Characterization of an Aminopeptidase and a Proline Iminopeptidase from Cabbage Leaves

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Z. Naturforsch. **63c**, 105 – 112 (2008); received July 25/October 11, 2007

Aminopeptidase, preferring phenylalanine-*p*-nitroanilide as substrate, and proline iminopeptidase, highly-specific for proline-*p*-nitroanilide, were isolated from cabbage leaves (*Brassica oleraceae* var. *capitata)*. As pH optima, 7.2 – 7.5 for aminopeptidase activity and 8.0 – 8.5 for proline iminopeptidase were determined. Both peptidases were strongly inhibited by *p*-chloromercuribenzoic acid, heavy metal ions and urea. The molecular weights were determined by gel filtration to be 56 and 204 kDa, respectively. The iminopeptidase was decomposed during SDS electrophoresis to four subunits of 50 kDa. Minor impurities of myrosinase-associated protein (~70 kDa) were found in both preparations. Preliminary data of their amino acid sequences showed similarities to those of aminopeptidases N (family M1) and proline iminopeptidases (family S33).

Key words: Cabbage, Aminopeptidase, Proline Iminopeptidase

Introduction

Aminopeptidases (EC 3.4.11), catalyzing the cleavage at the *N*-terminus in polypeptides, have been extensively reported in various organs of most plant species and were classified into two groups on the basis of their biochemical properties (Walling and Gu, 1996). The main group comprises thermolabile aminopeptidases of molecular weights of 60 – 130 kDa manifesting a preferable hydrolysis of hydrophobic *p*-nitroanilides at neutral pH value and strong inhibition by sulfhydryl reagents (Walling and Gu, 1996; Arima *et al.*, 2000). The second group are large (150 – 330 kDa), heat stable metallopeptidases with alkaline pH optima, inhibited by EDTA, 1,10-phenanthroline and bestatin (Walling and Gu, 1996; Yamauchi *et al.*, 2001).

In this paper, we report on the isolation, partial purification and initial characterization of an aminopeptidase and a proline iminopeptidase from cabbage leaves – a new available source of food-grade exopeptidases.

Materials and Methods

Plant material and reagents

Fresh cabbage (*Brassica oleraceae* var. *capitata*) was obtained from the Experimental Station for Vegetables, Sadovo town, Plovdiv region, Bulgaria.

L-Amino acid *p*-nitroanilides and benzoyl-arginine-*p*-nitroanilide (Bz-Arg-*p*-NA) were purchased from Sigma, St. Louis, MO, USA. All other reagents were of analytical grade.

Enzyme assays

The aminopeptidase activity against aminoacyl-*p*-nitroanilides and Bz-Arg-*p*-NA was assayed according to Chrispeels and Boulter (1975). After incubation for 10 min at 30 °C in 0.05 M sodium phosphate buffer (pH 7.0), the liberated *p*-nitroaniline was measured at 410 nm using a spectrophotometer (UV-VIS Spectrophotometer, Shimadzu 1240). The iminopeptidase activity was assayed spectrophotometrically at 410 nm against L-proline-*p*-nitroanilide (Pro-*p*-NA) according to Yoshimoto and Tsuru (1985) in 0.1 M Tris (trishy-

0939 – 5075/2008/0100 – 0105 $ 06.00 © 2008 Verlag der Zeitschrift für Naturforschung, Tübingen · http://www.znaturforsch.com · D

droxymethylaminomethane)-HCl buffer (pH 8.0) for 20 min at 30 °C. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitroaniline per minute.

Protein determination

Soluble protein was determined by the biuret reaction following precipitation with 20% (w/v) trichloroacetic acid (TCA). Bovine serum albumin (BSA) was used to prepare a standard curve (Gornall *et al.*, 1949).

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels using Tris-glycine buffer, pH 8.3, according to Laemmli (1970). Rabbit muscle myosin (205 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) were used as molecular weight marker proteins. The gels were stained by Coomassie Brilliant Blue R-250 [0.1 g solved in acetic acid/methanol/water, 10:50:40 (v/v)].

Molecular weight estimation

The molecular weights of the enzymes were determined by gel filtration on a Sephadex G-200 column (1.5 × 146 cm) using as elution medium 0.05 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl. Cytochrome c (12 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and ferritin (440 kDa) were used as standard proteins. The void volume was determined with Dextran Blue. The molecular weights of peptidases were also determined by SDS-PAGE.

Enzyme purification

Fresh leaves from 1500 g cabbage (*Brassica capitata* L.) were cut, milled and pressed with a juice squeezer (Braun MW30, Melsungen, Germany) yielding a native juice, further squeezed through two layers of gauze and centrifuged at 6000 × *g* for 30 min at 5 °C (MLW K24 D, Leipzig, Germany). The supernatant was transferred to the crude extract. Solid ammonium sulfate was added to the crude extract, and the precipitate, formed between 25 and 70% (w/v) saturation, was collected by centrifugation at 2000 × *g* for 30 min at 5 °C. The precipitate was dissolved in 25 mM Tris/HCl, pH 7.2, containing 5 mM EDTA (buffer A) and dialyzed 4–5 h against the same buffer. The suspension was then clarified by centrifugation at 4000 × *g* for 15 min and the pellet was discarded. The dialyzed solution was applied on a Toyopearl QAE-550C column (2 × 13 cm, Tosoh Co., Tokyo, Japan), pre-equilibrated with buffer A. Non-bound proteins were washed from the column with buffer A and further 400 ml of a 0–0.5 M NaCl gradient in the same buffer were applied at a flow rate of 1 ml min^{-1}. Fractions of 5 ml were collected. Both peptidases were eluted at a concentration of about 0.25 M NaCl. The active fractions were pooled and concentrated by ultrafiltration (Amicon UM-30 membrane).

Separation of aminopeptidase from proline iminopeptidase

The pooled active and concentrated fractions were loaded onto a Sephadex G-200 column (1.5 × 146 cm), equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl (buffer B). Fractions of 3.3 ml were collected at a flow rate of 0.1 ml min^{-1} and analyzed for aminopeptidase and proline iminopeptidase activity, and both readily separated fractions were concentrated by ultrafiltration (Amicon UM-30 membrane).

Aminopeptidase purification

The aminopeptidase sample was loaded for rechromatography onto a Sephadex G-200 column, equilibrated with buffer B. The active fractions were pooled, concentrated to 500 μl using a Centricon-30 microconcentrator (5000 × *g*, 20 min, and 5 °C) and injected onto a Mono Q HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden), washed with 25 mM Tris/HCl, pH 7.2 (buffer C). The column was eluted with a 0–0.5 M NaCl gradient in buffer C. The aminopeptidase, eluted at 0.23 M NaCl, was concentrated using a Centricon-30 microconcentrator (5000 × *g*, 20 min, and 5 °C) and passed through a Thermo Quest Elite C18 column, equilibrated with 0.1% trifluoroacetic acid (TFA) (buffer D). The enzyme was eluted with a 0–80% acetonitrile (ACN) gradient in buffer D.

Proline iminopeptidase purification

The iminopeptidase sample was loaded onto a second Sephadex G-200 column, equilibrated with buffer B. The active fractions were concentrated to 350 μl using a Centricon-30 microconcentrator (5000 × *g*, 20 min, and 5 °C). The concentrated pool from the Sephadex G-200 run was injected onto a Mono Q HR 5/5 column and washed with buffer C. The column was eluted with a 0–0.5 M NaCl gradient in buffer C. The proline iminopeptidase, eluted at 0.1 M NaCl, was concentrated using a Centricon-30 microconcentrator (5000 × *g*, 20 min, and 5 °C) and passed through a Thermo Quest Elite C18 column, equilibrated with buffer D. The enzyme was eluted with a 0–80% ACN gradient in buffer D.

Identification of aminopeptidase by MALDI-TOF mass spectrometry

The protein bands were excised from the Coomassie-stained SDSgel, and protein identification was performed by MALDI-TOF spectrometry. After tryptic digestion, MALDI-TOF analysis was performed on a Bruker Reflex III instrument (Bruker Daltonik, Bremen, Germany), equipped with an N_2 337 nm laser and gridless pulsed ion extraction (Shevchenko *et al.*, 1996).

Sequence verifications of some fragments were performed by nanoelectrospray tandem mass spectrometry on a Q-Tof I mass spectrometer (Micromass, Manchester, England), equipped with a nanoflow electrospray ionization source. Gold-coated glass capillary nanoflow needles were obtained from Proxeon (Type Medium NanoES spray capillaries, Odense, Denmark). Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software (Boston, USA) from Matrix Science (Perkins *et al.*, 1999).

Identification of proline iminopeptidase by amino acid sequence analysis

The sample of proline iminopeptidase was dissolved in 1 ml of 5 mM ammonium bicarbonate buffer, pH 8.2, and incubated with 50 μl bovine trypsin solution (1 mg ml^{-1}) at room temperature for 15 h, followed by further addition of 50 μl trypsin solution. Then, the reaction mixture (trypsin/iminopeptidase, 1:30 w/w) was incubated overnight at 37 °C. The generated peptides were separated by gel filtration on a Superdex 300 column (Pharmacia, Freiburg, Germany) at a flow rate of 2 ml min^{-1}, using water as eluent. The fractions were separated by reverse phase HPLC on a Nucleosil 100 RP-18 column (250 × 10 mm, 7 μm, Macherey-Nagel, Germany). The peptides were eluted (detection at 214 nm) at a flow rate of 1 ml min^{-1} by applying the following gradient: 90% buffer D, 10% buffer E [ACN/water/TFA, 80:20:0.085 (v/v)] for 10 min, then 10–100% buffer E in 70 min at a flow rate of 1 ml min^{-1}. The collected fractions were further subjected to amino acid sequence analysis. Peak fractions were dried and after dissolving in water/methanol/formic acid, 60:40:0.1 (v/v) subjected to automated Edman *N*-terminal sequencing (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

Characterization of enzymes

Aminopeptidases were kept at different temperatures from 15 °C to 60 °C. At various times, 0.1-ml aliquots of aminopeptidase and proline iminopeptidase solutions were removed and assayed for activity against 1.5 mM L-leucine-*p*-nitroanilide (Leu-*p*-NA) and 0.8 mM Pro-*p*-NA, respectively, at 30 °C as described previously (Chrispeels and Boulter, 1975; Yoshimoto and Tsuru, 1985).

All inhibitors and metal ions were prepared in 50 mM Tris/HCl buffer (pH 7.5). In the case of 1,10-phenanthroline and phenylmethylsulfonyl fluoride (PMSF), the inhibitors were first dissolved in dimethylformamide and then made up to the desired concentration by adding 50 mM Tris/HCl buffer, pH 7. 5. To determine the degree of inhibition of aminopeptidase and proline iminopeptidase, 0.1-ml aliquots of the enzymes were added to 0.1 ml of inhibitor or metal ion solution and maintained at 20 °C for 30 min.

The pH optima and stabilities of the aminopeptidase and proline iminopeptidase were determined using Leu-*p*-NA and Pro-*p*-NA, respectively, as substrates. For pH values between 5.0 and 7.0, 25 mM citrate-phosphate buffer, between 7.0 and 8.0, 25 mM sodium phosphate buffer, between 8.5 and 9.0, 25 mM Tris/HCl buffer, and between 9.0 and 10.0 glycine-NaOH buffer were used. Following the preincubation for 20 min, a 0.1-ml aliquot was removed and assayed against Leu-*p*-NA and Pro-*p*-NA.

Results

The purification procedures for aminopeptidase and proline iminopeptidase are summarized in Table I. The enzymes from crude the extract were purified by a combination of ammonium sulfate precipitation and chromatographic procedures.

The enzymes from the crude extract were precipitated with ammonium sulfate (25–70%), then the precipitate was resuspended in buffer A and the solution obtained was further subjected to a Toyopearl QAE-550C column. Although the loss of aminopeptidase and proline iminopeptidase activities demonstrated that minimal purification was achieved by this step, it is still considered to be worthwhile as it removed most of the contaminating proteins and polyphenols. The aminopeptidase (molecular weight ~60 kDa) was separated readily from the proline iminopeptidase (molecular weight ~200 kDa) *via* gel filtration chromatography on a Sephadex G-200 column. The enzyme fractions contained about 23% and 15% of the applied protein, respectively.

The active enzyme fractions were chromatographed on a second Sephadex G-200 column and the eluted aminopeptidase and iminopeptidase were concentrated and passed through FPLC and HPLC columns. Despite the significant loss of enzymatic activities after reversed-phase HPLC, it was considered to be a successful step before performing the amino acid sequence analysis due to the removal of contaminating proteins. The active fractions were concentrated using a Centricon-30 microconcentrator and applied to SDS-PAGE (Fig. 1A).

The protein bands with molecular weights around 56 and 66 kDa were excised and subjected to tryptic digestion and MALDI-TOF MS/MS analysis. The foremost peptide with the sequence "EQVLISNGNVVR" was determined for the aminopeptidase band in SDS-PAGE (molecular weight 56 kDa). A similarity search with the *lalign* program (http://www.ch.embnet.org/software/LALIGN_form.html) showed that the conservative region "L-SNGN" exists also in other aminopeptidases like the aminopeptidases N of *Arabidopsis thaliana* (Lin *et al.*, 2001), *Oryza sativa* (japonica cultivar group) (Chow *et al.*, 2004) and different microorganisms (Foglino *et al.*, 1986; Tettelin *et al.*, 2000; Buell *et al.*, 2003) (Table II).

The foremost peptide with the sequence "SNPTADGSAQEAFVTSVNSR" was determined for the second band in SDS-PAGE (molecular weight 66 kDa), which belongs to a myrosinase-associated protein of *Brassica napus* (Taipalensuu *et al.*, 1997).

The pure HPLC fraction manifesting proline iminopeptidase activity was subjected to automated Edman *N*-terminal sequencing, but no result could be achieved. As the *N*-terminus of proline iminopeptidase is blocked, internal amino acid sequences were analyzed after tryptic cleavage treatment. Two peptides were identified with the following sequences: "APVAAAVFTF" and "QSFAVDSATL". A similarity search with the *lalign* program showed that the sequence of the longer peptide is highly similar to proline iminopeptidase 2 of *Arabidopsis thaliana* (At3g61540 protein, unpublished) and different microorganisms (Kitazono *et al.*, 1994a, b, 1996; Leenhouts

Table I. Summary of the purification procedures of aminopeptidase and proline iminopeptidase (*).

Purification step	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Yield (%)	Relative purification
Crude extract	6048	100	0.017	100	1
		70*	0.012*		
Ammonium sulfate	448	67	0.15	67	8.8
fractionation		57*	0.13*	78*	10.8*
QAE 550C	46	30.7	0.67	30.7	39.4
		20.5*	0.45*	29*	37.5*
Sephadex G 200	10.7	26.5	2.48	26.5	146
	6.9*	17.5*	2.54*	25*	211.7*
Sephadex G 200	6.3	18	2.86	18	168
	3.1*	15*	4.8*	21*	400*
Mono Q HR 5/5	1.05	8	7.6	8	450
	0.85*	7*	10*	17*	700*
Thermo Quest Elite	0.53	0.7	1.32	0.7	78
C18	0.32*	0.6*	1.9*	0.9*	156*

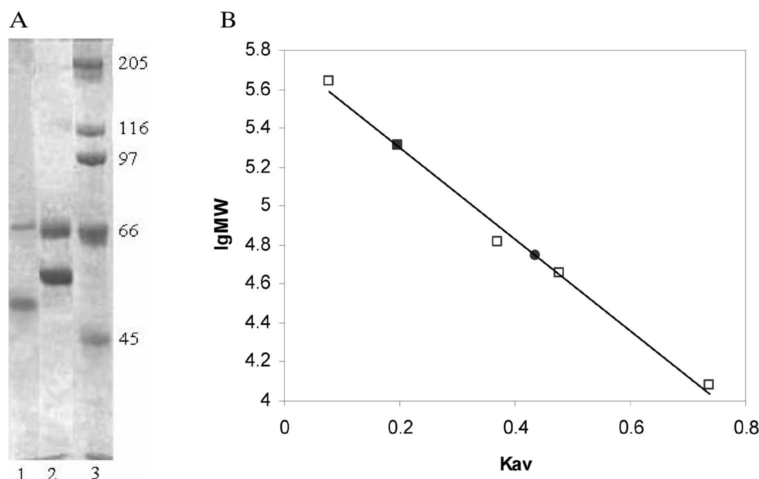

Fig. 1. (A) SDS-PAGE of proline imin5opeptidase (lane 1), aminopeptidase (lane 2) and protein markers (line 3). Proteins were stained with Coomassie Brilliant Blue R-250. (B) Plot of the mobility against molecular mass on a logarithmic scale for aminopeptidase (●), proline iminopeptidase (■) and molecular marker proteins (□) on a gel filtration column (Sephadex G-200).

Table II. Amino acid sequence alignment of aminopeptidase (AP) and proline iminopeptidase (IP) from cabbage and of similar proteins from other organisms. Accession numbers of amino acid sequences of similar proteins are AAG52429 and At3g61540 (*A. thaliana*), AAU03101 (*Oryza sativa*), AAA24318 (*E. coli*), AAF41777 (*Neisseria meningitides*), AAO57257 (*Pseudomonas syringae* pv. *tomato),* BAA09605 (*Hafnia alvei*), P46547 (*Aeromonas sobria)* and CAA04698 (*Propionibacterium freudenreichii* subsp. *Shermanii)*. Identical amino acid residues are shown in filled boxes (*lalign* program).

Origin	Sequence	(region)	Reference
Cabbage AP	EQVLISNGNVVR		This study
Arabidopsis APN	VLLSNGNLI	(235–243)	Lin *et al.*, 2001
Oryza sativa APN	VLLSNGNLI	(157–165)	Chow *et al.*, 2004
E. coli APN	LLSNGNRV	(155–162)	Foglino *et al.*, 1986
Neisseria APN	YPVLLSNGNKID	(147–158)	Tettelin *et al.*, 2000
Pseudomonas syringae pv. *tomato*	ILLSNGNPI	(160–168)	Buell *et al.*, 2003
Cabbage IP	APVAAAVFTFF		This study
Arabidopsis thaliana IP	PVAAAVY	(453–459)	unpublished
Hafnia alvei IP	PVVAAVY	(368–375)	Kitazono *et al.*, 1996
Aeromonas sobria IP	PVACAVY	(367–373)	Kitazono *et al.*, 1994a, b
Propionibacterium freudenreichii IP	APCAAAIY	(352–359)	Leenhouts *et al.*, 1998

et al., 1998) (Table II). The amino acid sequence of the second decapeptide showed similarities to the myrosinase-associated protein of *Brassica napus* (Taipalensuu *et al.*, 1997). It was suggested that the second visible protein band in SDS-PAGE (Fig. 1A) with a molecular weight around 70 kDa is caused by a myrosinase-associated protein of cabbage.

When stored in the absence of protective reagents, the purified enzymes were rather unstable. Little or no activity was observed after 2 days for aminopeptidase and 3 days for proline iminopeptidase at 4 °C. In the presence of 50% glycerol, solutions of the peptidases in 50 mM phosphate or Tris/HCl buffers, pH 7.5, could be kept at 4 °C for several months without loss of activity.

The purified enzymes have no endopeptidase activity, tested against the Bz-Arg-*p*-NA substrate and casein. The aminopeptidase preferentially hydrolyzed phenylalanine-*p*-nitroanilide (Phe-*p*-NA) and Leu-*p*-NA. There was little or no activity against alanine-*p*-nitroanilide (Ala-*p*-NA) and Pro-*p*-NA. Proline iminopeptidase hydrolyzed only Pro-*p*-NA.

Analysis of the activity of the aminopeptidases in crude cabbage extract showed a preference for substrates containing aromatic side chains. The *p*-nitroanilides of Phe, Leu and Pro were clearly the most preferred substrates, and lysine-*p*-nitroanilide (Lys-*p*-NA) was the only other substrate with substantial levels of aminopeptidase activity. There was little activity against Ala-*p*-NA. This is an evidence for the presence of multiple forms of aminopeptidases in fresh cabbage. After the final step of purification, the purified aminopeptidase had little or no activity against Ala-*p*-NA and Lys-*p*-NA.

The temperature optimum for the aminopeptidase was 35 – 40 °C. After 120 min at 50 °C no aminopeptidase activity remained. The proline iminopeptidase was moderately heat-stable with a temperature optimum of 45 °C. At 50 °C it started to become labile. The enzyme began to lose activity after about 10 min at 50 °C and by 120 min, 50% of its initial activity was lost.

Maximal activity for the aminopeptidase was observed at pH 7.5 and for the purified proline iminopeptidase between pH 8.0 and 8. 5. On standing overnight at 4 °C, the aminopeptidase and proline iminopeptidase were stable in phosphate buffer between pH 6.5 and 7.5 and pH 7.5 and 8.5, respectively.

The sulfhydryl group inhibitor *p*-chloromercuribenzoic acid (*p*-CMB) was potent for activities of both enzymes. The requirement of a sulfhydryl group for the enzymatic activities was supported by the strong inhibition observed following an incubation of the enzymes with the heavy metal ions Ag^+ and Zn^{2+}. Also PMSF, an inhibitor of serine proteinases, inhibited both peptidases, but this inhibitor also affected some cysteine proteinases (Storey and Wagner, 1986). No or little effect on the enzymatic activities was observed, when they were preincubated with EDTA, Mg^{2+} or Ca^{2+} ions. A light inhibition was observed in the presence of 2 M NaCl for both enzymes, while ethanol caused mainly a strong inhibition of proline iminopeptidase activity.

The molecular weights of the aminopeptidase and proline iminopeptidase were estimated to be about 56,000 and 206,000, respectively, by gel filtration using a Sephadex G-200 column (Fig. 1B). In SDS-PAGE of the aminopeptidase fraction two major protein bands at 66 kDa and 56 kDa were visible. The proline iminopeptidase fraction migrated as two bands on SDS-polyacrylamide gel in the presence of 2-mercaptoethanol. The molecular weights of the minor and major protein bands were determined to be 70 and 50 kDa, respectively, from the linear plot of standard proteins. These results indicated that the native proline iminopeptidase is composed of four subunits of 50 kDa.

Discussion

A new aminopeptidase and a proline iminopeptidase from cabbage leaves were identified, partially purified and characterized, and a content of about 20 and 5 mg per kg leaves, respectively, could be calculated. Both are true aminopeptidases requiring a free L-amino group. Both enzymes are sensitive to the inhibition by the thiol antagonist *p*-CMB.

The isolated and characterized aminopeptidase contains some impurities, but it was shown to be free of the other aminopeptidase species present in cabbage. So far, only leucine aminopeptidases (LAPs) have been well identified in plants, while other aminopeptidases could not be characterized, despite many studies with amino acid chromogenic substrates and peptides. In cabbage leaves, large amounts of aminopeptidases were found, probably not belonging to the LAP group, preferentially Phe-*p*-NA as substrate is hydrolyzed. Characterization of the isolated aminopeptidase demonstrated that it closely resembles aminopeptidases purified from other plant species, particularly those from pea seeds (Elleman, 1974), apricot seeds (Ninomiya *et al.*, 1981) and primary leaf of wheat (Waters and Dalling, 1984). In each of these species, multiple molecular forms of aminopeptidase, showing different substrate specificities, have been identified. The molecular weights of the aminopeptidases in pea, apricot and wheat are 58, 56 and 57 kDa, respectively. These prefer substrates with a hydrophobic amino acid, particularly Phe, Leu and Tyr, in the *N*-terminal position; they are highly sensitive to *p*-CMB and heavy metal ions, insensitive to EDTA and 1,10-phenanthroline, and have pH optima near 7.0 with the exception of the aminopeptidase from wheat, with a pH optimum at 7.6. The corresponding enzyme from cabbage also possesses most of these properties and shows maximum activity at pH 7.5.

The new proline iminopeptidase has some properties similar to other plant iminopeptidases (Ninomya *et al.*, 1982; Waters and Dalling, 1983;

Ovando *et al.*, 2004), such as an alkaline pH optimum, sensitivity against the sulfhydryl group inhibitor *p*-CMB and heavy metal ions, and high molecular weight. Among the amino acid *p*-nitroanilides tested, the enzyme was highly specific for Pro-*p*-NA. The purified proline iminopeptidase differed from the iminopeptidase from peanut seeds, as it shows appreciable activity against other amino acid chromogenic substrates, such as Ala-, Met- and Leu-2-naphthylamides. On the other hand, PMSF inhibits the proline iminopeptidase at moderate rates. Kitazono *et al.* (1994b) identified a serine residue in the active site by site-directed mutagenesis. The possibility can not be discarded that proline iminopeptidases from plants may be actually serine proteases as those isolated from other sources, in which SH groups are part of their active sites (Ovando *et al.*, 2004; Kitazono *et al.*, 1996). Thus, based on amino acid sequencing and biochemical characterization, we conclude that the purified proline iminopeptidase is a member of the iminopeptidase family (family S33).

Acknowledgements

We would like to thank Deutsche Forschungsgemeinschaft (DFG) for granting a scholarship and supporting this work.

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