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Characterisation of leucyl aminopeptidase from *Solanum tuberosum* tuber

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ABSTRACT

Potato juice (a waste product from the starch industry) is a potential source of novel enzymes for food applications. For use in the production and improvement of food protein hydrolysates, commercially available exopeptidases, predominantly aminopeptidases, are recommended. The present study was performed to explore possible biotechnological interest of leucyl aminopeptidase (LAP) activity in the potato tuber. The LAP from potato tuber was purified and characterised. Specific LAP activity was increased 200-fold by purification of the crude extract. The purified enzyme had a pH optimum of 9.0 and temperature optimum of 45 °C. LAP hydrolysed leucine-, alanine- and lysine-*p*-nitroanilide to a similar degree. The most efficient inhibitor was 1,10-phenanthroline. Almost all divalent cations tested inhibited the enzyme activity, while Co^{2+} stimulated LAP activity by over 100%. The purified LAP had a molecular weight of 90 kDa with an isoelectric point of 5.45. Sodium dodecylsulfate–polyacrylamide gel electrophoresis revealed one band of 48 kDa.

1. Introduction

Potato (*Solanum tuberosum*) is the world's fourth most important crop after rice, wheat and corn, and its importance is growing (Jørgensen, Bauw, & Welinder, 2006). Potato juice (a waste product from industrial starch manufacture, which represents an environmental problem) is a potential resource of novel proteins, especially enzymes, for biotechnological, pharmaceutical or food applications.

Aminopeptidases are exopeptidases that catalyse the cleavage of *N*-terminal amino acids from polypeptides and proteins. They are classified according to their substrate specificity, their location, their sensitivity to inhibitors and their metal cation cofactor requirement (Barrett, Rawlings, & Woessner, 1998). Of the various aminopeptidases leucyl aminopeptidases (LAPs) hydrolyse a wide variety of peptides and amides and have been identified in the tissue of numerous plant, microorganism and animal species.

Practically, the enzyme has been shown to have many food applications. Since the formation of carcinogens is the most serious problem in the production of protein acid hydrolyzates, the enzymatic production of amino acid mixtures from proteins using peptidases has been paid considerable attention in the food protein processing industry (Deejing, Yoshimune, Lumyong, & Moriguchi, 2005; Tang, Wang, & Yang, 2009). Recently, it has been shown that enzymes have high potential for hydrolysis of chicken breast meat (Chi, Lyu, Lin, & Huang, 2008) and for the proteolysis of enzyme-modified Cheddar cheese (Azarnia, Lee, Yaylayan, & Kilcawley, 2010). The use of animopeptidases in these industrial processes not only contributes to flavour of the final product but also to the improvement of nutritional value (Sanz, 2007).

Although many industrial peptidases have been developed, commercial aminopeptidases are limited in number (Deejing et al., 2005). For realisation of this potential, purification and biochemical characterisation of aminopeptidase from various sources is necessary. Accordingly, the kinetics and mechanisms of peptidase should be studied to improve their applications, as controlled peptidolysis is essential for stable qualities in industrial and biotechnological products (Nielsen, 2002). Attempts to use other enzymes from plant sources in food processing have also been made (Fahmy et al., 2008).

We have recently purified and partially characterised LAP from two cultivars (Agria and Desiree) of*S. tuberosum* tuber (Vujčić, Dojnov, Milovanović, & Božić, 2008), where it was shown that there were no differences in enzyme properties between those two varieties. The purified enzyme from both varieties had a molecular mass of 90 kDa, i.e., 48 kDa after sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and an isoelectric point of 5.45. The most efficient inhibitor was 1,10-phenanthroline. Results showed novel properties of LAP from plants.

The present study was performed to extend our previous work on LAP activity in the potato tuber. We purified LAP from cultivar

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Kennebec and characterised in detail its enzymological properties (catalytic properties, substrate specificity, pH and temperature optima, thermal stability and its susceptibility to inhibition) and molecular properties (molecular mass and isoelectric point). Possible biotechnological interest of this enzyme was also discussed.

2. Materials and methods

2.1. Materials and reagents

One year old potatoes (*S. tuberosum*), cultivar Kennebec, were purchased from local markets. 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).

2.2. Preparation of crude protein extracts

Potato tubers were minced using 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 determined by the Bradford assay at all purification stages as well.

2.3. LAP activity assays

Enzyme activity targeting leucine-*p*-nitroanilide (L*p*NA) was determined spectrophotometrically by measuring the absorbance at 410 nm (Erlanger, Kokowsky, & Cohen, 1961). Reaction mixture contained 50 µl of crude midgut extract or purified enzyme in 0.5 ml of 50 mM Tris buffer (pH 9.0) and 1.0 mM L*p*NA in 2% *N*,*N*-dimethylformamide. Incubations (at 37 °C) lasted 10 min. All reactions were terminated by adding 0.1 ml 30% acetic acid; absorbance was monitored using a Philips UV–Vis–NIR PU 8630 spectrophotometer. 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. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the mean).

2.4. LAP purification

LAP was purified as previously described (Vujčić et al., 2008) with slight modifications. Crude tuber extract from potato cultivar Kennebec, (500 ml; 14.4 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 5 mM Tris–HCl buffer (pH 6.8) in 0.9% NaCl.

Depigmentated material (650 ml) and desalted fraction with LAP activity (10.6 mg protein/ml) was applied to a DEAE Sepharose Fast Flow column (XK, 5.0 × 26 cm, Pharmacia) equilibrated with the 10 mM Tris–HCl buffer (pH 6.8). All proteins were eluted using a linear salt gradient, ranging from 0 to 1 M NaCl, using the same buffer at a flow rate of 150 ml/h. Fractions (each 10 ml) were collected and assayed for protein and LAP activity.

One hundred millilitres of a fraction containing LAP activity were lyophilised and 2 × 2.0 ml (29.5 mg protein/ml) were subjected to gel filtration on Superose 12 prep. column (HR 16/60, Pharmacia) on a fast protein liquid chromatography (FPLC) system (Pharmacia). The column was previously equilibrated with 10 mM Tris–HCl in 50 mM NaCl (pH 6.8) at a flow rate of 0.7 ml/min. One-millilitre fractions were collected and assayed for LAP activity. The obtained LAP preparation was used for enzyme characterisation.

2.5. Molecular mass and isoelectric point

The molecular weight (MW) of native LAP was determined by gel filtration on a Superose 12 FPLC column (HR 10/30, Pharmacia). 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 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% beta-mercaptoethanol 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. Electrophoresis was performed using a Hoefer SE 620 electrophoretic unit (Hoefer, San Francisco, CA) at 4 °C until the tracking dye reached the lower gel margin. Low molecular weight markers (LMW-SDS marker kit, GE Healthcare) were used as molecular mass standards. After the run gels were stained with Coomassie Brilliant Blue (CBB).

Isoelectric focusing was performed using Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology) 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) 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 staining with L*p*NA (Božić & Vujčić, 2005). In brief, the gel was washed twice (each 10 min duration) with distilled water and equilibrated with 50 mM Tris–HCl buffer (pH 8.5) twice (each 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 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 ethanol containing 1-naphthylamine, followed by gentle agitation, until a distinct pink-coloured azo dye formed (up to 5 min).

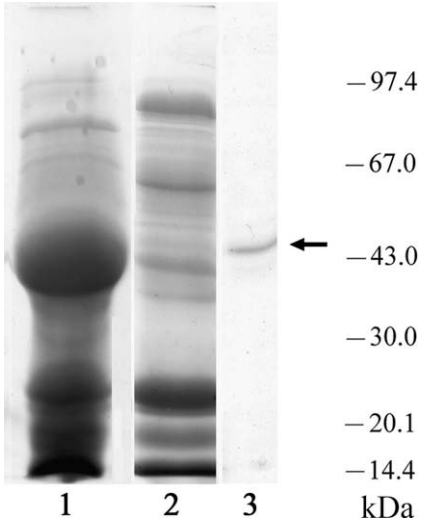

Fig. 1. Purification of potato tuber LAP (SDS–PAGE profiles). Lane 1: crude potato tuber extract. Lane 2: proteins after DEAE Sepharose FF. Lane 3: proteins after FPLC Superose 12. Lane kDa: positions of standard proteins molecular masses. Arrow indicates position of the band referred to LAP.

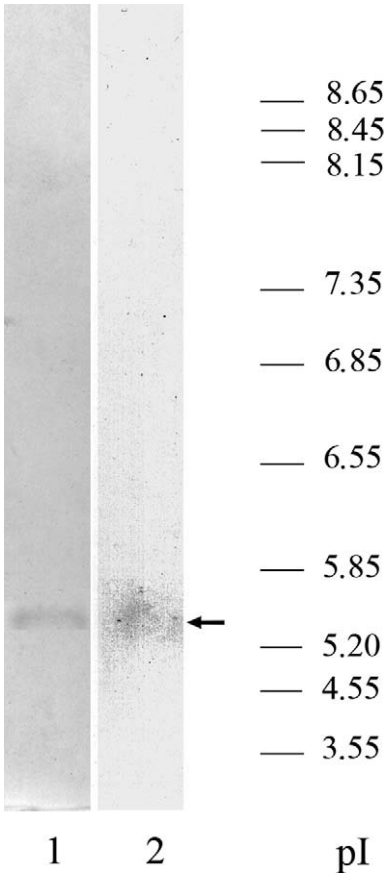

Fig. 2. Isoelectric focusing of potato tuber LAP. Lane 1: Commassie Brilliant Blue staining. Lane 2: In-gel activity staining. Lane pI: positions of standard proteins p*I* values. Arrow indicates position of the band referred to LAP.

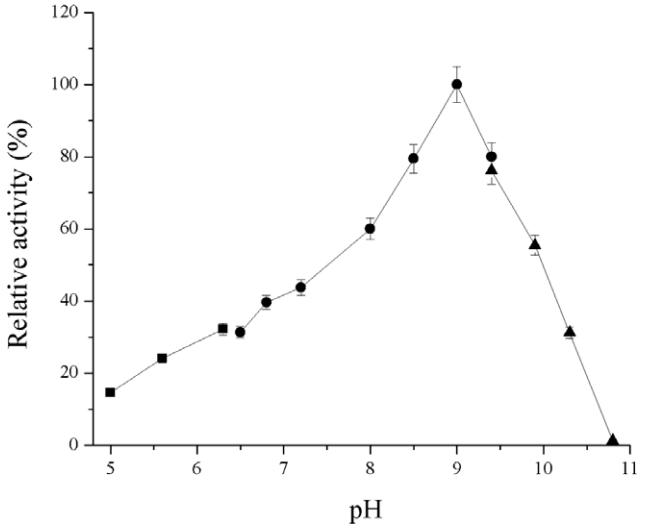

Fig. 3. The effect of pH on the activity of potato tuber LAP. Buffers used: ■ – acetate; ● – Tris–HCl and ▲ – phosphate. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

2.6. K_M and V_{max} values

Initial reaction rates were determined using *p*-nitroanilide substrates, L*p*NA, methionine-*p*-nitroanilide (M*p*NA), alanine-*p*-nitroanilide (A*p*NA) and lysine-*p*-nitroanilide (K*p*NA), in the concentration range from 1.1 μM to 1.1 mM. Incubation mixtures contained 10 μl of purified enzymes in 0.2 ml of 50 mM Tris–HCl buffer pH 9.0 and appropriate substrate concentration. Substrate hydrolysis was monitored at 405 nm at 30 s intervals for 30 min at 30 °C by using an LKB 5060-006 micro plate reader. The data were processed using non-linear regression analysis by the GraphPad Prism 5.02 program (Božić, Vujčić, Nenadović, & Ivanović, 2003).

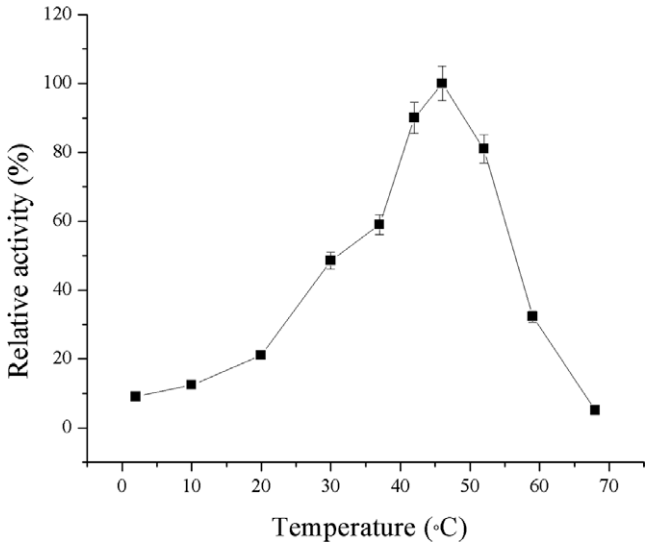

Fig. 4. The influence of temperature on the activity of potato tuber LAP. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

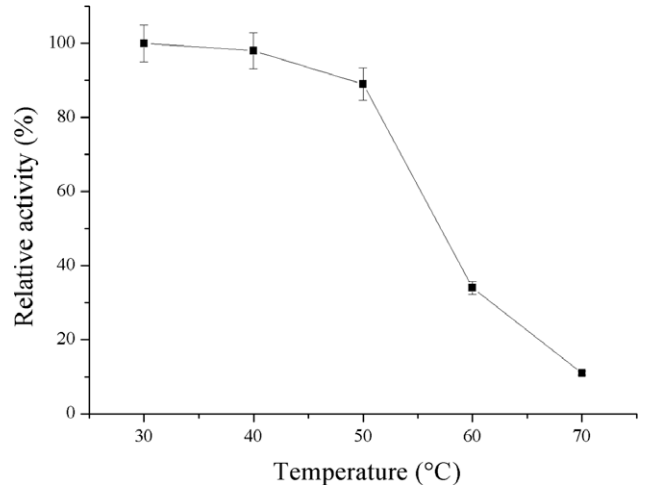

Fig. 5. Thermal stability of potato tuber LAP. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

2.7. 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; sodium phosphate, pH 9.0–11.0). Controls containing only substrate indicated that the buffers did not induce substrate hydrolysis.

2.8. 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 Tris–HCl buffer (pH 9.0) in the temperature range of 2–70 °C. 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. Ten microlitres of the purified enzyme were mixed with 50 mM Tris–HCl buffer (pH 9.0) and incubated for 15 min at each temperature. After that enzyme activity was monitored at 37 °C, as described.

2.9. Inhibitor studies

The effects of various agents (1,10-phenanthroline, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), cysteine, citrate and iminodiacetic acid (IDA)) on LAP activity were studied. LAP was preincubated with different inhibitors (final inhibitor concentrations were 2 mM) for 15 min at 37 °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 30% acetic acid. After that enzyme activity was monitored at 37 °C, as described.

2.10. 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 0.2 mM and 2 mM) for 15 min at 37 °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 30% acetic acid. After that enzyme activity was monitored at 37 °C, as described.

2.11. Statistical analysis

Each data point represents the mean of three independent assays. Statistical significance was determined by standard deviation (SD). The data in Figs. 3–5 are presented as the mean ± standard error of the mean (SEM). The data in the tables are presented as percentages, taking the control value as 100%.

3. Results and discussion

3.1. Purification of the major LAP

The isolation and purification of the major LAP from potato cultivar Kennebec was monitored by ability of LAP to hydrolyse the substrate L*p*NA. The purification procedure consisted of several chromatography steps and the result of the purification is summarised in Table 1 and Fig. 1. LAP was purified 200-fold with a yield of 1.2% and was homogenous, according to SDS–PAGE (Fig. 1, lane 3).

3.2. Characteristics of the LAP

The molecular weight of the enzyme from potato tuber was calculated from the plot of log (molecular weight) *versus Rm*, using standard proteins as markers after SDS–PAGE. The apparent molecular weight of the enzyme was calculated to be 48 kDa (Fig. 1, lane 3). Purified LAPs rechromatographed on the Superose 12-FPLC column yielded a single peak of activity corresponding to 90 kDa (results not shown). The p*I*, as determined by isoelectric focusing, was 5.45 (Fig. 2). A Single band in IEF confirmed the homogeneity of the major LAP.

Aminopeptidases are represented by an extraordinarily broad spectrum of enzymes and exhibit molecular weights ranging from 53–140 kDa per subunit and exist as monomers, hexamers and octamers (Taylor, 1993). In terms of molecular weights, according to SDS–PAGE, potato tuber LAP bears resemblance to LAP-N (Tu, Park, & Walling, 2003) and LAP-A from tomato (Gu & Walling, 2000), but mostly to *Bacillus stearothermophilus* LAP, having MW of 44.5 kDa after SDS–PAGE and a MW of 83–98 kDa after Sephacryl S-300 column chromatography (Kuo, Hwang, Lai, Yang, & Lin, 2003). On the basis of the experiment described here, potato tuber LAP is a dimeric enzyme. According to molecular weight and isoelectric point, LAP from potato cultivar Kennebec is the same enzyme form as the major LAPs found in cultivars Agria and Desiree (Vujčić et al., 2008).

LAP reaction kinetics using four different *p*-nitroanilide substrates (L*p*NA, M*p*NA, A*p*NA and K*p*NA) was according to Michaelis–Menten principles. The lowest K_M value, 9.25 μM, was observed with L*p*NA (Table 2). The highest V_{max} value of 464 μmol/min per ml of enzyme was for A*p*NA. The V_{max}/K_M ratio was the highest (1.503) for L*p*NA. Our data regarding the substrate specificity of the potato tuber LAP showed that the enzyme expressed a higher preference for non-polar amino acids leucine and alanine than methionine, while preference for basic amino acids lysine was almost comparable to those observed for leucine and alanine.

Optimum LAP activity using L*p*NA as a substrate was observed between pH 8.5 and 9.5. Maximum LAP activity was observed at pH 9.0 (Fig. 3). No enzymatic activity was detected in buffers with a pH below pH 4.5 or above 11.0. Maximum LAP activity was observed at pH 9.0. This alkaline pH optimum was similar to the pH optima of LAPs isolated from other plant (Herbers, Prat, & Willmitzer, 1994; Mikkonen, 1992) and bacterial species (Izawa, Ishikawa, Tanokura, Ohta, & Hayashi, 1997; Raksakulthai & Haard, 2003).

The temperature optimum of LAP was determined from 2 to 70 °C, monitoring the hydrolysis of L*p*NA. LAP had maximal activity at 45 °C (Fig. 4). In relation to maximum, the enzyme was 49% active at 30 °C and 32% at 60 °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 & Weiler, 1992) and 60 °C for tomatoes (Gu, Holzer, & Walling, 1999) were much higher, compared to potato tuber LAP (45 °C), but it was the same as the temperature optimum found for LAP from some microorganisms, like *Pseudomonas fluorescens* (Gobbetti, Corsetti, & Fox, 1995) and *Streptomyces lividans* (Aphale

Table 1
Purification of potato tuber leucyl aminopeptidase activity.

Purification stage	Total protein (mg)	Total activity (*U*)	Specific activity (*U*/mg)	Purification (-fold)	Yield (%)
Crude extract	6360	30.6	4.8×10^{-3}	1	100
DEAE Sepharose FF	118	2.90	24.6×10^{-3}	5.1	9.5
FPLC Superose 12	0.38	0.36	947.4×10^{-3}	197.4	1.2

Table 2
Kinetic parameters of potato tuber LAP. Each K_M and V_{max} value represents the mean ± standard error.

Substrate	K_M (μM)	V_{max} (μmol/min/ml)	V_{max}/K_m ratio
L*p*NA	9.25 ± 0.19	13.9 ± 0.15	1.503
A*p*NA	360 ± 28.1	464 ± 24.5	1.291
K*p*NA	98.8 ± 6.31	96.6 ± 7.23	0.978
M*p*NA	83.3 ± 5.11	0.75 ± 0.05	0.009

Table 3
The effect of different compounds on potato tuber leucyl aminopeptidase activity. L*p*NA was used a substrate. Values represents the mean ± standard error.

Compound	Final concentration (mM)	Residual activity (%)
EGTA	2	98.1 ± 0.1
EDTA	2	95.2 ± 0.1
Citrate	2	96.2 ± 0.3
IDA	2	92.3 ± 0.5
Cysteine	2	80.4 ± 1.2
1,10-Phenanthroline	2	25.2 ± 0.5

& Strohl, 1993). The thermal stability of LAP was determined at 30, 40, 50, 60 and 70 °C. Heat inactivation of enzyme started at 50 °C (Fig. 5). LAP retained 35% of activity after heating at 60 °C and subsequent renaturation. The temperature, at which LAP was inactivated to half its original rate, was 57 °C. Commercially available exopeptidases are predominantly aminopeptidases and normally recommended for use in the production and improvement of food protein hydrolysates. According to their pH (4–10) and temperature optima (30–70 °C) food grade commercially available aminopeptidases are a broad spectrum of different enzymes depending on manufacturer (Raksakulthai & Haard, 2003).

We found that the most efficient inhibitor of LAP activity was 1,10-phenanthroline (Table 3), inhibiting enzyme activity by 75%. Cysteine inhibited LAP activity by 20%. EDTA, EGTA, sodium citrate and IDA had no effect on LAP activity. Unlike LAP expressed in *E. coli* (Herbers et al., 1994) but in common with some of the LAPs isolated from plants, the purified major potato tuber LAP was insensitive to inhibition by EDTA (Kolehmainen & Mikola, 1971; Ogiwara, Amano, Satoh, & Shioi, 2005). It was also insensitive to the metal chelators citrate and IDA. 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 & Weiler, 1992) and of *Geobacillus thermoleovorans* (Deejing et al., 2005).

The presence of Co^{2+} was activator for LAP activity; 0.2 mM Co^{2+} activated LAP by over 100% (Table 4). The presence of other cations (Cd^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}) was inhibitory for LAP activity; 2 mM Hg^{2+} almost completely inhibited LAP activity. Other cations, with the exception of Mg^{2+}, were also strong inhibitors of LAP activity; 2 mM Co^{2+} ions were inhibitory for the major LAP from *S. tuberosum* (Vujčić et al., 2008). However, Co^{2+} ions have a stimulatory effect on the activity of many metallopeptidases, i.e., microbial, plant or animal aminopeptidases (http://www.brenda-enzymes.org/). 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 & Nagase, 1994).

Table 4
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.

Compound	Final concentration (mM)	Residual activity (%)
$CoCl_2 \cdot 6H_2O$	2	147.1 ± 0.4
	0.2	205.2 ± 0.6
$MgCl_2 \cdot 6H_2O$	2	59.0 ± 1.2
	0.2	88.3 ± 1.4
$CuSO_4 \cdot 5H_2O$	2	24.3 ± 0.1
	0.2	71.5 ± 0.1
$MnCl_2 \cdot 4H_2O$	2	18.5 ± 0.3
	0.2	59.7 ± 0.4
$Cd(OAc)_2 \cdot 2H_2O$	2	19.2 ± 0.3
	0.2	23.3 ± 0.2
$ZnSO_4 \cdot 7H_2O$	2	3.8 ± 0.1
	0.2	5.9 ± 0.0
$HgCl_2$	2	3.2 ± 0.2
	0.2	5.1 ± 0.2

4. Conclusion

Exopeptidases play an important role in food production. As commercial aminopeptidase are limited in number (Deejing et al., 2005) purification and biochemical characterisation (kinetics and mechanisms) of aminopeptidase from various sources is necessary. The LAP present in the soluble fraction of potato tuber extract, although it displayed many properties typical of a cytosolic LAP (EC 3.4.11.1) belonging to M17 peptidase family and of an aminopeptidase from M1 peptidase family, also displayed some novel properties among plant LAPs. The LAP from potato tuber represented in this study has interesting characteristics, alkaline pH optimum, high affinity toward *p*NA derivatives of hydrophobic and basic amino acids, and temperature optimum and stability which they share with some microbial LAPs used in the production of food protein hydrolysates (Raksakulthai and Haard, 2003). Therefore, the potato tuber LAP may have a considerable potential for application in protein hydrolysis.

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