Purification of xyloglucan endotransglycosylases (XETs): a generally applicable and simple method based on reversible formation of an enzyme–substrate complex

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We describe a novel and general, mechanism-based, method for purification of xyloglucan endotransglycosylases (XETs) from crude plant extracts. Putative isoforms, obtained by step-wise precipitation with $(NH_4)_2SO_4$, were incubated with tamarind xyloglucan (≈ 1 MDa) to form stable xyloglucan–XET complexes with apparent molecular masses > 500 kDa on gel-permeation chromatography (GPC). Subsequent addition of xyloglucan-derived oligosaccharides (a mixture of XET acceptor substrates) caused a shift in the GPC elution volume of the activity back to that expected of a ≈ 32 kDa protein, presumably by completing the transglycosylation reaction and so freeing the enzyme from the xyloglucan (donor substrate). This simple two-step method enabled the isolation of each XET activity attempted [various $(NH_4)_2SO_4$ cuts from extracts of cauliflower florets and mung bean seedlings], in pure form as judged by SDS/PAGE.

Key words: hemicellulose, oligosaccharide, plant cell wall, transglycosylation.

INTRODUCTION

Xyloglucan endotransglycosylases (XETs) are a family of cell-wall enzymes with proposed roles in the assembly [1] and modification [2] of the xyloglucan-cellulose network. They act by cleaving a donor xyloglucan somewhere along its length, and by joining the newly formed potentially reducing end to the non-reducing end of an acceptor xyloglucan or xyloglucan-derived oligosaccharide [3–5]. Some XETs can also hydrolyse xyloglucan [6].

High XET activity has been correlated with regions of tissue extension in many plants, including pea stems [7], maize roots [8], barley leaves [9] and *Arabidopsis* stems [10]. Production of XET mRNA correlates with elongation in *Arabidopsis* stems [11] and also with elongation in response to growth-promoting treatments with auxins [12], gibberellins [11] and brassinosteroids [13]. Increased levels of XET also correlate with fruit softening in kiwi [14] and tomato [15] and with the mobilization of seed reserves in nasturtium [6]. Flooding-induced formation of aerenchyma in maize roots is closely linked to expression of an XET-like protein [16,17]. Thus XETs appear to participate in several diverse aspects of plant growth and development.

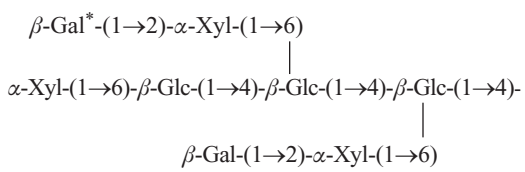
Crude plant extracts contain numerous isoforms of XET differing in isoelectric point, as revealed by zymogram techniques [18,19], and the *Arabidopsis* genome contains at least 10 XET-related (XTR) sequences [5,21]. Amino acid sequences predicted from *XTR* DNA sequences fall into three groups [20]. Group III, which includes the nasturtium seed XET, is the only group that can hydrolyse xyloglucan [6,21] and that shows a preference for a non-fucosylated donor [10]. One member of group II, TCH4, has been studied enzymologically [22]. However, nothing is known of the enzymological differences between groups I and II, which are classified solely on sequence similarity. Such differences could potentially include preferences for mid-chain or near-terminal cleavage of the donor substrate, for substrates with different chain-lengths or composition (e.g. degree of galactosylation, fucosylation or acetylation), and for pH and temperature. Individual *XTR* genes differ in the spatial and temporal patterns of their expression, probably reflecting different biological roles [20].

Progress in characterizing individual XET isoforms, and thus in elucidating their physiological significance, would be greatly assisted by the availability of a simple and effective purification scheme. Previous purification techniques have relied on empirical combinations of anion-exchange, cation-exchange, hydrophobic-interaction and gel-permeation chromatography (GPC) [23,24]. Here we present a novel purification technique that exploits the stable association formed between XET and its donor substrate [25] and provides a simple and generally applicable method for purifying XETs from plant tissues.

MATERIALS AND METHODS

Materials

Xyloglucan can be prepared from tamarind flour [23] and digested to oligosaccharides with cellulase [18]. We obtained tamarind xyloglucan and a mixture of tamarind xyloglucan-derived oligosaccharides (principally XLLG, XXLG and XXXG; see below) as generous gifts of Mr. K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan. XLLG has the structure:



XXLG lacks the Gal residue marked (*), and XXXG lacks both Gal residues. [1-^{3}H]XLLGol, in which the reducing terminal Glc moiety of XLLG is replaced by [^{3}H]glucitol, was prepared as described previously [26].

Abbreviations used: XET, xyloglucan endotransglycosylase; GPC, gel-permeation chromatography; V_0, void volume; V_t, included volume; XTR, XET-related.

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Enzyme extraction

Mung bean seedlings and cauliflower florets (400 g) were homogenized in ice-cold buffer A [600 ml; 350 mM succinate(Na^+)/16.7 mM $CaCl_2$/1.67 mM dithiothreitol (pH 5.5)], using an AtoMix blender. The homogenate was then left at 4 °C for 2 h with occasional mixing, strained through muslin and centrifuged at 2500 ***g*** for 30 min.

Ammonium sulphate precipitation

Proteins were precipitated from 100 ml of crude extract in a step-wise manner at 4 °C with increasing saturation of $(NH_4)_2SO_4$. The precipitates were collected by centrifugation at 12000 ***g*** for 10 min at 4 °C, re-dissolved in 3 ml of 60 % buffer A and flash-frozen in liquid N_2 in 0.5 ml aliquots.

Assay of XET activity

XET activity was assayed using an adaptation of the method of [2]. Protein solution (10 μl in 60 % buffer A) was added to 20 μl of an assay mixture containing 0.67 % (w/v) tamarind xyloglucan and 250 μM [^{3}H]XLLGol (approximately 320 kBq/μmol) in 60 % buffer A. The reaction was allowed to run for 40 min before being quenched with 20 μl of 50 % formic acid; the products were air-dried on Whatman 3MM paper and washed in running water overnight. The paper was then re-dried and the bound [^{3}H]xyloglucan assayed using OptiScint HiSafe scintillation fluid (Wallac, Milton Keynes, Bucks, U.K.). XET activity is expressed in katals (1 kat = 1 mol of product per s) per 30 μl assay.

Assay of xyloglucan

Xyloglucan was assayed by the method described in [24]. To 200 μl of sample (typically containing xyloglucan at 0.02–1.0 mg/ml), 1 ml of 20 % Na_2SO_4 was added, followed by 200 μl of a solution containing 0.5 % I_2 and 1 % KI. The A_{620} was measured within 10 min.

GPC

Bio-Gel A-0.5M columns (bed volume ≈ 90 ml, internal diameter 2.5 ml) were run at ≈ 0.5 ml/min at 4 °C. Columns were equilibrated in buffer B [100 mM succinate(Na^+) (pH 5.5)/10 mM $CaCl_2$] and either 1 M NaCl or 10 % glycerol. Fractions of 1.5 ml were collected and assayed for XET activity.

Polyacrylamide gels

Proteins were separated by SDS/PAGE in a 14 % (w/v) gel [27]. Samples were solubilized in sample buffer to give a final concentration of 5 % SDS/10 % glycerol/0.02 % Bromophenol Blue/62.5 mM Tris/HCl (pH 6.8)/50 mM iodoacetamide [28] and boiled for 5 min before loading. Proteins were visualized using a silver stain.

Optimized purification method

A sample (0.5 ml) containing XET activity was incubated in 0.2 % (w/v) tamarind xyloglucan for 30 min at 4 °C in buffer B. The solution was then spiked with Blue Dextran (0.8 mg) and subjected to GPC in buffer B containing 1 M NaCl. The void fractions (blue) were pooled, concentrated by ultrafiltration using dialysis tubing and poly(ethylene glycol) 20000, and fractionated on a second GPC column in buffer B containing 10 % glycerol and 210 μM xyloglucan oligosaccharides.

RESULTS

Ammonium sulphate precipitation of XET activities from crude extracts

Step-wise addition of $(NH_4)_2SO_4$ to crude extracts of cauliflower florets and mung bean seedlings revealed distinct precipitable XET activities (Figure 1). These may represent distinct isoforms of XET and so were treated separately.

Xyloglucan-dependent shift in elution pattern of XET from GPC columns

XET activity in a crude extract of cauliflower florets eluted near the totally included volume (V_t) on Bio-Gel A-0.5M (fractionation range 10–500 kDa; Figure 2, ●). The peak of activity extended past the V_t and was broader than the peak of internal marker [^{3}H]glucose (Figure 2, –·–). This suggests a slight affinity of XET for the agarose of the column. Similar behaviour on GPC has been observed for a xyloglucan hydrolase/endotransglycosylase [23] but is not sufficient for enzyme purification, as shown by the protein profile (Figure 2, –·–).

XETs form remarkably long-lived, probably covalent, complexes with xyloglucan [25] and we have isolated such complexes as a step towards enzyme purification. After incubation of the crude extract with 0.2 % tamarind xyloglucan (≈ 1 MDa), the activity eluted almost solely in the void volume (V_0; Figure 2, ■), indicating that a stable, high-molecular-mass complex had been formed. The complex remained in V_0 when the eluent contained 2 M NaCl, 2 M guanidinium hydrochloride or 2 M urea (results not shown), as expected for a covalent XET–xyloglucan bond.

SDS/PAGE of the V_0 material revealed numerous bands; fewer bands were present when the eluent contained NaCl

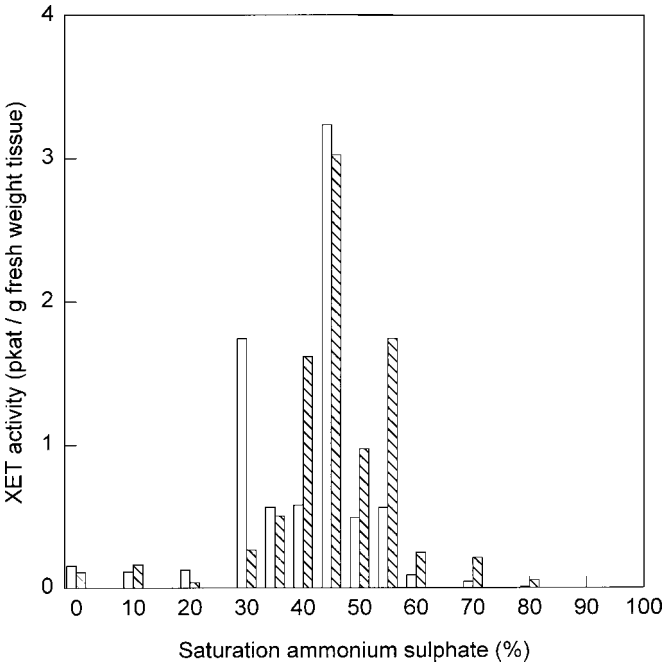

Figure 1 Ammonium sulphate fractionation of XET activity

Crude extracts of cauliflower florets (open bars) and mung bean seedlings (hatched bars) were fractionated by step-wise addition of $(NH_4)_2SO_4$ at 4 °C. Each pellet was resuspended in buffer B and assayed for XET activity.

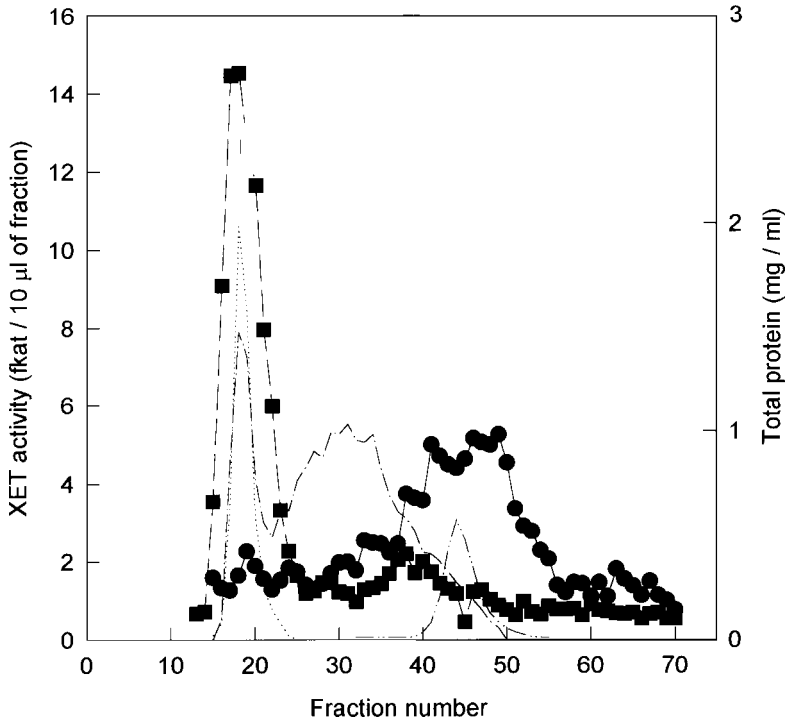

Figure 2 GPC of crude cauliflower XET in the presence and absence of xyloglucan

Cauliflower extract was chromatographed on Bio-Gel A-0.5M in the absence of xyloglucan (●), and after a pre-incubation in 0.2% (w/v) tamarind xyloglucan for 30 min at 4 °C (■). The V_0 was determined using 2 MDa Blue Dextran (· · · · ·) and the V_t with [^{3}H]glucose (–· ·–· ·), both on an arbitrary *y*-axis scale. Protein concentration (–·–·–) was estimated with the Bio-Rad dye-binding protein assay: measurements are shown for the '■' data; a very similar profile (results not shown) was obtained for the '●' data.

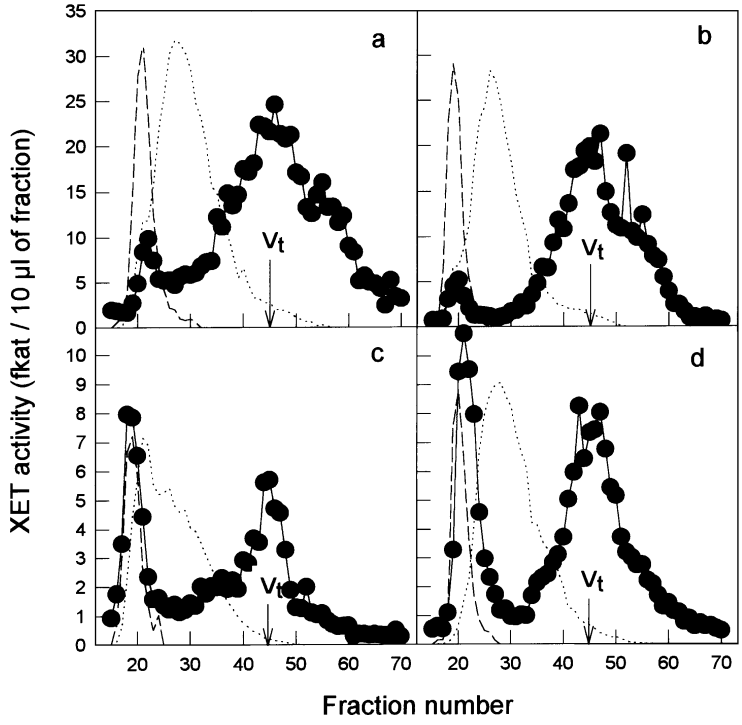

Figure 3 Disruption of XET–xyloglucan complexes in the presence of xyloglucan-derived oligosaccharides

$(NH_4)_2SO_4$-precipitated XET activities, complexed with xyloglucan as in Figure 2, were re-chromatographed in the presence of oligosaccharides. The graphs show XET activity (●), the void marker Blue Dextran (– – –; on an arbitrary *y*-axis scale), the V_t (arrows) and xyloglucan (· · · · · · · ; also on an arbitrary *y*-axis scale). The activities used were those precipitated from cauliflower extract by 45% saturated $(NH_4)_2SO_4$ (**a**), and from mung bean extract by 45% (**b**), 35% (**c**) and 55% (**d**) saturated $(NH_4)_2SO_4$.

Table 1 Typical yield and degree of purification of XET activity precipitated by 40–45% saturated $(NH_4)_2SO_4$

*This activity was assayed after dialysis to remove the oligosaccharides from the column eluate.

Fraction	Total volume (ml)	Protein quantity (mg)	Specific activity (pkat/mg)	Fold purification	Recovery (%)
$(NH_4)_2SO_4$ pellet	1.00	31.2	17.0	1	100
Void from first Bio-Gel column	8.25	8.75	25.6	1.51	42.2
Pure XET from second Bio-Gel column	26.4	0.0124	3640*	214	8.51

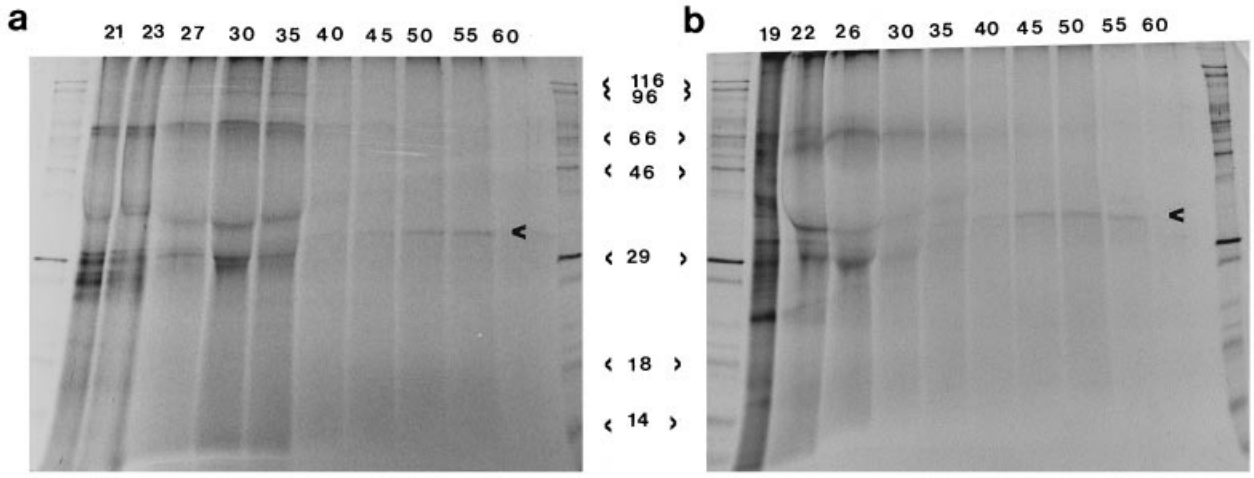

Figure 4 SDS/PAGE of the fractions shown in Figures 3a (a) and 3b (b)

Lanes are labelled with GPC fraction numbers. Large arrowheads indicate the purified XETs; small arrowheads show marker proteins (in kDa).

(results not shown), which was therefore added in subsequent purification work.

Purification of XET isoenzymes

To purify the putative XET isoenzymes, we added xyloglucan-derived oligosaccharides, which disrupted the XET–xyloglucan complex and shifted the activity back into the included volume.

$(NH_4)_2SO_4$-precipitated XET activity was mixed with xyloglucan and the complex was isolated by GPC as above. The void fractions were concentrated and re-chromatographed on Bio-Gel A-0.5M in the presence of 10% glycerol and 210 μM xyloglucan-derived oligosaccharides. (As the XETs were progressively purified, their activity became unstable; this problem could be minimized by addition of 10% glycerol.) During the running of this column, the oligosaccharides presumably acted as acceptors in an XET-catalysed transglycosylation reaction, allowing the XET to fall away from the xyloglucan and therefore to elute near the V_t, well resolved from the majority of the xyloglucan (Figure 3). The occurrence of the transglycosylation event is supported by the oligosaccharide-dependent partial degradation of the xyloglucan, shown by the shifting of the xyloglucan away from V_0 (Figure 3). This method was used successfully to purify all $(NH_4)_2SO_4$-precipitated XET samples tested (Figure 3).

The recovery of activity was typically $\approx$ 10%, with $\approx$ 200-fold purification (Table 1). This compares well with the 10.5% recovery and an apparent 9.5-fold purification reported by Edwards et al. [23] and the 24% recovery and 87-fold purification reported by Nishitani and Tominaga [4], both using more complex and less generally applicable procedures. We have not critically tested the capacity of our method, but we assume that at least one active XET molecule would bind per xyloglucan chain, giving a capacity of 10 nmol ($\approx$ 0.3 mg) of enzyme per column run. It can also be assumed that only active enzyme molecules would be obtained by our method. In support of this idea, the specific activity of the XET purified from mung bean (3600 pkat/mg) was 400 times higher than that of an *Arabidopsis* XET purified from transgenic *Escherichia coli* [22].

SDS/PAGE of the GPC fractions of the major $(NH_4)_2SO_4$-precipitable peak from cauliflower (Figure 3a) indicated that the V_0 (fractions 21–23) contained a large array of polypeptides (Figure 4a). Fractions 27–35 contained several polypeptides, presumably those that had been associated specifically with the xyloglucan and co-shifted with the xyloglucan from V_0. Fractions 45–55 contained a single $\approx$ 32 kDa polypeptide corresponding to a pure cauliflower XET. Similar results were obtained with mung bean (Figure 4b).

DISCUSSION

This paper reports a novel, general and simple method for purifying XET activities to homogeneity. Previously characterized XETs are $\approx$ 32 kDa [4,6,29], and would therefore be expected to be totally included on Bio-Gel A-0.5M. However, on addition of xyloglucan ($\sim$ 1 MDa), XET activity shifted to the void volume owing to the formation of a stable xyloglucan–XET

complex [25]. Most proteins in a crude plant extract have molecular mass < 500 kDa and do not bind xyloglucan; this step therefore achieves a substantial purification of the XET. Final purification was achieved by addition of oligosaccharides (XET acceptor substrates) to dissociate the complex. The XET activity now shifted back to near the totally included volume, as expected for a 32 kDa protein, yielding a final product that appeared to be homogeneous by SDS/PAGE.

The method reported will be valuable for the purification of XET isoenzymes both from crude plant extracts and from transgenic expression systems. This will enable enzymological characterization of pure XET proteins and hence give insight into their biological roles.

We thank Dr. J. E. Thompson for constructive discussions and Mrs. J. G. Miller for excellent technical assistance. We thank the Biotechnology and Biological Sciences Research Council for financial support.

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Received 6 October 1998/4 January 1999; accepted 17 February 1999