Planta (Berl.) 112, 301—308 (1973)
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Enzymic Activities and Galactomannan Mobilisation in Germinating Seeds of Fenugreek (*Trigonella fœnum-graecum* L. *Leguminosae*)

Secretion of α-galactosidase and β-mannosidase by the Aleurone Layer*

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Received March 5/April 4, 1973

Summary. The activities of α-galactosidase, β-mannosidase and α-mannosidase were determined in extracts from the endosperm and from the embryo of fenugreek seeds at different stages of germination.

Endosperm homogenates contained little or no activity of the above enzymes in the early stages of germination, before the reserve galactomannan began to be mobilised. The onset of galactomannan breakdown coincided with the appearance of α-galactosidase and β-mannosidase activities, which increased throughout the period of galactomannan degradation and then remained constant. A similar rise in α-galactosidase and β-mannosidase activities occurred during galactomannan breakdown in *dry-isolated endosperms* incubated under germination conditions. The increase could be suppressed by metabolic inhibitors which also inhibit galactomannan breakdown.

Embryo homogenates contained high α-galactosidase, high α-mannosidase and some β-mannosidase activity at all stages of germination.

No "oligomannosyl β-1,4 phosphorylase" activity could be detected either in the endosperm or in the embryo.

It is concluded that the galactomannan of fenugreek is broken down by a series of hydrolases secreted by the aleurone layer of the endosperm. They include α-galactosidase, β-mannosidase and probably also *endo*-β-mannanase.

Introduction

Plants belonging to several of the tribes of the *Leguminosae* have albuminous seeds, *i.e.* seeds which retain an endosperm when mature. The leguminous endosperms are storage organs and contain reserve polysaccharides of the galactomannan type. These all have a common basic structure, consisting of a linear "backbone" of β-1,4 linked D-mannopyranose residues to which the D-galactopyranose units are attached α-1,6 (Smith and Montgomery, 1959). The ratio galactose: mannose

* This is part four in a series of papers dealing with galactomannan metabolism. Part three: Planta (Berl.) **106**, 44–60 (1972).

varies from tribe to tribe and is chemotaxonomically significant (Reid and Meier, 1970; Kooiman, 1972).

According to Reese and Shibata (1965), the complete hydrolytic breakdown of a leguminous galactomannan would require at least three different enzymes, namely α-galactosidase (EC 3.3.1.22), *endo*-β-mannanase and β-mannosidase (EC 3.2.1.25). The α-galactosidases are very common constituents of leguminous seeds (Dey and Pridham, 1972), but are by no means confined to those which store galactomannan. The β-mannanases are less common and have been reported to be present in a few leguminous seeds all of which contain galactomannan (Whistler and Smith, 1952; Courtois and Le Dizet, 1963; Hylin and Sawai, 1964; Clermont-Beaugiraud and Percheron, 1968). That of fenugreek (Clermont-Beaugiraud and Percheron, 1968) is a typical *endo*-polysaccharidase, capable of degrading β-1,4 linked mannan chains only as far as mannobiose. No β-mannosidase has ever been detected in leguminous seeds as far as we are aware. Extracts of germinating fenugreek (Clermont-Beaugiraud, 1968) and a number of other galactomannan-containing leguminous seeds (Sømme, 1970, 1971) have been examined and found to be devoid of β-mannosidase activity. Her failure to detect β-mannosidase led Sømme (1970) to suggest phosphorolysis as an alternative to hydrolysis for the mechanism of galactomannan breakdown, and a recent note by Foglietti and Percheron (1972) seems to support this hypothesis. They found that crude homogenates of germinating fenugreek seeds were apparently capable of catalysing the reversible phosphorolysis of β-1,4 linked mannooligosaccharides with a degree of polymerisation greater than two.

We considered that a phosphorolytic breakdown of the polysaccharide was incompatible with the mechanism which we were recently able to establish for galactomannan mobilisation in germinating fenugreek and related leguminous seeds (Reid, 1971; Reid and Meier, 1972). In particular phosphorolysis would not explain the following observations:

1. The galactomannan is broken down to free galactose and mannose, which are taken up from the endosperm into the cotyledons where they are transformed into sucrose and transitory starch (Reid, 1971).

2. The enzymes responsible for the degradation of the polysaccharide are extracellular. They are synthesised by the cells of the aleurone layer and secreted into the non-living, galactomannan-containing storage tissue (Reid and Meier, 1972). We know of no extracellular phosphorylases.

In the present paper we report that the enzymes responsible for the breakdown of fenugreek galactomannan are hydrolases and prove that two of them, α-galactosidase and β-mannosidase, are produced by the aleurone layer. A third hydrolytic enzyme, *endo*-β-mannanase, is also involved in the breakdown process and will be described in a later communication.

Materials und Methods

Seeds. The fenugreek seeds were from plants grown during 1971 in Freiburg (Switzerland) from the seeds described in the previous two publications in this series. They were larger and heavier (ca. 18 mg/seed), and contained more galactomannan per seed than the parent English-grown seeds (weight ca. 12 mg/seed).

Germination and Incubation Conditions. The methods of seed germination and incubation of dry-isolated endosperms have been described elsewhere (Reid and Meier, 1972). As before, all manipulations and incubations were carried out under sterile conditions.

Hydrolase Preparations. Endosperms. Batches of 10 endosperms (+seed coats) manually removed from seeds at different stages of germination *or* batches of 20 dry-isolated half endosperms (+seed coats) which had been incubated under germination conditions, were ground in a Potter homogeniser with 3 ml of McIlvaine buffer, pH 5.0. This caused complete disintegration of the endosperm but the seed coats remained intact at the bottom of the homogeniser cylinder. The supernatant suspension was used as the enzyme preparation. *Embryos.* The endosperms were removed from batches of 10 germinating seeds and the cotyledons rinsed in buffer to eliminate adhering endosperm material. The embryos were added to buffer (3 ml) and ground to a fine suspension which was used as the enzyme preparation. Embryos from resting seeds were obtained by dissection of the dry seed.

Hydrolase Assays. The substrates were the commercially obtained *p*-nitrophenyl glycosides. All incubations were carried out at pH 5.0, which is within 0.3 pH units of the experimentally determined optima for fenugreek endosperm α-galactosidase and β-mannosidase (Reid, unpublished), and is also close to the published optima for the α-galactosidase (Courtois and Percheron, 1961) and α-mannosidase (Beaugiraud *et al.*, 1968) obtained from homogenates of whole, germinating fenugreek seeds. The incubation mixture consisted of McIlvaine buffer, pH 5.0 (0.25 ml), enzyme (0.1 ml) and 0.01 M substrate (0.05 ml). Incubation was at 25° during 15 min in the case of α-galactosidase determinations, and during 60 min for the others. The reactions were stopped by the addition of 0.1 M Na_2CO_3 (5 ml) and the absorptions read at 400 nm against a blank to which the substrate had been added at the end of the incubation time. Embryo preparations were clarified by centrifugation before measurement of the optical density. A unit of enzyme activity is the amount that hydrolyses 1 μmole of substrate per minute under the conditions of assay. The millimolar extinction coefficient of *p*-nitrophenol in 0.1 M Na_2CO_3 at 400 nm was taken to be 1.84×10^4.

Assay of Oligomannosyl β-1,4 Phosphorylase. This was carried out under conditions corresponding as closely as possible to those described by Foglietti and Percheron (1972). However the seeds were incubated under sterile conditions and then dissected into endosperm and embryo which were assayed separately. The seeds were allowed to germinate for 48 hours. Batches of 10 endosperms or embryos were ground in a Potter homogeniser with 3 ml of a Tris (0.05 M)-EDTA (0.01 M) buffer, pH 7.5. The homogenate was pressed through cloth and the residue re-extracted with the same buffer containing 1% Triton X-100. The combined extracts were allowed to stand 1 hour at 4°, centrifuged (1400 g; 15 min), and the supernatant used as the enzyme preparation. The incubation mixture contained KF (0.1 μMole), AMP (0.1 μmole), mannose-1-phosphate (2.5 μmole) as substrate, mannotriose (2.5 μmole) as acceptor, and enzyme preparation (0.2 ml), in a total volume of 0.5 ml. To compensate for phosphate release due to phosphatase action the controls contained enzyme but no acceptor. After incubation for 24 hours at 37°, inorganic phosphate was determined by the method of Fiske and Subbarow.

Results

1. Hydrolase Activities in Endosperms and Embryos of Germinating Seeds. The activities of α-galactosidase, β-mannosidase and α-mannosidase were measured in embryo and in endosperm homogenates from seeds at different stages of germination. Although an α-mannosidase cannot be directly involved in galactomannan breakdown, Beaugiraud *et al.* (1968), have nevertheless isolated one from germinating fenugreek seeds, and α-mannosidase activity has been found to be high in other germinating leguminous seeds (Sømme, 1970, 1971). Endosperm and embryo tissues were examined separately because the galactomannan is present only in the former, and because they perform quite different metabolic functions during germination (Reid, 1971; see also "Introduction").

In the *endosperm* (Fig. 1 A) the early stages of germination are marked by the virtual absence of any activity of the three enzymes assayed. Alpha galactosidase activity begins to increase after about 20–24 hours germination, and β-mannosidase activity begins to rise a few hours later. Both activities increase steeply until about 60 hours germination time and then remain constant. The activity of α-mannosidase in the endosperm increases only slightly throughout germination.

Extracts of resting and germinating *embryos* (Fig. 1 B) contain high α-galactosidase, high α-mannosidase and low β-mannosidase activities which change only slightly during germination.

2. Hydrolase Activities in Isolated Endosperms Incubated under Germination Conditions: Inhibition by Cycloheximide, Abscisic Acid, 5-Fluorouracil and Actinomycin D. Reid and Meier (1972) have shown that when dry-isolated fenugreek endosperms (devoid of embryo) are incubated under germination conditions the galactomannan is broken down to free galactose and mannose. The breakdown can be prevented by 2,4-dinitrophenol and cycloheximide, but it is only slightly inhibited by actinomycin C. We have since found (mss. in preparation) that abscisic acid is also a powerful inhibitor and that actinomycin D and 5-fluorouracil cause little or no inhibition of galactomannan degradation. The metabolic inhibitors act on the cells of the aleurone layer, which are the only living cells in the endosperm. Rijven (1972) has recently reported similar results concerning the influence on galactomannan breakdown of all the above-named and other inhibitors. He finds no inhibition by actinomycin D at a concentration of 16 μg/ml, but also mentions "substantial and statistically significant" inhibition at a concentration of 50 μg/ml. We have used even higher concentrations than this and observed only slight inhibition.

Fig. 2 shows the effects of cycloheximide, abscisic acid, fluorouracil and actinomycin D on the activities of α-galactosidase, β-mannosidase and

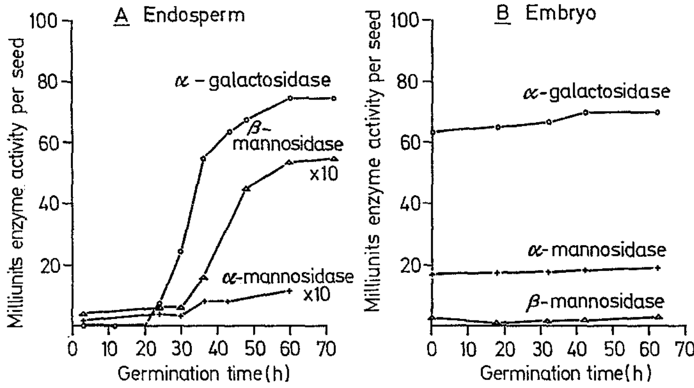

Fig. 1 A and B. Per seed activities of α-galactosidase, β-mannosidase and α-mannosidase in endosperms and in embryos of germinating fenugreek seeds. Dry, punctured seeds were set out to germinate at time zero; they were fully swollen after 5 hours

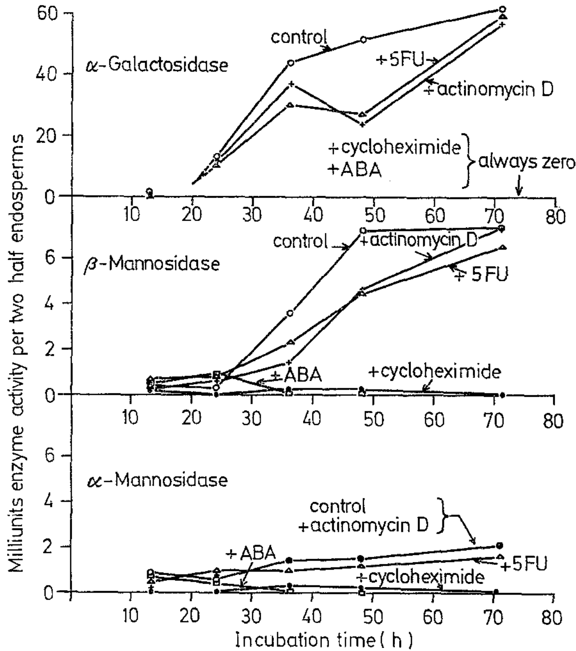

Fig. 2. Development of glycosidase activities in dry-isolated half-endosperms incubated under germination conditions with water (control), cycloheximide (2×10^{-5}M), abscisic acid (ABA, 10^{-5}M), 5-fluorouracil (5FU, 10^{-3}M) and actinomycin D (100 μg/ml)

α-mannosidase in dry-isolated fenugreek endosperms incubated under germination conditions.

The concentrations of cycloheximide and abscisic acid used were known to cause virtually complete inhibition of galactomannan breakdown in the same system. In the untreated controls the enzymic activities develop as in the endosperm of intact seeds (compare Figs. 1A and 2). In the presence of cycloheximide or of abscisic acid, however, the dramatic rise in the activities of α-galactosidase and β-mannosidase is completely suppressed. Fluorouracil and actinomycin D diminish the rates of increase of the enzymic activities, but the final activities attained approach those in the controls.

3. Activity of Oligomannosyl β-1,4 Phosphorylase. Oligomannosyl phosphorylase was assayed separately in extracts of endosperms and embryos from seeds germinated for 48 hours, at which time galactomannan mobilisation was well advanced and all enzymes involved in the breakdown process might be expected to be detectable. The extraction and assay procedures were as nearly as possible identical with those described by Foglietti and Percheron (1972), whilst retaining our small scale, sterile germination conditions. The "biosynthesis" assay of these authors was used in which the release of inorganic phosphate from mannose-1-phosphate is measured relative to a control lacking acceptor (mannotriose). No phosphorolytic activity was detected.

Discussion

The embryo and the endosperm homogenates both contain α-galactosidase, β-mannosidase and α-mannosidase activities, but only the α-galactosidase and β-mannosidase activities in the *endosperm* extracts vary significantly during germination and can be associated with galactomannan degradation.

The correlation between the changes in these two activities and the different stages of galactomannan mobilisation is so close as to leave little doubt about their direct involvement in the breakdown process. The increase in α-galactosidase activity begins about 16 hours after full swelling of the seed, which corresponds exactly to the germination time at which free galactose could first be detected in the endosperm by Reid (1971). The beginning of the rise in β-mannosidase activity similarly coincides with the first detectable diminution of the amount of galactomannan in the endosperm, and the period of rapidly increasing enzymic activity is virtually identical with the period of galactomannan breakdown (Reid, 1971; Reid and Meier 1972). Confirmation that α-galactosidase and β-mannosidase are two of the enzymes responsible for galactomannan breakdown *in vivo* is obtained by assaying their activities in homoge-

nates of dry-isolated endosperms incubated under germination conditions in the absence and in the presence of metabolic inhibitors (see "Results 2"). The activities of α-galactosidase and β-mannosidase are affected by cycloheximide and abscisic acid, 5-fluorouracil and actinomycin D to the same extent as is galactomannan breakdown. The inhibitors act on the cells of the aleurone layer, which must be the source of the enzymes.

Alpha galactosidase and β-mannosidase are only two of the three hydrolytic enzymes necessary for the complete breakdown of fenugreek galactomannan to free galactose and mannose. The third enzyme, *endo-β*-mannanase, could not be assayed in crude homogenates. We have, however, shown it to be present in the endosperm of the germinating seeds and shall describe it in a later communication. Our failure to detect oligomannosyl β-1,4 phosphorylase activity either in the endosperm or in the embryo suggests that this enzyme is not involved in galactomannan mobilisation. It has, so far, been assayed only in crude, whole seed homogenates (Foglietti and Percheron, 1972) and the presumed products, mannose-1-phosphate and mannooligosaccharides have not been rigorously identified. The status of the enzyme requires further investigation.

It is interesting to note that the embryo of the seed yields very high α-galactosidase activities for which the galactomannan cannot be the natural substrate. The embryo α-galactosidase may be responsible for the hydrolysis of the raffinose-stachyose oligosaccharides, which are also mobilised during germination (Reid, 1971). It will be interesting to determine whether the α-galactosidases of the endosperm and the embryo are distinct enzymes with different substrate specificities. There is no connection between the high α-mannosidase activity in embryo extracts and galactomannan metabolism. The α-mannosidases are widely distributed in the plant and animal kingdoms, but their physiological function is largely unknown.

We conclude that the galactomannan of fenugreek is broken down by a series of *hydrolases* secreted by the aleurone layer of the endosperm, and that the hydrolases include α-galactosidase, β-mannosidase and probably also *endo-β*-mannanase. These results are in complete agreement with our earlier observations on the mechanism of galactomannan mobilisation in fenugreek and related leguminous seeds.

We acknowledge the financial support of the Swiss National Research Foundation.

References

Beaugiraud, S., Percheron, F., Courtois, J.E., Lanchec, C.: Osidases des semences germées de fenugrec. Etude d'une α-mannosidase. Bull. Soc. Chim. biol. (Paris) **50**, 621–631 (1968).

Clermont-Beaugiraud, S.: Etude de deux osidases: β-mannanase et α-mannosidase des semences germées de fenugrec. Thèse, Université de Paris (1968).

Clermont-Beaugiraud, S., Percheron, F.: Etude du mode d'action de la β-mannanase des semences germées du fenugrec. Bull. Soc. Chim. biol. (Paris) **50**, 633–640 (1968).

Courtois, J. E., Le Dizet, P.: Recherches sur les galactomannanes IV. — Action de quelques préparations enzymatiques sur les galactomannanes de trèfle et gleditschia. Bull. Soc. Chim. biol. (Paris) **45**, 743–747 (1963).

Courtois, J. E., Percheron, F.: L'α-galactosidase des semences germées de fenugrec. Bull. Soc. Chim. biol. (Paris) **43**, 167–175 (1961).

Dey, P. M., Pridham, J. B.: Biochemistry of α-galactosidases. Advanc. Enzymol. **36**, 91–130 (1972).

Foglietti, M.-J., Percheron, F.: Présence d'une oligomannoside β-1,4 orthophosphate mannosyl transférase (oligomannosyl β-1,4 phosphorylase) dans les graines germées de fenugrec *Trigonella foenum graecum L.* C. R. Acad. Sci. (Paris) (D) **274**, 130–132 (1972).

Hylin, J. W., Sawai, K.: The enzymatic hydrolysis of *Leucaena glauca* galactomannan. Isolation of crystalline galactomannan depolymerase. J. biol. Chem. **239**, 990–992 (1964).

Kooiman, P.: The relationship between monosaccharide composition and specific optical rotation of galactomannans from plant seeds. Carbohydrate Res. **25**, 1–9 (1972).

Reese, E. T., Shibata, Y.: β-Mannanases of fungi. Canad. J. Microbiol. **11**, 167–183 (1965).

Reid, J. S. G.: Reserve carbohydratic metabolism in germinating seeds of *Trigonella* foenum-*graecum* L. (*Leguminosae*). Planta (Berl.) **100**, 131–142 (1971).

Reid, J. S. G., Meier, H.: Chemotaxonomic aspects of the reserve galactomannans in leguminous seeds. Z. Pflanzenphysiol. **62**, 89–92 (1970).

Reid, J. S. G., Meier, H.: The function of the aleurone layer during galactomannan mobilisation in germinating seeds of fenugreek (*Trigonella foenum-graecum* L.), crimson clover (*Trifolium incarnatum* L.) and lucerne (*Medicago sativa* L.): a correlative biochemical and ultrastructural study. Planta (Berl.) **106**, 44–60 (1972).

Rijven, A. H. G. C.: Control of the activity of the aleurone layer of fenugreek, *Trigonella foenum-graecum* L. Acta bot. neerl. **21**, 381–386 (1972).

Smith, F., Montgomery, R.: The chemistry of plant gums and mucilages. New York: Reinhold 1959.

Sømme, R.: Glycosidases and monosaccharides in germinated leguminous seeds. Acta chem. scand. **24**, 72–76 (1970).

Sømme, R.: The correlation between the mono-, oligo- and polysaccharides and the glycosidases present in clover seeds. Acta chem. scand. **25**, 759–761 (1971).

Whistler, R. L., Smith, C. G.: A crystalline mannotriose from the enzymatic hydrolysis of guaran. J. Amer. chem. Soc. **74**, 3795–3796 (1952).