BIOLOGIA PLANTARUM 42 (1): 81-87, 1999

Mobilization of reserve proteins during early stages of seed germination in *Fagopyrum esculentum*

M.K. ROUT and N.K. CHRUNGOO*

Department of Botany, North Eastern Hill University, Shillong - 793 022, India

Abstract

Hydrolysis of 13S globulin, the main storage protein in grains of common buckwheat (*Fagopyrum esculentum* Moench), proceeds in at least two phases during germination. The first stage, involving a limited proteolytic cleavage of the protein, is associated with increased activity of proteases having maximum activity at pH 7.6. The second stage, involving further hydrolysis of the partially cleaved protein, starts after 12 h of imbibition. During this phase, activity of proteases increased and activity maximum shifted to pH 5.6. Nevertheless, 13S globulin retains its antigenic identity till the emergence of radicle and plumule. Thus, it may not be the major source of amino acids utilized by the germinating seed during the initial stages of imbibition.

Additional key words: buckwheat, 13S globulin, proteolytic activity.

Introduction

The physiological and biochemical processes underlying germination and early seedling growth are important to the establishment of a plant in its environment (Bewley and Black 1978). An important aspect of seed germination is the hydrolysis and mobilization of storage compounds, the products of which are used by the growing seedling prior to the development of autotrophy. Storage proteins synthesized during seed maturation, are degraded during germination to small peptides and amino acids that are subsequently transported to the growing seedling. In legume seeds, limited proteolysis of the storage protein starts after 48 - 72 h of imbibition (Bewley and Black 1985, Shutov and Vaintraub 1987). Proteolysis of the main storage protein in seeds of non-leguminous plants starts almost simultaneously with the onset of imbibition (Hara *et al.* 1976, Hay *et al.* 1991). In pumpkin seeds, initial proteolysis is carried out by a metalloprotease already present in dry seeds

Received 1 December 1997, *accepted* 17 May 1998.
Acknowledgements: The authors are grateful to Head, Department of Botany, for laboratory facilities. MKR acknowledges the award of a research fellowship by UGC, New Delhi.
* Corresponding author; fax: (+91) 364 250076, e-mail: nchrungoo@hotmail.com

(Hara and Matsubara 1980a,b). The presence of a metalloprotease has also been demonstrated in dormant seeds of common buckwheat, an economically important crop because of the high protein content of its grains (Elpidina *et al.* 1991).

The present investigation was undertaken to determine the pattern of proteolytic cleavage of 13S globulin, the legumin-type seed storage protein of common buckwheat, during early stages of seed germination.

Materials and methods

Grains of common buckwheat (*Fagopyrum esculentum* Moench) were obtained from the North Eastern Regional Station of National Bureau of Plant Genetic Resources, Shillong, India. Healthy grains were surface sterilized for 15 min in 0.25 % sodium hypochlorite followed by rinsing with sterile deionized water. The grains were germinated on a moist filter paper (*Whatman No. 1*) under continuous white light (30 μmol m^{-2} s^{-1}) at 25 ± 2 °C. Samples were harvested in triplicate at periodic intervals till the completion of radicle and plumule emergence. Embryo and endosperm portions of the grains were separated manually. Dry matter content of the embryo and endosperm was determined by drying a known fresh mass of the material at 100 °C for 72 h.

A known fresh mass of the embryo and endosperm portions was also fixed in boiling 80 % ethanol. The fixed tissue was macerated and then separated into alcohol soluble and alcohol insoluble fractions by centrifugation. Total free amino acids were estimated from the alcohol soluble fractions according to Rosen (1957). Isolation of 13S globulin and preparation of antibodies against the protein has been described previously (Rout and Chrungoo 1996). Ouchterlony double diffussion test of the protein was carried out in 0.8 % agarose gel with antiserum directed against buckwheat 13S globulin according to Hudson and Hay (1989). Total protein extracts from grains harvested at various stages of germination were subjected to electrophoresis under non-denaturing conditions followed by blotting to nitrocellulose membrane according to Towbin *et al.* (1979). The 13S globulin was identified by incubating the nitrocellulose sheets with antiserum directed against the 280 kD (13S) globulin followed by incubation with goat anti-rabbit IgG.HRP conjugate. The content of 13S globulin in grains at different stages of germination was determined by ELISA according to the procedure described by Harlow and Lane (1988).

Freshly harvested mass of the endosperm, from grains harvested at periodic intervals, was homogenized separately in chilled glass distilled water in a chilled pestle and mortar to obtain a 20 % (m/v) homogenate. The homogenate was strained through two folds of muslin and centrifuged at 6 000 *g* for 15 min at 4 °C. Protease activity was estimated from the supernatant fraction according to Benyon and Bond (1989) using azocasein as the substrate. Soluble protein was estimated from endosperm and embryo tissues of the harvested grains according to Bradford (1976).

Results and discussion

Immediately upon exposure to water, the grains showed a rapid increase in the moisture content. The pattern of increase in moisture content of the endosperm followed a typical hyperbolic curve without any lag phase. In the embryo, however, the uptake of water showed a lag upto 18 h of imbibition after which it followed a pattern similar to that observed in endosperm (Table 1). There was a six fold decrease

Table 1. Changes in the moisture content [%] in the endosperm and embryo of grains of common buckwheat during germination (mean ± SE, $n = 3$).

Imbibition [h]	Endosperm	Embryo
0	18.2 ± 1.21	20.0 ± 0.91
2	57.1 ± 3.32	25.0 ± 0.82
6	70.0 ± 3.48	25.0 ± 0.71
12	75.0 ± 2.58	22.3 ± 0.90
18	85.7 ± 2.79	40.0 ± 1.52
24	93.3 ± 3.31	58.3 ± 1.78
36	93.7 ± 4.25	64.3 ± 3.10
48	94.1 ± 4.22	68.7 ± 2.89
72	94.4 ± 3.25	76.5 ± 3.08

in the dry matter content of endosperm during the 72 h of imbibition. Dry mass of the embryo, however, showed a two fold increase upto 12 h after which it decreased marginally till 72 h (Fig. 1).

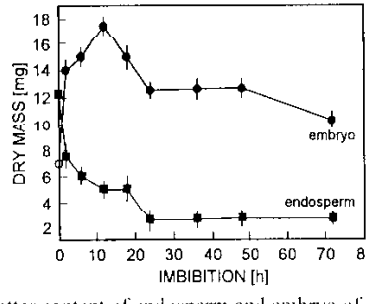

Fig. 1. Changes in the dry matter content of endosperm and embryo of grains of common buckwheat during germination. Means ± SE; $n = 3$.

Ouchterlony double diffussion test of total protein extracts, from grains harvested at various stages of germination, with antibodies raised against buckwheat 13S globulin revealed that the buckwheat 13S globulin retained its antigenic identity upto 72 h of imbibition (Fig. 2*A*). Western blot analysis of the protein further revealed that the electrophoretic mobility of the protein increased only after 12 h of imbibition. However, the protein showed antigenic cross reactivity even at 96 h of imbibition (Fig. 2*B*).

There was a nearly four fold decrease in the level of free amino acids in the endosperm during the initial 6 h of imbibition. Between 6 and 36 h the amino acid content showed a more than 10 fold increase. Beyond 36 h the content of free amino acids in the endosperm decreased markedly. In the embryo, the level of free amino acids did not change markedly upto 12 h of imbibition after which it increased steadily (Fig. 3*A*). An almost four fold increase in the content of soluble protein was

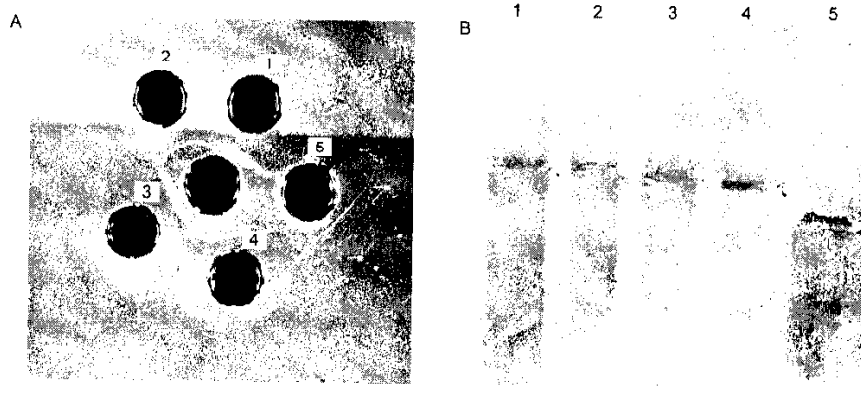

Fig. 2*A*. Ouchterlony double immunodiffussion of crude protein extract from buckwheat grains with undiluted antiserum raised against 280 kD (13S) buckwheat globulin. *Central well*: undiluted antiserum raised against the 280 kD (13S) buckwheat globulin; extract from dry (*well* 1), 24 h (*well* 2), 48 h (*well* 3), 72 h (*well* 4), and 96 h (*well* 5) imbibed grains. 2*B*. Western blot of total protein extract of grains with antiserum directed against 13S globulin. *Lane* 1: dry grains, *lanes* 2,3,4, and 5: grains imbibed for 12, 24, 48, and 96 h, respectively.

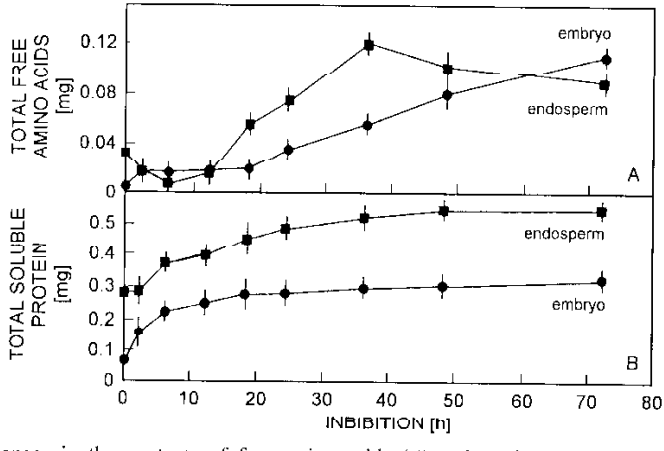

Fig. 3. Changes in the contents of free amino acids (*A*) and total soluble proteins (*B*) in the endosperm and embryo tissues of grains of common buckwheat during germination. Means ± SE; $n = 3$.

observed in the endosperm during the initial 36 h of imbibition. Beyond 36 h, the soluble protein content decreased markedly. In the embryo, soluble protein levels increased six fold during the 72 h of imbibition. However, during the initial 6 h, the increase was more than four fold (Fig. 3*B*).

When expressed as percent of total protein, no significant change was observed in the content of 13S globulin during the initial 12 h of imbibition. Beyond 12 h, the content of 13S globulin decreased gradually (Fig. 4*A*).

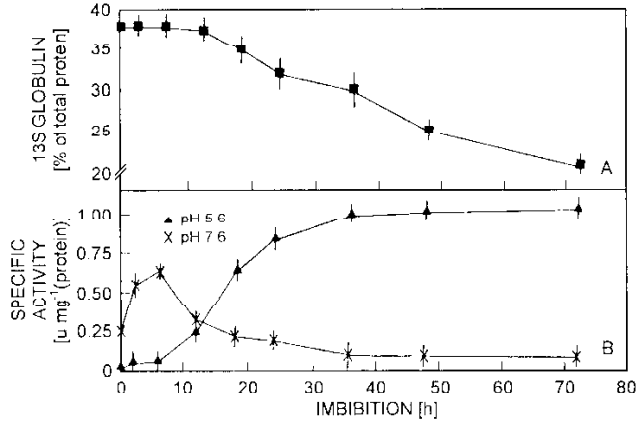

Fig. 4*A*. Changes in the content of 13S globulin [%(total proteins)] in grains of common buckwheat during germination. 4*B*. Changes in proteolytic activity assayed at pH 5.6 and 7.6 in the endosperm tissues of grains of common buckwheat during germination. Means ± SE; $n = 3$.

The protease activity from endosperm tissues showed two peaks, one at pH 5.6 and the other at pH 7.6. At pH 7.6, even the unimbibed grains showed significant levels of proteolytic activity. The activity at this pH showed more than two fold increase during the initial 6 h of imbibition after which it decreased and nearly disappeared after 36 h of imbibition. However, when the activity was assayed at pH 5.6, no significant proteolytic activity could be detected in endosperm tissues of buckwheat grains during the initial 6 h of imbibition. Beyond 6 h, the activity showed a more than four fold increase till 72 h of imbibition (Fig. 4*B*).

The changes in the dry matter content of the endosperm and embryo reveal a rapid uptake of water and mobilization of dry matter from endosperm to the embryo during the early stages of germination. Hydrolysis of reserve proteins in the endosperm and remobilization of the released peptides and amino acids to the embryo for maintenance of growth are essential features of seed germination (Fincher 1989, Vierstra 1993). Changes in the electrophoretic mobility of 13S globulin during germination of buckwheat grains indicates that the protein does not undergo much proteolytic cleavage during the early stages of germination. Results of the Western blot assay clearly established that the protein retained its antigenic identity even upto 96 h. Untill this time the process of radicle and plumule emergence is nearly complete. It can thus be inferred that the 13S globulin retained its structure even upto

the time of completion of radicle and plumule emergence. These results are in agreement with the observations of Dunaevsky and Belozersky (1989). They have observed that buckwheat 13S globulin retained its structural integrity till 3 d of seed germination. Similar observations have been made for soybean β-conglycinins (Bryant *et al.* 1995). The decrease in the content of free amino acids in the endosperm during initial 6 h of imbibition could be ascribed to their utilization in the synthesis of soluble proteins.

Changes in the pattern of proteolytic activity in buckwheat grains during germination indicate the involvement of two different enzyme systems in the hydrolysis of 13S globulin. During initial 6 h, the 13S globulin is subjected to limited proteolysis by proteases active at pH 7.6. The 2nd stage, involving further hydrolysis of the partially cleaved protein by proteases with activity maximum at pH 5.6, starts after 6 h of imbibition. Such a biphasic pattern of proteolysis conforms to the type observed in cotyledons of pumpkin seeds (Hara *et al.* 1976) and endosperm of maize (Hay *et al.* 1991). Kamata *et al.* (1991) have shown that degradation of soybean glycinins proceeds by an initial cleavage of the sites that are exposed on the surface of the protein complex. Other sites embedded in the core of the protein become accessible to proteolysis only after the initial cleavages. Callis (1995) has suggested that the initial proteolytic cleavage is more specific and rate limiting than subsequent hydrolysis to free amino acids by less specific endo- and exopeptidases. Considered along with changes taking place in the content of 13S globulin and the contents of free amino acids and soluble protein these results indicate that the 13S globulin may not be the major source of amino acids utilized by grains of common buckwheat during initial 6 h of imbibition. Contributions towards this pool as a result of proteolysis of 13S globulin start much later.

References

Benyon, R.J., Bond, J.S. (ed.): Proteolytic Enzymes: A Practical Approach. - IRL Press, Oxford 1989.

Bewley, J.D., Black, M.: Physiology and Biochemistry of Seeds in Relation to Germination. Vol. I. Development, Germination and Growth. - Springer-Verlag, Berlin 1978

Bewley, J.D., Black, M.: Physiology of Development and Germination. - Plenum Press, New York 1985.

Bradford, M.A.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Bryant, J.L., Hosangadi, P., Beachy, R.N.: The β-conglycinins of soyabean remain assembled in a 7S conformation while undergoing proteolytic cleavage during germination and early seedling growth. - Plant Cell Physiol. **36**: 1059-1065, 1995.

Callis, J.: Regulation of protein degradation. - Plant Cell **7**: 845-857, 1995.

Dunaevsky, Y.E., Belozersky, M.A.: Proteolysis of the main storage protein of buckwheat seeds at early stages of germination. - Physiol. Plant. **75**: 424-428, 1989.

Elpidina, E.N., Voskoboynikova, N.E., Belozersky, M.A., Dunaevsky, Y.E.: Localization of metalloproteinase and its inhibitor in the protein bodies of buckwheat seeds. - Planta **185**: 46-52, 1991.

Fincher, G.B.: Molecular and cellular biology associated with endosperm utilization in germinating cereal grains. - Annu. Rev. Plant Physiol. Plant mol. Biol. **40**: 305-346, 1989.

Hara, I., Matsubara, H.: Pumpkin (*Cucurbita* sp.) seed globulin V. Proteolytic activities involved in globulin degradation in ungerminating seeds. - Plant Cell Physiol. **21**: 219-232, 1980a.

Hara, I., Matsubara, H.: Pumpkin (*Cucurbita* sp.) seed globulin VI. Proteolytic activities appearing in germinating cotyledons. - Plant Cell Physiol. **21**: 233-245, 1980b.

Hara, I., Wada, K., Matsubara, H.: Pumpkin (*Cucurbita* sp.) seed globulin II. Alterations during germination. - Plant Cell Physiol. **17**: 815-823, 1976.

Harlow, E., Lane, D.: Antibodies: a Laboratory Manual. - Cold Spring Harbor Laboratory Press. Cold Spring Harbor 1988.

Hudson, L., Hay, F.C.: Practical Immunology. - Blackwell. London 1989.

Kamata, Y., Fukuda, M., Sone, H., Yamauchi, F.: Relationship between limited proteolysis of glycinin and its conformation. - Agr. biol. Chem. **55**: 149-155, 1991.

Rosen, M.K.: A modified ninhydrin colorimetric method for analysis of amino acids. - Arch. Biochem. Biophys. **67**: 10-15, 1957.

Rout, M.K., Chrungoo, N.K.: Partial characterization of the lysine rich 13S globulin from buckwheat (*Fagopyrum esculentum* Moench): Its antigenic homology with seed proteins of other crops. - Biochem. mol. Biol. int. **40**: 587-595, 1996.

Shutov, A.D., Vaintraub, I.A.: Degradation of storage proteins in germinating seeds. - Phytochemistry **26**: 1557-1566, 1987.

Towbin, H., Staehelin, T., Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. - Proc. nat. Acad. Sci. USA **76**: 4350-4354, 1979.

Vierstra, R.D.: Proteolysis in plants: mechanisms and functions. - Plant mol. Biol. **32**: 275-302, 1993.