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β-D-MANNANASES IN GERMINATING LUCERNE (ALFALFA) SEEDS*

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ABSTRACT

Extraction and fractionation of the β-D-mannanases in germinating lucerne produced four fractions if extracts were stored prior to chromatography. However, when extracts were applied rapidly to DEAE-cellulose, one of the fractions of high molecular weight (separated subsequently by gel chromatography) was not detected, indicating that it was formed after maceration. On storage, the amount of the lower-molecular-weight, unbound fraction from chromatography on DEAE-cellulose did not increase relative to that bound. During germination, the bound and unbound fractions both increased initially and then decreased, with only small changes in the ratio of each. The results suggest that three of the β-D-mannanase fractions are formed *in vivo* prior to maceration.

INTRODUCTION

Multiple forms of β-D-mannanase have been separated from extracts of germinating legume seeds[1]. Several species contained three activities that could be separated by column chromatography, and four were present in lucerne. However, in another examination of this last species[2], only one fraction was detected. Two bands have been separated by poly(acrylamide) gel-electrophoresis from clover seed extracts[3], and the single fraction prepared by chromatography from locust bean[1] has also been separated by the same technique into two bands of closely similar mobility.

In larger seeds which can be readily dissected, β-D-mannanase activity has been found only in the endosperm[1] which is composed largely of galactomannan reserve[4,5]. α-D-Galactosidase is also required for hydrolysis and, although whole seeds show multiple activities[6,7], only one form of this enzyme has been isolated from the endosperms of a range of legume seeds[7]. In the relatively undifferentiated tissue of the endosperm[4,5], it is not unreasonable to expect only one form of β-D-mannanase as well. However, multiple forms of another endo enzyme, alpha amylase, have been detected both prior to and after germination of cereal grains[8,9].

Apart from being isozymes because of genetic factors or from differentiation of tissue, multiple activities in a germinating seed could be the result of limited

*Dedicated to Professor Stephen J. Angyal on the occasion of his retirement.

proteolysis[10], modifying sections of the protein away from the active site. This modification could occur either *in vivo*, during germination before disruption of the tissue, or after maceration, that is, during extraction and preparation. The major activity in legume-seedling endosperm is of higher molecular weight than the minor activities, a finding consistent with this possibility. Protein aggregation may also occur in extracts under some conditions[11,12]. In the isolation of β-D-mannanases from legume seeds, relatively long storage and dialysis procedures (27–48 h)[1–3] have been used, and post-maceration proteolysis could have occurred.

The present work was undertaken to re-investigate the number of β-D-mannanase activities that can be separated from germinating lucerne seed and to monitor the changes that occur on germination.

RESULTS AND DISCUSSION

Extracts from seeds that had imbibed for 3 days at 25° were extracted as previously described[1], using a different batch of seed, and fractionated on DEAE-cellulose. Two fractions were separated as found earlier: fraction A that eluted before application of the salt gradient and which accounted for 20–25% of total activity and contained most of the protein; and fraction B that eluted at a concentration of potassium chloride of 0.35M. When the unbound fraction was re-chromatographed, no activity was retarded. Fraction A was separated by chromatography on Sephadex G-100 into two fractions, A_1 and A_2. Fraction A_1 had a higher mol. wt. (excluded on Sephadex G-100) than A_2 (23,000). The A_2 peak was further separated on CM-cellulose with a salt gradient, giving two fractions, $A_{2\alpha}$ and $A_{2\beta}$. More-consistent results were obtained by using *O*-(phosphono)cellulose (Fig. 1). Two-thirds of the protein that was not bound to the column was separated from the bound β-D-

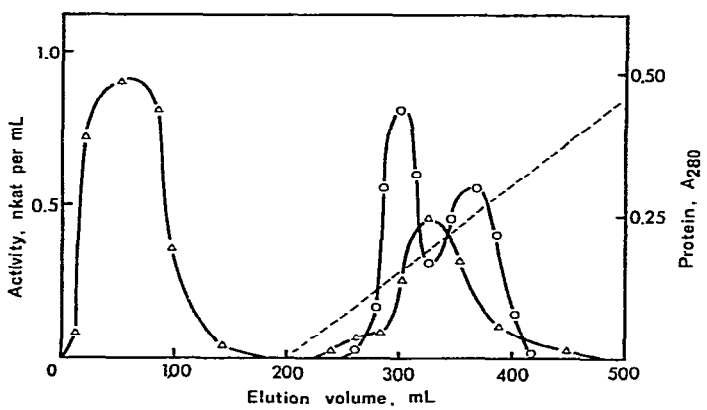

Fig. 1. Chromatography on *O*-(phosphono)cellulose of lucerne β-D-mannanase A_2: β-D-mannanase activity on soluble mannan (○) and absorbance at 280 nm (△). The enzyme solution was applied after chromatography on DEAE-cellulose and Sephadex G-100. The gradient (0–300 mM potassium chloride, total volume 300 mL) is indicated by ---.

mannanase activities. The specific activity of fraction $A_{2\alpha}$ was 3.5 and of fraction $A_{2\beta}$ 5.1 nKat/mg. Re-chromatography of each fraction gave a peak at the same elution volume as previously obtained. The two fractions had the same mol. wt., but differed in the K_m values[1] with carob galactomannan, 2.4 and 0.7mM. The major activity isolated (B) corresponds to the single activity isolated by Villarroya and Petek[2]. Possible reasons that can be suggested for the differing results are: different periods of germination, sensitivity of assay, recoveries, cultivars, and extraction procedures (compare ref. 13).

The highly viscous galactomannan that is also extracted interferes with fractionation; previous methods[1-3] have involved incubation and dialysis steps that allow depolymerisation of this galactomannan by endogenous hydrolases, but could also result in proteolytic modification of the β-D-mannanases. Procedures that shortened the extraction and fractionation times were then examined. Initially, incubation overnight at room temperature was replaced by a period[13] of one h at 40° followed by dialysis for 24 h after precipitation by ammonium sulphate. In chromatography on DEAE-cellulose, two peaks were still obtained in a ratio similar to that obtained after overnight incubation. The method was then further modified. Firstly, a much lower weight of seed per unit of extraction volume was combined with increased sensitivity of detection of column fractions. Secondly, the ammonium sulphate precipitation and dialysis steps were omitted; and thirdly, the initial extract was kept for one h at pH 5.0 at 2°. After centrifugation, the supernatant solution could be chromatographed directly on DEAE-cellulose. This procedure allowed chromatography after a relatively short period (2 h) following maceration of the seed. Eluted fractions were assayed with Remazol Brilliant Blue-dyed galactomannan[13] and by estimation of reducing sugar released from mannan, using *p*-aminobenzoic hydrazide[14]. In the latter method, samples were dialysed before assaying, to remove endogenous sugars.

Using smaller samples of seed, a comparison was made of the recoveries of fractions A and B by two methods of preparation; in the first, incubation at 40° for one h, precipitation by ammonium sulphate, and dialysis for 24 h, followed by chromatography on DEAE-cellulose and, in the second, incubation at pH 5.0 for one h at 2°, centrifugation, and application of the supernatant solution to DEAE-cellulose. The results are shown in Table I. Activity was assayed as release of reducing sugar from β-D-mannan, as measured with *p*-aminobenzoic hydrazide, but assay with Remazol Brilliant Blue-galactomannan gave similar results. Assay with this substrate showed that centrifugation of the original macerate gave an 85% recovery of activity. The recovery of total activity after this step with the shorter method was nearly complete (94%). The proportion of fraction A was higher with the shorter treatment, consistent with A's not being a proteolytic degradation-product. The percentage of the A fraction was lower than that in the earlier fractionation methods using larger weights of seed, and giving a higher B:A ratio. However, the concentrated extracts from these larger seed-samples gave much lower recoveries of total mannanase, 2 nKat per g of seed[15], as compared with 20 nKat per g of seed

TABLE I

COMPARISON OF PROCEDURES FOR PREPARATION OF β-D-MANNANASE A AND B

Step	*Total activity (nKat)*	*Specific activity (pKat/mg)*	*Recovery (%)*	
			Per step	*Overall*
Incubation for 1 h at 40°, precipitation by ammonium sulphate, dialysis, and chromatography on DEAE-cellulose				
Extract after centrifugation	68	113		
Incubation at 40°, precipitation by $(NH_4)_2SO_4$, and dialysis	57	645	83	83
Chromatography on DEAE-cellulose				
fraction A	2.7	6.4	4.8	4.0
fraction B	48	936	85	71
total	51		90	75
Incubation for 1 h at 2° at pH 5.0 and chromatography on DEAE-cellulose				
Extract after centrifugation	68	113		
Chromatography on DEAE-cellulose				
fraction A	6.0	44	8.8	
fraction B	58	197	85	
total	64		94	

obtained by using the smaller samples, making any comparison difficult, as fraction B may have been lost preferentially during processing.

Fraction A was then chromatographed on Sephadex G-100. The extract prepared after storing at 40° and dialysing gave two fractions, A_1 (high mol. wt.) and A_2 (with an apparent mol. wt. of 23,000), but only the second fraction (of lower mol. wt.) could be detected in the extract that had been kept at pH 5.0 and 2° for one h. Thus it appears that the fraction of high mol. wt. (A_1) is an artefact of the extraction procedure. Aggregated forms of α-D-galactosidase from plant seeds have been detected, and aggregation was influenced by pH (ref. 11) and the presence of potassium ions[12]. Another possible cause is association with polysaccharide.

The changes in the levels of β-D-mannanases A and B were then determined at various times of germination. Samples were prepared at 1.5, 2.5, 3.5, and 5.5 days after imbibition, by using the extraction method involving storage at pH 5.0 and 2° for one h. At 5.5 days, seedlings were approximately 2 cm in total length and the galactomannan had been depleted. It has been shown previously[1] that total content of β-D-mannanase rises from nonsignificant levels prior to imbibition to a peak, followed by a decrease. Disappearance of galactomannan is coincident with maximum levels of β-D-mannanase[1,7].

Chromatography on DEAE-cellulose gave high recoveries, except at 5.5 days, when very little endosperm remained (Table II). β-D-Mannanase A was present at

TABLE II

LEVELS OF ACTIVITY OF β-D-MANNANASE FRACTIONS A AND B[a]

Time of germination (days)	*Total extracted*	*Fraction A*	*Fraction B*
1.5	388	15	370
2.5	216	10	222
3.5	123	8	122
5.5	60	4	17

[a]nKat per g of original seed.

all times of germination, and it initially increased and then decreased, as did the total β-D-mannanase and mannanase B. There was a small increase in the ratio of activities A:B. The values in Table II were determined as release of reducing sugar, but assay with Remazol Brilliant Blue-galactomannan gave a similar pattern. The maximum level of total activity occurred earlier (1.5 days) than previously reported[1] (2–3 days). Although the same variety, and temperature of incubation (25°) were used, the batches of seeds were purchased at different times and visual examination indicated that the germination rates differed. As the β-D-mannanase A fractions have a lower mol. wt. than B (23,000–29,000 by gel chromatography), A could be an *in vivo* degradation-product of B. The results do not preclude this, but if A is a breakdown product, then it is rapidly changed *in vivo* into enzymically inactive products.

The presence of four β-D-mannanase activities in germinating lucerne has been confirmed, but one fraction is apparently an artefact of extraction. The changes in the levels of fractions A and B with time and on germination are consistent with the lesser fraction, that is the one unbound to DEAE-cellulose, not being an *in vitro* or *in vivo* proteolytic breakdown-product.

EXPERIMENTAL

Plant material. — Lucerne seeds (*Medicago sativa* cv. Hunter River) were soaked in 0.5% sodium hypochloride for 10 min, washed, and germinated in wet filter paper at 25° in the dark.

Preparation of Bangalow Palm D-*mannan.* — Seeds were extracted with strong alkali under an atmosphere of nitrogen, and purified by precipitation as the copper complex[1]. The final product was reduced with sodium borohydride.

Assay of β-D-mannanase with Bangalow Palm D-*mannan.* — A solution of D-mannan in 10% sodium hydroxide (0.2 g per 2 mL) was prepared and immediately diluted, neutralized with acetic acid, and then further diluted to 200 mL with 100mM acetate buffer, pH 5.0. Enzyme solution (0.1 mL) was incubated at 30° with substrate (1.0 mL). The reaction was stopped by addition of *p*-aminobenzoic hydrazide (2 mL)[14] [1 vol. of solution A: *p*-aminobenzoic hydrazide (3.8 g) in conc. hydro-

chloric acid (4.3 mL) diluted to 100 mL with water] mixed immediately before use with 4 vol. of solution B [sodium hydroxide (20 g), trisodium citrate (14.7 g), and calcium chloride hexahydrate (2.19 g), diluted to 1 L)]. Colour was developed by heating for 6 min at 100°, the tubes cooled, and absorbance measured at 420 nm. A calibration curve was constructed by using D-mannose.

Assay of β-D-mannanase with Remazol Brilliant Blue-dyed carob galactomannan. — Substrate was prepared[13] by treatment with one dye molecule per 50 hexose residues. Enzyme solution (0.5 mL) was incubated with dyed substrate at a concentration of 0.75% at 30°. The reaction was stopped by addition of ethanol (3.0 mL), the solution centrifuged, and the absorbance of the supernatant solution read at 590 nm.

Extraction and separation of β-D-mannanases. — In the method involving incubation overnight at room temperature, followed by precipitation with ammonium sulphate and dialysis, the method of McCleary and Matheson[1] was used, and in that involving incubation for 1 h at 40°, followed by precipitation by ammonium sulphate and dialysis, the method was that of McCleary[13].

In the third method, the germinated seeds (10–20 g dry wt. of seeds) were macerated for 2 min in 10mM acetate buffer, pH 5.0 (300 mL) in an Ultra-turrax apparatus. After filtering through 5 layers of cheesecloth, the residue was re-extracted with buffer (200 mL). The filtrates were combined, adjusted to pH 5.0, and centrifuged (25,000*g*, 15 min, 2°). The supernatant solution was stored for 60 min at 2° and centrifuged again. The supernatant solution was applied to a column of DEAE-cellulose that had been pre-equilibrated with 10mM acetate buffer, pH 5.0. Fraction A consisted of the eluate and washings with 500 mL of the same buffer. Fraction B was eluted with a 0–500mM gradient of potassium chloride in 10mM acetate buffer. Eluates were concentrated by using a PM 10 membrane (Amicon). On chromatography on Sephadex G-100, fractions were eluted with 10mM acetate buffer, pH 5.0, containing 100mM potassium chloride. For chromatography on CM-cellulose or *O*-(phosphono)cellulose, the column was pre-equilibrated with 10mM acetate buffer, pH 5.0, and protein was eluted with a 0–300mM gradient of potassium chloride in this buffer.

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