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Research Paper

# Xyloglucan endotransglucosylase/hydrolases (XTHs) are inactivated by binding to glass and cellulosic surfaces, and released in active form by a heat-stable polymer from cauliflower florets

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ABSTRACT

Xyloglucan endotransglucosylase (XET) activity, which cuts and re-joins hemicellulose chains in the plant cell wall, contributing to wall assembly and growth regulation, is the major activity of XTH proteins. During purification, XTHs often lose XET activity which, however, is restored by treatment with certain cold-water-extractable, heat-stable polymers (CHPs), *e.g.* from cauliflower florets. It was not known whether the XTH-activating factor (XAF) present in CHPs works by promoting (*e.g.* allosterically) XET activity or by re-solubilising sequestered XTH proteins. We now show that XTHs in dilute solution bind to diverse surfaces (*e.g.* glass and cellulose), and that CHPs can re-solubilise the bound enzyme, re-activating it. Cell walls prepared from cauliflower florets, mung bean shoots and *Arabidopsis* cell-suspension cultures each contained endogenous, tightly bound, inactive XTHs, which were likewise rapidly solubilised (within 0.5 h) and thus activated by cauliflower XAF. We present a convenient quantitative assay for XAF acting on the native sequestered XTHs of *Arabidopsis* cell walls; using this assay, we show that CHPs from all plants tested possess XAF activity. The XAF activity of diverse CHPs does not correlate with their conductivity, showing that this activity is not a simple ionic effect. The XAF action of cauliflower CHPs was augmented by NaCl, although NaCl alone was much less effective than a CHP solution of similar conductivity, confirming that the cauliflower polymers did not simply exert a salt effect. We suggest that XAF is an endogenous regulator of XET action, modulating cell-wall loosening and/or assembly *in vivo*.

## 1. Introduction

In dicot primary cell walls, the major tension-bearing structure, limiting cell expansion, is widely proposed to include a xyloglucan–cellulose complex (Fry, 1989; Hayashi, 1989; McCann et al., 1990; Passioura and Fry, 1992; Fenwick et al., 1999; Park and Cosgrove, 2015). Enzymes of xyloglucan metabolism are therefore of interest to our understanding of the control of wall strength and extensibility. In particular, the transient cleavage and rearrangement of xyloglucan chains is achieved by GH16-family enzymes named xyloglucan endotransglucosylase/hydrolases (XTHs), which are ubiquitous in land plants. The major activity of most XTHs is xyloglucan endotransglucosylase (XET; EC 2.4.1.207; Baydoun and Fry, 1989; Smith and Fry, 1991; Farkaš et al., 1992; Fry et al., 1992; Nishitani and Tominaga, 1992), though a few exhibit predominantly xyloglucan *endo*-hydrolase activity (XEH; Shi et al., 2015). The XET reaction involves the covalent 'cutting and pasting' of xyloglucan chains, which has been shown by *in-vivo* density labelling to occur during — and probably contribute to the mechanism of — cell-wall assembly (Thompson et al., 1997) and loosening (Thompson and Fry, 2001). Roles for XET activity in growth control, both stimulatory and inhibitory, have been demonstrated multiple times (Osato et al., 2006; Miedes et al., 2013; Maris et al., 2009; Liu et al., 2007; Van Sandt et al., 2007).

Although there have been many studies of XTHs and their enzymic activities, little is known about how XTH action is regulated *in vivo*, which may be crucial for plant cell growth control. The ionic

*Abbreviations:* BCP, boiled cauliflower preparation (as defined by Takeda and Fry 2004); CHP, cold water-extractable heat-stable polymer; MES, morpholinoethanesulphonic acid; PL, polylysine; XAF, XTH activating factor; XET, xyloglucan endotransglucosylase (activity); XTH, xyloglucan endotransglucosylase/hydrolase (protein); XXXGol, $NaBH_4$-reduced heptasaccharide of xyloglucan ($xylose_3$·$glucose_3$·glucitol)

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environment of the enzyme within the apoplast may be one factor controlling the action of XTHs (Taleisnik et al., 2009; Han et al., 2013) and other wall enzymes (Goldberg et al., 1992; Almeida and Huber, 2007). Takeda and Fry (2004) reported the presence of an endogenous polymeric factor, extractable from cauliflower florets, that promotes the XET activity of a crude, de-salted cauliflower XTH preparation as well as heterologously produced XTH24. In the present paper, we refer to this factor as XAF (=XTH activating factor). The polymer was solubilised from the florets in cold water, and — unlike most proteins — it remained soluble after boiling; the preparation was therefore termed 'boiled cauliflower preparation' (BCP; Takeda and Fry, 2004). Although the active principle responsible for XAF activity was not identified, BCP preparations containing it were rich in arabinogalactan-proteins. In agreement with this, gum arabic (a crude arabinogalactan-protein preparation) exerted some XAF activity (Takeda and Fry, 2004). In contrast, cauliflower pectin and hemicellulose preparations had little effect. Therefore it was suspected that XAF was attributable to the arabinogalactan-protein content of BCP. Many inorganic and organic salts exhibited XAF activity, and the order of effectiveness of metal chlorides was trivalent > divalent > monovalent, suggesting an effect due to ionic strength. In addition, some but not all anionic polysaccharides exhibited high XAF activity — *e.g.* carboxymethylcellulose, pectin, gum arabic and hypochlorite-oxidised (thus anionic) xyloglucan, but not alginate, λ-carrageenan, homogalacturonan and methylglucuronoxylan (Takeda and Fry, 2004; Takeda et al., 2008). Thus, XAF activity was not simply a non-specific effect of any anionic polymers.

Takeda and Fry (2004) defined BCP as the total cold-water-extractable, heat-stable preparation from cauliflower florets. BCP, thus defined, contains numerous low-$M_r$ substances including both inorganics ($K^+$, $Ca^{2+}$, phosphate etc.) and organics (sugars, citrate, amino acids etc.), as well as the relatively small amount of cold-water-extractable polymers that remained soluble on heating (≈8% of the total BCP dry weight). About half the XAF activity present in BCP was attributable to these polymers, and their effect was much higher than would have been predicted from their ionic strength (assayed by conductivity) in comparison with inorganic salts. Thus, the BCP polymers exerted a high XAF activity that was not due simply to their ionic strength. In the present work, we have used only the polymeric fraction, and 'BCP polymers' in the terminology of Takeda and Fry (2004) are referred to here as CHPs (cold-water-extractable, heat-stable polymers).

Takeda and Fry (2004) did not determine whether the ability of the XAF, present in CHP, to restore lost XET activity was due to a promotion (*e.g.* allosteric) of the activity of the enzyme or to a re-solubilisation of enzyme that had been sequestered in some way. As a step towards characterising XAF, we have now distinguished between these possibilities. We investigated the ability of XTHs to bind to various artificial and natural surfaces — including cellulose and native cell walls — and the ability of CHP and/or NaCl to re-solubilise (and thereby re-activate) bound enzyme. In this manuscript we also present a convenient new assay for XAF activity on *Arabidopsis* cell walls that contain native bound XTHs.

## 2. Materials and methods

### 2.1. Materials

Heterologously expressed AtXTH24, produced in baculovirus-infected insect cells in Sf-900 II serum-free medium (Invitrogen, Carlsbad, California) as described by Campbell and Braam (1999), was kindly supplied by Dr Janet Braam (Rice University, TX, USA). The total protein concentration in the collected medium was 325 μg $ml^{-1}$, as estimated by the Bradford micro-assay (Bradford, 1976). *Tamarind* xyloglucan was a generous gift of Mr K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan. XXXGol was bought from Megazyme. [$^3$H]XXXGol was from EDIPOS (http://fry.bio.ed.ac.uk/edipos.html) and when used carrier-free had specific radioactivity approximately 100 MBq μmol$^{-1}$. It was routinely used as 0.5 or 1 kBq per assay. Other general chemicals were bought from Sigma.

### 2.2. Preparation of CHP

Cauliflower florets from a supermarket were vigorously homogenised in a blender (300 g in approximately 100 ml de-ionised $H_2O$). The homogenate was filtered through three layers of Miracloth and the filtrate was incubated at 100 °C for 1 h then filtered through Miracloth again. The filtrate (crude extract) was frozen, thawed, mixed well, and centrifuged at 4000 rpm for 30 min, then the clear supernatant was mixed with 2.3 vol of 96% ethanol. After a second centrifugation, the supernatant was discarded and the pellet was further washed with, sequentially, 80% and 96% (v/v) ethanol. The pellet was air-dried (yield: 1.8–3.0 mg dry polymer per g fresh weight cauliflower), re-dissolved in a minimum volume of de-ionised water, and freeze-dried. The dried pellet was re-dissolved in water or 0.2 M MES ($Na^+$), pH 5.5, at a concentration of 2 mg/ml (total dry weight of pellet per ml buffer), labelled CHP, and stored at −20 °C until use.

An identical procedure was applied to a selection of other plant materials (listed in Table 1). In the case of cell-suspension cultures, the whole culture (200 ml; 7–9 days old) was homogenised in the blender with no additional water. For determination of ionic strengths, dried CHPs were re-dissolved at 2 mg/ml in pure water and the conductivity was read with a Jenway 4060 conductivity meter.

### 2.3. Preparation of Arabidopsis cell walls containing native bound XTHs

*Arabidopsis thaliana* cell-suspension cultures were grown in the medium of May and Leaver, (1993) with 2% (w/v) glucose in place of sucrose. Cells were grown under continuous low-intensity fluorescent lighting (about 25 μmol $m^{-2}$ $s^{-1}$) in 500-ml conical flasks with constant shaking at 150 rpm. Sub-culturing was performed weekly by transfer of approximately 20 ml of old culture into 180 ml of fresh medium. A 200-ml portion of 7–9-d culture was homogenised for 2 min with a hand-held food mixer, then passed through Miracloth. The cells on the Miracloth were washed with 1 l of ice-cold water, squeezed semi-dry and frozen overnight at −20 °C. The cells were thawed and re-washed on Miracloth with another 1 l of ice-cold water. The cells were squeezed again and re-suspended in 100 ml de-ionised water. While the cell suspension was kept stirring, 1.5-ml aliquots were made and the aliquots were stored frozen at −20 °C for further use. Representative aliquots were freeze-dried for dry weight determination.

### 2.4. Radiochemical XET assay

Unless otherwise stated, the enzyme (in solution and/or bound to a glass or other surface) was supplied with a reaction mixture to give final concentrations of: 80–500 kBq/ml [$^3$H]XXXGol, 100 μM non-radioactive 'carrier' XXXGol (thus final specific radioactivity of acceptor substrate = 0.8–5.0 MBq/μmol), 0.3% tamarind xyloglucan, 0.25% w/v chlorobutanol and 100 mM MES ($Na^+$, pH 5.5). In some experiments, NaCl or other agents were also present. CHP, when present, was routinely at a final concentration of about 1.5 mg/ml. After incubation at room temperature for various times (1–24 h, depending on the aims of the experiment), a 20-μl aliquot was added to 20 μl 45% formic acid and dried on a 4 × 4 cm square of Whatman no. 3 paper (marked in pencil on a large sheet), which was then washed overnight in running tap-water and re-dried. The $^3$H-labelled polysaccharide product, remaining on the paper, was assayed by liquid scintillation counting (with 2 ml OptiScint scintillant per paper square; counting efficiency approx. 28%).