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Endo-β-Mannanase, the Leguminous Aleurone Layer and the Storage Galactomannan in Germinating Seeds of *Trigonella foenum-graecum* L. *

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Abstract. The activity of *endo*-β-mannanase in the endosperm of fenugreek seeds at different stages of germination varies pari passu with storage galactomannan breakdown. *Endo*-β-mannanase is similarly associated with the galactomannan breakdown which occurs when isolated fenugreek half-endosperms are incubated under "germination" conditions. Metabolic inhibitors, *acting on the aleurone layer* reduce *endo*-β-mannanase production in the half-endosperms in proportion to their inhibition of galactomannan breakdown.

It is concluded (a) that *endo*-β-mannanase activity, like galactomannan breakdown, is regulated by the fenugreek aleurone layer and (b) that the *endo*-β-mannanase is almost certainly instrumental in bringing about galactomannanan hydrolysis in vivo.

Key words: Aleurone layer – Endo-β-mannanase – Endosperm – Galactomannan – Germination – Leguminous seeds.

Introduction

Storage galactomannan mobilisation in the germinating fenugreek seed, (*Leguminosae*, tribe *Trifolieae*) has recently been described (Reid, 1971; Reid and Meier, 1972; 1973). The polysaccharide, which is stored in the endosperm of the seed, is broken down rapidly and completely to its constituent monosaccharides which are taken up by the embryo and transformed to sucrose and starch (Reid, 1971). Galactomannan breakdown in the fenugreek endosperm is autonomous in the sense that it is not regulated by factors emanating from the embryo of the seed. Consequently, if half-endosperms are isolated from dry unimbibed seeds and incubated under germination conditions, galactomannan breakdown will occur (Reid and Meier, 1972). The *aleurone layer* of the fenugreek endosperm is responsible for the production of the enzymes which bring about the breakdown of the polysaccharide (Reid, 1971; Reid and Meier, 1972). Two hydrolases, α-galactosidase (EC 3.2.1.22), and β-mannosidase (EC 3.2.1.25) have been shown to be closely associated with galactomannan degradation in the fenugreek endosperm and to be produced by the aleurone layer (Reid and Meier, 1973); there seems little doubt that they are involved in galactomannan hydrolysis in vivo. Yet an α-galactosidase and a β-mannosidase acting in concert would not be sufficient to catalyse the rapid and complete depolymerisation of fenugreek galactomannan which is actually observed (Reid, 1971). Reese and Shibata (1965) have correctly pointed out that the complete enzymic hydrolysis of a leguminous galactomannan (Smith and Montgomery, 1959) to free monosaccharides would require a *minimum* of *three* hydrolases namely an α-galactosidase, a β-mannosidase and an *endo*-β-mannanase.

We now present evidence that an *endo*-β-mannanase does participate in the in vivo hydrolysis of fenugreek galactomannan.

Materials and Methods

The fenugreek seeds, purchased from Messrs. Siegfried AG, Zofingen, Switzerland, were selected for uniformity of size and colour. Their germination characteristics were identical with those described previously (Reid and Meier, 1972). Conditions of seed germination and the sterile preparation and incubation of isolated half-endosperms have been described (Reid and Meier, 1972, 1973).

The *endo*-β-mannanase assay procedure was considered to be critical. The method finally selected is described below followed by a brief summary of the experimental basis underlying its choice.

* This is part five in a series of papers dealing with galactomannan metabolism. Part four: Planta (Berl.) **112**, 301–308 (1973)

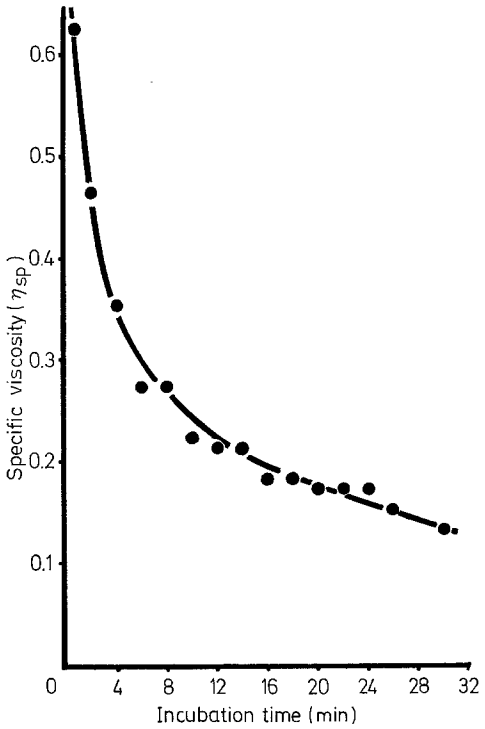

Fig. 1. A typical specific viscosity (η_{sp}) – time curve, following the addition of a fenugreek endosperm enzyme preparation to a solution of carob seed galactomannan

Routine Assay Procedure. Endo-β-mannanase was determined in homogenates (Potter) of endosperms, embryos or isolated half-endosperms prepared in McIlvaine phosphate-citrate buffer (1 ml per endosperm, embryo or two half-endosperms), pH 5.0 (optimum at pH 4.90). Homogenate (0.2 ml) was mixed with 2.8 ml of a 0.1% solution of carob seed galactomannan[1] (the substrate) at time 0, and 2.0 ml of the mixture transferred to a micro capillary viscometer thermostated at 30°. The number of units of *endo-β*-mannanase activity in 0.2 ml of homogenate was taken to be 10 times the reciprocal of the number of minutes required for the specific viscosity (η_{sp}) to fall to half its value at time 0. $[\eta_{sp}=(t-t_0)/t_0$, where t_0 is the viscometer flow time, in seconds, for the solvent and t the flow time for the solution.

Experimental Basis of Assay. Linearity. Measured activity was found to be proportional to enzyme amount for "half viscosity times" up to 30 minutes.

Specificity I. The specific viscosity – time curves obtained all showed the very steep initial drop in viscosity typical of *endo* – but not of *exo*-polysaccharidase activity (Fig. 1).

II. An enzyme preparation from fenugreek endosperm, which contained ca. 10 units "endo-β-mannanase" (measured viscometrically) per ml (0.4 ml) was added to 2.6 ml of a 0.1% suspension (prepared by sonication) of the sparingly water-soluble β1→4 linked *D*-mannan "A" from date endosperm (Meier, 1958). The mixture was placed in one stirred cell of an Amicon model MMC pressure dialyser, fitted with a PM 10 membrane (nominal cut-off at 10,000 daltons) and thermostated at 30°. A second cell contained a control mixture with heat-denatured enzyme. Low molecular weight components were collected by pressure dialysis against distilled water (pH 5) immediately, and then after 2 h, 4 h, 6 h and 34 h. In each case a 10 ml sample was collected: this represents just over 3 times the total volume in the cell and is equivalent to a 95% recovery of a low molecular weight solute. Pressure dialyses took ca. 10 minutes. The first dialysate was rejected as it contained salt and possibly other low molecular weight contaminants from enzyme and/or substrate; the 2 h, 4 h, 6 h and 34 h dialysates were examined by paper chromatography in the solvent system ethyl acetate: pyridine: water 2:1:2 (Table 1). In each case mannobiose and a series of β1→4 linked manno-oligosaccharides were detected. Such a pattern of breakdown for a pure β1→4 linked *D*-mannan can be reconciled only with the presence of an *endo-β*-mannanase.

Table 1. Semi-quantitative determination of manno-oligosaccharides released from date mannan A by a fenugreek β-mannanase preparation

Enzyme (0.4 ml) and 0.1% date mannan suspension (2.6 ml) were stirred in a pressure dialyser cell thermostated at 30°. Low molecular weight sugars were collected at intervals by pressure dialysis against distilled water (pH 5), and analysed semiquantitatively by paper chromatography. No sugars were detected in a control experiment containing heat-denatured enzyme

Incubation time	2 h	4 h	6 h	34 h
mannobiose	tr	tr	tr	1
mannotriose	5	5	tr	5
mannotetraose	5	5	tr	5
mannopentaose and higher	3	3	tr	1

tr = trace; 1, 2 ... 10 = 0.1, 0.2 ... 1.0 times the intensity of the spot produced by 50 μg mannobiose standard in the silver nitrate reaction (Trevelyan et al., 1950)
Small amounts of mannose were also produced

III. The very high α-galactosidase activities which develop in the fenugreek endosperm during galactomannan degradation (Reid and Meier, 1973) could feasibly interfere with an *endo-β*-mannanase assay by "stripping" the α-galactose units from the galactomannan substrate and altering its viscosity characteristics. Although it has been reported (McCleary and Matheson, 1975) that a "moderate amount" of galactose-stripping has virtually no effect on the viscosity of carob seed galactomannan solutions, a check nevertheless had to be made on the contribution of endogenous α-galactosidase to the measured viscosity change. A fenugreek endosperm preparation was obtained which had both very high α-galactosidase activity measured spectrophotometrically (Reid and Meier, 1973) and moderate "*endo-β*-mannanase" activity measured viscometrically. Heat denaturation treatment (65°–90 s: to be published) abolished the "*endo-β*-mannanase" activity completely whilst reducing α-galactosidase activity only slightly *and not affecting the ability of the α-galactosidase to accept galactomannan as substrate* (Table 2). Thus the contribution even of high endogenous α-galactosidase to the viscometric assay is negligible.

Results and Discussion

Endo-β-mannanase activity was determined in homogenates prepared from the endosperm and from the

[1] Fenugreek seed galactomannan was not used because its β1→4 linked *D*-mannan "backbone" is almost fully substituted by α-D-galactopyranosyl residues: it is therefore liable to attack by *endo-β*-mannanase only in the presence of high α-galactosidase activity (see Reid and Meier, 1973). Carob seed galactomannan's "backbone" is less heavily galactose-substituted and it can serve directly as a substrate for *endo-β*-mannanase (Dea and Morrison, 1975)

Table 2. Hydrolase activities in a fenugreek endosperm enzyme preparation before and after selective heat denaturation

Enzyme activity	Before heat denaturation	After heat denaturation
"endo-β-mannanase" (viscometric)[a]	5.5	0
α-galactosidase (substrate: p-nitrophenylgalactoside)[b]	653	458
α-galactosidase (substrate: fenugreek galactomannan)[c]	94.5	65.6

[a] Units per ml enzyme preparation
[b] Milli-units per ml enzyme preparation (Reid and Meier, 1972)
[c] μg D-galactose released per hour per ml enzyme preparation. A 1% (w/v) fenugreek galactomannan solution was incubated with enzyme preparation (0.1 ml) for 2 hr at 30°. The D-galactose released was determined in the "optical test" with D-galactose dehydrogenase (Kurz and Wallenfels, 1974)

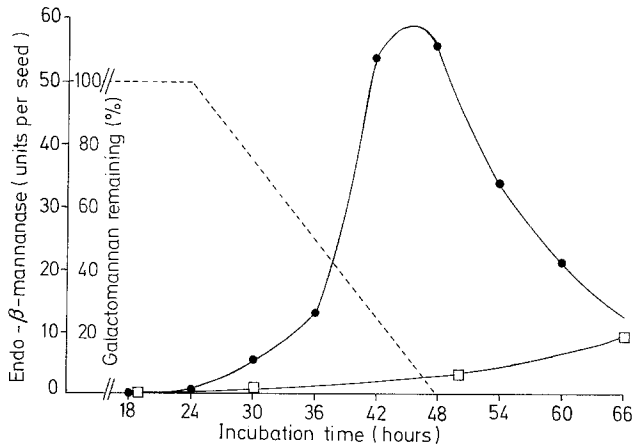

Fig. 2. *Endo-β*-mannanase activity in the endosperm (●–●–●) and in the embryo (□–□–□) of germinating fenugreek seeds. The dashed line (-----) indicates the average time-course of galactomannan mobilisation under identical germination conditions

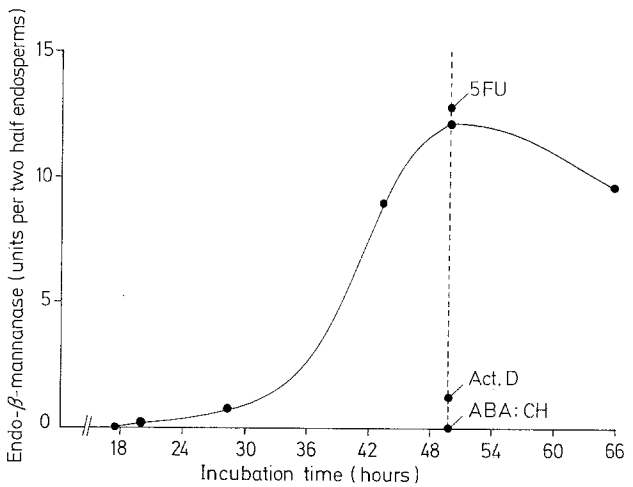

Fig. 3. Development of *endo-β*-mannanase activity in dry isolated fenugreek half-endosperms incubated under germination conditions, and the effect of inhibitors present throughout the incubation period. [5FU = 5-fluorouracil, 10^{-4} M; Act D = Actinomycin D, 10^{-4} M; ABA = abscisic acid, 10^{-5} M; CH = cycloheximide, 10^{-5} M]

embryo of fenugreek seeds at different stages of germination using a viscometric assay whose specificity had been carefully checked (see Materials and Methods). From Figure 2, which correlates the measured activities with the known (Reid and Meier, 1972) time course of galactomannan breakdown in fenugreek endosperm, there can be little doubt that *endo-β*-mannanase activity *in the endosperm* varies in step with galactomannan breakdown. This is suggestive of an in vivo link between galactomannan breakdown and *endo-β*-mannanase activity; indeed such an in vivo link has been inferred for several leguminous seeds from parallel variation of galactomannan breakdown and β-mannanase activities in enzyme preparations derived from *whole seeds* (Sioufi, Percheron and Courtois, 1970; McCleary and Matheson, 1975). Yet the *pari passu* variation of enzymic activity with a physiological phenomenon can be fortuitous even if, as in Figure 2, the variation of activity is in the correct physiological compartment. For this reason further evidence for an in vivo association between *endo-β*-manannase and galactomannan breakdown was sought using the isolated fenugreek half-endosperm system (Reid and Meier, 1972, 1973).

When dry-isolated fenugreek half-endosperms are incubated under "germination" conditions—i.e. on filter discs floating on water—the galactomannan in them is broken down normally as far as the monosaccharides; these diffuse into and accumulate in the water (Reid and Meier, 1972). Galactomannan breakdown in isolated half-endosperms can be modified by applying inhibitors dissolved in the water. The inhibitors act on the cells of the aleurone layer which are the only living cells in the endosperm (Reid and Meier, 1972; Rijven, 1972). Clearly from Figures 2 and 3 the patterns of galactomannan breakdown in attached and in isolated fenugreek endosperms are similar. *Endo-β*-mannanase activity therefore parallels galactomannan breakdown in isolated endosperms just as it does in the endosperm of intact germinating seeds. Furthermore (Fig. 3) the inhibitor 5-fluorouracil has no effect on the maximum level[2] of *endo-β*-

[2] The maximum level of *endo-β*-mannanase achieved in the half-endosperm system is lower by a factor of five than in the endosperm of intact seeds (Figs. 1 and 2). This may be due to some measure of feedback regulation by the products of galactomannan hydrolysis in the case of the half endosperms

mannanase activity achieved in the isolated endosperm system whilst cycloheximide and abscisic acid both inhibit the development of the enzyme activity completely; actinomycin D reduces the level of *endo-β*-mannanase but does not suppress it completely. At the concentrations marked in Figure 3 cycloheximide and abscisic acid inhibit galactomannan breakdown in isolated fenugreek endosperms totally, actinomycin D partially and 5-fluorouracil not at all (Reid and Meier, 1972; Rijven 1972; Reid and Davies, unpublished).

Although the data presented here show that there is a very close connection indeed between *endo-β*-mannanase activity and galactomannan breakdown in fenugreek endosperm, they fall just short of *proving* a *cause and effect* relationship. (This would require a demonstration that specific inhibition of *endo-β*-mannanase caused perturbation of galactomannan breakdown rather than the converse). Nevertheless there can be no serious doubt that an *endo-β*-mannanase is responsible, along with an α-galactosidase and a β-mannosidase, for the in vivo hydrolysis of fenugreek seed galactomannan.

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