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**Functional and chemical characterisation of XAF: a heat-stable plant polymer that activates xyloglucan endotransglucosylase/hydrolase (XTH)**

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Running title: Xyloglucan endotransglucosylase/hydrolase activating factor (XAF)

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1 **Abstract**

2 **Background and aims:** Xyloglucan endotransglucosylase/hydrolase (XTH) proteins that  
3 possess xyloglucan endotransglucosylase (XET) activity contribute to cell-wall assembly and  
4 remodelling, orchestrating plant growth and development. Little is known about *in-vivo* XET  
5 regulation, other than at the *XTH* transcriptional level. Plants contain ‘cold-water-extractable,  
6 heat-stable polymers’ (CHPs) which are XTH-activating factors (XAFs) that desorb and  
7 thereby activate wall-bound XTHs. Since XAFs may control cell-wall modification *in vivo*,  
8 we have further explored their nature.

9 **Methods:** Material was cold-water-extracted from 25 plant species; proteins were  
10 precipitated by heat-denaturation, then CHP was ethanol-precipitated. For XAF assays, CHP  
11 (or sub-fractions thereof) was applied to washed *Arabidopsis thaliana* cell walls, and  
12 enzymes thus solubilised were assayed radiochemically for XET activity. In some  
13 experiments, the CHP was pre-treated with trifluoroacetic acid (TFA), alkali (NaOH) or  
14 glycanases.

15 **Key results:**

- 16 • CHP specifically desorbed wall-bound XTHs, but not  $\beta$ -glucosidases, phosphatases or  
17 peroxidases.
- 18 • CHP preparations from 25 angiosperms all possessed XAF activity but had no  
19 consistent monosaccharide composition.
- 20 • Of eleven individual plant polymers tested, only gum arabic and tamarind xyloglucan  
21 were XAF-active, albeit less so than CHP.
- 22 • On gel-permeation chromatography, XAF-active cauliflower CHP eluted with  
23 molecular weight ~7,000–140,000, though no specific sugar residue(s) co-eluted  
24 exactly with XAF activity.

- 1 • Cauliflower XAF activity survived cold alkali and warm dilute TFA (which break  
2 ester and glycofuranosyl linkages respectively), but was inactivated by hot 2M TFA  
3 (which breaks glycopyranosyl linkages).
- 4 • Cauliflower XAF activity was remarkably stable to diverse glycanases and  
5 glycosidases.

6 **Conclusions:** XAFs are naturally occurring heat-stable polymers that specifically desorb  
7 (thereby activating) wall-bound XTHs. Their XAF activity considerably exceeds that of gum  
8 arabic and tamarind xyloglucan, and they were not identifiable as any major plant  
9 polysaccharide. We propose that XAF is a specific, minor, plant polymer that regulates  
10 xyloglucan transglycosylation *in vivo*, and thus wall assembly and restructuring.

11  
12 **Key words:** Cell wall, XET (xyloglucan endotransglucosylase activity), XTH (xyloglucan  
13 endotransglucosylase/hydrolase), *Arabidopsis thaliana*, *Brassica oleracea* (cauliflower),  
14 XAF (XET activating factor), functional properties, sugar composition, enzymic digestion,  
15 plant polymer (heat-stable), wall-bound enzymes.

16  
17 **APPENDIX: Abbreviations**

18 AGP, arabinogalactan–protein; CHP, cold-water-extractable heat-stable polymer; MES,  
19 morpholinoethanesulphonic acid; PL, polylysine; PyAW, pyridine/acetic acid/water (1:1:98;  
20 pH 4.7, unless otherwise stated); XAF, XTH activating factor; XET, xyloglucan  
21 endotransglucosylase (activity); XTH, xyloglucan endotransglucosylase/hydrolase (protein);  
22 XXXGol, borohydride-reduced heptasaccharide of xyloglucan (xylose<sub>3</sub>.glucose<sub>3</sub>.glucitol).

23  
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27 *M&M* 1299  
28 *Results* 2969  
29 *Discussion & Conclusions* 2672  
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31 *References* 1905  
32 *Total (Intro, M&M, Res, Discn, Concl, Legends)* 9820

## 1 INTRODUCTION

2 The susceptibility of the primary cell wall to turgor-driven expansion is the principal factor  
3 that controls plant cell growth (Cosgrove, 1993; Fenwick *et al.*, 1999). The tensile skeleton of  
4 the PCW is established through the interlinking of cellulose microfibrils and non-cellulosic  
5 matrix (Fry, 1989; Hayashi, 1989; McCann *et al.*, 1990; Carpita and Gibeaut, 1993), and the  
6 loosening of this network is integral to cell expansion (Passioura and Fry, 1992). In the  
7 primary walls of dicots and non-poalean monocots, xyloglucan and pectin are the most  
8 abundant matrix polysaccharides (Pauly *et al.*, 1999). The major tension-bearing structure in  
9 such walls is often proposed to be a xyloglucan–cellulose complex, possibly via local  
10 xyloglucan/cellulose nodes (Park & Cosgrove, 2015).

11 Enzymes of xyloglucan metabolism, especially those that cleave or “cut and paste” the  
12 backbone, are of interest because of their important role in controlling wall assembly,  
13 extensibility and turnover. Six GH families from micro-organisms include xyloglucan  
14 endohydrolases: GH 5, 7, 12, 16, 44 and 74 (Gilbert *et al.*, 2008). In contrast, the only plant  
15 enzymes known to cleave the xyloglucan backbone are the xyloglucan  
16 endotransglucosylase/hydrolases (XTHs; EC.2.4.1.207), which are in family GH16 (Rose *et*  
17 *al.*, 2002). Arabidopsis has 33 XTHs (Yokoyama & Nishitani, 2001), all but two of which  
18 possess essentially only xyloglucan endotransglucosylase (XET), the “cutting-and-pasting”  
19 activity, whereas XTH31 and XTH32 exert predominantly the hydrolytic (“cutting only”)  
20 activity (Zhu *et al.*, 2012).

21 XET action was first noted *in vivo* (Baydoun & Fry, 1989; Smith & Fry, 1991) and the XET  
22 activity of extracted enzymes was then detected *in vitro* (Farkaš *et al.*, 1992; Fry *et al.*, 1992;  
23 Nishitani & Tominaga, 1992). XET-active enzymes have been found in all land-plants tested  
24 (Fry *et al.*, 1992; Stratilová *et al.*, 2010) and in some charophytes (Fry *et al.*, 2008).

1 XET action *in vivo* can re-structure pairs of existing wall-bound xyloglucan chains  
2 (Thompson & Fry 2001) and can attach newly secreted xyloglucan chains to existing wall-  
3 bound ones (Thompson *et al.*, 1997). XTH proteins may thereby contribute to both wall-  
4 loosening, facilitating cell expansion, and wall assembly, depending on the molecular size,  
5 location and age of the participating xyloglucan chains (Maris *et al.*, 2009; Thompson & Fry,  
6 2001; Van Sandt *et al.*, 2007; Nishitani and Matsuda, 1982; Osato *et al.*, 2006). Correlative  
7 evidence supporting a role for XTHs in wall loosening includes the observation that  
8 extractable XET activity correlates with various aspects of plant physiology, such as seedling  
9 growth (Farkaš *et al.*, 1992; Fanutti *et al.*, 1993), later cell expansion (Fry *et al.*, 1992),  
10 somatic embryogenesis (Hetherington and Fry, 1993) and fruit ripening (Redgwell and Fry,  
11 1993; Miedes and Lorences, 2009; Brummell, 2006, Goulao *et al.*, 2007). Correlative  
12 evidence for the role of XTHs in wall assembly or tightening includes the finding that  
13 expression of *AtXTH22* (formerly known as *TCH4*), a touch-inducible protein, was rapidly  
14 upregulated by hormones (IAA and 24-epibrassinolide) and by touch, darkness, heat shock  
15 and cold shock, leading to alterations in plant elongation (Braam, 1992, Braam & Davis,  
16 1990; Xu *et al.*, 1995). Lee *et al.* (2005) confirmed that several *XTH* genes are up- and down-  
17 regulated in touched and darkness-treated arabidopsis, correlating with changes in growth  
18 rate.

19 More direct evidence for positive roles of specific XTHs in growth comes from molecular  
20 biological experiments: for example, a decrease in *AtXTH18* mRNA abundance by RNAi  
21 resulted in a significant reduction in the epidermal cell length of the arabidopsis primary root  
22 (Osato *et al.*, 2006); and higher expression of a *Brassica campestris* homologue of *AtXTH19*  
23 in arabidopsis evoked a pronounced increase in cell expansion (Maris *et al.*, 2009).

24 Although many studies focusing on the regulation of XTHs have monitored *XTH* gene  
25 expression and extractable XET enzyme activity, little is known about how the action of pre-

1 formed XTH molecules may be regulated *in vivo*. Such regulation may be important for wall  
2 assembly and growth regulation.

3 The attachment and detachment of XTHs to and from the primary cell wall may be important  
4 for governing their action *in vivo*. We assume that an XTH molecule that is firmly bound to  
5 the wall would be able to act on very few (or no) xyloglucan chains, because of the exact  
6 siting of the enzyme relative to that of its polysaccharide substrate — especially relative to  
7 the very rare (one per polysaccharide molecule) non-reducing terminal glucose residue which  
8 must serve as the acceptor substrate during the XTH-catalysed interpolymeric  
9 transglycosylation reaction. Thus, firmly wall-bound, immobile XTHs may exert little or no  
10 influence on wall assembly and remodelling. On the other hand, a solubilised (thus diffusible)  
11 XTH molecule is able to forage for xyloglucan substrates throughout the wall matrix and act  
12 sequentially on several xyloglucan chains, thereby having an appreciable effect on cell-wall  
13 properties.

14 Takeda and Fry (2004) discovered that endogenous cold-water-extractable, heat-stable  
15 polymer(s) (CHP) from cauliflower florets act as an XTH-activating factor (XAF), promoting  
16 the XET activity of XTHs. The effects of CHP were weakly mimicked by certain anionic  
17 polysaccharides e.g. hypochlorite-oxidised (thus anionic) xyloglucan,  
18 carboxymethylcellulose (CMC) and citrus pectin, and by gum arabic; in contrast, certain  
19 other polyanions (e.g. alginate,  $\lambda$ -carrageenan, homogalacturonan and  
20 methylglucuronoxylan) had the opposite effect. The results suggested that a limited range of  
21 acidic wall polysaccharides may contribute to the regulation of XET action *in vivo* (Takeda  
22 and Fry, 2004; Takeda *et al.*, 2008).

23 XTHs have a tendency to bind to various surfaces, including chromatography columns  
24 (Hrmova *et al.*, 2007) and cellulose (Sharples *et al.*, 2017). The activity of cellulose-  
25 associated XTH was promoted by 18 out of 4216 tested xenobiotics (especially

1 anthraquinones and flavonoids; Chormova *et al.*, 2015), though none of these compounds had  
2 such an effect when all components were cellulose-free (thus soluble), suggesting that the  
3 promotion of activity was only observed when XTH–cellulose interactions were occurring.  
4 Sharples *et al.* (2017) showed that cauliflower CHP exerts its XAF activity principally by  
5 (re-)solubilising XTHs from surfaces (including cellulose, glass-fibre, glass and plastics) to  
6 which these enzymes tend to bind. Likewise, and of more direct botanical relevance, cell  
7 walls prepared from cauliflower florets, mung bean shoots and arabidopsis cell-cultures each  
8 contained endogenous, tightly bound, inactive XTHs, which were rapidly solubilised, and  
9 consequently activated, by the XAF of cauliflower CHP. A convenient quantitative assay for  
10 XAF acting on the natively sequestered XTHs of arabidopsis cell walls was developed and  
11 this is exploited in the present paper. We have therefore been able to investigate further the  
12 physiology and biochemistry of the unidentified endogenous CHPs that possess XAF activity  
13 — agents that solubilise XTHs from their binding sites in the cell wall, activating them and  
14 enabling xyloglucan re-structuring *in vivo*.

15

## 16 **MATERIALS AND METHODS**

### 17 **Materials**

18 The following were from Sigma–Aldrich Life Science (Gillingham, Dorset, UK): horseradish  
19 peroxidase (193 purpurogallin U mg<sup>-1</sup> solid), Driselase,  $\alpha$ -amylase (from *Bacillus*  
20 *licheniformis*), larch arabinogalactan, gum arabic, cellulose powder, carboxymethylcellulose,  
21 citrus pectin, birch-wood xylan, homogalacturonan (‘polygalacturonic acid’), blue dextran,  
22 soluble starch, polylysine, bovine serum albumin (BSA) and general chemicals e.g. buffers.  
23 The Driselase was partially purified as described by Fry (2000). Tamarind xyloglucan was a  
24 generous gift of Mr K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan. Nasturtium



1 (*Tropaeolum majus*) xyloglucan was isolated as before (McDougall & Fry, 1989).  
2 Xylohexaose, arabino-octaose, potato galactan (containing 3% arabinose residues), cellulase  
3 (unable to digest xyloglucan; from *Aspergillus niger*),  $\beta$ -mannanase (*Bacillus* sp.),  $\alpha$ -  
4 glucosidase (yeast maltase) and endopolygalacturonase (*Aspergillus aculeatus*) were from  
5 Megazyme, Bray, Ireland. A  $\beta$ -1,3-galactosidase ('exo- $\beta$ -1,3-galactanase') from *Clostridium*  
6 *thermocellum* was bought from NZYTech (Haltwhistle, Northumberland, UK). XEG was a  
7 generous gift from Novozymes, Bagsværd, Denmark. [ $^3\text{H}$ ]XXXGol was from EDIPOS  
8 (<http://fry.bio.ed.ac.uk/edipos.html>) and had specific radioactivity  $\sim 100 \text{ MBq } \mu\text{mol}^{-1}$ . Merck  
9 silica-gel  $20 \times 20 \text{ cm}$  TLC plates were from VWR. Solvents and scintillants were from Fisher  
10 Scientific.

11

## 12 **Preparation of CHPs**

13 CHPs were prepared from cauliflower florets and 24 other plant materials as described  
14 (Sharples *et al.*, 2017). In brief, the tissue was homogenised in cold water and filtered, and  
15 the soluble material was incubated at  $100^\circ\text{C}$  for 1 h, and filtered again. The filtrate was frozen,  
16 thawed, and centrifuged at 4000 rpm for 30 min, and polymers were precipitated from the  
17 clear supernatant with 70% ethanol (16 h at  $4^\circ\text{C}$ ). The dried pellet (CHP) was re-dissolved  
18 water or buffer, usually at  $2 \text{ mg ml}^{-1}$ , and stored at  $-20^\circ\text{C}$  until use. Conductivity was read  
19 with a Jenway 4060 conductivity meter.

20

## 21 **XAF assay**

22 Crude cell walls from *Arabidopsis thaliana* cell-suspension cultures were isolated, water-  
23 washed, and used in XAF assays as before (Sharples *et al.*, 2017). In brief, the cell walls were  
24 dispensed into the wells of a 96-well plate (giving the equivalent of 15–18  $\mu\text{g}$  dry weight per

1 well, though the cell walls were not routinely dried), re-washed in water, and incubated in 66  
2  $\mu\text{l}$  (final volume) of a putative XAF solution [unless otherwise stated, made up in 200 mM  
3 MES ( $\text{Na}^+$ , pH 5.5) and 75 mM NaCl]. After 30 min shaking at 20°C, the cell-wall  
4 suspension was centrifuged and supernatant assayed for XET activity (based on Fry *et al.*,  
5 1992): 20  $\mu\text{l}$  of supernatant was transferred into a new 96-well plate, and mixed with 20  $\mu\text{l}$  of  
6 radioactive XET reaction mixture (containing [ $^3\text{H}$ ]XXXGol, tamarind xyloglucan, BSA and  
7 chlorobutanol) so that the final reaction mixture (40  $\mu\text{l}$ ) contained 100 mM MES, 37.5  $\mu\text{M}$   
8 NaCl, 2  $\text{mg ml}^{-1}$  xyloglucan and 2.5  $\text{mg ml}^{-1}$  BSA and 0.25% chlorobutanol. The quantity of  
9 [ $^3\text{H}$ ]XXXGol was 1.0 kBq per 40- $\mu\text{l}$  assay for Fig. 1, and 0.5 kBq for all other experiments.  
10 After 16 h incubation at 20°C, the yield of [ $^3\text{H}$ ]polysaccharide (XET reaction product) was  
11 assayed.

12

### 13 **Assay of four enzyme activities potentially solubilised from arabidopsis walls**

14 A 1.5-ml aliquot of arabidopsis cell-wall suspension (approx 0.45 mg dry weight) was  
15 sequentially incubated (30 min each, with gentle shaking) in (i) 7.5 ml 0.075 M NaCl  
16 containing 0.2 M MES, pH 5.5; (ii) 7.5 ml CHP (2  $\text{mg ml}^{-1}$ ) in (i); and (iii) 7.5 ml 1 M NaCl  
17 containing 0.2 M MES, pH 5.5. After each 30-min incubation, the suspension was  
18 centrifuged, all the supernatant was removed and kept, and the residual cell walls were  
19 resuspended in the next extractant.

20 Each extract was assayed for four enzyme activities:

21  *$\beta$ -Glucosidase.* The extract (500  $\mu\text{l}$ ) was added to 500  $\mu\text{l}$  5 mM *p*-nitrophenyl  $\beta$ -D-  
22 glucopyranoside in 0.2 M MES, pH 5.5. At the desired time-point, the reaction was stopped  
23 by addition of 1 ml 1 M  $\text{Na}_2\text{CO}_3$  and the  $A_{400}$  of the released *p*-nitrophenol was read.

24 *Phosphatase.* As above but with *p*-nitrophenyl phosphate ( $\text{Na}^+$ ) as substrate.

1 *Peroxidase*. Extract (100  $\mu$ l) was added to 3 ml of a reaction mixture containing 133 mM *o*-  
2 dianisidine and 133 mM H<sub>2</sub>O<sub>2</sub>, 167 mM NaH<sub>2</sub>PO<sub>4</sub> and 133 mM MES (Na<sup>+</sup>, final pH 5.5), and  
3 incubated at 20°C for 30 min. A<sub>420</sub> was monitored every 30 s (Fry, 2000). HRP (1 ng in 100  
4  $\mu$ l sample) was used as a positive control.

5 *XET*. The extract (20  $\mu$ l) was added to 20  $\mu$ l of a reaction mixture containing 0.5 kBq  
6 [<sup>3</sup>H]XXXGol (0.5 kBq), 0.4% (w/v) tamarind xyloglucan, 0.25% BSA and 0.5% (w/v)  
7 chlorobutanol. After incubation for 0, 4, 8, 16 or 24 h, the reaction was stopped with formic  
8 acid and the procedure was continued as described for the XAF assay.

9

## 10 **Acid hydrolysis and TLC**

11 A ~200- $\mu$ g portion of each of the 25 CHP preparations was incubated in 200  $\mu$ l of 2 M  
12 trifluoroacetic acid (TFA) at 120°C for 1 h. The hydrolysate was dried and redissolved in  
13 water, then the whole ~100  $\mu$ g was analysed by thin-layer chromatography (TLC).

14 Column fractions of cauliflower CHP were also subjected to TFA hydrolysis essentially as  
15 above. In addition, the same fractions were digested with Driselase: the sample was incubated  
16 with 0.17% Driselase in PyAW for 24 h and the digestion was stopped by heating to 120°C  
17 for 1 h.

18 Dionex HPLC methodology (high-pressure anion-exchange chromatography) was as  
19 described by O'Rourke *et al.* (2015). For HPLC of Driselase digests, the yield of each sugar  
20 was corrected for the small yield (if any) produced by Driselase autolysis. TLC was on 20  $\times$   
21 20 cm silica-gel plates. The solvent was ethyl acetate/pyridine/acetic acid/water (6:3:1:1) and  
22 sugars were stained with thymol/H<sub>2</sub>SO<sub>4</sub> (Jork *et al.*, 1994). Sugar spots on TLCs were  
23 quantified with Photoshop software. The ellipse tool (fixed size 0.87  $\times$  0.61 cm) was centred  
24 on the spot of interest, and the 'mean intensity' was measured in the green channel (which is

1 complementary to the more-or-less magenta stained spots). To correct for the background  
2 colour of the plate, we subtracted that ‘mean intensity’ from a blank zone at the same  $R_F$  on  
3 the same chromatogram (a typical blank mean was 220 pixels), and the corrected result is  
4 plotted on graphs as “Photoshop pixels”. A high “Photoshop pixels” value, corrected in this  
5 way, indicates an intense TLC spot. For example, the most intense galactose spot (given by  
6 lettuce leaf CHP) gave a value of 181 (= 220 – 39), whereas the least intense one (spinach  
7 leaf CHP) gave a value of 81 (= 220 – 139).

8

### 9 **Enzymic digestion of cauliflower CHP**

10 The susceptibility of cauliflower CHP to the following hydrolytic enzymes was tested. The  
11 following experimental details refer to the two experiments described in Fig 9a.

12 CHP (2 mg ml<sup>-1</sup>) was incubated with Driselase (3 µg ml<sup>-1</sup>), XEG (8 µg ml<sup>-1</sup>), β-galactosidase  
13 (0.0013 U µl<sup>-1</sup>) or cellulase, mannanase, endo-polygalacturonase, α-glucosidase (all at  
14 0.0167 U µl<sup>-1</sup>) in PyAW (pH 4.7) at 20°C for 24 h. Each enzyme reaction in experiment 1  
15 was stopped by heating at 120°C for 1 h and the digest centrifuged. The supernatant was then  
16 dried *in vacuo*, and the residue was redissolved in water and assayed for XAF activity. In  
17 experiment 2, the β-1,3-galactosidase reaction was done as above but in PyAW (3:11:2000,  
18 pH 5.6) at 55°C for 4 h; the α-glucosidase digestion was done in 1% lutidine and 0.3% acetic  
19 acid, pH 6.6, at 20°C for 48 h and stopped by addition of 100 µl formic acid; and the α-  
20 amylase reaction was exactly as in experiment 1.

21

### 22 **Gel-permeation column chromatography**

1 Bio-Gel P-2 and Sepharose CL-6B columns with bed volume 100 ml were used. These were  
2 washed with approximately two column volumes of PyAW (1:1:98) containing 0.5%  
3 chlorobutanol. A 4-ml sample containing CHP (2 mg ml<sup>-1</sup>) plus internal markers (0.1 mg  
4 blue dextran, 0.5 mg glucose and sometimes 0.3 kBq [<sup>14</sup>C]glucose) was applied, and 2-ml  
5 fractions were collected with PyAW as eluent. The A<sub>280</sub> and A<sub>620</sub> of each fraction was  
6 measured, and fractions were then dried in a SpeedVac and re-dried from 100 µl of water.

7

## 8 **RESULTS**

### 9 **Cauliflower CHP acts synergistically with CaCl<sub>2</sub> in XAF assays**

10 The ability of CHP to solubilise XTHs from arabidopsis cell walls was mimicked by NaCl  
11 (Sharples *et al.*, 2017) and we now show a similar effect with CaCl<sub>2</sub> (Fig. 1). The effect  
12 plateaued above about 30 mM CaCl<sub>2</sub>, but the CHP effect did not plateau even at the highest  
13 concentration tested (1.8 mg ml<sup>-1</sup>; Fig. 1). The relative effect of CHP was greatest (34-fold  
14 promotion) in the absence of CaCl<sub>2</sub>, but strong CHP effects (4.6- to 9.1-fold promotion) —  
15 and much higher absolute XET activities — were still detected in the presence of 15 mM  
16 CaCl<sub>2</sub>, indicating synergy between CHP and the inorganic salt (Table S1). This observation,  
17 together with the previous finding that certain anionic polysaccharides promote the XET  
18 activity of de-salted XTH preparations particularly well if a sub-optimal concentration of salt  
19 is also present (Takeda & Fry, 2004), led us to assay XAF activity in all subsequent  
20 experiments by suspending the washed arabidopsis cell walls in a solution containing 75 mM  
21 NaCl [buffered with 200 mM MES (Na<sup>+</sup>), pH 5.5, which itself has a low ionic strength and  
22 has been shown (Takeda & Fry, 2004; confirmed in the present work) to have no appreciable  
23 XAF activity]. The data show that CHP can solubilise XTHs from washed arabidopsis walls  
24 and that solubilisation causes these enzymes to acquire detectable XET enzymic activity.

1

## 2 **BSA minimises binding of solubilised XTHs to tube walls**

3 Dilute XTH solutions tend to lose XET activity by binding to tube walls (Hrmova *et al.*,  
4 2007; Sharples *et al.*, 2017). In glassware, this tendency was minimised if the glass surface  
5 was blocked by polylysine pre-treatment; however, this proved unreliable in the case of  
6 plastic vessels. We therefore tested several agents for their ability to minimise the loss of  
7 XTHs in three types of plastic tube (Fig. 2) and thus to enable a steady reaction rate during  
8 XET assays conducted in such tubes. Solubilised arabidopsis XTHs were incubated in the  
9 tube for 5.5 h in the presence **or** absence of the agent to be tested, and then any remaining  
10 soluble enzyme was assayed for XET activity. BSA had the strongest ability to maintain  
11 soluble XET activity, presumably by preventing solubilised XTHs from binding to the tube  
12 walls; Triton X-100 was also somewhat effective (Fig. 2). Additional NaCl, and pre-  
13 treatment of the plastic with polylysine were ineffective (Fig. 2), unlike in glass tubes  
14 (Sharples *et al.*, 2017). BSA was the only agent which led to the measured XET activity  
15 being proportional to the concentration of added enzyme: in all three types of plastic,  
16 reducing the concentration of the crude enzyme solution from 50% (v/v) to 15% (v/v)  
17 decreased the measured XET reaction rate by about 70%, as expected (Fig. 2). Therefore,  
18 BSA (2.5 mg ml<sup>-1</sup>) was included in the reaction mixture used in all subsequent XET assays.

19

## 20 **The XAF activity of cauliflower CHP specifically solubilises XTHs**

21 CHPs from across the plant kingdom solubilise XET activity from washed arabidopsis cell  
22 walls (Sharples *et al.*, 2017). We next tested whether they also solubilise other enzyme  
23 activities. To answer this, we examined which arabidopsis wall enzyme activities were  
24 solubilised by, sequentially: low salt, low salt plus cauliflower CHP, and high salt (Fig. 3).

1 After each extractant, all the solution was removed from the cell walls and the next extractant  
2 was then applied. Moderate activities of phosphatase and peroxidase were solubilised by low  
3 salt alone; after low salt, CHP in low salt solubilised almost no additional activity of these  
4 two enzymes, even though large amounts of them remained within the walls, as demonstrated  
5 by the effectiveness of subsequently applied high salt. Very little (3% of the total)  $\beta$ -  
6 glucosidase was solubilised by low salt alone, after which CHP in low salt solubilised an  
7 additional 15%; again, however, by far the most effective extractant was high salt (82% of  
8 the total activity), which thus had a strong effect that CHP was incapable of. In contrast,  
9 solubilisation of XET activity differed strongly: low salt alone solubilised very little, after  
10 which CHP in low salt solubilised much more, and subsequent high salt solubilised no further  
11 XET activity (Fig. 3). Thus, cauliflower CHP exerted a unique effect, relatively specific for  
12 solubilisation of XTHs.

13

#### 14 **CHP has a stronger XAF effect on more dilute cell-wall suspensions**

15 The concentration of arabidopsis cell walls had a strong influence on the effective XAF  
16 activity of  $2 \text{ mg ml}^{-1}$  cauliflower CHP. The effect of CHP increased from a 1.16-fold  
17 promotion to an 8-fold promotion as the cell wall concentration was decreased from 183 to  
18  $18 \mu\text{g per } 66 \mu\text{l}$  (Fig. 4a). The effect then remained almost unchanged at  $\sim 8$ -fold as the cell-  
19 wall concentration was decreased from 18 to  $8 \mu\text{g per } 66 \mu\text{l}$  (Fig. 4a). In the absence of buffer  
20 and NaCl, water solubilised very little XET activity, even from the highest concentration of  
21 cell walls (see  $\Delta$  datapoint in Fig. 4a). These data led us to select  $15\text{--}18 \mu\text{g walls per } 66 \mu\text{l}$   
22 as the routine concentration when testing CHP samples for XAF activity in subsequent  
23 experiments.

24

## 1 **The dose–response curve of CHP indicates two distinct XAF effects**

2 As expected, the XAF activity of cauliflower CHP was concentration-dependent; however,  
3 the relationship was not linear (Fig. 4b). The shape of the curve suggests two distinct effects  
4 of CHP: one saturating at very low CHP concentrations ( $K_m$  roughly  $0.035 \text{ mg ml}^{-1}$ ), and  
5 the other not saturating until much higher concentrations.

6

## 7 **The sugar composition of CHPs from diverse plants does not correlate with their XAF** 8 **activities**

9 The above work confirms that cauliflower floret CHP has XAF activity. We have also shown  
10 that CHPs from all other plant materials tested possess XAF activity when assayed on  
11 arabidopsis walls (Sharples *et al.*, 2017). The XAF activities of CHPs were independent of  
12 their conductivity (Sharples *et al.*, 2017; and present manuscript Fig. S1). Thus the XAF  
13 activity is not due simply to an ionic effect of the charged polymers present in CHPs. Note  
14 that most of the XAF values in Fig. S1 are within the range (500–2500 cpm per 16 h) where  
15 XAF activity is approximately proportional to cauliflower CHP concentration (Fig. 4b); thus  
16 cpm as reported in Fig. S1 is likely to be on an approximately linear scale. The highest ionic  
17 strengths of  $2 \text{ mg ml}^{-1}$  CHP solutions (those from spinach leaves and tobacco stems) were  
18 equivalent to  $\sim 15 \text{ mM NaCl}$ , a concentration at which NaCl itself has negligible XAF activity.  
19 This confirms that the XAF activity of CHPs is not a simple ionic effect, and the results  
20 suggest that specific polymers in CHPs are responsible for XAF activity.

21 To characterise further these specific polymers, we acid-hydrolysed each CHP preparation,  
22 revealing that they were all rich in galactose and arabinose residues (Fig. 5). The Man content  
23 varied from very high (e.g. in asparagus and spring-onion leaf CHPs) to almost undetectable  
24 (in spinach and tobacco leaf CHPs). Glucose, xylose and rhamnose contents also varied



1 widely (Fig. 5). Moderate proportions of uronic acids were detectable in most CHPs, and a  
2 spot corresponding to the lactone of glucuronic acid (formed from anionic glucuronate during  
3 acid hydrolysis) was abundant in some samples. The CHPs from asparagus and spring onion  
4 leaves contained an unidentified sugar (Unk1; possibly an *O*-methylhexose), and most of the  
5 CHPs yielded one or two fast-migrating sugars (Unk2 and Unk3) plus two slow-migrating  
6 ones (probably aldobiouronic acids) (Fig. 5).

7 There was no positive correlation between the XAF activity (always assayed at 2 mg ml<sup>-1</sup>  
8 CHP) and the levels of any given sugar residue in the different CHPs (Fig. S2). Indeed,  
9 galactose, arabinose and possibly xylose residues showed significant negative correlations.

10

11 **All authentic polysaccharides tested have much lower XAF activity than cauliflower**  
12 **CHP**

13 To define further which polymers in cauliflower CHP might be responsible for XAF activity,  
14 we assayed a selection of eleven authentic polysaccharides. None of these (even though  
15 tested at 5 mg ml<sup>-1</sup>) was more than 28% as effective as 2 mg ml<sup>-1</sup> cauliflower CHP (Fig. 6).  
16 Unexpectedly, tamarind xyloglucan exhibited some XAF activity, i.e. appeared able to  
17 solubilise XTHs from arabidopsis walls (Fig. 6). This effect was not simply due to the ability  
18 of the additional xyloglucan (contributing an extra 2.5 mg ml<sup>-1</sup> after dilution into the reaction  
19 mixture), to serve as donor substrate in the XET assay: the reaction mixture routinely  
20 contained 2 mg ml<sup>-1</sup>, an optimal xyloglucan concentration. Changing from 2 to 4.5 mg ml<sup>-1</sup>  
21 certainly would not cause the 11-fold promotion in measured XET reaction rates suggested  
22 by the difference between buffer only (sample 13) and +tamarind xyloglucan (sample 1);  
23 indeed, higher concentrations of non-radioactive xyloglucan may decrease the production of  
24 <sup>3</sup>H-labelled products as the additional non-radioactive xyloglucan competes with the

1 [<sup>3</sup>H]XXXGol as acceptor substrate (Purugganan *et al.*, 1997). Nasturtium-seed xyloglucan,  
2 which had been purified by Cu<sup>2+</sup> precipitation (McDougall & Fry, 1989), lacked the XAF  
3 activity of tamarind-seed xyloglucan (Fig. 6).

4 Gum arabic, an anionic mucopolysaccharide possessing type-II arabinogalactan side-chains,  
5 was about as effective as tamarind xyloglucan in the XAF assay, agreeing with its ability to  
6 ‘re-activate’ XTHs that had been lost from solution (Takeda & Fry, 2004). Another type-II  
7 arabinogalactan but lacking a protein core, from larch, had no XAF activity.

8 Another anionic polysaccharide, carboxymethylcellulose (CMC), was only weakly effective,  
9 and a further one, homogalacturonan, was inactive — both observations again agreeing with  
10 the data of Takeda & Fry (2004).

11

## 12 **Size distribution and sugar residue composition of XAF-active CHP fractions**

13 When cauliflower CHP was size-fractionated on Sepharose CL-6B (Fig. 7a), most XAF  
14 activity eluted in the  $K_{av}$  range 0.34–0.82 (indicating molecular weight  $\approx$  140,000 to 7,000 by  
15 reference to dextran standards; Steele *et al.*, 2002), where  $K_{av}$  0 and  $K_{av}$  1 are defined by the  
16 elution positions of blue dextran and glucose respectively (Fig. 7b). Thus cauliflower XAF  
17 has a fairly broad range of sizes, but the smallest ( $M_r < 7,000$ ) and largest ( $M_r > 140,000$ )  
18 polymers in CHP have little or no activity.

19 We attempted to identify CHP constituents that correlate with XAF activity. Certain fractions  
20 absorbed at 280 nm (ultraviolet), indicating proteins or phenolic groups (Fig. 7b), but these  
21 were not the main XAF-active fractions. Because some of the last-eluting fractions with high  
22 XAF-activity overlapped with the second peak of  $A_{280}$  (which itself did not appear to be  
23 associated with a discrete activity peak), we pooled the relatively early-eluting active  
24 fractions to use as partially purified XAF in the subsequent analyses (e.g. in Fig. 8a; see later).

1 Acid hydrolysis of the Sepharose fractions released at least nine monosaccharides, which  
2 were quantified by HPLC (Fig. 7c, d). All active fractions contained arabinose and galactose,  
3 and fraction 31+32, which had the highest XAF activity, also had highest galactose and  
4 arabinose levels. However, the levels of these sugars in individual fractions were not  
5 proportional to XAF activity. For example, fraction 39+40 had high XAF activity but little  
6 arabinose and galactose. The best correlation with XAF activity was generally shown by  
7 xylose and fucose, components of xyloglucan, although fraction 29+30 had high XAF  
8 activity without detectable fucose.

9 Digestion with Driselase (Fig. 7e, f) instead of acid also gave arabinose and galactose. These  
10 sugars are not efficiently released by Driselase from cell-wall glycoproteins such as  
11 arabinogalactan-proteins (AGPs) and extensins, and thus the majority of the Driselase-  
12 generated arabinose and galactose probably arose from polysaccharides such as pectins,  
13 xyloglucan and arabinoxylans. The XAF peak overlapped with the peaks of Driselase-  
14 generated isoprimeverose [ $\alpha$ -xylosyl-(1 $\rightarrow$ 6)-glucose], glucose and fucose, again consistent  
15 with xyloglucan (Fig. 7e, f). Xylose and xylobiose [ $\beta$ -xylosyl-(1 $\rightarrow$ 4)-xylose] in Driselase  
16 digests, which arise from xylans rather than xyloglucan (Thompson & Fry, 1997), correlated  
17 less well with XAF activity.

18 In conclusion, the major XAF peak overlapped with the xyloglucan peak on gel-permeation  
19 chromatography, though these peaks did not closely match (Fig. 7). No other major  
20 polysaccharide class showed better co-elution. However, authentic xyloglucans had zero or  
21 much less XAF-activity than cauliflower CHP (Fig. 6) and therefore xyloglucans are unlikely  
22 to be the major XAF-active polymers of cauliflower. More probably, XAF activity is due to  
23 minor polymers that make little contribution to total sugar composition.

24

1 **Furanosyl and ester linkages are not essential for XAF activity, but pyranosyl-like**  
2 **linkages are**

3 The XAF activity of cauliflower CHP was completely destroyed by ‘severe’ acid hydrolysis  
4 (conditions routinely used for analytically converting polysaccharides to monosaccharides: 2  
5 M TFA, 120°C, 60 min; Fig. 7a). To further define the acid sensitivity of XAF, we treated  
6 cauliflower CHP for various times under ‘mild’ acid (0.1 M TFA at 85°C) or ‘moderate’ acid  
7 conditions (2.0 M TFA, 100°C), and then re-assayed for XAF activity (Fig. 8a). Mild acid did  
8 not affect XAF within 60 min, whereas the moderate acid destroyed it with a half-life of  
9 about 8 min.

10 We used two authentic oligosaccharides to demonstrate the effects of the mild and moderate  
11 acid: furanosidically linked arabino-octaose (Araf-8) and pyranosidically linked xylohexaose  
12 (Xylp-6). Mild acid rapidly cleaved Araf-8, such that the octasaccharide had ~50%  
13 disappeared within 4 min and been completely hydrolysed to the monosaccharide within 64  
14 min (Fig. 8b). Mild acid cleaved Xylp-6 more slowly, ~50% of the hexasaccharide remaining  
15 intact after 32 min. In the moderate acid (Fig. 8c), Araf-8 and Xylp-6 were both completely  
16 hydrolysed to the monosaccharide, taking <4 and ~32 min respectively. Concurrently, the  
17 only monosaccharide released from cauliflower CHP by mild acid was arabinose (Fig. 8b)  
18 (the major furanosidically linked sugar in plant polysaccharides and glycoproteins),  
19 paralleling the release of arabinose from Araf-8. Moderate acid released all arabinose from  
20 CHP in <4 min, and then gradually released galactose and galacturonate (detectable by 32  
21 min; Fig. 8c). The XAF data in Fig. 8a thus show that highly acid-labile (furanosidically  
22 linked) residues are not required for XAF activity; however, pyranose-linked sugar residues  
23 (or other residues with similar acid-resistance) are essential.

1 Dilute alkali at room temperature cleaves ester bonds (Euranto, 1969). However, the XAF  
2 activity of cauliflower CHP survived at least 8 h in 0.48 M NaOH at room temperature (Fig.  
3 8a). Therefore ester-linked groups are not essential for XAF activity.

4

#### 5 **XAF-active cauliflower CHP withstands all polysaccharide-digesting enzymes tested**

6 Susceptibility to enzymic digestion can indicate the nature of an unidentified active principle,  
7 and this approach was applied to the XAF activity of cauliflower CHP. Eight commercial  
8 enzyme preparations were applied to the CHP; the enzymes were then denatured and the  
9 remaining CHP was re-assayed for XAF activity (Fig. 9a). Xyloglucan endoglucanase (XEG)  
10 caused a moderate loss of XAF activity, superficially suggesting that part of the XAF activity  
11 was due to xyloglucan. However, Driselase, which is an enzyme mixture capable of digesting  
12 essentially all plant cell-wall polysaccharides except rhamnogalacturonan-II (Fry, 2011),  
13 caused only a slight loss of XAF activity, indicating that the majority of the XAF activity was  
14 not due to any major wall polysaccharide, including xyloglucan.

15 Cellulase (of a type unable to digest xyloglucan),  $\beta$ -mannanase,  $\alpha$ -amylase,  $\alpha$ -glucosidase,  
16 endopolygalacturonase and  $\beta$ -1,3-galactosidase ('exo-galactanase') did not inactivate XAF,  
17 indicating that the activity was not dependent on a cellulose-like polymer, mannan, starch or  
18 homogalacturonan, nor terminal 1,3-linked galactose residues of (arabino)galactans. The  
19 activity of the tested enzymes was verified by the ability of most of them to release mono-  
20 and/or oligosaccharides from certain CHP components: this included  $\alpha$ -amylase and  $\beta$ -1,3-  
21 galactosidase (Fig. 9e),  $\beta$ -mannanase and endopolygalacturonase (similar TLCs; not shown).  
22  $\alpha$ -Glucosidase (maltase) did not release glucose from the starch present in CHP (Fig. 9e), but  
23 it did partially hydrolyse commercial maltohexaose (data not shown). [Although the ' $\beta$ -1,3-  
24 galactosidase' (CtGan43A; GH43) is stated by the manufacturers to be an exo-acting

1 galactanase, which should thus yield only the free monosaccharide galactose, we found a  
2 predominance of oligosaccharide products, indicating endo-hydrolysis (Fig. 9e).] Denatured  
3 cellulase and  $\alpha$ -glucosidase themselves exerted slight XAF activity (Fig. 9a).

4 Among the enzymes tested, only XEG showed some (moderate) ability to inactivate XAF;  
5 therefore we tested its effect, both before and after denaturation with formic acid, on three  
6 XAF-active polymers: cauliflower CHP, tamarind xyloglucan and gum arabic (Fig. 9c).

7 Surprisingly, CHP, which again lost a proportion of its XAF activity when treated with XEG,  
8 was equally inactivated by acid-denatured XEG. The acid treatment completely abolished the  
9 XEG activity itself, as shown by the inability of denatured XEG to destroy the XAF activity  
10 of tamarind xyloglucan (Fig. 9c). The moderate XAF activity of gum arabic, reported in Fig.  
11 6 and confirmed here, was unaffected by XEG (either native or denatured; Fig. 9c). We also  
12 confirmed that the XEG preparation did not release detectable mono- or oligosaccharides  
13 from gum arabic, whereas it completely digested xyloglucan (Fig. S3). In conclusion, the  
14 susceptibility of cauliflower CHP to enzymes differed substantially from tamarind  
15 xyloglucan's and gum arabic's.

16 The ability of XEG to reduce the XAF activity of CHP was re-confirmed in Fig. 9b, which  
17 shows by gel-permeation chromatography that XEG partially inactivates all XAF-active size  
18 classes of CHP. Again, the size distribution of XAF activity approximately agreed with that  
19 of xyloglucan (fractions 15–21), as shown by TLC of the XEG digestion products (Fig. 9d).

20 The oligosaccharide profiles generated from CHP fractions 15–21 were typical of dicot  
21 vegetative tissue xyloglucan: they appeared to include XXXG, *O*-acetyl-XXFG (not resolved  
22 from XXLG and/or XLXG), XXFG, *O*-acetyl-XLFG and XLFG. XEG also yielded a trace of  
23 free glucose, especially from the  $K_{av}$ -0 material (fractions 9+10 in this experiment). The  
24 presence of contaminating  $\beta$ -glucosidase and  $\alpha$ -amylase in the XEG preparation was shown  
25 by its ability to release glucose from both celohexaose and maltohexaose during prolonged

1 incubations at a high enzyme concentration ( $333 \mu\text{g ml}^{-1}$ ; Fig. 9e), and the presence of a trace  
2 of contaminating  $\beta$ -galactosidase was also demonstrated (Fig. S3b).

3 In summary, the major XAF-active components of cauliflower CHP largely resisted all  
4 carbohydrate-digesting enzymes tested. Thus, although CHP contains abundant sugar  
5 residues, and acid hydrolysis of pyranosyl linkages destroys XAF activity, we did not find  
6 any carbohydrase preparation — even the highly potent fungal enzyme mixture ‘Driselase’  
7 — capable of completely destroying it. Saccharide structures necessary for XAF activity  
8 must be quantitatively minor components of total CHP.

9

## 10 **DISCUSSION**

### 11 Functional characteristics of XAF

12 Since XAF is an endogenous regulator of xyloglucan transglycosylation, potentially  
13 modulating cell-wall loosening and/or assembly *in vivo*, we have now further explored the  
14 nature and action of this unidentified plant polymer. Our principal source was cauliflower  
15 floret CHP — a preparation containing high-molecular-weight substances that were cold-  
16 water extractable and not coagulated by subsequent boiling, thus likely to be polysaccharides  
17 or heavily glycosylated proteins. The present work follows up that of Sharples *et al.* (2017),  
18 who found that CHP is able to desorb XTHs from both inert and biological surfaces,  
19 including glass, plastics, cellulose and plant cell walls. In the present paper, we provide new  
20 information on the physiology and chemistry of the XAF-active CHP.

21 We show that  $\text{CaCl}_2$  can augment the ability of CHP to solubilise XET activity from  
22 arabidopsis walls. The  $\text{CaCl}_2$  and CHP effects are synergistic rather than additive (Fig. 1;  
23 Table S1), indicating that they have different modes of action; cauliflower CHP does not  
24 simply act as a non-specific polyanion, capable of breaking ionic bonds that hold XTHs in the

1 cell wall. This conclusion is supported by confirmation that the XAF activities of CHPs from  
2 25 species of plant do not correlate with their conductivities (Fig. S1).

3 The XAF activity of CHP is also not due simply to a general protein effect. For example,  
4 BSA does not solubilise XTHs from arabidopsis cell walls (data not shown), and is thus not  
5 itself XAF-active. However, BSA does help to keep previously solubilised XTHs in solution,  
6 preventing their re-adsorption to the washed arabidopsis cell walls or to vial surfaces (Fig. 2).  
7 A detergent (Triton X-100) and high salt do not have this effect. We therefore routinely  
8 added BSA to minimise the subsequent loss of solubilised XTHs due to rebinding to the  
9 washed arabidopsis walls and/or the tube surfaces.

10 The action of XAF in solubilising XTHs is dose-dependent, as expected; however, the dose-  
11 response curve is not linear (Fig. 4b). The shape of the curve suggests two distinct effects of  
12 CHP: one saturating at very low CHP concentrations ('Michaelis constant',  $K_m \approx 0.035 \text{ mg}$   
13  $\text{ml}^{-1}$ ), and the other not saturating until much higher concentrations ( $K_m \approx 4 \text{ mg ml}^{-1}$ ). This  
14 observation may indicate that some XTH-wall bonds are labile and easily broken by low  
15 concentrations of CHP, whereas others are stronger and require higher a CHP concentration.  
16 Strong XTH-wall bonding could be either a characteristic of certain XTH isozymes, or a  
17 feature of the specific wall components to which they are attached. It is also tenable that there  
18 could be two or more XAFs differing in  $K_m$ .

19 The relative XAF effect of CHP is stronger when acting on dilute cell-wall suspensions (<20  
20  $\mu\text{g per } 66 \mu\text{l}$ ) than on higher wall concentrations (Fig. 4a). As a baseline, we note that pure  
21 water solubilised almost no XTH from washed arabidopsis cell walls, even at the highest  
22 concentration of walls ( $\Delta$  datapoint). Compared with this, the routine NaCl/MES medium  
23 alone solubilised large amounts of XTH from concentrated suspensions of cell walls ( $\circ$   
24 datapoints), but almost none from lower concentrations. The  $\circ-\circ$  curve resembles a  
25 titration: a likely explanation for this is that the NaCl initially solubilises a constant



1 proportion of the wall's XTH, but when the concentration of this is low almost all of it binds  
2 to the plastic surface of the 96-well plate during the 0.5 h incubation before the solution is  
3 transferred into the XET assay mixture containing BSA. On the other hand, at higher cell-  
4 wall concentrations (above about 20 µg walls per 66 µl), enough enzymes have been  
5 solubilised by the NaCl/MES to saturate all the plastic's binding sites. Increasing the wall  
6 concentration beyond this threshold results in all additional solubilised XTH remaining in  
7 solution. Contrasting with this scenario, when 2 mg ml<sup>-1</sup> CHP is added (● datapoints),  
8 almost all solubilised XTH always remains in solution, regardless of its concentration,  
9 because the plastic's sites are already occupied by the CHP polymers. To maximise the  
10 effective XAF activity of CHP preparations, we therefore routinely kept the cell wall  
11 concentration below the threshold of 20 µg per 66 µl.

12

### 13 Cauliflower floret XAF solubilises XTHs but not three other wall enzyme activity classes

14 The conclusion that the XAF activity of cauliflower CHP is due to a unique CHP–XTH  
15 interaction rather than to a general protein/salt/detergent effect is supported by the  
16 observation that CHP solubilises only XET activity (i.e., XTHs) rather than any of the other  
17 tested wall enzyme activities including β-glucosidase, peroxidase or phosphatase (Fig. 3).

18 The latter three activities, like XTHs, are well established to be ionically bound within plant  
19 cell walls (Wei *et al.*, 2015; Jamet *et al.*, 2006; Minic *et al.*, 2007), and we confirmed that  
20 they are present in salt-extractable form in arabidopsis walls, albeit unaffected by CHP.

21

### 22 Sugar composition of XAF-active CHPs from diverse plants

23 Sharples *et al.* (2017) detected XAF activity in the CHPs obtained from all plants tested,  
24 including monocots and dicots and at various stages of plant development. Our new results

1 confirm that the XAF activities of diverse CHP preparations are not simply determined by  
2 their ionic strengths (Fig. S1).

3 Furthermore, there is no positive correlation between the levels of any given monosaccharide  
4 residue in diverse CHPs and their measured XAF activities (always assayed at 2 mg ml<sup>-1</sup>; Fig.  
5 5). All CHP preparations are rich in galactose and arabinose residues, which may be derived  
6 from arabinogalactans of type I [i.e. based on a (1→4)-β-D-galactan backbone, as found in  
7 the neutral side-chains of the pectic domain rhamnogalacturonan-I] and/or type II [based on a  
8 (1→3)-β-D-galactan backbone; AGP-related] (Seymour and Knox, 2002), and possibly also  
9 from the hemicelluloses xyloglucan plus arabinoxylan (though these are a less likely major  
10 source because these two hemicelluloses tend to contain mainly galactose or arabinose  
11 respectively, not both (Shibuya *et al.*, 1983, Scheller and Ulvskov, 2010). The consistently  
12 high galactose and arabinose content, in both high- and low-XAF-activity CHPs, makes it  
13 impossible to positively ascribe XAF activity to polymers containing these residues. Indeed,  
14 galactose and arabinose residues showed a significant negative correlation with XAF activity  
15 (Fig. S2). This may indicate that the active principle is not one of the major galactose- and  
16 arabinose-rich polymers, and that the major polymers effectively dilute out the true but  
17 quantitatively minor XAF-active principle with inert material. This idea does not preclude the  
18 possibility that the quantitatively minor active principle is a specific polymer rich in galactose  
19 and arabinose.

20 There is a 5-fold range of xylose residue content, the highest concentrations being found in an  
21 eclectic range of species (dicots and a monocot) and tissues: arabidopsis stems, flowers and  
22 cell-cultures, rose cell-cultures, carrot leaves, mature celery petioles, tobacco stems and  
23 crocus flowers. Xylose may possibly arise from water-extractable xyloglucans, which are  
24 reported to be present in some tissues (Jacobs & Ray, 1975; de Castro *et al.*, 2015). The  
25 negative correlation between xylose content and XAF activity could possibly indicate that the

1 active principle does not contain xylose; alternatively, as argued for galactose and arabinose,  
2 it is possible that XAF is a minor xylose-containing polymer and that co-occurring major  
3 xylose-containing polymers effectively dilute out the active principle.

4 Some CHP preparations, especially those from the Asparagales (asparagus, onion, snowdrop,  
5 crocus), have a high mannose residue content. Cold-water-extractable mannose-rich polymers  
6 include glucuronomannans (Kato *et al.*, 1977) and some galactomannans (Moreira & Filho,  
7 2008), whereas most  $\beta$ -(1→4)-mannans tend to be inextractable in cold, neutral water.

8 However, some highly XAF-active CHPs (e.g. from spinach and tobacco leaf CHPs) were  
9 almost devoid of mannose, so glucuronomannans etc. are unlikely to be the XAF active  
10 principle. Likewise, the levels of uronic acid, rhamnose, glucose and three unidentified sugar  
11 residues failed to correlate with XAF activity (Fig. 5, Fig. S2).

12 One approach that might characterise the elusive XAF-active polymers of cauliflower CHP is  
13 to fractionate them and look for a specific size-class of polymers exhibiting XAF activity.

14 The results (Fig. 7) show that the active principle has a wide  $M_r$  range (~7,000–140,000) so it  
15 cannot be ascribed to any specific glycoprotein. The broad  $M_r$  range indicates polydispersity  
16 — e.g. XAF is a population of a polysaccharide or a glycoprotein with variation in its  
17 carbohydrate moieties. Furthermore, no specific building block (either TFA- or Driselase-  
18 released; Fig. 7c–f) shows a size distribution mimicking that of XAF activity (Fig. 7a) and  
19 that might thus be deemed necessary or sufficient for XAF activity.

20

#### 21 Eleven authentic plant polymers exhibit little or no XAF activity

22 Another approach that might lead to the identification of the XAF of CHP is to test various  
23 authentic polysaccharides or glycoproteins for XAF activity. Of eleven authentic polymers

1 tested at 5 mg ml<sup>-1</sup>, only gum arabic and tamarind xyloglucan possessed appreciable XAF  
2 activity (Fig. 6), though they were less effective than 2 mg ml<sup>-1</sup> cauliflower floret CHP.

3 Gum arabic is an AGP with a protein core to which numerous polysaccharide units (type-II  
4 arabinogalactans) are attached: these consist of a (1→3)-β-D-galactan backbone with long  
5 (1→3)-α-L-arabinan chains attached to the 6-position of some of the backbone residues; in  
6 addition, short side-chains containing α-D-galacturonate, β-D-glucuronate, α-L-rhamnose, α-  
7 L-arabinose are attached to some 2-, 4- and 6-positions of the galactan backbone (Nie et al.,  
8 2013; Lopez-Torrez et al., 2015; Andersen et al., 2017). Larch arabinogalactan, which in  
9 contrast to gum arabic has no XAF activity, is another type-II arabinogalactan; it is also  
10 (slightly) anionic and has a (1→3)-β-D-galactan backbone, but differs from gum arabic in  
11 lacking a protein core and in having only short side-chains (β-D-galactose, α-L-arabinose and  
12 β-D-glucuronic acid) attached only at the 6-position (Willför *et al.*, 2002). Likewise, a  
13 (1→4)-β-D-galactan (related to type-I arabinogalactan) from potato lacks XAF activity. Our  
14 results show that the AGP gum arabic possesses XAF activity, whereas other  
15 (arabino)galactans lacking a protein core do not.

16 The XAF activity of tamarind-seed xyloglucan was unexpected. It is possible that the wall-  
17 bound XTHs are held within the walls by an association with endogenous xyloglucan but can  
18 dissociate from this and re-attach to soluble exogenous xyloglucan. Curiously, nasturtium-  
19 seed xyloglucan does not exhibit XAF activity. Both tamarind- and nasturtium-seed  
20 xyloglucans are non-ionic and devoid of fucose residues; the main structural difference  
21 between them is that the major octasaccharide building block is XXLG in tamarind and  
22 XLXG in nasturtium (Fanutti *et al.*, 1996). It is possible that XTHs have a greater propensity  
23 to bind to xyloglucans with the XXLG unit. Another difference between the two xyloglucan  
24 preparations is that only the nasturtium xyloglucan had been purified by Cu<sup>2+</sup> precipitation

1 (McDougall & Fry, 1989). It might be speculated at this point that only the tamarind  
2 xyloglucan preparation is contaminated by traces of heat-stable plant glycoproteins with XAF  
3 activity; however, the latter hypothesis is discredited by the results of Fig. 9c (see below).

4

#### 5 Stability of cauliflower XAF to acid and alkali

6 A further way of defining the nature of XAF is to identify a specific treatment that destroys  
7 its activity. For example, loss of activity upon treatment with cold dilute alkali would suggest  
8 the involvement of an essential ester-linked moiety (Euranto, 1969) such as a methyl, acetyl,  
9 feruloyl or *p*-coumaroyl ester, all of which occur in certain plant polysaccharides (Fry, 2000).  
10 However, the XAF activity of cauliflower CHP survives in 0.48 M NaOH at 20°C for at least  
11 8 h (Fig. 8a), suggesting that XAF does not have an indispensable ester group.

12 In contrast, the XAF activity of cauliflower CHP is completely destroyed by the ‘severe’ acid  
13 conditions routinely used for monosaccharide residue analysis of cell wall polysaccharides (2  
14 M TFA, 120°C, 60 min; Fig. 7a). This could indicate the presence of an essential glycosidic  
15 (or potentially peptide) bond within XAF. Susceptibility to graded acid hydrolysis potentially  
16 gives clues to the nature of the XAF-active components since different types of glycosidic  
17 linkage differ in acid lability — in particular, furanosyl linkages are more labile than  
18 pyranosyl.

19 XAF activity survives mild acid treatment (0.1 M TFA at 85°C) for at least an hour (Fig. 8a),  
20 conditions which completely hydrolyse the furanose sugar linkages in the model compound  
21 arabino-octaose (Fig. 8b). Thus, XAF does not have an indispensable glycofuranose residue  
22 — the principal examples of which in plant polymers are arabinose (e.g. in arabinogalactans,  
23 rhamnogalacturonan-I and arabinoxylans; Kotake et al., 2016), apiose and aceric acid (in  
24 rhamnogalacturonan-II; Stevenson et al., 1988), fructose (in fructans; Ritsema & Smeekens,

1 2003) and ribose (in RNA). Indeed, arabinose is the sole monosaccharide released in  
2 detectable amounts from cauliflower CHP under these mild acid conditions.

3 Moderately severe acid treatment (2 M TFA at 100°C) does reduce XAF activity in a time-  
4 dependent manner with a half-life of ~8 min and complete loss by 32 min (Fig. 8a),  
5 concomitant with the release of galactose and galacturonic acid from CHP, and cleavage of  
6 the pyranosidically linked model substrate xylohexaose (Fig. 8c). An 8-min half-life under  
7 these conditions would be exceptionally short for all but the most acid-labile peptide linkages  
8 such as Asp-Pro (Rittenhouse & Marcus 1984). The data therefore suggest the presence in  
9 CHP of XAF-essential sugar pyranose linkages, which are present in almost all plant  
10 polysaccharides except arabinans and fructans. Indeed, cauliflower CHP does contain a wide  
11 range of pyranose-linked sugar building blocks including those diagnostic of  
12 arabinogalactans or AGPs (giving high levels of galactose on hydrolysis), xyloglucan  
13 (glucose, xylose, galactose and fucose), xylans (xylose), mannans (mannose), pectins  
14 (galacturonic acid, rhamnose and galactose) and starch (glucose) (Fig. 7c–f).

15

#### 16 Stability of cauliflower XAF to seven specific polysaccharide hydrolases and Driselase

17 If XAF activity is due to a specific type of polysaccharide present in CHP, this activity  
18 should be lost upon digestion with an appropriate glycanase or glycosidase. However, our  
19 data show that cauliflower XAF is remarkably stable to all eight such hydrolase preparations  
20 tested. Only XEG causes a modest loss of XAF activity, although most of the XAF  
21 withstands prolonged XEG treatment (Fig. 9b) under conditions that fully digest tamarind  
22 xyloglucan (Fig. S3b). Between them, the enzymes tested should be capable of hydrolysing  
23 most plant polysaccharides. Remarkably, cauliflower XAF activity also withstands Driselase,  
24 a highly potent commercial mixture of basidiomycete enzymes that digests plant primary cell

1 walls to mono- and disaccharides (typically to 98% completion; Gray *et al.*, 1993). The  
2 resistance of XAF activity to all these hydrolases, both pure and mixed, excludes the great  
3 majority of common plant polysaccharides as XAF candidates.

4 Denatured cellulase and  $\alpha$ -glucosidase themselves exert slight XAF activity (Fig. 9a),  
5 possibly owing to the presence of heat-stable (glyco?)-proteins present in these enzyme  
6 preparations. Biological effects of inactive enzymes, e.g. mutated xylanases (Enkerli *et al.*,  
7 1999) and fragmented invertases (Basse *et al.*, 1992), have been reported before. Such effects  
8 of the utilised enzymes were not sufficient to interfere in the interpretation of our study of  
9 XAF activity.

10 Curiously, the partial destruction of cauliflower CHP's XAF activity by native XEG was  
11 equally caused by acid-denatured XEG (Fig. 9c). The thoroughness of the acid denaturation  
12 is confirmed by the fact that the denatured XEG was unable to destroy the XAF activity of  
13 tamarind xyloglucan. Thus the effect of XEG on cauliflower CHP may be due to a minor  
14 contaminating enzyme which resists denaturation by acid treatment (Fig. 9c). As expected,  
15 XEG does not affect the XAF activity of gum arabic. The data show that the XAF activity of  
16 tamarind xyloglucan is indeed due to xyloglucan, and not a contaminating polymer, and that  
17 the XAF activity of gum arabic is not due to contaminating xyloglucan. Importantly,  
18 cauliflower CHP contains at least two XAFs: one type (a minority) that is destroyed by  
19 denatured XEG and is thus not xyloglucan, plus a second type that resists active XEG, and is  
20 thus also not xyloglucan.

21

## 22 **Conclusion**

23 Our study demonstrates a potential role for XAF — a specific, quantitatively minor, plant  
24 polymer — in the control of cell-wall properties, e.g. extensibility and thus cell expansion

1 and/or wall assembly, by solubilisation of xyloglucan endotransglucosylase/hydrolases from  
2 their binding sites in the cell wall. XAF does not solubilise or activate other wall enzymes,  
3 including peroxidase,  $\beta$ -glucosidase or phosphatase. We suggest that XAF, present in the  
4 apoplast, may modulate the action of endogenous XTHs. XAF may thus be a hitherto  
5 overlooked factor regulating the action restructuring of xyloglucan *in vivo*.

6

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10

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13

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19

20



## Figure legends

1

2 Fig. 1. Cauliflower CHP and CaCl<sub>2</sub> synergistically solubilise XET activity from arabidopsis  
3 cell walls.

4 Washed arabidopsis cell walls were incubated for 30 min in 66 µl of buffer [0.18 M MES  
5 (Na<sup>+</sup>), pH 5.5] containing various combinations of CHP and CaCl<sub>2</sub>. After centrifugation, 20  
6 µl of supernatant was incubated with 20 µl of XET reaction mixture also containing 0.5%  
7 BSA and the yield of [<sup>3</sup>H]polysaccharide at 16 h was determined ±SE (n = 4). Data from two  
8 representative experiments (Expt 1, dashed lines; Expt 2, solid lines), covering different  
9 concentration ranges, are shown.

10

11 Fig. 2. Ability of various agents to prevent loss of solubilised XTHs due to binding to tube  
12 walls.

13 XTHs were solubilised from washed arabidopsis cells in 180 mM MES (Na<sup>+</sup>, pH 5.5),  
14 containing 338 mM NaCl, for 1 h. The enzyme solution was then diluted into sufficient 180  
15 mM MES (pH 5.5), containing various additives, to give 15% or 50% of the initial enzyme  
16 concentration (in a total final volume of 20 µl) in three types of container (see *x*-axis): a well  
17 of a 96-well plate (96WP), or a 0.5-ml Eppendorf tube (Epp), or a PCR tube. The additives  
18 were as indicated in the box. When NaCl was the additive, it was *in addition* to the 50 or 169  
19 mM carried over with the enzyme extract. In the case of polylysine, the containers had been  
20 pretreated by filling with 0.5% (w/v) polylysine, incubating for 16 h, then water-washing and  
21 re-drying prior to addition of the enzyme extracts; thus no soluble polylysine remained.

22 The 20-µl solutions were then incubated in these containers for 5.5 h, permitting possible  
23 binding to the tube walls, after which 20 µl of XET substrate mixture was added; the XET  
24 reaction-products (radioactive polysaccharide) were measured after a further 16 h. Data show

1 the mean of two determinations  $\pm$  range. Asterisks indicates data which are significantly  
2 different from the relevant 'untreated' sample: \*,  $p \leq 0.01$ ; \*\*,  $p \leq 0.001$ .

3

4 Fig. 3. Enzyme activities solubilised from arabidopsis cell walls by cauliflower CHP or high  
5 salt.

6 Washed *Arabidopsis* cell walls were incubated in 0.2 M MES (Na<sup>+</sup>), pH 5.5, containing,  
7 sequentially, (i) 0.075 M NaCl, (ii) 2 mg/ml CHP with 0.075 M NaCl, and (iii) 1.0 M NaCl,  
8 for 30 min in each solution. After each extractant, all the solution was removed from the cell  
9 walls and the next extractant was then applied. Aliquots of each extract were assayed for (a)  
10  $\beta$ -d-glucosidase, (b) phosphatase, (c) peroxidase and (d) XET activity. In (a) and (b) the  
11 yellow *p*-nitrophenol product was assayed at 400 nm; in (c) the reddish peroxidase product  
12 was assayed at 420 nm [and a standard of commercial horseradish peroxidase (HRP) was also  
13 assayed]; in (d) [<sup>3</sup>H]polysaccharide formed by XET activity was measured. The deceleration  
14 of reaction rate in (c) was not reversed by additional H<sub>2</sub>O<sub>2</sub> (data not shown) and may indicate  
15 gradual denaturation of the solubilised peroxidase. Error bars represent SE (n = 4).

16

17 Fig. 4. Solubilisation of arabidopsis cell-wall-bound XTH by cauliflower CHP: effect of  
18 varying cell wall and CHP concentrations.

19 (a) Effect of cell wall concentration. A suspension of washed arabidopsis cell walls (3 to 66  
20  $\mu$ l) was washed several further times with water and the washings were removed. The slightly  
21 moist wall pellet (equivalent to 8.3–183  $\mu$ g dry weight) was incubated in 66  $\mu$ l of 0.075 M  
22 NaCl containing 0.2 M MES with 2 mg/ml CHP (solid symbols) or without CHP (open  
23 symbols) for 30 min. Solubilised enzymes were then assayed for XET activity by the normal

1 method for 16 h in the presence of 0.25% BSA.  $\Delta$ = water (without CHP) used in place of  
2 NaCl/MES buffer. Data are mean of four determinations  $\pm$  SE.

3 (b) Effect of CHP concentration. Details as in (a), but the cell-wall concentration was always  
4 20  $\mu$ g per 66  $\mu$ l and the CHP concentration was varied. Data are mean of two determinations  
5  $\pm$  range. The curve is fitted according to the equation for two superimposed hyperbolae,

$$6 \quad y = [(V_{\max 1} \times x) / (K_{m1} + x)] + [(V_{\max 2} \times x) / (K_{m2} + x)],$$

7 with  $K_{m1} = 0.035$  mg/ml and  $K_{m2} = 39$  mg/ml.

8

9 Fig. 5. Sugar residue composition of diverse CHPs.

10 (a) CHPs (100  $\mu$ g) were hydrolysed in 2 M TFA at 120°C, and the sugars were analysed by  
11 TLC with thymol staining.

12 (b) XAF activity of a 2 mg/ml CHP solution (y-axis shows Bq of radioactive polysaccharide  
13 formed in 16 h by the XTHs solubilised from arabidopsis walls).

14 Samples were: arabidopsis (1, stem; 2, leaf; 3, flower); snowdrop (4, leaf; 5, flower; 6, stem);  
15 crocus (7, leaf; 8, flower); cell-cultures (9, rose; 10, arabidopsis; 11, spinach); carrot (12,  
16 root; 13, leaf); 14, spinach leaf; 15, asparagus shoot; celery (16, mature petiole; 17, young  
17 whole leaf); 18, watercress shoot; 19, lettuce leaf; 20, parsley leaf; spring onion (21, basal  
18 stem + leaf; 22, leaf); tobacco (23, leaf; 24, stem); 25, cauliflower floret. Sample 26 was a  
19 TFA-only control. Scientific names listed in Fig. S1. Abbreviations: ABUAs, aldobionuronic  
20 acids; Ara, arabinose; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose;  
21 GlcA, glucuronic acid; GlcA(L), glucuronolactone; Man, mannose; Rha, rhamnose; Rib,  
22 ribose; Unk, unknown; Xyl, xylose. Black sugar labels are authentic markers; green labels  
23 (left) are sugars derived from the plant CHPs.

1 Fig. 6. XAF activity of cauliflower CHP and various commercial polysaccharides.  
2 Authentic polysaccharides were dissolved (or suspended in the case of cellulose) at 5 mg/ml  
3 in the standard NaCl/MES buffer. Cauliflower CHP was dissolved in the same buffer but at 2  
4 mg/ml. Each polysaccharide solution/suspension was assayed in triplicate for XAF activity  
5 — the ability to solubilise XET activity from washed arabidopsis walls. Data are means  $\pm$  SE  
6 (n=3). Asterisk indicate statistically significant difference from the buffer sample: \*,  $p \leq 0.05$ ;  
7 \*\*,  $p \leq 0.001$ .

8 Polysaccharides tested: tamarind xyloglucan; nasturtium xyloglucan; larch-wood  
9 arabinogalactan; gum arabic (*Acacia*); cellulose powder; CMC, carboxymethylcellulose;  
10 esterified citrus fruit pectin; potato galactan; birch-wood xylan; homogalacturonan; soluble  
11 starch; cauliflower CHP. Samples 'buffer' and 'water' had no added polysaccharide: Buffer,  
12 buffer in water; Water, water only.

13

14 Fig. 7. Size fractionation and sugar residue composition of XAF-active cauliflower CHPs.

15 Cauliflower CHPs were passed through Sepharose CL-6B. For panels **a** and **c-f**, the fractions  
16 were paired and tested for XAF activity and sugar residue composition; for example,  
17 fractions 31 + 32 were pooled and the result is plotted at 31.5 on the  $x$ -axis. Arrows indicate  
18 void volume ( $V_0$ ;  $K_{av}$  0) and totally included volume ( $V_i$ ;  $K_{av}$  1). Vertical dashed lines  
19 demarcate the major XAF-active fractions.

20 (a) XAF activity before and after treatment with TFA: 3.8% of each paired fraction was dried  
21 and an equivalent portion was hydrolysed (in 2 M TFA at 120°C for 1 h) then dried, after  
22 which both samples were assayed for XAF activity. Data are mean of two determinations  
23  $\pm$ range.

1 (b) All fractions were assayed individually for the internal markers blue dextran and  
2 [<sup>14</sup>C]glucose and for endogenous UV-absorbing components ( $A_{280}$ ). The  $K_{av}$  0.32–0.66 zone  
3 was pooled for further analysis, e.g. in Fig. 8a.

4 (c, d) Sugars released by acid hydrolysis (TFA): 1.8% of each paired fraction was hydrolysed  
5 (in 2 M TFA at 120°C for 1 h) and analysed by HPLC. The peak of glucose in fractions 17–  
6 20 is mainly derived from the added blue dextran.

7 (e, f) Sugars released by enzymic hydrolysis (dris): 0.28% of each paired fraction was  
8 digested with Driselase and analysed by HPLC. Abbreviations as in Fig. 5, and Xyl2 =  
9 xylobiose.

10

11 Fig. 8. Acid lability and alkali stability of XAF-active cauliflower CHPs.

12 (a) The relatively high- $M_r$ , XAF-active fractions of cauliflower CHP eluting from Sepharose  
13 CL-6B (equivalent to the  $K_{av}$  0.32–0.66 zone marked in Fig. 7b) were pooled, dried, treated  
14 with either 0.1 M TFA (85°C) or 2.0 M TFA (100°C) or 0.48 M NaOH (20°C), and then  
15 assayed for XAF activity. Data are mean of at least three determinations  $\pm$ SE.

16 (b,c) Effect of the two acid treatments on authentic oligosaccharides and the sugar  
17 components of XAF-active fractions of cauliflower CHP eluting from Sepharose CL-6B [b,  
18 hydrolysis in 0.1 M TFA (85°C); c, hydrolysis in 2.0 M TFA (100°C) ]. The products were  
19 resolved by TLC and stained with thymol–H<sub>2</sub>SO<sub>4</sub>. S = substrate in water; 0'–64' = substrate  
20 in TFA heated for the time indicated in minutes.

21

22 Fig. 9. Digestion of cauliflower CHP by various commercial enzymes.

1 (a) Effect of enzymes on XAF activity of CHP. Dried CHP (0.4 mg) was incubated with 300  
2  $\mu$ l solution of the named enzymes [in PyAW (pH 4.7) at 20°C for 24 h unless otherwise  
3 stated]; (1) and (2) refer to two independent experiments: in (2), the galactosidase was used at  
4 pH 5.6 and 55°C for 4 h and the  $\alpha$ -glucosidase was in 1% lutidine and 0.3% acetic acid, pH  
5 6.6, at 20°C for 48 h. None = buffer in place of enzymes; this result was set as 100% XAF  
6 activity within each experiment; in these cases, 'CHP + enzyme' (black bar) was CHP in  
7 buffer and 'enzyme only' (grey bar) was buffer alone. Data are means of at least 4 assays  $\pm$   
8 SE. Asterisks indicate statistically significant difference from the 'none (1)' sample: \*,  $p <$   
9 0.05; \*\*,  $p <$  0.001.

10 (b) Effect of XEG on XAF activity of individual CHP fractions from a Sepharose column  
11 (same column run as shown in Fig. 7). Fractions from a Sepharose CL-6B column were  
12 treated with 5.2  $\mu$ g/ml XEG for 1.5 h at 20°C, then the reaction was stopped by heating at  
13 120°C for 70 min; after centrifugation, the supernatant was assayed for XAF activity. Equal  
14 volumes of each Sepharose fraction were assayed for XAF activity without XEG treatment (–  
15 XEG). Data are mean of two determinations  $\pm$ range.

16 (c) Effect of XEG on three different XAF-active substrates. XEG (7.8  $\mu$ g/ml; in PyAW  
17 1:1:98) was incubated with CHP (2 mg/ml), tamarind xyloglucan (XyG; 5 mg/ml), gum  
18 arabic (GA; 5 mg/ml) or water ('none'), and incubated at 20°C for 1.5 h. Controls were  
19 without XEG ('untreated') or with XEG that had been denatured in 22% formic acid at 20°C  
20 and then freed of the acid *in vacuo*. Enzyme remaining after incubation with active XEG was  
21 denatured in 22% formic acid. Each solution was then assayed for XAF activity, and the yield  
22 of [<sup>3</sup>H]polysaccharide is reported. Data are mean of two determinations  $\pm$  range.

23 (d) Xyloglucan oligosaccharides produced by XEG digestion of the Sepharose fractions.  
24 One-sixth of each fraction shown in (b) was analysed by TLC; panels (b) and (d) are aligned.

1 (e) TLC of digestion products. The  $\alpha$ -amylase,  $\beta$ -galactosidase and  $\alpha$ -glucosidase CHP  
2 digests (from panel (a), experiment 2) were analysed by TLC. Controls lacked CHP ('enz  
3 only') or enzyme ('CHP only'). In addition, authentic disaccharides (C6, cellohexaose; M6,  
4 maltohexaose) were treated with XEG (83  $\mu$ g/ml , PyAW 1:1:98, 24 h, as in panel a).

5

1 Caption for Supplementary Table

2 Table S1. Effect of CaCl<sub>2</sub> and cauliflower CHP on solubilisation of XET activity from  
3 arabidopsis cell walls

4

5 Captions for Supplementary Figures

6 Figure S1. Lack of a strong relationship between ionic strength of diverse CHPs and their  
7 XAF activity.

8 Figure S2. Scattergrams showing the relationship between sugar residue composition of  
9 diverse CHPs and their XAF activity.

10 Fig. S3. Effect of XEG on gum arabic and xyloglucan.



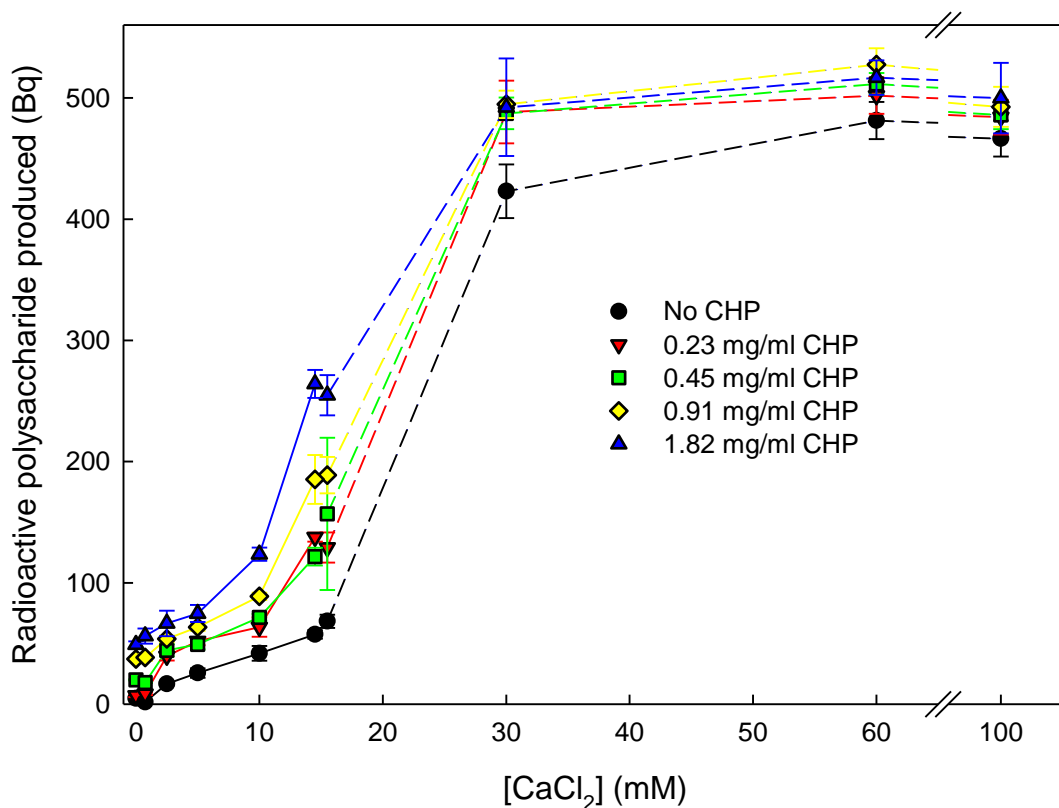


Fig. 1. Cauliflower CHP and CaCl<sub>2</sub> synergistically solubilise XET activity from arabidopsis cell walls.

Washed arabidopsis cell walls were incubated for 30 min in 66  $\mu$ l of buffer [0.18 M MES (Na<sup>+</sup>), pH 5.5] containing various combinations of CHP and CaCl<sub>2</sub>. After centrifugation, 20  $\mu$ l of supernatant was incubated with 20  $\mu$ l of XET reaction mixture also containing 0.5% BSA and the yield of [<sup>3</sup>H]polysaccharide at 16 h was determined  $\pm$  SE (n = 4). Data from two representative experiments (Expt 1, dashed lines; Expt 2, solid lines), covering different concentration ranges, are shown.

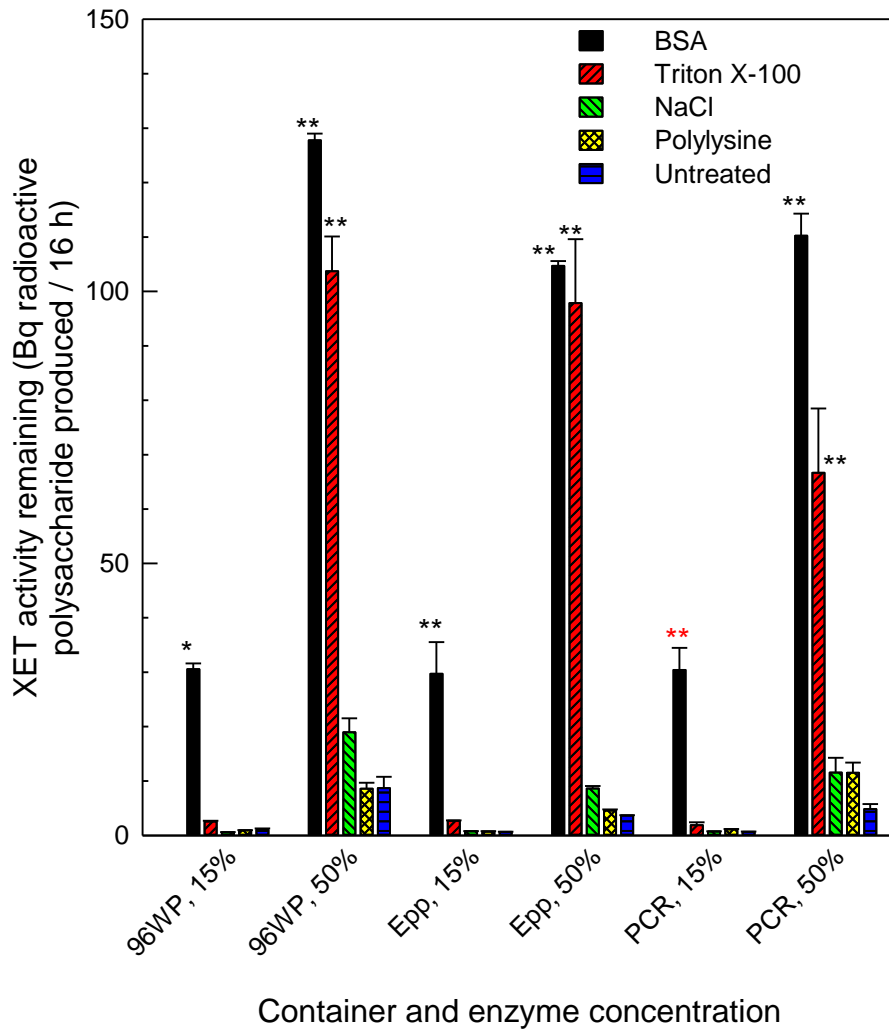


Fig. 2. Ability of various agents to prevent loss of solubilised XTHs due to binding to tube walls.

XTHs were solubilised from washed arabidopsis cells in 180 mM MES (Na<sup>+</sup>, pH 5.5), containing 338 mM NaCl, for 1 h. The enzyme solution was then diluted into sufficient 180 mM MES (pH 5.5), containing various additives, to give 15% or 50% of the initial enzyme concentration (in a total final volume of 20  $\mu$ l) in three types of container (see x-axis): a well of a 96-well plate (96WP), or a 0.5-ml Eppendorf tube (Epp), or a PCR tube. The additives were as indicated in the box. When NaCl was the additive, it was *in addition* to the 50 or 169 mM carried over with the enzyme extract. In the case of polylysine, the containers had been pretreated by filling with 0.5% (w/v) polylysine, incubating for 16 h, then water-washing and re-drying prior to addition of the enzyme extracts; thus no soluble polylysine remained.

The 20- $\mu$ l solutions were then incubated in these containers for 5.5 h, permitting possible binding to the tube walls, after which 20  $\mu$ l of XET substrate mixture was added; the XET reaction-products (radioactive polysaccharide) were measured after a further 16 h. Data show the mean of two determinations  $\pm$  range. Asterisks indicates data which are significantly different from the relevant 'untreated' sample: \*,  $p \leq 0.01$ ; \*\*,  $p \leq 0.001$ .

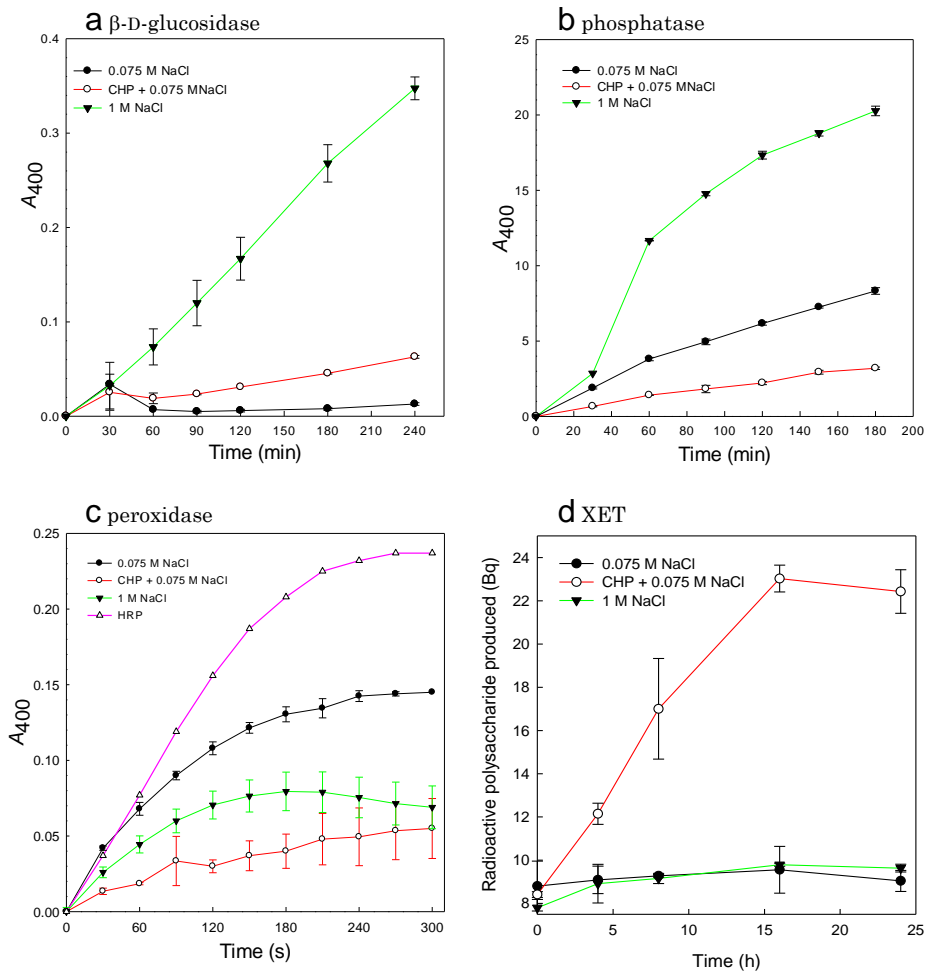


Fig. 3. Enzyme activities solubilised from arabidopsis cell walls by cauliflower CHP or high salt.

Washed *Arabidopsis* cell walls were incubated in 0.2 M MES ( $\text{Na}^+$ ), pH 5.5, containing, sequentially, (i) 0.075 M NaCl, (ii) 2 mg/ml CHP with 0.075 M NaCl, and (iii) 1.0 M NaCl, for 30 min in each solution. After each extractant, all the solution was removed from the cell walls and the next extractant was then applied. Aliquots of each extract were assayed for (a)  $\beta$ -D-glucosidase, (b) phosphatase, (c) peroxidase and (d) XET activity. In (a) and (b) the yellow *p*-nitrophenol product was assayed at 400 nm; in (c) the reddish peroxidase product was assayed at 420 nm [and a standard of commercial horseradish peroxidase (HRP) was also assayed]; in (d) [ $^3\text{H}$ ]polysaccharide formed by XET activity was measured. The deceleration of reaction rate in (c) was not reversed by additional  $\text{H}_2\text{O}_2$  (data not shown) and may indicate gradual denaturation of the solubilised peroxidase. Error bars represent SE ( $n = 4$ ).

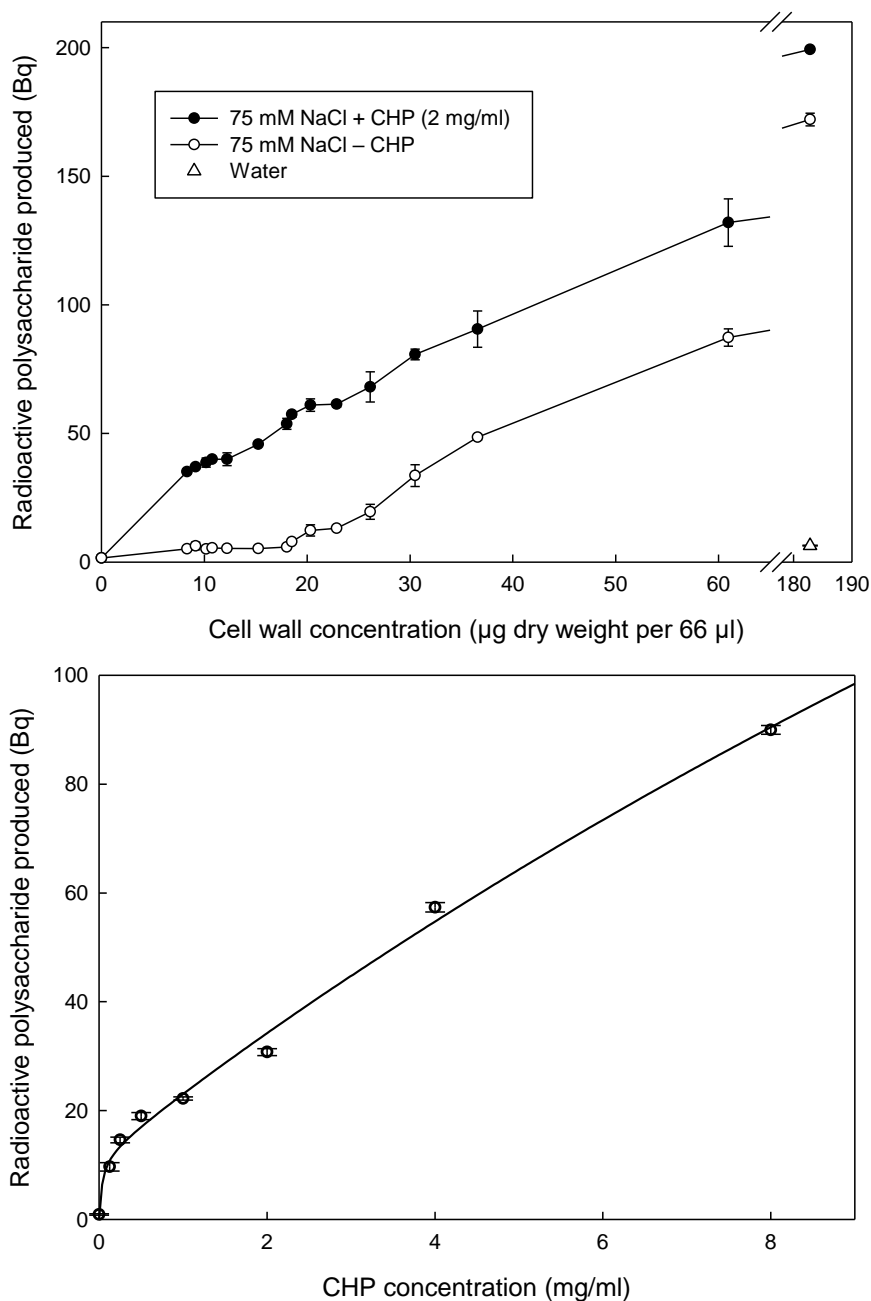


Fig. 4. Solubilisation of arabidopsis cell-wall-bound XTH by cauliflower CHP: effect of varying cell wall and CHP concentrations.

(a) Effect of cell wall concentration. A suspension of washed arabidopsis cell walls (3 to 66 µl) was washed several further times with water and the washings were removed. The slightly moist wall pellet (equivalent to 8.3–183 µg dry weight) was incubated in 66 µl of 0.075 M NaCl containing 0.2 M MES with 2 mg/ml CHP (solid symbols) or without CHP (open symbols) for 30 min. Solubilised enzymes were then assayed for XET activity by the normal method for 16 h in the presence of 0.25% BSA.  $\Delta$  = water (without CHP) used in place of NaCl/MES buffer. Data are mean of four determinations  $\pm$  SE.

(b) Effect of CHP concentration. Details as in (a), but the cell-wall concentration was always 20 µg per 66 µl and the CHP concentration was varied. Data are mean of two determinations  $\pm$  range. The curve is fitted according to the equation for two superimposed hyperbolae,

$$y = [(V_{\max 1} \times x) / (K_{m1} + x)] + [(V_{\max 2} \times x) / (K_{m2} + x)],$$

with  $K_{m1} = 0.035$  mg/ml and  $K_{m2} = 39$  mg/ml.

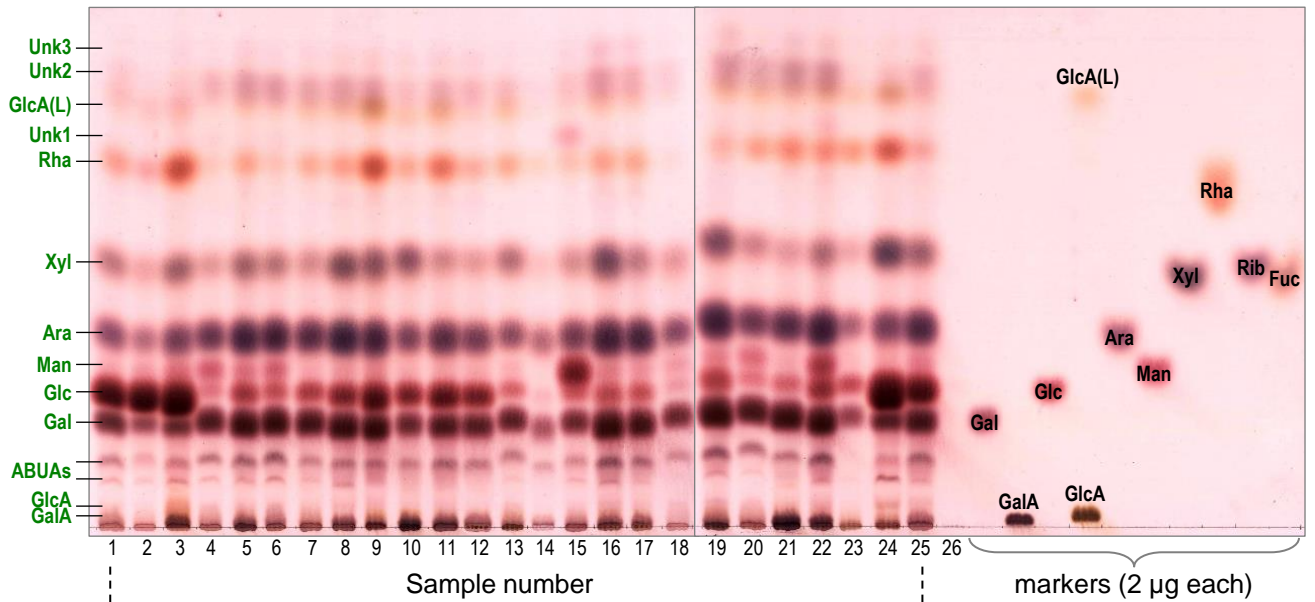
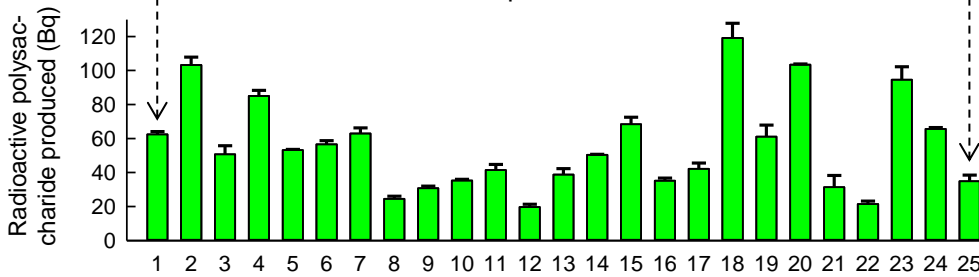
**a****b**

Fig. 5. Sugar residue composition of diverse CHPs.

(a) CHPs (100  $\mu$ g) were hydrolysed in 2 M TFA at 120° C, and the sugars were analysed by TLC with thymol staining.

(b) XAF activity of a 2 mg/ml CHP solution (*y*-axis shows Bq of radioactive polysaccharide formed in 16 h by the XTHs solubilised from arabidopsis walls).

Samples were: arabidopsis (1, stem; 2, leaf; 3, flower); snowdrop (4, leaf; 5, flower; 6, stem); crocus (7, leaf; 8, flower); cell-cultures (9, rose; 10, arabidopsis; 11, spinach); carrot (12, root; 13, leaf); 14, spinach leaf; 15, asparagus shoot; celery (16, mature petiole; 17, young whole leaf); 18, watercress shoot; 19, lettuce leaf; 20, parsley leaf; spring onion (21, basal stem + leaf; 22, leaf); tobacco (23, leaf; 24, stem); 25, cauliflower floret. Sample 26 was a TFA-only control. Scientific names listed in Fig. S1.

Abbreviations: ABUAs, aldobiouronic acids; Ara, arabinose; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; GlcA(L), glucuronolactone; Man, mannose; Rha, rhamnose; Rib, ribose; Unk, unknown; Xyl, xylose. Black sugar labels are authentic markers; green labels (left) are sugars derived from the plant CHPs.

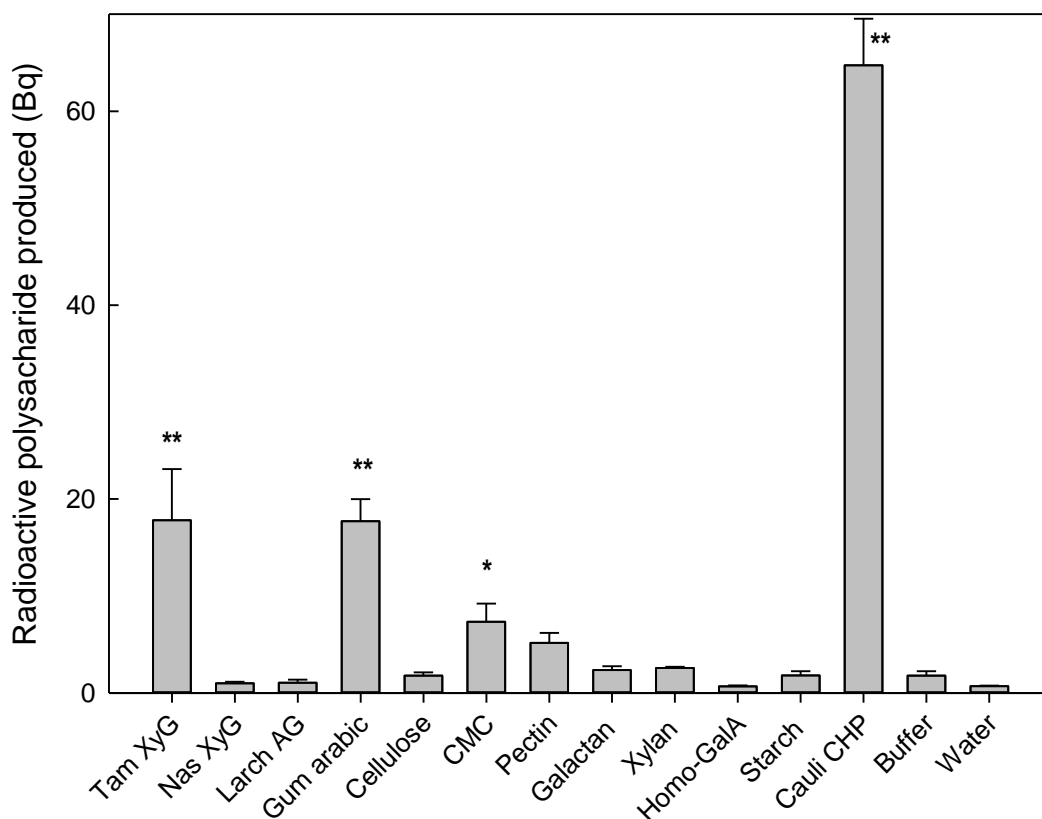


Fig. 6. XAF activity of cauliflower CHP and various commercial polysaccharides.

Authentic polysaccharides were dissolved (or suspended in the case of cellulose) at 5 mg/ml in the standard NaCl/MES buffer. Cauliflower CHP was dissolved in the same buffer but at 2 mg/ml. Each polysaccharide solution/suspension was assayed in triplicate for XAF activity — the ability to solubilise XET activity from washed arabidopsis walls. Data are means  $\pm$  SE (n=3). Asterisk indicate statistically significant difference from the buffer sample: \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.001$ .

Polysaccharides tested: tamarind xyloglucan; nasturtium xyloglucan; larch-wood arabinogalactan; gum arabic (*Acacia*); cellulose powder; CMC, carboxymethylcellulose; esterified citrus fruit pectin; potato galactan; birch-wood xylan; homogalacturonan; soluble starch; cauliflower CHP. Samples 'buffer' and 'water' had no added polysaccharide: Buffer, buffer in water; Water, water only.

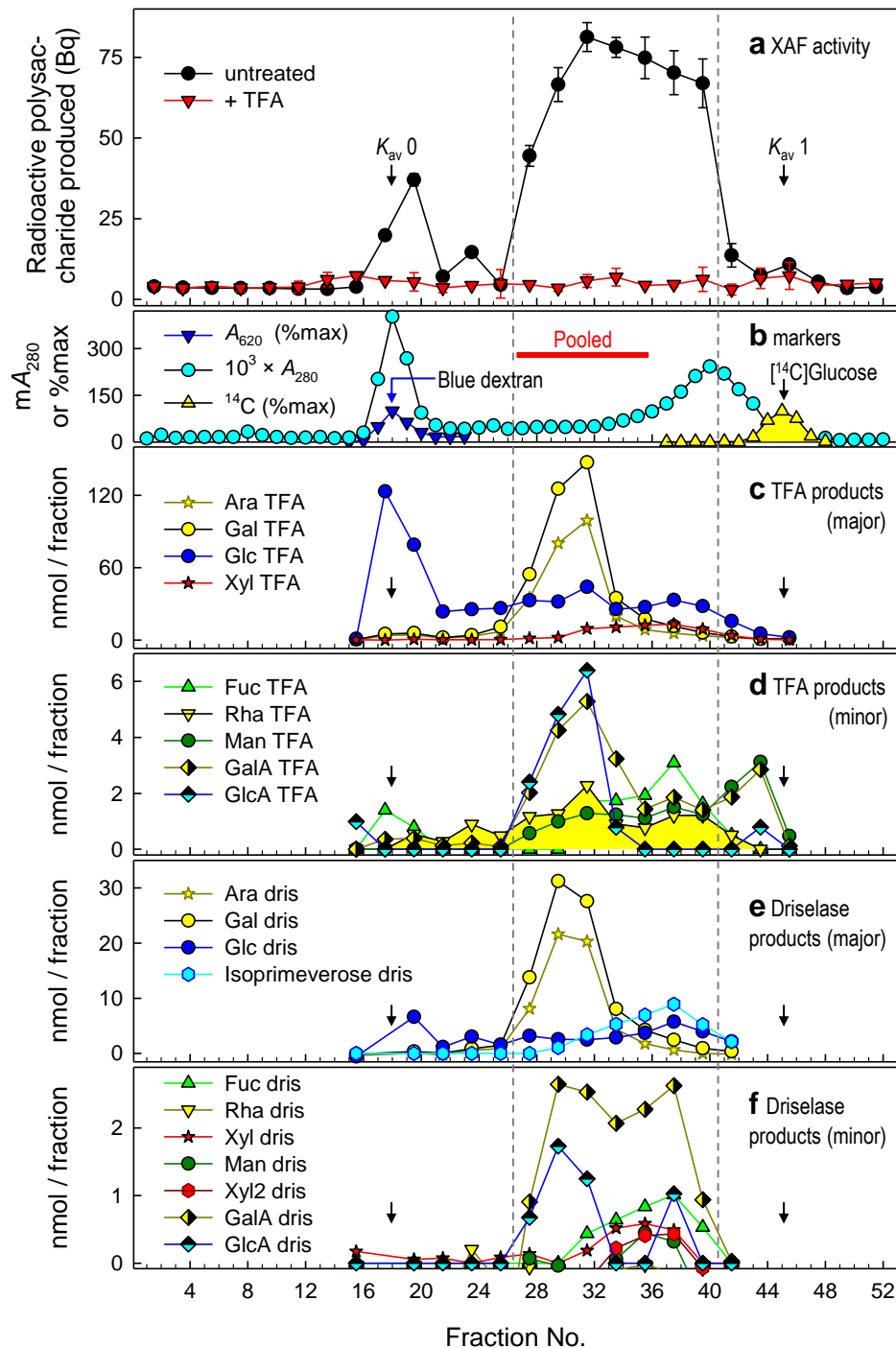


Fig. 7. Size fractionation and sugar residue composition of XAF-active cauliflower CHPs.

Cauliflower CHPs were passed through Sepharose CL-6B. For panels **a** and **c–f**, the fractions were paired and tested for XAF activity and sugar residue composition; for example, fractions 31 + 32 were pooled and the result is plotted at 31.5 on the x-axis. Arrows indicate void volume ( $V_0$ ;  $K_{av}$  0) and totally included volume ( $V_i$ ;  $K_{av}$  1). Vertical dashed lines demarcate the major XAF-active fractions.

(a) XAF activity before and after treatment with TFA: 3.8% of each paired fraction was dried and an equivalent portion was hydrolysed (in 2 M TFA at 120°C for 1 h) then dried, after which both samples were assayed for XAF activity. Data are mean of two determinations  $\pm$  range.

(b) All fractions were assayed individually for the internal markers blue dextran and [ $^{14}$ C]glucose and for endogenous UV-absorbing components ( $A_{280}$ ). The  $K_{av}$  0.32–0.66 zone was pooled for further analysis, e.g. in Fig. 8a.

(c, d) Sugars released by acid hydrolysis (TFA): 1.8% of each paired fraction was hydrolysed (in 2 M TFA at 120°C for 1 h) and analysed by HPLC. The peak of glucose in fractions 17–20 is mainly derived from the added blue dextran.

(e, f) Sugars released by enzymic hydrolysis (dris): 0.28% of each paired fraction was digested with Driselase and analysed by HPLC. Abbreviations as in Fig. 5, and Xyl2 = xylobiose.

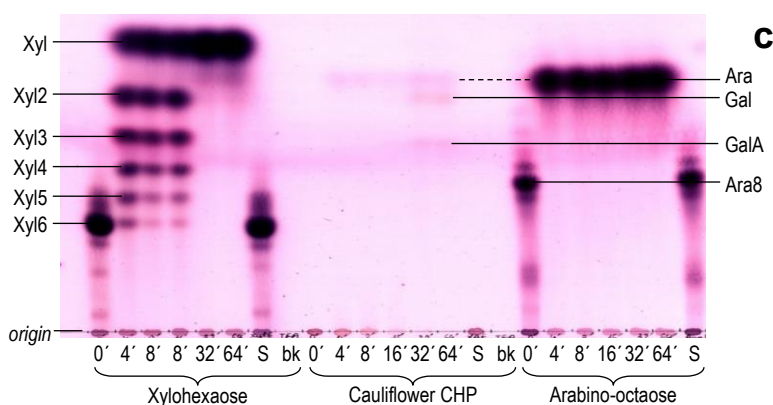
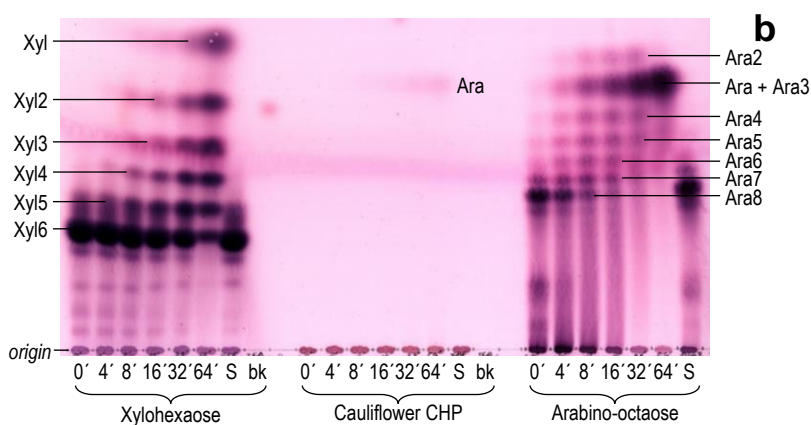
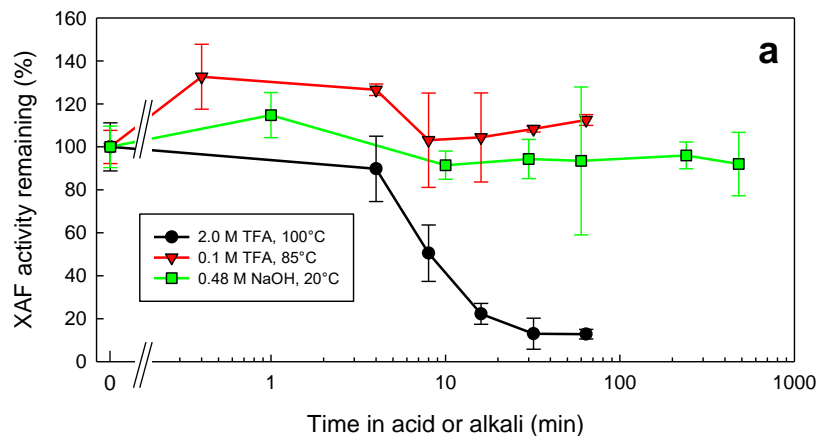


Fig. 8. Acid lability and alkali stability of XAF-active cauliflower CHPs.

(a) The relatively high- $M_r$ , XAF-active fractions of cauliflower CHP eluting from Sepharose CL-6B (equivalent to the  $K_{av}$  0.32–0.66 zone marked in Fig. 7b) were pooled, dried, treated with either 0.1 M TFA (85°C) or 2.0 M TFA (100°C) or 0.48 M NaOH (20°C), and then assayed for XAF activity. Data are mean of at least three determinations  $\pm$ SE.

(b,c) Effect of the two acid treatments on authentic oligosaccharides and the sugar components of XAF-active fractions of cauliflower CHP eluting from Sepharose CL-6B [b, hydrolysis in 0.1 M TFA (85°C); c, hydrolysis in 2.0 M TFA (100°C)]. The products were resolved by TLC and stained with thymol- $H_2SO_4$ . S = substrate in water; 0'–64' = substrate in TFA heated for the time indicated in minutes.



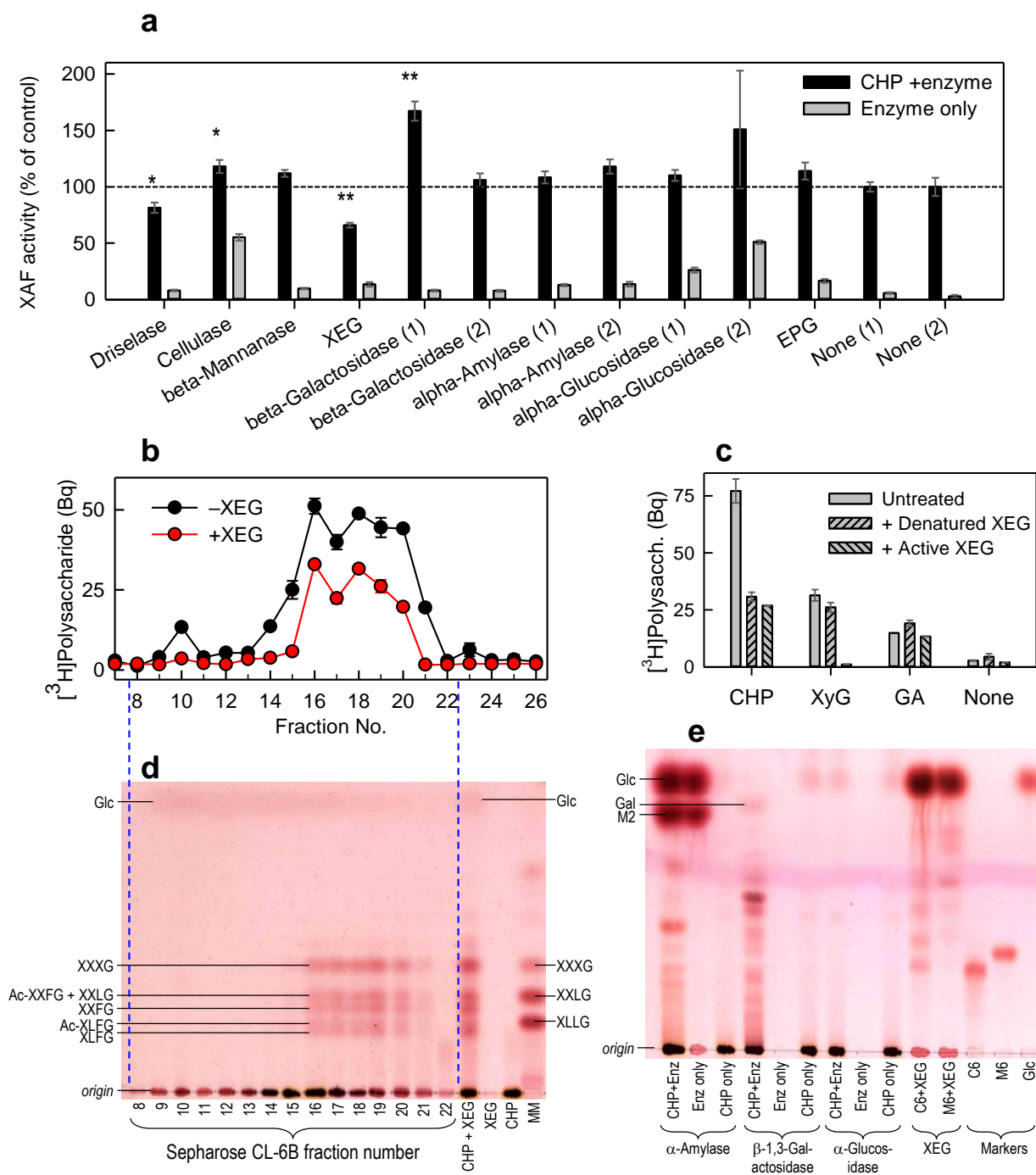


Fig. 9. Digestion of cauliflower CHP by various commercial enzymes.

(a) Effect of enzymes on XAF activity of CHP. Dried CHP (0.4 mg) was incubated with 300  $\mu$ l solution of the named enzymes [in PyAW (pH 4.7) at 20°C for 24 h unless otherwise stated]; (1) and (2) refer to two independent experiments: in (2), the galactosidase was used at pH 5.6 and 55°C for 4 h and the  $\alpha$ -glucosidase was in 1% lutidine and 0.3% acetic acid, pH 6.6, at 20°C for 48 h. None = buffer in place of enzymes; this result was set as 100% XAF activity within each experiment; in these cases, 'CHP + enzyme' (black bar) was CHP in buffer and 'enzyme only' (grey bar) was buffer alone. Data are means of at least 4 assays  $\pm$  SE. Asterisks indicate statistically significant difference from the 'none (1)' sample: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ .

(b) Effect of XEG on XAF activity of individual CHP fractions from a Sepharose column (same column run as shown in Fig. 7). Fractions from a Sepharose CL-6B column were treated with 5.2  $\mu$ g/ml XEG for 1.5 h at 20°C, then the reaction was stopped by heating at 120°C for 70 min; after centrifugation, the supernatant was assayed for XAF activity. Equal volumes of each Sepharose fraction were assayed for XAF activity without XEG treatment (-XEG). Data are mean of two determinations  $\pm$  range.

(c) Effect of XEG on three different XAF-active substrates. XEG (7.8  $\mu$ g/ml; in PyAW 1:1:98) was incubated with CHP (2 mg/ml), tamarind xyloglucan (XyG; 5 mg/ml), gum arabic (GA; 5 mg/ml) or water ('none'), and incubated at 20°C for 1.5 h. Controls were without XEG ('untreated') or with XEG that had been denatured in 22% formic acid at 20°C and then freed of the acid *in vacuo*. Enzyme remaining after incubation with active XEG was denatured in 22% formic acid. Each solution was then assayed for XAF activity, and the yield of [ $^3$ H]polysaccharide is reported. Data are mean of two determinations  $\pm$  range.

(d) Xyloglucan oligosaccharides produced by XEG digestion of the Sepharose fractions. One-sixth of each fraction shown in (b) was analysed by TLC; panels (b) and (d) are aligned.

(e) TLC of digestion products. The  $\alpha$ -amylase,  $\beta$ -galactosidase and  $\alpha$ -glucosidase CHP digests (from panel (a), experiment 2) were analysed by TLC. Controls lacked CHP ('enz only') or enzyme ('CHP only'). In addition, authentic disaccharides (C6, cellobiose; M6, maltose) were treated with XEG (83  $\mu$ g/ml, PyAW 1:1:98, 24 h, as in panel a).