Functional and chemical characterisation of XAF

submitted to *Ann Bot* 30 July 2018

response received 2 January 2019

revision submitted 10 February 2019

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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Abstract

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

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

Key results:

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

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

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

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

APPENDIX: Abbreviations

AGP, arabinogalactan–protein; CHP, cold-water-extractable heat-stable polymer; MES, morpholinoethanesulphonic acid; PL, polylysine; PyAW, pyridine/acetic acid/water (1:1:98; pH 4.7, unless otherwise stated); XAF, XTH activating factor; XET, xyloglucan endotransglucosylase (activity); XTH, xyloglucan endotransglucosylase/hydrolase (protein); XXXGol, borohydride-reduced heptasaccharide of xyloglucan ($xylose_3.glucose_3.glucitol$).

Word count

Abstract	*292*
Intro	1083
M&M	1299
Results	2969
Discussion & Conclusions	2672
Fig. legends	1797
References	*1905*
Total (Intro, M&M, Res, Discn, Concl, Legends)	**9820**

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

anthraquinones and flavonoids; Chormova *et al.*, 2015), though none of these compounds had such an effect when all components were cellulose-free (thus soluble), suggesting that the promotion of activity was only observed when XTH–cellulose interactions were occurring.

Sharples *et al.* (2017) showed that cauliflower CHP exerts its XAF activity principally by (re-)solubilising XTHs from surfaces (including cellulose, glass-fibre, glass and plastics) to which these enzymes tend to bind. Likewise, and of more direct botanical relevance, cell walls prepared from cauliflower florets, mung bean shoots and arabidopsis cell-cultures each contained endogenous, tightly bound, inactive XTHs, which were rapidly solubilised, and consequently activated, by the XAF of cauliflower CHP. A convenient quantitative assay for XAF acting on the natively sequestered XTHs of arabidopsis cell walls was developed and this is exploited in the present paper. We have therefore been able to investigate further the physiology and biochemistry of the unidentified endogenous CHPs that possess XAF activity — agents that solubilise XTHs from their binding sites in the cell wall, activating them and enabling xyloglucan re-structuring *in vivo*.

MATERIALS AND METHODS

Materials

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

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

Preparation of CHPs

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

XAF assay

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

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

Assay of four enzyme activities potentially solubilised from arabidopsis walls

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

Each extract was assayed for four enzyme activities:

β-Glucosidase. The extract (500 µl) was added to 500 µl 5 mM *p*-nitrophenyl β-D-glucopyranoside in 0.2 M MES, pH 5.5. At the desired time-point, the reaction was stopped by addition of 1 ml 1 M Na_2CO_3 and the A_{400} of the released *p*-nitrophenol was read.

Phosphatase. As above but with *p*-nitrophenyl phosphate (Na^+) as substrate.

Peroxidase. Extract (100 µl) was added to 3 ml of a reaction mixture containing 133 mM *o*-dianisidine and 133 mM H_2O_2, 167 mM NaH_2PO_4 and 133 mM MES (Na^+, final pH 5.5), and incubated at 20°C for 30 min. A_{420} was monitored every 30 s (Fry, 2000). HRP (1 ng in 100 µl sample) was used as a positive control.

XET. The extract (20 µl) was added to 20 µl of a reaction mixture containing 0.5 kBq [^{3}H]XXXGol (0.5 kBq), 0.4% (w/v) tamarind xyloglucan, 0.25% BSA and 0.5% (w/v) chlorobutanol. After incubation for 0, 4, 8, 16 or 24 h, the reaction was stopped with formic acid and the procedure was continued as described for the XAF assay.

Acid hydrolysis and TLC

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

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

Dionex HPLC methodology (high-pressure anion-exchange chromatography) was as described by O'Rourke *et al.* (2015). For HPLC of Driselase digests, the yield of each sugar was corrected for the small yield (if any) produced by Driselase autolysis. TLC was on 20 × 20 cm silica-gel plates. The solvent was ethyl acetate/pyridine/acetic acid/water (6:3:1:1) and sugars were stained with thymol/H_2SO_4 (Jork *et al.*, 1994). Sugar spots on TLCs were quantified with Photoshop software. The ellipse tool (fixed size 0.87 × 0.61 cm) was centred on the spot of interest, and the 'mean intensity' was measured in the green channel (which is

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

Enzymic digestion of cauliflower CHP

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

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

Gel-permeation column chromatography

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

RESULTS

Cauliflower CHP acts synergistically with $CaCl_2$ in XAF assays

The ability of CHP to solubilise XTHs from arabidopsis cell walls was mimicked by NaCl (Sharples *et al.*, 2017) and we now show a similar effect with $CaCl_2$ (Fig. 1). The effect plateaued above about 30 mM $CaCl_2$, but the CHP effect did not plateau even at the highest concentration tested (1.8 mg ml^{-1}; Fig. 1). The relative effect of CHP was greatest (34-fold promotion) in the absence of $CaCl_2$, but strong CHP effects (4.6- to 9.1-fold promotion) — and much higher absolute XET activities — were still detected in the presence of 15 mM $CaCl_2$, indicating synergy between CHP and the inorganic salt (Table S1). This observation, together with the previous finding that certain anionic polysaccharides promote the XET activity of de-salted XTH preparations particularly well if a sub-optimal concentration of salt is also present (Takeda & Fry, 2004), led us to assay XAF activity in all subsequent experiments by suspending the washed arabidopsis cell walls in a solution containing 75 mM NaCl [buffered with 200 mM MES (Na^+), pH 5.5, which itself has a low ionic strength and has been shown (Takeda & Fry, 2004; confirmed in the present work) to have no appreciable XAF activity]. The data show that CHP can solubilise XTHs from washed arabidopsis walls and that solubilisation causes these enzymes to acquire detectable XET enzymic activity.

BSA minimises binding of solubilised XTHs to tube walls

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

The XAF activity of cauliflower CHP specifically solubilises XTHs

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

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

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

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

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

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

The sugar composition of CHPs from diverse plants does not correlate with their XAF activities

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

XAF-active cauliflower CHP withstands all polysaccharide-digesting enzymes tested

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

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

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

Among the enzymes tested, only XEG showed some (moderate) ability to inactivate XAF; therefore we tested its effect, both before and after denaturation with formic acid, on three XAF-active polymers: cauliflower CHP, tamarind xyloglucan and gum arabic (Fig. 9c). Surprisingly, CHP, which again lost a proportion of its XAF activity when treated with XEG, was equally inactivated by acid-denatured XEG. The acid treatment completely abolished the XEG activity itself, as shown by the inability of denatured XEG to destroy the XAF activity of tamarind xyloglucan (Fig. 9c). The moderate XAF activity of gum arabic, reported in Fig. 6 and confirmed here, was unaffected by XEG (either native or denatured; Fig. 9c). We also confirmed that the XEG preparation did not release detectable mono- or oligosaccharides from gum arabic, whereas it completely digested xyloglucan (Fig. S3). In conclusion, the susceptibility of cauliflower CHP to enzymes differed substantially from tamarind xyloglucan's and gum arabic's.

The ability of XEG to reduce the XAF activity of CHP was re-confirmed in Fig. 9b, which shows by gel-permeation chromatography that XEG partially inactivates all XAF-active size classes of CHP. Again, the size distribution of XAF activity approximately agreed with that of xyloglucan (fractions 15–21), as shown by TLC of the XEG digestion products (Fig. 9d). The oligosaccharide profiles generated from CHP fractions 15–21 were typical of dicot vegetative tissue xyloglucan: they appeared to include XXXG, *O*-acetyl-XXFG (not resolved from XXLG and/or XLXG), XXFG, *O*-acetyl-XLFG and XLFG. XEG also yielded a trace of free glucose, especially from the K_{av}-0 material (fractions 9+10 in this experiment). The presence of contaminating β-glucosidase and α-amylase in the XEG preparation was shown by its ability to release glucose from both cellohexaose and maltohexaose during prolonged

incubations at a high enzyme concentration (333 μg ml^{-1}; Fig. 9e), and the presence of a trace of contaminating β-galactosidase was also demonstrated (Fig. S3b).

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

DISCUSSION

Functional characteristics of XAF

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

We show that $CaCl_2$ can augment the ability of CHP to solubilise XET activity from arabidopsis walls. The $CaCl_2$ and CHP effects are synergistic rather than additive (Fig. 1; Table S1), indicating that they have different modes of action; cauliflower CHP does not simply act as a non-specific polyanion, capable of breaking ionic bonds that hold XTHs in the

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

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

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

The relative XAF effect of CHP is stronger when acting on dilute cell-wall suspensions (<20 µg per 66 µl) than on higher wall concentrations (Fig. 4a). As a baseline, we note that pure water solubilised almost no XTH from washed arabidopsis cell walls, even at the highest concentration of walls (△ datapoint). Compared with this, the routine NaCl/MES medium alone solubilised large amounts of XTH from concentrated suspensions of cell walls (○ datapoints), but almost none from lower concentrations. The ○—○ curve resembles a titration: a likely explanation for this is that the NaCl initially solubilises a constant

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

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

The conclusion that the XAF activity of cauliflower CHP is due to a unique CHP–XTH interaction rather than to a general protein/salt/detergent effect is supported by the observation that CHP solubilises only XET activity (i.e., XTHs) rather than any of the other tested wall enzyme activities including β-glucosidase, peroxidase or phosphatase (Fig. 3). The latter three activities, like XTHs, are well established to be ionically bound within plant cell walls (Wei *et al.*, 2015; Jamet *et al.*, 2006; Minic *et al.*, 2007), and we confirmed that they are present in salt-extractable form in arabidopsis walls, albeit unaffected by CHP.

Sugar composition of XAF-active CHPs from diverse plants

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

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

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

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

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

Some CHP preparations, especially those from the Asparagales (asparagus, onion, snowdrop, crocus), have a high mannose residue content. Cold-water-extractable mannose-rich polymers include glucuronomannans (Kato *et al.*, 1977) and some galactomannans (Moreira & Filho, 2008), whereas most β-(1→4)-mannans tend to be inextractable in cold, neutral water. However, some highly XAF-active CHPs (e.g. from spinach and tobacco leaf CHPs) were almost devoid of mannose, so glucuronomannans etc. are unlikely to be the XAF active principle. Likewise, the levels of uronic acid, rhamnose, glucose and three unidentified sugar residues failed to correlate with XAF activity (Fig. 5, Fig. S2).

One approach that might characterise the elusive XAF-active polymers of cauliflower CHP is to fractionate them and look for a specific size-class of polymers exhibiting XAF activity. The results (Fig. 7) show that the active principle has a wide M_r range (~7,000–140,000) so it cannot be ascribed to any specific glycoprotein. The broad M_r range indicates polydispersity — e.g. XAF is a population of a polysaccharide or a glycoprotein with variation in its carbohydrate moieties. Furthermore, no specific building block (either TFA- or Driselase-released; Fig. 7c–f) shows a size distribution mimicking that of XAF activity (Fig. 7a) and that might thus be deemed necessary or sufficient for XAF activity.

Eleven authentic plant polymers exhibit little or no XAF activity

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

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

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

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

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

Stability of cauliflower XAF to acid and alkali

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

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

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

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

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

Stability of cauliflower XAF to seven specific polysaccharide hydrolases and Driselase

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

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

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

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

Conclusion

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

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

Funding

This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC) [grant number BB/D00134X/1] and the Vietnamese Government.

Acknowledgements

We thank Mr Tim Gregson for the HPLC work.

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Figure legends

Fig. 1. Cauliflower CHP and $CaCl_2$ synergistically solubilise XET activity from arabidopsis cell walls.

Washed arabidopsis cell walls were incubated for 30 min in 66 µl of buffer [0.18 M MES (Na^+), pH 5.5] containing various combinations of CHP and $CaCl_2$. After centrifugation, 20 µl of supernatant was incubated with 20 µl of XET reaction mixture also containing 0.5% BSA and the yield of [^{3}H]polysaccharide at 16 h was determined ±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^+, 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 µl) 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-µl solutions were then incubated in these containers for 5.5 h, permitting possible binding to the tube walls, after which 20 µl 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 ± 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 (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) β-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}H]polysaccharide formed by XET activity was measured. The deceleration of reaction rate in (c) was not reversed by additional H_2O_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. △= water (without CHP) used in place of NaCl/MES buffer. Data are mean of four determinations ± 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 ± range. The curve is fitted according to the equation for two superimposed hyperbolae,

$$\boldsymbol{y = [(V_{max1} \times x) / (K_{m1} + x)] + [(V_{max2} \times x) / (K_{m2} + x)],}$$

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

Fig. 5. Sugar residue composition of diverse CHPs.

(a) CHPs (100 µg) 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 ± 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 ±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 ±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 µl 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 α-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 ± 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 µg/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 ±range.

(c) Effect of XEG on three different XAF-active substrates. XEG (7.8 µg/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 ± 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 α-amylase, β-galactosidase and α-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, cellohexaose; M6, maltohexaose) were treated with XEG (83 µg/ml , PyAW 1:1:98, 24 h, as in panel a).

Caption for Supplementary Table

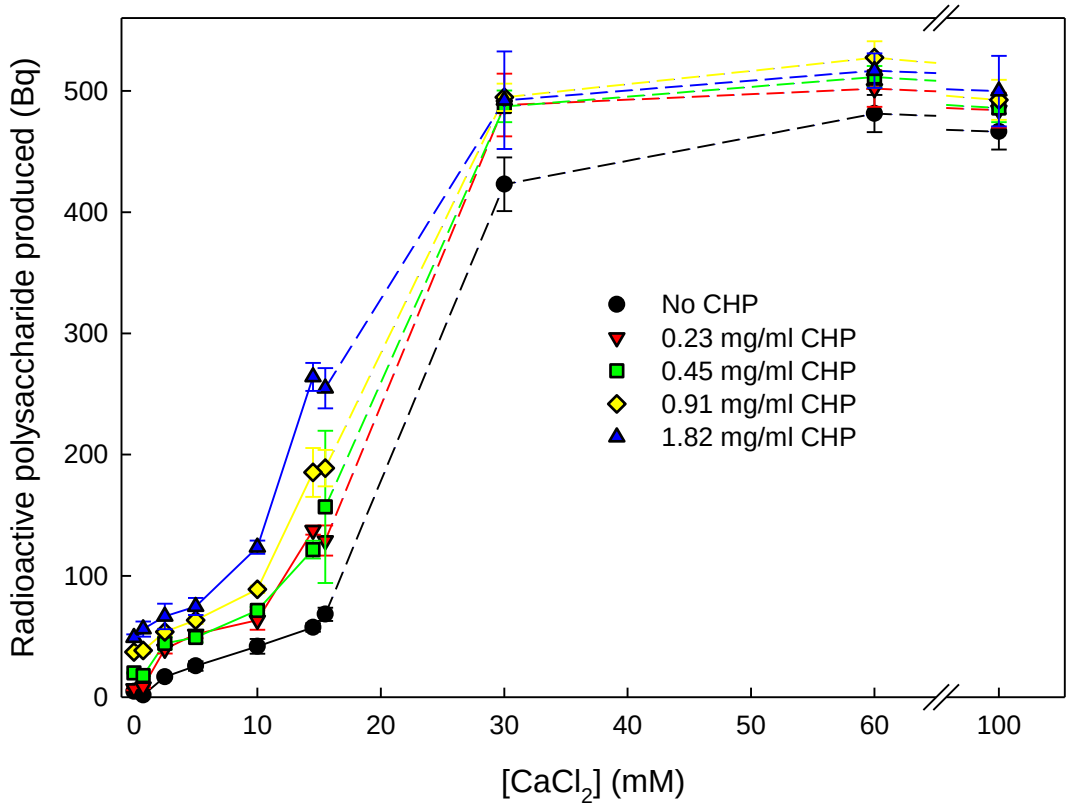
Table S1. Effect of $CaCl_2$ and cauliflower CHP on solubilisation of XET activity from arabidopsis cell walls

Captions for Supplementary Figures

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

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

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



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Washed arabidopsis cell walls were incubated for 30 min in 66 µl of buffer [0.18 M MES (Na^+), pH 5.5] containing various combinations of CHP and $CaCl_2$. After centrifugation, 20 µl of supernatant was incubated with 20 µl of XET reaction mixture also containing 0.5% BSA and the yield of [^{3}H]polysaccharide at 16 h was determined ±SE (n = 4). Data from two representative experiments (Expt 1, dashed lines; Expt 2, solid lines), covering different concentration ranges, are shown.

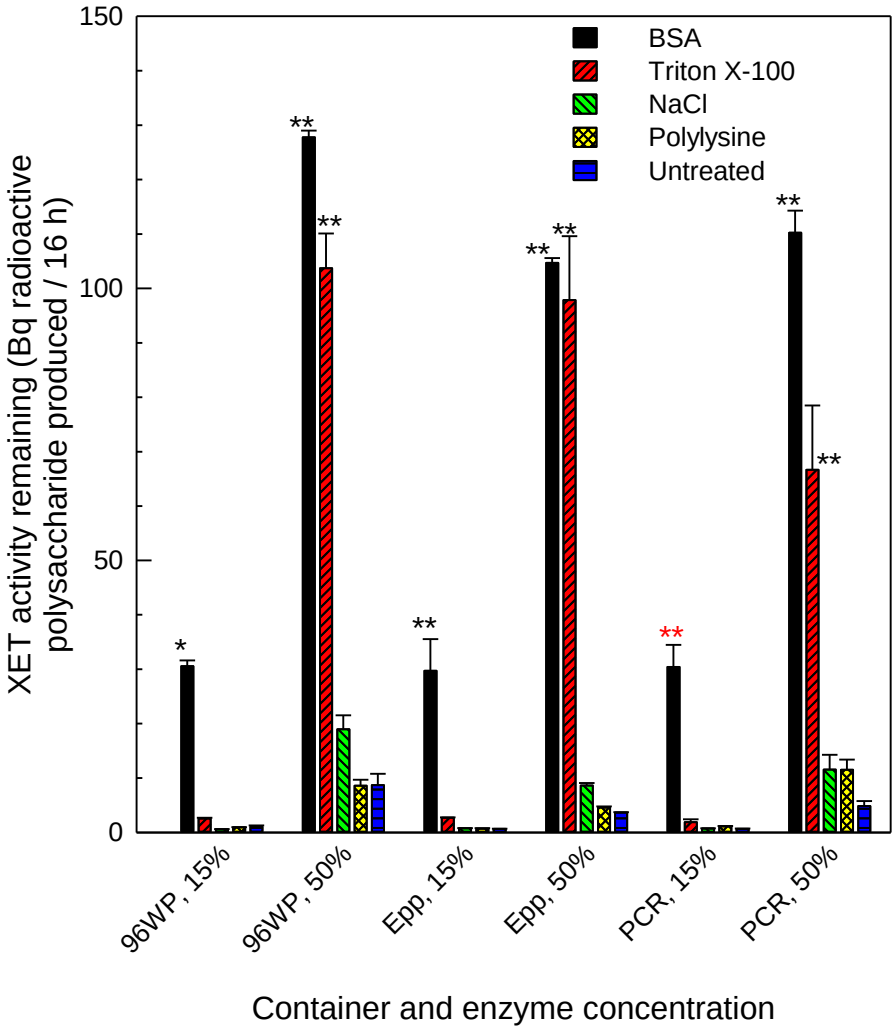

Fig. 2. Ability of various agents to prevent loss of solubilised XTHs due to binding to tube walls.

XTHs were solubilised from washed arabidopsis cells in 180 mM MES (Na^+, 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 µl) 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.

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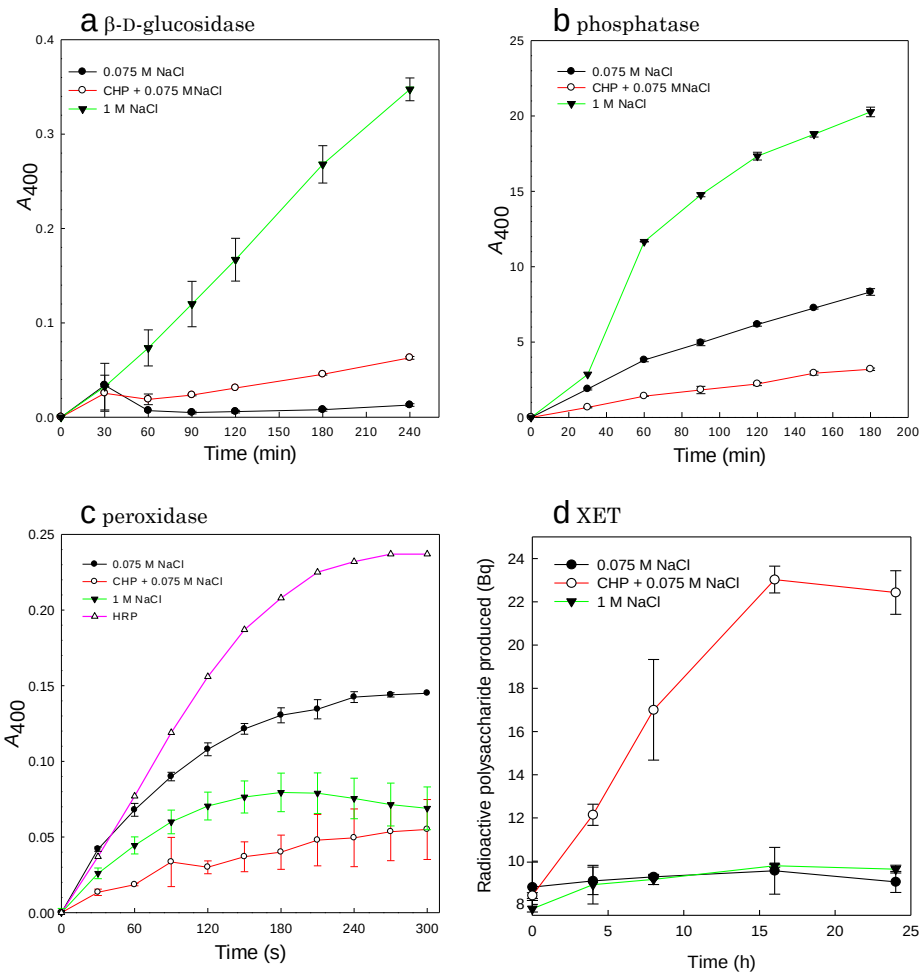
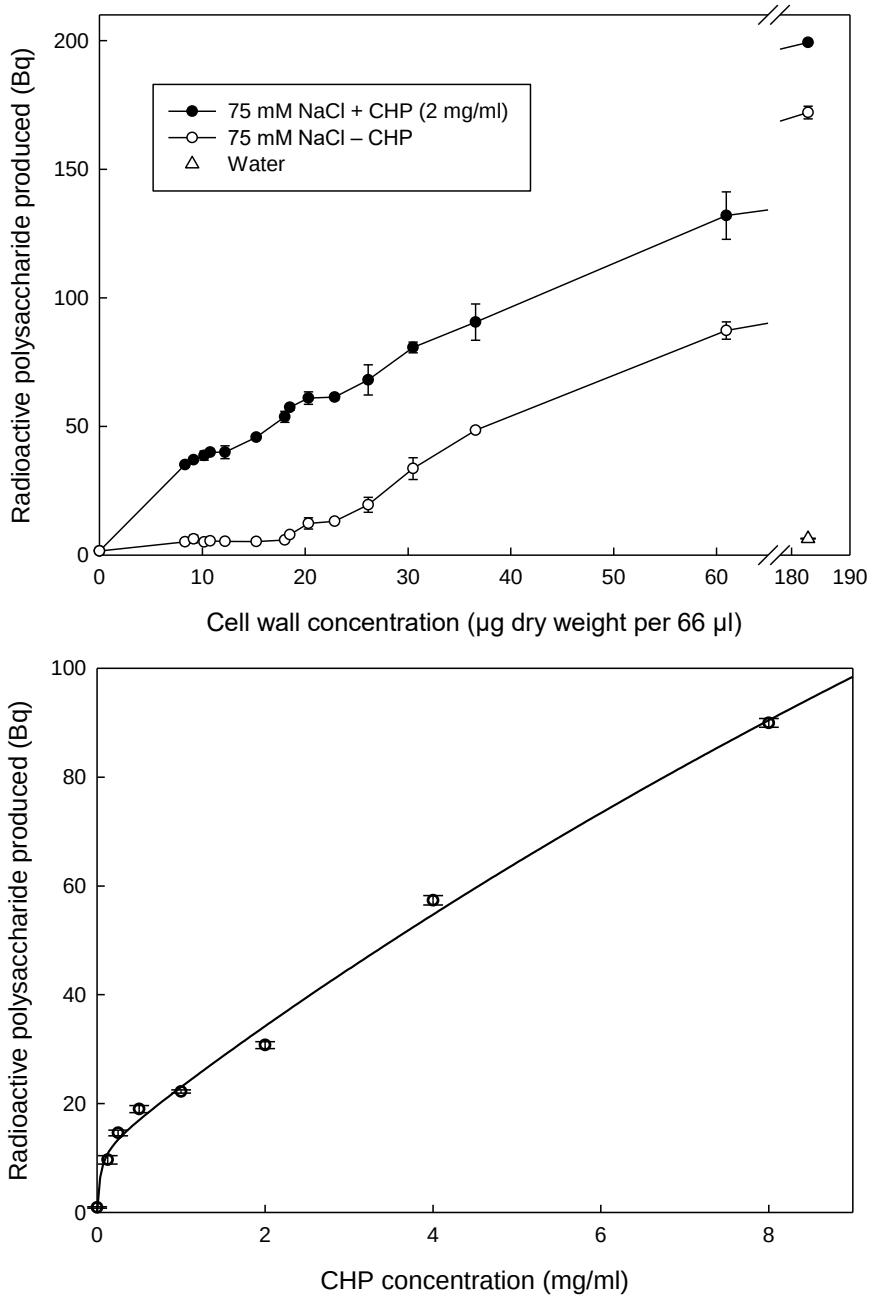


Fig. 3. Enzyme activities solubilised from arabidopsis cell walls by cauliflower CHP or high salt.

Washed *Arabidopsis* cell walls were incubated in 0.2 M MES (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) β-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}H]polysaccharide formed by XET activity was measured. The deceleration of reaction rate in (c) was not reversed by additional H_2O_2 (data not shown) and may indicate gradual denaturation of the solubilised peroxidase. Error bars represent SE (n = 4).



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(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. Δ = 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,

$$y = [(V_{max1} \times x) / (K_{m1} + x)] + [(V_{max2} \times x) / (K_{m2} + x)],$$

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

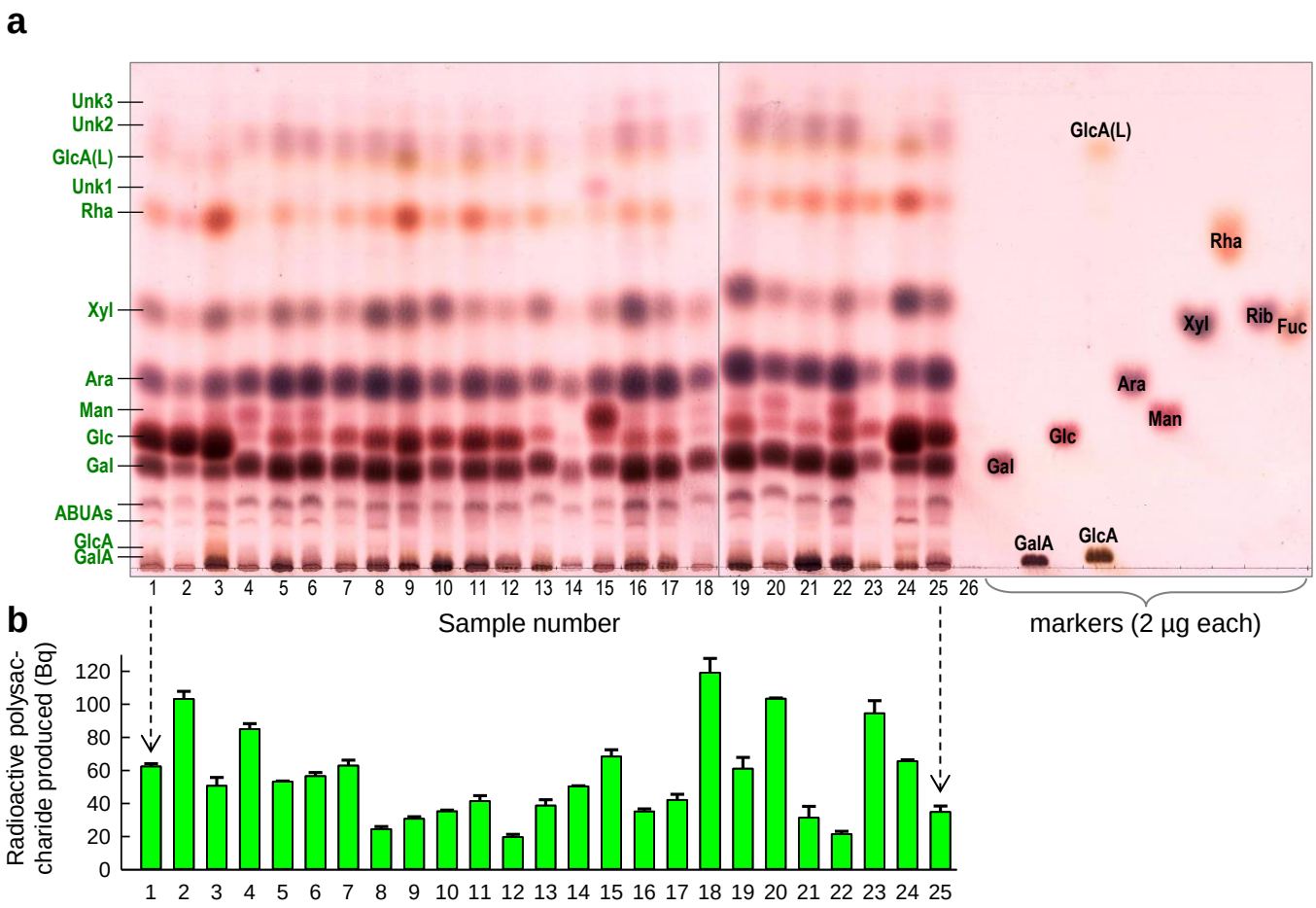

Fig. 5. Sugar residue composition of diverse CHPs.

(a) CHPs (100 µg) 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.

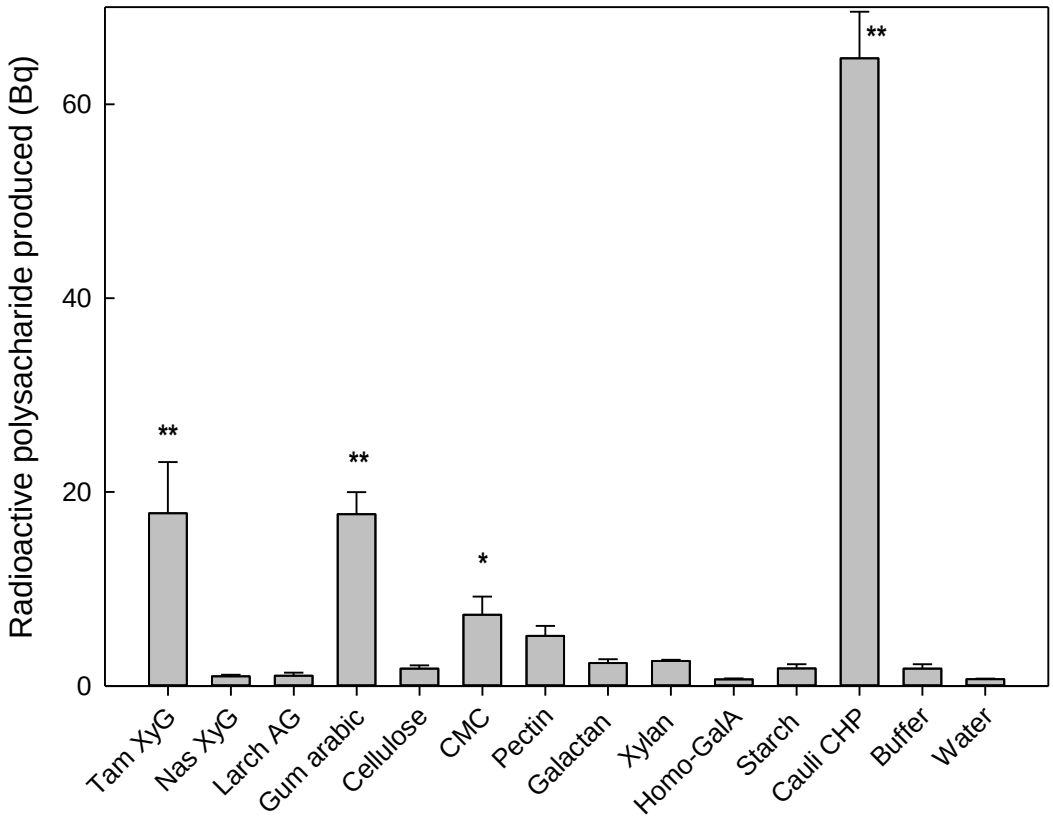

Fig. 6. XAF activity of cauliflower CHP and various commercial polysaccharides.

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 ± 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.

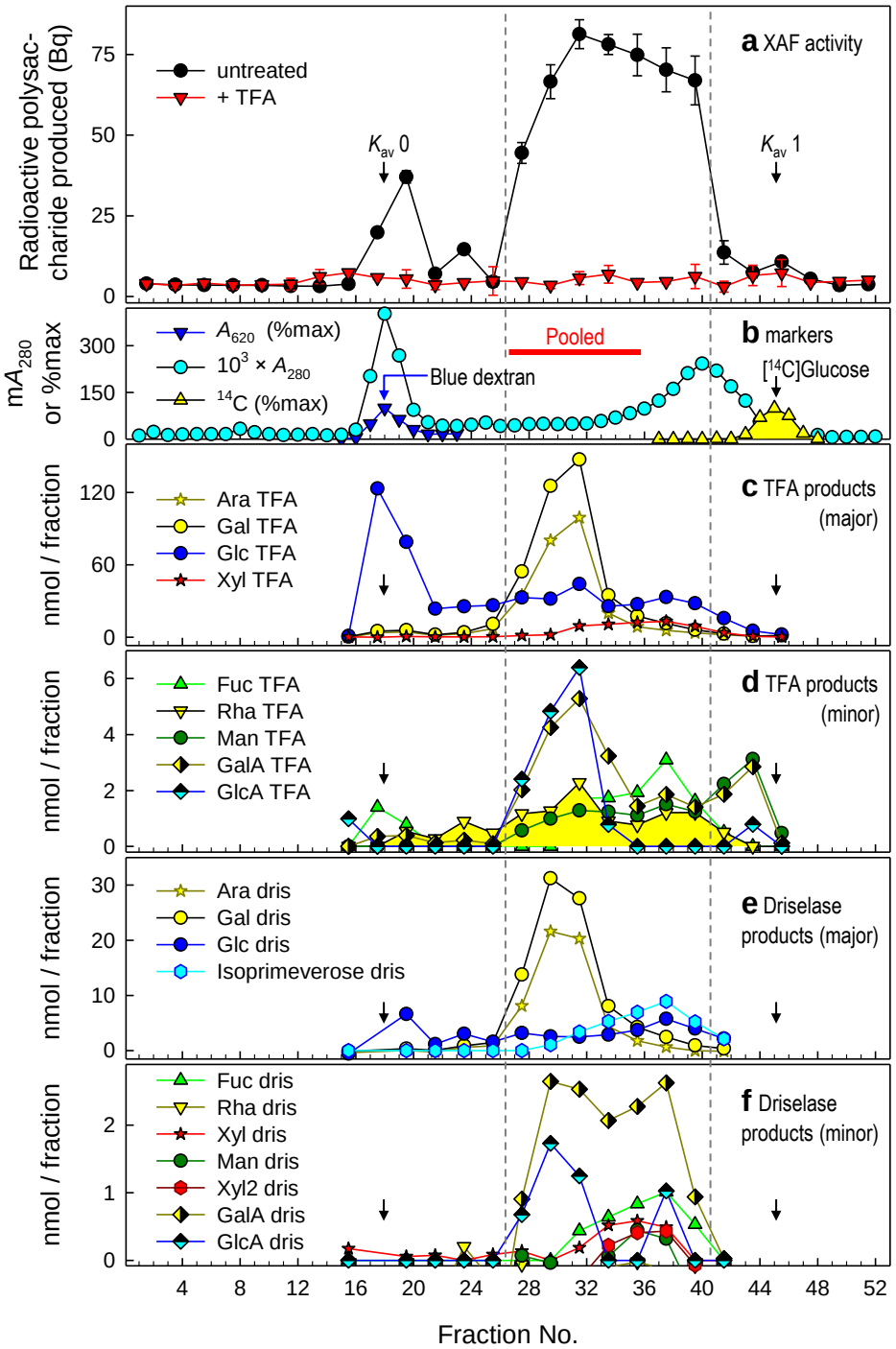

Fig. 7. Size fractionation and sugar residue composition of XAF-active cauliflower CHPs.
Cauliflower CHPs were passed through Sepharose CL-6B. For panels **a** and **c–f**, the fractions were paired and tested for XAF activity and sugar residue composition; for example, fractions 31 + 32 were pooled 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 ±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.

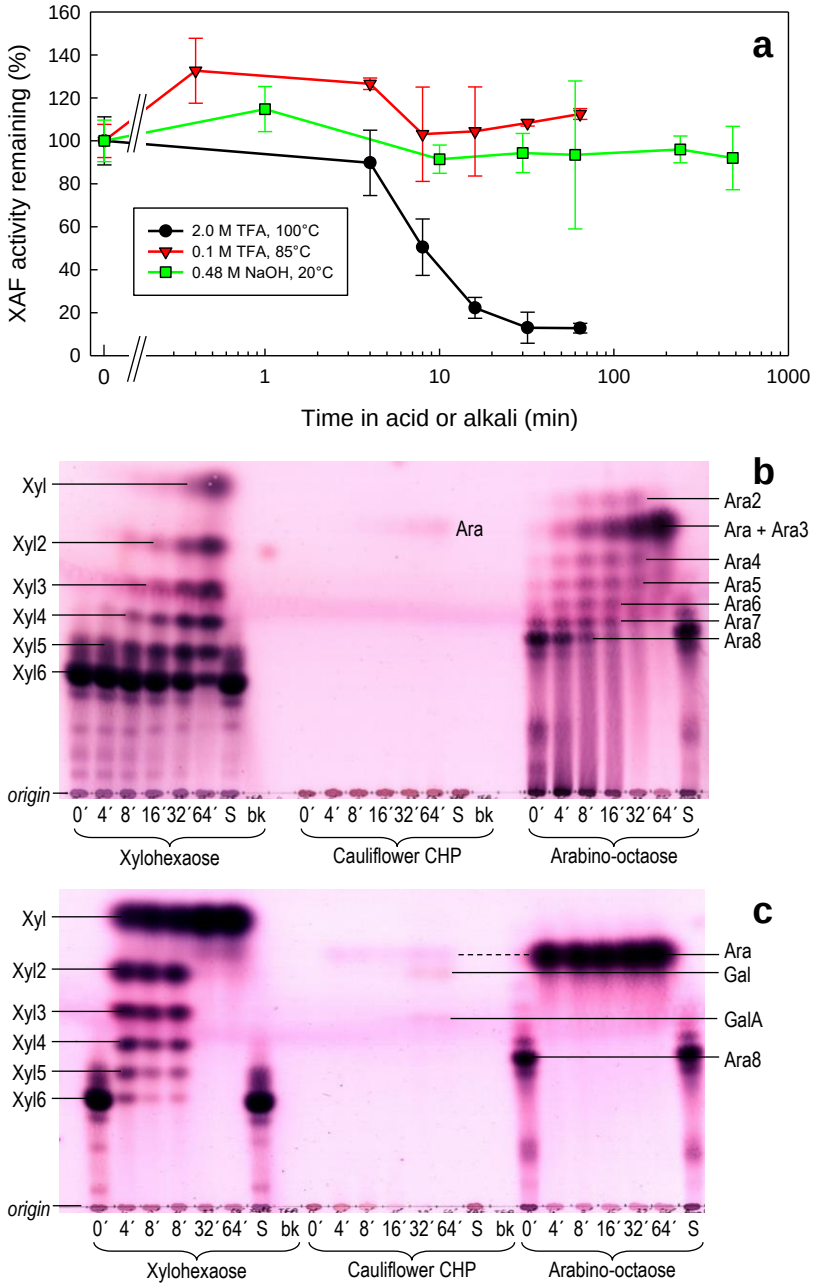

Fig. 8. Acid lability and alkali stability of XAF-active cauliflower CHPs.
(a) The relatively high-M_r, XAF-active fractions of cauliflower CHP eluting from Sepharose CL-6B (equivalent to the K_{av} 0.32–0.66 zone marked in Fig. 7b) were pooled, dried, treated with either 0.1 M TFA (85°C) or 2.0 M TFA (100°C) or 0.48 M NaOH (20°C), and then assayed for XAF activity. Data are mean of at least three determinations ±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.

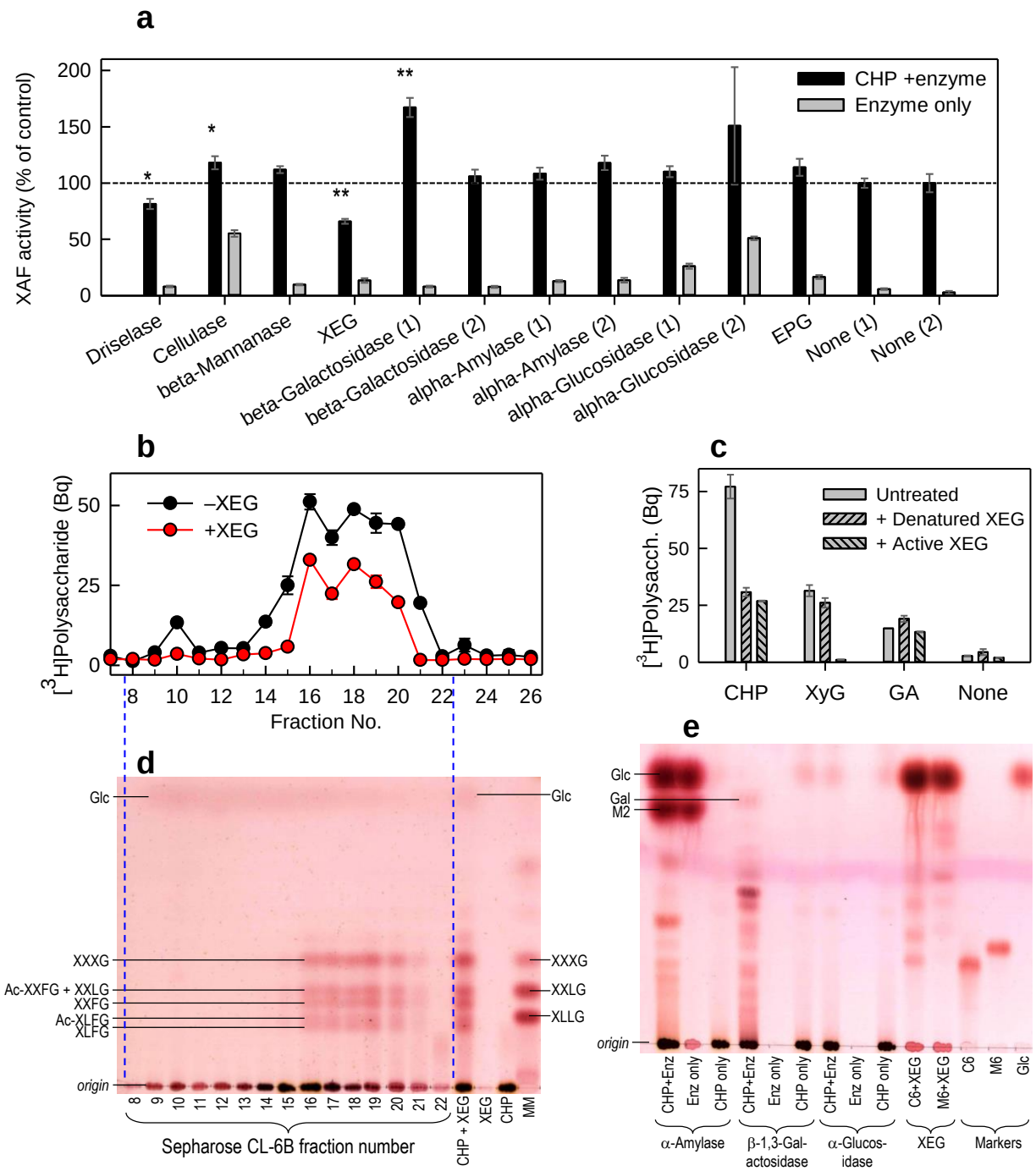

Fig. 9. Digestion of cauliflower CHP by various commercial enzymes.

(a) Effect of enzymes on XAF activity of CHP. Dried CHP (0.4 mg) was incubated with 300 µl 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 α-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 ± 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 µg/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 ±range.

(c) Effect of XEG on three different XAF-active substrates. XEG (7.8 µg/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 ± 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 α-amylase, β-galactosidase and α-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, cellohexaose; M6, maltohexaose) were treated with XEG (83 µg/ml , PyAW 1:1:98, 24 h, as in panel a).