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ASPARTIC PROTEINASES FROM BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH) SEEDS - PURIFICATION AND PROPERTIES OF THE 47 kDa ENZYME

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Abstract - Aspartic proteinases from buckwheat seeds are analyzed. Three forms of 47 kDa, 40 kDa and 28 kDa, were purified from mature buckwheat seeds, while two forms of 47 kDa and 28 kDa were detected in developing buckwheat seeds using pepstatin A affinity chromatography. A form of 47 kDa was selectively precipitated from other forms by ammonium sulfate precipitation. This enzyme resembles the chymosin-like pattern of proteolytic activity, as it was shown using BSA and κ-casein as substrates, clarifying its ability for milk-clotting. The 47 kDa aspartic proteinase form is localized in the membrane fraction.

Key words: Buckwheat, aspartic proteinase, pepstatin A affinity chromatography.

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INTRODUCTION

Aspartic proteinases (APs) are a widely distributed class of proteases present in animals, microbes, viruses, and plants. Plant APs have been purified from monocotyledons (Doi *et al.*, 1980; Belozersky *et al.*, 1989; Sarkkinen *et al.*, 1992), dicotyledons (Polanowski *et al.*, 1985; Rodrigo *et al.*, 1989) as well as from gymnosperms (Bourgeous and Malek 1991). Such enzymes have been found in distinct parts of different plants. Thus, APs have been isolated from seeds (Doi *et al.*, 1980; Belozersky *et al.*, 1989; Stachowiak *et al.*, 1994; Asakura *et al.*, 1995; D'Hondt *et al.*, 1993; Hiraiwa *et al.*, 1997; Mutlu and Gal 1999), grains (Runeberg-Roos *et al.*, 1991; Sarkkinen *et al.*, 1992), flowers (Heimgartner *et al.*, 1990; Cordeiro *et al.*, 1994; Verissimo *et al.*, 1996), pollen (Radlowski *et al.*, 1996) and leaves (Rodrigo *et al.*, 1989, 1991; Kuwabara and Suzuki, 1995; Schaller and Ryan, 1996; Guevara *et al.*, 2001).

The functions of APs in different tissues of different plants are not fully understood. In seeds, these enzymes are believed to participate in various proteolytic processes during seed development and germination, although their exact functions have still not been elucidated. To date, it is known that APs take part in the maturation of 2S albumins from *Brassica* (D'Hondt *et al.*, 1993) and *Arabidopsis* (Gruis *et al.*, 2002) and in the initiation of hydrolysis of wheat seed storage proteins (Belozersky *et al.*, 1989). In barley, numerous data suggest that phytepsin (barley AP) performs different functions in protein processing and turnover in different stages of the plant life cycle (Paris *et al.*, 1996; Runeberg-Ross *et al.*, 1994; Törmäkangas *et al.*, 1994). Moreover, phytepsin has recently been inked with programmed cell death in the developing tracheal elements of barley roots (Lindholm *et al.*, 2000; Runeberg-Roos and Saarma, 1998). Roles for APs in the degradation of pathogenic proteins (Rodrigo *et al.*, 1991) as well as in the development of tracheal elements and sieve cells (Runeberg-Roos and Saarma 1998) have also been reported. The precise function of AP in these processes remains to be elucidated.

One of the important features of plant aspartic proteinases is their ability to clot milk, which makes them very useful for biotechnological purposes. Besides the

worldwide known vegetable rennet from *Cynara*, there are also a limited number of other plant species used in cheese making (Yousif *et al.*,1996; Lopes *et al.*, 1997).

In a previous work of ours, we found that crude buckwheat seed protein extract showed pepstatin A-sensitive proteolytic activity, which was attributed to an aspartic proteinase of 47 kDa (Timotijević *et al.*, 2003). In the present paper, the 47 kDa polypeptide is analyzed in more detail after purification by pepstatin A affinity chromatography. To addition to this, the presence of other forms of APs detected in developing and mature buckwheat seeds is demonstrated.

MATERIALS AND METHODS

Plant material

Seeds of buckwheat (*Fagopyrum esculentum* Moench) were taken from plants grown in field conditions, frozen in liquid nitrogen, and stored at -20° C.

Protein extract preparation

Buckwheat seeds were ground to a fine powder in liquid nitrogen in a mortar with a pestle. An acidic protein extract from buckwheat seed powder was prepared by homogenization in three volumes of acidic buffer (1 M NaCl, 1% Triton X-100, and 3 mM $NaHSO_3$ with a supplement of polyvinyl-polypyrolidone, adjusted to pH 4.0 with HCl). After homogenization for 5 min at room temperature, the suspension was again adjusted to pH 4.0 and stirred gently for 20 min before centrifugation at 10000 g for 20 min at 4^0C. The supernatant was filtered through cotton, kept for 1-2 days at 4^0C, and stored at -20^0 C if not immediately processed.

Purification of aspartic proteinase from buckwheat seed by affinity chromatography

Purification of aspartic proteinases from buckwheat was performed according to Sarkkinen *et al.* (1992). The acidic extracts prepared from mature or developing buckwheat seeds (5 ml) were loaded onto a pepstatin A-agarose (Sigma) column (1.2 cm long, 0.3 cm i.d.) previously equilibrated with 5 ml of 0.1 M sodium acetate buffer (pH 4) containing 3 mM $NaHSO_3$. The column was washed with: 4 ml of 0.1 M sodium acetate (pH 4), 0.5 M NaCl, and 0.2 mM DTT; 8 ml 0.1 M Na acetate (pH 4), 1.5 M NaCl, and 0.2 mM DTT; and 4 ml 0.1 M potassium phosphate (pH 7.5), 0.5 M NaCl, and 0.2 mM DTT. During elution with 0.1 M sodium bicarbonate (pH 10.0) containing 0.5 M NaCl and 0.2 mM DTT, fractions of 500 or 300 μl were collected.

Ammonium sulfate precipitation

In order to purify 47 kDa buckwheat seed AP, the acidic protein extract from developing buckwheat seeds was brought to 80% saturation with solid ammonium sulfate. After centrifugation (10 min at 10000g and 4° C), the pellet was resuspended in 0.1 M sodium acetate buffer (pH 4) with 3 mM $NaHSO_3$ and dialyzed against the same buffer. The resulting extract was chromatographed on pepstatin A-agarose. Proteolytic activity in the obtained fractions was analyzed by SDS-PAGE of the products of BSA digestion.

Enzyme assay

Activity of proteinases in the protein fractions was determined using bovine serum albumin (BSA) as the substrate at pH 3.1 (50 mM citrate buffer with 0.2 M NaCl) with or without pepstatin A (10 μM). The standard assay mixture contained 50 μl of protein fraction protein extract, 30 μl of citrate buffer (pH 3.1), and 20 μg of BSA. The reactions were allowed to proceed for 3 h at 37° C and stopped by the addition of the sample buffer for SDS-PAGE (see below) followed by heating at 100° C for 5 min. The rate of proteolysis and pattern of BSA digestion were estimated by analysis of the electrophoregram. Reaction with 1 μg of chymosin from commercial rennet was performed in the same buffer and conditions.

Activity of AP in protein fractions was also determined using κ-casein as the substrate and products of hydrolysis were resolved by SDS-PAGE (see below). Reaction mixtures contained 50 μl of the protein fraction, 45 μl of 5% κ-casein in buffer for digestion (50 mM sodium citrate, pH 5.0, and 0.2 M NaCl), with or without 10 μM pepstatin A as inhibitor. The reactions were allowed to proceed for 3 h at 37°. The same buffer and conditions were used for reaction with 1 μg of chymosin from commercial rennet.

SDS-PAGE

Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide (Laemmli, 1970). They were treated in denaturing buffer with SDS and β-mercap-

toethanol (optional) and heated before SDS-PAGE. Gels were stained either with 0.2% Coomassie Brilliant Blue R before destaining in 50% methanol, 10% acetic acid, or silver nitrate using the Plusone Silver Staining Kit (*Amersham Pharmacia Biotech*).

Immunoblot analyses

The polyclonal antibodies against barley phytepsin (HvAP) used in this work for AP identity examination were provided by Dr Kirsi Törmäkangas from the University of Helsinki. Antibodies were prepared as described by R u n e b e r g - R o o s *et al.*, (1994).

For immunoblotting, samples were electrophoresed and transferred to PVDF (polyvinylidene difluoride) membrane in 25 mM Tris-HCl containing 192 mM glycine and 20% (v/v) methanol (pH 9.2) using an electrophoretic transfer cell (Bio Rad) at 12 V for 2 hours. The PVDF sheet was soaked for 2 h in a solution containing 100 mM Tris-HCl (pH 8.0) and 1% (w/v) BSA. The membrane was washed three times with 100 mM Tris-HCl (pH 8.0) containing 0.2% (v/v) Tween 20 (TBST) and then incubated overnight with HvAP antibodies diluted with 100 mM Tris-HCl (pH 8.0) and 1% BSA. After three washes with TBST solution, the blot was allowed to react for 1 h with goat anti-rabbit antibodies (1:7500 v/v) labeled with alkaline phosphatase (Sigma). Bound antibody was detected using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) according to the procedure recommended by the manufacturer (Sigma).

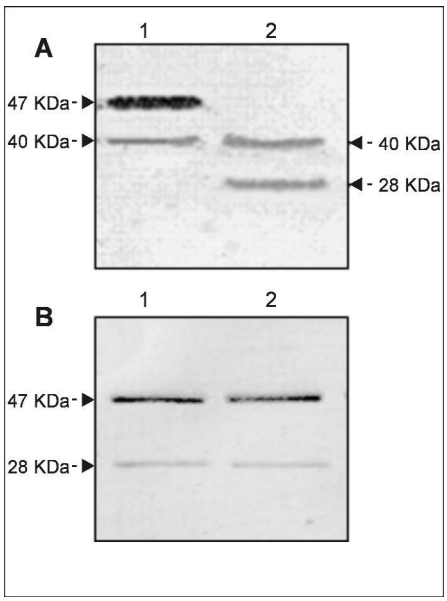

Fig. 1. SDS-PAGE analysis of the fractions obtained after purification of acidic protein extracts of mature (1A) and developing (1B) buckwheat seeds. Fractions (500 µl) were eluted from the column and resolved on 15% SDS-PAGE. The gel was silver stained. Molecular masses of proteins are indicated by arrows.

Analysis of protein glycosylation was performed using the Immun-Blot kit (Bio Rad) according to the manufacturer's instructions after SDS-PAGE and electotransfer to the PVDF membrane.

Preparation of microsomal fraction

Preparation of the microsomal fraction was performed according to F a r o *et al.* (1999). Extracts from developing buckwheat seeds (17-21 DAF) were prepared in buffer containing 8.0 % sucrose, 2mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris, and 20 mM HEPES, pH 7.4. The extract was centrifuged at 10 000g for 15 min at 4 ^{0}C in a Sorvall centrifuge. The supernatant was centrifuged at 100 000g for 60 min at 4^{0}C in a Beckman ultracentrifuge, and the resulting pellet was washed with the extraction buffer and resuspended in the SDS-PAGE loading buffer. The supernatant was subjected to SDS-PAGE also, and SDS-PAGE, electrotransfer, and immunodetection were performed as described above.

RESULTS AND DISCUSSION

Purification of buckwheat aspartic proteinases by affinity chromatography

The presence of specific pepstatin A sensitive acidic proteolytic activity in protein extracts from mature and developing seeds of buckwheat was reported in a previous paper of ours. Immunoblot analysis using cross-reactive antibodies raised against barley phytepsin suggested that this proteolytic activity with features characteristic of aspartic proteinases could be attributed to the 47 kDa heterodimeric polypeptide (T i m o t i j e v i ć *et al.*, 2003). To confirm this assumption, we separated total protein extracts from mature and developing buckwheat seeds using pepstatin-A affinity chromatography, the method specific for isolation of aspartic proteinases. Fractions eluted from the affinity column were separated on SDS-PAGE, silver stained, and analyzed by Western blot as described in Materials and Methods.

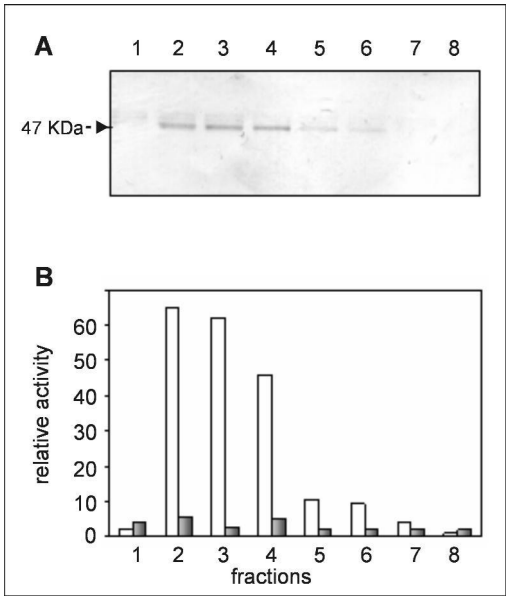

Fig. 2. SDS-PAGE analysis of fractions obtained after purification of acidic protein extract of developing buckwheat seeds by affinity chromatography followed by ammonium sulfate precipitation. Fractions (300 µl) were eluted from the column and resolved on 15% SDS-PAGE. The gel was silver-stained (2A). Histogram of the relative proteolytic activity of the eluted fractions without (white bars) and with pepstatin A (gray bars) (2B).

Three polypeptides of 47 kDa, 40 kDa, and 28 kDa were eluted from the pepstatin A affinity column after applying mature buckwheat seed protein extract (Fig. 1A). These polypeptides cross-reacted with antibodies against barley phytepsin, further confirming their aspartic proteinase-like features (not shown). When the same purification procedure was applied to the protein extract of developing buckwheat seeds, the polypeptide of 40 kDa was missing (Fig. 1B). The polypeptide of 47 kDa corresponds to previously detected buckwheat AP (T i m o t i j e v i ć *et al.*, 2003). That was the only AP form detected in crude protein extract of buckwheat seeds using antiphytepsin antibodies, probably due to its greather quantity in comparison with the 28 and 40 kDa polypeptides. The 28 kDa polypeptide could be a form of AP corresponding to that detected previously by B e l o z e r s k y *et al.* (1984). A polypeptide of with mol. wt. of 40 kDa appeared during late stages of buckwheat seed development and may represent a new form of APs in buckwheat seeds with specific functions. Different forms of APs identified in a buckwheat seeds may have specific substrate specificity appropriate to their specific functions. It is possible that tissue and cellular localization of those APs in furture experiments will offer more elements for elucidation of the function of these proteinases during buckwheat seed development and maturation.

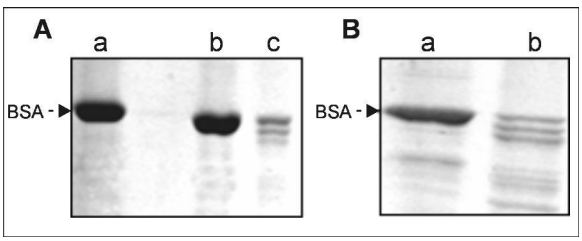

Fig. 3. SDS-PAGE analysis of BSA hydrolysis profile obtained with chymosin from commercial rennet compared to that obtained with purified 47 kDa AP of developing buckwheat seed. 3A: BSA (a), BSA+chymosin incubated in buffer of pH 3.1 with (b) or without (c) pepstatin A. 3B: BSA+47 kDa protein from developing buckwheat seed in buffer of pH 3.1 with (a) or without (b) pepstatin A. The 12 % PAA gel was stained with Coomassie brilliant blue R 250.

Purification of the 47 kDa aspartic proteinase

In order to characterize the 47 kDa AP form in more detail, we tried to separate the given polypeptide out of other buckwheat APs. This was achieved by pepstatin A chromatography after ammonium sulfate precipitation of the acidic protein extract from developing buckwheat seeds. Fractions obtained were analyzed on SDS-PAGE, the results of wich are shown on Fig. 2A. The proteolytic activity of those fractions was investigated in reactions with BSA as the substrate, and products of its degradation were separated by SDS-PAGE. Relative activities were estimated from the amount of degraded BSA determined by computer analysis of electrophoretogram using the Scion Image densitometry program (based on "NIH Image for Macintosh" by Wayne Rasband, National Institutes of Health, USA; modified for Windows by Scion Corporation, 1997) (Fig. 2B). The hydrolytic activity of fractions was significantly inhibited in the presence of 10 µM pepstatin, confirming their AP ability.

In addition, it was found that the purified 47 kDa enzyme is glycosylated (data not shown), as has already been reported for barley phytepsin and some other APs (C o s t a *et al.*, 1997; H e i m g a r t n e r *et al.*,1990).

Specificity of proteolytic activity of the purified 47kDa enzyme

In order to specify the mode of proteolytic action of the purified 47 kDa enzyme, BSA and κ-casein were used as substrates and the chymosin-like pattern of hydrolysis was observed (Figs. 3 and 4). The most important feature

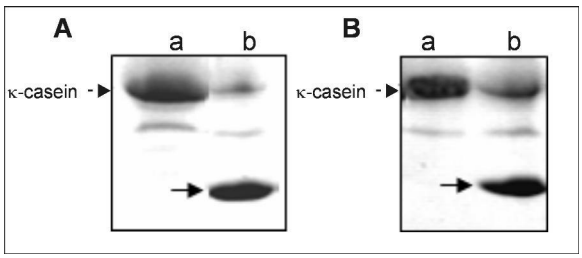

Fig. 4. SDS-PAGE analysis of κ-casein hydrolysis by chymosin from commercial rennet and by 47 kDa AP purified from developing buckwheat seeds. 4A: κ-casein+chymosin incubated in buffer of pH 3.1 with (a) and without (b) pepstatin A. 4B: κ-casein + 47 kDa protein from developing buckwheat seeds in buffer of pH 3.1 with (a) and without (b) pepstatin A. Positions of κ-casein and the 14 kDa product of its proteolytic degradation are marked with arrows. The 12 % PAA gel was stained with Coomassie brilliant blue R 250.

of chymosin is specific and limited hydrolysis of the peptide bond between Phe_{105}-Met_{106} amino acid in κ-casein, which is the key substrate in milk coagulation. The product of proteolytic action of the 47 kDa buckwheat AP on κ-casein as substrate was the same polypeptide of about 14 kDa that was obtained after chymosin action (Fig. 4). This feature explains previously detected milk-cloting activity (T i m o t i j e v i ć *et al.*, 2003). The growing demand for non-animal sources of agents to be used in food production has encouraged the search for substitutes from sources such as microorganisms and plants. In connection with this, it would be of particular interest to investigate the possibility of cloning the genes encoding buckwheat aspartic proteinase, which could be used for the specific engineering of lactic bacteria.

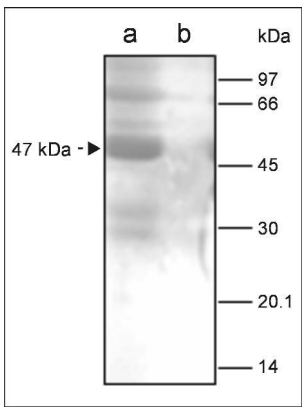

Fig. 5. Immunodetection of aspartic proteinase in subcellular fractions of developing buckwheat seeds. Proteins of microsomal (a) and cytosol (b) fractions were resolved by SDS-PAGE, electroblotted on PVDF membrane and immunodetected by barley antiphytepsin antibodies as described in Materials and Methods.

Subcellular localization of the 47 kDa enzyme

Subcellular fractionation studies were carried out to determine localization of the 47 kDa AP. Protein extract from developing buckwheat seeds was subjected to differential centrifugation, and the resulting pellet and supernatant were analyzed by immunobloting using cross reactive antibodies raised against barley phytepsin. The 47 kDa AP was detected in the pellet of the 100 000g fraction of developing buckwheat seeds (Fig. 5), indicating that the given enzyme is associated with microsomal fractions. This finding suggests that the specific buckwheat AP in question is localized in the protein bodies or vacuoles, as has been shown for most plant Aps. More specific tissue localization studies are needed in order to elucidated the specific function of this enzyme as well as other AP forms identified in the paper.

Abbreviations used

AP, aspartic proteinase; DAF, days after flowering; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mol. wt., molecular weight; PVDF, poly-(vinylidene difluoride); NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; HvAP, *Hordeum vulgare* L. aspartic proteinase.

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АСПАРТИЧНЕ ПРОТЕИНАЗЕ СЕМЕНА ХЕЉДЕ (*FAGOPYRUM ESCULENTUM* MOENCH) – ПРЕЧИШЋАВАЊЕ И СВОЈСТВА ЕНЗИМА 47 kDa

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Анализиране су аспартичне протеиназе семена хељде. Употребом пепстатин А афинитетне хроматографије, из зрелог семена издвојене су три форме аспартичних протеиназа, од 47 kDa, 40 kDa и 28 kDa, док је у екстракту незрелог семена одсуствовала форма од 40 kDa. Протеин од 47 kDa накнадно је раздвојен од осталих форми када је хроматографији претходила амонијум-сулфатна преципитација. Показано је да тип протеолитичког деловања пречишћене форме ензима одговара деловању химозина, аспартичне протеиназе анималног порекла, чиме би се могла објаснити његова способност да коагулише млеко. Ензим је локализован у мембранској ћелијској фракцији.