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Characterization of an Aspartic Proteinase Activity in Buckwheat (*Fagopyrum esculentum* Moench) Seeds

GORDANA S. TIMOTIJEVIC,† SVETLANA R. RADOVIC,§ AND VESNA R. MAKSIMOVIC*,†

Institute of Molecular Genetics and Genetic Engineering and Faculty of Biological Science, Belgrade, Yugoslavia

The pepstatin A sensitive acidic proteolytic activity of total protein extracts of buckwheat seeds has been analyzed in developing, mature, and germinating seeds by activity measurements as well as by electrophoretic and immunochemical techniques. Immunoblot analysis using cross-reactive antibodies raised against barley phytepsin suggested that specific proteolytic activity could be attributed to a 47 kDa heterodimeric polypeptide, composed of two subunits: 31 and 16 kDa polypeptides. The analysis of time course expression revealed that the 47 kDa heterodimer accumulated during seed maturation starting from 12 days after pollination and was also present at the beginning of germination. Milk-clotting activity of this proteinase was also indicated.

KEYWORDS: Buckwheat; aspartic proteinase; milk-clotting activity; immunodetection; "in-gel" assay

INTRODUCTION

Aspartic proteinases (APs) (EC 3.4.23) constitute one of the four superfamilies of proteolytic enzymes. They are a widely distributed class of proteinases present in animals, microbes, viruses, and plants, showing acidic pH optima for enzymic activity, inhibition by pepstatin A, and preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues. Much of our knowledge about the role of APs comes from studies on microbial and animal proteases, whereas the data about the biological functions of these enzymes in plants have become available only recently (*1*).

Plant APs have been purified from monocotyledonous and dicotyledonous species, as well as from gymnosperms, and they are either heterodimeric or monomeric. They are found in distinct parts of different plants: in seeds, grains, leaves, flowers, and roots. In seeds, these enzymes are believed to participate in various proteolytic processes during seed development and germination, although their exact functions are still unknown. To date, it is known that APs take part in maturation processes as well as in the initiation of hydrolysis of storage proteins (*2*−*4*). Possible roles for aspartic proteinases in protein degradation during organ senescence and cell death have also been suggested (*5*−*7*).

Buckwheat (*Fagopyrum esculentum* Moench) is a very interesting species, because of the high nutritive value of its seed proteins. While working on buckwheat seed storage proteins we detected a specific acidic proteolytic activity in the total protein extract (*8*). In this study we investigated this activity further and found that it could be attributed to an aspartic proteinase, the function of which still has to be elucidated. The observed milk-clotting ability of protein extracts from immature seeds could also be related to this specific proteinase, which makes this enzyme very interesting for biotechnological purposes.

MATERIALS AND METHODS

Plant Material and Germination. Buckwheat (*F. esculentum* Moench cv. Darja) was grown in field conditions. Various plant organs, as well as developing and mature seeds, were collected and, if not used immediately, frozen in liquid nitrogen and kept at −70 °C. Before germination, seeds were surface sterilized with 1% (w/v) sodium hypochlorite. Seeds were germinated on filter paper at 25 °C in the dark.

Preparation of Proteinase Extracts. Seeds and the other plant material were frozen in liquid nitrogen and ground in a mortar to a fine powder with a pestle. Proteins were extracted in buffer A [50 mM Tris-HCl, pH 7.5, 0.2 M NaCl (1 mass/2 vol)] on ice for 1 h and centrifuged at 7000*g*.

Enzyme Assay and Inhibition by Pepstatin A. Proteinase activity was measured using bovine serum albumin (BSA) as the substrate at pH 3.1 (50 mM citrate buffer with 0.2 M NaCl) in the presence of 100 μM antipain, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) as inhibitors of cysteine, serine, and metallo-type proteinases. The standard assay mixture contained 50 μL of protein extract, 10 μL of 2% BSA, and 50 μL of sodium citrate buffer. Citrate buffers at various pH values were used for the assay of pH dependence. The reactions were allowed to proceed for 3 h at 37 °C and stopped by the addition of 100 μL (mass/vol) of trichloroacetic acid (10% TCA). After 30 min, the mixture was centrifuged in a microfuge for 15 min. Absorbance of TCA soluble components in the supernatant was

* Address correspondence to this author at Vojvode Stepe 444a, P.O. Box 446, 11000 Belgrade, Yugoslavia (fax ++38113974808; e-mail heljda@sezampro.yu).
† Institute of Molecular Genetics and Genetic Engineering.
§ Faculty of Biological Science.

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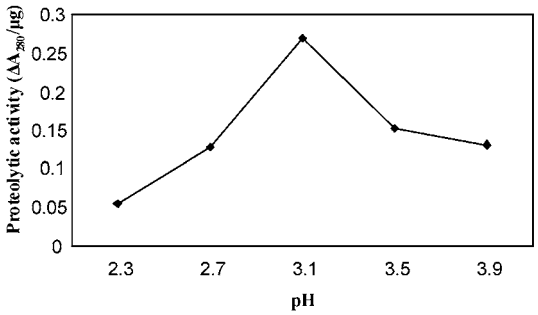

Figure 1. Effect of pH on proteolytic activity of protein extracts from buckwheat seeds. Protein extracts of mature buckwheat seeds were incubated at 37 °C with BSA as a substrate, in the presence of PMSF, antipain, and EGTA. Proteolytic activity was shown as described under Materials and Methods.

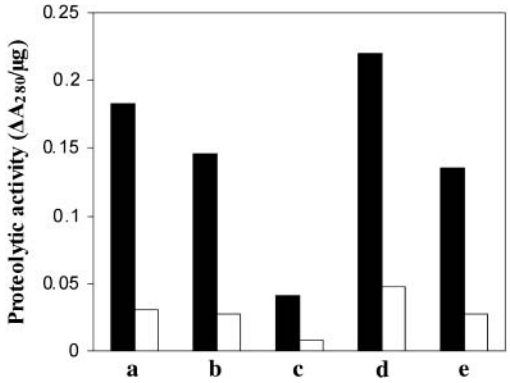

Figure 2. Proteolytic activity of protein extracts from different buckwheat organs: (a–e, pairs of bars from left to right) (a) leaf; (b) germinating seeds; (c) seeds in stage 9–11 DAP, (d) 17–21 DAP, and (e) 28 DAP. Protein extracts were incubated at 37 °C/pH 3.1 with BSA as a substrate. White bars represent data in the presence of PMSF, antipain, EGTA, and pepstatin A; black bars are the same as white bars but in the absence of pepstatin A.

determined at 280 nm. A mixture containing all components of the reaction, immediately treated with 10% TCA, and the reaction mixture without enzyme subjected to standard assay conditions were used as controls. The AP activity was shown as A_{280} per microgram of total proteins in the protein extracts after the standard assay, corrected for A_{280} measured for both controls (in **Figures 1** and **2** given as ΔA_{280}).

To confirm the aspartic proteinase activity, 10 μM pepstatin A was added to the protein extracts, and the mixtures were preincubated on ice for 15 min before the proteinase activity was assayed.

Protein concentration was determined according to the method of Bradford (*9*).

Gel Electrophoresis. Samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide (*10*). Samples were treated in denaturing buffer with SDS and β-mercaptoethanol (optional) and heated before SDS-PAGE. Gels were stained with 0.2% Coomassie Brilliant Blue R and destained in 50% methanol/10% acetic acid. Seminative gels (10% acrylamide) were copolymerized with 0.5% BSA. Samples were treated in 25 mM Tris, pH 6.8/0.08% SDS, without β-mercaptoethanol. After electrophoresis, the gels were washed first with 1% Triton X-100 in 25 mM Tris-HCl, pH 6.8, and then in the sodium citrate buffer, pH 3.1. After incubation in this buffer for 12 h at 37 °C, proteolytic activity appeared as negative bands upon staining with Coomassie Brilliant Blue.

Immunoblot Analysis. The polyclonal antibodies used in this work were provided by Dr. Kirsi Törmäkangas from the University of Helsinki. Antibodies were prepared as described in Runeberg-Roos et al. (*11*).

For immunoblotting, samples were electrophoresed and transferred to poly(vinylidene difluoride) (PVDF) 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 h. 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 the 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 antibody (1:7500 v/v) labeled with alkaline phosphatase (Sigma). Bound antibody was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT)) according to the procedure recommended by the manufacturer (Sigma).

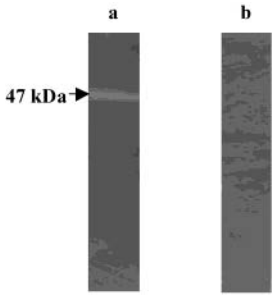

Figure 3. In-gel proteinase assay of protein extract from buckwheat seed: BSA-copolymerized SDS-PAGE of total protein extract from buckwheat seed (17–21 DAP), stained with Coomassie brilliant blue, after incubation in appropriate conditions for proteinase activity without pepstatin A (lane a) or with addition of 10 μM pepstatin A (lane b).

Milk-Clotting Activity. Proteinase extract in buffer A (100 μL) was added to 10 mL of standardized milk (3% fat) containing 10 mM $CaCl_2$ and incubated at 37 °C in a test tube as recommended previously (*6*).

RESULTS

The ability of total protein extracts of buckwheat seeds to degrade BSA in acidic conditions has been detected previously (*8*). Under the same conditions enzyme activity was noticed with casein, hemoglobin, and buckwheat seed storage proteins as substrates. Because it was shown that the observed proteolytic activity was pepstatin A sensitive, we supposed that it could be attributed to an aspartic proteinase. In this paper, the protease activity was further characterized in terms of optimal pH and susceptibility to inhibitors, as well as by immunoblot and "in-gel" assay identification.

The proteolytic activity of buckwheat seed extract as well as the effects of pH on general substrate hydrolysis were studied using BSA as indicated under Materials and Methods. In the presence of inhibitors specific to cysteine, serine, and metallo-type proteinases, the highest proteolytic activity of mature seed extract was obtained at pH 3.1 (**Figure 1**).

In addition to mature seeds, this specific proteolytic activity was detected in developing and germinating seeds, as well as in leaves. In fact, the highest activity was measured using the protein extract from seeds at the midmaturation stage of development [17−21 days after pollination (DAP)]. When 10 μM pepstatin A was added to the reaction mixture, ~80% of the total acid proteolytic activity was inhibited in all organs (**Figure 2**), suggesting that this acidic proteolytic activity could be mainly attributed to aspartic proteinase(s).

To identify the protein fraction(s) present in buckwheat seeds that could be attributed to an aspartic proteinase, the total protein extract from developing seeds (17−21 DAP) was subjected to an "in-gel" assay with incorporated BSA as the substrate. The proteolytic activity of proteinase bands became visible after incubation of the gel in appropriate conditions, optimal for the investigated proteolytic activity (**Figure 3a**). A band representing a polypeptide of molecular weight (MW) of 47 kDa was

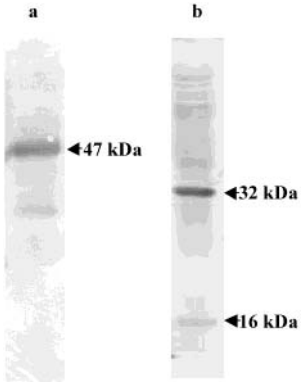

Figure 4. Immunodetection of AP in total protein extracts: total protein extracts from buckwheat seed (17–21 DAP) resolved on SDS-PAGE under (a) nonreducing or (b) reducing conditions.

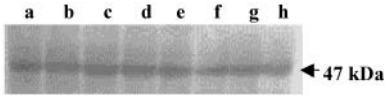

Figure 5. Accumulation of buckwheat AP during seed development: (lane a) 12 DAP; (lane b) 15 DAP; (lane c) 17 DAP; (lane d) 19 DAP; (lane e) 21 DAP; (lane f) 23 DAP; (lane g) 25 DAP; (lane h) 28 DAP. Equal amounts of proteins, extracted from seeds at different stages of maturation, were loaded on 15% polyacrylamide gel, transfered onto PVDF membrane, and immunodetected with anti-phytepsin antibodies.

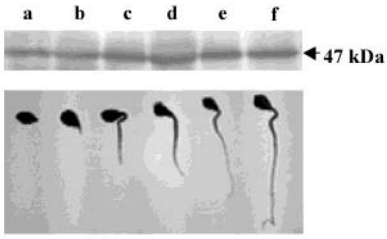

Figure 6. Presence of aspartic proteinase during germination of buckwheat seeds: (lane a) 0 days after imbibition (DAI); (lane b) 1 DAI; (lane c) 2 DAI; (lane d) 3 DAI; (lane e) 4 DAI; (lane f) 5 DAI. Equal amounts of proteins extracted from buckwheat seeds at different stages of germination were loaded on 15% polyacrylamide gel, transferred onto PVDF membrane, and immunodetected with anti-phytepsin polyclonal antibodies.

detected. In the presence of pepstatin A there were no visible bands, indicating the absence of proteolytic activity (**Figure 3b**). Therefore, the 47 kDa polypeptide could represent the buckwheat aspartic proteinase.

Cross-reactivity with antibodies raised against barley phytepsin *Hordeum vulgare* L. aspartic proteinase (HvAP) was used for further identification and characterization of the buckwheat aspartic proteinase. After SDS-PAGE of total protein extract from developing buckwheat seeds under nonreducing conditions and immunodetection, strong reactivity was detected with a polypeptide of MW of 47 kDa (**Figure 4**). Polypeptides of MW of 31 and 16 kDa were detected under reducing conditions, suggesting a heterodimeric nature for the 47 kDa polypeptide.

The expression pattern of this proteinase during maturation and germination of buckwheat seeds was investigated using the above-mentioned immunodetection after SDS-PAGE of equal amounts of proteins isolated from seeds at different stages of maturation and germination. The 47 kDa dimer was detected in all investigated stages (**Figures 5** and **6**).

As the amount of protein is dramatically increased during seed maturation due to accumulation of storage proteins, the almost equal amount of AP detected by Western blot indicated that the observed time course for AP was overlapping with the kinetics of synthesis of seed storage proteins. The 47 kDa dimer accumulated from 12 DAP to the end of seed maturation (**Figure 5**). The same heterodimer was also present throughout the investigated period of germination (8 days) (**Figure 6**).

Milk-Clotting Activity of Protein Extract from Buckwheat Seed. The ability of total protein extracts of buckwheat seeds to degrade casein in acidic conditions has been detected previously (*8*). Regarding this finding, the milk-clotting ability of such extracts was tested as indicated under Materials and Methods. The expected milk-clotting ability of extracts from developing, mature, and germinating seeds was confirmed. The most prominent activity was detected in an extract from developing seeds (17−21 DAP), which is in accordance with the highest proteolytic activity measured for that extract. The clotting time for the extract from developing buckwheat seed was ∼8 h at 37 °C.

DISCUSSION

Aspartic proteinase activity has been detected in different plant organs and tissues, mostly in seeds (*12*−*21*). Pepstatin A sensitive acid proteolytic activity, detected previously in total protein extracts of buckwheat seeds, was further characterized here. According to our results it could be attributed to an aspartic proteinase. This specific activity was noticed in the protein extracts from immature, mature, and germinating buckwheat seeds. The maximum activity observed at pH 3.1 (**Figure 1**) is in accordance with data obtained for other enzymes of this class (*1*).

To investigate further the features of buckwheat aspartic proteinase, we used antibodies raised against barley phytepsin, as well as "in-gel" proteinase assay. The 47 kDa polypeptide in total protein extracts from developing buckwheat seeds formed a band (**Figure 4a**). This cross-reactivity is not surprising. A sequence search between plant APs revealed that homology in the N- and C-terminal regions is significant (*22*). Under reducing conditions, antibodies against phytepsin recognized two polypeptides of MW of 31 and 16 kDa after SDS-PAGE (**Figure 4b**), suggesting that the buckwheat enzyme is composed of two polypeptides linked by disulfide bond(s). These data differ from those published previously by Belozersky et al. (*23*), which showed that aspartic proteinases from dormant buckwheat seed (therein named carboxyl proteinases I and II) have MW of 27.8 kDa. One of the possible explanations for the noticed discrepancy could be the existence of different forms of proteins with the same proteolytic abilities but differently abundant in dormant compared to immature seed. The dimeric type of AP that we found in buckwheat seed has been observed in various plant species. For example, barley phytepsin contains two isoforms of the enzyme, each with two subunits (*24*). Three different heterodimeric enzymes from dried cardoon flowers and two from fresh flowers have been isolated (*6*, *25*). The aspartic proteinase from figleaf gourd has been isolated and shows two protein bands of 30 and 11 kDa (*26*). Several plant APs are monomeric and have a broad range of sizes, such as 60−65 kDa from rice seeds (*27*) and 28 kDa from *Brassica napus* (*2*). An aspartic proteinase from castor bean contains both a heterodimeric (32 + 16 kDa) and a monomeric form (29 kDa). The identity of the 47 kDa polypeptide from developing buckwheat seed as an aspartic proteinase was also confirmed by an "in-gel" proteinase assay (**Figure 3**).

To obtain information related to the possible function(s) of aspartic proteinase in buckwheat seed, the expression time course of the 47 kDa polypeptide during seed development and

germination was investigated. It was shown that this polypeptide accumulated from the 12 DAP stage to maturity. The observed time course overlapped with the kinetics of synthesis of seed storage proteins (*28*), which pointed to a possible role for this proteinase in the maturation of seed storage proteins. However, the presence of aspartic proteinase at the beginning of germination suggests that this enzyme could be also active in other processes, which include regulated protein degradation. Localization of an aspartic proteinase in protein bodies of dry buckwheat seed, as well as its ability to degrade the 13S buckwheat storage globulin in vitro (*29*), is an indication that AP could be involved in the degradation of storage proteins in vivo. Clearly, it is not unreasonable to suppose that different forms of APs could have different roles in specific physiological and stress conditions, as well as in different buckwheat organs or tissues. To date, it is known that APs take part in maturation processes of 2S albumins from *Brassica* (*2*) and *Arabidopsis* (*30*) and in the initiation of hydrolysis of wheat seed storage proteins (*3*). In barley, numerous data suggest that phytepsin performs different functions in protein processing and turnover in different stages of the plant life cycle (*11*, *31*, *32*). Moreover, phytepsin has recently been associated with programed cell death in the developing tracheary elements of barley roots (*33*, *34*). Roles for APs in the degradation of pathogenesis-related proteins (*15*), as well as in the development of tracheary elements and sieve cells (*34*), have also been reported. The precise function of AP in these and other basic processes should be investigated in the future.

Besides the involvement of APs in fundamental processes, this class of enzyme is very interesting because of the ability of some of them to clot milk. This feature of AP from flowers of *Cynara cardunculus* was utilized in the manufacture of Portuguese cheese (*6*, *35*, *36*). As observed in this work, the protein extract from buckwheat seed has milk-clotting ability. This was most prominent for the extract from developing seeds. Having in mind that the highest proteolytic activity specific to an aspartic proteinase was also observed in the seed extract from the 17−21 DAP stage, it is possible that the milk-clotting ability could be attributed to aspartic proteinase. This indication should be investigated in detail with purified enzyme in the future. For the time being, we propose buckwheat seed extract to be very promising in cheese production, especially as an alternative/substitute for animal chymosine. Moreover, cloning of the gene for buckwheat AP and its introduction in the appropriate type of lactic acid bacteria is another biotechnological opportunity.

ABBREVIATIONS USED

AP, aspartic proteinase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MW, molecular weight; DAP, days after pollination; DAI, days after imbibition; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 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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