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 Hg2+, Cu2+, Fe2+ and Fe3+, but sulphydryl 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 Co2+, and they required Mg2+, Ca2+, Mn2+ or Co2+ 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 carboxymethylcellullose (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; NFC, 4-nitrophenyl β-D-cellobioside; NPG, 4-nitrophenyl β-D-glucopyranoside.

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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 three or more experiments. Protein was determined by the procedure of Miller et al., 1960). Assays for cellulolytic activity against filter paper, Avicel, swollen celluloses, and cellobiose, lichenan (from Cetaria islandica, Sigma), curdlan (from Alcaligenes faecalis var. nitrogenes, 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 pmol of product min⁻¹ under the standard assay conditions.

Electrophoretic analysis. PAGE was performed on 12% (w/v) polyacrylamide slab gels (70 mm × 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 CMCase agar sheet, essentially according to the method of Béguin (1983). The CMCase agar sheet (5 mm thickness) was composed of 2% (w/v) CMCase, 3% (w/v) NaCl, 0.1 mM-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 0.1 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% Na₂SO₄, 0.015% CaCl₂, 2H₂O and 0.5% Na₂CO₃. Calcium chloride and Na₂CO₃ 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-CaCl₂ (buffer A). The dialysate was applied to a column of DEAE-Trystal pap 560S (4.4 cm × 40 cm; Tosoh) equilibrated with buffer A containing 0.2 M-NaCl. The adsorbed materials were eluted with a 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 collected, 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 × 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 × 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 (669000); horse spleen ferritin (440000); bovine liver catalase (232000); rabbit muscle aldolase (158000); bovine serum albumin (67000); hen egg ovalbumin (43000); bovine pancreas chymotrypsinogen A (25000). 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 (200000); Escherichia coli β-galactosidase (116250); rabbit muscle phosphorylase b (97400); bovine serum albumin (66200); hen egg white ovalbumin (42699); bovine carbonic anhydrase (31000); soybean trypsin inhibitor (21500).

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 cellohexaose (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 acetic/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 NaBH₄, and then treated with anhydrous acetic acid at 100 °C for 30 min, according to the alditol-aceate procedure (Björndal et al., 1970). Gas-liquid 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 × 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 dialyzed 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 CMCases from Bacillus sp. KSM-635

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free culture</td>
<td>1224</td>
<td>19833</td>
<td>16-2</td>
<td>100</td>
<td>1-0</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650S</td>
<td>395</td>
<td>15889</td>
<td>40-2</td>
<td>80</td>
<td>2.5</td>
</tr>
<tr>
<td>Bio-Gel A-0-5m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-H</td>
<td>327</td>
<td>11061</td>
<td>33-8</td>
<td>56</td>
<td>(2-1)</td>
</tr>
<tr>
<td>E-L</td>
<td>40</td>
<td>2366</td>
<td>59-2</td>
<td>12</td>
<td>(3-7)</td>
</tr>
</tbody>
</table>

**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 glucosaminotetra-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 sulphydryl inhibitors, such as N-ethylmaleimide, monooiodoacetate and 4-chloromercuribenzoate (1 mM) either had no effect or caused moderate levels of
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-phosphofluoridate, 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 mM) 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 97.8% and 95.4%, respectively. Avicel, filter paper, swollen celluloses, cellobiose, curdlan (β-1,3,1,6-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 CMCases
Assays were performed at 30 °C in 0.1 M-glycine/NaOH buffer (pH 9.5) with 0.86 µg E-H or with 0.63 µg 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%.

<table>
<thead>
<tr>
<th>Substrate (1%)</th>
<th>E-H activity</th>
<th>E-L activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U mg⁻¹</td>
<td>Rel. act. (%)</td>
</tr>
<tr>
<td>CMC</td>
<td>23.5</td>
<td>100</td>
</tr>
<tr>
<td>Avicel</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Filter paper</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>H₃PO₄-swollen cellulose</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>NaOH-swollen cellulose</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Lichenan</td>
<td>19.7</td>
<td>84</td>
</tr>
<tr>
<td>Curdlan</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Laminarin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NPC* at pH 9.5</td>
<td>0.3</td>
<td>1-3</td>
</tr>
<tr>
<td>at pH 7.0</td>
<td>0-8</td>
<td>3-4</td>
</tr>
<tr>
<td>NPC* at pH 9.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>at pH 7.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

tr, Very low but not negligible activity (<1.1 U mg⁻¹).
* Assays were performed with 4 µmol 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 µg E-H or 0.63 µg 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 Kₘ 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, Kₘ 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 laureate (soap), sodium tripolyphosphate, 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 microorganisms 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 & Chambliess, 1984; Dhillion 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 CMCases 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 CMCases, 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
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 CMCases 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 CMCases 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 CMCases 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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References


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 \textit{P. multivesiculatum}. Non-sedimentable (i.e. soluble) malate dehydrogenase (decarboxylating) has also been detected in the rumen ciliates \textit{Dasytricha ruminantium}, \textit{Isotricha prosto}ma and \textit{I. intestinalis} (Yarlett et al., 1981, 1983a). However, this particular activity within the trichomonad flagellates (Müller, 1980), mixed rumen ophryoscolecid ciliates (Snyers et al., 1982) and the rumen fungus \textit{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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### References


