Purification and properties of an acid endo-1,4- β -glucanase from *Bacillus* sp. KSM-330

KATSUYA OZAKI* and SUSUMU ITO

Tochigi Research Laboratories of Kao Corporation, 2606 Akabane, Ichikai, Tochigi 321-34, Japan

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A novel acid cellulase (endo-1,4- β -glucanase, EC 3.2.1.4) was found in a culture of *Bacillus* sp. KSM-330 isolated from soil. One-step chromatography on a column of CM-Bio-Gel A yielded a homogeneous enzyme, as determined by silver staining of both sodium dodecyl sulphate (SDS) and nondenaturing gels. The enzyme had a molecular mass of 42 kDa, as determined by SDS-polyacrylamide gel electrophoresis. The isoelectric point was higher than pH 10. The N-terminal amino acid sequence of the enzyme was Val-Ala-Lys-Glu-Met-Lys-Pro-Phe-Pro-Gln-Gln-Val-Asn-Tyr-Ser-Gly-Ile-Leu-Lys-Pro. This enzyme had an optimum pH for activity of 5.2, being active over an extremely narrow range of pH values, from 4.2 to 6.9; below and above these pH values no activity was detectable. The optimum temperature at pH 5.2 was around 45 °C. The enzyme efficiently hydrolysed carboxymethylcellulose (CMC) and lichenan, but more crystalline forms of cellulose, curdlan, laminarin, 4-nitrophenyl- β -D-glucopyranoside and 4-nitrophenyl- β -D-cellobioside were barely hydrolysed. The enzymic activity was inhibited by Hg²⁺ but was not affected by other inhibitors of thiol enzymes, such as 4-chloromercuribenzoate, N-ethylmaleimide and monoiodoacetate. N-Bromosuccinimide abolished the enzymic activity, and CMC protected the enzyme from inactivation by this tryptophan-specific oxidant. It is suggested that a tryptophan residue(s) is involved in the mechanism of action of the *Bacillus* cellulase and that the inhibition of enzymic activity by Hg²⁺ is ascribable to interactions with the tryptophan residue(s) rather than with thiol group(s).

Introduction

Cellulolytic enzymes have been reported in several bacterial genera, namely *Clostridium* (Garcia-Martinez *et al.*, 1980; Ng & Zeikus, 1981; Wu *et al.*, 1988), *Cellulomonas* (Stoppok *et al.*, 1982; Nakamura & Kitamura, 1983), *Ruminococcus* (Gardner *et al.*, 1987), *Bacteroides* (Saddler & Khan, 1979) and *Acetivibrio* (Saddler *et al.*, 1980). Members of the genus *Bacillus* also produce cellulases (e.g. Tewari & Chahal, 1977; Horikoshi *et al.*, 1984; Robson & Chambliss, 1984; Dhillon *et al.*, 1985; Au & Chan, 1987). However, cellulases from *Bacillus* have received very limited attention, probably because most of these enzymes hydrolyse carboxymethylcellulose (CMC) but barely hydrolyse crystalline forms of cellulose and, furthermore, they have had no commercial applications until recently.

We have reported that the properties of alkaline carboxymethylcellulases (CMCase, endo-1,4- β -glucanase, EC 3.2.1.4) from some isolates of *Bacillus* conform

to the essential requirements for enzymes that can be used as effective additives in laundry detergents (Kawai *et al.*, 1988; Ito *et al.*, 1989; Shikata *et al.*, 1990). One of the isolates is currently used for the large-scale industrial production of an alkaline CMCase for this purpose (Ito *et al.*, 1989). Therefore, cellulases from *Bacillus* have attracted our attention both because of our interest in their mechanisms of action (Okoshi *et al.*, 1990; Yoshimatsu *et al.*, 1990) and because of our interest in some genetic aspects of these enzymes (Ozaki *et al.*, 1990). The genes for some alkaline cellulases have been sequenced (Horikoshi & Fukumori, 1988; Ozaki *et al.*, 1990), as have some genes for neutral cellulases from *Bacillus* (MacKay *et al.*, 1986; Robson & Chambliss, 1987; Nakamura *et al.*, 1987). However, the nucleotide sequences of the genes for alkaline cellulases are too similar to those of the neutral enzymes to explain the difference in pH optima of these enzymes (Horikoshi & Fukumori, 1988; Ozaki *et al.*, 1990).

In order to clarify the mechanism of action of cellulases, with a special focus on differences in pH optima, we isolated a *Bacillus* strain, KSM-330, that produces a CMCase that is active over a very narrow

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

range of acidic pH values, with the optimum pH being 5.2. The aim of this study was to purify and characterize the acid CMCase in the hope that the enzymic properties of this enzyme could later be applied to a genetic search for the amino acid sequence and tertiary structure that determine the pH optima for activity of cellulases from *Bacillus*.

Methods

Organism. The organism used was *Bacillus* sp. KSM-330. It was obtained from the stock culture collection of the Microbiology Laboratory of Tochigi Research Laboratories of Kao Corporation, Japan. This strain was originally isolated from a soil sample collected in Japan. Morphological and taxonomical characteristics of the isolated strain were examined according to the methods of Gordon *et al.* (1973) and Claus & Berkeley (1986).

Bacillus sp. KSM-330 was propagated at 30 °C in a medium composed of (w/v): 1% Polypepton (Daigo-Eiyo), 0.5% yeast extract (Difco), 0.1% KH_2PO_4 , 0.25% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1% carbon source (pH 6.8).

Preparation of crude enzyme. *Bacillus* sp. KSM-330 was propagated in the growth medium with 1.0% (w/v) maltose at 30 °C with shaking. After a 24 h incubation, cells were removed by centrifugation (9800 g, 10 min) at 4 °C. All further manipulations were also conducted at this temperature. The culture supernatant obtained (0.6 l) was concentrated to a small volume by ultrafiltration on an H1P3-20 hollow-fibre module (3 kDa cutoff; Amicon). The concentrate (70 ml) was dialysed four times over the course of 20 h against 40 vols each time of 10 mM-sodium phosphate buffer (pH 7.2) and was then used as the crude enzyme preparation.

Purification of the enzyme. The crude enzyme preparation was applied immediately to a column of CM-Bio-Gel A (3.2 cm × 20 cm; Bio-Rad) equilibrated with 10 mM-phosphate buffer (pH 7.2). The column was first washed with 350 ml of the equilibration buffer and then proteins were eluted with a 2 litre linear gradient of 0 to 0.3 M-NaCl in the equilibration buffer, at a flow rate of 35 ml h⁻¹. Fractions of 5.0 ml were collected from the start of the gradient. The elution pattern was monitored by measuring the absorbance at 280 nm (A_{280}) of each fraction, and then the CMCase activity in each fraction was measured. CMCase activity in some of these fractions was also checked by both protein and activity stains after nondenaturing PAGE. Two peaks of CMCase activity, a major peak A (tubes nos 284–306) and a minor peak B (tubes nos 271–280), were eluted and were separately pooled and used as the final preparations of purified enzymes. The material in the major peak A was designated Endo-K.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% (w/v) polyacrylamide slab gels (70 mm × 80 mm, 1 mm thickness) by the method of Reisfeld *et al.* (1962), at a constant current of 40 mA, with β -alanine/acetic acid buffer (pH 4.5) as the running buffer. Sodium dodecyl sulphate (SDS)-PAGE was carried out on 12% (w/v) polyacrylamide slab gels with 25 mM-Tris/192 mM-glycine buffer (pH 8.3) that contained 0.1% (w/v) SDS as the running buffer, after the enzyme preparations had been heated at 95 °C for 5 min in 62.5 mM-Tris/HCl buffer (pH 6.8) that contained 2.0% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol (Laemmli, 1970). Isoelectric focusing of Endo-K was performed by PAGE, as described by Wrigley (1971). The purified enzyme (13 µg) was subjected to electrophoresis on a column (0.5 cm × 8.5 cm) of 5% (w/v) acrylamide gel that contained 13.3% (v/v) glycerol and 1.9% (v/v)

Pharmalyte (pH range 3–10; Pharmacia), at a constant voltage of 300 V for 14 h at 10 °C, with the cathode in the upper chamber (20 mM- H_3PO_4) and the anode in the lower (0.1 M-NaOH). The isoelectric point was determined by cutting out zones of gel, eluting them and measuring the pH of the eluates.

Activity staining of CMCase in the slab gel was performed essentially by the methods of Béguin (1983) and Yoshimatsu *et al.* (1990). The slab gel after nondenaturing PAGE was laid on top of a sheet of 0.8% (w/v) agar that contained 2% (w/v) CMC, 3% (w/v) NaCl and 50 mM-sodium citrate/citric acid buffer (pH 5.2) and was left for 2 h at 30 °C. The CMC-agar sheet was washed in 0.5 M-glycine/NaOH buffer (pH 9.5) for 15 min, stained by flooding with 0.1% (w/v) Congo red for 15 min and then destained in 1.0 M-NaCl for 15 min, at room temperature. The bands of protein that were associated with CMCase activity were seen as clear zones on the CMC-agar sheet, which formed a dark-red background. Proteins were located by the silver-staining method (Oakley *et al.*, 1980).

Determination of molecular mass. Molecular mass was established both by SDS-PAGE and by gel filtration on a column of Bio-Gel P100-medium. The Bio-Gel column (1.6 cm × 92 cm) was equilibrated with 10 mM-phosphate buffer (pH 7.2) supplemented with 0.1 M-NaCl and calibrated by elution of molecular mass markers (Pharmacia) that included bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and RNAase A (13.7 kDa). The phosphate buffer was used as elution buffer and the eluate was collected in 1 ml fractions at a flow rate of 5.8 ml h⁻¹. For the determination of molecular mass by SDS-PAGE, molecular mass markers for SDS-PAGE (Bio-Rad) were used: hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

Enzyme assays. Assays for cellulolytic activity of Endo-K against CMC (degree of substitution 0.68, Sanyo Kokusaku Pulp), Avicel (Merck), phosphoric acid-swollen cellulose, lichenan (from *Cetraria islandica*, Sigma), laminarin (from *Laminaria digitata*, Sigma) and curdlan (from *Alcaligenes faecalis* var. *mixogenes*, Wako Pure Chemical) were performed essentially according to published procedures (Kawai *et al.*, 1988; Ito *et al.*, 1989). The acid-swollen cellulose was prepared from cellulose powder (Toyo Roshi) by the method of Tomita *et al.* (1974). For the CMCase assay, 0.1 ml of enzyme solution and 0.9 ml of 1.1% (w/v) CMC in 0.11 M-citrate buffer (pH 5.2) were mixed and incubated for 20 min at 40 °C. The reducing sugar formed was quantified as glucose by the dinitrosalicylic acid procedure (Miller *et al.*, 1960). Depolymerization (liquefaction) of CMC was measured viscometrically at 40 °C in the above reaction using an Ubbelohde viscometer, and was also detected by the liquefaction of the CMC-agar sheet used for activity staining. Lichenan-hydrolysing activity was measured in the same way as the assay for CMCase described above.

For the assays of hydrolytic activities against the other cellulosic substrates, each reaction mixture contained 0.1 ml of a suitably diluted solution of Endo-K, 0.9 ml of 0.11 M-citrate buffer (pH 5.2) and 50 mg substrate. This mixture was incubated for 2–12 h at 40 °C with stirring. After incubation, solids were removed by centrifugation at 20 °C (8000 g, 10 min) and the enzymic activity of the supernatant was measured as described by Miller *et al.* (1960).

Assays for hydrolytic activities against 4-nitrophenyl- β -D-glucopyranoside, (NPG, Sigma) and 4-nitrophenyl- β -D-cellobioside (NPC, Sigma) were done with 8 mM-substrate and a sample of Endo-K in 1.0 ml of 0.1 M-citrate buffer (pH 5.2); reactions were incubated for an appropriate length of time (up to 4 h) at 40 °C. Each reaction was stopped by the addition of 0.4 ml 1 M- Na_2CO_3 and the 4-nitrophenol liberated was quantified at 410 nm, using an ϵ value of 12000 l mol⁻¹ cm⁻¹. For the assay of cellobiase, 0.1 ml enzyme solution was mixed with 0.9 ml 1.1% (w/v) cellobiose in 0.11 M-citrate buffer (pH 5.2). The mixture was incubated for 10 min at 40 °C, and the amount of glucose

formed was determined by use of glucose oxidase/peroxidase (Glucose C-Test; Wako Pure Chemical).

Protein was determined by the procedure of Bradford (1976) with bovine plasma albumin as protein standard and a protein assay kit (Bio-Rad). One unit (U) of enzymic activity was defined as the amount of protein that produces 1.0 μmol of product min^{-1} under the standard assay conditions.

Chromatographic analysis of the products of hydrolysis of cello-oligosaccharides. Samples (50 mg) of cello-oligosaccharide, namely cellobiose through cellohexaose (Seikagaku Kogyo), were dissolved in 1.0 ml 0.1 M-citrate buffer (pH 5.2). A portion (0.1 ml) of the reaction mixture was incubated with a sample of Endo-K (5.6 mU) at 40 °C for 16 h and an aliquot (10 μl) of the hydrolysate was subjected to thin-layer chromatography on a silica gel 60 plate (20 cm $\times$ 20 cm; Merck), 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 heating the plate at 80 °C for 15 min after spraying with aniline/diphenylamine/phosphoric acid solution (Bailey & Bourne, 1960).

Amino-terminal sequence of Endo-K. The purified enzyme (37.8 μg , equivalent to approx. 0.9 nmol) was concentrated and desalted by centrifugation in a centrifuge tube concentrator (Centricon 10, Amicon). The N-terminal amino acid residues of the enzyme in this preparation were determined by automated Edman sequencing with a 477A sequenator (Applied Biosystems; Hunkapiller *et al.*, 1983). For the fractionation and detection of phenylthiohydantoin (PTH) derivatives of amino acids, a 120A PTH-amino acid analyser (Applied Biosystems) was used.

Reaction of Endo-K with *N*-bromosuccinimide (NBS). Titration of Endo-K with NBS (Wako Pure Chemical) was done essentially by the method of Spande & Witkop (1967). To 60 μl deionized water, 10 μl 0.5 M-acetate buffer (pH 4.0), 20 μl enzyme solution (26 μg protein, 1.35 U) and, finally, 10 μl 10 mM-acetate buffer (pH 4.0) containing NBS at a suitable concentration were added. After 5 min incubation at 30 °C, a 10 μl sample was withdrawn and quenched by the addition of 0.59 ml acetate buffer containing 15 mM-tryptophan; the residual CMCase activity in this fraction was then determined. The remaining mixture (90 μl) was used to measure the change in A_{280} of the NBS-treated enzyme in a Beckman DU-65 spectrophotometer with a 5-karat microcell. Appropriate controls with 50 mM-acetate buffer (pH 4.0) containing NBS were included for measurements of background absorbance. The number of tryptophan residues oxidized (N) was calculated from the following equation: $N = (1.31 \times \text{change in } A_{280}) / (5500 \times \text{molarity of enzyme})^{-1}$, where the molecular mass adopted was 42 kDa in the case of Endo-K and $5500 \text{ l mol}^{-1} \text{ cm}^{-1}$ is the molar absorption coefficient of tryptophan at 280 nm.

Results

Taxonomic characterization of the isolated strain

The isolate, KSM-330, was an obligately aerobic, spore-forming (cylindrical, subterminal to paracentral, without a swollen sporangium), Gram-positive, motile and rod-shaped bacterium (0.9–1.0 $\mu\text{m} \times$ 2.5–3.0 μm). The organism was positive for formation of acetoin (V-P test), reduction of nitrate, decomposition of casein and growth in 7% (w/v) NaCl. It seemed likely that this isolate was a relative of *B. subtilis*, although it was negative for hydrolysis of starch and production of acid from arabinose, xylose and mannitol.

Purification

When *Bacillus* sp. KSM-330 was grown on the medium that contained 1% (w/v) CMC, CMCase activity (1.22 U ml^{-1}) was maximal after 24 h, corresponding to the late-exponential or stationary phase of growth. In order to simplify the purification of the CMCase, the production of the enzyme during growth in the presence of metabolizable carbohydrates other than CMC was examined. When cells were grown on 1% (w/v) maltose, glucose and fructose separately, the CMCase activity after 24 h was 1.86, 1.23 and 1.82 U ml^{-1} , respectively, and the activity in cells grown in control medium (without carbohydrate) was 1.04 U ml^{-1} . Therefore, the medium supplemented with maltose was chosen for the production of CMCase by *Bacillus* sp. KSM-330.

On chromatography of the crude enzyme on a CM-Bio-Gel A column, two peaks of CMCase activity, a major peak A (Endo-K; tubes nos 284–306) and a minor peak B (tubes nos 271–280), were detected, with an activity ratio of approximately 15:1. The degree of purification and total recovery are summarized in Table 1. The specific activities were 51.6 and 58.3 U (mg protein) $^{-1}$ for peak A and peak B, respectively. A very high overall yield of CMCase activity was achieved (71.4% of the initial activity).

Homogeneity, molecular mass and N-terminal amino acid sequence

The two purified preparations of enzyme were tested for purity by nondenaturing PAGE and SDS-PAGE. Both nondenaturing PAGE and SDS-PAGE gave single bands of protein when proteins were visualized by silver staining. The enzyme in peak A migrated slightly faster than that in peak B during nondenaturing PAGE, but both enzymes migrated at almost the same rate during SDS-PAGE. Each band of protein, detected by staining after nondenaturing PAGE, coincided fairly well with the band visualized by activity staining with Congo red.

The molecular masses of the two enzymes were estimated to be 39 kDa by gel chromatography on a column of Bio-Gel P100-medium. SDS-PAGE also gave the same subunit molecular mass of 42 kDa for both enzymes. These results indicate that both enzymes have a monomeric structure. Both enzymes showed almost the same profiles in terms of pH for activity, stability to changes in pH, temperature for activity and stability to heating (data not shown). Endo-K, the material in the major peak A, was chosen for further study.

The isoelectric point of Endo-K was higher than pH 10. The N-terminal amino acid sequence of Endo-K, determined on a gas-phase protein/peptide sequencer, was Val-Ala-Lys-Lys-Glu-Met-Lys-Pro-Phe-Pro-Gln-Gln-Val-Asn-Tyr-Ser-Gly-Ile-Leu-Lys-Pro.

Table 1. Purification of CMCase activities from *Bacillus* sp. KSM-330

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Crude enzyme	64	1036	16.2	100	1.0
CM-Bio-Gel A					
Peak A	13.4	692	51.6	66.8	3.2
Peak B	0.82	47.8	58.3	4.6	3.6

Table 2. Substrate specificity of Endo-K

Assays for CMC- and lichenan-hydrolysing activities were performed at 40 °C in 0.1 M-citrate buffer (pH 5.2) with 0.58 µg enzyme under the standard reaction conditions. For the assays of the other substrates, 11.5 µg enzyme was used in each experiment. The numbers in parentheses show the relative activities, the activity against CMC being taken as 100%.

Substrate (1%)	Endo-K activity (U mg ⁻¹)
CMC	51.6 (100)
Avicel	0.005 (0.0097)
H ₃ PO ₄ -swollen cellulose	0.139 (0.268)
Lichenan	59.9 (115.6)
Curdlan	0
Laminarin	0
NPC*	0
NPG*	0

* Assays were done with 8 µmol substrate and 5.8 µg enzyme.

Substrate specificity of Endo-K

The purified preparation of Endo-K was examined for its ability to hydrolyse various carbohydrates and aryl-glycosides at 40 °C and at pH 5.2 in 0.1 M-citrate buffer (Table 2). Of the carbohydrate substrates tested, CMC (β -1,4-linkage) and lichenan (β -1,3;1,4-linkage) were efficiently hydrolysed by Endo-K at almost the same rate. The enzyme rapidly decreased the viscosity of a solution of CMC (data not shown). Avicel, phosphoric-acid-swollen cellulose, cellobiose, curdlan (β -1,3-linkage), laminarin (β -1,3;1,6-linkage), NPG and NPC were practically unhydrolysable by Endo-K.

Cello-oligosaccharides were hydrolysed by Endo-K to an equilibrium end-point (16 h of incubation) at 40 °C and pH 5.2 in 0.1 M-citrate buffer. The enzyme acted on cellopentaose to generate cellobiose and cellotetraose, and on cellohexaose to generate cellobiose, cellotriose and cellotetraose, as the major products of hydrolysis. With cellotetraose as substrate, a large amount remained intact, with the products of hydrolysis being glucose, cellobiose and cellotriose. The enzyme was not active on cellobiose or cellotriose.

Effect of pH on the activity and stability of Endo-K

The effect of pH on the activity of Endo-K was determined at 40 °C in various buffers ranging from pH 2.1 to pH 11.9, as shown in Fig. 1(a). Maximal activity toward CMC was observed at pH 5.2 in 0.1 M-citrate buffer. The enzyme was active over a very narrow range of pH values, between 4.2 and 6.9, with less than 5% of the maximum activity detectable above and below these pH values. A pH-activity curve with a similar profile was obtained with lichenan as substrate. To determine the short-term stability of the enzyme activity with changes in pH, Endo-K was preincubated at 40 °C for 10 min in various buffers ranging from pH 2.5 to pH 10.7 and then assayed at pH 5.2 and 40 °C. The enzyme was stable to incubation over a broad range between pH 3.2 and pH 9.5 (Fig. 1b). The activity was lost when the enzyme was incubated at pH 2.5 or pH 10.7 under these conditions.

Effect of temperature on the activity and stability of Endo-K

The dependence on temperature of Endo-K activity toward CMC was determined by measuring the activity at various temperatures in 0.1 M-citrate buffer (pH 5.2). Maximal activity with CMC as substrate was detected at around 45 °C. At 10 °C more than 25% of the maximum activity was detected, whereas at temperatures above 60 °C the enzyme was inactive. The stability to heating of Endo-K was examined after the enzyme had been heated at various temperatures for 10 min in 0.1 M-citrate buffer (pH 5.2). The enzyme was quite stable in the absence of additives up to 50 °C. Above this temperature, the stability of the enzyme decreased rapidly, with less than 5% of the original activity remaining after 10 min at 60 °C.

Effects of chemical reagents

Endo-K and each chemical (1 mM) were mixed and incubated at 30 °C for 30 min in 1.0 ml of 50 mM-MOPS buffer and an aliquot (0.1 ml) was used for the assay of

Fig. 1. Effect of pH on the activity and stability of Endo-K. Sodium citrate/HCl buffer (pH 2.5–4.2), sodium citrate/citric acid buffer (pH 3.2–6.3), sodium phosphate buffer (pH 6.1–7.5), Tris/HCl buffer (pH 7.0–8.8), glycine/NaOH buffer (pH 8.7–10.1) and $\text{Na}_2\text{HPO}_4/\text{NaOH}$ buffer (pH 9.3–10.7) were used. (a) pH-activity curve for Endo-K. Assays were done at 40 °C in the indicated buffers at 0.1 M, at various pH values, with 0.43 μg enzyme, using CMC (●) and lichenan (○) as substrates. Each enzyme activity at pH 5.2 in 0.1 M-citrate buffer was taken as 100%. (b) pH-stability curve for the enzyme. Enzyme (243 mU) was preincubated at the indicated pH in 1.0 ml of 50 mM-buffer for 10 min at 40 °C. Aliquots of 0.1 ml were withdrawn and the residual CMCase activity was measured at 40 °C and pH 5.2 in 0.1 M-citrate buffer with 0.47 μg enzyme. Control activity (100%) was 24.3 mU under standard conditions of the assay.

the residual CMCase activity in 0.1 M-citrate buffer (pH 5.2). The enzyme was not significantly affected by the following cations: Ca^{2+} , Mg^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , Cd^{2+} , Zn^{2+} , Pb^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Na^+ and K^+ . The notable exception was Hg^{2+} (40% inhibition). Reagents specific for thiol groups, such as *N*-ethylmaleimide, monoiodoacetate and 4-chloromercuribenzoate (1 mM) had little if any effect on the enzyme. The chelating reagents, EDTA and EGTA, each added at 1 mM, were without effect, suggesting that these compounds did not chelate a possible divalent cation(s) required for the activity of Endo-K. Phenylmethylsulphonyl fluoride and diisopropylfluorophosphate, strong inhibitors of serine enzymes, also had almost no effect. However, NBS, at a concentration of less than 0.1 mM, abolished the activity of Endo-K completely at pH 4.0 in 50 mM-acetate buffer.

Interaction of *N*-bromosuccinimide with Endo-K

Strong inhibition of Endo-K activity by NBS suggests a crucial involvement of tryptophan residue(s) at the active site of the enzyme. As shown in Fig. 2(a), the peak of absorbance of Endo-K at 280 nm decreased in the presence of increasing amounts of NBS. This decrease was accompanied by an increase in the A_{250} of the enzyme. This result suggests that oxidation of tryptophan occurred in the enzyme molecule (Spande & Witkop, 1967). The effect of NBS on the enzyme activity and the number of tryptophan residues oxidized are shown in Fig. 2(b), as a function of the amount of NBS added. The inactivation of enzyme activity by NBS at each concentration was complete within 5 min and further inactivation did not occur after an additional 10–20 min of incubation. There was a correlation

between the loss of activity and the number of tryptophan residues oxidized. The molar concentration of NBS required for complete inactivation of one mole of Endo-K activity was calculated to be approximately 4, and the measured titre of tryptophan residues in the enzyme finally reached 11.4 (data not shown). The complete inactivation of Endo-K by the addition of a low molar excess of NBS indicates that one of the tryptophan residues is essential for activity. Fig. 2(c) shows that the presence of CMC efficiently protected the enzyme from inactivation by NBS added up to a concentration of 12 μM . This result provides further evidence for the crucial involvement of a tryptophan residue in the mechanism of action of Endo-K.

Discussion

Some species of *Bacillus*, such as *B. subtilis*, *B. firmus*, *B. pumilus*, *B. cereus*, *B. polymyxa* and *B. brevis* are known to produce endo-1,4- β -glucanase (CMCase) activities (Knösel, 1971; Fogarty & Griffin, 1973; Fogarty *et al.*, 1974; Thayer, 1978). In the present study, we also isolated an obligately aerobic strain of *Bacillus*, KSM-330, which produces an acid CMCase. However, very few attempts have been made to purify cellulases from *Bacillus* to homogeneity, possibly because of the multiple forms of the enzymes and/or the complexity of the cellulase system(s), or because of formation of fragmented enzymes by proteolytic events during growth of the organisms or purification of the enzymes. Ito and his colleagues have isolated a number of strains of *Bacillus* that produce alkaline CMCases suitable for use as detergent additives (Kawai *et al.*, 1988; Ito *et al.*, 1989;

Fig. 2. Effect of NBS on Endo-K activity. Experimental details are described both in the Methods and elsewhere in the text. (a) Change in ultraviolet absorption of Endo-K. Absorption curves: A, not treated (without NBS); B, treated with 50 μ M-NBS; C, treated with 100 μ M-NBS. (b) Titration of Endo-K with NBS. ○, Number of tryptophan residues, estimated by the method of Spande & Witkop (1967); ●, residual activity. (c) Protection of Endo-K by CMC from inactivation by NBS in 50 mM-acetate buffer (pH 4.0). Endo-K (5.9 μ g protein, corresponding to 0.14 nmol of enzyme) and 0.1% (w/v) CMC were preincubated for 20 min in 50 mM-acetate buffer (pH 4.0) before the addition of NBS at the concentrations indicated. Then the residual enzyme activity was measured. ●, Control activities; ○, residual activities in the presence of 1.0% (w/v) CMC.

Shikata *et al.*, 1990), and some of these enzymes have been purified to homogeneity with high yield and characterized (Okoshi *et al.*, 1990; Yoshimatsu *et al.*, 1990). An alkaline CMCase from the alkalophilic *Bacillus* sp. no. 1139 was purified and characterized to a limited extent by Fukumori *et al.* (1985), and a neutral CMCase from *B. subtilis* AU-1 was purified to homogeneity by Au & Chan (1987).

In this study, we purified an acid CMCase, Endo-K, from *Bacillus* sp. KSM-330. In order to purify this enzyme with high yield, we used chromatography on CM-Bio-Gel A, instead of chromatography on CM-cellulose or dialysis with cellulose membranes. In addition, cultures were harvested in the late-exponential phase of growth to avoid the formation of fragmented enzymes by proteolytic events at the N- or C-terminus of Endo-K. The pH-activity curve of the purified enzyme obtained in this way shows a very narrow range of pH values at which the enzyme is active, namely, 4.2–6.9, the optimum pH being around 5.2. Such an unusually narrow range of pH values for CMCase activity has not been reported for other bacterial stains. Endo- and exo-1,4- β -glucanases from a fungus, *Trichoderma harzianum*, also have relatively narrow ranges of pH for activity, but the pH-stability curves of these enzymes are very narrow compared with that of Endo-K (Sidhu *et al.*, 1986). Endo-K hydrolyses CMC and lichenan but displays only very low activity with Avicel and acid-swollen cellulose as substrates. The presence of a β -1,4-bond appears to be required for enzyme activity, since curdian, a predominantly β -1,3-linked glucan, and laminarin, a predominantly β -1,6-linked glucan, were not degraded. Hence, it would probably be more appropriate to refer to Endo-K as an endo-1,4- β -glucanase rather than as a cellulase. The N-terminal amino acid sequence of the enzyme was determined, and the first 20 amino acids of Endo-K match the sequence of amino acids 56 to 75 (numbered starting at the N-terminal methionine) deduced from the open reading frame of the cloned gene for the enzyme (K. Ozaki & S. Ito, unpublished observations).

Endo-K activity from *Bacillus* sp. KSM-330 was completely abolished by NBS. This result provides evidence for the crucial involvement of a tryptophan residue(s) in the active site of this enzyme. Our results provide the first evidence, to our knowledge, that a tryptophan residue(s) is precisely involved in the action of bacterial cellulase, although a tryptophan residue(s) has been suggested as essential for the action of fungal cellulases (Pettersson, 1968; Hurst *et al.*, 1977; Clarke, 1987). The overall absence of inhibition of Endo-K activity by commonly used thiol reagents suggests that the inhibition by Hg^{2+} is not related to interactions of this heavy metal ion with cysteine residues in the enzyme molecule, and that Endo-K does not contain any thiol

groups that are necessary for enzyme activity. Thus, sensitivity toward Hg^{2+} may be the result of the interaction with a tryptophan residue(s) at the active site of the Endo-K molecule, as suggested by Eriksson & Pettersson (1968) and Hurst *et al.* (1977). In fact, the genetic analysis of the gene for Endo-K cloned from *Bacillus* sp. KSM-330 shows that the enzyme is devoid of cysteine residues (K. Ozaki & S. Ito, unpublished data). The insensitivity toward monoiodoacetate also suggests a noncritical role for imidazole, methionine or amino groups (Hurst *et al.*, 1977). The slight stimulation (about 20%) by dithiothreitol and 2-mercaptoethanol, however, complicates the explanation of the contradictory results with Hg^{2+} and the thiol reagents tested. A similar stimulatory effect by 2-mercaptoethanol has been observed with an alkaline endo-1,4- β -glucanase from *Bacillus* sp. KSM-635, which is devoid of cysteine residues at the active site (Ozaki *et al.*, 1990; Yoshimatsu *et al.*, 1990).

In order to investigate further the unique pH-activity pattern of Endo-K at the molecular level and the mechanisms of action of this enzyme, we have cloned the gene for Endo-K in *Escherichia coli*. The deduced amino acid sequence of Endo-K is being compared with those of both neutral and alkaline CMCase of microbial origin, with a specific focus on differences in pH optima for activity of these enzymes.

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