Extremophiles (1997) 1:151–156

ORIGINAL PAPER

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Thermostable alkaline cellulase from an alkaliphilic isolate, *Bacillus* sp. KSM-S237

Received: April 28, 1997 / Accepted: May 24, 1997

Abstract Thermostable alkaline cellulase (endo-1,4-β-glucanase, EC 3.2.1.4) activity was detected in the culture medium of a strictly alkaliphilic strain of *Bacillus*, designated KSM-S237. This novel enzyme was purified to homogeneity by a two-step column-chromatographic procedure with high yield. The *N*-terminal amino acid sequence of the purified enzyme was Glu-Gly-Asn-Thr-Arg-Glu-Asp-Asn-Phe-Lys-His-Leu-Leu-Gly-Asn-Asp-Asn-Val-Lys-Arg. The enzyme had a molecular mass of approximately 86 kDa and an isoelectric point of pH 3.8. The enzyme had a pH optimum of 8.6–9.0 and displayed maximum activity at 45°C. The alkaline enzyme was stable up to 50°C and more than 30% of the original activity was detectable after heating at 100°C and at pH 9.0 for 10 min. The enzyme hydrolyzed carboxymethylcellulose, lichenan (β-1,3;1,4-linkage), and *p*-nitrophenyl derivatives of cellotriose and cellotetraose. Crystalline forms of cellulose (Avicel and filter paper), H_3PO_4-swollen cellulose, NaOH-swollen cellulose, curdlan (β-1,3-linkage), laminarin (β-1,3;1,6-linkage), and xylan were barely hydrolyzed at all.

Key words *Bacillus* · Alkaliphilic · Alkaline · Thermostable · Cellulase · Purification

Introduction

The members of *Bacillus* often produce extracellular enzymes of industrial importance (Priest 1977; Horikoshi 1996). We have isolated many alkaliphilic and neutrophilic strains of *Bacillus* that produce alkaline cellulases (endo-1,4-β-glucanase, EC 3.2.1.4), the properties of which fulfill the essential requirements for enzymes to be used in laundry detergents (Ito 1997). Alkaline cellulases from *Bacillus* strains are effective for the removal of soils from cotton fabrics without degradation of the cotton fibers (Hoshino and Ito 1997). A mutant of one of our isolates, *Bacillus* sp. KSM-635 (Ito et al. 1989; Yoshimatsu et al. 1990), is currently exploited for the large-scale industrial production of an alkaline cellulase (Ito et al. 1991). The alkaline cellulase based detergents are now being marketed in Japan and Asian countries.

Most of the alkaline cellulases from *Bacillus* reported to date are not very thermostable (Horikoshi et al. 1984; Fukumori et al. 1985; Okoshi et al. 1990; Shikata et al. 1990; Yoshimatsu et al. 1990), and therefore are not economical or suitable for home laundering at high temperature. During the course of screening for detergent cellulase-producers, we found a mesophilic, alkaliphilic strain of *Bacillus* that produced a thermostable, alkaline cellulase. In this report, we describe the purification and some properties of the novel enzyme from the mesophilic, alkaliphilic isolate.

Materials and methods

Organism and culture conditions

The organism used was *Bacillus* sp. KSM-S237, which was originally isolated from a soil sample collected in Okinawa, Japan. Its morphological and taxonomic characteristics were examined according to the methods of Gordon et al. (1973) and Nielsen et al. (1995).

Bacillus sp. KSM-S237 was propagated at 30°C in a medium composed of (w/v): 0.1% carboxymethylcellulose (CMC; A10MC from Nihon Pulp, Tokyo, Japan), yeast extract (Difco, Detroit, MI, USA), 2.0% Polypepton S (Nihon Pharmaceutical, Tokyo, Japan), 1.0% meat extract (Wako Pure Chemical, Kyoto, Japan), 0.5% sodium glutamate,

Communicated by K. Horikoshi

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0.15% K_2HPO_4, 0.01% $CaCl_2 \cdot 2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $MnSO_4 \cdot 4H_2O$, and 0.5% Na_2CO_3. Solutions of $CaCl_2$ and Na_2CO_3 were separately autoclaved. The organism was grown with shaking at 30°C for 40 h in 50-ml aliquots of medium in 500-ml flasks. Cells were harvested by centrifugation (12000 × *g*, 15 min) at 5°C. All further manipulations were also done at this temperature. The supernatant obtained was used for purification of the enzyme.

Enzyme assays

The routine assays for carboxymethylcellulase (CMCase) activity and hydrolytic activities toward cellulosic and non-cellulosic polysaccharides were performed in 0.1 M glycine-NaOH buffer (pH 9.0), as described previously (Ito et al. 1989). After the reaction mixture had been incubated at 40°C for an appropriate period, the reducing sugar formed was quantified as glucose by the dinitrosalicylic acid procedure (Miller 1959). Hydrolytic activities toward *p*-nitrophenyl (PNP) β-D-cello-oligosaccharides, including PNP β-D-glucopyranoside (PNPG1), PNP β-D-cellobioside (PNPG2), PNP β-D-cellotrioside (PNPG3), PNP β-D-cellotetroside (PNPG4), and PNP β-D-cellopentaoside (PNPG5) (Seikagaku Kogyo, Tokyo, Japan), were measured at 40°C with 5 mM of each substrate in the same buffer. The *p*-nitrophenol liberated was determined at 400 nm, using an ε value of 15000. One unit of enzyme activity was defined as the amount of protein that produced 1 μmol product per min under these conditions. Protein was quantified by a modification of the method of Lowry et al. (1951) using the DC protein assay kit (Bio-rad, Richmond, CA, USA), with bovine serum albumin as the standard.

Purification of the enzyme

The crude enzyme was prepared by treating the supernatant of centrifuged cultures with ammonium sulfate and isolating the fraction that precipitated between 60% and 90% saturation. The precipitates were collected by centrifugation (12000 × *g*, 20 min). A small amount of 10 mM Tris-HCl buffer (pH 7.5) was added to the precipitates, which were then dialyzed overnight against the same buffer. The dialyzed retentate was applied to a column (2.5 cm inner diameter (i.d.) × 23 cm) of diethylaminoethyl (DEAE)-Toyopearl 650M (Tosoh, Tokyo, Japan) that had been equilibrated with 10 mM Tris-HCl buffer (pH 7.5). After the column had been washed with 250 ml of the equilibrating buffer, proteins were eluted in 4-ml fractions at a flow rate of 0.7 ml·cm^{-2}·min^{-1} with a 0.8-l gradient of 0–1.0 M NaCl in the same buffer. The active fractions (tubes nos. 85–91) were pooled and diluted to 200 ml with 10 mM Tris-HCl buffer (pH 7.5). The diluted solution was put directly on a column (1.5 cm i.d. × 20 cm) of DEAE-Toyopearl 650S equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Elution was done in 2-ml fractions at a flow rate of 0.42 ml·cm^{-2}·min^{-1} with a 0.6-l gradient of 0–0.6 M NaCl in the equilibrating buffer. The active fractions (tubes nos. 115–118) were combined, diluted with 200 ml of 10 mM Tris-HCl buffer (pH 7.5), and then concentrated to a small volume by ultrafiltration on a PM10 membrane (Amicon, Denver, MA, USA). The resultant concentrate was used for further experiments as the final preparation of enzyme.

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was done on 7.5% polyacrylamide slab gels, according to the method of Davis (1964), with 5 mM Tris/38 mM glycine buffer (pH 8.5) as the running buffer. Sodium dodecyl sulfate (SDS) PAGE (SDS-PAGE) was done on 12.5% polyacrylamide slab gels with 25 mM Tris-glycine buffer (pH 8.3) plus 0.1% SDS (Laemmli 1970). Isoelectric focusing of the purified enzyme was performed on 5% polyacrylamide slab gels that contained 1.9% Biolyte (pH range 3–5; Bio-rad), essentially as described by Wrigley (1971). The standard markers with known isoelectric point (pI; Bio-rad) included amyloglucosidase (pI 3.5), methyl red (pI 3.75), phycocyanin (pI 4.65), and β-lactoglobulin (pI 5.1). Protein bands were visualized by staining with Coomassie Brilliant Blue dye G-250 (Wako).

Activity staining of cellulase in the slab gel was done essentially by the Congo red-CMC procedure (Yoshimatsu et al. 1990), using CMC-agar sheets adjusted to pH 10 with 0.1 M glycine-NaOH buffer.

Amino-terminal sequencing

The N-terminal amino acid sequence of the purified enzyme was analyzed with an automated protein sequencer (model 477A; Applied Biosystems, Foster City, CA, USA). The sample (approximately 10 μg) was blotted onto a polyvinylidene difluoride membrane using a ProSpin cartridge (Applied Biosystems).

Estimation of molecular mass

Molecular mass was estimated both by gel filtration on a column of Sephacryl S-200 (1.5 cm i.d. × 80 cm; Pharmacia, Uppsala, Sweden) and by SDS-PAGE (12.5% polyacrylamide). The Sephacryl S-200 column (1.5 cm i.d. × 80 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.5) plus 2 mM $CaCl_2$ and 0.1 M NaCl, and calibrated by elution of standard protein markers which included alcohol dehydrogenase (150 kDa; Sigma, St. Louis, MO, USA), bovine serum albumin (67 kDa; Pharmacia), ovalbumin (43 kDa; Pharmacia), and carbonic anhydrase (29 kDa; Sigma). For the determination of molecular mass by SDS-PAGE, molecular mass markers (Bio-rad) were used: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

Results and discussion

Taxonomic characterization of strain KSM-S237

The isolate, designated KSM-S237, was a facultative anaerobe, which was spore-forming (cylindrical, subterminal endospores with a swollen sporangium), Gram-positive, motile, and rod-shaped (0.6–0.8 μm × 3.0–5.0 μm) with peritrichous flagella. It was capable of growing over the pH range of 9–12, but not at pH 7, and the range of temperature for growth was between 10°C and 40°C. The strict alkaliphile was positive for utilization of citrate (in Koser's medium); reduction of nitrate; production of oxidase and catalase; hydrolysis of starch, casein, and esculin; and liquefaction of gelatin; and was negative for acid fastness, denitrification, formation of indole and H_2S, production of urease, and growth on D-xylose. It seems likely that this isolate is a relative of *Bacillus agaradhaerens* (Nielsen et al. 1995).

Purification of the enzyme

The CMCase was purified to homogeneity from cultures of alkaliphilic *Bacillus* sp. KSM-S237 by precipitation with ammonium sulfate and the subsequent two-step column-chromatographic procedure. Figure 1a shows that the preparation of purified enzyme was homogeneous, as judged by nondenaturing PAGE. The band of the purified protein, detected by staining after nondenaturing PAGE, coincided fairly well with the single band seen by activity staining (Fig. 1b). The purification and total recovery of the enzyme are summarized in Table 1. Approximately 236-fold purification to a specific activity of 49.4 U/mg protein was obtained for the CMCase activity when measured at pH 9.0 and at 40°C.

Molecular mass, isoelectric point, and N-terminal amino acid sequence

The molecular mass of the purified enzyme was estimated to be approximately 86 kDa, as determined both by gel filtration on Sephacryl S200 and by SDS-PAGE. The pI of the enzyme was estimated to be pH 3.8 by isoelectric focusing under native conditions.

The N-terminal amino acid sequence of the purified enzyme, determined by Edman sequencing, was Glu-Gly-Asn-Thr-Arg-Glu-Asp-Asn-Phe-Lys-His-Leu-Leu-Gly-Asn-Asp-Asn-Val-Lys-Arg. The sequence is identical to those of alkaline cellulases, belonging to family A5, from *Bacillus* sp. strain 1139 (Fukumori et al. 1986) and *Bacillus* sp. KSM-64 (Sumitomo et al. 1992), but is completely different from the sequence of alkaline cellulase belonging to family A2, from *Bacillus* sp. KSM-635 (Ozaki et al. 1990). Therefore, the *Bacillus* sp. KSM-S237 cellulase may belong to the cellulases of family A5.

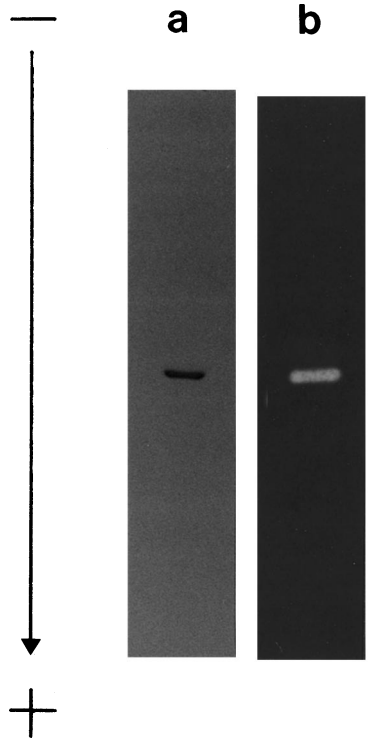

Fig. 1. Nondenaturing polyacrylamide gel electrophoresis (PAGE) (**a**) and activity staining (**b**) of the purified enzyme from *Bacillus* sp. KSM-S237. Approximately 5.6 μg of protein was put on each gel. Migration was from *top* (anode) to *bottom* (cathode). After electrophoresis, each gel was stained for protein and activity. After cutting the gels containing the marker dye Bromophenol blue, the gels were photographed

Table 1. Purification of carboxymethylcellulase (CMCase) activity from alkaliphilic *Bacillus* sp. KSM-S237

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude enzyme	1892	401	0.21	100	1
Ammonium sulfate (60%–90%)	64.8	270	4.1	67	19.5
DEAE-Toyopearl 650M	6.7	253	37.8	63	180
DEAE-Toyopearl 650S	3.4	168	49.4	42	236

DEAE, diethylaminoethyl.

Substrate specificity

As shown in Table 2, the purified enzyme hydrolyzed CMC, lichenan (β-1,3;1,4-linkage), PNPG3, and PNPG4. The enzyme rapidly decreased the viscosity of solutions of CMC. The enzyme had an apparent K_m of 11.4 mg/ml for CMC with an apparent V_{max} of 135 units/mg protein, when measured at 40°C and at pH 9.0 in 0.1 M glycine-NaOH buffer. Crystalline forms of cellulose (Avicel and filter paper), H_3PO_4-swollen cellulose, NaOH-swollen cellulose, curdlan (β-1,3-linkage), laminarin (β-1,3;1,6-linkage), xylan, PNPG1, PNPG2, and PNPG5 were barely hydrolyzed at all.

Effect of pH on the activity and stability of the enzyme

The purified CMCase was an alkaline enzyme, having a pH optimum of 8.6–9.0 in Britton-Robinson buffer, as shown in Fig. 2a. The CMCase activity was apparent over a wide range of pH values, with more than 90% of the maximum activity detected between pH 7.5 and pH 9.5. More than 30% of the maximum activity was observed even at pH 11.

To examine the stability of the alkaline CMCase at different pH values, the enzyme was preincubated at 30°C for 30 min in the various buffers (10 mM) and then residual activities were assayed at 40°C and at pH 9.0 in 0.1 M glycine-NaOH buffer (Fig. 2b). The enzyme was very stable, with more than 80% of the original activity detected throughout the wide pH range from 5 to 11.

Effect of temperature on the activity and stability of the enzyme

The optimum temperature for the reaction with CMC as substrate was detected at around 45°C at pH 9.0 in 0.1 M glycine-NaOH buffer, as shown in Fig. 3. $CaCl_2$ (5 mM) shifted the optimum temperature to 55°C, and the enzymatic activity was 1.15-fold greater than that at 45°C. No activity was observed at 70°C in the absence of Ca^{2+}, or at 80°C in the presence of Ca^{2+}.

Table 2. Substrate specificity of the purified enzyme

Substrate (1.0%, w/v)	Relative activity (%)
Carboxymethylcellulose	100
Avicel	0
H_3PO_4-swollen cellulose	0
NaOH-swollen cellulose	0
Lichenan	212
Curdlan	0
Laminarin	0
Xylan	0
PNPG1[a]	0
PNPG2[a]	1
PNPG3[a]	11
PNPG4[a]	4
PNPG5[a]	0.5

Assays were done at 40°C and at pH 9.0 in 0.1 M glycine-NaOH buffer.
[a] A 5-mM solution of each substrate was used for assays.

The thermal stability of the purified enzyme was assessed in 0.1 M glycine-NaOH buffer (pH 9.0) after heating for 10 min at various temperatures. In the absence of 5 mM $CaCl_2$, the enzyme was stable up to 45°C, and above this

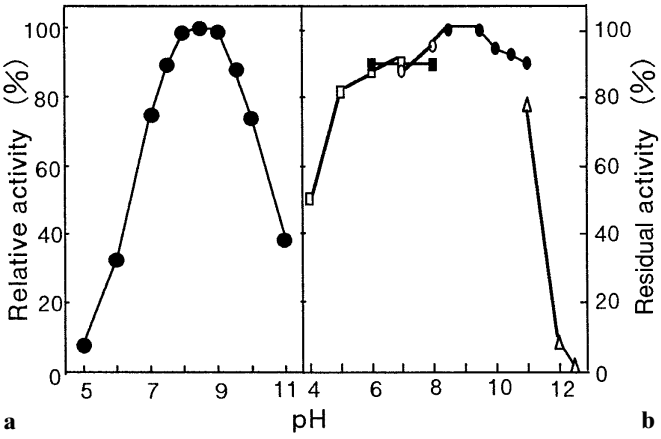

Fig. 2. Effect of pH on the activity (**a**) and stability (**b**) of the purified enzyme. **a** The effect of pH on the activity (*closed circles*) was determined with carboxymethylcellulose (CMC) as substrate at 40°C in 40 mM Britton-Robinson buffer ranging from pH 5 to pH 11. The maximum enzyme activity, at around pH 8.6–9.0 in this buffer, was taken as 100%. **b** To determine the enzyme stability with changes in pH, the purified enzyme was incubated at 30°C for 30 min in various buffers (10 mM) and then the residual activities were assayed at 40°C and at pH 9.0 in 0.1 M glycine-NaOH buffer. The values are shown as percentages of the maximum activity, taken as 100%. The buffers used for preincubation were acetate (pH 4.1–6.1, *open squares*), phosphate (pH 6.2–8.1, *closed squares*), Tris-HCl (pH 7.1–9.0, *open circles*), glycine -NaCl (pH 8.5–11, *closed circles*), and phosphoric acid-NaOH (pH 11–12.3, *open triangles*)

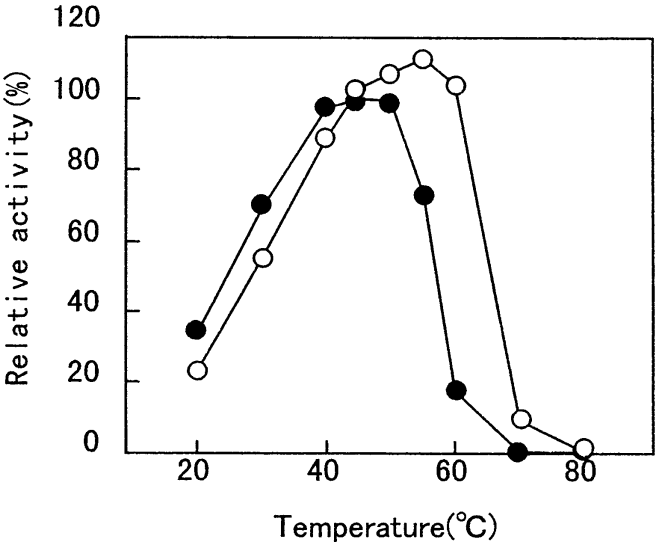

Fig. 3. Optimum temperature for the CMC-hydrolyzing activity of the purified enzyme. The purified enzyme was added to 1.0% CMC in 0.1 M glycine-NaOH buffer (pH 9.0). After a 20-min reaction at various temperatures, each activity was stopped by heating for 5 min in the presence of dinitrosalicylic acid solution. The enzymatic activities are expressed as percentages of the activity at 45°C in the absence of Ca^{2+} ions. *Closed circles*, activity in the absence of $CaCl_2$; *open circles*, that in the presence of 5 mM $CaCl_2$

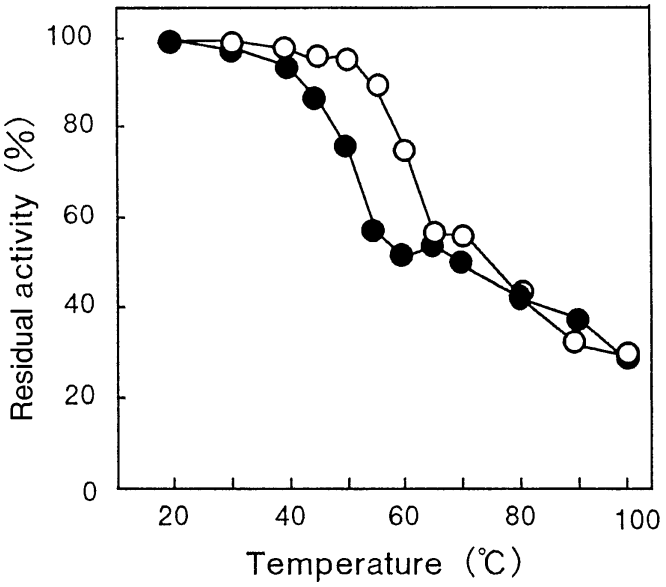

Fig. 4. Thermal stability of the purified enzyme. The purified enzyme was preincubated for 10 min at various temperatures in 0.1 M glycine-NaOH buffer (pH 9.0). Heating was stopped by cooling in a ice bath. Aliquots suitably diluted with the same buffer were used for measurement of the residual activity at 40°C for 20 min. The enzymatic activity, after heating at 30°C for 10 min, was taken as 100%. *Open circles*, residual activity in the presence of 5 mM $CaCl_2$; *closed circles*, that in the absence of $CaCl_2$

temperature the residual activity decreased gradually. The response to temperature was unusual in that there was a shoulder around 65°C in the temperature stability curve and more than 30% of the original activity remained even after heating at 100°C (boiling) for 10 min, as shown in Fig. 4. $CaCl_2$ protected the enzyme from thermal inactivation between 45°C and 65°C and consequently the enzyme was stable up to 55°C, but above 65°–70°C Ca^{2+} had no effect on the stability of the enzyme. These results indicate that the present enzyme is apparently the most thermostable among the *Bacillus* cellulases reported to date (Horikoshi et al. 1984; Fukumori et al. 1985; Au and Chan 1987; Yoshimatsu et al.1990; Okoshi et al. 1990; Shikata et al. 1990).

Effects of metal ions and various reagents

The enzyme activity was inhibited completely by Fe^{2+} but not by Hg^{2+} ions (each at 1 mM), and various sulfhydryl inhibitors had either no effect or a slight inhibitory effect. It was stimulated by Co^{2+} ions rather than Ca^{2+} ions. The stimulation by Co^{2+} may be a common characteristic of some *Bacillus* cellulases (Au and Chan 1987; Yoshimatsu et al. 1990; Okoshi et al. 1990). The enzyme was resistant to various surfactants (each added at 0.05%), such as SDS (0.01%), linear-alkylbenzene sulfonate, alkyl sulfate α-sulfonate, and polyoxyethylene alkyl ether, and to chelating agents (each at 5 mM), such as sodium citrate, zeolite (0.05%), ethyleneglycoltetraacetic acid (EGTA), and ethylenediaminetetraacetic acid (EDTA). These properties of the enzymes conformed to the essential requirements for enzymes that can be used as effective additives in laundry detergents.

In conclusion, the alkaline cellulase from *Bacillus* sp. KSM-S237 is unique in that it is very thermostable among the *Bacillus* alkaline cellulases so far reported, and may be the most suitable for use in detergents.

Acknowledgments We thank Dr. K. Horikoshi of Toyo University for helpful and critical discussions about our results.

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