The Plant Journal (1993) 3(3), 415–426

14471.BT

Purification, characterization, and cell wall localization of an α-fucosidase that inactivates a xyloglucan oligosaccharin

Christopher Augur[1], Nicole Benhamou[2], Alan Darvill[1], and Peter Albersheim[1,*]
[1]Complex Carbohydrate Research Center and the Department of Biochemistry, The University of Georgia, 220 Riverbend Road, Athens, GA 30602, USA, and [2]Université Laval, Département de Phytologie, Faculté des Sciences de l'Agriculture, Ste Foy, Québec, GIK 7P4, Canada

Summary

An α-fucosidase that releases fucosyl residues from oligosaccharide fragments of xyloglucan, a plant cell wall hemicellulosic polysaccharide, was purified to homogeneity from pea (*Pisum sativum*) epicotyls using a combination of cation exchange chromatography and isoelectric focusing. The α-fucosidase has a molecular mass of 20 kDa according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The α-fucosidase has an isoelectric point of 5.5. The substrate specificity of the α-fucosidase was determined by high performance anion exchange chromatographic analysis of oligosaccharide substrates and products. The enzyme hydrolyzes the terminal α-1,2-fucosidic linkage of oligosaccharides and does not cleave *p*-nitrophenyl-α-L-fucoside. The enzyme does not release measurable amounts of fucosyl residues from large polysaccharides. The subcellular localization of α-fucosidase in pea stems and leaves has been studied by immunogold cytochemistry. The α-fucosidase accumulates in primary cell walls and is not detectable in the middle lamella or in the cytoplasm of 8-day-old stem tissue and 14-day-old leaf tissue. α-Fucosidase activity was readily detected in extracts of 8-day-old stem tissue. No significant α-fucosidase acitivity or immunogold labeling of the α-fucosidase was detected in 2- and 4-day-old stem tissue indicating that production of α-fucosidase is developmentally regulated.

Introduction

The cell walls of higher plants contain a family of highly branched polysaccharides called hemicelluloses. Hemicelluloses are functionally defined as those polysaccharides that form strong non-covalent associations with cellulose microfibers. The predominant hemicelluloses in the primary cell walls of higher plants are arabinoxylan and xyloglucan. Xyloglucan is thought to be a load-bearing structure in the primary cell wall because of its proposed role in cross-linking cellulose microfibers (Fry, 1989a). The dynamic nature of the cross-linking is hypothesized to be the major factor controlling the rate of cell wall expansion, thereby regulating plant cell growth. Oligosaccharide fragments of the xyloglucan of primary cell walls can regulate auxin-stimulated growth in pea stem segments (Emmerling and Seitz, 1990; McDougall and Fry, 1988, 1989a, 1989b, 1990; York *et al.*, 1984). One of these oligosaccharides, a fucose-containing nonasaccharide (XG9, Figure 1), was shown, in the nanomolar range, to inhibit 2,4-dichlorophenoxyacetic acid (2,4-D)-induced growth of pea stem segments. 2,4-D is an auxin analog. A heptasaccharide (XG7) lacking the fucosyl-galactosyl disaccharide side chain of XG9 was unable to inhibit 2,4-D-induced growth. An octasaccharide, identical to XG9 but lacking the terminal fucosyl residue, was also unable to inhibit 2,4-D-stimulated growth (McDougall and Fry, 1989b), indicating that the terminal α-linked L-fucosyl residue of XG9 is required for its inhibitory activity. Thus, an α-fucosidase that cleaves the Fuc–α-1,2-Gal linkage could be an essential component of the growth regulation process as it could control the levels of fucose-containing xyloglucan 'oligosaccharins'.

Enzymes that modify xyloglucan oligosaccharides have been detected in plants (O'Neill *et al.*, 1988) and have been purified and characterized (Fanutti *et al.*, 1991; O'Neill *et al.*, 1989b). A β-galactosidase and a xyloglucan-specific β-1,4-D-*endo*glucanase, have been purified from nasturtium cotyledons (Edwards *et al.*, 1986, 1988). The galactosidase is only able to remove terminal non-reducing galactosyl residues from xyloglucan oligosaccharides. Therefore, XG9 is not a substrate for either of these enzymes. A xyloglucan oligosaccharide-specific α-xylosidase has been extracted from pea stems (O'Neill *et al.*, 1989b) and germinated nasturtium seeds (Fanutti *et al.*, 1991). The xylosidases from both sources have been purified to homogeneity. The enzymes specifically cleave only the XG9 α-xylosidic linkage furthest from its reducing end, yielding XG8′ (oligosaccharide **10**, Figure 1). XG8′ was as effective as XG9 in inhibiting 2,4-D-stimulated growth of pea stem segments (Augur *et al.*, 1992). Although there is no previous report on the purification of an α-fucosidase from plants, α-fucosidase activity (EC 3.2.1.51)

Received 22 May 1992; revised and accepted 28 July 1992.
*For correspondence (fax +1 706 542 4412).

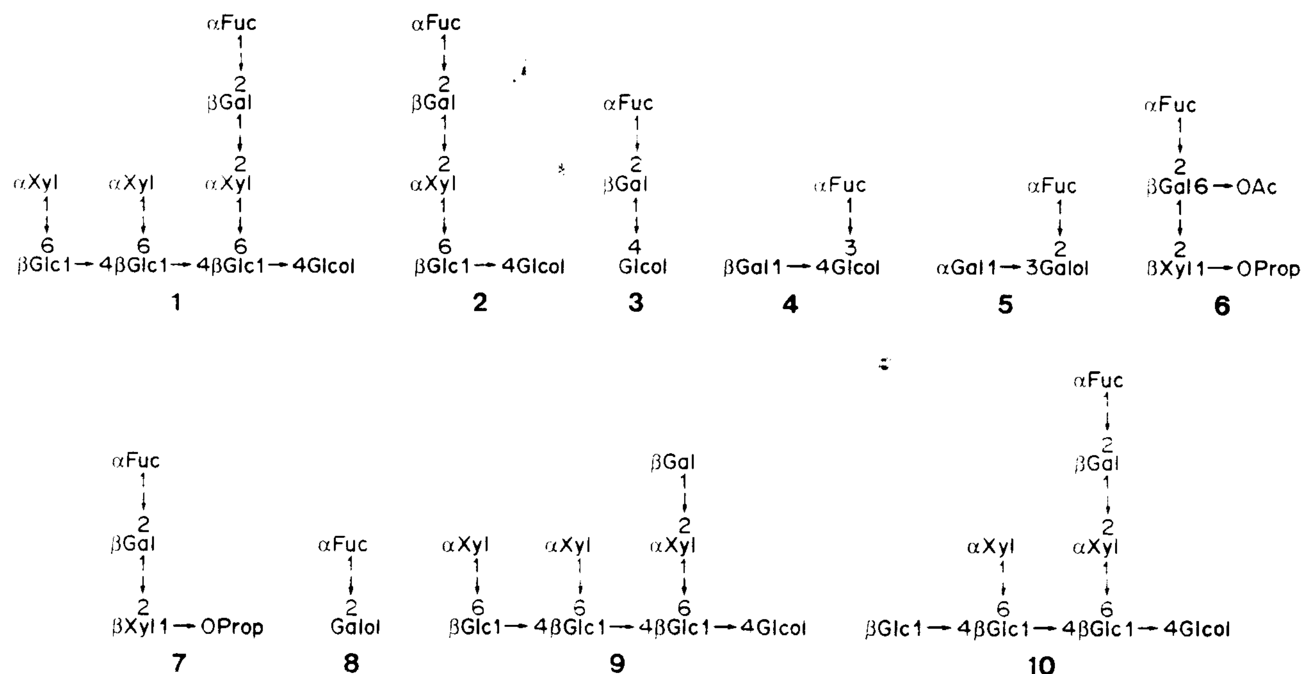

Figure 1. Structures of fucose-containing oligosaccharides and oligosaccharide alditols.
Oligosaccharides **1–5** and **8–10** are oligoglycosyl alditols prepared by converting the reducing glucose residue of each oligosaccharide to its corresponding glucitol. Oligosaccharides **1** and **2** were generated by digestion of xyloglucan with a fungal β-1,4-endoglucanase. Oligosaccharide **9** is oligosaccharide **1** minus fucose. Oligosaccharide **10** is oligosaccharide **1** minus a single xylose residue. Compounds **6** and **7** were chemically synthesized.

has been detected in extracts of almond seed (Kobata, 1982; Ogata-Arakawa *et al.*, 1977) and etiolated pea stems (O'Neill *et al.*, 1988). Recently, Farkas and co-workers (Farkas *et al.*, 1991) reported that the α-fucosidase activity in pea stems was unaffected by auxin pretreatment and that α-fucosidase activity in germinating nasturtium seeds is developmentally regulated. In the present study, we report on the purification to homogeneity, substrate characterization, and immunogoid localization of an α-fucosidase from etiolated pea stems.

Results

Purification of the enzyme

Pea seedings were harvested 8 days after sowing, as α-fucosidase activity was known to be present in pea epicotyl tissue at that age (O'Neill *et al.*, 1988). The α-fucosidase was partially purified by selective extraction from pea epicotyls; the first two low salt washes (0.1 M and 0.05 M sodium acetate, pH 4.5) were discarded as previous data indicated that the majority of cell wall localized enzymes are released from cell wall material by 1.0 M sodium acetate but not by 0.1 M sodium acetate (O'Neill *et al.*, 1988). The α-fucosidase was further purified by a combination of CM-Sepharose (Figure 2) and preparative isoelectric focusing (Figure 3). The purification steps were monitored by SDS–PAGE as shown in Figure 4. On CM-Sepharose, the analysis of fractions obtained after chromatography of 8-day-old pea seedling extract showed a single, broad peak of α-fucosidase activity using reduced 2'-fucosyl-lactose as substrate (Figure 2). Fractions from the CM-Sepharose column, containing α-fucosidase activity, were pooled and subjected to two consecutive isoelectric focusing steps (Figure 3a and b). Fractions from the second isoelectric focusing step were analyzed by SDS–PAGE (data not shown). Fractions 3–8

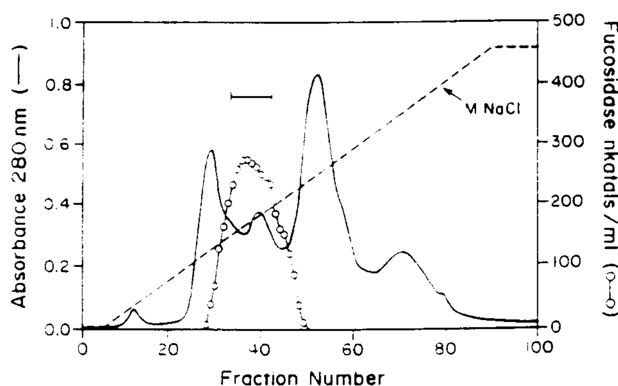

Figure 2. Cation exchange chromatography of crude dialyzed pea stem extract on carboxymethyl (CM)-Sepharose.
α-Fucosidase activity was assayed as described in Experimental procedures. Fractions 35–41 (8 ml each, see bar) were pooled and dialyzed before preparative isoelectric focusing.

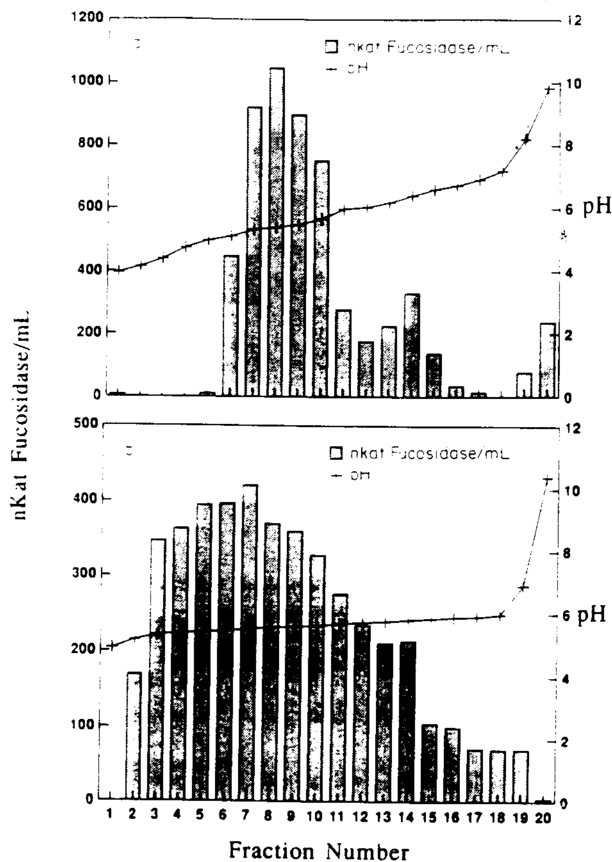

Figure 3. Preparative isoelectric focusing of CM-Sepharose purified α-fucosidase.
(a) Fractions 7–11 (2.5 ml each) were pooled, brought to a final volume of 50 ml with H_2O, reloaded into the rotofor chamber, and a similar procedure (no additional ampholytes were added for the second purification) was repeated (b), yielding pure α-fucosidase in fractions 3–8, as determined by SDS–PAGE.

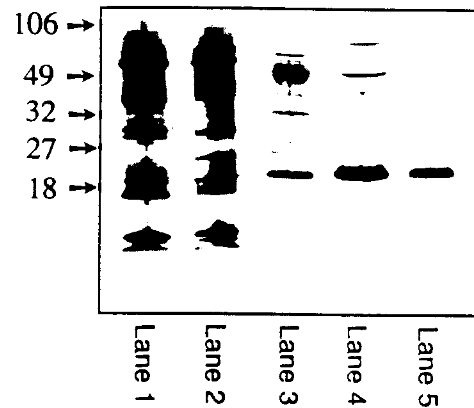

Figure 4. SDS–PAGE of α-fucosidase-containing preparations.
Fucosidase samples at various stages of purification were subjected to denaturing gel electrophoresis and silver stained. The samples loaded were as follows: lane 1, 0.1 M sodium acetate, pH 5.0, extract of pea tissue; lane 2, 0.05 M sodium acetate, pH 5.0, extract of residue from pea tissue after extraction with 0.1 M buffer; lane 3, pooled α-fucosidase-containing fractions from CM-Sepharose chromatography; lane 4, pooled α-fucosidase-containing fractions from first preparative isoelectric focusing; lane 5, purified α-fucosidase from second run in preparative isoelectric focusing chamber. The positions of molecular weight markers are shown on the left side of the figure.

(Figure 3b), spanning the range pI= 5.4 to pI= 5.6, each contained a single, α-fucosidase active, protein band with an apparent molecular mass of 20 000. In the other α-fucosidase-containing fractions (1,2, and 9–20), protein contaminants of higher molecular weight were observed.

It is not possible to construct a conventional purification table for α-fucosidase, since the activity in the 1 M buffer extract, containing the crude enzyme preparation, could not be assayed due to interference from reducing sugars in the plant extract. A fourfold increase in specific activity of α-fucosidase was observed between the cation exchange chromatography and the second isoelectric focusing purification.

Substrate specificity of α-fucosidase

An initial goal of this work was to determine whether the α-fucosidase from pea stems is able to hydrolyze the terminal fucosyl residues of xyloglucan oligosaccharides and polysaccharides. It was of particular interest to determine whether the α-fucosidase could remove the terminal fucosyl residue of XG9. XG9 was incubated with α-fucosidase and the products separated by high performance anion exchange (HPAE) chromatography as described in Experimental procedures. The HPAE peak eluting at 3 min (Figure 5, trace b) co-eluted with an L-fucose standard. The HPAE peak with a retention time of 25 min (Figure 5, trace b) co-eluted with XG9 (Figure 5, trace a). The peak eluting at 28 min was identified as XG8 (Figure 1) by fast atom bombardment mass spectroscopy (data not shown) as the observed [M+H] pseudomolecular ion corresponded in mass to XG8. The presence of fucose

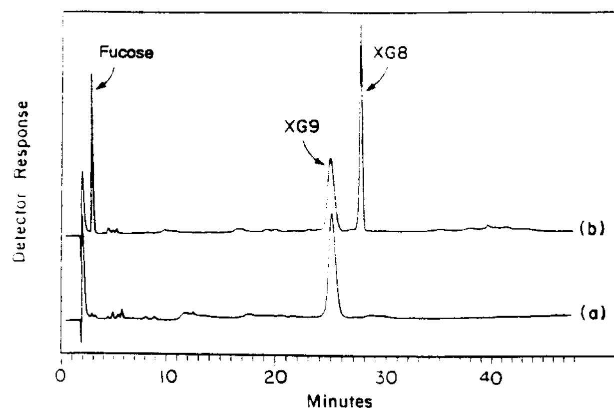

Figure 5. Separation of xyloglucan oligosaccharides using high performance anion exchange.
The chromatography was on a Dionex CarboPac PA-1 column (4 × 250 mm) with PAD detection. (a) Chromatography of about 2 μg of XG9. (b) Chromatography of products generated by α-fucosidase digestion of XG9.

Table 1. α-Fucosidase substrate specificity

Substrate[a]	Normalized initial rate[b]	% Fucose released in 20 min	% Fucose released in 120 min
Rhamnogalacturonan I	0		0
Rhamnogalacturonan II	0		0
SEPS Xyloglucan	0		0
XG25	2		1
Oligosaccharide **1**	23		6
Oligosaccharide **2**	4		2
Oligosaccharide **3**	68	5	
Oligosaccharide **4**	0		0
Oligosaccharide **5**	0		0
Oligosaccharide **6**	100	9	
Oligosaccharide **7**	57	4	
Oligosaccharide **8**	8		4
PNP–α-L-Fucopyranoside	0		0

[a]See text for description of substrates. The structures of oligosaccharides **1–8** are shown in Figure 1. All of the structures except oligosaccharide **6** and PNP–α-L-fucopyranoside were reduced to their corresponding oligoglycosyl alditols prior to assay of α-fucosidase activity.

[b]Initial rates were normalized to that of oligosaccharide **6**. The initial rate of hydrolysis of the fucosyl residue of oligosaccharide **6** was 1.5 μM fucose h^{-1} μg^{-1} fucosidase.

in oligosaccharides is known to decrease their HPAE retention time (Hardy and Townsend, 1988), which explains why XG8 (devoid of fucose) has a greater retention time than XG9. Recently, McDougall and Fry (1991a) reported that XG8 does in fact elute later than XG9 on a CarboPak PA-1 anion exchange column.

The ability of the purified α-fucosidase to cleave the α-fucosidic linkages of a variety of oligosaccharides is summarized in Table 1. In these experiments, the reactions were carried out under conditions where the substrate is not limiting (less than 10% hydrolysis of substrate). Complete hydrolysis of substrate can be achieved by adding less substrate or more enzyme (see for example Figure 5, where the substrate was hydrolyzed approximately 60%). Three suspension-cultured sycamore cell wall polysaccharides (rhamnogalacturonan I, rhamnogalacturonan II, and xyloglucan), each of which contains terminal α-fucosyl residues and are, therefore, potential substrates for α-fucosidase *in vivo*, were tested for their ability to act as substrates. Rhamnogalacturonan I (RG I), which is a pectic polysaccharide that is solubilized from plant cell walls by treatment with α-1,4-endopolygalacturonase, has a molecular weight between 10^5 and 10^6 (York *et al.*, 1985). Rhamnogalacturonan II (RG II) which is a pectic polysaccharide that is also solubilized from plant cell walls by treatment with α-1,4-endopolygalacturonase, is composed of approximately 30 glycosyl residues. RG II contains terminal 2-*O*-methyl fucose (York *et al.*, 1985). α-Fucosidase does not release measurable amounts of fucosyl residues from RG I, RG II, or xyloglucan (Table 1). A mixture of xyloglucan oligosaccharide alditols, each with about 25 glycosyl residues (XG25) and two purified xyloglucan-derived oligosaccharides (oligosaccharides **1** and **2**, Figure 1; Table 1), as well as several commercially available fucose-containing oligosaccharides and oligosaccharide alditols (compounds **3–5**, **8**, and *p*-nitrophenyl- α-L-fucopyranoside), and chemically synthesized oligosaccharides (compounds **6**, **7**) were also tested as substrates of α-fucosidase. *p*-Nitrophenyl-α-L-fucopyranoside is not hydrolyzed by the enzyme. The α-fucosidase also does not hydrolyze the α-1,3-L-fucosidic linkage of compound **4** (Figure 1) or the α-1,2-L-fucosidic linkage of compound **5**. The presence of a 6-*O*-acetyl group on the galactosyl residue of compound **6** made it a better substrate for α-fucosidase.

Specificity of the rabbit antiserum raised against pea seedling α-fucosidase

Polyclonal antibodies were raised in rabbits against the α-fucosidase in order to study the distribution of the α-fucosidase in pea stem and leaf tissue. The specificity of the antiserum used for the immunolocalization studies was tested on gel blots of SDS–PAGE pea epicotyl protein extracts (Figure 6). The antiserum reacted specifically with the α-fucosidase (Figure 6, lanes 2, 3 and 4). Little cross-reactivity was detected with other proteins in the extracts. The use of rabbit pre-immune serum instead of the α-fucosidase antiserum did not reveal any polypeptide on immunoblots (data not shown). The preponderance of antibody in the antiserum recognizing the α-fucosidase were periodate insensitive (Figure 7) suggesting that the antibodies recognize the protein moiety of α-fucosidase. The signal and titer of untreated and periodate treated α-fucosidase were identical (Figure 7).

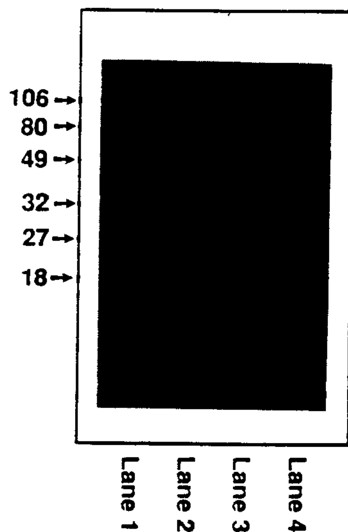

Figure 6. Western blot of α-fucosidase-containing extracts from pea epicotyl tissue.
Lane 1, molecular weight markers (the masses of the molecular weight markers are indicated on the left side of the figure); lane 2, 0.1 M sodium acetate extract of pea stem tissue; lane 3, 1 M sodium acetate extract of pea stem tissue; lane 4, α-fucosidase purified to homogeneity.

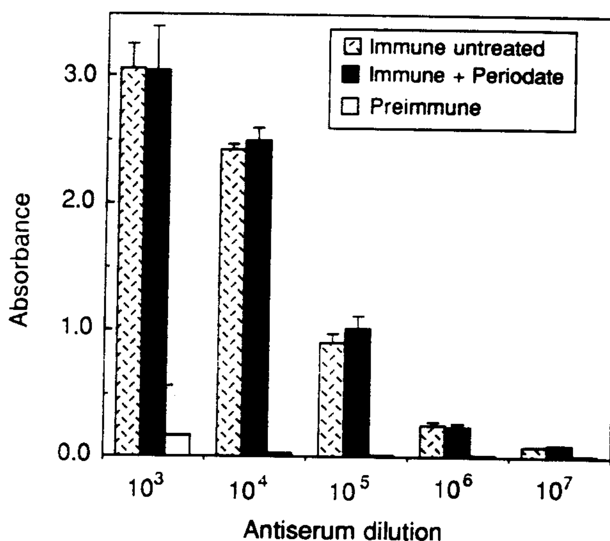

Figure 7. Effect of pretreatment of α-fucosidase with periodate on the subsequent binding of α-fucosidase-antiserum.
Each datum is the average of two replicates. The mean absorbance value ± SE, for untreated α-fucosidase and periodate-treated α-fucosidase is given for each of the antiserum dilution series. The absorbance was measured as follows: A(405 nm)−A(492 nm). The absorbance at 492 nm was subtracted from the absorbance at 405 nm, to correct for the presence of paranitrophenyl phosphate.

Subcellular localization of α-fucosidase in leaf and stem cells

The α-fucosidase was localized by post-embedding immunocytochemistry. Ultrastructural preservation and antigen immobilization were achieved by fixing the tissues with glutaraldehyde. Non-specific deposition of gold particles on the sections was prevented by pre-incubating the ultrathin sections with normal goat serum prior to treatment with the antiserum. In addition, the background staining over both the tissue sections and the embedding resin was reduced significantly by washing the sections with Tris-HCl buffer containing 0.5 M salt and 1% (w/v) BSA as an inert protein. Then, ultrathin sections of samples from 8-day-old pea stem and 14-day-old leaf were incubated with anti-fucosidase antiserum and goat-anti-rabbit (GAR)-gold antibodies. The result was an intense labeling of cell walls (Figures 8 and 9a).

Epidermal cell walls of pea stems showed strong labeling, as judged by the number of gold particles distributed over the entire wall (Figure 8b). The density of label is lower in the middle lamella and particularly in the corners of the walls (Figure 8a). Epidermal cell walls of pea leaves were also labeled (Figure 8 c–d). Again, the gold particles were not evenly distributed over the walls as the particles were preferentially associated with the wall layer closest to the cytoplasm (Figure 8d); the middle lamella region of the epidermal wall had greatly reduced label (Figure 8e).

Labeling with the anti-fucosidase antiserum was confined to the primary walls in the mesophyll tissue of pea leaves (Figure 8c). Once again, fewer gold particles were attached to the middle lamella of stems and leaves than to the rest of the primary cell walls. The cytoplasm, organelles, and vacuoles were not labeled in the leaf or stem tissue (Figure 8). A similar labeling pattern was found in the parenchyma cells of pea stems (Figure 9a). Here again, the middle lamella was less densely labeled than the rest of primary walls (Figure 9a).

Labeling with the anti-fucosidase antiserum-GAR-gold antibodies was virtually abolished by competition for the rabbit antibodies, with purified α-fucosidase (Figure 9b and c). This was achieved by adding an excess of purified α-fucosidase to the anti-fucosidase antiserum and incubating overnight before using the depleted antiserum for immunocytochemical labeling. Similarly, incubation of the sections directly with GAR-gold antibodies, the anti-fucosidase antiserum step being omitted, yielded negative results (data not shown).

Sections from 2- and 4-day stems rather than 8-day stems were incubated with the anti-fucosidase antiserum and with GAR-gold antibodies (Figure 10 a–d). No significant labeling was detected over primary walls of epidermal and parenchyma cells of 2- and 4-day stems. The absence of the fucosidase in 2- and 4-day-old stems was confirmed by enzymatic studies. These studies involved attempting to purify the α-fucosidase from 2- and 4-day-old pea seedlings using the extraction procedure developed with 8-day-old pea seedlings (see Experimental procedures). α-Fucosidase activity in CM-Sepharose-chromatographed extracts of 2-, 4- and 8-day-old pea seedlings was assayed

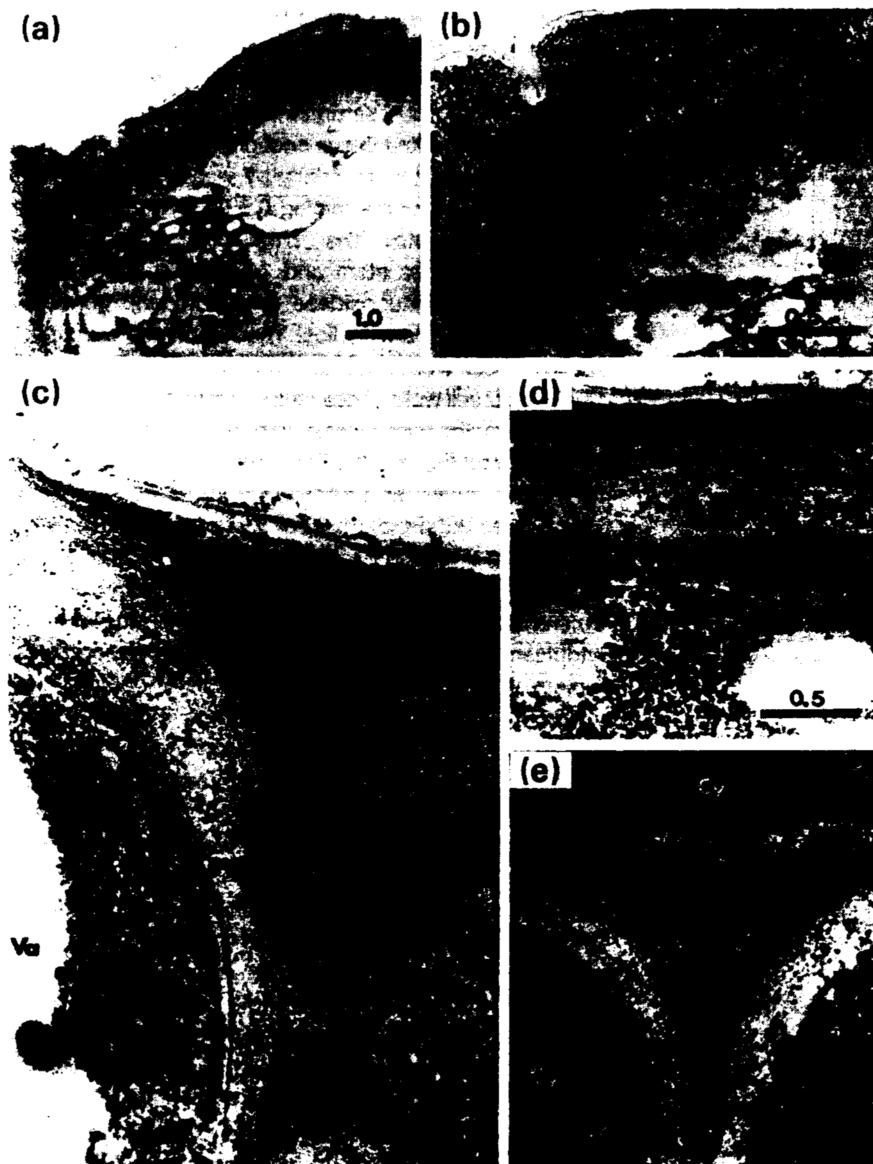

Figure 8. Transmission electron micrographs of pea stem (a and b) and leaf (c–e) tissues. Sections were incubated with anti-α-fucosidase antibodies followed by goat-anti-rabbit-gold antibodies (10 nm).
(a) Gold label is found exclusively over the epidermal wall. Bar = 1 μm.
(b) Enlarged portion of (a) showing the accumulation of gold particles in the epidermal cell wall. Bar = 0.5 μm.
(c) Leaf epidermal cell; labeling occurs over the primary wall but with reduced intensity in the middle lamella. Bar = 0.25 μm.
(d) Leaf epidermal cell. The outer region of the epidermal wall shows reduced labeling. Bar = 0.5 μm.
(e) Cross-section of leaf mesophyll cells, again with reduced labeling in the middle lamella. Bar = 0.25 μm.
The abbreviations used are: EW, epidermal wall; PW, primary wall; Cy, cytoplasm; ML, middle lamella; Va, vacuole.

for using reduced 2′-fucosyl lactose as a substrate. α-Fucosidase activity was detected in the extract from 8-day-old pea seedlings, whereas no hydrolysis of the fucosidic linkage was detected in extracts from 2- and 4-day-old pea seedlings. Under the conditions tested, α-fucosidase in the extract from 8-day-old seedlings hydrolyzed 35% of L-fucose from reduced 2′-fucosyl lactose.

Discussion

We have described a procedure including ion-exchange chromatography and isoelectric focusing, for the purification to homogeneity of pea epicotyl α-fucosidase. Several criteria were used to assess purity including SDS–PAGE, N-terminal amino acid sequencing (manuscript in preparation), and generation of monospecific antisera against the purified fucosidase. To our knowledge this is the first report on the purification to homogeneity of a plant α-fucosidase.

The molecular mass of α-fucosidase under denaturing conditions was 20 kDa, which represents the smallest known α-fucosidase. Under denaturing conditions, an observed molecular mass of 40 000–73 000 has been reported for purified α-fucosidases isolated from a number of prokaryotic and eukaryotic sources (Alhadeff *et al.*, 1975; Kochibe, 1973; Sano *et al.*, 1992; Tsuji *et al.*, 1990). α-Fucosidases from mammalian tissues (Alhadeff *et al.*, 1975; Opheim and Touster, 1977) and marine mollusks (D'Aniello *et al.*, 1982; Nishigaki *et al.*, 1974) are able to hydrolyze artificial substrates such as *p*-nitrophenyl-α-L-fucopyranoside and have broad aglycon specificities.

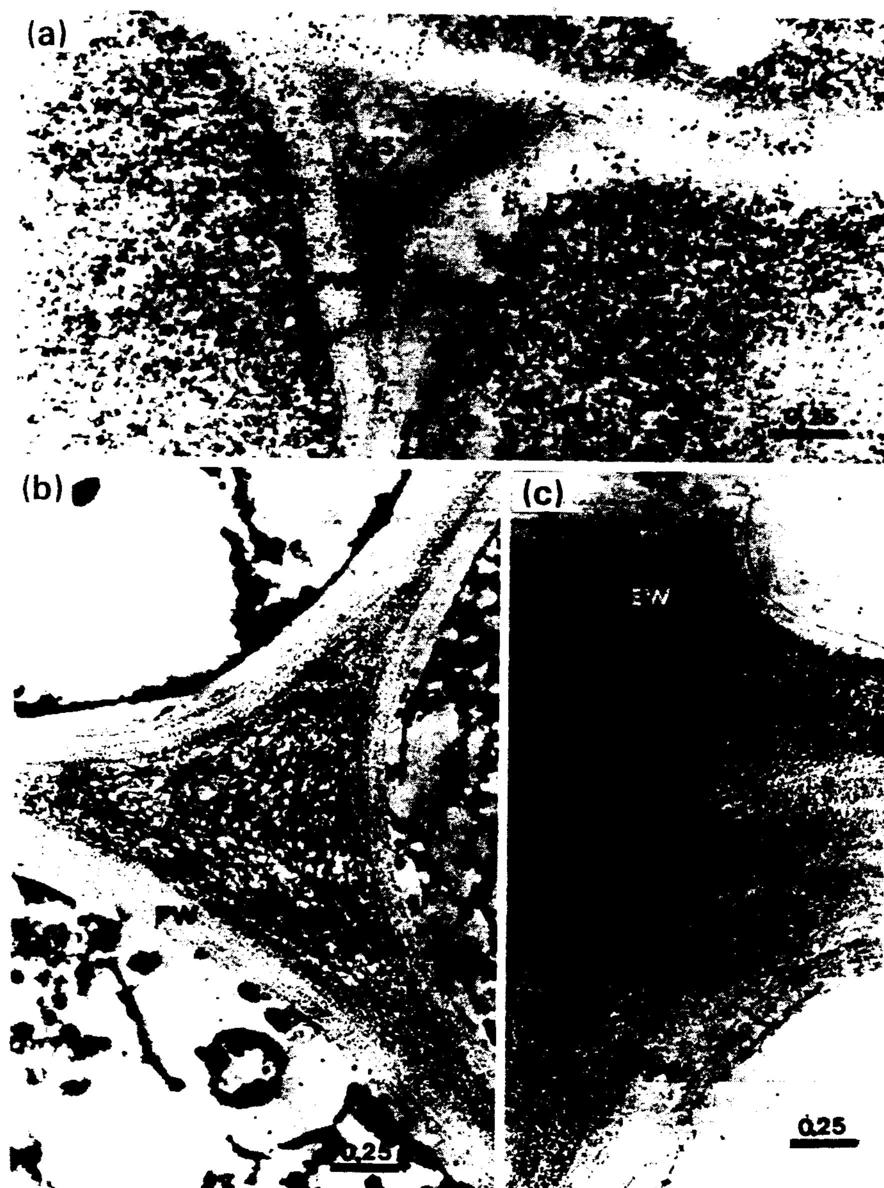

Figure 9. Electron micrographs of pea stem sections.
(a) Transmission electron micrograph of stem parenchyma cells immunolabeled with anti-α-fucosidase antibodies followed by GAR-gold antibodies (10 nm). Labeling is associated with the primary wall, but absent from the middle lamella. Bar = 0.25 μm.
(b) Transmission electron micrograph of stem parenchyma tissue. Section of control stem incubated with anti-α-fucosidase antibodies that were preadsorbed with an excess of purified α-fucosidase. No label above background is found over the primary wall. Bar = 0.25 μm.
(c) Transmission electron micrograph of stem epidermal tissue. Section of control stem incubated with anti-α-fucosidase antibodies that were preadsorbed with an excess of purified α-fucosidase. Bar = 0.25 μm.
IS, intercellular space. For other abbreviations see Figure 8.

They are able to hydrolyze α-1,2; α-1,3; α-1,4; and α-1,6-L-fucosidic linkages. In contrast, microbial (Aminoff and Fukurawa, 1970; Bhal, 1970; Kochibe, 1973) and almond emulsin (Ogata-Arakawa *et al.*, 1977; Yoshima *et al.*, 1979) α-fucosidases have narrow aglycon specificities and cannot hydrolyze artificial substrates. α-Fucosidase from pea epicotyls falls into the latter category. The α-fucosidase from pea seedlings, which hydrolyzes oligosaccharide substrates containing Fuc-α-1,2-Gal linkages (Table 1), was unable to hydrolyze *p*-nitrophenyl-α-L-fucopyranoside.

The reduced disaccharide, Fuc-α-1,2-Gal (Figure 1; oligosaccharide **8**), is a relatively poor substrate for the α-fucosidase, and reduced Fuc-α-1,3-Gal (oligosaccharide **5**) is not a substrate. Thus, attachment of the α-fucosyl residue to O-3 rather than O-2 of galactose abolished the activity of α-fucosidase.

The α-fucosidase hydrolyzed compound **7** more slowly than compound **6**. These two compounds only differ by the presence of a 6-*O*-acetyl group on the galactosyl residue of compound **6**. It is interesting to note that 50% of the galactosyl residues of xyloglucan oligosaccharides that were enzymatically released from purified sycamore cell walls are substituted with a 6-*O*-acetyl group (Kiefer *et al.*, 1989). Thus, *O*-acetyl groups may affect the half life of xyloglucan oligosaccharins *in vivo*.

The solubilized primary cell wall polysaccharides RG I, RG II, and xyloglucan are not substrates for the α-fucosidase (Table 1). On the other hand, xyloglucan oligosaccharides containing fucosyl residues are substrates

Figure 10. Transmission electron micrographs of 2-day-old (a and b) and 4-day-old (c and d) stem parenchyma cells. Sections were incubated with anti-α-fucosidase antibodies followed by GAR-gold antibodies (10 nm). No labeling could be detected. (a) Bar = 0.5 μm. (b) Bar = 0.25 μm. (c) Bar = 0.5 μm. (d) Bar = 0.25 μm. For abbreviations see Figure 8.

for the α-fucosidase. The ability of α-fucosidase to hydrolyze fucosyl-containing xyloglucan oligosaccharides but not fucosyl-containing polysaccharides may arise from differences in conformation between the oligo- and polysaccharides. Levy and co-workers (1991) have preliminary NMR data indicating that the fucosyl residue of XG9 is in close contact with its reducing glucose residue; these results were also predicted by theoretical potential energy calculations. These data suggest that the terminal α-linked fucosyl residues of the xyloglucan polysaccharide may not be accessible for hydrolysis by the α-fucosidase due to steric hindrance.

Xyloglucan polysaccharide, which is not a substrate for α-fucosidase, has been localized throughout the entire wall in suspension-cultured sycamore cells (Moore *et al.*, 1986). A small portion of the wall polymers are cleaved during auxin-induced growth (Labavitch and Ray, 1974) and fucose-containing oligosaccharides such as XG9, which are substrates for α-fucosidase, apparently arise by the partial hydrolysis of pre-formed xyloglucan (McDougall and Fry, 1991b). There is also suggestive evidence in the literature (Albersheim *et al.*, 1992; Fry, 1989b; York *et al.*, 1984) that these fucosylated xyloglucan oligosaccharides are involved in feed back inhibition of auxin-induced growth. Therefore, during growth, an α-fucosidase that hydrolyzes the glycosidic bond of the

α-fucosyl residues of these oligosaccharins could control their *in situ* concentrations. However, it is not known how the α-fucosidase level is regulated.

The evidence that hydrolytic enzymes, such as α-fucosidase, play a role in plant growth regulation is only correlative; direct evidence remains to be obtained. The possibility that α-fucosidase controls the levels of growth inhibitory XG9, and the lack of information concerning the localization of hydrolytic enzymes in general and of the α-fucosidase in particular, prompted us to use immunocytochemical methods to visualize the distribution of the α-fucosidase during growth. Three stages of seedling development were studied (days 2, 4, and 8). At days 2 and 4, when the stem cells are elongating rapidly, no α-fucosidase is present in the primary cell walls of stem tissue. At day 8 when elongation has slowed, the α-fucosidase is abundantly present in the cell walls, of stem and leaf tissues. The results obtained from the immunolocalization studies correlate with those at the enzyme activity level. No α-fucosidase activity could be detected in extracts of 2- and 4-day-old stems of pea seedlings, even after incubating reduced 2′-fucosyl lactose with the partially purified extracts for 6 h. Thus, α-fucosidase is developmentally regulated. These results are in agreement with those of Farkas *et al.* (1991) who determined that α-fucosidase activity increases with the age of the cotyledons of nasturtium seedlings, peaking at day 12.

In conclusion, we have purified to homogeneity a cell wall localized α-fucosidase that is able to regulate oligosaccharin activity. The present study adds to our understanding of how oligosaccharins function in the regulation of growth.

Experimental procedures

Plant material

Peas (*Pisum sativum* var. Alaska) were grown in vermiculite in the dark at 25°C for 2, 4, and 8 days as previously described (O'Neill *et al.*, 1989b). Etiolated pea stem material above the cotyledons was harvested, weighed, and frozen in liquid nitrogen.

Protein determination

Protein concentration was determined by the Bio-Rad assay system (Bradford, 1976) according to the manufacturer's procedures. BSA was used as the standard.

Enzyme assay

α-Fucosidase activity was assayed by a modification of the Kato and Kinoshita (1980) fluorescence method for the general detection of reducing sugars (O'Neill *et al.*, 1989a, 1989b). Reduced 2′-fucosyl lactose (Sigma), in which the glucose residue at the reducing terminus has been converted to glucitol (York *et al.*, 1985), was used as the substrate during all steps of α-fucosidase purification. For the assay of column fractions, 10 μl of enzyme solution was added to 10 μg of reduced 2′-fucosyl-lactose in a total volume of 50 μl containing 200 mM sodium acetate, pH 6.0, with 0.02% sodium azide. After incubation of the mixture at 30°C for 1 h, the enzyme reaction was stopped by adding 300 μl of a solution of 4.67% (v/v) ethanolamine and 4.67% (w/v) boric acid and heating the mixture for 10 min in a 150°C aluminum heating block. Samples were then cooled and the fluorescence read with a Perkin–Elmer model 650–15 fluorescence spectrophotometer, using excitation and emission wavelengths of 357 and 443 nm, respectively. L-Fucose (Sigma) was used as the sugar standard for quantitation. For the assay of extracts from 2-, 4-, and 8-day-old seedlings, 8 μg from each extract was added to 10 μg of reduced 2′-fucosyl lactose in a total volume of 55 μl containing 200 mM sodium acetate, pH 6.0, 200 mM sodium chloride, and 0.02% sodium azide. After incubation of the mixture at 30°C for 6 h, the enzyme reaction was stopped, and the sample heated and the fluorescence read as described above.

Enzyme purification

Eight-day-old pea stem tissue (1.5 kg) was homogenized for 2 min in 4 l of ice-chilled 100 mM sodium acetate, pH 5.0, containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVP, Sigma) and 0.3% (v/v) β-mercaptoethanol (Sigma). The homogenate was filtered through a nylon cloth (400 mesh). The retentate was collected and resuspended in 2 l of 50 mM sodium acetate, pH 5.0, containing 1% PVP and 0.3% β-mercaptoethanol. The suspension was filtered through a nylon cloth and the retentate was suspended in 2.5 l of 1 M sodium acetate, pH 5.0. The suspension was maintained at 0°C for 1 h to allow release of wall associated proteins, and filtered through a nylon cloth. The soluble fraction, which contained the 'crude' enzyme, was dialyzed against 25 mM sodium acetate, pH 5.0. The crude enzyme was centrifuged at 13 000 ***g*** for 1 h prior to cation exchange chromatography on CM-Sepharose.

Cation exchange chromatography

The resulting supernatant (about 2.5 l) was applied at 3 ml min^{-1} to a 2.5 × 9 cm CM-Sepharose fast flow (Pharmacia) column equilibrated in 25 mM sodium acetate, pH 5.0. The column was washed with 25 mM sodium acetate, pH 5.0, until the absorbance of the eluate at 280 nm was equal to that of the buffer. The column was eluted at 3 ml min^{-1} with a 500 ml linear gradient starting with 25 mM sodium acetate, pH 5.0, and ending with the same buffer containing 500 mM NaCl. Fractions (8 ml) were collected and assayed for α-fucosidase activity. The fractions containing α-fucosidase activity (35–41, Figure 2) were pooled and dialyzed against H_2O prior to preparative isoelectric focusing.

Isoelectric focusing

Preparative isoelectric focusing was performed using a Rotofor cylindrical focusing chamber (BioRad) with a capacity of approximately 50 ml. The focusing chamber is divided into 20 discrete compartments by a membrane core. Ampholytes, pH 5–7 (Biolyte 5/7, BioRad), were added to the enzyme solution (about 45 ml) to 1% (v/v). This solution was loaded into the Rotofor chamber and maintained at 4°C for 20 min before being electrofocused at 12 W constant power for 4 h with a model 3000Xi power supply (BioRad). The contents of the focusing chamber were collected into 20 2.5 ml fractions. Each fraction was analyzed for pH and α-fucosidase activity. α-Fucosidase-containing fractions

(7–11, Figure 3a) were pooled, brought to a final volume of 50 ml with H_2O, reloaded into the Rotofor chamber, and the electrofocusing procedure repeated. After 4 h, 20 2.5 ml fractions were collected and analyzed for pH and α-fucosidase activity (Figure 3b). The proteins in each of the fractions from both the electrofocusing procedures were monitored by SDS–PAGE (data not shown).

Preparation of extracts from 2-, 4-, and 8-day-old etiolated pea stem

Enzyme purification. Two-day-old stem tissue (50 g) was homogenized for 2 min in 400 ml of ice-chilled 100 mM sodium acetate, pH 5.0, containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVP, Sigma) and 0.3% (v/v) β-mercaptoethanol (Sigma). The homogenate was filtered through a nylon cloth (400 mesh). The retentate was collected and resuspended in 300 ml of 50 mM sodium acetate, pH 5.0, containing 1% PVP and 0.3% β-mercaptoethanol. The suspension was filtered through a nylon cloth and the retentate was suspended in 150 ml of 1 M sodium acetate, pH 5.0. The suspension was maintained at 0°C for 1 h to allow release of wall-associated proteins, and then filtered through a nylon cloth. The filtrate, was dialyzed against 25 mM sodium acetate, pH 5.0, and then centrifuged at 13 000 ***g*** for 1 h prior to cation exchange chromatography on CM-Sepharose.

Cation exchange chromatography. The centrifugation supernatant (about 150 ml) was applied at 3 ml min^{-1} to a 0.5 × 4 cm CM-Sepharose fast flow (Pharmacia) column equilibrated in 25 mM sodium acetate, pH 5.0. The column was washed with 25 mM sodium acetate, pH 5.0, until the absorbance of the eluate at 280 nm was equal to that of the buffer. The column was eluted at 3 ml min^{-1} with 25 mM sodium acetate, pH 5.0, containing 200 mM NaCl. A 40 ml fraction containing the 200 mM NaCl-released fraction, was collected and concentrated 10-fold in a Centriprep 10 (Amicon) in preparation for α-fucosidase activity assay. The purification procedure was repeated for the 4- and 8-day-old pea seedlings.

Substrate specificity of α-fucosidase

RG I, RG II, xyloglucan, XG25 and XG9 were purified from sycamore cell walls as described (York *et al.*, 1985). All the compounds tested (except compound **6**, Figure 1, and *p*-nitrophenyl-α-L-fucoside) were reduced to their corresponding alditols, as described in Augur *et al.* (1992), prior to being used in the α-fucosidase activity assay. For the assay of α-fucosidase activity, 0.05 α-fucosidase units (one unit of α-fucosidase is defined as the amount of enzyme required to produce 1 μM reducing fucose per hour at 30°C and at pH 6.0, using 10 μg of reduced 2′-fucosyllactose as substrate) was added to 2.5–5.0 μg of fucose equivalent substrate in a total volume of 50 μl containing 200 mM sodium acetate, pH 6.0, with 0.02% sodium azide. After incubation of the mixture at 30°C for 1 h, the enzyme reaction was stopped by adding 300 μl of a solution of 4.67% (v/v) ethanolamine and 4.67% (w/v) of boric acid and heating the mixture for 10 min in a 150°C aluminum heating block. The samples were cooled and the fluorescence read, using excitation and emission wavelengths of 357 and 443 nm, respectively. L-Fucose was used as the sugar standard for quantitation.

The ability of α-fucosidase to hydrolyze *p*-nitrophenyl-α-L-fucoside (Sigma) was assayed using 20 μg of substrate and 0.05 units of α-fucosidase in a total volume of 50 μl containing 200 mM sodium acetate, pH 6.0, with 0.02% sodium azide. The reaction mixture was incubated for 3 h at 30°C. The reaction was terminated by adding 1 ml of 0.2 M sodium carbonate and the absorbance was measured at 400 nm.

α-Fucosidase treatment of XG9

XG9 (40 μg) was incubated (30°C for 3 h) in 110 μl of 100 mM sodium acetate, pH 6.0, containing 0.02% sodium azide and 0.1 unit of α-fucosidase. The resulting digest was passed through a column (0.5 × 2 cm) of Dowex 50 (Sigma) to remove sodium ions and then lyophilized to remove acetic acid. XG8, XG9, and free fucose were separated by HPAE chromatography on a CarboPac PA-1 column using a Dionex metal-free BioLc liquid chromatograph interfaced to an AutoIon Series 450 data station (Dionex Corp., Sunnyvale, CA). The carbohydrates in the effluent were detected using a Dionex pulsed amperometric detector (PAD) equipped with a gold working electrode. The amperometric detector was operated in the integrated amperometry mode at 500 nAmps sensitivity. The following pulse potentials (E_1–E_3) and durations (t_1–t_3) were used for detection of the oligosaccharides: E_1 = 0.05 V (t_1 = 500 msec); E_2 = 0.6 V (t_2 = 80 msec); E_3 = −0.6 V (t_3 = 50 msec). The column was eluted with 100 mM NaOH (0–5 min) followed by a 40 ml linear gradient of sodium acetate (0–60 mM) in 100 mM NaOH (5.1–45 min). The column was then eluted isocratically with 60 mM sodium acetate in 100 mM NaOH (45.1–50 min) and re-equilibrated for 10 min in 100 mM NaOH prior to the next injection. The flow rate was 1 ml min^{-1}.

The eluant solutions were prepared using ultrapure water, carbonate-free aqueous 50% sodium hydroxide (J.T. Baker, Phillipsburg, NJ), and sodium acetate trihydrate (Biochimika grade, Fluka, Ronkonkom, NY). The solutions were filtered (0.2 μm Nylon 66 membranes, Rainin, Woburn, MA) and degassed with helium using an eluant degas module (Dionex, Sunnyvale, CA) prior to their use. To facilitate the detection of carbohydrates and to minimize base-line drift, 400 mM NaOH was added post-column at a flow rate of 0.6 ml min^{-1} using a pressurized Dionex reagent delivery system.

Production of anti-α-fucosidase antibodies

Antiserum to the α-fucosidase was prepared by emulsifying the purified enzyme (100 μg in 100 μl H_2O) with an equal volume of Freund's complete adjuvant (Pierce, Rockford, IL) and injecting the resulting emulsion into the foot pad of a rabbit. After 4 weeks, the rabbit was injected with an emulsion containing 50 μg of purified enzyme, 100 μg of H_2O, and 100 μl of Freund's incomplete adjuvant (Pierce). After 6 weeks, the rabbit was bled from the ear artery and serum was obtained by centrifugation at 1500 ***g*** for 25 min at room temperature. The serum was stored in aliquots at −20°C. The specificity of the antiserum raised against the purified α-fucosidase was assessed by Western blotting after gel electrophoresis of the soluble proteins in the crude pea epicotyl extracts and by enzyme-linked immunosorbent assay (ELISA). The ELISA assay was carried out as previously described (Hahn *et al.*, 1987).

SDS–Gel electrophoresis and Western blotting

SDS–gel electrophoresis, to monitor the α-fucosidase purification steps, was performed in 20% (w/v) polyacrylamide gels using the Phast system from Pharmacia. SDS–gel electrophoresis, for Western blotting, was performed in 15% (w/v) polyacrylamide gels as described (Schägger and Jagow, 1987). Electrophoretically

separated proteins were transferred to a nitrocellulose membrane (0.45 μm, Bio-Rad) as described (Towbin *et al.*, 1979). For immunodetection, free polypeptide binding sites were blocked with TBS (50 mM Tris/500 mM sodium chloride, pH 7.6) containing 0.5% (w/v) BSA. The membrane was then incubated for 2 h in primary antibody diluted (1:10^4) in TBS. Antigen–antibody reactions were visualized by the indirect alkaline phosphatase technique as described in the Bio-Rad technical sheet.

Electron microscopy

Plant material. Peas were grown as described above. Stem segments (6 mm long starting 3 mm from the maximum curvature of the apical hook) were cut from 2-, 4- and 8-day-old (dark grown) etiolated pea seedlings. Eight-day-old stem segments were washed with a freshly prepared incubation medium consisting of 1% sucrose, 0.02% benzylpenicillin (potassium salt, Sigma), and 5 mM potassium phosphate (pH 6.1). Eight-day-old stem segments were incubated in 2 ml of the same incubation medium, for 4 h on a rotary snaker before tissue processing. Leaf sections of non-etiolated 14-day-old peas were also collected for electron microscope studies.

Tissue processing. Pea stem and leaf segments (about 1 mm^3) were fixed in 3% (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2, for 2 h at room temperature, then dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were collected on formvar-coated nickel grids and processed for immunogold labeling (Benhamou *et al.*, 1990). From each experiment, 10–15 sections from each of three stems and three leaves were examined visually with a JEOL 1200 EX electron microscope at 80 kV.

Immunocytochemical labeling. Ultrathin sections were first floated for 5 min on a drop of phosphate-buffered saline (PBS), pH 7.2, containing 0.5% (w/v) BSA and then transferred to a drop of normal goat serum (diluted 1:10 in PBS–BSA) for 30 min at room temperature. Sections were incubated for 2 h at 37°C on a drop of anti-α-fucosidase antiserum (diluted 1:20 in PBS–BSA) and then washed for 10 min with TrisHCl-BSA-NaCl (0.05 M Tris HCl (pH 8.2), 1% (w/v) BSA, 0.5 M sodium chloride). Sections were then incubated on a drop of colloidal gold (10 nm)-conjugated goat antiserum raised against rabbit immunoglobulins (GAR-gold antibodies) (diluted 1:10 in TrisHCl-BSA-NaCl) for 1 h at room temperature, washed with PBS (pH 7.4), rinsed with distilled water, and finally contrasted with uranyl acetate and lead citrate prior to examination under the electron microscope.

Immunocytochemical controls. The specificity of labeling was assessed by including two controls. In the first, purified α-fucosidase (40 ng) was incubated overnight at 4°C in a 20 μl droplet of anti-α-fucosidase antiserum (diluted 1:20 in PBS–BSA). An ultrathin section was then floated on the droplet for 2 h at 37°C. The immunocytochemical labeling procedure was then followed as described above. In the second, each ultrathin section was incubated directly with GAR-gold antibodies, the anti-α-fucosidase antiserum step being omitted.

Quantification of labeling. The density of labeling associated with cell walls and cell corners was calculated and expressed as the number of gold particles per μm^2. Areas were determined with a ZEISS MOP image analysis system. The amount of labeling over a specific area (Sa) was estimated by the point counting method established by Weibel (1969) on a photographic enlargement. The density of labeling (Ns) is calculated as follows: Ns = Ni/Sa, where Ns represents the number of gold particles (Ni) per unit surface (Sa).

Acknowledgements

The authors thank Andy Whitcombe for providing RG I and RG II polysaccharides, William York for providing XG25, and Dr P. Sinaÿ for the synthetic trisaccharides (compounds **6** and **7**). The authors also thank Sylvain Noel, Sally Giles and Karen Howard for their excellent technical and secretarial assistance. This work was supported in part by US Department of Energy grant DE-FG09-85ER13425, and by Department of Energy grant DE-FG09-87ER13810 as part of the USDA/DOE/NSF Plant Science Centers program.

References

Albersheim, P., Darvill, A., Augur, C., *et al.* (1992) Oligosaccharins: Oligosaccharide regulatory molecules. *Acc. Chem. Res.* **25**, 77–83.

Alhadeff, J., Miller, A., Wenaas, H., Vedvick, T. and O'Brien, J. (1975) Human liver α-L-fucosidase. Purification, characterization, and immunochemical studies. *J. Biol. Chem.* **250**, 7106–7113.

Aminoff, D. and Fukurawa, K. (1970) Enzymes that destroy blood group specificity. Purification and properties of α-fucosidase from *Clostridium perfringens*. *J. Biol. Chem.* **245**, 1659–1669.

Augur, C., Yu, L., Sakai, K., Ogawa, T., Sinaÿ, P., Darvill, A. and Albersheim, P. (1992) Further studies on the ability of xyloglucan oligosaccharides to inhibit auxin-induced growth. *Plant Physiol.* **99**, 180–185.

Bahl, O. (1970) Glycosidases of *Aspergillus niger*. Purification and properties of α-1,2-fucosidase. *J. Biol. Chem.* **245**, 299–304.

Benhamou, N., Joosten, M. and De Wit, P. (1990) Subcellular localization of chitinase and its potential substrate in tomato root tissues infected by *Fusarium oxysporum f. sp. radicis-lycopercisi*. *Plant Physiol.* **92**, 1108–1120.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

D'Aniello, A., Hakimi, J., Cacace, G. and Ceccarini, C. (1982) The purification and characterization of α-fucosidase from the hepatopancreas of *Octopus vulgaris*. *J. Biochem.* **91**, 1073–1080.

Edwards, M., Dea, I.C.M., Bulpin, P.V. and Reid, G. (1986) Purification and properties of a novel xyloglucan-specific endo-(1→4)-β-D-glucanase from germinated nasturtium seeds (*Tropaeolum majus* L.). *J. Biol. Chem.* **261**, 9489–9494.

Edwards, M., Bowman, Y.J.L., Dea, I.C.M. and Reid, G. (1988) A β-D-galactosidase from nasturtium (*Tropaeolum majus* L.) cotyledons. *J. Biol. Chem.* **263**, 4333–4337.

Emmerling, M. and Seitz, N.V. (1990) Influence of a specific xyloglucan-nonasaccharide derived from cell walls of suspension-cultured cells of *Daucus carota* L. on regenerating carrot protoplasts. *Planta*, **182**, 174–180.

Fanutti, C., Gidley, M.J. and Reid, G. (1991) A xyloglucan-oligosaccharide-specific α-D-xylosidase or exo-oligoxyloglucan-α-xylohydrolase from germinated nasturtium (*Tropaeolum majus* L.). *Planta*. **184**, 137–147.

Farkas, V., Hanna, R. and MacLachlan, G. (1991) Xyloglucan oligosaccharide α-L-fucosidase activity from growing pea

stems and germinating nasturtium seeds. *Phytochemistry*, **30**, 3203–3207.

Fry, S. (1989a) Analysis of crosslinks in the growing cell-walls of higher plants. In *Modern Methods of Plant Analysis*, New Series, 10 (Linskens, H.F. and Jackson, J.F., eds). Berlin: Springer, pp 12–36.

Fry, S. (1989b) The structure and functions of xyloglucan. *J. Exp. Bot.* **40**, 1–11.

Hahn, M., Lerner, D., Fitter, M., Norman, P. and Lamb, C. (1987) Characterization of monoclonal antibodies to protoplast membranes of *Nicotiana tabacum* identified by an enzyme-linked immunosorbent assay. *Planta*, **171**, 453–465.

Hardy, M. and Townsend, R. (1988) Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *PNAS USA*, **85**, 3289–3293.

Kato, Y. and Kinoshita, T. (1980) Fluorometric detection and determination of carbohydrates by high-performance liquid chromatography using ethanolamine. *Anal. Biochem.* **106**, 238–243.

Kiefer, L., York, W., Darvill, A. and Albersheim, P. (1989) Structure of plant cell walls XXVII. Xyloglucan isolated from suspension-cultured sycamore cell walls is O-acetylated. *Phytochemistry*, **28**, 2105–2107.

Kobata, A. (1982) α-L-Fucosidases from almond emulsin. *Methods Enzymol.* **83**, 625–631.

Kochibe, N. (1973) Purification and properties of α-fucosidase from *Bacillus fulminans*. *J. Biochem.* **74**, 1141–1149.

Labavitch, J.M. and Ray, P.M. (1974) Relation between promotion of XG metabolism and induction of elongation by IAA. *Plant Physiol.* **54**, 499–502.

Levy, S., York, W., Stuike-Prill, R., Meyer, B. and Staehelin, A. (1991) Simulation of the static and molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *Plant J.* **2**, 195–215.

McDougall, G. and Fry, S. (1988) Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan. *Planta*, **175**, 412–416.

McDougall, G. and Fry, S. (1989a) Anti-auxin activity of xyloglucan oligosaccharides: the role of groups other than the terminal α-L-fucose residue. *J. Exp. Bot.* **40**, 233–238.

McDougall, G. and Fry, S. (1989b) Structure–activity relationships for xyloglucan oligosaccharides with anti-auxin activity. *Plant Physiol.* **89**, 883–937.

McDougall, G. and Fry, S. (1990) Xyloglucan oligosaccharides promote growth and activate cellulase: evidence for a role of cellulase in cell expansion. *Plant Physiol.* **93**, 1042–1048.

McDougall, G. and Fry, S. (1991a) Purification and analysis of growth-regulating xyloglucan-oligosaccharides by high-pressure liquid chromatography. *Carbohydr. Res.* **219**, 123–132.

McDougall, G. and Fry, S. (1991b) Xyloglucan nonasaccharide, a naturally occurring oligosaccharin, arises *in vivo* by polysaccharide breakdown. *J. Plant Physiol.* **137**, 332–336.

Moore, P., Darvill, A., Albersheim, P. and Staehelin, A. (1986) Immunogold localization of xyloglucan and rhamnogalacturonan I in the cell-walls of suspension cultured sycamore cells. *Plant Physiol.* **82**, 787–794.

Nishigaki, M., Muramatsu, T., Kobata, A. and Maeyama, K. (1974) The broad aglycon specificity of α-L-fucosidases from marine gastropods. *J. Biochem.* **75**, 509–517.

Ogata-Arakawa, M., Muramatsu, T. and Kobata, A. (1977) α-L-Fucosidases from almond emulsin: characterization of the two enzymes with different specificities. *Arch. Biochem. Biophys.* **181**, 353–358.

O'Neill, R., White, A., York, W., Darvill, A. and Albersheim, P. (1988) A gas chromatographic-mass spectrometric assay for glycosylases. *Phytochemistry*, **27**, 329–333.

O'Neill, R., White, A.R., York, W., Darvill, A. and Albersheim, P. (1989a) A fluorescence assay for enzymes that cleave glycosidic linkages. *Anal. Biochem.* **177**, 11–15.

O'Neill, R., Albersheim, P. and Darvill, A. (1989b) Purification and characterization of a xyloglucan oligosaccharide-specific xylosidase from pea seedlings. *J. Biol. Chem.* **264**, 20 430–20 437.

Opheim, D.J. and Touster, O. (1977) The purification and characterization of rat liver lysosomal α-L-fucosidase. *J. Biol. Chem.* **252**, 739–743.

Sano, M., Hayakawa, K. and Kato, I. (1992) Purification and characterization of α-fucosidase from *Streptomyces* species. *J. Biol. Chem.* **267**, 1522–1527.

Schägger, H. and Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of protein in the range of 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfers of proteins from polyacrylamide gels to nitrocellulose: procedure and some applications. *PNAS USA*, **76**, 4350–4354.

Tsuji, Y., Yamamoto, K. and Toshikura, T. (1990) Purification and some properties of a novel α-fucosidase capable of acting on α-1,6-L-fucosidic linkages of *Bacillus circulans*. *J. Biochem.* **108**, 235–240.

Weibel, W.R. (1969) Stereological principles for morphometry in electron microscopy cytology. *Int. Rev. Cytol.* **26**, 235–244.

York, W., Darvill, A. and Albersheim, P. (1984) Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. *Plant Physiol.* **75**, 295–297.

York, W., Darvill, A., McNeil, M., Stevenson, T. and Albersheim, P. (1985) Isolation and characterization of plant cell walls and cell wall components. *Methods Enzymol.* **118**, 3–40.

Yoshima, H., Takasaki, S., Ito-Mega, S. and Kobata, A. (1979) Purification of almond emulsin α-L-fucosidase I by affinity chromatography. *Arch. Biochem. Biophys.* **194**, 394–398.