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The molecular and biochemical characteristics of proline iminopeptidase from rye seedlings (*Secale cereale* L.)

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Abstract

A proline iminopeptidase (EC. 3.4.11.5) was isolated from shoots of 3 day old seedlings. The purification procedure consisted of 5 steps: acid precipitation, gel filtration on Sephadex G-200, ion-exchange chromatography on Sepharose CL 6B, twice repeated hydrophobic chromatography on Phenyl-Sepharose HP. The enzyme was purified 404.8-fold, with the specific activity of 8.5 units·mg^{-1}of protein with recovery yield of 3 %. The purified enzyme had a molecular mass of 225 kDa estimated by gel filtration and 55.4 kDa by SDS PAGE. This indicates that native enzyme is composed of four subunits. The enzyme was specific for proline β-naphtylamide among various amino acid β-naphtylamides.

An optimal activity was observed at 37 °C at pH 7.75. The enzyme was thermostable up to 37 °C for 30 min. The enzyme was strongly inhibited by pHMB, E-64, heavy metal ions and partially by PMSF, DFP. The results suggest that cysteine and serine residues may participate in the enzyme activity.

List of abbreviations: 2-ME, 2-mercaptoethanol
PAGE, polyacrylamide gel electrophoresis;
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
EDTA, ethylenediaminetetraacetic acid;
E-64, transepoxysuccinyl-L-leucyloamido-(4-guanidino)butane;
pHMB, *p*-hydroxymercuribenzoic acid;
βNA, β-naphthylamide;
PMSF, phenyl methylsulfonyl fluoride;
DFP, diisopropyl fluorophosphate

Introduction

Plant aminopeptidases catalyze the hydrolysis of peptide bonds on N-terminus of peptide substrates (Varshavsky and Byrd 1997, Schaller 2004). Great diversity of these enzymes in terms of kinetic and molecular properties, sub-cellular localization and metabolic functions was a base for introducing various classification systems. One of them distinguishes neutral, acidic and basic aminopeptidases (Taylor 1993). Neutral aminopeptidases is a common, abundant enzyme group in all living organisms with optimum pH 6.5 - 7.5, In terms of substrate preferences neutral aminopeptidases have been divided into three subgroups. The first consists of enzymes showing the highest affinity to substrates with leucine, tyrosine, tryptophane and phenylalanine on N-terminus. They are monomers with molecular mass in range of 56 - 76 kDa and belong to cysteine enzymes (Couton *et al.* 1991, Yamaoka *et al.* 1994). The second subgroup consists of aminopeptidases preferring substrates with alanine or leucine on N-terminus. They are metalloenzymes with molecular mass varying from

14 to 390 kDa (Casano *et al.* 1989, Gu and Walling 2002). The last and less investigated subgroup of neutral aminopeptidases are iminopeptidases (proline aminopeptidases). They have been traced in many plants species, both mono- and dicotyledonous (van der Valk *et al.* 1989, Isola and Franzoni. 1996). However, up to the present only three iminopeptidases from higher plants have been purified: from wheat 127-fold (Waters and Dalling 1983), apricot 1 700-fold (Ninomiya *et al.* 1982) and peanut 300-fold (Oviedo Ovando *et al.* 2004). Much more effort was made to purify iminopeptidases in procaryota (Atlan *et al.* 1994, Kitazono *et al.* 1996, Medrano *et al.*1998, Inoue *et al*. 2003), but the obtained results show only sensible differences between plant and bacteria enzymes. Similarity in amino acid sequences was very low, not exceeding 30 %. Plant iminopeptidases have cysteine –SH groups in the active site and are characterized by high substrate specificity towards proline at N-terminus. An important role in enzyme activity is also played by group –OH of serine present in enzymatic protein (Oviedo Ovando *et al.* 2004). The metabolic functions of proline aminopeptidases have not been investigated yet. It can be supposed that, together with other aminopeptidases, they degrade proteins and peptides (Caldwell and Sparrow 1990, Simpson 2001). Low ability of the known aminopeptidases to liberating proline from protein molecule may be the indication of that. Moreover, some authors do not preclude that iminopeptidases take part in maintaining high level of proline under abiotic stress (Dubey and Rani 1990, Walling 2006). As our current knowledge about plant proline iminopeptidases is very insufficient, in this paper we tried to determine the molecular and kinetic properties of iminopeptidase and recognize the activity regulating processes of that enzyme in rye seedlings leaves. These investigations are starting points for further studies on determining the exact metabolic functions of this enzyme.

Material and Methods

Plant material

Shoots of 3-day old rye seedlings var. Dańkowskie złote were used for the experiments. Rye seeds were imbibed on moistened filter paper in Petri dishes in darkness at the temperature 22 °C. After 3 days seeds germination shoots of seedlings were cut and stored frozen -80 °C prior to extraction.

Purification procedure

All steps of the purification procedure were performed at 0 - 4 °C. Shoots of the seedlings (30 g) were cut into 1 cm long pieces and homogenized in 50 mM Tris-HCl buffer,1:5 (w/v), pH 7.5 containing 2 mM 2-ME, filtered through several layers of miracloth. The filtrate was centrifuged for 15 min at 20,000 g. The pellet was discarded and supernatant was acidified to pH 5.2 with 2M acetic acid according to method described by Waters and Dalling (1983). After 24 h at 2 °C, acid - participated proteins were removed by centrifugation at 20,000 g for 15 min. The volume of supernatant obtained was reduced to approximately 5 ml using Amicon with a PM 100 membrane. The concentrated extract was applied on the Sephadex G-200 column 2.6 x 100 cm equilibrated with the 50 mM Tris-HCl buffer, pH 7.5 containing 2 mM 2-ME. The column was washed with the same buffer at a rate of 0.5 $ml \cdot min^{-1}$ and 5-ml fractions were collected. Fractions were analyzed for soluble protein and iminopeptidase activity. Following chromatography on Sephadex G-200, the fractions showing activity against Pro-ß-NA were pooled and concentrated as above to about 2.5 ml. The preparation obtained was loaded onto a column 1.5 x 10 cm of Sepharose CL 6B equilibrated with 10 mM Tris-HCl buffer, pH 7.5 containing 2 mM 2-ME. The column was washed with 250 ml of equlibrainting buffer and iminopeptidase protein was eluted with linear NaCl gradient $0 \rightarrow 0.3$ M. 3 ml fractions active against Pro-ß-NA were collected, concentrated to 1 ml and loaded onto Phenyl-Sepharose HP column 1.5 x 5 cm equilibrated with 50 mM Tris-HCl – 1 M ammonium sulfate buffer, pH 7.5 containing 2mM 2-ME at the flow rate 2 $ml \cdot min^{-1}$ the column was first washed with equilibrating buffer and then subjected to a descending gradient from $1.0 \rightarrow 0$ M $(NH_4)_2SO_4$. The last step of enzyme purification was the rechromatography of active fractions on the same Phenylsepharose HP column equilibrated with 50 mM Tris-HCl - 2 M ammonium sulfate buffer, pH 7.5 containing 2mM 2-ME. The absorbed proteins were eluted with linear gradient of

$(NH_4)_2SO_4$ 2.0→0 M at the flow rate 2 ml·min^{-1}. The active fractions were used as a purified iminopeptidase.

Assay of enzyme activity

Iminopeptidase activity was determined by the method of Kolehmainen and Mikola (1971) using amino acid β-naphtylamides. In routine assay the reaction was carried in 1ml of 50 mM Tris- HCl buffer, pH 7.5, containing 0.1 mM amino acid β-naphtylamide at 37 °C. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmole naphtylamine per one min.

Detection enzyme activity in polyacrylamide gel

The gels after electrophoresis were incubated in 1mM solution of substrate dissolved in 50 mM Tris- HCl buffer, pH 7.5 for 30 min. The gels were stained in the same buffer containing 0.1 % Fast Garnet GBC salt (Kolehmainen i Mikola 1971).

Determination of K_m value

Km values for amino acid β-naphtylamides were determined graphically by the method of Lineweaver and Burk (1934). The substrate concentrations used ranged from 0.015 to 1.5 mM.

Molecular mass estimation

The molecular mass of the iminopeptidase was estimated by the gel filtration on a Sepharose 2B column 2.5 x 100 cm. The column was equilibrated with 100 mM Tris-HCl buffer, pH 7.5 containing 2 mM 2-ME. The standards, blue dextran (2000 kDa), apoferritin (480 kDa), catalase (240 kDa), bovine albumin (137.5 kDa), were made up to a concentration of 1 mg·ml^{-1} in equilibrating buffer containing 0.1 % (w/v) sucrose.

Native PAGE

Electrophoresis under non-denaturing conditions was performed on 7.0 % polyacrylamide gel by the method of Laemmli (1970), in Tris-glycine buffer, pH 8.3.

SDS PAGE

Electrophoresis was carried out according to Laemmli (1970) in 12 % slab polyacrylamide gel with 4 % stacking gel using SDS-PAGE markers standard kit Sigma: Trypsinogen (24 kDa), Carbonic anhydrase (29 kDa), Glyceraldehyde-3-phosphate dehydrogenase (36 kDa), Ovalbumin (45 kDa), Glutamic dehydrogenase (55 kDa), Albumin (66 kDa), Phosphorylase b (97 kDa), β-Galactosidase (116 kDa), Myosin (205 kDa). Proteins bands were visualized according to Blum *et al.* (1987).

Determination of proteins

Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Results and Discussion

Purification

The purification procedure used for proline iminopeptidase from rye seedlings consists of 5 steps (acid precipitation, gel filtration, anion-exchange chromatography, twice repeated hydrophobic chromatography) and the results are presented in Table 1. The applied procedure allowed to obtain 404.8-fold purified enzymatic preparation with the specific activity of 8.5 units·mg^{-1} protein. Acid precipitation was the first step, as all traditional methods of initial purification, like ammonium sulfate precipitation, protamine sulphate or acetone precipitation did not give expected results. All above - metioned methods led to almost 80 % decrease in initial enzyme activity (data not shown). Chromatography on Sephadex G-200 and Phenyl-Sepharose HP appeared to be the most effective steps of the purification with 8-and 5.5- fold increase of the specific activity, respectively. In addition, chromatography on Sephadex G-200 was a necessary step, as it was the only way to separate various aminopeptidases with broad substrate specificity from proline iminopeptidases. As a result of using partially original purification procedure one of the highest described factor of purification of proline iminopeptidase in higher plants was obtained (Ninomiya *et al.* 1982, Waters and Dalling 1983, Oviedo Ovando *et al.* 2004). In fact, the real purifi-

Table 1. Summary of the purification of proline iminopeptidase from 3 day – old rye shoots.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units·mg^{-1}protein)	Purification (fold)	Yeld (%)
Crude extract	332.40	6.770	0.021	1	100
Acid precipitation	129.09	5.551	0.043	2	82
Sephadex G-200	5.620	1.883	0.335	15.9	28
Sepharose CL6B	1.330	0.682	0.513	24.4	10
1st Phenyl-Sepharose HP	0.200	0.541	2.710	129.0	8
2nd Phenyl-Sepharose HP	0.024	0.204	8.500	404.8	3

cation of the enzyme seems to be higher than 405-fold because of presence of other aminopeptidases in crude extract which use Pro-βNA as a substrate. The homogeneity of the enzymatic preparation was checked with PAGE method under non-denaturating conditions. As shown at Fig. 1, one band of protein corresponds with one band of enzyme activity.

Characteristics of the enzyme

The enzyme activity was determined at the presence of three different buffers in pH range of 6.75 - 8.25 (Fig. 2). Using Tris-HCl or Robinson's buffer in the incubation mixture was showed that the optimum pH of the enzyme was 7.75. Moreover, it was found, that in the pH range 7.5 - 8.0 the enzyme activity submitted only little changes. For the phosphate buffer optimum pH was lower (7.5) and the level of enzyme activity within tested pH range slightly differentiated. Similar results were obtained for apricot (Ninomiya *et al.* 1982) and peanut seeds iminopeptidase (Oviedo Ovando *et al.* 2004), whereas optimum pH both for the crude and partially purified enzyme preparation from wheat leaves was 7.4 (Waters and Dalling 1983). Optimum temperature of studied enzyme was 37 °C, and its increase by 5 °C over the optimum caused almost 30 % drop of activity (Fig. 3).

Table 2. Substrate specificity and kinetic parameters of proline iminopeptidase.

Substrate	Relative activity (%)	Km (M)
Pro-β-NA	100	$1.56 \cdot 10^{-5}$
Phe-β-NA	7.6	$2.19 \cdot 10^{-4}$
Gly-β-NA	6.4	$3.19 \cdot 10^{-4}$
His-β-NA	6.3	$3.45 \cdot 10^{-4}$
Ala-β-NA	6.1	$6.56 \cdot 10^{-4}$
Leu-β-NA	4.7	-
Arg-β-NA	3.2	-
Trp-β-NA	0	-
Tyr-β-NA	0	-
Met-β-NA	0	-
Glu-β-NA	0	-
Ile-β-NA	0	-
Val-β-NA	0	-

Table 3. Effect of inhibitors and metal ions on the proline iminopeptidase activity. The enzyme was preincubated in 50 mM Tris-HCl buffer, pH 7.5 containing different reagents for 120 min. at 4 °C. After preincubation, the activity toward Pro-βNA was assayed by standard procedure.

Reagent	Concentration (mM)	Relative activity (%)
None	-	100
1,10-phenantroline	10	99
EDTA	10	98
PMSF	1.0	51
E-64	1.0	16
pHMB	0.1	2
DFP	1.0	49
Pepstatine A	0.15	91
$ZnCl_2$	0.5	6
$CuCl_2$	0.5	9
$PbCl_2$	0.5	9
$MgCl_2$	0.5	102
$MnCl_2$	0.5	101
$CaCl_2$	0.5	95

Fig. 1. Electrophoretic pattern of purified proline iminopeptidase:, gel stained for protein (1) and for activity (2).

Thermostability of the enzyme has been studied in the range of 25 - 60 °C (Fig. 3). 60 minute pre-incubation at 25 - 37 °C did not have any influence on enzyme activity, whereas at the temperature of 45 - 55 °C most of the enzyme was inactivated. The comparison of thermostability of rye proline iminopeptidase with those from other organisms is relatively difficult, due to lack of similar studies for higher plants. Moreover, exceptionally differentiated thermostability of proline iminopeptidases from microorganisms was observed (Kitazono *et al.* 1996, Medrano *et al.*1998, Inoue *et al*. 2003).

Substrate specificity was determined with different β-naphtylamide substrates under standard conditions (Table 2). Among fourteen substrates used, proline - βNA allowed for achieving the highest activity. In contrast to many aminopeptidases functioning in plant cell, rye iminopeptidase shows relatively narrow substrate specificity (Yamaoka *et al.* 1994, Gu *et al*. 2002). Furthermore, high activity in presence of Pro-β-NA is accompanied by much lower K_m value for the substrate in comparison with other substrate used (Table 2). This data ensure, that studied enzyme is a proline iminopeptidases showing high preferences towards substrates with proline at N-terminus. It is known that iminopeptidases prefer substrates with proline at the N-terminus, but in the case of enzyme from rye shoots the replacement of Pro-β-NA with Leu-β-NA or Ala-β-NA allows for achieving only 5-6 % activity, whereas from peanut seeds - 12 and 37 %, respectively (Oviedo Ovando *et al.* 2004). The above observations may indicate the important metabolic functions of this enzyme, as specific release of proline serves the plant growth and development processes. On the other hand it may be one of the mechanisms of osmotic adaptation of plants

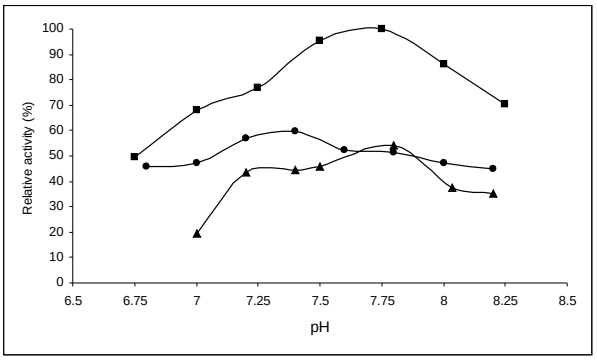

Fig. 2. Effect of pH on proline iminopeptidase activity. The enzyme activity was measured at 37 °C in 50 mM concentration of various buffers including Robison's buffer (■), Tris-HCl buffer (s), phosphate buffer (●). The activity toward Pro-βNA was expressed as a percentage of the maximum activity.

to stress conditions. Many earlier studies under water deficit (Delauney and Verma 1993), NaCl salinity (Dubey and Rani 1990) or high ozone concentration (Eckey-Kaltenbach *et al.* 1994) revealed that accumulation of proline as a key amino acid responsible for the osmoregulation and being also a scavenger of free radicals formed during stress was observed (van der Hoorn and Jones 2004). It is interesting that regardless that study, increased level of aminopeptidase activity under high ozone concentration was recorded (Eckey-Kaltenbach *et al.* 1994). The explanation of this phenomenon requires further research, as the aminopeptidase ac-

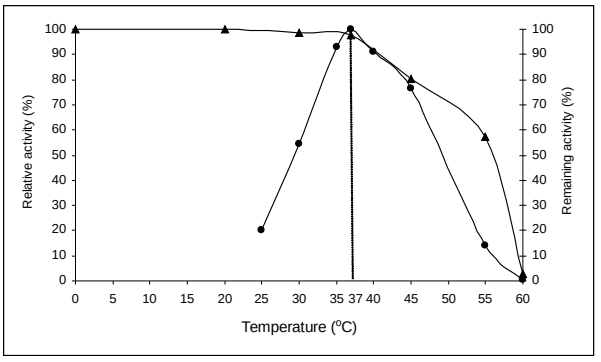

Fig. 3. Effect of temperature and thermal stability on proline iminopeptidase activity. The effect of temperature (●) was determinated at various temperature using standard assay described in Materials and methods. For the thermal stability (s), the remaining activity was determinated as above after preincubation of the enzyme for 30 min. at different temperatures.

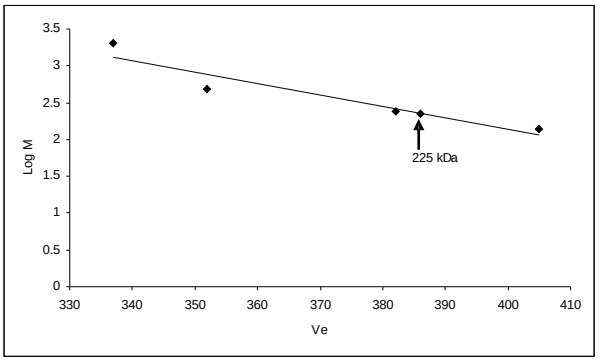

Fig. 4. Plot of molecular mass of standard proteins and proline iminopeptidase against the Ve value determinated by molecular gel filtration on Sepharose 2B. Standards: Blue Dextran (2000 kDa), Apoferritin (480 kDa), Catalase (240 kDa), Bovine Albumine (137,5 kDa).

tivity increase may also reflect the intensifying process of removal damaged proteins, formed due to excessive accumulation of reactive oxygen forms (van der Hoorn and Jones 2004). Besides determination of the kinetic properties of rye proline iminopeptidase, the aim of study was to recognize the structure of active site and mechanisms of enzyme activity regulation. Experiments carried with diagnostic inhibitors showed the highest inhibition with E-64 and pHMB (Table 3). Various heavy metal ions showed similar influence, which at concentration of 0.5 mM decreased the level of activity in 90 % in comparison with control (Table 3). The experiments show an important role of cysteine residues in forming of enzyme-substrate complex. Similar results were obtained for other up to now studied higher plant iminopeptidases (Oviedo Ovando *et al.* 2004) and were also the reason for including them into cysteine enzymes. The lack of EDTA and Pepstatine A influence on the enzyme excludes presence of aspartic acid -COOH groups and metal ions in the active site of proline iminopeptidase. Also, interesting is the over 50 % decrease of activity caused by DFP (Table 3). It suggests an important role of serine -OH groups in maintaining the activity of the enzyme. Perhaps, in rye iminopeptidases, cysteine -SH and serine -OH groups participate in the enzyme activity and additionally cysteine residues stabilize subunits composition. Similar observations were obtained for proline iminopeptidase isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. (Atlan *et*

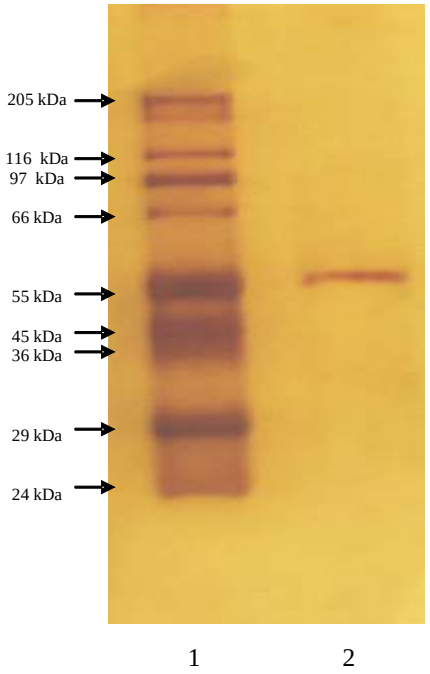

Fig. 5. SDS-PAGE of purified proline iminopeptidase.
1- molar mass markers: Trypsinogen (24 kDa), Carbonic anhydrase (29 kDa), Glyceraldehyde-3-phosphate dehydrogenase (36 kDa), Ovalbumin (45 kDa), Glutamic dehydrogenase (55 kDa), Albumin (66 kDa), Phosphorylase b (97 kDa), β-Galactosidase (116 kDa), Myosin (205 kDa).
2- purified enzyme

al. 1994), *Flavobacterium meningosepticum (*Kitazono *et al*. 1996), *Xanthomonas campestris* pv. *Citri (*Medrano *et al*. 1998). Despite many similarities between higher plant-origin iminopeptidases, also significant differences can be observed. One of them was the molecular mass. The estimated by gel filtration molecular mass of the native rye proline iminopeptidase was 225 kDa (Fig. 4) whereas for enzyme of wheat was 400 kDa (Waters and Dalling 1983). SDS-PAGE showed that studied enzyme was built of a single type of subunits, of 55.4 kDa (Fig. 5). According to these results the native enzyme may be composed of four subunits. The multimeric structure of the iminopeptidases appeared to be a common characteristic of these enzymes in most eukaryote and prokaryote. The molecular mass of higher plants and animal iminopeptidases in denatured state were found to be 55 to

56 kDa (Ninomiya *et al.* 1982, Mathushima *et al* 1991, Oviedo Ovando *et al.* 2004), whereas enzymes of prokaryotes 34-63 kDa (Atlan *et al.* 1994, Kitazono *et al.* 1996). The first were described as tetrmers or hexamers (Ninomiya *et al.* 1982, Waters and Dalling 1983, Oviedo Ovando *et al.* 2004) but the second as trimmers, dimmers or monomers (Atlan *et al.* 1994, Kitazono *et al.* 1996).

The above differences may be due to the fact, that the enzymes were purified from different organisms, which are genetically far from each other. The generalization of our current knowledge is difficult mainly because of the insufficient data on this enzyme especially in higher plants. We plan to carry out more experiments regarding the gene expression of this enzyme under control and abiotic stress conditions in different plant species.

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