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Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells

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ABSTRACT

The extraction of xyloglucan from *Rosa* suspension-culture cell walls (not depectinated in hot aqueous solutions) with alkali was dependent on the temperature, with 2–3 times more being extracted at 37° than at 25°. At 25°, 6M NaOH was the optimal concentration, but it extracted only 25–30% of the xyloglucan within 24 h; efficient extraction took 2–3 weeks. After pre-treatment of cell walls with 6M NaOH at 25°, water extracted more xyloglucan in 24 h than did additional 6M NaOH.

INTRODUCTION

Xyloglucan, a hemicellulose which typically accounts for 20–25% of the dry mass of the growing cell wall in dicotyledons[1–3], is tightly hydrogen-bonded to the cellulosic microfibrils[4–8] and, by bridging adjacent microfibrils, it may contribute to inextensibility of the cell wall[2,3,9,10]. Cell wall-bound xyloglucan is thought to be subject *in vivo* to enzymic hydrolysis[11] and endo-transglycosylation[12,13]. The ability of auxin and H^+ to promote the cleavage of xyloglucan may be related to their ability to enhance cell expansion[9,11,14–18].

Hemicelluloses are usually extracted from cell walls with concentrated aqueous NaOH or KOH at room temperature (Table I). Prior to the extraction, the secondary cell walls are often delignified with hot acidified chlorite[19,20], and primary cell walls are usually depectinated by heating (with or without a chelating agent) at pH 3–7. Either of these pre-treatments may cause some depolymerisation of the hemicelluloses, thereby facilitating their extraction. There appear to be no reports of efficient extraction of xyloglucan by alkali without a heat pre-treatment; with a heat pre-treatment, extraction may[21] or may not[6,22] be efficient (Table I). No systematic study appears to have been reported of the factors that affect the efficiency of the extraction of xyloglucan.

Because of the diversity of published methods for the extraction of hemicelluloses (Table I), we have investigated the time courses of the extraction of xyloglucan from unheated cell walls of *Rosa* cell cultures with aqueous NaOH under various conditions.

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TABLE I

Published methods for the extraction of hemicelluloses, especially xyloglucan

Alkali	*Concn* (M)	*Extraction*			*Tissue*	*Treatment of cell walls before extraction with alkali*
		Time (h)	*Temp* (deg)	*Efficiency*[a]		
KOH	3.6	2 × 2	65–70	n.e.	Maple wood sawdust[20]	No pretreatment
NaOH	2.5	24	25	n.g.	Annual plants[36]	90 min in acidified $NaClO_2$ at 70–75°
KOH	0.9–4.3	16	n.g.	n.g.	Oat leaves[37]	4 h in hot acidified $NaClO_2$
KOH	4.0	12	25	eff[b]	Pea stem[14]	5 h in 25mM H_2SO_4 at 100°
KOH	4.0	2	n.g.	83%[c]	Bean-pod parenchyma[38]	Ball mill, detergent, phenol–acetic acid–H_2O, hot aqueous 2% hexametaphosphate, 4 h in acidified $NaClO_2$ at 70°, 1M KOH
KOH	4.3	2 × 18	room temp	n.g.	Mung-bean hypocotyls[39]	3 × 3 h in benzene–ethanol (2:1) at 50°, 3 × 2 h in H_2O at 80–85°, 6 × 1 h in aqueous 37mM oxalate at 80–85°, 2 × 18 h in 0.7M KOH at room temp.
KOH	0.7–4.3	n.g.	n.g.	eff[d]	Soyabean cell cultures[21]	Hot water, aqueous 0.5% ammonium oxalate, 0.7M KOH
NaOH	6.0[e]	18	room temp	>90%[f]	Potato tuber[33]	2 h in oxalate/citrate buffer (pH 4) at 100°, 30 h in 0.1M Na_2CO_3 at 2°
NaOH	2.5–4.3	18	20	n.e.	Rose cell cultures[6]	3 × 2 h in aqueous 2% NaEDTA at 70°, 3 h in aqueous 0.5% ammonium oxalate at 80°, 15 h in methyl sulphoxide at 20°, 15 h in aqueous 1% $NaBH_4$ at 20°, 18 h in 1.25M NaOH at 20°
KOH	4.3	2 × 2	25–30	98%[g]	Pea stem[7]	3 × 30 min in aqueous 70% EtOH at 70°, 5 × 30 min(?) in 0.1M EDTA (pH 7) at 85°, 5 × 3 h sonication in 0.7M KOH at <30°
KOH	1.8	2 × 2	25–30	n.e.		
KOH	0.7–4.3	2 × 1 in each	4	30%[h]	Tomato cell cultures[35]	Overnight in 0.1M NaEDTA (pH 7) at 4°

[a] Key: eff, apparently efficient; n.e., not efficient; n.g., no information given. [b] Glucose and mannose were the only sugar residues left unextracted, suggesting efficient extraction of xyloglucan. [c] Based on extraction of xylose residues. [d] Xyloglucan was claimed to be completely extracted with 4.3M KOH. [e] Containing 4% H_3BO_3. [f] Based on polysaccharides extracted by NaOH relative to trifluoroacetic acid-hydrolysable (*i.e.*, non-cellulosic) polysaccharides recovered in the residue. [g] Estimated from hemicellulose-bound [^{3}H]fucose residues. [h] Estimated from data for the solubilisation of non-cellulosic neutral sugar residues in normal cells. A further ~ 26% was solubilised by H_2O after treatment with 4.3M KOH.

EXPERIMENTAL

Plant material. — Cell suspension cultures of rose (*Rosa* sp., cv. "Paul's Scarlet") were grown as described[23]. On day 9 after sub-culturing, *i.e.*, in the linear phase of growth, L-[1-^{3}H]arabinose (92 TBq/mol) was added to a final concentration of 592 kBq/mL. Radioactivity from L-[^{3}H]arabinose is incorporated efficiently and specifically into L-arabinose and D-xylose residues, and the xyloglucan becomes heavily labelled[24]. Portions of the culture were withdrawn under sterile conditions after various periods, then filtered through a nylon gauze, and the cells collected were rinsed with water, frozen in liquid nitrogen, and stored at $-20°$.

Fractionation of suspension-cultured rose cells. — Frozen, ^{3}H-labelled cells were suspended in 20mM Tris–HCl (pH 7.0) containing 2% of Triton X-100 and 40mM NaF (~ 30 mL/g fresh weight) and immediately sonicated at 20–30° in an MSE "Soniprep". When cell disruption was judged to be essentially complete (by use of Evans' Blue as a cytoplasmic dye), the suspension was filtered through a nylon gauze, the cell walls collected were rinsed with the same buffer (6 × 3 mL) and re-suspended in 4.5M guanidinium thiocyanate (GTC, pH 7.5), and the suspension was stirred for 24 h at 25°, centrifuged, rinsed with 4.5M GTC solution (6 × 3 mL), and then rinsed with H_2O. GTC typically extracted 7–10% of the total cell wall-bound [^{3}H]xyloglucan (data not shown). The residual cell walls were rinsed with H_2O (3 × 3 mL) in a 10-mL disposable plastic chromatography column (BioRad "Poly Prep" column), and the filtrates and cell wall residues were kept for analysis.

For testing of the alkali-extractability of the GTC-inextractable xyloglucan, the cell wall residues were suspended in water and dispensed in small portions (each representing 100–250 mg of the initial fresh weight of cells) into Poly Prep columns and filtered. 0–9M NaOH (5 mL) containing 1% of $NaBH_4$ was added to each column, which was capped, and incubated at a controlled temperature with occasional agitation for 18 h. The filtrates were then collected.

Radiochemical analysis of xyloglucan extracted. — Extracts were neutralised, dialysed against water at 4°, and incubated at 37° for 48 h in 0.2% Driselase [a mixture of hydrolytic enzymes from *Irpex lacteus* obtained from Sigma, and freed of non-proteinaceous material[25]] in acetic acid–pyridine–water (1:1:98, pH 4.7) containing 0.05% of 1,1,1-trichloro-2-methylpropan-2-ol to minimise microbial contamination. Samples of the hydrolysates were analysed by p.c. Radioactivity co-chromatographing with α-D-xylopyranosyl-(1→6)-D-glucose (XG2) was used as a measure of [^{3}H]xyloglucan[25].

P.c. — Whatman 3MM paper was used with butan-1-ol–acetic acid–water (12:3:5) and then ethyl acetate–pyridine–water (8:2:1) in the same dimension (16 h in each solvent)[25]. Markers (arabinose, xylose, xylobiose, and XG2) were stained with either $AgNO_3$–NaOH or aniline hydrogenphthalate[25,26].

Determination of radioactivity. — Strips of the paper chromatograms were soaked in toluene containing 0.5% of PPO and 0.05% of POPOP, and assayed for ^{3}H by liquid scintillation counting at an efficiency of ~ 7%. Aqueous solutions (if necessary neutralised with acetic acid) were mixed with 10 vol. of toluene–Triton X-100 (2:1) containing

0.33% of PPO and 0.033% of POPOP, and assayed at an efficiency of ~ 35%. Usually, measurements were made in triplicate. Values of ^3H/g were calculated on a cell fresh-weight basis.

Cellulose-swelling experiments. — Portions (0.4 g) of cellulose powder (Microgranular, Sigma) were mixed with aqueous NaOH (10 mL), incubated at various temperatures for 24 h, then centrifuged for 5 min at 1500 r.p.m. and room temperature. The volume of each resulting pellet was measured.

Dissolution of xyloglucan in aqueous NaOH. — *Tropaeolum*-seed xyloglucan, extracted with 2M NaOH containing 0.05% of $NaBH_4$ at 100°, was purified by Cu^{2+} precipitation[27] and freeze-dried. Suspensions of portions (10 mg) in water (5 mL) or aqueous NaOH (5 mL) were shaken gently for 16 h at 25°, then centrifuged (5 min at 500*g*) in order to remove any insoluble polysaccharide, and 20-μL portions of each supernatant solution were assayed for hexose residues.

Determination of hexose and uronic acid residues. Hexose was assayed by the anthrone method[28], and uronic acids by the *m*-hydroxybiphenyl method[29]. Alkali reduced the formation of colour in these assays. Therefore, the crude extracts (in either 6M NaOH or water) were adjusted to 3M NaOH before addition of the reagents. Standard curves were obtained under the same conditions.

RESULTS

Effect of temperature on the extraction of xyloglucan. — A large increase in the yield of total hemicellulose and xyloglucan extracted by 6M NaOH occurred between 25° and 37° (Fig. 1). The dependence on temperature was less pronounced in the ranges 0–25° and 37–100°. The residue, inextractable at 100°, contained < 0.5% of the total cell

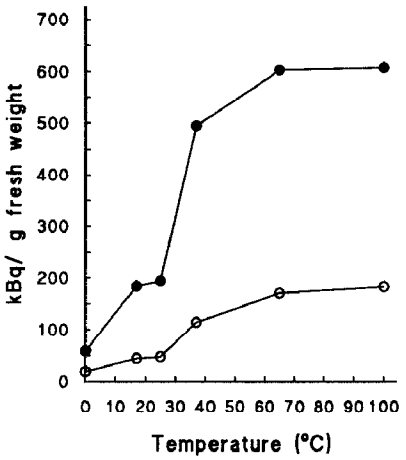

Fig. 1. Effect of temperature on the extraction of total [^3H]polymer (●) and [^3H]xyloglucan (○) with alkali. Rose cells were exposed to L-[^3H]arabinose for 2 h and then samples of GTC-washed cell walls were incubated for 24 h in 6M NaOH at the temperatures indicated (results in triplicate). The [^3H]hemicellulose that was not xyloglucan yielded, on hydrolysis with Driselase, mainly [^3H]arabinose together with traces of [^3H]xylose, [^3H]xylobiose, and chromatographically immobile material.

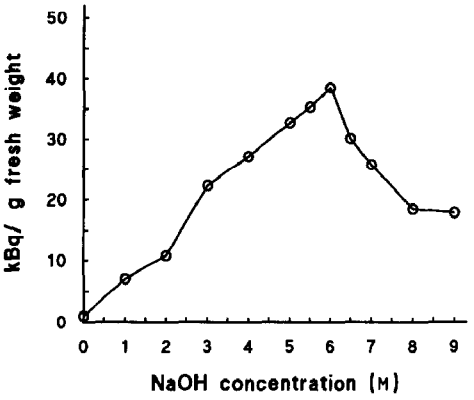

Fig. 2. Effect of the concentration of NaOH on the extraction of [^{3}H]xyloglucan. Rose cells were exposed to L-[^{3}H]arabinose for 8 h and then samples of GTC-washed cell walls were incubated for 24 h at 25 ± 2° with NaOH at the concentrations indicated (results in sextuplicate).

wall-bound ^{3}H, and digestion of this residue with Driselase yielded negligible [^{3}H]XG2, indicating that extraction of xyloglucan was essentially complete.

Effect of the concentration of NaOH on the extraction of xyloglucan. — The most effective extractant at 25° was 6M NaOH; both higher and lower concentrations were considerably less effective during a treatment for 24 h (Fig. 2). Most studies have used 4.0–4.3M alkali.

Effect of the concentration of NaOH on the swelling of cellulose. — At 25°, the most effective concentration of NaOH for the swelling of cellulose was ~ 6M; higher concentrations (7–9M) had slightly less effect (Fig. 3). This loss of effect paralleled, but was less pronounced than, the decrease in extraction of [^{3}H]xyloglucan seen in 6–9M NaOH (Fig. 2).

At 37°, the optimal concentration of NaOH for the swelling of cellulose was again 6M, although the shape of the dose/response curve was different from that at 25° (Fig. 3).

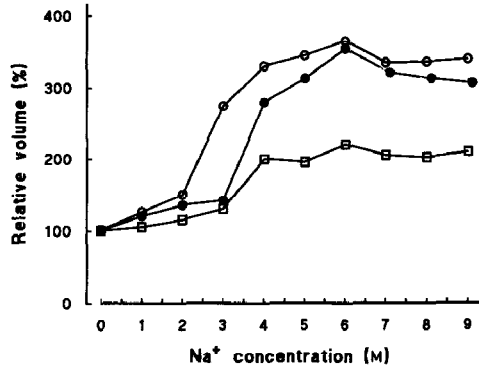

Fig. 3. Effect of the concentration of NaOH and the temperature on the swelling of cellulose powder. After incubation for 24 h, the suspension was cooled to ~ 25° and then centrifuged for 10 min at 500*g*, and the volume of the cellulosic pellet was determined: ●, 25°; ○, 37°; □, 70°.

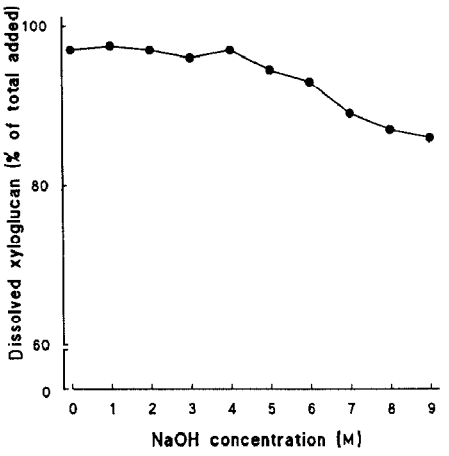

Fig. 4. Dissolution of xyloglucan as a function of the concentration of NaOH at 25° (results in quintuplicate).

Since the measurements of the volume of the cellulose pellet were made at ~25°, the extra swelling caused by the treatment at 37° was evidently permanent. At 70°, NaOH caused the cellulose pellet to collapse.

Cellulose was swelled by KOH about as effectively as by NaOH, and also exhibited an optimum at ~6M (data not shown).

Dissolution of xyloglucan in aqueous NaOH. — Freeze-dried *Tropaeolum* xyloglu-

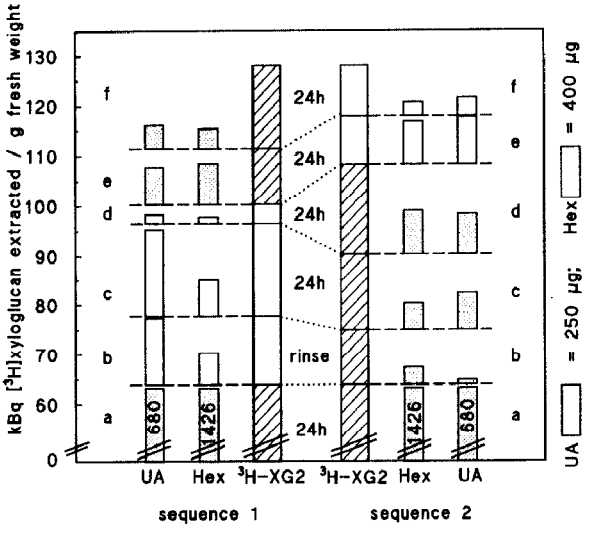

Fig. 5. Pattern of extraction of [^{3}H]xyloglucan (^{3}H-XG2), polysaccharide-bound hexose (Hex), and polysaccharide-bound uronic acid (UA) during two alternative treatments (sequences 1 and 2) of GTC-washed cell walls. The sequences of extractants used (a–f) are shown: ■, water at 25°; ▨ and □, 6M NaOH–1% $NaBH_4$ at 25°. Treatment (a), common to both sequences, was 24 h in 6M NaOH/1% $NaBH_4$. The rinsing step (sequence 1, step b), which took ~ 1 h, was carried out with water (5 × 1 mL) until the filtrate was neutral. Step (b) of sequence 2 was an equivalent alkaline rinse.

can was essentially completely soluble in water, although it dissolved less completely in NaOH at $> \sim 5$M (Fig. 4).

Effect of pre-treatment with alkali on the extractability of [^{3}H]xyloglucan and other polysaccharides. — Pre-treatment of cell walls with 6M NaOH rendered some [^{3}H]xyloglucan and glycuronans extractable with water (Fig. 5; *cf.* Fig. 2). The first steps of sequence 1 (alkali→water) extracted more material than the corresponding steps of sequence 2 (alkali→alkali) (Fig. 5). This effect was particularly strong for glycuronans. By the fourth extraction step of sequence 1, the extraction of xyloglucan with water had decreased markedly compared with NaOH (sequence 2). However, subsequent treatment with alkali (sequence 1) restored extractability of xyloglucan and glycuronans. During the six extraction steps of the two sequences, approximately the same total amount of [^{3}H]xyloglucan was extracted.

DISCUSSION

The extraction of xyloglucan from *Rosa* cell walls was markedly dependent on the temperature. At 37°, 2–3 times more [^{3}H]xyloglucan could be extracted by alkali within 24 h than at 25° (Fig. 1). Extraction at 25° was slow, each of six sequential 18–36-h treatments extracting a substantial increment of the total wall-bound [^{3}H]xyloglucan (data not shown). Other reports on the influence of temperature on the extraction of hemicelluloses[20] have dealt largely with cell wall material pre-treated with hot aqueous solutions, which may have caused some degradation of the hemicellulose, and they have generally involved[30] the temperature range 0–20° rather than 25–37°. For extraction of xyloglucan, alkali at 18–25° for 2–24 h is used typically (Table I), sometimes after treatment with alkali at 0–4°. In cell walls of cultured *Rosa* cells (not depectinated in hot aqueous solutions), and probably also in some other types of cell wall, this practice extracts $< 30\%$ of the total xyloglucan. If the extractable xyloglucan differs functionally from that which is inextractable, analyses of the former alone could lead to erroneous physiological conclusions.

The concentration of the alkali was also a critical factor (Fig. 2). Carpita[31] showed that the extraction of glucuronoarabinoxylan and (1→3),(1→4)-β-D-glucan from graminaceous cell walls occurs at low and high concentrations of alkali, respectively. Similarly, increasing concentrations of NaOH up to ~ 4M extracted progressively more hemicellulose (xylan and mannan) from bleached wood pulp[32] (concentrations of $>$ 4M extracted slightly less hemicellulose). The most efficient concentration of NaOH for the extraction of xyloglucan (at 25° and 37°) was 6M; at higher or lower concentrations, there was a substantial decrease in the efficiency of the extraction. Alkali has been used at 6M in a few studies of primary cell walls[33]; however, the data reported here show that the concentrations used in many other studies (0.7–4.3M, Table I) would have left 25–80% of the xyloglucan unextracted.

The extraction of xyloglucan appeared to be related to the swelling of cellulose, as reflected by the effects of varying the concentration of the NaOH (Figs. 2 and 3) and temperature (Figs. 1 and 3). This finding supports the suggestion[34] that the swelling of

cellulose in alkali explains why aqueous alkali can extract xyloglucan. Once extracted from the cell wall, xyloglucan is slightly more soluble in water than in concentrated aqueous NaOH (Fig. 4), which may explain in part why the decrease of xyloglucan extracted at concentrations of NaOH >6M exceeds the decrease in the swelling of the cellulose (Figs. 3 and 4).

Once the cell walls had been swollen by 6M NaOH, more xyloglucan was extracted during the next 24 h by water than by 6M NaOH (Fig. 5). This effect again may be due in part to the higher solubility of xyloglucan in water than in concentrated alkali (Fig. 4). Alkali can also affect cell wall-bound pectins in a way that renders them subsequently extractable in water[35].

Thus, the efficient extraction of xyloglucan from unheated primary cell walls requires higher temperatures, much longer times, and/or more concentrated NaOH than has been used traditionally. The physicochemical basis for the low extractability of some xyloglucan is of considerable interest to an understanding of the architecture of the primary cell wall.

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