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A Xyloglucan-Specific Endo-1,4-β-Glucanase Isolated from Auxin-Treated Pea Stems[1]

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A xyloglucan-specific endo-1,4-β-glucanase was isolated from the apoplast fraction of auxin-treated pea *(Pisum sativum)* stems, in which both the rate of stem elongation and the amount of xyloglucan solubilized were high. The enzyme was purified to apparent homogeneity by sequential cation-exchange chromatographies, affinity chromatography, and gel filtration. The purified enzyme gave a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the molecular size was determined to be 77 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 70 kD by gel filtration. The isoelectric point was about 8.1. The enzyme specifically cleaved the 1,4-β-glucosyl linkages of the xyloglucan backbone to yield mainly nona- and heptasaccharides but did not hydrolyze carboxymethylcellulose, swollen cellulose, and (1→3,1→4)-β-glucan. By hydrolysis, the average molecular size of xyloglucan was decreased from 50 to 20 kD with new reducing chain ends in the lower molecular size fractions. This suggests that the enzyme has endo-1,4-β-glucanase activity against xyloglucan. In conclusion, a xyloglucan-specific endo-1,4-β-glucanase with an activity that differs from the activities of cellulase and xyloglucan endotransglycosylase has been isolated from elongating pea stems.

Xyloglucan-modifying enzymes in the primary cell wall are one of the most probable contributors to the loosening of the cellulose microfibril network, which renders the wall susceptible to turgor-driven expansion (Hayashi, 1989). Auxin promotes the turnover of xyloglucan in segments of pea *(Pisum sativum)* epicotyls; promotion begins within 15 min, and the effect increases with increasing IAA concentration and corresponds to the increase in elongation rate (Labavitch and Ray, 1974a, 1974b). Auxin- and/or acid-induced growth has also been associated with xyloglucan degradation in *Pisum* sp. (Terry et al., 1981), *Vigna* sp. (Nishitani and Masuda, 1982), *Pinus* sp. (Lorences and Zara, 1987), *Avena* sp. (Inouhe et al., 1984), and *Oryza* sp. (Revilla and Zarra, 1987).

The endo-1,4-β-glucanase activities responsible for degradation of xyloglucan in the primary wall were associated with auxin-induced expansion of cells in pea stems (Hayashi et al., 1984). The enzyme hydrolyzes the internal 1,4-β-glucosyl linkages of cellulose derivatives such as carboxymethylcellulose and cellodextrins and, in addition, both nonfucosylated and fucosylated xyloglucans. However, two endo-1,4-β-glucanases have recently been isolated from suspension-cultured poplar cells, which specifically cleaved the 1,4-β-glucosyl linkages of carboxymethylcellulose, swollen cellulose, and lichenan, but which showed very little degradation of xyloglucan (Nakamura and Hayashi, 1993; Ohmiya et al., 1995). Therefore, there may be at least two or three kinds of endo-1,4-β-glucanases, differing in their specificities for cellulose and xyloglucan, that are involved in the degradation of these β-glucans in higher plants. In addition, the pea endo-1,4-β-glucanase activity responsible for xyloglucan degradation might have been due to contamination with a xyloglucan-specific endo-1,4-β-glucanase (Hayashi et al., 1984).

Xyloglucan endotransglycosylase activity has been shown to correlate positively with growth (Fry et al., 1992), and expansins have been shown to catalyze cell wall loosening in vitro (McQueen-Mason and Cosgrove, 1994). A xyloglucan-specific endo-1,4-β-glucanase has been isolated from germinating *Nasturtium* seeds for degrading a storage nonfucosyl xyloglucan (Edwards et al., 1986), namely amyloid polysaccharide, but not from elongating stems for hydrolyzing xyloglucan in the primary wall. The endo-1,4-β-glucanase was later found to have a stronger transglycosylation activity with xyloglucan oligosaccharides than hydrolase activity. The kinetic data (Farkas et al., 1992) suggest that the xyloglucan endotransglycosylase reaction involves two substrates (donor and acceptor) in a ping-pong mechanism, in which the enzyme cleaves a donor xyloglucan to release a smaller xyloglucan with a reducing end in the first step, and transfers the remainder to an acceptor oligosaccharide. If the concentration of the acceptor is low, the enzyme works as a hydrolase by transferring the newly released part of the donor molecule onto water. This enzyme had very little activity against fucosylated xyloglucans that are present in the primary walls of dicotyledonous plants.

To clarify the mechanism of xyloglucan degradation in auxin-treated pea stems, we must determine whether there are xyloglucan-specific endo-1,4-β-glucanases that differ from cellulases. The aim of this study was to characterize xyloglucanase activities in pea.

MATERIALS AND METHODS

Sodium borotritide (185 GBq $mmol^{-1}$) was obtained from Amersham. DEAE- and SP-Toyopearl were obtained

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Abbreviation: IBA, indol-3-butyric acid.

from Tosoh (Tokyo), and other materials for chromatography were obtained from Pharmacia. Carboxymethylcellulose, lichenan, laminarin, dextran molecular markers, and Sepharose CL-6B were obtained from Sigma. Xyloglucan from *Tamarindus indica* was kindly provided by Mr. K. Yamatoya (Dainippon Pharmaceutical, Osaka, Japan). Pea xyloglucan (50 kD) treated with sodium borohydride has been previously described (Hayashi et al., 1994). The *Aspergillus oryzae* enzyme preparation was a generous gift from Dr. A. Endo (Tokyo University of Agriculture and Technology, Japan). This enzyme mixture is capable of hydrolyzing noncellulosic polysaccharides into monosaccharides and isoprimeverose (6-*O*-α-D-xylopyranosyl-D-Glc), in which all of the xylosyl residues of xyloglucan are recovered (Hayashi et al., 1981).

Seeds of pea (*Pisum sativum* L. var Alaska) were soaked for 18 h in water, sown in moistened vermiculite, and grown in darkness at 28°C. When the third internode was more than 1 cm long (7 or 8 d after sowing), segments (10 mm long) of this internode were harvested. They were soaked in ice-cold water for 30 min, incubated at 25°C in the dark, and shaken in a Petri dish (10 cm in diameter) that contained 10 mL of water with 20 μM IBA (Merck, Tokyo).

Determination of Stem Length and Xyloglucan Content after Auxin Treatment

The lengths of 20 segments were measured after incubation with IBA using a binocular microscope. An apoplast solution from the segments was collected by centrifugation according to the method of Terry et al. (1981). About 40 segments that had been auxin-treated were packed into a plastic syringe barrel fitted with a disc of nylon mesh. The segments were infiltrated with ice-cold water for 3 min under a vacuum and then centrifuged at 1500*g* at 20°C for 10 min to collect the apoplastic solution. This procedure was repeated twice. About 2.5 mL of a wall solution obtained from 200 segments was boiled for 5 min and incubated with *A. oryzae* enzyme preparation (2 mg) in 20 mM sodium acetate buffer, pH 5.0, at 40°C for 12 h. The reaction was stopped by boiling the mixture for 5 min and the mixture was applied to a charcoal column (0.5 × 5 cm). The column was then washed with 10 mL of water and eluted with 5 mL of 10% ethanol. The eluate from the column was concentrated and subjected to paper chromatography with solvent A (described below). The area corresponding to isoprimeverose on the paper was excised and eluted with water, and its sugar content was determined by the phenol/sulfuric acid method (Dubois et al., 1956). The xyloglucan content was estimated from the amount of isoprimeverose (Hayashi et al., 1981).

Sequential Enzyme Extraction

Auxin-treated segments were packed into plastic syringe barrels, and apoplast preparations were made as described above but with only a single repeat extraction. About 200 mL of the wall solution was obtained from 15,000 segments (fresh weight 600 g), and this was brought to 80% saturation with ammonium sulfate (wall solution). After the wall solution was removed, the segments were ground in a mortar in the presence of liquid nitrogen, and the resulting powder was homogenized in a blender with 20 mM sodium phosphate buffer, pH 6.2. The mixture was centrifuged at 12,000 rpm for 15 min, and the residue was again homogenized twice with the buffer and centrifuged. The combined supernatant was adjusted to 80% saturation with solid ammonium sulfate (buffer-soluble fraction). The segment residues were homogenized twice with 20 mM sodium phosphate buffer (pH 6.2) containing 1 M NaCl in a blender and the mixtures were centrifuged. This procedure was repeated once. The resulting supernatant was brought to 80% saturation with ammonium sulfate (buffer-insoluble fraction).

Purification of Xyloglucan-Degrading Enzyme Activity

All procedures were done at 4°C. The ammonium sulfate precipitate from both the wall solution and the buffer-insoluble fraction was dissolved in 20 mM sodium phosphate buffer (pH 6.2) and dialyzed against the same buffer. Crude enzyme preparations in 150 mL of buffer were applied to an SP-Toyopearl column (2.6 × 16 cm) that had been equilibrated with 400 mL of 20 mM sodium phosphate buffer (pH 6.2). Each column was washed with 400 mL of the same buffer and eluted with a linear gradient of 0 to 0.5 M NaCl in the buffer (total 400 mL). Fractions containing xyloglucanase activity (between 0.1 and 0.2 M NaCl) were combined, dialyzed against the buffer containing 0.2 M NaCl, and applied to a Con A-Sepharose 6B column (2 × 3 cm) that had been equilibrated with 20 mM sodium phosphate buffer (pH 6.2) containing 0.2 M NaCl. The column was successively eluted with 60 mL of the same buffer and 60 mL of the buffer containing 0.5 M methyl-α-mannoside.

The fractions eluted with 0.5 M methyl-α-mannoside, which contained xyloglucanase activity, were combined, dialyzed against 20 mM potassium acetate buffer (pH 5.0), and applied to a Mono-S column (0.5 × 5 cm) equilibrated with 10 mL of 20 mM potassium acetate buffer (pH 5.0) containing 0.15 M NaCl. The column was eluted with 5 mL of the same buffer, followed by a linear gradient of 0.15 to 0.65 M NaCl in the buffer (total 20 mL). The fractions eluted with 0.15 M NaCl, which contained xyloglucanase activity, were combined, dialyzed against the buffer, and applied to a Superdex 200 column (1.6 × 60 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.2) containing 0.5 M NaCl. The column was eluted with the same buffer. The fractions containing xyloglucanase activity (fraction nos. 41–50) were combined, dialyzed against 20 mM potassium acetate buffer (pH 5.0), and applied to another Mono-S column (0.5 × 5 cm) equilibrated with 20 mM potassium acetate buffer (pH 5.0). The enzyme activity was eluted with a linear gradient of 0.1 to 0.25 M NaCl in the buffer. The enzyme in fractions containing activity (eluting between 0.16 and 0.17 M NaCl) migrated as a single band on SDS-PAGE.

Assay for Xyloglucan Endo-1,4-β-Glucanase

The reaction mixture contained an enzyme preparation, 400 μg of pea xyloglucan, and 10 mM sodium phosphate

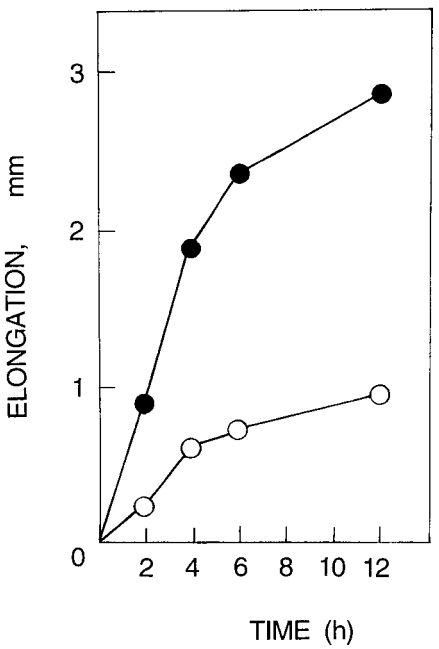

Figure 1. Time course of elongation of pea-stem segments treated with IBA. Third-internode segments (1 cm long) were soaked in ice-cold water for 30 min and incubated at 25°C in the dark with or without 20 μM IBA, as described in "Materials and Methods." The lengths of segments were measured using a binocular microscope. ●, 20 μM IBA; ○, control.

buffer (pH 6.2) in a total volume of 200 μL. The mixture was incubated at 35°C for 2 h. After incubation, 170 μL of the mixture was assessed for reducing power, and 20 μL was assessed for xyloglucan content. Reducing power was measured by the neocuproine method (Dygert et al., 1965), and xyloglucan content was determined by decolorization in the amount of the xyloglucan-iodine complex by use of the iodine-sodium sulfate method (Kooiman, 1960). One unit of enzyme activity is defined as the amount of enzyme that causes 1 nmol of reducing power as Glc in 10 mM sodium phosphate buffer (pH 6.2) in 1 min at 35°C.

Assay for Xyloglucan Endotransglycosylase

Xyloglucan endotransglycosylase activity was determined according to the method described by Fry et al. (1992). The standard reaction mixture contained 200 μM XXXGol (4.63 kBq), the enzyme preparation (usually about 30 μg of protein), 40 μg of pea xyloglucan, and 20 mM Mes/NaOH (pH 6.0), in a total volume of 16 μL. After the sample was incubated for 1 h at 25°C, the reaction was stopped by the addition of 100 μL of 20% (w/v) formic acid. This solution was dried on Whatman 3MM filter paper (5 × 5 cm), which was then washed in running tap water for more than 1 h to remove free [^{3}H]XXXGol. The paper was again dried and its radioactivity determined by scintillation counting.

Assay for Cellulose Endo-1,4-β-Glucanase

Endo-1,4-β-glucanase activity was assayed viscometrically at 35°C, with 50 μL of enzyme preparation plus 0.9 mL of 10 mM sodium phosphate buffer containing 0.3% (w/v) carboxymethylcellulose in semimicroviscometers. One unit of activity is defined as the amount of enzyme required to cause 0.1% loss in viscosity in 2 h under such conditions (Nakamura and Hayashi, 1993).

Chromatofocusing

An enzyme preparation that was partially purified by a gel-filtration step (Superdex 200) was applied to a column (0.5 × 20 cm) of Mono-P equilibrated with 48 mL of 25 mM Tris-acetic acid buffer (pH 9.0). The column was eluted with 60 mL of polybuffer 96-acetic acid buffer (pH 6.0), in which the pH gradient was 9.0 to 6.0. Aliquots were analyzed for pH or for the activity of xyloglucan endo-1,4-β-glucanase.

Characterization of Xyloglucan Degradation

One milligram of pea xyloglucan was incubated with the enzyme in 10 mM sodium phosphate buffer (pH 6.2) in a total volume of 500 μL with a few drops of toluene at 35°C for 12 or 24 h. After the reaction 15 μL of 0.5 N NaOH and 37 MBq sodium borotritide (in 50 μL of 0.1 N NaOH) were added to the mixture and the reaction mixture was further incubated for 12 h to tritiate reducing ends. The mixture was then neutralized with 2 N acetic acid, concentrated to dryness, and successively dried three times from methanol. It was then dissolved in 400 μL of 0.1 N NaOH, subjected to gel filtration on a column (0.6 × 110 cm) of Sepharose CL-6B, and eluted with 0.1 N NaOH. Aliquots were assayed for radioactivity in Aquasol (NEN) or for the amount of polysaccharide by the phenol/sulfuric acid method (Dubois et al., 1956).

General Methods

Paper chromatography was performed with 1-propanol: ethyl acetate:water, 3:2:1, v/v (solvent A). Sugars were visualized using the silver nitrate reagent (Robyt and French, 1963). Carbohydrate was determined by the phenol/sulfuric acid method (Dubois et al., 1956). Xyloglucan oligosaccharides were determined by the system for anion-exchange chromatography (CarboPak PA-1) with pulsed amperometric detection as previously described (Ohsumi and Hayashi, 1994). Protein was estimated using the Coomassie Plus protein assay reagent (Pierce). SDS-PAGE was carried out essentially according to the method of Laemmli

Table I. *Effect of IBA on the amount of xyloglucan liberated into the wall solution of pea stems*

Time	Xyloglucan	
	+IBA	−IBA
h	*μg/100 sections*	
0	8	8
2	87	17
4	65	21
6	57	19
12	20	11

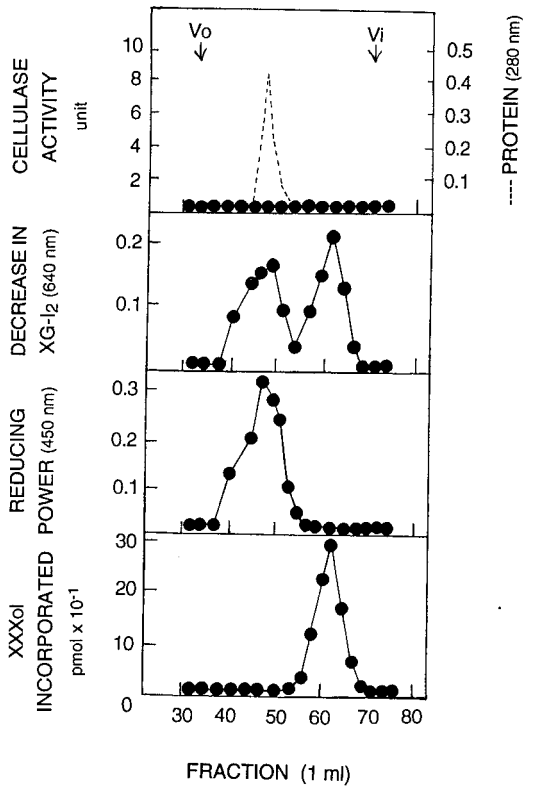

Figure 2. Gel-filtration pattern (Superdex 200) of partially purified xyloglucan endo-1,4-β-glucanase preparation. Aliquots were assayed for the activities of cellulose endo-1,4-β-glucanase (cellulase), xyloglucan endo-1,4-β-glucanase (decrease in xyloglucan-iodine complex and reducing power), and xyloglucan endotransglycosylase (XXXGol incorporated). Vo, Blue dextran; Vi, Glc.

(1970). Proteins were visualized on gels by the silver-stain method.

RESULTS AND DISCUSSION

Amounts of Xyloglucan Liberated into Wall Solution in Auxin-Treated Pea Stems during Elongation

The segments of the third internode of pea stems incubated in 20 μM IBA elongated in response to IBA treatment, and the amount of xyloglucan in the apoplastic solution increased. The rate of elongation of the segments was highest from 0 to 4 h of incubation and declined thereafter (Fig. 1). The amount of xyloglucan in the apoplast solution was highest in segments that were rapidly elongating (2 or 4 h) and increased with IBA treatment (Table I). These findings suggest that elongation of the segments is associated with solubilization of xyloglucan, which is in full agreement with earlier observations (Labavitch and Ray, 1974a, 1974b) that auxin promotes both the elongation of pea stem and the turnover of xyloglucan in the primary wall.

Purification of Xyloglucan-Degrading Enzyme Activity

Pea stem segments were treated with 20 μM IBA for 2 to 4 h to give maximal rates of segment elongation and amounts of solubilized xyloglucan (Fig. 1; Table I). The xyloglucan-degrading enzyme activity was determined from either the increase in reducing power or the extent of decolorization in the xyloglucan-iodine complex. Attempts to determine the activity in crude enzyme preparations (wall solution, buffer-soluble, and buffer-insoluble fractions) were unsuccessful. After gel filtration or ion-exchange chromatography of each fraction, the activity could be determined but its distribution in each fraction was not reproducible. When the recovery of the wall solution was low, the buffer-insoluble fraction had high activity. The activity was distributed in the wall solution and the buffer-insoluble fraction, and only low activity was detected in the buffer-soluble fraction in spite of the large amount of total protein. Therefore, both the wall solution and the buffer-insoluble fraction were combined and used for the purification of the enzyme.

Xyloglucan endo-1,4-β-glucanase was eluted from a column of Mono-S with a linear gradient of NaCl from 0.15 to 0.65 M in 20 mM potassium acetate buffer, pH 5.0. Two peaks of endo-1,4-β-glucanase activity were eluted with the buffer containing 0.15 and 0.25 M NaCl, the activity in each peak being 70 and 30% of the total activity, respectively. The major peak of activity, eluted by 0.15 M NaCl, was subjected to the next purification step.

A single peak of protein (70 kD) with xyloglucan endo-1,4-β-glucanase activity for increase in reducing power was obtained after gel filtration on Superdex 200 in 20 mM sodium phosphate buffer (pH 6.2) containing 0.5 M NaCl, whereas two peaks for decrease in xyloglucan-iodine complex were obtained (Fig. 2). One of them, with the lower molecular size of 1.5 kD, corresponded to the activity of xyloglucan endotransglycosylase. Therefore, xyloglucan endo-1,4-β-glucanase was separated from the transglycosylation activity for xyloglucan. Endo-1,4-β-glucanase activity against carboxymethylcellulose was not detected in any of the eluates. These findings indicate that the activity of xyloglucan endo-1,4-β-glucanase was isolated separately from the activities of xyloglucan endotransglycosylase and

Table II. *Purification of xyloglucan endo-1,4-β-glucanase*

Purification Step	Total Protein	Total Activity	Specific Activity	Purification	Recovery
	mg	*units*	*units mg⁻¹*	*fold*	*%*
SP-Toyopearl	77.9	268.0	3.4	1	100
Con A-Sepharose	12.0	61.3	5.1	1.5	23
Mono-S	0.93	15.0	16.1	4.7	5.6
Superdex	0.10	6.3	63.0	18.3	2.4
Mono-S	0.0034	4.4	1294	376	1.6

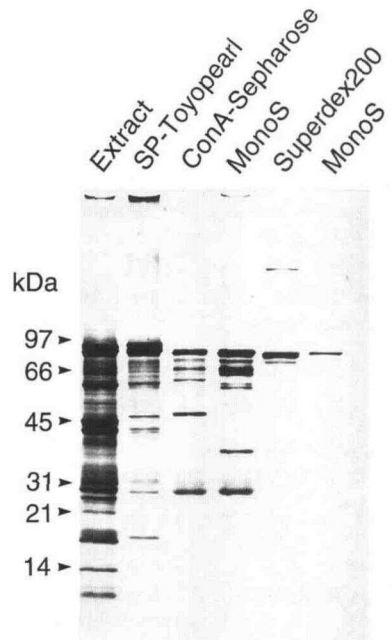

Figure 3. SDS-PAGE of xyloglucan endo-1,4-β-glucanase at various stages of purification. Proteins were visualized by silver-staining and the molecular masses (kD) are indicated on the left side of the photograph. Marker proteins are phosphorylase B (97 kD), BSA (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21 kD), and lysozyme (14 kD).

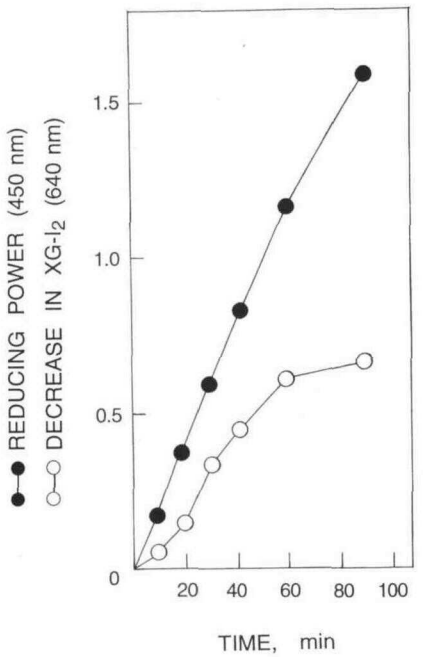

Figure 4. Time course for xyloglucan degradation by xyloglucan endo-1,4-β-glucanase. The reaction mixture contained an enzyme preparation, 400 μg of xyloglucan, and 10 mM sodium phosphate buffer (pH 6.2) in a total volume of 200 μL. The mixture was incubated at 35°C; part (170 μL) of the mixture was subjected to the determination of increased reducing power and part (20 μL) of the mixture was subjected to the determination of xyloglucan decline ($XG\text{-}I_2$).

cellulase. The specific activity of the purified enzyme was 1294 units/mg enzyme (Table II). The enzymatic activity was purified 376-fold relative to the activity in the crude preparation.

Properties of Xyloglucan-Degrading Enzyme

The purified enzyme gave a single protein band on SDS-PAGE. The molecular size was estimated to be 77 kD by SDS-PAGE (Fig. 3) and 70 kD by gel filtration on Superdex 200 (Fig. 2). The enzyme may be a single polypeptide chain. The lower molecular size obtained by gel filtration may be due to a compact tertiary structure of the nondenatured enzyme. The pI of the enzyme, as determined by chromatofocusing, was 8.1.

Hydrolysis of xyloglucan proceeded proportionally to the length of the reaction period at least up to 60 min. The enzyme caused an increase in the reducing power of the incubation mixture at a constant rate, whereas the decrease in amount of xyloglucan-iodine complex gave a sigmoidal curve (Fig. 4). This is due to effects of molecular size on color intensity of the xyloglucan-iodine complex. The colorization did not occur well when the fragments of xyloglucan generated were below 20 kD. The apparent K_m value for pea xyloglucan was 0.64 mg mL^{-1}. The purified enzyme was active between pH 4.5 and 8.0, with an optimum pH of 6.2. The temperature optimum for the enzyme activity was approximately 35°C.

The purified enzyme hydrolyzed pea and *T. indicus* xyloglucans but not carboxymethylcellulose or phospho-swollen cellulose (Table III). The gel-filtration pattern of xyloglucan after treatment with the purified enzyme indicated that the average molecular size had decreased from 50 to 20 kD, with an increase in oligosaccharides during the time course (Fig. 5). Since the accessible chain ends of the polysaccharide are reduced, subsequent treatment with sodium borotritide did not introduce any detectable new chain ends into the 0 time sample. However, after enzyme treatment, reducing chain ends in the xyloglucan were distributed throughout the molecular size profile, with more in the lower-molecular-size fractions, suggesting typical endohydrolysis.

Hydrolysis of pea xyloglucan with the enzyme yielded oligosaccharides identified as XLFG, XXFG, XLLG, XXLG, XXXG, and XXG in a molar ratio of 5.8:32.5:2.1:10.5:42.7:6.4, respectively, by the system for anion-exchange chromatography. Since the molar ratio of the oligosaccharide units was in accord with their relative amounts of pea xyloglu-

Table III. *Substrate specificity of xyloglucan endo-1,4-β-glucanase*

Substrate	Relative activity
	%
Pea xyloglucan	100
T. indicus xyloglucan	100
Carboxymethylcellulose	0
Phospho-swollen cellulose	0
Lichenan	0
Laminarin	0

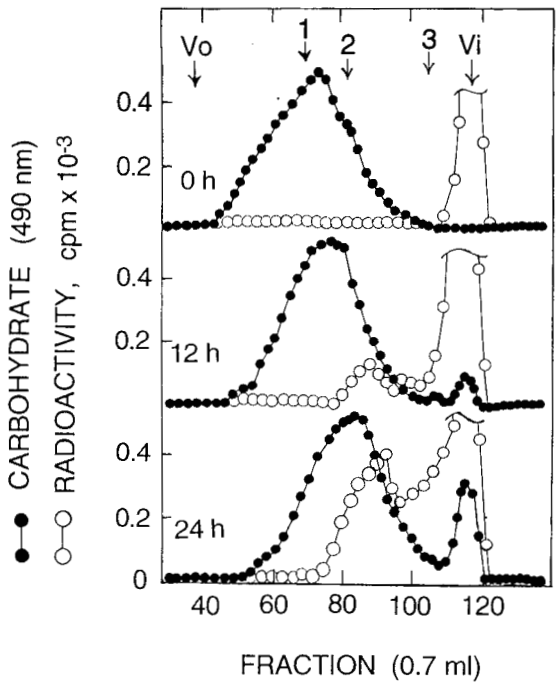

Figure 5. Gel-filtration pattern of the products treated with sodium borotritide after hydrolysis of pea xyloglucan with purified xyloglucan endo-1,4-β-glucanase. Molecular sizes of dextran markers: 1, 70 kD; 2, 39.1 kD; and 3, 8.8 kD. Vo, Blue dextran; Vi, Glc.

can, the enzyme probably cleaves internal linkages adjacent to unsubstituted Glc units at random.

The xyloglucan endo-1,4-β-glucanase hydrolyzes internal linkages adjacent to unsubstituted Glc units of both nonfucosylated and fucosylated xyloglucans, introducing free reducing end groups at these positions. It has neither hydrolase activity on carboxymethylcellulose nor transglycosylase reaction on xyloglucans. Therefore, the enzyme is different from either cellulases from auxin-treated pea stems (Hayashi et al., 1984) or the xyloglucan-specific endo-1,4-β-glucanase specific for nonfucosylated xyloglucan (Edwards et al., 1986). In fact, the enzyme has a different pI (8.1) from pea cellulases (pI 5.2 and 6.9 for buffer-soluble and -insoluble cellulases, respectively) and xyloglucan-specific endo-1,4-β-glucanase (pI 5.0) from germinated *Nasturtium* seeds, although chromatofocusing often underestimates the pI of proteins. Nevertheless, it has a molecular size resembling pea cellulase (70 kD for buffer-insoluble cellulase).

This enzyme is associated with stem elongation (and potentially wall-loosening) in pea, and its activity is higher following treatment with auxin. An enzyme preparation from the cell walls of soybean stems degrades xyloglucan by a two-step mechanism (Koyama et al., 1981), in which xyloglucan is hydrolyzed into oligosaccharides in the first step. In addition, the xyloglucan oligosaccharides formed could enhance the degradation and solubilization of xyloglucan with xyloglucan endotransglycosylase (Fry et al., 1992). The purified pea endo-1,4-β-glucanase should now allow N-terminal microsequence determination as a first step toward the molecular cloning of this enzyme gene.

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