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MANNANASE FROM *LACTUCA SATIVA*: METABOLIC REQUIREMENTS FOR PRODUCTION AND PARTIAL PURIFICATION

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Key Word Index—*Lactuca sativa*; Compositae; mannanase; endosperm mobilization; enzyme synthesis and secretion.

Abstract—Endo-(1→4)-β-mannanase is produced by endosperms isolated from ungerminated Grand Rapids lettuce achenes when they are incubated in a sufficiently large volume of buffer (LV); this production is inhibited if incubation takes place either in a small volume (SV) or with ABA included in the large volume (LV + ABA). This report describes changes in protein and RNA synthesis concominant with mannananase production, the effects of inhibitors specific to these metabolic events on enzyme production, and partial purification of mannase from lettuce endosperms. After 18 hr or more of incubation, protein synthesis is greater in endosperms incubated in a LV than in endosperms incubated in either a SV or a LV + ABA. The peak of protein synthesis in endosperms in a LV precedes that of mannanase activity by *ca* 4 hr. Mannanase production is cycloheximide-sensitive, indicating that increased mannanase activity is dependent of protein synthesis. RNA synthesis is invariant with incubation conditions. Nevertheless, mannanase production can be reduced, although not prevented, by either actinomycin D or α-amanitin; these results suggest a transcriptional requirement for mannanase production. Partial purification of mannanase was achieved by DEAE-chromatography and affinity chromatography. Mannanase activity in the incubation medium of isolated endosperms can be attributed to two polypeptides of apparent M_r of 46 000 which may represent isozymes of the enzyme. These M_r values are similar to those reported for mannanases from diverse sources. Mannanase is only a minor product protein synthesis in isolated lettuce endosperms.

INTRODUCTION

The lettuce endosperm is a two-cell thick envelope encasing the embryo in intact achenes. Mannan-based carbohydrate reserves are stored in the thickened cell walls of the endosperm, and are mobilized early after germination (i.e. after radicle protrusion) is completed [1, 2]. Activity of endo-(1→4)-β-mannanase (EC 3.2.1.78) is required for degradation of mannan-based reserves [3], and the production of this enzyme in lettuce seeds is temporally regulated [4]. Mannanase in lettuce seeds is produced exclusively in the endosperm, wherein its activity increases markedly following germination. Enzyme production in endosperms isolated from intact, ungerminated seeds is controlled in large part by endogenous ABA [5]. Isolated endosperms will produce mannanase when they are incubated in a liquid volume sufficient to leach the ABA from the tissue (a large volume, LV). Enzyme production is prevented by either maintenance of the endosperms in a small volume of liquid (SV) or by addition of 10^{-6} M ABA to the medium (LV + ABA [6]).

We are taking advantage of these simple manipulations in volume and ABA addition in our investigations into the basis of the temporal regulation of mannanase production in lettuce seeds. To this end, we have tested the effects of inhibitors of protein and RNA synthesis on this event, and also have purified lettuce mannanase sufficiently for comparison to mannanases from other seed types and as a preliminary step towards antibody production.

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RESULTS AND DISCUSSION

Metabolic parameters of enzyme production

The kinetics of endo-(1→4)-β-mannanase production in endosperms isolated from the lettuce seed harvest used in this study is presented in Fig. 1A to serve as a reference to related metabolic events. The lag time prior to a detectable increase in enzyme activity in endosperms incubated in a LV is 20 hr in these seeds, and maximum activity occurs after 30 hr of incubation. There is no significant change in mannanase activity throughout the incubation period when endosperms are incubated in either a SV or a LV + ABA, as previously reported [6]. The peak of mannanase activity in a LV was achieved some 12 hr earlier in the studies of Halmer [7], although the maximum level of activity reported by him was lower by *ca* 40%.

Protein synthesis was assayed by measurement of the incorporation of ^{35}S-methionine into trichloroacetic acid (TCA)-precipitable material from both tissue extracts and incubation media, thus ensuring the inclusion of any labelled secreted proteins. Since the majority of mannanase activity is found in the incubation medium of isolated endosperms [6], this is an important consideration. After

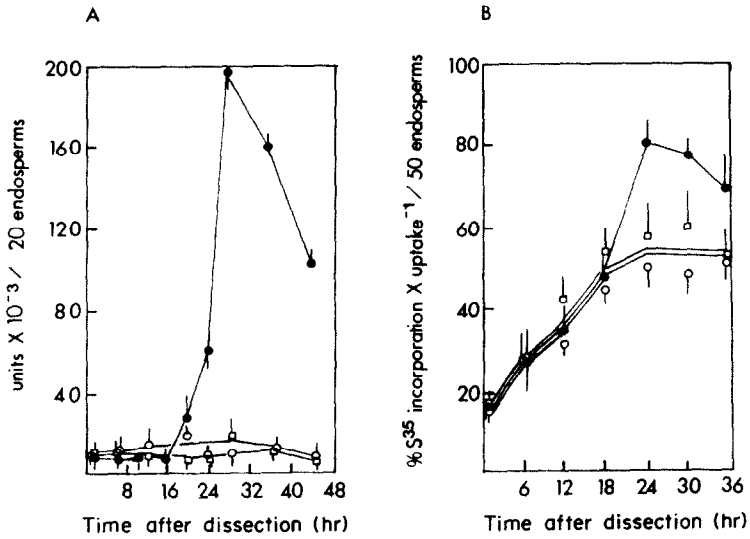

Fig. 1. Time course of mannanase activity (A) and protein synthesis (B) in isolated endosperms. Isolated endosperms were incubated in a LV (●), in a SV (○), or in a LV + ABA (□). Mannanase activity values are the mean of at least duplicate samples; triplicate samples at least were used to estimate protein synthesis.

the first 18 hr of incubation, endosperms incubated in a LV show a higher rate of protein synthesis relative to endosperms not engaged in mannanase production (Fig. 1B). This peak in protein synthesis precedes by about 4 hr that of mannanase production, and hence the production of the enzyme is unlikely to account for this difference. Halmer [7] has shown that protein synthesis increases in isolated endosperms placed in a LV up to the time of the start of mannanase production. We have shown in addition that this increase is incubation volume dependent.

Mannanase production in endosperms incubated in a LV is sensitive to cycloheximide (Table 1), and therefore is dependent upon protein synthesis. Enzyme production is decreased by as little as 0.1 mM (2.81 μg/ml) cycloheximide, although there is no measurable decrease in protein synthesis at this concentration. This result suggests that mannanase production comprises but a small proportion of total protein synthesis, so that a decline in enzyme synthesis does not necessarily result in a detectable reduction in ^{35}S-methionine incorporation. Also, lettuce mannanase may have an amino acid composition similar to that described for lucerne mannanase [9], in which methionine is represented in less than 1% of the amino acid residues. Cycloheximide at 10 mM completely prevents any increase in mannanase activity over the incubation period. These results are in agreement with the report that D_2O-labelling of isolated lettuce endosperms shows mannanase to be synthesized *de novo* in a LV [7]. It should be emphasized that these data do not demonstrate that regulation of mannanase production occurs at the level of synthesis of the enzyme, however. It does not rule out the possibility that mannanase is synthesized under all three incubation conditions, yet fails to accumulate in endosperms incubated in a SV or a LV + ABA due to a higher turnover rate and/or to inactivation of the enzyme. Indeed, our preliminary data indicate that turnover rate

Table 1. The effects of various concentrations of cycloheximide on mannanase production by the endosperms of lettuce seed

Concentration Cycloheximide (mM)	Mannanase activity (units × 10^{-3}/20 endosperms)	(% control)	[^{35}S] Methionine incorporation $\left(\frac{\%\text{ incorporation}}{\text{uptake}}\right)$	(% control)
0.0	9.2 ± 1.9	100	30 ± 4	100
0.1	5.8 ± 0.6	63	35 ± 3	117
1.0	4.0 ± 0.6	43	39 ± 6	130
10.0	0.5 ± 0.1	6	17 ± 2	56
50.0	0.8 ± 0.5	8	5 ± 5	17

*1 unit = a decrease in initial flow time of 1% hr^{-1} (see Experimental).

Cycloheximide was included from the start of incubation of isolated endosperms in a LV. Enzyme activity and protein synthesis were assayed in samples consisting of the combined extract and medium from the endosperms after 30 hr of incubation. Each value is the mean of at least three replicates.

is the primary point of regulation of mannanase production in isolated lettuce endosperms (not shown).

The incorporation of ^{3}H-uridine into trichloroacetic acid-precipitable material (i.e. RNA synthesis) does not vary significantly with incubation conditions up to 24 hr after dissection. At this time, total protein synthesis is maximal and mannanase production has ensued, so any changes in total RNA synthesis relevant to enzyme production would be expected to occur earlier. Mannanase production or its inhibition by ABA does not, then, involve any gross increases in RNA synthesis.

Mannanase production in isolated endosperms can be reduced, but not prevented, by inclusion of either cordycepin, actinomycin D [7] or α-amanitin with incubation. Actinomycin D at 50 μg/ml throughout a 30-hr incubation of endosperms in a LV reduces mannanase activity to 30% of that observed without the inhibitor, and reduces ^{3}H-uridine incorporation to 43% of control values. Increasing the concentration to as high as 100 μg/ml does not cause any further inhibition of either mannanase production or uridine incorporation. Lower concentrations do not inhibit enzyme prodution at all, although RNA synthesis is reduced somewhat. α-Amanitin is entirely ineffective at concentrations lower than 100 μg/ml. At 100 μg/ml, mannanase activity is similarly reduced to 30% of control values although RNA synthesis is reduced to only 70% of control values in this instance. Since mRNA synthesis generally represents 20–40% of total RNA synthesis in a typical eukaryotic cell, it is tempting to conclude that α-amanitin is specifically affecting mRNA synthesis in the endosperm cells and that the high concentration necessary to obtain this effect reflects difficulties in uptake of the inhibitor. Since complete inhibition of mannanase production could not be achieved by inclusion of the RNA synthesis inhibitor (as found by Halmer [7]) further studies using them could not be justified and this question was not directly addressed. Nevertheless, these experiments demonstrate a transcriptional requirement of some sort for mannanase production exists in isolated endosperms incubated in a LV. In our hands, cordycepin delayed the synthesis of mannanase by12 hr (not shown). Hence the reduction in enzyme activity demonstrated by Halmer [7] up to 16 hr after incubation could be due to a delay in synthesis of the enzyme rather than its total suppression of its production.

Purification of lettuce endo-β-mannanase

Endo-β-mannanase was partially purified from the proteins secreted into the medium during incubation of lettuce endosperms in a LV. The use of isolated endosperms removes α-galactosidase, which is produced by the lettuce embryo [2], from the initial sample; this enzyme commonly co-purifies with mannanase [9]. Since the bulk of mannanase is secreted, it is unnecessary to extract the endosperms. An initial 12 hr pre-incubation of the isolated endosperms served both to leach out ABA [5] and to remove any early-secreted proteins from the starting sample. The endosperms were then incubated for 24 to 30 hr in fresh buffer containing ^{35}S-methionine. A fluorograph of the labelled protein population in an aliquot of the initial sample is shown in Fig. 2A.

DEAE-chromatography separates mannanase from the bulk of the protein in the sample (Fig. 2B). The enzyme is released from the column when the NaCl concentration of the elution buffer reaches 275 mM, at which point there is release of two radioactively-labelled proteins of *ca* M_r 45 000 (Fig. 2C). There are a number of highly labelled proteins in these fractions also (e.g. *ca* 38 000) but they are unlikely to be mannanase since their elution profile does not parallel that of increased mannanase activity (Fig. 2B vs 2C, and they are present in later non-active fractions).

Further purification was achieved by affinity chromatography of the pooled fractions. Mannanase appears to bind tightly to the column (due to hydrophobic affinity binding), since no activity is detectable in the flow-through of the column wash. Active enzyme can be recovered from the column either by addition of 0.2% soluble mannan or of 1 M sodium chloride to the column buffer (Fig. 3A, B). Elution with mannan results in recovery of about 20% of the activity loaded onto the column. Increasing the concentration of mannan in the elution buffer did not improve this yield. The active eluate contains two polypeptides of similar M_r (46 000 and 43 000) when resolved by 1-dimensional SDS-PAGE and stained with Coomassie (Fig. 3C, lane 1). The low recovery of activity does not result from inhibition of the enzyme by yeast mannan (α 1,2-; 1,3- and 1,6-linked mannan), since inclusion of increasing amounts of this mannan in a standard assay mixture has not effect on the measurable activity. Higher recovery of enzyme activity can be achieved by elution of bound proteins with buffer containing sodium chloride at 1 M, but this also results in co-elution of other proteins not previously observed (Fig. 3C, lane 3), which also apparently bind to mannan residues. Since, however, the two proteins marked in Fig. 3 by arrows are the only proteins common to eluates obtained by both methods, it is apparent that one or both of them is responsible for mannanase activity. These two proteins separate under our electrophoretic conditions at similar positions to those of the 3 polypeptides of purified lucerne mannanase (Fig. 3C, lane 2). Mannanases purified from such diverse species as *Bacillus subtilis*, *Aspergillus nigens* [9] and *Chlorella* [10] also are of similar size. It is not unlikely that the two polypeptides in the lettuce extract are isozymes of mannanase, since all legume seeds examined to date (these being the only higher plant group in which mannanase has been studied other than lettuce) contain multiple mannanases, distinguished by M_r and/or pI [3].

Attempts at further resolution of lettuce mannanase by gel filtration chromatography either after DEAE-chromatography or affinity chromatography resulted in complete loss of enzyme activity. It is likely that this result, and also the low recovery of activity from affinity chromatography, is caused by partial denaturation of the protein by dilution effects. Similar difficulties in increasing the specific activity of a preparation with purification steps have also been noted in the purification of mannanase from lucerne [11] and *Chlorella* [10].

In Fig. 3C, lane 4, is presented a fluorograph of the labelled proteins present in a aliquot of the active eluate from a mannan affinity column eluted with 1 M sodium chloride. Both the 46 and 43 000 proteins that co-elute with mannanase activity incorporate ^{35}S-methionine, which is consistent with new synthesis of the mannanase protein in isolated endosperms incubated in a LV [7]. The amino acid composition of mannanase from lettuce is likely to be similar to that of lucerne, at least with respect to methionine (i.e. $<1\%$ methionine residues [11]), since the incorporation of label into either polypep-

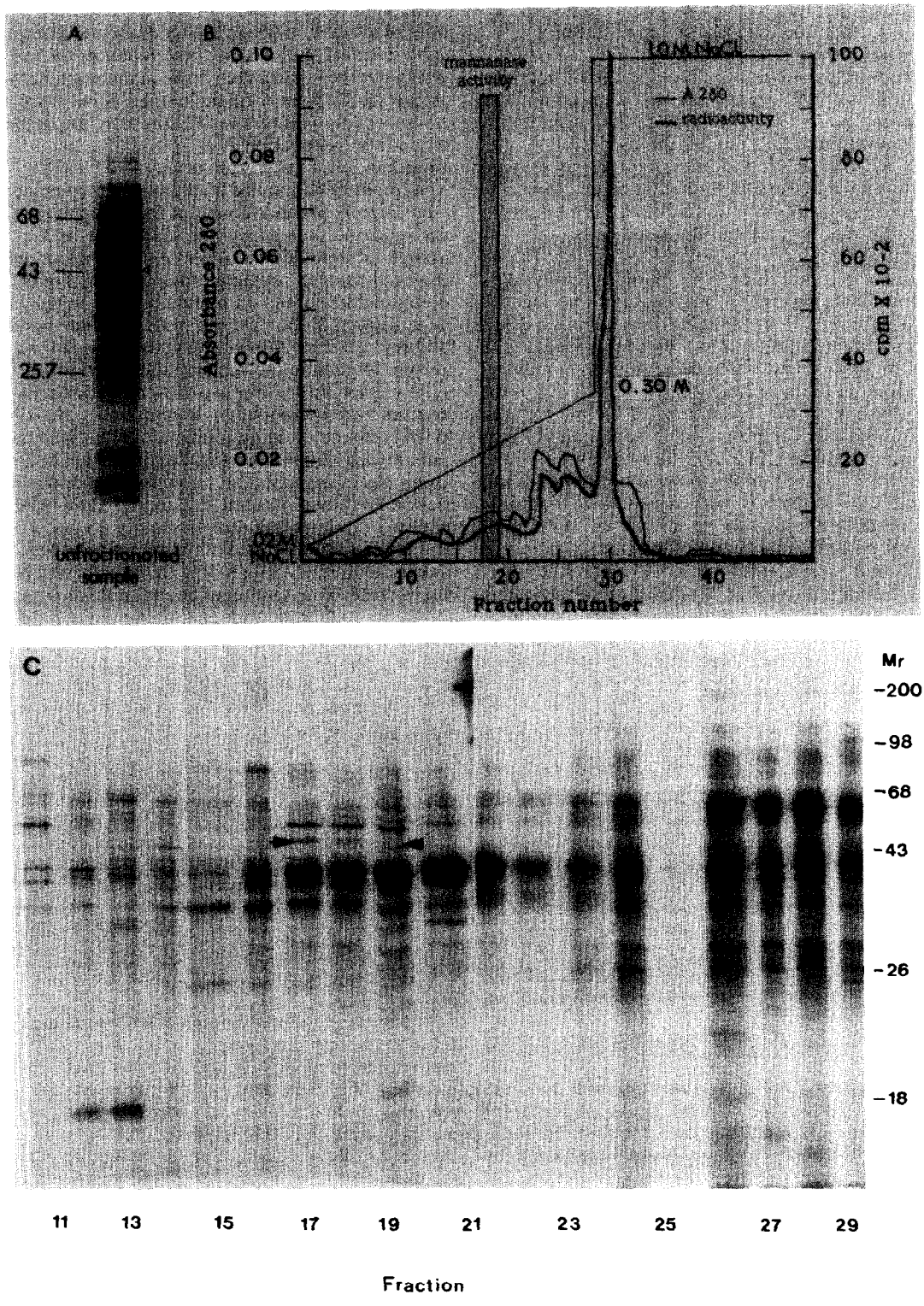

Fig. 2. Partial purification of lettuce endo-β-mannanase by DEAE chromatography. (A). Fluorograph of ^{35}S-methionine-labelled proteins in the starting sample of the incubation medium from 1000 endosperms incubated for 36 hr in 40 ml. (B). The elution pattern of the DEAE column when increasing salt concentrations are included in the elution buffer. Each 5 ml fraction was tested for A_{280} (solid line), radioactivity (hatched line) and mannanase activity (bar). The bar represents only the presence of the enzyme, and is not meant to indicate the amount of activity. (C). Fluorograph of labelled fractions of proteins eluting from the column with increasing salt concentration. The arrows shows the position at which mannanase separates, and the numbers on the right M_r in kd.

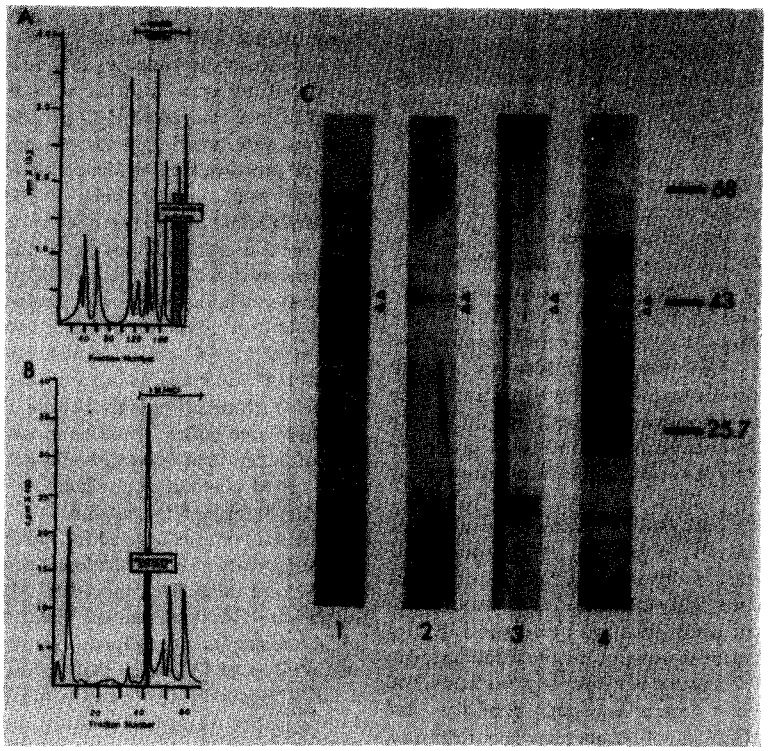

Fig. 3. Further enrichment of lettuce endo-β-mannanase by mannan-AH-Sepharose affinity chromatography. The elution patterns of radioactivity (line) and mannanase activity (box) when columns are eluted with either soluble yeast mannan (A) or with 1 M NaCl (B) are shown. (C). Lane 1 is the Coomassie Blue stained profile of the active fraction obtained by elution with 0.2% soluble mannan. The entire fraction was loaded, except that portion used to measure enzyme activity and radioactivity. Lane 2 is purified lucerne mannanase. Lane 3 is the silver-stained profile of the active fraction from a column eluted with 1 M NaCl. One-third of the total fraction was loaded. Lane 4 is a fluorograph of the remaining two-thirds of this elute. Arrows refer to the position of the polypeptides identified as lettuce mannanase. M_r in kd is shown to the right of the gel profiles.

tide is quite small. Also apparent in the sample are a number of highly labelled contaminants that are not observable using silver-staining. They are probably those polypeptides noted in the discussion of Fig. 2C which do not elute in parallel with mannanase activity. It is noteworthy also that the exposure time of the fluorograph in Fig. 3 is much longer (2 weeks vs 3 days) in comparison to the fluorographs in Fig. 2 in order to maximize the detection of the 46 and 43 000 bands. The relative amounts of these contaminants in the protein population, therefore, is much reduced after the affinity chromatography step.

In contrast to the claims of Halmer [7], we contend that mannanase is only a minor product of the lettuce endosperms and it is not concentrated as a secreted protein. It is undetectable in the initial starting sample by Coomassie staining (not shown) and forms only a minor band in the fluorograph (arrow, Fig. 2A). The latter may be due in part to the relatively low incorporation of ^{35}S-methionine into mannanase. We have labelled isolated endosperms with ^{14}C-labelled leucine and aspartate (not shown), which are more highly represented in lucerne mannanase, yet the incorporation of isotope into the 46 and 43 000 polypeptides is only marginally improved in the case of leucine, and not at all when aspartate is used.

That mannanase is such a minor product is surprising in light of the presumed role of the endosperm in lettuce seeds but, unlike Halmer [7], we have never found it to be a major protein in either the endosperm cells or in the proteins secreted into the surrounding medium. Mannanase activity increases as a post-germinative event [4], and germination can take place in the absence of mannanase activity [12]. Evidently the activity of the enzyme is such that only small amounts of the protein need to be produced in order to provide enough activity to effect mobilization of the galactomannan reserves present in the endosperm cell walls. This tissue thus is quite unlike the well-described endosperms of monocots, in which most of the protein synthetic machinery of the associated aleurone layers is harnessed for the production of the key enzyme of degradation, α-amylase [13].

Further resolution of the mechanism of regulation of mannanase production in lettuce seeds rests on the development of appropriate molecular probes. The enrichment procedure presented here should facilitate the production of antibodies against mannanase, as well as subsequent screening requirements. The small amount of protein used, and the difficulties in dissecting very large numbers of lettuce endosperms militate against rapid progress.

EXPERIMENTAL

Plant material. Lettuce achenes (*Lactuca sativa* cv Grand Rapids) were obtained from the Ferry Morse Seed Co. (Mountain View, CA, 1983 harvest) and stored at 4° until use. Imbibition and dissection of the seeds was as previously described [6]. Isolated endosperms were quickly rinsed with sterile H_2O prior to incubation under appropriate conditions at 25° in darkness. Conditions typically were: (i) large volume (LV), 1 ml 10mM 2(*N*-morpholino)-ethanesulphonic acid (MES; Sigma, St. Louis, U.S.A). pH 5.0 per 20 endosperms or 3 ml per 50; (ii) small volume (SV), 20 μl buffer per 20 endosperms or 50 μl per 50; (iii) LV + ABA, as described for LV but with addition of 10^{-6} M +/- *cis, trans* abscisic acid (Sigma). All buffers were filter-sterilized prior to use. LV and LV + ABA samples were incubated in sterile 35 mm diam. plastic Petri-dishes; SV samples were incubated in glass spot-plates which were surface sterilized with EtOH just prior to use. All vessels were placed in a sealable plastic container lined with moistened paper towelling for the incubation period to prevent moisture loss.

Mannanase activity determinations. Endosperms were extracted in ice cold 0.1 M Tris–glycine (pH 7.8) in a Kontes–Duall ground-glass homogenizer (size 20) on ice. Extracts were clarified by low speed centrifugation, and mannanase activity was determined by the viscometric assay described previously [6]. Viscosity measurements were taken using a Cannon-Manning semi-micro viscometer (size 75), using as substrate a 0.1% soln of galactomannan from locust bean gum (Sigma) purified as described by McCleary [14]. A unit of enzyme activity is defined as a decrease in initial flow-time of 1% hr^{-1}. Activity is expressed on a per endosperm basis, since insufficient protein was present to measure.

Where quick determination of the presence or absence of mannanase was required, mannanase activity was first detected by reaction with *p*-hydroxybenzoic acid hydrazide (PAHBAH; Sigma) as described by McClearly [14], and then confirmed by the viscometric assay.

Protein synthesis. Protein synthesis was measured as incorporation of ^{35}S-methionine (> 800 Ci/mmol, New England Nuclear, Boston, USA) into TCA-precipitable material as described in ref. [12]. Isolated endosperms (50) were incubated under appropriate conditions without label for various periods, rinsed × 3 with sterile H_2O, and then incubated for 1 hr in fresh sterile buffer containing 50 μ Ci ^{35}S-methionine. After labelling, the incubation medium was removed and the endosperms rinsed × 3 with sterile H_2O. The medium and the rinse water were combined and retained to correct for incorporation into secreted proteins. The endosperms were extracted as described for mannanase determinations. Duplicate aliquots (100 μl) of each sample were counted in a dioxane-based scintillation cocktail. Quench-corrected values were expressed as percentage incorporation of uptake.

RNA synthesis. Samples were prepared as described above, except that labelling was with 20 μ Ci ^{3}H-uridine (35–50 Ci/mmol). Endosperms were rinsed × 3 with an excess of sterile H_2O after incubation in label, then immediately frozen in liquid N_2. Endosperms remained in liquid N_2 until immediately prior to extraction, when they were ground in 1 ml extraction buffer [7 M urea, 135 mM NaCl, 10 mM Tris–HCl (pH 7.2), 2 mM DTT, 7 mM EDTA and 2% (w/v) SDS] in a ground glass homogenizer (Kontes– Duall, size 21) at room temp. Extracts were clarified of cell-wall material by low-speed centrifugation, made to 200 μg/ml with salmon sperm DNA (Sigma) as carrier, then spotted onto glass-fibre discs (Whatman GC) which were processed as described in ref. [15]. Values are expressed as percentage incorporation of uptake, after correction for quenching.

Purification of endo-β-mannanase. The purification scheme was based on that described in ref. [9] for lucerne seed mannanase. Two samples of 500 endosperms were isolated on separate days, and rinsed at least × 5 with excess sterile H_2O before incubation for 12 hr in 20 ml sterile H_2O per 250 endosperms. The endosperms were rinsed again at this point to remove any early secreted proteins, then incubated for a further 24–30 hr in fresh sterile buffer containing 300 μCi ^{35}S-methionine (> 800 Ci/mmol, NEN) per 1000 endosperms.

At the end of incubation, the medium was collected and the endosperms rinsed × 3 with 5 ml sterile H_2O. The medium and rinse water of both samples were combined and clarified by low-speed centrifugation. The buffer was changed to 20 mM Tris–MES (pH 8.0) and the sample coned to 3 ml under ultra-filtration (CS-10 filters, Millipore). This was applied to a DEAE-BioGel A (BioRad) column (0.8 cm i.d. × 30 cm) equilibrated with the same buffer. The column was washed with 3 column vols of buffer, then bound protein was eluted by passage of a 0.02–0.30 M continuous gradient (150 ml) total vol. generated with NaCl) through the column.

Each fraction was assayed for radioactivity, A_{280}, and mannanase activity. Those fractions with enzyme activity were pooled, and the buffer changed to 20 mM Tris–MES (pH 5.0) under ultra-filtration as above. This sample (0.5 ml) was applied to a mannan-affinity column. The matrix was prepared as described in ref. [9] except that 1 g yeast mannan was linked to 1.5 g AH-Sephracyl by the carbodiimide method recommended by the matrix manufacturer. After loading of the sample, unbound proteins were eluted with 15 column vols of 20 mM Tris–MES (pH 5.0); bound proteins were eluted by the same buffer but with addition of either 0.2% (w/v) solubilized yeast mannan or 1 M NaCl.

The mannanase-containing fractions from the affinity CC step were desalted and concentrated by ultrafiltration as above when elution was with soluble mannan. The concentrated sample was then prepared for PAGE as described earlier. When elution was with 1 M NaCl, the sample was concentrated and desalted with an A25 conen filter (Amicon), then diluted with 1 vol of 2 × gel loading buffer prior to electrophoresis [17]. After electrophoresis, proteins were stained with Coomassie Blue R or with silver [16].

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REFERENCES

1. Halmer, P., Bewley, J. D. and Thorpe, T. A. (1978) *Planta* **139**, 1.
2. Leung, D. W. M., Reid, J. S. G. and Bewley, J. D. (1979) *Planta* **146**, 335.
3. McCleary, B. V. and Matheson, N. K. (1975) *Phytochemistry* **14**, 1187.
4. Halmer, P., Bewley, J. D. and Thorpe, T. A. (1976) *Planta* **130**, 189.
5. Dulson, J. and Bewley, J. D. (1988) *Plant Physiol.* **87**, 660.
6. Halmer, P. and Bewley, J. D. (1979) *Planta* **144**, 333.
7. Halmer, P. (1989) *Phytochemistry* **28**, 371.
8. Leung, D. W. M. and Bewley, J. D. (1983) *Planta* **157**, 274.

9. McCleary, B. V. (1978) *Phytochemistry* **17**, 651.
10. Loos, E. and Meindl, D. (1985) *Planta* **166**, 557.
11. Villarroya, T. and Petek, F. (1976) *Biochem. Biophys. Acta* **438**, 200.
12. Powell, A. D., Dulson, J. and Bewley, J. D. (1984) *Planta* **162**, 40
13. Jacobsen, J. V., Higgins, T. J. V. and Zwar, J. A. (1979) in *The Plant Seed* (Rubenstein, I., Phillips, R. L., Green, C. E., and Gengenbach, B. G., eds), pp. 241–261. Academic Press, New York.
14. McClearly, B. V. (1978) *Carbohyd. Res.* **67**, 213.
15. Yu, E. L. and Fiegelson, P. (1971) *Analyt. Biochem.* **39**, 319.
16. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307.
17. Laemmli, U. K. (1970) *Nature* **227**, 680.