Ten isoenzymes of xyloglucan endotransglycosylase from plant cell walls select and cleave the donor substrate stochastically

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To map the preferred cleavage sites of xyloglucan endotransglycosylases (XETs; EC 2.4.1.207) along the donor substrate chain, we incubated the enzymes with tamarind (*Tamarindus indica*) xyloglucan (donor substrate; ≈ 205 kDa; 21 μM) plus the nonasaccharide [^{3}H]XLLGol ($Gal_2 \cdot Xyl_3 \cdot Glc_3 \cdot$ [^{3}H]glucitol; acceptor substrate; 0.6 μM). After short incubation times, to minimize multiple cleavages, the size of the ^{3}H-labelled transglycosylation products (determined by gel-permeation chromatography) indicated the positions of the cleavage sites relative to the non-reducing terminus of the donor. There was very little difference between the size profiles of the products formed by any of ten XETs tested [one native XET purified from cauliflower (*Brassica oleracea*) florets, four native XET isoenzymes purified from etiolated mung-bean (*Phaseolus aureus*) shoots, native XETs purified from lentil (*Lens culinaris*) and nasturtium (*Tropaeolum majus*) seeds, and three insect-cell-produced thale-cress (*Arabidopsis thaliana*) XETs (EXGT, TCH4 and MERI-5)]. All such product profiles showed a good fit to a model in which the enzyme chooses its donor substrate independently of size and attacks it, once only, at a randomly selected cleavage site. The results therefore do not support the hypothesis that different XET isoenzymes are adapted to produce longer or shorter products such as might favour either the efficient integration of new xyloglucan into the cell wall or the re-structuring of old xyloglucan within an expanding wall.

Key words: cauliflower, mung bean, *Arabidopsis*, nasturtium, lentil.

INTRODUCTION

Xyloglucan in primary cell-wall architecture

Xyloglucan endotransglycosylases (XETs) are a family of cell-wall enzymes that cleave the backbone of a xyloglucan molecule (donor substrate) and join the newly formed, potentially reducing end on to the non-reducing end of an acceptor substrate, which can be another xyloglucan chain or a xyloglucan-derived oligosaccharide [1–5]. Some XETs can also hydrolyse xyloglucan [4,6]. Xyloglucan has a linear β-(1 → 4)-D-glucan backbone, along most of which three contiguous Glc residues carrying α-D-xylosyl (or more complex) side chains are followed by a single Glc residue lacking a side chain [7]. Xyloglucans can thus be regarded as being built up of repeat units based on cellotetraose. Whether catalysing hydrolysis or transglycosylation, XETs appear to cleave the substrate at one of the Glc residues lacking a side chain [8].

Current models of the primary cell walls of dicotyledonous plants propose that cellulose microfibrils are held in position within the hydrated matrix by xyloglucan molecules. Xyloglucans are thought to hydrogen-bond to cellulose, tethering adjacent microfibrils and so forming a coherent xyloglucan–cellulose network [9–11]. Cell growth involves expansion of the cell wall, usually without significant thinning, and so weakening, of the structure. This must involve modification of the existing xyloglucan–cellulose network within, and incorporation of newly synthesized xyloglucan into, the expanding cell wall. It has been proposed that XETs may play a role in both these processes, either cleaving the tethers between cellulose microfibrils to enable re-arrangement, or catalysing transglycosylation reactions of newly secreted xyloglucans such that they become integrated into the existing xyloglucan–cellulose network [5]. Also, in the walls of cultured rose (*Rosa* sp.) cells, about one-third of the total xyloglucan is covalently linked to acidic pectins [12]; it seems possible that some XETs are adapted to catalyse transglycosylation with xyloglucan as donor substrate and pectin as acceptor (= xyloglucan-to-pectin transglycosylation) and thereby generate such xyloglucan–pectin conjugates.

XET expression in relation to physiological activity

Intense XET action detected *in vivo* [13] and high enzyme activity as assayed in tissue extracts [2,14–17] have been correlated with regions of cell expansion in many plants, and with rapidly dividing meristematic cells in thale cress (*Arabidopsis thaliana*) [18]. In addition, XET mRNAs production is elevated in elongating stems [19], especially in response to growth-promoting treatments with auxins [20], gibberellins [19] and brassinosteroids [20,21], supporting the proposed involvement in growth-related cell-wall modification. Increased levels of XETs have also been shown to correlate with fruit softening in kiwifruit (*Actinidia deliciosa*) [22], persimmon (*Diospyros kaki*) [23] and tomato (*Lycopersicon esculentum*) [24–26], and with the mobilization of

Abbreviations used: XET, xyloglucan endotransglycosylase; XLLGol, $Gal_2 \cdot Xyl_3 \cdot Glc_3 \cdot$glucitol {$\alpha$-D-Xyl*p*-(1 → 6)-$\beta$-D-Glc*p*-(1 → 4)-[$\beta$-D-Gal*p*-(1 → 2)-$\alpha$-D-Xyl*p*-(1 → 6)]-$\beta$-D-Glc*p*-(1 → 4)-[$\beta$-D-Gal*p*-(1 → 2)-$\alpha$-D-Xyl*p*-(1 → 6)]-$\beta$-D-Glc*p*-(1 → 4)-D-glucitol}; XTR, XET-related; XLLG, $Glc_4 \cdot Xyl_3 \cdot Gal_2$ {α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-[β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*-(1 → 4)-[β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*-(1 → 4)-D-Glc}; XXLG, $Glc_4 \cdot Xyl_3 \cdot Gal$ {α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-[α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*-(1 → 4)-[β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*-(1 → 4)-D-Glc}; XXXG, $Glc_4 \cdot Xyl_3$ {α-D-Xyl*p*-(1 → 6)-β-D-Glc*p*-(1 → 4)-[α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*-(1 → 4)-[α-D-Xyl*p*-(1 → 6)]-β-D-Glc*p*-(1 → 4)-D-Glc}; GPC, gel-permeation chromatography.

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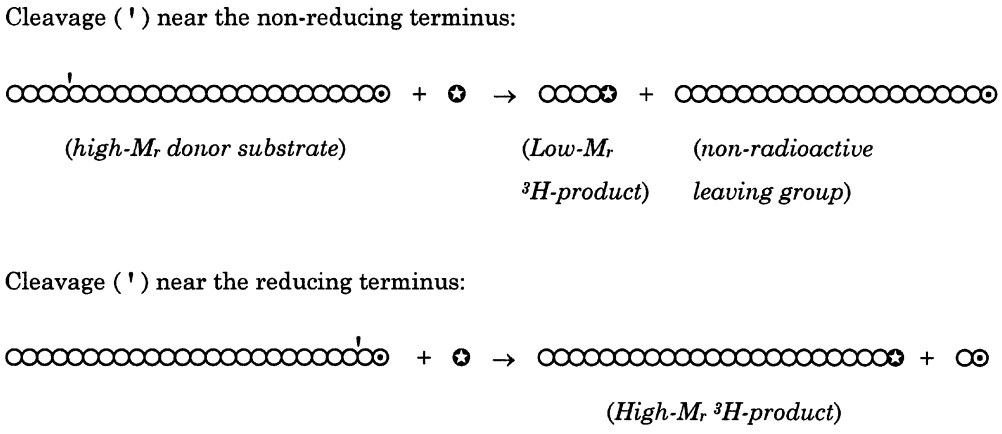

Scheme 1 Use of a ^{3}H-labelled, cellotetraose-based oligosaccharide (✪) as acceptor substrate to report the cleavage site of XET in the donor substrate

The reducing terminus is shown at the right-hand end of each chain. Each ○ represents a cellotetraose repeat unit of xyloglucan; ⊙ = initial reducing terminus.

xyloglucan seed reserves in nasturtium (*Tropaeolum majus*) [4]. Anti-XET antibody staining is high at the cell junctions during the production of air spaces between mesophyll cells [18], and an XET gene is up-regulated during the formation of aerenchyma in maize (*Zea mays*) roots growing in flooded soil [27]. The plant partner of a mycorrhizal symbiosis produces an XET-related (XTR) mRNA. This expression is closely linked to modification of the host-cell walls to allow interaction with the fungal cells [28]. These observations strengthen the correlative evidence of XET's involvement in cell wall modification.

XET isoenzymes

The *XET* gene family is extensive, with the *Arabidopsis* genome containing at least 21 *XTR* sequences [29–31]. Crude plant extracts show numerous isoforms of XET, differing in pI [32], solubility in $(NH_4)_2SO_4$ solutions, pH optimum and K_m for the acceptor substrate [33,34]. DNA sequencing has enabled classification of *XET* and *XTR* sequences into three classes: I, II and III. It has been proposed that the enzymes encoded by these gene classes may differ catalytically. For example, class III, which includes the nasturtium-seed XET, was thought to contain the only XET isoforms that could hydrolyse xyloglucan [16,29]. However, an XET isoform recently purified from ripening kiwi fruit can also hydrolyse xyloglucan, and has a cDNA sequence that places it in class I [6]. The XET produced in ripening tomato fruit is unable to hydrolyse xyloglucan and has a cDNA sequence that places it in class II [25]. It is possible, therefore, that classification of XETs by cDNA sequences alone may be misleading. Characterization of the catalytic activities of individual XET isoforms will be very informative. Four *Arabidopsis* XETs, produced in insect cells, have been shown to differ in temperature optima and substrate preferences [35].

Site(s) at which XETs cleave their donor substrate

One possible reason for the large number of XET isoenzymes is that they may differ in preferred cleavage site along the donor substrate's backbone. An XET predominantly contributing to the integration of newly secreted xyloglucan into the pre-existing xyloglucan–cellulose network might be expected to cleave the donor substrate close to the reducing end. This could minimize waste by ensuring that the leaving group (Scheme 1), which is liable to be excluded from the wall network as a by-product, is relatively small. Conversely, an XET isoenzyme predominantly contributing to the re-structuring of the existing xyloglucan–cellulose network during wall loosening might be expected to have a preference for cleavage in a more mid-chain position, where the enzyme's action would be likely to result in the cleavage of a microfibril-tethering segment of xyloglucan. XETs involved in the mobilization of storage xyloglucan after germination might tend to cleave the substrate near to a terminus, so as to promote the formation of low-M_r products amenable to further hydrolysis to monosaccharides by glycosidases.

Methodological approach of the present work

To test these hypotheses, we have now characterized the cleavage-site preferences of ten diverse XET isoenzymes: the expression of *MERI-5* and *TCH4* [20,36] and the synthesis of cauliflower floret isoenzymes [33,34] occur in dense cytoplasmic tissues predominantly involved in the assembly of new cell walls and thus in the integration of newly secreted xyloglucan into the wall network; isoenzymes from etiolated mung-bean shoots [34] are from a tissue actively involved in the loosening of existing wall material necessary for rapid cell expansion (vacuolation), and the XET EXGT is also produced in such tissues [37]; *TCH4* is expressed in several tissues of *Arabidopsis* in response to mechanical stimulation or cold shock, which may also trigger the re-structuring of existing wall material (and/or integration of new xyloglucan) [18,20]; and the lentil and nasturtium (NXG1) seed enzymes are involved in the mobilization of cotyledonary xyloglucan reserves after germination [38]. *Arabidopsis* EXGT is similar to azuki-bean (*Vigna angularis*) EXGT and so falls into class I [31,35], TCH4 and MERI-5 are both class II XETs, and NXG1 is a class III XET [29].

Small, ^{3}H-labelled acceptor substrates can be used to map the preferred cleavage sites of different XETs. When a high-M_r donor substrate and a small, ^{3}H-labelled acceptor substrate are used, the size of the ^{3}H-labelled reaction product provides information on the distance from the non-reducing terminus of the donor substrate at which XET-catalysed cleavage occurred (Scheme 1). Although free oligosaccharides are probably not the usual acceptor substrates of most XETs *in vivo*, it is unlikely that the nature of the acceptor substrate available has any influence on the site at which the donor substrate is attacked. The

experimental use of small, ^{3}H-labelled acceptor substrates is therefore considered a valid means of probing the sites at which the high-M_r donor substrate is cleaved.

Repeated cleavage of individual donor substrate molecules would confuse this information. The conditions of our XET assays were therefore designed to minimize multiple cleavages in any single xyloglucan molecule, and thus to enable us to trace the products of single cleavage events. To this end, reaction mixtures contained a high concentration of xyloglucan as donor substrate, a low concentration of ^{3}H-labelled oligosaccharide (of high specific radioactivity) as acceptor, and a low concentration of purified XET isoenzyme.

MATERIALS AND METHODS

Materials

Tamarind-seed xyloglucan was generously provided by Mr. K. Yamatoya (Dainippon Pharmaceutical Co., Osaka, Japan), as was an oligosaccharide mixture (principally the nona-, octa- and hepta-saccharides XLLG, XXLG and XXXG, i.e. $Glc_4 \cdot Xyl_3 \cdot Gal_2$, $Glc_4 \cdot Xyl_3 \cdot Gal$ and $Glc_4 \cdot Xyl_3$ respectively), produced from the tamarind xyloglucan by digestion with cellulase. The full structures of these oligosaccharides are given in the Abbreviations footnote and, for brevity, are not repeated here.

Preparation of $Gal_2 \cdot Xyl_3 \cdot Glc_3 \cdot$ glucitol (XLLGol) and [^{3}H]XLLGol

A portion of the oligosaccharide mixture was reduced with excess $NaBH_4$ in 1 M NH_3, and the reduced nonasaccharide (XLLGol) fraction was purified by gel-permeation chromatography (GPC) on Bio-Gel P-2. Purity was checked by TLC on silica-gel in propan-1-ol/nitromethane/water (5:2:3, by vol.); carbohydrates were detected on the plate by spraying with ethanol containing 0.5 % (w/v) thymol and 5 % (v/v) H_2SO_4 and heating at 105 °C for 10 min.

An additional, non-reduced, portion of the oligosaccharide mixture was fractionated by GPC on Bio-Gel P-2. The nonasaccharide (XLLG) fraction was further purified by preparative paper chromatography and then reduced with NaB^3H_4; the [^{3}H]XLLGol produced was purified on Bio-Gel P-2 followed by preparative paper chromatography; final specific radioactivity 80 MBq/μmol.

Isolation of size-homogeneous xyloglucan

Tamarind xyloglucan, previously dissolved at 1 % (w/v) in hot water and stored at −40 °C, was diluted to 0.33 % in 6 M NaOH containing 1 % $NaBH_4$ (final concns.), shaken for 16 h at 37 °C, neutralized with acetic acid, dialysed against pyridine/acetic acid/water (1:1:23, by vol.; pH ≈ 4.7) and then subjected to GPC on Sepharose CL-6B (column bed volume 600 ml, internal diameter 2.5 cm) that was equilibrated and eluted in the same solvent. Those fractions of the xyloglucan eluted between k_{av} 0.15 and 0.35 were pooled, freeze-dried and stored in an air-tight container at 20 °C (we define k_{av} values of 0 and 1 by the peak centres of 5–40 MDa dextran and sucrose respectively).

XET isoenzymes used

XET isoenzymes from growing tissues were prepared as before [33]. In brief, XET activities in $(NH_4)_2SO_4$-precipitated fractions from cauliflower florets and etiolated mung-bean shoots were complexed with high-M_r xyloglucan, isolated by GPC on Bio-Gel A-0.5M, de-complexed by addition of xyloglucan oligosaccharides, re-isolated at a lower M_r by GPC on the same column [33], and then further fractionated by cation-exchange chromatography on Whatman CM22 carboxymethylcellulose. Our nomenclature of these isoenzymes is exemplified by M55b, where 'M' stands for mung bean, '55' is the activity precipitated by 55 %-satd. $(NH_4)SO_4$ and 'b' is the second peak of activity eluted during cation-exchange chromatography [34].

TCH4, EXGT and MERI-5 are *Arabidopsis* XETs produced in insect cell lines as described previously [35]. TCH4 and EXGT were purified, while the preparation of MERI-5 was an unpurified sample of the insect-cell-suspension medium. As no XET activity was detected in the medium of untransformed control cell lines, MERI-5 was taken to be the only XET activity in the culture medium.

XETs were purified from germinated nasturtium and lentil seeds by the method of Sulová and Farkaš [39], exploiting selective adsorption of xyloglucan–XET complexes to cellulose followed by liberation of XET by a mixture of xyloglucan oligosaccharides (DP7–9, where DP is degree of polymerization).

Determination of site of cleavage of donor substrate

To 1 ml of enzyme solution (approx. 5–10 pkat, as measured with 0.45 % xyloglucan as donor and 167 μM XLLGol as acceptor substrate [33]) was added 2 ml of a substrate mixture to give final concentrations of 0.45 % xyloglucan (205 kDa; ≈ 21 μM), 0.6 μM [^{3}H]XLLGol, 100 mM succinate (Na^+, pH 5.5), 15 mM $CaCl_2$ and 10 % glycerol. After various time intervals at 20 °C, 500 μl aliquots were added to 500 μl of 50 % formic acid, which stopped the reaction. Each sample was subjected to GPC on Sepharose CL-6B [column bed volume 300 ml; internal diameter 1.25 cm; equilibrated and eluted with pyridine/acetic acid/water (1:1:23, by vol.)] and 2.0 ml fractions were collected. A 1.0 ml portion of each fraction was assayed for ^{3}H radioactivity after the addition of 10 ml of OptiPhase HiSafe scintillation fluid (Wallac, Milton Keynes, Bucks., U.K.); duplicate 0.5 ml portions were assayed for total xyloglucan by the anthrone method [40].

RESULTS AND DISCUSSION

Isolation and characterization of 'size-homogeneous' tamarind xyloglucan

Calibration of a Sepharose CL-6B column (Figure 1) indicated a linear relationship between k_{av} and $\log M_r$, approximated by:

$$k_{av} = -0.369(\log M_r) + 2.238$$

for M_r values within the range $(8–1000) \times 10^3$. We acknowledge that M_r values from a calibration curve prepared mainly using dextrans are not absolute when applied to xyloglucans and that it is possible that the relationship between k_{av} and $\log M_r$ within the range used [$(8–1000) \times 10^3$; Figure 1b] is not precisely linear; the M_r data reported in the present paper should therefore be regarded as nominal.

When stored at −40 °C, aqueous solutions of tamarind xyloglucan tended to form aggregates of apparent $M_r > 5 \times 10^6$ (excluded by Sepharose CL-4B; results not shown). To reverse this trend, we treated the xyloglucan with alkali followed by preparative GPC on Sepharose CL-6B. Fractions with k_{av} between 0.35 and 0.15 [apparent $M_r \approx (4.7–1.3) \times 10^5$] were selected as a relatively size-homogeneous population, well clear of the void volume of Sepharose CL-6B. This fraction was stored

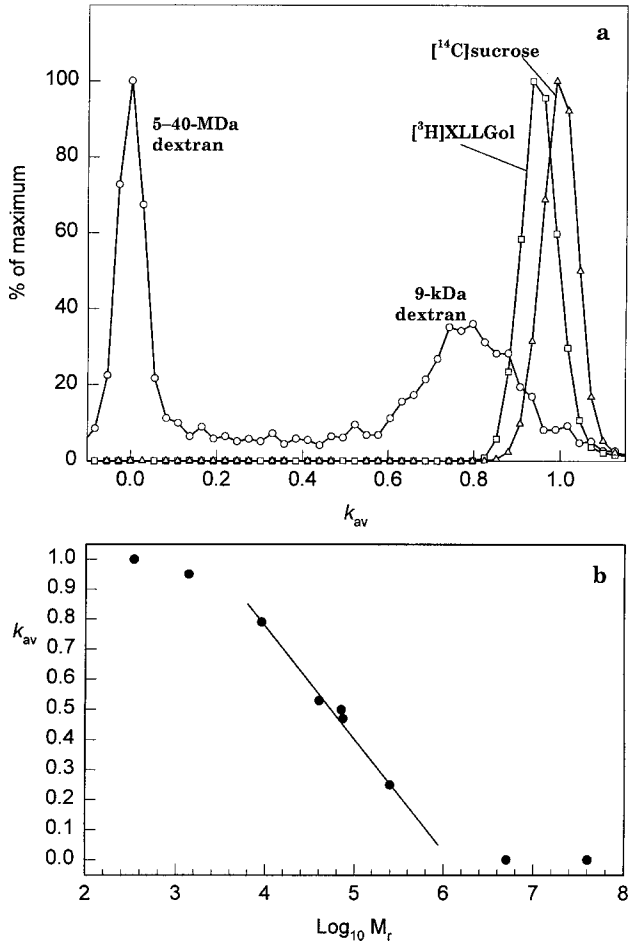

Figure 1 Calibration of Sepharose CL-6B by markers of known M_r

(**a**) Representative fractionation of a mixture containing two dextran preparations [M_r (5–40) $\times 10^6$ and 9.0×10^3, detected by the anthrone reaction; ○], the nonasaccharide [^{3}H]XLLGol (M_r 1388; □) and [^{14}C]sucrose (M_r 342; △). (**b**) Calibration curve based on the results in (**a**) and data from several other similar runs. k_{av} values of 0 and 1 are defined by the elution patterns of the 5–40 MDa dextran and sucrose respectively.

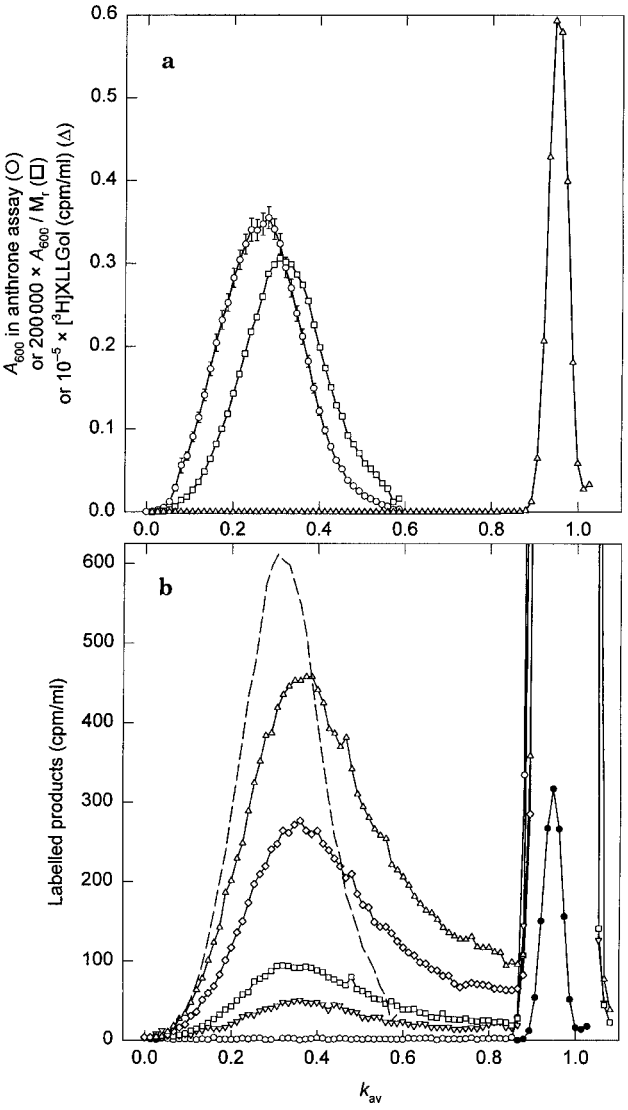

Figure 2 Sepharose CL-6B chromatography of size-homogeneous tamarind xyloglucan (donor substrate) and of its transglycosylation products

(**a**) Chromatography of donor substrate. The data are the means of 32 separate runs on the same column. ○, Mass profile (A_{600} reading in the anthrone assay, mean ± S.E.M.); □, molar profile (200000 × A_{600}/M_r, where M_r is estimated for each fraction by reference to Figure 1b). The elution profile of [^{3}H]XLLGol (△) is shown for comparison. (**b**) ^{3}H-labelled transglycosylation products formed by the action of cauliflower isoenzyme C45a on size-homogeneous tamarind xyloglucan plus [^{3}H]XLLGol after various times (○, 0 min; ▽, 2 min; □, 5 min; ◇, 15 min; △, 30 min). The zero-time data were also plotted on a 200-fold condensed scale (●). The molar profile of the non-radioactive, size-homogeneous xyloglucan (donor substrate) is shown for comparison (– – –; arbitrary scale).

as a freeze-dried powder and re-dissolved in hot water immediately before each of the experiments reported here.

When re-chromatographed on Sepharose CL-6B, the size-homogeneous xyloglucan was eluted as a relatively narrow peak of anthrone-positive material (Figure 2a, ○). The median M_r, plotted in this way, was ≈ 280000 (i.e. 50 % of the mass of the xyloglucan was associated with molecules of $M_r > 280000$). This curve is based on measurements of total hexose residues (A_{600} in the anthrone assay), and we refer to it as a 'mass profile' (that is, the y-axis is proportional to the mass of product having the M_r in question).

By contrast, in a graph showing the ^{3}H-labelled products formed by the action of XET, the y-axis is proportional to the number of mol of product having the M_r in question, since each ^{3}H-labelled xyloglucan molecule produced has only one ^{3}H atom, however long the polysaccharide chain is. We therefore refer to such a curve as a 'molar profile' rather than a mass profile.

To make the anthrone profiles comparable with the ^{3}H profiles, we estimated the M_r of each Sepharose fraction (from Figure 1b) and calculated A_{600}/M_r, which is proportional to the molar concentration of anthrone-reactive polysaccharides. The resulting molar profile (Figure 2a, □) is shifted towards lower M_r values compared with the mass profile. The median M_r of the potential donor substrate on a molar basis was ≈ 205000 (that is, 50 % of the molecules had $M_r > 205000$).

The use of this donor xyloglucan with a narrow size range (median $M_r \approx 205000$) also ensures the absence of any small non-radioactive xyloglucan fragments with the potential to compete with the radiolabelled oligosaccharides as acceptors for XET. The reaction conditions were therefore standardized so

that any preferences shown by certain XET isoenzymes towards differently sized acceptors would not affect the interpretation of the elution patterns of radioactive transglycosylation products from the Sepharose CL-6B column.

Size profile of ^{3}H-labelled transglycosylation products of isoenzyme C45a

Data are shown in detail for one representative XET, namely the cauliflower isoenzyme C45a (Figure 2b). The ^{3}H-labelled products formed after 2–30 min incubation (▽, □, ◇, △) showed (molar) profiles on Sepharose CL-6B very different from that of the acceptor substrate, [^{3}H]XLLGol (○, ●; $k_{av} \approx 0.95$). This indicates that transglycosylation occurred with polysaccharide as donor and oligosaccharide as acceptor substrate (= polysaccharide-to-oligosaccharide transglycosylation). The ^{3}H profiles were also shifted considerably towards higher k_{av} values than the molar profile of the non-radioactive donor substrate (Figure 2b, – – –). This shows that the products were of appreciably lower M_r than the donor, indicating that, during polysaccharide-to-oligosaccharide transglycosylation, cleavage was not targeted very near the reducing terminus of the donor substrate.

It is important to estimate the proportion of xyloglucan molecules undergoing transglycosylation during these incubations. When isoenzyme C45a was incubated with the substrate mixture for 30 min, 94.5% of the [^{3}H]XLLGol (acceptor substrate) remained unchanged and thus was eluted at $k_{av} \approx 0.95$. Since only 5.5% of the XLLGol molecules participated in transglycosylation events and there was initially a ≈ 35-fold molar excess of xyloglucan (donor) over XLLGol (acceptor), we estimate that only about 0.16% of the potential donor molecules underwent polysaccharide-to-oligosaccharide transglycosylation.

The yield of ^{3}H-labelled reaction products increased with time (Figure 2b), indicating that the enzyme remained active throughout the incubation period. However, at all four time points sampled (2–30 min), the profiles of ^{3}H-labelled products were superimposable. If an appreciable proportion of the xyloglucan chains had undergone two or more transglycosylation events with [^{3}H]XLLGol as acceptor substrate, the ^{3}H-labelled products present at 30 min would have been of lower M_r than those present at 2 min. The data therefore show that, as expected from the low enzyme activities used, a negligible number of xyloglucan molecules encountered an XET molecule more than once during the course of the experiment.

More significantly, the data rule out the hypothesis that each xyloglucan–enzyme encounter initiates a series of local reactions on the same donor chain, which would have resulted in a progressive decrease in mean M_r of the radioactive product. Since this was not observed, we conclude that the enzyme dissociates from the reaction products after each polysaccharide-to-oligosaccharide transglycosylation event. This agrees with the finding that xyloglucan oligosaccharides are able to cause the liberation of XET from xyloglucan–XET complexes [41].

Lack of detectable change in M_r distribution of total xyloglucan

We considered the possibility that rapid polysaccharide-to-polysaccharide transglycosylation could have appreciably changed the M_r profile of the initially formed ^{3}H-labelled reaction products. Any such changes would have fogged the picture of transglycosylation sites as revealed by the ^{3}H profile.

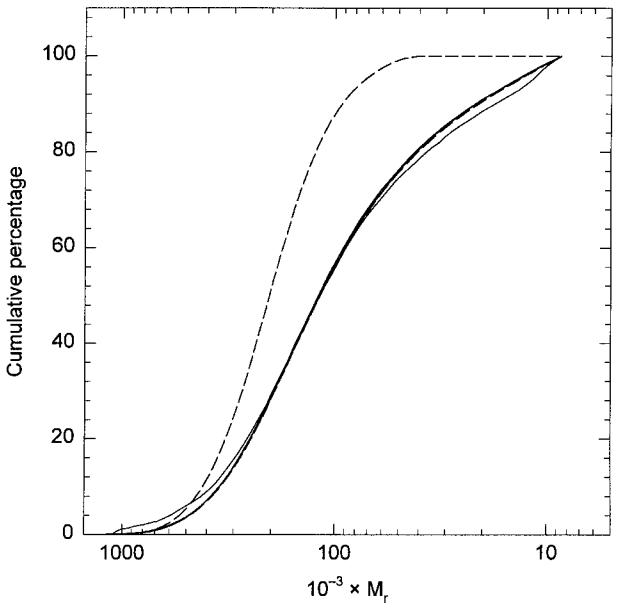

Figure 3 M_r distribution of ^{3}H-labelled products formed by cauliflower isoenzyme C45a

The curves are calculated from the data shown in Figure 2(b). Products of $M_r < 8000$ are omitted. ——, Four, partially superimposed, continuous lines representing the products formed after 2, 5, 15 and 30 min; only one (2 min) differs perceptibly from the others. Each curve consists of 63 data points joined by straight lines; it was not practicable to use symbols. – – –, The right-hand broken line represents the mean of the ^{3}H-labelled products of all ten isoenzymes at all time points tested (i.e. the all-isoenzyme mean); the left-hand broken line shows the mean molar profile of the non-radioactive xyloglucan (donor substrate). Data for the *x*-axis were calculated from the k_{av} values in Figure 2 using the calibration curve shown in Figure 1(b).

However, the profile of total xyloglucan (anthrone assay) did not perceptibly change during incubation with any of the ten XETs (results not shown), whereas the ^{3}H-labelled products were of considerably lower M_r than the non-radioactive donor substrate (Figure 2b). Therefore the possibility of rapid polysaccharide-to-polysaccharide transglycosylation can be discounted.

Scarcity of very small transglycosylation products

On Sepharose CL-6B, molecules of M_r 2500–8000 (k_{av} 0.9–0.8), which would indicate cleavage of the donor substrate at a site 1–5 cellotetraose-based repeat units from the non-reducing end, would not be reliably separated from the large amount of remaining unchanged XLLGol (M_r 1388) and would therefore be overlooked. We therefore analysed representative samples by GPC on Bio-Gel P-10, which has a lower M_r fractionation range than Sepharose CL-6B. This study showed that very few radioactive products had an $M_r < 10^4$ (tested for C45, M35a and lentil XETs; results not shown). Therefore, the XETs showed no preference for cleavage very near the non-reducing end of the donor substrate.

Comparison of ten XET isoenzymes

By plotting molar profiles as a cumulative percentage of ^{3}H-labelled products versus M_r, we can compare quantitatively the

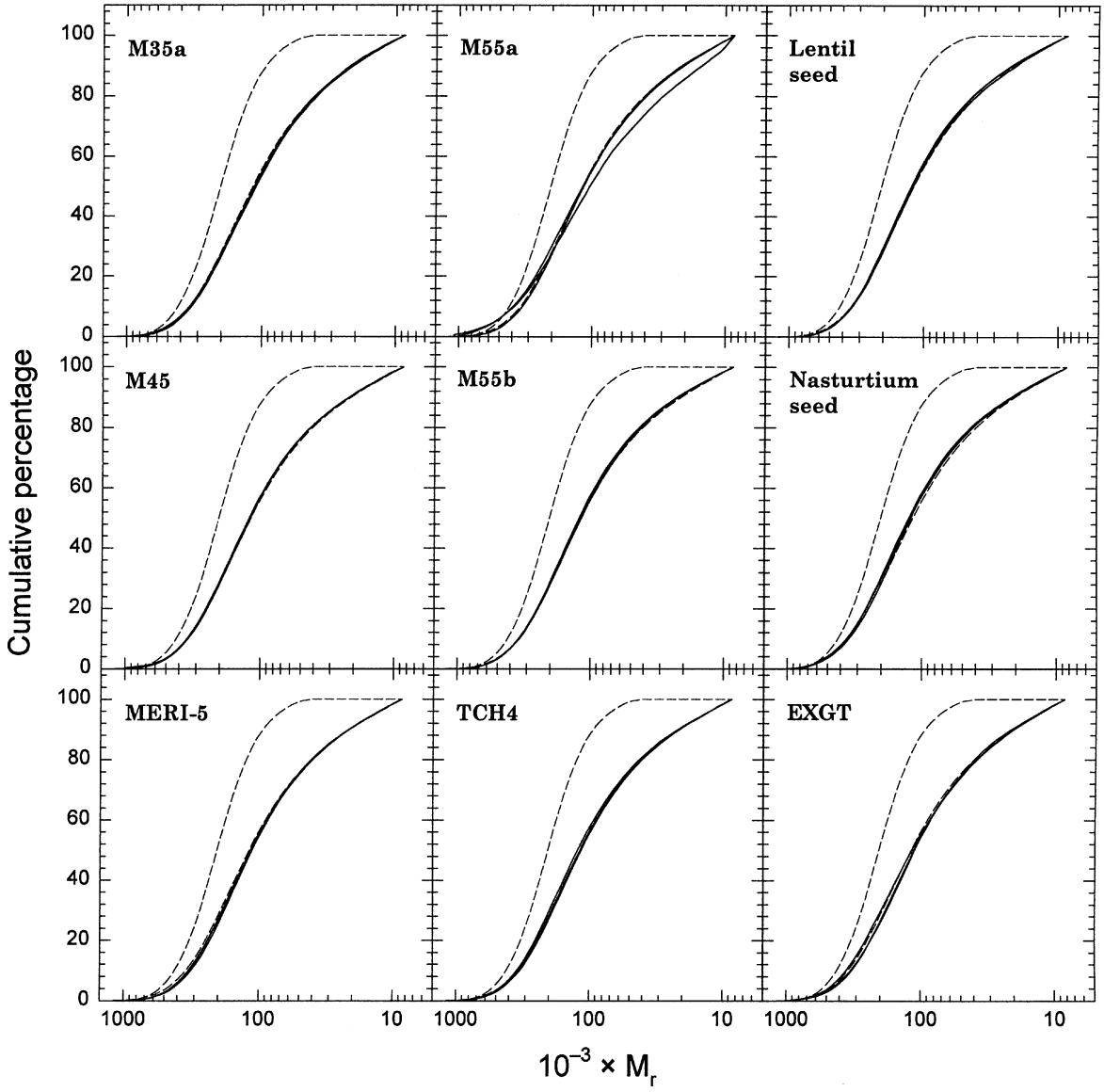

Figure 4 M_r distribution of ^{3}H-labelled products formed by nine different XET isoenzyme preparations

—, Products formed after 5, 15, 30 and 60 min (isoenzyme M35a), 30, 60 and 90 min (M45), 5, 15 and 30 min (M55a), 15, 30 and 60 min (M55b), 30, 60 and 90 min (MERI-5), 15, 30 and 60 min (TCH4), 30, 60 and 90 min (EXGT), 30, 60 and 90 min (lentil seed) and 30, 60 and 90 min (nasturtium seed). – – –, As for Figure 3.

size distributions of the different isoenzymes' transglycosylation products. Such plots are presented for each of the ten XET isoenzymes: Figure 3 shows the data in detail for isoenzyme C45a, and Figure 4 summarizes the data for the other nine isoenzyme preparations. On each plot, the mean molar profile of the total non-radioactive xyloglucan (donor substrate) and the mean molar profile of the ^{3}H-labelled reaction products (of all ten isoenzymes at all time-points sampled; referred to hereafter as the all-isoenzyme mean) are shown as two broken lines for comparison.

Native isoenzyme C45a, purified from cauliflower florets, gave three curves (5, 15 and 30 min incubation times) indistinguishable from each other and from the all-isoenzyme mean (Figure 3). Only the 2 min curve deviated somewhat, probably owing to error in measuring the very small quantities of radioactive product formed within 2 min (cf. Figure 2b, $\bigtriangledown$).

The four native isoenzymes purified from mung-bean shoots (M35a, M45, M55a, M55b) showed small (Figure 4) but significant (Table 1) differences from the all-isoenzyme mean. Products formed by M35a were slightly smaller than the all-isoenzyme mean. Products formed by M45 and M55b were slightly larger than the all-isoenzyme mean, especially at the low-M_r end of the range (Table 1).

Of the three heterologously produced *Arabidopsis* XET isoenzymes, TCH4 showed no significant differences from the all-isoenzyme mean (Figure 4). Products formed by MERI5 and EXGT were slightly smaller than the all-isoenzyme mean, especially at the high- and low-M_r ends of the range respectively (Table 1).

Both the native seed isoenzymes, from lentil and nasturtium, showed a tendency to favour higher-M_r products in comparison with the all-isoenzyme mean (Figure 4; Table 1).

Table 1 Size distribution of the ^{3}H-labelled products formed by specific XETs

Results shown are selected data from Figures 3 and 4, showing significance of deviation from the all-isoenzyme mean. The M_r cut-offs quoted were selected arbitrarily for each XET to emphasize any deviation from the all-isoenzyme mean.

Isoenzyme	Products with M_r exceeding	Percentage of such products: Formed by the specific isoenzyme*	Formed by the all-isoenzyme mean†	P
M35a	113000	50.25 ± 0.25	51.56 ± 0.27	< 0.01
M45	70800	68.25 ± 0.09	67.23 ± 0.26	< 0.01
M55b	51800	76.11 ± 0.22	75.07 ± 0.26	< 0.01
MERI-5	247000	18.37 ± 0.38	20.14 ± 0.21	< 0.001
EXGT	70800	66.05 ± 0.08	67.23 ± 0.26	< 0.001
Lentil	113000	52.82 ± 0.27	51.56 ± 0.27	< 0.01
Nasturtium	113000	53.90 ± 0.27	51.56 ± 0.27	< 0.001

* Mean for all time points ± S.E.M. ($n = 3$, except isoenzyme M35a, where $n = 4$).
† Mean for all time points ± S.E.M. ($n = 32$).

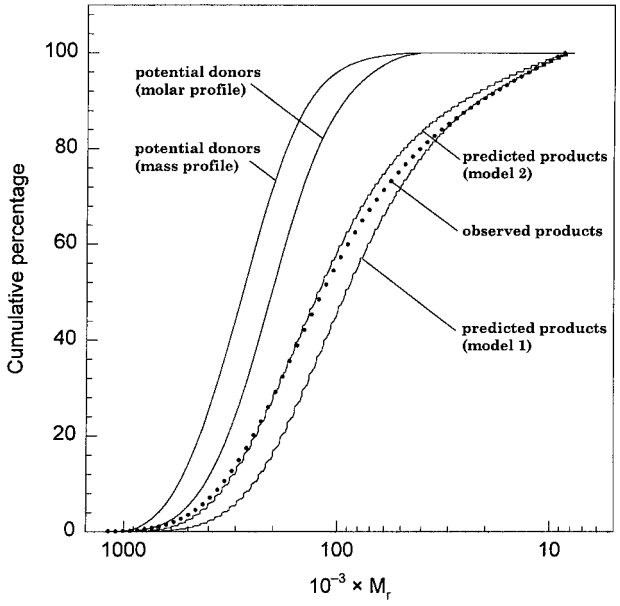

Figure 5 M_r distribution of ^{3}H-labelled products observed experimentally and predicted by two models

The two curves on the left are the molar profile and mass profile of the non-radioactive xyloglucan (total population of potential donor substrates). The dotted curve (· · · ·) represents the observed ^{3}H-labelled transglycosylation products (mean for all ten isoenzymes at all time points tested). This is compared with the ^{3}H-products predicted by two models. Model (1) assumes that donor molecules are chosen in proportion to their molar concentration; model (2) assumes that they are chosen in proportion to their weight/volume concentration. Both models assume that XET cleaves the selected donor molecule, once only, at a randomly selected point along the chain-length. In both models, predicted products of $M_r < 8000$ have been omitted, as was done with the observed data; this resulted in the omission of 3.75% (model 1) or 1.81% (model 2) of the total predicted radioactive products.

The largest divergence from the all-isoenzyme mean was given by nasturtium-seed enzyme. The magnitude of this divergence was, however, still rather small: the median M_r of the all-isoenzyme mean reaction products was estimated at 118000, whereas the median M_r of the nasturtium enzyme products was 124000. Thus our main conclusion is of a close similarity between the products of all ten isoenzymes tested.

Modelling the action pattern of XETs on the donor substrate

To model the action pattern of the XETs, we averaged the data for the products of all ten isoenzymes at all time points (Figure 5). The median M_r (on a molar basis) of the potential donor substrate molecules was ≈ 205000, whereas that of the transglycosylation products was ≈ 120000. This suggests that XETs tend to cleave the donor substrate on average near the middle of the donor substrate.

Two simple models were considered that could potentially describe the action pattern of the XETs. Model (1) proposes that XET chooses its donor substrates from among the total population of xyloglucan molecules in proportion to their molar concentration; thus a 300 kDa chain is as likely to be chosen as a 150 kDa one if chains of these sizes are equimolar. Model (2) proposes that XET chooses its donor substrates in proportion to their weight-per-volume concentration; thus a 300 kDa chain is twice as likely to be chosen as a 150 kDa one if chains of these sizes are equimolar. Both models propose that, once a particular xyloglucan chain has been chosen, the XET cleaves it at a cellotetraose repeat unit that is randomly selected from the whole chain length.

We approximated the size distribution of the products that would be predicted by the two models. Let M_i be the M_r of xyloglucan in Sepharose fraction i (Figure 1b). In Figure 2(a) (□), let f_{ni}% be the percentage (of the total number of potential donor substrate molecules) that is eluted in fraction i. In Figure 2(a) (○), let f_{mi}% be the percentage (of the total mass of potential donor substrate) that is eluted in fraction i. For simplicity, we assume that the enzyme has a 10% chance of cleaving within each one-tenth of the length of the selected donor chain. Under these conditions, the radioactive reaction products formed (from those donor molecules that would elute in fraction i) are predicted to contribute to the total radioactive products approximately as follows.

In model (1), molecules characteristic of fraction i are predicted to constitute f_{ni}% of all the cleaved donor molecules. Furthermore, $0.1f_{ni}$% of all the radioactive product molecules formed would result from cleavage of these donor molecules somewhere within the first one-tenth of the chain length (measuring from the non-reducing terminus) and thus have an M_r within the range 0–$0.1M_i$ (approximated in Figure 5 as $M_r = 0.05M_i$); similarly, $0.1f_{ni}$% of all product molecules would have an M_r between $0.1M_i$ and $0.2M_i$ (approximated as $0.15M_i$), $0.1f_{ni}$% would be $\approx 0.25M_i$, … and $0.1f_{ni}$% would be $\approx 0.95M_i$.

In model (2), molecules characteristic of fraction *i* are predicted to constitute f_{mi} % of all the cleaved donor molecules. Furthermore, $0.1f_{mi}$ % of all the radioactive product molecules formed would have an M_r between 0 and $0.1M_i$ (approximated in Figure 5 as $M_r = 0.05M_i$), $0.1f_{mi}$ % of them would be between $0.1M_i$ and $0.2M_i$ (approximated as $0.15M_i$), $0.1f_{mi}$ % would be $\approx 0.25M_i$, ... and $0.1f_{mi}$ % would be $\approx 0.95M_i$.

The results of these approximations (Figure 5) show that, for most of its length, the curve generated by model (1) fails to predict the observed pattern of ^{3}H-labelled transglycosylation products. In contrast, for much of its length, the curve generated by model (2) correctly predicts the observed pattern of ^{3}H-labelled products, especially those of relatively high M_r.

The slight deviation of observed from predicted data at the lower end of the M_r range suggests that somewhat more low-M_r products are formed than model (2) predicts. This could theoretically be because XET has a slight tendency to select small donor substrate molecules more frequently than predicted by the weight/volume concentration of such molecules. Schröder et al. [6] have suggested that a kiwifruit XET has a preference for shorter donor substrates. However, there are also reports of XETs that are unable to use donors of $M_r < 10000$ [3] or < 60000 [42].

An alternative explanation of our observation is that XETs do not cleave totally randomly along the length of the donor chain, but preferentially near the non-reducing terminus, thus producing a slight excess of small ^{3}H-labelled products. Tabuchi et al. [42] reported that an XET from mung-bean stems cleaves 600 kDa xyloglucan chains predominantly 50 kDa from the non-reducing end. However, our data show that any such tendency is very slight and that three-quarters of the products formed by our enzymes were of a size indicating cleavage more than 50 kDa from the non-reducing end. It is clear from Figure 5 that the general trend of the ten isoenzymes tested by us is for XETs to select their donor substrates in proportion to their weight/volume concentration, with little regard to M_r in the range tested [$\approx (1–5) \times 10^5$], and to cleave the donor substrate at essentially any distance from the non-reducing terminus.

Conclusion

Our data show that there is no strong inherent tendency of any of the ten XETs tested to cleave the donor substrate near to or far from either terminus. The data therefore do not support the hypothesis that different isoenzymes are adapted to produce longer or shorter products such as might favour either the wall-integration of newly secreted xyloglucan or the re-structuring of previously wall-integrated xyloglucan. We have, however, tested the XETs on substrates in aqueous solution; it remains possible that donor substrates within a xyloglucan–cellulose network will exhibit different cleavage patterns.

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