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MULTIPLE ISOZYMES OF *ENDO*-β-D-MANNANASE IN DRY AND IMBIBED SEEDS

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Key Word Index—Monocot; dicot; gymnosperm; seed; isozyme; *endo*-β-D-mannanase.

Abstract—A gel overlay method for detecting isozymes of *endo*-β-D-mannanase (EC 3.2.1.78) separated by isoelectric focusing has been developed for use with plant extracts. The seeds of 19 selected species of monocots, dicots, and gymnosperms (and all cultivars thereof tested) expressed *endo*-β-D-mannanase activity when imbibed and most also contained enzyme activity in the dry state. Generally, the number of isozymes increased when the seed imbibed water. This was most evident when the seed was left intact instead of being separated into the embryo and nutritive tissue (if present) prior to imbibition. Usually, more isozymes were present in the endosperm/megagametophyte than in the embryo regardless of whether the seed was intact or separated. In some cases, isozymes were recovered from the incubation water in which the seeds or seed parts had been imbibed. Such isozymes were considered to be mature forms of the enzyme that were present extracellularly and had leaked from the cell walls into the surrounding medium. The roots, shoots, and leaves of flowering alfalfa also contained *endo*-β-D-mannanase. In the roots there was one very active isozyme, identical in p*I* to that present in the imbibed alfalfa seed.

INTRODUCTION

Mannan hemicelluloses have been found in the cell walls of a variety of plant species (e.g. *Lactuca sativa* L. [1]; *Phaseolus vulgaris* [2]; *Cucurbita maxima* Duch. [3]; *Avena sativa* [4]; *Lupinus angustifolius* [5]). The siphonouos green algae have microfibrils of mannan, replacing cellulose [6]. Mannans also serve as storage carbohydrates in many seeds (legumes [7]) and in the perennial organs of some plants [8–10]. *endo*-β-D-Mannanase (EC 3.2.1.78), an *endo*-enzyme which hydrolyses mannans and galactomannans, has been implicated as an important enzyme in the germination of seeds of tomato (*Lycopersicon esculentum*) [11] and lettuce (*Lactuca sativa*) [1] and in the post-germinative mobilization of endosperm cell walls, e.g. lettuce [12] and fenugreek [13].

Given the ubiquity of long-chain, substituted mannans in cell walls, and the specialized role they have as carbon reserves in some stages of the life-cycle of many plants, it would be expected that the partial or complete hydrolysis of these hemicelluloses is under rigid control. One mechanism by which the cell may regulate the degradation of mannans is by producing several variants of the same *endo*-enzyme, each with unique affinities and substrate-binding requirements. The importance of such heterogeneity in regulation has been demonstrated for other enzymes in plant cells [14]. Because of the lack of an effective method for detecting multiple forms of this enzyme in crude homogenates, only two studies have been undertaken previously to identify or study the isozymes of *endo*-β-D-mannanase in plants. *endo*-β-D-Mannanase has been purified from alfalfa seed [15] and tomato pericarp [16]. The p*I*s determined were 4.5 and 4.4 for alfalfa seeds and 9.3 for tomato pericarp.

Here, we report the development and improvement of an overlay and staining method, previously used with fungal extracts [17], for the separation and detection of plant *endo*-β-D-mannanase isozymes and demonstrate its effectiveness using seeds of several species, and non-seed parts of one species.

RESULTS AND DISCUSSION

Monocotyledons

All extracts from the seeds of monocotyledonous plants, whether imbibed intact or following separation into embryo and endosperm, had at least one isozyme of *endo*-β-D-mannanase in the pH range used for this study (Tables 1–4). The 4-hr-imbibed embryo usually contained more isozymes than the endosperm, not counting the isozymes detected in the imbibition water (the exception was barley; Tables 1 and 2), whereas, in the 24–48-hr-imbibed seeds (either separated or intact), the embryo usually contained fewer isozymes than the endosperm (the exceptions were separated barley parts and intact rice; Tables 3 and 4). Intact grains leaked isozymes of certain p*I* values (barley, 4.6; oats, 6.6, and rice, 4.0 or 4.2)

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Table 1. Isoelectric points of isozymes from the embryo of early imbibed seeds of monocots, dicots and gymnosperms*

Species	Seed part	Dry or imbibed	Number of isozymes	pI of isozymes
(A) *Monocots*				
Barley cv Himalaya	Embryo	4 hr	1	5.6W
Oats	Embryo	4 hr	8	3.9, 4.3, 4.4, 4.8, 4.95, 5.0, 5.3W, 6.5?
Wheat cv Frederick	Embryo	4 hr	11	4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 5.0, 5.1, 5.3W, 6.5?
Rice cv Bengal	Embryo	4 hr	3	4.9, 5.3W, 6.5?
(B) *Dicots*				
Tomato				
cv Money Maker	Embryo	24 hr	UD	
cv Caruso	Embryo	24 hr	UD	
Lettuce				
cv Grand Rapids	Embryo	Dry	UD	
cv New York	Embryo	Dry	UD	
Fenugreek	Embryo	4 hr	UD	
Pea	Embryo	4 hr	UD	
Soybean	Embryo	4 hr	1	5.6W
Alfalfa	Embryo	4 hr	UD	
(C) *Gymnosperms*				
Black spruce	Embryo	Dry	2	5.4?, 6.9W
White spruce	Embryo	Dry	3	4.8, 5.2, 6.9W
Sitka spruce	Embryo	Dry	5	4.8, 4.9, 5.0, 5.4W, 6.2?
Jack pine	Embryo	Dry	2	4.7?, 6.9W
Red pine	Embryo	Dry	2	5.3, 6.9W
Douglas fir	Embryo	Dry	3	4.6, 6.1, 6.9W
Nordmann fir	Embryo	Dry	1	6.9W
Eastern larch	Embryo	Dry	1	6.9W

*Isozymes that were indistinctly detected are signified by a ? appearing after their pIs. W immediately after a pI value signifies the appearance of the isozyme at the corresponding point on the overlay at which sample wick was placed on the IEF gel. UD, no isozymes were detectable.

into the imbibition water. Isozymes which appeared in the imbibition water in which separated seed parts were imbibed can be considered as secreted forms of *endo-β*-D-mannanase.

For both the embryo and the endosperm, the number of isozymes detected was greater when the seeds were left intact (the exceptions were barley embryo and rice endosperm) than when the parts were dissected and incubated separately. Of the monocot seeds investigated after only a short time of imbibition, wheat had the greatest number of isozymes in both the embryo and endosperm, 11 and 9, respectively (Tables 1 and 2). Of the monocot seeds studied under imbibed and separated conditions, rice had the greatest number of isozymes in both the embryo (8) and endosperm (11) (Tables 3 and 4). For the intact, imbibed seeds, the greatest number of isozymes was 13 for the oat embryo and 23 for the wheat endosperm. Both oat and wheat endosperm contained 14 more isozymes under the 24-hr-imbibed intact conditions that in the 4-hr-imbibed state. Representative contact prints of the isozymes of the endosperm from imbibed intact seeds are given in Fig. 1.

Dicotyledons

Of all the seeds surveyed from the dicotyledonous plants, only the soybean seed contained a detectable isozyme of *endo-β*-D-mannanase in the dry or early-imbibed (4 hr) state (Table 1). In marked contrast, almost all extracts from imbibed seeds contained at least one isozyme; the exceptions were notably Iceberg and Prizehead Leaf cultivars of lettuce and ungerminated pea embryos (Tables 3 and 4).

Separation of the embryo from the endosperm had pronounced effects on the number of detectable isozymes in both tomato and lettuce cultivars. For lettuce, the intact seed produced more isozymes than seeds separated into their respective parts (Tables 3 and 4; exceptions were cv Iceberg and cv Prizehead Leaf which contained no detectable isozymes even after imbibition of the intact seed). For lettuce, no isozymes in the pH range used could be detected in either part when the embryo and endosperm were separated. The number of isozymes in the whole imbibed intact seeds from lettuce cultivars ranged from 0 to 14. For tomato, the endosperm contained or released as many, if not more isozymes when the parts were separated (with the exception of cv Caruso in which there were no isozymes in either part) than did the intact seed. The number of isozymes in the imbibed intact seed of different tomato cultivars ranged from 4 to 6 (Table 3; Fig. 2).

Fenugreek and carob seeds, which characteristically have a largely galactomannan-containing endosperm, contained 5 and 9 isozymes, respectively, within the endo-

Table 2. Isoelectric points of isozymes from the endosperm or megagametophyte of early imbibed seeds of monocots, dicots and gymnosperms*

Species	Seed part	Dry or imbibed	Number of isozymes	pI of isozymes
(A) *Monocots*				
Barley cv Himalaya	Endo	4 hr	5	4.3, 4.4, 4.6, 5.6W, 6.5
Oats	Endo	4 hr	8 [3]	4.4?, 4.5, 4.6, 5.0, 5.1?, 5.3W, 6.0?, 6.5
Wheat cv Frederick	Endo	4 hr	9 [1]	4.6, 4.8, 4.9, 5.1?, 5.2?, 5.3W, 5.5, 6.0?, 6.5
Rice cv Bengal	Endo	4 hr	1 [12]	[3.4], [3.7], [3.9], [4.1], [4.3], [4.4], [4.8], [4.9], [5.1], [5.3], [5.4], [5.6W], 6.5
(B) *Dicots*				
Tomato				
cv Money Maker	Endo	24 hr	UD	
cv Caruso	Endo	24 hr	UD	
Lettuce				
cv Grand Rapids	Endo	Dry	UD	
cv New York	Endo	Dry	UD	
Fenugreek	Endo	4 hr	UD	
Alfalfa	Endo	4 hr	UD	
(C) *Gymnosperms*				
Black spruce	Meg	Dry	4	4.7, 4.75, 4.9, 5.4W
White spruce	Meg	Dry	3	4.7, 4.9, 5.4W
Sitka spruce	Meg	Dry	5	4.8, 4.9, 5.0, 5.4W, 6.2
Jack pine	Meg	Dry	2	4.2, 5.4W
Red pine	Meg	Dry	1	5.4W
Douglas fir	Meg	Dry	16	4.1, 4.15, 4.2, 4.3, 4.35, 4.4, 4.6, 4.7, 4.95, 5.0, 5.1, 5.15, 5.2, 5.25, 5.3, 5.4W
Nordmann fir	Meg	Dry	2	5.2, 5.4W
Eastern larch	Meg	Dry	2	4.7, 5.4W

*Endo, designates endosperm. Meg. designates megagametophyte. See Table 1 for details.

Isozymes appearing in detectable quantities in the incubation water of seeds or seed parts are designated by square brackets.

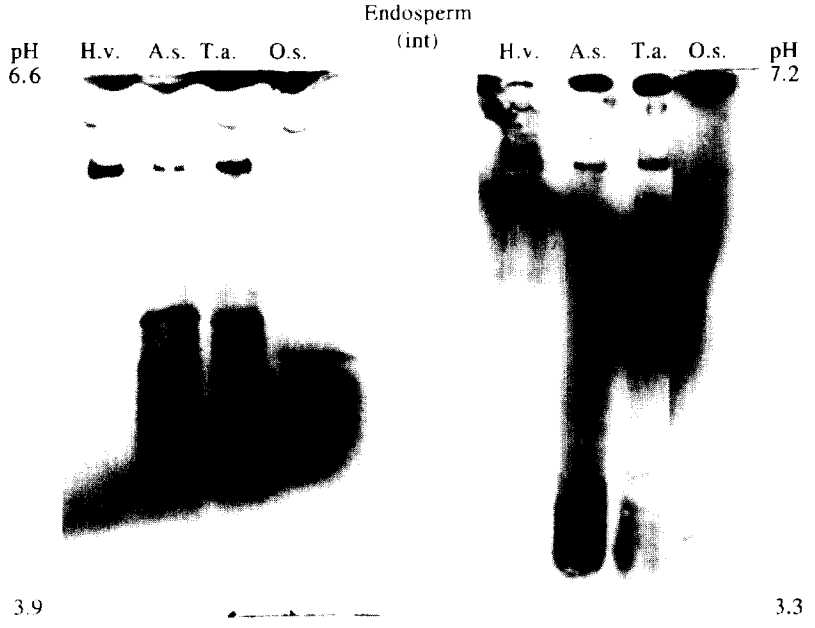

Fig. 1. Contact prints of *endo*-β-D-mannanase isozymes from the overlay of a 3.9–6.6 (left) and a 3.3–7.2 (right) pH-range IEF gel. The gel used for the contact print on the left was made using 4–6 range ampholytes while that on the right was made from 5–7 range ampholytes. The pH values of the IEF gel which are given are those extrapolated from the graph of the measured pH gradient. The endosperm samples of *Hordeum vulgare* (H.v.), *Avena sativa* (A.s.), *Triticum aestivum* (T.a.), and *Oryza sativa* (O.s.) were taken from seeds imbibed intact until the time of harvest.

Table 3. Isoelectric points of isozymes from the embryo or from imbibed seeds of monocots and dicots*

Species	Seed part	Hour of dissection	Hour of harvest	Number of isozymes	pI of isozymes
(A) *Monocots*					
Barley cv Himalaya	Embryo (Sep)	6	24	5	4.3?, 6.1, 6.2W, 6.3, 7.4
Oats	Embryo (Sep)	6	24	4	4.2, 6.3W, 7.1, 7.3
Wheat cv Frederick	Embryo (Sep)	6	24	5	4.2, 6.3W, 7.1, 7.3, 7.4
Rice cv Bengal	Embryo (Sep)	6	48	8 [1]	4.9, 5.0, [6.3], 6.3W, 6.5, 6.6, 6.7, 7.1, 7.3
Barley cv Himalaya	Embryo (Int)	NA	24	4	4.1, 4.3, 6.3W, 7.4?
Oats	Embryo (Int)	NA	24	13	4.1, 4.4, 4.65, 4.8, 4.9, 4.95, 5.0, 5.05, 5.1, 6.2W, 6.3W, 7.1, 7.3
Wheat cv Frederick	Embryo (Int)	NA	24	7	4.2?, 4.7, 4.8, 6.2W, 6.3W, 7.1, 7.4
Rice cv Bengal	Embryo (Int)	NA	48	7	4.1?, 4.7, 5.5, 6.1W, 6.8, 7.1, 7.3
(B) *Dicots*					
Tomato					
cv Money Maker	Embryo (Sep)	24	96	UD [4]	[4.4], [4.8], [4.9], [4.95]
cv Caruso	Embryo (Sep)	24	96	UD	
cv Vendor	Embryo (Sep)	24	96	UD [1]	[5.45]
cv Burpee's Big Boy	Embryo (Sep)	24	96	UD	
cv Scotia	Embryo (Sep)	24	96	UD [1]	[4.9]
cv Cold Set	Embryo (Sep)	24	96	UD	
cv Manitoba	Embryo (Sep)	24	96	UD [3]	[4.3], [4.4], [4.5]
cv Stokesalaska	Embryo (Sep)	24	96	UD	
cv Money Maker	WSeed (Int)	NA	96	5	5.0, 5.3, 5.4, 5.6, 5.8
cv Caruso	WSeed (Int)	NA	96	5	5.0, 5.3, 5.4, 5.6, 5.8
cv Vendor	WSeed (Int)	NA	96	5	5.0, 5.3, 5.4, 5.6, 5.8
cv Burpee's Big Boy	WSeed (Int)	NA	96	6	5.0, 5.3, 5.4, 5.5, 5.6, 5.8
cv Scotia	WSeed (Int)	NA	96	4	5.3, 5.4, 5.6, 5.8
cv Cold Set	WSeed (Int)	NA	96	5	5.0, 5.3, 5.4, 5.6, 5.8
cv Manitoba	WSeed (Int)	NA	96	6	5.0, 5.3, 5.4, 5.5, 5.6, 5.8
cv Stokesalaska	WSeed (Int)	NA	96	4	5.3, 5.4, 5.6, 5.8
Lettuce					
cv Grand Rapids	Embryo (Sep)	4	24	UD	
cv New York	Embryo (Sep)	4	24	UD	
cv Iceberg	Embryo (Sep)	4	24	UD	
cv May King	Embryo (Sep)	4	24	UD	

cv Prizehead Leaf	Embryo (Sep)	4	24	UD	
cv Oak Leaf	Embryo (Sep)	4	24	UD	
cv Romaine	Embryo (Sep)	4	24	UD	
cv Grand Rapids	WSeed (Int)	NA	24	9	4.25, 4.3, 4.40, 4.45, 4.5, 4.6, 4.7, 4.8, 4.9
cv New York	WSeed (Int)	NA	24	7	4.25, 4.3, 4.4, 4.45, 4.5, 4.6, 4.65
cv Iceberg	WSeed (Int)	NA	24	UD	
cv May King	WSeed (Int)	NA	24	7	4.3, 4.4, 4.45, 4.6, 4.65, 4.8, 4.9
cv Prizehead Leaf	WSeed (Int)	NA	24	UD	
cv Oak Leaf	WSeed (Int)	NA	24	14	4.0, 4.05, 4.15, 4.2, 4.25, 4.3, 4.4, 4.45, 4.5, 4.6, 4.65, 4.8, 4.9, 4.95
cv Romaine	WSeed (Int)	NA	24	9	4.25, 4.3, 4.35, 4.45, 4.5, 4.6, 4.65, 4.8, 4.9
Fenugreek	Embryo (Sep)	4	24	1	Varies with gel
Carob	Embryo (Sep)	6	96	ND	ND
Pea	Embryo (Int) (UG)	NA	48	0	
	(Int) (G)	NA	48	1	5.4W
Soybean	Embryo (Int) (UG)	NA	48	6 [17]	[4.4], [4.6], [4.8], [4.9], 5.0, [5.0], [5.2], 5.3, [5.3], [5.4], [5.6], [5.7], [5.8], [6.0], 6.2W, [6.2], [6.3W], 6.6?, [6.9], 7.0, 7.1, [7.1], [7.5]
	Embryo (Int) (G)	NA	48	2 [13]	[4.4], [4.6], [4.8], [4.9], [5.0], [5.2], [5.3], [5.4], [5.6], [5.7], [5.8], [6.0], [6.2], [6.3W], [6.9], 7.1, [7.1], 7.2W, [7.5]
Alfalfa	Embryo (Int)	NA	24	3	4.0, 4.2?, 5.1W

*Seeds which were imbibed intact for the full incubation period until harvest (hr of harvest) and then separated into embryos and endosperms are designated by (Int). Isozymes detected in incubation water from intact seeds are of uncertain origin and are therefore reported for both the embryo and the endosperm. Seeds which were imbibed and dissected at specific times (hr of dissection) into component parts and then incubated for a prescribed period until harvest (hr of harvest) are designated by (Sep). Isozymes that were indistinctly detected are signified by a ? appearing after their p*I*s. W immediately following a p*I* value signifies the appearance of the isozyme at the corresponding point on the overlay at which sample wick was placed on the IEF gel. Isozymes appearing in detectable quantities in the incubation water of seeds or seed parts are designated by square brackets. UD, no detectable isozymes. ND, isozymes not determined. UG, seed had not completed germination. G, seed had completed germination. NA, not applicable. WSeed, whole seed.

Table 4. Isoelectric points of isozymes from the endosperm of imbibed seeds of monocots and dicots*

Species	Sep or Int	Hour of dissection	Hour of harvest	Number of isozymes	p*I* of isozymes
(A) *Monocots*					
Barley cv Himalaya	Sep	6	24	4 [1]	4.2, [4.4], 6.1?, 6.2W, 7.2
Oats	Sep	6	24	9 [3]	4.3, 5.0, 5.1, 5.15, 5.2, 6.1?, 6.2W, [6.3], 6.4, [6.8], [6.9W], 7.1
Wheat cv Frederick	Sep	6	24	8 [3]	4.6?, 4.8, 4.9, 5.1, 5.2, 6.1?, 6.2W, [6.2], [6.3], [6.4], 7.1
Rice cv Bengal	Sep	6	48	11 [6]	3.9, [4.3], 4.6, 4.7, 4.75, 4.8, 4.9, 5.0?, 5.1, 6.0, [6.1], [6.2], [6.4], 6.2W, [6.9W], 7.1, [7.8]
Barley cv Himalaya	Int	NA	24	10 [1]	3.9?, [4.6], 5.1, 5.4, 5.8?, 5.9?, 6.1, 6.2W, 6.4, 6.6, 7.1
Oats	Int	NA	24	22 [1]	3.9, 4.3, 4.4?, 4.5, 4.6, 4.7, 4.75, 4.8, 4.9, 4.95, 5.0, 5.1, 5.2?, 5.5, 5.7, 5.8, 5.9, 6.2W, 6.3, 6.5, [6.6], 7.1, 7.4
Wheat cv Frederick	Int	NA	24	23	4.3, 4.4?, 4.5, 4.6, 4.7, 4.8, 4.9, 4.95, 4.97, 5.0?, 5.1?, 5.2, 5.3?, 5.5, 5.6?, 5.7, 5.8?, 5.9, 6.0, 6.1, 6.2W, 7.1, 7.4
Rice cv Bengal	Int	NA	48	6 [2]	[4.0?], [4.2], 4.7, 4.8, 4.9, 6.2W, 7.1, 7.4
(B) *Dicots*					
Tomato					
cv Money Maker	Sep	24	96	1 [4]	[4.0], [4.1], 4.2, [4.4], [4.5]
cv Caruso	Sep	24	96	UD	
cv Vendor	Sep	24	96	3 [8]	[4.3], [4.5], [4.80], [4.85], [4.90], [5.0], 5.1, [5.3], 5.35, 5.7, [5.9]
cv Burpee's Big Boy	Sep	24	96	3 [1]	5.1, 5.35, 5.7, [5.9]
cv Scotia	Sep	24	96	3 [3]	[4.3], [4.85], 5.1, 5.35, 5.7, [5.9]
cv Cold Set	Sep	24	96	3 [2]	5.1, [5.3], 5.35, 5.7, [5.9]
cv Manitoba	Sep	24	96	3 [6]	[4.80], [4.82], [4.85], [5.0], 5.1, [5.3], 5.35, 5.7, [5.9]
cv Stokesalaska	Sep	24	96	3 [14]	[4.5], [4.75], [4.80], [4.85], [4.9], [5.0], 5.1, [5.2], [5.3], 5.35, [5.4], [5.45], [5.49], [5.5], [5.6], 5.7, [5.9]
Lettuce					
cv Grand Rapids	Sep	4	24	UD	
cv New York	Sep	4	24	UD	
cv Iceberg	Sep	4	24	UD	
cv May King	Sep	4	24	UD	
cv Prizehead Leaf	Sep	4	24	UD	
cv Oak Leaf	Sep	4	24	UD	
cv Romaine	Sep	4	24	UD	
Fenugreek	Sep	4	24	5 [5]	4.4, [4.4], 4.5, [4.5], 4.8, [4.8], 5.0, [5.0], 5.3, [5.3]
Carob	Sep	6	96	9 [4]	4.25, 4.3, 4.4, [4.4], 4.5, [4.5], 4.6, [4.6], 4.7, [4.7], 5.0, 5.05, 5.2
Alfalfa	Int	NA	24	1	4.0

*See Table 3 for details.

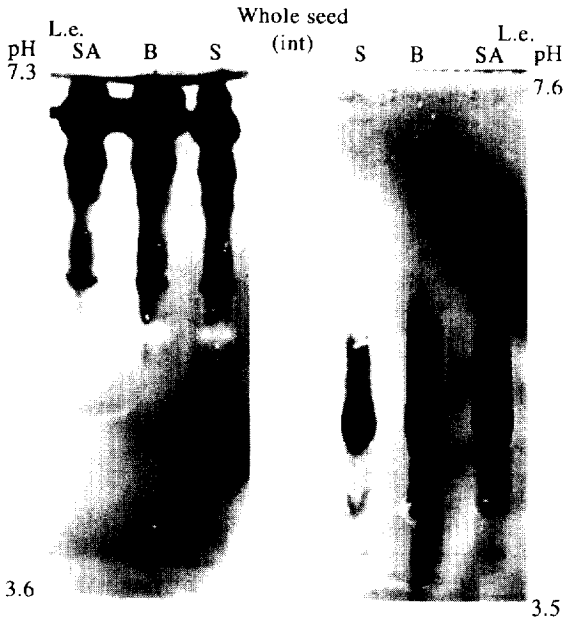

Fig. 2. Contact prints of *endo*-β-D-mannanase isozymes from the overlay of a 3.6–7.3 (left) and a 3.5–7.6 (right) pH-range IEF gel. The gel use for the contact print on the left was made using 4–6 range ampholytes while that on the right was made from 5–7 range ampholytes. The pH values of the IEF gel which are given are those extrapolated from the graph of the measured pH gradient. *Lycopersicon esculentum* (L.e.) seeds were imbibed intact until the time of harvest. Cultivar abbreviations used are: SA, Stokesalaska; B, Burpee's Big Boy; and S, Scotia.

sperm after imbibition (Tables 3 and 4). The position of the fenugreek embryo isozyme varied from gel to gel even though their pH range was identical. We have no explanation for this.

Mature pea and soybean seeds are without an endosperm (or only a residual one is present). Extracts from both seeds contained *endo*-β-D-mannanase isozymes, although there were none present in pea seeds which had been imbibed but had not completed germination (Table 3). Soybean embryos greatly increased in the number of isozymes present after imbibition, if the isozymes within the imbibition water are also considered (the extract itself increased from 1 to 6 and the surrounding liquid increased from 0 to 17; Tables 1 and 3).

Three isozymes were found within the embryo and one within the endosperm once alfalfa seed had been imbibed (Tables 3 and 4). The number of isozymes concurs with that reported by McCleary [15] although the p*I*s are slightly different. Those reported here are apt to be more accurate since the gradient used was narrower (2 pH units) than that used by McCleary (7 pH units). A single isozyme of p*I* 4.0 was detected in the imbibition water and is presumably a secreted form of the enzyme. The alfalfa vegetative parts also contained a number of isozymes (Table 5). Within the shoot there were the greatest number of isozymes, followed by the root and the leaf. The most active isozyme (p*I* 4.0) within the root had a counterpart with the same p*I* in the endosperm of the seed (Fig. 3).

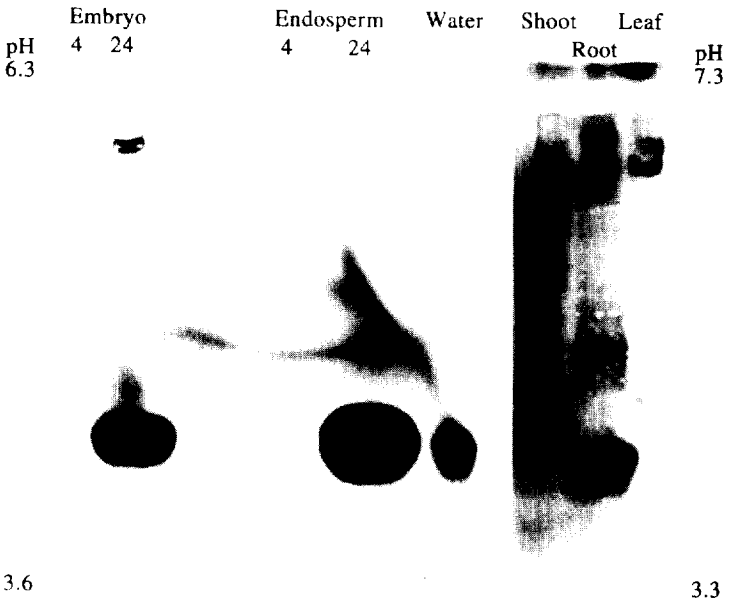

Fig. 3. Contact prints of *endo*-β-mannanase isozymes from the overlay of a 4–6 pH-range IEF gel (left 3.6–6.3; right 3.3–7.3). The gels used for the contact print were made using 4–6 range ampholytes. The pH values of the IEF gel which are given are those extrapolated from the graph of the measured pH gradient. *Medicago sativa* seeds were imbibed intact (4 hr and 24 hr) and the parts dissected at the time of harvest. The vegetative tissues were harvested from mature flowering plants.

Table 5. Isozymes of the vegetative parts of alfalfa plants (in flower)*

Part	Number of isozymes	pI of isozymes
Shoots	13	3.45, 3.5?, 3.7?, 4.1?, 4.8?, 5.0?, 5.1?, 5.7W, 6.1?, 6.8, 7.0, 7.4?
Roots	11	3.8, 3.9, 4.6, 4.9?, 5.7W, 5.8, 6.1, 6.6?, 6.8, 7.0, 7.4?
Leaves	8	4.0?, 4.3, 5.7W, 5.8?, 6.1?, 6.8, 7.0, 7.4?

*Isozymes that were indistinctly detected are signified by a ? appearing after their pIs. W immediately following a pI value signifies the appearance of the isozyme at the corresponding point on the overlay at which sample wick was placed on the IEF gel.

Table 6. Isoelectric points of isozymes from the embryo of imbibed seeds of gymnosperms*

Species	Sep or Int	Hour of dissection	Hour of harvest	Number of isozymes	pI of isozymes
Black spruce	Sep	8	172	2	5.4, 6.2?
White spruce	Sep	8	172	1	6.2?
Sitka spruce	Sep	8	172	1	6.2?
Jack pine	Sep	8	172	2	5.4W, 6.1
Red pine	Sep	8	172	2	5.4W, 6.1
Douglas fir	Sep	8	172	3	5.4W, 6.0, 6.15
Nordmann fir	Sep	8	172	2	5.4W, 6.0
Eastern larch	Sep	8	172	1	6.0
Black spruce	Int	NA	172	4 [2]	[3.9], 4.3, 4.5, 4.6, 5.1W, [5.1W]
White spruce	Int	NA	172	4 [4]	[3.9], [4.1], 4.4, 4.5, [4.5], 4.6, 5.1W, [5.1W]
Sitka spruce	Int	NA	172	5 [1]	4.3?, 4.5, [4.5], 4.6, 4.65, 5.1W
Jack pine	Int	NA	172	2 [1]	[5.1W?], 5.9, 6.2W
Red pine	Int	NA	172	3 [2]	[3.9], 4.0, 4.6?, [4.7], 5.1W
Douglas fir	Int	NA	172	5 [9]	3.5, [3.7], [4.1], [4.2], 4.3, [4.4], [4.5], 4.6, [4.8], [5.0], [5.1W], [5.6], 6.1W, 6.7
Nordmann fir	Int	NA	172	3 [17]	[3.7], [3.9], [3.95], 4.0, [4.0], [4.1], [4.2], [4.3], [4.4], [4.5], [4.55], [4.6], [4.7], [4.8], [4.9], 5.1W, [5.1W], [5.5], 6.2W, [6.3]
Eastern larch	Int	NA	172	1 [9]	[3.9], [4.0], [4.1], [4.2?], [4.3?], [4.4?], [4.5?], [4.6?], 5.1W, [5.1W]

*See Table 3 for details.

Gymnosperms

All extracts of seeds from the different gymnosperms surveyed contained at least one isozyme in the embryo and the megagametophyte of the seed in the dry state (Tables 1 and 2). There was no consistent trend to the increase or decrease in the number of isozymes upon imbibition of the seeds (Tables 1, 2, 6 and 7). Four fewer isozymes were detected in Sitka spruce embryos, imbibed separately from the megagametophyte than in the embryos in mature, dry seeds. Black spruce and Douglas fir embryos imbibed in the intact seed contained two more isozymes than embryos in mature, dry seeds. Relative to the megagametophytes from mature, dry seeds, Nordmann fir contained 13 (imbibed separately) and 10 (imbibed intact) more isozymes; whereas in Douglas fir there were nine (imbibed separately) and seven (imbibed intact) fewer isozymes. In other instances the number of isozymes was unaffected by imbibition (e.g. eastern larch embryo separate or intact and black spruce megagametophyte intact).

The isozymes from the megagametophyte of four different gymnosperm species are shown in Fig. 4. This is a typical separation of isozymes achieved using a pH range 4–6 and 5–7 for extracts from dry seeds of white spruce, jack pine, red pine and Douglas fir.

Table 7. Isoelectric points of isozymes from the megagametophyte of imbibed seeds of gymnosperms*

Species	Sep or Int	Hour of dissection	Hour of harvest	Number of isozymes	p*I* of isozymes
Black spruce	Sep	8	172	5 [6]	[4.2], [4.3], 4.3?, [4.4], 4.4, [4.5], [5.1], [5.4W?], 5.4W, 6.0, 6.7
White spruce	Sep	8	172	4	4.7, 4.9, 5.4W?, 6.0
Sitka spruce	Sep	8	172	4	4.9, 5.4W?, 6.0, 6.5
Jack pine	Sep	8	172	7 [4]	[4.2], 4.2, [4.3?], [4.4], 4.4, 4.45, 5.4W?, 6.0, [6.1W?], 6.2, 6.7
Red pine	Sep	8	172	3 [1]	6.0, [6.1?], 6.2, 6.6
Douglas fir	Sep	8	172	7 [2]	[3.9], 3.9, [5.0?], 5.2?, 5.3?, 5.4W, 6.0, 6.5?, 6.7?
Nordmann fir	Sep	8	172	15 [12]	[3.9], 3.9, 4.0, [4.2?], [4.25], 4.25, [4.3], 4.3, [4.4], 4.4, [4.5], 4.5?, 4.55, 4.6, [4.7], [4.9], 4.9, 4.95, [5.0], [5.1], 5.1, 5.4W, 6.0, [6.1W], 6.5?, [6.6], 6.7?
Eastern larch	Sep	8	172	7 [2]	4.3?, 4.4?, 4.55?, [4.6], [4.7], 4.7, 5.4W?, 5.5, 6.5?
Black spruce	Int	NA	172	4 [2]	[3.9], 4.3, 4.6, 4.65?, 5.1W, [5.1W]
White spruce	Int	NA	172	4 [4]	[3.9], [4.1], 4.3, 4.5, [4.5], 4.6, 5.1W, [5.1W]
Sitka spruce	Int	NA	172	4 [1]	4.3, 4.5, [4.5], 4.6, 5.1W
Jack pine	Int	NA	172	1 [1]	5.1W, [5.1W?]
Red pine	Int	NA	172	9 [2]	[3.9], 4.4, 4.5, 4.6, 4.65, 4.7, [4.7], 4.9?, 5.1W, 5.7?, 6.8
Douglas fir	Int	NA	172	9 [9]	3.5, 3.6, [3.7], [4.1], 4.2, [4.2], [4.4], 4.5, [4.5], 4.6, 4.7, [4.8], 4.9, [5.0], 5.1W, [5.1W], [5.6], 6.8
Nordmann fir	Int	NA	172	12 [17]	3.4, [3.7], [3.9], [3.95], 4.0?, [4.0], 4.1?, [4.1], [4.2], 4.3, [4.3], 4.4, [4.4], 4.5?, [4.5], [4.55], 4.6, [4.6], 4.7, [4.7], 4.8, [4.8], [4.9], 5.1W, [5.1W], [5.5], 5.7?, 6.1?, [6.3]
Eastern larch	Int	NA	172	8 [9]	3.4, 3.9, [3.9], 4.0, [4.0], [4.1], 4.2, [4.2?], [4.3?], [4.4?], [4.5?], [4.6?], 4.8?, 5.1W, [5.1W], 6.5, 6.7

*See Table 3 for details.

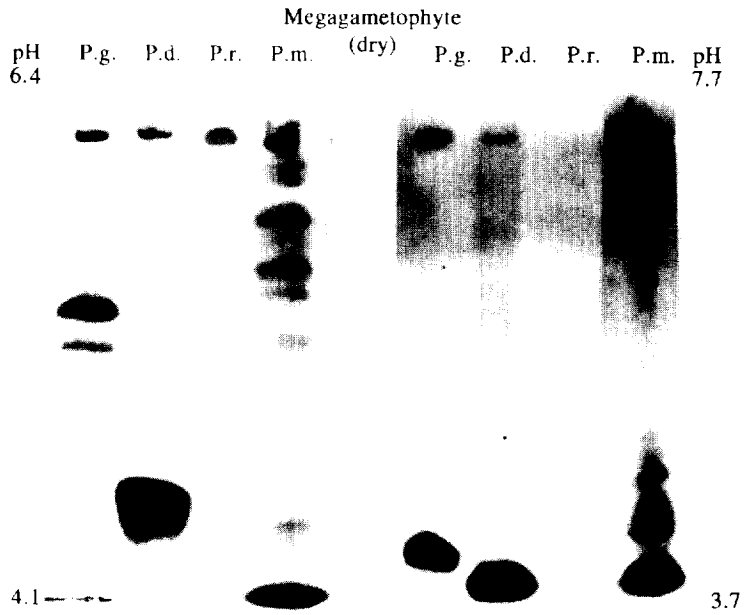

Fig. 4. Contact prints of *endo*-β-mannanase isozymes from the overlay of a 4.1–6.4 (left) and a 3.7–7.7 (right) pH-range IEF gel. The gel used for the contact print on the left was made using 4–6 range ampholytes while that on the right was made from 5–7 range ampholytes. The pH values of the IEF gel which are given are those extrapolated from the graph of the measured pH gradient. The endosperms of dry seeds from *Picea glauca* (P.g.), *Pinus divaricata* (P.d.), *Pinus resinosa* (P.r.) and *Pseudotsuga menziesii* (P.m.) were used.

CONCLUSION

The presence of *endo*-β-D-mannanase within certain seeds which contain a large store of galactomannan reserves (e.g. *Trigonella foenum-graecum*) [18, 19] was expected, but its presence in other seeds, which seemingly have little requirement for an enzyme responsible for cleaving mannan chains, was not. For example, the megagametophyte of the conifers is not known to be a store of mannan or galactomannan (it contains protein and lipid) and yet a number of isozymes of *endo*-β-D-mannanase are present. As well, *endo*-β-D-mannanase was detected within all of the vegetative tissues of a mature, flowering alfalfa plant. The number of isozymes reported here may be fewer than are present within the tissues, since to maintain good resolution of the isozymes, the pH ranges used were small (two pH units) and limited (only 4–6 and 5–7). Also, despite the extreme sensitivity of the technique [20], it is possible that relatively inactive isozymes were not detected.

The differences in the p*I* values of the isozymes in the seed and plant parts suggests that the *endo*-β-D-mannanase is variable in its amino acid composition, or in the amount or type of glycosylation, or both. The active site and enzyme structure are presumbably not affected by these changes. *endo*-β-D-Mannanase isozymes could be produced from a multi-gene family or, if there is a single gene for *endo*-β-D-mannanase, the isozymes could be a group of post-translationally modified extracellular enzymes, e.g. by differential glycosylation, or limited proteolysis. Post-translational modification has been used to explain enzyme polymorphism of xanthine dehydrogenase and aldehyde oxidase in *Drosophila melanogaster* [21]. Whatever the reason for the variability in the p*I* of *endo*-β-D-mannanase, this enzyme is widely distributed throughout the seeds of angiosperms and gymnosperms in many different forms. It is also present in vegetative tissues. Its known function is to mobilize mannan-containing reserves (usually galactomannans in the endosperm) as a source of nutrients for the growing seedling. In some seeds it may serve an important function in partially degrading cell wall mannans to allow elongation of the embryo, thus completing germination.

EXPERIMENTAL

Plant sources assayed. The term 'seed' was used collectively throughout this document to refer to kernels, caryopses, achenes, and true seeds. The embryo and (if present) its surrounding tissue (endosperm or megagametophyte) from dry and emerged seeds of dicots: tomato (*Lycopersicon esculentum* Mill.); lettuce (*Lactuca sativa* L.); soybean (*Glycine max* (L.) Merrill); pea (*Pisum sativum* L.); alfalfa (*Medicago sativa* L.); carob (*Ceratonia siliqua* L.); and fenugreek (*Trigonella foenum-graecum* L.); monocots: wheat (*Triticum aestivum* L. cv Frederick); barley (*Hordeum vulgare* L. cv Himalaya); oats (*Avena sativa* L.); and rice (*Oryza sativa* L. cv Bengal); and gymnosperms: white spruce (*Picea glauca* (Moench) Voss); black spruce (*Picea mariana* (Mill.) B.S.P.); jack pine (*Pinus banksiana* Lamb.); red pine (*Pinus resinosa*

Ait.); eastern larch (*Larix laricina* (Du Roi) C. Koch); Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco); and Nordmann fir (*Abies nordmanniana* (Steven) Spach.) were assayed for isozymic variants. Isozymes from shoots, roots, and leaves of mature, flowering alfalfa plants were also assayed.

Imbibition conditions. Seeds with an endosperm or megagametophyte were dissected into these parts in the dry state or after a brief imbibition period. Those seeds which were too brittle to dissect dry were imbibed for 4 to 6 hr and then dissected to determine the isozymes of the seed parts. If the seeds were imbibed prior to dissection, a 1 ml aliquot of imbibition water was obtained to check for enzyme leaching form the intact seed.

Germination conditions. (i) Intact seeds: Seeds were placed on a single sheet of Whatman No. 1 filter paper in 100 × 15 mm Petri dishes, moistened with H_2O, plus 0.1% (w/v) Benlate fungicide in the case of the gymnosperms [22]. The dishes were placed at 25° in the dark until the time of harvest. A 1 ml aliquot of imbibition H_2O was obtained to determine if isozymes leaked out into the surrounding liquid. (ii) Isolated seed parts: Angiosperm seeds were imbibed for 4–24 hr before they were dissected into embryo and endosperm (when present) plus testa. Seed parts were placed on a filter paper moistened with H_2O in Petri dishes and germinated as above. These seed parts, as well as a 1 ml aliquot of imbibition water, were harvested at designated times.

Conifer embryos are dependent on the megagametophyte tissue for elongation and normal seedling development [23]. To separate these parts and keep the embryos alive for the prescribed length of time, embryos were dissected from surface-sterilized seeds (7 min in 1% bleach followed by 5 × 5 min washes in sterile H_2O) under sterile conditions, placed on 3% (w/v) potato dextrose agarose and incubated at 25° in the dark until harvest. The corresponding megagametophytes were similarly incubated on filter paper moistened with 0.1% (w/v) Benlate in H_2O until harvest.

Enzyme extraction. Dry seed parts or parts from seeds that were just completing germination were pulverized in liquid N_2 (if this was found to aid enzyme extraction) in a mortar and pestle. 0.5–1 ml of extraction buffer (0.1 M Na citrate, 0.2 M NaPi buffer, pH 7) per 100 mg seeds was mixed with the powder, the suspension ground together to a slurry, transferred to 2 ml microtubes, and centrifuged at 14 000 *g* for 10 min at 4°.

Enzyme assay. An attempt was made to use a gel diffusion assay [20] to quantify *endo*-β-D-mannanase activity and to use this information to load gels on an equal activity basis. When this did not provide uniform activity among the isozymes appearing in the different lanes on the overlays, loading was by trial and error and activity was compared ocularly. This sometimes necessitated rerunning the same extracts several times. When extracts had low enzyme activity, 20 μl, the maximum vol. possible, was loaded on the gel.

Protein concn. Extracts of seed parts incubated separately after dissection often had very low activity and were therefore concd 4 × by lyophilizing the extract to 25% of its original vol. Failure to detect isozymes after concn was reported as undetectable in the pH range (4–7) tested.

Isoelectric focusing. Proteins were subjected to isoelectric focusing, using Bio-lyte ampholytes pH 5.0–7.0 and 4.0–6.0 using an LKB Bromma 2117 Multiphor flatbed IEF unit and 0.5 mm thick, 5% acrylamide gels. After focusing, the pH distribution was measured by cutting 1 cm^2 slices of gel beginning at the cathode, parallel to the pH gradient, and placing these squares in 2 ml H_2O in test tubes covered with Parafilm and shaking the whole vigorously for at least 12 hr. The pH of the H_2O was then measured and the gradient was estimated from the curve by interpolation and extrapolation.

Isozyme detection. The IEF gel was placed in contact with a 0.5 mm thick overlay (14.4 ml 0.1% (w/v) locust bean gum in 0.1 M Na citrate/0.2 M Na phosphate buffer pH 5, 5 ml 30% T: 2.5%C acrylamide, 0.1 ml TEMED, 0.5 ml 2% (w/v) ammonium persulphate), which was formed with a gel-bond backing. The portions of the IEF gel under the paper electrode wicks were lifted from the gel bond and discarded. The IEF gel and the overlay were placed in contact with each other and tightly appressed by gently rolling an ink print-roller over the overlay. The two gels were incubated with the overlay on the bottom for 16 hr at 37° in a closed container with a wet paper towel to maintain high humidity; the gels were then separated, the IEF gel was discarded after its outline was cut into the overlay. Development of the isozyme band pattern on the overlay required 20 min of shaking in H_2O, 30 min of staining with 0.5% Congo Red in H_2O, 5 min of destaining with 95% EtOH, and finally washing in 1 M NaCl. Contact prints of the overlays were made by using the gel as a negative [24], placing it directly on the photographic paper, and exposing it for the length of time required to give the best contrast. The isozyme patterns thus obtained were never as distinct as on the original gels.

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REFERENCES

1. Dutta, S., Bradford, K. J. and Nevins, D. J. (1994) *Plant Physiol.* **104**, 623.
2. Sasaki, K., Nagahashi, G., Gretz, M. R. and Taylor, I. E. P. (1989) *Plant Physiol.* **90**, 686.
3. Sakurai, N., Tanaka, S. and Kuraishi, S. (1987) *Plant Cell Physiol.* 28, 1051.
4. Wada, S. and Ray, P. M. (1978) *Phytochemistry* **17**, 923.
5. Monro, J. A., Bailey, R. W. and Penny, D. (1976) *Phytochemistry* **15**, 175.
6. Kaihou, S., Hayashi, T., Otsuru, O. and Maeda, M. (1993) *Carbohydr. Res.* **240**, 207.

7. McCleary, B. V. (1979) *Carbohydr. Res.* **67**, 213.
8. Tampion, J. (1972) *Enzymologia* **42**, 253.
9. Wozniewski, T., Blaschek, W. and Franz, G. (1991) *Phytochemistry* **30**, 3579.
10. Wozniewski, T., Blaschek, W. and Franz, G. (1992) *Phytochemistry* **31**, 3365.
11. Groot, S. P. C., Kieliszewska-Rokicka, B., Vermeer, E. and Karssen, C. M. (1988) *Planta* **174**, 500.
12. Halmer, P. and Bewley, J. D. (1979) *Planta* **144**, 333.
13. Reid, J. S. G., Davies, C. and Meier, H. (1977) *Planta* **133**, 219.
14. Dennis, E. A., Rhee, S. G., Billah, M. M. and Hannun, Y. A. (1991) *FASEB J.* **5**, 2068.
15. McCleary, B. V. (1978) *Phytochemistry* **17**, 651.
16. Pressey, R. (1989) *Phytochemistry* **28**, 3277.
17. Torrie, J. P., Senior, D. J. and Saddler, J. N. (1990) *Appl. Microbiol. Biotechnol.* **34**, 303.
18. Reid, J. S. G. and Meier, H. (1970) *Phytochemistry* **9**, 513.
19. Campbell, J. McA. and Reid, J. S. G. (1982) *Planta* **155**, 105.
20. Downie, B., Hilhorst, H. W. M. and Bewley, J. D. (1994) *Phytochemistry* **36**, 829.
21. Finnerty, V. and Johnson, G. (1979) *Genetics* **91**, 695.
22. Lock, W., Sutherland, J. R. and Sluggett, L. J. (1975) *Tree Planters' Notes* **26**, 16.
23. Sasaki, S. and Kozlowski, T. T. (1969) *Ann. Botany* **33**, 473.
24. Mullen, R. T. and Gifford, D. J. (1993) *Plant Physiol.* **103**, 477.