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Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants

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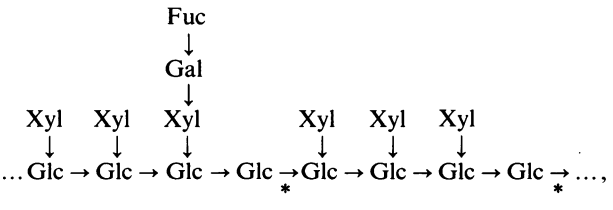
1. Cell-free extracts of all plants tested contained a novel enzyme activity (xyloglucan endotransglycosylase, XET) able to transfer a high-M_r portion from a donor xyloglucan to a suitable acceptor such as a xyloglucan-derived nonasaccharide ($Glc_4Xyl_3GalFuc$; XG9). 2. A simple assay for the enzyme, using [^{3}H]XG9 and based on the ability of the [^{3}H]polysaccharide product to bind to filter paper, is described. 3. The enzyme was highly specific for xyloglucan as the glycosyl donor, and showed negligible transglycosylation of other polysaccharides, including CM-cellulose. 4. The K_m for XG9 was 50 μM; certain other ^{3}H-labelled xyloglucan oligosaccharides also acted as acceptors, and certain non-radioactive xyloglucan oligosaccharides competed with [^{3}H]XG9 as acceptor; the minimum acceptor structure was deduced to be:

Xyl Xyl
↓ ↓
Glc → Glc → Glc

5. The pH optimum was approx. 5.5 and the enzyme was less than half as active at pH 7.0. The enzyme was slightly activated by Ca^{2+}, Mg^{2+}, Mn^{2+}, spermidine, ascorbate and 2-mercaptoethanol, and inhibited by Ag^+, Hg^{2+}, Zn^{2+} and La^{3+}. 6. XET activity was essentially completely extracted by aqueous solutions of low ionic strength; Triton X-100, Ca^{2+}, La^{3+}, and Li^+ did not enhance extraction. Negligible activity was left in the unextractable (cell-wall-rich) residue. 7. The enzyme differed from the major cellulases (EC 3.2.1.4) of pea in: (a) susceptibility to inhibition by cello-oligosaccharides, (b) polysaccharide substrate specificity, (c) inducibility by auxin, (d) requirement for salt in the extraction buffer and (e) activation by 2-mercaptoethanol. XET is therefore concluded to be a new enzyme activity (xyloglucan:xyloglucan xyloglucanotransferase; EC 2.4.1.-). 8. XET was detected in extracts of the growing portions of dicotyledons, monocotyledons (graminaceous and liliaceous) and bryophytes. 9. The activity was positively correlated with growth rate in different zones of the pea stem. 10. We propose that XET is responsible for cutting and rejoining intermicrofibrillar xyloglucan chains and that it thus causes the wall-loosening required for plant cell expansion.

INTRODUCTION

Xyloglucan is a structural polysaccharide of plant cell walls (Hayashi, 1989; Fry, 1989*a*). It is composed of a backbone of β-(1→4)-D-glucopyranose residues, about 75% of which have a single α-D-xylopyranose residue attached at O-6. Some of the xylose residues are β-D-galactopyranosylated at O-2, and (except in the storage xyloglucans of certain seeds, e.g. *Tropaeolum*) some of the galactose residues are α-L-fucopyranosylated at O-2. A repeating motif of xyloglucan appears to be:



the linkages marked '*' being cellulase-labile. The galactose residues may also be *O*-acetylated.

Xyloglucan can hydrogen-bond to cellulose microfibrils (Hayashi *et al.*, 1987) and may cross-link them (Fry, 1989*b*; McCann *et al.*, 1990), restraining cell expansion. The enzymic cleavage of xyloglucan is thought to loosen the wall, enabling cell growth. For instance, it has been shown that xyloglucan decreases in mean M_r and is partially solubilized during auxin- or acid-induced growth (Labavitch & Ray, 1974; Gilkes & Hall, 1977; Nishitani & Masuda, 1983; Hayashi *et al.*, 1984; Wakabayashi *et al.*, 1991). In further support of this hypothesis, treatment of stem segments with xyloglucan-binding lectins or antibodies, which might be expected to restrict the access of an endogenous degradative enzyme to wall-bound xyloglucan, blocks auxin-induced growth (Hoson & Masuda, 1987, 1989).

The enzyme responsible for xyloglucan cleavage was assumed to be cellulase (Hayashi *et al.*, 1984; Fry, 1989*b*; McDougall & Fry, 1990), which hydrolyses the β-(1→4)-D-glucan backbone of xyloglucan. However, hydrolysis is irreversible whereas bond breakage during growth is reversible (Taiz, 1984), which argues against the involvement of cellulase-catalysed hydrolysis. A possible explanation of this discrepancy arises from the finding that xyloglucan is subject *in vivo* to endotransglycosylation, i.e., a xyloglucan chain (□□□□□...) can be cleaved and then transferred to a different (acceptor) chain (*A*) (Baydoun & Fry, 1989; Smith & Fry, 1991) (Scheme 1). Such cleavage could still loosen the wall, enabling growth, but would be followed by a repair step, as proposed by Albersheim (1974), so its loosening effect would be reversible.

Abbreviations used: XET, xyloglucan endotransglycosylase; XG9 etc., xyloglucan-derived oligosaccharides (see Fig. 2); 2,4-D, 2,4-dichlorophenoxyacetic acid.

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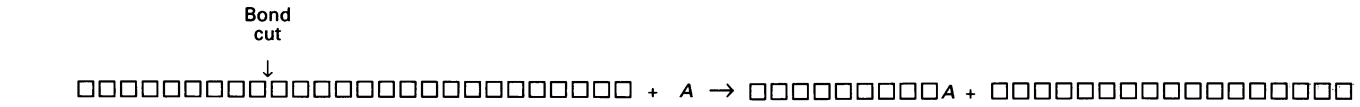


Scheme 1.

The enzyme responsible for this endotransglycosylation is of considerable interest for studies of the mechanism and control of plant cell expansion. In the present paper we report the widespread occurrence in plants of a new enzyme activity, xyloglucan endotransglycosylase (XET), which catalyses this reaction. We show that it differs from cellulase and propose that it is central to the mechanism of plant growth.

EXPERIMENTAL

Plant material

Peas (*Pisum sativum*, cv. Alaska) were soaked overnight in running tap water and then germinated in moist vermiculite at 25 °C in the dark. Analyses were performed on day 7 or 8, when the third internode was rapidly elongating. Tomato seedlings (cv. Ailsa Craig) were greenhouse-grown to a height of approx. 15 cm. Other plants were collected wild or from gardens in the vicinity of Edinburgh except *Marchantia*, which was collected by Loch Caolisport, Argyll.

Oligosaccharides

[1-^{3}H]XG9, i.e. XG9 labelled at position 1 of the reducing terminal glucose moiety (12 TBq/mol), was prepared as described previously (Smith & Fry, 1991). [1-^{3}H]XG7f ($Glc_3Xyl_2GalFuc$) and [1-^{3}H]XG5 ($Glc_2XylGalFuc$) were prepared from [1-^{3}H]XG9 as described by McDougall & Fry (1991) and purified by paper chromatography in ethyl acetate/acetic acid/water (10:5:6, by vol.). [^{3}H]XG2 was prepared by treatment of [*pentosyl*-^{3}H]-xyloglucan with 'Driselase' and purified by paper chromatography in ethyl acetate/pyridine/water (8:2:1, by vol.) (Fry, 1988). Non-radioactive XG9, XG9n and XG7 were prepared and h.p.l.c.-purified by the methods of McDougall & Fry (1990, 1991). XG3 was a generous gift from Dr. Y. Kato, Sendai, Japan. Cello-oligosaccharides and monosaccharides were from Sigma Chemical Co., Poole, Dorset, U.K.

Polysaccharides

Xyloglucan from *Tropaeolum* seeds and from *Rosa* cell suspension cultures was purified by Cu^{2+} precipitation (Rao, 1959). Rhamnogalacturonans were prepared from the walls of cultured *Acer* cells by the methods of Darvill *et al.* (1978) and McNeil *et al.* (1980). *Fagus* glucuronoxylan (Kohn *et al.*, 1986*a*) and *Secale* arabinoxylan (Kohn *et al.*, 1986*b*) were generous gifts from Dr. A. Ebringerová, Bratislava, Czechoslovakia. *Hordeum* arabinoxylan was as used by Ahluwalia & Fry (1986). Other polysaccharides were purchased from BDH, Sigma, Koch-Light and Bauer & Pfaltz.

Enzymes

Trichoderma cellulase and *Irpex lacteus* 'Driselase' were from Sigma Chemical Co. The 'Driselase' was partially purified as before (Fry, 1988).

Extraction of XET

Rapidly growing plant tissue (fresh or frozen) was used as the source of XET. For peas, the third (= uppermost) internodes were cut from 7–8-day etiolated seedlings, mixed with a little acid-washed sand, and ground with a pestle and mortar in ice-cold extractant [usually distilled water, though we have recently obtained higher activities using 10 mM-$CaCl_2$/10 mM-ascorbate/20 mM-Mes (Na^+), pH 6.0] at 1 ml/g fresh weight. The homogenate was centrifuged at 2000 ***g*** for 5 min, and the supernatant was used as the XET preparation.

Standard XET assay

All the experiments used the following basic methods except where otherwise stated. The reaction mixtures (total volume 40 μl) contained 2 mg of xyloglucan/ml, 1.4 kBq [^{3}H]XG9 (2.9 μM), 50 mM-Mes (Na^+), pH 6.0, and 10 μl of enzyme extract. After 1 h at 25 °C, the reaction was stopped by the addition of 100 μl of 20% (w/v) formic acid; the solution was dried on to a 5 cm × 5 cm square of Whatman 3MM filter paper, which was washed for at least 30 min in running tap water to remove unchanged [^{3}H]XG9. The square of paper was then redried at 60 °C, rolled into a cylinder with the loaded side facing outwards, placed in a 22 ml scintillation vial, soaked with approx. 2 ml of scintillant [0.5% 2,5-diphenyloxazole/0.05% 1,4-bis(5-phenyloxazol-2-yl)benzene in toluene] and assayed for ^{3}H by scintillation counting. The counting efficiency was approx. 44% for [^{3}H]xyloglucan and approx. 7% for [^{3}H]oligosaccharides, presumably because the latter penetrated the paper fibres whereas the former coated them. Since most of the assays were conducted with oligosaccharide concentrations well below the K_m, product formation is reported as Bq of [^{3}H]polymer formed per kBq of [^{3}H]oligosaccharide supplied.

Assay of endotransglycosylation of polysaccharides other than xyloglucan

For the testing of donor substrate specificity, the reaction products (after addition of formic acid) were applied to 46 cm × 57 cm sheets of Whatman 3MM paper and chromatographed for 18 h in ethyl acetate/acetic acid/water (10:5:6, by vol.). Unchanged [^{3}H]XG9 had R_F approx. 0.1, whereas the polysaccharides were immobile (Fry, 1988); > 97% of the ^{3}H was accounted for in these two zones. Polymeric material (R_F = 0.00) was assayed for ^{3}H as already described and corrected for the value obtained in the absence of added polysaccharide.

Assay of protein

The protein content of plant extracts was assayed by the Coomassie Blue G dye-binding method as modified by Read and Northcote (1981).

Auxin induction of cellulases

Day-8 etiolated pea seedlings were sprayed with 0.1% 2,4-dichlorophenoxyacetic acid (2,4-D) in a solution containing 0.1% Tween 80 and 0.1 M-NaCl (pH adjusted to 7.0) and grown in the dark for a further 4 days. This caused the growing zone of the third internode to swell laterally whereas control stems (sprayed with Tween/NaCl alone) continued to elongate. Whole third internodes were excised from treated and untreated plants on day 12, and extracted with the buffer used by Wong *et al.* (1977) (20 mM-sodium phosphate, pH 6.2, containing 1% glucose and 5% glycerol) followed by the same buffer containing

1 M-NaCl. Each extractant was used at 1 ml/g initial fresh weight. For assay of cellulase (CM-cellulase), the enzyme extract (0.5 ml) was mixed with 2 ml of 0.8 % (w/v) CM-cellulose in the low-salt buffer and incubated at 25 °C. At intervals (0–3 h), approx. 1.2 ml of the reaction mixture was sucked into a 1 ml pipette and the time taken for the meniscus to flow from the 1.0 ml to the 0.5 ml mark was recorded. The initial mean flow time was 13.2 s.

RESULTS

Demonstration of XET activity

XET activity was readily detected in pea stem homogenates (Fig. 1). XET was assayed by its ability to transfer part of a large (M_r approx. 2×10^5) non-radioactive xyloglucan molecule to a [^{3}H]nonasaccharide ([^{3}H]XG9; for structure, see Fig. 2), forming a [^{3}H]polymer (Scheme 1; A = [^{3}H]XG9). The [^{3}H]polymer produced in the reaction was distinguished from the [^{3}H]XG9 starting material in three ways: (1) the [^{3}H]polysaccharide bound tightly to cellulose (filter paper) during prolonged washing in water (e.g. Fig. 1), (2) it precipitated with ethanol (results not shown) and (3) it was immobile on paper chromatography (as in Table 4). For most of the following investigations, method (1) was used.

The [^{3}H]XG9 became the reducing terminus of the newly labelled polysaccharide, as demonstrated by the fact that its (reducing terminal) [1-^{3}H]glucose unit could be oxidized in warm NaOH to yield ^3H_2O and could be reduced by $NaBH_4$ to yield a [^{3}H]glucitol moiety (results not shown; cf. Smith & Fry, 1991). Thus the [^{3}H]XG9 was the acceptor for XET-catalysed transglycosylation and the polysaccharide was the donor. The ^{3}H could be released from the [^{3}H]polysaccharide, in the form of a product chromatographically identical with [^{3}H]XG9, by digestion with *Trichoderma* cellulase [endo-β-(1→4)-D-glucanase]. Since there are no cellulase-labile sites in XG9, this indicates that the non-radioactive portion of the polysaccharide (shown in bold in Scheme 2) contributed a β-(1→4)- linkage to the product and thus that the acceptor hydroxy group of XG9 was O-4 of the glucose residue (underlined in Scheme 2) furthest from the reducing terminus. The enzyme assayed *in vitro* in this work thus corresponded closely to the characteristics of the endotransglycosylation *in vivo* of xyloglucans reported earlier (Smith & Fry, 1991).

Occurrence of XET in the plant kingdom

XET activity was extractable from the growing portions of all plants tested (Table 1). These included dicotyledons, graminaceous monocotyledons, a liliaceous monocotyledon, a moss and a liverwort (both sporophyte and gametophyte). XET thus seems likely to be present in all land plants. The high activity in the Gramineae is perhaps surprising in view of the relatively low xyloglucan content of these plants (for details, see Fry, 1988).

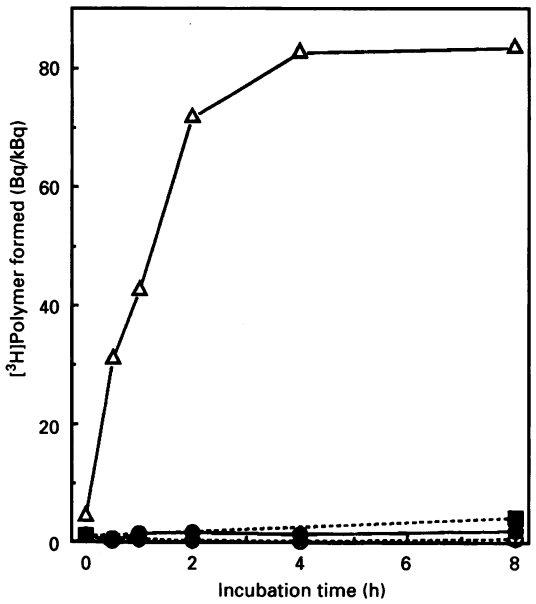


Fig. 1. Time-course for action of pea XET on xyloglucan + [^{3}H]XG9

The buffer was 50 mM-citrate + 50 mM-phosphate (Na^+). ○, pH 2.0; △, pH 6.0; ●, pH 6.0, enzyme boiled for 5 min before assay; ■, pH 6.0, no xyloglucan added.

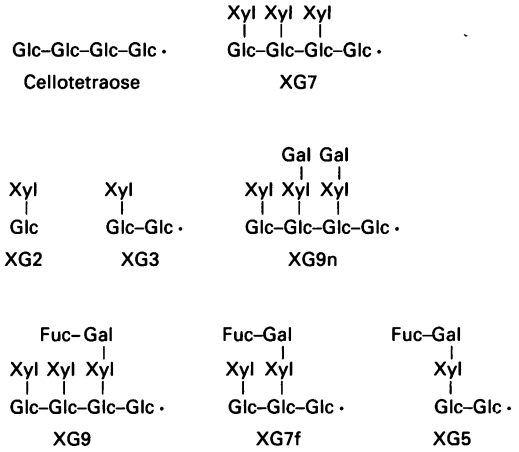
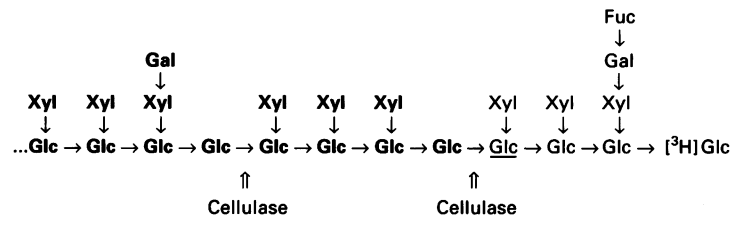


Fig. 2. Proposed structure of XG9 and related oligosaccharides

For further details, see Bauer *et al.* (1973), McDougall & Fry (1990, 1991) and Smith & Fry (1991). Glc· = reducing terminus.



Scheme 2.

Table 1. Extraction of XET activity from growing plant tissues

The extractant used was 10 mM-$CaCl_2$/10 mM-sodium ascorbate/20 mM-sodium Mes, pH 6.0. Young rapidly growing portions of each plant were used. Each assay contained the quantity of protein (range 2.6–44.0 μg) extracted from 5 mg fresh weight of tissue. Values are corrected for the apparent rate of [^{3}H]polymer formation in enzyme-free blanks (11.8±2.1 Bq/kBq per h per 5 mg fresh weight).

Plant	Organ extracted	XET activity (Bq/kBq per h)	
		Per 5 mg fresh wt.	Per μg of soluble protein
Bryophyta: Hepaticae			
Marchantia polymorpha (liverwort)	Seta	24.4	9.38
Marchantia polymorpha (liverwort)	Thallus	19.9	6.86
Bryophyta: Musci			
Mnium hornum (moss)	Gametophyte	64.5	18.4
Monocotyledons: Liliaceae			
Allium schoenoprasum (chives)	Peduncle	102	10.2
Monocotyledons: Gramineae			
Zea mays (maize)	Leaf+stem	161	10.4
Holcus lanatus (Yorkshire fog)	Leaf+stem	324	18.5
Bromus erectus (brome grass)	Leaf+stem	110	9.57
Dicotyledons			
Lupinus polyphyllus (lupin)	Leaf	55.1	3.44
Pisum sativum (pea)	Etiolated stem	97.1	2.21
Anthriscus sylvestris (cow parsley)	Leaf	33.7	2.25
Acer pseudoplatanus (sycamore)	1st year stem	39.4	6.06
Lycopersicon esculentum (tomato)	Leaf+stem	272	14.3
Taraxacum officinale (dandelion)	Peduncle	33.3	5.12

Enzymic characteristics

The enzyme was inactivated by boiling (Fig. 1). The reaction rate was fairly constant for the first 2 h, but then rapidly decreased; routine assays were conducted for 1 h (Fig. 1). The initial reaction rate was proportional to the amount of plant extract added (results not shown). The pH optimum was approx. 5.5, and the enzyme was less than half as active at pH 7.0 (Fig. 3). Non-radioactive XG9 decreased the incorporation of [^{3}H]-XG9 into xyloglucan (Fig. 4; see also Table 3) and the apparent K_m was 50 μM-XG9. The optimum xyloglucan concentration was approx. 2 mg/ml (approx. 10 μM); higher concentrations presumably competed with the [^{3}H]XG9 as an acceptor. XET activity was slightly enhanced by 1 mM-spermidine and 2-mercaptoethanol at various concentrations and 10 mM-Ca^{2+}, -Mg^{2+}, -Mn^{2+} and -ascorbate. It was partially inhibited by

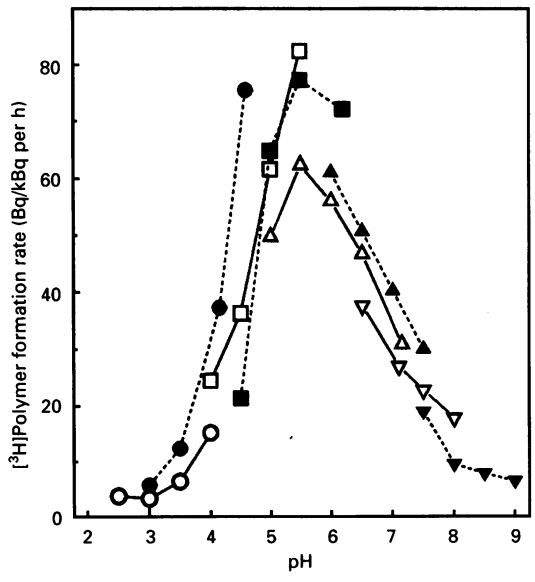


Fig. 3. pH-dependence of action of pea XET on xyloglucan+[^{3}H]XG9

The buffers used were (all 50 mM): ○, citrate; ●, formate; □, acetate; ■, phthalate; △, Mes; ▲, phosphate; ▽, Hepes (all Na^+ salts); or ▼, Tris (acetate salt).

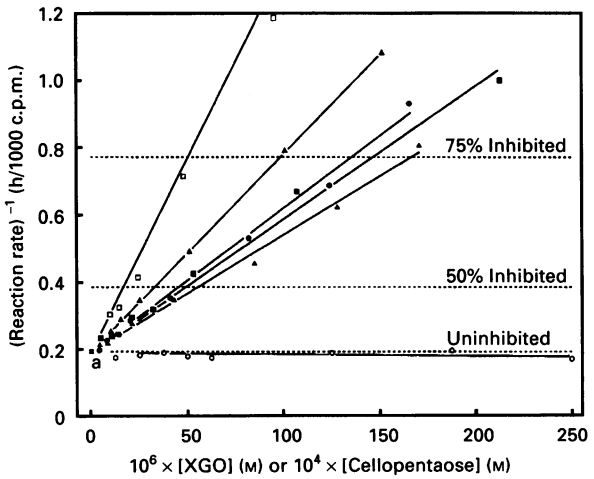


Fig. 4. Inhibition of the XET-catalysed incorporation of [^{3}H]XG9 into xyloglucan by added non-radioactive oligosaccharides

A pea stem extract was incubated with 2 mg of xyloglucan/ml+2.2 μM-[^{3}H]XG9 either alone or supplemented with 0–220 μM non-radioactive xyloglucan oligosaccharides (XGO] or with 0–25 mM-cellopentaose. The ordinate shows the reciprocal of the initial incorporation rate (h/1000 c.p.m.); the uninhibited rate was 5180 c.p.m./h. □, XG9n; △, XG7; ■, ●, ▲, three different samples of XG9; ○, cellopentaose. The zero oligosaccharide control value (a) shows the mean±S.E.M. of ten determinations.

Table 2. Activators and inhibitors of XET

Pea stem extract was assayed for XET activity in the presence of the additives listed. The XET activity of the controls in this experiment was 37.4±1.5 Bq/kBq per h. The results are means±S.E.M. for 3–4 determinations.

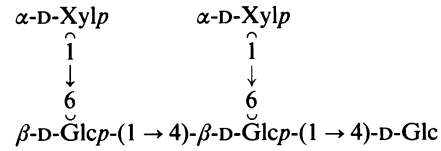
Additive	Concentration (mM)	XET activity (% of control)
None	–	100±4
$AgNO_3$	1	26±2
$Hg(OAc)_2$	1	30±3
$ZnCl_2$	10	49±3
$LaCl_3$	10	74±4
EDTA	10	82±8
$CaCl_2$	10	126±1
$MgSO_4$	10	127±4
Ascorbic acid	10	106±1
2-Mercaptoethanol	2.5	127±4
2-Mercaptoethanol	5	133±1
2-Mercaptoethanol	10	134±1
2-Mercaptoethanol	20	132±1
Spermidine	1	122±4
Putrescine	1	104±1
NaF	10	95±4
Gluconolactone	10	98±2

1 mM-Ag^+ and -Hg^{2+} and 10 mM-La^{3+} and -Zn^{2+}. It was unaffected by 10 mM-D-gluconolactone and by chelating agents (Table 2).

Acceptor substrate specificity

The acceptor substrate specificity was investigated in two ways (Table 3). First, several alternative [^{3}H]oligosaccharides were tested as potential acceptors in place of [^{3}H]XG9. Removal of the non-reducing terminal Xyl→Glc unit of [^{3}H]XG9 (by the method of McDougall & Fry, 1991) (yielding [^{3}H]XG7f; see Fig. 2) did not diminish acceptor activity, but removal of the next Xyl→Glc unit {yielding [^{3}H]XG5 (Glc_2XylGalFuc)} abolished activity, suggesting that a non-reducing terminal Xyl→Glc group was essential. The disaccharide [^{3}H]XG2 [α-D-Xyl*p*-(1→6)-D-Glc; isoprimeverose], however, was not an acceptor.

Second, various non-radioactive oligosaccharides were tested as competitors of the reaction with [^{3}H]XG9. Estimated I_{50} values were 19 μM-XG9n and 33 μM-XG7 (Fig. 4) and these oligosaccharides thus appeared to be better substrates than XG9 itself. Their activity shows that the fucose and galactose residues of XG9 were not required for acceptor activity. Cellopentaose did not compete at concentrations up to 25 mM (Fig. 4), nor did cellotetraose (equivalent to the Glc_4 backbone of XG9) at 5 mM (Table 3). Xylose, glucose, cellobiose, maltose, maltotetraose and XG3 (Xyl→Glc→Glc) also did not compete. Thus the simplest structural unit that we can propose for an acceptor is:



It is not yet clear whether both xylose residues are required.

Donor substrate specificity

Numerous polysaccharides were tested as potential donor substrates (Table 4). In this experiment, paper chromatography was used to separate the unchanged [^{3}H]XG9 from [^{3}H]polysaccharides (if any) because few polysaccharides bind to filter paper as tightly as xyloglucans. Nevertheless, xyloglucan was the only polysaccharide to act as an efficient donor substrate (Table 4). *Tropaeolum* seed xyloglucan (which lacks fucose) was somewhat better than *Rosa* callus xyloglucan (which is fucose-rich). No other β-glucans showed significant activity: cellulose, CM-

Table 3. Acceptor substrate specificity of XET from pea stems

The [^{3}H]XG9 in the standard XET assay (using *Tropaeolum* xyloglucan as donor) was either supplemented by a non-radioactive oligosaccharide or replaced by a different [^{3}H]oligosaccharide (0.14–1.9 kBq per assay).

Acceptor substrate	Rate (Bq/kBq per h)
[^{3}H]XG9 ($Glc_4Xyl_3GalFuc$)	46.0
[^{3}H]XG9+5 mM-cellotetraose	53.8
[^{3}H]XG9+25 mM-cellopentaose	49.0
[^{3}H]XG9+50 mM-cellobiose	41.4
[^{3}H]XG9+10 mM-xylose	49.9
[^{3}H]XG9+130 μM-XG9 ($Glc_4Xyl_3GalFuc$)	12.7
[^{3}H]XG9+130 μM-XG9n ($Glc_4Xyl_3Gal_2$)	5.8
[^{3}H]XG9+130 μM-XG7 (Glc_4Xyl_3)	9.2
[^{3}H]XG2 [α-D-Xyl*p*-(1→6)-D-Glc]	0.4
[^{3}H]XG5 (Glc_2XylGalFuc)	2.8
[^{3}H]XG7f ($Glc_3Xyl_2GalFuc$)	51.2

Table 4. Donor substrate specificity of XET from pea stems

Each polysaccharide was tested at 1.25 mg/ml using [^{3}H]XG9 as acceptor. All the polysaccharides formed clear solutions at pH 6.0 (those marked * by autoclaving for 15 min) except cellulose and chitosan (suspensions) and nigeran, the three xylans and *Hordeum* arabinoxylan (hazy solutions).

Substrate	Rate (Bq/kBq per h)
Blank (no added polysaccharide)	0.0
Cellulose [β-(1→4)-D-glucan], microgranular	0.1
CM-cellulose* (Na^+ salt)	1.0
Methylcellulose	−0.2
Xanthan gum* (*Xanthomonas campestris*)	1.1
Xyloglucan (*Trapaeolum* seed)	47.9
Xyloglucan (*Rosa* cultures)	24.0
Laminarin [β-(1→3)-D-glucan]	−0.4
β-(1→3), (1→4)-D-Glucan (*Hordeum*)*	−0.1
Lichenan* [β-(1→3), (1→4)-D-glucan] (*Cetraria*)	−1.0
Dextran [α-(1→6)-D-glucan]	0.3
Nigeran* [α-(1→3), (1→4)-D-glucan]	−0.4
Starch* [α-(1→4), (1→6)-D-glucan]	0.0
Glucuronoxylan* (*Fagus* wood)	5.4
Xylan* [β-(1→4)-D-xylan] (*Avena*) (Sigma)	1.3
Xylan* (Pfaltz & Bauer)	−0.1
Xylan* (Koch–Light)	1.0
Arabinoxylan* (*Secale*)	−0.6
Arabinoxylan* (*Hordeum*)	−0.4
Guar* [D-galacto-β-(1→4)-D-mannan]	0.1
Mannan (*Saccharomyces*)	0.0
Inulin* [β-(2→1)-D-fructan] (*Dahlia*)	−0.5
Chitosan	0.4
Polygalacturonic acid [α-(1→4)-D-galacturonan]	0.3
Pectin (*Citrus*)	0.9
Rhamnogalacturonan-I (*Acer* cell cultures)	1.8
Rhamnogalacturonan-II (*Acer* cell cultures)	0.4
α-(1→5), (1→3)-L-Arabinan (*Beta*)	0.2
β-(1→4)-D-Galactan	0.2
Arabinogalactan (*Larix*)	0.1

cellulose, methylcellulose, xanthan, β-(1→3),(1→4)-D-glucan, and laminarin were all inactive. *Fagus* wood glucuronoxylan [a β-(1→4)-linked D-xylan with α-D-glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid side chains] appeared to be 11% as effective as *Tropaeolum* xyloglucan, and several other xylan-related polysaccharides were therefore tested; however, three samples of β-(1→4)-D-xylan and two of arabinoxylan did not act.

The very high specificity of XET for xyloglucans resembles that of an endo-β-glucanase from *Tropaeolum* cotyledons (Edwards *et al.*, 1986), which also exhibits transglycosylase activity (C. Fanutti & J. S. G. Reid, personal communication).

Relationship with cellulase

XET differed from the major cellulases [endo-(1→4)-β-D-glucanases] of pea stems in several respects. First, cello-oligosaccharides that inhibit the hydrolysis of CM-cellulose by pea cellulase had no effect on XET (Table 3; Fig. 4). Cellopentaose at 2 mM gives 40–60% inhibition of pea cellulases (Wong *et al.*, 1977) but even 25 mM-cellopentaose did not affect XET.

Second, XET failed to transfer CM-cellulose or β-(1→3),(1→4)-D-glucan to [^{3}H]XG9 (Table 4), although these polysaccharides are efficiently hydrolysed by pea cellulases (Wong *et al.*, 1977).

Third, the auxin 2,4-D induced a large increase in concentrations of buffer- and salt-extractable cellulases, as reported by Hayashi *et al.* (1984), but caused some decrease in extractable XET activity (Table 5). XET extracted from 2,4-D-treated pea stems was inhibited by 50 μM-XG9n almost as effectively as was XET extracted from control stems (cf. Fig. 4), indicating that the lower apparent activity in the latter was not due to the presence of elevated concentrations of co-extracted xyloglucan oligosaccharides.

Fourth, a high-salt extract from 2,4-D-treated tissue that had been thoroughly pre-extracted with low-salt buffer contained low XET but high cellulase activity (Table 5).

Fifth, the XET activity of pea stem extracts was increased by approx. 30% on addition of 5–20 mM-2-mercaptoethanol (Table 2). We were unable to confirm the inhibitory effect of 2-mercaptoethanol on pea cellulases reported by Wong *et al.* (1977). Nevertheless, 2-mercaptoethanol did not promote cellulase activity whereas it did promote XET activity.

XET is not bound to cell walls

Most of the XET in pea stems was not ionically bound to the cell wall. Of several reagents that solubilize wall-bound enzymes (Fry, 1988), only 20 mM-$LaCl_3$ extracted any XET activity (a trace) after thorough pre-extraction with water (Table 6). Triton X-100 also failed to extract any additional activity, arguing against entrapment of XET within membranous vesicles.

Relatively little XET appeared to be covalently bound in the cell wall. The water-washed residue of pea stem homogenates showed little ability to incorporate [^{3}H]XG9 into either wall-bound or soluble xyloglucan (Table 6).

Table 5. XET and cellulase activity of extracts from pea stems grown with or without auxin

Pea seedlings were sprayed with or without 2,4-D and grown on for 4 days, and then the third internode was extracted sequentially with low- and high-salt buffers. The extracts were assayed for XET using xyloglucan+[^{3}H]XG9 and for cellulase using CM-cellulose.

Pretreatment	Extractant	XET (Bq/kBq per h)	Cellulase $\Delta\eta/t$* (s/h)
−2,4-D	Buffer	24.3	0.0
−2,4-D	Buffer+1 M-NaCl	4.8	1.0
+2,4-D	Buffer	7.8	8.8
+2,4-D	Buffer+1 M-NaCl	2.3	5.3

* $\Delta\eta$ = decrease in viscometer flow time (initially 13.2 s); t = incubation time.

Table 6. Extraction of XET from pre-extracted pea stems

Tissue from the third internode of day-8 etiolated pea stems was thoroughly pre-extracted by grinding and washing in distilled water, and then shaken gently for 3 h at 20 °C in the extractant indicated (1 ml/g original fresh weight). Buffer [20 mM-Mes (Na^+), pH 6.0] was present in all extractants. Controls showed that neither the XET-catalysed reaction nor the binding of [^{3}H]xyloglucan to filter paper was affected by these extractants at the final concentrations used. In the last two samples ('no extractant'), the wall-rich residue from tissue that had been thoroughly pre-extracted with water was resuspended in the assay solution (xyloglucan+[^{3}H]XG9) at 1 g initial fresh weight/ml; after incubation, the solution was tested for soluble [^{3}H]xyloglucan (solution) by the filter paper binding assay, and the insoluble residue (walls) was washed in water and assayed for bound ^{3}H.

Extractant	XET activity (Bq/kBq per h)
Control (not pre-extracted)	82.6
Buffer (Mes, pH 6.0)	0.0
1% Triton X-100	0.0
40 mM-$CaCl_2$	0.3
100 mM-$CaCl_2$	0.3
250 mM-$CaCl_2$	0.1
20 mM-$LaCl_3$	2.1
40 mM-$LaCl_3$	0.6
100 mM-$LaCl_3$	0.0
2.0 M-LiCl	0.5
2.7 M-LiCl	0.0
No extractant (solution)	0.2
No extractant (walls)	5.7

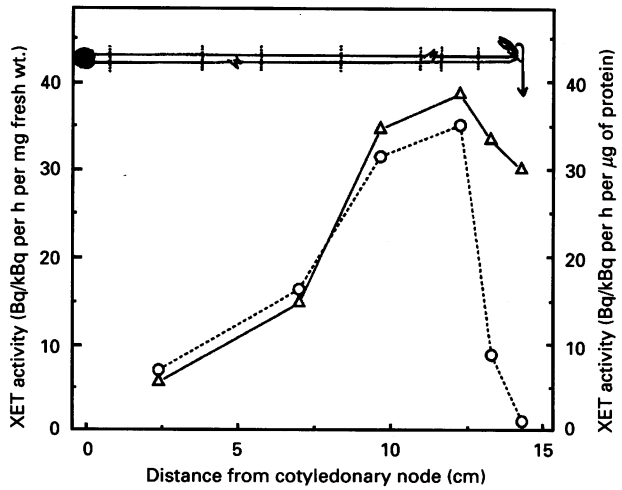


Fig. 5. Distribution of extractable XET activity along the pea stem

Day-7 etiolated pea seedlings were dissected into segments as indicated in the drawing and each segment was weighed and assayed for extractable XET and soluble protein. △, XET activity per mg fresh weight of tissue; ○, XET activity per μg of extractable protein.

Correlations between XET activity and growth rate

We observed several correlations between extractable XET activity and growth rate, as expected if XET catalyses a wall-loosening reaction. First, XET activity was highest in the third (= fastest growing) pea internode, a short distance back from the apical bud (Fig. 5), and substantially lower in the first internode. This relationship was seen whether XET activity was expressed per unit fresh weight of tissue or per μg of extracted protein. Second, excised pea stem segments lost XET activity during an 18 h incubation, paralleling their fall in growth rate (K. F. Renwick & S. C. Fry, unpublished work). Third, the XET activity of cultured spinach cells peaked during the most rapid phase of cell expansion (R. C. Smith & S. C. Fry, unpublished work).

DISCUSSION

Xyloglucan-derived oligosaccharides can activate plant (but not fungal) 'cellulase' in a viscometric assay using xyloglucan (but not CM-cellulose) as substrate (Farkaš & Maclachlan, 1988; McDougall & Fry, 1990). The oligosaccharides (1–100 μM) were observed to cause an immediate but short-lived burst of xyloglucan depolymerization. A second burst occurred upon the addition of a second dose of oligosaccharide (McDougall & Fry, 1990), suggesting that the oligosaccharides exerted a stoichiometric rather than regulatory effect. In the light of the present results, it seems likely that the reaction detected viscometrically was not hydrolysis of the xyloglucan but XET-catalysed transglycosylation, with the oligosaccharides acting as acceptors. In support of this, the structural requirements for an oligosaccharide to act as an acceptor for XET (assayed radiochemically; Table 3) match closely the requirements for promotion of the enzymic depolymerization of xyloglucan (assayed viscometrically; E. P. Lorences & S. C. Fry, unpublished work).

According to this interpretation, in the absence of oligosaccharides the enzyme would catalyse an 'idling' reaction in which the new (potentially reducing) terminus of each cleaved xyloglucan chain (□□□□□□...) is transferred to the non-reducing terminus of another chain (■■■■■■...), resulting in no net change in mean M_r and therefore no loss of viscosity:

□□ + ■■■■■■■■■■■■■■■■■■■■■■■■■■■■ ⟷
□□□□□□□□□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■ + □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□

Evidence for the occurrence of such an 'idling' reaction has been presented (Figs. 7a and 7b of McDougall & Fry, 1990). The gradual fall in viscosity which is nevertheless observed in the absence of oligosaccharides could be due partly to the presence of cellulase, which catalyses the hydrolysis of xyloglucan, and partly to a possible limited ability of XET to catalyse hydrolysis at low substrate concentrations. The strong 'stimulation' of xyloglucan depolymerization seen (by viscometry) upon addition of 1–100 μM-oligosaccharides would be due to their ability to act as low-M_r acceptor substrates, competing with the idling reaction. The non-reducing termini of the xyloglucan used in viscometry studies (approx. 20 μM) were roughly equimolar with the added oligosaccharides. Thus the following reaction would compete with the idling reaction:

□□ + ● ⟷
□□□□□□□□□□□● + □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□,

where ● = oligosaccharide. Since the oligosaccharide is much smaller than the polysaccharide, transglycosylation with an oligosaccharide as acceptor has practically the same effect on viscosity as does endohydrolysis:

□□ + H_2O ⟷
□□□□□□□□□□□ + □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□

Although we have assayed XET with oligosaccharides as acceptors, we suggest that *in vivo* the usual acceptor is polymeric wall-bound xyloglucan. XET would cleave a xyloglucan chain, locally weakening the cell wall and allowing incremental expansion (Fry, 1989*b*), then rejoin the cut portion to the non-reducing terminus of a different xyloglucan chain, restoring the strength of the wall fabric (Smith & Fry, 1991). The enzyme could also play a role in the initial integration of new xyloglucan chains into the architecture of the wall by linking them to the ends of older chains.

Certain oligosaccharides (e.g. 1 μM-XG9) promote cell extension when added to pea stem segments, and this was interpreted as being due to a promotion of the cell wall-loosening action of cellulase (McDougall & Fry, 1990). We now suggest that the growth-enhancing action of xyloglucan oligosaccharides is due to their ability to compete with structural xyloglucans as acceptors during the action of XET, thus interfering in the rejoining process and keeping the wall 'loose'. Consistent with this idea is the observation that XG9n is somewhat more effective than XG9 both in promoting extension (McDougall & Fry, 1990) and as a substrate for XET (Table 3). Also, XG5, which is not an acceptor for XET (Table 3), seems to be unable to enhance extension (McDougall & Fry, 1989).

Thus, we propose that XET contributes to growth promotion in two ways. (1) In the absence of oligosaccharides it causes a polysaccharide-to-polysaccharide transglycosylation, reversibly loosening the cell wall and permitting molecular creep. (2) In the presence of suitable oligosaccharides it causes a polysaccharide-to-oligosaccharide transglycosylation, effectively cleaving the polymer and resulting in a more permanent wall-loosening.

XET could also play an important role in the numerous other situations where a partial lysis of the cell wall occurs, e.g. in ripening fruit, in the abscission zone, in the mobilization of storage xyloglucan during and after germination, in cell separation after mitosis, and in the formation of perforation plates and sieve plates in developing xylem and phloem cells respectively.

Our results demonstrate the widespread occurrence of XET, a new enzyme activity, in the growing tissues of higher plants and bryophytes. The action of XET suggests that it is central to the mechanism of plant growth, reversibly rendering the cell wall

more extensible. The simple assay developed for XET should facilitate future studies of its occurrence and function.

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