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Characterization of Carboxymethylcellulase(CMCase) Produced by Recombinant *E. coli* Containing CMCase Gene from *Cellulomonas* sp. YE-5

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Abstract

CMCase produced by recombinant *E. coli* JM109(pCEH#4) containing CMCase gene from *Cellulomonas* sp. YE-5 was purified to 24.3 fold and 2.6% yield by ammonium sulfate precipitation, DEAE-cellulose column chromatography and gel filtration on Sephadex G-100. The optimum pH and temperature for CMCase activity were pH 7.0 and 50°C. The enzyme was stable between pH 5.0 and 10.0, and up to 60°C. The molecular weight of the enzyme was estimated to be approximately 40,000 daltons by SDS-PAGE. Analysis of the amino acid composition showed that the enzyme contained many glycines and acidic amino acids. The enzyme was an endo-type CMCase and the final enzyme reaction product from hydrolysis of CM-cellulose by the enzyme was cellobiose. K_M value determined with CM-cellulose was 1.28mM.

Key words: recombinant *E. coli*, *Cellulomonas* sp YE-5, carboxymethylcellulase(CMCase)

INTRODUCTION

Cellulose is the most abundant carbohydrate available from plant biomass(1,2) with an estimated synthesis rate of 4×10^{10}tons/year(3). It has attracted the interest of many microorganisms, which use it as a carbon source and more recently, of biotechnologists, who wish to use it as a renewable source of fuels and chemicals. The enzymatic conversion of cellulose into glucose is far from simple due to the physical nature of the substrate. In its native form, cellulose is composed of largely crystalline fibers in which the hydrogen bonds hold the individual molecules together. Furthermore, the fibers are embedded in a matrix of hemicellulose and lignin, which further reduces their accessibility to cellulolytic enzymes(4). For the mineralization of the crystalline polysaccharide, cellulolytic microbes produce a number of enzymes with different functions(5-8). The polysaccharide is degraded mainly by microorganisms in the aerobic as well as in the anaerobic ecosystems (9-15). Most of the studies on microbial cellulolysis have been done using aerobic bacteria, fungi, and thermophilic anaerobic bacteria. Based on biochemical studies on various microbial enzymes, the cellulolytic enzymes have been classified into three basic groups; endo-1,4-β-glucanase(EC 3.2.1.4), exo-1,4-β-glucanase(EC 3.2.1.91), and β-1,4-glucosidase(EC 3.2.1.21)(16). The industrial feasibility of the enzymatic processing of cellulose does not only depend on the large-scale production of well-identified enzymes, but also require further basic research to find out how existing cellulase systems work. In recent years, recombinant DNA technology and protein chemistry provided information about the structure and function of cellulase with an accelerating pace. Choi et al.(1992) purified CMCase from *Cellulomonas* sp. YE-5 which was isolated from soil(17). A gene for CMCase was cloned into *E. coli*(18) and investigated for the chemical and physical properties of the purified CMCase from donor and recombinant strain. In this report, we described the purification and characterization of CMCase produced by recombinant *E. coli* containing CMCase gene from *Cellulomonas* sp. YE-5.

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MATERIALS AND METHODS

Bacterial strains and media

Cellulomonas sp. YE-5 was used as the source of cellulase genes(17). For purification of CMCase, *Cellulomonas* cells were grown in 1.2% solka floc, 0.5% yeast extract, 0.6% urea, 0.2% K_2HPO_4, 0.15% $MgSO_4 \cdot 7H_2O$, 0.1% bactopeptone and pH 7.0, and *E. coli* strains were grown in 1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.4% nutrient broth, pH 7.0 at 37°C for 21hrs. Selection for ampicillin resistance was done on LB plates containing 100μg of ampicillin per ml.

Enzyme purification

Culture broth was used for the purification of CMCase and purification procedures were carried out at 4°C. Bacterial cells were removed by centrifugation (8,000rpm, 10min). The crude enzyme solution was brought to 40% saturation with ammonium sulfate, and centrifuged at 8,000rpm for 10min. The resulting supernatant was further fractionated with ammonium sulfate in 70% saturation. The resulting precipitates were dissolved in small amounts of 20mM phosphate buffer (pH 7.5) and dialyzed sufficiently, and then concentrated with an ultrafiltration kit(Model KMC-86S, KMC Co.). The solution was applied to a column(4.0×20cm) of DEAE-cellulose, which was previously equilibrated with a 20mM phosphate buffer(pH 7.5). The enzyme was eluted with a linear gradient of 0~0.5M NaCl using the same buffer. CMCase rich fractions were pooled, concentrated by an ultrafiltration kit, and subject to gel filtration using a Sephadex G-100 column(1.8×80cm) and Sephadex G-100(1.8×60cm), which was previously equilibrated with the same buffer.

Protein determination

Protein concentration was measured by the method of Lowry(19), with bovine serum albumin as a standard.

Enzyme assay

CMCase activity was assayed by incubation for 50 min at 50°C in a 2%(w/v) solution of CM-cellulose in 0.2M phosphate buffer(pH 7.0). A sample was withdrawn, and reducing sugar was determined with dinitrosalicylic acid(DNS) reagent. One unit of enzyme activity was defined as the amount of enzyme required to the release of 1μmole of glucose equivalent per minute.

CMCase activity was also determined by viscometry assay. Reaction mixture containing 2%(w/v) CM-cellulose in phosphate buffer(pH 7.0) was incubated for 0 to 90min at 50°C. The incubation was stopped by boiling for 10min to inactivate the enzyme. Viscosity was determined at room temperature with a Haake viscometer(Sensor system NV). Enzymatic activity was proportional to the increase in fluidity.

Analysis of the amino acid composition

Amino acid analysis was performed on samples hydrolyzed with 6N HCl at 105°C for 24 hours using a modification of the Pico-Tag system(Waters Associations, Inc.).

RESULTS AND DISCUSSION

Purification of enzyme

The purification of the enzyme was performed by ammonium sulfate precipitation, DEAE-cellulose column chromatography, and gel filtration by Sephadex G-100 and 2nd Sephadex G-100. The overall purification procedures were summarized in Table 1. The enzyme was purified to 24.3 folds and 2.6% yield. Polyacrylamide gel electrophoresis(PAGE) of the purified enzyme in the presence of sodium dodecyl sulfate(SDS) yielded

Table 1. Purification of CMCase from *E. coli* JM109(pCEH#4)

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	992.0	61.9	0.06	100.0	1.0
Ammonium sulfate precipitation	316.0	41.1	0.13	66.4	2.1
DEAE-cellulose chromatography	63.7	24.9	0.39	40.2	6.5
Sephadex G-100 column chromatography	8.3	7.4	0.89	12.0	14.8
2nd Sephadex G-100 column chromatography	1.1	1.6	1.45	2.6	24.3

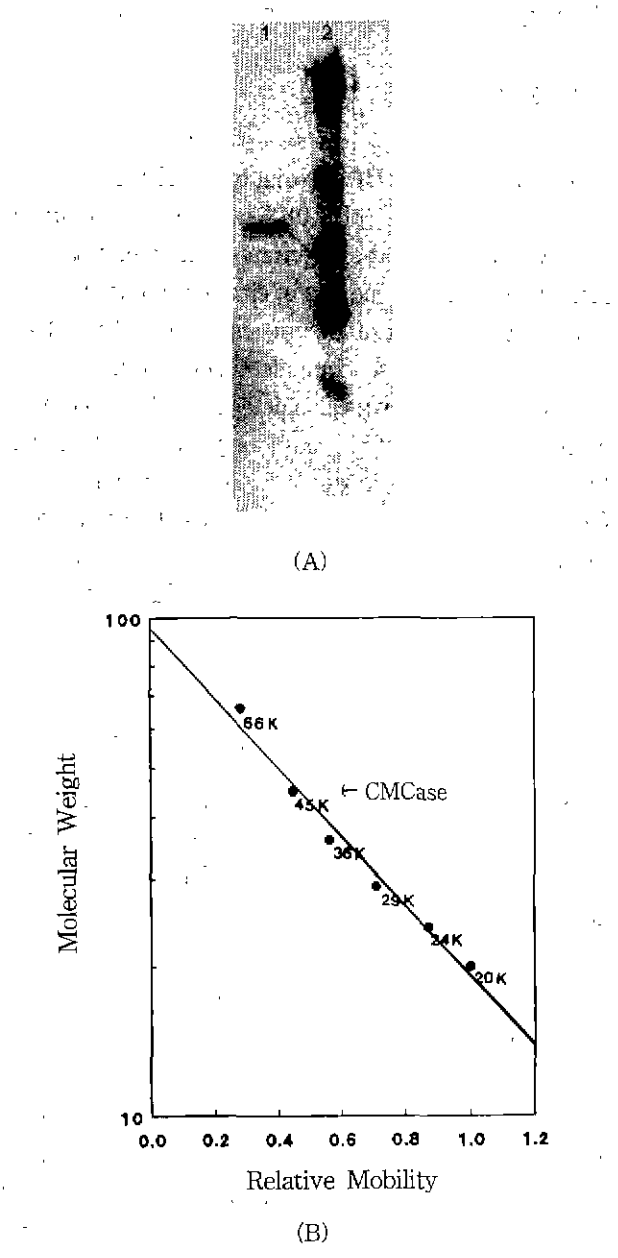


Fig. 1. (A) SDS-Polyacrylamide gel electrophoresis pattern of the CMCase.
Electrophoresis on 12.5% acrylamide gel in the presence of SDS was performed. Lane 1, the purified CMCase; lane 2, protein standard.
(B) Molecular weights estimation of the CMCase by SDS-PAGE.
Molecular weights of marker proteins(from top to bottom) are albumin bovine(66,000), albumin egg(45,000), glyceraldehyde-3-phosphate dehydrogenase(36,000), carbonic anhydrase(29,000), trypsinogen(24,000) and *trypsin inhibitor*(20,000).

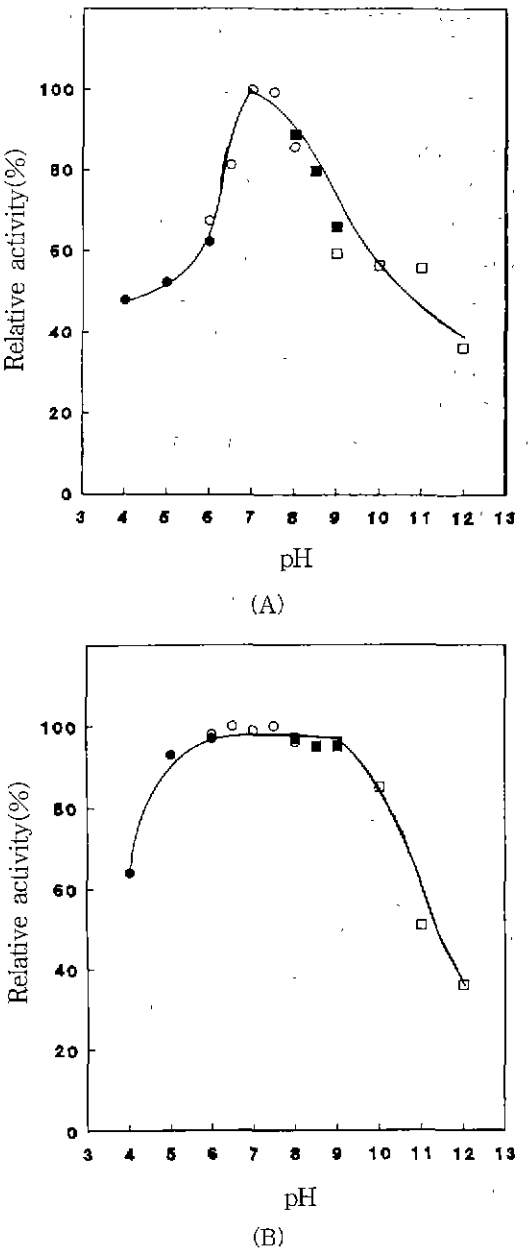


Fig. 2. Effect of pH on the activity(A) and stability(B) of CMCase.
The following buffers were used: pH 3.0~6.0, 0.2M citrate phosphate buffer(●), pH 6.0~8.0, 0.2M Na-phosphate buffer(○), pH 8.0~9.0, 0.2M Tris- HCl buffer(■), pH 9~12, 0.2M glycine-NaOH buffer (□).

a single band. The molecular weight of the enzyme was estimated to be about 40,000 daltons by SDS-PAGE as shown in Fig. 1, whereas that of CMCase isolated previously from *Cellulomonas* sp. YE-5 was 46,000~48,000 daltons(17). The difference was significant and might be due to proteolysis in *E. coli*.

Effect of pH on activity and stability

The effect of pH on the activity and stability of the enzyme was shown in Fig. 2. The highest activity was estimated at pH 7.0, which was the optimal pH noted as compared to host strain *Cellulomonas* sp. YE-5(17)

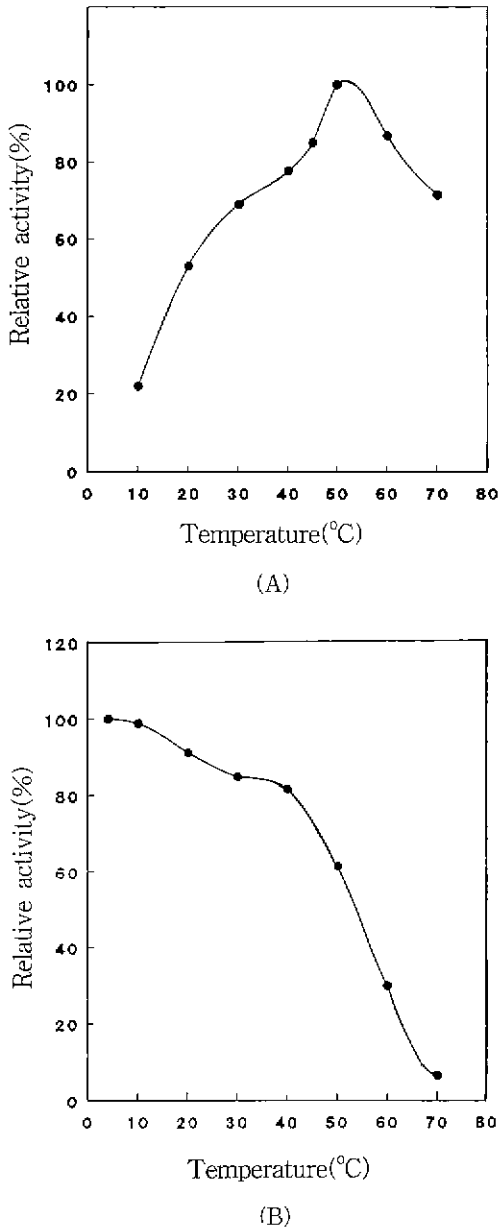


Fig. 3. Effect of temperature on the activity(A) and stability(B) of CMCase.

and *Clostridium thermocellum*(20). In contrast, the optimal pH of *Clostridium acetobutylicum*, *Bacillus* sp. KSM-522 and *E. coli* DH1(pWS11) were 5.2, 6.0 and 5.5 (21-23). The stability of the enzyme was also assessed by treatment at 4°C for 24 hours in buffer solutions of various pHs. The enzyme was relatively stable within the pH range from 5.0 to 10.0. Activity fell dramatically below pH 5.0 and above pH 10.0.

Effect of temperature on enzyme activity and stability

The effect of temperature on the activity and stability of the enzyme was shown in Fig. 3. The maximum activity of the enzyme was observed at 50°C at pH 7.0. To investigate thermal stability, the enzyme was incubated at several different temperatures for 1hr at pH 7.0 and residual enzyme activity was determined by DNS reagent. The results showed that the enzyme was relatively stable below 50°C and 60% of initial activity was lost at 60°C.

The analysis of amino acid composition

As shown in Table 2, CMCase had relatively high glycine content. In addition, the contents of as Asx and Glx was higher than those of other amino acids, which is similar to the strains such as *Clostridium cellulolyticum*(11) and *Clostridium themocellum*(24).

Reaction products from hydrolysis of CM-cellulose

Reaction mixture used to determine hydrolysis products was incubated for 12hrs, and the enzyme was removed by ethanol precipitation, followed by concentration of the supernatant. Hydrolysis products were separated by silica gel TLC plate(Merck Co.). Sample and sugar standards were applied to the plate by spotting 5μl samples. It was developed with butanol-ethanol-water(2 : 2 : 1), sprayed with aniline phthalate,

Table 2. Amino acid composition of CMCase expressed from *E. coli* JM109(pCEH#4)

Amino acids	Composition	
	Mole percent (% mol)	Number of residues
Ala	4.5	22
Cys	N.D.	N.D.
Asx	10.0	34
Glx	6.9	21
Phe	4.1	11
Gly	9.9	58
His	3.7	11
Ile	5.8	20
Lys	4.7	15
Leu	6.0	20
Met	2.4	7
Pro	6.0	23
Arg	5.9	15
Ser	5.3	22
Thr	10.9	38
Val	11.0	39
Tyr	2.9	7
Total	100.0	364

N.D.: not detected

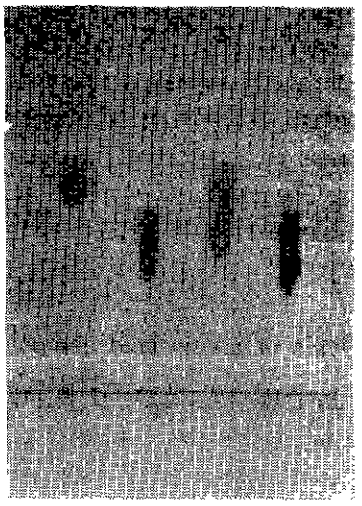


Fig. 4. TLC chromatogram of the reaction product from the hydrolysis of CM-cellulose by CMCase.
Lane 1, glucose; lane 2, cellobiose; lane 3, glucose +cellobiose; lane 4, reaction product

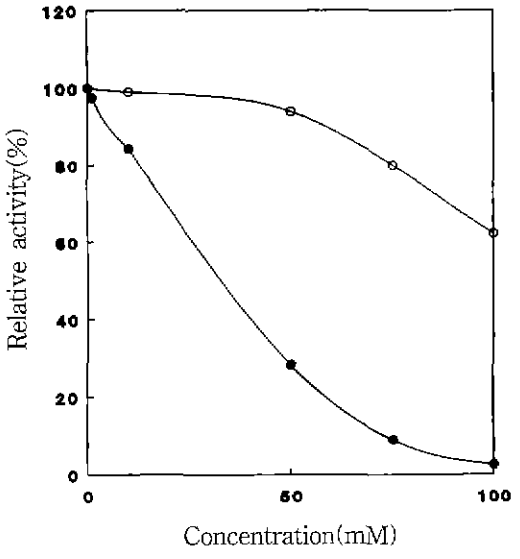
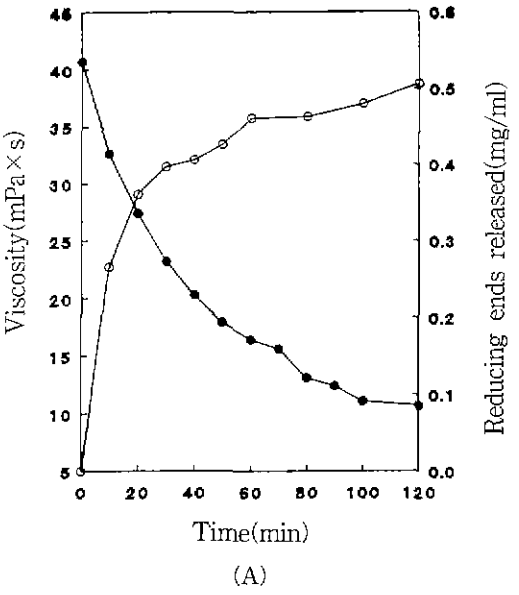


Fig. 5. Effect of glucose and cellobiose on the activity of CMCase.
Glucose(○), Cellobiose(●)

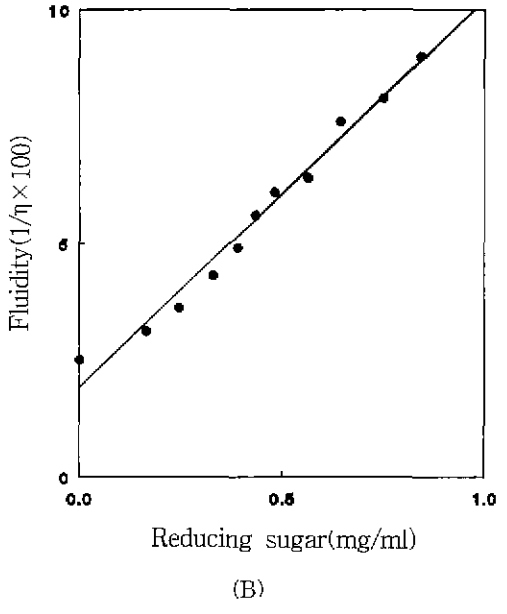
and heated at 100°C for 2~3min. Cellobiose was the main product from the hydrolysis of CM-cellulose by CMCase(Fig. 4). Glucose was not formed as a product of the complete reaction of CM-cellulose with the enzyme.

Effect of hydrolysis products on enzyme activity

The effect of glucose and cellobiose on CMCase activity was examined(Fig. 5). The enzyme was significantly affected by cellobiose, whereas glucose had little effect on CMCase activity.



(A)



(B)

Fig. 6. Relationship between specific fluidity and generation of reducing groups during hydrolysis of CM-cellulose.
The viscosity of the reaction mixtures was determined as described in MATERIALS and METHODS. Reducing sugars were determined with DNS reagent.

Mode of action

Enzyme hydrolysis of CM-cellulose result in a loss of viscosity concomitant with an increase of reducing sugar residues. Any hydrolytic event, regardless of position, generates a new reducing group, while only endonuclease activity cause a rapid drop in viscosity. An endoglucanase is therefore predicted to cause a steep increase in fluidity when plotted versus the increase in

reducing sugar residues. However, a significant lower slope is expected for an exoglucanase(20,25). The curve shown in Fig. 6 indicated that the enzyme catalyzed internal cleavage as expected for endoglucanase.

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