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Enhancement of the viscometric endocellulase activity of *Polyporus arcularius* CMCase IIIa by cellobiose and cellooligosaccharides

Abstract Purification and viscometric characterization of three CMCases from *Polyporus arcularius* were carried out. The three CMCases, I, II, and IIIa, were estimated to have molecular masses of 39.1 kDa, 36.3 kDa, and 24.3 kDa, respectively. The addition of cellobiose and cellooligosaccharides to the reaction mixtures of CMCase I and II inhibited viscometric endocellulase activity. Following the addition of 20 mM cellobiose, CMCase I and II activities fell to about 30%–36% of their activity in the absence of cellobiose. CMCase IIIa activity, on the other hand, increased in proportion to the increase in cellobiose or cellooligosaccharide concentration. Maximal enhancement of CMCase IIIa activity was observed following the addition of cellobiose, whereas less enhancement was observed with cellooligosaccharides spanning more than two glucoside units. The addition of 20 mM cellobiose resulted in an increase greater than 500% in CMCase IIIa activity. Inhibition of CMCase I and II by cellobiose and cellooligosaccharides may be the result of competition between the substrate and the reaction products. One of the reaction products of CMCase IIIa may bind to a site other than the active site of the enzyme, thus enhancing CMCase IIIa activity.

Key words CMCases · Cellobiose · Enzyme enhancement · Enzyme inhibition · *Polyporus arcularius* · Viscometric CMCase activity

Introduction

The enzymatic hydrolysis of cellulose into various cellooligosaccharides is a complex process involving the synergistic action of a number of cellulolytic enzymes with different substrate specificities. For example, the culture filtrates of the basidiomycetous mushrooms *Polyporus arcularius* Fr., *Hebeloma vinosophyllum* Hongo, and *Coprinus cinereus* Schaeff: Fr. showed endo- and exocellulases with β-glucosidase activities, the maximum activities of which were detected at acidic, subneutral, and alkaline ranges, respectively (Enokibara et al. 1993). Further, these fungi may degrade cellulose by synergistic action of different cellulase components. Cellulases are traditionally divided into two major groups: the endo-1,4-β-glucanases (e.g., endocellulase, CMCase, or 1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4) and the cellobiohydrolases (e.g., exocellulase; EC 3.2.1.91) (Schülein 1988). Endocellulases hydrolyze the β-1,4-glucosidic linkages of cellulose in a random manner, thereby producing new substrates for cellobiohydrolases, thus releasing cellobiose. Microbial endocellulases have been isolated from a number of different sources, including fungi (Okada et al. 1968; Okada 1985; Idogaki and Kitamoto 1992; Enokibara et al. 1992) and bacteria (Yoshikawa et al. 1970; Nakazawa and Kitamura 1983). Endocellulase activity is usually determined by measuring the amount of reducing cellooligosaccharides liberated from carboxymethyl cellulose (CMC) into the reaction mixture using colorimetric methods, such as the Somogyi–Nelson method (Somogyi 1952). However, longer-chain CMC fragments that are actually inert with the Somogyi–Nelson reagent may be also produced as a result of endodegradation. Thus, the colorimetric method cannot be used to evaluate the production of longer-chain nonreducing sugars as a result of endocellulase activity. Fortunately, viscometric analysis can be applied to evaluate endocellulase activity because the hydrolysis of internal bonds within polymer molecules alters the viscosity of a solution (Almin and Eriksson 1967a,b). The decrease in viscosity resulting from endocellulase activity is primarily due to fragmentation of CMC by cleavage of glucosidic linkages remote from the glucoside chain end of the substrate. In contrast, exocellulases, which act on CMC near the glucoside chain end, have little effect on viscosity. Endocellulase activity determined by viscometric methods is most often expressed in terms of arbitrary viscometric

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units based on the initial rate of decline in specific viscosity or the initial rate of increase in specific fluidity. In a preliminary experiment for estimating the inhibitory effect of accumulation of enzyme reaction products in the viscometric enzyme assay using different endocellulase fractions from various cellulase-producing fungi such as *Coriolus versicolor* (L.: Fr.) Quél, *P. arcularius*, *Penicillium funiculosum* Thom, and *Trichoderma viride* Pers. Ex Gray, we found that the two CMCases (CMCases I and II) isolated from *P. arcularius* and the most of CMCase fractions from other fungi were expectedly inhibited by the addition of reaction products. However, an endocellulase (CMCase IIIa) from *P. arcularius* was strongly enhanced in viscometric enzyme activity by the addition of reaction products into the reaction mixture. Similar increase of viscometric CMCase activity by the addition of endoproducts was also observed with some fractions of endocellulases among the fungi tested in the preliminary experiment.

In the present article, we present determination of the viscometric characteristics of a novel CMCase IIIa, in contrast with the viscometric characteristics of two other endocellulases, CMCase I and II, from *P. arcularius*.

Materials and methods

Microorganism

Polyporus (*Favolus*) *arcularius* ATCC 24461 was used in the present study. The stock was subcultured on a potato dextrose agar (PDA) slant, and kept at 2°C in a refrigerator.

Purification of CMCases

Three major CMCases were purified from 2000 ml of the culture filtrate of *P. arcularius*. To produce cellulases of the fungus, a medium containing 5.0 g avicel, 2.0 g peptone, 0.1 g yeast extract, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $CaCl_2$ in 1000 ml distilled water at pH 5.8 was used. For the preparation of inoculum, several pieces of the mycelial fragments dissected from the slant culture were inoculated into 20 ml of the seed culture medium in a 100-ml Erlenmeyer flask. In the seed culture medium, 5.0 g/l avicel was replaced by 20 g/l glucose. After growth for 10 days at 24°C, the inoculum mycelia were aseptically homogenized with a Warling blender, and 1 ml homogenate was inoculated into 100 ml culture medium in a 1000-ml shaking flask. Then, 20 flasks were incubated at 24°C for 12 days with reciprocal shaking at 120 strokes/min to produce crude cellulases in the culture filtrates.

For further purification, a potassium phosphate (KP) buffer (pH 7.0) supplemented with 2 mM reduced glutathione (GSH) was used unless otherwise described. After removal of the fungus by filtration, the culture filtrate was concentrated to one-tenth of the original volume by lyophylization, after which solid ammonium sulfate was added to achieve 80% saturation; this was followed by

agitation for 2 h. The resulting precipitate was collected by centrifugation, dissolved in 0.02 M KP buffer, and dialyzed against the same buffer overnight. The dialysate was charged on a DEAE-cellulose (DE52) column (4.1 × 40 cm) equilibrated with 0.02 M KP buffer, and the column was eluted using a linear gradient of 0.02–0.4 M KP buffer. Protein peak fractions that did not adsorb to the column (peak 1) were used for further purification of CMCase I. Fractions demonstrating protein peaks at buffer concentrations approximating 0.06 M and 0.08 M were separately pooled for purification of CMCase II and CMCase IIIa, respectively.

The fractions containing CMCase I were put into Centrifro CF25 (Amicon, Beverley, MA, USA), and concentrated by centrifugation. The concentrate was then charged on a DEAE-Toyopearl column (2.4 × 24 cm) equilibrated with 0.02 M KP buffer and eluted using a linear gradient of 0–0.4 M KP buffer. The active fractions were pooled and solid ammonium sulfate was added to achieve 30% saturation. The enzyme solution was then charged on a Butyl Toyopearl column (1.4 × 12 cm) equilibrated with 0.1 M KP buffer containing 30% ammonium sulfate. A linear elution gradient was established using 30%–0% ammonium sulfate dissolved in 0.1 M KP buffer, and the active fractions were pooled and concentrated by centrifugation with a Centrifro CF25. The enzyme solution was then charged on a Bio-gel P-60 column (1.2 × 45 cm), and the active fractions were pooled as purified CMCase I fraction. The purification of CMCase II was performed by successive DEAE-Toyopearl, Butyl-Toyopearl, Sephadex G-75, and repeat DEAE-Toyopearl column chromatography, in a similar manner to the purification of CMCase I, after which active fractions from the final column were pooled as purified CMCase II. CMCase IIIa was purified by DEAE-Toyopearl, followed by two rounds of Butyl-Toyopearl chromatography. A summary of the purification steps followed to isolate the three CMCases from *P. arcularius* is outlined in Table 1.

Determination of cellulase activity

Colorimetric assay of CMCase activity was used as the control assay method for cellulase activity. The reaction mixture for CMCase activity contained 0.6% CMC, 50 μmol sodium acetate buffer (pH 4.5), and 20 μl enzyme solution in a total volume of 1.0 ml. After incubation at 37°C for 10 min, reducing sugars released from the substrate were measured as glucose using the Somogyi–Nelson method (Somogyi 1952). One unit of enzyme activity was defined as the quantity of enzyme required for liberating 1 μmol reducing sugar equivalents per minute. The protein concentration of the reaction mixture was determined by the method of Lowry et al. (1951).

Viscometric assay of CMCase activity was used to assess endoglucanase activity. The reaction mixture contained 1.5% CMC, 100 μmol sodium acetate buffer (pH 4.5), and 20 μl enzyme solution in a total volume of 2.0 ml. The mixture was incubated at 37°C for 10 min, and the reaction was

Table 1. Purification of CMCase I, II, and IIIa from *Polyporus arcularius*

| Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)
Culture filtrate	2,507.0	992.0	2.53	100.0
Ammonium sulfate precipitation (80% sat.)	1,568.0	472.0	3.32	62.5
DEAE cellulose (DE-52)				
Peak 1	187.4	57.1	3.28	7.5
Peak 2	390.0	136.0	2.86	15.6
Peak 3	227.0	123.0	1.85	9.1
Peak 1 (CMCase I)				
DEAE-Toyopearl	54.5	14.3	3.81	2.2
Butyl-Toyopearl	36.9	2.2	16.7	1.5
Bio-gel P-60	24.2	1.1	22.0	1.0
Peak 2 (CMCase II)				
DEAE-Toyopearl	146.0	17.0	8.59	5.8
Butyl-Toyopearl	79.7	3.7	21.5	3.1
Sephadex G-75	52.5	2.9	18.1	2.1
DEAE-Toyopearl	25.5	0.8	31.9	1.0
Peak 3 (CMCase IIIa)				
DEAE-Toyopearl	94.7	9.66	9.80	3.8
First Butyl-Toyopearl	9.7	0.80	12.1	0.4
Second Butyl-Toyopearl	6.8	0.40	17.0	0.3
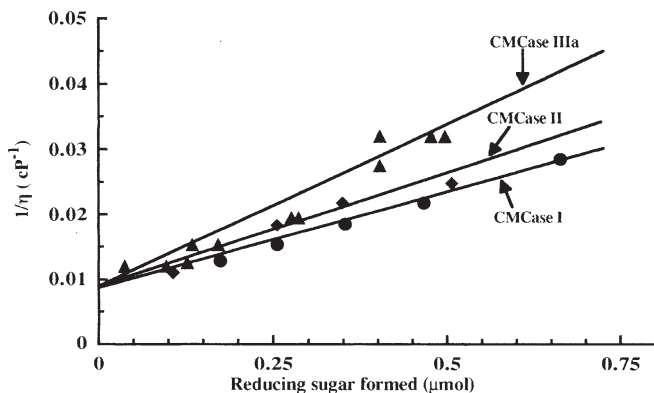
Fig. 1. Relationship between increased reducing power and fluidity of the reaction mixture for CMCase I, II, and IIIa: ●, CMCase I; ◆, CMCase II; ▲, CMCase IIIa. The amount of reducing sugar formed was determined by colorimetry, and fluidity (1/cP) was determined using a rotary viscometer

stopped by placement in a boiling water bath for 10 min. After cooling, the viscosity of the mixture was determined at 20°C using a rotary viscometer (Type E; Tokyo Instrument, Tokyo, Japan). The enzyme activity of each cellulase was related to the reciprocal of η (1/cP), and these results corresponded to the colorimetric measurements of CMCase I, II and IIIa activities (Fig. 1).

Electrophoresis and molecular mass estimation

Electrophoresis of each enzyme was performed on a polyacrylamide gel (native PAGE) using a CD-8 apparatus (Tokyo Kagaku Sangyo, Tokyo, Japan) at 4°C for 1 h at a current of 20 mA per gel. A mixture of 0.025 M Tris and 0.192 M glycine (pH 8.3) was used as a buffer. Each gel was stained for protein with 1% Amid black dissolved in 7%

acetic acid and decolored with 7% acetic acid. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed using the same apparatus as was used for native PAGE. CMCases and marker proteins were incubated in 10 mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 2% 2-mercaptoethanol for 5 min in a boiling water bath before electrophoresis.

Chemicals

Calboxymethyl cellulose sodium salt (CMC) were purchased from Nacalai Tesque, Tokyo, Japan. The CMC product was actually free from reducing sugar contaminants. Cellobiose was purchased from Sigma Chemical (St. Louis, MO, USA). Cellooligosaccharides were the products of Seikagaku (Tokyo, Japan).

Results and discussion

Purity and molecular mass estimation of the three CMCases

The homogeneity of purification of the three enzymes isolated from *P. arcularius* was confirmed by native PAGE, in which only one protein band corresponding to CMCase I, II, and IIIa was observed in each lane. Thus, it was concluded that adequate purification of the three CMCases was achieved. Molecular mass estimation of the three native enzymes was done by SDS-PAGE (Fig. 2). The molecular masses of CMCase I, II, and IIIa were estimated to be 39.1, 36.3, and 24.3 kDa, respectively. These values are similar to those reported for CMCases isolated from a basidiomycete, *C. versicolor* (L.: Fr.) Quél. (Idogaki and Kitamoto 1992).

Optimal pH and temperature for CMCase activity

The optimal pH for hydrolysis of CMC by CMCase I and II, as determined by viscometric analysis, was 4.4–4.6, while optimal hydrolysis by CMCase IIIa was observed at pH 4.9. The optimal temperature for CMCase I and II activities, as determined by viscometric analysis, was 68°C, whereas CMCase IIIa showed maximum activity at 52°C. We also determined the optimal pH and temperature of CMCase activity via colorimetric analysis and attained nearly identical values for all three enzymes.
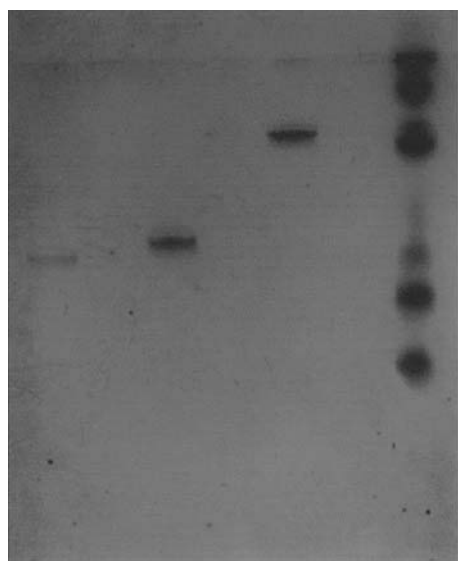
Lane 1   Lane 2   Lane 3   Molecular markers

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis of three purified CMCases from *Polyporus arcularius*. Lanes 1, 2, and 3 were CMCases I, II, and IIIa, respectively

Substrate specificities

The substrate specificities of the three CMCases were determined by colorimetric analysis (Table 2). All three CMCases were active on amorphous cellulose (phosphate-swollen cellulose) and less active on Avicel. Moreover, the relative activity of CMCase IIIa against amorphous cellulose was 1.4 times greater than that of either CMCase I or II using CMC as the standard substrate. All three enzymes actively degraded CMC and the CMCase I and II were highly active against cellooligosaccharides, such as hexaose and pentaose. On the other hand, CMCase IIIa was less active against cellooligosaccharides than CMCase I and II. Based on these results, it appears that CMCase IIIa might have greater endodegradative capability than the other CMCases, resulting in the production of more nonreducing long-chain cellulose moieties. The CMCase activities of all three enzymes increased as cellooligosaccharides of increasing glucoside chain length were used as substrates. However, none of the enzymes hydrolyzed cellobiose. The apparent colorimetric K_m values of CMCase I, II, and IIIa against CMC were 0.35, 0.26, and 0.26 mg/ml, respectively. These values were three to four times higher than the apparent K_m values of cellulases isolated from *Aspergillus niger* van Tiegh. (Okada 1985) and *T. viride* (Okuda 1976), but less than half of those isolated from a wood-rotting basidiomycete, *C. versicolor* ((Idogaki and Kitamoto 1992).

The observed relationship between reducing power and fluidity (1/cP) during the hydrolysis of CMC by CMCase I, II, and IIIa, is shown in Fig. 1. CMCase IIIa produced the greatest increase in fluidity as a function of reducing power, followed by CMCase II. CMCase I produced the least increase in fluidity as a function of reducing power among all three enzymes. This result suggests that CMCase IIIa has the greatest endodegradative capacity of all the CMCases, resulting in the production of a large number of nonreducing longer-chain cellulose fragments, followed by

Table 2. Colorimetric determination of the relative activities of three *Polyporus arcularius* CMCases against various substrates

Substrate	Relative activity (%)		
	CMCase I	CMCase II	CMCase IIIa
0.5% concentration			
Avicel	2.4	3.2	2.3
H_3PO_4-swollen cellulose	18.5	18.4	25.9
CMC	100	100	100
0.1% concentration			
CMC	100	100	100
Cellohexaose	214	257	182
Cellopentaose	170	194	182
Celloteraose	70.4	158	51.4
Cellotriose[a]	1.1	1.5	3.9
Cellobiose[a]	0	0	0

The reaction was carried out by using 0.05 units of CMCase I or II, or 0.02 units of CMCase IIIa, respectively

Reducing sugars released from various substrates other than cellotriose and cellobiose were measured as glucose by the Somogyi–Nelson method

[a] Glucose released from each substrate was measured using the glucose oxidase method

Table 3. Inhibition or enhancement of viscometric *Polyporus arcularius* CMCase I, II, and IIIa activity upon exposure to cellooligosaccharides or glucose

Enzyme	Additive (5 mM)	Relative activity (%)
CMCase I	None	100
	Glucose	88
	Cellobiose	58
	Cellotriose	38
	Celloteraose	28
	Cellopentaose	11
	Cellohexaose	7
CMCase II	None	100
	Glucose	92
	Cellobiose	59
	Cellotriose	42
	Celloteraose	32
	Cellopentaose	20
	Cellohexaose	12
CMCase IIIa	None	100
	Glucose	102
	Cellobiose	384
	Cellotriose	248
	Celloteraose	233
	Cellopentaose	161
	Cellohexaose	69

The reaction was carried out by using 0.05 units of CMCase I or II, or 0.02 units of CMCase IIIa, respectively; relative activity in terms of 1/cP was determined using a rotary viscometer

Table 4. Effect of cellobiose concentration on viscometric *Polyporus arcularius* CMCase I, II, and IIIa activity

Enzyme	Cellobiose (mM)	Relative activity (%)
CMCase I	0	100.0
	1	78.5
	5	57.7
	10	42.0
	20	30.9
CMCase II	0	100.0
	1	87.3
	5	58.7
	10	43.6
	20	36.0
CMCase IIIa	0	100.0
	1	141.0
	5	226.0
	10	400.0
	20	522.0

The reaction was carried out by using 0.05 units of CMCase I or II, or 0.02 units of CMCase IIIa; respectively
Relative activity in terms of 1/cP was determined using a rotary viscometer

CMCase II and CMCase I. Thus, it seems reasonable to suggest that CMCase IIIa might degrade the inside chain, producing longer-chain cellulose fragments, whereas CMCase I and II might degrade shorter-chain reducing glucoside fragments produced from the endocellulase reaction of CMCase IIIa.

Effect of accumulated reaction products on CMCase activity, as determined by viscometric analysis

Cellulase-mediated hydrolysis might be inhibited by the accumulation of cellobiose and cellooligosaccharides within the reaction mixture. However, to date, this has not been reported. We investigated whether the end products of CMCase reactions might inhibit CMCase activity by adding 5 mM cellobiose, cellooligosaccharide, or glucose to the reaction mixtures of CMCase I, II, and IIIa, followed by viscometric analysis. The results are shown in Table 3.

Inhibition of CMCase I and II activities by cellooligosaccharides was confirmed. The inhibitory effect of cellooligosaccharides on the cellulase reaction was directly related to the number of glucoside chains contained within the cellooligosaccharides added to the reaction mixture. Interestingly, enhancement of CMCase III activity by accumulation of reaction products was observed. Therefore, we concluded that endocellulases from *P. arcularius* could be divided into one of two categories, based on whether viscometric results show inhibition or enhancement of CMCase activity upon exposure to reaction products. Although we did not determine kinetic analysis of the inhibitory effect of cellooligosaccharides, we assumed that inhibition may be the result of competition between the substrate and the reaction products, and it strengthens when the number of the glucoside units of cellooligosaccharides was increased. However, a reaction product might also enhance activity by binding to a site other than the active site of some enzymes such as CMCase IIIa. The most dramatic enhancement of CMCase IIIa activity was observed following the addition of cellobiose; however, this effect was reduced when cellooligosaccharide chains involving more than two glucoside units were used. Glucose did not show any effect on CMCase activity.

Effect of cellobiose concentration on the viscometric endocellulase activity of the three CMCases

From the preceding results, it appears that accumulated reaction products enhance *Polyporus arcularius* CMCase IIIa activity. To confirm the effect of concentration on CMCase activity, different concentrations of cellobiose as a model reaction product were added to the reaction mixtures of CMCases I, II, and IIIa, after which CMCase activity was determined via viscometric analysis (Table 4).

The activities of CMCase I and II decreased as greater concentrations of cellobiose were added to the reaction mixture. Following the addition of 20 mM cellobiose, CMCase I and II activities fell to about 30%–36% of their activity in the absence of cellobiose. CMCase IIIa activity, on the other hand, increased in proportion to the increase in cellobiose concentration. The addition of 20 mM cellobiose resulted in a greater than 500% increase in CMCase IIIa activity.

In conclusion, we identified a novel CMCase, CMCase IIIa from a basidiomycete, *P. arcularius*, and we demonstrated enhancement of CMCase IIIa viscometric endocellulase activity in the presence of increasing concentrations of cellobiose and cellooligosaccharides, using viscometric analysis. In preliminary experiments, cellobiose was

not observed to inhibit the activity of a partially purified enzyme fraction from *T. viride*; rather, mild enhancement of viscometric CMCase activity was observed. However, several other fractions from this fungus were observed to inhibit viscometric CMCase activity. Therefore, we expect that CMCases similar to CMCase IIIa will be found in other microorganisms in the future. Cloning and expression of the gene encoding *Polyporus arcularius* CMCase IIIa are in progress.

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