Xyloglucan endotransglycosylase: evidence for the existence of a relatively stable glycosyl–enzyme intermediate

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Xyloglucan endotransglycosylases (XETs) catalyse the breakdown of xyloglucan molecules predominantly by transglycosylation. In this process, fragments of cleaved polysaccharide are preferentially transferred to other xyloglucan molecules or their oligosaccharide subunits, with overall retention of the anomeric configuration of the glycosidic bond. In accordance with the theory, we propose that the cleavage and re-formation of the glycosidic bond in xyloglucan involves the formation of a glycosyl–enzyme intermediate which decomposes by transfer of the glycosyl moiety to a suitable carbohydrate acceptor. XETs from nasturtium seed cotyledons, mung bean hypocotyls and cauliflower florets interacted with xyloglucan to form complexes

of high M_r as judged by gel-permeation chromatography. The nasturtium enzyme also showed evidence of XET–xyloglucan complex-formation according to anion-exchange chromatography and adsorption of the complex to filter paper on the basis of affinity of its xyloglucan moiety for cellulose. The XET–xyloglucan complex was stable in water, 6 M urea and acidic and alkaline buffers (pH 2.5–9.5), but readily decomposed by transferring its glycosyl moiety to xyloglucan-derived oligosaccharides or by incubation with the strong nucleophile imidazole at pH 3.8–9.6. These results strongly support the assumption that XET forms a relatively stable covalently linked glycosyl–enzyme intermediate.

INTRODUCTION

The xyloglucan-specific endo-(1 → 4)- β -D-glucanase from nasturtium (*Tropaeolum majus*) cotyledons was first characterized as an enzyme involved in the degradation of seed storage xyloglucan after germination [1,2]. An interesting property of the enzyme is that it degrades xyloglucan not only by hydrolysis but also partly by a transglycosylation mechanism, endocleaving β -(1 → 4)-linked glucan main chains of xyloglucan molecules and transferring the newly formed reducing ends of polysaccharide fragments to C-4 positions of the glucose units at the non-reducing ends of other xyloglucan molecules or their oligosaccharide subunits; transglycosylation predominates over hydrolysis when acceptor substrate concentrations are high enough [3,4]. Similar enzyme activities had been detected in cell-suspension cultures [5,6] and were also characterized [7,8] and purified [9] from the growing organs of numerous plants; they are collectively termed xyloglucan endotransglycosylases (XETs) or xyloglucan–xyloglucosyltransferases (EC 2.4.1.207). Since xyloglucan is an important hemicellulosic component of primary cell walls in dicotyledonous plants, where it may cross-link the microfibrils of cellulose [10–12], the existence of an enzyme capable of endocleaving and rejoining xyloglucan chains would seem likely to be of essential importance for cell wall expansion and thus plant growth [13].

Assuming that the transglycosylation catalysed by XET follows the general two-step mechanism known for other transglycosylating glycanases [14], we proposed [3] that, in the course of the reaction with xyloglucan, the enzyme forms a glycosyl–enzyme intermediate with the newly formed reducing end of the cleaved xyloglucan. The XET–xyloglucan intermediary complex thus formed could then decompose by transferring the attached glycosyl to either water (hydrolysis) or, preferably, another molecule of xyloglucan or a xyloglucan-derived oligosaccharide

(XGO) (transglycosylation). The predominance of transglycosylation over hydrolysis is indicated by the fact that the decomposition of xyloglucan by XET is greatly stimulated by XGOs [3,15,16].

The aim of the present work was to probe the validity of the proposed mechanism by demonstrating the existence of the putative XET–xyloglucan intermediate complex and to investigate its nature.

EXPERIMENTAL

Substrates

Tamarind (*Tamarindus indica*) seed xyloglucan was prepared as described by Edwards et al. [1] with some modifications: the precipitation with Fehling's solution was omitted and, instead, the crude xyloglucan was purified by batch adsorption of coloured impurities on DEAE-cellulose (acetate form). The M_r of the prepared xyloglucan was in the range 10^5 – 10^6 as determined by gel filtration on Sepharose CL-6B (Pharmacia).

[³H]Xyloglucan was prepared by feeding suspension-cultured spinach (*Spinacia oleracea* L.) cells with L-[1-³H]arabinose. The medium was as described previously [17] except that it contained 0.2 % glucose and 60 MBq of L-[1-³H]arabinose (Amersham; specific radioactivity 92 TBq/mol). The medium (5 ml) was sterilized by autoclaving, transferred to a 60 ml Sterilin polycarbonate vessel, inoculated with 0.2–0.3 g fresh weight of 11-day-old spinach cells and incubated aseptically under standard culture conditions [17] for 6 days. The cells were harvested by centrifugation, washed with water and extracted in sequence with 7 % (v/v) formic acid, water and phenol/acetic acid/water (2:1:1, w/v/v). The extracts were rejected and the sediment was further extracted in succession with 7 ml of 10 % EDTA (sodium salt), pH 6.5, at 100 °C for 2 h (pectin fraction; 1.1 MBq), 10 ml

Abbreviations used: XET, xyloglucan endotransglycosylase; XGO, xyloglucan-derived oligosaccharide; XLLGol, reduced nonasaccharide of xyloglucan (Xyl₃-Gal₂-Glc₃-Glucitol).

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of 0.5 M NaOH/0.5 % NaBH₄ at 25 °C for 16 h (hemicellulose 1 fraction; 3.9 MBq), 6 M NaOH/0.5 % NaBH₄ at 25 °C for 16 h (hemicellulose 2 fraction; 3.8 MBq) and 6 M NaOH/0.5 % NaBH₄ at 37 °C for 16 h (hemicellulose 3 fraction; 0.17 MBq). The alkaline extracts were neutralized with acetic acid, dialysed against several changes of distilled water for 24 h at 4 °C and lyophilized. The hemicellulose 2 fraction, which contained radioactive xyloglucan, was further purified by passage through a column of DEAE-Spheron (Lachema, Brno, Czech Republic) in 20 mM Tris/HCl buffer, pH 7.2. The material not retained by the column (about 40 % of the total applied radioactivity) was dialysed against water and concentrated by ultrafiltration (M_r cut-off 10000) to a final volume of about 1 ml. It yielded [³H]isoprimeverose (a diagnostic marker for xyloglucan) on hydrolysis with Driselase (Sigma) and analysis by paper chromatography [18]. The mass of [³H]xyloglucan obtained was too small for the specific radioactivity to be estimated.

XGOs (a mixture of oligomers of degree of polymerization 7–9) were obtained by hydrolysis of xyloglucan with partially purified *Trichoderma reesei* cellulase as described previously [19]. A xyloglucan-derived nonasaccharide (XLLG [20]) was prepared by the method of Lorences and Fry [16] and converted into its radioactive alditol ([1-³H]XLLGoI) by reduction with NaB³H₄. Its specific radioactivity was about 20 MBq/μmol.

Enzymes

XET was purified to homogeneity from extracts of germinated nasturtium (*Tropaeolum majus*) seeds by a combination of (NH₄)₂SO₄ precipitation, anion-exchange chromatography, hydrophobic-interaction chromatography and gel-permeation chromatography [19]. Its purity was checked by SDS/PAGE; it yielded a single band on silver staining of the gel.

Mung bean hypocotyls and cauliflower florets were extracted as follows. Finely chopped plant tissue (400 g fresh weight) was mixed with 600 ml of 350 mM sodium succinate buffer, pH 5.5, containing 10 mM CaCl₂ and 5 mM L-cysteine, and blended in an 'AtoMix'. The homogenate was stood at 4 °C for 2 h with occasional mixing, then strained through four layers of muslin followed by Miracloth. The filtrate was centrifuged at 2500 g at 0 °C for 30 min. The supernatant was subjected to (NH₄)₂SO₄ fractionation. Protein that precipitated between 40 and 45 % saturation was redissolved in 200 mM succinate buffer, pH 5.5, containing 10 mM CaCl₂ and demonstrated by the radiometric assay to have high XET activity.

Immunochemical methods

Mouse polyclonal antibodies were prepared against purified XET by conventional methods [21]. The mice were immunized with three intraperitoneal 0.5 ml injections (25–30 μg of XET protein) at 3-week intervals. The first dose contained complete Freund's adjuvant, and the remaining two doses were in physiological saline. The immune serum was further purified by fractional precipitation with (NH₄)₂SO₄ and dialysis. An ELISA method using peroxidase-conjugated swine anti-mouse IgG serum (USOL, Prague, Czech Republic) was employed for detection and quantification of XET. Standardization was achieved by serial dilution of the enzyme in buffer.

Other methods

The activity of XET was assayed colorimetrically [19] and/or radiometrically [8]. Proteins were determined by use of the Folin reagent, and carbohydrates by the phenol/H₂SO₄ method [22]. Radioactivity was measured by liquid-scintillation counting using

a toluene-based liquid-scintillation cocktail; for assay of aqueous solutions, the scintillant was mixed with Triton X-100 in a ratio of 2:1 (v/v).

RESULTS

Gel-permeation chromatography of nasturtium XET

As a first approach, we attempted the isolation of the putative XET–xyloglucan intermediary complex by different chromatographic techniques. Purified XET (280 μg of protein) was mixed with 6.4 mg of xyloglucan and, after incubation for 30 s at 37 °C, the mixture was cooled to 0 °C and applied to a column of Bio-Gel P-60 (2.2 cm × 100 cm) and eluted with 0.05 M phosphate buffer, pH 7, containing 0.15 M NaCl, at 4 °C. The pH of the eluting buffer was chosen so that it differed from the pH optimum of the enzyme (4.5–5.0 [23]) in order to inhibit undesirable progress of the reaction during chromatography. A control mixture contained XET without xyloglucan. ELISA showed that the XET was eluted with a substantially higher apparent M_r in the presence of xyloglucan (Figure 1B) than in its absence (Figure 1A). Pooled fractions containing XET and xyloglucan (Figure 1B) were concentrated by ultrafiltration or lyophilization and rechromatographed on the same column. The XET was re-eluted in exactly the same fractions (Figure 2A) as during the first chromatography. However, if the XET–xyloglucan fractions were incubated with 1 mg/ml XGOs at pH 5 and 37 °C for 30 min before rechromatography, the XET was eluted in the same position as the free enzyme (Figure 2B).

To probe the pH stability of the XET–xyloglucan complex we incubated the lyophilized fractions in 0.1 M glycine/HCl buffer, pH 2.5, and/or 0.1 M glycine/NaOH buffer, pH 9.5, at 37 °C for 30 min, and then rechromatographed the material on a Bio-Gel P-60 column eluted with the respective buffers containing 0.15 M NaCl. Whereas at pH 2.5 the complex did not dissolve well in the

Figure 1 Elution profile of (A) XET and (B) XET + xyloglucan mixture on Bio-Gel P-60

The void (V_0) and totally included (V_t) volumes were determined with horse spleen ferritin ($M_r \sim 450\,000$) and [³H]glucose respectively.

Figure 2 Rechromatography on Bio-Gel P-60 of (A) XET-xyloglucan complex recovered from the peak fractions in Figure 1(B) and (B) elution of the same complex incubated with XGOs before chromatography

The conditions were as in Figure 1.

buffer and did not penetrate into the column, at pH 9.5 the position of the XET-xyloglucan peak in the elution profile was the same as at pH 7 (not shown).

Figure 3 Elution profile of mung bean XET activity on Sephadex G-75 in the presence and absence of xyloglucan

The column was pre-equilibrated with buffer (200 mM succinate buffer, pH 5.5, containing 10 mM CaCl_2) with (■) or without (●) 0.1% (w/v) tamarind xyloglucan, and elution was continued with the same solution at 4 °C. The enzyme sample to be chromatographed in the presence of xyloglucan was preincubated in buffer containing 0.1% xyloglucan at 4 °C for 30 min before loading on to the column. The void (V_0) and totally included (V_t) volume were determined with tamarind xyloglucan ($M_r \sim 10^6$) and [^3H]glucose respectively; the column bed volume was about 80 ml.

Figure 4 DEAE-Spheron chromatography of (A) [^3H]xyloglucan (○) and XET (●) separately

In (B) the [^3H]xyloglucan and XET were mixed before application to the column.

Gel-permeation chromatography of XETs from growing plant tissues

XET extracted from mung bean hypocotyls exhibited a xyloglucan-induced shift in elution volume on gel-permeation chromatography on Sephadex G-75. On a column pre-equilibrated with 0.1% xyloglucan in 200 mM succinate buffer, pH 5.5, containing 10 mM CaCl_2 , most of the activity (assayed radio-metrically) was eluted in the void volume, whereas the same enzyme, when chromatographed in the absence of added xyloglucan, was eluted as a broad peak within the partially included volume (Figure 3). Thus XETs from a tissue undergoing rapid cell expansion showed an ability, comparable with that of the seed enzyme, to interact with xyloglucan permanently enough to cause a shift in chromatographic behaviour. Similar data were obtained for XET activity extracted from cauliflower florets (results not shown). This chromatographic shift could potentially form the basis of a method for affinity purification of XETs.

Anion-exchange chromatography of XET-xyloglucan complex

The nasturtium enzyme (35 μg of protein in 100 μl of 0.05 M citrate/phosphate buffer, pH 5.5) was mixed with 4.55 kBq of purified [^3H]xyloglucan at 37 °C for 30 s, cooled to 0 °C, applied to a column (1 cm $\times$ 2.5 cm) of DEAE-Spheron equilibrated with 0.02 M Tris/HCl buffer, pH 7.2, and eluted with a salt gradient (0–1 M NaCl) in the same buffer at 4 °C. In control experiments, the XET and [^3H]xyloglucan were chromatographed separately. The presence of XET in the fractions was detected by ELISA; radioactivity was measured by liquid-scintillation counting. Whereas the radioactive xyloglucan was not retained by the column, the XET was eluted with approx. 0.24 M NaCl (Figure 4A). When mixed before the chromatography, a portion of

Table 1 Efficiency of various treatments in desorbing XET from the XET–xyloglucan complex adsorbed on filter paper

The conditions were as described in the Experimental section. Results are means $\pm$ S.E.M.

Treatment	Desorption of XET (%)
Water	12 $\pm$ 6
Buffer, pH 2.5	2 $\pm$ 4
Buffer, pH 9.5	20 $\pm$ 5
Aq. XGOs (1 mg/ml)	78 $\pm$ 12
6 M Urea, pH 7.0	74 $\pm$ 10
0.1 M Imidazole, pH 9.6	68 $\pm$ 16

Figure 5 Time course of XET release (∇) from a XET–xyloglucan complex adsorbed on cellulose, the incorporation of [$1\text{-}^3\text{H}$]XLLGol from the incubation medium into the insoluble phase ($\circ$) and the remaining radioactivity in the medium ($\bullet$)

Strips (1 cm $\times$ 1.5 cm) of Whatman 3MM filter paper were impregnated with 70 μl of mixture containing 22.5 μg of XET and 0.23 mg of xyloglucan and dried in a stream of air at ambient temperature. The strips were washed twice in water and immersed into duplicate solutions containing 0.1 M succinate buffer, pH 5.0, and 8 kBq (0.4 nmol) of [$1\text{-}^3\text{H}$]XLLGol in 0.5 ml and incubated at 35 $^{\circ}\text{C}$. At time intervals, the strips were removed, thoroughly washed in cold distilled water, dried and assayed for the incorporated radioactivity. Aliquots (100 μl) from the incubation mixtures were taken to determine the remaining radioactivity and the desorbed XET.

[^3H]xyloglucan was coeluted with XET at about 0.19 M NaCl, which differed from the elution position of both the free enzyme and the free polysaccharide (Figure 4B). The same result was obtained when the chromatography was performed in the buffer containing 6 M urea (not shown).

Adsorption of XET–xyloglucan complex on cellulose

In this experiment, we took advantage of the well-known fact that xyloglucan binds strongly to cellulose [10]. It was assumed that the XET–xyloglucan complex would also exhibit strong affinity towards cellulose. The enzyme (50 μg of protein) was mixed with 1 mg of unlabelled xyloglucan or 1 mg of [^3H]xyloglucan (1.2 kBq) in 200 μl of 0.05 M citrate/phosphate buffer, pH 5.5, and the mixture applied to a strip (1 cm $\times$ 4 cm) of Whatman 3MM filter paper and dried in a stream of air at ambient temperature. A control mixture contained XET without xyloglucan. Desorption of the XET was carried out by incubating the paper strips at 30 $^{\circ}\text{C}$ for 30 min with two 2 ml portions of water, buffer (0.1 M citrate/phosphate, pH 2.5, or 0.1 M car-

Figure 6 Release of XET (XET_R) as a function of time (t) from the cellulose-adsorbed XET–xyloglucan complex into 0.1 M imidazole at pH 3.8 ($\circ$) and pH 9.6 ($\bullet$) respectively and 30 $^{\circ}\text{C}$

Strips (1 cm $\times$ 1.5 cm) of Whatman 3MM filter paper were impregnated with 70 μl of mixture containing 9.5 μg of XET and 0.1 mg of xyloglucan. The broken line has been fitted to the first-order kinetics equation $\text{XET}_R = A(1 - e^{-kt})$ where k is the apparent rate constant and A is the percentage of enzyme released at the steady state. The calculated values were $k = 0.057 \text{ min}^{-1}$ and $A = 53.6$.

bonate, pH 9.5), 6 M urea, 0.1 M imidazole, pH 3.8 and 9.6, or a solution (1 mg/ml) of XGOs (degree of polymerization 7–9) in water. XET was determined in the combined washings by ELISA and [^3H]xyloglucan radiometrically.

Table 1 shows that desorption with acidic or alkaline buffer was low and similar to that obtained with water. In controls, in which XET was applied to the paper without xyloglucan, practically all the enzyme applied could be washed out with water. Up to 90 % release of XET from the paper-adsorbed complex could be achieved by incubating the paper strips for 2 h at 30 $^{\circ}\text{C}$ with XGOs (Table 1). Efficient desorption of XET from the complex adsorbed on cellulose was also achieved with 6 M urea at pH 7. With this treatment, however, both the enzyme protein and xyloglucan were desorbed to the same extent. A major portion (up to 85 %) of the enzyme could also be removed from the adsorbed complex with 0.1 M imidazole, pH 9.6.

The liberation of XET from the adsorbed XET–xyloglucan complex by incubation with XGOs was time-dependent and concurrent with the incorporation of xyloglucan nonasaccharide [$1\text{-}^3\text{H}$]XLLGol from the medium into the insoluble phase (Figure 5). There was neither release of XET nor incorporation of radioactivity into the insoluble phase observed when the enzyme was heat-denatured before application to the paper.

When the cellulose-adsorbed XET–xyloglucan complex was incubated with 0.1 M imidazole adjusted to pH 3.8 or pH 9.6, the liberation of the enzyme protein proceeded in a time-dependent manner with pseudo-first-order kinetics and the rate constant $k = 0.057 \text{ min}^{-1}$, independently of pH, and over 56 % of the XET originally applied was released at equilibrium (Figure 6). The imidazole did not solubilize the xyloglucan portion of the complex from cellulose, indicating that the linkage between the two moieties in the complex had been broken.

It is noteworthy that the enzyme desorbed from the complex by XGOs has retained its activity fully whereas the enzyme released by imidazole was largely inactive.

DISCUSSION

According to a general scheme valid for retaining glycanases (i.e. those acting with the net retention of the anomeric configuration of the glycosidic bond), the catalytic reaction proceeds by a double-displacement mechanism and involves participation of two carboxy groups, usually the side-chain carboxy groups of aspartic and glutamic acid residues, in the catalytic site of the enzyme (for a review see ref. [14]). At optimum pH for the reaction, one of the carboxy groups is protonated and functions as a general acid by donating a proton to the leaving aglycone, whereas the other carboxy group is dissociated and stabilizes the transitory oxocarbenium ion formed in the first reaction step by formation of an ester linkage with the remaining glycosyl. In the second step, the glycosyl-enzyme intermediate decomposes by transfer of the glycosyl residue to an acceptor molecule which could be either water (hydrolysis) or an alcohol such as a sugar (transglycosylation).

Several lines of evidence obtained in this work support the existence of a relatively stable (i.e. resistant to water attack) covalently linked enzyme-glycosyl intermediary complex in the reaction catalysed by the XET. This assumption is supported by following observations.

(1) There was a shift in the position of the XET peak towards higher M_r values during gel-permeation chromatography when the enzyme was mixed with xyloglucan (.....) before the application to the column (Figures 1 and 3). The elution position of the XET-xyloglucan peak was unchanged on rechromatography. However, when the XET-positive fractions were incubated with XGOs before rechromatography, the complex decomposed, and XET was eluted from the column in the same position as the free enzyme (Figures 2A and 2B). The liberation of the enzyme from the complex can be explained as being due to transfer of bound glycosyl to the oligosaccharide acceptor according to the scheme:

(2) During anion-exchange chromatography, the [^3H]xyloglucan alone did not bind to DEAE-Spheron (Figure 4A), but a portion of the radioactivity was retained when applied to the column in combination with XET. The bound [^3H]xyloglucan was coeluted from the column with XET somewhat earlier than the elution position of the free enzyme, indicating that the charge/mass ratio of the enzyme was decreased, presumably because of its firm association with xyloglucan (Figures 4A and 4B). The covalent nature of the association between the enzyme and [^3H]xyloglucan is indicated by the observation that the elution pattern of the mixture did not change when the chromatography was performed in the presence of 6 M urea.

(3) XET alone only bound poorly to cellulose paper but it was firmly bound when applied in combination with xyloglucan. We explain this behaviour as being due to formation of a complex between XET and xyloglucan and its subsequent binding to

cellulose via its xyloglucan moiety. The enzyme from the adsorbed complex could be released in a time-dependent manner by incubation with XGOs or with the strong nucleophilic agent imidazole. In the first case, the liberation of the enzyme could be regarded as the result of an exchange between XET and the oligosaccharide (XGO*) mediated by transglycosylation (Scheme 1). The ability of complexed XET to catalyse the exchange reaction shows that the glycosyl-enzyme complex was trapped on the paper in the native fully competent state. Xyloglucan glycosyls associated non-covalently with XET could not be transferred to oligosaccharide acceptor. Preliminary results showed that the adsorption of the XET-xyloglucan complex on cellulose and the subsequent release of the enzyme by incubation with XGOs can be effectively exploited in the purification of XET from plant extracts.

Assuming that an ester link exists between the enzyme and the glycosyl residue in the complex [14], the release of XET by imidazole can be explained as being due to a nucleophilic attack of imidazole on the ester bond with the formation of an *N*-imidazolyl-protein complex in the active site of the enzyme [24]. In this context, it is interesting to note that imidazole and its derivatives have been described as effective active-site-directed inhibitors of glycosidases [24,25]. Further work will be needed to verify this assumption.

Somewhat surprising is the stability of the glycosyl-enzyme complex at alkaline pH, which is contrary to what would be expected if the ester bond were present, as it is, for example, in the case of sucrose phosphorylase [26] or levansucrase from *Bacillus subtilis* [27]. A possible explanation for the observed pH stability of the glycosyl-XET complex could be that, in addition to covalent linkage, the complex is stabilized through hydrophobic interactions between the enzyme and its substrate xyloglucan. It is known that some polysaccharidases contain, besides the catalytic domains, specific binding domains remotely positioned on the protein molecule and rich in the aromatic amino acids tyrosine and tryptophan whose hydrophobic interactions with pyranose units in the polysaccharide confer additional specificity and stability to enzyme-substrate complexes. Well-documented examples include cellulases [28,29] and xylanases [30] from different sources.

With regard to the postulated role of XET in plant cell wall expansion [13], one could envisage that the formation of stable XET-xyloglucan complexes in the plant cell wall might represent a means of converting the enzyme into a latent state in the plant cell wall until suitable acceptors, e.g. newly arriving xyloglucan molecules [31] or fragments of xyloglucan formed by the action of a non-specific or xyloglucan-specific (1 $\rightarrow$ 4)- β -D-glucanase [32], become available.

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Scheme 1 Exchange between XET and radioactive oligosaccharides (XGOs*) in the cellulose-adsorbed XET-xyloglucan (XG) complex

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