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α-D-Glucuronidases from the xylanolytic thermophiles *Clostridium stercorarium* and *Thermoanaerobacterium saccharolyticum*

Karin Bronnenmeier, Harald Meissner, Susanne Stocker and Walter L. Staudenbauer

Author for correspondence: Karin Bronnenmeier. Tel: +49 89 2105 2637. Fax: +49 89 2105 2360.

Lehrstuhl für Mikrobiologie, Technische Universität München, Arcisstraße 16, D-80290 München, Germany

α-D-Glucuronidases were purified from the xylanolytic thermophiles *Clostridium stercorarium* and *Thermoanaerobacterium saccharolyticum*. This enzyme activity was found to be intracellular in each organism, with *T. saccharolyticum* producing much greater total activity. The specific activities of the purified enzymes (10 U mg^{-1} *T. saccharolyticum*; 1·7 U mg^{-1} *C. stercorarium*) differed by a factor of approximately 5. For the determination of enzyme activities, 4-*O*-methyl-α-D-glucuronosyl-xylotriose was used as a substrate and the glucuronic acid released by α-D-glucuronidase action was quantified by a colorimetric procedure. 4-*O*-Methyl-α-D-glucuronosyl-xylotriose was the hydrolysis product that accumulated after exhaustive degradation of 4-*O*-methyl-α-D-glucuronoxylan with xylanases of *C. stercorarium*. Hydrolysis of side chains in high-molecular-mass glucuronoxylan could not be detected. Neither of the enzymes was able to hydrolyse the chromogenic aryl-substrate *p*-nitrophenyl-α-D-glucuronoside. Both α-D-glucuronidases have a dimeric structure, with monomeric molecular masses of 72 and 76 kDa for *C. stercorarium* and of 71 kDa for *T. saccharolyticum*. The pI was estimated to be 4·3 for each enzyme. While both enzymes exhibited a similar pH optimum (pH 5·5–6·5) they differed in their thermostabilities. At 60 °C, half-lives of 14 and 2·5 h, respectively, were determined for the α-D-glucuronidases of *C. stercorarium* and *T. saccharolyticum*. This description of α-D-glucuronidase activity in thermophilic anaerobic bacteria extends our knowledge of these enzymes, previously purified and characterized only in fungi.

Keywords: α-D-glucuronidases, *Clostridium*, *Thermoanaerobacterium*, thermoactive enzymes, xylan

INTRODUCTION

Xylans are the most abundant noncellulosic polysaccharides in angiosperms, where they account for 20–30% of the dry weight of woody tissues (Aspinall, 1980). Xylans from different sources exhibit considerable variation in composition and structure (Coughlan & Hazlewood, 1993). However, most xylans consist of a homopolymeric backbone chain of β-1,4-linked D-xylopyranosyl residues. The backbone may be substituted with α-1,3-linked L-arabinofuranosyl and α-1,2-linked 4-*O*-methylglucuronic acid residues as well as with acetic, *p*-coumaric and ferulic acids. Complete hydrolysis of these complex heteropolysaccharides requires the interaction of various main-chain- and side-chain-cleaving activities. Endo-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), β-D-xylosidases (1,4-β-D-xyloside xylohydrolase, EC 3.2.1.37) and possibly exo-β-1,4-xylanases (1,4-β-D-xylan xylohydrolase) are involved in the hydrolysis of the xylan backbone. Removal of side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55) and α-D-glucuronidases (EC 3.2.1). Esterase activities are responsible for the liberation of acetyl, coumaryl and feruloyl substituents (Coughlan & Hazlewood, 1993).

Xylanolytic enzymes are produced by a wide variety of micro-organisms. However, due to the lack of a suitable

Abbreviations: mGlcAX3, 4-*O*-methyl-α-D-glucuronosyl-xylotriose; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid.

routine assay system, α-D-glucuronidase has been isolated and characterized from only a few, and exclusively fungal, sources (Puls *et al.*, 1987; Khandke *et al.*, 1989; Uchida *et al.*, 1992; Siika-aho *et al.*, 1994). An α-D-glucuronidase has not been purified from bacteria up to now. We selected the saccharolytic thermoanaerobic bacteria *Clostridium stercorarium* and *Thermoanaerobacterium saccharolyticum* as candidates for α-D-glucuronidase production. *C. stercorarium* (Madden, 1983) produces a complete enzyme system for the degradation of arabinoxylan, which includes xylanase, β-D-xylosidase and α-L-arabinofuranosidase activities (Bronnenmeier *et al.*, 1990; Schwarz *et al.*, 1990). The species *T. saccharolyticum* (Lee *et al.*, 1933a) contains two closely related isolates from geothermal sources originally described as *Thermoanaerobacter* strain B6A (Weimer *et al.*, 1984) and strain B6A-RI (Lee *et al.*, 1993a). Both strains hydrolysed xylan extensively (Weimer, 1985; Lee *et al.*, 1993b), and strain B6A was shown to produce and utilize uronic acids in addition to monosaccharide degradation products during growth on various xylan preparations (Hespell, 1992).

This paper reports the identification, purification and characterization of α-D-glucuronidases from *C. stercorarium* and *T. saccharolyticum* strain B6A.

METHODS

Organisms and culture conditions. *Clostridium stercorarium* NCIB 11754 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, UK. It was grown under anaerobic conditions at 65 °C in GS-2 medium (Johnson *et al.*, 1981) with 0·5 % xylan from birchwood (Sigma) as carbon source.

Thermoanaerobacterium saccharolyticum B6A (Weimer *et al.*, 1984; Lee *et al.*, 1993a) was obtained from R. Hespell (National Center for Agricultural Utilisation Research, Peoria, USA). It was grown under anaerobic conditions at 60 °C in CM5 medium (Weimer *et al.*, 1984) supplemented with 0·2 % yeast extract and 0·3 % tryptone. Xylan from birchwood (0·5 %) was added as carbon source.

Since the xylan preparation used proved to be completely soluble, the increase in OD_{600} could be used to follow growth of *C. stercorarium* and *T. saccharolyticum* cultures. After the stationary phase was reached, cultivation was continued for 12 h before the cells were harvested.

E. coli JM83(WS1259) and *E. coli* JM109(WS1231), which bear the *C. stercorarium* genes *xynA* and *celX* described previously (Schwarz *et al.*, 1990), were grown at 37 °C in Luria broth (LB) containing (per litre): 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 g glucose, pH 7·5. Ampicillin was added at a concentration of 100 μg ml^{-1}.

Determination of α-D-glucuronidase activity

Preparation of the substrate. Since a requirement for a low-molecular-mass glucuronoxylan substrate has been reported for the fungal α-D-glucuronidases characterized thus far (Puls *et al.*, 1987; Puls & Poutanen, 1989; Khandke *et al.*, 1989; Uchida *et al.*, 1992), we prepared 4-*O*-methyl-α-D-glucuronosyl-xylotriose (aldotetraouronic acid, mGlcAX3) from 4-*O*-methyl-α-D-glucuronoxylan. 4-*O*-Methyl-α-D-glucuronoxylan (3·77 g, Sigma) was incubated with crude extracts (20 ml each, derived from approximately 10 g of cells) of *E. coli* JM83(WS1259) and *E. coli* JM109(WS1231) (Schwarz *et al.*, 1990), expressing xylanase A and celloxylanase X from *C. stercorarium*, in 50 mM ammonium acetate, pH 6·0, with 0·02 % sodium azide, at 60 °C for 78 h. The hydrolysate was cleared by centrifugation and incubated for 15 min at 100 °C in order to remove ammonium acetate. The enzymic degradation of the xylan substrate resulted in the formation of xylose, xylobiose, and two acidic oligosaccharides. Fractionation of the xylan hydrolysate was achieved by anion-exchange chromatography. The hydrolysate (310 ml) was applied to a K 50/60 column packed with AG 1-X8, 100–200 mesh, AC^- (Bio-Rad) and equilibrated with distilled water. Neutral sugars were eluted with distilled water (2700 ml); the acidic oligosaccharides were released from the column with 80 mM ammonium acetate, pH 5·9 (2700 ml). Fractions (20 ml) were collected and assayed for reducing sugars. α-D-Glucuronosyl-xylotriose was identified by TLC and HPLC using mGlcAX3 purchased from Megazyme as a standard. Furthermore, the putative mGlcAX3 was shown to contain hexuronic acid with the colorimetric reagent 3,5-dimethylphenol (Scott, 1979).

Enzyme assay. α-D-Glucuronidase activity was determined by measuring the release of 4-*O*-methyl-α-D-glucuronic acid from mGlcAX3 with a modification of the colorimetric procedure described by Khandke *et al.* (1989), which is based on the method developed by Milner & Avigad (1967). We reduced the concentration of the acidic substrate to 0·7 mM. In order to increase the sensitivity of the assay the absorbance was read at 710 nm. One unit (U) of activity is defined as the amount of enzyme required to release 1 μmol 4-*O*-methyl-α-D-glucuronic acid min^{-1}.

Buffers used for the determination of the pH profiles were 100 mM ammonium acetate (pH 3·5–5·5), 100 mM sodium succinate (pH 5·5–7·0), 100 mM Tris/HCl (pH 7·0–8·5), and 100 mM AMPSO (pH 8·5–9·5). The pH values of buffers were adjusted at the temperatures of use.

Assays for other xylanolytic enzyme activities. α-L-Arabinofuranosidase and β-xylosidase activities were determined by measuring the release of *p*-nitrophenol from the corresponding *p*-nitrophenyl glycoside (Sigma) at 60 °C in 0·1 M sodium succinate, pH 6·0. One unit of activity is defined as the amount of enzyme liberating 1 μmol *p*-nitrophenol min^{-1}. The hydrolysis of *p*-nitrophenyl α-D-glucuronoside was determined with an equivalent assay procedure. The substrate was synthesized via catalytic oxidation of *p*-nitrophenyl α-D-glucopyranoside (Sigma) as described by Marsh (1952) and Marsh & Levvy (1958).

Xylanase activity was assayed by determination of the reducing sugars released during incubation for an appropriate time at 60 °C in a 0·5 % (w/v) solution of xylan from oat spelts (Sigma) in 0·1 M sodium succinate, pH 6·0. One unit (U) of enzyme corresponds to the release of 1 μmol xylose equivalent min^{-1}.

Enzyme purification

C. stercorarium. The cells from a 16 l *C. stercorarium* culture were harvested by centrifugation, washed three times with 50 mM Tris/HCl, pH 8·0, and suspended in 20 mM Tris/HCl, pH 8·0. A cell extract was prepared by passage of lysozyme-treated cells (incubated for 30 min at 37 °C with 1 mg lysozyme ml^{-1}) through an Aminco French pressure cell. The lysate was incubated at 37 °C with DNase I and RNase (each 20 μg ml^{-1}). Debris was removed by centrifugation and the cleared cell extract was loaded on a Pharmacia Q Sepharose FF column. Anion-exchange chromatography was performed as described

in the legend of Fig. 2(a). Solid NaCl was added to the pooled fractions (43–59) up to a concentration of 2 M. Hydrophobic interaction chromatography (HIC) was carried out on a Pharmacia Phenyl-Sepharose HP column as described in the legend of Fig. 2(b). After dialysis against equilibration buffer, pooled fractions (28–34) of the HIC column were loaded on a Pharmacia Mono Q HR 10/10 column equilibrated with 20 mM L-histidine.HCl, pH 6·0. Elution was effected with a 160 ml linear gradient (0–0·4 M LiCl) in equilibration buffer at a flow rate of 4 ml min^{-1}. Fractions (26–31) were pooled and concentrated to 1·2 ml in a Centriprep centrifugal microconcentrator (Amicon). Gel filtration was carried out on a Pharmacia Superdex 200 prep-grade HiLoad 16/60 column equilibrated with 20 mM Tris/HCl, pH 7·0, containing 150 mM NaCl, at a flow rate of 1 ml min^{-1}. Apparent molecular masses were estimated from the partition coefficients as described previously (Bronnenmeier & Staudenbauer, 1988). Chromatofocusing was performed on a Pharmacia Mono P HR 5/20 column equilibrated with 25 mM L-histidine.HCl, pH 5·3. The column was eluted with 44 ml Pharmacia Polybuffer 74, diluted 1:10 and adjusted to pH 3·8 with HCl. Following dilution with 4 vols 20 mM ammonium acetate, pH 5·0, the pooled fractions of the Mono P column were loaded on a Pharmacia Mono Q HR 5/5 column equilibrated with the same buffer. Elution was effected with a 20 ml linear gradient (0–0·4 M NaCl) in equilibration buffer at a flow rate of 1 ml min^{-1}.

T. saccharolyticum. A crude extract of a 20 l *T. saccharolyticum* culture was prepared as described above. Anion-exchange chromatography was performed on a Pharmacia XK 50/20 column packed with 110 ml Q Sepharose FF and equilibrated with 20 mM Tris/HCl, pH 8·0, containing 150 mM NaCl. Elution was effected with a 1100 ml linear gradient (0·15–0·50 M NaCl) in equilibration buffer at a flow rate of 9 ml min^{-1}. Pooled fractions were dialysed against 50 mM sodium phosphate buffer, pH 7·0, containing 1·2 M ammonium sulfate and loaded on a Pharmacia Phenyl Sepharose HP HiLoad 16/10 column equilibrated with the same buffer. Elution was performed with a 230 ml linear gradient (1·2–0·0 M ammonium sulfate) in equilibration buffer at a flow rate of 4 ml min^{-1}. After dialysis against 20 mM Tris/HCl, pH 6·0, pooled fractions were loaded on a Pharmacia Mono Q HR 10/10 column equilibrated with the same buffer. FPLC anion-exchange chromatography was carried out as described previously (Bronnenmeier *et al.*, 1991). Gel filtration was performed on a Pharmacia Superdex 200 prep-grade HiLoad 16/60 column equilibrated with 100 mM ammonium acetate, pH 6·0, at a flow rate of 1·5 ml min^{-1}. Chromatofocusing was carried out on a Pharmacia Mono P HR 5/20 column equilibrated with 25 mM Bis-Tris/HCl, pH 6·5. The column was eluted with 50 ml Pharmacia Polybuffer 74, diluted 1:10 and adjusted to pH 3·0 with HCl at a flow rate of 1 ml min^{-1}.

Analytical methods

Protein concentrations were measured by the method of Sedmak & Grossberg (1977). Reducing sugars were determined with the 3,5-dinitrosalicylic acid reagent (Wood & Bhat, 1988). Uronic acids were detected with the colorimetric reagent 3,5-dimethylphenol as described by Scott (1979). SDS-PAGE was performed in 10% (w/v) polyacrylamide slab gels in the presence of 0·1% SDS according to Laemmli (1970). Acidic sugars and oligosaccharides were analysed by HPLC (Beckman System Gold) at 45 °C on an Aminex HPX-87H column (Bio-Rad) equipped with a cation H guard column. Sulfuric acid (5 mM) was used as eluent at a flow rate of 0·8 ml min^{-1}. TLC was carried out as described previously (Bronnenmeier & Staudenbauer, 1990). Sugars were detected by spraying the plates with a reagent containing 1·23 g *p*-anisidine and 1·66 g phthalic acid in 100 ml ethanol.

RESULTS

Cellular localization

Hydrolysis of xylan with concentrated culture supernatant of *T. saccharolyticum* or *C. stercorarium* resulted in the formation of xylose, xylobiose and a third component later identified as mGlcAX3 (Fig. 1). Accumulation of this acidic oligosaccharide suggests a deficiency of α-D-glucuronidase activity in culture supernatants. mGlcAX3, as well as xylobiose, was completely degraded when crude extract from either *T. saccharolyticum* or *C. stercorarium* cells was added to the reaction mixture (Fig. 1). These findings indicate an intracellular localization of the α-D-glucuronidase activities of both organisms.

Enzyme purification

The purification of the α-D-glucuronidase present in extracts of cells of *C. stercorarium* grown on xylan from birchwood is summarized in Table 1. The α-D-glucuronidase could be clearly separated from other components of the xylanase system by a combination of anion-exchange chromatography on Q Sepharose Fast

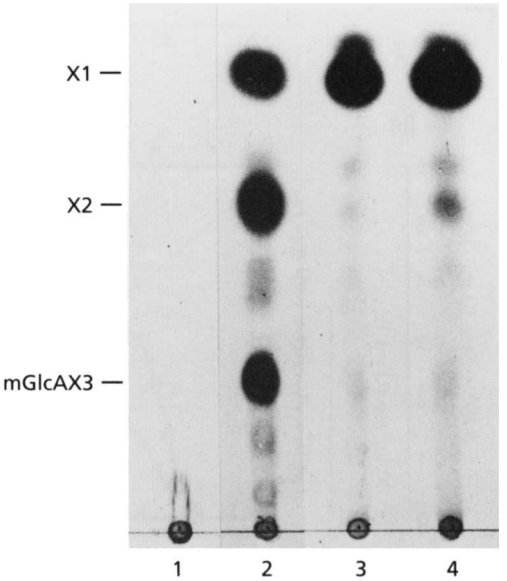

Fig. 1. **TLC analysis of the hydrolysis of 4-*O*-methyl-α-D-glucuronoxylan with crude extracellular and intracellular enzymes. Lanes: 1, 4-*O*-methyl-α-D-glucuronoxylan; 2, 4-*O*-methyl-α-D-glucuronoxylan hydrolysed with culture supernatant of *T. saccharolyticum*; 3, 4-*O*-methyl-α-D-glucuronoxylan pre-incubated with culture supernatant and further hydrolysed with crude extract of *T. saccharolyticum*; 4, 4-*O*-methyl-α-D-glucuronoxylan pre-incubated with culture supernatant of *T. saccharolyticum* and further hydrolysed with crude extract of *C. stercorarium*. The figure shows representative results for one of at least three experiments.**

Table 1. Purification of α-D-glucuronidase from crude extract of *C. stercorarium*

Purification step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (mU mg^{-1})	Purification (-fold)
Crude extract	148	1468	7·2	4·9	1·0
Q Sepharose FF	320	171	2·6	15·2	3·1
Phenyl-Sepharose	75	31	1·3	43·3	8·8
Mono Q pH 6·0	21	7	0·5	71·4	14·6
Superdex 200	18	2	0·3	150·0	30·6
Mono P	2·3	0·3	0·1	333·3	68·0
Mono Q pH 5·0	1·0	0·03	0·05	1646·9	336·1

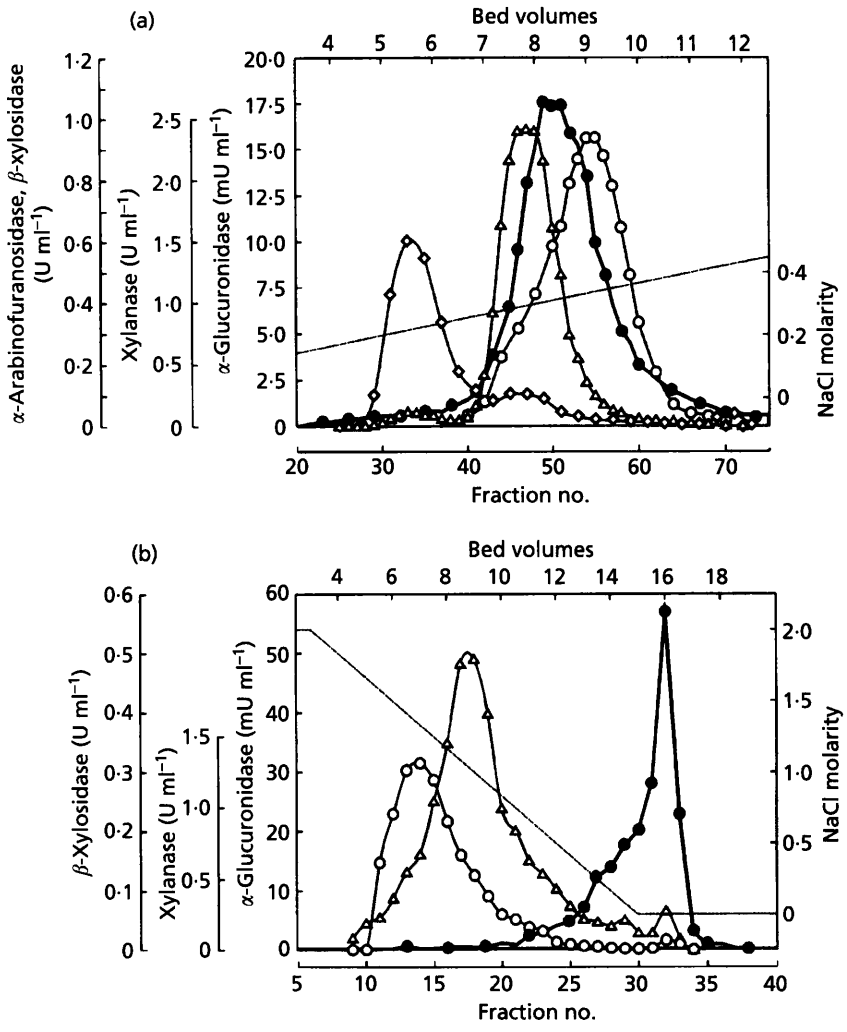

Fig. 2. Fractionation of *C. stercorarium* enzymes involved in xylan depolymerization by Q Sepharose anion-exchange chromatography (a) and Phenyl-Sepharose hydrophobic interaction chromatography (b). The figure shows representative results for one of two independent experiments. (a) Crude extract was applied to a Q Sepharose Fast Flow XK 50/20 column (bed height 6 cm) equilibrated with 20 mM Tris/HCl, pH 8·0, containing 100 mM NaCl. Elution was performed with a 2040 ml linear gradient (0·1–0·6 M NaCl) in 20 mM Tris/HCl, pH 8·0, at a flow rate of 10 ml min^{-1}. Fractions (20 ml) were assayed for α-D-glucuronidase (●), α-L-arabinofuranosidase (◇), β-D-xylosidase (△) and xylanase (○). (b) The α-D-glucuronidase pool of the Q Sepharose column (fractions 43–59), adjusted to 2 M NaCl, was applied to a Phenyl-Sepharose HP HiLoad 16/10 column equilibrated with 20 mM Tris/HCl, pH 8·0, containing 2 M NaCl. Elution was performed with a 276 ml linear gradient (2·0–0·0 M NaCl) in 20 mM Tris/HCl, pH 8·0, at a flow rate of 5 ml min^{-1}. Fractions (11·5 ml) were assayed for α-D-glucuronidase (●), β-D-xylosidase (△) and xylanase (○). In both (a) and (b) the NaCl gradient is shown by a finely dotted line.

Flow and hydrophobic interaction chromatography on Phenyl-Sepharose High Performance columns (Fig. 2a, b). Further purification was achieved by anion-exchange chromatography on Mono Q, chromatofocusing on Mono P and gel filtration on a Superdex 200 column. On the latter column, the enzyme migrated as a peak with an apparent molecular mass of 124 kDa. Analysis of the purified enzyme by SDS-PAGE revealed two protein bands with molecular masses of 72 and 76 kDa (Fig. 3), indicating an oligomeric structure of the α-D-glucuronidase. Due to the fact that proteins elute from a Mono P chromatofocusing column at a pH close to their isoelectric point, the pI of the purified enzyme was estimated to be 4·3.

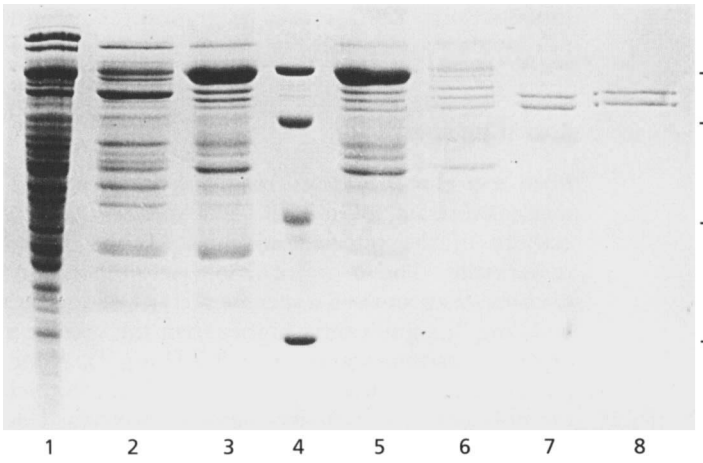

Fig. 3. SDS-PAGE of the fractions obtained during purification of the α-D-glucuronidase of *C. stercorarium*. Lanes: 1, crude extract; 2, pooled fractions from the Q Sepharose column; 3, pooled fractions from the Phenyl-Sepharose column; 4, molecular mass markers (values in kDa on the right); 5, pooled fractions from the Mono Q column at pH 6·0; 6, pooled fractions from the Superdex column; 7, pooled fractions from the Mono P column; 8, fraction 22 from the Mono Q column at pH 5·0.

Table 2. Purification of α-D-glucuronidase from crude extract of *T. saccharolyticum*

Purification step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Purification (-fold)
Crude extract	50	1479	259	0·2	1·0
Q Sepharose FF	250	173	103	0·6	3·0
Phenyl-Sepharose	100	64	67	1·1	5·5
Mono Q	14	12	39	3·3	16·5
Superdex 200	6	4	24	6·6	33·0
Mono P	3	0·5	4·8	9·6	48·0

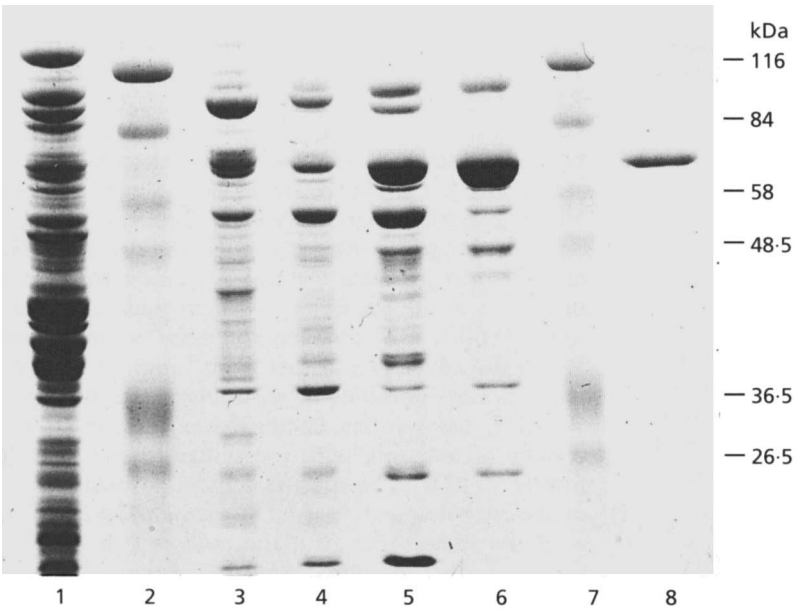

Fig. 4. SDS-PAGE of the fractions obtained during purification of the α-D-glucuronidase of *T. saccharolyticum*. Lanes: 1, crude extract; 2 and 7, molecular mass markers (values in kDa on the right); 3, pooled fractions from the Q Sepharose column; 4, pooled fractions from the Phenyl-Sepharose column; 5, pooled fractions from the Mono Q column; 6, pooled fractions from the Superdex column; 7, fraction 41 from the Mono P column.

The α-D-glucuronidase of *T. saccharolyticum* was isolated from the crude extract by a similar purification procedure (Table 2). The purified enzyme ran as a single 71 kDa band in SDS-PAGE (Fig. 4). During gel filtration on a Superdex 200 column, the enzyme eluted as a single peak with an apparent molecular mass of 118 kDa, suggesting a dimeric subunit structure. The pI was determined as 4·3 by chromatofocusing.

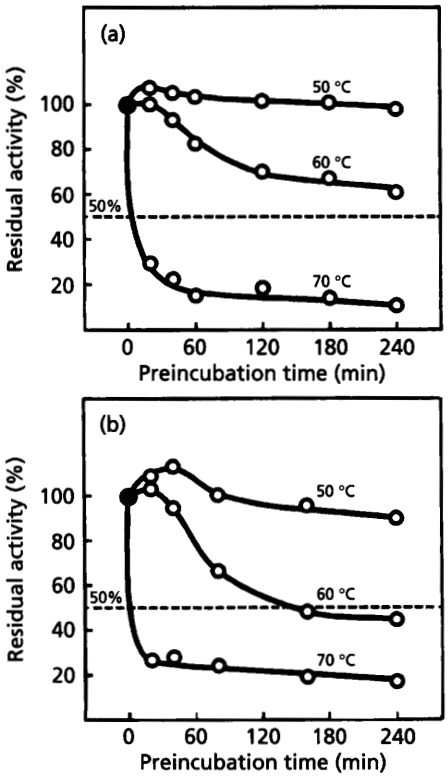

Fig. 5. Thermal stability of α-D-glucuronidases from *C. stercorarium* (a) and *T. saccharolyticum* (b) in the absence of substrate. Purified enzymes were incubated in 100 mM sodium succinate, pH 6·0, at various temperatures. At the times indicated, samples were withdrawn for the determination of α-D-glucuronidase activity with the standard assay procedure at 60 °C for the *C. stercorarium* enzyme and at 50 °C for the enzyme of *T. saccharolyticum*. The results shown are representative of three experiments. One hundred percent residual activity of the enzymes of *C. stercorarium* and *T. saccharolyticum* corresponds to 0·14 mU and 1·7 mU, respectively.

Effects of pH, temperature and salts on enzyme activity

Both α-D-glucuronidases were maximally active at pH 6·0 and retained at least 40 % activity at pH values ranging from 5 to 8. When the enzymes were assayed for 180 min in 100 mM sodium succinate, pH 6·0, the highest activity was observed at 60 °C for the *C. stercorarium* and at 50 °C for the *T. saccharolyticum* α-D-glucuronidase. In 100 mM sodium succinate buffer, pH 6·0, at an enzyme concentration of 6·4 μg ml^{-1}, and without stabilizing additives, the α-D-glucuronidase from *C. stercorarium* was stable at 50 °C (Fig. 5). At 60 °C the enzyme exhibited a half-life of about 14 h. The enzyme of *T. saccharolyticum* was less thermostable, showing a half-life of 2–3 h at 60 °C. Addition of bovine serum albumin (10 mg ml^{-1}) and/or Ca^{2+} (20 mM) did not stabilize the purified enzyme. The purified α-D-glucuronidases of both organisms were strongly inhibited by $CuCl_2$. At a concentration of 1 mM, the *C. stercorarium* enzyme suffered a 74 % loss of activity and the enzyme from *T. saccharolyticum* lost 80 % of its initial activity. $ZnCl_2$ caused a significant reduction (40 %) in enzyme activity only for the *C. stercorarium* enzyme.

Substrate specificity

Both α-D-glucuronidases readily hydrolysed the oligomeric substrate mGlcAX3. The differences in specific activity of the purified enzymes (Tables 1 and 2) is remarkable. The α-D-glucuronidase preparation of *T. saccharolyticum* showed a specific activity of approximately 10 U mg^{-1}, significantly higher than the specific activity of the *C. stercorarium* enzyme (1·7 U mg^{-1}). None of the purified enzymes was able to release glucuronic acid from the polymer 4-*O*-methyl-α-D-glucuronoxylan. Under the conditions of the assay, the lower detection limit was 3·7 mU mg^{-1} and 0·5 mU mg^{-1} for the *T. saccharolyticum* and *C. stercorarium* enzymes, respectively. Since chromogenic aryl-glycosides are commonly used for the determination of hydrolase activity we synthesized a *p*-nitrophenyl-α-D-glucuronide according to Marsh (1952) and Marsh & Levvy (1958). Neither the α-D-glucuronidase of *C. stercorarium* nor that of *T. saccharolyticum* was able to hydrolyse the α-glycosidic bond of the aryl-substrate. The lower detection limit was 4·2 mU mg^{-1} and 0·6 mU mg^{-1} for the *T. saccharolyticum* and *C. stercorarium* enzymes, respectively, under the conditions of the *p*-nitrophenyl-α-D-glucuronidase assay.

DISCUSSION

α-D-Glucuronidase activity has been detected in a variety of fungi (Puls, 1992). The enzymes from *Thermoascus aurantiacus* (Khandke *et al.*, 1989), *Agaricus bisporus* (Puls *et al.*, 1987; Puls, 1992), *Aspergillus niger* (Uchida *et al.*, 1992), *Trichoderma reesei* (Puls, 1992), and *Trichoderma reesei* RUT C-30 (Siika-aho *et al.*, 1994) have been purified thus far. Amongst bacteria, α-D-glucuronidase activity has been identified in *Streptomyces olivochromogenes* (MacKenzie *et al.*, 1987), *Fibrobacter succinogenes* (Smith & Forsberg, 1991), *Clostridium acetobutylicum* (Trudeau *et al.*, 1992) and *Thermotoga maritima* (Bronnenmeier *et al.*, 1995). The enzymes of *C. stercorarium* and *T. saccharolyticum* reported in this paper are the first α-D-glucuronidases which have been purified to homogeneity from bacterial sources. While the enzymes purified from fungi all have a comparably high monomeric molecular mass of more than 100 kDa, the purified bacterial enzymes described here consist of subunits with molecular masses of approximately 70 kDa. The specific activity towards mGlcAX3 of the enzyme purified from *C. stercorarium* (1·7 U mg^{-1}) is of the same order of magnitude as that reported for the α-D-glucuronidase of the fungus *Thermoascus aurantiacus* (2·2 U mg^{-1}) towards the same substituted xylo-oligomer (Khandke *et al.*, 1989). The specific activity of the *T. saccharolyticum* enzyme (10 U mg^{-1}) is comparable to that reported recently for the purified enzyme of *Trichoderma reesei* RUT C-30 (Siika-aho *et al.*, 1994).

Hydrolysis of side-chains in high-molecular-mass glucuronoxylan could not be detected with α-D-

glucuronidase preparations of *C. stercorarium* and *T. saccharolyticum*. This is consistent with the intracellular localization of both enzymes. The enzymes from *Agaricus bisporus* (Puls, 1992), *Fibrobacter succinogenes* (Smith & Forsberg, 1991) and *Streptomyces olivochromogenes* (Fontana *et al.*, 1988) were also unable to release glucuronic acid from the polymeric substrate. Minor activity against long-chain glucuronoxylan was reported for the enzyme of *Trichoderma reesei* RUT C-30 (Siika-aho *et al.*, 1994). In contrast, the α-D-glucuronidase from *Aspergillus niger*, *Schizophyllum commune* and *Thermoascus aurantiacus* effected the liberation of 4-*O*-methyl-α-D-glucuronic acid from glucuronoxylan (Coughlan & Hazlewood, 1993; Khandke *et al.*, 1989). In all cases, substituted xylo-oligomers proved to be better substrates with respect to relative rates of hydrolysis. The synthetic *p*-nitrophenyl-α-D-glucuronoside was not cleaved by the enzymes from *C. stercorarium* and *T. saccharolyticum*. A lack of aryl-α-D-glucuronidase activity has also been reported for the purified enzyme of *Aspergillus niger* 5-16 (Uchida *et al.*, 1992) and a crude xylanolytic enzyme preparation of *Streptomyces olivochromogenes* (Fontana *et al.*, 1988).

The isolation of α-D-glucuronidase from thermophilic anaerobes indicates that these bacteria produce complete sets of xylanolytic enzymes for the degradation of glucuronoxylan. Since α-D-glucuronidase proved to be an intracellular enzyme, the complete hydrolysis of glucuronoxylan must result from the co-operative action of intra- and extracellularly located enzymes in these bacteria. The high-molecular-mass xylan is degraded outside the cells by the main-chain hydrolysing enzymes endoxylanase and β-xylosidase. The products – xylose, xylobiose and 4-*O*-methyl-α-D-glucuronosyl-xylotriose – are transported into the cells, where the side-chain glucuronosyl residues are removed by α-D-glucuronidase action. The resulting xylotriose and the extracellularly produced xylobiose may then be further degraded to xylose by an intracellular β-xylosidase (K. Bronnenmeier, unpublished results).

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REFERENCES

Aspinall, G. O. (1980). Chemistry of cell wall polysaccharides. In *The Biochemistry of Plants*, pp. 473–500. Edited by N. E. Tolbert. New York: Academic Press.

Bronnenmeier, K. & Staudenbauer, W. L. (1988). Purification and properties of an extracellular β-glucosidase from the cellulolytic thermophile *Clostridium stercorarium*. *Appl Microbiol Biotechnol* **28**, 380–386.

Bronnenmeier, K. & Staudenbauer, W. L. (1990). Cellulose hydrolysis by a highly thermostable endo-1,4-β-glucanase (Avicelase I) from *Clostridium stercorarium*. *Enzyme Microb Technol* **12**, 431–436.

Bronnenmeier, K., Ebenbichler, C. & Staudenbauer, W. L. (1990). Separation of the cellulolytic and xylanolytic enzymes of *Clostridium stercorarium*. *J Chromatogr* **521**, 301–310.

Bronnenmeier, K., Rücknagel, K. P. & Staudenbauer, W. L. (1991). Purification and properties of a novel type of exo-1,4-β-glucanase (Avicelase II) from the cellulolytic thermophile *Clostridium stercorarium*. *Eur J Biochem* **200**, 379–385.

Bronnenmeier, K., Kern, A., Liebl, W. & Staudenbauer, W. L. (1995). Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. *Appl Environ Microbiol* **61**, 1399–1407.

Coughlan, M. P. & Hazlewood, G. P. (1993). β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. *Biotechnol Appl Biochem* **17**, 259–289.

Fontana, J. D., Gebara, M., Blumel, M., Schneider, H., MacKenzie, C. R. & Johnson, K. G. (1988). α-4-*O*-Methyl-D-glucuronidase component of xylanolytic complexes. *Methods Enzymol* **160**, 560–571.

Hespell, R. B. (1992). Fermentation of xylans by *Butyrivibrio fibrisolvens* and *Thermoanaerobacter* strain B6A: utilization of uronic acids and xylanolytic activities. *Curr Microbiol* **25**, 189–195.

Johnson, E. A., Madia, A. & Demain, A. L. (1981). Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. *Appl Environ Microbiol* **41**, 1060–1062.

Khandke, K. M., Vithayayathil, P. J. & Murthy, S. K. (1989). Purification and characterization of an α-D-glucuronidase from a thermophilic fungus, *Thermoascus aurantiacus*. *Arch Biochem Biophys* **274**, 511–517.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lee, Y.-E., Jain, M. K., Lee, C., Lowe, S. E. & Zeikus, J. G. (1993a). Taxonomic distinction of saccharolytic thermophilic anaerobes: description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfurigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., respectively; and transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*. *Int J Syst Bacteriol* **43**, 41–51.

Lee, Y.-E., Lowe, S. E. & Zeikus, J. G. (1993b). Regulation and characterization of xylanolytic enzymes of *Thermoanaerobacterium saccharolyticum* B6A-RI. *Appl Environ Microbiol* **59**, 763–771.

MacKenzie, C. R., Bilous, D., Schneider, H. & Johnson, K. G. (1987). Induction of cellulolytic and xylanolytic enzyme systems in *Streptomyces* spp. *Appl Environ Microbiol* **53**, 2835–2839.

Madden, R. A. (1983). Isolation and characterization of *Clostridium stercorarium* sp. nov., cellulolytic thermophile. *Int J Syst Bacteriol* **33**, 837–840.

Marsh, C. A. (1952). The synthesis of conjugated hexuronic acids. *J Chem Soc* 1578–1582.

Marsh, C. A. & Levvy, G. A. (1958). The synthesis of aryl glycosiduronic acids. *Biochem J* **68**, 617–621.

Milner, Y. & Avigad, G. (1967). A copper reagent for the determination of hexuronic acids and certain ketohexoses. *Carbohydr Res* **4**, 359–361.

Puls, J. (1992). α-Glucuronidases in the hydrolysis of wood xylans. In *Xylans and Xylanases*, pp. 213–224. Edited by J. Visser, G. Beldman, M. A. Kusters-van Someren & A. G. J. Voragen. Amsterdam: Elsevier.

Puls, J. & Poutanen, K. (1989). Mechanism of enzyme hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. In *Enzyme Systems for Lignocellulose Degradation*, pp. 151–165. Edited by M. P. Coughlan. London: Elsevier Applied Science.

Puls, J., Schmidt, O. & Granzow, C. (1987). α-Glucuronidase in two microbial xylanolytic systems. *Enzyme Microb Technol* **9**, 83–88.

Schwarz, W. H., Adelsberger, H., Jauris, S., Hertel, C., Funk, B. & Staudenbauer, W. L. (1990). Xylan degradation by the thermophile *Clostridium stercorarium*: cloning and expression of xylanase, β-D-xylosidase, and α-L-arabinofuranosidase genes in *Escherichia coli*. *Biochem Biophys Res Commun* **170**, 368–374.

Scott, R. W. (1979). Colorimetric determination of hexuronic acids in plant materials. *Anal Chem* **51**, 936–941.

Sedmak, J. J. & Grossberg, S. E. (1977). A rapid, sensitive assay for protein using Coomassie brilliant blue G250. *Anal Biochem* **79**, 544–552.

Siika-aho, M., Tenkanen, M., Buchert, J., Puls, J. & Viikari, L. (1994). An α-glucuronidase from *Trichoderma reesei* RUT C-30. *Enzyme Microb Technol* **16**, 813–819.

Smith, D. C. & Forsberg, C. W. (1991). α-Glucuronidase and other hemicellulase activities of *Fibrobacter succinogenes* S85 grown on crystalline cellulose or ball-milled barley straw. *Appl Environ Microbiol* **57**, 3552–3557.

Trudeau, D. G., Bernier, R. L., Gannon, D. J. & Forsberg, C. W. (1992). Isolation of *Clostridium acetobutylicum* strains and the preliminary investigation of the hemicellulolytic activities of isolate 3BYR. *Can J Microbiol* **38**, 1120–1127.

Uchida, H., Nanri, T., Kawabata, Y., Kusakabe, L. & Murakami, K. (1992). Purification and characterization of intracellular α-glucuronidase from *Aspergillus niger* 5-16. *Biosci Biotech Biochem* **56**, 1608–1615.

Weimer, P. J. (1985). Thermophilic anaerobic fermentation of hemicellulose and hemicellulose-derived aldose sugars by *Thermoanaerobacter* strain B6A. *Arch Microbiol* **143**, 130–136.

Weimer, P. J., Wagner, W., Knowlton, S. & Ng, T. K. (1984). Thermophilic anaerobic bacteria which ferment hemicellulose: characterization of organisms and identification of plasmids. *Arch Microbiol* **138**, 31–36.

Wood, T. M. & Bhat, K. M. (1988). Methods for measuring cellulase activities. *Methods Enzymol* **160**, 87–112.

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