Purification and characterization of alkaline endo-1,4- β -glucanases from alkalophilic *Bacillus* sp. KSM-635

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Two carboxymethylcellulases (CMCase, 1,4- β -D-glucan glucanohydrolase, EC 3.2.1.4), designated E-H and E-L, were purified to homogeneity from a culture filtrate of the alkalophilic *Bacillus* sp. KSM-635, by chromatography on DEAE-Toyopearl 650S and gel filtration on Bio-Gel A-0.5m. The purified CMCases both contained approximately 2–3% (w/w) glucosamine. Molecular masses deduced from SDS-PAGE were 130 kDa for E-H and 103 kDa for E-L. The pH optima of the enzymes were both about 9.5, and their optimum temperatures were around 40 °C. Activities of both enzymes were inhibited by Hg^{2+} , Cu^{2+} , Fe^{2+} and Fe^{3+} , but sulphhydryl inhibitors, such as *N*-ethylmaleimide, monoiodoacetate and 4-chloromercuribenzoate, had either no effect or a slightly inhibitory effect. *N*-Bromosuccinimide was strongly inhibitory, suggesting that a tryptophan residue is essential for the activity of the CMCases from *Bacillus*. In addition, the activities of both E-H and E-L were stimulated by Co^{2+} , and they required Mg^{2+} , Ca^{2+} , Mn^{2+} or Co^{2+} for stabilization. Both enzymes efficiently hydrolysed carboxymethylcellulose (β -1,4-linkage) and lichenan (β -1,3; 1,4-linkage), but crystalline cellulosic substrates, curdlan (β -1,3-linkage), laminarin (β -1,3; 1,6-linkage) and 4-nitrophenyl- β -D-glucopyranoside were hydrolysed very little, if at all. 4-Nitrophenyl- β -D-cellobioside was hydrolysed by both enzymes to liberate 4-nitrophenol, and their hydrolysis rates were higher at neutral pH than at alkaline pH.

Introduction

The industrial application of cellulases has been recognized mainly in terms of their potential for use in the saccharification of cellulosic materials, and much attention has been focused on *Trichoderma* (e.g. Mandels, 1982; Berghem *et al.*, 1976; Beldman *et al.*, 1985) and other cellulolytic fungi (Kubo & Nisizawa, 1983; Rao *et al.*, 1986). Several bacteria have also been studied for their ability to produce cellulolytic enzymes, and these bacteria include *Clostridium* (Ng & Zeikus, 1981; Creuzet *et al.*, 1983), *Cellulomonas* (Nakamura & Kitamura, 1983), *Ruminococcus* (Halliwell & Bryant, 1963; Gardner *et al.*, 1987) and *Cytophaga* (Chang & Thayer, 1977). Most of the information on the properties of cellulases and the mechanisms of their activity has come from studies of cellulases from these organisms. Many authors have also reported that members of the

genus *Bacillus* produce cellulases and hemicellulases in culture (Fogarty *et al.*, 1974; Priest, 1977), but to date, little is known about the production and the properties of *Bacillus* enzymes that hydrolyse carboxymethylcellulose (CMC) (Tewari & Chahal, 1977; Horikoshi *et al.*, 1984; Dhillon *et al.*, 1985; Chan & Au, 1987), and most of these enzymes are not capable of hydrolysing crystalline forms of cellulose.

Recently, we isolated several strains of *Bacillus* that produce alkaline carboxymethylcellulases (CMCase, EC 3.2.1.4), the properties of which fulfil the essential requirements for enzymes to be used in laundry detergents (Kawai *et al.*, 1988; Ito *et al.*, 1989; Shikata *et al.*, 1990). In this report, we describe a detailed study of alkaline CMCases from one of the isolates, alkalophilic *Bacillus* sp. KSM-635 (Ito *et al.*, 1989). This study was undertaken not only to enhance our immediate understanding of such enzymes, but also to provide a foundation for future investigations of the structure, mechanism of action and biosynthesis of the enzymes. For these purposes, we purified and characterized the alkaline CMCases found in cultures of *Bacillus* sp. KSM-635.

Abbreviations: CMC, carboxymethylcellulose; CMCase, carboxymethylcellulase; NPC, 4-nitrophenyl β -D-cellobioside; NPG, 4-nitrophenyl β -D-glucopyranoside.

Methods

Organism. The organism used was *Bacillus* sp. KSM-635. A mutant form of this organism is currently used for the industrial production of an alkaline CMCase which serves as an effective additive in laundry detergents (Ito *et al.*, 1989).

Enzyme assays. Cellulase activity was routinely assayed by measuring the rate of formation of reducing sugars from CMC (A01MC; degree of substitution 0.68; Sanyo Kokusaku Pulp) as a substrate, in 0.1 M-glycine/NaOH (pH 9.5) as the reaction buffer (Ito *et al.*, 1989). The reducing sugars liberated from CMC were quantified as glucose by the 3,5-dinitrosalicylic acid procedure (Miller *et al.*, 1960). Assays for cellulolytic activity against filter paper, Avicel, swollen celluloses, cellobiose, lichenan (from *Cetraria islandica*, Sigma), curdlan (from *Alcaligenes faecalis* var. *mixogenes*, Wako Pure Chemical), laminarin (from *Laminaria digitata*, Sigma), 4-nitrophenyl β -D-glucopyranoside (NPG, Sigma), and 4-nitrophenyl β -D-cellobioside (NPC, Sigma) were performed according to published procedures (Kawai *et al.*, 1988; Ito *et al.*, 1989). The value of each enzymic activity reported is the mean of three or more experiments. Protein was determined by the procedure of Bradford (1976) with bovine plasma albumin as protein standard and a Bio-Rad protein assay kit (Bio-Rad). One unit (U) of enzyme activity was defined as the amount of protein that produces 1.0 μ mol of product min^{-1} under the standard assay conditions.

Electrophoretic analysis. PAGE was performed on 12% (w/v) polyacrylamide slab gels (70 mm $\times$ 80 mm, 1 mm thickness) by the method of Davis (1964) with 5 mM-Tris/38 mM-glycine (pH 8.3) as the running buffer. SDS-PAGE was also carried out on slab gels (Laemmli, 1970) after the enzyme preparation had been heated at 95 °C for 5 min in 62.5 mM-Tris/HCl buffer (pH 6.8) that contained (w/v) 2% SDS/10% glycerol/5% 2-mercaptoethanol. The running buffer used was 25 mM-Tris/glycine buffer (pH 8.3) that contained 0.1% (w/v) SDS. Proteins were visualized by silver staining (Oakley *et al.*, 1980).

Active staining of CMCase activity with Congo red dye. Protein bands associated with CMCase activity were visualized by staining with Congo red after PAGE, by laying the slab gels at 30 °C for 30 min on top of a CMC-agar sheet, essentially according to the method of Béguin (1983). The CMC-agar sheet (5 mm thickness) was composed of 2% (w/v) CMC, 3% (w/v) NaCl, 0.1 M-glycine/NaOH buffer (pH 9) and 0.8% (w/v) agar (Difco). This sheet was stained with 0.1% (w/v) Congo red solution for 15 min and then destained for 15 min in 1.0 M-NaCl solution, at room temperature. The protein bands with CMCase activity were located as clear zones on the sheet of red background.

Preparation of cultures. *Bacillus* sp. KSM-635 was grown in a medium that contained (w/v): 1.5% meat extract (Lab-Lemco powder, Oxoid), 0.4% yeast extract (Difco), 1.5% sucrose, 0.07% Na_2SO_4 , 0.015% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.5% Na_2CO_3 . Calcium chloride and Na_2CO_3 were autoclaved separately. The organism was cultivated with shaking at 30 °C for 2 d in 50 ml aliquots of medium in 500 ml flasks. Cells were removed by centrifugation, and the supernatant obtained was used as the cell-free culture medium.

Purification of two forms of alkaline CMCase. Purification was done at 4 °C. The cell-free supernatant from a 3 l culture was concentrated by ultrafiltration on a PM-10 membrane (10 kDa cutoff, Amicon) and then dialysed overnight against 10 mM-Tris/HCl buffer (pH 7.5) containing 5 mM- CaCl_2 (buffer A). The dialysate was applied to a column of DEAE-Toyopearl 650S (4.4 cm $\times$ 40 cm; Tosoh) equilibrated with buffer A containing 0.2 M-NaCl. The adsorbed materials were eluted with a 6 l linear gradient of 0.2 to 0.4 M-NaCl in the equilibrating

buffer; 10 ml fractions were collected at a flow rate of 40 ml h^{-1} . The fractions corresponding to the peak of CMCase activity were eluted at 250 mM-NaCl. The active fractions (tubes nos 480–560) were combined, and then concentrated by ultrafiltration (PM membrane). The concentrate was divided into portions, and each portion was chromatographed sequentially on a column of Bio-Gel A-0.5m (2.5 cm $\times$ 90 cm; Bio-Rad) equilibrated with buffer A containing 0.1 M-NaCl. Elution was carried out with the equilibrating buffer at a flow rate of 20 ml h^{-1} . Fraction size was 4 ml. In some of these fractions, CMCase activity was checked by both protein and activity staining after nondenaturing PAGE. Two peaks of CMCase activity were eluted from the Bio-Gel A-0.5m column and the active fractions with high specific activity (tubes nos 60–70 and 74–80) were pooled separately and used as the final preparations of the enzymes.

Determination of molecular masses. Molecular masses were estimated by gel filtration on a column of Bio-Gel A-1.5m (1.5 cm $\times$ 70 cm; Bio-Rad) with buffer A containing 0.1 M-NaCl as the equilibrating and elution buffer. The eluate was collected in 1 ml fractions at a flow rate of 4.4 ml h^{-1} . The purified enzymes and marker proteins were chromatographed separately on the defined column. The molecular mass markers (Pharmacia) contained the following proteins (molecular masses are given in Da): bovine thyroglobulin (669 000); horse spleen ferritin (440 000); bovine liver catalase (232 000); rabbit muscle aldolase (158 000); bovine serum albumin (67 000); hen egg ovalbumin (43 000); bovine pancreas chymotrypsinogen A (25 000). Molecular masses were also determined by SDS-PAGE under the conditions described above on 12% polyacrylamide slab gels. The marker proteins (Bio-Rad), were as follows (molecular masses in Da): rabbit skeletal muscle myosin (200 000); *Escherichia coli* β -galactosidase (116 250); rabbit muscle phosphorylase b (97 400); bovine serum albumin (66 200); hen egg white ovalbumin (42 699); bovine carbonic anhydrase (31 000); soybean trypsin inhibitor (21 500).

Chromatographic analysis of the products of hydrolysis of cello-oligosaccharides. Excess purified enzyme (30 mU) was incubated with 0.5 mg cello-oligosaccharide, namely, cellobiose (G2) through cellobiose (G6) (Seikagaku Kogyo), at 30 °C for 12 h in 50 mM-pyrophosphate buffer (pH 9.0). The reactions were stopped by the addition of ethanol. The products of hydrolysis were analysed by ascending chromatography on Whatman no. 1 filter paper, in a solvent system of *n*-butanol/pyridine/water (6:4:3, by vol.). After one to four ascents of solvent at room temperature, the chromatogram was developed by an alkaline acetone/silver nitrate dip procedure (Trevelyan *et al.*, 1950).

Determination of carbohydrate. Each purified enzyme was hydrolysed by treatment with 2.2 M-trifluoroacetic acid at 100 °C for 7 h, reduced by NaBH_4 and then treated with anhydrous acetic acid at 100 °C for 30 min, according to the alditol-acetate procedure (Björndal *et al.*, 1970). Gas-chromatographic determination of sugar alcohols in the preparations was carried out at 200 °C with He as the flow gas (60 ml min^{-1}), a 3% (w/v) ECNSS-M column (4 mm $\times$ 2 m; Gasukuro Kogyo) and a flame-ionization detector (300 °C). The carbohydrate (as glucosamine) was also estimated by the phenol/sulphuric acid procedure (Hodge *et al.*, 1962) and by the Morgan-Elson procedure (Levy & McAllan, 1959).

Divalent cation assays. Each preparation of enzyme (4 ml) was treated with 10 mM-EDTA and 10 mM-EGTA at 5 °C for 12 h prior to the assay. The treated solution was diluted to 40 ml by addition of 20 mM-glycine/NaOH buffer (pH 9.0), and then concentrated to 2 ml in a centrifuge tube concentrator (Centricon-10, Amicon). A divalent cation was added at a final concentration of 5 mM to the concentrate, and the mixture was dialysed at 5 °C for 12 h against 500 vol 20 mM-glycine/NaOH buffer (pH 9.0) containing the divalent cation at 5 mM.

Table 1. Purification of the CMCase from *Bacillus* sp. KSM-635

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Cell-free culture	1224	19833	16.2	100	1.0
DEAE-Toyopearl 650S	395	15889	40.2	80	2.5
Bio-Gel A-0.5m					
E-H	327	11061	33.8	56	(2.1)
E-L	40	2366	59.2	12	(3.7)

Results

Purification

In order to obtain a high yield of purified CMCase, the cell-free supernatant of a culture of *Bacillus* sp. KSM-635 was fractionated directly on a column of DEAE-Toyopearl 650S, immediately after dialysis. The subsequent gel filtration on Bio-Gel A-0.5m separated the CMCase activity of the eluate into two fractions, designated E-H and E-L. The first peak of activity (E-H) contained 70% of the total amount of CMCase applied to the column. Specific activities against CMC substrate were 34 U (mg protein)⁻¹ for E-H and 59 U (mg protein)⁻¹ for E-L. When Ca²⁺ was omitted from buffer A, it was difficult to purify and separate these enzymes and there was an appreciable loss of activity. The degree of purification and total recovery were 2.1-fold and 56% for E-H, and 3.7-fold and 12% for E-L, as summarized in Table 1. A very high overall yield of CMCase activity was achieved (68% of the initial activity).

Homogeneity and absorption spectra

The preparations of purified enzymes, E-H and E-L, were homogeneous, as judged by nondenaturing PAGE and SDS-PAGE. Each band of protein, detected by staining after nondenaturing PAGE, coincided fairly well with the single band visualized by active staining with Congo red. The purified enzymes had absorption maxima at 280 nm in 10 mM-phosphate buffer (pH 7.2). In addition to absorption at 280 nm due to simple protein, the spectra of both enzymes had a shoulder at 290 nm, possibly attributable to absorption by tryptophan residues in the enzyme molecules (Clarke, 1987).

Molecular masses and isoelectric points.

The molecular masses of E-H and E-L were estimated to be 500 kDa and 100 kDa, respectively, by gel-filtration chromatography on a column of Bio-Gel A-1.5m. SDS-PAGE gave molecular masses for E-H and E-L of

130 kDa and 103 kDa, respectively. These results indicate that E-H exists as a tetramer and that E-L has a monomeric structure. Gel focusing of the enzymes gave isoelectric points for E-H and E-L of less than pH 4 in both cases, as estimated by the method of Wrigley (1971).

Carbohydrate in the enzymes

E-H and E-L both contained carbohydrate. They reacted positively with the Morgan-Elson reagent, indicating that the carbohydrate was an amino sugar. In order to identify the carbohydrate moiety of each enzyme, the carbohydrate was reduced by NaBH₄, acetylated and then analysed by gas chromatography on an ECNSS-M column. A main peak corresponding to glucosaminitol tetra-acetate was detected in the case of both enzymes, with the same retention time as an authentic sample and the carbohydrate in E-H and E-L was thus identified as glucosamine. Both enzymes contained approximately 2–3% (w/w) glucosamine. The nature of the intramolecular glucosidic linkage of the amino sugar was not determined.

Effects of chemical reagents

The effect of divalent cations on E-H and E-L was examined by first treating the enzymes with EDTA and EGTA to remove any endogenous divalent cations, and then adding various divalent cations to the reaction mixture (see Methods). Co²⁺ (5 mM) stimulated the CMCase activities of both enzymes 1.3-fold, but the other divalent cations (Ca²⁺, Mg²⁺ and Mn²⁺) had either no effect or slightly stimulatory effects.

When the native preparations of E-H and E-L were incubated with various cations and chemicals (1 mM) for 20 min at 30 °C and pH 9.5 in 0.1 M-glycine/NaOH buffer, Hg²⁺, Cu²⁺, Pb²⁺, Fe²⁺ and Fe³⁺ inhibited the activities of both enzymes by 30–50%, but other sulphhydryl inhibitors, such as *N*-ethylmaleimide, monoiodoacetate and 4-chloromercuribenzoate (1 mM) either had no effect or caused moderate levels of

Fig. 1. Protective effects of some cations on the thermal stability of the purified CMCases. Enzyme (0.33 U) was preheated at 45 °C for 10 min in 0.1 M-glycine/NaOH buffer (pH 9.5) in the presence of the indicated cations at various concentrations. ○, Ca²⁺; ●, Mg²⁺; □, Mn²⁺; ■, Co²⁺; △, Na⁺; ▲, K⁺. After the heat treatment, samples were immediately cooled in an iced-water bath and aliquots (0.1 ml) were used to determine the residual activities of E-H (a) or E-L (b) at 40 °C in 0.1 M-glycine/NaOH buffer (pH 9.5). The residual activities of both enzymes without additives were nearly zero, and the control activities (without heating) were taken as 100%.

inhibition. Under the same assay conditions, Co²⁺ stimulated the activities of both enzymes approximately 1.3-fold, but Ca²⁺, Mg²⁺ and Mn²⁺ had no effect. *N*-Bromosuccinimide (10 µM) completely abolished the activities of E-H and E-L at pH 6.0 in 50 mM-acetate buffer. The following reagents (1 mM each) were without effect on the activity of the *Bacillus* enzymes under our assay conditions at alkaline pH; di-isopropyl-phosphor-fluoridate, phenylmethanesulphonyl fluoride and sodium azide.

Effect of temperature and pH on the activity and stability of each enzyme

The optimum temperatures for reactions were examined. The two CMCases exhibited a similar temperature-activity profile, the optimum temperature being around 40 °C in 0.1 M-glycine/NaOH buffer (pH 9.5). Thermal stabilities of E-H and E-L were assessed in 0.1 M-glycine/NaOH buffer (pH 9.5) after heating for 10 min at various temperatures. Both enzymes were stable up to 35 °C. Complete loss of enzyme activity was observed after heating to 45 °C in each case. When Ca²⁺ was present, the inactivation temperatures shifted from 45 °C to 50 °C for E-H and from 45 °C to 55 °C for E-L. Several inorganic ions also protected the two enzymes from inactivation at 45 °C, as shown in Fig. 1. The presence of Mg²⁺, Mn²⁺ and Ca²⁺ (at more than 0.1 mM) markedly reduced the heat inactivation of the enzymes. Co²⁺ ions were somewhat less effective than these three ions. Na⁺ and K⁺ (at more than 0.2 M) effectively

protected the CMCases from thermal inactivation at 45 °C.

The effect of pH on the activities of E-H and E-L was determined at 40 °C in various buffers ranging from pH 3 to pH 13. Both enzymes hydrolysed CMC and were active over a wide range from pH 6 to pH 12.5, the maximum activities being observed at pH 9.5 in both cases in 0.1 M-glycine/NaOH buffer. To determine the stability of enzyme activities with changes in pH, E-H and E-L were preincubated at 5 °C for 3 h in various buffers and then assayed at pH 9.5 and 40 °C. Both enzymes were very stable to incubation over a range between pH 6 and pH 11. The enzyme activity was completely lost when either enzyme was incubated at pH 4.5 or pH 12 under these conditions.

Substrate specificity

The purified enzymes were tested for their ability to hydrolyse various carbohydrates and aryl-glucosides at 30 °C and at pH 9.5 or pH 7.0 (Table 2). Of the carbohydrate substrates tested, CMC (β-1,4-linkage) and lichenan (β-1,3; 1,4-linkage) were efficiently hydrolysed by E-H and E-L at pH 9.5 in 0.1 M-glycine/NaOH buffer. The rates of hydrolysis by E-L of these substrates were twice those of E-H. At equilibrium, the degrees of hydrolysis of CMC by E-H and E-L reached 9.78% and 9.54%, respectively. Avicel, filter paper, swollen celluloses, cellobiose, curdlan (β-1,3-linkage), laminarin (β-1,3; 1,6-linkage) and NPG were practically unhydrolysable by either enzyme. This substrate specificity is

Table 2. Substrate specificity of CMCase

Assays were performed at 30 °C in 0.1 M-glycine/NaOH buffer (pH 9.5) with 0.86 μ g E-H or with 0.63 μ g E-L under the standard reaction conditions. Each value is the mean of three determinations, and the activity of E-H against CMC was taken as 100%.

Substrate (1%)	E-H activity		E-L activity	
	U mg ⁻¹	Rel. act. (%)	U mg ⁻¹	Rel. act. (%)
CMC	23.5	100	43.8	186
Avicel	tr	tr	tr	tr
Filter paper	tr	tr	tr	tr
H ₃ PO ₄ -swollen cellulose	tr	tr	tr	tr
NaOH-swollen cellulose	tr	tr	tr	tr
Lichenan	19.7	84	42.5	181
Curdlan	0	0	0	0
Laminarin	0	0	0	0
Cellobiose	0	0	0	0
NPC* at pH 9.5	0.3	1.3	0.6	2.6
at pH 7.0	0.8	3.4	1.4	6.0
NPG* at pH 9.5	0	0	0	0
at pH 7.0	0	0	0	0

tr, Very low but not negligible activity (<1.1 U mg⁻¹).

* Assays were performed with 4 μ mol substrate in 0.5 ml 0.1 M-glycine/NaOH buffer (pH 9.5) or 0.1 M-phosphate buffer (pH 7.0), with 0.86 μ g E-H or 0.63 μ g E-L as enzyme.

consistent with the classification of the *Bacillus* enzymes as β -1,4-endoglucanases. Slight, but not negligible, activity against NPC was observed with either enzyme. The maximum activities of the two enzymes toward NPC were both observed at pH 7.0 in 0.1 M-phosphate buffer, rather than at alkaline pH. The double-reciprocal plots against NPC yielded apparent K_m values of 0.26 mM with a V of 1.14 U (mg protein)⁻¹ at pH 7.0, and 1.1 mM with a V of 0.54 U (mg protein)⁻¹ at pH 9.5 for E-H; and, for E-L, K_m values of 0.27 mM with V of 1.52 U (mg protein)⁻¹ at pH 7.0, and 1.0 mM with a V of 0.83 U (mg protein)⁻¹ at pH 9.5.

The actions of E-H and E-L on cello-oligosaccharides (G2 through G6) were examined after completion of the reactions, reached after 12 h at 30 °C in 50 mM-pyrophosphate buffer (pH 9.0). E-H and E-L were alike, in that they acted on G3 through G6, yielding G2 and G1 as the major products of the hydrolysis. Glucose (G1) was not found as a product of the complete reaction of cellobiose (G2) with these enzymes, indicating again that there was no β -1,4-glucosidase (cellobiase) activity in the preparations.

Effects of surfactants and chelating agents

The effects on the activities of E-H and E-L of various surfactants and chelating agents used in laundry detergents were examined at 40 °C and pH 9.5 in 0.1 M-glycine/NaOH buffer. Neither enzyme was inhibited at all by alkyl sulphate, α -carboxymethyl alkyl sulphate, α -olefin sulphonate, polyoxyethylene alkyl sulphate,

polyoxyethylene alkyl ether, secondary alkyl sulphonate, sodium laurate (soap), sodium triphosphosphate, zeolite 4A or sodium citrate, each added at a final concentration of 0.05% (w/v). EDTA and EGTA, each added at 1 mM, slightly inhibited the activities of both enzymes (by about 10% in each case).

Discussion

The genus *Bacillus* represents a group of micro-organisms that produce extracellular enzymes of industrial importance (Fogarty *et al.*, 1974; Priest, 1977; Sippola & Mäntsälä, 1981; Zemek *et al.*, 1981). They are also known for their ability to produce cellulolytic enzymes (Tewari & Chahal, 1977; Horikoshi *et al.*, 1984; Lobson & Chambliss, 1984; Dhillon *et al.*, 1985; Chan & Au, 1987; Kawai *et al.*, 1988; Ito *et al.*, 1989; Shikata *et al.*, 1990), but relatively few attempts have been made to purify and characterize the cellulases that they produce. An alkaline CMCase was purified and characterized to a limited extent by Fukumori *et al.* (1985), who used the alkalophilic *Bacillus* sp. no. 1139. Okoshi *et al.* (1990) purified two alkaline CMCase and one neutral enzyme from the neutrophilic *Bacillus* sp. KSM-522, and Au & Chan (1987) isolated one neutral CMCase as the major component from a culture of *Bacillus subtilis* AU-1.

In this study, alkaline CMCase, E-H and E-L, were purified to homogeneity with high yield from cultures of the alkalophilic *Bacillus* sp. KSM-635. The use of a crude preparation of the *Bacillus* sp. KSM-635 cellulase was

extended by us to inclusion in laundry detergents (Ito *et al.*, 1989). The purified CMCase, E-H and E-L, are obviously alkaline enzymes with pH optima at pH 9.5, and they are very stable to incubation at pH 6–11 and against various detergents and chelating agents of laundry detergents. These characteristics of the enzymes make them most suitable for use as effective laundry detergent additives. E-H and E-L contained glucosamine (presumably *N*-acetylglucosamine). There are some reports concerning the physiological function of carbohydrate moieties of glycoproteins (Olden *et al.*, 1982). For example, the carbohydrate moiety of glucoamylase from a strain of *Flavobacterium* sp. (Takegawa *et al.*, 1988) has been shown to contribute to the high stability of the enzyme against temperature and pH, and to protect it from proteolytic degradation. Protection against proteolytic degradation afforded by the carbohydrate moiety was also reported by Bernard *et al.* (1983) who studied bovine pancreatic ribonucleases A and B. Such carbohydrate effects could be applicable to the CMCase from *Bacillus* sp. KSM-635, which are relatively stable against surfactants and chelating agents.

The purified preparations of E-H and E-L both hydrolysed only CMC and lichenan and did not hydrolyse crystalline celluloses, curdlan and laminarin. Hence, it is probably more appropriate to refer to these enzymes as endo-1,4- β -glucanases rather than as cellulases. In fact, E-H and E-L rapidly decreased the viscosity of solutions of CMC (data not shown). E-H appears to exist as an aggregate of four identical CMCase subunits with a molecular mass of 130 kDa. We have cloned the gene that encodes a *Bacillus* sp. KSM-635 CMCase into *Escherichia coli* HB101, using pBR322 (Ozaki *et al.*, 1990). There is an open reading frame of 2823 bp, which encodes 941 amino acid residues that give a calculated molecular mass of 104626 Da. The molecular mass of the plasmid-encoded CMCase is very close to that of E-L, as estimated both by gel filtration on Bio-Gel A-1.5m (100 kDa) and by SDS-PAGE (103 kDa). E-H and E-L, with molecular masses of 130 kDa and 103 kDa, are distinguishable from each other with respect to rates of hydrolysis of CMC, lichenan and NPC, but showed essentially the same pH-activity, pH-stability, temperature-activity and temperature-stability profiles, and their activities were reduced to almost the same extents by various inhibitors and inorganic ions. A possible explanation for the occurrence of these isoenzymes is that they are encoded by different genes. However, as glycoproteins frequently give erroneous molecular mass values when these are estimated by commonly used methods, the reason for the observed discrepancy in molecular masses is not clear.

Microbial extracellular enzymes are generally known to require Ca^{2+} and some divalent cations for activity

and stabilization. Co^{2+} , rather than Ca^{2+} , seemed to stimulate the activity of E-H and E-L. The stimulation by Co^{2+} of cellulase activity is very unusual among extracellular enzymes and has been reported only for CMCase of *Bacillus subtilis* AU-1 (Au & Chan, 1987) and *Bacillus* sp. KSM-522 (Okoshi *et al.*, 1990). Protection by high concentrations of Na^+ and K^+ from thermal inactivation of E-H and E-L cannot readily be explained, but it could arise from some interactions of the cations with free carboxyl groups of some amino acid residues in the CMCase molecules, or from changes in the gross conformation of the enzymes. Both enzymes were strongly inhibited by Hg^{2+} , and such inhibition has been noted with many cellulases, including that of *Bacillus* sp. no. 1139 (Fukumori *et al.*, 1985). Heavy metal ions are generally thought to inactivate enzymes by forming covalent salts with cysteine moieties in the enzyme molecule. However, amino acid analysis did not detect cysteine in the CMCase proteins of *Bacillus* sp. KSM-635 (data not shown). Since the genetic analysis of the cellulase genes of *Bacillus* sp. no. 1139 (Fukumori *et al.*, 1986) and *Bacillus* sp. KSM-635 (Ozaki *et al.*, 1990) has also shown that these CMCase are devoid of free thiol groups and disulphide linkages, interaction with thiol groups can be ruled out in these cases. The inhibition by Hg^{2+} could arise from the interaction with tryptophan residues at the active site of the CMCase molecules, as suggested by Eriksson & Pettersson (1968) and Hurst *et al.* (1977). In fact, E-H and E-L have a shoulder absorption at 290 nm, which may be due to tryptophan residues (Clarke, 1987), and the activities of E-H and E-L were completely inhibited by *N*-bromosuccinimide at concentrations below 10 μM .

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when large cell numbers needed for biochemical localization are not available.

Although malate dehydrogenase (decarboxylating) activity was detectable in the whole cell homogenate, the enzyme was not organelle-associated in *P. multivesiculatum*. Non-sedimentable (i.e. soluble) malate dehydrogenase (decarboxylating) has also been detected in the rumen ciliates *Dasytricha ruminantium*, *Isotricha prostoma* and *I. intestinalis* (Yarlett *et al.*, 1981, 1983a). However, this particular activity within the trichomonad flagellates (Müller, 1980), mixed rumen ophryoscolecids ciliates (Snyers *et al.*, 1982) and the rumen fungus *Neocallimastix patriciarum* (Yarlett *et al.*, 1986) is sedimentable. Further studies on the occurrence and distribution of this enzyme in other rumen ciliates are therefore warranted, as there would appear to be species differences in the complement of hydrogenosomal enzymes.

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