Kinetic Evidence of the Existence of a Stable Enzyme-Glycosyl Intermediary Complex in the Reaction Catalyzed by Endotransglycosylase

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Abstract. Xyloglucan-endotransglycosylase (XET) is an enzyme involved in the metabolism of xyloglucan (XG) in plant cell walls and seeds. This enzyme acts both as a hydrolase and as a transglycosylase by transfering the fragments of xyloglucan molecules to other XG molecules or xyloglucan-derived oligosaccharides (XGOs). In this work, we studied the kinetics of interaction between XET and XG. The equilibrium in the reaction of XG degradation by XET was found to depend on the initial enzyme concentration and the availability of suitable glycosyl acceptors. After reaching the equilibrium, the addition to the reaction mixture of XET or XGOs caused further degradation of XG, and a new equilibrium with a higher degree of XG depolymerization was established. These results indicated that in the course of XG depolymerization, the enzyme is bound in a relatively stable temporarily inactive enzyme-glycosyl complex and this complex is decomposed by transfering its glycosyl moiety to suitable oligosaccharide acceptor. Mouse polyclonal antibody against XET linked to AffiGel 10 (Affi-Ab) adsorbed both XET and XET-XG complex but not [^{3}H]XG alone. XET immobilized onto Affi-Ab was able to bind [^{3}H]XG and catalyze transglycosylation in presence of XGOs.

Key words: Xyloglucan — Endotransglycosylase — Oligosaccharides — Enzyme-substrate complex.

Abbreviations: XG – xyloglucan, XET – xyloglucan-endotransglycosylase, XGOs – xyloglucan derived oligosaccharides, Affi-Ab – AffiGel 10 linked with antibodies against XET.

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Introduction

Xyloglucan (XG) is the major hemicellulose in the primary cell walls of dicotyledonous plants where it is associated by a strong hydrogen binding with cellulose microfibrils (Hayashi 1989) XG molecule is long enough to simultaneously form hydrogen-bonds with more than one cellulose microfibril and it has been proposed that XG forms tethers between cellulose microfibrils (Fry 1989) The breakage of xyloglucan chains would therefore have an important effect on wall plasticity and the enzymes capable of splitting and reconnecting the XG chains may have an essential importance for the plant growth (Fry et al 1992) Such enzyme activities have been detected in many plants and they are closely related to various physiological aspects of plant growth such as cell expansion (Fry et al 1992) fruit ripening (Maclachlan and Brady 1994) or seed germination (Edwards et al 1986) and were collectively termed xyloglucan endotransglycosylases (XETs) EC 2 4 1 207 This type of enzyme activity was also found in cell suspension cultures of plant cells (Takeda et al 1996 Nakamura and Hayashi 1993)

In some seeds (e g , nasturtium *Tropaeolum majus* L), XG serves as storage polysaccharide Its degradation during seed germination is brought about by a cooperative action of four enzymes (Edwards et al 1986) xyloglucan specific endo-(1-4)-β-D-glucanase β galactosidase, α-xylosidase, and β-glucosidase Xyloglucan specific endo-(1-4)-β-D-glucanase was found to be in fact a transglycosylase and for this reason it was later termed xyloglucan endotransglycosylase, XET (Farkaš et al 1992) In the absence of XGOs, the enzyme acts as a hydrolase but the XG depolymerization is very slow In previous papers (Farkas et al 1992, Sulova and Farkaš 1996) we proposed that an intermediary complex forms between XET and XG in the course of transglycosylation The present paper provides kinetic evidence for a relatively high stability of the assumed XET XG complex

Materials and Methods

Substrates

Tamarind (*Tamarindus indica*) seed xyloglucan was isolated by alkaline extraction according to Edwards et al (1985) with some modification In the last step, the dialyzed crude XG was dissolved in water and purified by batch treatment with DEAE cellulose in acetate form The M_r of the prepared XG was in the range 10^5 10^6 as determined by gel filtration

[^{3}H]xyloglucan was prepared by feeding suspension cultured spinach (*Spinacia oleracea* L) cells with L-[1-^{3}H]arabinose (Amersham, specific radioactivity 92 TBq/mol) as described elsewhere (Sulova et al 1998)

Enzyme purification

XET was purified to homogeneity from extracts of germinated (at 25°C 12 h/12 h light/night regime), nasturtium *Tropaeolum majus* L seeds by ammonium sulfate precipitation anion-exchange chromatography, hydrophobic-interaction chromatography and gel permeation chromatography (Sulova et al 1995) The degradation of XG was followed colorimetrically or viscometrically as described previously (Sulova et al 1995)

The time course of XG degradation by XET was fitted by non-linear regression according to equation (1)

$$w = w_{\infty}(1 - e^{-kt}) \quad (1)$$

where w and w_{∞} represent the portion of XG degraded at times t and $t \to \infty$ respectively k is the rate constant

Preparation of antibodies

Mouse polyclonal antibody against XET was prepared according to Harlow and Lane (1988) Mice were immunized with three intraperitoneal injections (0 5 ml 25 30 μg of XET) at three week intervals The first dose contained complete Freud's adjuvant the next two were in physiological solution The immune serum was further purified by ammonium sulfate precipitation, dialyzed and concentrated by ultrafiltration

Binding of XET and [^{3}H] XG to AffiGel 10

Binding of antibodies against XET to AffiGel 10 0 5 ml of AffiGel 10 (BioRad) was incubated with 1ml of antibody's solution (5 7 mg of protein/ml, 0 5 mol/l MOPS pH 7 2) at room temperature for 4 h The amount of bound protein was calculated as the difference between protein concentrations in the solution before and after the binding The gel with bound antibodies was centrifuged and washed three times with MOPS containing 0 15 mol/l NaCl and then 10 volumes of 100 mmol/l ethanolamine were added and incubated at room temperature overnight under mixing After blocking, ethanolamine was removed by centrifugation, and AffiGel antibody (Affi-Ab) was thoroughly washed with MOPS NaCl XET was bound to Affi-Ab in 0 5 mol/l MOPS pH 7 2 for 4 h The amount of XET bound was detected as the decrease of XET activity in the solution after binding or using the ELISA method

Results

Effect of enzyme concentration on the reaction equilibrium

The time-course of XG degradation by XET could be described as a pseudo first order reaction characterized by a rectangular hyperbole (Fig 1 4) Good agreement between measured and computed data was obtained when the experimental

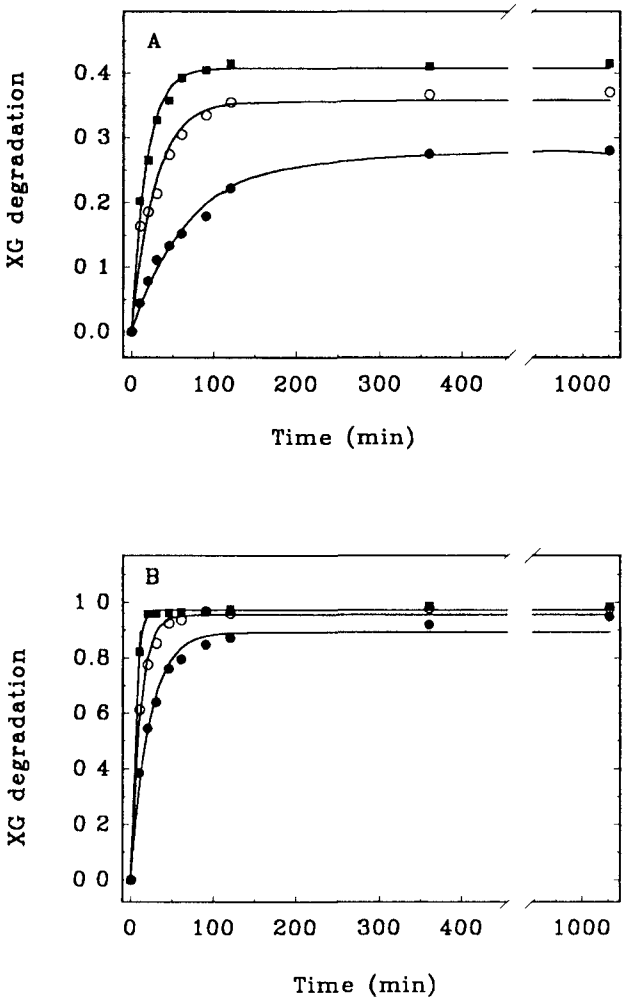


Figure 1. Time-course of XG degradation and its dependence on XET concentration in the absence and in the presence of XGOs *A* Two ml reaction mixture containing 2 mg of XG in 50 mmol/l citrate-phosphate buffer, pH 5 5 and (●) 2 8 μg XET, (○) 5 6 μg XET, (■) 11 2 μg XET *B* The same conditions as in *A* but in the presence of 1 mg/ml XGOs

data were fitted by equation (1) The kinetic parameters obtained by non-linear regression are shown in Table 1 The values of w_∞ depended on the initial enzyme concentration and the degree of XG degradation at equilibrium increased with the increasing XET concentration (Fig 1 4) Addition to reaction mixture of XGOs at

Table 1. XG degradation by XET in several concentrations kinetic parameters A in the absence and B in the presence of 1 mg/ml XGOs respectively

Amount of XET (μg)		w_∞	k (s^{-1})
A	2.8	$0.274 \pm 2.32 \times 10^{-3}$	$0.0143 \pm 3.07 \times 10^{-4}$
	5.6	$0.357 \pm 3.90 \times 10^{-3}$	$0.0355 \pm 1.36 \times 10^{-3}$
	11.2	$0.408 \pm 1.89 \times 10^{-3}$	$0.0560 \pm 1.11 \times 10^{-3}$
B	2.8	$0.893 \pm 5.80 \times 10^{-3}$	$0.0454 \pm 1.14 \times 10^{-3}$
	5.6	$0.955 \pm 3.53 \times 10^{-3}$	$0.0910 \pm 1.184 \times 10^{-3}$
	11.2	$0.969 \pm 1.89 \times 10^{-3}$	$0.1810 \pm 3.31 \times 10^{-3}$

w_∞ and k were obtained by nonlinear regression of experimental data from Fig. 1 according to equation (1) and represent the computed value ± S.E.M. (for 9 degrees of freedom)

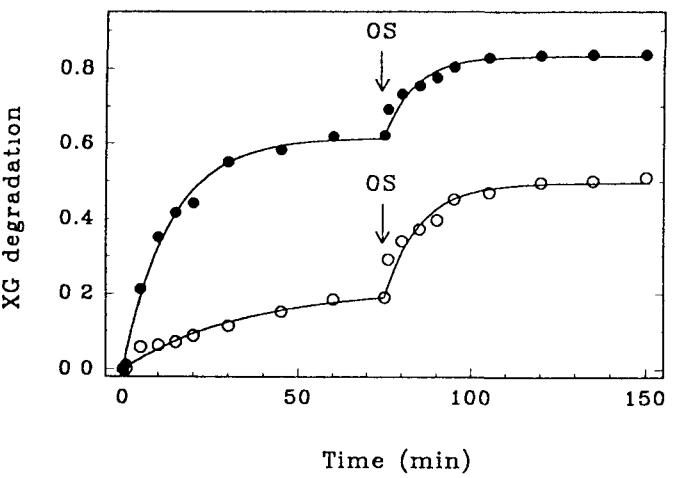


Figure 2. Effect of the addition of XGOs in the course of XG degradation by XET. Four ml reaction mixture containing 1 mg XG in 50 μmol/l citrate-phosphate buffer pH 5.5 and 5.6 μg XET (○) reaction started without XGOs (●) mixture containing 0.8 mg XGOs. At times indicated by arrows 0.4 mg XGOs were added to each reaction mixture

reaction equilibrium initiated further XG breakdown and shifted the equilibrium towards higher values. Saturating XGOs concentration was about 0.6 mg/ml (data not shown). At higher XGOs concentrations (1 mg/ml) the equilibrium values were no more enzyme concentration dependent (Fig. 1*B*). The effect of XGOs added in the course of the reaction is illustrated in Fig. 2. Addition of XET (Fig. 3) to the reaction mixture at equilibrium also had a similar effect. In both cases additional

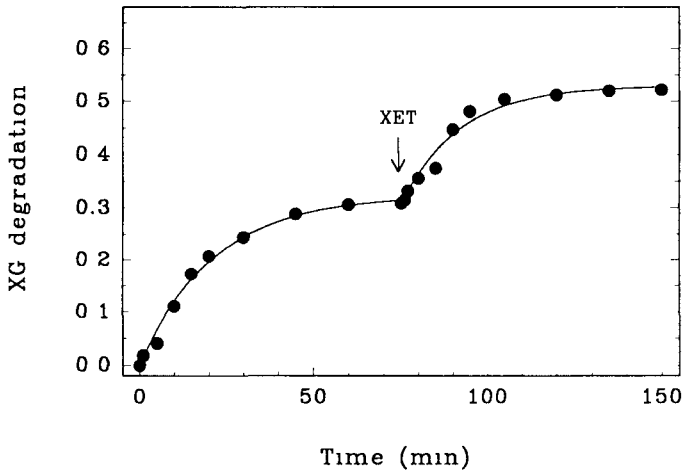


Figure 3. Effect of stepwise enzyme addition in the course of XG breakdown by XET Four ml reaction mixture containing 4 mg XG in 50 mmol/l citrate-phosphate buffer pH 5 5 and 11 6 μg XET the addition of XET (5 6 μg) is marked by the arrow

XG degradation was initiated and a new equilibrium was established at a higher level

[^{3}H]-XG and XET binding to Affi-Ab

In these experiments binding of XET and [^{3}H]XG alone or of XET [^{3}H]XG complex to AffiGel 10 linked with antibodies against XET was studied (Fig 5) The specificity of the antibody to XET was determined by Western blotting, and is documented in Fig 4 The antibody only stained a single protein band (M_r 25.000) in the crude extract from nasturtium seedlings corresponding to XET (Edwards et al 1986) XG alone did not bind to AffiGel or Affi-Ab (Fig 5) In contrast XET alone was binding onto Affi-Ab very extensively Immobilized XET obtained in this way retained its residual enzymatic activity (about 80 to 90% of that of the native enzyme) and could bind [^{3}H]XG from the solution (Fig 5) Moreover, when XET was preincubated with [^{3}H]XG under similar concentration ratios as mentioned above, addition of Affi-Ab to the preincubation mixture resulted in similar binding of XET as observed with the enzyme alone but the binding of [^{3}H]XG was enhanced (Fig 5) About 15% of bound [^{3}H]XG was released into the solution after the incubation of Affi-Ab-XET-[^{3}H]XG complex with 1 mg/ml XGOs in 0 5 mol/l MOPS, pH 7 2 whereas practically no radioactivity was released when the complex was incubated in the buffer alone this indicates that the enzyme had retained its transglycosylase activity even when bound on the carrier in the complex

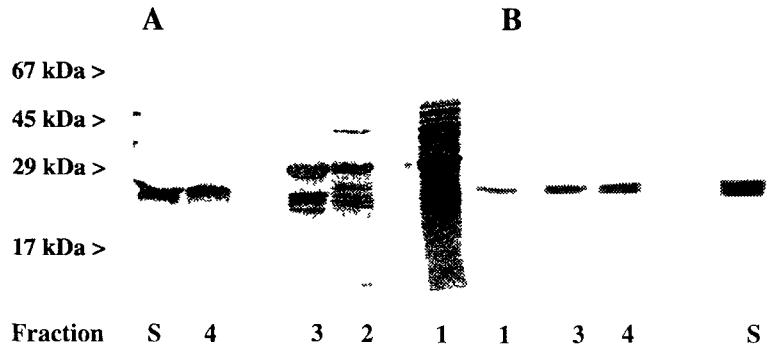


Figure 4. SDS PAGE and Western blot of fractions from individual purification stages *A* SDS PAGE electrophoresis 1 – crude extract, 2 – anion exchange chromatography (DEAE Spherion), 3 – hydrophobic interaction chromatography (phenyl sepharose), 4 – size exclusion chromatography (BioGel P 60), S – standard XET *B* Western blot of individual purification steps for symbols see *A*

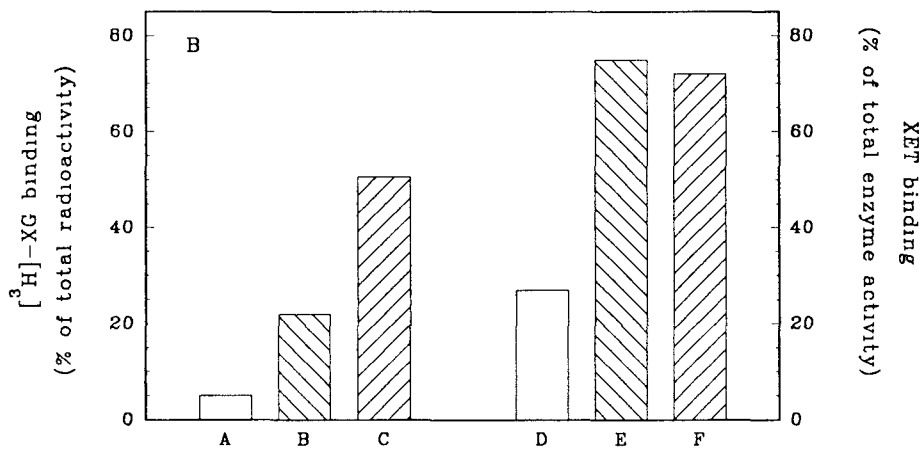
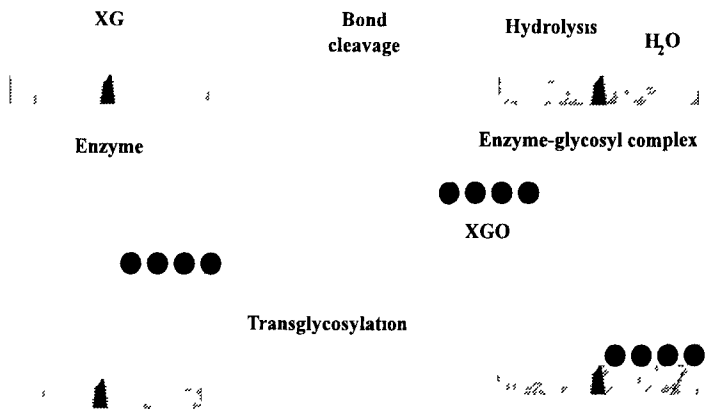


Figure 5. Binding of XET and [^{3}H]XG to Affi-Ab *A* binding of [^{3}H]XG to Affi-Ab in the absence of XET, *B* binding of [^{3}H]XG to XET immobilized on the carrier, *C* binding of [^{3}H]XG to Affi-Ab after preincubation with XET *D* nonspecific binding of XET to AffiGel 10, *E* immobilization of XET on Affi-Ab, *F* binding of XET after preincubation with [^{3}H]XG

Discussion

In our previous work (Farkaš et al. 1992) we have proposed a hypothetical mechanism of XET interaction with substrate (Scheme 1)



Scheme 1

This mechanism was based on the assumption that after the interaction of XET with XG the substrate molecule is split into two parts, the first part containing the newly formed nonreducing end is released from the binding site of the enzyme, but the second one containing the potentially new reducing end remains attached to the active site of the enzyme forming a relatively stable and temporarily inactive enzyme-glycosyl intermediate which is decomposed by transfering the glycosyl moiety to a suitable polysaccharide or oligosaccharide acceptor (transglycosylation).

The existence of the stable enzyme glycosyl intermediate is supported by the observation that the equilibrium of XG degradation by XET appeared to be directly proportional to the enzyme concentration (Fig. 1A). At equilibrium, all the enzyme was probably bound to an intermediary complex with XG. Apparently, the complex is relatively stable against the attack of water so that the enzyme bound in this complex becomes temporarily inactive. Equilibrium could be disbalanced by further addition of XET (Fig. 3) and/or XGOs (Fig. 2). After the addition of a new portion of XET, the reaction continues until practically all newly added

enzyme molecules become a part of the complex. On the other hand, added XGOs are probably promoting the reaction by acting as glycosyl acceptors. Transglycosylation is accompanied by the release of the enzyme from the complex so that new binding to substrate molecule can take place. When the concentration of free XGOs in reaction mixture is decreasing to zero the reaction rate is very low and equilibrium is established at a higher level.

The ability of Affi-Ab to bind firmly [^{3}H] xyloglucan in complex with XET from the solution gives a further support to the existence of a stable XET:XG complex. An interesting physiological implication of these findings would be that the formation of inactive XET:XG complex could provide a means for temporary inactivation of the enzyme or its conversion into a latent state in the plant cell wall during periods of limited growth (Sulová et al. 1998).

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References

Edwards M., Dea I. C. M., Bulpin P. V., Reid J. S. G. (1985): Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeolum majus L.* following germination. Planta **163**, 133—140

Edwards M., Dea I. C. M., Bulpin P. V., Reid J. S. G. (1986): Purification and properties of a novel xyloglucan-specific endo-(1-4)-β-D-glucanase from germinated nasturtium seeds (*Tropaeolum majus L.*) J. Biol. Chem. **261**, 9489—9494

Farkaš V., Sulová Z., Stratilová E., Hanna R., Maclachlan G. (1992) Cleavage of xyloglucan by nasturtium seeds xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides Arch. Biochem. Biophys. **298**, 365—370

Fry S. C. (1989) The structure and function of xyloglucan J. Exp. Botany **40**, 1—11

Fry S. C., Smith R. C., Renwick F. K., Martin D. J., Hodge S. K. M., Mathews K. J. (1992): Xyloglucan-endotransglycosylase, a new wall-loosening enzyme activity from plants Biochem. J. **282**, 821—828

Harlow E., Lane D. (1988): Antibodies A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 92—137

Hayashi T. (1989): Xyloglucans in the primary cell wall Annu. Rev. Plant. Physiol. Plant Mol. Biol. **40**, 139—168

Maclachlan G., Brady C. (1994) Endo-1,4-β-glucanase, xyloglucanase and xyloglucan endo-transglycosylase activities versus potential substrates in ripening tomatoes Plant Physiol. **105**, 965—974

Nakamura S., Hayashi T. (1993) Purification and properties of an extracellular endo-(1-4)-β-D-glucanase from suspension-cultured poplar cells Plant Cell Physiol. **34**, 1009—1013

Sulová Z., Farkaš V. (1996): Mechanism of interaction of xyloglucan-endotransglycosylase with the substrate. Chem. Papers **90**, 601—602

Sulová Z., Lednická M., Farkaš V. (1995). A colorimetric assay for xyloglucan-endotransglycosylase from germinating seeds. Anal. Biochem. **229**, 80—85

Sulova Z Takačova M Steele N M Fry S C Farkas V (1998) Xyloglucan endotransglycosylase evidence for existence of a relatively stable glycosyl-enzyme complex intermediate Biochem J (in press)

Takeda T Mitsuishi Y Sakai F Hayashi T (1996) Xyloglucan endotransglycosylation in suspension-cultured poplar cells Biosci Biotechnol Biochem **60** 1950 1955

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