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Enzymically attaching oligosaccharide-linked 'cargoes' to cellulose and other commercial polysaccharides via stable covalent bonds

1   **Enzymically attaching oligosaccharide-linked 'cargoes' to cellulose and other commercial**

2   **polysaccharides via stable covalent bonds**

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25

29 **Abstract**

30 ~~Cellulose is a fundamental component of biomass and of many high-technology materials.~~ The

31 *Equisetum* enzyme hetero-trans-β-glucanase (HTG) covalently grafts native plant cellulose

32 (donor-substrate) to ~~(oligo)~~xyloglucan~~s~~ (acceptor-substrates), potentially offering a novel

33 'green' method of cellulose functionalisation. However, the range of cellulosic and non-

34 cellulosic donor substrates that can be utilised by ~~current ignorance of HTG's donor-~~

35 ~~substrate~~HTG is unknown, ~~range~~ limit~~ing~~s our insight into its biotechnological potential. Here

36 we show that HTG ~~was able to bond~~ binds all celluloses tested (papers, tissues, hydrogels,

37 bacterial cellulose) to radioactively- or fluorescently-labelled xyloglucan-heptasaccharide

38 (XXXGol; acceptor-substrate). Glycol-chitin, glycol-chitosan and chitosan also acted as donor

39 substrates, but less effectively than cellulose~~, donor-substrates~~. Cellulose-XXXGol conjugates

40 were formed throughout ~~a~~ the volume of a block of hydrogel~~'s volume~~, demonstrating

41 penetration. Plant-derived celluloses (cellulose Iβ) became more effective donor-substrates after

42 'mercerisation' in ≥ 3M NaOH; the opposite was true for bacterial cellulose Iα. Cellulose-

43 XXXGol bonds resisted boiling 6 M NaOH, demonstrating strong glycosidic bonding. In

44 conclusion, HTG stably grafts native and processed celluloses to xyloglucan-oligosaccharides,

45 which may carry valuable 'cargoes', exemplified by sulphorhodamine. We thus demonstrate

46 HTG's biotechnological potential to modify various cellulose-based substrates such as ~~cellulosic~~

47 textiles, pulps, papers, packaging, sanitary products and hydrogels.

48

49

1. Introduction

Plant cell walls are complex polysaccharide composites, crucial for plant function and survival [1]. Wall polysaccharides fall into three classes: cellulose, hemicelluloses and pectin [2],[3]. Cellulose molecules typically comprise 1,000–10,000 (1→4)-linked β-D-glucose residues and these unbranched chains, synthesised at the plasma membrane, aggregate into microfibrils [4]. Pectin is a complex anionic polysaccharide composed of four major interlinked domains, which are rich in galacturonic acid residues [5]. Major land-plant hemicelluloses possess a β-(1→4)-linked backbone of β-D-glucose and/or β-D-mannose or β-D-xylose residues. Some hemicelluloses carry side chains: for example, xyloglucan, the most abundant hemicellulose in most land-plant primary cell walls [1], carries (1→6)-linked α-D-xylose side-chains, some of which themselves also bear β-D-Gal or α-L-Fuc-(1→2)-β-D-Gal attached to *O*-2. One plant hemicellulose — mixed-linkage β-glucan (MLG) — has ~25–30% β-(1→3)-bonds interspersed with the β-(1→4)-bonds of cellotriose and cellotetraose sequences. These three polysaccharide classes are considered to form dense networks [6].

Plant cell walls also contain numerous enzymes whose substrate specificity suggests that they act to restructure the wall's polysaccharides [7]. Here we focus on transglucanases which belong to glycoside hydrolase family 16 (GH16) [8],[9],[10]. The most intensively studied GH16s are xyloglucan endotransglucosylase/hydrolases (XTHs) [11],[12], which possess one or both of two activities: xyloglucan endotransglucosylase (XET; EC 2.4.1.207) and xyloglucan endohydrolase (XEH; EC 3.2.1.151). XET activity cuts a xyloglucan chain (donor substrate) and grafts it onto the non-reducing end of a neighbouring xyloglucan (or oligosaccharide thereof; the acceptor substrate), thus bringing about a xyloglucan:xyloglucan homo-transglucanase reaction [13],[14]. Land-plant genomes typically encode more than 30 XTHs [15]. Other examples of homo-transglycanase activities reported in plant extracts are trans-β-xylanase [16] and trans-β-mannanase [17].

75   In addition to enzymes that catalyse homo-transglycosylation reactions, recent studies

76   report that certain transglucanases can cleave cellulose chains [18],[19], soluble cellulose

77   derivatives [20],[21], or MLG, and subsequently attach them via glycosidic bonds to the

78   xyloglucan acceptor substrate, creating hybrid products e.g. cellulose–xyloglucan or MLG–

79   xyloglucan. Since the donor differs qualitatively from the acceptor, such reactions are termed

80   hetero-transglycosylation; they have considerable biotechnological potential, giving a means of

81   covalently attaching commercially significant polysaccharides (cellulose or MLG) to xyloglucan

82   or its oligosaccharides. The latter can, in principle, carry valuable 'cargoes' which thereby

83   become permanently attached to the cellulose or MLG.

84   In most reports of "cellulose" heterotransglucosylation, the substrate tested was an

85   artificial, soluble, cellulose derivative (e.g. cellulose acetate, sulphocellulose,

86   hydroxyethylcellulose or phosphoric acid-treated amorphous cellulose [19],[22]. In contrast, a

87   heterotransglucanase that acts on unmodified cellulose, a major industrial raw material and the

88   world's most abundant organic substance, could have great commercial impact. The only

89   hetero-transglucanase known to exhibit cellulose:xyloglucan endotransglucosylase (CXE)

90   activity on native insoluble plant cellulose (cotton-sourced filter paper) is hetero-trans-β-

91   glucanase (HTG), a highly acidic transglucanase discovered recently in the early-diverging

92   'fern' genus *Equisetum* [18]. All other tested acidic transglucanases from *Equisetum* are XTHs

93   with very predominantly homo-transglucanase activity [23]. Besides CXE activity, *Ef*HTG

94   catalyses two further transglucosylation reactions at high rates — MLG:xyloglucan

95   endotransglucosylase (MXE) and ~~'classical'~~ordinary XET activity [18],[24],[25].

96   The aim of the present study was to provide the first thorough investigation of the

97   influence of the cellulose type and origin on hetero-transglucosylation by HTG. We also further

98   explored the range of donor substrates tolerated by HTG, and we tested the chemical robustness

99   of the cellulose–xyloglucan bonds formed by this unique enzyme.

100

2. Experimental

2.1. Plant sources and materials

Equisetum fluviatile was obtained from a pond outside the Institute of Molecular Plant Sciences at the University of Edinburgh or from the Pentland Hills (Edinburgh, UK). Polysaccharides purchased from Sigma-Aldrich were as follows: chitin (from shrimp shells; C7170), chitosan 1 (low M_r, 75–85% deacetylated; 448869), chitosan 2 (medium M_r, 75–85% deacetylated; 448877), chitosan 3 (high M_r, > 75% deacetylated; 419419) chitosan 4 (from crab shells, ~80% deacetylated; C0792), chitosan 5 (from shrimp shells, ≥ 75% deacetylated; C3646), glycol chitosan (DP > 400, ≧ 60% deacetylated; G7753), fucoidan (from *Fucus Vesiculosus*, F5631), alginate (from brown macroalgae; W201502), λ-carrageenan (from *Gigartina aciculaire* and *G. pistillata*; C3889,) laminarin (M_r ~4500; L9634), pectin (from Citrus fruit, ≥ 85% esterified; P9561), homogalacturonan (polygalacturonic acid, from citrus fruit, 85-90%; P7276), arabinogalactan (from larch wood; 10830), xylan (from birchwood, M_r 98,066, 92% soluble, 88% xylose, 10% hexuronic acids, < 10% arabinose; X0502), xylan (from oat spelts, ~90% xylose, < 10% arabinose; 95590), 4-*O*-methylglucuronoxylan (from *Fagus sylvatica,* M-5144; 96% soluble, 13% hexuronic acids), α-mannan (from <u>*Saccharomyces cerevisiae*</u>; M7504) and microcrystalline cellulose (M_r ~29,000; 11365). Polysaccharides from Megazyme were: wheat arabinoxylan (Ara:Xyl ratio 38:62, M_r 192,000; P-WAXYM), arabinan (from sugar beet pulp; Ara: Gal: Rha: GalA = 88: 3: 2: 7; P-ARAB), linear arabinan (debranched, Ara: GalA: Rha: GalA = 97.5:0.4:0.1:2, M_r 18,000; P-DBAR), arabinogalactan (from larch wood, Gal: Ara: Other sugars = 81: 14: 5, M_r 47,000; P-ARGAL), galactan (from potato, Gal: Ara: Rha: GalA = 87: 3: 4: 6; P-GALPOT), arabinoxylan medium viscosity (from wheat; M_r 323,000; P-WAXYM), lichenan (MLG from Iceland moss, 1,4:1,3-β-D linkage ratio 2:1; P-LICHN), MLG low viscosity (from barley, M_r 179,000, 1,4:1,3-β-D linkage ratio 3:1; P-BGBL), MLG medium viscosity (from barley, M_r 251,000; P-BGBM) and high viscosity (from barley, M_r 495,000; P-BGBM), rhamnogalacturonan I (from potato fiber; GalA: Rhamnose: Arabinose: Xylose:

127 Galactose: Other Sugars = 61.0: 6.2: 2.5: 0.5: 23.1: 6.7; P-RHAM1), β-mannan (from Carob

128 seed, sodium borohydride-reduced, Man: Gal = 97: 3), medium viscosity galactomannan (from

129 guar, Gal: Man = 38: 62, M_r 380,000 P-GGMMV) and glucomannan low viscosity ('KGM',

130 from konjac, Man: Glu ratio 60: 40, M_r 950,000; P-GLCML). *Tamarindus indica* seed

131 xyloglucan (M_r ~2×10^6) was donated by Dainippon Pharmaceutical Co. (Osaka, Japan).

132 Cellulose Iβ donor substrates were obtained from various sources (Table S1). [^{3}H]XXXGol

133 (standard xyloglucan-oligosaccharide nomenclature [26]), XXXGol–sulphorhodamine

134 (XXXGol–SR; Fig. 1a), prepared as previously described [27],[28], and XXXGol–SR-

135 impregnated papers were from EDIPOS (http://fry.bio.ed.ac.uk//edipos.html).

136

2.2. Preparation of bacterial cellulose Iα

138 Culturing *Komagataeibacter xylinus* (traditionally known as *Acetobacter xylinum* or

139 *Gluconacetobacter xylinus*) for bacterial cellulose production was as previously described [29].

140 Briefly, the bacteria were grown on solid Hestrin–Schramm (HS) agar medium for 7 d at 30°C

141 and a colony was transferred into 6 ml of fresh HS liquid medium and incubated for 7 d more.

142 Next, 0.5 ml was inoculated into 4.5 ml of fresh liquid medium and incubated 3 d, and 1.5-cm-

143 diameter well-plates were filled with 2 ml of 1:14 inoculum:HS fresh medium and incubated for

144 another 3 d. A pellicle of bacterial cellulose was harvested from the surface of each well, soaked

145 for 10 min in 50% ethanol, boiled in water (2×20 min) and immersed in 0.1 M NaOH at 90°C

146 (2×20 min), which removes organic residues but does not appreciably convert cellulose I to II.

147 Finally, the films were neutralised by water-washing.

148

2.3. Production of glycol chitin from glycol chitosan

150 Glycol chitosan was dissolved in water (0.5%, w/v) at 100°C for 20 min. After cooling to 20°C,

151 100 μl of collidine/acetic anhydride (2:1) was added to 19.9 ml glycol chitosan solution and

152 incubated on a wheel for 1 d. Then, 4 ml of 1 M NaOH was added, resulting in a pH of ~12.6.

153 After 6 h ~~rotation~~on a rotating wheel, the pH was adjusted to 5.5 with acetic acid. A 20-μl

154 portion of the products and 20 μl of untreated glycol chitosan solution (0.16% and 0.46%, w/v)

155 were loaded as spots onto Whatman No. 3 paper; the papers where washed in 70% (v/v) ethanol,

156 dipped through 0.2% (w/v) ninhydrin in acetic acid/acetone (0.5:99.5) and heated at 105°C for

157 30 min, which stains amino groups.

158

2.4. Preparation of cellulose hydrogels

160 Cellulose hydrogels were prepared as previously described [30]. Microcrystalline cellulose was

161 mixed with water and swelled for 2 h at 5°C. Aqueous NaOH was precooled to −6°C, then the

162 swelled cellulose was added (final concentrations 5% w/v in 2 M NaOH) and the mixture was

163 stirred at 1000 rpm at −6°C for 2 h. The solution was poured into cylindrical moulds (3 cm

164 diameter, 0.5 cm height) and kept for 2 h at 50°C; in these conditions cellulose gels irreversibly

165 [31]. Disc-shaped gels were placed in water, which was regularly renewed over several day until

166 a neutral pH was obtained.

167

2.5. Heterologous protein production and *Equisetum* enzyme extraction

169 Production of *Ef*HTG in *Pichia pastoris* strain SMD1168H was as described before [18].

170 Extraction of native enzyme from *Equisetum* followed a previous protocol [24]. In brief, ~100 g

171 freshly harvested tissue was ground in 500 ml ice-cold extraction buffer [0.3 M succinate (Na$^+$,

172 pH 5.5) containing 3% (w/v) polyvinylpolypyrrolidone] and after centrifugation (10,000 g, 45

173 min) the supernatant was stored at −80°C.

174

2.6. Assaying radioactivity

176 ^{3}H in aqueous solutions and ^{3}H bound to various insoluble cellulosic substrates was quantified

177 by scintillation counting in ScintiSafe 3 scintillation cocktail (Fisher Scientific, Loughborough,

178 UK) or in GoldStar 'O' scintillation cocktail (Meridian, Chesterfield, UK) respectively.

2.7. Radiochemical assay of various transglycanase activities with non-cellulosic donor substrates

Two partially overlapping experiments (a and b) were conducted. In both cases the reaction mixture (final volume 20 μl) contained ~~5 μl~~25% (v/v) ~~of~~ filtrate from *Pichia* cultures expressing *Ef*HTG, 0.1 M succinate (Na$^+$, pH 5.5), 0.1% (w/v) bovine serum albumin (BSA), 1.0 or 2.0 kBq acceptor substrate ([^{3}H]XXXGol) and 0.5% (w/v) donor substrate (xyloglucan or MLG for XET or MXE activity respectively; or alternative polysaccharides for surveying possible novel hetero-transglycanase activities). Mixtures were incubated for 24 ~~h~~ or 48 h at 20°C, during which period the reactions are approximately linear, and the enzyme was then denatured by addition of 6 μl of 90% formic acid. XET and MXE products were then dried on Whatman No. 3 paper, to which they hydrogen-bond, washed in running tap-water overnight, and quantified for radiolabelled high-M_r products by scintillation-counting. In experiment (a), the products (if any) formed from polysaccharides other than xyloglucan and MLG were washed in 72% ethanol until the supernatant was non-radioactive (removal of free [^{3}H]XXXGol). ^{3}H left in the pellets was quantified by scintillation-counting. In experiment (b), any ^{3}H-labelled polymeric products formed in heterotransglycanase reactions were quantified by a glass fibre blotting method followed by scintillation-counting [32] (Fig 3 a and b respectively).

2.8. Radiochemical assay of heterotransglucanase activity with cellulose as donor (CXE activity)

For CXE assays, various cellulose samples were tested after water-washing and drying ('untreated', cellulose I) or after alkali-pretreatment ('mercerisation', cellulose II). For the latter, cellulosic materials including bacterial cellulose were routinely incubated in 6 M NaOH at 20°C for 4 h, washed 4× in copious tap water, then washed in 5% (v/v) acetic acid for 0.5 h, followed by washing in running tap water for 16 h and dried. This converts cellulose I to cellulose II

205 [33],[34]. *Equisetum* cellulose was prepared from an alcohol-insoluble residue [35] by removal

206 of hemicelluloses with 6 M NaOH (37°C, 3×1 day), and was thus only available as cellulose II.

207 Each type of cellulose (20–29 mg) was soaked with 20 µl reaction mixture (as described in the

208 previous paragraph) with the addition of 0.1 or 1.0% BSA but lacking any deliberately added

209 donor substrate other than the cellulose. For cellulosic paper from print products (e.g.

210 ~~books~~newspapers), 20-mg pieces lacking ink or colour were used. Controls contained heat-

211 inactivated enzymes and these values were subtracted from the non-mock groups, thus

212 correcting values for any possible unspecific [^{3}H]XXXGol binding.

213   After the CXE reaction had been stopped (in the linear range) with 6 µl of 90% formic

214 acid, cellulose pieces were washed in 6 M NaOH for 12 h at 20°C, 6 M NaOH for 0.5 h at

215 100°C, and then in running tap-water overnight, dried and assayed for bound ^{3}H. Besides

216 washing out all unreacted [^{3}H]XXXGol, this alkali treatment would solubilise any XET- or

217 MXE-products potentially formed from endogenous hemicelluloses present in crude *Equisetum*

218 enzyme preparations. Some of the cellulosic substrates were water-washed (freed of unreacted

219 [^{3}H]XXXGol) in dialysis tubes, preventing loss of material due to fibre disintegration.

220

2.9. Fluorescent dot-blot assay of heterotransglucanase activity with cellulose as donor

(CXE activity)

223 Two methods of attaching XXXGol–SR (acceptor substrate) to cellulose were compared. Native

224 *Equisetum fluviatile* enzyme (precipitated in 30% saturated ammonium sulphate and redissolved

225 at various concentrations in 0.2 M succinate buffer containing 1% BSA) was either (a) pre-

226 mixed with XXXGol–SR and then pipetted onto dry filter paper or (b) pipetted directly on~-~to dry

227 papers that had previously been impregnated with XXXGol–SR at ~1 µmol/m^2 such that 4 µl of

228 aqueous solution (giving a ~9-mm diameter spot) would lead to an XXXGol–SR concentration

229 of ~20 µM. In both cases, the paper had been marked out in 96-well format and either pretreated

230 in 6 M NaOH, or not, before application of the enzyme and/or acceptor substrate. ~~In both cases,~~

231 tThe volume of solution applied to each 'well' position on the paper was 4 μl. After 12 h

232 enzymic reaction under humid conditions (with the paper tightly pressed between two cellulose

233 acetate stationery sheets), the remaining unreacted XXXGol–SR was washed out with formic

234 acid/ethanol/water (1:1:1) for 1 h, and the paper was re-dried. Covalently bound fluorescent

235 products were photographed under 254-nm UV and then the fluorescence intensity of the spots

236 wereas quantified in ImageJ. In a test of the stability of the cargo's attachment to the paper, each

237 paper was then bathed in 6 M NaOH for 12 h, neutralised by briefly rinsinged in water, dried,

238 and re-photographed under UV, and the fluorescence intensity of the spot was again quantified

239 as beforeabove.

240

2.10. Determining the minimal NaOH pretreatment required to produce cellulose

optimised as a CXE substrate

243 Whatman No. 1 paper (with a pre-printed grid in 96-well-plate format) was pretreated with 0 – 6

244 M NaOH at 20°C for various times, then washed with water, slightly acidified with 1% acetic

245 acid, and re-washed in running tap-water for 6 h followed by deionised water, and air-dried. The

246 alkali-pretreated papers were tested as substrates for native HTG (CXE activity) obtained from

247 *Equisetum fluviatile* plants.

248

2.11. Testing the alkali-stability of HTG-generated MXE, XET and CXE products

250 Transglucanase reactions were performed with *Pichia*-produced *Ef*HTG as above, with 6 M

251 NaOH-pretreated Whatman No. 1 filter paper (for CXE), MLG (for MXE activity) or

252 xyloglucan (for XET) as donor substrate, and [^{3}H]XXXGol as acceptor. We then tested the

253 alkali-stability of the three radiolabelled products obtained (cellulose–[^{3}H]XXXGol, MLG–

254 [^{3}H]XXXGol and xyloglucan–[^{3}H]XXXGol; washed rid of unreacted [^{3}H]XXXGol with water

255 or 75% ethanol for cellulose and the hemicelluloses respectively). For the CXE product, 0.25-

256 mg pieces (each ~1 kBq) of the cellulose–[^{3}H]XXXGol were incubated at 100°C for 1 h in 100

257 μl of 0–6 M NaOH, then cooled and diluted to 1 ml with water. The solution was centrifuged

258 (12,000 g for 2 min) and the supernatant was slightly acidified with acetic acid, then assayed for

259 ^{3}H; the pelleted paper was suspended in 2 ml of 5% acetic acid, and likewise assayed for ^{3}H.

260 The proportion of the total ^{3}H (from cellulose–[^{3}H]XXXGol) that remained water-insoluble was

261 thereby calculated. The water-soluble XET and MXE products (1 kBq in ~4-μl) were mixed

262 with 100 μl of 0–6 M NaOH, and incubated at 100°C for 1 h. After cooling, 100 μl of 50%

263 acetic acid was added and 100 μl of the acidified solution was dried onto a 6×4-cm piece of

264 Whatman No. 3 paper, which was then washed in running tap-water for 72 h, dried and assayed

265 for hydrogen-bonded [^{3}H]hemicellulose.

266 The free acceptor substrate was also incubated in various concentrations of NaOH at

267 100°C for 1 h, then slightly acidified with acetic acid, dried, redissolved in water and assayed

268 for remaining non-volatile (heat-stable) ^{3}H. Additional samples of [^{3}H]XXXGol that had been

269 treated in 0 and 6 M NaOH were analysed by paper chromatography on Whatman No. 1 in ethyl

270 acetate/acetic acid/water (10:5:6) for 40 h. The chromatogram was cut into strips, which were

271 assayed for ^{3}H.

272

2.12. CXE assays on cellulose hydrogels

274 Blocks of hydrogel (20 mm^3) were equilibrated in 200 mM succinate (Na$^+$; pH 5.5), then soaked

275 with 30 μl reaction mixture [10 μl native *Equisetum* protein extract or *Pichia*-produced *Ef*HTG,

276 with additives to give a final composition of 133 mM succinate buffer (Na$^+$; pH 5.5) containing

277 1 kBq [^{3}H]XXXGol, 1.0% BSA and 0.5% chlorobutanol] and incubated for two weeks at 20°C

278 (linear reaction range). After the reaction had been stopped, the hydrogel was washed 3× in

279 water, then heated in 2 M TFA at 120°C for 1 h. The ^{3}H in the TFA supernatant and pelleted

280 cellulose were separately quantified by scintillation counting.

281 To visualise whether *Pichia*-produced *Ef*HTG can form CXE products inside cellulose

282 hydrogel blocks, we used a 20-mm^3 piece of buffer-equilibrated gel (2×2×5 mm) to which was

283 added 10 µl of enzyme solution plus 10 µl of 7.2 µM XXXGol–SR. Thus, if uniformly

284 distributed within the gel, the XXXGol–SR concentration was 1.6 µM. Controls lacked the

285 enzyme. After the reaction had been stopped at 72 h, the gel blocks were rinsed for 3 days in

286 water, then sectioned with a razor blade (giving a slice ~2×5×0.6 mm) and examined with a

287 Leica DM2000 LED microscope equipped with a Leica DFC7000 T camera and Leica EL6000

288 external light source. Incorporated SR was visualised with a GFP filter cube (excitation band

289 pass (BP) 470/40 nm, emission BP 525/50 nm) using LAS X software. Minimal adjustments to

290 contrast were applied equally across entire image plates.

291

3. Results

*3.1. Ef*HTG acts on a wide range of cellulosic donor substrates derived from plants and bacteria

So far, *Ef*HTG's CXE activity had only been tested on cotton cellulose Iβ (Whatman No. 1 filter paper) [18],[36]. To explore whether *Ef*HTG can act on other forms of cellulose, we tested a variety of papers and other commercial cellulosic products, which had undergone different industrial treatments (Table S1). Each cellulose sample was used with and without a pretreatment in 6 M NaOH. Exposure to NaOH turns the cellulose I crystal structure to Na-cellulose; water washing converts it to cellulose II hydrate and the following drying yields cellulose II [37]. NaOH treatment and conversion to cellulose II may cause a partial cellulose depolymerisation by oxidation and subsequent β-alkoxy-elimination [38],[39].

*Ef*HTG acted on most cellulose samples tested, but at different rates (Fig. 1b). Of plant-derived cellulose samples that were not pretreated with alkali, the most effective donor substrates were less-processed papers (paper napkins, handkerchieves, toilet paper, *The Sun* newspaper, a glossy brochure and Whatman filter papers), whereas the least effective included cotton wool, cotton fabric (lab-coat), absorbent sanitary products, and certain papers (*The Guardian* newspaper, lens-cleaning tissue, printer paper, thin brochure paper and lab-book paper).

The highly modified cellulose derivative, nitrocellulose, was not an effective donor substrate for HTG, as previously reported for other cellulose derivatives such as carboxymethylcellulose [24] and water-soluble cellulose acetate [18].

All substrates based on plant-derived, unsubstituted cellulose supported higher CXE activities after pretreatment with 6 M NaOH (and thus presumably converted to the cellulose II allomorph; Fig. 1b). There was no simple relationship between the substrate effectiveness after alkali-pretreatment and that observed without NaOH pretreatment. Therefore, there was wide variation in fold-stimulation by alkali pretreatment: for example, the glossy brochure paper was

13/35

318 enhanced only 1.8-fold, whereas Whatman No. 1 filter paper was enhanced 13.5-fold, and some

319 samples (e.g. printer paper and cotton wool) were enhanced over 60-fold.

320   Only bacterial cellulose Iα, which differs conformationally from the cellulose Iβ

321 predominating in plants [40], served as a better substrate in its native form than after

322 pretreatment with 6 M NaOH for 4 h. Although 6 M NaOH converts bacterial cellulose Iα to

323 cellulose II within 3 min, complete mercerisation takes longer than for Iβ as the interdigitation

324 between microfibrils is different and the crystallinity is higher than in plant-derived cellulose

325 [34],[41]; this may have been responsible for our observed decrease in substrate effectiveness.

326 Indeed, native bacterial cellulose Iα, despite its high crystallinity, yielded even more CXE

327 product than the non-alkali-pretreated cellulose Iβ of Whatman filter papers (Fig. 1b). The

328 native bacterial cellulose Iα as used had been freed of contaminants by a brief treatment with 0.1

329 M NaOH at about 90°C; these alkaline conditions are too mild to produce appreciable amounts

330 of cellulose II [40]. Pretreating the bacterial cellulose it with 6 M NaOH at 20°C caused the

331 never-dried bacterial cellulose to shrink drastically (Fig. 1b), as also reported previously [34].

332

3.2. Alkali pretreatment enhances the donor substrate effectiveness instantaneously but

requires ≥3 M NaOH

335 We next investigated the NaOH concentration and duration required for enhancing plant-derived

336 cellulose as a donor substrate. Alkali pretreatment of (cotton-derived) Whatman No. 1 filter

337 paper at 20°C increased the subsequently observed CXE reaction rate. The minimal effective

338 alkali concentration for this was 3 M NaOH (Fig. 2; Fig. S1), which agrees with the cut-off

339 reported for the conversion of plant cellulose I to cellulose II by NaOH [42]. The same is true

340 for the conversion of bacterial cellulose I to cellulose II by NaOH at 22°C [34]. With 3–6 M

341 NaOH, the improvement of the donor substrate for CXE activity (presumed to be due to

342 formation of the type II cellulose allomorph) at 20°C occurred in < 4 min — thus essentially

343 instantaneously — and did not appreciably increase during the next 16 h (Fig. S1). The I → II

344 conversion of bacterial cellulose by 6.25 M NaOH at 22°C was ~~even~~ complete within 3 min as

345 was also reported before [34]. The measured CXE activity was highest on papers pretreated with

346 4.5 or 6.0 M NaOH.

347   In the experiments shown in Fig. 2 and Fig. S1, conducted with native *Equisetum* enzyme,

348 the optimal NaOH pretreatment led to a 1.8–2.5-fold increase in CXE activity. This is

349 substantially less than the 13.5-fold increase seen with *Pichia*-produced *Ef*HTG (Fig. 1b, dark

350 bar referring to Whatman No 1). We suggest that this difference between *Pichia*-produced

351 *Ef*HTG and the native *Equisetum* extract is because the latter contains not only HTG but also

352 expansins, hydrogen-bond-cleaving proteins which, to some extent, mimic NaOH in disrupting

353 the conformation of cellulose I. Added expansins have indeed been found to enhance the CXE

354 action of *Pichia*-produced *Ef*HTG on alkali-nonpretreated cellulose [36], presumably due to

355 weakening the lateral adhesion of load-bearing polysaccharides to the cellulose surface.

356

3.3. Dot-blot method for covalently attaching a fluorescent 'cargo' to filter paper

358 Two methods of attaching a fluorescently labelled xyloglucan-oligosaccharide–

359 sulphorhodamine conjugate (XXXGol–SR), as acceptor substrate, to cellulose were compared.

360 Native *Equisetum fluviatile* enzyme (at various concentrations) was either (a) pre-mixed with

361 XXXGol–SR and then pipetted onto filter paper or (b) pipetted directly onto dry papers which

362 had previously been impregnated with XXXGol–SR. (In both cases, the paper had been either

363 alkali-pretreated, or not, before application of enzyme and/or acceptor substrate.) After 12 h

364 enzymic reaction under humid conditions, covalently attached XXXGol–SR was monitored by

365 its fluorescence.

366   All enzyme concentrations tested achieved high incorporation of fluorescence under both

367 regimes (a and b), showing that the enzyme was highly active (Fig. S2a). The highest enzyme

368 concentrations produced a fluorescent ring rather than a uniformly fluorescent disc, probably

369 because the high protein concentration, which remained close to the point of application on the

paper, partially quenched the sulphorhodamine's fluorescence. This was particularly the case in method (b), where the applied enzyme solution would have carried some of the ~~previously applied~~ impregnated XXXGol–SR towards the periphery of the blot, leaving a ring-shaped 'tide-mark'. Image analysis showed that this ring-shaped fluorescence signal at high enzyme concentrations resulted, for most samples, in a slightly lower total fluorescence intensity of spots than with the signal at lower enzyme loadings, probably owing to quenching of sulphorhodamine's fluorescence by proteins (Fig. S2b). Advantageously, this suggests that a high and evenly distributed cargo incorporation can be achieved with relatively small amounts of enzyme. As expected, the alkali-pretreated papers (cellulose II) gave more intense spots of product (Fig. S2). Thus, both regimes (a and b) are valuable — (a) giving more uniformly fluorescent discs, and (b) being preferred for conveniently testing diverse enzyme preparations.

Moreover, the fluorescent cellulose–XXXGol–cargo linkage was largely stable upon further harsh alkali treatment (Fig. S2): 60–80% of fluorescence was retained after post-treatment in 6 M NaOH for 12 h. Papers not pretreated with alkali retained slightly more fluorescence: ~75–80% (not pretreated) *vs*. ~60–70% (pretreated).

3.4. Cellulose hydrogel as a donor substrate

We also tested a cellulose hydrogel, which is a swelled, hydrophilic network built of coagulated cellulose II and pores filled with water. Hydrogels' dry counterparts, aerogels, are of low density (around 0.1 g/cm^3 [30]); the fraction of the pores' volume in a hydrogel is thus above about 90%. The size of the pores in cellulose aerogels varies from a few tens of nanometres to a few micrometres [30], and we presume they are similar in cellulose hydrogels. *Ef*HTG proved able to incorporate the fluorescently labelled acceptor substrate XXXGol–SR deeply into the gel structure (Fig. 1c), showing that the enzyme was able to permeate the hydrogel. Incorporation into the hydrogel was confirmed quantitatively with [3H]XXXGol as acceptor substrate (Fig. 1d). A fraction of the 3H-labelled CXE hydrogel products even resisted hot TFA treatment (Fig.

1d), which might have been expected to hydrolyse the XXXGol moiety of cellulose–

397 [³H]XXXGol if it behaved the same as free [³H]XXXGol. This observation confirms that the

398 enzyme can deeply penetrate the hydrogel.

399

3.5. Range of non-cellulosic donor substrates

401 After testing numerous cellulosic donor substrates, we explored whether a set of different

402 polysaccharides can serve as donor substrates. We confirmed that two plant-derived

403 hemicelluloses, xyloglucan and MLG, are good donor substrates (XET and MXE activity,

404 respectively; Fig. 3a,b). Lichenan, which is a lichen-sourced MLG with very few cellotetraose

405 sequences, was slightly active, as reported before [18]. However, the other plant-derived

406 hemicelluloses — including β-mannan, glucomannan, galactomannan, arabinogalactan,

407 glucuronoxylan and laminarin (an algal polysaccharide which chemically resembles plant

408 callose) — did not support any appreciable activity compared with xyloglucan or MLG (Fig.

409 3a,b). One commercial xylan preparation from oat exhibited some activity (~5% of XET

410 activity; Fig. 3a), which, however, probably resulted from contamination by commercial oat

411 MLG since birch xylan was not a substrate. Other xylan preparations did not serve as donor

412 substrate (Fig. 3a,b). Various other polysaccharides were almost inactive, including plant pectic

413 components, seaweed polymers, and yeast α-mannan (Fig. 3b).

414 Interestingly, however, various commercial preparations of chitosan [(1→4)-linked

415 polysaccharide of β-D-glucosamine] consistently generated ethanol-insoluble transglycanase

416 products (chitosan–[³H]XXXGol conjugates), amounting to ~10% of the observed XET activity

417 (Fig. 3a). The degree of acetylation in chitosan (a polymer of non-acetylated β-(1→4)-D-

418 glucosamine) preparations (~75–85%) did not affect their suitability as a donor substrate. (Fig.

419 3a). In contrast, chitin [(1→4)-linked polysaccharide of *N*-acetyl-β-D-glucosamine] did not

420 serve as a transglycanase substrate (Fig. 3a). However, water-soluble glycol chitin, which we

421 produced by *N*-acetylating commercially available glycol chitosan (Fig. 3c), ~~. Efficient *N*-~~

422 ~~acetylation was confirmed by the absence of ninhydrin staining in glycol chitin preparations~~

423 ~~(Fig. 3c). Glycol chitin~~ was utilised as a donor substrate (activity was up to ~8% of XET

424 activity; Fig. 3d). Glycol chitosan even supported a transglycosylation rate of up to ~15% of

425 XET activity (Fig. 3d). Efficient *N*-acetylation was confirmed by the absence of ninhydrin

426 staining in glycol chitin preparations (Fig. 3c).

427

3.6. Cellulose–xyloglucan bonds are highly stable in hot alkali

429 For the polysaccharide–xyloglucan bonds of the hetero-transglucanase products to be valuable

430 industrially, they need to be chemically robust. We tested this by incubating cellulose–

431 [^{3}H]XXXGol, MLG–[^{3}H]XXXGol and xyloglucan–[^{3}H]XXXGol conjugates (i.e., CXE, MXE

432 and XET products respectively) in 0.1–6.0 M NaOH at 100°C for 1 h. Even after heating in the

433 most severe alkali tested (6 M NaOH; pH > 14.7), over 80% of the radioactivity remained

434 associated with the respective polysaccharide in each case (Fig. 4a) — as judged by water-

435 insolubility (cellulose–[^{3}H]XXXGol) or by retention of the ability to hydrogen-bond to paper

436 (both the hemicellulose–[^{3}H]XXXGol conjugates). The free [^{3}H]XXXGol oligosaccharide itself

437 showed a similar stability, with > 80% of the radioactivity remaining non-volatile, and thus not

438 exchanged as volatile ^{3}H$_2$O (Fig. 4a). We therefore suggest that the apparent loss of

439 polysaccharide–XXXGol was mainly due to the slight instability of the C-1–^{3}H bond within

440 XXXGol rather than to any instability of the polysaccharide–XXXGol glycosidic bond.

441   This idea was supported by the observation that [^{3}H]XXXGol retained its original mobility

442 on paper chromatography (Fig. 4b). Although ~14% of the ^{3}H became volatile (presumably as

443 ^{3}H$_2$O) after heating under the most extreme alkaline conditions, the remaining non-volatile

444 material maintained the standard R_F of a heptasaccharide ([^{3}H]XXXGol), indicating that

445 glycosidic bonds were not appreciably cleaved.

4. Discussion

4.1. Enzymic covalent modification of cellulose

Hetero-trans-β-glucanase (*Ef*HTG) can catalyse the formation of covalent bonds between

insoluble cellulose and soluble xyloglucan oligosaccharides [18]. Here we show that the enzyme

is effective on broad range of cellulose substrates ~~preparations~~(Fig. 1b, Fig. 3a+b), and that in

all plant-derived celluloses studied the enzymic reaction is enhanced if the cellulose is pre-

converted from the Iβ to the II allomorph (shown diagrammatically in Fig. 5a). Another factor

improving NaOH-treated cellulose as a CXE substrate might be alkaline 'peeling' (progressive

removal of glucose moieties from the reducing end in the form of saccharinic acids), but mid-

chain cellulose hydrolysis does not occur at room temperature [43]. Potentially, peeling could

loosen up the cellulose network and make more cellulose chains accessible for enzymic

modification, explaining higher CXE activities after NaOH exposure. However, treatment with

4.5 M NaOH (~5 min) at room temperature was optimal (Fig. 2) and it is unlikely that

appreciable cellulose degradation occurs under such conditions [44]. Increasing the NaOH

concentration to 6 M and the treatment time to 4 h did not further enhance the cellulose as a

substrate (Fig. 2). We thus conclude that the better suitability of plant derived-celluloses after

NaOH treatment is primarily due to cellulose I to cellulose II conversion.

The covalent cellulose–XGO bonds formed are highly stable, resisting solubilisation even

in boiling alkali (Fig. 4a) and partially withstanding hot acid (Fig. 1d). The process of loading

cellulose with a chemical 'cargo', potentially transferrable to the biotechnology industry, is

illustrated in Fig. 5b.

A c~~C~~ellulose-acting transglucanase activity for the *Arabidopsis At*XTH3 protein has been

shown before.[19] However, *At*XTH3 showed appreciable CXE activity only with cellulose that

had been rendered amorphous by pretreatment with phosphoric acid, and not with

microcrystalline cellulose. Moreover, CXE is not the major activity of *At*XTH3, representing

only ~45% of its predominant XET activity.

472   Here we confirm that *Ef*HTG is preferentially a <u>hetero</u>-transglucanase (its CXE activity is

473   200–300% of its XET activity [18]), and we show that it can act on celluloses with various

474   degrees of crystallinity. This includes the cellulose of cotton 'fibres' (specialised seed

475   trichomes; crystallinity ~60% [45]) and bacterial cellulose Iα. Interestingly, the native bacterial

476   cellulose of biofilms functioned as a better CXE donor substrate than all untreated plant-derived

477   cellulose samples tested, including Whatman filter paper, which is manufactured from cotton

478   (Fig. 1b). Cellulose biofilms produced by *Komagataeibacter xylinus* are dominated by cellulose

479   Iα and exhibit a higher crystallinity (~75–95%, depending on the method used [41],[46]), M_r and

480   water-holding capacity than land-plant cellulose [45]. The crystalline fraction of the latter is

481   dominated by cellulose Iβ. In both crystal forms, cellulose chains form flat ribbons, where

482   glucose monomers are locked in position by hydrogen-bonds between –OH groups and ring-O

483   atoms [47]. In a cellulose microfibril, these chains lie parallel and form hydrogen-bonds edge-

484   to-edge, creating sheets, which are stacked onto each other and held in place via weak C–H to O

485   hydrogen-bonding [48].

486   The main reason for the different properties of cellulose Iα and Iβ is the relative

487   displacement of the sheets in the chain direction [48]. The very high water-holding capacity of

488   bacterial cellulose (more than 100 times its dry weight [40]; compared with 2.0 ± 0.1 times for

489   Whatman paper No. 1 paper), suggests an easy accessibility of cellulose chains and might

490   explain its high suitability as a CXE donor substrate. The water holding capacity of bacterial

491   cellulose remains considerably higher than that of paper even after drying [49]. In contrast to the

492   apparently open structure of bacterial cellulose, NaOH pretreatment caused its dramatic

493   compaction (Fig. 1b), diminishing its ability to absorb water and consequently its suitability as a

494   substrate. For all other celluloses tested, NaOH-pretreatment, which converts cellulose I into the

495   allomorph cellulose II with antiparallel arrangement of cellulose chains, consistently increased

496   the CXE activity catalysed by *Ef*HTG. Still, several tested cellulosic substrates (e.g. newspaper

497 or filter papers) supported high CXE activity without NaOH pretreatment, suggesting

498 differences in accessibility.

499   *A priori*, it might be postulated that our 6 M NaOH pretreatments washed out various

500 industrial additives (e.g. other polysaccharides, sizing agents and inorganics) from some of the

501 cellulose samples and that this purification is what enhanced the donor substrate effectiveness

502 for CXE activity. However, the cellulosic substrates that were not NaOH-pretreated were always

503 washed in water, which would be expected to remove ~~some~~ the main contaminants.

504 Furthermore, pure paper (e.g. Whatman filter papers) always supported much higher CXE

505 activity when pretreated with NaOH, suggesting that the alkali effect is due to conversion of

506 cellulose I to II, which should apply to all papers. For printed paper (e.g. newsprint) we always

507 used unprinted areas.

508

4.2. Potential biotechnological applications

510 As discussed above, *Ef*HTG is a predominant hetero-transglucanase that grafts various kinds of

511 insoluble cellulose (donor substrate) onto xyloglucan oligosaccharides (acceptor substrate). We

512 anticipate several industrial processes where HTG might be applied to produce cellulose-based

513 products with new properties and functionality. The xyloglucan oligosaccharide can easily be

514 loaded with a valuable cargo (dye, tracer molecule, indicator, drug etc.) at the hemiacetal group

515 of its reducing terminus. This group is far removed from the −OH group at C-4 of the

516 oligosaccharide's non-reducing terminal glucose residue, which is the site selected by *Ef*HTG as

517 its acceptor substrate; therefore the 'cargo' does not sterically block the hetero-

518 transglycosylation reaction. We illustrated this by showing that both the smallest possible cargo

519 (tritium) as well as a 'bulky' fluorophore (sulphorhodamine; Fig. 1a) can be efficiently

520 incorporated into e.g. paper (Fig. 1b, Fig. S2). Consequently, *Ef*HTG can be used to attach

521 various cargoes to commercial celluloses without the use of toxic or environmentally harmful

522 chemical treatments.

523   Since plant cellulose is the world's most abundant organic substance, numerous

524   biotechnological applications can be envisaged for bulk cellulosic materials (wood-pulp, cotton

525   fabrics, linen, etc.), for example in the manufacture of paper, tissue, sanitary materials,

526   packaging and textile industries. Among numerous other potential examples, the covalently

527   introduced 'cargoes' could be fluorophores such as the sulphorhodamine used in the present

528   work (Fig. S2), which would be valuable for publicly invisible quality control. Other cargoes

529   could include visible dyes and various indicators. For instance, in food-packaging materials,

530   covalently bound indicators that would not 'leak out' could report the contained foodstuff's pH

531   and its history of exposure to cumulative temperature/time status, oxygen, redox status, salts or

532   toxic metals, biogenic amines and sulphides (formed from proteins in food that is spoilt),

533   antibodies (potentially revealing undesirable microbes or their by-products, e.g. aflatoxins).

534   Essentially any indicator that is available as a 'dipstick' (such as ~~such as~~ pH paper, indicator

535   paper and test strips for water quality assessment) could in principle be permanently (covalently

536   and chemically stably) bonded to cellulosic packaging by *Ef*HTG. Other cargoes could include

537   antibiotics, drugs, disinfectants, positively and negatively charged groups, and tamper-proof

538   markings. The covalent attachment of such cargoes to cellulosic products can be achieved by

539   use of *Ef*HTG via a low-cost procedure, using reagents likely to be acceptable in food and

540   medical applications. Furthermore, mixing cargoes would allow the attachment of multiple

541   groups to the cellulosic material in a single step, e.g. allowing the production of universal pH

542   indicator strips, where the blended indicator dyes do not leak out. The cellulose–

543   oligosaccharide–cargo product can withstand remarkably harsh treatments, e.g. with hot,

544   concentrated alkali (Fig. 1b, Fig. 4a, Fig. S2) or even hot acid (Fig. 1d). HTG-modified

545   cellulosic materials would be expected to be less environmentally persistent than fossil fuel-

546   based plastics, and potentially replace them in some applications.

547   Not only bulk sources of cellulose, but also cellulosic hydrogels and bacterial cellulose are

548   suitable resources for small-scale, high-technology applications, for instance in medicine and

549 pharmacy [50],[51], or for manufacturing chromatographic matrices. Hetero-transglucosylation

550 could provide a cheap and environmentally friendly method to functionalise inert cellulosic

551 substrates, e.g. introducing xyloglucan-oligosaccharide-conjugated antibiotics into wound

552 dressings [52] or personal protection equipment. The newly discovered ability of HTG to act on

553 chitin and chitosan may also offer biomedical applications where these polysaccharides are

554 widely used.

555

5. Conclusion

557 This paper deals with cellulose and other plant cell-wall polysaccharides — sustainable,

558 degradable ~~polymers based on natural~~ and renewable resources, — and explores ~~specifically~~

559 ~~these polymers'~~their enzymic functionalisation. ~~conjugates and the creation~~This has high

560 potential to create various ~~of~~ bioactive cellulose or chitin surface~~s~~es and hydrogels. We found

561 that a hetero-transglucanase acts on most plant-derived cellulose Iβ substrates and on bacterial

562 cellulose Iα, as well as on cellulose II including that in the form of a hydrogel. This offers great

563 biotechnological potential, allowing the covalent grafting of cellulose to (oligo)xyloglucans,

564 offering a novel 'green' ~~method~~ technology of functionalising cellulose and other biomass

565 components. We pioneer the enzyme-based functionalisation of cellulose for commercial

566 exploitation by exploring HTG's range of natural substrates, discovering ~~some~~ new

567 commercially intriguing examples (chitin and chitosan), and we characterise the enzyme's

568 ability to utilise diverse commercial cellulose-based substrates~~preparations of cellulose~~,

569 including papers, pulps, textiles, hydrogels and bacterial pellicles, and revealing an unexpected

570 difference between cellulose allomorphs Iα and Iβ. We also report on the very high stability of

571 the functionalised cellulose conjugates that HTG generates , especially their high resistance to

572 hot, concentrated alkali. These conjugates can carry industrially valuable 'cargoes', thus

573 providing a biotechnological tool to manufacture commercial cellulose-based products useful in

574 fabrics, pulps, papers, packaging, sanitary and medical products, and hydrogels.

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Declaration of competing interest

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605

Abbreviations

607   BSA, bovine serum albumin; CXE, cellulose:xyloglucan endotransglucosylase; HTG, hetero-

608   trans-β-glucanase; MLG, mixed-linkage β-D-glucan; MXE, MLG:xyloglucan

609   endotransglucosylase; SR, sulphorhodamine; TFA, trifluoroacetic acid; XET, xyloglucan

610   endotransglucosylase (homotransglucanase); XGO, xyloglucan oligosaccharide; XTH,

611   xyloglucan endotransglucosylase/hydrolase; XXXGol, reduced heptasaccharide of xyloglucan

612   (xylose$_3$.glucose$_3$.glucitol)

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756

Figure captions

758 **Fig. 1. Activities of *Ef*HTG on insoluble cellulosic donor substrates.** (a) <u>Two</u> Glc$_4$-based

759 xyloglucan oligosaccharides used as acceptors substrates: [^{3}H]XXXGol and XXXGol<u>—</u>SR. <u>R$_1$,</u>

760 <u>tritium-labelled glucitol moiety; R$_2$, sulphorhodamine-labelled glucitol moiety</u>; (b) CXE activity

761 on various plant-derived cellulosic substrates and bacterial cellulose pretreated, or not, with 6 M

762 NaOH. "X" indicates that this sample was not measured (*Equisetum* cellulose was available

763 only as cellulose II as it is not possible to isolate hemicellulose-free cellulose without prior

764 alkali treatment); n=3±SD. Insets show bacterial cellulose films in water before (–NaOH) and

765 after (+NaOH) treatment with 6 M NaOH; scale bar = 1 cm. Acceptor substrate, 1 kBq

766 [^{3}H]XXXGol; enzyme, *Pichia*-produced *Ef*HTG. (c) XXXGol–SR (acceptor substrate)

767 incorporation by *Ef*HTG into 20-mm^3 pieces of hydrogel (approximately 2×2×5 mm), as

768 visualised in cross-section by fluorescence microscopy; scale-bar = 2 mm. Above, with enzyme;

769 below, without. (d) CXE activity on cellulose hydrogels plus [^{3}H]XXXGol; the formed products

770 were separated into a hot TFA-extractable fraction (SN TFA) and a non-extractable fraction

771 (pellet TFA); n=2±SD. Enzyme: *Pichia*-produced *Ef*HTG or native HTG extracted from

772 *Equisetum fluviatile* plants.

773

774 **Fig. 2. Estimating the duration and concentration of alkali treatment required to improve**

775 **the donor efficiency of cellulose.** CXE reactions were set up with variously NaOH-pretreated

776 samples of Whatman No. 1 paper (~29 mg) as donor substrate, and 2 kBq [^{3}H]XXXGol as

777 acceptor, in the presence of 1% BSA. The enzyme was a native *Equisetum fluviatile* extract

778 (precipitated in 30% saturated ammonium sulphate) and the enzymic reaction time was 0.5 h.

779 The donor substrate had been pretreated with 0–6 M NaOH, for 4 min to 4 h as shown on the *x*-

780 axis. The CXE activity is quoted per unit of enzyme extracted from 1 g fresh weight of
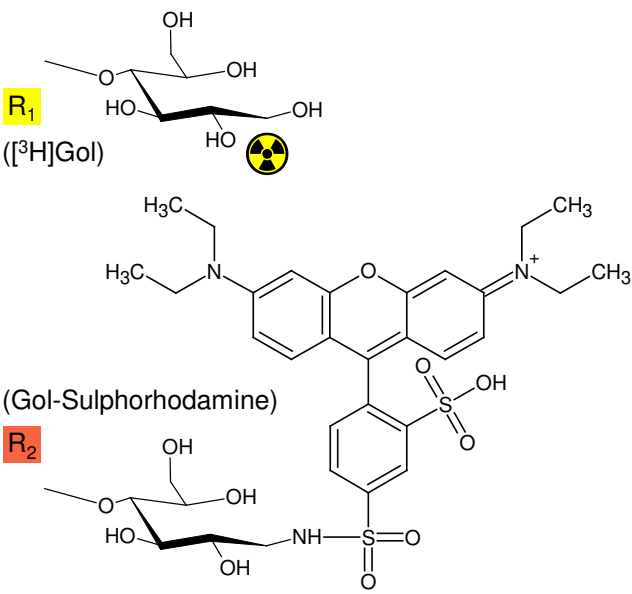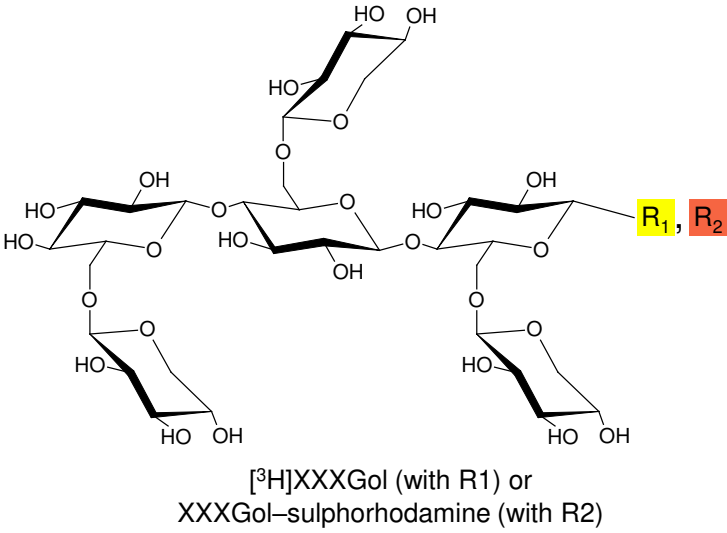
781 *Equisetum* tissue per 0.5 h.

782

783 **Fig. 3. Testing transglycanase activity on various non-cellulosic polysaccharides.** The

784 reaction mixtures contained 0.5% 'donor' polysaccharide, 1 or 2 kBq [³H]XXXGol as acceptor

785 substrate, *Pichia*-produced *Ef*HTG and 0.1% BSA. All values are corrected for a zero-enzyme

786 control. (a) Experiment a: radioactive products harvested by ethanol precipitation; 1 kBq

787 acceptor; n=4±SD. (b) Experiment b: products harvested by the glass-fibre blotting method; 2

788 kBq acceptor; n=3±SD [the 'control' shown on the *x*-axis had enzyme but no donor substrate

789 (n=8±SD)]. Abbreviations: l.v., m.v., h.v. = low-, medium- and high-viscosity mixed-linkage β-

790 glucan. (c) Glycol chitosan (characterised by a higher degree of ninhydrin-stainable free amino

791 groups, arrows) and glycol chitin (with acetylated amino groups) were both prepared at 0.16%

792 and 0.46% (w/v), as indicated, and spotted on to filter paper (plusses vs. minuses). (d) Donor

793 substrates: soluble glycol chitin or glycol chitosan; data expressed as a percentage of the same

794 enzyme's XET activity. Absolute XET values are shown above columns in kcpm / 24 h ± SD;

795 n=4. Enzyme: *Pichia*-produced *Ef*HTG.

796

797 **Fig. 4. Stability of CXE, MXE and XET products in hot alkali.** (a) Radiolabelled

798 transglycanase products were manufactured *in vitro* with alkali-pretreated filter paper, MLG or

799 xyloglucan as donor substrates for CXE, MXE and XET activities respectively, using *Pichia*-

800 produced *Ef*HTG as the enzyme. The polymeric products, freed of unreacted [³H]XXXGol, were

801 then treated with 0.1–6.0 M NaOH at 100°C for 1 h; controls received hot or cold water. The

802 CXE product was monitored for remaining insoluble radioactivity (thus cellulose); soluble MXE

803 and XET products were monitored for their continued ability to hydrogen-bond to paper (thus

804 polymeric hemicelluloses). Free [³H]XXXGol was also treated with alkali as above and

805 monitored for remaining non-volatile radioactivity (thus not exchanged as ³H₂O). Solid lines are

806 linear regressions on the log scale. The structural diagrams use the following symbols: blue

807 circles, β-Glc; yellow circles, β-Gal; orange stars, α-Xyl; pink zigzag, [³H]glucitol. (b) Paper

808 chromatography of free [³H]XXXGol after alkali treatment. [³H]XXXGol was treated with 6 M
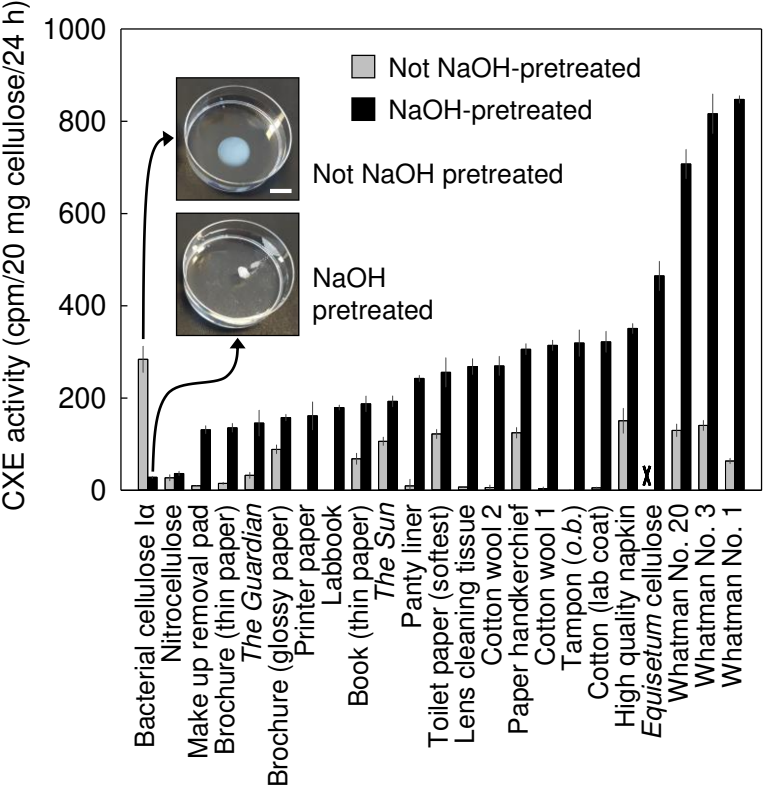
809 NaOH at 100°C for 1 h, and the remaining non-volatile material was run by chromatography on

810 Whatman No. 1 paper in ethyl acetate/acetic acid/water (10:5:6) for 40 h (black profile). A

811 control received cold water (red line). Glucose, maltose and maltoheptaose (markers) were

812 stained with $AgNO_3$.

813

814 **Fig. 5. Interpretation of the observed processes.** (a) Cellulose allomorphs before and after

815 mercerisation in alkali. Left: untreated filter paper with cellulose I; 70–80% ordered

816 (crystalline); 20–30% less ordered (amorphous [42]). Right: mercerised filter paper with

817 cellulose II; less ordered, ~60% crystalline; thermodynamically more stable, with inter-sheet

818 hydrogen bonding. (b) Cellulose heterotransglucosylation by *Ef*HTG as a new tool to load a

819 'cargo' stably onto cellulose. The cargo (e.g. a dye) can be covalently attached to the cellulosic

820 substrate (e.g. paper, cellulose hydrogel) at random positions via a xyloglucan oligosaccharide

821 (XXXGol) linker. Glucose residues of cellulose involved in breakage and re-formation of bonds
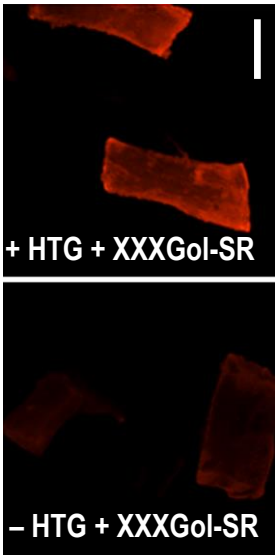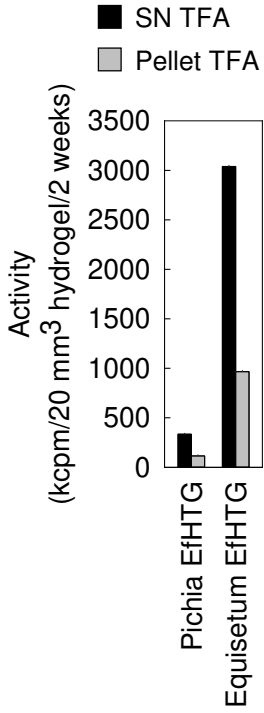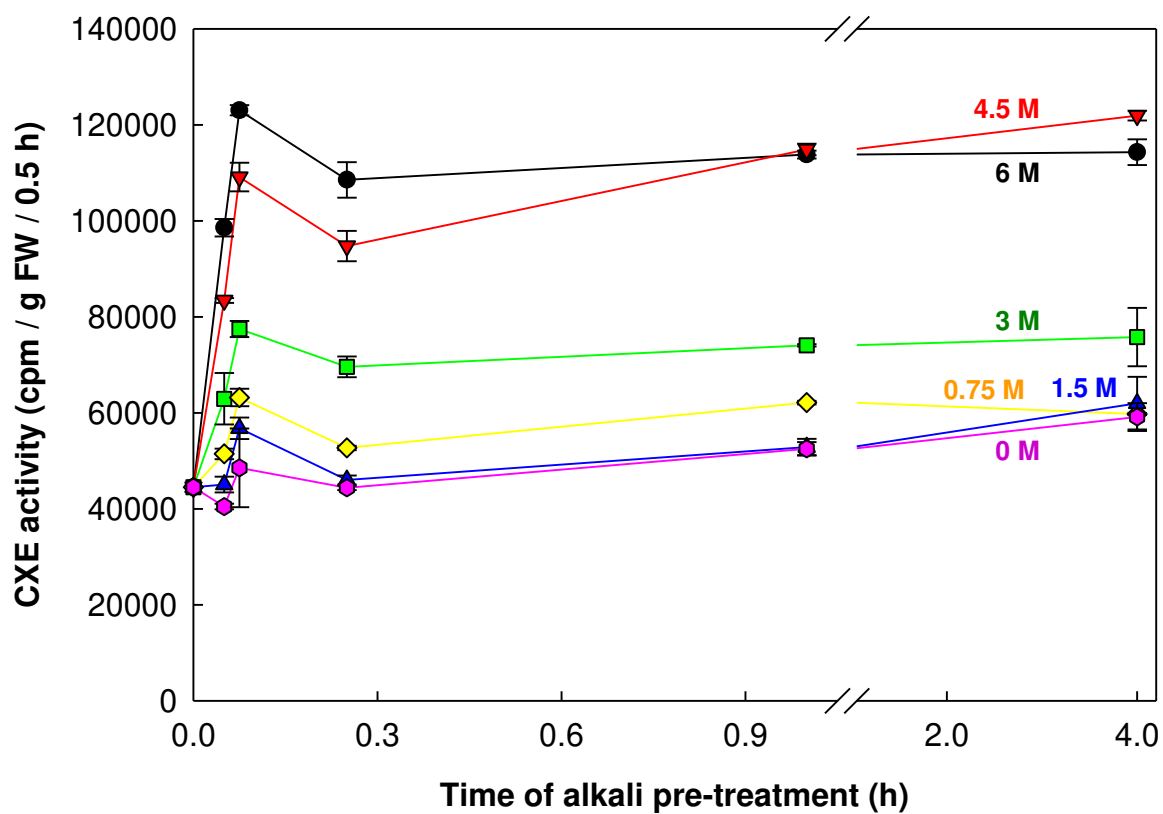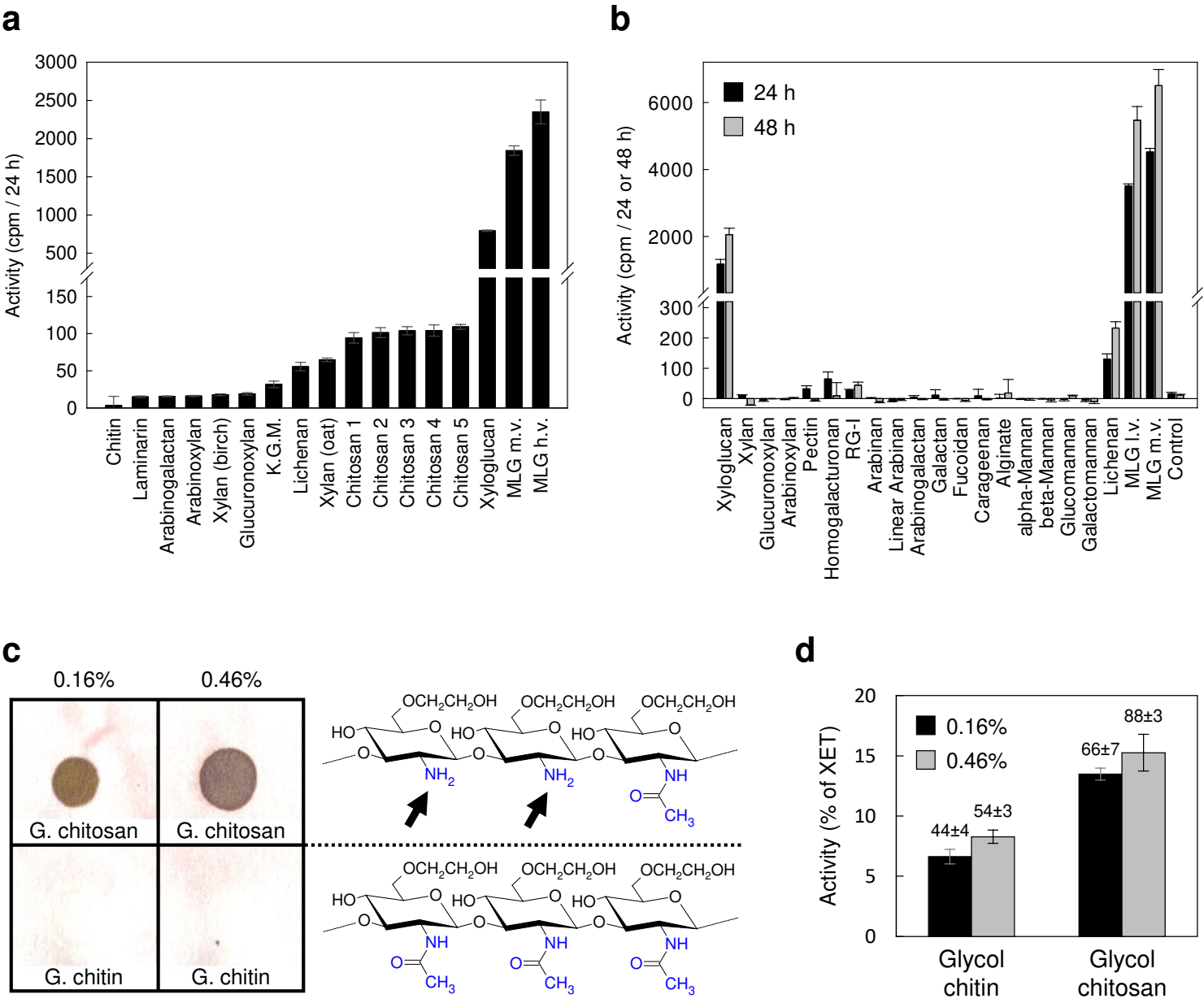
822 are marked with stars.

Fig_1_revision

a

R_1
([³H]Gol)

(Gol-Sulphorhodamine)
R_2

[³H]XXXGol (with R1) or
XXXGol–sulphorhodamine (with R2)

b

CXE activity (cpm/20 mg cellulose/24 h)

Not NaOH-pretreated
NaOH-pretreated

Not NaOH pretreated

NaOH pretreated

Bacterial cellulose Iα
Nitrocellulose
Make up removal pad
Brochure (thin paper)
The Guardian
Brochure (glossy paper)
Printer paper
Labbook
Book (thin paper)
The Sun
Panty liner
Toilet paper (softest)
Lens cleaning tissue
Cotton wool 2
Paper handkerchief
Cotton wool 1
Tampon (*o.b.*)
Cotton (lab coat)
High quality napkin
Equisetum cellulose
Whatman No. 20
Whatman No. 3
Whatman No. 1

c

+ HTG + XXXGol-SR

– HTG + XXXGol-SR

d

SN TFA
Pellet TFA

Activity (kcpm/20 mm³ hydrogel/2 weeks)

Pichia EfHTG
Equisetum EfHTG

Fig_2

Fig_3_revision

a

b

c

d

Fig_4

a

b

Fig_5

a

Cellulose-I — parallel

Crystal 'down'   Crystal 'up'

Amorphous

Mercerisation in >3M NaOH

Cellulose-II — antiparallel

Crystal 'up' and 'down'   Crystal 'up' and 'down'   Crystal 'up' and 'down'

Amorphous

(i) Untreated filter paper (100% area)

(ii) Alkali-treated filter paper (~78% area)

b

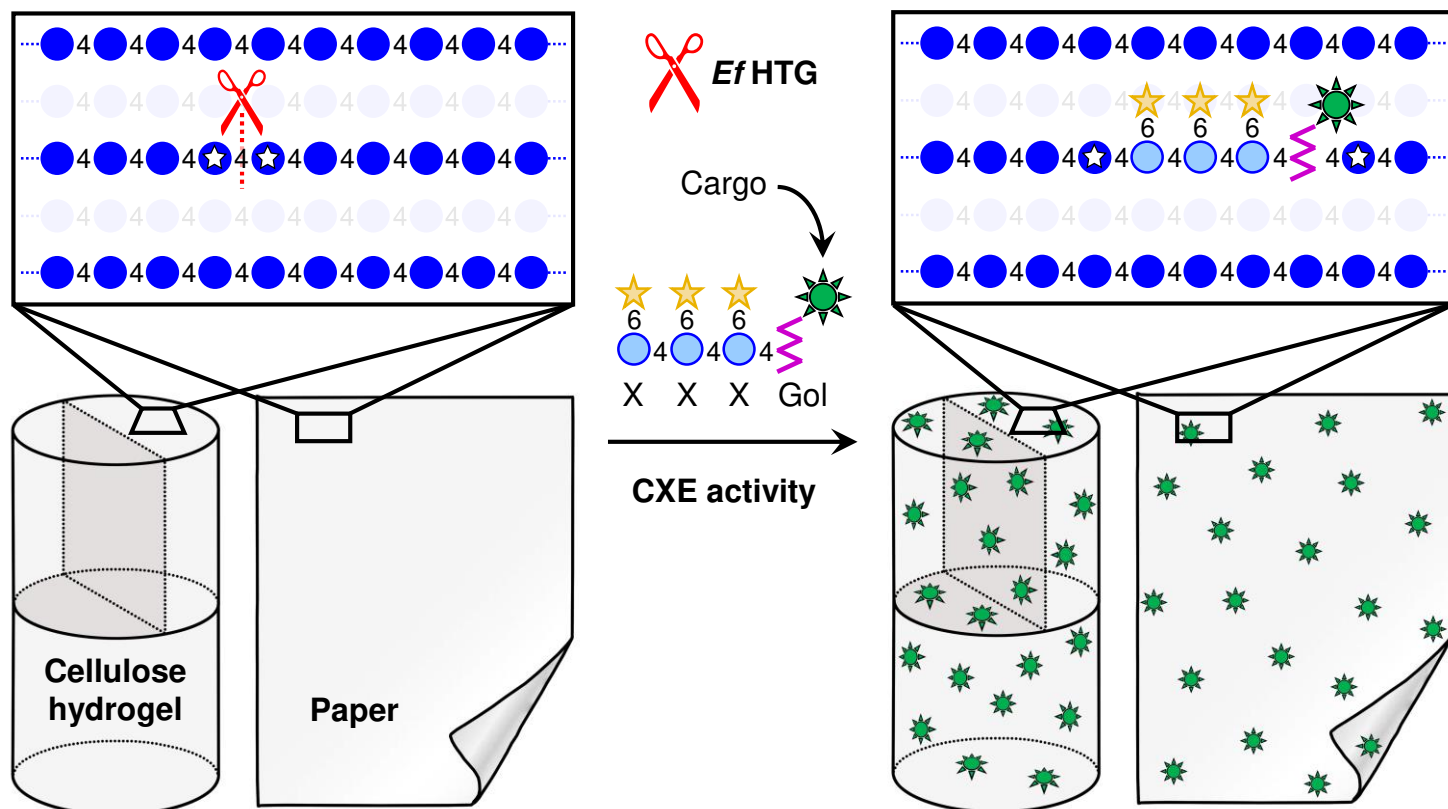*Ef* HTG

Cargo

CXE activity

Cellulose hydrogel   Paper

1 *International Journal of Biological Macromolecules*

2 Herburger et al.

3 **Enzymically attaching oligosaccharide-linked 'cargoes' to cellulose and other commercial**

4 **polysaccharides via stable covalent bonds**

5

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