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Purification and partial characterization of *Phaseolus vulgaris* seed aminopeptidase

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Abstract

The aminopeptidase activity of *Phaseolus vulgaris* seeds was measured using L-Leu-p-nitroanilide and the L-aminoacyl-ß-naphthylamides of Leu, Ala, Arg and Met. A single peak of aminopeptidase activity on Leu-ß-naphthylamide was eluted at 750 µS after gradient elution chromatography on DEAE-cellulose of the supernatant of a crude seed extract. The effluent containing enzyme activity was applied to a Superdex 200 column and only one peak of aminopeptidase activity was obtained. SDS-polyacrylamide gel electrophoresis (10%) presented only one protein band with molecular mass of 31 kDa under reducing and nonreducing conditions. The aminopeptidase has an optimum pH of 7.0 for activity on all substrates tested and the highest $Vmax/K_M$ ratio for L-Leu-ß-naphthylamide. The enzyme activity was increased 40% by 0.15 M NaCl, inhibited 94% by 2.0 mM Zn^{2+}, inhibited 91% by sodium p-hydroxymercuribenzoate and inhibited 45% by 0.7 mM o-phenanthroline and 30 µM EDTA. Mercaptoethanol (3.3 mM), dithioerythritol (1.7 mM), Ala, Arg, Leu and Met (70 µM), p-nitroaniline (0.25 mM) and ß-naphthylamine (0.53 mM) had no effect on enzyme activity when assayed with 0.56 mM of substrate. Bestatin (20 µM) inhibited 18% the enzyme activity. The aminopeptidase activity in the seeds decayed 50% after two months when stored at 4ºC and room temperature. The enzyme is leucyl aminopeptidase metal- and thiol group-dependent.

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- *Phaseolus vulgaris* aminopeptidase
- Bean seed aminopeptidase
- Leucyl aminopeptidase

Hydrolysis and mobilization of storage proteins occur during seed germination. Despite the intensive studies on proteolytic activity during seed germination the mechanism of protein degradation is still not well understood. The reserve proteins are broken down by endopeptidases, aminopeptidases and carboxypeptidases yielding amino acids, which are reutilized in the synthesis of new functional proteins. The purification and characterization of these proteases should improve the understanding of the regulation, structure and function of these enzymes in seeds.

Aminopeptidases (EC 3.4.11.XX) are enzymes that participate in the final stages of protein degradation and hydrolyze peptide bonds yielding amino acids from N-terminal peptides and proteins and act on some artificial substrates like aminoacyl-ß-naphthylamides (AA-NA) and aminoacyl-p-nitroanilide (AA-Nan). They are found in many subcellular organelles, in the cytoplasm and in the cell membranes (1). Aminopeptidases

have been purified and characterized from several plant sources such as soybean seeds (2), *Enterolobium contortisiliquum* seeds (3), barley (4) and mung bean cotyledons (5).

In the present study we describe the purification and characterization of one aminopeptidase from *Phaseolus vulgaris* seed crude extract which presented arylamidase activity on the L-Leu-p-nitroanilide (Leu-Nan) and AA-NA of Leu, Ala, Arg and Met. The hydrolysis of AA-NA was measured by the method of Höpsu et al. (6) and that of Leu-Nan by the method of Tuppy at al. (7). Enzyme activity was determined under the following conditions: 0.56 mM substrate, 20 mM sodium phosphate buffer (NaPB), pH 7.0, at 37°C in a final volume of 3 ml. One enzyme unit (U) is defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per minute at 37°C under the conditions of the assay for each particular substrate.

Phaseolus vulgaris seeds (15.47 g) were swollen in water and, after the tegument was removed, homogenized in a blender with 83 ml 0.02 M NaPB, pH 7.0. The crude extract was filtered and centrifuged at 9000 *g* for 30 min at 4°C. The supernatant which contained 505 U L-Leu-ß-naphthylamide (Leu-NA) activity was submitted to chromatography on DEAE Cellex D, equilibrated and washed with 20 mM NaPB, pH 7.0. Elution with a linear gradient of 20 mM to 200 mM NaPB, pH 7.0, provided only one peak with activity on AA-NA of Leu, Arg, Ala and Met as substrate. The effluent containing activity, eluted at 0.7 mS, was lyophilized and filtered on a Superdex 200 column, equilibrated and developed with 0.1 M Tris-HCl, pH 8.0, containing 0.15 M NaCl. Only one peak with activity on Leu-NA (112 U) was obtained. Molecular mass estimated by gel filtration was 31 kDa. Protein was determined using Coomassie brilliant blue (8). Bovine serum albumin was used as standard.

SDS-polyacrylamide gel electrophoresis, performed on 10% gel with reduced and nonreduced samples under the conditions described by Laemmli (9) and stained with silver nitrate, showed only one protein band of 31 kDa. Phosphorylase b (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and soybean trypsin inhibitor (21.5 kDa) were used as standards.

Enzyme activity was stable for about 30 days at 4°C in 10 mM sodium phosphate buffer, pH 7.0. The optimum pH of activity was 7.0 for Leu-Nan and AA-NA of Leu, Ala, Met and Arg as substrates, using different buffers with pH varying from 4.0 to 9.5. The enzyme preparation did not hydrolyze Glu-NA in the presence of 3.3 mM $CaCl_2$ (10).

Kinetic parameters for the aminopeptidase were determined using Leu-Nan and different AA-NA as substrates (Table 1). Vmax and K_M values were estimated by the double-reciprocal Lineweaver-Burk plots of initial velocity data (11). Statistical analysis was performed using the variance of velocity by the method of Wilkinson (12). The highest catalytic efficiency (0.15 nmol mg^{-1} s^{-1} μM^{-1}) was obtained with Leu-NA, showing that the enzyme is similar to aminopeptidases of mung been (5) and soybean cotyledons (13).

To study the effect of aging seeds on the aminopeptidase activity, seeds were kept at room temperature and at 4°C. The aminopeptidase activity on Leu-NA was determined using a crude extract prepared from seeds of both groups as described before, at different times. After two months the enzyme activity decreased 50% in both groups.

The effect of different chemicals on the aminopeptidase activity is shown in Table 2. The rate of Leu-NA hydrolysis was increased 40% by 0.15 M NaCl. Divalent cations such as Ca^{2+} and Mg^{2+} (2.0 mM) had no effect on the enzyme activity, while Mn^{2+} and Co^{2+} (0.5 mM) activated it by about 25%. The enzyme was strongly inhibited by 0.5 mM Zn^{2+}.

Chelating agents such as ethylenedia-

Table 1 - Kinetic parameters of *Phaseolus vulgaris* seed aminopeptidase acting on ß-naphthylamides (NA) of several amino acids and on L-Leu-p-nitroanilide (Leu-Nan).

Experiments were performed in 20 mM sodium phosphate buffer, pH 7.0, at 37ºC with 8.4 to 2.2 mM substrate concentration. Data are reported as means ± SEM for 5 determinations each on 3 preparations.

Substrate	K_M (µM)	Vmax (nmol mg^{-1} s^{-1})	$Vmax/K_M$ (nmol mg^{-1} s^{-1} μM^{-1})
Leu-NA	19.2 ± 0.1	2.78 ± 0.43	0.15
Arg-NA	29.8 ± 0.1	3.29 ± 0.79	0.11
Met-NA	89.5 ± 0.3	4.61 ± 0.36	0.05
Ala-NA	164.0 ± 0.5	3.14 ± 0.27	0.02
Leu-Nan	123.1 ± 0.2	7.25 ± 0.03	0.06

Table 2 - Effect of various chemicals on the aminopeptidase activity of *Phaseolus vulgaris* seeds.

Assays were carried out at 37ºC in 20 mM sodium phosphate buffer, pH 7.0. The enzyme was pre-incubated with each chemical for 15 min at 37ºC, before the addition of substrate. Leu-NA, L-Leu-ß-naphthylamide; Leu-Nan, L-Leu-p-nitroanilide; o-PHE, o-phenanthroline; EDTA, ethylenediaminetetraacetic acid; pOHMB, sodium p-hydroxymercuribenzoate; 2-ME, 2-mercaptoethanol; DTE, dithioerythritol.

	Chemical	Concentration (mM)	Residual activity (%)	Substrate
Ions	NaCl	150.0	140.0	Leu-NA
	$ZnCl_2$	0.5	6.0	Leu-NA
	$MgCl_2$	0.2	100.0	Leu-NA
	$CaCl_2$	2.0	100.0	Leu-NA
	$MnCl_2$	0.5	120.0	Leu-NA
	$CoCl_2$	0.5	125.0	Leu-NA
Chelating agents	o-PHE	0.7	57.0	Leu-NA
	EDTA	0.03	56.0	Leu-NA
Sulfhydryl group reagents	pOHMB	0.08	9.0	Leu-Nan
	2-ME	3.3	100.0	Leu-Nan
	DTE	1.7	100.0	Leu-Nan
Antibiotic	Bestatin	0.02	82.0	Leu-NA
L-amino acids	Ala	0.07	100.0	Ala-NA
	Arg	0.07	100.0	Arg-NA
	Leu	0.07	100.0	Leu-NA
	Met	0.07	100.0	Met-NA
Cyclic compounds	p-nitroaniline	0.25	98.0	Leu-NA
	ß-naphthylamine	0.53	100.0	Leu-Nan

minetetraacetic acid (EDTA) and o-phenanthroline (o-PHE) inhibited the aminopeptidase activity by about 54%, suggesting that the enzyme is a metal-dependent aminopeptidase.

The enzyme activity was inhibited 91% by sodium p-hydroxymercuribenzoate (pOHMB), suggesting the presence of an important thiol group in the catalytic center of the enzyme. The enzymes from *Vigna radiata* (5) and soybean cotyledons (13) also require a free sulfhydryl group for their activity. *Phaseolus vulgaris* aminopeptidase was not inhibited by -S-S- group reagents as 2-mercaptoethanol (2-ME) and dithioerythritol (DTE). These data agree with the results of SDS-PAGE, with and without dithiothreitol, suggesting the presence of only one pro-

tein chain in the enzyme.

Bestatin (0.02 M), a known inhibitor of leucyl aminopeptidases (14), only partially inhibited (18%) *Phaseolus vulgaris* aminopeptidase.

Since the aromatic product and the amino acids resulting from the reaction between the aminopeptidase and the aromatic substrate may inhibit the enzyme activity, we studied the effect of Leu, Arg, Ala, Met, ß-naphthylamine and p-nitroaniline on the activity of the enzyme. Table 2 shows that the amino acids studied, p-nitroaniline and ß-naphthylamine, have no regulatory effect on enzyme activity and both substrates (AA-NA and AA-Nan) may be used for the determination of *Phaseolus vulgaris* seed aminopeptidase activity. Soybean seed aminopeptidase is inhibited by p-nitroaniline (Ki = 2.71 mM) but not by ß-naphthylamine (6.7 mM), while *Enterolobium contortisiliquum* seed aminopeptidase is inhibited by ß-naphthylamine (Ki = 0.62 mM) and not by 3.3 mM p-nitroaniline (15).

A metal- and thiol group-dependent leucyl aminopeptidase with a molecular mass of 31 kDa was purified to homogeneity from *Phaseolus vulgaris* seed. In contrast to other leucyl aminopeptidases (3,16), it is activated by chloride ions. There are many differences between the aminopeptidase activity purified from *Phaseolus vulgaris* and that present in *Glycine max* seeds (2). The best substrate for activity in soybean is Lys-NA with optimum pH 9.6, molecular mass 65 kDa. It is not inhibited by Zn^{2+} (0.9 mM), pOHMB (50.0 µM), EDTA (33.0 µM), oPHE (1.0 mM) and is inhibited 50% by DTE (2.3 mM).

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