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DE NOVO SYNTHESIS OF MANNANASE BY THE ENDOSPERM OF *LACTUCA SATIVA*

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Key Word Index—*Lactuca sativa*; Compositae; seeds; germination; endosperm mobilization; mannanase; density labelling.

Abstract—The increase in endo-(1→4)-β-mannanase activity in isolated lettuce endosperms was inhibited by 1 μg/ml cycloheximide added before or during enzyme production. *In vivo* density labelling using 50% D_2O increased mannanase buoyant density from 1.308 to 1.319. The production of the enzyme is therefore based upon *de novo* synthesis.

Mannanase synthesis in isolated endosperms was prevented by addition of 10 μM cordycepin or 20 μg/ml actinomycin D during the *ca* 4 hr period before enzyme production began. These inhibitors had little effect at later times. Enzyme production was similarly inhibited when endosperms were isolated from non-germinated seeds at 24 hr, instead of 4 hr, after sowing. It is suggested that (i) mannanase production is dependent on the completion of transcriptional events and (ii) in germinating seeds these events are part of the control mechanism through which the germinating axis initiates mannanase production.

Mannanase secreted by isolated lettuce endosperms has been characterized by native gel filtration chromatography and two-dimensional gel electrophoresis. The secreted mannase was present as three charge-isomers, with pIs between 4.75 and 4.9. Estimates of M_r by chromatography and electrophoresis were 19 and 46 000, respectively. Reasons for favouring the 46 000 estimate are presented.

INTRODUCTION

The mature lettuce embryo is enveloped by a two-cell-layer endosperm. Thick cell walls, largely containing mannan, comprise about half the endosperm dry weight, and provide a substantial source of carbohydrate food reserve for the embryo immediately after germination [1]. The main protein, lipid and phytate reserves are mobilized afterwards [2]. Endosperm mobilization is accompanied by considerable increase in activities of two key cell-wall-hydrolysing enzymes: endo-(1→4)-β-mannanase (EC 3.2.1.78) in the endosperm, and β-mannosidase (EC 3.2.1.25) in the cotyledons [1–3]. Mobilization also involves α-galactosidase (EC 3.2.1.22), which is already present at high levels in mature seed endosperms [4].

In intact seeds, the increase in mannanase activity depends upon the removal or inactivation of an inhibitor in the endosperm. This is achieved by the cotyledons under the control of the germinating axis. However, in endosperms dissected out from ungerminated seeds, full-scale production of mannanase can be promoted simply by incubation in enough water to leach out the inhibitor [5, 6]. Under these conditions, the endosperm cell walls undergo incomplete degradation, and oligo-saccharides are released into the incubation medium [4].

In this paper, I report on the use of a density-labelling technique to establish that mannanase production is based upon *de novo* enzyme synthesis. Also, application of inhibitors of nucleic acid synthesis has indicated that there is a necessary transcription stage after the removal of the inhibitor before mannanase synthesis can start.

Mannanase has also been partially characterized, by gel filtration chromatography and two-dimensional gel electrophoresis (native IEF/SDS-PAGE). An elegant feature of the procedure has been to use the endosperm tissue itself to semi-purify mannanase on a micro-scale. By analysing material secreted from isolated endosperms, mannanase has been identified in a mixture of *ca* 16 polypeptides, amounting to less than a microgram of protein.

RESULTS

De novo *synthesis of mannanase*

In isolated endosperms, the mannanase level begins to increase *ca* 6 hr from the start of incubation, and continues to do so for *ca* 12 hr more. By this time most of the activity is found in the incubation medium (Fig. 1). In contrast, general protein synthesis begins within 2 hr after the start of incubation (Table 1). The production of enzyme was found to be greatly reduced after the addition of cycloheximide, at 1 or 10 μg/ml, up to at least 12 hr from the start of incubation (Fig. 2), indicating that continued protein synthesis is necessary for mannanase production.

A density labelling experiment was then carried out to see whether mannanase production is based on *de novo*

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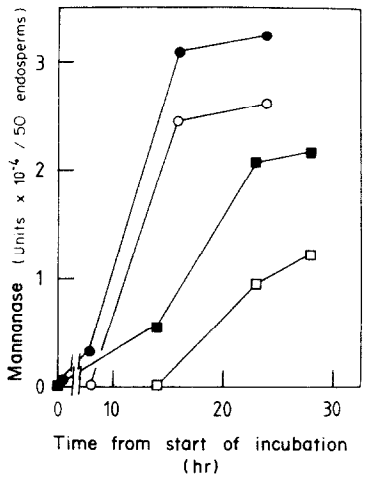

Fig. 1. Time course of production (●, ■) and appearance in the incubation medium (O, □) of mannanase on incubation of 50 isolated lettuce endosperms in 2.0 ml buffered H_2O (●, O), or H_2O:D_2O (1:1) (■, □).

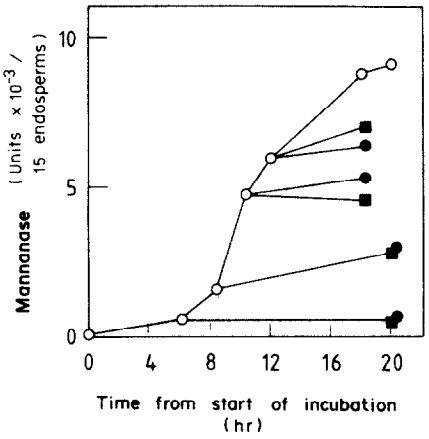

Fig. 2. Inhibition of mannanase production by 15 lettuce endosperms, isolated 4 hr from sowing in response to addition of cycloheximide at various times. (●) 1 μg/ml; (■) 10 μg/ml; (O) no addition. The extract and incubation medium were combined before assay.

enzyme synthesis. The buoyant densities of the enzyme produced and secreted by isolated endosperms in the presence of either H_2O or D_2O–H_2O (1:1) were compared by isopycnic centrifugation in caesium chloride density gradients. Enzyme production and secretion was slower in the presence of D_2O (Fig. 1). After incubation in D_2O, the distribution of mannanase activity in the gradient was uniformly shifted, so that the peak was moved from specific gravity 1.308 to 1.319 (Fig. 3A, B). This 0.83% increase in density, together with the unimodal shift of enzyme distribution in the gradient, provide clear evidence that mannanase production is based overwhelmingly upon *de novo* synthesis of the enzyme, rather than upon the activation of a performed precursor.

The peak locations of unlabelled mannanase and barley α-amylase activities were very close, but mannanase was distributed more widely in the gradient (a less steep slope in Fig. 3A). The variance ratio of the distributions of mannanase and α-amylase was 2.6:1.

Requirement of RNA synthesis

Since mannanase is synthesized *de novo*, the possibility that there is also a requirement for synthesis of its mRNA

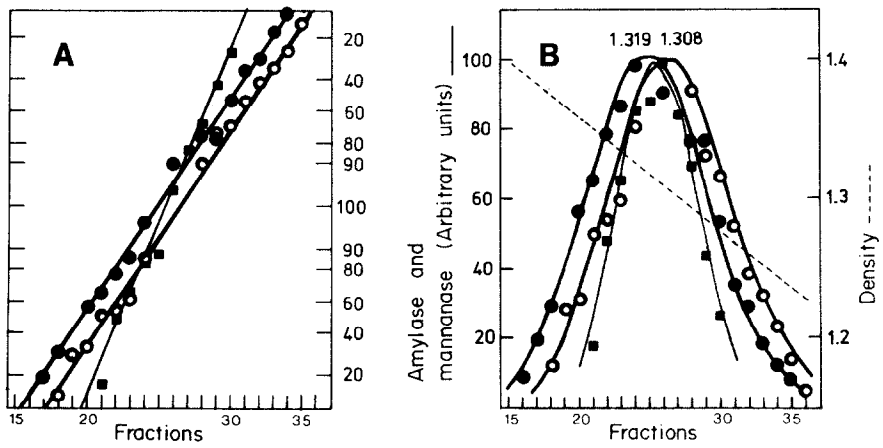

Fig. 3A, B. Distribution of activity in CsCl density gradients of mannanase secreted by isolated lettuce endosperms incubated for 24 hr in H_2O (O) or H_2O–D_2O (1:1) (●). Also shown is the distribution of barley α-amylase activity (■). (A) data plotted on Gaussian-scale graph; (B) data plotted on linear-scale graph. In (A), activities in fractions in the first (i.e. denser) half of each distribution are plotted in the lower part of the graph; those in the second half in the upper part of the graph. The coordinate axis, drawn symmetrically about its mid-point, has been transformed so that a normally distributed population, plotted by this procedure, will lie on a straight line. Each set of assay data in turn was multiplied by a constant so that plotted values lay on a straight line. The position of peak activity was found by the ordinate corresponding to a co-ordinate of 100, and the lines were also used to generate the 'best-fit' curves plotted in (B).

Table 1. Protein synthesis by lettuce endosperms, isolated 4 hr from sowing, during and after the lag phase before mannanase production begins. Batches of endosperms were incubated for various periods before 2-hr labelling by addition of ^{3}H-leucine

Period of labelling after start of incubation (hr)	Radioactivity (Bq × 10^{-6}) Total taken up	TCA precipitated
0–2	19.2	9.0
2–4	30.6	18.6
5–7	29.4	18.6
8–10	30.0	19.8
No labelling (^{3}H-leu added to extract)	(30.0)	1.2

was investigated. This was achieved by following the time course of enzyme production after addition of cordycepin (3′-deoxyadenosine, 10 μM) at various times during the incubation of endosperms, isolated 4 hr from sowing (Fig. 4). Mannanase synthesis was prevented if cordycepin was added during the lag phase, up to about 4 hr from the start of incubation, but was little affected after 6 hr, once activity had begun to rise. A similar decline in response to actinomycin D (16 μM), was seen, except that it occurred sooner. The same pattern was found with tenfold higher concentrations of inhibitors, but the lowest concentrations required to demonstrate the effect were: 5 μM for cordycepin, or 8 μM for actinomycin D. These did not completely prevent the production of mannase, even when added at the start of incubation.

When endosperms were isolated from non-germinated seeds 24 hr after sowing, instead of 4 hr (as in the above experiments), mannanase production was still substantially reduced by 10 μM cordycepin and 16 μM actinomycin D from 56 units × 10^{-2} per 15 endosperms to 19 units × 10^{-2} and 21 units × 10^{-2} per 15 endosperms, respectively. At this stage, mannanase production also

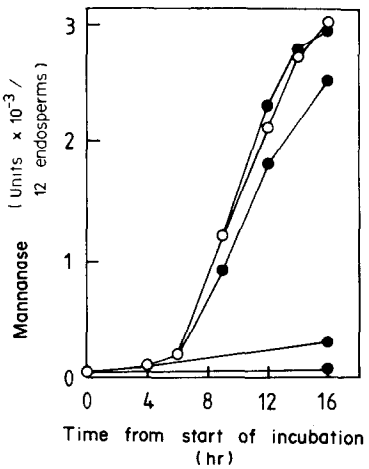

Fig. 4. Inhibition of mannanase production (O) by 12 lettuce endosperms, isolated 4 hr from sowing, in response to addition of 10 μM cordycepin (●) at various times. The extract and incubation medium were combined before assay.

Table 2. Inhibition of total and poly(A)$^+$-RNA synthesis in lettuce endosperms by cordycepin (10 μM) and actinomycin D (16 μM). Batches of 20 endosperms were incubated for 5 hr in MES buffer, then for 1.5 or 3.0 hr in 1.0 ml fresh buffer containing [2, 5′,8-^{3}H]adenosine. Extracted RNA was fractionated by oligo-dT-cellulose chromatography. To certain batches either cordycepin or actinomycin D was added, along with the radiotracer. Results shown mean of two replicates.

Additions	Incubation time (hr)	Radioactivity (Bq × 10^{-6}) Total RNA	poly(A)$^+$-RNA	Total uptake
None	1.5	2.55	0.44	516
	3.0	4.20	0.78	—
Cordycepin	1.5	2.22	0.41	516
	3.0	2.88	0.55	—
Actinomycin D	1.5	1.65	0.30	570
	3.0	2.04	0.37	—

depended on the leaching out of an inhibitor (probably abscisic acid), i.e. incubation in 2.0 ml rather than 0.1 ml.

These concentrations of cordycepin (10 μM) and actinomycin D (16 μM) were found to be sufficient to inhibit RNA synthesis, even when added at the end of the lag phase, after 5 hr incubation (Table 2). RNA synthesis, measured by the incorporation of ^{3}H-adenosine into the nucleic acid fraction, was little affected during the first 1.5 hr after addition of the inhibitors, but was reduced during the next 1.5 hr. Moreover, the synthesis of poly (A$^+$)-RNA was inhibited to the same extent as that of total RNA. Total uptake of ^{3}H-adenosine was not inhibited, however.

These results suggest, but do not prove, that mannanase synthesis in the isolated endosperm depends on transcriptional events that are completed after inihibitor removal in the lag phase before synthesis begins. The 'transcriptional events' do not occur in intact ungerminated seeds.

Electrophoretic characterization of mannanase

Under the conditions used in these experiments, about 80% of mannanase activity is secreted by isolated endosperms into the incubation medium [5]. Electrophoresis under denaturing conditions (SDS-PAGE) of the proteins secreted after 23 hr showed the presence of at least eight stainable bands, including a major one with apparent M_r about 46 000, corresponding to a very minor band in extracts of freshly dissected endosperms (Fig. 5). The band became heavily labelled when endosperms were incubated with ^{3}H-leucine (Fig. 6, Panel 7).

A two-dimensional procedure was then devised to see which protein components(s) carried mannanase activity. The first dimension was isoelectric focussed under non-denaturing conditions. This resolved at least 10 stainable bands. Direct assay of eluates of gel slices showed that three bands, with apparent pIs of 4.75, 4.85 and 4.9, accounted for all the recoverable mannanase activity (indicated by the broad bar in Fig. 6, panels 1,2). In the second dimension (SDS-PAGE), all three bands moved at the same rate as the major 46 000 component (arrowed in

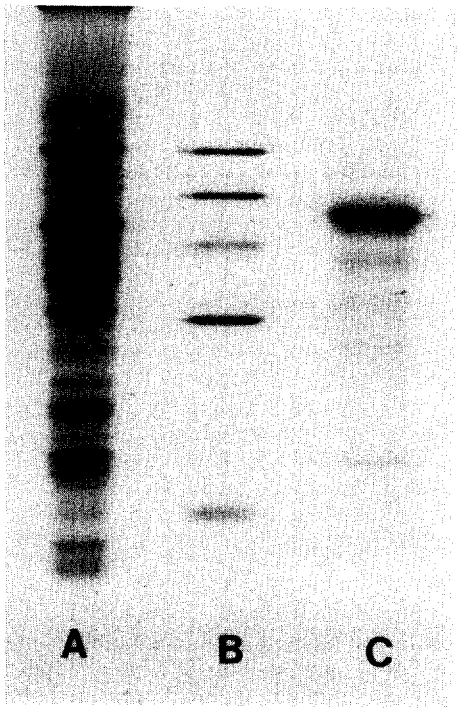

Fig. 5A-C. Coomassie-stained gels after SDS-PAGE of proteins from isolated lettuce endosperms. (A) Buffer-extract of 30 lettuce endosperms before incubation. (B) M_r markers (kd): Catalase (60), pig heart fumarase (49), rabbit muscle aldolase (40), carbonic anhydrase (29), myoglobin (18) (Sigma). *C* Material secreted by isolated endosperms between 5 hr and 23 hr of incubation of 140 endosperms.

Fig. 6, panel 4). Other components, not containing mannanase and with different pIs, also ran close to the 46 000 band. In all, 12 stainable proteins were seen.

To provide an additional identification criterion, the endosperms were incubated with ^{3}H-leucine to label all *de novo* synthesized proteins, now known to include mannanase. In the fluorograph (Fig. 6, Panels 5, 6, 7), as expected, the triplet mannanase bands were the main radioactively labelled protein components. Two less heavily labelled bands with a M_r 47 000, and one ca 45 000, all with pI 4.8–4.85, were also detected. In all, the incubation medium contained 16 or more labelled proteins.

In summary, two-dimensional PAGE indicated that mannanase exists as three charge isomers, has an apparent M_r of 46 000, and is the major protein secreted by endosperms.

Gel filtration chromatography of native mannanase

Labelled material secreted by isolated endosperms, similar to that used in the last section, was subjected to gel filtration chromatography to provide an alternative characterization method. A polyacrylamide gel was used, rather than a dextran gel which might give problems because of mannanase binding non-specifically to polysaccharide. Conditions were non-denaturing.

Mannanase activity eluted in a single, symmetrical peak, co-chromatographing with the major peak of

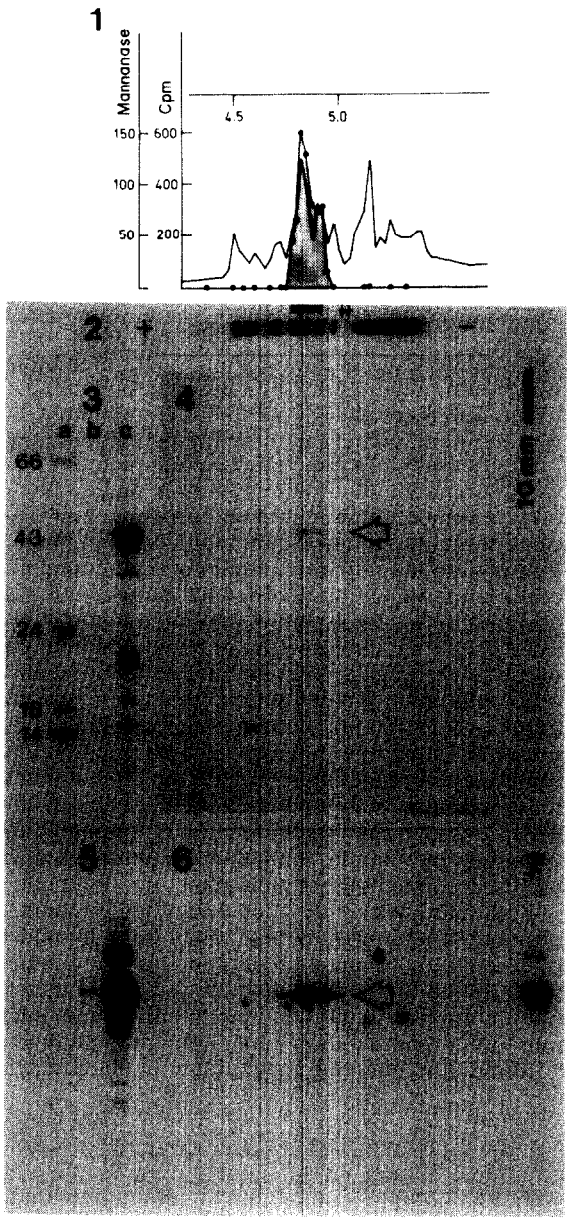

Fig. 6. Two-dimensional IEF/SDS-PAGE of ^{3}H-labelled mannanase secreted by isolated lettuce endosperms between 5 and 23 hr of incubation with ^{3}H-leucine. Panel 1. Distribution of radioactivity (——) and mannanase activity (●----● and shaded) in an IEF gel. The pH gradient was linear between 4.5 and 5.0 at the positions shown. Panel 2. Stained IEF gel. Mannanase activity is confined to three adjacent bands, marked by thick bar, pI 4.75–4.90. Arrows indicate the position of the two isozymes of β-lactoglobulin, in a replicate gel. Panel 3. One-dimensional SDS-PAGE. (a) M_r markers (kd)-bovine plasma albumin (66), ovalbumin (43), pepsin (35), trypsinogen (24), β-lactoglobulin (18), egg white lysozyme (14) (Sigma); (b) material concentrated from fractions indicted in Fig. 7B; (c) original sample, in double quantity. The three bands containing mannanase activity co-electrophoresed with an apparent M_r of 46 kd (large arrow). Panel 4. Two-dimensional electrophoretogram. Panel 5. 7-day fluorograph of Panel 3b, c. Panel 6. 7-day fluorograph of Panel 4. Panel 7. 3-day fluorograph of Panel 3c. The most heavily labelled proteins (arrowed) are those with mannanase activity.

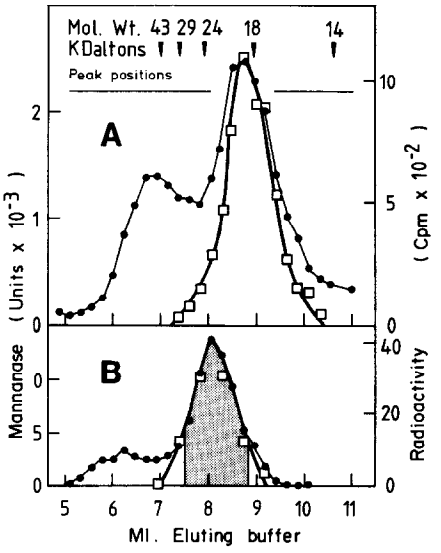

Fig. 7. Polyacrylamide gel-filtration chromatography of ^{3}H-labelled mannanase, prepared as for Fig. 6. Eluting buffers, pH 5.0, were; A. citrate, B. pyridine–acetic acid. Mannanase activity (□——□) co-chromatographed with the main peak of radioactivity (●---- ●). The fractions indicated in (B) were concentrated and subjected to SDS-PAGE in Fig. 6 (Panels 3b and 5). In (A) the peak positions of M_r marker proteins (kd) are shown: ovalbumin (43), carbonic anhydrase (29), trypsin (24), myoglobin (18), ribonuclease A (14) (Sigma).

radioactivity (Fig. 7A). This peak had an apparent M_r of *ca* 19 000. This material was then electrophoresed alongside the IEF gel in Fig. 6. For technical reasons, it was necessary to use a different eluting buffer, to desalt the protein without losing mannanase activity; this slightly changed the elution volumes, but not the elution pattern (Fig. 7B).

On SDS-PAGE the chromatographic peak containing mannanase and radioactivity co-electrophoresed with the protein earlier identified as mannanase, with apparent M_r 46 000 (Fig. 6, panel 5). In Fig. 6, panel 3, there was a trace of stainable protein, but this cannot be reproduced photographically. Thus the '19 000' mannanase peak found by chromatography and the '46 000' denatured mannanase band found by SDS-PAGE correspond to each other.

DISCUSSION

Previous work has demonstrated the inhibitory control by the axis and cotyledons of the germinating lettuce seed over the production of endo-(1→4)-β-mannanse in the endosperm [5]. In the work presented here, the processes leading to mannanase production have been studied using dissected endosperms incubated in sufficient liquid to leach out the inhibitor and promote the response.

Endosperm isolation and incubation is associated with an immediate protein synthesis, at a high rate, which continues for at least 8 hr (Table 1). Mannanase production, however, only begins after a 6-hr lag phase, and is based on *de novo* synthesis, a point proved by *in vivo* density-labelling with D_2O (Fig. 3). The main control over mannanase production is therefore not post-translational, i.e. not by the activation of a pre-existing mannanase zymogen. Control might therefore lie over the translation of pre-existing mRNA, or might be at the transcriptional stage.

During the lag phase, events necessary for mannanase production occur, which can be blocked by actinomycin D or cordycepin at levels that inhibit RNA synthesis (Fig. 4). After the lag phase these chemicals do not stop mannanase production. This suggests, but does not prove, that transcription is a prerequisite for mannanase production in isolated endosperms. Whatever their nature, the cordycepin- and actinomycin D-sensitive events seem to depend on the prior removal of the natural inhibitor which controls mannanase production in intact lettuce seeds. If inhibitor removal is delayed, by dissecting endosperms from dark-incubated seeds after 24 hr, mannanase production remains sensitive to actinomycin D and cordycepin (Table 4). Thus the 'events' do not occur in a constitutive temporal manner in all seeds, whether germinated or not, during the period 4 to 10 hr, from the start of imbibition. Rather they seem to lie on the same pathway that is controlled by the removal of the inhibitor, and leads to mannanase synthesis.

This production of mannanase in the lettuce endosperm bears similarities to the control of the production of mobilizing enzymes, most notably α-amylase, in certain cereal aleurone layers. There, *de novo* α-amylase synthesis is initiated and maintained in response to an external supply of gibberellin from the embryo [e.g. 7]. Synthesis begins after a lag phase during which the level of translatable amylase mRNA rises markedly [8].

Mannanase is the main protein released into the incubation medium by isolated lettuce endosperms (Fig. 6). This major secretion of mannanase reflects the 60% mannose content of the endosperm cell wall [2]. Some of the other released proteins may be additional cell-wall-degrading enzymes secreted into the wall in intact seeds.

The M_r of mannanase has been estimated as 46 000 by SDS-PAGE (Figs 5, 6) and 19 000 by native gel-filtration chromatography (Fig. 7). Since these two 'forms' co-electrophorese (Fig. 6), the discrepancy must be the consequence of a methodological artefact. It is interesting to note here that the value obtained by caesium chloride density gradient analysis coincides with the lower estimate for mannanase size. The distribution variance of mannanase activity compared to barley α-amylase activity is 2.6 (Fig. 3). Since distribution variance is inversely proportional to molecular size, and α-amylase is about 41 000 [9, 10], a size of about 16 000 for mannanase is suggested.

How to explain these discrepancies? One possibility is that mannanase is heavily glycosylated, so that the charge:mass ratio in the presence of SDS would be lower than for a pure protein, and the migration rate in electrophoresis would be correspondingly slow. However, the buoyant density of mannanase (1.308; Fig. 3) indicates that the enzyme is not glycosylated to a detectable degree. An alternative explanation is that, under native conditions, mannanase has an atypically compact shape for its size. After this work was completed, it became apparent that the pH 5.0, chosen for chromatography and density gradient centrifugation because it is near-optimal for mannanase activity, was fortuitously very close to its pI (Fig. 6). Near to their pIs "many globular proteins adopt configurations much closer to

impenetrable spheres, than rods or random coils" [11]. Such a phenomenon could explain an under-estimate of true molecular size under the conditions used.

Secreted mannanase has been shown by IEF to be an acidic protein, with a pI about 4.8, with charge isomers, within a pI range of 0.15 units (Fig. 6, Panels 1, 2). These probably correspond to single amidation differences within one polypeptide, which might have arisen after secretion and during experimental handling.

Very few other plant endo-(1→4)-β-mannanases have been characterized. Galactomannan-storing endospermic legumes produce multiple forms of endo-(1→4)-β-mannanase. Honey locust (*Gleditsia triacanthos*) and carob (*Ceratonia siliqua*) have one and three mannanases respectively, all *ca* 22 000; soybean (*Glycine max*) has two 23 000 isozymes and one of 27 000; and lucerne (*Medicago sativa*) is similar to soybean but with another of 100 000 isozyme [12]. The last-named isozyme in lucerne has a pI of 4.5 [13]. By contrast, the lettuce endosperm has only three close charge-isomers of one protein. This reflects differences in the physiology of mobilization of lettuce and legume endosperms. In lettuce, the endosperm can be regarded as a simple two-cell-thick autolysing aleurone layer. In legumes, endosperms typically contain several additional layers of galactomannan-storing cells, and cell-wall-degrading enzymes may be produced by cells in the endosperm and embryo, as well as in the aleurone layer [14].

In conclusion, the work described in this paper has made clear that it is the synthesis of mannanase that is being controlled in germinating lettuce seeds. Synthesis begins after a lag phase, following the removal of inhibitor, during which events necessary for subsequent mannanase synthesis occur. These events are sensitive to cordycepin and actinomycin D, but their nature is not yet clear. Questions now arise about whether control is exerted before or after the production of translatable mannanase mRNA. The gel electrophoretic separation of mannanase devised in this work could prove an important experimental tool for molecular biological investigations along these lines.

EXPERIMENTAL

Seed, dissection and incubation of endosperms. Lettuce achenes (*Lactuca sativa* L., cv. Grand Rapids: Ferry Morse seed Co., Mountain View, Cal., U.S.A.; 1970 harvest) were stored at 4° in a dry sealed jar, and in 1978/79, when this work was carried out, had retained 99% germination in light at 24°. Endosperms were dissected from 'seeds' preimbibed in darkness for 4–5 hr at 24°, as described in ref. [5], and incubated in batches of up to 75 in 2.0 ml 10 mM MES, pH 5.8, in 35-mm diameter plastic Petri dishes in darkness at 24°.

Mannanase extraction and assay. Extraction of endosperms was in ice-cold Na^+-phosphate-citrate (PC) buffer, pH 7.0, and assay by a viscometric procedure, as described in ref. [5]. The incubation medium was either assayed separately, or combined with the extracted fraction before assay. A simplified assay was devised to cope rapidly with the screening of fractions obtained after density gradient centrifugation, gel chromatography and gel electrophoresis work: fractions containing activity then were reassayed in the standard procedure. Assays were run using 2 mg/ml galactomannan for 15 min at room temp. and enzyme action stopped by addition of Na_2CO_3 to 50 mM in a final vol. of 1–1.5 ml. Decrease in viscosity, due to mannanase activity, was detected by a reduction in the time of flow of 200 μl of this soln down a vertical microcapillary tube. The upper end of the capillary was inserted into the tapered end of a 1 ml capacity disposable plastic pipet tip, which thereby served as a small funnel. Flow times were in the range from *ca* 30 down to *ca* 20 sec.

Density labelling of mannanase. Two batches of 50 isolated endosperms were each incubated in 2.0 ml MES buffer dissolved in D_2O–H_2O (1:1, v/v) (New England Nuclear Corp., Boston, Mass., U.S.A.). Two control batches were incubated in buffer made up in water alone. After 28 hr, the incubation medium, containing secreted mannanase, was clarified by centrifugation (10 min, 12000 rpm) and ultrafiltration through 0.2 μM Unipore polycarbonate membranes (Biorad Laboratories, Richmond, Cal., U.S.A.). The two samples, containing *ca* 10^4 units mannanase, were made to 35 mM buffer, 1:5 (v/v D_2O–H_2O and 0.49 g/ml CsCl (specific gravity of mixture *ca* 1.36). Barley α-amylase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was included, to provide an internal standard. Six ml of these samples were centrifuged to isopycnic equilibrium for 50 hr at 4° and 48 000 rpm in cellulose nitrate tubes in a SW65 rotor (Beckman Instruments, Palo Alto, Cal, U.S.A.). Fractions (150 μl) were collected by gravity-feed from punctured tubes, and assayed for mannanase and for amylase by the method in ref. [15]. The specific gravity of each fraction was determined by refractometry, using tabulated values for CsCl (EM Laboratories Inc., Elmsford, N. Y., U.S.A.), allowing for the presence of D_2O. Each location of peak enzyme activity within the gradient was calculated graphically by fitting data to a Gaussian distribution (Fig. 3A), and peak specific gravity estimated by interpolation (linear regression) using the 10 nearest fraction densities.

^{3}H-Labelling and extraction of total protein. Batches of 50 endosperms were incubated in MES buffer for periods up to 8 hr, before a 2-hr labelling by addition of 1.9×10^5 Bq (5 μCi) L-[4,5-^{3}H]leucine (2.22×10^{12} Bq/mol: Amersham Radiochemical Corp., Chicago, Illinois, U.S.A.) to the incubation medium. Endosperms were rinsed in 10 mM unlabelled leucine, before extraction in 1.0 ml PC buffer. After clarification by centrifugation, total radioactivity in the supernatant was counted. Then the supernatant was made up to 10% w/v TCA, the ppt., collected by centrifugation, was rinsed with 2×2.0 ml Me_2CO, dried, redissolved in 8 M urea, and counted for radioactivity.

^{3}H-Labelling and extraction of RNA, and separation of poly $(A)^+$ and poly $(A)^-$ RNA. Batches of 20 endosperms were incubated for 5 hr in MES buffer, then in fresh buffer containing 3.7×10^5 Bq(10 μCi) [2,5', 8-^{3}H] adenosine (181×10^7 Bq/mol: Amersham), and 10 μM cordycepin or 16 μM actinomycin D (Sigma) as appropriate, for 1.5 or 3 hr. Endosperms were then rinsed in 10 mM unlabelled adenosine, and extracted using a ground glass homogenizer in 1.0 ml extraction buffer: (0.1 M NaCl, 0.01 M EDTA, 0.1 M Na-glycine, pH 7.9). Protein was removed from the supernatant after centrifugation by vigorous mixing with 1.0 ml phenol–isoamyl alcohol–$CHCl_3$ (25:1:24) saturated with buffer, followed by back extraction of the organic solvent fraction with 1.0 ml buffer, mixing of the two aqueous fractions, and re-extraction from them with the organic solvent mixture. RNA was pptd from the final aq. fraction at −20° for 16 hr in 66% EtOH containing 27.5 mM KCl, and redissolved at 60° in 0.3 ml dissolution buffer (0.5 M NaCl in 10 mM Tris–HCl pH 7.9). One portion was counted to measure total radioactivity in the sample; another sample was clarified by centrifugation and applied to a 12-mm long, 9-mm diameter column of oligo-dT-cellulose (Boehringer-Mannheim Biochemicals, Indianapolis, Ind., U.S.A.) pre-equilibrated in the dissolution buffer. The

immediate column eluate was reapplied three times to ensure full finding of poly(A)$^+$ RNA. Poly(A)$^-$ RNA was eluted in a total of 5.0 ml dissolution buffer, then 3.0 ml buffer containing 0.25 M NaCl: poly(A)$^+$ RNA was eluted in 3.0 ml buffer without NaCl. Radioactivity in fractions was counted by scintillation spectrometry.

Two-dimensional electrophoresis: native isoelectric focusing (IEF) and SDS-PAGE. Mannanase was ^{3}H-labelled by incubation of eight batches of 75 endosperms each in 2.0 ml M buffer containing 5 μg/ml chloramphenicol (Sigma) and 50 μCi L-[4,5-^{3}H]leucine (2.22×10^{12} Bq/mol: Amersham). The endosperms were preincubated for 5 hr in buffer alone, to remove contaminating proteins derived from the fragments of embryo tissue: no mannanase was produced during this period. After 18 hr of radio-labelling, the incubation medium was clarified by centrifugation, and concentrated, diluted and reconcentrated three times by pressure-ultrafiltration (Amicon Model 3 Microvolume Stirred Cell with PM10 membrane, nominal exclusion limit, 10 kd: Amicon Corp., Lexington, Mississippi, U.S.A.). No mannanase activity was detectable in the liquid that passed through the membrane. The concentrated sample was made to 200 μl: 40 μl was mixed with 40 μl double-strength SDS-sample-suspension buffer, and used for direct SDS-PAGE; 160 μl was mixed with 20 μl glycerol and 20 μl 40% w/v pH 3–10 Biolyte ampholytes (Biorad Laboratories, Richmond, Cal., U.S.A.) and portions subjected to IEF.

Isoelectric focusing was carried out in 3.5-mm internal diameter, 125-mm-long silanized glass tubes containing 110-mm-long photopolymerized polyacrylamide gels (5% T, 5.3% C; containing 5% glycerol, 1.2% Biolyte 3/5 ampholytes, 0.8% Biolyte 3/10 ampholytes, 0.05% riboflavin: all w/v: Biorad) preconditioned overnight at 3°. Replicate samples were loaded at the cathodic end of 4 gels, and overlayed with spacer soln (1% 3/10 ampholytes, 5% glycerol). Electrofocusing was at 250 V for 17 hr, then 500 V for 1.5 hr, at 3°. (Anodic loading gave a similar pattern on IEF, but resulted in degradation artifacts on SDS-PAGE). Cathode buffer was 65 mM ethanolamine: anode buffer was 50 mM glutamate adjusted to pH 2.5 with HCl. In one replicate gel, β-lactoglobulin (Sigma) was added as internal standard: the pIs of the two isomers were taken as 5.35 and 5.45 [16].

The pH gradient in a replicate blank gel was measured using a microcombination electrode placed in eluates of 5 mm segements of gel in deaerated 0.1 M NaCl at 3° (Micro-electrodes, Londonderry, N. H., U.S.A.). Gels were frozen using solid CO_2, and stored at −80° until needed.

Four gels were used in the experiment illustrated in Fig. 2. Two gels, one with and one without β-lactoglobulin as internal standard, were fixed in 50% (w/v) TCA for 4 hr, and stained using $CuSO_4$/Coomassie Brilliant Blue (Sigma) [16], scanned at 595 nm in a spectrophotometer, photographed, destained, and cut into 1-mm slices for counting radioactivity, using NCS tissue solubilizer (Amersham; according to manufacturer's directions). The distribution of radioactivity was used to align the information gained from all four gels. A third gel was longitudinally bisected before taking 1-mm slices. One of each pair of half-slices was eluted with 100 μl 0.1 M Na-citrate buffer, pH 5.0, for 16 hr at 3°, and the eluate assayed for mannanase. The other half-slice was used for counting radioactivity. The forth replicate gel was used to generate the second electrophoretic dimension. Equilibration was in two changes of SDS suspension buffer for 30 min at room temp., then 10 min at 60° (1% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.001% bromophenol blue in 125 mM Tris–HCl buffer, pH 6.8). An aliquot of the original sample was included alongside. A 1.5-mm-thick slab SDS-polyacrylamide gel was made in a Biorad Model 220 apparatus, using the Laemmli buffer system [17] with modifications to the polyacrylamide gel formulation. The 100 mm separating gel was 17% T, 0.6%, C, containing 0.1% SDS in 300 mM Tris–HCl buffer, pH 8.8; the 25 mM stacking gel was 5% T, 0.6% C, containing 0.1% SDS in 125 mM Tris–HCl buffer, pH 6.8; and the electrode buffer was 0.1% SDS in 50 mM Tris–glycine buffer, pH 8.3. Electrophoresis was at 20 mA constant current until the marker dye reached the bottom of the gel. The fixed gel (50% w/v TCA) was stained (0.25% Coomassie Blue-R250 in MeOH–H_2O–HOAc, 5:4:1 (by vol.) for 3 hr; 0.025% dye in the same solvent for 3 hr; destained in MeOH–H_2O–HOAc, 2:7:1), and photographed. Then the gel was prepared for fluorography by infiltration with 2.5-diphenyl oxazole (Sigma) in DMSO [18], and exposed at −80° against Kodak X-omat R film, which was developed using Kodak KLX developer (Eastman-Kodak, Rochester, N. Y., U.S.A.).

Native polyacrylamide gel filtration chromatography. The incubation medium, containing material secreted by 600 isolated endosperms incubated with ^{3}H-leucine (Amersham) between 3 and 22 hr from dissection, was concentrated by pressure ultrafiltration, as described above. One-sixth of this material was diluted to 400 μl in 100 mM Na-citrate buffer, pH 5.0, and chromatographed on a 7-mm internal diameter, 480-mm-long silanized glass column BiogelP-60 (Biorad), eluted with 16 μl/h mm^2 buffer at 3°. Exclusion vol was 6.2 ml: free leucine eluted at 17.0 ml. Fractions (125 μl) were assayed for mannanase, and the location of peak activity found by plotting data on Gaussian-scale graph paper. The column was calibrated for molecular size using protein markers (Sigma; see Fig. 3), and the Bradford assay [19] or direct absorption spectrometry to measure protein.

In the experiment where it was desired to recover material after chromatography for use in subsequent electrophoresis, the eluting buffer was changed to pyridine–HOAc (about 60 mM and 40 mM), pH 5.0. This volatile buffer was used to avoid the problem found with citrate, where it was difficult to desalt without losing enzyme activity.

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