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Differences in catalytic properties between native isoenzymes of xyloglucan endotransglycosylase (XET)

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Abstract

Four isoenzymes of xyloglucan endotransglycosylase (XET; EC 2.4.1.207) were isolated from sprouting mung bean seedlings (M35, M45, M55a, M55b) and two from cauliflower florets (C30, C45). Purification in each case was by ammonium sulphate precipitation, reversible formation of a covalent xyloglucan–enzyme complex, and cation-exchange chromatography. The isoenzymes differed in pH optimum (range 5.0–6.5), K_m for the nonasaccharide XLLGol ($Gal_2.Xyl_3.Glc_3$.glucitol) as acceptor substrate, ability to utilise diverse oligosaccharides as acceptor substrate, and ability to bind to carboxymethyl-cellulose (and thus possibly to other polyanions such as pectin in the cell wall). None of the isoenzymes was particularly cold-tolerant, unlike one XET (TCH4) of *Arabidopsis*. The two cauliflower isoenzymes had higher K_m values for XLLGol (70–130 μM) than the four mung bean isoenzymes (16–35 μM). We suggest that this difference is related to the major roles of the XETs in these two tissues: integration of new xyloglucan into the walls of the densely cytoplasmic cauliflower florets, and re-structuring of existing wall material in the rapidly vacuolating bean shoots.

Keywords: *Brassica oleracea* var. botrytis; Compositae; Cauliflower; *Vigna radiata*; Leguminosae; Mung bean; Isoenzyme activity characterisation; Xyloglucan endotransglycosylases; Xyloglucan; Oligosaccharide; Transglycosylation; Cell walls

1. Introduction

Xyloglucan is a major structural polysaccharide of the primary cell walls of higher plants and as such is thought to play a central role in determining many of the biologically relevant properties of the wall (Fry, 1989a; Hayashi, 1989). It appears to hydrogen-bond to the cellulose microfibrils and possibly to tether them, thus limiting cell expansion (Fry, 1989b; McCann et al., 1990), and a proportion of the wall's xyloglucan is covalently bonded to pectic polysaccharides (Thompson and Fry, 2000). Enzyme activities that cleave xyloglucan are thus of considerable interest as potential wall-loosening agents which could be important in the mechanism of cell expansion growth, fruit softening during ripening and cell separation during abscission. One enzyme that cleaves xyloglucan is xyloglucan endotransglycosylase (XET; EC 2.4.1.207) (Baydoun and Fry, 1989; Smith and Fry, 1991; Fry et al., 1992; Nishitani and Tominaga, 1992). XET cleaves a glycosidic linkage in the β-glucan backbone of xyloglucan (= donor substrate) with the concomitant formation of a xyloglucan–XET covalent complex (Sulová et al., 1998); this is followed some time later by the transfer of the xyloglucan portion from this complex on to the non-reducing terminus of an acceptor molecule, which can be either another xyloglucan chain or an oligosaccharide thereof.

XET activity and XET gene expression are found in most, if not all, expanding plant cells (Fry et al., 1992; Pritchard et al., 1993; Nishitani and Tominaga, 1992; Xu et al., 1995; Antosiewicz et al., 1997; Smith et al.,

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1996; Oh et al., 1998). The occurrence of XET protein in primary cell walls has been detected immunologically (Antosiewicz et al., 1997); in addition, it has been shown that active XET and accessible donor substrate chains co-occur in primary cell walls (Ito and Nishitani, 1999; Vissenberg et al., 2000). Often XET activity appears to correlate with rapid cell expansion, suggesting a causal role in this process. Although moderate XET activity often remains detectable in tissues that have stopped expanding (Campbell and Braam, 1999a), this observation does not argue against a role for XET in cell expansion since it is likely that other factors e.g. phenolic cross-linking (Müsel et al., 1997; Fry et al., 2000) over-ride the ability of XET to loosen the wall in mature tissues.

Ammonium sulphate precipitation of proteins from cauliflower florets revealed the existence of several discrete classes of XET activity (Steele and Fry, 1999), and isoelectric focusing of similar extracts resolved at least eight XET activity bands differing widely in isoelectric point (Iannetta and Fry, 1999). The *Arabidopsis* genome encodes at least 21 XET-related proteins (XTRs, defined as sequences resembling known XETs, whether or not the translation product has yet been shown to possess XET activity) (Xu et al., 1996; Nishitani, 1997; Campbell and Braam, 1999a).

One of the most extensively studied XETs is TCH4 of *Arabidopsis*. Accumulation of *TCH4* mRNA is promoted during mechanical stimulation (e.g. by wind) of the *Arabidopsis* plant (Braam and Davis, 1990). However, a *TCH4*::*GUS* transgene is also expressed in expanding tissues in the absence of deliberate mechanical stimulation (Xu et al., 1995). In addition, an antibody raised against TCH4 (but probably also recognising other XETs) labelled not only *Arabidopsis* meristems but also developing pith parenchyma and mesophyll cells at the positions of future cell junctions (Antosiewicz et al., 1997). The involvement of XETs in the re-modelling of cell wall contacts during the production of air spaces is also supported by a correlation between XET expression and the production of aerenchyma in flooded maize roots (Saab and Sachs, 1996).

The various XTR genes other than *TCH4* are expressed in a tissue-specific manner and in response to different stimuli, e.g. heat-shock, cold, hormone treatments (Aubert and Herzog, 1996; Clouse, 1996) and mechanical stimulation (Purugganan et al., 1997; Nishitani, 1997). However, little is known of the catalytic differences, if any, between the various XTRs, and it is therefore difficult to comment on their individual biological roles.

Potential sources of the variation between native XETs isolated from diverse plant tissues include (a) the genetically defined amino acid sequence and (b) the co- or post-translationally determined degree of glycosylation. The *N*-glycan is important for enzyme activity in TCH4 but not in two other *Arabidopsis* XETs — EXGT and XTR9 (Campbell and Braam, 1998, 1999b).

One potential catalytic difference between isoenzymes is in their substrate affinity. The K_m of TCH4 for a low-M_r acceptor substrate (XLLGol; for nomenclature, Section 4.1 and Fry et al., 1993) is 73 μM, whereas its K_m for high-M_r xyloglucan as acceptor substrate is ~0.3 μM (Purugganan et al., 1997), indicating a much higher affinity for the polysaccharide. This suggests that TCH4 preferentially catalyses interpolymeric rather than polysaccharide-to-oligosaccharide transglycosylation. Comparison of XETs with respect to their K_m values is complicated by the fact that different laboratories have used different oligosaccharides as acceptor substrates. XET from ripening kiwi fruit has a K_m of 100 μM for XXXGol (Schröder et al., 1998). Unpurified XET activity (probably mixed isoenzymes) from pea stems has apparent K_m values of 19, 50 and 33 μM for XLLG, XXFG and XXXG, respectively (Fry et al., 1992) and of ~300 μM for the pentasaccharide XXG (Lorences and Fry, 1993). Unpurified XET activity (again probably mixed isoenzymes) from the medium of suspension-cultured poplar cells has K_m values of 320 μM for the Glc_4-based heptasaccharide, XXXGol; 230 μM for the Glc_8-based tetradecasaccharide, XXXGXXXGol; and higher values for larger acceptors. No transglycosylase activity was detected with oligosaccharides having backbones of Glc_{16} or longer (Takeda et al., 1996). In view of the diverse assay conditions used by different authors, a systematic study was required to determine the affinities of different XETs to a standardised substrate.

Another parameter that might vary between XET isoenzymes is temperature-dependence. TCH4 is surprisingly cold-tolerant: its optimum temperature is ca. 12° and at −5° it still exhibits 55% of its maximal catalytic rate (Purugganan et al., 1997), suggesting a role in the adaptation of growing *Arabidopsis* to cold conditions. TCH4 is exceptional in its cold-tolerance: total XET activity (mixed isoenzymes) from *Arabidopsis* has a temperature optimum of ca. 30°. More studies were therefore needed of the temperature-dependence of diverse XETs.

All XETs characterised to date have pH optima in keeping with their proposed location in the cell wall. Unpurified XETs from pea stems have an average pH optimum of ca. 5.5 (Fry et al., 1992). Purified TCH4 has an optimum of pH 6.0–6.5 (Purugganan et al., 1997), mung bean stem XET of 5.8–6.0 (Tabuchi et al., 1997), and ripening kiwi fruit XET of 5.5–5.8 (Schröder et al., 1998).

Although many XET and XTR sequences have been identified and their expression patterns documented, at least at the mRNA level, information on the catalytic

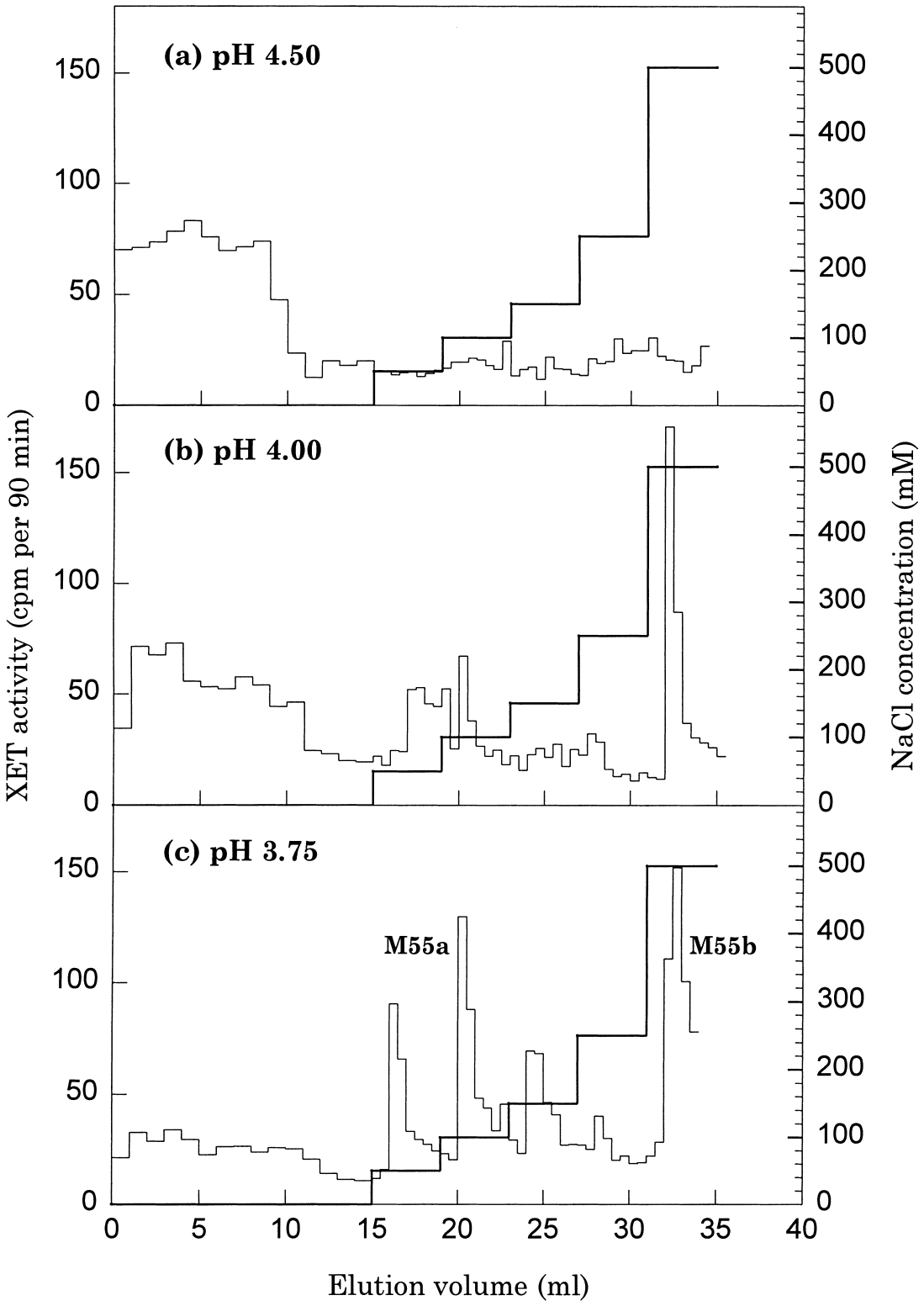

Fig. 1. Effect of pH on binding to CM-cellulose and subsequent elution of XET activity. Aliquots of fraction M55 (= bean shoot XET precipitated by 55% saturated ammonium sulphate) were applied to CM-cellulose in buffers of pH 4.50, 4.00 or 3.75 and then eluted (——) with a step gradient of NaCl (——).

properties of this large group of cell wall enzymes is not so plentiful. The aim of the present study was therefore to perform a concerted set of enzymatic analyses on a group of pure, native, XET isoenzymes from cauliflower florets and shooting mung beans. Both these tissues have high XET activity but they form an interesting contrast: cauliflower florets are rich in small, densely cytoplasmic cells, where wall assembly predominates; on the other hand, mung bean hypocotyls are rich in rapidly expanding, vacuolated cells, where the re-structuring of existing wall material may predominate. The results highlight catalytic differences between these isoenzymes in their kinetic properties with respect to pH optima, K_m for XLLGol as acceptor substrate, and ability to utilise xyloglucan-derived oligosaccharides of various sizes.

2. Results

2.1. Purified XET activities

Stepwise ammonium sulphate precipitation from crude extracts of cauliflower florets and shooting mung beans revealed five distinct fractions of XET activity (Steele and Fry, 1999). Cauliflower activities were precipitated by 30% (C30) and 45% (C45) saturated ammonium sulphate, and mung bean activities by 35% (M35), 45% (M45) and 55% (M55) saturated ammonium sulphate (see Fig. 1 of Steele and Fry, 1999). The activities were purified to size-homogeneity by a mechanism-based method (Steele and Fry, 1999) which proved applicable to all five XET fractions. Representative SDS gels, showing a single 32-kDa polypeptide in each XET preparation, are given in the previous paper (Steele and Fry, 1999). However, all known XETs appear to be of approximately this size; therefore SDS gels by themselves do not confirm isoenzyme purity.

2.2. Cation-exchange chromatography

The five size-homogeneous XET preparations were subjected to cation-exchange chromatography at pH 3.75, chosen because this was the highest pH at which the majority of the XET activity would bind to CM-cellulose. For example, M55 revealed two isoforms: neither of them bound to CM-cellulose at pH 4.5; M55b was able to bind at pH 4.0 and required 500 mM NaCl for elution; M55a required pH 3.75 for binding and was readily eluted with ca. 100 mM NaCl (Fig. 1). Thus, at pH 3.75, both isoforms bound to CM-cellulose and application of a salt gradient enabled their separation.

Of the other XET preparations, some eluted from CM-cellulose as single peaks, others as multiple peaks. C30 eluted in 40 mM NaCl (Fig. 2(a)); C45 eluted in 500 mM (C45a) or 1 M NaCl (C45b) (Fig. 2(b)); M35 eluted in 50 mM (M35a) or 100 mM NaCl (M35b) (Fig. 2(c)); and M45 eluted in 50 mM (Fig. 2(d)). Multiple peaks may be artefacts due to the use of step-gradients; nevertheless, they were kept separately and examined for possible catalytic differences.

2.3. Tissue source of mung bean XETs

The shooting mung beans from which the enzymes were prepared included the cotyledons. Some of the mung bean XET isoenzymes could have arisen from the cotyledons (Farkaš et al., 1992; Fanutti et al., 1993). Tissue-prints (Fry, 1997) of sections of mung bean seedlings showed that XET activity was more abundant in the growing tissues of the hypocotyls and leaves than in the cotyledons (data not shown). This finding indicates that the isoenzymes investigated were principally associated with primary wall metabolism rather than mobilisation of any seed storage xyloglucan.

2.4. Temperature dependence

The temperature–activity profiles of the CM-cellulose-purified XET isoforms showed that all had a broad optimum (24–34°) and were heat-labile (Fig. 3). The activity at 3° (relative to that at the optimum temperature) varied over a three-fold range. However, none of these isoenzymes exhibited the exceptional cold-tolerance noted for TCH4 (Xu et al., 1995).

2.5. pH dependence

The pH–activity profiles of the CM-cellulose-purified isoforms fell into three classes (Fig. 4). Some isoforms had a relatively sharp pH optimum at either 5.0–5.5 (C30, M35a, M35b, M55b) or at ca. 6.5 (C45a, C45b) (Fig. 4(a)); others had a broad pH optimum of ca. 5.0–7.0 (M45 and M55a) (Fig 4b). This finding clearly demonstrates catalytic differences between the various XET isoforms.

2.6. K_m values

The K_m of each isoenzyme for XLLGol as acceptor substrate was determined from a plot of the reciprocal of the rate of ^{3}H-incorporation versus the total concentration of XLLGol (Fig. 5), the dose of [^{3}H]XLLGol being kept constant. The data approximated straight lines, as predicted by simple Michaelis–Menten kinetics. A first-order regression was extrapolated to the y-axis, giving an estimate of 1/(maximum

rate of ^{3}H-incorporation). The concentration of XLLGol that halves the maximum rate of ^{3}H-incorporation (i.e., the intersect with the *x*-axis) is an estimate of the apparent K_m of the isoenzyme for XLLGol as acceptor substrate.

The results (Fig. 5) show that the mung bean shoot XETs had a higher affinity for XLLGol as acceptor substrate (K_m in the range 16–35 μM) than did the cauliflower floret XETs (K_m 71–130 μM). This again demonstrates catalytic differences, this time mainly correlating with the botanical source of the enzyme.

2.7. Oligosaccharide acceptor preferences

Each cation-exchange-purified XET preparation was tested against a panel of oligosaccharidyl-[^{3}H]alditols (with backbones ranging from [^{3}H]Glc_2-ol to [^{3}H]Glc_{16}-ol) as potential acceptor substrates. [^{3}H]Glc_{16}-ol-based substrates were the largest that could be tested by the filter paper-binding method of Fry et al. (1992); larger oligosaccharides are themselves able to bind to the paper (Vincken et al., 1995) and thus could not be distinguished from transglycosylation products formed by reaction with the donor

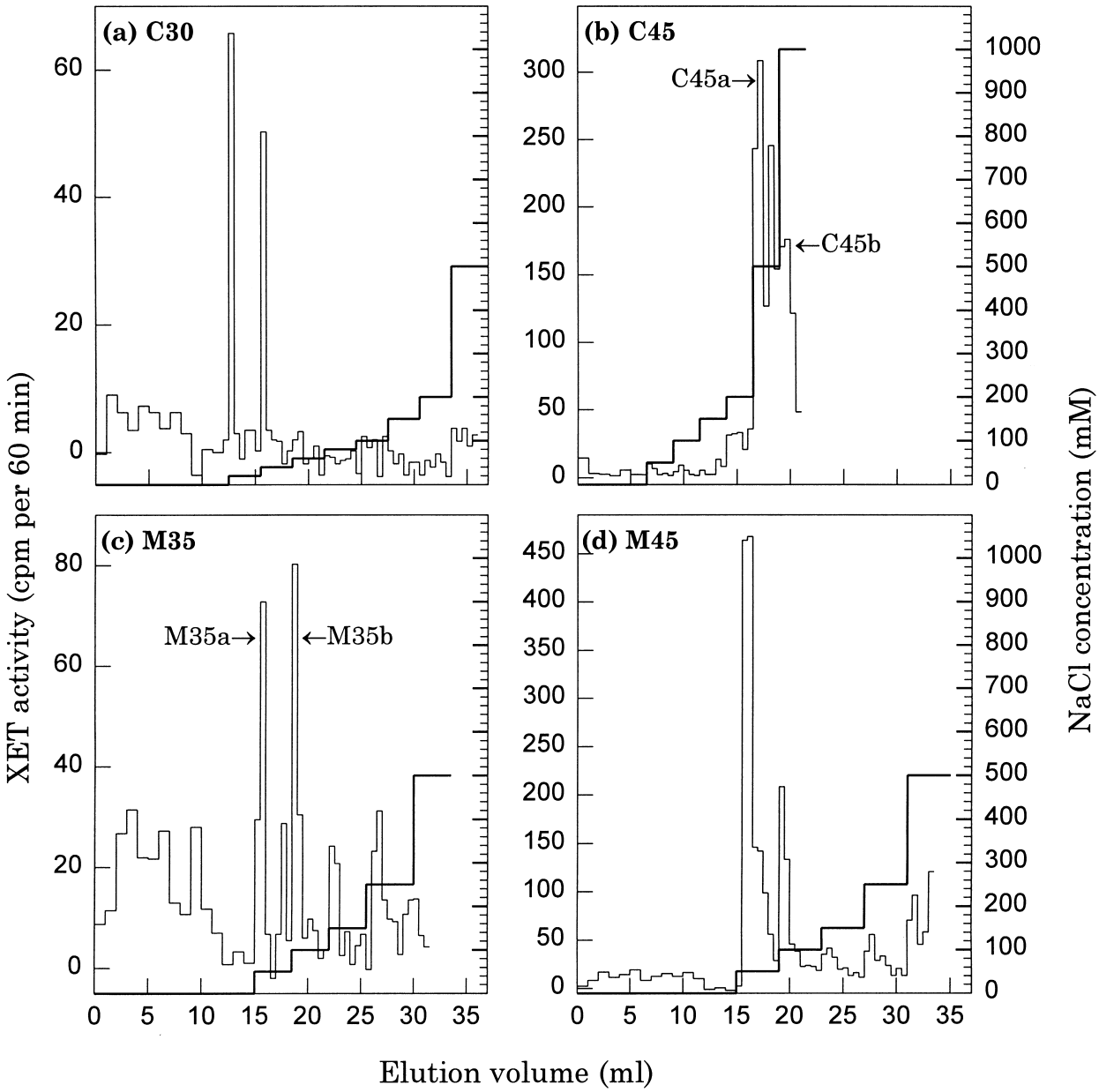

Fig. 2. Binding of various XET preparations to CM-cellulose at pH 3.75 and subsequent elution. Aliquots of XET preparations from cauliflower (C30, C45) or mung bean shoots (M35, M45) were applied to CM-cellulose at pH 3.75 and then eluted (——) with a step gradient of NaCl (——) in the same buffer.

(tamarind xyloglucan). Each ^{3}H-oligomer was tested at a low absolute concentration, well below any reported K_m values for oligomeric acceptor substrates. Thus, rates expressed as Bq of ^{3}H-polymeric product formed per kBq of ^{3}H-acceptor substrate per hour do not permit calculation of K_m or V_{max} values but will provide qualitative information on the structural requirements of the isoenzymes' acceptor sites and will indicate the relative activity of a given XET on each oligomer at low concentration.

Only one of the eight XETs (M55a) had any, slight, activity on the smallest oligomer tested — the Glc_2-ol-based trimer, XGol (Fig. 6). All eight isoforms had low but significant activity on the Glc_3-ol-based pentamer, XXGol. Activities on the Glc_4-ol-based heptamer, XXXGol, were 9–19 times greater than on XXGol. Thus, there was a pronounced preference for oligosaccharides with backbones of DP 4 > 3 > 2.

Galactosylation (XXXGol → XLLGol) was consistently beneficial for acceptor substrate function. However, the isoforms differed in their sensitivity to galactosylation of the acceptor substrate, showing a 1.7–3.6-fold preference for the bis-galactosylated XLLGol over the corresponding non-galactosylated molecule, XXXGol.

All XETs tested were able to utilise Glc_8-ol- and Glc_{16}-ol-based oligomers as acceptor substrates, although there was wide variation in the isoforms' preferences for these larger substrates. C30 preferred the Glc_{16}-ol-based oligomers over XLLGol, whereas the other isoforms preferred XLLGol. Curiously, the Glc_{12}-ol-based oligomers appeared to be less favoured by all XETs than the Glc_8-ol- and Glc_{16}-ol-based oligomers.

These results confirm that catalytic differences exist

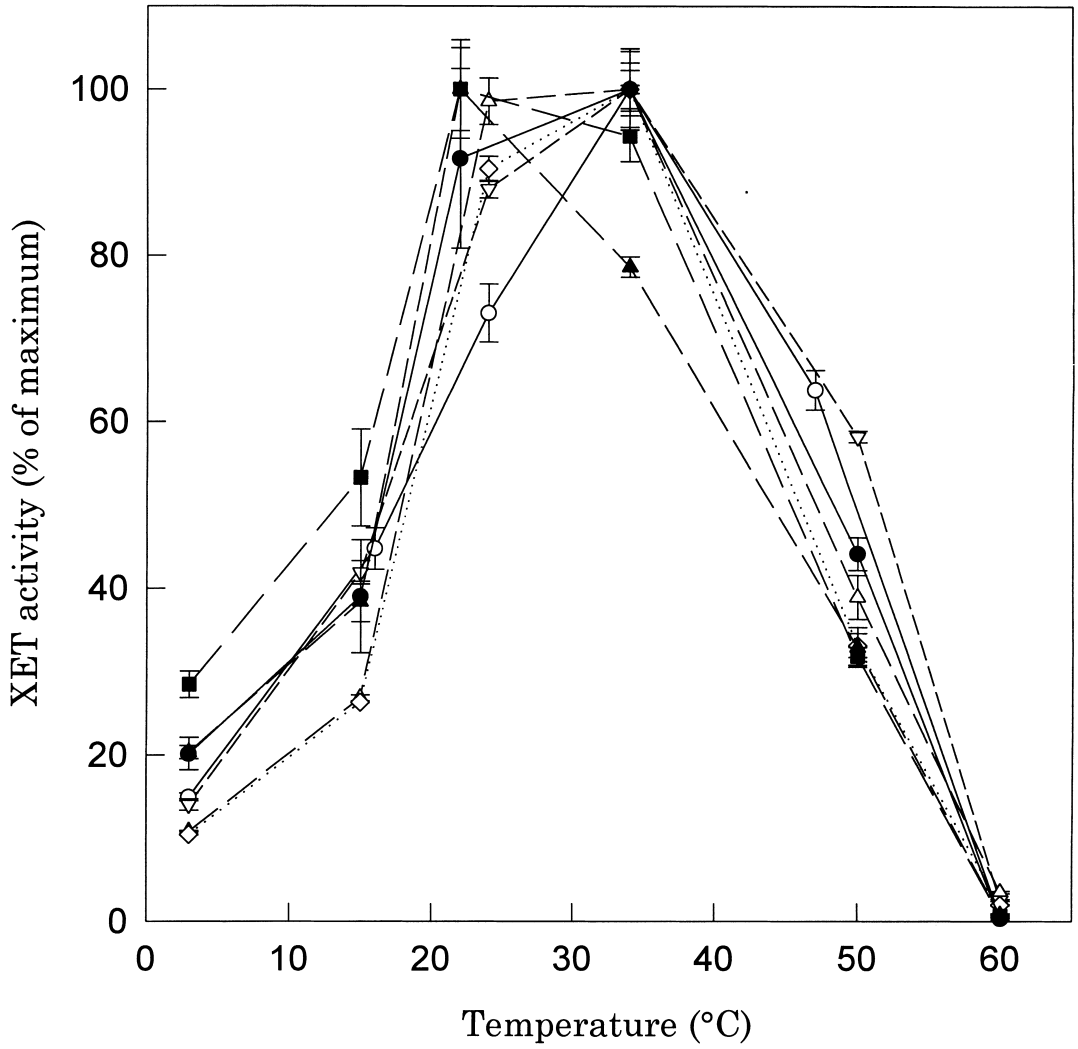

Fig. 3. Effect of temperature on the activity of XET isoenzymes purified by ion-exchange chromatography. Open symbols represent mung bean preparations: ○ —— ○, M35a; □— —□, M35b; △– – –△, M45; ▽ - - - - - ▽, M55a; ◇ ····· ◇, M55b. Filled symbols represent cauliflower preparations: ● —— ●, C30; ■— —■, C45a; ▲– – –▲, C45b. Each assay was performed in triplicate and s.e. is shown.

between the various XET isoenzymes purified, indicating that they may serve different roles in muro.

3. Discussion

A fractionation scheme based on differential precipitation with ammonium sulphate followed by cation-exchange chromatography has resolved a series of isoenzymes from both cauliflower and mung bean. Each preparation was size-homogeneous (~32 kDa) according to SDS gels (Steele and Fry, 1999), showing that few or no proteins other than XETs remained; however, SDS gels cannot establish whether each preparation contained only a single isoenzyme since most XETs are of a similar size

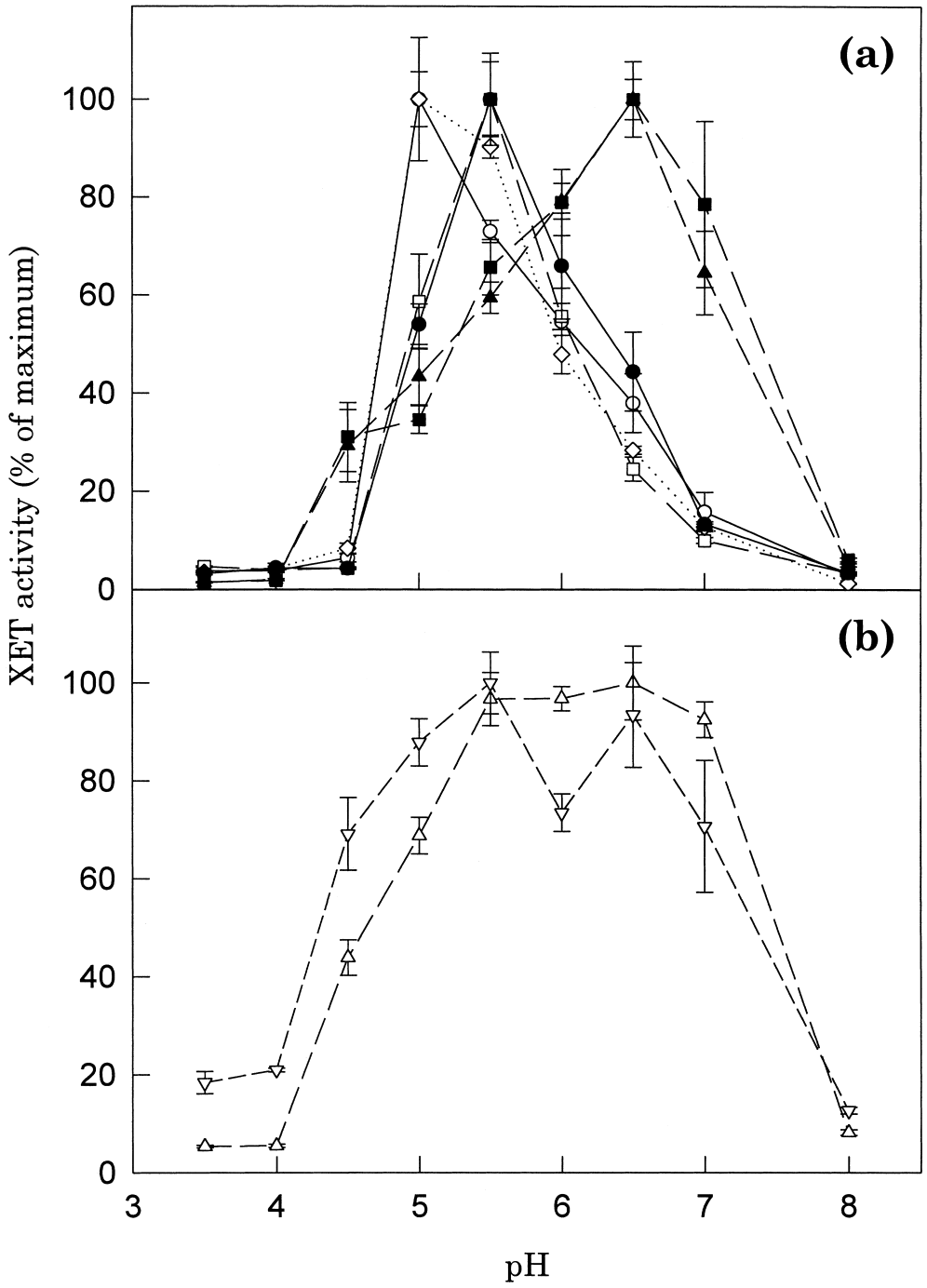

Fig. 4. Effect of pH on the activity of XET isoenzymes purified by ion-exchange chromatography. Symbols are as in Fig. 3. Each assay was performed in triplicate and s.e. is shown.

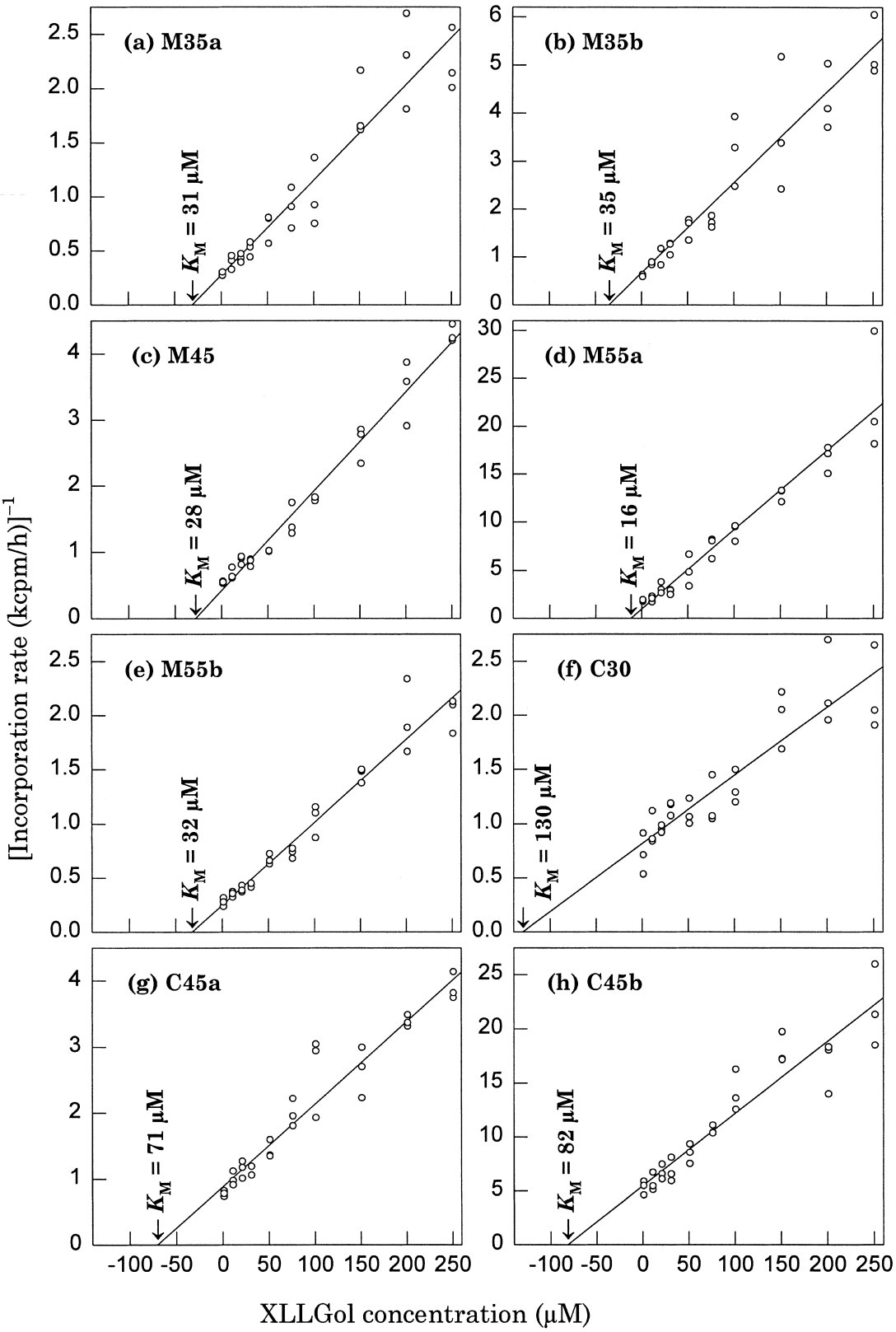

Fig. 5. Affinity for XLLGol as acceptor substrate of XET isoenzymes purified by ion-exchange chromatography. Each assay was performed with a standard dose of [^{3}H]XLLGol plus various concentrations of non-radioactive XLLGol. The *y*-axis shows the reciprocal of the rate of incorporation of ^{3}H into polymeric products. The intersect of the regression line with the *x*-axis is an estimate of $-K_m$ of the isoenzyme for XLLGol. Isoenzymes tested were (a) M35a, (b) M35b, (c) M45, (d) M55a, (e) M55b, (f) C30, (g) C45a and (h) C45b.

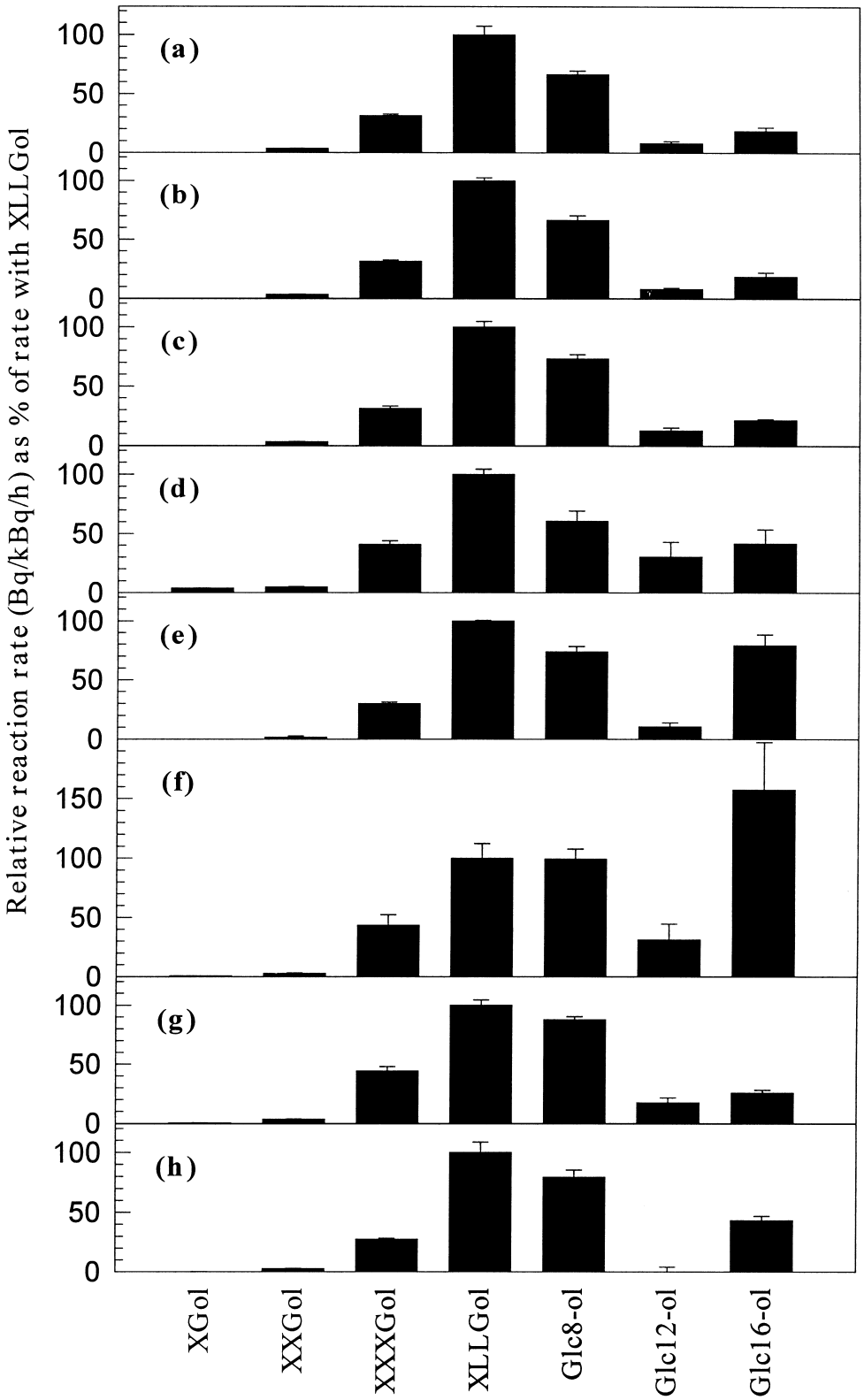

Fig. 6. Relative effectiveness of various reduced oligosaccharides as acceptor substrates of XET isoenzymes purified by ion-exchange chromatography. Each potential acceptor substrate was tested at high specific radioactivity; data are presented as Bq of ^{3}H-labelled polymeric products formed per kBq of starting material during a 1-h incubation; background binding of each oligosaccharide to Whatman 3MM paper was subtracted. Each assay was performed in triplicate and s.e. is shown. Isoenzymes tested were (a) M35a, (b) M35b, (c) M45, (d) M55a, (e) M55b, (f) C30, (g) C45a and (h) C45b.

(Campbell and Braam, 1999a). Isoenzyme purification therefore relied on cation-exchange properties. The possibility remains that some of the preparations contained more than one individual isoenzyme; nevertheless, we did detect catalytic difference between some of the isoenzyme preparations. This is the main conclusion of the present paper.

Cauliflower florets, which are rich in small, densely cytoplasmic cells, yielded at least two isoenzymes (C30 and C45). C45 was only partially eluted (C45a) from the cation-exchange column by 500 mM NaCl; however, C45b (eluted by 1 M NaCl) did not show any appreciable catalytic differences from C45a and may thus simply have been the tail of the C45 peak. C30 did show catalytic differences from C45: the former had a greater ability to utilise Glc_{16}-ol-based acceptor substrates, a much lower tenacity of binding to CM-cellulose and a lower pH optimum.

Mung bean shoots, which are rich in rapidly expanding, vacuolated cells, yielded at least four isoenzymes (M35, M45, M55a and M55b). M35 was only partially eluted (as M35a) from CM-cellulose by 50 mM NaCl, but M35b (eluted by 100 mM NaCl) did not show any clear catalytic differences from M35a. However, M55a and M55b did differ from each other not only in affinity for the cation-exchange resin (eluted by 100 and 500 mM, respectively), but also in acceptor substrate preference and in pH profile. M45 also differed catalytically from M35, M55a and M55b.

The four mung bean isoenzymes all had a considerably higher affinity for XLLGol as acceptor substrate than did either of the cauliflower isoenzymes (Fig. 5). A high affinity for low-DP acceptor substrates, as exhibited by the mung bean isoenzymes, may indicate a role in wall re-structuring, which is necessary during the wall loosening that underlies the enormous cell expansion occurring in a shooting bean sprout. The cauliflower isoenzymes, in contrast, may prefer polymeric acceptors and thus contribute principally to the integration of new wall material (Thompson et al., 1997) into the small, dividing cells of the cauliflower floret. In support of this interpretation, it has been found that, in cultured rose cells, 1 mM exogenous XLLG only partially inhibited the interpolymeric transglycosylation associated with integration of newly secreted xyloglucan, but strongly inhibited the interpolymeric transglycosylation involved in 're-structuring' of existing wall material (Thompson et al., 1997; Thompson and Fry, unpublished); this suggests that the XETs involved in wall assembly have a lower affinity for small acceptor molecules than do those involved in wall re-structuring.

The pH–activity profiles revealed marked differences between XET isoenzymes. Optima of pH 5.0–5.5 would be characteristic of wall enzymes. The higher pH optima (~6.5), exhibited by other isoenzymes, may suggest action in different sub-cellular locations, possibly including endo-membrane vesicles during the transport of xyloglucans from their site of synthesis in the Golgi bodies (Driouich et al., 1994) to their destination in the wall. Such an activity (C45) might be expected to be abundant in the densely cytoplasmic cauliflower floret cells. None of the XET isoenzymes tested here exhibited any unusual cold-tolerance of the type seen with TCH4 (Purugganan et al., 1997). However, the isoenzymes did differ in their affinity for CM-cellulose, suggesting that they may differ in binding to acidic pectin and thus in their placement within the cell wall in vivo.

In conclusion we have confirmed the presence in vivo of multiple XET isoenzymes, whose existence had been suspected on the basis of studies of mRNA expression (Xu et al., 1996; Nishitani, 1997). Furthermore, we have shown that the isoenzymes exhibit differences with respect to functionally relevant features such as acceptor substrate affinity and specificity, pH optimum, and ability to bind acidic polysaccharides. The precise physiological role of each isoenzyme remains to be elucidated but the present work provides a foundation for the hypothesis that different XETs do indeed serve different roles.

4. Experimental

4.1. Materials

Xyloglucan, prepared from tamarind flour by a method similar to that of Edwards et al. (1986), was a generous gift of Mr. K. Yamatoya, Dainippon Pharmaceutical, Osaka, Japan. Xyloglucan oligosaccharides are named according to the abbreviated nomenclature of Fry et al. (1993): each (1 → 4)-linked glucose residue (and the reducing terminal glucose group) of the oligosaccharide's backbone is given a 1-letter code according to its substituents (if any); the name of the oligosaccharide consists of these code letters listed in sequence from the non-reducing terminus of the backbone. The code letters used in the present paper are (all residues in the pyranose ring-form): G = β-D-Glc; X = α-D-Xyl-(1 → 6)-β-D-Glc; L = β-D-Gal-(1 → 2)-α-D-Xyl-(1 → 6)-β-D-Glc; Gol = D-glucitol. An oligosaccharide mixture (principally XLLG, XXLG and XXXG, but also containing several larger and smaller oligosaccharides), produced from tamarind xyloglucan by digestion with cellulase, was also kindly provided by Mr. Yamatoya. A sample of the trisaccharide, XG, was kindly provided by Dr. Y. Kato, Hirosaki University, Japan.

4.2. Buffers

The following buffers were used: A, 100 mM succinate (Na^+), 15 mM $CaCl_2$, 10% glycerol (pH 5.5); B, 20 mM acetate (Na^+), 10% glycerol (pH 5.0); C, as B with the pH adjusted to pH 3.75 by addition of HOAc; D, pyridine–HOAc–H_2O (1:1:98, by volume, pH 4.7) containing 0.5% 1,1,1-trichloro-2-methylpropan-2-ol as an anti-microbial agent; E, 350 mM succinate (Na^+), 16.7 mM $CaCl_2$, 1.67 mM dithiothreitol (pH 5.5); F, 100 mM succinate (Na^+), 18 mM acetate (Na^+), 9% glycerol, (pH 5.5).

4.3. Fractionation of oligosaccharides

A portion of the oligosaccharide mixture (500 mg in 20 ml buffer 'D') was chromatographed on a 750-ml bed-volume column of Bio-Gel P-2 in buffer 'D'. A portion (5 μl) of each fraction was subjected to TLC on silica gel in PrOH–nitromethane–H_2O (5:2:3) and stained with ethanolic orcinol–H_2SO_4. A second portion (20 μl) was analysed by PC in EtOAc–HOAc–H_2O (10:5:6) and stained with aniline hydrogen-phthalate (Fry, 2000). Besides traces of glucose and xylose, thirteen carbohydrate spots (**A**–**M**) were detected by TLC and collected individually or in pools (Table 1).

Fractions **E**, **F** and **G** (Table 1) were further purified by preparative PC on Whatman 3MM developed in BuOH–pyridine–H_2O (4:3:4) for 48 h by the descending method. The major bands were eluted by the method of Eshdat and Mirelman (1972); yields were: XXXG, 18.9 mg; XXLG, 51.7 mg; XLLG, 64.0 mg. Pool **I**–**K** and pool **L**–**M** were separately re-chromatographed on Bio-Gel P-4 and portions of the eluate were analysed by TLC as before; the fractions which gave the same major TLC spots as after the Bio-Gel P-2 run were pooled and freeze-dried.

4.4. Preparation of oligosaccharidyl-[1-^{3}H]alditols

To an aq. soln of each purified oligosaccharide preparation was added an aliquot of 10 mM NaB^3H_4 (326 MBq/μmol) in 1 M NH_3. Wherever possible, quantities were 10 mg oligosaccharide in 100 μl H_2O + 100 μl NaB^3H_4 soln (326 MBq). After incubation at 25° for 16 h, an excess of HOAc was added and the solns were left open in a fume cupboard for several hours to allow $^3H_2$ gas to dissipate.

[^{3}H]XGol (prepared from Dr Kato's XG sample) was further purified by PC in EtOAc–HOAc–H_2O (10:5:6) for 36 h. The Glc_4-ol-based compounds were further purified by preparative PC in BuOH–pyridine–H_2O (4:3:4) for 72 h; their R_{M7} values were [^{3}H]XXXGol = 1.02, [^{3}H]XXLGol = 0.91, [^{3}H]XLLGol = 0.78, the marker maltoheptaose (M7) migrating 33 cm. Higher-DP ^{3}H-compounds (produced from pools **I**–**K** and **L**–**M**) were applied to 1-mm-thick layers of silica-gel and developed in PrOH–nitromethane–H_2O (5:2:3). All chromatograms were fluorographed after dipping through 7% PPO in Et_2O (Randerath, 1970). The major radioactive spots were eluted with 0.5% aq. 1,1,1-trichloro-2-methylpropan-2-

Table 1
Fractionation on Bio-Gel P-2 of oligosaccharides from tamarind seed xyloglucan

Spot	K_{av} on Bio-Gel P-2	Staining intensity on TLC	Apparent 'DP' (Glc-units)[a]		R_{M7}[b]		Proposed identity	Yield on drying (mg)
			by TLC	by PC	on TLC	on PC		
Glc	1.00	+	1.0	1.0			Glucose	
A	0.82	+++	3.2	2.3			XG	35
B	0.66	+	2.8	~3				} 21
C	0.64	+	4.0	~3				
D	0.59	+	3.2	~3				1
E	0.44	+++	~5	~7			XXXG	38
F	0.39	++++	~6	~7			XXLG	107
G	0.33	+++++	~8	~7			XLLG	149
H	0.25	++			0.60	ns		6
I	0.13	+			0.51	0.2	Glc_8-based	
J	0.11	++			0.39	0.2	Glc_8-based	} 25
K	0.09	++			0.32	0.2	Glc_8-based	
L	0.03	+			0.10	ns	Glc_{12}-based	} ~2
M	0.01	++			0.00	ns	Glc_{16}-based	

[a] Apparent degree of polymerisation (DP) in 'Glc-units' relative to malto-oligosaccharides (DP 1–8) analysed on the same chromatogram. Solvents used were propan-1-ol–nitromethane–water (5:2:3) for TLC and ethyl acetate–acetic acid–water (10:5:6) for PC.

[b] R_{M7} = chromatographic mobility relative to that of maltoheptaose; ns = not detectably stained.

ol; 0.33 vol. of EtOH was added to the eluate and the solution stored at 4°. The two main fractions in the **L–M** pool (the putative [^{3}H]Glc_{12}-ol- and [^{3}H]Glc_{16}-ol-based oligosaccharides, respectively) were separated from each other by gel-permeation chromatography on Bio-Gel P-6.

[^{3}H]XXGol was prepared by partial digestion of [^{3}H]XXXGol with Driselase followed by fractionation of the products on Bio-Gel P-2. The products were [^{3}H]XXGol, [^{3}H]XGol and a trace of [^{3}H]glucitol. Radiochemical purity of the isolated [^{3}H]XXGol was established by TLC on silica-gel in BuOH–HOAc–H_2O (2:1:1), ^{3}H being monitored on a radio-isotope thin layer analyser (RITA; LabLogic, Sheffield).

The [^{3}H]XGol, [^{3}H]XXXGol, [^{3}H]XXLGol and [^{3}H]XLLGol had sp. act ca. 80 MBq/μmol. The [^{3}H]Glc_8-ol-, [^{3}H]Glc_{12}-ol- and [^{3}H]Glc_{16}-ol-based oligomers had sp. act ca. 6.5, 3.9 and 2.0 MBq/μmol, respectively.

4.5. Preparation of non-radioactive XLLGol

A further portion of XLLG was reduced with an excess of non-radioactive $NaBH_4$ in 1 M NH_4OH, and the XLLGol formed was purified by GPC on Bio-Gel P-2. On TLC, the product was stainable with orcinol–H_2SO_4 but not with aniline hydrogen-phthalate, indicating that it had been successfully reduced.

4.6. Preparation of XET isoenzymes

Five putative isoenzymes of XET were prepared as described by Steele and Fry (1999). Cauliflower florets and shooting mung beans (400 g fr. wt) were homogenised in ice-cold buffer 'E' in an AtoMix blender. The homogenate was left at 4° for 2 h with occasional mixing, strained through four layers of muslin and centrifuged at 2500 $\times g$ for 10 min.

XET activities, pptd by step-wise addition of ammonium sulphate at 4°, were purified to size-homogeneity (as judged by SDS–PAGE) by a two-column method (Steele and Fry, 1999) that exploits the stable association formed between XET and its donor substrate. Each activity, eluted from the second column in buffer 'A', was dialysed into buffer 'B' overnight at 4°. The pH was adjusted to pH 3.75 (forming buffer 'C') by addition of glacial acetic acid immediately before cation-exchange chromatography.

Whatman CM22 carboxymethyl-cellulose (CM-cellulose) was washed in 0.5 M NaOH for 30 min followed by 0.5 M HCl for 30 min. To achieve a high column flow-rate, we then mixed the CM-cellulose with a large volume of buffer C in a beaker and allowed it to settle for 30 s for every cm in height of the suspension. The supernatant was rejected and the process repeated three times. The resulting cation-exchange resin was stored at room temperature in buffer C containing 1% NaN_3 until needed, when 1-ml beds were prepared in BioRad 'PolyPrep' columns. The column was rinsed in 5 vol. of buffer C containing 1 M NaCl, followed by 5 vol. of buffer C.

Each size-homogeneous preparation of XET (~40 ml in buffer C) was applied to a column, and eluted with 5 vols. of buffer C followed by a step-wise gradient of NaCl in buffer C as indicated on the graphs. Each XET-containing eluate was immediately adjusted to pH 5.5 by addition of $\frac{1}{9}$ vol. of 1 M succinate (forming buffer 'F') and quickly frozen in liquid nitrogen.

4.7. Assay of XET activity

XET activity was assayed using a method similar to that of Fry et al. (1992). Enzyme solns were diluted in buffer 'F' such that all samples contained approximately the same activity when assayed with [^{3}H]XLLGol as acceptor substrate. The appropriately diluted enzyme (10 μl) was added to 20 μl of a substrate mixture to give (final concentrations) 0.45% (w/v) tamarind xyloglucan and 167 μM [^{3}H]XLLGol (specific radioactivity adjusted to 102 kBq/μmol by addition of non-radioactive XLLGol) in buffer 'F', pH 5.5. Incubation was at 20° (unless otherwise stated) for 1 h, during which time the reaction rate was approximately constant. The reaction was stopped by addition of 20 μl 50% formic acid; the products were dried on Whatman 3MM paper and washed in running water overnight. The paper was then re-dried and the bound [^{3}H]xyloglucan assayed using OptiScint Hisafe scintillation fluid (Wallac, Milton Keynes, Bucks, UK).

For investigation of pH–activity profiles, the substrate mixtures were modified to contain 100 mM succinic acid and 100 mM HEPES, adjusted with NaOH to pH values such that when 20 μl was mixed with 10 μl of enzyme soln the final pH had the required value.

For determination of K_m values, the substrate mixtures were designed such that the final reaction mixture always contained 0.45% xyloglucan and 1.02 kBq of [^{3}H]XLLGol (80 MBq/μmol) in buffer 'A'; various doses of non-radioactive XLLGol were also included to give the final desired concentrations. The K_m value of each isoenzyme was determined from the concentration of non-radioactive XLLGol required to give half the maximal rate of incorporation of ^{3}H into high-M_r products.

For the investigation of oligosaccharide preferences, the substrate mixtures were such that the final reaction mixtures contained 0.45% xyloglucan in buffer 'F', plus a low concentration of a high-specific-radioactivity (carrier-free) oligosaccharidyl-[^{3}H]alditol. The ^{3}H dose per assay was 1.9 kBq [^{3}H]XGol, 1.1 kBq [^{3}H]XXGol, 1.8 kBq [^{3}H]XXXGol 1.8 kBq

[^{3}H]XLLGol, or 1.5 kBq [^{3}H]Glc_8-ol-based, 0.3 kBq of [^{3}H]Glc_{12}-ol-based or 0.1 kBq of [^{3}H]Glc_{16}-ol-based oligosaccharides. Data for enzyme-free controls were subtracted for each substrate tested.

4.8. Tissue printing

XET activity test-papers were as described by Fry (1997) and Iannetta and Fry (1999). Longitudinal sections of mung bean seedlings were briefly pressed against the moist papers, which were then incubated under humid conditions for 1 h. The distribution of the fluorescent reaction-products of XET activity was monitored under 254-nm UV (Fry, 1997).

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References

Antosiewicz, D.M., Purugganan, M.M., Polisensky, D.H., Braam, J., 1997. Cellular localization of *Arabidopsis* xyloglucan endotransglycosylase-related proteins during development and after wind stimulation. Plant Physiology 115, 1319–1328.

Aubert, D., Herzog, M., 1996. A new cDNA encoding a xyloglucan endotransglycosylase-related polypeptide (AtXTR8) preferentially expressed in seedling, root and stem of *Arabidopsis thaliana*. Plant Science 121, 187–196.

Baydoun, E.A.-H., Fry, S.C., 1989. In vivo degradation and extracellular polymer-binding of xyloglucan nonasaccharide, a natural anti-auxin. Journal of Plant Physiology 134, 453–459.

Braam, J., Davis, R.W., 1990. Rain-, wind- and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell 60, 357–364.

Campbell, P., Braam, J., 1998. Co- and/or post-translational modifications are critical for TCH4 XET activity. Plant J. 15, 553–561.

Campbell, P., Braam, J., 1999a. Xyloglucan endotransglycosylases: diversity of genes, enzymes and potential wall-modifying functions. Trends in Plant Science 4, 361–366.

Campbell, P., Braam, J., 1999b. In vitro activities of four xyloglucan endotransglycosylases from *Arabidopsis*. Plant Journal 18, 371–382.

Clouse, S.D., 1996. Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant Journal 10, 1–8.

Driouich, A., Levy, S., Staehelin, L.A., Faye, L., 1994. Structural and functional organisation of the Golgi apparatus in plant cells. Plant Physiology and Biochemistry 32, 731–749.

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.). Journal of Biological Chemistry 261, 9489–9494.

Eshdat, Y., Mirelman, D., 1972. An improved method for the recovery of compounds from paper chromatograms. Journal of Chromatography 65, 458–459.

Fanutti, C., Gidley, M.J., Reid, J.S.G., 1993. Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1 → 4)-β-D-glucanase) from the cotyledons of germinated nasturtium seeds. Plant Journal 3, 691–700.

Farkaš, V., Sulová, Z., Stratilová, E., Hanna, R., Maclachlan, G., 1992. Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. Archives of Biochemistry and Biophysics 298, 365–370.

Fry, S.C., 1989a. The structure and functions of xyloglucan. Journal of Experimental Botany 40, 1–11.

Fry, S.C., 1989b. Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. Physiologia Plantarum 75, 532–536.

Fry, S.C., 1997. Novel 'dot-blot' assays for glycosyltransferases and glycosylhydrolases: optimization for xyloglucan endotransglycosylase (XET) activity. Plant Journal 11, 1141–1150.

Fry, S.C., 2000. In: The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Reprint Edition. The Blackburn Press, Caldwell, NJ, USA [ISBN 1-930665-08-3].

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., Matthews, K.J., 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochemical Journal 282, 821–828.

Fry, S.C. Willis, S.C. Paterson, A.E.J., 2000. Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta, in press.

Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., White, A.R., 1993. An unambiguous nomenclature for xyloglucan-derived oligosaccharides. Physiologia Plantarum 89, 1–3.

Hayashi, T., 1989. Xyloglucans in the primary cell wall. Annual Review of Plant Physiology and Plant Molecular Biology 40, 139–168.

Iannetta, P.P.M., Fry, S.C., 1999. Visualization of the activity of xyloglucan endotransglycosylase (XET) isoenzymes after gel electrophoresis. Phytochemical Analysis 10, 140–238.

Ito, H., Nishitani, K., 1999. Visualization of EXGT-mediated molecular grafting activity by means of a fluorescent-labeled xyloglucan oligomer. Plant and Cell Physiology 40, 1172–1176.

Lorences, E.P., Fry, S.C., 1993. Xyloglucan oligosaccharides with at least two α-D-xylose residues act as acceptor substrates for xyloglucan endotransglycosylase and promote the depolymerisation of xyloglucan. Physiologia Plantarum 88, 105–112.

McCann, M.C., Wells, B., Roberts, K., 1990. Direct visualization of cross-links in the primary plant cell wall. Journal of Cell Science 96, 323–334.

Müsel, G, Schindler, T., Bergfeld, R., Ruel, K., Jacquet, G., Lapierre, C., Speth, V., Schopfer, P., 1997. Structure and distribution of lignin in primary and secondary cell walls of maize coleoptiles analysed by chemical and immunological probes. Planta 201, 146–159.

Nishitani, K., 1997. The role of endoxyloglucan transferase in the organization of plant cell walls. International Review of Cytology 173, 157–206.

Nishitani, K., Tominaga, R., 1992. Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. Journal of Biological Chemistry 267, 21058–21064.

Oh, M.H., Romanow, W.G., Smith, R.C., Zamski, E., Sasse, J., Clouse, S.D., 1998. Soybean BRU1 encodes a functional xyloglucan endotransglycosylase that is highly expressed in inner epicotyl tissues during brassinosteroid-promoted elongation. Plant and Cell Physiology 39, 124–130.

Pritchard, J., Hetherington, P.R., Fry, S.C., Tomos, A.D., 1993. Xyloglucan endotransglycosylase activity, microfibril orientation

and the profiles of cell wall properties along growing regions of maize roots. Journal of Experimental Botany 44, 1281–1289.

Purugganan, M.M., Braam, J., Fry, S.C., 1997. The *Arabidopsis TCH4* xyloglucan endotransglycosylase: substrate specificity, pH optimum, and cold tolerance. Plant Physiology 115, 181–190.

Randerath, K., 1970. An evaluation of film detection methods for weak β-emitters particularly tritium. Analytical Biochemistry 34, 188–205.

Saab, I.N., Sachs, M.M., 1996. A flooding-induced xyloglucan endotransglycosylase homolog in maize is responsive to ethylene and associated with aerenchyma. Plant Physiology 112, 385–391.

Schröder, R., Atkinson, R.G., Langenkämper, G., Redgwell, R.J., 1998. Biochemical and molecular characterisation of xyloglucan endotransglycosylase from ripe kiwifruit. Planta 204, 242–251.

Smith, R.C., Fry, S.C., 1991. Endotransglycosylation of xyloglucans in plant cell suspension cultures. Biochemical Journal 279, 529–535.

Smith, R.C., Matthews, P.R., Schünmann, P.H.D., Chandler, PM., 1996. The regulation of leaf elongation and xyloglucan endotransglycosylase by gibberellin in 'Himalaya ' barley (*Hordeum vulgare* L.). Journal of Experimental Botany 47, 1395–1404.

Steele, N.M., Fry, S.C., 1999. Purification of xyloglucan endotransglycosylases (XETs): a generally applicable and simple method based on reversible formation of an enzyme–substrate complex. Biochemical Journal 340, 207–211.

Sulová, Z., Takáčová, M., Steele, N.M., Fry, S.C., Farkaš, V., 1998. Xyloglucan endotransglycosylase: evidence for the existence of a relatively stable glycosyl–enzyme intermediate. Biochemical Journal 330, 1475–1480.

Tabuchi, A., Kamisaka, S., Hoson, T., 1997. Purification of xyloglucan hydrolase/endotransferase from cell walls of azuki bean epicotyls. Plant and Cell Physiology 38, 653–658.

Takeda, T., Mitsuishi, Y., Sakai, F., Hayashi, T., 1996. Xyloglucan endotransglycosylation in suspension-cultured poplar cells. Biosci. Biotech. Biochem. 60, 1950–1955.

Thompson, J.E. Fry, S.C., 2000. Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells. Planta, in press.

Thompson, J.E., Smith, R.C., Fry, S.C., 1997. Xyloglucan undergoes inter-polymeric transglycosylation during binding to the plant cell wall in vivo: evidence from $^{13}C/^{3}H$ dual labelling and isopycnic centrifugation in caesium trifluoroacetate. Biochemical Journal 327, 699–708.

Vincken, J.-P., De Keizer, A., Beldman, G., Voragen, A.G.J., 1995. Fractionation of xyloglucan fragments and their interaction with cellulose. Plant Physiology 108, 1579–1585.

Vissenberg, K. Martinez-Vilchez, I.M. Verbelen, J.-P. Miller, J.G. Fry, S.C., 2000. In vivo co-localization of xyloglucan endotransglycosylase activity and its donor substrate in the elongation zone of *Arabidopsis* roots. The Plant Cell 12, 1–10.

Xu, W., Purugganan, M.M., Polisensky, D.H., Antosiewicz, D.M., Fry, S.C., Braam, J., 1995. *Arabidopsis TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. Plant Cell 7, 1555–1567.

Xu, W., Campbell, P., Vargheese, A.K., Braam, J., 1996. The *Arabidopsis XET*-related gene family: environmental and hormonal regulation of expression. Plant Journal 9, 879–889.