Purification and Properties of a New *endo*-Cellulase from *Robillarda* sp. Y-20

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A cellulolytic enzyme was purified from a crude enzyme preparation from *Robillarda* sp. Y-20 by consecutive column chromatography. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis and contained 10.5% carbohydrate as glucose. The enzyme had a molecular weight of 56,000, an isoelectric point of 3.80, an optimum pH of 5.0, and an optimum temperature of 60°C. It was stable from pH 4.0 ~ 7.0 at 37°C for 1 hr and below 50°C for 30 min.

The amino-terminal amino acid sequence of the enzyme was

4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Tyr-Ala-Gly-Val-Ala-Glu-Ser-Ser-Gly-Glu-Phe-Gly-Val-Trp-Ser.

The enzyme was inactivated by 1 mM Fe^{3+} , Hg^{2+} , or *N*-bromosuccinimide. It was active on sodium carboxymethyl cellulose (CMC) and cellooligosaccharides (cellotriose to cellohexaose), but not on either cellobiose or microcrystalline cellulose (Avicel) under our assay conditions. The *K_m* value of the enzyme for CMC was 0.060%.

Cellulolytic enzymes have been purified from many microorganisms and their enzymatic properties have been investigated. One component of the cellulase system is 1,4- β -D-glucan 4-glucanohydrolase [EC 3.2.1.4], sometimes called carboxymethyl cellulase (CMCase) or *endo*-cellulase. *Robillarda* sp. Y-20, isolated in our laboratory, is a fungus with sodium carboxymethyl cellulose (CMC) hydrolyzing activity in the culture filtrate higher than that of *Trichoderma reesei* QM9414, reaching 4.9-fold¹⁾ and Ujiie and Sasaki reported the purification and enzymatic properties of one *endo*-cellulase, tentatively called CMCase II, and two β -glucosidases from a crude enzyme preparation.²⁾

We have isolated another cellulase component which acted on CMC, tentatively called CMCase I, from the same preparation

from *Robillarda* sp. Y-20.

Here we report the purification and enzymatic properties of CMCase I, and discuss the differences between CMCases I and II.

MATERIALS AND METHODS

Materials. CMC was supplied by Daiichi Industrial Pharmaceutical Co., Ltd.; the degree of substitution was 0.51. Cellooligosaccharides from cellobiose (G_2) to cellohexaose (G_6) were obtained from Seikagaku Kogyo Co., Ltd. and microcrystalline cellulose (Avicel SF) from Asahi Chemical Industry Co., Ltd. All other chemicals were commercial products of analytical grade.

Enzyme source. A crude enzyme powder of *Robillarda* sp. Y-20, prepared and furnished by Meiji Seika Kaisha, Ltd., was used as a starting material for the purification of the enzyme. This powder was prepared by 70% ethyl alcohol precipitation of the culture filtrate, followed by dialysis and lyophilization of the precipitated materials.

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Abbreviations: G_1 , D-glucose; G_2 to G_6 , cellooligosaccharides from cellobiose to cellohexaose; SDS, sodium dodecyl sulfate; NBS, *N*-bromosuccinimide; EDTA, ethylenediaminetetraacetic acid.

Enzyme assay method. CMC-saccharifying activity was used as the standard assay of cellulase. The reaction mixture contained 0.05 ml of enzyme solution, 0.2 ml of 0.1 M acetate buffer (pH 5.0), and 0.25 ml of 1% CMC solution. After incubation at 37°C for an appropriate period, the reducing sugar produced was measured by the method of Somogyi³⁾ and Nelson.⁴⁾ One unit of the enzyme activity was defined as the amount that liberated reducing sugar equivalent to 1 μ mol of D-glucose per min under our standard assay conditions.

Analytical methods. Protein content was measured by the method of Lowry *et al.*⁵⁾ with bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring proteins in column effluents. The amount of carbohydrate was measured by the phenol-sulfuric acid method⁶⁾ with D-glucose as a standard.

Electrophoresis. Polyacrylamide disc gel electrophoresis was done by the method of Davis⁷⁾ except that the running gel contained 0.125% CMC. After the electrophoresis, one gel was stained for protein with 0.4% Coomassie Brilliant Blue R-250 and the other was stained for the enzyme activity by congo red⁸⁾ as follows: the gel was incubated in 0.1 M acetate buffer (pH 5.0) at 37°C for 30 min, dipped in 0.2% congo red solution for 15 min, and washed with 1 M sodium chloride solution. The visualized bands were stabilized with 7.5% acetic acid. SDS-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn.⁹⁾ Samples were first treated at room temperature for 24 hr in 1% SDS and 4.8% 2-mercaptoethanol. Electrofocusing was done on a polyacrylamide gel column as described by Wrigley¹⁰⁾ using Ampholyte of pH 2.5~5.0 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) at a constant voltage of 400 V for 2.5 hr. Two sample gels each containing 42.4 μ g of protein were run simultaneously. After electrofocusing, one gel was stained for protein and the other was cut into 2.5 mm pieces, then extracted overnight with 1 ml of deionized water. The enzyme activity and pH of each extract were measured.

Amino-terminal sequence analysis. Amino-terminal amino acid sequencing was done with an automated protein sequencer/PTH analyzer system (Model 477A/120A, Applied Biosystems).

Hydrolysis of soluble substrates. The reaction mixture, composed of 0.4 ml of 1% substrate solution and 0.4 ml of the enzyme solution (0.32 U) in 0.01 M acetate buffer (pH 5.0), was incubated at 37°C. At times, 200- μ l samples were withdrawn and heated at 100°C for 1 min. After cooling, 20 μ l of the samples was used for high-performance liquid chromatography (HPLC), as described elsewhere,¹¹⁾ to identify each cellooligosaccharide produced by the enzyme action. The amounts of glucose and cellobiose produced from CMC were found by the peak area method.¹²⁾

Measurement of K_m for CMC. A mixture of 0.25 ml of 0.25~1.0% CMC solution and 0.2 ml of 0.1 M acetate buffer (pH 5.0) was equilibrated at 37°C. The reaction was started by the addition of 0.05 ml of the purified enzyme (0.04 U) warmed to the same temperature. At times, 0.1-ml samples were removed, and the reducing sugar liberated was measured by standard assay conditions. The K_m value was calculated from Lineweaver-Burk plots.¹³⁾

RESULTS

Purification of the enzyme

The following procedures were done at 4°C except that column chromatography was done at room temperature.

Step 1: Butyl-Toyopearl 650S column chromatography. The crude enzyme powder (2 g) was dissolved in 50 ml of 0.01 M acetate buffer (pH 5.0). The crude enzyme solution was centrifuged at 15,000 rpm (27,300 $\times g$) for 15 min to remove insoluble materials. To the resultant supernatant (48.5 ml), ammonium sulfate was added to 40% saturation. The suspension was left for 1 hr and centrifuged to remove precipitated proteins. The supernatant (52.0 ml), added to the same volume of 0.01 M acetate buffer (pH 5.0), was divided into two equal parts. Each portion (52.0 ml) was put on a Butyl-Toyopearl 650S column (1.5 $\times$ 18 cm) preequilibrated with 0.01 M acetate buffer (pH 5.0) containing 20% saturation ammonium sulfate and eluted stepwise with the solutions shown in Fig. 1. The CMCase activity was eluted as two peaks (P-A, P-B), fractions belonging to the major peak (P-A) were combined, and ammonium sulfate was added to 80% saturation. After centrifugation, the precipitated proteins were dissolved in and dialyzed against 0.01 M acetate buffer (pH 5.0).

Step 2: 1st DEAE-Toyopearl 650S column chromatography. The dialyzate (12.1 ml) was put on a DEAE-Toyopearl 650S column (1.5 $\times$ 18.5 cm) previously equilibrated with 0.01 M acetate buffer (pH 5.0). Elution was done successively with 200 ml of 0.01 M acetate buffer (pH 5.0) [P-A1], 300 ml of 0.01 M acetate buffer (pH 5.0) containing 0.05 M NaCl [P-A2], 200 ml of 0.01 M acetate buffer (pH 5.0) containing 0.1 M NaCl [P-A3], and 200 ml of

FIG. 1. A Typical Elution Profile of the CMC-Saccharifying Activity on Butyl-Toyopearl 650S Column Chromatography.

The flow rate was kept at 0.55 ml/min. The fractions of 10 ml each were collected and assayed for protein by the absorbance at 280 nm (○) and for CMC-saccharifying activity (●).

FIG. 2. Elution Patterns of the CMC-Saccharifying Activity on 2nd DEAE-Toyopearl 650S Column Chromatography.

The flow rate was kept at 1.38 ml/min and fractions of 5 ml each were collected. Symbols are the same as in Fig. 1.

0.01 M acetate buffer (pH 5.0) containing 0.5 M NaCl [P-A4]; the flow rate was kept at 5 ml (= volume of one fraction)/3.62 min. The CMCase activity was eluted with each of the above four fractions (P-A1~4). When fractions belonging to each CMCase activity peak were pooled, dialyzed against 0.01 M acetate buffer (pH 5.0), and chromatographed again on a DEAE-Toyopearl 650S column, only P-A3 gave a homogeneous CMCase.

Step 3: 2nd DEAE-Toyopearl 650S column chromatography. The dialyzate (P-A3, 70.8 ml) was put on a DEAE-Toyopearl 650S column (1.5 × 18.5 cm) preequilibrated with 0.01 M acetate buffer (pH 5.0) containing 0.05 M NaCl and eluted by a linear salt gradient with the results shown in Fig. 2. The active fractions (55~58) were combined and dialyzed against 0.01 M acetate buffer (pH 5.0).

Table I summarizes the purification pro-

TABLE I. SUMMARY OF THE PURIFICATION PROCEDURES

Step	Total protein (mg)	Total activity toward CMC (U)	Specific activity (U/mg)	Yield (%)
Crude enzyme	304	3705	12.2	100
40% (NH ₄) ₂ SO ₄	182	2855	15.7	77.1
Butyl-Toyopearl 650S				
P-A	25.9	1048	40.5	28.3
P-B	18.5	343	18.5	9.3
P-A				
80% (NH ₄) ₂ SO ₄	29.5	708	24.0	19.1
1st DEAE-Toyopearl 650S				
P-A1	6.74	23.4	3.5	0.63
P-A2	2.82	109	38.7	2.9
P-A3	1.45	21.3	14.6	0.57
P-A4	9.77	229	23.4	6.2
P-A3				
2nd DEAE-Toyopearl 650S	0.95	16.1	17.0	0.43

cedures. The final purified enzyme (P-A3), called CMCase I, had a specific activity of 17.0 units per mg of protein in a final activity yield of 0.43%, and was used in the following experiments as the purified enzyme.

Homogeneity of the enzyme

The purified enzyme showed a single protein band on polyacrylamide disc gel electrophoresis (Fig. 3A). This single band of protein had CMCase activity as shown in Fig. 3B. The crude enzyme preparation gave at least 9 CMCase activity bands (No. 1~9) as shown in Fig. 3C. The purified enzyme (CMCase I) corresponded to band No. 3 in the crude enzyme preparation by its relative mobility.

Molecular weight and carbohydrate content

On SDS-polyacrylamide gel electrophoresis, the purified enzyme also gave a single band of protein as shown in Fig. 3D. The molecular weight was estimated to be 56,000 by comparison of its relative mobility on SDS-polyacrylamide gel electrophoresis with those of standard proteins; phosphorylase *B* (mol. wt. 92,500), bovine serum albumin (mol. wt. 66,200), ovalbumin (mol. wt. 45,000), carbonic anhydrase (mol. wt. 31,000), soybean trypsin inhibitor (mol. wt. 21,500), and lysozyme (mol. wt. 14,400).

The carbohydrate content was calculated to

FIG. 3. Polyacrylamide Gel Electrophoresis.

A sample of the purified enzyme (6.3 μ g) or the crude enzyme preparation (835 μ g) was put on a gel column of 7.5% polyacrylamide and run at 4°C under a constant current of 3 mA per tube for 2 hr. A, protein staining of the purified enzyme; B, activity staining of the purified enzyme; C, activity staining of the crude enzyme preparation; D, SDS-polyacrylamide gel electrophoresis was done in 7.5% polyacrylamide using 1.65 μ g of the purified enzyme.

be 10.5% as glucose.

Isoelectric point

The purified enzyme showed one major protein band that had CMCase activity, and 6 to 7 minor bands that had no CMCase activity. The isoelectric point of the major band was estimated to be pH 3.80. The reason why the purified enzyme is isoelectrophoretically heterogeneous is unclear.

Amino-terminal amino acid sequence

The amino-terminal sequence of the enzyme was

4 5 6 7 8 9 10 11
Tyr-Ala-Gly-Val-Ala-Glu-Ser-Ser-
12 13 14 15 16 17 18
Gly-Glu-Phe-Gly-Val-Trp-Ser.

Sequencing was done twice with different enzyme preparations and exactly the same results were obtained. For the first three amino acids no specific amino acid was found in both sequencings.

Optimum pH and pH stability

The effects of pH on the activity of the purified enzyme were studied under standard assay conditions using citrate buffer (0.1 M) at pH 2.5~3.0, acetate buffer (0.1 M) at pH 3.5~6.0, and Tris-maleate buffer (0.1 M) at pH 6.5~8.0. CMCase I had its maximum activity at pH 5.0, and about 75% of the maximum activity at pH 4.0 and 6.5 (Fig. 4A).

Enzyme solution (0.05 ml, 0.04 U) was incubated with 0.2 ml of buffers indicated above at 37°C for 1 hr. Then 0.05 ml of the reaction mixture was withdrawn, and the re-

maining activity was measured by the addition of 0.2 ml of 0.5 M acetate buffer (pH 5.0) and 0.25 ml of 1% CMC solution. The results are shown in Fig. 4B. The enzyme was completely stable between pH 4.0 and 7.0. It was rapidly inactivated at pHs below 3.5.

Optimum temperature and temperature stability

The effects of temperature on the activity of the purified enzyme were studied at various temperatures under the standard assay conditions. CMCase I had its maximum activity at 60°C (Fig. 5A).

Enzyme solution (0.05 ml, 0.04 U) was preincubated with 0.2 ml of 0.1 M acetate buffer (pH 5.0) at various temperatures for 30 min. After the preincubation, the remaining activity was measured by the addition of 0.25 ml of 1% CMC solution at 37°C. CMCase I was completely stable at temperatures below 50°C (Fig. 5B). The enzyme retained about 50% of its original activity on heating at 60°C, but was completely inactivated by heating at 65°C, under the conditions used.

Effects of metal ions and other reagents

The reaction mixture containing 0.05 ml of the enzyme solution (0.04 U) plus 0.2 ml of buffered metal or reagent solution (or buffer alone) was held at 37°C for 10 min. Then the remaining activity was measured by the addition of 1% CMC solution. The final concentration of each metal ion or reagent in the reaction mixture was 1 mM. Fe^{3+} and Hg^{2+} inhibited the enzyme activity almost completely, and NBS did considerably (Table II). CMCase I was not inhibited by 1 mM EDTA or

FIG. 4. Effects of pH on the Activity and Stability.

Experimental details are described in the text. A, effects on the activity; B, effects on the stability.

iodoacetamide. This suggests that metals and sulfhydryl reagents are not essential for the enzymatic action, as are the cases with cellulases from *Aspergillus niger*¹⁴⁾ and *Trichoderma viride*.^{15,16)}

Hydrolysis products from soluble substrates

The amounts of celooligosaccharides produced from soluble substrates by the action of CMCase I are shown in Table III. The enzyme could attack neither G₂ nor Avicel. G₃ to G₆ were hydrolyzed to G₂ as the predominant

product. Transfer actions were also observed in the early stages of the reaction; G₄ and G₅ from G₃, G₅ and G₆ from G₄, and G₆ and G₇ from G₅ were formed. This enzyme hy-

TABLE II. EFFECTS OF METALS AND OTHER REAGENTS ON THE CMCase ACTIVITY

Metals were used as the chloride salt except for Fe³⁺ and Ni²⁺ sulfate.

Materials	Remaining activity (%)
None	100
Mg ²⁺	84
Fe ³⁺	1
Co ²⁺	60
Ba ²⁺	75
Zn ²⁺	64
Ca ²⁺	99
Ni ²⁺	89
Hg ²⁺	0
Cu ²⁺	71
NBS	35
EDTA	105
SDS	107
Iodoacetamide	113

FIG. 5. Effects of Temperature on the Activity and Stability.

Experimental details are described in the text. A, effects on the activity; B, effects on the stability.

TABLE III. CELLOOLIGOSACCHARIDES FORMATION BY THE PURIFIED ENZYME

Substrate	Time (min)	Products (wt., %)						
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇
G ₃	0			100				
	5	0.3	2.7	91.9	3.6	1.5		
	30	3.1	17.8	70.3	3.7	5.1		
	120	2.9	37.1	57.0	2.9			
G ₄	0				100			
	5		6.0	1.6	88.7		3.8	
	30	1.8	36.8	9.2	38.8	5.1	8.3	
	120	2.2	61.0	26.3	8.0	2.6		
G ₅	0					100		
	5		4.1	9.9	3.8	82.2		
	30	0.7	20.4	25.6	9.3	29.4	7.0	7.5
	120	1.1	47.0	32.9	14.0	5.0		
G ₆	0						100	
	5		4.9	7.6	8.8	3.6	75.1	
	30	0.7	19.6	23.4	25.1	10.9	20.2	
	120	1.4	48.1	19.0	15.1	4.0	2.3	
CMC	5	4.4	6.1					
	30	4.0	15.1					
	120	4.5	18.1					

TABLE IV. A SUMMARY OF THE VALUES OF K_m AND SPECIFIC ACTIVITY FOR CMC OF *Endo*-CELLULASES FROM DIFFERENT FUNGI

Enzyme source	K_m (%)	Specific activity (U/mg)
<i>Robillarda</i> sp. Y-20 CMCase I	0.060	17.0
CMCase II ²⁾	—	72.3
<i>Asperigillus niger</i> cellulase ¹⁴⁾	0.086	116.83
<i>Trichoderma viride</i> cellulases		
II-A ¹⁵⁾	0.081	29.83
II-B ¹⁵⁾	0.096	4.95
III ¹⁶⁾	0.054	20.00

drolyzed CMC to G_1 and G_2 (Table III). Certain amounts of oligosaccharides, probably containing carboxymethyl glucose, were detected on paper chromatography (data not shown) but were not detected by HPLC.

The relative initial hydrolysis rates for cellooligosaccharides were calculated from their decrease during the first 5 min; 100 minus residual substrate (mol, %). Taking initial rate of G_6 as 100, CMCase I hydrolyzed more rapidly in the order of G_6 (100) > G_5 (65) > G_4 (40) > G_3 (22).

K_m value for CMC

Table IV summarizes the K_m values of CMCase I and some purified *endo*-cellulases from other fungal sources, with the specific activities of the individual enzymes. CMCase I was close to *T. viride* cellulase III in its action on CMC. The specific activity of CMCase II was about 4.25 times higher than that of CMCase I.

DISCUSSION

Ujii and Sasaki isolated a cellulase component (CMCase II) from a crude enzyme preparation of *Robillarda* sp. Y-20 by isoelectric focusing chromatography and ion exchange chromatography.²⁾ In this report, we isolated a new cellulase component (CMCase I) by running hydrophobic chromatography and ion exchange chromatography. Final activity yields of CMCase I and CMCase II were

TABLE V. COMPARISON OF TWO CMCASES FROM *Robillarda* sp. Y-20

Properties	I	II ²⁾
Molecular weight	56,000	59,000
Sugar content (%)	10.5	0
pI	3.80	3.5
Trp in protein	+	—
Optimum pH	5.0	4.0 ~ 5.0
Stable pH	4.0 ~ 7.0	4.0 ~ 7.0
Optimum temperature (°C)	60	55
Stable temperature (°C)	~ 50	~ 50
Inhibition by (1 mM)	Fe ³⁺ , Hg ²⁺ , NBS	Hg ²⁺ , NBS
Minimum substrate of hydrolysis	G_3	G_3

0.43% (Table I) and 30.6%,²⁾ respectively, and it would seem that the former was a minor CMCase component, and the latter was a major one in the crude enzyme preparation.

The crude enzyme preparation of *Robillarda* sp. Y-20 gave at least 9 CMCase activity bands on polyacrylamide disc gel electrophoresis, and CMCase I corresponded to band No. 3 by its relative mobility (Fig. 3). CMCase II, purified by a different procedure from the same preparation by Ujii and Sasaki,²⁾ perhaps corresponded to the band(s) No. 1 and/or 2 since the final activity yield was 30.6%; the bands of No. 1 and 2 were very close to each other.

Table V is a comparison of the enzymatic properties of CMCases I and II. Though the two enzymes showed similarity in most of these properties, three main differences were observed; (1) CMCase I contained 10.5% carbohydrate as glucose while no carbohydrate moiety was associated with CMCase II. (2) CMCase I was almost completely inhibited by 1 mM Fe³⁺ while CMCase II wasn't. (3) CMCase I contained tryptophan in its amino acid sequence while no tryptophan was present in CMCase II by amino acid component analysis.

CMCase I could attack neither G_2 nor Avicel, but hydrolyzed cellooligosaccharides (G_3 to G_6) to G_2 as the predominant product

(Table III); the yields of G_2 were about 37 to 61% within the test conditions. This result is different from the case of CMCase II; it produced G_1 as the main product from G_4 to G_6 , and equal amounts of G_1 and G_2 from G_3 .²⁾ CMCase I showed not only hydrolysis but a transfer reaction for G_3 to G_5 (Table III). Cellooligosaccharides, the original substrate combined with G_1 or G_2 , were produced by transglycosylation of CMCase I; especially for G_3 , transglycosylation seemed to be stronger than hydrolytic action in the early stages of the reaction. In case of the action for CMC, CMCase I produced G_1 and G_2 ($G_2 > G_1$, Table III) while CMCase II formed G_1 to G_3 ($G_1 > G_2, G_3$).²⁾ From these results, it is suggested that the action mode of CMCase I is different from that of CMCase II. It seems that CMCase I is a cellobiose-producing cellulase from soluble substrates, while CMCase II is a glucose-producing enzyme. So, CMCase I is considered to play some part in cellulose degradation using the crude enzyme preparation that has high CMC hydrolyzing activity.¹⁾

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