Purification and properties of a 1,3-1,4- β -D-glucanase (lichenase, 1,3-1,4- β -D-glucan 4-glucanohydrolase, EC 3.2.1.73) from *Bacteroides succinogenes* cloned in *Escherichia coli*

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A 1,3-1,4- β -D-glucanase (lichenase, 1,3-1,4- β -D-glucan 4-glucanohydrolase, EC 3.2.1.73) from *Bacteroides succinogenes* cloned in *Escherichia coli* was purified 600-fold by chromatography on Q-Sepharose and hydroxyapatite. The cloned enzyme hydrolysed lichenin and oat β -D-glucan but not starch, CM(carboxymethyl)-cellulose, CM-pachyman, laminarin or xylan. The enzyme had a broad pH optimum with maximum activity at approx. pH 6.0 and a temperature optimum of 50 °C. The pH of elution from a chromatofocusing column for the cloned enzyme was 4.7 (purified) and 4.9 (crude), compared with 4.8 for the mixed-linkage β -D-glucanase activity in *B. succinogenes*. The M_r of the cloned enzyme was estimated to be 37200 by gel filtration and 35200 by electrophoresis. The K_m values estimated for lichenin and oat β -D-glucan were 0.35 and 0.71 mg/ml respectively. The major hydrolytic products with lichenin as substrate were a trisaccharide (82%) and a pentasaccharide (9.5%). Hydrolysis of oat β -D-glucan yielded a trisaccharide (63.5%) and a tetrasaccharide (29.6%) as the major products. The chromatographic patterns of the products from the cloned enzyme appear to be similar to those reported for the mixed-linkage β -D-glucanase isolated from *Bacillus subtilis*. The data presented illustrate the similarity in properties of the cloned mixed-linkage enzyme and the 1,3-1,4- β -D-glucanase from *B. subtilis* and the similarity with the 1,4- β -glucanase in *B. succinogenes*.

INTRODUCTION

Bacteroides succinogenes is one of the major cellulolytic bacteria in the rumen. The mechanism by which this organism degrades fibrous plant material in the rumen is thought to involve a complex of enzymes including endoglucanases, exoglucanases, xylanases and cellobiohydrolase (Forsberg *et al.*, 1981; Groleau & Forsberg, 1981; Schellhorn & Forsberg, 1984). Resolution and characterization of the components of this enzyme complex have been difficult (Groleau & Forsberg, 1983) because of aggregate formation and the apparent heterogeneity of molecular size and charge of some of the enzymes in the complex [a problem encountered in other cellulolytic organisms as well (Saddler & Khan, 1980; Ng & Zeikus, 1981)].

Cloning of DNA from *B. succinogenes* in *Escherichia coli* and screening for components of the fibre-degrading complex of the organism has led to the isolation of a number of endoglucanases (Crosby *et al.*, 1984; Taylor *et al.*, 1987) and a xylanase (Sipat *et al.*, 1987). This approach can provide several advantages over purification and characterization of the enzymes present in the native organism. First, post-translational alterations such as glycosylation or *N*-terminal cleavage (which may play a role in the export of the enzyme) may not occur in the host organism (i.e. *E. coli*). Secondly, the possibility exists for the isolation of separate clones containing each of the individual components of the complex. Each

component can then be studied as a separate entity without the need for complex isolation procedures. In the present case the 1,3-1,4- β -D-glucanase (lichenase, 1,3-1,4- β -D-glucan 4-glucanohydrolase, EC 3.2.1.73) from *B. succinogenes* was not known to be a separate enzyme in the fibre-degrading-enzyme complex until it was isolated by cloning (Irvin & Teather, 1988). The object of this work was to isolate this mixed-linkage β -D-glucanase from the *E. coli* clone and to compare its properties with other mixed-linkage β -glucanases and with other enzymes of the fibre-degrading complex of *B. succinogenes*.

EXPERIMENTAL

Materials

Lichenin (isolated from the lichen *Cetraria islandica*, approx. 57% β -D-glucan on the basis of reducing-sugar production after complete hydrolysis with β -D-glucanase), CM-cellulose (low viscosity), xylan, cellobiose, laminarin, Congo Red, dithiothreitol (DTT), Bistris, Bistris Propane, bovine serum albumin (fraction V, 3.3% water) and protein molecular-mass standards for gel filtration were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Polybuffer 74, Q-Sepharose, PBE 94 Exchanger and protein molecular-mass standards for electrophoresis were from Pharmacia (Dorval, Que., Canada). Hydroxyapatite was from Behring Diagnostics (La Jolla, CA, U.S.A.) and cellodextrin standards

Abbreviations used: CM-, carboxymethyl; DTT, dithiothreitol; f.p.l.c., fast protein liquid chromatography.

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(cellotriose, cellotetraose, cellopentaose) were from V-Labs (Covington, LA, U.S.A.). Oat β -D-glucan preparation was purified from oat gum as described by Wood (1986). The material used was 78% β -D-glucan as determined with the β -glucan assay procedure of McCleary & Glennie-Holmes (1985). Solvents and buffers for h.p.l.c. and f.p.l.c. were prepared with water purified with a Millex RO-15 and Milli-Q system (Millipore Ltd., Mississauga, Ont., Canada). All other chemicals were of reagent grade.

Growth of organisms

The original clone carrying the 1,3-1,4- β -D-glucanase gene (Irvin & Teather, 1988) was isolated from a *B. succinogenes* S-85 gene bank cloned in *E. coli* (Crosby *et al.*, 1984). The 1,3-1,4- β -D-glucanase clone used in these experiments (pJ15) has been described elsewhere (Irvin & Teather, 1988). Large batches (60 litres) of cells were grown on LB medium [Tryptone (10 g/litre; Oxoid)/yeast extract (5 g/litre; Oxoid)/NaCl (10 g/litre)] at 37 °C in a 75-litre fermenter (New Brunswick Scientific, Edison, NJ, U.S.A.). When the culture-fluid absorbance at 420 nm reached 2, cells were concentrated on a Millipore Pellicon Cassette concentrator (Millipore, Mississauga, Ont., Canada) with a 10000 M_r cut-off membrane. Once cells and soluble proteins had been concentrated to 1 or 2 litres, the suspension was diluted with water (5 vol.) and re-concentrated. This procedure was repeated three times in all, and the final suspension was centrifuged (16000 g , 10 min) to remove bacterial cells. The aqueous extract was freeze-dried to serve as a potential source of enzyme.

Bacteroides succinogenes strain S-85 was grown in the defined medium 10 of Caldwell & Bryant (1966), as modified by Teather (1982a). A portion (1 ml) of a cell suspension stored in glycerol (Teather, 1982b), was inoculated into flasks containing 10 ml of medium with 0.2% milled filter paper (Smith *et al.*, 1973) as carbon source. The culture was scaled up through intermediate cultures in 500 ml serum bottles and a 6 litre bench-top fermenter (Virtis Co., Gardiner, NY, U.S.A.) to 60 litres in the 75-litre fermenter. Once all cellulose had been degraded (in the 75-litre fermenter), cells were harvested by continuous centrifugation (Cepa Model Z41 instrument; New Brunswick Scientific, Edison, NJ, U.S.A.). The culture supernatant was concentrated on a Millipore Pellicon Cassette concentrator (10000 M_r cut-off) and dialysed against 25 mM-potassium phosphate buffer, pH 6.8, containing 1 mM-DTT. The culture supernatant was further concentrated to 250 ml in a stirred cell (Amicon Corp., Lexington, MA, U.S.A.) by using a YM10 (10000 M_r cut-off) membrane. The concentrated culture supernatant was centrifuged at 146000 g (50 rotor; Spinco centrifuge Model L2-65B; Beckman Instruments, Palo Alto, CA, U.S.A.) for 2 h. The final supernatant was subsequently dialysed on the Minitan concentrator (Millipore) (10000 M_r cut-off) to change to 25 mM-Bistris buffer (pH 6.3 at 6 °C) for application to a chromatofocusing column.

Enzyme purification

The separation of the proteins by ion-exchange chromatography, hydroxyapatite chromatography and chromatofocusing, as well as the M_r estimation, were performed on an f.p.l.c. apparatus (Pharmacia). Sample injection was with a MV-7 sample injection valve,

protein was detected at 280 nm with a UV-2 dual-wavelength/dual-light-path monitor, and fractions were collected in a Frac-300 fraction collector. The details of each procedure are described in the legend to the corresponding Figures. Fractions containing 1,3-1,4- β -D-glucanase activity, assayed as described below by Congo Red binding, were combined, concentrated, and dialysed on a Minitan ultrafiltration system.

Enzyme assay

The hydrolysis of lichenin or oat β -D-glucan, as measured by the production of reducing sugar (Nelson, 1944) was used as the routine assay of activity during enzyme purification, as well as in studies of pH and temperature optimum, substrate specificity and enzyme kinetics on the purified enzyme. The reaction, initiated by addition of enzyme, was incubated for 30 min at 39 °C, unless otherwise stated. The reaction mixture consisted of substrate (3 mg/ml) in 1 ml of 25 mM-potassium phosphate buffer, pH 6.8. Enzyme addition was varied to produce an A_{540} of ≥ 1.0 in the assay. Glucose was used as a standard. A unit of activity in the reducing-sugar assay was that amount of enzyme which produced 1 μ mol of reducing sugar (glucose equivalent)/min.

Enzyme activity in column-chromatography fractions was assayed with the Congo Red binding assay (Wood *et al.*, 1988), with either lichenin or oat β -D-glucan as substrate for the cloned *E. coli* enzyme. In chromatofocusing experiments with *B. succinogenes* extracts, xylan was also used as substrate in the Congo Red binding assay, whereas β 1,3-1,4- β -D-glucanase and 1,4- β -D-glucanase were assayed with the reducing-sugar assay with oat β -D-glucan and CM-cellulose respectively.

Protein assays

Protein, in concentrated fractions, was assayed by the Lowry method (Lowry *et al.*, 1951) as modified by Miller (1959). Protein in column-chromatography fractions was estimated by u.v. absorption as described by Groves *et al.* (1968) or in fractions from chromatofocusing columns by the Coomassie Blue assay (Bradford, 1976). All protein methods used bovine serum albumin as standard.

Substrate specificity

The crude *E. coli* extract was assayed for activity toward soluble starch, CM-cellulose, CM-pachyman, xylan, laminarin, lichenin and oat β -D-glucan. Soluble starch (30 mg; Fisher Scientific, Nepean, Ont., Canada) was dissolved in 50 mM-Mes buffer, pH 6.5, containing 5 mM-Na₂S₂O₅ and the reaction started by addition of crude *E. coli* 1,3-1,4- β -D-glucanase (500 μ l, 1 mg/ml). The mixture was incubated at 40 °C for 20 h, diluted to 30 ml, and after centrifugation, aliquots (0.1 ml) were assayed for glucose by using glucose oxidase as described by McCleary & Glennie-Holmes (1985) and provided in the Biocon β -glucan assay kit (Biocon U.S.A., Lexington, KY, U.S.A.). Aliquots were also analysed by h.p.l.c. using a Bio-Rad HPX-42A column maintained at 85 °C and eluted with distilled deionized water at a flow rate of 0.4 ml/min. Sugars were detected by an automated orcinol/H₂SO₄ method (P. J. W. will supply full details on request).

CM-cellulose and CM-pachyman were dissolved to give 0.5% (w/v) solutions in 50 mM-Mes buffer, pH 6.5,

containing 5 mM- NaN_3 . The crude *E. coli* enzyme (100 μl , 1 mg/ml) was added to 1 ml aliquots of substrate and the viscosity loss determined in Cannon–Manning Ubbelohde-type U-tube viscometers in which the efflux time of solvent was about 7.5 s.

The specificity of the crude, as well as the purified, enzyme was also determined, using the Nelson (1944) reducing-sugar assay described above, with CM-cellulose, laminarin, xylan, lichenin and oat β -D-glucan.

M_r determination

The M_r of the purified 1,3-1,4- β -D-glucanase was determined on a Superose 12 HR 10/30 column (Pharmacia). The void volume of the column (V_0) was estimated with Blue Dextran eluted with 0.1 M-NaCl in 25 mM-potassium phosphate buffer, pH 6.8. M_r standards used were aprotinin (M_r 6500), cytochrome *c* (M_r 12400), carbonic anhydrase (M_r 29000), bovine serum albumin (M_r 66000), alcohol dehydrogenase (M_r 150000), and β -amylase (M_r 200000). Standards and samples (injected in a total volume of 200 μl) were run in triplicate. Protein in the eluate was detected by u.v. absorption at 280 nm to obtain an estimate of V_e/V_0 .

Molecular weights were also determined by polyacrylamide-gel electrophoresis [10% (w/v) acrylamide] using the discontinuous SDS system of Laemmli & Favre (1973). The gels were fixed and stained in methanol/acetic acid/water (5:1:5, by vol.) containing 0.1% Coomassie Brilliant Blue R250, and destained in methanol/acetic acid/water (5:7:88, by vol.). M_r standards were β -lactalbumin (M_r 18400), soybean trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 29000), ovalbumin (M_r 45000), bovine serum albumin (M_r 66000), and phosphorylase *b* (M_r 97400).

Determination of temperature optimum

(a) **Crude enzyme.** The temperature optimum of the crude enzyme was studied at pH 6.0 using viscometry as described below. Assays (in duplicate) contained 25 μl aliquots of crude enzyme solution (0.1 mg/ml). Initial values (i.e. the first 6 min) of $d/dt(1/\eta_{sp.})$ (where $\eta_{sp.}$ is specific viscosity) were used for the 60 °C incubations, since the rate of change was decreasing by 10 min.

The stability of the crude enzyme was tested viscometrically at pH 6.0 using 25 μl of crude enzyme (0.1 ml/ml) held at 60 °C. Aliquots of enzyme were removed at intervals and incubated with 0.5% oat β -D-glucan at 37 °C. Activity was calculated as $d/dt(1/\eta_{sp.})$.

(b) **Purified enzyme.** Reaction mixtures were set up in triplicate with 0.014–0.019 unit of purified β -D-glucanase activity and with either lichenin or oat β -D-glucan (3 mg/ml) as substrate. Reaction mixtures were agitated in a shaking water bath over the temperature range 25–60 °C (5 °C increments) for 30 min. Activities were determined by the reducing-sugar assay and are expressed as a percentage of the maximum activity observed for each substrate.

Determination of pH optimum

(a) **Crude enzyme.** The activity of the crude enzyme preparation was examined in the pH range 4.5–7.5 by using oat β -D-glucan as substrate. Activity was determined both by reducing-sugar assay and by viscometry. Oat β -D-glucan was dissolved in water to give a 1%

solution, which was filtered through a coarse sintered-glass filter. The solution was maintained at 37 °C, and 0.5 ml aliquots mixed with buffer (0.2 M-sodium acetate, pH 4.5, 5.0 and 5.5, and 0.2 M-sodium/potassium phosphate, pH 6.0, 6.5, 7.0 and 7.5), also at 37 °C, then crude enzyme (0.1 mg/ml, dissolved in the appropriate buffer) was added. Portions (50, 25 or 10 μl) of enzyme solution were added, and efflux times were determined in a Cannon–Manning semi-micro Ubbelohde-type viscometer at 37 °C over approx. 12 min. Activity was determined as $d/dt(1/\eta_{sp.})$ with zero time taken as the moment of mixing. [Plots of $1/\eta_{sp.}$, reciprocal specific viscosity or fluidity, against time were linear, $r \geq 0.99$, and activity per unit weight of enzyme was determined from plots of $d/dt(1/\eta_{sp.})$ against the amount of enzyme used ($r \geq 0.99$)].

Reducing-sugar assays were carried in a similar fashion. Oat β -D-glucan (1% in water; 1.5 ml) was added to 1.5 ml of each buffer (0.1 M), crude 1,3-1,4- β -D-glucanase (10 μl , 1 mg/ml in each buffer) was added to each solution and, after 30 min at 37 °C, duplicate aliquots (1 ml each) were removed and the reaction stopped by dilution in 1 ml of 0.1 M- Na_2CO_3 in an ice bath. A control incubation contained no enzyme. Production of reducing sugar (as glucose) was determined by the above-mentioned automated ferricyanide method.

(b) **Purified enzyme.** Incubation mixtures contained 25 mM-sodium acetate, sodium malonate or sodium phosphate buffer to cover the range of pH from 5.00 to 7.75, with lichenin or oat β -D-glucan as substrate. Incubations (in triplicate), with 0.007–0.014 unit of activity/tube, were for 30 min at 40 °C. Activity (reducing-sugar production) is expressed as percentage of maximum to provide a comparison between experiments.

Enzyme kinetics

Triplicate incubations containing 0.005–0.010 unit of enzyme activity in 25 mM-sodium phosphate buffer, pH 6.5, were incubated at 50 °C for 30 min with crude substrate concentrations (lichenin or oat β -D-glucan) ranging from 0.1 to 5.0 mg/ml, followed by determination of reducing sugar produced. Triplicate no-enzyme blanks at each substrate concentration corrected for reducing-sugar reaction of the substrates. Data (means for three observations/substrate concentration) were analysed for K_m by the statistical method described by Cleland (1967). Values for K_m for each substrate were corrected for mixed-linkage β -D-glucan content (i.e. 57% for lichenin and 78% for oat glucan).

Product identification

Large-scale incubations (10 ml; 3 mg of substrate/ml of 10 mM-ammonium formate, pH 6.8 and 0.1 unit of enzyme) with either lichenin or oat β -D-glucan as substrate were incubated at 39 °C with agitation for 2 or 4 h. Reactions were stopped by heating in an autoclave for 15 min at 120 °C. After centrifugation (12000 *g* for 10 min) duplicate aliquots were assayed for reducing sugars as described above. The remaining sample was freeze-dried, redissolved in 0.5 ml of water and filtered with a 0.45 μm -pore-size Millex-HV4 filter (Millipore). Products were separated on Bakerflex cellulose sheets (J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.) with a solvent system of ethyl acetate/pyridine/water

(10:4:4, by vol.). Solvent was allowed to migrate to the top of the plate, and, after drying the sheet, the procedure was repeated three more times. Cellodextrin standards and reaction products were located by spraying with aniline phthalate and then heating at 105 °C for 20 min. Products were quantified by h.p.l.c. using a Waters carbohydrate column (no. 84038). Elution was with acetonitrile/water (3:1, v/v) at 30 °C at a flow rate of 1 ml/min for 5 min, followed by a linearly increased flow to 2 ml/min for an additional 3 min. Quantification was by peak area using glucose as a standard, and detection of peaks was by refractive index (model R401 differential refractometer; Waters).

RESULTS AND DISCUSSION

Assay of reducing-sugar production by the crude *E. coli* enzyme with CM-cellulose, xylan, laminarin and lichenin or oat β -D-glucan as substrates demonstrated that the enzyme was truly a mixed-linkage β -D-glucanase, since neither β -1,4 (i.e. CM-cellulose and xylan)- nor β -1,3-(laminarin)-linked polysaccharides were hydrolysed by the enzyme. The specificity of the crude *E. coli* enzyme preparation was confirmed by viscometric assays for hydrolysis of CM-cellulose and CM-pachyman: the viscosity of the substrates remained unchanged after 1.5 h in the presence of 10 times the amount of enzyme that was sufficient to decrease the efflux time of a 0.5% solution of oat β -D-glucan to that of water. The crude enzyme preparation also showed a complete lack of amylase activity when soluble starch was used as substrate.

The activities of the crude *E. coli* enzyme with lichenin (6.5 units/mg of protein) or oat β -D-glucan (6.1 units/mg of protein) were not substantially different. Since this was the case, the two substrates were used interchangeably for routine enzyme assays, depending upon their availability. Because of certain structural differences between lichenin and oat β -D-glucan (Wood, 1986), both substrates were used for purposes of comparison in experiments on pH and temperature optimum and for kinetics and product formation.

The activity of the high-speed supernatant of *B. succinogenes* culture fluid, with lichenin, oat β -D-glucan and CM-cellulose, was 0.59, 1.20 and 0.95 units/mg of protein respectively. These specific activities, with lichenin and oat β -D-glucan, are only about 10 or 20% of those of the crude enzyme extract of the pJ15 clone.

Enzyme purification

The 1,3-1,4- β -D-glucanase produced in *E. coli* was absorbed on an anion-exchange resin (Q-Sepharose; Pharmacia) at pH 7.8 and eluted before the curvilinear NaCl gradient had started (Fig. 1). Although the major portion of the activity (about 80%) appeared in one peak, three additional minor peaks of activity were also eluted. Fractions containing the major active peak plus a small trailing peak (i.e. eluant volume from 240 to 720 ml) were combined, concentrated and exchanged into 10 mM-potassium phosphate buffer, pH 7.8, on a tangential flow concentrator (Minitan) and then applied to a hydroxyapatite column (Fig. 2). The two peaks of activity obtained from the Q-Sepharose column (Fig. 1) were resolved on hydroxyapatite (Fig. 2) and the major enzyme component appeared to be associated with a single protein peak. A summary of the enzyme puri-

Fig. 1. Purification of cloned 1,3-1,4- β -D-glucanase on a 2.6×37 cm column of Q-Sepharose (Fast Flow)

A 10 ml portion (183 mg of protein) of cell extract was injected on to the column and eluted (4 ml/min) with a NaCl gradient (—) in 25 mM-potassium phosphate buffer, pH 7.8, containing 1 mM-DTT. The 1,3-1,4- β -D-glucanase activity ($\blacktriangle$) was determined by Congo Red binding with lichenin as substrate, and protein ($\circ$) was determined by u.v. absorbancy on fractions as described by Groves *et al.* (1968).

fication (Table 1) demonstrates that about a 600-fold purification of the major component was achieved. The recovery of enzyme activity from the hydroxyapatite column appears to indicate a stimulation of activity. Concentration of fractions containing the major active peak (elution volume 420–940 ml) to a volume of 50 ml (Minitan concentrator) resulted in recovery of 120% of the activity compared with the starting material. Further concentration of this fraction (to 5 ml) in a stirred cell (Amicon) resulted in recovery of only 36% of the protein placed in the cell, but activity was increased 4-fold relative to the starting activity. The reason(s) for this apparent activation or removal of inhibition is (are) unknown.

The pH of elution of the purified 1,3-1,4- β -D-glucanase from *E. coli* from a chromatofocusing column was 4.7 (results not shown). Chromatofocusing of the crude *E. coli* enzyme (Fig. 3) gave an elution pH of 4.88 (mean for four experiments) for the major active component. One minor component was eluted at 5.54, and a third component at < 4.1 was eluted from the PBE 94 column when NaCl was applied. The multi-component expression of the enzyme cloned in *E. coli* may have resulted from post-translational changes. *E. coli* can readily degrade abnormal proteins (Goldberg & Dice, 1974), and this process may have resulted in the components with a somewhat different net charge and lower M_r which we observed, or the protein may possess a signal sequence that is recognized by the *E. coli* protein-export system, albeit inefficiently. Three active bands with different M_r values were also observed by Taylor *et al.* (1987) when a 1,4- β -D-glucanase from *B. succinogenes* was cloned in *E. coli*.

Fig. 2. Purification of the cloned 1,3-1,4- β -D-glucanase on a 2.6 cm $\times$ 80 cm column of high-resolution hydroxyapatite

A portion (35 mg) of protein (representing the major active peak) from several runs on a Q-Sepharose column was applied to hydroxyapatite in 10 mM-potassium phosphate buffer, pH 7.8, containing 1 mM-DTT. Elution (3 ml/min) was with a gradient of 10–300 mM-potassium phosphate (—). Protein (○) was estimated by absorption at 280 nm, and enzyme activity (▲) was determined by Congo Red binding with lichenin as substrate.

Table 1. Purification of 1,3-1,4- β -D-glucanase produced in *E. coli*

A unit of activity is the production of 1 μ mol of reducing sugar/min.

Fraction	Volume (ml)	Protein (mg)	β -D-Glucanase activity (units)		Recovery (%)
			Total	Specific	
Cell extract	40	732.0	1.65×10^3	2	100
Q-Sepharose fraction	45	34.7	1.14×10^3	32.8	69
Hydroxyapatite fraction	5	4.9	6.72×10^3	1372.3	400

A β -D-glucanase specific for 1,3-1,4- β -D-glucans had not been previously demonstrated to exist in *B. succinogenes*. Therefore the crude soluble culture fluid from this organism was also subjected to chromatofocusing under the conditions used for the *E. coli* enzyme (Fig. 4). Column fractions were assayed for 1,3-1,4- β -D-glucanase (oat β -D-glucan substrate), 1,4- β -D-glucanase (CM-cellulose substrate) and xylanase (oat spelt's xylan) activities. The pH of elution for the 1,3-1,4- β -D-glucanase activity in *B. succinogenes* was 4.76 (mean for four experiments), whereas that for the 1,4- β -D-glucanase activity (measured with CM-cellulose) was the same (4.79; mean for four experiments). Since oat β -D-glucan (and other mixed-linkage glucans) are degraded by 1,4- β -D-glucanases, this does not demonstrate the presence of the 1,3-1,4- β -D-glucanase in *B. succinogenes*. However, the fact that the 1,4- β -D-glucanase is eluted at the pH where the 1,3-1,4- β -D-glucanase would be expected to be eluted would explain why the 1,3-1,4- β -D-glucanase has not been previously identified as a distinct enzyme activity. The

similarity in pH of elution from a chromatofocusing column for the 1,3-1,4- β -D-glucanase and the 1,4- β -D-glucanase activities suggests that these enzymes would not be readily resolved when applying conventional techniques (i.e. ion-exchange chromatography or electrophoresis) to purification of crude extracts of *B. succinogenes*. The pH of elution of the xylanase in *B. succinogenes* appears sufficiently distinct from that of the β -D-glucanases to permit ready separation.

Properties of the cloned 1,3-1,4- β -D-glucanase

The pH optimum of the crude enzyme, with oat glucan as substrate, was broad and had a maximum activity between pH 5.5 and 6.0. Even at the extremes of the pH values tested (i.e. 4.5 and 7.5) the activity did not fall below 80% of the maximum activity observed (results not shown). The pH optimum of the purified enzyme (1372 units/mg of protein) was very broad with both substrates (Fig. 5), but tended to be maximal between pH 5.75 and 6.25. There was a rapid decrease in activity

Fig. 3. Determination of pH of elution of a crude preparation of the cloned 1,3-1,4- β -D-glucanase on PBE 94 exchanger

PBE 94 was equilibrated with 25 mM-Bistris buffer (pH 6.3 at 6 °C; 10 column volumes) in a 10/30 HR column (Pharmacia). Elution (0.8 ml/min) was with Polybuffer 74 diluted 1:8 (v/v) with water and adjusted to pH 4.0 (6 °C) with HCl. From 230 to 330 ml elution buffer was changed with a linear gradient to 1 M-NaCl in 25 mM-Bistris buffer, pH 6.3. The pH (●) was measured in alternate fractions with a glass combination electrode. Protein (○) was determined by Coomassie Blue binding (Bradford, 1976), and 1,3-1,4- β -D-glucanase activity (▲) was measured by Congo Red binding, oat β -D-glucan being used as a substrate.

below pH 5.5 with lichenin as substrate. Additional experiments confirmed this observation. The pH optimum is similar to that observed for a mixed-linkage β -D-glucanase from *Bacillus subtilis* (pH 6.5) (Huber & Nevin, 1977) and to that of a 1,4- β -D-glucanase (CM-cellulose) from *B. succinogenes* (Huang & Forsberg, 1987).

There was a rapid increase in activity of the crude enzyme from 35 to 60 °C when measured by the viscometric assay (results not shown). At 70 °C the rate of change of fluidity was close to zero, but initial efflux times showed that considerable viscosity loss had taken place before the first measurement was completed (i.e. in 1 min). Enzyme-stability measurements with the crude enzyme at 60 °C demonstrated a 50% loss in activity in 10 min. The activity of the purified enzyme, as measured by reducing-sugar production during a 30 min incubation, was maximum at 50 °C with both substrates when measured at pH 6.5 (Fig. 6). In agreement with the temperature-stability measurements on the crude enzyme, activity over 30 min was only 20% of maximum at 60 °C, suggesting a rather rapid inactivation above the temperature optimum.

Kinetic experiments were run at the temperature and pH optima. The K_m was found to be 0.35 and 0.71 mg/ml for lichenin and oat β -D-glucan respectively (Fig. 7). The K_m for both substrates was substantially

lower than that of the mixed-linkage β -D-glucanase ($K_m = 3.4$ mg/ml) isolated from *B. subtilis* (Huber & Nevin, 1977).

The M_r estimates of the major active component were 37200 and 35200 as determined by gel filtration on a Superose 12 H/R column and by SDS/polyacrylamide-gel electrophoresis respectively. These estimates are in reasonable agreement with the expected M_r based on the size of the DNA fragment cloned in *E. coli* (Irvin & Teather, 1988) and show that the active enzyme probably contains a single polypeptide.

Reaction products and nature of bond cleavage

The reaction products of the cloned mixed-linkage β -glucanase with lichenin and oat β -glucan (Table 2) were studied by h.p.l.c. and t.l.c. With lichenin as substrate the major product (82 mol %) was a triose (perhaps 3-*O*- β -cellobiosyl-D-glucose; Reese & Perlin, 1963), which was eluted on h.p.l.c. or migrated on t.l.c. just ahead of cellobiose. Minor products included a component (9.5 mol %) with a retention time (h.p.l.c.) somewhat less than that of cellobiose. Other minor components included cellobiose and a tetraose. The major products from oat β -D-glucan hydrolysis were a triose (63.5 mol %) and a tetraose (29.6 mol %). This elution pattern of the products of the cloned enzyme with oat glucan was identical with that observed with the 1,3-1,4- β -D-

Fig. 4. Determination of pH of elution of *B. succinogenes* 1,3-1,4- β -D-glucanase, 1,4- β -D-glucanase and xylanase activities in a crude centrifuged culture supernatant on PBE 94 exchanger

Conditions were identical with those described in Fig. 3, except that enzyme activities were measured in the reducing-sugar assay with oat β -D-glucan (▲) and CM-cellulose (□) as substrates. Xylanase activity (▽) was measured with the Congo Red binding assay. Protein (○) and pH (●) were measured as in Fig. 3.

Fig. 5. pH optimum of 1,3-1,4- β -D-glucanase with lichenin (▲) or oat β -D-glucan (●) as substrate

Details of the procedure are given in the text.

glucanohydrolase from *B. subtilis*. The products from both substrates are probably the same as those reported for the enzyme from *B. subtilis* (Huber & Nevin, 1977). From these results and the known structure of the substrates, the cloned enzyme would seem to be similar to other 1,3-1,4- β -D-glucanases in its general pattern of action (Reese & Perlin, 1963): that is, the hydrolysis site in the substrate is probably a β -1,4 glucopyranosidic linkage of a 3-*O*-substituted β -D-glucopyranose unit.

General conclusions

The cloned 1,3-1,4- β -D-glucanase was shown to be a mixed-linkage-specific β -D-glucanase by virtue of its substrate specificity. Comparison of the cloned enzyme with the 1,3-1,4- β -D-glucanase and the 1,4- β -D-glucanase activities in *B. succinogenes* by chromatofocusing revealed that all three enzymes probably have the same pI. The general physical properties of the cloned enzyme are very similar to those of a 1,4- β -D-glucanase isolated from *B. succinogenes* by Huang & Forsberg (1987).

The pH and temperature optimum and the M_r are almost identical for these two enzymes. These similarities in properties provide one explanation of why resolution of the two enzyme activities from *B. succinogenes* has not been achieved.

Fig. 6. Temperature optimum of 1,3-1,4- β -D-glucanase with lichenin (▲) or oat β -D-glucan (●) as substrate

Details of the procedure are given in the text.

Fig. 7. Effect of lichenin (▲) or oat β -D-glucan (●) concentration on the activity of the purified cloned 1,3-1,4- β -D-glucanase

Details of the assays are given in the text.

Table 2. Reaction products of 1,3-1,4- β -D-glucanase action on lichenin or oat β -D-glucan as substrate

Details of the incubation and isolation procedure for products from cloned 1,3-1,4- β -D-glucanase action are described in the text.

Substrate	Time (h)	Saccharide ...	Proportion (mol %)			
			Di-	Tri-	Tetra-	Penta-
Lichenin	2		4.1	83.0	3.2	9.8
	4		6.7	80.7	3.4	9.2
	Mean ...		5.4	81.8	3.3	9.5
Oat β -D-glucan	2		3.4	64.8	28.2	3.6
	4		4.1	62.1	30.9	2.9
	Mean ...		3.8	63.5	29.6	3.3

The catalytic properties of the cloned 1,3-1,4- β -D-glucanase appear to be similar to those of the mixed-linkage β -D-glucanase isolated from *B. subtilis* (Huber & Nevin, 1977), although the K_m differs markedly. Because of the high specific activity of the cloned enzyme and the absence of other glucanase activities, this enzyme, even in the unpurified state, provides a potentially powerful tool to establish the structure of mixed-linkage polymeric β -glucans.

The appearance of minor 1,3-1,4- β -D-glucanase components which we observed in crude extracts of *E. coli* seems to have occurred when other glucanases were cloned in this bacteria (Taylor *et al.*, 1987). The occurrence of proteolytic enzymes which degrade foreign protein in *E. coli* is well established (Goldberg & Dice, 1977), and this process could explain this apparent artefact. It is also possible that the gene possesses a leader sequence that is recognized by the protein-export system of *E. coli*. It has been suggested that post-translational changes, such as proteolytic cleavage of fragments from some of the enzymes of the cellulase complex, could provide a specific and deliberate mechanism by which specificity and total activity of these enzymes is directed and controlled (Knowles *et al.*, 1987). Further work is necessary to determine whether the multiple glucanase components observed in the *E. coli* clone result from the operation of such a mechanism.

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REFERENCES

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Caldwell, D. R. & Bryant, M. P. (1966) *Appl. Microbiol.* **13**, 794-801
- Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **28**, 1-32
- Crosby, B., Collier, B., Thomas, D. Y., Teather, R. M. & Erfle, J. D. (1984) in *Fifth Canadian Bioenergy R & D Seminar* (Hasnain, S., ed.), pp. 573-576, Elsevier Applied Science Publishers, London

- Forsberg, C. W., Beveridge, T. J. & Hellstrom, A. (1981) *Appl. Environ. Microbiol.* **42**, 886–896
- Goldberg, A. L. & Dice, J. F. (1974) *Annu. Rev. Biochem.* **43**, 835–869
- Groleau, D. & Forsberg, C. W. (1981) *Can. J. Microbiol.* **27**, 517–530
- Groleau, D. & Forsberg, C. W. (1983) *Can. J. Microbiol.* **29**, 504–517
- Groves, W. E., Davis, F. C., Jr. & Sells, H. B. (1968) *Anal. Biochem.* **22**, 195–210
- Huang, L. & Forsberg, C. W. (1987) *Appl. Environ. Microbiol.* **53**, 1034–1041
- Huber, D. J. & Nevin, D. J. (1977) *Plant Physiol.* **60**, 300–304
- Irvin, J. & Teather, R. M. (1988) *Appl. Environ. Microbiol.*, in the press
- Knowles, J., Lehtovaara, P. & Teeri, T. (1987) *Tibtech.* **5**, 255–261
- Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McCleary, B. V. & Glennie-Holmes, M. (1985) *J. Inst. Brew.* **91**, 285–290
- Miller, G. L. (1959) *Anal. Chem.* **31**, 964
- Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380
- Ng, T. K. & Zeikus, J. G. (1981) *Biochem. J.* **199**, 341–350
- Reese, T. E. & Perlin, A. S. (1963) *Biochem. Biophys. Res. Commun.* **12**, 194–197
- Saddler, J. N. & Khan, A. W. (1980) *Can. J. Microbiol.* **26**, 760–765
- Schellhorn, H. E. & Forsberg, C. W. (1984) *Can. J. Microbiol.* **30**, 930–937
- Sipat, A., Taylor, K. A., Lo, R. Y. C., Forsberg, C. W. & Krell, P. J. (1987) *Appl. Environ. Microbiol.* **53**, 477–481
- Smith, W. R., Yu, I. & Hungate, R. E. (1973) *J. Bacteriol.* **114**, 729–737
- Taylor, K. A., Crosby, W., McGavin, M., Forsberg, C. W. & Thomas, D. Y. (1987) *Appl. Environ. Microbiol.* **53**, 41–46
- Teather, R. M. (1982*a*) *Appl. Environ. Microbiol.* **43**, 298–302
- Teather, R. M. (1982*b*) *Appl. Environ. Microbiol.* **44**, 499–501
- Wood, P. J. (1986) in *Oats: Chemistry and Technology* (Webster, F. H., ed.), pp. 121–152, American Association of Cereal Chemists, St. Paul, MN
- Wood, P. J., Erfle, J. D. & Teather, R. M. (1988) *Methods Enzymol.* **160**, 59–74

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