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Characterisation of the aminopeptidase from non-germinated winter rape (*Brassica napus* L.) seeds

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ABSTRACT

Rapeseed plays a crucial role in food and fuel industry. Since aminopeptidases take part in many physiological processes in all organisms, it is important to learn their role and characteristics in economically relevant plants. Extracts of non-germinated winter rape seeds were screened for aminopeptidase activity. Substrate specificity, the influence of pH and temperature, as well as effect of protease inhibitors and chosen metal ions on the aminopeptidase activity were determined. The approximate molecular weight estimated by NATIVE-PAGE and SDS-PAGE electrophoresis was ~60 kDa. The partially purified enzyme as well as the aminopeptidases present in crude extract cleaved preferentially Phe-*p*NA. The activity profiles toward several substrates were also determined. Maximum activity was observed at pH 6.5 and temperature of 40 °C for Phe-*p*NA as a substrate. Two visible picks in the pH profile toward Phe-*p*NA, together with other results (IEF) suggest the presence of more than one aminopeptidase, having similar molecular mass. Much lower activity and broad pH profiles were observed for Leu- and Ala-*p*NA as substrates.

1. Introduction

Brassicaceae is an economically important family of plants containing rapeseed, cabbage, broccoli, brussels, radish and mustard. Among them rapeseed plays a crucial role, because of its application in food and also in fuel industry. According to the USDA report (January 2015) (United States Department of Agriculture, 2015), rapeseed is the second largest oilseed crop worldwide (production of 71.94 mln MT) and the third largest source of vegetable oil. Additionally, the post-extraction protein meal is widely used as a source of proteins for animal feed (production of 40.02 mln MT).

Plant aminopeptidases take part in many crucial physiological processes such as protein turnover, maturation and degradation. It has been proven that they are involved in processes of amino acids turnover, germination, aging, plant defense, stress response and meiosis (Walling, 2006).

Several information concerning aminopeptidases isolated from important oilseed crops have been published. Aminopeptidase isolated from sunflower seeds (*Helianthus annus* L.) (Tishinov, Stambolieva, Petrova, Galunsky, & Nedkov, 2009) was a 80 kDa, monomeric enzyme, with broad substrate specificity, and preference for hydrophobic, bulky side chains (e.g. phenylalanine – the most preferred substrate). The enzyme was also active in hydrolysis of 4-nitroanilides of other L-amino acids such as Ala, Val, Ile, Leu. It is interesting that Pro-*p*NA was also cleaved by that enzyme. Substrates with positively charged side chains of amino acids were not hydrolysed. The aminopeptidase showed optimum activity within the pH range of 7.5–8.0 and at the temperature of 45–50 °C. The other aminopeptidase, isolated from expanded soybean cotyledons, was a 85 kDa monomer, with high affinity for hydrophobic terminal amino acids. Increased activity was observed during seedlings growth (Couton, Sarath, & Wagner, 1991). Peanut cotyledons were also screened for aminopeptidase activity. Five aminopeptidases were identified (Isola & Franzoni, 1996). Among them, one was an iminopeptidase with only Pro-*p*NA activity, while other revealed broad activities toward many amino acids *p*-nitroanilides. High aminopeptidase activity was observed in resting seeds, with decreasing tendency after imbibition. The molecular mass of the aminopeptidase active toward Leu-*p*NA (APa) was in the range of 55–60 kDa. Leucine aminopeptidase was also identified in cotyledons of germinating peanuts (Basha & Cherry, 1978).

Several plants of *Brassicaceae* family (cabbage, brussels, kohlrabi, broccoli, cauliflower and chinese cabbage) were screened for aminopeptidase and iminopeptidase activities (Marinova & Tchorbanov, 2008; Marinova et al., 2008). The molecular mass of studied enzymes was similar for all studied plants: aminopeptidases ~60 ± 3 kDa and iminopeptidases 200 ± 3 kDa. The pH

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optimum of 7.2–7.5 for aminopeptidases and of 8.0–8.5 for iminopeptidases was determined. Aminopeptidases predominantly hydrolyzed phenylalanine- and leucine-4-nitroanilides, with little or no activity toward alanine-4-nitroanilide. Proline iminopeptidase hydrolysed only proline-4-nitroanilide. Both peptidases were successfully applied in the production of soy protein hydrolysates, and high degree of hydrolysis (36–38%) was obtained (Marinova, Thi Kim Cun, Tchorbanov, 2008).

There are only few information about rapeseed (*Brassica napus* L.) aminopeptidases in the literature. Alanine specific aminopeptidase, with the highest activity toward L-alanine-4-(phenylazo)-phenylamide, was isolated from germinated rapeseed (Barth & Hermann, 1974; Hermann, Hermann, Neubert, Huebner, & Barth, 1979). L-leucine, glycine- and L-lysine derivatives were also hydrolyzed. The molecular weight was estimated for 79 kDa and the pH optimum for 8.0–8.5. Addition of metal ions such as Hg^{2+}, Zn^{2+}, Cu^{2+},Co^{2+}, Mn^{2+} and Mg^{2+} (Hermann & Barth, 1976) decreased the activity of the enzyme.

Herein, we report for the first time characterisation of the enzymes with aminopeptidase activity, present in extract of non-germinated winter rape seeds cv. Bellevue, as well as in partially purified sample. Substrate specificity, pH and temperature optimum, as well as thermal stability are widely discussed. The activities of aminopeptidases treated with several metal salts and protease inhibitors are compared. Isoelectric focusing (IEF) study as well as estimation of molecular mass are also presented.

2. Experimental

2.1. Materials and reagents

L-leucine *p*-nitroanilide (Leu-*p*NA), L-alanine *p*-nitroanilide (Ala-*p*NA), bestatin, 1,10-phenanthroline, ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride (PMSF) L-3-carboxy-*trans*-2,3-epoxy-propionyl-L-leucine-4-guanidinobutyl-amide (E-64), Sephadex G-25, dithiothreitol (DTT), DEAE-Sepharose, Sephacryl HR 300 were purchased from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin fraction V and glycine were obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris), 2-mercaptoethanol (BME), polyvinylpyrrolidone (PVP) were purchased from Acros Organics (Geel, Belgium). Dimethyl sulfoxide (DMSO), ammonium sulfate, sodium chloride, and *n*-hexane were from POCH (Gliwice, Poland). Chemicals for electrophoresis, protein molecular weight markers and ready IEF gel, pH 5–8 were purchased from Bio Rad (Hercules, CA, USA). IEF standard was from Sigma Aldrich (St. Louis, MO, USA), Coomassie R-350 was purchased from GE Healthcare (Upsala, Sweden) and trichloroacetic acid (TCA) from Carl Roth (Karlsruhe, Germany). Glycine *p*–nitroanilide (Gly-*p*NA), L-methionine *p*-nitroanilide (Met-*p*NA), L-phenylalanine *p*–nitroanilide (Phe-*p*NA), L-proline *p*-nitroanilide (Pro-*p*NA) were synthesized according to the known procedures (Pansare & Kirby 2009; Rijkers, Adams, Hemker, & Tesser 1995). Gly-L-Phe *p*-nitroanilide (Gly-Phe-*p*NA), L-Phe-Gly *p*-nitroanilide (Phe-Gly-*p*NA), Gly-Phe-Gly-Phe *p*-nitroanilide (Gly-Phe-Gly-Phe-*p*NA) were kindly provided by Dr. Maciej Makowski from the University of Opole. Inorganic salts $AlCl_3$, $BaCl_2$, $CaCl_2$, $CdCl_2$, $CuCl_2$, $FeSO_4$, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, $ZnCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA) or POCH (Gliwice, Poland) in highest available purity. All other chemicals were of high analytical grade and used as purchased. Winter rapeseed cv. Bellevue was obtained from Bayer CropScience, Poland.

2.2. Equipment

Spectrophotometer UV/VIS Jasco V-650; centrifuge 5804R Eppendorf, Mini-PROTEAN® Tetra Cell vertical electrophoresis system Bio Rad, BioLogic LP and BioFrac fraction collector from BIO-RAD, vortex PV-1 from Grant-bio, homogenizer ErgoMix Bosch.

2.3. Synthesis of chosen amino acids p-nitroanilides

Boc-protected amino acids (glycine, L-phenylalanine, L-methionine) (4 mmol) were coupled with *p*-nitroaniline using phosphoryl chloride in pyridine, according to the procedure described in the literature (Rijkers et al. 1995). *N-Boc* amides were deprotected with HCl in EtOAc, at room temperature. Obtained solid was recrystallized from 2-propanol. Pure compounds (based on NMR spectroscopy and melting points) were obtained with the yields of 64% (0.843 g) L-Phe-*p*NA, 58% (0.453 g) Gly-*p*NA, and 12% (0.130 g) L-Met-*p*NA respectively.

L-Phenylalanine-*p*-nitroanilide: ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.88 (s, 1H, NHCO), 8.19 (d, 2H, ArH, *ortho* to NO_2), 7.74 (d, 2H, ArH), 7.27 (m, 5H, Ph), 3.77 (dd, 1H, CHCO), 3.35 (dd, 1H, CH_2Ph), 2.81 (dd 1H, CH_2Ph); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9 (CO), 143.5 (CNO_2 (ipso)), 143.4 (CNHCO (ipso)), 137.1, 129.2, 128.9, 127.18 (Ph), 125.1 (ArC (*ortho* to NO_2)), 118.8 (ArC), 56.7 (CHCO), 40.4 (CH_2Ph).

Glycine-*p*-nitroanilide: ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.45 (s, 1H, NHCO), 8.17 (d, 2H, ArH, *ortho* to NO_2), 7.94 (d, 2H, ArH), 3.91 (s, 2H, CH_2CO), ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.9 (CO), 143.6 (CNO_2 (ipso)), 142.2 (CNHCO (ipso)), 124.0 (ArC (*ortho* to NO_2)), 118.4 (ArC), 41.0 (CHCO).

L-Methionine-*p*-nitroanilide: ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.08 (s, 1H, NHCO), 8.21 (d, 2H, ArH, *ortho* to NO_2), 7.77 (d, 2H, ArH), 3.70 (dd, 1H, CHCO), 2.69 (m, 2H, CH_2S), 2.34 (m 1H, CH_2CH), 2.14 (s, 3H CH_3S), 1.86 (m 1H, CH_2CH), ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.2 (CO), 143.5 (CNO_2 (ipso)), 143.4 (CNHCO (ipso)), 125.1 (ArC (*ortho* to NO_2)), 118.8 (ArC), 54.5 (CHCO), 33.4 (CHCH_2), 30.7 (CH_2S), 15.3 (CH_3S).

L-proline *p*-nitroanilide was obtained using procedure described by Pansare and Kirby (Pansare & Kirby 2009). Briefly, *Boc*-L-proline (8 mmol) was coupled with *p*-nitroaniline using isobutyl chloroformate and 4-methylmorpholine in THF. The obtained *N*-Boc-protected amide was deprotected with trifluoroacetic acid (TFA) in CH_2Cl_2, at room temperature. The resulting solid was recrystallized (EtOAc/hexane) to give 0.380 g (21% yield) of L-Pro-*p*NA.

L-Proline-*p*-nitroanilide: ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.18 (s, 1H, NHCO), 8.20 (d, 2H, ArH, *ortho* to NO_2), 7.78 (d, 2H, ArH), 3.90 (dd, 1H, CHCO), 3.13 (dt, 1H, CH_2N), 3.00 (dt 1H, CH_2N), 2.24 (m, 1H, CH_2CH), 2.04 (m, 2H, NH, CH_2CH), 1.77 (m, 2H, CH_2CH_2NH).^{13}C NMR (100 MHz, $CDCl_3$): δ 174.1 (CO), 143.6 (CNO_2 (ipso)), 143.3 (CNHCO (ipso)), 125.0 (ArC (*ortho* to NO_2)), 118.7 (ArC), 61.0 (CHCO), 47.4 (CH_2NH), 30.7 (CH_2CH), 26.3 (CH_2CH_2NH).

2.4. Crude extract preparation and purification

200 g of rape seeds (winter rapeseed cv. Bellevue) were soaked in 750 cm^3 of 50 mM Tris-HCl buffer (pH 8.0), containing 50 mM NaCl and 1% PVP, for 24 h. The resulting mixture was homogenized (homogenizer ErgoMix Bosch, 10 min) and the obtained slurry was centrifuged at 4 °C, at 10 000 rpm (12 208 rcf), for 30 min. In order to separate the oil fraction, the supernatant was carefully decanted and extracted several times with cold *n*-hexane. Organic fraction was rejected. Aqueous fraction was saturated with ammonium

sulfate up to 35%. The mixture was centrifuged (4 °C, 12 208 rcf, 30 min), and the precipitate was rejected (inactive toward Leu-*p*NA). Additional portion of ammonium sulfate was added to the supernatant, to give 65% saturation. The mixture was centrifuged again (under the same conditions), the supernatant was rejected, while the active precipitate was resuspended in 150 cm^3 of 50 mM Tris-HCl buffer (pH 8.0), containing 50 mM NaCl and 10 mM BME. The obtained crude extract was desalted on the Sephadex G-25 column (2.5 × 30 cm; flow rate 2.0 cm^3/min). Active fractions were pooled, concentrated and loaded onto DEAE Sepharose column (1.5 × 20 cm; flow rate 1.0 cm^3/min; 50 mM Tris-HCl buffer; pH 8.0; NaCl gradient from 50 mM to 250 mM; 1 cm^3 fractions were collected). The aminopeptidase active fractions were eluted at NaCl concentration of ~130 mM. They were collected, concentrated and loaded on the gel filtration Sephacryl HR300 column (1.5 × 50 cm; 50 mM Tris-HCl buffer; pH 8.0, containing 50 mM NaCl and 10 mM BME; flow rate 0.2 cm^3/min). 1 cm^3 fractions were collected and the hydrolytic activity toward Leu-*p*NA was determined. The active fractions were pooled, concentrated, desalted (buffer exchange in order to remove NaCl) and loaded onto ion exchange column Macro Prep High Q (1.5 × 20 cm; 50 mM Tris-HCl buffer pH 8.0, NaCl gradient from 0 mM to 300 mM; flow rate 1 cm^3/min; 1 cm^3 fractions). The aminopeptidase active fractions were eluted at NaCl concentration of ~180 mM. Active fractions (toward Leu-*p*NA as a substrate) were collected and concentrated. Protein concentration was determined using Bradford assay (Bradford 1976).

2.5. Aminopeptidase activity assay

Partially purified extracts were assayed for catalytic activity using L-leucine *p*-nitroanilide as a substrate, according to the slightly modified method described by Appel et al. (Appel, Van Oudheusden, & Bergmeyer, 1974). In this assay, the hydrolysis of Leu-*p*NA was measured spectrophotometrically (37 °C, 10 min), by monitoring the formation of *p*-nitroaniline at 405 nm. Briefly, enzyme extract (25 µl) was added to the mixture containing 950 µl of 50 mM potassium phosphate buffer (PBS) (pH 7.0), 25 µl of 52 mM Leu-*p*NA in DMSO (final concentration of substrate in the sample – 1.3 mM). The measured value was converted to enzymatic units, where one unit was defined as the conversion of 1 µmol of substrate per 1 min, at 37 °C. The hydrolytic activity of studied enzyme toward Ala-*p*NA, Gly-*p*NA, Met-*p*NA, Pro-*p*NA, Phe-*p*NA, Gly-Phe-*p*NA, Phe-Gly-*p*NA and Gly-Phe-Gly-Phe-*p*NA as substrates was determined by the same method. In all cases L-amino acid derivatives were used as substrates.

2.6. Estimation of molecular mass

Polyacrylamide gel electrophoresis (PAGE) was performed under non-denaturing conditions in 7.0% polyacrylamide gels in the absence of SDS. 10 identical samples of extract were loaded on the gel. After electrophoresis, two strips of the gel were cut off and incubated in 50 mM Tris–HCl buffer (pH 8.0), containing 5 mM Leu-*p*NA or Phe-*p*NA, for 30 min, at room temperature. Third strip was stained with Coomassie Brilliant Blue for 1 h, and destained in methanol-acetic acid-water mixture. The rest of the unstained gel was cut into strips according to the bands stained with Coomassie Brilliant Blue. Unstained gel strips were placed in microcentrifuge tubes and immersed in 1 ml of 50 mM Tris–HCl buffer (pH 8.0). Gel pieces were fragmented using clean blade and stirred overnight at room temperature, using magnetic stirrer. Proteins extracted from the gel strips were then centrifuged. Elution was repeated by adding fresh portion of buffer. Filtrates from each unstained gel strip were combined. Each protein eluate was concentrated separately on Amicon Centrifugal Unit (Merck). Concentrated protein eluates were subjected to SDS-PAGE electrophoresis. The molecular weight was finally estimated in 12% polyacrylamide gel using Precision Plus Protein Dual Xtra Standards (Bio-Rad) as a molecular weight standards (250-10 kDa). Proteins in the gel were stained with Coomassie Brilliant Blue.

2.7. Isoelectric focusing (IEF) procedure

Purified protein sample was diluted with 1 part of 50% glicerol and applied on the ready IEF gel pH 5–8. IEF standard was also applied. NaOH solution (50 mM) was applied as a cathode buffer, and 7 mM H_3PO_4 as an anode buffer. IEF running conditions: 100 V for 1 h, 250 V for 1 h and 500 V for 30 min. IEF gel staining was performed using the following stock solutions: A- 0.2% w/v $CuSO_4$ + 20% acetic acid; B – 60% v/v methanol; C – 0.4% w/v solution of Coomassie R-350 in 60% methanol. Conditions of IEF gel staining procedure: fixing (10 min in 20% w/v TCA); washing (2 min with the mixture of A + B 1:1 v/v); staining (15 min, 50 °C, A + C 1:1 v/v); destaining (A + B 1:1); impregnating (5% glicerol and 10% acetic acid).

2.8. Determination of K_m and V_{max} values

Kinetic parameters were determined using either Leu-*p*NA or Phe-*p*NA as substrates. Eight concentrations of each substrate were used (0.026–1.30 mM, of Leu-*p*NA, and 0.026–0.780 mM of Phe-*p*NA respectively). Kinetic parameters were calculated using the software Hyper32.

2.9. Thermal stability and temperature optimum

Thermal stability of the studied enzyme was measured in 50 mM PBS buffer, at several temperatures (25, 30, 35, 37, 40, 45, 50, 55 °C). Enzyme was incubated at each temperature for 15, 30, 45, and 60 min. The aminopeptidase residual activity was determined with Phe-*p*NA as a substrate. Temperature optimum was determined in 50 mM PBS buffer (pH 7.0), after 10 min of incubation at chosen temperature (from the range of 25–65 °C), using Phe-*p*NA as a substrate.

2.10. pH dependence

The effect of pH on the rapeseed aminopeptidase activity was determined at 37 °C, using Phe-*p*NA, Leu-*p*NA or Ala-*p*NA as substrates, in 50 mM buffers: sodium acetate (pH 5.0–5.5), PBS (pH 6.0–7.0), Tris-HCl (pH 7.5–8.5) and Na_2CO_3-$NaHCO_3$ (pH 9.0–10.0).

2.11. Effect of metal ions

The influence of metal salts of Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+} and Zn^{2+} on the activity of rapeseed aminopeptidase was determined using two concentrations of each salt (1 mM and 0.1 mM). The enzyme was incubated with each salt solution for 2 h at 4 °C. Control sample without any metal salt was also prepared. Enzyme activity was determined using the assay previously described, with Phe-*p*NA as a substrate.

2.12. Effect of proteolytic enzymes inhibitors

In order to determine the influence of standard protease inhibitors, the enzyme was incubated with various concentrations of EDTA, EGTA, 1,10-phenantroline, bestatin, E-64 and PMSF, at 30 °C for 30 min. Control measurement in the absence of inhibitors was also performed. The residual enzyme activity was determined using the assay previously described.

3. Results and discussion

3.1. Characterisation of crude extract and partially purified aminopeptidase

Preliminary experiments revealed that the crude extract obtained from dry rape seeds, after separation of oil fraction and precipitation with ammonium sulfate, showed the highest specific activity (65.1 mU/mg) toward Phe-*p*NA as a substrate, but was also active in hydrolysis of Leu-*p*NA (25.0 mU/mg), Ala-*p*NA (15.1 mU/mg), and Pro-*p*NA (7.1 mU/mg). The activity profiles obtained during gel filtration of crude extract are depicted in Fig. 1. As can be observed, the hydrolytic activity toward several L-amino acids accumulates in the same region. Three peaks (with the middle peak partially separating into three additional parts) can be observed in Phe-*p*NA activity profile. Also in Leu- and Ala-*p*NA profiles 4–5 signals can be noticed, but they are not separated. The Native-gel electrophoresis of crude extract showed that the R_f values of bands corresponding to all substrates hydrolysed (Ala-, Phe-, Leu-, Pro-*p*NA) (Supplementary materials, Fig. SI 1) were the same suggesting, that either there is one enzyme with broad activity or more aminopeptidases with the similar molecular mass.

Partial purification of extract (131.17-fold purification) increased the specific activity of studied enzyme to 788 mU/mg toward Leu-*p*NA (Table 1). The highest activity was obtained for Phe-*p*NA as a substrate (4944.2 mU/mg). The approximate molecular mass, estimated by Native-gel electrophoresis (Supplementary materials, Fig. SI 2) was about 60 kDa. It revealed, that there is only one clear protein band showing the hydrolytic activity toward both Phe-*p*NA as well as Leu-*p*NA. The estimated molecular mass was lower than that described for aminopeptidase isolated by A. Barth from germinated rapeseed (79 kDa) (Barth & Hermann, 1974). Similar molecular mass was determined for several cereal aminopeptidases isolated from seeds, e.g. barley (58 kDa) (Oszywa, Makowski, & Pawełczak 2013). The molecular mass of aminopeptidase within the same range was also determined in other oilseed plants e.g. peanut cotyledons (55–60 kDa) (Isola & Franzoni 1996). Aminopeptidases isolated from soybean cotyledons as well as sunflower seed had higher molecular weights – 85 kDa and 80 kDa respectively (Couton et al., 1991; Tishinov et al., 2009). Isoelectric focusing showed that at least 4–5 visible protein bands with pI values of 5.3–6.7 can be observed in the partially purified aminopeptidase (Supplementary materials, Fig. SI 3). The aminopeptidase that gives the most visible band has slightly acidic pI of 6.25. Similar pI values were obtained for aminopeptidases in wound-induced aminopeptidase proteins of tomato (pI 6.2–6.4 for 60 kDa LAP-A and pI 5.6–5.8 for 55 kDa aminopeptidases) (Gu, Chao, & Walling 1996), as well as LAP from *Solanum tuberosum* tubers (pI 5.45) (Vujcic, Dojnov, Milovanovic, & Bozic 2008). The K_m value of 334.3 μM and V_{max} of 76.82 μM/s for Leu-*p*NA, as well as 247.5 μM and 403 μM/s for Phe-*p*NA were determined, showing higher affinity of the enzyme toward phenylalanine substrate.

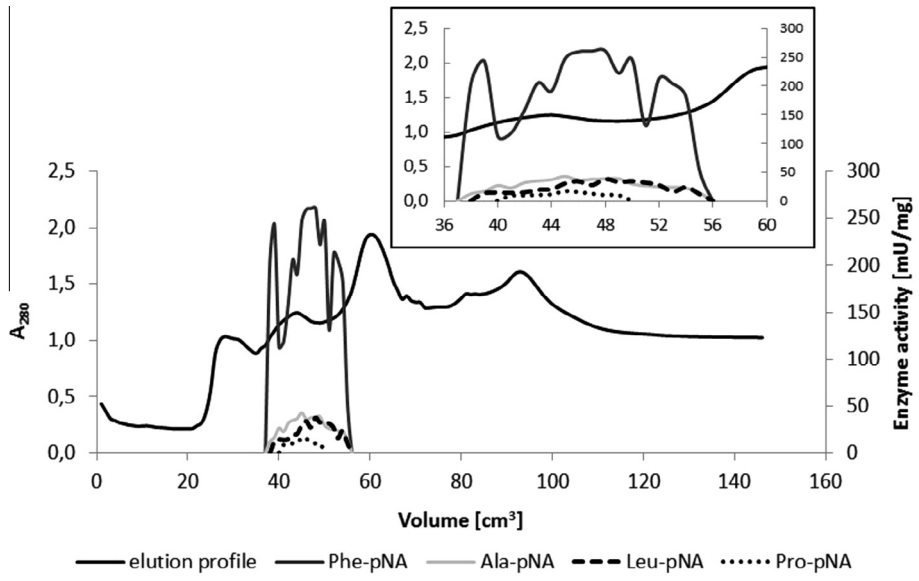

Fig. 1. The profile of aminopeptidase activity during gel filtration of winter rape seeds cv. Bellevue crude extract. Column: Sephacryl HR 300 (BioRad) 1.5 × 50 cm; flow rate 0.20 cm^3/min, volume of fractions 1 cm^3. Buffer Tris-HCl 50 mM, pH 8.0; Substrates: Phe-*p*NA, Ala-*p*NA, Leu-*p*NA and Pro-*p*NA.

Table 1
Partial purification of aminopeptidase isolated from winter rape seeds cv. Bellevue.

Purification step	Total activity* [U]	Total protein [mg]	Specific activity* [U × mg^{-1} protein]	Purification (-fold)	Recovery [%]
Crude extract	63.33	10089.0	0.006	1.0	100.0
35% $(NH_4)_2SO_4$	51.46	3976.0	0.013	2.17	81.3
65% $(NH_4)_2SO_4$	25.60	1031.0	0.025	4.16	40.4
Sephadex G-25	25.56	955.0	0.027	4.50	40.4
DEAE – Sepharose	12.76	65.6	0.195	32.33	20.2
Sephacryl HR 300	5.38	20.1	0.268	44.67	8.5
MacroPrep High Q	1.97	2.5	0.788	131.17	3.1

* The activity was assayed with Leu-*p*NA as a substrate, at pH 7.0 (PBS buffer).

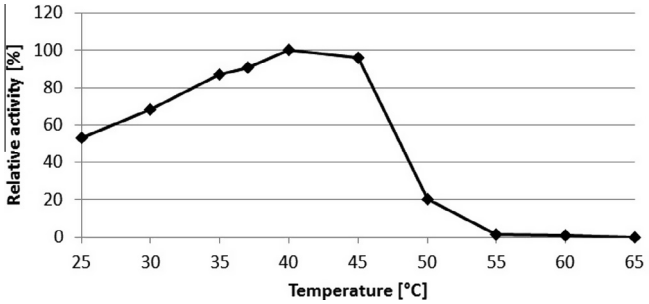

Fig. 2. The influence of temperature on the activity of partially purified aminopeptidase from winter rape seeds cv. Bellevue. Incubation time 10 min.; pH 7.0; the residual aminopeptidase activity was determined using Phe-*p*NA as a substrate.

3.2. Temperature optimum and thermal stability

The influence of temperature on the activity and stability of partially purified aminopeptidase was determined in the range of 25–65 °C, using Phe-*p*NA as a substrate (Fig. 2). The maximum activity of the aminopeptidase toward Phe-*p*NA can be observed at 40 °C. The enzyme remains over 80% of activity over the temperature range of 35–45 °C, and at 25 °C it shows ~52% of its maximum activity toward Phe-*p*NA. It is completely inactive over 55 °C. Similar results were obtained for Leu-*p*NA (maximum activity at 40 °C), but for Ala-*p*NA the maximum activity was shifted to 45–50 °C. Both temperatures correlate well with the optima of aminopeptidases from other species: 52 °C for aminopeptidase from barley seeds (Oszywa et al., 2013), and 35–45 °C for aminopeptidases from several *Brassicaceae* plants (Marinova & Tchorbanov, 2008). The thermal stability of the aminopeptidase was determined by incubation for 15, 30, 45 and 60 min at the temperatures of 25, 30, 35, 37, 40, 45, 50 and 55 °C (Fig. 3). The study revealed, that the enzyme is stable in the temperature range of 25–40 °C. It remains over 40% of activity after incubation for 60 min at 45 °C. The dramatic loss of activity is observed at the temperatures above 45 °C.

3.3. pH optimum and substrate specificity

The pH dependence of aminopeptidase activity in the range of pH 5.0–10.0, for Phe-, Leu- and Ala-*p*NA as substrates, was determined (Fig. 4). The obtained curve is bell shaped for Phe-*p*NA, with the maximum at pH 6.5, but smaller peak can be observed at pH 9.0, suggesting the presence of more than one enzyme isoforms in the sample. Similar pH optima were determined for sunflower seeds as well as chosen *Brassicaceae* plants aminopeptidases (pH 7.2–8.0), *Solanum tuberosum* tubers LAP (pH 9.0) and iminopeptidases (pH 8.0–8.5) (Marinova & Tchorbanov, 2008; Marinova et al., 2008; Tishinov, Petrova, & Nedkov, 2010; Vujcic et al. 2008).

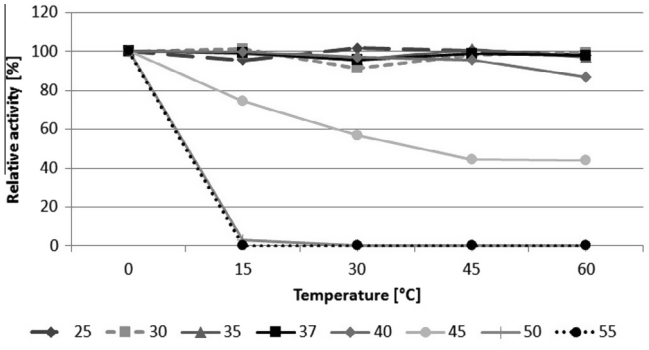

Fig. 3. Thermal stability of partially purified aminopeptidase from winter rape seeds cv. Bellevue. Enzyme was incubated at different temperatures (25–55 °C) for 15, 30, 45, and 60 min. The residual aminopeptidase activity was determined using Phe-*p*NA as a substrate.

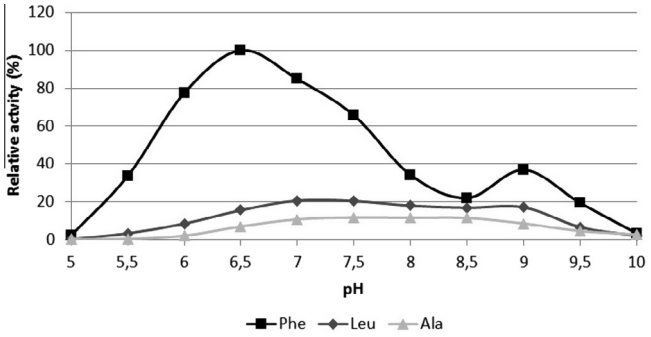

Fig. 4. The pH dependence of partially purified rapeseed aminopeptidase activity from winter rape seeds cv. Bellevue. Enzyme activity was assayed with Phe-*p*NA, Leu-*p*NA and Ala-*p*NA as substrates.

Table 2
Substrate specificity of aminopeptidase from winter rape seeds cv. Bellevue in crude extract and partially purified enzyme at the optimum pH 6.5. Final substrate concentration – 1.3 mM; PBS buffer pH 6.5; temperature 37 °C.

Substrate	Relative activity* [%]	
	Purified enzyme	Crude extract
Phe-*p*NA	**100**	3.4
Leu-*p*NA	16.0	0.7
Ala-*p*NA	6.7	0.4
Gly-*p*NA	2.7	0.1
Met-*p*NA	8.3	0.3
Pro-*p*NA	9.0	0.2
Gly-Phe-*p*NA	0.2	0.1
Phe-Gly-*p*NA	0	0.01
Gly-Phe-Gly-Phe-*p*NA	0	0

* The activity of purified enzyme with Phe-*p*NA was taken as 100%.

Substrate specificity of partially purified enzyme was also determined at optimum pH (pH 6.5 for Phe-*p*NA cleveage), using 4-nitroanilides of chosen amino acids. Two dipeptides and one tetra-peptide were also applied as potential substrates of studied enzyme (Table 2). Additionally, substrate specificity of crude extract at pH 6.5 was determined, in order to compare results with partially purified sample. The obtained data indicate, that the enzyme catalyzes preferentially the hydrolysis of Phe-*p*NA and also Leu-*p*NA. This indicates that amino acids with larger hydrophobic side chains are much better substrates. Specific activities toward Ala- and Gly-*p*NA are much lower. It is also worth mentioning, that the enzyme is active in cleavage of Met-*p*NA and Pro-*p*NA. Among the studied dipeptides only Gly-Phe-*p*NA was hydrolysed. No detectable activity of partially purified enzyme was observed for Gly-Phe-Gly-Phe-*p*NA.

3.4. Protease inhibitors

The influence of the most efficient protease inhibitors on the rapeseed aminopeptidase was determined in the presence of Phe-*p*NA as a substrate (Supplementary materials, Table SI 1). We found that the inhibitory effect of chelating agent EDTA was observed after 30 min of incubation (50%, 73%, 84% of residual activity, for the concentrations of 33 mM, 10 mM and 1 mM, respectively). The results indicate, that the studied enzyme requires some metal ions for full activity. The other chelating agents did not show such a significant influence, and 1,10-phenantroline had even slightly activating effect.

Phenylmethylsulfonyl fluoride known serine proteases inhibitor also lowered the enzyme activity (46% and 64% of residual activity for 4 mM and 2 mM concentration of PMSF, respectively). Bestatin, which is a typical leucine aminopeptidase inhibitor, showed only minimum inhibitory effect (at the concentration of 0.1 mM, the residual aminopeptidase activity was 94%). Inhibitor of cysteine proteases (E-64) did not show any significant inhibition (96% of residual activity for 0.1 mM concentration). Proteases can be classified according to the mechanism of hydrolysis of peptide bond. Therefore four groups of peptidases can be distinguished: metalloproteases, serine/threonine proteases, cysteine and aspartyl proteases. The obtained results suggest that serine plays a crucial role in the activity of rapeseed aminopeptidase and that metal ions, required for full enzymatic activity, are tightly bound to the active site. Similar influence of typical protease inhibitor was also observed in other plants. PMSF was one of most effective inhibitors towards purified Phe-AP from shoots of 3-week-old pea plants, however EDTA and 1,10-phenantroline had slightly activating influence on this aminopeptidase (Pyrzyna, Szawłowska, Bielawski, & Zdunek-Zastocka, 2011). In the case of crude extract of the whole fruit of *A. deliciosa* (kiwifruit), the presence of 1 mM of 1,10-phenanthroline, EDTA and iodoacetamide inhibited aminopeptidase activity while the 1 mM PMSF had no effect (Premarathne & Leung, 2010). 1,10-Phenantroline was also efficient inhibitor of leucine aminopeptidase from *Solanum tuberosum* tuber, while EDTA, EGTA and sodium citrate had no effect on aminopeptidase activity (Vujcic et al., 2010).

3.5. Metal ions

Among the tested metal ions, the aminopeptidase from dry rape seeds is slightly activated by Ba^{2+}, Na^{+} ions at the concentration of 0.1 mM. There is no significant influence of Ca^{2+}, Mg^{2+} or Al^{3+} ions, while the presence of 0.1 mM Mn^{2+}, Ni^{2+}, Fe^{2+}, Cd^{2+} have mild inhibitory effect (Supplementary materials, Fig. SI 4). The significant inhibition was observed for Zn^{2+} (residual activity 65.4% and 60.3% for 0.1 and 1 mM $ZnCl_2$ respectively). Chosen metal ions were also tested at the concentration of 1 mM. The addition of Ca^{2+}, Mg^{2+} or Al^{3+} did not influence enzyme activity, while in the presence of 1 mM of Cu^{2+} and Ni^{2+} significant inhibition was observed (residual activity 40.8% and 32.9% respectively). Among aminopeptidases described in the literature, Mg^{2+} and Mn^{+2} activated the enzymes from pigeonpea (*Cajanas cajan*) (Lomate & Hivrale, 2011) or barley seeds (Oszywa et al., 2013) while other heavy metals inhibited them. Strong inhibitory effect of Zn^{2+} and Cu^{2+} as well as Pb^{2+} was noticed for prolyl aminopeptidase from shoots of triticale seedlings (Szawłowska, Zdunek-Zastocka, & Bielawski, 2011). Interestingly, similar effect of Ca^{2+} and Zn^{2+} ions was observed for the aminopeptidase hydrolyzing acidic amino acids from *N*-terminus of proteins, isolated from soybean cotyledons (Asano, Nakamura, Kawai, Miawa, & Nio, 2010). The effect of addition of metal ions on the activity of aminopeptidase is widely known. Even if the enzyme requires metal ions for its full activity, inhibitory effect of the excess of metal can be observed in some metalloaminopeptidases (Lowther & Matthews, 2002).

3. Conclusions

Extract of non-germinated winter rape seeds cv. Bellevue was screened for the aminopeptidase activity. It revealed broad substrate specificity, with the highest activity toward Phe-*p*NA. Also in the partially purified sample, phenylalanine was preferentially hydrolysed. PMSF, known serine protease inhibitor and EDTA (metalloprotease inhibitor) were most efficient, suggesting that serine plays a crucial role in the catalytic process and that metal ions are required for full enzymatic activity. The enzyme was slightly activated by Ba^{2+}, Na^{+} ions and no influence of Ca^{2+}, Mg^{2+} or Al^{3+} ions was observed. The addition of Cu^{2+} Zn^{2+} and Ni^{2+} had significant inhibitory effect. The pI values as well as pH and temperature optima were quite similar to other plant aminopeptidases and iminopeptidases. The obtained results suggest that there are few aminopeptidases (including prolyl aminopeptidase) present in the partially purified sample, having similar molecular weight and slightly different pI values.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.03.097.

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