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In vitro reconstitution of the proteolytic system responsible for hydrolysis of the 13S globulin in buckwheat seeds

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The reconstruction of the proteolytic system responsible for the profound hydrolysis of the main storage protein of buckwheat seeds, 13S globulin, has been carried out in vitro. It was found that subsequent action of a metalloproteinase from dry buckwheat seeds and cysteine proteinase and carboxypeptidase from growing seedlings led to a profound hydrolysis of the storage protein. The 13S globulin that was not hydrolyzed with metalloproteinase was not degraded by the proteolytic enzymes of growing seedlings. A scheme is proposed, which describes the functioning of the proteolytic system hydrolyzing the storage protein in growing buckwheat seedlings in vivo.

Buckwheat; Proteolysis; Reconstruction; Storage protein

1. INTRODUCTION

Degradation of storage substances (storage proteins in particular) is an essential stage of metabolism in growing seedlings. For a long time the study of storage protein proteolysis has been limited either by searching for an enzyme capable of attacking the storage protein in vitro or showing changes in protease activity correlating with those in the storage protein content (for reviews see [1,2]). However, our results [3–5] in total with a number of literature data [6–8] indicate that storage protein degradation is a complex process involving a successive action of several proteolytic enzymes. This degradation may be carried out by proteases from both dry seeds (i.e. synthesized at maturation) and seedlings (i.e. activated or synthesized at seedling growth). Besides, the above proteases reveal their apparent specificity to the protein. Some of them attack the initial storage protein, others hydrolyze the in vivo or in vitro modified storage protein.

In vitro experiments have proved that dry buckwheat seeds contain a metalloproteinase capable of carrying out limited proteolysis of 13S globulin isolated from dry seeds [4]. On the other hand, from growing seedlings we isolated a cysteine proteinase and carboxypeptidase, performing profound hydrolysis of in vivo modified during germination 13S globulin, isolated from 3-day-old seedlings [5]. It should be noted that neither the metalloproteinase from dry seeds nor proteases from seedlings could each separately perform profounded hydrolysis of initial storage protein.

In the present work we tried to carry out reconstruction of a whole system responsible for 13S globulin proteolysis using purified enzymes of both dry seeds and seedlings.

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2. EXPERIMENTAL

2.1. *Plant material*

Dry seeds and seedlings of buckwheat (*Fagopyrum esculentum* Moench) cv. Shatilovskaya-5 were used. Seedlings were grown for 3 days at 20°C in the dark in a moist chamber.

2.2. *Isolation and assay of 13S globulin*

13S globulin was isolated from dry buckwheat seeds by acetone fractionation and gel-filtration on Sepharose 6B [3]. The degree of 13S globulin hydrolysis was determined by PAGE analysis at pH 8.9 for 1 h at 600 V and 5 mA per tube [10]. Gels were stained with Coomassie G-250 in 3.5% chloric acid, and excess dye was removed with 7% acetic acid [11]. Gels were scanned in a Gilford-250 (UK) spectrophotometer at 550 nm.

2.3. *Isolation of proteases*

The metalloproteinase was isolated from dry seeds by ammonium sulfate precipitation, gel-filtration on Sepharose 6B, chromatography on DEAE-cellulose DE-52, gel-filtration on Ultra-gel AcA54 and FPLC chromatography on a Mono S ion-exchange column [4].

Cysteine proteinase and carboxypeptidase were isolated from 3-day-old buckwheat seedlings by ammonium sulfate fractionation, gel-filtration on Sephadex G-150 and ion-exchange chromatography on DEAE-Toyopearl 650 M [5].

2.4. *Protein determination*

Protein was assayed according to Lowry et al. [12]

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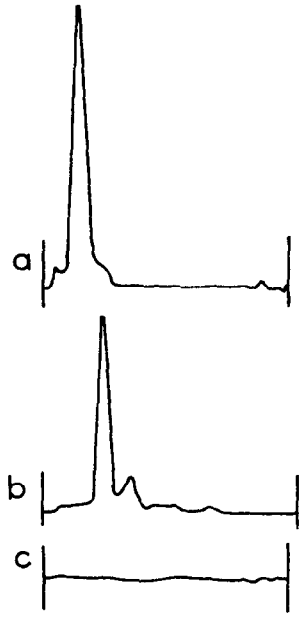

Fig. 1. Densitograms of the preparations of 13S globulin before and after hydrolysis with proteases from dry seeds and growing seedlings after native electrophoresis. (a) 13S globulin from dry seeds; (b) 13S globulin after treatment with the metalloproteinase; (c) 13S globulin after successive treatment with the metalloproteinase and the preparation of cysteine proteinase and carboxypeptidase.

3. RESULTS AND DISCUSSION

To hydrolyze the storage protein with the metalloproteinase, 200 μg (20 mg/ml) of 13S globulin isolated from dry seeds was treated with 10 μg (0.8 mg/ml) of a purified preparation of the metalloproteinase in 10 mM K,Na-phosphate, pH 7.0, 0.025% NaN_3, for 24 h at 37°C. As can be seen in Fig. 1b, the first stage of 13S globulin proteolysis is accompanied by a typical increase in electrophoretic mobility of this protein. By the end of the incubation the electrophoretic mobility of 13S globulin usually stopped changing since the metalloproteinase performed only a limited proteolysis of the protein. After that, the storage protein modified at the first stage of proteolysis was treated with the preparation of cysteine proteinase and carboxypeptidase isolated from 3-day-old buckwheat seedlings. The preparation caused complete degradation of 13S globulin from growing seedlings. The procedure was performed using microdialysis since cysteine proteinase hydrolyzed the modified 13S globulin only when reaction products were constantly removed from the incubation medium. The reaction mixture (120 μl) containing 200 μg of the modified 13S globulin and 20 μg (1.0 mg/ml) of the preparation including cysteine proteinase and carboxypeptidase in 10 mM citrate-phosphate, 3 mM dithiothreitol, 0.025% NaN_3, pH 5.3, was placed into plastic micro-test Tubes 3810 (1.5 ml volume; Eppendorf, Germany) fitted with a dialysis membrane 260-9U (Sigma, USA) secured by a bored cap [9]. The reaction mixture was dialyzed against the same buffer containing dithiothreitol for 4–24 h at 25°C. Fig. 2 shows changes in the 13S globulin content recorded at fixed intervals of hydrolysis of the modified protein. It follows from the results obtained that while proteolysis of the 13S globulin pretreated with metalloproteinase is completed for 18 h, the initial 13S globulin from dry seeds is not practically hydrolyzed under the same conditions. After such hydrolysis of the modified 13S globulin the protein could not be detected on electrophoretograms obtained after native electrophoresis (Fig. 1c).

Thus we managed to achieve a profound hydrolysis of the main storage protein of buckwheat seeds by successive action of the metalloproteinase from dry buckwheat seeds, active at ph 7.0, and the complex of enzymes (cysteine proteinase and carboxypeptidase) from seedlings, active at pH 5.3, which was not possible in the case of separate action of each of the proteolytic enzymes from dry seeds and seedlings. Profound hydrolysis of the initial 13S globulin achieved in the described reconstructed in vitro system argues for the possibility of functioning of such a system in vivo in growing buckwheat seedlings.

Analysis of the literature data available allows us to suggest that a similar sequence of action of proteases is realized in pumpkin seeds [6,7,13], whereas in vetch seeds the sequence of action of proteases involved in the proteolysis of legumin, storage protein of these seeds, is different. First, legumin is attacked by protease A, synthesized during seedling growth, and only then protease B and some others, already present in dry seeds, enter into the hydrolysis [8].

Proceeding from the experimental results obtained in this work and earlier [4,5] we propose a scheme for 13S globulin proteolysis (Fig. 3). According to the scheme the cleavage of the initial 13S globulin from dry buckwheat seeds is initiated by a metalloproteinase, also present in dry seeds, and performing at pH values close to neutral only limited hydrolysis of the storage protein.

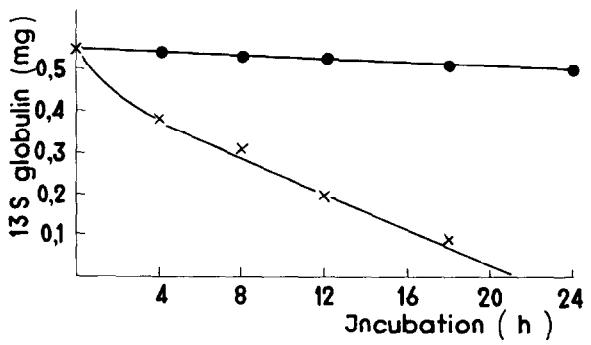

Fig. 2. Effect of the premodification of the 13S globulin on its further hydrolysis with the preparation of cysteine proteinase and carboxypeptidase. (a) cleavage of the 13S globulin unmodified with metalloproteinase; (b) cleavage of the 13S globulin pretreated with metalloproteinase.

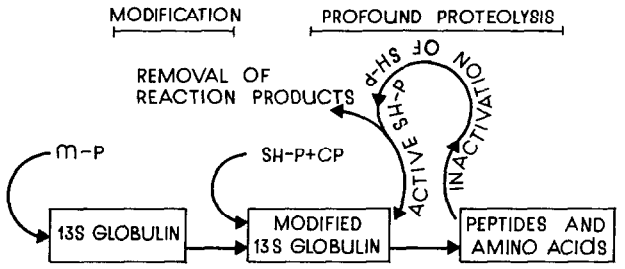

Fig. 3 The scheme of proteolysis of the 13S globulin in growing buckwheat seedlings. M-P, metalloproteinase, SH-P, cysteine proteinase, CP, carboxypeptidase.

Further the modified 13S globulin is attacked by cysteine proteinase, synthesized during germination. This proteinase performs, under more acidic conditions, profound hydrolysis only of the modified storage protein and only when reaction products are constantly removed from the incubation medium. To make the proteolysis more complete it is important that carboxypeptidase be involved at the second stage of 13S globulin degradation. Hence the above mentioned successive actions of the studied proteases result in profound hydrolysis of the storage protein in growing buckwheat seedlings.

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