THE CELLULASE OF *TRICODERMA VIRIDE*

Purification and Properties of a Number of Carboxymethylcellulase

By

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Reese, Siu and Levinson (1950) posturated that the enzyme system from cellulolytic micro-organisms are made up of two types of enzyme ; one type (C_1-enzyme) degrades highly ordered forms of cellulose such as natural cotton and the other (C_X-enzymes) are capable of attacking only chemically modified forms of cellulose. Many works have been carried out on these enzymes after that. The multiplicity of C_X-enzymes has been found in several micro-organisms (Reese and Gilligan, 1953 ; Grims, 1957 ; Haga, 1958 ; Slorvick and King, 1960 ; Shikata, 1963), whereas a single enzyme responsible for the hydrolysis of native and derived celluloses and cellobiose has been obtained by some workers (Whitaker, 1953 ; Kooiman, Roelofsen and Sweeris, 1954 ; Hollo and Szilagyi, 1957 ; Toyama, 1959). More recently (Selby and Maitland, 1967) C_1- and C_X-components (a cellobiase and a carboxymethylcellulase) bave been separated from culture filtrate of *Tricoderma viride*. The highly purified C_1-component is no longer able to solubilize cotton ; recombination with the C_X-components, however, restores all the activity of the orginal culture filtrate.

The C_X-components from *T. viride* probaly are composed a cellobiase and some carboxymethylcellulases. The difference in action towards substrate between the carboxymethylcellulases is unknown. The present paper reports on the purification and properties of a few enzymes belonging to the C_X-components from *T. viride*.

MATERIALS and METHODS

Crude enzyme preparation. Culture filtrate of *T. uiride*, which was cultured with bran, was saturated by 75% with $(NH_4)_2SO_4$ at room temperatures. The precipitate formed was collected and three volumes of water were added to it. The mixture was centrifuged and the supernatant was freeze-dried. The dried powder was supplied by Meijiseika Ltd., Tokyo.

Substrates. The soluble carboxymethylcellulose (SCMC) and hydrocellulose used here were obtained from commercial source. Cellooligosaccharides prepared by acetolysis from powdered filter paper by the method of Miller, Dean and Blum (1960).

Measurement of SCMC-lipuidizing activity. The stock solution of SCMC was prepared by continuously shaking an aqueous suspension of the powder overnight at room temperatures, any undissolved residue being removed by filtering through a glass filter under reduced pressure. The mixture of 2 ml. of 1% SCMC solution, 2 ml. of 0.4 M acetate buffer (pH 4.5), 0.5 ml of the enzyme solution and 0.5 ml. of water was poured into an Ostwald viscometer (water-flow time, 17.8 seconds at 30°C) and the flow time was measured at varied intervals during 15 minutes incubation at 30°C. The enzyme activity was represented in $d(1/\eta_{sp})/dt$, where the dimention of time was minute.

Measurement of SCMC-saccharifying activity. The mixture of 0.25 ml. of 1% SCMC aqueous solution and 0.5 ml. of 0.4 M acetate buffer (pH 4.5) was incubated with 0.25 ml. of enzyme solution for 1 hr. at 30°C. The increase in reducing power was measured by the Somogyi-Nelson's method (1945, 1944).

Measurement of the activity towards hydrocellulose, The measurement was carried out in the same way as in SCMC. Here 1% hydrocellulose suspension was used as substrte solution and the reaction mixture was shaken between incubation.

Measurement of the activities towards cellobiose and cellooligosaccarides. The reaction mixture was composed in the same way as in SCMC except that the substrate concentration was 4%. Nine ml. of water were added to the reaction mixture after 30 min. incubation at 30°C and 0.5 ml. of the diluted solution was determined by the above method.

Measurement of the activity towards p-Nitrophenyl-β-D glucoside (PNG). The reaction mixture contained 0.1 ml. of 0.0136 M PNG, 0.2 ml. of 0.4 M acetate buffer (pH 4.5) and 0.1 ml. of enzyme solution. After incubation at 30°C for 30 min., 10 ml. of 0.1 M Na_2CO_3 were added to the mixture. The amount of p-nitrophenol liberated was then calculated from the extinction at 420 mμ.

Chromatographic assay of the enzymic reaction products. Paper chromatography with the enzyme digests was carried out on Toyo Roshi filter paper No. 50 in the decending way. After development with the organic phase of a 6 : 4 : 3 mixture of n-butanol, pyridine and water (v/v), the paper strip was air-dried and sugars were detected on paper with $AgNO_3$-NaOH method (Smith, 1954).

RESULTS

Purification of enzyme. Twenty g. of the crude enzyme powder were suspended in 60 ml. of water and the suspension was stirred for 8 hr. at room

temperatures. The supernatant from centrifugation of the suspension at 8000 r. p. m. for 20 min. at 0°C was dialized against tap water at 10°C for 70 hr. The protein concentration of the dialyzed solution was 7 mg./ml. After applying the extract containg 500 mg. of protein to a ion exchange column (Amberlite CG-50, 5 × 80 cm.), it was washed with 2 l. of 0.2 M acetate buffer (pH 3.5) at a rate of 5 ml. per min. The first step of elution was carried out with 2 l. of 0.3 M acetate buffer (pH 5.5) and the second was done with 2 l. of 0.4 M acetate buffer (pH 5.5) at same rate as it of washing. Both of the eluted fraction contained SCMC-saccharifying and -liquidizing activity. The acetivity towards cellobiose was contained in the second fraction (CG-II) but not in the first. Table I shows the activities of CG-II fraction towards substrates.

activity \ substrate	cellobiose	P N G	SCMC
% of hydrolysis	12	25	
reducing sugar formed (γ as glucose)			48
$d(1/\eta_{sp})/dt$			0.08

Table I. The final enzyme concentration of the reaction mixtures were 0.003%.

After dialysis, 100 mg. of freeze-dried powder of the CG-II fraction were gained and it was placed on the following purification. A 5 × 60 cm. column of CM-Sephadex C-50 was bufferrized with 0.2 M acetate buffer (pH 4.0) and 5 g. of CG-II was applyed to the column. The step-wise elution was carried out successively with each 2 l. of 0.2 M (pH 4.0), 0.4 M (pH 4.5) and 0.6 M (pH 5.0) acetate buffer at a rate of 2 ml. per min. ; fractions of 50 ml. were collected. The distribution of enzymic activities was shown in Fig. 1. Enzymic activities were found in fraction 10 to 40 (CM-I), 45 to 75 (CM-II), 85 to 100 (CM-III) and 101 to 110 (CM-IV). The activities and the yields of proteins were shown in Table II.

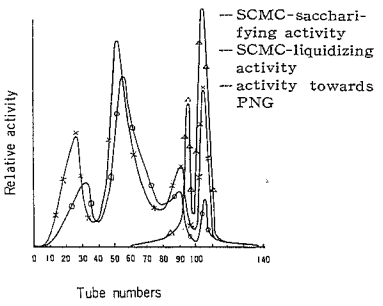

Fig. 1. CM-Sephadex column chromatography of CG-II fraction.

	CM-I	CM-II	CM-III	CM-IV
% of hydrolysis of cellobiose	36	7	40	51
% of hydrolysis of PNG	0	0	52	60
SCMC-saccharifying activity (γ as glucose)	65	106	110	44
SCMC-liquidizing activity $d(1/\eta_{sp})/dt$	0.04	0.08	0.03	0.01
yield of protein (g. from 5 g. of CG-II)	2.46	0.33	0.21	0.29

Table II The final enzyme concentrations of the reaction mixtures were 0.003% in all measurements.

Further purifications were carried out with the CM-II and -IV fractions. A 2.5 × 40 cm. column of DEAE-Sephadex A-50 was bufferrized with 0.05 M acetate buffer (pH 4.0) and 0.3 g. of the CM-II was applyed to the column. The column was eluted step-wisely with each 300 ml. of 0.05 M, 0.1 M, 0.2 M 0.4 M, 0.8 M, 1.0 M and 1.5 M acetate buffer (pH 4.0) at a rate of 0.5 ml. per min. ; fractions of 10 ml. were collected. Fig. 2 showe the distribution of protein. The greater part of protein was removed in fraction 8 to 20 (DEAE-II) and the enzymic activities were concentrated into this fraction,

After applying 0.26 g. of CM-IV to a 2.5 × 40 cm. column of DEAE-Sephadex A-50 bufferrized with 0.02 M acetate buffer (pH 4.5), it was eluted step-wisely with each 400 ml. of 0.02 M, 0.04 M and 0.06 M acetate buffer (pH 4.5). Fig 3. showe the distribution of protein. Two peaks of protein appeared here and the enzymic activities were found in the first peak (DEAE-IV) but not in the second. Table III shows the enzymic activities and protein yield of DEAE-II and IV fractions.

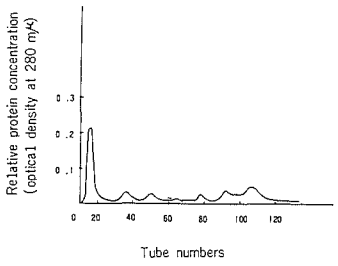

Fig. 2. DEAE-Sephadex column chromatography of CM-11 fraction.

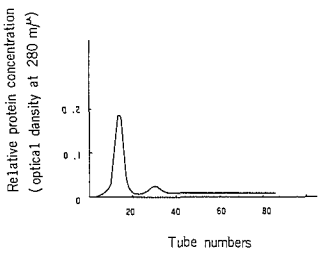

Fig. 3. DEAE-Sephadex column chromatography of CM-IV fraction.

	DEAE-II	DEAE-IV
% of hydrolysis of cellobiose	8.5	68
% of hydrolysis of PNG	8	67
% of hydrolysis of cellopentaose	22	53
% of hydrolysis of cellohexaose	21	52
SCMC-saccharifying activity (γ as glucose)	100	156
hydrocellose-saccharifying activity (γ as glucose)	9	2.5
SCMC-liquidizing activity $d(1/\eta_{sp})/dt$	0.40	0.03
yield of protein (g. from 0.3 g of CM-II and 0 26 g. of CM-IV)	0.1	0.06

Table III. The final enzyme concentrations of the reaction mixtures were 0.003% in all measurements.

Paperchromatography of reaction product. Fig. 4 shows the result when SCMC was digested by each purified enzyme for 6 hr. In Fig. 5, hydrocellulose, cellopentaose and cellohexaose were attacked by DEAE-II and -IV for 4 hr.

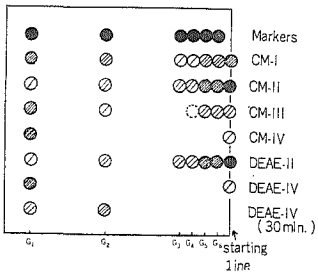

Fig. 4. Paper chromatograms of the reaction products from SCMC. $G_1, G_2, \cdots\cdots G_6$ represent glucose, cellobiose, ……cellohexaose respectively. DEAE-IV(30 min.) shows that the incubation time was 30 min.

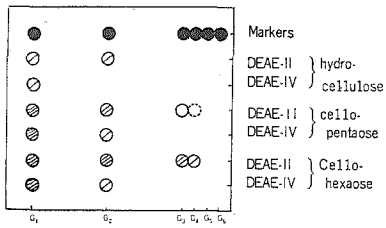

Fig. 5. Paper chromatograms of reaction products from hydrocellulose and cellooligosaccarides.

DISCUSSION

As shown in Table I and II, CG-II fraction has activities towards various substrates containing PNG, whereas CM-I attacks cellobiose but not PNG. The difference is sharp. It shows likely that there is CMCase which has not β-glucosidase activity but is able to hydrolize cellobiose. As shown in the table, the action of CM-II towards cellobiose is very weak, and then above mentioned discussion is inapplicable to the case.

Table II shows that the ratio SCMC-saccharifying activity to cellobiase activity is largest in CM-II, which has the strongest SCMC-liquidizing activity, and CM-IV is more active towards cellobiose and PNG than towards SCMC. It seems that these fractions contain each typical CMCase of exo- and endo-type, and then both fractions were subjected to further purification.

As shown in Table III, SCMC-liquidizing activity of DEAE-II increased by five times as large as it of CM-II. The results in Fig. 4 shows that main products from SCMC by the action of DEAE-II are cellooligosaccharides which have probably polymerization degrees, 7 and over. Therefore, the fraction is a endo-CMCase, which cuts off randomly SCMC and liquidizes it. As shown in Fig. 5, this enzyme attacks more strongly cellooligosaccharides of polymerization degree 5 and over than 4 and less.

It is shown in Table III that DEAE-IV has a strong β-glucosidase activity in addition to a SCMC-saccharifing activity. In Fig. 4, the spots on the staring line represent cellooligosaccharides, which have a polymerization degree 7 and over, and the spot of DEAE-IV is more pale than it of -II. The result shows that the main products from SCMC by the action of DEAE-IV have larger polymerization degree than it with DEAE-II. As shown in Fig. 5, DEAE-IV produces cellobiose from cellopentaose or cellhexaose. The spot of cellobiose appears at the initial stage of the hydrolysis of SCMC by DEAE-IV and disap-

pears at the final stage in Fig. 4. It is concluded that DEAE-IV is a exo-CMCase, which cuts off step-wisely cellobiose from SCMC and digests the produced cellobiose to glucose.

SUMMARY

It was seen that C_X-components from *Tricoderma viride* contain several CMCases. Two of them were fractionated and purified. It was concluded that one of them is endo-CMCase, which cuts off randomly SCMC to produce cellooligosaccharides (polymerization degree 7 and over) as main products and has a weak activity towards the products, and another is exo-CMCase, which hydrolyzes SCMC step-wisely at cellobiose unit and dose cellobiose prduced to glucose.

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