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Modifications of cell wall polysaccharides during auxin-induced growth in azuki bean epicotyl segments

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Changes in sugar compositions and the distribution pattern of the molecular weight of cell wall polysaccharides during indole-3-acetic acid (IAA)-induced cell elongation were investigated. Differential extraction of the cell wall and gel permeation chromatography of wall polysaccharides indicated that galactans, polyuronides, xylans, glucans and cellulose were present in the azuki bean epicotyl cell wall.

When segments were incubated in the absence of sucrose, IAA enhanced the degradation of galactans in both the pectin and hemicellulose fractions, whereas to some extent it enhanced the polymerization of xylans and glucans in the hemicellulose fraction and an increase in the amounts of polyuronides in the pectin fraction and of α-cellulose. In the presence of 50 mM sucrose, IAA caused large increases in the amounts of all the wall polysaccharides, and enhanced the polymerization of galactans, xylans and glucans in the hemicellulose fraction.

These results and an important role of galactan turnover in cell wall extension are discussed.

Key words: Azuki bean epicotyl — Cell extension — Cell wall polysaccharides — Galactans — Indole-3-acetic acid.

Many studies have demonstrated that an extensive turnover of cell wall polysaccharides occurs during cell growth in higher plants (*1*, *11*, *16*, *18*, *19*, *26*, *28*). Auxin is considered to alter polysaccharide metabolism and, thereby, to modify the cell wall structure, resulting in cell wall extension. It induces the synthesis of cell wall polysaccharides, both cellulosic and noncellulosic ones in oat coleoptiles (*2*, *3*, *13*, *21*), pea stems (*1*) and azuki bean epicotyls (*18*, *19*), that had been supplied with an adequate amount of glucose or sucrose as substrate. Under starved conditions, auxin-induced degradation of glucans in oat (*13*, *15*, *24*) and barley (*22*) coleoptiles and of galactans in azuki bean epicotyls (*19*) has been observed.

These investigations, however, have presented little information on the changes in the net amounts of or in molecular weights of individual wall polysaccharides that had undergone modifications. Our present study was undertaken to obtain information on auxin-induced changes in the net amount and in the molecular weights of individual wall polysaccharides of azuki bean epicotyl segments by means of differential extraction and gel permeation chromatography of the polysaccharides.

Abbreviations: IAA, indole-3-acetic acid; Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; UA, uronic acid; Xyl, xylose; $\bar{M}w$, average molecular weight.

Materials and methods

Growth experiments

Azuki bean (*Vigna angularis*) seedlings were grown for 6 days in light at 25°C as reported previously (*18*). From the epicotyls (8–10 cm long), 10-mm segments were excised 5 to 15 mm below the first leaves. These were kept in water at 25°C for 1–2 hr before incubation. Unless otherwise specified, lots of twenty segments were incubated for 20 hr at 25°C in light in a petri dish containing 4 ml of test solution. The test solution consisted of 10 mM K-phosphate buffer solution (pH 6.0) with or without 0.1 mM IAA and/or 50 mM sucrose. After incubation, we measured segment length microscopically, then killed the segments immediately in boiling methanol after which they were stored in methanol until use.

Cell wall fractionation

Methanol-killed segments were rehydrated and treated with 200 ppm Pronase-P (Kaken Kagaku Co.) for 18 hr to remove proteins, after which they were homogenized with a mortar and pestle, then washed with water, acetone and a methanol-chloroform mixture (1:1, v/v) and air-dried (*18*). The dried material was treated with 10 units of pancreatic α-amylase (Sigma Chemical Co.) to remove starch (*18*).

The holocellulose thus obtained was extracted 4 times for 1 hr each with 0.5% ammonium oxalate solution at 90°C. The extracts were combined and dialyzed against pure water to obtain the pectin fraction. The residue then was treated three times with 24% KOH solution for 24 hr at 25°C under a nitrogen atmosphere. The alkali extracts were combined and neutralized to pH 6.0 with acetic acid after which they were dialyzed overnight against pure water to obtain the hemicellulose fraction. After the alkali extraction, the residue was neutralized with acetic acid and washed successively with water and ethanol, then it was air-dried to obtain α-cellulose fraction.

Analysis of sugar compositions

The pectin and hemicellulose fractions were evaporated to dryness in a test tube at 50°C under a stream of filtered air after which they were hydrolyzed with 2 N trifluoroacetic acid at 121°C for 1.0 and 1.5 hr, respectively. The acid was removed by evaporation under a stream of filtered air at 50°C.

α-Cellulose was dissolved in 72% sulfuric acid (v/v) at room temperature for 1 hr then diluted with 29 volumes of water, after which it was heated at 121°C for 1 hr. After the hydrolysis, the sulfuric acid solution was neutralized with barium carbonate. The barium sulfate formed was removed by centrifugation; the supernatant was evaporated under a stream of filtered air.

Neutral sugars in these hydrolyzates were determined by gas-liquid chromatography as reported previously (*17*, *18*). The total sugar and uronic acid contents were determined by the phenol sulfuric acid method (*6*) and the carbozole sulfuric acid method (*8*), respectively.

Gel permeation chromatography

The dialyzed hemicellulose solution was lyophilyzed. The hemicellulose powder (5 mg) was dissolved in 1.0 ml of 50 mM Na-acetate buffer solution (pH 5.0)

at 90°C. After the trace amount of insoluble impurity had been removed by centrifugation at 3000 rpm for 30 min, the supernatant was applied to a Sepharose 4 B column (1.7×80 cm) equilibrated with 50 mM Na-acetate buffer solution (pH 5.0) and eluted with the same buffer solution at room temperature at a flow rate of 0.1 ml/min. Fractions of 2.2 ml were collected, and a 0.4 ml portion of each fraction was assayed for the total sugar content. The other portion (1.8 ml) was used in the determination of neutral sugar compositions by gas-liquid chromatography.

Gel permeation chromatography of the pectin (5 mg) was performed under the same conditions as for the hemicellulose. Fractions of 4.4 ml were collected and 0.4 and 0.6 ml portions of each fraction were assayed for their total sugar and uronic acid contents. The other portion (3.4 ml) was used in the determination of neutral sugar compositions by gas-liquid chromatography.

For gel permeation chromatography of the wall polysaccharides, only one elution system (50 mM Na-acetate buffer solution, pH 5.0) was used. Possibly, the use of different systems would produce different chromatograms, since the apparent molecular weights of polysaccharides in solution usually change depending upon such properties of the solvent as pH, ionic strength, etc. Thus, we need to investigate the polysaccharides under various conditions for chromatography.

The calibration curve used to estimate the molecular weight of the cell wall polysaccharides was derived from authentic dextrans ($\bar{M}w=1\times10^4$, 4×10^4, 7×10^4, 2×10^5, 5×10^5, 2×10^7) purchased from Pharmacia Fine Chemicals and Sigma Chemical Co. The $\bar{M}w$ of cell wall polysaccharides was estimated with the following formula:

$$\bar{M}w=\sum w_i\cdot M_i / \sum w_i \qquad \begin{array}{ll} 9\leq i\leq 32 & \text{for pectic polymers} \\ 18\leq i\leq 64 & \text{for hemicellulosic polymers} \end{array}$$

where w_i is the sugar content of the ith fraction and M_i is the molecular weight of the ith fraction estimated from the calibration curve.

Results

The azuki bean epicotyl cell wall was fractionated into three fractions, i.e. pectin, hemicellulose and α-cellulose. The sugar compositions of these three fractions are shown in Fig. 1. The pectin fraction was 35.1% of the total cell wall polysaccharides and was chiefly composed of UA, Gal, Ara and Rha. The hemicellulose fraction (22.1% of the total polysaccharides) contained Glc, Xyl and Gal as its major components and Ara, UA, Fuc and Rha as its minor components. The α-cellulose fraction was composed mainly of Glc with a small amount of Man.

Table 1 shows IAA-induced elongation of the epicotyl segments and changes in the sugar compositions in the three cell wall fractions during IAA-induced elongation in the presence or absence of 50 mM sucrose, which enhanced this cell elongation. In the absence of sucrose, the Gal content in both pectin and hemicellulose decreased during the incubation period. The decrease in the Gal content in the pectin was significantly enahnced by IAA treatment. Under the conditions used in this study, IAA caused a significant increase in the amounts of Glc in α-cellulose and of UA in pectin. Little or no change in the amounts of the other sugars was observed in IAA-

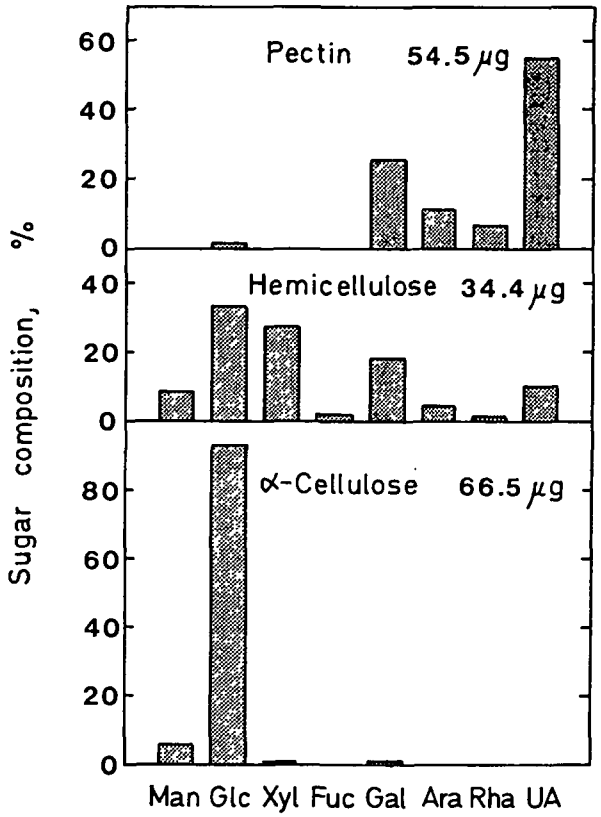

Fig. 1. *Sugar compositions of azuki bean cell wall fractions.* Epicotyl segments were killed in boiling methanol then fractionated into pectin, hemicellulose and α-cellulose. After these fractions had been hydrolyzed by acid, the sugar compositions each were determined by the carbazole-H_2SO_4 method and gas-liquid chromatography (See **Materials and methods**). Figures given with each graphs indicate the total amount of polysaccharides in the respective fraction.

treated or buffer-treated segments. In contrast, when segments were incubated in the presence of 50 mM sucrose, IAA greatly increased the amounts of Glc in α-cellulose, Gal and UA in pectin, and Glc, Xyl and Gal in hemicellulose.

IAA-induced changes in the amounts of the sugar components in pectin and hemicellulose, i.e. differences in the sugar compositions between ± IAA-treated segments in the presence or absence of 50 mM sucrose, were calculated from the data in Table 1 and are illustrated in Fig. 2.

To examine changes in the molecular weight of component polysaccharides in pectin and hemicellulose, we fractionated these fractions further by gel permeation chromatography on a Sepharose 4 B column. Fig. 3 shows elution patterns of the total carbohydrate and the major component sugars in the pectin fraction. Gal was eluted in the first peak at a high $\bar{M}w$ (ca. 10^6) and was accompanied by Ara (Fig. 3A). Acidic polymers (UA) appeared in two peaks: one of the peaks at the higher $\bar{M}w$ overlapping with the peaks of neutral sugars. These data indicate that

Table 1 *Effects of IAA and sucrose on elongation and on the sugar compositions of the cell walls of azuki bean epicotyl segments*

		Treatments[a]				
		Initial	Buffer	IAA	Sucrose	IAA+sucrose
Segment length (mm)[b]		10.32±0.04	10.64±0.04	14.82±0.09	10.77±0.04	16.65±0.16
Fractions	Sugars	Sugar content (μg/segment)[c]				
Pectin	Total	53.6	46.0	49.7	68.4	80.6
	UA	30.0 ±2.6	33.5 ±2.1	39.0 ±1.1	45.3 ±1.9	53.1 ±1.3
	Gal	14.0 ±0.7	4.8 ±0.2	2.9 ±0.5	12.7 ±0.6	16.3 ±0.9
	Ara	6.0 ±0.4	3.5 ±0.3	3.6 ±0.2	6.0 ±0.1	5.9 ±0.3
	Rha	3.6 ±0.2	4.2 ±0.2	4.2 ±0.2	4.4 ±0.3	5.3 ±0.3
Hemicellulose	Total	32.1	33.0	33.5	41.8	64.9
	Glc	10.2 ±2.0	11.2 ±0.7	11.8 ±0.8	13.2 ±1.1	21.9 ±1.5
	Xyl	8.5 ±0.8	9.7 ±0.6	10.1 ±0.7	11.6 ±0.8	18.2 ±1.1
	Gal	5.6 ±0.8	4.0 ±0.6	3.8 ±0.4	6.7 ±1.0	12.1 ±1.4
	Ara	1.4 ±0.2	1.5 ±0.2	1.5 ±0.2	2.4 ±0.4	3.1 ±0.6
	Man	2.6 ±0.6	2.4 ±0.4	2.3 ±0.4	2.7 ±0.5	3.1 ±0.6
	UA	3.1 ±0.5	3.5 ±0.3	3.3 ±0.3	4.4 ±0.4	5.2 ±0.5
	Fuc	0.7 ±0.1	0.7 ±0.1	0.7 ±0.1	0.8 ±0.1	1.3 ±0.2
α-Cellulose	Glc	61.9 ±0.4	69.3 ±2.3	79.3 ±2.0	81.3 ±1.5	131.1 ±0.5

[a] Segments (initial segments) were excised from 6-day-old azuki bean epicotyls then incubated for 20 hr in 10 mM K-phosphate buffer solution (pH 6.0) in the presence or absence of 10^{-4} M IAA and/or 50 mM sucrose.

[b] After incubation, segment length was measured. Mean values with standard errors are given (n=20).

[c] Twenty segments used for each treatment were killed with boiling methanol and subjected to cell wall fractionation. The sugar compositions of each fraction were determined (See **Materials and methods**). Mean values of the four different experiments are given with standard errors (n=4).

the pectin fraction contained at least three types of polysaccharides, presumably galactans (or arabinogalactans) and two types of polyuronides.

When segments were incubated in the absence of sucrose, the amount of galactan decreased. The decrease was enhanced by treatment with IAA. By contrast, in the presence of sucrose, a slight decrease in the amount of galactans was observed, and IAA to some extent increased the amount. During incubation, the UA peak at the lower $\bar{M}w$ increased in comparison with the UA peak at the higher $\bar{M}w$. IAA caused an increase in the amount of the polyuronides in the absence as well as in the presence of sucrose.

For ready comparison of the distribution pattern of the molecular weights of pectic polysaccharides between ±IAA-treated segments, the % of Gal or the UA content in the eluate was plotted against the fraction number, i.e. the areas under the elution curves were standardized as shown in Fig. 4. Clearly IAA caused a decrease in the molecular weight of the galactans in the absence of sucrose, but it had little effect on their molecular weights in the presence of sucrose. IAA had

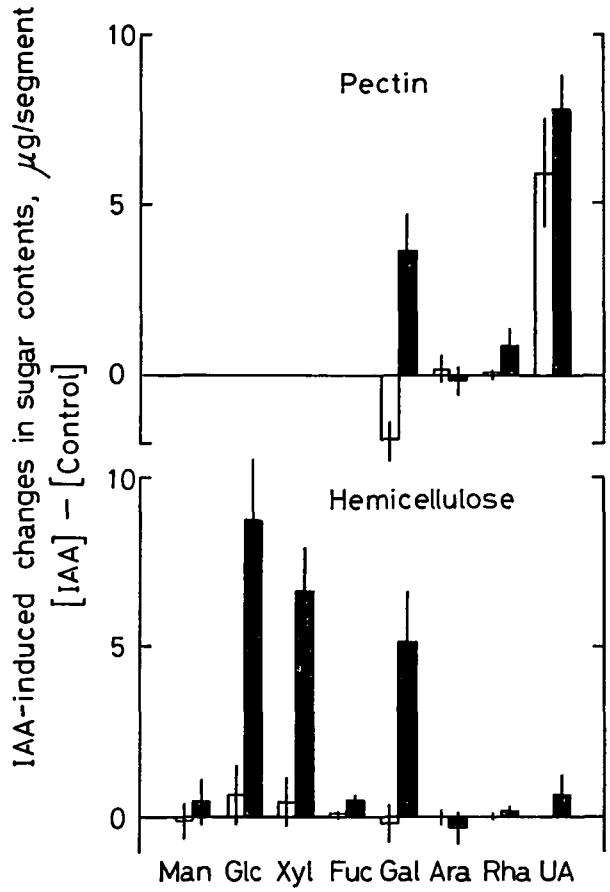

Fig. 2. *Effect of IAA on changes in the sugar compositions of pectic and hemicellulosic fractions in azuki bean epicotyl segments.* Differences in individual sugar contents in pectin and hemicellulose between IAA-treated and buffer-treated segments (light bars) and between IAA+sucrose-treated and sucrose-treated (dark bars) were calculated from the data shown in Table 1. Vertical lines indicate standard errors (n=4).

little effect on the molecular weights of the polyruonides, though their amounts increased during IAA-induced elongation (cf. Fig. 3 and 4). Provided that Gal was the main component of galactans, the $\bar{M}w$ of the respective polymers could be estimated tentatively from the elution pattern of Gal. Thus, the $\bar{M}w$ of the galactans in the intial, buffer-treated and IAA-treated segments in the absence of sucrose were calculated to be 1.6×10^6, 1.1×10^6 and 6.9×10^5.

Fig. 5 shows gel permeation chromatograms of the hemicellulose. Gal, Glc and Xyl were eluted in three distinct peaks: presumably, galactans ($\bar{M}w$=ca. 1.1×10^6), glucans ($\bar{M}w$=ca. 2.9×10^5) and xylans ($\bar{M}w$=ca. 1.5×10^5). The areas under the elution curves of these three neutral sugars were standardized as were the pectic Gal and UA and are shown in Fig. 6. The galactan peak decreased, as in the pectin fraction, during incubation in the absence of sucrose (Fig. 5). IAA promoted not

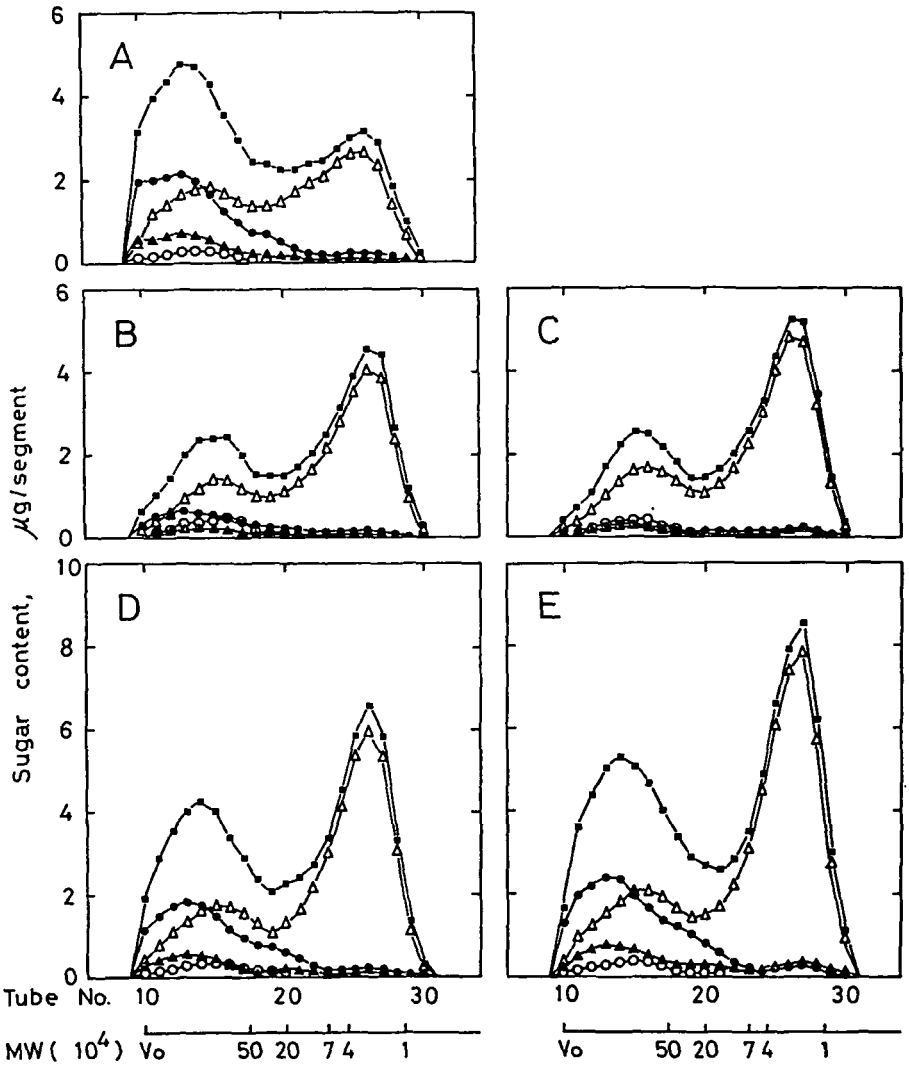

Fig. 3. *Gel permeation chromatograms of pectin fractions.* Epicotyl segments (initial segments) were incubated for 20 hr in K-phosphate buffer solution with or without 10^{-4} M IAA and/or 50 mM sucrose, then their pectin fractions were extracted. Five milligrams of pectin dissolved in 50 mM Na-acetate buffer solution (pH 5.0) was applied to a Sepharose 4 B column which then was eluted with the same buffer solution. Individual sugar contents in the eluate was determined by the carbazole-H_2SO_4 method and gas-liquid chromatography. (See **Materials and methods**). Symbols: A, initial; B, buffer; C, IAA; D, sucrose; E, sucrose+IAA; ●, Gal; ▲, Ara; ○, Rha; △, UA; ■, total sugar.

only the decrease in galactans but the reduction of the $\bar{M}w$ of polymer (Fig. 6). By contrast, in the presence of sucrose, IAA caused an increase in the $\bar{M}w$ of the galactans as well as an increase in their amounts. The $\bar{M}w$ of the galactans, estimated from the elution pattern of Gal, changed as follows: initial, 1.1×10^6; buffer-treated 8.4×10^5; IAA-treated, 5.8×10^5; sucrose-treated, 1.1×10^6; sucrose+IAA-treated, 1.4×10^6.

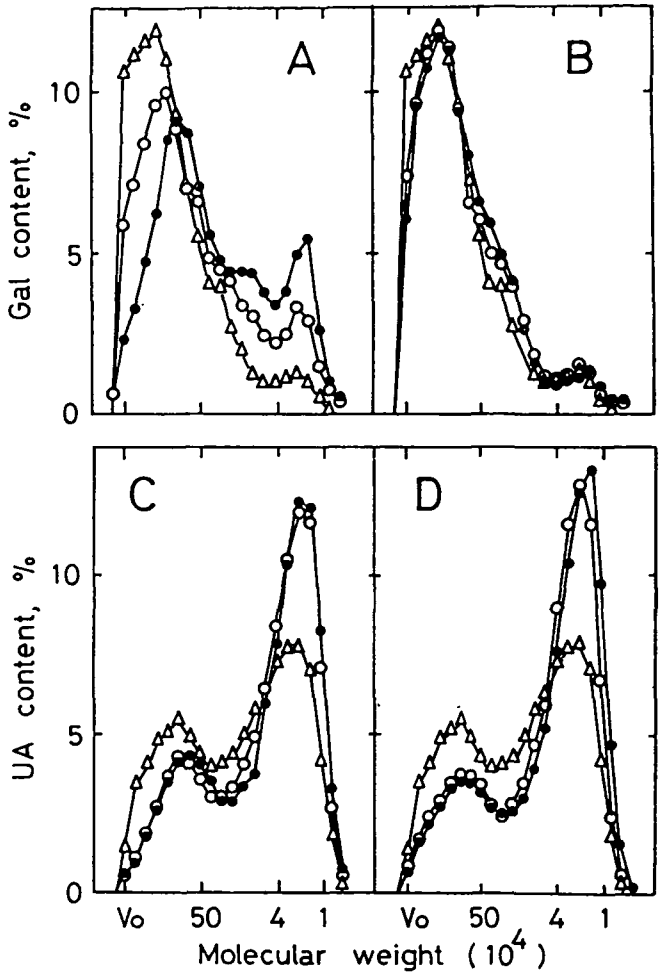

Fig. 4. *Effect of IAA on the elution patterns of Gal and UA in pectin.* Peak areas for Gal and UA in the chromatograms shown in Fig. 3 were standardized. A, C: △, initial; ○, buffer; ●, IAA; B, D: △, initial; ○, sucrose; ●, IAA+sucrose.

With respect to glucans and xylans, IAA caused a substantial increase in the $\bar{M}w$ as well as the amounts of these polymers in the presence of sucrose. Note the high peak for Glc and Xyl at the void volume in IAA+sucrose-treated segments (Fig. 5 and 6). The $\bar{M}w$ of the glucans and xylans were estimated to be 4.1×10^5, 3.0×10^5 in the sucrose-treated segments and 7.0×10^5, 5.1×10^5 in the sucrose+IAA-treated segments. In the absence of sucrose, unlike galactans, no decrease in the $\bar{M}w$ or the amounts of glucans or xylans was observed. IAA tended to cause an increase in their $\bar{M}ws$ under these conditions.

Discussion

The results presented here indicate a specific turnover of cell wall poly-

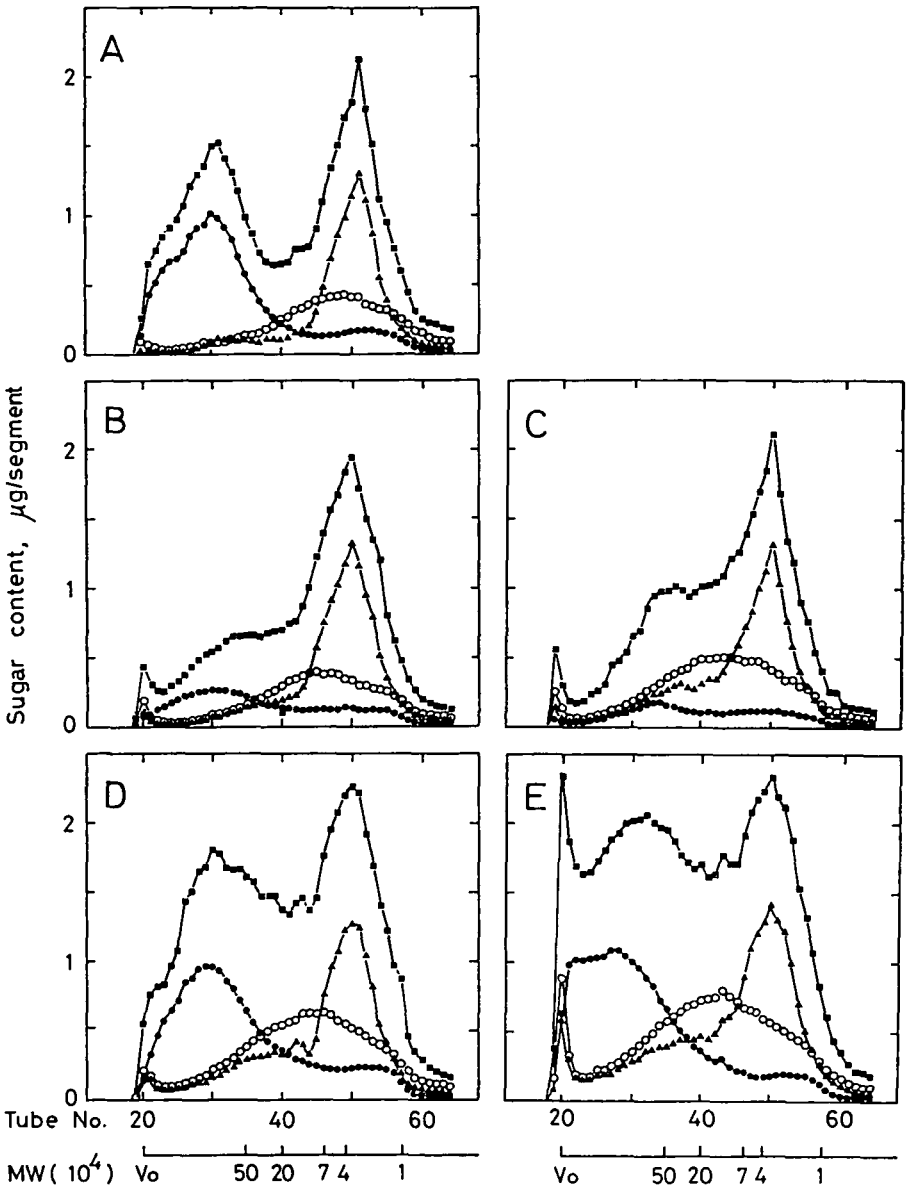

Fig. 5. *Gel permeation chromatograms of the hemicellulose fractions.* Epicotyl segments (initial segments) were incubated for 20 hr in K-phosphate buffer solution with or without 10^{-4} M IAA and/or 50 mM sucrose, then their hemicellulose fractions were extracted. Five milligrams of hemicellulose dissolved in 50 mM Na-acetate buffer solution (pH 5.0) was applied to a Sepharose 4 B column then eluted with the same buffer solution. Sugar contents in the eluate were determined by gas-liquid chromatography (See **Materials and methods**). Symbols: A, initial; B, buffer; C, IAA; D, sucrose; E, IAA+ sucrose; ●, Gal; ○, Glc; ▲, Xyl; ■, total sugar.

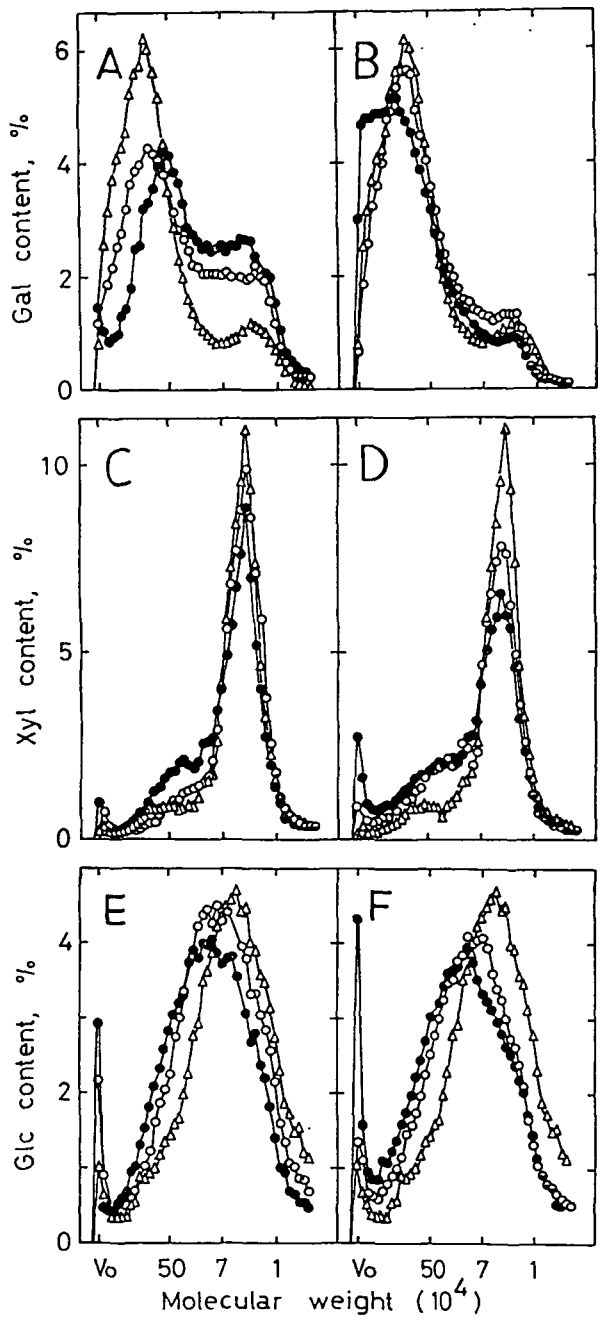

Fig. 6. *Effect of IAA on the elution pattern of Glc, Xyl and Gal in hemicellulose.* Peak areas of Glc, Xyl and Gal on the chromatograms shown in Fig. 5 were standardized. A, C, E: △, initial; ○, buffer; ●, IAA; B, D, F: △, initial; ○, sucrose; ●, sucrose+IAA.

saccharides during cell elongation, i.e. degradation, polymerization and synthesis of individual wall polysaccharides depending upon the application of IAA as well as the supply of sucrose as substrate. Extensive turnover of cell wall galactans has been indicated in pea epicotyls (*7*, *11*, *16*), azuki bean epicotyls (*18*, *19*) and sycamore cells (*26*). We previously have reported IAA-induced changes in the Gal content of the cell wall in azuki bean epicotyl segments (*19*). Our present study has shown that changes in the Gal level in the cell wall are ascribable to changes in the high $\bar{M}w$ galactans in both pectin and hemicellulose.

In suspension-cultured sycamore cell walls, galactans are thought to be located between rhamnogalacturonans (pectic polymers) (*27*) and xyloglucans (hemicellulosic polymers) (*4*) which, in turn, are attached to cellulose microfibrils (*11*). Thus, the extensive turnover of the azuki bean galactans (Table 1 and Fig. 2, 3 and 5) suggests the degradation and reconstruction of galactans located between cellulose microfibrils in the cell wall. When segments were incubated without sucrose, degradation of the galactan became apparent, and was enhanced by IAA, whereas in the presence of sucrose this degradation was overcome by the synthesis of the polymer, which also was enhanced by IAA. However the fact that substantial degradation of the galactans in the wall did occur in buffer-treated segments, which elongated only a little, negates the possibility that galactan degradation itself is the primary reaction responsible for wall extension. Probably, the processes critical for wall extension take place during the turnover of the galactans.

The metabolic turnover of polyuronides in the pectin fraction of the azuki bean cell wall differed from that of the galactans: IAA stimulated an increase in the amount of polyuronides in both the presence and absence of sucrose. The difference may be the result of different precursor pools (*26*). The pectin fraction contained two distinct species of polyuronides, as indicated in Fig. 3, presumably complex rhamnogalacturonans and galacturonans. These results are consistent with the report by Stoddart et al. (*25*) of the presence of two types of acidic components, i.e. complex polygalacturonans carrying neutral sugar blocks and polygalacturonans with only traces of neutral sugars, in the pectin preparation from sycamore tissues. During incubation of azuki bean segments, the ratio of rhamnogalacturonans to galacturonans decreased. This change in the ratio of the amounts of the two acidic components may be attributable partly to their interconversion; interconversion between acidic components has been reported in the scyamore pectic fraction (*26*).

When the azuki bean epicotyl segments were supplied with sucrose, IAA promoted substantial increases in the amounts and the $\bar{M}w$ of the hemicellulosic polysaccharides; galactans, xylans and glucans. In the absence of sucrose, however, IAA did not increase the amount of these polysaccharides. Thus, sucrose as a substrate plays a crucial role in the synthesis of hemicellulose during IAA-induced elongation. A similar effect of sucrose was found in the IAA-inudced synthesis of cellulose. The promotive effect of sucrose on IAA-induced elongation may be attributed, at least partly, to the role of sucrose as the substrate for cell wall synthesis.

Labavitch and Ray (*11*, *12*), using pulse-chase experiments, have reported that IAA causes liberation of xyloglucans into the cold water fraction from pea stem segments. The xyloglucans liberated might become the precursors for cell wall xyloglucan synthesis and might be incorporated into the cell wall xyloglucan chains

when the cell is to elongate. Indeed promotion of β-glucan synthetase activity by IAA has been reported in pea stem tissues (*20*).

For *Avena* coleoptile segments, a monocotyledonous plant part, Loescher and Nevins (*13*) and Sakurai and Masuda (*22*) have reported an IAA-induced decrease in the Glc content in the cell wall. Recently, Sakurai et al. (*24*) showed that the decrease in the Glc level resulted from the IAA-induced degradation of β-glucans ($\bar{M}w$=ca. 2×10^6) within the wall. In azuki bean epicotyl segments, however, no degradation of β-glucans was observed; instead, IAA enhanced galactan degradation. Generally, the cell wall compositions of monocots differ greatly from those of dicots (*13*); many dicot cell walls do not contain the β-1,3 β-1,4 mixed glucans found in monocot walls (*29*), but are abundant in galactans and xyloglucans in comparison with monocot walls (*9*, *27*). Considering these differences in the cell wall composition of monocots and dicots, we may place glucan degradation in the monocots as comparable to galactan degradation in the dicots when IAA causes cell wall extension. The amount of pectic substances in the primary wall of monocots has been reported to be small in comparison with that found in dicots (*5*). In addition, the role of pectic substances in auxin-induced cell extension is controversial, partly because there are little biochemical data on the changes in pectic substances during auxin-induced cell extension. As reported here, polyuronides and galactans in the pectin fraction in the azuki bean cell wall undergo great changes during auxin-induced cell wall extension, which suggests that they play important roles in cell wall extension.

It is not clear, however, whether the IAA-induced turnover of cell wall polysaccharides described here is the primary reaction of IAA-induced cell wall loosening. The biochemical nature of cell walll loosening is under investigation.

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