Fruit, Vegetable and Cereal Science and Biotechnology ©2008 Global Science Books

Purification and Properties of the Major Leucyl Aminopeptidase from *Solanum tuberosum* Tubers

Zoran Vujčić[1*] • Biljana Dojnov[2] • Aleksandra Milovanović[2] • Nataša Božić[2]

[1] Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia
[2] Centre of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia
Corresponding author: * zvujcic@chem.bg.ac.yu

ABSTRACT

Zymogram analysis revealed three isoforms of leucyl aminopeptidase (LAP) using leucine-*p*-nitroanilide as a substrate in the crude extracts of different potato tuber cultivars. The major LAP isoform, detected in all analyzed potato cultivars, was also active using methionine-*p*-nitroanilide, alanine-*p*-nitroanilide and lysine-*p*-nitroanilide as a substrates. The major LAP from potato tuber was subsequently purified and characterized. Specific LAP activity increased 160-fold by purification of the crude extract. The purified enzyme had a pH optimum of 9.0 and temperature optimum of 40°C. LAP hydrolyzed leucine-*p*-nitroanilide with a K_M of 0.048 mM and a V_{max} of 31.56 mM /min. Among a number of inhibitors tested, the most efficient was 1,10-phenanthroline, while ethylene glycol tetraacetic acid (EGTA) stimulated LAP activity. Almost all divalent cations tested, significantly inhibited the enzyme activity. LAP, purified by gel-filtration on Superose 12-FPLC column, had molecular mass of 90 kDa and isoelectric point of 5.45. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed one band of 48 kDa. LAP from potato tubers displayed some novel properties among plant LAPs.

Keywords: isoform, LAP, plant, potato tuber, zymogram
Abbreviations: **A*p*NA**, alanine-*p*-nitroanilide; **CBB**, Commassie Brilliant Blue; **DEAE**, diethylaminoethyl group; **EDTA**, ethylene diamine tetraacetic acid; **EGTA**, ethylene glycol tetraacetic acid; **FPLC**, fast protein liquid chromatography; **HEPES**, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; **IDA**, imino-diacetic acid; **K*p*NA**, lysine-*p*-nitroanilide; **LAP**, leucyl aminopeptidase; **LAP-A**, leucyl aminopeptidase-acid form; **LAP-N**, leucyl aminopeptidase-neutral form; **LMW**, low molecular weight; **L*p*NA**, leucine-*p*-nitroanilide; **M*p*NA**, methionine-*p*-nitroanilide; **SDS-PAGE**, sodium dodecylsulphate-polyacrylamide gel electrophoresis; **SEM**, standard error of the mean

INTRODUCTION

Aminopeptidases are exopeptidases that catalyse the cleavage of N-terminal amino acids from polypeptides and proteins. They have a broad specificity, are widely distributed in many cells and tissues and are involved in several important biological functions. Aminopeptidases are classified according to their substrate specificity, their location, their sensitivity to inhibitors and their metal cation cofactor requirement (Barrett *et al.* 1998). Of the various aminopeptidases leucyl aminopeptidases (LAPs) hydrolyse a wide variety of peptides and amides and have been identified in the tissues of numerous plant, microorganism and animal species.

Generally, two classes of LAPs have been reported for most plant species (Herbers *et al*. 1994). The first group comprises thermolabile aminopeptidases with molecular weight of approximately 60-90 kDa and a neutral pH optimum. They are strongly inhibited by heavy metals and SH-reagents. The second group refers to enzymes similar to plant LAPs but isolated from animals. These are large (250-330 kDa), homohexameric metallopeptidases that are inhibited by ethylene diamine tetraacetic acid (EDTA) and bestatin. They are heat-stable and possess an alkaline pH optimum. LAPs with alkaline pH optimum have been biochemically purified from a number of plants (Matsui *et al*. 2006).

The exact role of LAPs in plants is not known. These enzymes take part in some important processes, such as protein mobilization from cotyledons after germination, and protein turnover required for cell maintenance in vegetative and reproductive organs (Tu *et al*. 2003). LAPs are involved in rapid turnover of proteins required in wounding or pathogen attack (Hildmann *et al*. 1992; Herbers *et al*. 1994; Gu and Walling 2000).

Although LAPs (LAP-N and LAP-A, neutral and acidic forms of plant leucyl aminopeptidases) have been found in potato (Tu *et al*. 2003), to date relatively little information is available concerning the presence and properties of such enzymes in the tuber. Potato leaf and root tissues contain the highest level of aminopeptidase activites (Santarius and Belitz 1978). However very low levels have been observed in tubers (Santarius and Belitz 1978; Hildmann *et al*. 1992), which would indicate that purification of the enzyme from tubers is difficult. LAP encoded by a potato cDNA homologous to a LAP from bovine lens expressed in *Escherichia coli* was characterized (Herbers *et al*. 1994) and possible role in potato was analyzed.

Potato (*Solanum tuberosum*) is the world's fourth most important crop after rice, wheat and corn, and its importance is growing (Jørgensen *et al*. 2006). Potato juice (waste product from industrial starch manufacture) is a potential resource of novel proteins for biotechnological, pharmaceutical or food applications. For realization of this potential, biochemical characterization of individual proteins such as LAPs from potato tubers, is necessary.

In the present study, we purified major LAP from potato tuber and characterized its enzymological properties (catalytic properties, substrate specificity, pH and temperature optimum, thermal stability and its susceptibility to inhibition) and molecular properties (molecular mass and isoelectric point). The results show novel properties of LAP from potatoes.

Received: 29 May, 2008. Accepted: 8 September, 2008.

Original Research Paper

MATERIALS AND METHODS

Materials and reagents

One year old potatoes (*S. tuberosum*) were purchased from local markets for enzyme purification. Different (red and white) potato tuber cultivars ('Aladin', 'Agria', 'Desiree', 'Cleopatra', 'Kennebec', 'Kondor' and 'Virgo') were gifts from the Centre of Potato (Guča, Serbia). All reagents and solvents used were of the highest available purity and at least of analytical grade. They were purchased unless otherwise stated, from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Preparation of crude protein extracts

Potato tubers were homogenized in a domestic juice extractor (Jack La Lanne's power juicer) in the presence of sodium bisulfate (0.5 g/kg of tuber) to prevent browning. Starch was sedimented for 30 min at 4°C, and the supernatant was decanted and centrifuged at 10,000 × *g* for 15 min.

The concentration of proteins was determined by the Bradford assay with bovine albumin as a standard (Bradford 1976). The concentration of proteins was subsequently determined by the Bradford assay at all purification stages as well.

LAP activity assays

Enzyme activity targeting leucine-*p*-nitroanilide (L*p*NA) was determined spectrophotometrically by measuring the absorbance at 410 nm (Erlanger *et al*. 1961). Reaction mixtures contained 10 µl of potato crude extract or purified enzyme in 0.5 ml of 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer pH 8.0 and 1.0 mM L*p*NA in 2% N,N-Dimethyl-formamide. Incubations (at 30°C) lasted 15 and 30 min for crude extract and purified enzyme, respectively. All reactions were terminated by adding 0.1 ml 30% acetic acid. Enzyme activity was expressed in *U* which was defined as the amount of enzyme hydrolysing 1 µmol of *p*-nitroanilide per min at 30°C.

Native vertical PAGE

PAGE (Davis 1964) was performed in a 4% w/v stacking gel (1.5 × 16.0 × 0.15 cm) and 10% w/v running gel (5.5 × 16.0 × 0.15 cm). Samples were mixed with 3X sample buffer. Electrophoresis was performed using a Hoefer SE 620 (Hoefer, San Francisco, CA, USA) at a constant 80 V for 2 h at 4°C until the tracking dye approached the lower gel margin.

Activity staining of tuber LAP from red and white potato cultivars

Different (red and white) potato tuber cultivars ('Aladin', 'Agria', 'Desiree', 'Cleopatra', 'Kennebec', 'Kondor' and 'Virgo') were subjected to native vertical PAGE and after which LAP activity was detected by zymogram analysis (Božić and Vujčić 2005). The gel was washed twice (each 10 min duration) with distilled water and equilibrated with 50 mM Tris-HCl buffer, pH 8.5 (two 5 min duration). Thereafter, the gel was dipped into L*p*NA solution in 50 mM Tris buffer pH 8.5 and incubated at 30°C for 10 min. Diazotisation of liberated *p*-nitroaniline was performed at room temperature by immersing the gel into freshly prepared 0.1% w/v sodium nitrite solution in 1 M HCl for 2 min. Excess sodium nitrite in the gel was removed using 1% w/v urea (30 s exposure with gentle agitation). The diazotized gels were then immersed into 22% v/v ethanol containing 0.025% w/v 1-naphthylamine followed by gentle agitation until a distinct pink-coloured azo dye formed (up to 5 min).

Activity staining of LAP isoforms using different aminoacyl-p-nitroanilides

In order to detect leucyl aminopeptidase isoforms with different specificity in tuber of white potato cultivar Agria, the same detection procedure described for L*p*NA was applied for methionine-*p*-nitroanilide (M*p*NA), alanine-*p*-nitroanilide (A*p*NA) and lysine-*p*-nitroanilide (K*p*NA).

LAP purification

Crude tuber extract (700 mL) from potato cultivar Agria (white potato), (7.55 mg protein/mL) was loaded onto a Sephadex G 25 coarse column (4.6 × 60 cm, Pharmacia, Uppsala, Sweden) for pigment removal. The column was equilibrated with 10 mM Tris HCl buffer pH 7.0 in 0.9% NaCl.

Solid $(NH_4)_2SO_4$ was added to 20% saturation to 850 mL of depigmentated material and loaded onto a Phenyl Sepharose Fast Flow column (3.5 × 15 cm) equilibrated with the 10 mM Tris HCl buffer (pH 7.0) in 20% $(NH_4)_2SO_4$ buffer at a flow rate of 10.0 ml/min. The retained proteins were eluted with 10 mM Tris HCl buffer pH 7.0. Fractions (each 5 mL) were collected and immediately assayed for protein using absorbance monitoring at 280 nm and LAP activity.

Seventy mililitres of desalted fraction with LAP activity (18.6 mg protein/mL) was applied to a DEAE Sepharose Fast Flow column (XK, 2.6 × 20 cm, Pharmacia, Uppsala, Sweden) equilibrated with the 10 mM Tris HCl buffer pH 7.0. All proteins were eluted using a linear salt gradient ranging from 0 to 1M NaCl using the same buffer at a flow rate of 30 mL/h. Fractions (each 5 mL) were collected and assayed for protein and LAP activity.

Fraction (1.5 mL) containing LAP activity (4.1 mg protein/mL) was subjected to gel filtration on Superose 12 prep column (HR 16/60, Pharmacia, Uppsala, Sweden) on a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The column was previously equilibrated with 10 mM Tris HCl in 50 mM NaCl pH 7.2 at a flow rate of 0.7 mL/min. Fractions (1.0 mL) were collected and assayed for LAP activity. Several preparative native (6%) polyacrylamide gel electrophoresis (Božić *et al*. 2008) were used to separate LAP activities from the fraction obtained after Superose 12 FPLC chromatography. A total amount of 87.9 µg protein of fraction per electrophoresis was used. Electrophoresis was performed using a Hoefer SE 620 electrophoretic unit (Hoefer, San Francisco, CA, USA) at 4°C until the tracking dye reached the lower gel margin. Following electrophoresis, LAP activity was detected by in-gel activity staining. The gel was washed twice (10 min each) with distilled water and then equilibrated with 50 mM Tris-HCl buffer, pH 8.5 (two 5 min incubations). Thereafter, the gel was dipped into 2 mM L*p*NA solution in 50 mM Tris buffer pH 8.5 and incubated at 30°C for 10 min. The most intensely stained (yellow) part of the gel was excised and homogenized in two volumes of water to facilitate protein extraction. The obtained LAP preparation (25.9 µg protein/mL) was used for enzyme characterization.

The same procedure was applied for purification of LAP from the potato cultivar 'Desiree' (red potato).

Molecular mass and isoelectric point

The molecular mass (MM) of native LAP was determined by gel filtration on Superose 12 FPLC column (HR 10/30, Pharmacia, Uppsala, Sweden). The column was calibrated with aldolase (158,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da) and cytochrome *c* (13,000 Da).

The apparent molecular mass of the LAP subunits was determined by SDS-PAGE by comparison with standards. Samples were prepared with reducing sample buffer (0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mercapthoethanol and 0.002% bromophenol blue) with heating for 3 min in a boiling water bath. Electrophoresis was carried out according to Laemmli (1970) using 10% acrylamide. LMW-SDS marker kit (GE Healthcare, UK) was used as molecular mass standards. After the run gels were stained with Commassie Brilliant Blue (CBB).

Isoelectric focusing was performed using Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) according to the manufacturer's instruction. Focusing was carried out on 7.5% acrylamide gel with ampholytes in a pH range 3.0-10.0, at 7 W constant power for 1.5 h at 10°C. Broad p*I* kit (GE Healthcare, UK) was used as isoelectric point (p*I*) markers. After the run, one part of the gel was CBB stained and another processed for LAP activity with L*p*NA (Božić and Vujčić 2005).

K_M and V_{max} values

Initial reaction rates were determined using L*p*NA as a substrate in the concentration range from 1.8 to 55 µM. Incubation mixtures contained 10 µl of purified enzymes from the cultivar 'Agria' (white cultivar) and 'Desiree' (red cultivar) in 0.5 ml of 50 mM HEPES buffer pH 8.0 and approriate substrate concentration. Substrate hydrolysis was monitored at 410 nm at 30 s intervals for 30 min at 30°C. The data were processed using non-linear regression analysis by the GraphPad Prism 5.01 program (Božić *et al*. 2003).

pH optimum studies

To determine the pH optimum of LAP activity against L*p*NA, 10 µl of the purified enzyme and a series of 50 mM buffers in the pH range from 4.0 to 12.0 were used [acetate, pH 3.8-6.3; Tris HCl, pH 6.5-9.0; phosphate, pH 9.0-11.0 (made from $Na_2HPO_4 \times 2H_2O$)]. Controls, contained only substrate, indicated that the buffers did not induce substrate hydrolysis.

Temperature optimum and thermal stability studies

To determine the temperature optimum of LAP activity against L*p*NA, 10 µl of the purified enzyme were assayed in 50 mM HEPES buffer, pH 8.0 at 2°C and in the temperature range of 10 to 60°C at 10°C intervals. Controls, contained only substrate, indicated that the temperature did not induce substrate hydrolysis.

Thermal stability was ascertained at 30, 40, 50, 60 and 70°C. Aliquots of 10 µl of the purified enzyme were mixed with 50 mM HEPES buffer, pH 8.0 and incubated for 15 min at each temperature. Enzyme activity was then monitored at 30°C, as described.

Inhibitor studies

The effects of various agents [1,10-phenanthroline, EDTA, ethylene glycol tetraacetic acid (EGTA), cysteine, citrate, imidazole and imino-diacetic acid (IDA)] on LAP activity were studied. LAP was preincubated with individual inhibitors (final inhibitor concentrations were 2 mM) for 15 min at 30°C prior to the addition of L*p*NA (final substrate concentrations were 1 mM). All reactions were terminated after 30 min by adding 0.1 ml acetic acid (30%). Substrate hydrolysis was monitored at 410 nm.

Effect of divalent metal cations

$ZnSO_4$, $MgSO_4$, $MnCl_2$, $Cd(CH_3COO)_2$, $HgCl_2$, $CuCl_2$ and $CoCl_2$ were used as the sources of divalent metal cations. LAP was preincubated with different salts (final salt concentrations were 2 mM) for 15 min at 30°C prior to the addition of L*p*NA (final substrate concentrations were 1 mM). All reactions were terminated after 30 min by adding 0.1 ml acetic acid (30%). Substrate hydrolysis was monitored at 410 nm.

Statistical analysis

Each data point concerning activity measurements represents the mean of three independent assays. The data in **Tables 2-3** and in **Figs. 5–7** are presented as the mean ± standard error of the mean (SEM). The data on these figures are presented as percentages, taking the control value as 100%.

RESULTS AND DISCUSSION

Activity staining of tuber LAP from red and white potato cultivars

LAP activity from the tubers of seven white and red potato cultivars ('Desiree', 'Virgo', 'Cleopatra', 'Agria', 'Kondor', 'Kennebec' and 'Aladin') was examined using zymogram analysis after native PAGE employing L*p*NA as a substrate (**Fig. 1**). In total, three isoforms were detected. However, the three isoforms were not detected in all the cultivars tested. Two isoforms were detected in the samples from the red cultivar 'Desiree' (low and middle mobility) (**Fig. 1**, lane 1) and the white cultivar 'Agria' (high and middle mobility) (**Fig. 1**, lane 4). Other cultivars had one isoform (middle mobility). Since this isoform was detected in all cultivars it was termed as major and was chosen for purification and characterization.

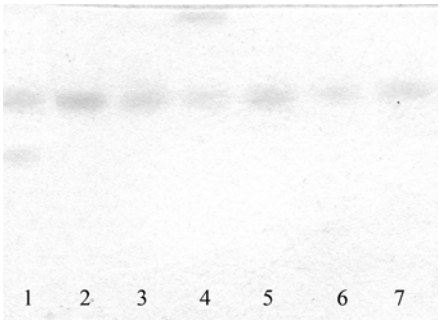

Fig. 1 Zymogram analysis of LAP isoforms from different potato cultivars (after native PAGE). Lane 1: 'Desiree'. Lane 2: 'Virgo'. Lane 3: 'Cleopatra'. Lane 4: 'Agria'. Lane 5: 'Kondor'. Lane 6: 'Kennebec'. Lane 7: 'Aladin'.

Fig. 2 Zymogram analysis of LAP isoforms from white potato cultivar Agria (after native PAGE) employing four different substrates. Lane 1: alanine-*p*-nitroanilide. Lane 2: lysine-*p*-nitroanilide. Lane 3: leucine-*p*-nitroanilide. Lane 4: methionine-*p*-nitroanilide.

Isoforms may arise via post-translational modifications (for example; differential glycosylation) or via the existence of multigene families. Multiple isoforms can provide increased capability for organism to respond on different physiological conditions or environmental demands (Wagner *et al*. 2002; Božić *et al*. 2008).

Activity staining of LAP isoforms using different A*p*NAs

LAP substrate specificity from the tuber of the white potato cultivar Agria was examined using zymogram analysis after native PAGE employing A*p*NA, K*p*NA, L*p*NA and M*p*NA as substrates (**Fig. 2**). The visualized bands were the result of identical reaction conditions for all four substrates. Analysis of bands demonstrates the specificity in substrate choice between the isoforms. Potato tuber leucyl aminopeptidase was not strictly specific to L*p*NA hydrolysis, although both isoforms against this substrate were visible in the zymogram resolved by native PAGE (**Fig. 2**, lane 3). M*p*NA was also an efficient substrate, giving two less intense isoforms of leucyl aminopeptidase in comparison to L*p*NA (**Fig. 2**, lane 4). A*p*NA was most efficient substrate for major LAP activity (higher mobility isoform) (**Fig. 2**, lane 1). Although K*p*NA was not expected to be an efficient substrate for leucyl aminopeptidases because these enzymes have a higher affinity for nonpolar amino acids, a single band of enzyme activity corresponding to major LAP activity was obtained (**Fig. 2**, lane 2).

Purification of the major LAP

The isolation and purification of the major LAP from two potato cultivars, white and red, was monitored by the ability of LAP to hydrolyse the substrate L*p*NA. The purification procedure consisted of several chromatography steps and one preparative electrophoresis. The results of the purification of white potato cultivar 'Agria' are summarized in

Table 1 Purification of major potato tuber leucyl aminopeptidase activity.

Purification stage	Total protein (mg)	Total activity (*U*)	Specific activity (*U*/mg)	Purification (-fold)	Yield (%)
Crude extract	5285.0	35.18	6.7×10^{-3}	1	100
Phenyl Sepharose FF	1302.0	13.31	10.2×10^{-3}	1.5	37.8
DEAE Sepharose FF	82.0	2.90	35.4×10^{-3}	5.3	8.2
FPLC Superose 12	2.4	0.98	408.3×10^{-3}	60.9	2.8
Native PAGE eluate	0.7	0.75	1071.4×10^{-3}	159.9	2.1

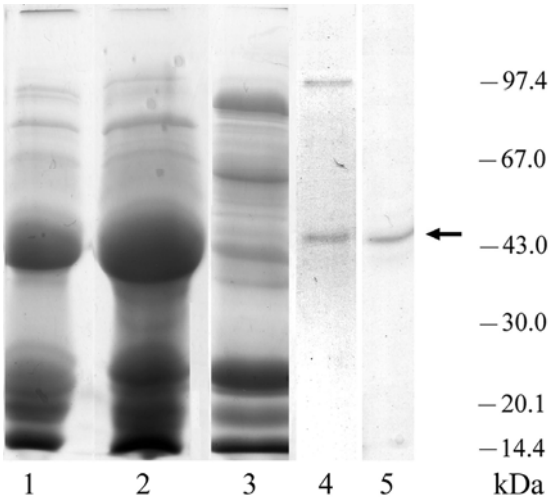

Fig. 3 Purification of major potato tuber LAP (SDS-PAGE profiles). Lane 1: crude potato tuber extract. Lane 2: proteins after Phenyl Sepharose FF. Lane 3: proteins after DEAE Sepharose FF. Lane 4: proteins after FPLC Supharose 12. Lane 5: purified LAP obtained after native PAGE gel extraction. Lane kDa: positions of standard proteins molecular masses. LMW-SDS marker kit (GE Healthcare) was used as molecular mass standards. The arrow indicates positions of the band referred to LAP.

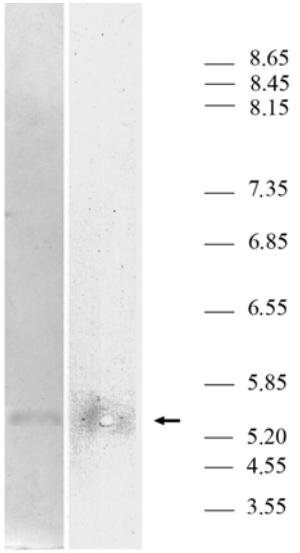

Fig. 4 Isoelectric focusing of major potato tuber LAP. Lane 1: Commassie Brilliant Blue staining. Lane 2: In-gel activity staining. Lane pI: positions of standard proteins p*I* values. Broad p*I* kit (GE Healthcare) was used as p*I* markers. The arrow indicates positions of the band referred to LAP.

Table 1 and **Fig. 3**. The result of purification of red potato cultivar 'Desiree' was not shown since it was practically the same as for white cultivar. LAP was purified 160-fold with a yield of 2.1% and was homogenous according to SDS-PAGE (**Fig. 3**, lane 5).

Characteristics of the LAP

The molecular mass of the enzymes from both potato cultivars was calculated from the plot of *log* molecular mass *versus Rm* using standard proteins as markers after SDS-PAGE. The apparent molecular mass of both enzymes was calculated to be 48 kDa (**Fig. 3**, lane 5). Purified LAPs re-chromatographed on the Superose 12-FPLC column yielded a single peak of activity corresponding to 90 kDa (results not shown). Aminopeptidases are represented by an extraordinarily broad spectrum of enzymes. They exhibit molecular weights ranging from 53–140 kDa per subunits and exist as monomers, hexamers and octamers (Taylor 1993). In terms of molecular masses, according to SDS-PAGE, the major potato tuber LAP bears resemblance to LAP-N (Tu *et al*. 2003), LAP-A from tomato (Gu and Walling 2000), and LAP expressed in *E. coli* (Herbers *et al*. 1994).

The p*I* for major LAP from both potato cultivars was determined after isoelectric focusing from the plot of pH gradient *versus* mobility of standard proteins in relation to anode. The p*I* for both major LAP was calculated to be 5.45 (**Fig. 4**). A single band in IEF confirmed the homogeneity of the major LAP. Acidic p*I* was also found for kidney bean LAP (Mikkonen 1992) and for tomato LAP-A (Matsui *et al*. 2006).

LAP reaction kinetics using L*p*NA was according to Michaelis-Menten principles. The values obtained for the white potato cultivar LAP were V_{max}, 31.56 mM/min and K_M, 48 μM, while V_{max}/K_M ratio were the same for both LAPs purified.

Optimum LAP activity using L*p*NA as a substrate was observed between pH 8.0 and 9.5. Maximum LAP activity

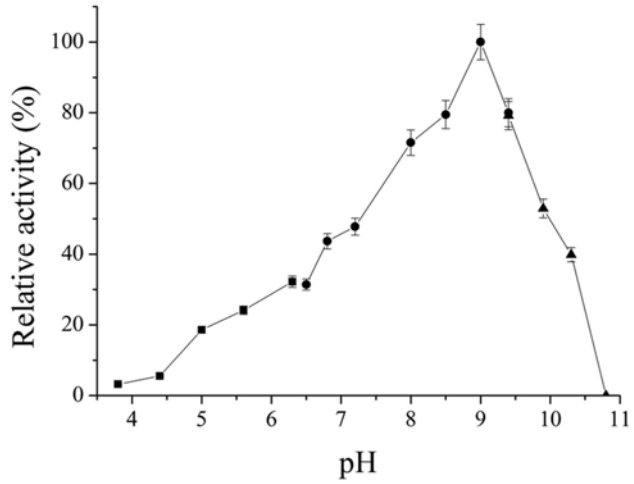

Fig. 5 The effect of pH on the activity of potato tuber LAP. Buffers used: ■ – acetate; ● – Tris HCl and ▲– phosphate. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

was observed at pH 9.0±0.4 (**Fig. 5**). No enzymatic activity was detected in buffers with a pH below pH 4.5 or above 11.0. This alkaline pH optimum was similar to the pH optima of LAPs isolated from the different plant species (Mikkonen 1992; Herbers *et al*. 1994; Ogiwara *et al*. 2005).

The temperature optimum of LAP was determined from 2 to 60°C, monitoring the hydrolysis of L*p*NA. LAP had distinct maximal activity at 40°C (**Fig. 6**). In relation to the maximum, the enzyme was 45±2% active at 30°C. The thermal stability of LAP was determined at 30, 40, 50, 60 and 70°C. Heat inactivation of the enzyme started at 50°C (**Fig. 7**). LAP retained 20±1% of its activity after heating on 60°C and subsequent renaturation. The temperature, which inactivated to half the original rate, was 55°C. The temperature optimum of LAP from *S. tuberosum* expressed in *E. coli* (65°C) (Herbers *et al*. 1994) and LAPs from other species [70°C for *Arabidopsis thaliana* (Bartling and Weiler 1992) and 60°C for tomatoes (Gu *et al*. 1999)] were higher compare to potato tuber LAP (40°C).

We found that the most efficient inhibitor of LAP acti-

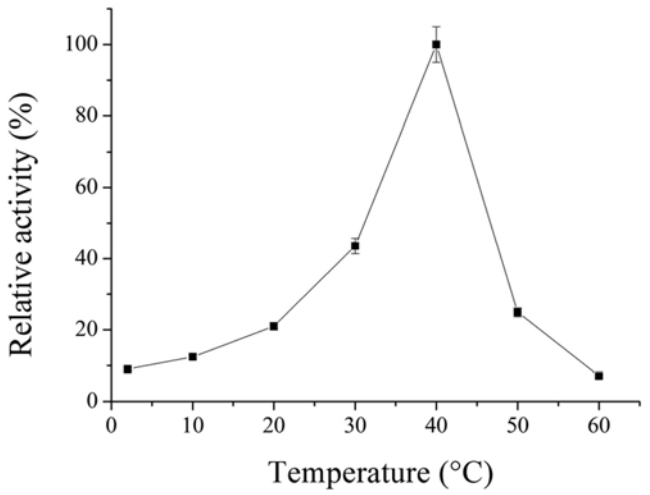

Fig. 6 The influence of temperature on the activity of potato tuber LAP. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

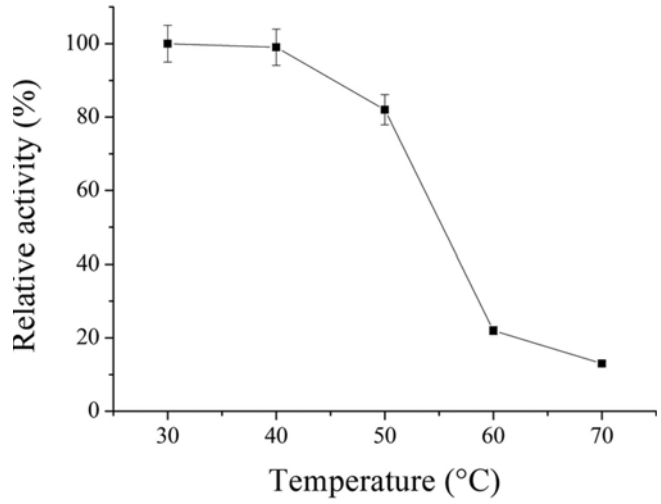

Fig. 7 Thermal stability of potato tuber LAP. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

Table 2 The effect of different compounds on potato tuber leucyl aminopeptidase activity. L*p*NA was used a substrate. Values represent the mean ± standard error value.

Compound	Final concentration	Residual activity (%)
EGTA	2	107.5 ± 0.1
Imidazole	2	100.0 ± 0.1
EDTA	2	100.0 ± 0.1
Citrate	2	96.8 ± 1.1
IDA	2	95.9 ± 0.5
Cysteine	2	80.4 ± 1.8
1,10-phenanthroline	2	26.7 ± 0.5

vity was 1,10-phenanthroline (**Table 2**), which inhibited enzyme activity by over 70%. Cysteine inhibited LAP activity by 20%. However, EGTA was a weak activator, activating LAP activity by 7%. Unlike LAP expressed in *E. coli* (Herbers *et al.* 1994), but common with some of the LAPs isolated from plants, the purified major potato tuber LAP was insensitive to inhibition by EDTA (Kolehmainen and Mikola 1971; Ogiwara *et al*. 2005). It was also insensitive to imidazole and the metal chelators citrate and IDA. However, EGTA activated LAP, similarly like it activated one insect cytosol LAP (Božić *et al*. 2008). Evidence that the major LAP is a metallopeptidase was provided by inhibition of its activity after incubation with the chelating agent, 1,10-phenanthroline. 1,10-phenanthroline (2 mM) was also a strong inhibitor of *A. thaliana* LAP activity (Bartling and Weiler 1992) and of barley leaves LAP activity (Desimone *et al*. 2000).

Table 3 The effect of metal divalent cations on potato tuber leucyl aminopeptidase activity. L*p*NA was used a substrate. Values represent the mean ± standard error value.

Compound	Final concentration	Residual activity (%)
$MgCl_2\,6H_2O$	2	70.4 ± 1.8
$CoCl_2\,6\,H_2O$	2	24.4 ± 0.4
$Cd(OAc)_2\,2H_2O$	2	20.8 ± 0.3
$MnCl_2\,4H_2O$	2	20.1 ± 0.3
$ZnSO_4\,7H_2O$	2	17.0 ± 0.3
$CuSO_4\,5H_2O$	2	14.0 ± 0.0
$HgCl_2$	2	5.2 ± 0.1

The presence of cations (Co^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}) was inhibitory to LAP activity (**Table 3**). LAP activity was almost completely inhibited by 2 mM Hg^{2+}. Other cations, with exception of Mg^{2+}, were also strong inhibitors of LAP activity. High concentrations of Zn^{2+} (in the mM range) often inhibit metallopeptidases due to the formation of zinc monohydroxide that bridges the catalytic Zn^{2+} ion to a side chain in the active site of the enzyme (Salvesen and Nagase 1994).

While many mammalian and microbial LAPs have evolved ancillary functions that supplement their role as peptidases, there is evidence for plant LAPs exhibiting secondary activities (Matsui *et al*. 2006), such as their role in defense. The surprising diversity of plant LAP functions and their impacts on cellular and physiological activities makes these enzymes particularly interesting for further investigations.

ACKNOWLEDGEMENTS

This work was supported by the Serbian Ministry of Science and Technological Development (project grant number 142026B).

REFERENCES

Barrett AJ, Rawlings ND, Woessner JF (1998) Metallopeptidases. In: Barrett AJ, Rawlings ND, Woessner JF (Eds) *Handbook of Proteolytic Enzymes*, Academic Press, New York, pp 987-1551

Bartling D, Weiler WE (1992) Leucine aminopeptidase from *Arabidopsis thaliana* Molecular evidence for a phylogenetically conserved enzyme of protein turnover in higher plants. *European Journal of Biochemistry* **205**, 425-431

Božić N, Ivanović J, Nenadović V, Bergström J, Larsson T, Vujčić Z (2008) Purification and properties of major midgut leucyl aminopeptidase of *Morimus funereus* (Coleoptera, Cerambycidae) larvae. *Comparative Biochemistry and Physiology* **B 149**, 454-462

Božić N, Vujčić Z (2005) Detection and quantification of leucyl aminopeptidase after native electrophoresis using leucine-*p*-nitroanilide. *Electrophoresis* **26**, 2476-2480

Božić N, Vujčić Z, Nenadović V, Ivanović J (2003) Partial purification and characterisation of midgut leucyl aminopeptidase of *Morimus funereus* (Coleoptera, Cerambycidae) larvae. *Comparative Biochemistry and Physiology* **B 134**, 231-241

Bradford MM (1976) A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principe of protein – dye binding. *Analytical Biochemistry* **72**, 248-254

Davis JB (1964) Disc electrophoresis-II: method and applications to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404-427

Desimone M, Krüger M, Wessel T, Wehofsky M, Hoffmann R, Wagner E (2000) Purification and characterisation of an aminopeptidase from the chloroplast stroma of barley leaves by chromatographic and electrophoretic methods. *Journal of Chromatography B* **737**, 285-293

Erlanger FB, Kokowsky N, Cohen W (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics* **95**, 271-278

Gu YQ, Holzer MF, Walling LL (1999) Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato. *European Journal of Biochemistry* **263**, 726-735

Gu YQ, Walling LL (2000) Specificity of the wound-induced leucine aminopeptidase (LAP-A) of tomato activity on dipeptide and tripeptide substrates. *European Journal of Biochemistry* **267**, 1178-1187

Herbers K, Prat S, Willmitzer L (1994) Functional analysis of a leucine aminopeptidase from *Solanum tuberosum* L. *Planta* **194**, 230-240

Hildmann T, Ebneth M, Peña-Cortés H, Sánchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation

as a result of mechanical wounding. *The Plant Cell* **4**, 1157-1170

Jørgensen M, Bauw G, Welinder GK (2006) Molecular properties and activities of tuber proteins from starch potato cv. Kuras. *Journal of Agricultural and Food Chemistry* **54**, 9389-9397

Kolehmainen L, Mikola J (1971) Partial purification and enzymatic properties of an aminopeptidase from barley. *Archives of Biochemistry and Biophysics* **145**, 633-642

Laemmli KU (1970) Cleavage of structural proteins during the assembly of the head of bacteriofage T4. *Nature* **227**, 680-685

Matsui M, Fowler HJ, Walling LL (2006) Leucine aminopeptidase: diversity in structure and function. *Biological Chemistry* **387**, 1535-1544

Mikkonen A (1992) Purification and characterization of leucine aminopeptidase from kidney bean cotyledons. *Physiologia Plantarum* **84**, 393-398

Ogiwara N, Amano T, Satoh M, Shioi Y (2005) Leucine aminopeptidase from etiolated barley seedlings: Characterization and partial purification of isoforms. *Plant Science* **168**, 575-581

Salvesen G, Nagase H (1994) Inhibition of proteolytic enzymes. In: Beynon RJ, Bond JS (Eds) *Proteolytic Enzymes: A Practical Approach*, IRL Press, Oxford, pp 83-104

Santarius K, Belitz HD (1978) Proteinase activity in potato plants. *Planta* **141**, 145-153

Taylor A (1993) Aminopeptidases: structure and function. *The Federation of American Societies for Experimental Biology Journal* **7**, 290-298

Tu CJ, Park SY, Walling LL (2003) Isolation and characterization of the neutral leucine aminopeptidase (*LapN*) of tomato. *Plant Physiology* **132**, 243-255

Wagner W, Möhrlen F, Schnetter W (2002) Characterization of the proteolytic enzymes in the midgut of the European cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae). *Insect Biochemistry and Molecular Biology* **32**, 803-814