Ping-Pong Character of Nasturtium-seed Xyloglucan Endotransglycosylase (XET) Reaction

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Abstract. Plant xyloglucan endotransglycosylase (XET, EC 2.4.1.207) degrades its substrate by a transglycosylation mechanism while endo-cleaving xyloglucan (XG) molecules at their β-1,4-linked polyglucosyl main chain and transferring the newly generated reducing chain ends to hydroxyls at C-4 of non-reducing glucosyl ends of the main chains of other XG molecules or of low-M_r XG-fragments (OS). Kinetic data obtained with purified nasturtium seed (*Tropaeolum majus*, L.) XET while using high-M_r xyloglucan and ^{3}H-labeled XGOS alditols (DP 7–9) as substrates could be best fitted to the model for Ping-Pong Bi Bi reaction mechanism. Such mechanism is typical for transglycosylases operating with retention of the anomeric configuration of the formed glycosidic bond and involving the formation of a covalent glycosyl-enzyme reaction intermediate.

Key words: Xyloglucan — XET — Transglycosylation — Reaction mechanism — Reaction kinetics

Introduction

The enzyme xyloglucan endotransglycosylase (XET, EC 2.4.1.207) is ubiquitous in higher plants (Fry et al. 1992). Its proposed role is to catalyze reversible loosening of xyloglucan (XG) cross-links between cellulose microfibrils in the primary plant cell wall thereby permitting the turgor-driven expansion of the walls during growth. Such action would allow continuous reorganization of the cell walls without disturbing their integrity as well as anchoring of newly synthesized XG molecules into the preexisting cell wall structure (Fry 1993; Nishitani 1995; Thompson et al. 1997). XET enzymes are present also in seeds of some leguminous plants where their primary role appears to be the depolymerization of reserve xyloglucan during germination (Edwards et al. 1986; Sulová et al. 1995). Mechanism by which XET degrades or disproportionates molecules of xyloglucan is primarily transglycosylation: the β-(1–4) linked polyglucosyl main chains of XG are cleaved and portions of the XG molecules containing newly generated reducing ends are transferred to

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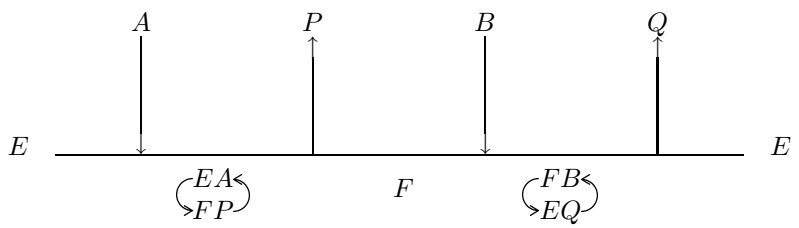
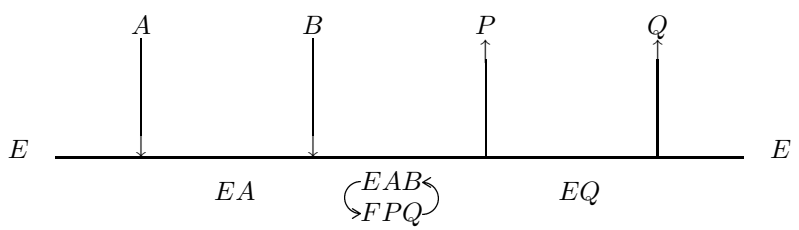
hydroxyls at C-4 of the glucosyl units at the non-reducing ends of other xyloglucan molecules or of low-M_r xyloglucan fragments (Farkaš et al. 1992; Fry et al. 1992; Nishitani and Tominaga 1992; de Silva et al. 1993). A mechanism for XET reaction has been postulated (Farkaš et al. 1992) according to which the reaction proceeds in several consecutive steps: 1. association of XET (E) with xyloglucan molecule (A); 2. cleavage of XG and release of the cleaved reducing-end portion of the substrate molecule (P) and formation of an intermediate covalent glycosyl-enzyme complex (F) with the rest of XG molecule containing the newly formed reducing end; 3. association of the glycosyl-enzyme complex (F) with the acceptor molecule (B) (which could be either XG or XG-derived oligosaccharide) into a transient complex (FB) followed by transglycosylation, i. e. the formation of a covalent linkage between the glycosyl residue from the (F) complex and the acceptor molecule (B), giving rise to enzyme-product complex (EQ); and 5. dissociation of the complex to product of transglycosylation (Q) and the free enzyme (E). During this sequence, the enzyme exists in two stable forms: as the free enzyme (E) and as a covalently glycosylated intermediate (F). According to Cleland's nomenclature (Cleland 1963), such sequence of reactions would correspond to Ping-Pong Bi Bi reaction mechanism, and could be schematically presented as folows:



(1)

An alternative mechanism suggested for XET isolated from poplar suspension cell culture (Takeda et al. 1996) is an Ordered Bi Bi reaction in which both xyloglucan as the glycosyl donor (A) and OS (oligosaccharide) as the glycosyl acceptor (B) associate with the enzyme to form a ternary complex (EAB) before the cleavage of XG and transglycosylation of the formed glycosyl residue to B can take place. Such sequence could be schematically expressed as follows:



(2)

In this case, the cleavage of (A) and transglycosylation of newly created reducing end of polyglycan chain to the acceptor (B) would occur practically in one step, without formation of a stable glycosyl-enzyme complex. While in the Ping-Pong

Bi Bi mechanism, the first product is released from the enzyme-substrate complex before the second substrate can bind, in the Ordered Bi Bi mechanism both substrates have to be bound to the enzyme in order for the reaction to proceed. According to the second model, the only stable form of the enzyme is the enzyme in the free state (E).

Recently, we provided indirect evidence for the existence of a stable glycosyl-enzyme intermediate complex in the reaction catalyzed by nasturtium-seed XET (Sulová et al. 1998; Sulová and Farkaš 1998). The kinetic evidence presented herein supports our previous hypothesis (Farkaš et al. 1992) on the reaction mechanism of the enzyme by confirming the Ping-Pong character of the transglycosylation reaction.

Materials and Methods

Substrates

Tamarind-seed xyloglucan (XG, M_r 5×10^5–2×10^6) was prepared as described elsewhere (Farkaš et al. 1992). A crude mixture of xyloglucan-derived oligosaccharides (OS, largely DP 7-9) was a gift from Mr. Kazuhiko Yamatoya, Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). The oligosaccharides (OS) were purified on Biogel P2 column eluted with water and further fractionated by preparative HPLC on TSK Gel Amide column (Tosoh), 21.5 mm i.d. × 300 mm eluted by 65 % (v/v) aqueous acetonitrile. The fractions corresponding to heptasaccharide XXXG, octasaccharide XLXG and nonasaccharide XLLG (nomenclature according to Fry et al. 1993) were identified by ^{1}H- and ^{13}C- NMR using the data published by York et al. (1990). The individual XGOS were tritiated by reduction with NaB^3H_4 using a standard procedure. Briefly, the individual oligosaccharides (2 mg, 1.5–1.6 μmoles) were dissolved in 200 μl 0.1 mol.l^{-1} NaOH and 100 μl (25 mCi, 4 μmoles) of NaB^3H_4 (Amersham) dissolved in water were added and the mixture was allowed to stand overnight at room temperature. Then, 10 μmoles of cold $NaBH_4$ in 200 μl water were added and the incubation was continued for additional 4 h. The solution was then passed through a column (2 ml) of Dowex 50 (H^+) and the filtrate was evaporated several times from methanol under stream of nitrogen. The radioactive alditols were further purified by TLC on aluminium foil-backed Silicagel M60 (Merck) plates (thickness 0.2 mm) using the solvent system n-butanol-ethanol-water (5 : 5 : 4, by vol.), run twice with intermittent drying. The sugars were located on plates by autoradiography and the areas corresponding to the respective [1-^{3}H]oligosaccharide alditols were cut out and eluted with 20 % (v/v) ethanol. After evaporation to dryness, the radioactive alditols were dissolved in 20 % ethanol and kept at 0–4 ℃. The reduced radioactive hepta-, octa- and nonasaccharides are termed [^{3}H]XXXGol, [^{3}H]XLXGol and [^{3}H]XLLGol respectively.

Enzyme

Nasturtium-seed xyloglucan endotransglycosylase was purified to homogeneity by

the method based on affinity entrappment of active glycosyl-enzyme complex as previously described (Sulová and Farkaš 1999).

XET assays

For assays of XET activity, the radiometric method of Fry et al. (1992) was adopted. Forty μl assay mixtures contained 50 mmol.l^{-1} succinate buffer (pH 5.5), 10 mmol.l^{-1} $CaCl_2$, 22 μg purified enzyme protein and varying concentrations of substrates (XG plus [1-^{3}H]XXXGol, [1-^{3}H]XLXGol or [1-^{3}H]XLLGol respectively). After 20 min incubation at 25℃, the reaction was terminated by addition of 200 μl of 20 % (v/v) formic acid and the radioactivity incorporated into xyloglucan was determined. The kinetic parameters were computed and statistically evaluated using Sigmaplot 2.0 program (Jandel Co.).

The initial rate of the reaction was measured under conditions when the concentration of one of the substrates was varied while the concentration of the other substrate was kept constant. XG served as the first substrate (A) while as the second substrate (B) radioactive XG oligosaccharides [1-^{3}H]XXXGol, [1-^{3}H]XLXGol and/or [1-^{3}H]XLLGol were used. The concentrations of stock solutions of radioactive oligosaccharides were adjusted to desired value with their cold counterparts. The measurements with each combination of substrates were performed in triplicate and the resulting values were averaged.

Data analysis

The first step in distinguishing between the ordered sequential and the ping-pong mechanism was to fit the dependence of the initial rate of the reaction v on the concentration of one of the substrates at different fixed concentrations of the other substrate to Eq. (1).

$$v = \frac{V'_{\max} c}{K'_{\mathrm{m}} + c} \tag{1}$$

where v is the initial rate of the reaction, c is the concentration of the varied substrate, and $V'_{\max}$ and K'_{m} are the apparent maximum rate and apparent Michaelis constant respectively, both being functions of the concentration of the fixed substrate. These constants were used to construct plots that would allow to discriminate between the two possible mechanisms.

$$v = \frac{V_{\max} ab}{K_{\mathrm{b}} a + K_{\mathrm{a}} b + ab} \tag{2}$$

$$v = \frac{V_{\max} ab}{K_{\mathrm{b}} a + K_{\mathrm{a}} b + ab + K_{\mathrm{ia}} K_{\mathrm{b}}} \tag{3}$$

Eq. (2) expresses the dependence of initial velocity of reaction on the concentration of the varied substrate for the ping-pong mechanism, whereas Eq. (3) is the same dependence for the ordered sequential mechanism. Equations (2) and (3) can

be transformed to linear form for fixed concentrations of (B). Then, the equation for the ping-pong mechanism takes the form:

$$1/v = \frac{1}{V_{\max}} \left(1 + \frac{K_{\mathrm{b}}}{b}\right) + \frac{K_{\mathrm{a}}}{V_{\max}} \cdot \frac{1}{a} \tag{4}$$

and that for the sequential ordered mechanism:

$$1/v = \frac{K_{\mathrm{a}}}{V_{\max}} \left(1 + \frac{K_{\mathrm{ia}} K_{\mathrm{b}}}{K_{\mathrm{a}} \cdot b}\right) \frac{1}{a} + \frac{1}{V_{\max}} \left(1 + \frac{K_{\mathrm{b}}}{b}\right) \tag{5}$$

From these equations it follows that when the reciprocal of the initial rate ($1/v$) is plotted against the reciprocal of the concentration of the varied substrate ($1/a$) for different fixed concentrations b of the second substrate, the initial rate equations give, for reactions obeying the ping-pong mechanism, a set of parallel lines while for ordered mechanism, the lines converge to a common intersect on, above or below the abscisa. The dependence of $V'_{\max}$ on the concentration of the fixed substrate is hyperbolic for both types of mechanism. Another notable distinction is that plots of $V'_{\max}$ *vs.* K'_{m} are linear and going through the axes' origin for reactions obeying ping-pong mechanism whereas they intersect the K'_{m} axis for sequential reactions with the intersect corresponding to the value of K_{ia} (Eisenthal and Cornish-Bowden 1974).

Based on the appearance of Lineveawer-Burk's plots, we tried to construct the rate equations giving the best fit to the plotted data. The goodness of the fit to suggested rate equations and to equations (2) and (3) was evaluated as described by Mannervik (1982). Kinetic data were fitted by nonlinear regression according to the respective equation. To the linear plot results of above computation were transformed numerically.

Results and Discussion

Xyloglucan endotransglycosylase operates with retention of the anomeric configuration of the formed glycosidic bond (Fry et al. 1992) which can be achieved *via* double-displacement at the anomeric center and involving the formation of a covalent enzyme-substrate (substituted enzyme) intermediate (Sinnott 1990). Evidence consistent with ping-pong reaction mechanism has accumulated in recent years for nasturtium-seed XET including the ability of XET to form a relatively stable intermediary complex with xyloglucan from which the enzyme can be released with a suitable glycosyl acceptor or the nucleophile imidazole (Sulová et al. 1998; Sulová and Farkaš 1999). The formation of a glycosyl-enzyme intermediate is characteristic for retaining glycanases operating by ping-pong mechanism (Sinnott 1990; Kempton and Withers 1992).

Detailed analysis of the obtained kinetic data revealed a rather complex pattern of the reaction mechanism of XET. When the reciprocals of the initial rates of the reaction were plotted against the reciprocals of the concentration of OS

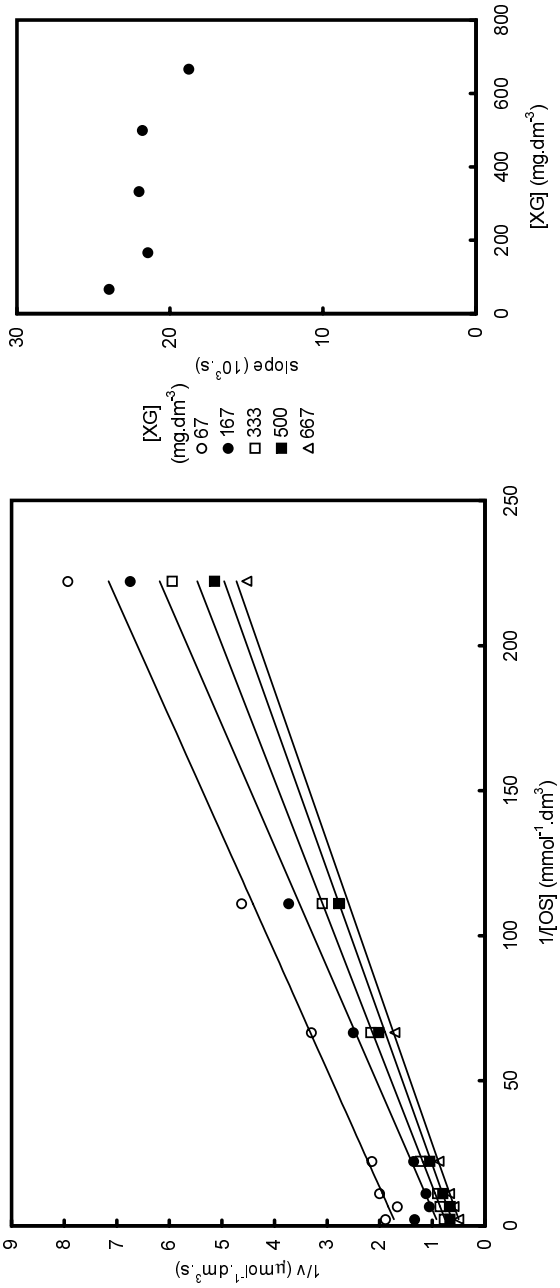


Figure 1. Plot of $1/v = f(1/[\mathrm{OS}])$ at different fixed concentrations of xyloglucan [XG] (a), and the dependence of slopes of the lines on [XG] (b). Data were fitted to Michaelis equation using nonlinear regression.

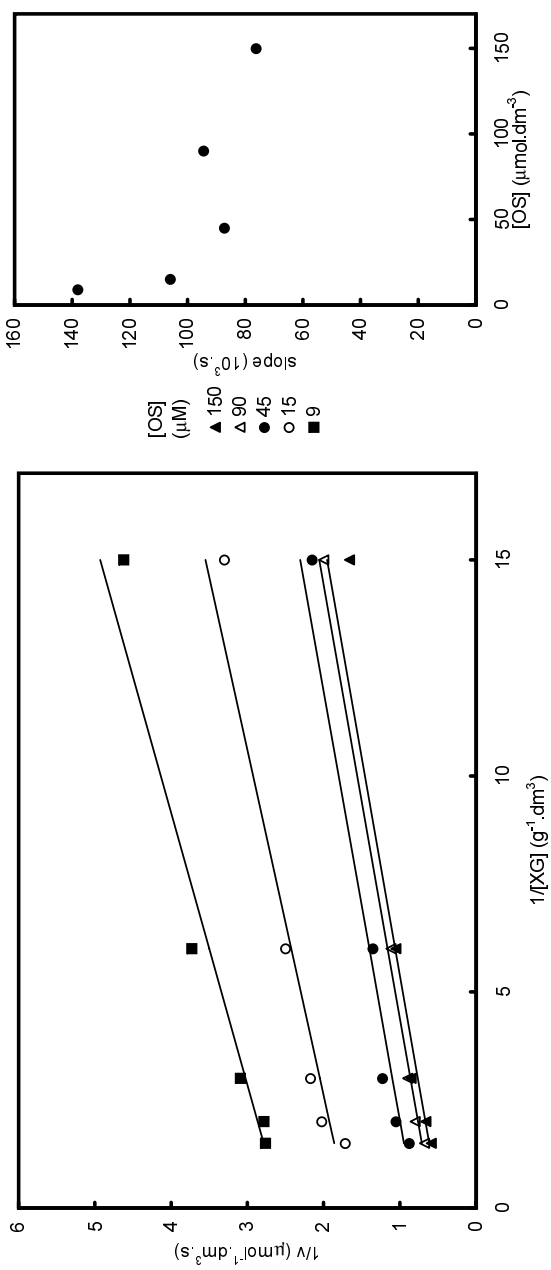


Figure 2. Plot of $1/v = f(1/[\mathrm{XG}])$ at different fixed concentrations of oligosaccharides [OS] (a), and the dependence of slopes of the lines on [OS] (b). Data were fitted to Michaelis equation using nonlinear regression.

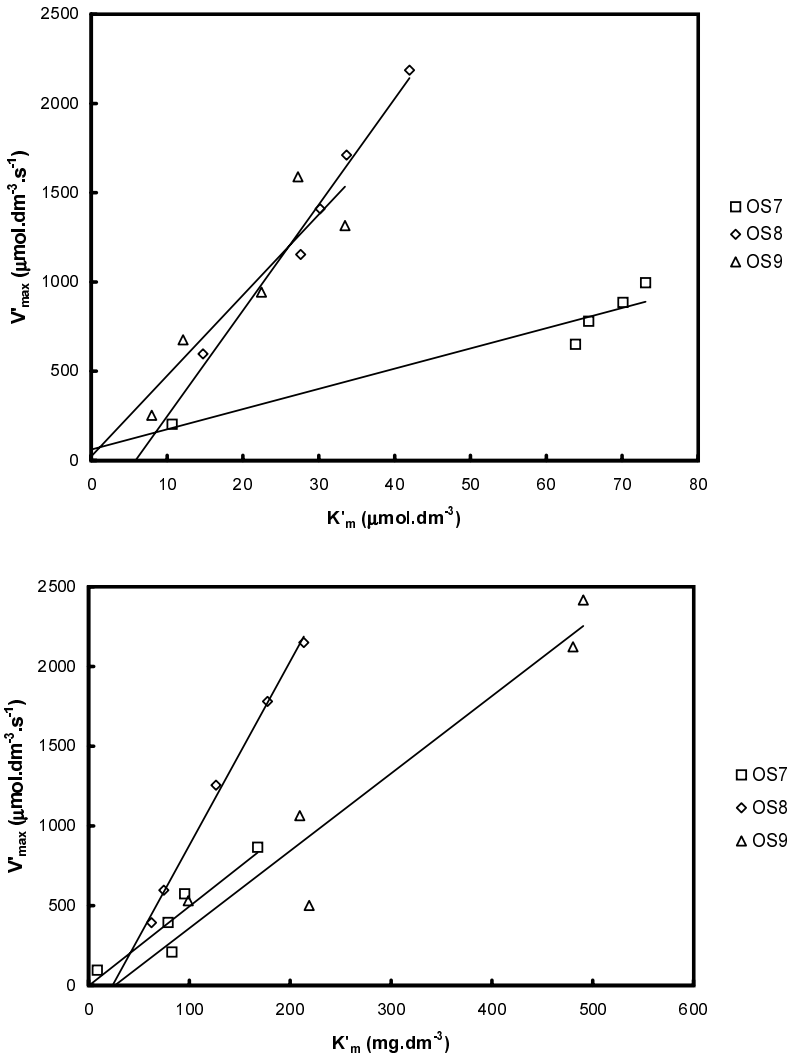


Figure 3. Plots depicting the functions $V'_{\max} = f(K'_{\mathrm{m}})$ at fixed concentrations of XG (upper graph) derived from Fig. 1, and at fixed concentrations of OS (lower graph) derived from Fig. 2.

(we present here only the plots obtained with octasaccharide XXLGol, the results with heptasaccharide XXXGol and nonasaccharide XLLGol were similar) at different fixed concentrations of XG a series of nearly parallel lines was obtained (Fig. 1a). Although the slopes of the lines did not differ significantly from each other (Fig. 1b), they cannot be assumed to be identical and due to deviations

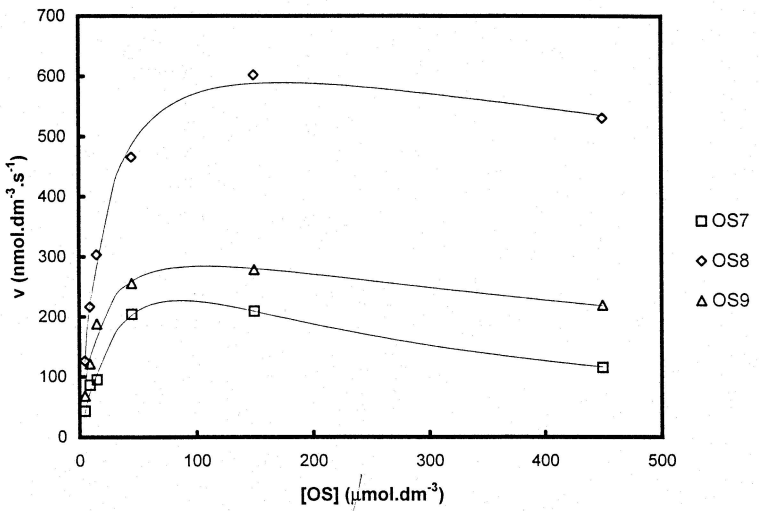


Figure 4. Function of $v = f([\mathrm{OS}])$ at the lowest concentration of XG used (67 mg.dm^{-3}) showing the inhibition of the reaction at high concentrations of OS. Data were fitted to the rate equation for a single substrate reaction exhibiting substrate inhibition.

caused just by experimental error because an overall trend of the decrease of the slope with the decreasing concentration of the xyloglucan as the fixed substrate can be seen (Fig. 1b). Similar patterns were obtained from the assays where [XG] was the variable and XXLGol served as the fixed substrate (Fig. 2a,b). However, the lines of dependencies of the apparent V'_{max} as the function of the apparent K'_{m} intersect the axes near their origin (Fig. 3). In these plots, the extreme concentrations of XG and XGOS at which the effect of substrate inhibition was apparent (see Fig. 4) were omitted. This can be observed with OS as well as XG used either as the fixed or the variable substrate, which is in agreement with patterns distinctive for the ping-pong mechanism. In the case of ordered sequential mechanism, the lines of these dependencies would intersect the K'_{m} axis.

In order to obtain better fit of the experimental data to kinetic equations, we took into consideration also the possible side-reactions (hydrolysis, dead-end inhibition) in the model of the reaction mechanism (Fig. 5). Namely, the appearance of the dependence of the reaction rate on the concentration of either oligosacharide at the lowest concentrations of xyloglucan was suggesting a substrate inhibition for all the three acceptor oligosaccharides (Fig. 4).

When the kinetic data were fitted using nonlinear regression (Mannervik 1982) to the rate equations for the sequential and/or ping-pong mechanisms, the constant term in the denominator of the sequential mechanism rate equation Eq. (3) was by 1–2 orders of magnitude smaller than the sum of other terms, thereby changing the equation to one characteristic for the ping-pong mechanism (Tab. 1). The regression

Table 1 ►

Model and rate equation	Acceptor OS
1 $v = \dfrac{V_{\max} \cdot [\mathrm{XG}][\mathrm{OS}]}{K_{\mathrm{mOS}} \cdot [\mathrm{XG}] + K_{\mathrm{mXG}} \cdot [\mathrm{OS}] + [\mathrm{XG}][\mathrm{OS}]}$	OS7 OS8 OS9
2 $v = \dfrac{V_{\max} \cdot [\mathrm{XG}][\mathrm{OS}]}{K_{\mathrm{mOS}} \cdot [\mathrm{XG}] + K_{\mathrm{mXG}} \cdot [\mathrm{OS}] + [\mathrm{XG}][\mathrm{OS}]} + K_{\mathrm{mOS}} \cdot K_{\mathrm{iXG}}$	OS7 OS8 OS9
3 $v = \dfrac{V_{\max} \cdot [\mathrm{XG}][\mathrm{OS}]}{K_{\mathrm{mOS}} \cdot [\mathrm{XG}] + K_{\mathrm{mXG}} \cdot [\mathrm{OS}] \left(1 + \dfrac{[\mathrm{OS}]}{K_{\mathrm{sOS}}}\right) + [\mathrm{XG}][\mathrm{OS}]}$	OS7 OS8 OS9
4 $v = \dfrac{V_{\max} \cdot [\mathrm{XG}][\mathrm{OS}]}{K_{\mathrm{mOS}} \cdot [\mathrm{XG}] \left(1 + \dfrac{[\mathrm{XG}]}{K_{\mathrm{iXG}}}\right) + K_{\mathrm{mXG}} \cdot [\mathrm{OS}] + [\mathrm{XG}][\mathrm{OS}]}$	OS7 OS8 OS9
5 $v = \dfrac{V_{\max} \cdot [\mathrm{XG}][\mathrm{OS}]}{K_{\mathrm{mOS}} \cdot [\mathrm{XG}] \left(1 + \dfrac{[\mathrm{XG}]}{K_{\mathrm{iXG}}}\right) + K_{\mathrm{mXG}} \cdot [\mathrm{OS}] \left(1 + \dfrac{[\mathrm{OS}]}{K_{\mathrm{sOS}}}\right) + [\mathrm{XG}][\mathrm{OS}]}$	OS7 OS8 OS9
6 $v = \dfrac{V_{\max} \cdot [\mathrm{XG}][\mathrm{OS}]}{K_1 + K_2[\mathrm{OS}] + K_3[\mathrm{OS}]^2 + K_4[\mathrm{XG}] + [\mathrm{XG}][\mathrm{OS}]}$	OS7 OS8 OS9

1 – ping-pong mechanism, 2 – ordered sequential mechanism, 3 – ping-pong mechanism with oligosaccharides acting as a competitive inhibitor with respect to xyloglucan, 4 – ping-pong mechanism with xyloglucan acting as a competitive inhibitor with respect to oligosaccharides, 5 – ping-pong mechanism with xyloglucan acting as a competitive inhibitor with respect to oligosaccharides, and with oligosaccharides acting as a competitive inhibitor with respect to xyloglucan, 6 – ping-pong mechanism with a branching point and with oligosaccharides acting as a competitive inhibitor with respect to xyloglucan.

gave reasonable values of constants and acceptable residuals and their distribution around zero (Tab. 1). A better agreement was obtained for oligosaccharides OS8 and OS9 when the data were fitted to the equation for the ping-pong mechanism with oligosaccharide acting as a competitive inhibitor with respect to xyloglucan. The values of dissociation constants K_s of oligosaccharides from the dead-end complex were increasing in the order from nonasaccharide (OS9) to heptasaccharide (OS7, Tab. 1). No significant improvement of the fit was obtained when either xyloglucan was considered as a competitive inhibitor with respect to oligosaccharides or when

◀ **Table 1**

Mean sum of squares	Parameter values ± S.D.					
(SS)/(n-p)	V_{max}	K_{mXG}	K_{mOS}	K_{iXG}	K_{sOS}	K_{sXG}
1.92E-03	1.40 ± 0.07	261 ± 29	111 ± 9			
9.00E-03	3.26 ± 0.17	392 ± 40	65 ± 5			
7.24E-03	3.52 ± 0.35	829 ± 126	67 ± 8			
1.93E-03	1.37 ± 0.09	249 ± 36	106 ± 13	9.45 ± 19.39		
9.09E-03	3.26 ± 0.20	393 ± 47	65 ± 8	−1.27 ± 19.91		
7.23E-03	3.32 ± 0.37	756 ± 132	58 ± 11	37.70 ± 45.50		
1.93E-03	1.42 ± 0.09	254 ± 31	115 ± 13		6.53 ± 12.45	
7.54E-03	3.58 ± 0.22	351 ± 38	82 ± 8		1.005 ± 0.278	
5.03E-03	4.33 ± 0.48	821 ± 128	103 ± 15		0.852 ± 0.167	
1.94E-03	1.40 ± 0.07	261 ± 29	111 ± 9			61 ± 2188
9.09E-03	3.25 ± 0.17	392 ± 40	65 ± 5			631 ± 4735
7.31E-03	3.52 ± 0.36	829 ± 128	68 ± 9			139 ± 4239
1.95E-03	1.42 ± 0.09	254 ± 31	115 ± 13		6.53 ± 12.80	209 ± 2196
7.62E-03	3.58 ± 0.22	351 ± 38	82 ± 8		1.005 ± 0.281	425 ± 4337
5.08E-03	4.33 ± 0.49	821 ± 129	103 ± 15		0.852 ± 0.169	499 ± 3537

(SS)/(n-p)	V_{max}	K_1	K_2	K_3	K_4
1.98E-03	1.39 ± 0.09	1532 ± 2199	234 ± 42	56 ± 81	110 ± 14
7.60E-03	3.52 ± 0.24	641 ± 1409	337 ± 46	347 ± 90	79 ± 10
4.91E-03	3.81 ± 0.45	4183 ± 2363	664 ± 125	858 ± 185	81 ± 15

Parameter units: V_{max} [mmol.dm^{-3}.min-1], K_{mXG} [mg.dm^{-3}], K_{mOS} [mmol.dm^{-3}], K_{sXG} [mg.dm^{-3}], K_{sOS} [mmol.dm^{-3}], K_{iXG} [mg.dm^{-3}], K_1 [mmol.mg.dm^{-6}], K_2 [mg.dm^{-3}], K_3 [mmol^{-1}.mg], K_4 [mmol.dm^{-3}];

multiple molecules of oligosaccharides were considered to be able to bind to the active site of the unsubstituted enzyme as a dead-end inhibitor (data not shown).

The above results could be interpreted as corresponding to a modified ping-pong mechanism with oligosaccharides acting as dead-end inhibitors and with two alternative fates for the glycosyl-enzyme intermediate: either hydrolysis or transglycosylation (Fig. 5). It is probable that the oligosaccharides in the dead-end complex XETOS (Fig. 5) were binding to the enzyme at substrate-binding subsites distant from the active site and therefore could hinder the effective binding of XG

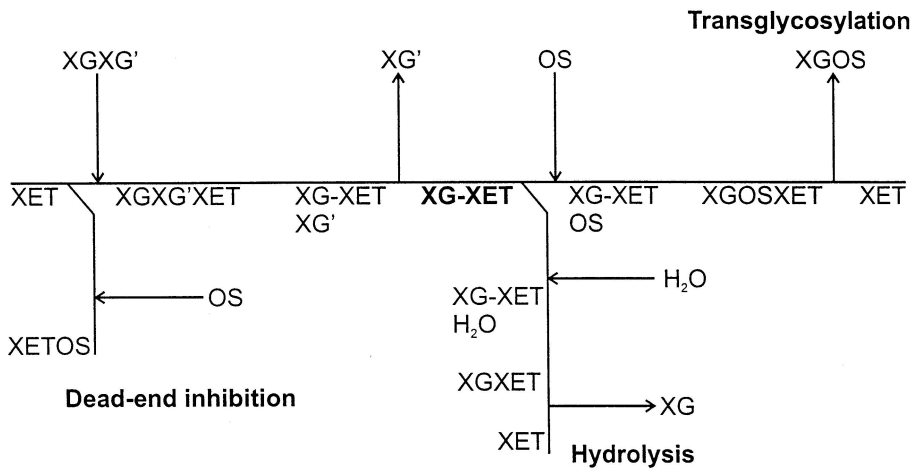


Figure 5. Scheme illustrating the branched reaction mechanism of XET.

to the enzyme. This seems to be a reasonable point since oligosaccharides share a structural similarity with xyloglucan and therefore should be able to bind to both substituted and unsubstituted forms of the enzyme.

Our failure to find an ideal fit for the measured kinetic parameters may be caused by factors such as: (i) Molecular heterogeneity of one of the substrates (XG) – the affinity of the enzyme to molecules of XG having different lengths may not be the same; (ii) Idle (undetectable) reaction, i. e. transfer of glycosyls from the XG-XET intermediate to other xyloglucan molecules, in other words, the molecules of XG could be acting both as glycosyl donors and glycosyl acceptors. This, however, seems improbable in the view of our preliminary findings (not shown here) that the ability of an oligosaccharide to act as the glycosyl acceptor decreased with its increasing size; (iii) errors caused by inability to determine accurately the reaction rate at low radioactivity counts in the assays where low concentrations of radioactive XGOS were used.

The apparent discrepancy of our results with those of Takeda et al. (1996) obtained with XET from extracts from suspension-cultured poplar cells could be explained by the fact that in the present work, the measurements were performed with purified enzyme rather than with crude extracts. It should be, however, taken into consideration that also the reactions obeying ping-pong mechanism may yield a set of convergent lines in double-reciprocal plots when the reaction contains a branching point, such as the hydrolysis of the intermediary glycosyl-enzyme complex (Nordlie 1982). Overlooking of such a possibility may lead to erroneous conclusions.

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