected at reaction 0 min represents undigested Met-enkephalin. This peak overlapped the peak corresponding to phenylalanine, and hence, phenylalanine was not determined. Tyrosine (peak c) and peak b were first detected. Peak b was identified as corresponding to the dipeptide Gly–Gly, since its retention time was identical to the retention time (13 min) of Gly–Gly (Fig.5, left below). Standard glycine eluted at 8.9 min on ion-exchange chromatography. Glycine was not detected in the digests. The pattern of the cleavage products released from Met-enkephalin is shown in the inset of Fig. 5B. These results indicated that the order of bond cleavage of Met-enkephalin by PCE is Tyr–Gly, Gly–Phe and then Phe–Met. To confirm the cleavage specificity towards Gly–Gly, the activity towards Z-Leu–Arg–Gly–Gly–MCA and Gly–Gly–MCA were examined. PCE did not hydrolyse these synthetic substrates at all (Table IV). The cleavage activity of PCE towards Gly–Gly–Gly and Gly–Gly–Gly–Gly was also

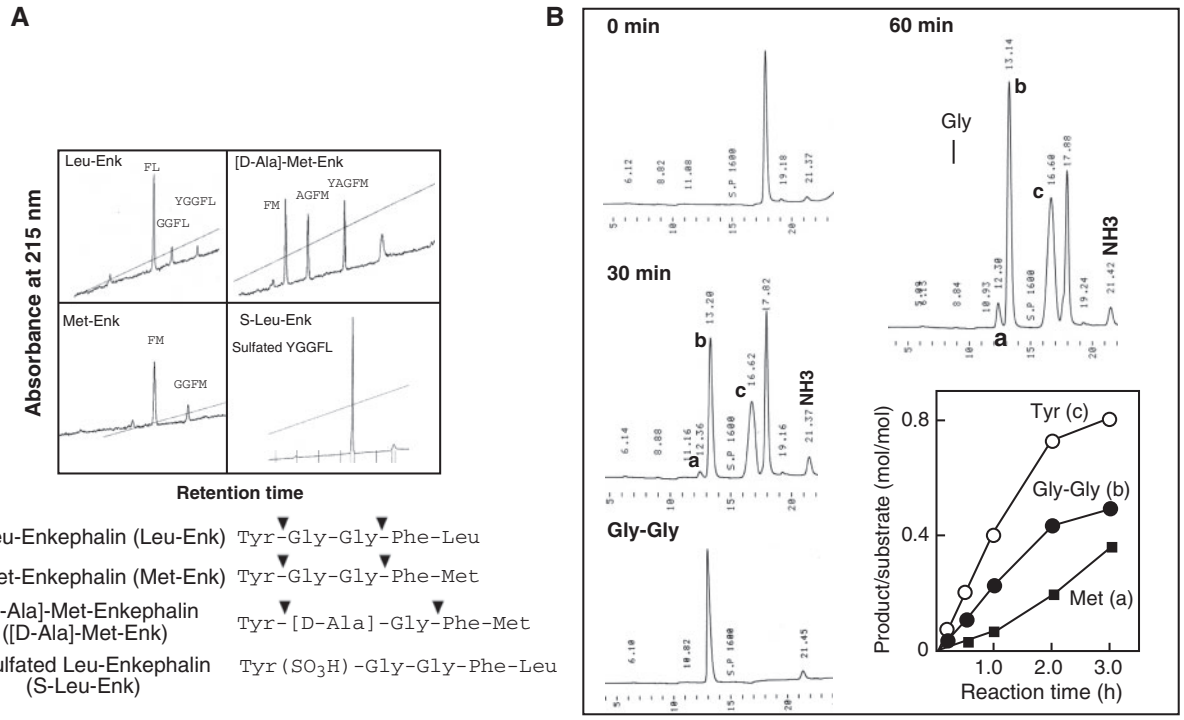

Fig. 5 Cleavage of model peptides by the purified enzyme. (A) HPLC profiles of Leu-enkephalin, Met-enkephalin, [D-Ala]-Met-enkephalin and sulphated Leu-enkephalin digests. The digestion mixtures were subjected to reverse phase-HPLC on a μRPC C2/C18 pc3.2/3 column. Peptides were eluted with a linear gradient of acetonitrile (broken line) in 0.1% trifluoroacetic acid. Cleavage sites are indicated by arrowheads. (B) Time-course of Met-enkephalin digestion by PCE. The digests were examined using an amino acid analyzer and elution profiles of cleavage products on ion-exchange chromatography are shown. The pattern of the cleavage products quantified is shown in the inset.

determined using an amino acid analyzer. These peptides were not cleaved by PCE (data not shown).

As mentioned above, β-casomorphin-5 (Tyr–Pro–Phe–Pro–Gly) and Tyr-bradykinin (Tyr–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg) were not cleaved by PCE, suggesting that Tyr–Pro and Tyr–Arg bonds are resistant to digestion by PCE. It is highly likely that PCE possesses strict selectivity with respect to the amino acid residue at the P1′ position. In contrast, although both BAM-12P (Tyr–Gly–Gly–Phe–Met–Arg–Arg–Val–Gly–Arg–Pro–Glu) and dynorphin A (Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys) possess the same amino terminal sequence (Tyr–Gly–Gly–Phe) to enkephalin, these peptides were not cleaved by PCE, unlike enkephalin. These results suggested that PCE also possesses selectivity with respect to the length of the peptide substrate.

Discussion

Plant aminopeptidases have been shown to play an important role in various cellular processes such as meiosis (*25*), mobilization of proteins during germination (*26*, *27*) and defence responses to infection and wounding (*28*). Most aminopeptidases purified from higher plants are metallo-enzymes such as leucine aminopeptidase (*29*), acidic amino acid-specific aminopeptidase (*27*) and aminopeptidase N (*30*). Metallo-exopeptidases preferentially hydrolysing amino-terminal phenylalanine have been isolated from Japanese cedar pollen (*16*), chick-pea cotyledons (*17*) and cabbage leaves (*31*).

In this study, we purified an aminopeptidase with preference for N-terminal aromatic amino acid residues from cotyledons of daikon radish. Analysis of the amino acid sequence showed that the PCE sequence is highly homologous with the sequence of the uncharacterized gene (TAIR ID: AT5G36210) product of *A. thaliana* (amino acid identity: 88%). Although, enzymatic analysis of the gene product of AT5G36210 has yet to be performed, this protease is predicted to belong to the POP family of serine proteases given its amino acid sequence. In the UniProt database, this protease is designated as being AHLP (ID: Q9FG66). Recently, Kaschani *et al.* (*32*) confirmed that the AT5G36210 gene encodes active serine protease using fluorophosphate-based probes in *A. thaliana*. However, its enzymatic properties have not been clarified. PCE possesses aminopeptidase activity and its activity is inhibited by the serine protease inhibitor, PMSF. High concentrations of thiol-modifying reagent were also found to have an inhibitory effect. Our results suggested that the inhibitory effect of SH-modifying agents is due to the presence of a cysteine residue in the vicinity of the active site. Modified cysteine residues might prevent substrate binding. Thus, sequence and enzymatic characterization of PCE clearly showed that AHLP is a serine-aminopeptidase with a preference for aromatic amino acids at the P1 position.

Blast analyses showed that PCE orthologues are widely distributed in plants and bacteria. PCE orthologues were also found in invertebrates such as lancelet and sea anemone, but not identified in vertebrates. Recently, bacterial 72 kDa-puromycin hydrolases, displaying a high similarity to the POP family of serine proteases, from *Streptomyces morookaensis* (UniProt ID: Q2HXD9) and *Streptomyces griseus* (UniProt ID: B1VUD8) were characterized at the enzyme level (*33*). Puromycin hydrolase (PMH) has aminopeptidase activity with preference for phenylalanine, but does not possess acylamino acid-releasing activity. Amino acid identities of PMH with daikon PCE, *Arabidopsis* OPB, AARE and Streptomyces POP were 33, 16, 19 and 20%, respectively. These results strongly suggested that, both plant PCE and bacterial PMH belong to the same subfamily of the POP family.

More recently, aminopeptidase (Eryngase) was purified from mushroom (*Pleurotus eryngii*) and characterized (*34*). Eryngase and PCE are similar with respect to molecular mass and catalytic properties. The amino-terminus of POP family proteases is generally blocked (*11–13*). Similarly, the amino-terminus of native eryngase is blocked. An internal sequence comprising a 50 kDa fragment (Ala–Pro–Tyr–Gly–Thr) generated by lysyl endopeptidase digestion was reported. A similar sequence is located in the amino-terminal region of PCE (amino acid numbers 14–18) and *Arabidopsis* AHLP (*16–20*). Although the threonine residue of Ala–Pro–Tyr–Gly–Thr is replaced by serine in PCE and AHLP, the Ala–Pro–Tyr–Gly–Thr/Ser sequence is highly conserved in the N-terminal region of these enzymes. These results strongly suggested that eryngase and PCE are the same members of the POP family.

The cleavage specificity of PCE was investigated using synthetic substrates and model peptides. The N-terminal Tyr–Gly bond of enkephalin was cleaved by the enzyme, whereas the N-terminal Tyr–Pro bond of β-casomorphin-5 and the Tyr–Arg bond of Tyr-bradykinin were not cleaved. The enzyme did not cleave the N-terminal Tyr–Gly bond of BAM-12P (12 amino acids) and dynorphin A (13 amino acids). It is highly likely that PCE possesses strict selectivity with respect to both the P1′ position and length of the peptide substrate. Additionally, it is noteworthy that the dipeptide Gly–Gly was released from enkephalin and released glycine was not detected. Endopeptidase and dipeptidyl aminopeptidase activities of PCE towards Gly–Gly were examined using synthetic substrates, Z-Leu–Arg–Gly–Gly–MCA and Gly–Gly–MCA. These substrates were not hydrolysed by PCE. Gly–Gly–Gly and Gly–Gly–Gly–Gly were also not cleaved by PCE, suggesting importance of P1′ residue. Further analysis is necessary to determine whether the Gly–Phe bond in enkephalin is cleaved by the endopeptidase or dipeptidyl aminopeptidase activity of the enzyme. OPB cleaves peptides consisting of no more than 30 amino acids at the carboxyl side of arginine or lysine (*11*). These results indicated that the length of peptide cleaved by PCE is shorter, compared with the length of peptide cleaved by OPB.

Arabidopsis PCE (AHLP) transcript exhibits ubiquitous expression (*Arabidopsis* eFP Browser,

http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). It is expressed in dry seed, imbibed seed, cotyledons, leafs, root, petal and pollen. It is likely that the enzyme plays a role in general peptide degradation, and not in special peptide processing.

In conclusion, the enzyme purified in this study is a novel member of the prolyl oligopeptidase family of serine protease, and is widely distributed among plants, bacteria and invertebrates. Of the synthetic substrates tested, the enzyme was most active towards Tyr–MCA. We propose the name 'tyrosyl aminopeptidase' for the uncharacterized protein ALHP.

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Conflict of interest
None declared.

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