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# Localization of a metalloproteinase and its inhibitor in the protein bodies of buckwheat seeds

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**Abstract.** Cotyledons of dry buckwheat (*Fagopyrum esculentum* Moench) seeds were used to study the cellular localization of a metalloproteinase which performs in vitro the initial limited proteolysis of the main storage protein of the seed, and of its proteinaceous inhibitor. Fractions of complex protein bodies (PB 1) and of the cytoplasm and membrane material (CMM) were obtained by fractionating cotyledons in a mixture of acetone and $CCl_4$. The greater part of the metalloproteinase activity was found to be localized in the PB 1 fraction, with a lesser amount in the CMM fraction, whereas the metalloproteinase inhibitor was localized almost entirely in the PB 1 fraction. The data obtained indicate that the complex protein bodies of dry buckwheat seeds contain the components of the proteolytic system responsible for the initial degradation of the main storage protein – the 13S globulin – of buckwheat seeds, i.e. 13S globulin, the metalloproteinase, and its inhibitor. This confirms that it is possibile for the metalloproteinase to perform a controlled proteolysis of the 13S globulin in vivo. The effect of divalent cations on the degradation of the 13S globulin was also studied. A mechanism is discussed whereby the proteolysis of 13S globulin is initiated by divalent cations released as a result of phytin decationization during seedling growth.

**Key words:** *Fagopyrum* – Metalloproteinase – Metalloproteinase inhibitor – Protein body – Seed germination – Storage protein (seed) – Zinc proteinase

## Introduction

One of the main processes of seed germination is the hydrolysis of storage proteins, the products of which are used by the growing seedling. Within the storage tissue of seeds the storage proteins are localized in specialized cellular organelles called protein bodies or aleurone grains (Pernollet 1978; Bewley and Black 1985; Sobolev 1985), inside which the storage proteins are hydrolyzed during seedling growth (Harris and Chrispeels 1975; Pernollet 1978; Bewley and Black 1985; Sobolev 1985). Investigation of the mechanism of storage-protein hydrolysis has shown that it proceeds in stages (Hara et al. 1976; Pernollet 1978; Shutov and Vaintraub 1987; Dunaevsky and Belozersky 1989a). Initially, in the majority of plants studied, there is a limited proteolysis of the seed-storage proteins by proteinases of narrow specificity. After that the modified proteins are completely degraded by other enzymes, some of which are synthesized de novo during seedling growth.

According to the available data there are two possible pathways of storage-protein hydrolysis. In legume seeds the limited proteolysis of storage proteins starts a few days after the beginning of seedling growth (Bewley and Black 1985; Shutov and Vaintraub 1987). During this time a cysteine proteinase, absent in dry seeds, is synthesized in the cytoplasm and transferred to the protein bodies where it initiates storage-protein hydrolysis. Further hydrolysis of the modified storage proteins in the legume seeds is carried out by the enzymes which are synthesized during seedling growth including the initiating enzyme, and also by enzymes present in dry seeds. In this case premature initiation of storage-protein proteolysis is impossible because of the absence of the necessary enzyme.

By contrast, investigations of the proteolysis of storage proteins in buckwheat (Dunaevsky et al. 1983; Dunaevsky and Belozersky 1989a, b) and pumpkin seeds (Hara et al. 1976; Hara and Matsubara 1980a, b) have shown that limited proteolysis of the main storage proteins in these two plants starts almost simultaneously with the onset of seedling growth and, apparently, is carried out by a metalloproteinase which is present in the dry seeds. The second stage of the hydrolysis is performed by enzymes appearing in the growing seedlings

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*Abbreviations:* CMM = cytoplasm and membrane material; PAGE = polyacrylamide gel electrophoresis; PB 1 = complex protein bodies with globoids

(Hara and Matsubara 1980b; Dunaevsky and Belozersky 1989b). In this case the protection of the storage proteins from premature hydrolysis by the metalloproteinase (mostly during seed maturation) is achieved by formation of an enzyme-inhibitor complex, the dissociation of which at the onset of seedling growth leads to enzyme activation and initiation of proteolysis. The metalloproteinase inhibitor found earlier in dry buckwheat seeds (Belozersky et al. 1982) may serve as such an inhibitor since it is capable of preventing proteolysis of the main storage protein in vitro.

To prove that the buckwheat metalloproteinase actually initiates storage-protein hydrolysis in vivo and that its activity is regulated by an endogenous inhibitor, it is necessary to demonstrated that both the enzyme and its inhibitor are simultaneously present in the protein bodies where storage-protein hydrolysis takes place. The purpose of the present work was to determine the subcellular localization of the $Zn^{2+}$-containing metalloproteinase from buckwheat seeds performing the limited proteolysis of the main storage protein, the 13S globulin, in vitro (Dunaevsky et al. 1983; Voskoboynikova et al. 1989) and of its inhibitor (Belozersky et al. 1982), and to study the possible ways by which the metalloproteinase is activated at the beginning of seedling growth.

## Material and methods

*Plant material.* Dry seeds of buckwheat (*Fagopyrum esculentum* Moench cv. Shatilovskaya 5) were used. The cotyledons and embryos were obtained by selection from manually crushed seeds. For germination experiments, seeds were grown in darkness at 20° C on wet filter paper in a moist chamber.

*Isolation of protein bodies and cytoplasmic material* was carried out according to the method of Elpidina et al. (1990) with some modifications. A 1.5- to 1.8-g sample of cotyledons together with intact embryos was ground in a mortar with a pestle in acetone under mild conditions at 4–8° C, as described in Elpidina et al. (1990). The homogenate was centrifuged at 17 000 · *g* for 30 min, the sediment carefully suspended in 8–9 ml of acetone, and the suspension allowed to stand for 1–2 min before decanting. Then, 3-ml samples of the resultant suspension were layered onto discontinuous density gradients of mixtures of acetone and $CCl_4$. Each gradient was composed of the following volumes of solution of a given density: 3 ml of 1.53 g · $cm^{-3}$, 6 ml of 1.505 g · $cm^{-3}$, 4 ml of 1.462 g · $cm^{-3}$, 5 ml of 1.406 g · $cm^{-3}$, 5 ml of 1.329 g · $cm^{-3}$, 6 ml of 1.298 g · $cm^{-3}$, and 5 ml of 1.26 g · $cm^{-3}$. The gradients were centrifuged at 80 000 · *g* for 2 h at 4° C in a Beckman L5 65B (Palo Alto, Cal., USA) ultracentrifuge in an SW 27 rotor. After centrifugation, 1- to 2-ml fractions were collected and examined microscopically. Fractions containing complex protein bodies with globoids (PB 1) were located at the bottom of the density zone of 1.505 g · $cm^{-3}$. The cytoplasm and the membrane material (CMM) were present in the fractions of the lightest zone with a density of 1.26 g · $cm^{-3}$. These fractions were pooled, precipitated with cold acetone, and stored under acetone at −20° C.

*Preparation of protein extracts of subcellular fractions, and ammonium-sulfate fractionation.* Combined acetone sediments of fractions, obtained after four to eight isolation procedures, were collected by centrifugation at 10 000 · *g* for 20 min, thoroughly separated from the supernatant, and resuspended in 1% NaCl solution in 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8. Extraction was done in the cold for 4–6 h under periodic stirring. The suspension was then dialyzed against 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8, and centrifuged at 10 000 · *g* for 15 min. Dry $(NH_4)_2SO_4$ was added to the supernatant to 80% saturation and the mixture was kept at 4° C overnight. The resultant precipitate was separated by centrifugation at 44 000 · *g* for 30 min, dissolved in a minimal volume of 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8, and dialyzed against the same buffer. The dialyzate was centrifuged at 10 000 · *g* for 15 min and the protein content assayed.

*Isolation of the metalloproteinase and its inhibitor.* The enzyme was purified as described previously (Voskoboynikova et al. 1989) using $(NH_4)_2SO_4$ fractionation, gel-filtration on Sepharose 6B and ion-exchange chromatography on diethylaminoethyl (DEAE)-Toyopearl.

The activity inhibiting the metalloproteinase was first observed in the fraction of trypsin inhibitors obtained by affinity chromatography, and the isolation procedure for the inhibitor consequently included affinity chromatography on trypsin-Sepharose 4B combined with $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography on DEAE-cellulose, as described in Belozersky et al. (1982).

*Preparation of protein extracts from seeds.* Buckwheat seeds were ground in an electric mill (seedlings were crushed in a mortar) and extracted with 10 mM Na,K-phosphate, 1% NaCl, 0.02% $NaN_3$, pH 6.8, for 1 h at 20° C. The extract was centrifuged (17 000 · *g*, 30 min), the precipitate discarded, and the supernatant used in the experiments.

*Assay of metalloproteinase activity.* The metalloproteinase activity was assayed by the trinitrophenylation technique (Dunaevsky and Belozersky 1989b) with denatured 13S globulin as the substrate (13S globulin was denatured by heating the solution in boiling water for 5 min). A 50- to 100-µl aliquot of a 0.2% solution of 13S globulin in 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8, was added to 50 µl of the enzyme solution (2–50 µg protein). The mixture was incubated for 18 h at 37° C, then 1 ml of a fresh solution of trinitrobenzenesulphonic acid (Serva, Heidelberg, FRG) (0.5 mg · $ml^{-1}$) in 100 mM Na,K-phosphate, pH 8.0, was added and the mixture was incubated for 30 min at 37° C. Finally, the optical density of the solution was measured at 420 nm. The activity of the enzyme preparations was measured twice – in the presence and in the absence of 10 mM EDTA. The difference between the two values was used for the calculation of enzyme activity. One unit of enzyme activity corresponded to the amount of enzyme that caused the formation of 1 nmol of $NH_2$-groups in 1 h under the incubation conditions, using glycine as a standard. The specific activity of the enzyme in the PB 1 and CMM fractions was expressed as the ratio of the total activity of the enzyme preparation, obtained by gel-filtration on Sepharose 6B, to the amount of protein in the fraction investigated, 13S-globulin content excluded.

The metalloproteinase activity in the presence of nondenatured 13S globulin was assayed by polyacrylamide gel electrophoresis (PAGE) at pH 8.9 by recording the increase in the electrophoretic mobility of the protein.

*Assay of the activity of the metalloproteinase inhibitor.* The activity of the inhibitor was determined by trinitrophenylation (Dunaevsky and Belozersky 1989b) with the denatured 13S globulin as a substrate for the metalloproteinase. A 50-µl aliquot of the inhibitor preparation (50–100 µg) in 10 mM Na,K-phosphate, pH 6.8, was added to 50 µl of the enzyme preparation (10–50 µg) in the same buffer so that the ratio enzyme: inhibitor was 1:5, 1:2 or 1:1 (w/w). The mixture was incubated for 1 h at 20° C and the activity of the metalloproteinase was assayed. One unit of inhibitor activity corresponded to the amount that prevented the formation of 1 nmol of $NH_2$-groups in 1 h during substrate hydrolysis under the incubation conditions. The specific activity of the inhibitor was calculated as for the metalloproteinase.

*Polyacrylamide gel electrophoresis.* Analysis by PAGE was carried out in 7.5% gels at pH 8.9 in glass tubes (0.5 cm diameter, 6.5 cm

long) (Davis 1964). Gels were stained with 0.02% Coomassie blue G–250.

*Gel chromatography* was performed on a Sepharose 6B (Pharmacia, Uppsala, Sweden) column (1.0 cm diameter, 50 cm long) equilibrated with 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8. A 2-ml aliquot of the extract of subcellular fraction containing 10–30 mg of protein was applied to the column and eluted at a flow rate of 1 ml · $min^{-1}$ at 4° C.

*Affinity chromatography*. Trypsin (Spofa, Prague, Czechoslovakia) was covalently bound to CNBr-activated Sepharose 4B (Pharmacia) according to a standard procedure (Cuatrecasas et al. 1968). A 7– to 10-mg sample of protein in 50 mM Na,K-phosphate, 500 mM NaCl, pH 7.0, was applied to a trypsin-Sepharose column (0.5 cm diameter, 10 cm long) equilibrated with the same buffer. Elution was carried out with 10 mM HCl containing 500 mM NaCl at a flow rate 1.0 ml · $min^{-1}$ at 4° C.

*Isolation and assay of 13S globulin*. The 13S globulin was obtained by fractionation of the extract of dry buckwheat seeds with 20% acetone followed by gel-filtration on a Sepharose 6B column as described in Dunaevsky and Belozersky (1989a).

Determination of the 13S globulin content in the extracts was done by enzyme-linked immunosorption assay (ELISA) with pyrophosphatase as a label (Baykov et al. 1988). The antibodies to the 13S globulin were purified by $Na_2SO_4$ precipitation followed by chromatography on DEAE-cellulose DE–52. Specific content of the 13S globulin was calculated as the ratio of the 13S globulin content to the residual protein in the fraction.

*Protein determination and concentration*. Protein concentration in solutions was measured according to Lowry et al. (1951) and spectrophotometrically at 280 nm.

Protein solutions were concentrated by ultrafiltration in Amicon (Oosterhout, The Netherlands) cells with the aid of YM–5, PM–5, YM–10 and PM–10 membranes.

*Microscopy*. The preparations of the subcellular fractions were examined under a light microscope equipped with a phase-contrast device. In some cases the preparations were stained by eosine and iodine dissolved in absolute ethanol.

## Results

*Localization of the metalloproteinase*. The intracellular localization of the metalloproteinase was determined by studying the protein extracts of complex protein bodies (PB 1) and of the cytoplasm and membrane material (CMM), obtained by fractionation of dry buckwheat cotyledons using a nonaqueous method with mixtures of acetone and $CCl_4$. A detailed description of the subcellular-fractionation method and results was presented earlier (Elpidina et al. 1990). The PAGE analysis demonstrated no metalloproteinase activity in the salt extract of the PB 1 fraction, i.e. there were no changes in the mobility of the endogenous 13S globulin after incubation of the extract for 48–72 h at 37° C. However, it was reported previously that the metalloproteinase appeared to be present in the form of a complex with its inhibitor in the extracts of dry seeds and, as a consequence of this, noticeable activity of the enzyme was detected only during the course of its purification and separation from the inhibitor (Voskoboynikova et al. 1989). Therefore, to establish the presence of metalloproteinase activity, salt extracts of the PB 1 and CMM preparations were fractionated with $(NH_4)_2SO_4$ and subjected to gel filtration on a Sepharose 6B column, and the protein content and metalloproteinase activity with the denatured 13S globulin were determined in the fractions obtained. Metalloproteinase activity was detected in both the PB 1 and CMM preparations. The active fractions of each preparation were pooled and the metalloproteinase specific activities and their ratios in PB 1 and CMM were determined (Table 1). Similar calculations of specific content were made for the 13S globulin as a standard. This globulin, as well as other storage proteins of dicotyledons, is located in the protein bodies (Pernollet 1978; Bewley and Black 1985; Sobolev 1985), and its presence in the CMM is caused by the partial destruction of protein bodies during homogenization of the cotyledons. The data of Table 1 show that the metalloproteinase is predominantly associated with the protein bodies; however, a considerable but smaller amount of enzyme activity is also present in the CMM fraction, since the ratio of enzyme specific activities in the two fractions is considerably lower than for the marker 13S globulin. The metalloproteinase preparation isolated from the PB 1 fraction performed the limited proteolysis of the nondenatured 13S globulin (Fig. 1), as shown by the characteristic increase in the electrophoretic mobility of the 13S globulin (Dunaevsky and Belozersky 1989a).

**Table 1.** Specific activities of metalloproteinase and its inhibitor in the PB 1 and CMM fractions from buckwheat cotyledons

| Preparation | Specific activity (units/mg protein) | | Ratio of specific activities PB 1/CMM |
|---|---|---|---|
| | PB 1 | CMM | |
| Metalloproteinase | 1.37 | 1.03 | 1.33 |
| Inhibitor | 14.23 | 3.36 | 4.24 |
| 13S globulin[a] | 2.52 | 0.57 | 4.42 |

[a] Specific content of the 13S globulin is given

*Localization of the metalloproteinase inhibitor*. Localization of the inhibitor of the metalloproteinase was also studied in the PB 1 and CMM preparations. Protein extracts of the two preparations were fractionated with $(NH_4)_2SO_4$ and subjected to affinity chromatography on trypsin-Sepharose. The eluates were concentrated, dialyzed against 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8, and the activity of the inhibitor was determined using the denatured 13S globulin as a substrate for the metalloproteinase. The results presented in Table 1 indicate that the metalloproteinase inhibitor is localized almost entirely in the protein bodies since the ratio of its specific activities in fractions PB 1 and CMM is close to the ratio of specific contents of the protein-body marker, the 13S globulin. The inhibitor obtained from the PB 1 fraction prevented hydrolysis of the nondenatured 13S globulin by the metalloproteinase, as indicated by the absence of a change in mobility of 13S globulin after electrophoresis (Fig. 2).

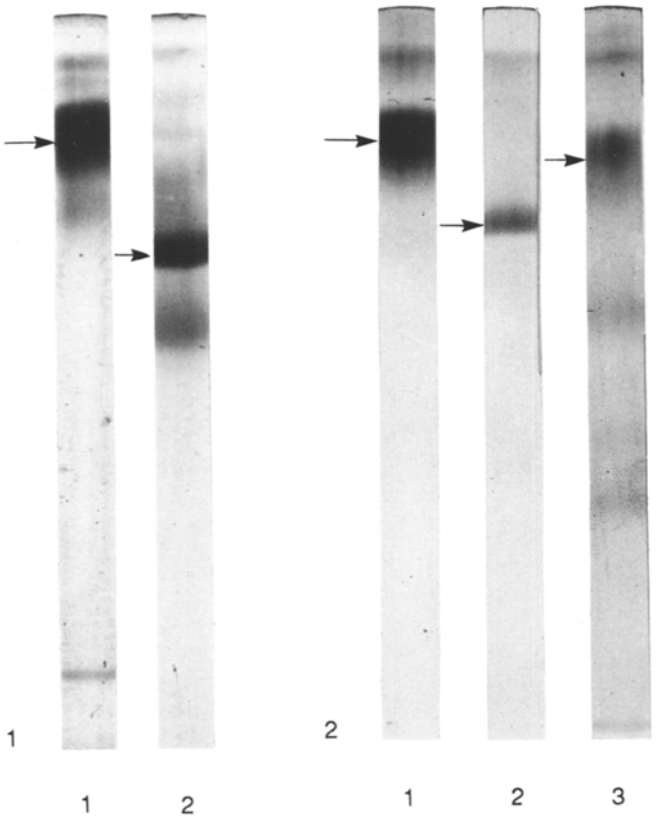


**Fig. 1.** Analysis by PAGE of nondenatured 13S globulin, treated with metalloproteinase from the PB 1 fraction of buckwheat cotyledons. *1*, nonhydrolyzed 13S globulin; *2*, 13S globulin treated with metalloproteinase isolated from the PB 1 fraction. *Arrows* indicate the position of the 13S globulin

**Fig. 2.** Analysis by PAGE of nondenatured 13S globulin, treated with metalloproteinase in the presence of its inhibitor from the PB 1 fraction of buckwheat cotyledons. *1*, nonhydrolyzed 13S globulin; *2*, 13S globulin treated with metalloproteinase; *3*, 13S globulin, treated with metalloproteinase in the presence of its inhibitor isolated from the PB 1 fraction. *Arrows* indicate the position of the 13S globulin

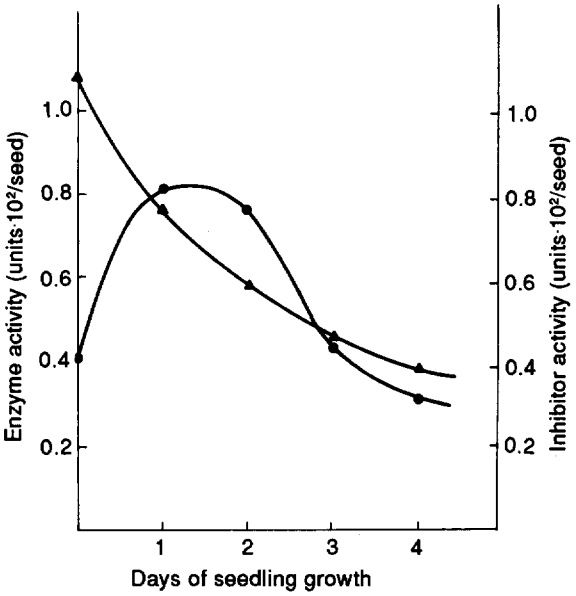


**Fig. 3.** Changes in metalloproteinase (●—●) and inhibitor (▲—▲) activities during growth of buckwheat seedlings. The activity of the metalloproteinase was determined in extracts of dry seeds and seedlings (100 μg) using denatured 13S globulin (100 μg) as described in *Material and methods*. The activity of the inhibitor was determined in the preparation obtained after fractionation of the extracts with $(NH_4)_2S_{04}$ followed by chromatography on trypsin-Sepharose. A 40-μg aliquot of purified metalloproteinase was incubated with 80 μg of the inhibitor preparation for 1 h at 25° C and the residual activity of the enzyme was assayed using denatured 13S globulin, as described in *Material and methods*.

*Activities of the metalloproteinase and its inhibitor during seedling growth.* Figure 3 shows the changes in the activities of the metalloproteinase and its inhibitor during seedling growth. The activity of the metalloproteinase was determined in extracts of seeds and seedlings, and the activity of the inhibitor was assayed in preparations obtained after fractionation of the extracts with $(NH_4)_2SO_4$ followed by affinity chromatography on trypsin-Sepharose. The activity of the metalloproteinase present in dry seeds approximately doubled during the first 2 d of seedling growth and then fell sharply to a value lower than the initial activity. By contrast, the activity of the inhibitor declined throughout seedling growth, the most substantial decrease occurring during the first 2 d.

*The effect of divalent cations on the activity of the metalloproteinase-inhibitor complex.* The interaction of the metalloproteinase with the inhibitor was investigated by studying the influence of several divalent cations on the metalloproteinase-inhibitor complex. Treatment of the enzyme-inhibitor complex with cations caused a noticeable cleavage of 13S globulin, while the activity of the control sample was insignificant (Table 2). When the enzyme preparation was treated with inhibitor which had already been dialyzed against the same cations, the ability to cleave the 13S globulin was also retained (data not shown).

**Table 2.** The effect of divalent cations on the activity of the metalloproteinase-inhibitor complex. Forty μg of purified metalloproteinase preparation (E) were incubated with 40 μg of purified inhibitor preparation (I) in 10 mM Na, K-phosphate, 0.02% $NaN_3$, pH 6.8, for 1 h at 25° C. The complex formed was dialyzed for 16–18 h at 4° C against the same buffer containing 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$ or 1 mM $CoCl_2$. The control samples were dialyzed against the same buffer without metal cations added. After dialysis, metalloproteinase activity was determined in the presence of denatured 13S globulin as described in *Material and methods*

| Reaction mixture | Enzyme activity (%) |
|---|---|
| E | 100 |
| E+I | 8 |
| E+I+$Zn^{2+}$ | 50 |
| E+I+$Mg^{2+}$ | 85 |
| E+I+$Co^{2+}$ | 36 |

Since $Mg^{2+}$ caused maximal recovery of the metalloproteinase activity, we tried to establish the concentration of $Mg^{2+}$ cations required for optimal recovery of

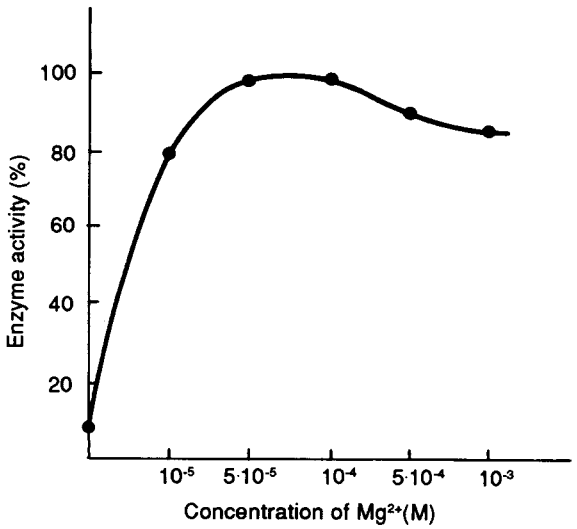


**Fig. 4.** The effect of $Mg^{2+}$ concentration on the metalloproteinase-inhibitor complex. Incubation conditions were the same as in Table 2

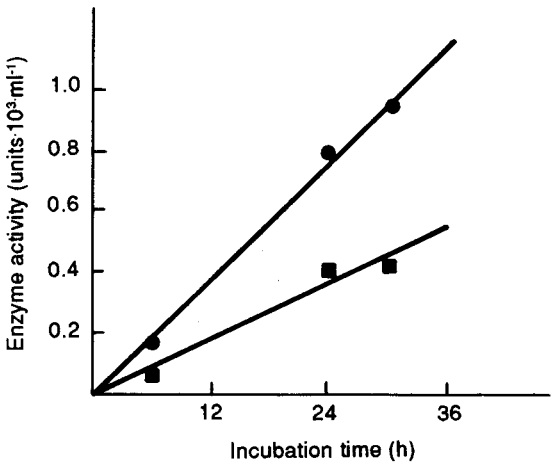


**Fig. 5.** The effect of $Mg^{2+}$ on the hydrolysis of denatured 13S globulin by an extract of dry buckwheat seeds. Two mg of the extract were dialyzed against 10 mM Na,K-phosphate, 0.02% $NaN_3$, pH 6.8, with (●—●) or without (■—■) 0.1 mM $MgCl_2$. During the course of dialysis aliquots were taken and the metalloproteinase activity was determined using denatured 13S globulin, as described in *Material and methods*

enzyme activity. It was found that practically 100% recovery of the enzyme activity could be obtained by 0.1 mM and 0.05 mM $Mg^{2+}$ (Fig. 4).

In addition to the effect of exogenous cations on the complex formed by purified preparations of the metalloproteinase and its inhibitor, the ability of divalent cations to affect the proteolytic activity of a crude extract of dry buckwheat seeds was also investigated. It was established that there was a significant increase in the proteolytic activity of the extract in the presence of $Mg^{2+}$ (Fig. 5).

## Discussion

The question whether the metalloproteinase from dry buckwheat seeds participates in the hydrolysis of the 13S globulin, and that its inhibitor regulates this process in vivo, depends to a large extent on the localization of these proteins at the site of the storage and hydrolysis of the 13S globulin in the protein bodies. Earlier (Elpidina et al. 1990) it was shown that the 13S globulin comprised 72–81% of the total amount of the PB 1-soluble proteins, and that the PB 1 fraction, containing globoids, dominated in the population of protein bodies of dry buckwheat cotyledons; therefore, PB 1 was the fraction where the activities of the metalloproteinase and its inhibitor were initially determined to reside. The CMM fraction was regarded to be an alternative site of localization of these proteins. The data in Table 1 and Figs. 1 and 2 now show that the PB 1 fraction of complex protein bodies, unlike the CMM fraction, is the predominant site of localization of both the metalloproteinase and its inhibitor. All the components of the proteolytic system which can perform the limited proteolysis of 13S globulin in vitro, i.e. the substrate – the 13S globulin, the enzyme – the metalloproteinase and its inhibitor, are present in this fraction. These data confirm that it is possibile for this system to carry out the initial hydrolysis of the 13S globulin in vivo during seedling growth. The fact that the metalloproteinase is present as a complex with its inhibitor is confirmed by the inability of extracts of the protein bodies to autolyze the 13S globulin. It should be noted that the absence or the low level of autolysis of storage proteins in extracts of protein bodies of dry seeds was also observed in legumes, namely mung beans (Harris and Chrispeels 1975) and French beans (Pusztai et al. 1977). However, the authors explained this fact by the absence in dry seeds of enzymes capable of hydrolysing storage proteins.

To the best of our knowledge our work with buckwheat is the first demonstration of the presence of a proteinase-inhibitor-substrate proteolytic system in protein bodies. This fact is consistent with the possibility of protection of the 13S globulin from premature hydrolysis by the metalloproteinase during seed maturation by formation of the enzyme-inhibitor complex; it also indicates the existence of a controlling mechanism for the hydrolysis of this protein.

There have been a number of studies of the localization of enzymes which hydrolyse storage proteins (St. Angelo et al. 1969; Hobday et al. 1973; Chrispeels et al. 1976; Baumgartner et al. 1978; Hara-Nishimura et al. 1982). Thus, the metalloproteinase responsible for the limited proteolysis of the main storage protein of pumpkin seeds was shown to be predominantly localized in the protein bodies (Hara-Nishimura et al. 1982). A proteolytic activity which at pH 4.3 hydrolyzed the storage protein edestin was detected in the protein-body fraction of dry hemp seeds (St. Angelo et al. 1969). Similarly, in pea seeds, 29% of the activity which at pH 5.5, hydrolyzed the azo-derivative of the storage protein was found to be present in the protein-body fraction (Hobday et al. 1973).

In a series of studies using mung-bean seeds it was demonstrated that vicilin peptidohydrolase, a cysteine endopeptidase which initiates the hydrolysis of vicilin, is absent from dry mung-bean seeds. During seedling

growth this enzyme is synthesized de novo in the cytoplasm and is transferred into the protein bodies where it hydrolyzes the storage protein (Chrispeels et al. 1976; Baumgartner et al. 1978). The inhibitor of this proteinase was found to be located in the cytoplasm and, in the authors' opinion, could not therefore participate in the regulation of storage-protein proteolysis (Baumgartner and Chrispeels 1976).

During buckwheat seedling growth, storage-protein proteolysis is apparently initiated by the destruction of the metalloproteinase-inhibitor complex. In fact we observed a sharp increase in metalloproteinase activity during the first 2 d of seedling growth (Fig. 3). This process was accompanied by a decrease in the activity of the metalloproteinase inhibitor that could be accounted for by the proteolysis of the inhibitor in the protein bodies. Similarly, it was shown that in mung-bean seeds a trypsin inhibitor, associated with the protein bodies, is subjected during seedling growth to a limited proteolysis by the enzyme localized in these organelles (Wilson and Tan-Wilson 1987).

It seems reasonable to assume that divalent cations play a substantial role in splitting the metalloproteinase-inhibitor complex since the enzyme is metal-dependent (Dunaevsky et al. 1983; Voskoboynikova et al. 1989) and protein bodies of buckwheat cotyledons contain sufficient amounts of various metal cations, including $Mg^{2+}$, $Zn^{2+}$, $Co^{2+}$ (Sokolov et al. 1981). In dry seeds most of the metal ions are associated with the phytin of the globoids (Pernollet 1978; Bewley and Black 1985; Sobolev 1985), making their active participation in metabolic processes unlikely. However, at the onset of seedling growth decationization and hydrolysis of phytin take place (Bewley and Black 1985; Dmitrieva and Sobolev 1984); the metal cations change to a soluble state and are then able to regulate proteolysis.

We have shown that, at the concentrations used, the divalent cations $Zn^{2+}$, $Mg^{2+}$ and $Co^{2+}$ are able to restore the activity of the metalloproteinase pretreated with the inhibitor (Table 2, Fig. 4), as well as to activate this enzyme in protein extracts of dry buckwheat seeds (Fig. 5). It is important to note that when the inhibitor preparation was dialyzed against the solution containing divalent cations its inhibitory activity towards the metalloproteinase was lost. These data allow us to propose the following mechanism of metalloproteinase activation in buckwheat seeds: divalent cations, released by phytin decationization, compete with $Zn^{2+}$ in the enzyme molecule for binding to the active site of the inhibitor and thus destabilize the enzyme-inhibitor complex, causing its dissociation, or preventing its formation.

It should be noted that in buckwheat and pumpkin seeds, where the enzymes which initiate storage-protein proteolysis are metalloproteinases, most protein bodies have globoids which contain phytin (Hara-Nishimura et al. 1982; Elpidina et al. 1990) and which during seedling growth provide most of the monovalent and divalent cations (Dmitrieva and Sobolev 1984; Bewley and Black 1985). Therefore, it seems probable that the role of metalloproteinases is to initiate the enzymatic proteolysis of storage proteins. By contrast, in legume seeds where most protein bodies lack globoids (Pernollet 1978; Sobolev 1985) and the amount of phytin in these organelles in considerably lower (Dieckert et al. 1962; Sobolev et al. 1976), cysteine proteinases, synthesized de novo, appear to act as the starting enzymes for storage-protein proteolysis (Bewley and Black 1985; Shutov and Vaintraub 1987).

Thus, our data confirm the assumption that in the complex protein bodies of buckwheat seeds the limited initial proteolysis of the main storage protein – the 13S globulin – during seedling growth is carried out by the metalloproteinase. The protein inhibitor of this enzyme and, apparently, divalent cations liberated by phytin decationization take part in the regulation of this process.

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