Jour. Microbiol. March 1996 p. 90-94

Vol. 34, No. 1

Purification of Carboxymethyl Cellulase from Hybrid between *Aspergillus niger* and *Penicillium verruculosum*

Young-Ki Yang*, Jung-Sup Lee, Hyung-Nam Park, Myung-Nim Moon, Hong-Sub Kim[1], Jong-Se Kim[1], Chae-Young Lim and Young-ha Rhee[2]

Department of Genetic Engineering,
[1]Department of Biology, College of Natural Science, Chosun University,
[2]Department of Microbiology, College of Natural Science, Chungnam National University

(Received August 10, 1995/Accepted December 30, 1995)

The carboxymethyl cellulase (CMCase) was purified from the induced culture filtrate of hybrid TAPW157-3 between *Aspergillus niger* and *Penicillium verruculosum* made by nuclear transfer. The enzyme was purified 80 fold with an overall yield 17% from the culture medium by ammonium sulfate fractionation, Sephadex G-75 gel permeation chromatography, and DEAE-ion exchange column chromatography. The molecular weight of the CMCase has estimated to be 32,000 daltons on SDS-polyacrylamide gel electrophoresis and Sephadex G-150 gel permeation chromatography. The purified enzyme functions optimally at pH 4.0 and 40°C. The Km value for carboxymethyl cellulose was 68 mM. The enzyme activity was increased by the presence of Mg^{2+} and Mn^{2+}.

Key words: *Aspergillus niger, Penicillium verruculosum,* **TAPW157-3, carboxymethyl cellulase, purification**

A large variety of enzymes are synthesized in all organisms and most of these enzymes are produced in small amounts and are a necessarity which are involved in cellular metabolism. However, in certain species of microorganisms some enzymes are produced in much larger amounts and secreted into medium (3, 4, 5, 6). In industrial processes, microorganisms are especially useful because of their higher specificity and efficiency when they are cultured in optimal pH and temperature. Cellulase in nature play an essential role in the carbon cycle (7, 8, 16, 23). Generally, cellulolytic microorganisms produce three types of cellulases i.e., endo-β-1,4-glucanase (CMCase, EC 3.2.1.4), exo-β-1,4-glucanase (cellobiohydrolase, EC 3.2.1.19) and β-1,4-glucosidase (cellobiase, EC 3.2.1.21). These cellulases seem to convert native cellulosic substrates to soluble sugar by synergistic action (3, 7, 10, 15). Carboxymethyl cellulases (CMCases) are the major secreted proteins produced by certain species of bacteria and fungi when the organisms are grown in a medium containing carboxymethyl cellulose as the sole carbon source (18, 21, 22). These enzymes from several bacterial and fungal species have uses in a number of industrial applications. Although mutation, protoplast fusion, and hybridization techniques have been frequently applied for the improvement of fungal strains in biotechnology, these techniques have revealed low efficiency and limitation of mass production. Nuclear transfer method was more effective than protoplast fusion (7). We have constructed intergeneric hybrids between *Aspergillus niger* and *Penicillium verruculosum* by nuclear transfer, and obtained a most active transformant, TAPW157-3 (24). In this study, we describe the purification of extracellular carboxymethyl cellulase from TAPW157-3 by ammonium sulfate fractionation, Sephadex G-75 gel permeation chromatography and DEAE-sephadex A-50 ion exchange chromatography. In addition, some physicochemical characteristics of the purified enzyme were analyzed.

Materials and Methods

Culture condition

We have constructed intergeneric hybrids between *Aspergillus niger* and *Penicillium verruculosum* by nuclear transfer (24). Transformant TAPW157-3 (ala^-) was cultured in PDA (potatoes 200 g, dextrose 20 g, agar 15 g/l) slant medium and incubated at 30℃ for 4 days. The conidial suspension (5×10^5 conidia/ml) were cultivated in the PDB (potatoes 200 g, dextrose 20 g/l) medium and then the produced mycelia were inoculated in CMC minimal medium (CMC 10g, minimal salt stock solution

* To whom correspondence should be addressed.

2ml, alanine stock solution 1 ml/l) and cultivated on rotary shaker at 30℃ for 48 hrs.

Enzyme assay and protein quantitation

Reducing sugar measured per 1.0 ml of the reaction mixture was estimated by the method of Somogyi and Nelson (20). The reaction mixture contained 0.5 ml of 1% CMC solution, and 0.5 ml of crude enzyme solution. The mixture was incubated at 40℃ for an appropriate period. The enzyme activity was assayed by measuring the absorbance at 540 nm. Under these conditions the rate of reducing sugar release was linear throughout the assay period. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmole of reducing sugar as glucose from the substrate per min under standard assay conditions. And the protein was determined by Lowry method (12), with bovine serum albumin (BSA) as a standard.

Purifcation of carboxymethyl cellulase

All purification steps of CMCase were carried out at 4℃.

Step-1. Preparation of crude enzyme solution

The mycelia produced from TAPW157-3 were inoculated in CMC minimal medium and cultivated at 30℃ for 24 hrs. After mycelium harvesting, the cell debris was removed by centrifugation at 5,000×*g* for 30 min and the supernatant collected.

Step-2. Ammonoum sulfate fractionation

Ammonium sulfate powder was added to the crude extracts until 55% saturated concentration was reached. the suspension was stirred for another 30 min and the precipitate was removed by centrifugation (10,000×*g*, 20 min). To the supernatant, 90% saturation concentration of the precipitate was collected by centrifugation and dissolved in 50 mM acetate buffer, pH 5.4.

Step-3. Sephadex G-25 gel filtration

To desalt, the fractionated protein (55~90%) was applied to a Sephadex G-25 column, previously equilibrated with 50 mM acetate buffer (pH 5.4) and then centrifuged at 5,000×*g* for 2 min.

Step-4. Sephadex G-75 gel permeation chromatography

The desalted protein solution was loaded onto a Sephadex G-75 column (2.1×60 cm) eluted with 50 mM acetate buffer (pH 5.4) at a flow rate of 3 ml/hr. Each 1.5 ml eluted proteins were collected.

Table 1. CMCase activities of transformants between *Aspergillus niger* and MPV 157 by nuclear transfer

Strains	CMCase units	CMCase units/mg protein	Protein content μg/ml
A. niger	0.51	1.75	292
MPV 157	0.41	1.26	326
TAPW157-1	0.54	1.61	335
TAPW157-2	0.43	1.26	340
TAPW157-3	0.89	2.81	317
TAPW157-4	0.68	2.19	310
TAPW157-5	0.67	2.11	336
TAPW157-6	0.84	2.75	306
TAPW157-7	0.87	2.55	341

Step-5. DEAE-Sephadex A-50 ion exchange chromatography

The protein obtained from Sephadex G-75 column chromatography was dialysed and loaded onto DEAE-Sephadex A-50 column (1.1×30 cm) equilibrated with 50 mM acetate buffer (pH 5.4). The protein bound to the column was eluted with linear gradient of 0~0.4 M NaCl containing 50 mM acetate buffer. The flow rate was 4.5 ml/hr and each 2.25 ml fraction were collected.

Determination of molecular weight

Molecular weight of the purified enzyme was determined by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (11). Protein bands were visualized by staining with 0.05% coomassie brilliant blue R-250. Molecular weight of native enzyme was determined by Sephadex G-150 column (1.45×73 cm) chromatography. Alcohol dehydrogenase (M.W. 150,000), bovine serum albumin (M.W. 66,000), and carbonic anhydrase (M.W. 29,000) were used as standard markers and void volume was estimated by using blue dextran.

Results and Discussion

Purification of carboxymethyl cellulase

The CMCase activities of transformants are shown in Table 1. TAPW157-3 among the transformants have the highest activity, which the specific enzyme activity of 2.81 unit/mg. Also the results of enzyme purification are summarized in Table 2. Throughout the overall process, the CMCase was approximtely purified 80-fold with a yield of about 17%. Almost all of the enzyme activity was recovered in the 55~90% ammonium sulfate precipitates. Therefore, the 55~90% fractions were dissolved in 50 mM acetate buffer (pH 5.4) and then desalted by Sephadex G-25 gel filtration. The specific activity was 171.9 unit/mg. When the desalted CMCase was eluted

Table 2. Purification of CMCase from TAPW157-3

Purification step	Total vol. (ml)	Total enzyme activity (unit)	Total protein (mg)	Specific activity (unit/mg)	X-fold	Yield (%)
Crude enzyme	800	5408.0	112.8	47.9	1.0	100
Ammonium sulfate	3	1821.8	10.6	171.9	3.6	41
Sephadex G-75	12	1305.0	4.0	326.3	6.8	30
DEAE-Sephadex A-50	3	763.0	0.2	3815.0	79.6	17

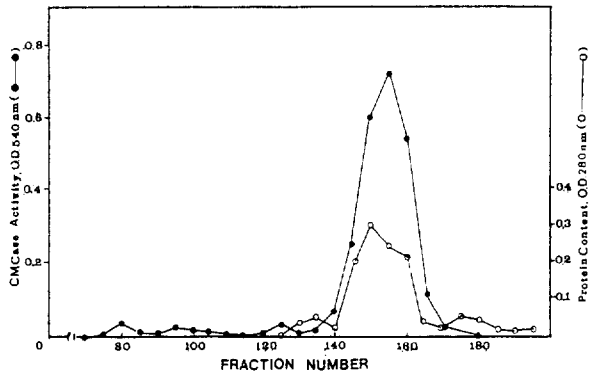

Fig. 1. Gel permeation chromatography on sephadex G-75 of 55~75% ammonium sulfate fraction. The column 1.45~73 cm) was eluted with 50 mM acetate buffer (pH 5.4). Flow rate; 3.0 ml/hr, 3.0 ml/fraction.

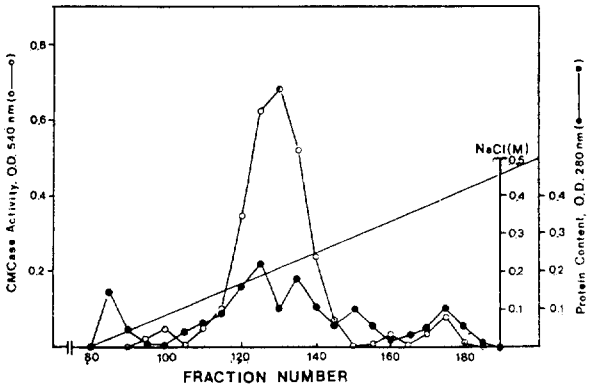

Fig. 2. Ion exchange chromatography of carboxymethyl cellulase on DEAE-Sephadex A-50. The column (1.0×20 cm) was eluted with a linear gradient of NaCl (0~0.5 M). Flow rate; 4.5 ml/hr, 3 ml/fraction.

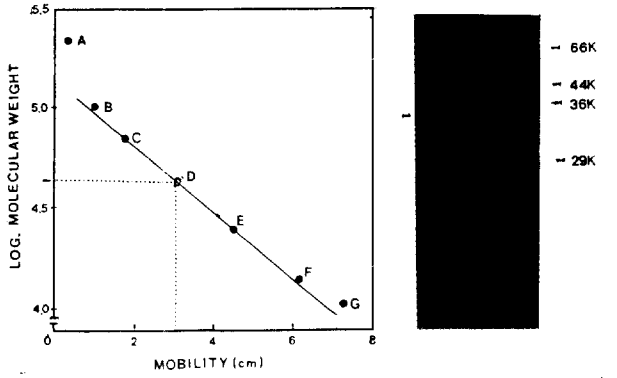

Fig. 3. Molecular weight estimation of CMCase by SDS-PAGE. A, Phosphorylase B (MW 97,400); B, Bovine serum albumin (MW 66,200); C, Ovalbumin(MW 45,000); D, Carbonic anhydrase (MW 29,000); E, β-lactoglobulin (MW 18,400); F, Lysozyme (MW 14,300).

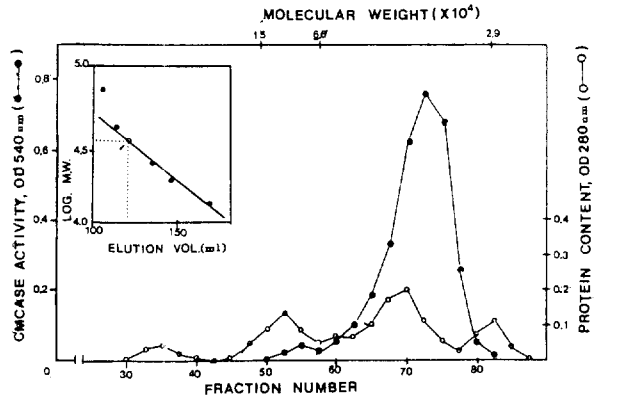

Fig. 4. Molecular weight estimation of CMCase by gel permeation chromatography on Sephadex G-150. The column (2.0×60 cm) was eluted with 50 mM acetate buffer (pH 5.4). Flow rate: 3.0 ml/hr, 1.5 ml/fraction.

from the Sephadex G-75 column with 50 mM acetate buffer (pH 5.4), the activity was detected at around 145-162 fractions and the specific activity was 326.3 unit/mg as shown in Fig. 1. The active fractions were collected and applied onto the DEAE-Sephadex A-50 column. The activity of enzyme was detected at around 131-134 fractions as shown in Fig. 2, and the specific enzyme activity was 3815.0 unit/mg. These results indicated that the purified enzyme was an anionic enzyme.

Determination of molecular weight

One major 32,000 dalton protein band was detected when the purified enzyme was electrophoresed on SDS-polyacrylamide gel (Fig. 3). Also, molecular weight of native enzyme was estimated to be 32,000 daltons by Sephadex G-150 gel permeation chromatography (Fig. 4). These results indicate that the enzyme is a 32,000 dalton monomer. Compared with the CMCase from other fungal species, CMCase from TAPW157-3 had a different molecular weight (14, 15).

Characteristics of carboxymethyl cellulase

Effect of temperature on the enzyme activity of the CMCase was measured at varying temperatures between

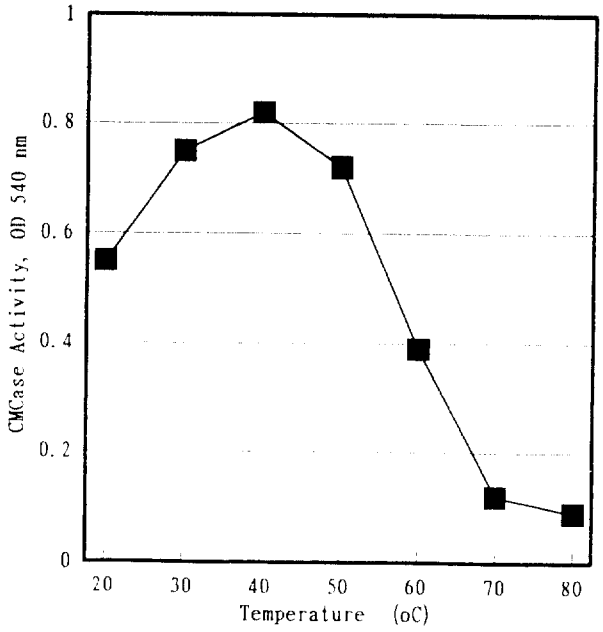

Fig. 5. Effect of temperature on CMCase activity. Reactions were carried out for 15 min in 100 mM acetate buffer (pH 6.4) containing 50 mM CMC.

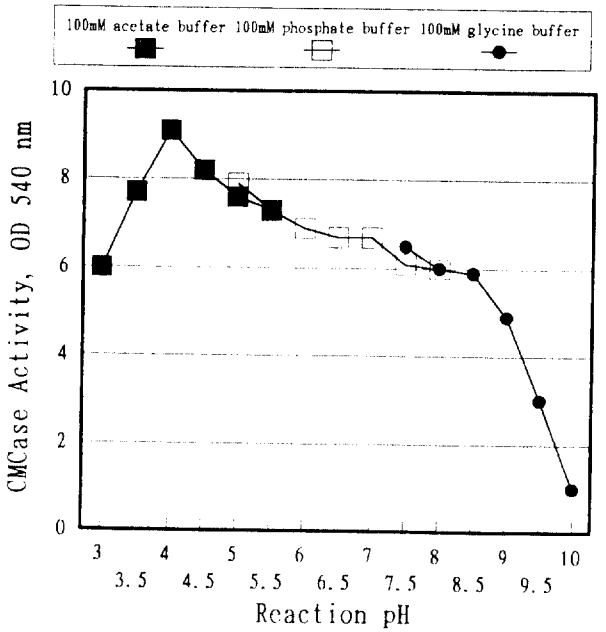

Fig. 6. Effect of pH on CMCase activity. Reaction were carried out for 15 min at 40℃ in various buffer containing 50 mM CMC.

20 and 80℃. As shown in Fig. 5, the optimal temperature for the enzyme activity was at 40℃, and it critically decreased above 60℃. This result of optimal temperature was similiar to *Aspergillus niger* (6, 15). However, the optimal temperature of TAPW157-3 CMCase was lower than those of CMCase from *Trichoderma viride, Fusarium limi, Aspergillus aculeatus,* and *Geotricum candidum* (10, 13, 14, 17). The effect of pH on the enzyme activity was examined in the range of pH 3.0 to 10.0. The enzyme

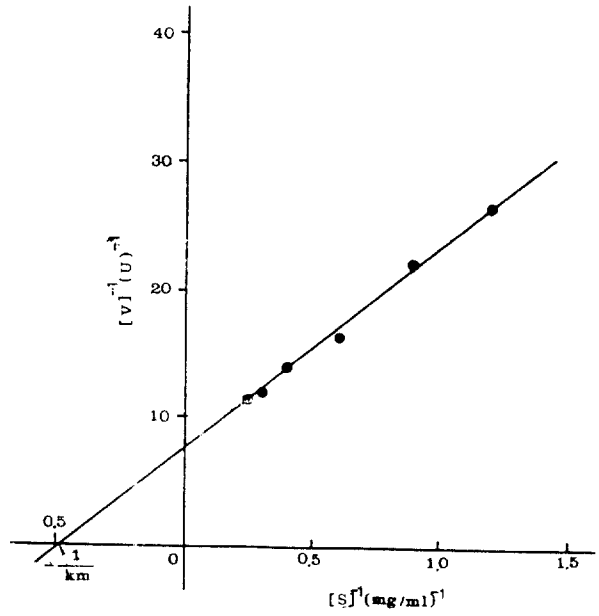

Fig. 7. Effect of substrate concentration by Lineweaver-Burk plot.

Table 3. Effect of divalent metal cations on CMCase activity of TAPW157-3

Metal ions	Conc. (mM)	Relative activity (%)
None	10	100.0
Ca^{2+}	10	86.4
Co^{2+}	10	70.4
Cu^{2+}	10	83.2
EDTA	10	79.4
Fe^{2+}	10	73.5
Mg^{2+}	10	150.2
Mn^{2+}	10	117.6
Ni^{2+}	10	81.4
Zn^{2+}	10	69.2

Measurements were carried out in the standard assay conditions after 5 min preincubation of purified CMCase with various effectors.

activity was measured under three different assay conditions with 100 mM acetate buffer at pH 3.0~5.0, 100 mM phosphate buffer at pH 5.5~8.0 and 100 mM glycine buffer at 8.5~10.0. As shown in Fig. 6, the optimal pH for the purified CMCase was shown to be 4.0. This result was similar with those of other CMCases from *Aspergillus* sp. (19), *Aspergillus niger* (15), and *Myrothecium verrucria* (18), but showed a difference in pH optima from *Fusarium lini* (17), *Geotrichum candidum* (13), *Trichoderma viride* (2), and *Trichoderma koningii* (9). The effect of several metal ions on the CMCase activity was assayed and presented in Table 2. The enzyme activity increased in the presence of Mg^{2+} and Mn^{2+} but Zn^{2+} was a srong inhibitor. This results showed a difference in other fungal species (2, 6, 9, 14) For the determination of Km values, the enzyme activity was assayed at various concentrations of the substrates. Km values were de-

termined from the Lineweaver-Burk plot. The calculated Km value was 2.19 mg/ml (68 mM). This value was lower than those of *Aspergillus niger* (2.5 mg/ml) and *Fusarium lini* (2~12 mg/ml) (15, 17).

Acknowledgement

This work was supported by the program (1990~1993) of Korea Research Foundation to encourage the activities of the Research Institutes belong to universities, for which authors are very grateful.

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