Agric. Chem. Biotechnol. 42(3), 107-112(1999)

Purification and Characterization of Carboxymethyl-cellulase Produced by *Bacillus* sp. KD1014

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Received August 23,1999

A carboxymethyl-cellulase (CMCase) was purified from the culture supernatant of *Bacillus* sp. KD1014 by ultrafiltration, ammonium sulfate precipitation, and a series of chromatography on QAE-Sephadex A-50, hydroxylapatite and Sephadex G-75. The purified CMCase was a single protein of 32 kDa, showed an optimum activity at 60°C and pH 6.0, and had a half-life of 23 min at 70°C. The enzyme activity was not influenced by metal ions such as Mg^{2+}, Fe^{3+}, K^{+}, Zn^{2+}, and Cu^{2+} at a concentration of 1.0 mM, partially inhibited by Mn^{2+} and Ag^{+}, and significantly inhibited by pentachlorophenol (PCP). The purified enzyme showed a 3.9-times higher activity on lichenan than on CMC, but hardly cleaved xylan, starch, avicel, laminarin, filter paper and levan. The results of activity staining of the purified enzyme separated by native and denaturing gel electrophoresis suggested that the CMCase might exist in dimeric, oligomeric or aggregated form as well as in monomeric form. The enzymatic cleavage products from cellotetraose indicated that the CMCase possessed transglycosylation activity.

Key words: *carboxymethyl-cellulase,* Bacillus *sp. KD1014, purification, thermostable enzyme, transglycosylation*

Cellulose-hydrolyzing enzymes have drawn much attention in regard to their role in utilizing enormous amount of cellulosic waste. Successful utilization of cellulosic material as a renewable carbon source depends mainly on the ability of cellulase to degrade cellulose to low molecular weight products. The hydrolysis products can then be converted to other useful products to be used as liquid fuel, feedstock, and food materials by biological and/or chemical processes. Recently cellulase has also been used in textile, paper, food and pharmaceutical industry for various purposes.[1]

Most cellulases are enzyme complexes consisted of carboxymethyl-cellulase (CMCase, endo-β-1,4-glucanase, endoglucanase), avicelase (exo-β-1,4-glucanase, exoglucanase), and β-1,4-glucosidase, and the interaction of the enzyme components is necessary for the complete hydrolysis of cellulose. Cellulolytic enzymes are produced by a variety of microorganisms in nature. Bacterial cellulases produced by *Cellulomonas*, *Clostridium*, *Bacillus*, *Ruminococcus*, *Caldocellum*, and *Pseudomonas* species are well studied.[1] Several cellulases, in particulary CMCases, have been reported to be produced by *Bacillus* species.[2-10] Recently, the use of a new endoglucanase from *Bacillus* sp. BP-23 for paper manufacture from cereal straw was reported.[11]

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Abbreviations: CMC, carboxymethyl-cellulase; CMCase, carboxymethyl-cellulase; DNS, 3,5-dinitrosalicylic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The advantages of using thermostable enzymes for various industrial applications have been well recognized and several thermostable endoglucanases have been reported from *Bacillus* species.[12] Previously, we reported the isolation of a thermostable CMCase-producing *Bacillus* strain.[13] In this paper, we describe the characteristics of the purified CMCase produced by *Bacillus* sp. KD1014.

Materials and Methods

Bacterial strain and culture medium. *Bacillus* sp. KD1014 was isolated from soil.[13] LBC [LB supplemented with 0.5% CMC] was used for enzyme production.

Reagents. Medium components were from Difco. CMC, xylan, 3,5-dinitrosalicylic acid (DNS), and Congo red were from Sigma, hydroxylapatite from Bio Rad, QAE- Sephadex A-50 and Sephadex G-75 from Pharmacia, and Avicel PH101 was from Fluka.

Enzyme assay. CMCase activity was determined by measuring the amount of reducing sugar released by DNS method[14] after 15 min of reaction at 50°C using 0.5% CMC in 50 mM sodium citrate (pH 5.5) as the substrate. Xylanase activity was determined by the same method except that 0.5% xylan was used as the substrate. One unit of the enzyme activity was defined as the amount of enzyme that released reducing sugar equivalent to 1.0 μmol of glucose per min under the above condition. Various substrates at a concentration of 0.5% were used to study the substrate specificity of the enzyme.

Enzyme purification. *Bacillus* sp. KD1014 was grown in LBC for 24 h at 45°C for CMCase production. All

purification procedures were performed at 4°C. After removal of cells by centrifugation for 30 min at 4,000 × g, the culture supernatant was concentrated by ultrafiltration (Amicon 400 m*l* kit, PM 10 membrane). The concentrate was then treated with 30~90% ammonium sulfate, and the precipitate obtained by centrifugation at 54,000 × g was dissolved in 50 mM Tris-HCl buffer (pH 7.0), and then dialyzed twice against the same buffer.

The dialysate was applied onto a QAE Sephadex A-50 column (4.0 × 28 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0). After eluting unbound proteins with the same buffer, bound proteins were eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer at 24 m*l*/h. Fractions (3.4 m*l* × 210) were collected and checked for their protein concentrations and for CMCase and xylanase activities.

Active fractions from the QAE-Sephadex A-50 chromatography (Q-I peak) were concentrated by ultrafiltration, equilibrated with 10 mM sodium phosphate (pH 6.8), and then eluted on a hydroxylapatite column (HA1, 3 × 5cm) with a linear gradient of 0.01~0.3 M sodium phosphate (pH 6.8) at 13.5 m*l*/h. Active fractions (HA1-II peak) were again chromatographed on a hydroxylapatite column (HA2, 3 × 5 cm) under the same condition.

Active fractions from the second hydroxylapatite chromatography (HA2-I peak) were concentrated, dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM KCl, and then eluted on a Sephadex G-75 column (1.8 × 44.5 cm) with the same buffer at 12 m*l*/h. Active fractions were collected and used as a purified enzyme for further studies.

Protein analysis. Protein concentration was determined by the method of Lowry *et al.*[15] or Bradford[16] with bovine serum albumin as the standard. Molecular weight of the purified CMCase was determined by SDS-PAGE on an 11% gel according to Laemmli.[17] Molecular weight standards were bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen from bovine pancreas (24 kDa) and trypsin inhibitor (20 kDa).

Activity staining (Zymogram). The purified enzyme was electrophoresed on 11% of native and SDS- polyacrylamide gel. For SDS-PAGE, the enzyme was mixed with the sample buffer and then heated for 3 min before loading. After electrophoresis, the gels were washed in 20% isopropanol (1 × 1 h) and then in 50 mM sodium citrate buffer (pH 5.5) (2 × 1 h). The washed gels were kept in contact with 1.5% agar gels containing 0.5% CMC for 1 h at 55°C. The agar gels were stained for enzyme activity with 0.1% Congo red and then destained with 1 M NaCl.[18] Protein staining was done with Coomassie brilliant blue.

Heat and pH stability of the enzyme. Heat stability of the enzyme was determined by measuring the remaining activity after treating the enzyme in the absence of the substrate at 50, 60, 65, 70 and 80°C in 50 mM sodium citrate buffer (pH 5.5) for designated time periods. pH stability of

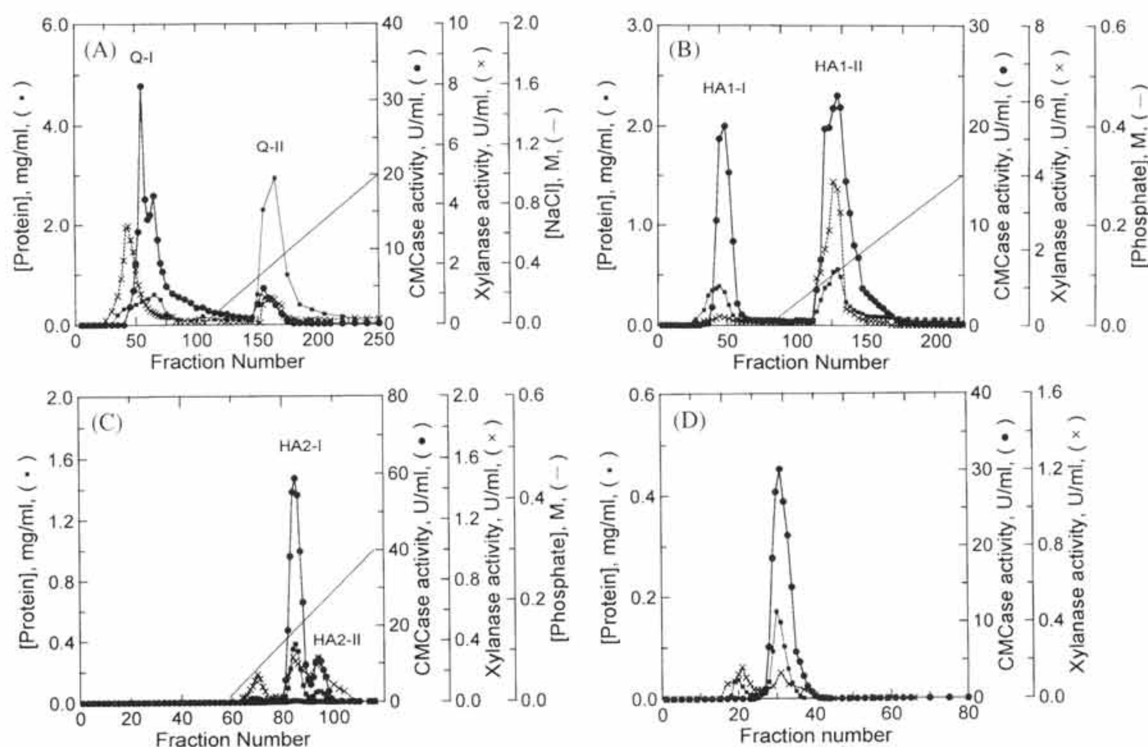

Fig. 1. Chromatography of CMCase from *Bacillus* sp. KD1014. A, QAE-Sephadex A-50; B, first hydroxylapatite; C, second hydroxyapatite; D, Sephadex G-75. Ammonium sulfate (30~90%) precipitate was chromatographed on A, and Q-I from A was chromatographed on B. HA1-II from B was chromatographed on C, and HA2-I from C was chromatographed on D.

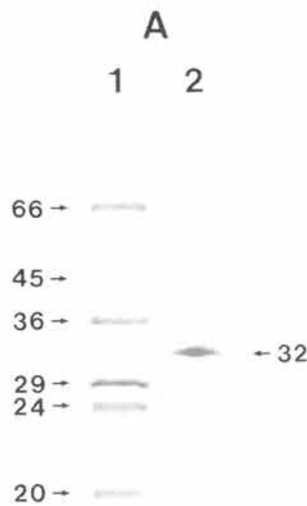

Fig. 2. SDS-PAGE of the purified CMCase. The numbers on leftside indicate molecular weights of standard proteins in kDa. Lane 1, size marker; lane 2, purified CMCase.

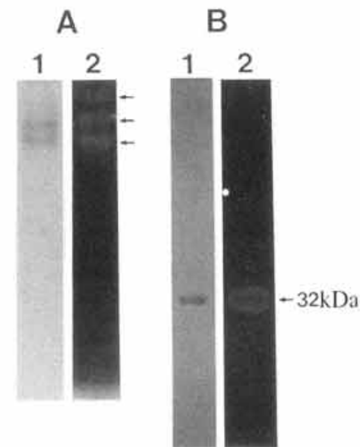

Fig. 3. Protein and activity staining of the CMCase purified from *Bacillus* sp. KD1014 after native (A) and SDS-PAGE (B). Lanes 1, protein staining; lanes 2, activity staining. The arrows indicate active bands on agar replica gel.

the enzyme was determined by measuring the remaining activity after keeping the enzyme in appropriate buffers for up to 24 h.

Analysis of reaction products by HPLC. Ten millimole cellotetraose (G4) was mixed with 50 mM potassium phosphate buffer (pH 5.8) and an appropiate amount of the enzyme (15 U/m*l*), and then incubated for 1 h at 50°C. The reaction products were applied on to a HPLC column [μBondapak NH_2 125Å (10 μm), 39 × 300 mm, Waters] and eluted with 73% acetonitrile.[6]

Results and Discussion

Purification of the enzyme. The culture supernatant of *Bacillus* sp. KD1014 was concentrated by ultrafiltration, precipitated with 30%~90% ammonium sulfate, and then separated on a QAE Sephadex A-50 column. Two CMCase activity peaks were obtained during the washing, and a little CMCase activity which could be eluted with increasing concentration of NaCl was remained bound to the resin (Fig. 1A). Major portion of the xylanase activity was eluted prior to the CMCase activity during the washing (Fig. 1A).

When major CMCase activity fractions from QAE-Sephadex A-50 (Q-I) were separated on a hydroxylapatite column, two CMCase activity peaks were obtained (Fig. 1B). One peak (HA1-I) was eluted during the washing and the other (HA1-II) with 0.07~0.13 M phosphate. Rechromatography of HA1-II on a hydroxylapatite column resulted in two CMCase activity peaks, a major peak (HA2-I) eluted with 0.13 M phosphate and a minor peak (HA2-II) eluted with 0.18 M phosphate (Fig. 1C). It was later found that HA1-I was resulted from overloading (data not shown).

Sephadex G-75 chromatography of the major active fractions from the second hydroxylapatite chromatography (HA2-I) resulted in a single CMCase activity peak (Fig. 1D). SDS-PAGE analysis of the active fractions showed a single protein band, and the molecular weight of the protein was estimated to be 32 kDa (Fig. 2).

The specific activity of the CMCase was increased by 2,050-times and 2.1% of the total activity was recovered by the purification procedures (Table 1). The results of the purification indicate that *Bacillus* sp. KD1014 produces one major CMCase and two minor CMCases.

Table 1. Purification of major CMCase from *Bacillus* sp. KD1014.

Purification steps	Total Volume (m*l*)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Supernatant	12,000	13,272	58,440	0.23	1.0	100.0
Ultrafiltration	626	10,329	7,238	1.4	6.2	77.8
Ammonium sulfate (30-90%)	48	4,658	740	6.3	27.4	35.1
QAE-Sephadex A-50	356	2,976	250	11.9	51.9	22.4
1st Hydroxylapatite	110	1,752	5	350.5	1,523.0	13.2
2nd Hydroxylapatite	3.7	569.3	1.4	421.7	1,833.0	4.3
Sephadex G-75	2.4	282.9	0.6	471.5	2,050.0	2.1

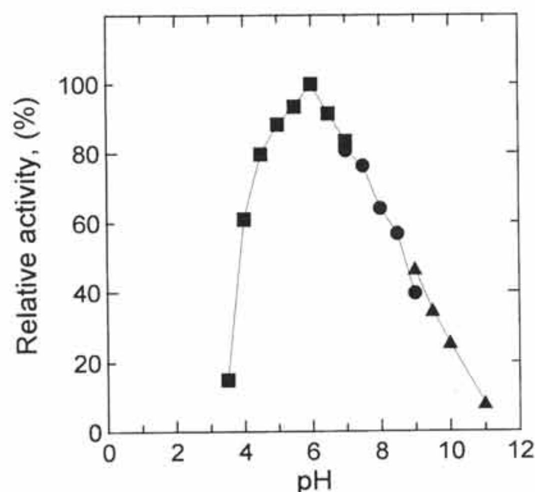

Fig. 4. Effect of pH on the CMCase activity. Symbols: ■, Na-citrate; ●, Tris-HCl; ▲, Glycine-NaOH buffer.

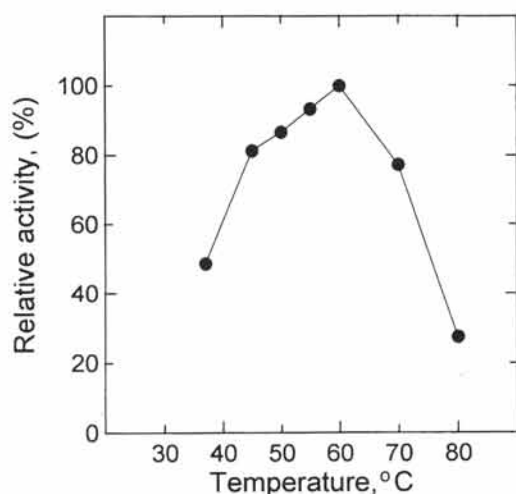

Fig. 5. Effect of temperature on the CMCase activity.

Activity staining. Native gel electrophoresis of the purified enzyme resulted in two major and a minor protein bands (Fig. 3A, lane 1). However, only a single protein band corresponding to 32 kDa was observed on SDS-gel (Fig. 3B, lane 1). As in the protein staining, three CMCase activity bands were observed on the agar replica of the native gel (Fig. 3A, lane 2) and only a single activity band of 32 kDa from that of the denaturing gel (Fig. 3B, lane 2). These results suggest a possibility that the CMCase from *Bacillus* sp. KD1014 may exist in dimeric, oligomeric or aggregated form as well as in monomeric form. Similar phenomenon has been reported with an α-amylase from *Bacillus licheniformis*.[19-20]

Influence of pH and temperature. The purified CMCase was active at a broad range of pHs, showing a maximal activity at pH 6.0 and more than 70% of the maximal activity at pHs 4.5~7.5 (Fig. 4). The enzyme was stable at pHs 5~9, retaining most of the activity after 24 h storage at 25°C (data not shown). The optimum temperature for the CMCase was

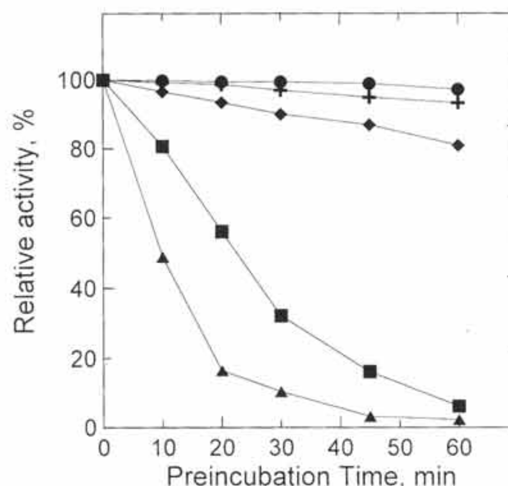

Fig. 6. Thermal stability of the purified CMCase. Symbols: ●, 50°C; +, 60°C; ◆, 65°C; ■, 70°C; ▲, 80°C.

60°C, and more than 75% of the maximal activity was obtained at temperatures 45~70°C (Fig. 5). When the enzyme was preincubated for 1 h, no decrease in activity was observed at 50°C, and 7 and 19% decrease at 60 and 65°C,

Table 2. Effect of various metal ions and reagents on activity of the purified CMCase from *Bacillus* sp. KD1014.

Reagents	Relative activity (%)	
	1.0 mM	0.1 mM
None	100.0	100.0
Metal ions		
$MnCl_2$	53.2	95.5
$ZnCl_2$	100.6	100.3
$CdCl_2$	95.1	102.9
$MgCl_2$	97.5	103.5
$BaCl_2$	99.1	101.0
$CoCl_2$	116.3	98.4
$SrCl_2$	98.2	94.0
$CaCl_2$	97.5	101.0
KCl	100.0	98.4
$FeCl_3$	105.0	103.2
$CuSO_4$	123.5	98.1
$AgNO_3$	84.8	97.8
Reagents[a]		
SDS	93.2	100.0
EDTA	97.8	97.1
NBS	99.1	101.0
Na_2HAsO_4	98.5	98.1
PCP	13.7	93.4
MIA	93.5	95.5
TCA	98.2	101.6

[a]Abbreviations: SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetate; NBS, n-bromosuccinimide; PCP, pentachlorophenol; MIA, monoiodoacetate; TCA, trichloroacetate.

Table 3. Substrate specificity of the purified CMCase from *Bacillus* sp. KD1014.

Substrate	Relative activity (%)
CMC	100.0
Filter paper[a]	2.7
Avicel	0.8
Xylan	2.4
Levan	1.2
Lichenan	384.8
Laminarin	1.1
Souble starch	2.5

[a]The size of the filter paper (Whatman No. 1) used was 1cm × 1cm and was reacted for 2 h at 50°C.

respectively (Fig. 6). The half-lives of the enzyme activity at 70 and 80°C were 23 and 10 min, respectively, and the enzyme was completely inactivated by preincubation for 1 h at these temperatures (Fig. 6).

Influence of metal ions and chemicals. No tested metals ions or chemicals inhibited the CMCase activity at a concentration of 0.1 mM. Metal ions Cd^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, Ca^{2+}, K^{+} and Fe^{3+} at 1.0 mM showed little influence on the activity, Co^{2+} and Cu^{2+} at 1.0 mM increased the activity by 16 and 24%, respectively, and Mn^{2+} and Ag^{+} decreased the activity by 47 and 15%, respectively (Table 2). One millimole of NBS, Na_2HAsO_4, TCA, and EDTA showed no significant influence on the enzyme activity, but PCP decreased the enzyme activity by 86%.

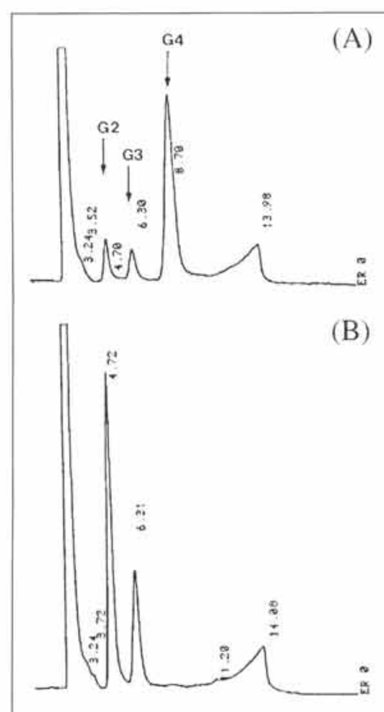

Fig. 7. HPLC chromatogram of reaction products of the purified CMCase on cellotetraose. A, before reaction; B, after reaction for 1 h at 50°C. Cellotetraose in 50 mM potassium phosphate buffer (pH 5.8) was treated with the purified enzyme for 1 h at 50°C. G4, cellotetraose; G3, cellotriose; G2, cellobiose.

Substrate specificity. The purified enzyme showed a 3.9-times higher activity on lichenan than on CMC (Table 3). This relative activity of the CMCase from *Bacillus* sp. KD1014 on lechenan is higher than those of CMCases from *Bacillus subtilis* BSE616[21] and from *Bacillus* sp. BP-23.[11] The CMCase from *Bacillus subtilis* BSE616 showed a 1.4-times higher activity on lichenan than on CMC,[20] and that from *Bacillus* sp. BP-23 showed a 0.23-times activity.[11] This might indicate that the CMCase from *Bacillus* sp. KD1014 is a new enzyme different from those of other *Bacillus* species. The purified CMCase could hardly hydrolyze laminarin, filter paper, Avicel, levan and xylan (Table 3).

Reaction products of cellotetraose. Cellobiose (G2) was found to be the major enzymatic cleavage product from cellotetraose (G4) through HPLC analysis (Fig. 7). Cellotriose (G3), one half of the amount of cellobiose, was produced by the reaction, though no glucose (G1) was produced. This suggests that the CMCase from *Bacillus* sp. KD1014 possesses transglycosylation activity and belongs to a retaining enzyme rather than to an inverting enzyme with regard to its catalytic mechanism.[1]

Acknowledgments. This work was supported in part by a G7 grant from the Ministry of Science and Technology, Korea.

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