Purification and Properties of Cellulases from an Alkalophilic *Streptomyces* Strain

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An alkalophilic *Streptomyces* strain, KSM-9, producing extracellular cellulases was isolated from soil. Three kinds of cellulases that preferentially hydrolyzed carboxymethylcellulose (CMC) were purified from the strain and designated as CMCase I, II and III. The optimum pH of CMCase I (M_r , 32,000) is 8.5 while those of CMCase II (M_r , 32,500) and III (M_r , 92,000) are at around pH 6.0. CMCase I hydrolyzed CMC in a more random fashion than the other two enzymes.

Most cellulases which are commercially available show optimum activity over a pH range of 4 to 6 and are usually inactive at alkaline pHs.^{1~3)} Recently, Horikoshi *et al.* reported cellulases with alkaline optimum pHs obtained from alkalophilic *Bacillus* strains^{4,5)} and cloned their genes.^{6,7)}

We screened alkalophilic microorganisms showing extracellular cellulase activity and isolated an alkalophilic *Streptomyces*, strain KSM-9. The strain secreted several cellulases, one of which exhibited an alkaline optimum pH for its activity. In this paper, we report the isolation and characterization of the strain, purification of its cellulases and some properties of the purified enzymes.

MATERIALS AND METHODS

Media. The alkaline agar medium for the isolation of alkalophilic microorganisms was prepared as follows. A solution containing 4.0% carboxymethylcellulose (CMC), 2.0% Bactopeptone (Difco), 2.0% meat extract (Difco), 0.2% KH_2PO_4 , 2.0% NaCl and 1.56% agar was autoclaved, and then an equal volume of 2.0% sterilized Na_2CO_3 was added just before pouring into petri dishes. The liquid medium for cultivation of the cellulase-producing strains contained 1.5% meat extract (Difco), 0.5% yeast extract (Difco) and 0.5% KH_2PO_4 , to which 0.5% sterilized Na_2CO_3 was added after autoclaving.

Isolation and characterization of the cellulase-producing

strains. A soil sample was suspended in a small volume of sterilized water and then spread on alkaline agar medium plates. The plates were incubated at 30°C for 5 days and colonies forming a shallow depression in the agar surface were isolated. The selected organisms were cultured in the liquid medium aerobically at 30°C for 4 days and then cellulase activity in the culture filtrate was measured.

The isolated *Streptomyces* strain, KSM-9, was characterized according to the 8th edition of Bergey's Manual of Determinative Bacteriology⁸⁾ and the International *Streptomyces* Project.⁹⁾ The micromorphology of the strain was observed under a scanning electronmicroscope, JEOL JSM-35C. Diaminopimeric acid in peptidoglycan was analyzed according to the method of Rhuland *et al.*¹⁰⁾

Cultivation for cellulase production. *Streptomyces* strain KSM-9 was cultured to the early stationary phase in 100 ml of the liquid medium in a 500 ml Erlenmeyer flask aerobically at 30°C for 3 days. The resultant culture was inoculated into 20 l of the same medium in a 30 l jar fermenter (Marubishi MSJ-U3) and then cultured with 1:1 aeration and 200 rpm agitation at 30°C for 4 days.

Chromatography and gel electrophoresis of the enzymes. A FPLC-liquid chromatography system (Pharmacia Fine Chemicals Co.) equipped with a column of Mono Q HR 5/5 or Superose 12 (Pharmacia Fine Chemicals Co.) was used for purification of the enzymes.

SDS-Polyacrylamide gel electrophoresis of the enzymes was performed according to Laemmli.¹¹⁾

Measurement of cellulolytic activity.

i) **CMC-saccharifying activity.** The reaction mixture contained 0.2 ml of 2.5% CMC solution, 0.1 ml of 0.5 M

phosphate buffer (pH 8.0), 0.1 ml of distilled water and 0.1 ml of the enzyme solution. The mixture was incubated at 40°C for 30 min, and then the released reducing sugar was determined with dinitrosalicylic acid.¹²⁾ One unit of the enzyme activity was defined as the amount of the enzyme releasing 1 μ mol of reducing sugar from CMC per minute.

ii) *CMC-liquefying activity*. The reaction mixture contained 3 ml of 1.0% CMC solution, 2 ml of 0.1 M phosphate buffer (pH 8.0), 3 ml of distilled water and 0.2 ml of the enzyme solution. The mixture was incubated in an Ostwald viscometer at 40°C and the viscosity was measured at appropriate intervals. One unit of the enzyme activity was defined as the amount of the enzyme causing a reduction of 1 centistoke viscosity unit per 5 min.

iii) *Avicelase activity*. The reaction mixture contained 0.9 ml of 2.0% Avicel in 0.1 M phosphate buffer (pH 8.0), and 0.1 ml of the enzyme solution. The mixture was incubated at 40°C for 200 min, and then the released reducing sugar was determined with dinitrosalicylic acid. One unit of the enzyme activity was defined as in the case of the CMC-saccharifying activity.

Protein concentration determination. The protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard by the method of Bradford.¹³⁾ On column chromatography, the protein concentration was monitored at A_{280} .

Molecular weight determination. Molecular weight estimation was carried out by SDS-polyacrylamide gel electrophoresis and gel-filtration column chromatography with Superose 12.

Inhibition by metal ions. The purified enzyme was first dialyzed against 0.1 M phosphate buffer (pH 8.0) containing 1 mM EDTA for 10 hr, and then against the same buffer without EDTA for 15 hr at 5°C. The dialyzed enzyme was incubated in the presence of various metal ions (5 mM) for 30 min at 40°C and then assayed under the standard conditions.

Chemicals. CMC was purchased from Wako Pure Chemical Industries. Avicel was purchased from Asahi Kasei Co., Ltd.

RESULTS

Isolation of Streptomyces strain KSM-9

Fourteen strains of alkalophilic bacteria were isolated as cellulase producers, among which a streptomycetes strain, KSM-9, was found to show the highest cellulolytic activity with CMC as a substrate. The taxonomical characteristics of strain KSM-9 are shown in

TABLE I. MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF *Streptomyces* STRAIN KSM-9

Spore chains	Straight
Spore shape	Spherical
Spore surface	Smooth
Melanoid pigment	Negative
Hydrolysis of starch	Positive
Liquefaction of gelatin	Positive
Reaction in litmus milk	Not coagulated
L,L-Diaminopimelic acid	Positive
meso-Diaminopimelic acid	Negative
Growth pH range	pH 8 ~ pH 9.5
Optimum pH	pH 8.5

Table I. From the data in Table I and other taxonomical characteristics (data not shown), KSM-9 appeared to show some similarity with *Streptomyces puniceus*, however, its distinct alkalophilic property discriminates them clearly.

Purification of cellulases from strain KSM-9

Ammonium sulfate was added to the twenty liter culture filtrate of strain KSM-9 to 70% saturation and then the precipitated cellulases were redissolved in 50 ml of distilled water. The solution was dialyzed for 18 hr against 7 l of 0.1 M phosphate buffer (pH 5.8) and then applied onto a DEAE-Toyopearl 650 M ion-exchange column (8 $\times$ 20 cm) equilibrated with the same buffer. The enzymes were eluted with 1.2 l of a linear gradient of NaCl (0.05 ~ 0.4 M) in 0.1 M phosphate buffer (pH 5.8) at a flow rate of 30 ml/hr.

The CMC-saccharifying activity was separated into two peaks, F1 and F2 (Fig. 1). Fraction F1 was applied to a column of Mono Q HR5/5 and then eluted with a linear gradient of NaCl, from 0.05 M to 0.3 M NaCl, in 0.1 M phosphate buffer (pH 5.8). The activity was separated further into two peaks (F1-1 and F1-2).

Fraction F2 was applied to a Mono Q HR 5/5 column and then eluted with a linear gradient of NaCl, from 0.05 M to 0.7 M in 0.1 M phosphate buffer (pH 5.8). A single peak of enzyme activity was obtained.

The resultant fractions (F1-1, F1-2 and F2),

which each gave a single peak of enzyme activity, were further subjected to gel filtration on a column of Superose 12 with 0.1 M phosphate buffer (pH 5.8). A single protein band was observed on SDS-polyacrylamide gel electrophoresis of each purified sample (data not shown).

The enzyme purification is summarized in Table II. The purified enzymes derived from fractions F1-1, F1-2 and F2 were designated as CMCase I, II and III, respectively.

Physicochemical properties of the enzymes

1) *Molecular weights.* The molecular weights of CMCase I, II and III were estimated to be 32,000, 32,500 and 92,000, respectively, by gel filtration on Superose 12 or by

SDS-polyacrylamide gel electrophoresis.

2) *Optimum temperature.* The CMC-saccharifying activity of CMCase I, II and III was measured at various temperatures, from 30 to 80°C, in 0.1 M phosphate buffer (pH 7.0). The amounts of CMCase I, II and III used were 0.35, 0.48 and 0.41 units, respectively. The optimum temperatures for all the enzymes were around 45~55°C (Fig. 2a).

3) *Heat stability.* Residual saccharifying activities of the three CMCases, in the same amounts as above, were examined after heat-treatment at various temperatures, from 30 to 90°C, for 30 min in 0.1 M phosphate buffer (pH 7.0). CMCase I showed the highest stability (Fig. 2b).

4) *Optimum pH.* The activities of CMCase I, II and III were measured at various pHs, from pH 4.0~12.0. The optimum pHs of CMCase I and the other two CMCases were found to be 8.5 and 6.0, respectively (Fig. 3a).

5) *pH stability.* The pH stability of the CMCases, in the same amounts as above, was also examined at various pHs, from 4.0~13.0. After incubation at 30°C for 24 hr, the residual activities were measured. The pH stability range of CMCase I and the other two CMCases were 6.0~8.0 and 6.0~7.0, respectively (Fig. 3b).

FIG. 1. DEAE-Toyopearl 650M Chromatography of CMCases from *Streptomyces* Strain KSM-9.

----, absorbance at 280 nm; —●—, CMCase activity; —, NaCl concentration (M). The experimental details are given in the text.

Catalytic properties of the enzymes

All the CMCases showed high saccharifying activity toward CMC but little activity toward

TABLE II. SUMMARY OF PURIFICATION OF CMCASES PRODUCED BY *Streptomyces* STRAIN KSM-9

Step		Total protein (A ₂₈₀)	Total activity (Units)	Specific activity (Units/A ₂₈₀)	Yield (%)
Culture filtrate		17296	880	0.051	100
Ammonium sulfate		1960	468	0.024	53.2
DEAE-Toyopearl	F1	250	180	0.72	42.5
	F2	56	194	3.5	
DEAE-Mono Q	F1-1	10	12	1.2	17.9
	F1-2	31	88	2.8	
	F2	2.5	59	23	
Superose 12	F1-1	1.3	8.7	6.7	6.8
	F1-2	2.8	59	21	
	F2	1.2	38	32

FIG. 2. Effect of Temperature on the Activities of CMCase I, II and III.

- (a) Temperature-activity curves for CMCase I (●), CMCase II (○) and CMCase III (■).
 (b) Heat-stability of CMCase I (●), CMCase II (○) and CMCase III (■).

FIG. 3. Effect of pH on the Activities of CMCase I, II and III.

- (a) pH-activity curves for CMCase I (●), CMCase II (○) and CMCase III (■).
 (b) pH-stability curves for CMCase I (●), CMCase II (○) and CMCase III (■). The buffers used were 0.1 M acetate buffer (pH 4.0~6.0), 0.1 M phosphate buffer (pH 6.0~8.0) and 0.1 M glycine-NaOH buffer (pH 8.0~11.0).

avicel or cotton. CMCase II showed the highest specific activity.

The degree of randomness as to the hydrolysis of CMC was examined by comparing the liquefying activity with the saccharifying activity. Each CMCase showed a definite ratio for the two activities (Fig. 4). On the basis of these data, CMCase I could be classified as a more-random type, and CMCase II and III as less-random types.

The effects of metal ions, *i.e.*, Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Fe^{2+} , Cu^{2+} and Zn^{2+} , on these CMCase activities were measured. The activities of CMCase II and III were slightly enhanced by calcium ions.

DISCUSSION

This paper described the isolation of alkalophilic *Streptomyces* strain KSM-9, which produces at least three different extracellular cellulases (CMCases I, II and III). CMCase I has some properties clearly different from those of the other two CMCases. CMCase I is the most active at an alkaline pH (pH 8.5) and 50% of its activity still remained at pH 10, while the optimum pHs of CMCase II and III are slightly acidic. In addition, CMCase I hydrolyzed CMC in a more random fashion than the other two enzymes.

Most of the hitherto reported cellulases

FIG. 4. Relationship between Liquefying Activity and Reducing activity during the Hydrolysis of CMC.

Symbols: CMCase I (●), CMCase II (○) and CMCase III (■).

derived from various microorganisms show optimum pHs in a neutral or slightly acidic range. However, several cellulases with alkaline optimum pHs have been found in alkalophilic *Bacillus* strains which are expected to be of industrial importance. CMCase I of *Streptomyces* strain KSM-9 is another alkalophilic cellulase, but in a different genus. Since the enzyme productivity of the parental strain is very low, we attempted to improve the yield

by recombinant DNA techniques. Successful cloning of the CMCase I gene and its structure will be reported elsewhere.

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