Purification and Characterization of L-Phenylalanine Aminopeptidase from Chick-Pea Cotyledons (*Cicer arietinum* L.)

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Abstract: Chick-pea (*Cicer arietinum* L.) cotyledons are unique source of aminopeptidase – 8-9 U/g cotyledons was observed using L-leucine-*p*-nitroanilide as substrate. The aminopeptidase was purified (65 kDa, pI 4.8) reaching a specific activity of 220 U/mg at pH 7.0-7.2 and 35-40°C. The determined constant of specificity k_{cat}/K_m during hydrolysis of N-unsubstituted amino acid-*p*-nitroanilides showed a decrease order: Phe>Leu>Pro>Ile>Val>Ala. The enzyme was strongly inhibited by *p*-chloromercuribenzoic acid as well as in a competitive rate by the antihypertensive peptides Ile-Pro-Pro and Val-Pro-Pro.

Keywords: Chick-pea cotyledons, aminopeptidase, kinetic analysis, antihypertensive peptides.

INTRODUCTION

The legume storage proteins are mobilized during germination and they are hydrolyzed by endopeptidases, aminopeptidases, and carboxypeptidases yielding amino acids, reutilized in the new proteins synthesis. Aminopeptidases (EC 3.4.11) participate in the final stages of protein degradation hydrolyzing peptide bonds from the N-terminus of the peptide. Peptidases have been described for many legume seeds but only a few have been purified to an extent sufficient for characterization [1, 2, 3]. New aminopeptidase and iminopeptidase were isolated and characterized from *Brassica oleraceae* var. *capitata* [4].

Protein hydrolysates are components of many food supplements but bitter peptides arise during enzymatic hydrolysis limit their use. The significance of high degree of protein hydrolysis for flavor formation was realized decades ago but little progress has been achieved, mainly because no suitable enzymes are available. Because of lack of nonspecific exopeptidases, the attention has been recently focused on aminopeptidases specific for hydrophobic amino acids having real effect on bitterness reducing. Phenylalanine containing peptides demonstrate more than 100-fold more bitter taste than the free phenylalanine [5].

The purpose of this paper is isolation, purification and initial characterization of L-phenylalanine aminopeptidase from chick-pea (*Cicer arietinum* L.) cotyledons. The study on this peptidase should improve the understanding of the regulation, structure, and function of these enzymes in seeds and, on the other hand, it can be used as new tool for modification of food peptides.

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MATERIALS AND METHODS

Plant Material and Reagents

Seeds of chickpea, *Cicer arietinum* L., were obtained from the Experimental Station for Vegetables, Sadovo town, Plovdiv region, Bulgaria.

L-Leu-*p*-nitroanilide, L-Pro-*p*-nitroanilide, L-Ala-*p*-nitroanilide, L-Lys-*p*-nitroanilide, L-Arg-*p*-nitroanilide and L-Phe-*p*-nitroanilide were from Sigma-Aldrich, SDS PAGE molecular weight calibration kit, 1,10-phenanthroline, phenylmethylsulfonyl fluoride (PMSF) and EDTA were from Merck, L-Val-*p*-nitroanilide, L-Ile-*p*-nitroanilide, D-Leu-*p*-nitroanilide, D-Phe-*p*-nitroanilide, N-Suc-L-Phe-*p*-nitroanilide and N-Ac-L-Phe-*p*-nitroanilide were from Bachem AG.. The tripeptides IleProPro and ValProPro were obtained by solid-phase synthesis [6]. All other reagents were of analytical grade. Ion-exchange Toyopearl QAE-550C was obtained from Tosoh Co., Tokyo, Japan. Sephadex G-200, DEAE-Cellulose and Mono Q HR 5/5 were from Amersham Pharmacia Biotech, Uppsala, Sweden. Reverse phase HPLC was performed on a Nucleosil 100 RP-18 column (250 x 10 mm; 7 μm; Macherey-Nagel, Germany).

Enzyme Assay

Aminopeptidase activity with *p*-nitroanilide substrates was assayed at 1.5 mM substrate concentration in 50 mM sodium phosphate buffer, pH 7.0, containing 10% (v/v) N,N-dimethylformamide at 30°C [7]. The liberated *p*-nitroaniline was measured at 410 nm on a spectrophotometer (UV-VIS Spectrophotometer, Shimadzu 1240). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitroaniline per minute.

Kinetic parameters K_m, V_{max}, k_{cat}/K_m and K_i for all substrates and inhibitors were determined as average values of three independent experiments.

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Table 1. Summary of the Purification Procedures for Aminopeptidase

Purification Step	Total Protein (mg)	Total Activity (U)	*Specific Activity(U/mg)*	*Yield %*	*Relative Purification*
Crude extract	6667	850	0.13	100	1
Ammonium sulfate fractionation	818	560	0.68	66	5.7
QAE 550C	186	330	1.8	39	14.8
DEAE-cellulose	37.7	177	4.7	21	39
Sephadex G 200	10.5	152	14.4	18	120
Sephadex G 200	3.4	80.5	23.7	9.5	200
Mono Q HR 5/5	0.15	33	220	4	1700

Protein Determination

Soluble protein was determined by the biuret reaction following precipitation with 20% (v/v) TCA or by the absorbance at 280 nm with bovine serum albumin as standard [8].

Electrophoretic Analysis

For estimation of purity of the enzyme preparations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gels using Tris-glycine buffer, pH 8.3 according to Laemmli [9]. Myosine, rabbit muscle (205 kDa), β-galactosidase (116 kDa), phosphorylase b, rabbit muscle (97 kDa), bovine serum albumin (66 kDa), egg ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) were used as molecular weight marker proteins. The gel was visualized by silver staining.

Native isoelectric focusing (IEF) was performed as described by Robertson *et al.* [10]. Trypsin inhibitor (pI 4.5), bovine serum albumine (pI 4.9), carbonic anhydrase (pI 6.0), myoglobin horse (pI 6.9) and cytochrome c (pI 10.1) were used as pI marker proteins. The gel was visualized by silver staining [11].

Enzyme Purification

Chickpea seeds (*Cicer arietinum* L.) were surface-sterilized by soaking in 1% (w/v) sodium hypochlorite for 10 min, washed in distilled water and then soaked overnight in water at 25°C. The seed coat and axis were removed and the cotyledons were homogenized in a Waring (MX 980, Ireland) in 25 mM Tris/HCl, pH 7.0 containing 10 mM 2-mercaptoethanol at the ratio of 1:5 (w/v). The slurry was stirred for 30 min and centrifuged at 4000 ×g for 10 min (MLW K24 D, Germany). Solid ammonium sulfate was added to the crude extract, and the precipitate, formed between 35 and 55% (w/v) saturation was collected by centrifugation at 4000 ×g for 10 min at 5°C. The precipitate was dissolved in 25 mM Tris/HCl, pH 7.2 containing 5 mM EDTA (buffer A) and dialyzed against the same buffer. The suspension was then clarified by centrifugation at 4000 ×g for 15 min and the pellet of contaminating proteins was discarded. The dialyzed solution was chromatographed on a Toyopearl QAE-550C column (13 cm × 2 cm) pre-equilibrated with buffer A. After the non-bounded proteins were washed from the column with equilibration buffer, 500 ml of a 0-0.5 M NaCl gradient was applied. Fractions of 6.6 ml were collected at a flow rate of 1 ml. min^{-1}. The aminopeptidase activities were eluted at about 0.2 M NaCl concentration. Fractions of aminopeptidases with high activity were pooled and concentrated by ultrafiltration (Amicon YM UM-30 membrane).

The sample after ion-exchange chromatography was loaded onto a second ion-exchange chromatography on DEAE-cellulose column (2.5 × 48 cm) equilibrated with 25 mM Tris/HCl, pH 7.2 (buffer B) and a 0-0.4 M NaCl gradient was applied. Fractions of 6 ml were collected at a flow rate of 0.3 ml. min^{-1}. The aminopeptidase activities were eluted at about 0.25 M NaCl concentration. Fractions of aminopeptidases with high activity were pooled and concentrated by ultrafiltration (Amicon YM UM-30 membrane) and applied on a Sephadex G-200 column (140 cm × 1.5 cm) equilibrated with 50 mM phosphate buffer pH 7.2, containing 0.15 M NaCl (buffer C). Fractions of 3 ml were collected at a flow rate of 0.1 ml. min^{-1} and analyzed for aminopeptidase activity using Leu-*p*-NA as substrate. Following chromatography on Sephadex G-200, the fractions showing activity against Leu-*p*-NA were combined and concentrated by ultrafiltration (Amicon UM-30 membrane). The sample was loaded onto a second Sephadex G-200 column equilibrated with buffer C. The active fractions were combined and concentrated to 1.0 ml with ultrafiltration and stored at 5°C in 50% glycerol.

The sample from the second Sephadex G-200 run was injected into Mono Q HR 5/5 column and washed with buffer B. The column was eluted with 0-0.5 M NaCl gradient in buffer B. The aminopeptidase eluted at 0.23 M NaCl was concentrated with Centricon-30 microconcentrations (5 000 ×g, 20 min, 5°C).

Characterization of Aminopeptidase

The temperature and pH optimums of aminopeptidase were determined using Leu-*p*-NA as substrate. Temperature optimum was estimated by determination of the enzyme activity at the specified temperature ranging from 15 to 60°C. pH optimum was determined in buffered solution with pH

values varying from 4.5 to 10.0. For pH values between 4.5 and 7.0, 25 mM citrate-phosphate buffer was used; between 7.0 and 8.0, 25 mM sodium phosphate buffer was used; between 8.5 and 9.0, 25 mM Tris/HCl buffer was used; and between 9.0 and 10.0 glycine-NaOH was used.

All inhibitors and metal ions were prepared in 50 mM Tris/HCl buffer (pH 7.0). In the case of 1,10-phenathroline and PMSF, the inhibitors were first dissolved in dimethylformamide and then made up to desired concentration by adding sodium phosphate buffer.

Enzyme Kinetics

Aminopeptidase activity against Phe-*p*-NA and Leu-*p*-NA was determined at substrate concentrations up to 1.5 mM, while the substrates Pro-*p*-NA, Ile-*p*-NA, Val-*p*-NA and Ala-*p*-NA were used at concentrations up to 0.5, 2.5, 3 and 4 mM, respectively (Table **2**). Kinetic parameters K_m and k_{cat}/K_m for all substrates were determined by nonlinear regression data analysis of the dependence of the initial rates on the substrate concentration using Enzfitter software [12].

Aminopeptidase activity against Ile-Pro-Pro and Val-Pro-Pro was determined at peptide concentration of 0.5 mM. Kinetic parameters K_m and V_{max} were determined by nonlinear regression data analysis of the dependence of the initial rates on the Phe-*p*-NA concentration using Enzfitter software [12].

Enzymatic Digestion

The sample of aminopeptidase was dissolved in 1 ml of 5 mM ammonium bicarbonate buffer, pH 8.2, and incubated with 50 µl trypsin solution (1 mg. ml^{-1}) at room temperature for 15h, followed by a further addition of 50 µl trypsin solution. Then the reaction mixture (trypsin:aminopeptidase ratio of 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. 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 [trifluoroacetic acid/water, 0.1:99 (v/v)], 10% buffer E [acetonitrile/water/trifluoroacetic acid, 80:20:0.085 (v/v)] for 10 min, then 10-100% E in 70 min. The collected fractions were further subjected to amino acid sequence analysis.

Amino Acid Sequences

Each fraction was further purified on a HPLC Nucleosil RP C18 column using buffer D as loading buffer and buffer E as eluting solution. The following conditions were used: 10% buffer E for 10 min; then 10-100% buffer E in 70 min at a flow rate of 1 ml. min^{-1}. Peak fractions were dried and after dissolving in water/methanol/formic acid 20:40:0.1 (v/v), they were subjected to automated Edman N-terminal sequencing (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

RESULTS

The purification procedures of aminopeptidase are summarized in Table **1**. The enzyme from crude extract has been purified by combination of ammonium sulfate precipitation and chromatographic methods. The procedure differs from the known approaches for aminopeptidase isolation from plant origin with application of ion-exchange chromatography in the presence of EDTA, resulting in the complete adsorption of pigments on the resin. The purified enzyme preparation was homogenous by SDS PAGE and had specific activity of 220 U. mg^{-1}. The achieved degree of purification was 1700-fold at 4% recovery of activity.

On SDS PAGE with silver staining the purified enzyme preparation showed one sharp band with molecular weight of 65 kDa, (Fig. **1**). The isoelectric point of the enzyme at the native IEF was 4.8.

The pure fraction manifesting aminopeptidase activity was subjected to automated Edman N-terminal sequencing, but no result could be achieved. As the N-terminus of aminopeptidase is blocked, internal amino acid sequences were analyzed after tryptic cleavage treatment. The treatment gave major peptide with the following sequence "VNLG LEEDGR". A similarity search with *lalign* program [13] showed that sequence of the peptide is highly similar to aminopeptidases of *Arabidopsis thaliana* [14, Rounsley *et al.* and Cheuk *et al.* unpublished] and *Oryza sativa* (japonica cultivar group) [15] (Table **2**).

Enzymes Properties

The purified enzyme has no endopeptidase activity tested by benzoyl-arginine-*p*-nitroanilide, N-acetyl-phenylalanine-*p*-nitroanilide, N-succinyl-phenylalanine-*p*-nitroanilide and casein as substrates. The aminopeptidase requires L-configuration of the α-carbon atom of the amino acid moiety. That is evidenced by the complete lack of activity towards

Table 2. Amino Acid Sequence Alignment of Aminopeptidase (AP) from Chick-Pea and Similar Proteins of Other Organisms
Accession numbers of amino acid sequences of similar proteins are CAA45040, AADO3380, AAP21153 (*A. thaliana*) and ABA97701 (*Oryza sativa)*. Identical amino acid residues are shown in filled boxes (*lalign* program).

Origin	Sequence (region)	Reference
Chick-pea AP	VNLGLEEDGR	This study
Arabidopsis thaliana LAP	VLGLFEDGR (156-164)	13
Arabidopsis thaliana AP	VLGLFEDGR (156-164)	unpublished
Arabidopsis thaliana AP	VLGLFEDGR (156-164)	unpublished
Oryza sativa AP	VVLGLHEDRR (161-170)	14

D-Phe-*p*-NA and D-Leu-*p*-NA. The aminopeptidase displayed broad substrate specificity with preference for bulky hydrophobic side chains of the amino acid residue (Table **3**). The enlargement of the side chains resulted in higher hydrolysis rates as the enzyme mostly preferred phenylalanine. No hydrolysis was detected with positively charged amino acids L-Lys-*p*-nitroanilide and L-Arg-*p*-nitroanilide.

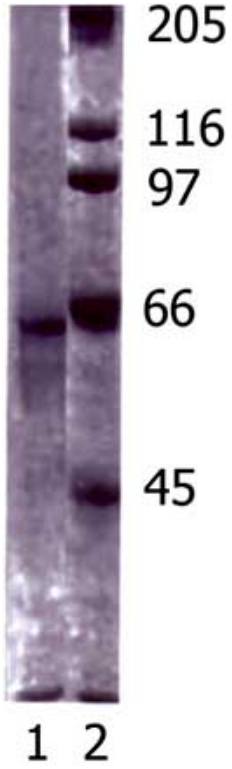

Figure 1. SDS-PAGE of aminopeptidase (lane 1) and protein markers (lane 2). Proteins were visualized by silver staining.

Effect of the ACE Inhibitors (Ile-Pro-Pro and Val-Pro-Pro) on the Enzyme Activity

It is well known that the two peptides, Ile-Pro-Pro and Val-Pro-Pro are biologicaly active peptides, which demonstrate their antihypertensive efects through the inhibition of angiotensin converting enzyme (ACE), an enzyme involved in the regulation of blood presure in target organs. These peptides are not substrates but demonstrate some inhibitor activity tested with the purified aminopeptidase. As it could be seen by the obtained data (Table **4**) in the two cases V_{max} remained almost the same but the K_m increases in the presence of inhibitor. The obtained values for V_{max} and K_m are an evidence for a competitive type of inhibition.

Effect of Temperature and pH on Aminopeptidase Stability

The enzyme had optimum activity at 35-40ºC and was rapidly inactivated above that temperature. The optimal pH for its catalytic action was 7.0-7.2.

Effect of Various Reagents on Enzymes Activity

The sulfhydryl group inhibitor *p*-CMB was potent inhibitor of aminopeptidase activitiy. In another hand, PMSF, inhibitor of serine-enzymes, also inhibit aminopeptidase. Nevertheless, this inhibitor also affects some cysteine-proteinases [16]. No or little effect on enzymes activities was observed when they were preincubated with EDTA, 1,10-phenantrolin, Mg^{2+} and Ca^{2+} ions, indicating no requirement for a metal ion in catalysis.

DISCUSSION

Biochemical characterization of plant aminopeptidases has identified two classes of enzymes. The first group comprises thermolabile aminopeptidases of molecular weight 60-130 kDa and a neutral pH optimum. They are strongly inhibited by sulfhydryl-reagents. Most of the aminopeptidases characterized so far belong to this group [1, 17]. Another group contains large (150-330 kDa) metallopeptidases that have an alkaline pH optima [17, 18]. They are further characterized to have high thermostability and are inhibited by EDTA, 1,10-phenanthroline and bestatin. In chick-pea seeds a highly active aminopeptidase exists and it is likely to belong to the first group of thermolabile aminopeptidases with low molecular weights. Characterization of the isolated aminopeptidase preparation demonstrated that it closely resembles the aminopeptidases purified from other plant species, particularly those from germinating barley grains [19], *Euonymous* leaves [20] and maize kernels [21]. The molecular weights of the aminopeptidases in barley, *Euonymous* leaves and maize are 65, 62.5 and 61 kDa, respectively. They prefer substrates with a hydrophobic amino acid, particularly Phe, Tyr and Leu in the N-terminal position; they are highly sensitive to *p*-CMB, insensitive to EDTA and 1,10-phenanthroline, and have pH optima near 7.0. The corre-

Table 3. Substrate Specificity and Kinetic Parameters of Aminopeptidase

Substrate	k_{cat}, s^{-1}	$10^4 K_m$, M	$10^{-2} k_{cat}/K_m$, $M^{-1}s^{-1}$
Phe-p-NA	230	5.11	4501
Leu-p-NA	102	3.84	2656
Pro-p-NA	18.55	1.19	1559
Ile-p-NA	3.68	16.7	22.04
Val -p-NA	3.08	17.6	17.50
Ala -p-NA	2.69	19.3	1.39

Table 4. Kinetic Parameters for Reaction with Ile-Pro-Pro and Val-Pro-Pro Catalyzed by the Chick-Pea Aminopeptidase

Kinetic Parameters, Inhibitors	10^4K_m, M		10^4V_{max}, $M^{-1}s^{-1}$		10^4K_i, M
	With Inhibitor	Without Inhibitor	With Inhibitor	Without Inhibitor	
IleProPro	15.4	5.11	4.66	4.59	2.4
ValProPro	11.6	5.11	4.34	4.59	3.8

sponding enzyme from chick-pea also possesses most of these properties and shows maximum activity at pH 7.0 – 7.2.

The reaction with Ile-Pro-Pro and Val-Pro-Pro catalyzed by the aminopeptidase is of specific interest, as they showed relatively high antihypertensive activity. During the last decades it has been discovered that specific peptides present in fermented milk have an ACE inhibiting capacity and can induce blood pressure reductions in hypertensive subjects [22]. Nowadays numerous *in vitro* and a few animal trials have demonstrated ACE inhibiting effects of different peptides obtained from a variety of protein sources [23]. A structure-function study of the various ACE inhibiting peptides has suggested that they often possess a Pro-Pro at their C-terminal sequence [24]. This finding is partly explained by the fact that ACE is a peptidyl dipeptidase (EC 3.4.15.1) unable to cleave peptide bonds involving proline. Thus from tripeptides having the structure Xaa- Pro-Pro the dipeptide Pro-Pro cannot be removed because the Xaa-Pro bond cannot be cleaved. It is therefore conceivable that if present in relatively high concentrations, tripeptides having the Xaa-Pro-Pro structure will inhibit ACE activity. As not only ACE, but almost all proteolytic enzymes, including aminopeptidases [17], have difficulties in cleaving Xaa-Pro bonds, the notion that the presence of proline residue at the P_2 position of peptide substrates results in aminopeptidase resistant molecules is almost self-evident. Furthermore, we investigate that these peptides exhibit some inhibitory activity tested with the chick-pea aminopeptidase. With the growing need for a more simple method that generates a bland tasting product with a high and reproducible yield of antihypertensive peptides this finding could be successfully used for affinity purification of proline containing peptides manifesting ACE inhibitory activity.

CONCLUSIONS

In this paper we report on purification and initial characterization of a new aminopeptidase from chick-pea seeds manifesting considerable aryl amidase activity. That enzyme can be directly applied in the debittering of protein hydrolysates, which is still ongoing problem. Protein hydrolysates have range applications in the healthcare sectors as food supplements.

Significant current interest exists in the hydrolytic release of bioactive peptide sequences, such as Ile-Pro-Pro and Val-Pro-Pro, encrypted within the primary structure of food proteins manifesting significant ACE inhibitory activity. Here, we report for the first time our finding on inhibition of these peptides in the presence of chick-pea aminopeptidase, which can be successfully used for affinity purification of proline containing peptides with ACE inhibitory activity. On the other hand a method for biospecific chromatography of aminopeptidases could be developed, using as ligands such inhibitors.

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