Bulgarian Chemical Communications, Volume 40, Number 4 (pp. 397–400) 2008

Brassicaceae plants as a new source of food grade peptidases

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Dedicated to Academician Ivan Juchnovski on the occasion of his 70th birthday

Received March 13, 2008, Revised March 30, 2008

Several plants of *Brassicaceae* (cabbage, Brussels sprouts, kohlrabi, broccoli, cauliflower and Chinese cabbage) were screened for aminopeptidase and iminopeptidase activities using L-leucine-*p*-nitroanilide and proline-*p*-nitroanilide as substrates, respectively. Significant levels of aminopeptidase (40–160 μU/g) and iminopeptidase (40–100 μU/g) activities with seasonal variability were detected. The exopeptidases were precipitated from the native juice by ammonium sulfate saturation (25–70%) and separated by gel filtration chromatography on Sephadex G-200. The aminopeptidases (molecular weight ~60 kDa) predominantly hydrolyzed phenylalanine-*p*-nitroanilide as substrate, while the iminopeptidases (molecular weight ~200 kDa) were strongly specific to the applied substrate. The pH optima, 7–7.5 for aminopeptidases activities and 8–8.5 for iminopeptidases activities, were determined and they demonstrated a temperature optimum at 40 and 45ºC, respectively.

The edible biomass from *Brassicaceae* plants is a new effective source of food grade exopeptidases meeting all the requirements with regard to contemporary ecological and economical concepts. The aminopeptidases and iminopeptidases can be used successfully for peptide modification under mild conditions during food processing, e.g. debittering of protein hydrolysates from substrates with high Q-value.

Key words: *Brassicaceae* plants, aminopeptidases, iminopeptidases, exopeptidases.

INTRODUCTION

Plant enzymes are essential for different biological processes and often meet all the requirements with regard to contemporary ecological and economical concepts. Aminopeptidases (EC 3.4.11) from plants have been isolated and characterized from various sources [1–5]. Most reports describe aminopeptidases from seeds and leaves. These enzymes catalyze the sequential removal of amino acids from the unblocked N-termini of peptides and proteins. In addition to their role in general protein and peptide metabolism, aminopeptidases have more specific functions. These include defense response to infection and wounding [6], protein maturation [7] and auxin transportation [8]. Iminopeptidases in plant kingdom (EC 3.4.13.8-9) are not so well investigated. Since plant aminopeptidases have little ability to release N-terminal proline-residues, iminopeptidases are important for the degradation of proline-containing peptides. Recently, the importance of proline-degrading proteolytic enzymes in celiac sprue treatment has been evaluated because of their unique ability to accelerate the decomposition of proline-rich gluten in the gut lumen [9].

The aim of this study was to describe aminopeptidase and iminopeptidase activities in *Brassicaceae* plants, their separation by gel chromatography and to determine the optimal conditions for their application.

EXPERIMENTAL

Materials and methods

The edible parts of fresh *Brassicaceae* plants – cabbage (*Brassica oleraceae* var. *capitata*), Brussels sprouts (*Brassica oleraceae* var. *gemmifera*), kohlrabi (*Brassica oleraceae* var. *caulorapa*), cauliflower (*Brassica oleraceae* var. *botrytis*), broccoli (*Brassica oleraceae* var. *cauliflora*) and Chinese cabbage (*Brassica campestris* var. *pekinensis*), were used.

The L-amino acid *p*-nitroanilides were purchased from Sigma, USA. All the other reagents were of analytical grade of purity. Sephadex G-200 was obtained from Pharmacia Biotech, Sweden.

Enzyme assay

The aminopeptidase activity using aminoacyl-*p*-nitroanilides as substrates was assayed according to the procedure of Chrispeels and Boulter [10]. After incubation for 10 min at 30ºC in 0.05 M phosphate buffer (pH 7.0), the amount of liberated *p*-nitro-

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aniline was measured at 410 nm on a spectrophotometer (UV-VIS spectrophotometer, Shimadzu 1240). The iminopeptidase activity was assayed spectrophotometrically at 410 nm in regard to L-proline-*p*-nitroanilide (Pro-*p*-NA) according to the approach of Yoshimoto and Tsuru [11] in 0.1 M Tris/HCl buffer (pH 8.0) for 20 min at 30ºC. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitroaniline per minute.

Protein determination

The amount of soluble protein was determined by the biuret reaction following precipitation with 20% (v/v) trichloroacetic acid (TCA). Bovine serum albumin (BSA) was used to prepare a standard curve [11].

Molecular weight estimation

The molecular weights of the enzymes were determined by gel filtration on a Sephadex G-200 column (1.5×146 cm) using as elution medium 0.05 M sodium phosphate buffer at pH 7.5 containing 0.15 M NaCl. Cytochrome C (12 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and ferritin (440 kDa) were used as standard proteins. The void volume was determined with Blue Dextran.

Enzyme extraction and purification

The fresh bio-mass of *Brassicaceae* plant was cut into pieces, milled and pressed giving a native juice. It was further precipitated with 25–75% ammonium sulphate (at 0°C), followed by centrifugation of the suspension at 6000×g for 30 min at 5ºC (MLW K24 D, Germany). The pellet was resolved in 0.05 M phosphate buffer at pH 7.5 containing 0.15 M NaCl (buffer A) and dialyzed against the same buffer for 4h. The suspension was then clarified by centrifugation at 4000×g for 15 min. The dialyzed solution (3 ml) was applied on a Sephadex G-200 column (1.5×146 cm) equilibrated with buffer A.

Characterization of the enzymes

The exopeptidases were incubated at different temperatures from 15 to 60ºC. At various moments of time, 0.1-ml aliquots of aminopeptidase and iminopeptidase solutions were removed and assayed for activity against 1.5 mM L-leucine-*p*-nitroanilide (Leu-*p*-NA) and 0.8 mM Pro-*p*-NA, respectively, at 30ºC as it was described previously. The effect of pH optima on the aminopeptidase and iminopeptidase activities was determined using Leu-*p*-NA and Pro-*p*-NA as substrates, respectively, and 25 mM citrate-phosphate buffers (pH 5.0–7.0); 25 mM sodium phosphate buffer (pH 7.0–8.0); 25 mM Tris/HCl buffer (pH 8.5–9.0); and 25 mM glycine-NaOH buffers (pH 9.0–10.0) were used. Following the preincubation for 20 min, a 0.1-ml aliquot was removed and assayed against Leu-*p*-NA and Pro-*p*-NA.

RESULTS AND DISCUSSION

The *Brassicaceae* plants are a new source of food quality grade peptidases and the levels of the enzymes activities for different representatives are summarized in Table 1. It is seen that the plants of the *Brassicaceae* family grown during the summer-autumn period have higher exopeptidase activities than that of those grown during the winter-spring. The cabbage seems to be the most attractive source according to its price and the yield from the fresh juice. Moreover, cabbage leaves can be used successfully for preparative production of the enzymes.

Table 1. Aminopeptidase and iminopeptidase activities with seasonal variations in the crude extracts of edible parts from some representatives of *Brassicaceae* plants.

Brassicaceae plants	Crude juice from 1 kg biomass, ml	Amino-peptidase activity, μU/g	Imino-peptidase activity, μU/g
Cabbage	600	100–130	40–60
Brussel sprouts	200	110–160	100–80
Kohlraby	700	80–110	40–50
Broccoli	500	130–150	100–90
Cauliflower	500	100–120	40–70
Chinese cabbage	600	40–60	40–60

* The first digits showed the enzymes activities measured for winter-spring plants and the second digits – for summer-autumn plants. Aminopeptidase and iminopeptidase activities were determined using L-leucine-p-nitroanilide and proline-p-nitroanilide as substrates, respectively.

The high solution viscosity of all extracts from *Brassicaceae* plants hindered the filtration process as well as the concentration by ultrafiltration. The exopeptidases from crude extracts were precipitated with ammonium sulfate (25–70%), then the precipitates were resuspended in buffer A and the solutions obtained were further passed through a Sephadex G-200 column (Fig. 1). Under the chosen conditions, the iminopeptidase was separated readily from the aminopeptidase independently of the enzyme source. The elution volumes and the ratio between the two enzymes do not vary much in all cases. Using globular proteins as markers the molecular weights of the studied enzymes were evaluated – 60 ± 3 kDa for aminopeptidases and 200 ± 7 kDa for the iminopeptidases (Fig. 1).

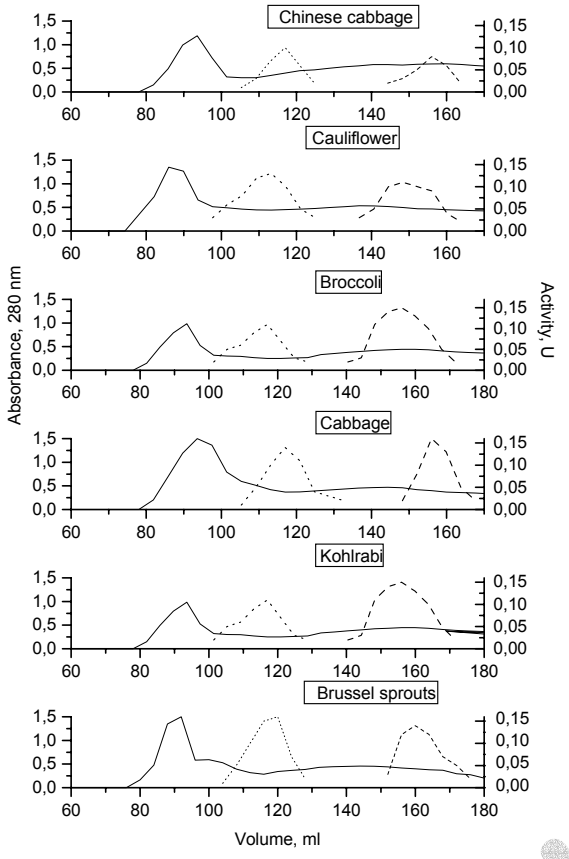

Fig. 1. Elution profiles (—) of exopeptidases from different plants of *Brassicaceae* family at the outlet of G-200 column (1.5×146 cm), equilibrated with 0.05 M sodium phosphate buffer pH 7.5 containing 0.15 M NaCl. The aminopeptidases (----) and iminopeptidases (·····) activities were assayed against L-leucine-*p*-nitroanilide and proline-*p*-nitroanilide, respectively.

During the tests with different L-aminoacyl-*p*-nitroanilides, it was found out that the aminopeptidases predominately hydrolyzed L-phenylalanine-*p*-nitroanilide as substrate while the iminopeptidases were strongly specific to the applied substrate. The action of the isolated enzymes was independent of the salt concentrations in the range 0–15% NaCl. The temperature of 35–45ºC and pH 7.5–8.5 appear to be optimal for the peptidases action (Fig. 2).

The initial characterization of the isolated aminopeptidases and iminopeptidases demonstrated that they closely resembled peptidases purified from other plant species [1]. The aminopeptidases are likely to belong to neutral aminopeptidases with preference to substrates with hydrophobic amino acids, as they have a neutral pH optima and they preferentially hydrolyzed L-phenylalanine-*p*-nitroanilide as substrate [12]. The iminopeptidases also possess most of the other plant iminopeptidases properties, such as an alkaline pH optimum, strong preference for hydrolyzing Pro-*p*-NA and high molecular weight [1, 12].

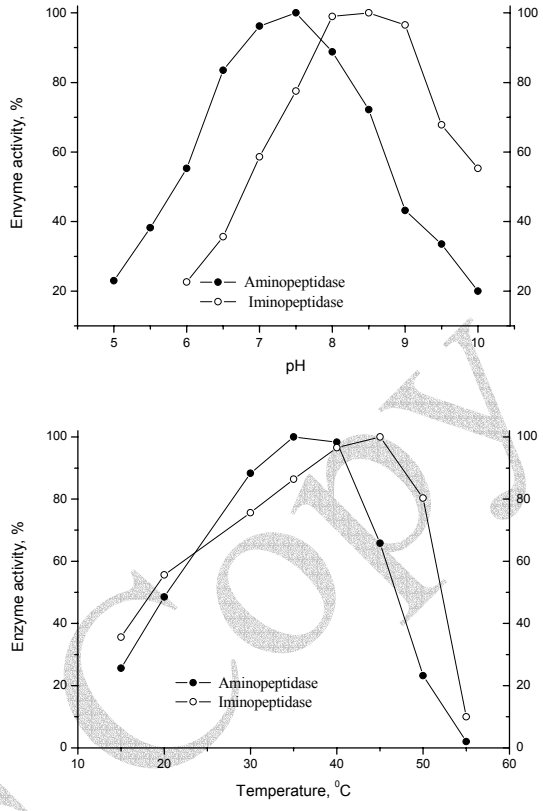

Fig. 2. (Top) pH optima of the aminopeptidases (full symbols) and iminopeptidases (open symbols). (Bottom) Temperature optima of the aminopeptidases (full symbols) and iminopeptidases (open symbols).

CONCLUSIONS

The obtained results manifest, that the edible parts of *Brassicaceae* plants are real source of peptidases especially as unique combination of aminopeptidases and iminopeptidases. The aminopeptidase action demonstrates a higher specificity to the more hydrophobic substrate – phenylalanine-*p*-nitroanilide. The optimal pH and temperature for peptidase activity were studied and the enzymes could be succesfully employed in reducing the bitterness associated with hydrolysis of food proteins in order to obtain hydrolysates as anti-ageing food ingredients.

***Acknowledgment**: The authors acknowledge financial support for these investigations by MES-NFSI of Bulgaria (Project CC-1509).*

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РАСТЕНИЯТА ОТ СЕМ. *BRASSICACEAE* КАТО ИЗТОЧНИК НА ХРАНИТЕЛНИ ПЕПТИДАЗИ

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Посветена на акад. Иван Юхновски по повод на 70-та му годишнина

Постъпила на 13 март 2008 г., Преработена на 30 март 2008 г.

(Резюме)

Различни представители на растенията от сем. *Brassicaceae* (бяло главесто зеле, брюкселско зеле, алабаш, броколи, карфиол и китайско зеле) бяха изследвани за аминопептидазна и иминопептидазна активност, използвайки като субстрати левцин-*p*-нитроанилид и пролин-*p*-нитроанилид. Бяха установени значителни нива на аминопептидазна (40–160 μU/g) и иминопептидазна (40–100 μU/g) активност със сезонна изменяемост. Екзопептидазите бяха утаени с амониев сулфат (25–70%) и разделени с помощта на гел-филтрационна хроматография на колона Sephadex G-200. Аминопептидазите (молекулна маса ~60 kDa) хидролизират предпочетено фенилаланин-*p*-нитроанилид, докато иминопептидазите (молекулна маса ~200 kDa) проявяват строга специфичност към използвания за скрининг субстрат. Бяха определени pH оптимумите за аминопептидазите 7–7.5 и за иминопептидазите 8–8.5, както и температурните им оптимуми – съответно 40 и 45ºC.

Ядивните части на растенията от сем. *Brassicaceae* са нов ефективен източник на хранителни екзопептидази, отговарящи на съвременните екологични и икономически норми. Аминопептидазите и иминопептидазите могат да бъдат успешно използвани за модифициране на пептиди при меки условия в процеса на обработка на храните напр. при обезгорчаване на белтъчни хидролизати от субстрати с висока Q-стойност.