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Cellulases of *Pseudomonas fluorescens* var. *cellulosa*

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Abstract

The substrate specificities and the mode of action of three cellulases from *Pseudomonas fluorescens* var. *cellulosa* were investigated. Two components were obtained by starch-zone electrophoresis of the culture filtrate, and another component was extracted from the cells by ultrasonic oscillation.

These cellulase preparations hydrolyzed CM-cellulose in more or less random fashion. The extracellular cellulases hydrolyzed cellooligosaccharides with DP above four, cellodextrins, colloidal hydrocellulose, and ground native cellulose. The hydrolysis products therefrom were composed of glucose, cellobiose, and at least two oligosaccharides of unknown structure in an approximate molar ratio of 1:10:10:2:3. Cellobiose, several β-glucosides, and methyl β-cellobioside were not hydrolyzed and vanillin β-cellobioside was only slightly hydrolyzed.

Using labeled glucose as acceptor, it was demonstrated that the formation of unknown oligosaccharides was due to the glycosyl-transferring activity of these cellulases.

An extracellular cellulase hydrolyzed colloidal hydrocellulose according to the equation; $p = 11.6\, t^{0.5} E^{4.0}$.

Introduction

In contrast to much effort to elucidate the nature of fungal cellulases[1~8], relatively little has been made with those of bacterial origin, although a variety of soil bacteria have been known to utilize cellulose as a sole carbon source.

Fujiwara and Terui[2] have formerly reported that a strain of *Bacillus hydrolyticus* produces an extracellular cellulase that causes considerable loosening of cellulose fiber with the formation of only a small amount of reducing sugars. The effect of this cellulase action was similar to the degradation of cellulose by weathering and this particular enzyme was named "Weathering cellulase". On the other hand, an extracellular enzyme from *Pseudomonas fluorescens* var. *cellulosa* showed the hydrolase activity toward cellulose fiber and produced a large amount of reduc-

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ing sugars[10]; this cellulase was named "Hydrolytic cellulase". The expressions of "Weathering" or "Hydrolytic" appear to belong to the current category of endo- or exo- splitting mechanisms for cellulose, respectively.

Recently, a very interesting study on bacterial cellulases have been reported by King *et al*[11], who found the presence of a cellulase component acting on cellopentaose in exowise mechanism in the culture of a rumen bacterium, *Cellvibrio gilvus*, in addition to several components of an endowise type. However, other detailed studies on the mode of action of bacterial cellulases have not been carried out thereafter. In the present paper, preparations of partially purified three cellulases from *Pseudomonas fluorescens* var. *cellulosa* and the mode of their action toward various substrates are reported.

Materials and Methods

1. Isolation of cellulolytic bacteria Several soil bacteria were isolated in the following way to compare their cellulolytic activities. A small amount of garden soil was inoculated on the culture broths of Omerianski[12] or Hutchinson and Clayton[13] which were enriched with partly immersed pieces of filter paper (1%). The cultures were kept at 37°C for several days until visible colonies appeared. After successive transfer of the cultures the growing cells were diluted to a suitable concentration and spread on the plates of nutrient agar containing starch (0.5%) and yeast extracts (0.01%). After incubation for a few days each of the colonies appeared was transferred to a broth of filter paper. In this way, about fifty strains of cellulolytic soil bacteria were isolated.

On the other hand, *Pseudomonas fluorescens* Migula var. *cellulosa* Ueda et Ishikawa, strain number 107[14] was obtained through the courtesy of Dr. K. Ueda, Institute of Biochemical Engineering Agricultural Faculty of Tokyo Kyoiku University. This bacterium has been isolated in 1948 by Ueda from the field soil. It is a Gram-negative straight rod from 0.5 to 0.7 μ in width, and from 1.2 to 3.0 μ in length with a single polar flagellum.

In addition, several strains of *Sporocytophaga* were kindly supplied by Dr. K. Akashi, Institute of Zootechnics, Agricultural Faculty of Kyusyu University.

2. Test conditions of cellulolytic activity Bacteria were cultured on a 10 ml medium of Omerianski or Huchinson *et al.* in a test tube containing 1% partially immersed filter paper. The cellulolytic activity was estimated by an extent of disintegration of the filter paper, and the time of culture required for complete crumbling was employed for their relative activity.

For the cultivation of *Pseudomonas*, 500 ml Roux bottles containing 200 ml of Dubos' mineral medium[15] with 1% filter paper were used. Culture of a large scale was made at 40°C in a 10 *l* jar-fermentor containing 6 *l*. Dubos'medium with 1% filter paper pulp under rotary agitation of 400 cycles per minute and aeration of 5

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to 10 liters per minute. Cellulase activity in the culture filtrate reached a maximum in general on the 4th day of culture after inoculation. Possible contamination with other bacteria was checked by incubation of a small portion of the culture on Dubos-starch agar plate.

3. Substrates Cellulose powder was prepared by grinding defatted commercial absorbent cotton dried on phosphorous pentoxide and sieved through an 1 mm mesh. Colloidal hydrocellulose was prepared according to the method of Willstätter *et al*[16]. Cellodextrins and cellooligosaccharides were prepared by partial acetolysis of absorbent cotton according to the method of Braun[17] modified by Whitaker[4].

A mixture of deacetylated oligosaccharides was fractionated on a column of activated charcoal (Shirasagi, Takeda Co.) and Celite 535 (1:1 w/w) by stepwise elution with increasing ethanol concentrations.[18] The cellooligosaccharides eluted were located by Anthrone method[19] and paper chromatography. Effluents containing sugar component of the same mobility were combined and concentrated under reduced pressure to a syrup. Individual cellooligosaccharides therein were crystallized from aqueous ethnol. It was identified by measuring the melting point, R_g value and the degree of polymerization by means of Anthrone method and by reducing value[20,21]. Cellodextrins were obtained from the water insoluble part of the saponified degradation products of cotton. An average degree of polymerization was determined to be approximately 20 by the method of Willstätter and Schudel[22]. Sodium carboxymethyl celluloses (CM-celluloses) with the average degrees of substitution ($\overline{DS}$), 0.613 and 0.410, were purchased from Wako Pure Chemicals Industry Ltd. *p*-Nitrophenyl β-D-glucoside and vanillin β-D-glucoside were prepared by the method of Glaser and Wulweck[23], methyl β-D-glucoside was prepared according to Königs and Knorr[24], vanillin β-D-cellobioside was synthesized by the method of Helferich and Weber[25], methyl β-D-cellobioside was synthesized according to Helferich *et al*[26]. C^{14}-glucose was purchased from Japan Radio-isotope Association.

4. Paper chromatography Reducing sugars in reaction mixtures were identified by paper chromatography with Toyo Roshi filter paper No. 51 using (a) ascending and descending techniques with the solvent system of *n*-buthanol-pyridine-water (6:4:3)[27] in most cases, (b) descending technique with the upper layer of isoamyl alcohol-pyridine-water (1:1:1)[4,28] for samples containing only cellooligosaccharides, and (c) descending technique with buthanol-pyridine-water (10:1:2) (personal communication from Dr. A. Yasumura*). In case of necessity, samples were treated with Amberlite IR 120 (H^+) and IR 410 (OH^-) resin before spotting. Alkaline silver nitrate reagent[29], or with a benzidine-trichloroacetic acid reagent[30] was used as detectors.

5. Crude enzyme preparations (a) Extracellular cellulase (Preparation I): *Pseudomonas* cells grown on 10 *l* culture medium were removed by centrifugation at 2000 × *g* for 30 min. The supernatant was concentrated to about one thirtieth of

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the initial volume under reduced pressure below 40°C and dialyzed against tap water at 5°C. The dialyzed solution was centrifuged at 50000 x g for 40 min, and lyophilyzed. The resulting fluffy white powder of about 4 grams (0.023 unit of CM-cellulose liquefying activity /μg protein) was stored *in vacuo* on silica gel at −40°C. In some cases, the crude enzyme was stored in a state of precipitate obtained by salting out with ammonium sulfate. For further purification, the lyophilyzed enzyme was dissolved in water at 0.2% and pH of the solution was adjusted to 5 with addition of 0.1 M. acetic acid. Solid ammonium sulfate was added to 35% saturation under constant stirring. The resulting precipitate was dissolved in water, dialyzed against 0.005 M. potassium phosphate buffer, pH 7.0 for 3 days at 0°C and lyophilyzed. This cellulase preparation showed a considerably high cellobiase activity.

(b) Cell-bound cellulase (Preparation IV): About 5 g of bacterial cells were obtained by centrifugation from 10 l culture medium after filtration through several layers of gauze. They were washed three times with 0.85 % saline, suspended in a small volume of water and treated by sonic oscillation at 10 k cycle for 15 min. The disrupt cell-suspension was then kept standing overnight with toluene at 0°C. A clear solution obtained by centrifugation at 50000 × g for 40 min was dialyzed against 0.005 M potassium phosphate buffer, pH 7.0. The preparation showed some cellobiase and β-glucosidase activities in addition to powerful cellulase activity (0.0036 unit of CM-cellulose liquefying activity /μg protein).

6. Enzyme assays (a) Measurement of cellulase activity: Colloidal hydrocellulose and CM-cellulose ($\overline{DS}$, 0.410), were employed for the cellulase assays. The reaction mixture was composed of two volumes of 1 % CM-cellulose solution (or 1 % suspension of colloidal hydrocellulose), one volume of 0.02 M sodium citrate buffer, pH 6.1, and one volume of enzyme solution. After suitable incubation time at 30°C., aliquots of the reaction mixture was removed for the determination of total reducing power (saccharifying activity, SA) by Schaffer-Hartmann-Somogyi titration method[31,32] or Nelson-Somogyi colorimetry[20,21]. In the latter method, absorbancy at 500 mμ was measured in a Hitachi Beckmann spectrophotometer DU.

Viscometric assay (liquefying activity, LA) was performed in an Ostwald viscometer (T_0 = 14.2 sec. for 2 ml of water) using CM-cellulose as substrate, and the flow rates of the reaction mixture were measured at suitable intervals. Although aqueous CM-cellulose gave a non-Newtonian liquid, changes in apparent specific fluidity (ϕ_{sp}) under these conditions were parallel with enzyme concentration. No further attempt was, therefore, made to elucidate the relationship between true and apparent specific fluidities.

(b) Unit of cellulase: The unit of cellulase was defined as an enzyme amount which increases the specific fluidity of reaction mixture by 1.0 during initial

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10^4 seconds*. The reaction mixture was composed of one volume of 1% CM-cellulose ($\overline{DS}$, 0.613), two volumes of 0.02 M sodium acetate buffer, pH 6.1, and one volume of enzyme solution[5,6]. The specific fluidity and reducing power were determined in the same way as above.

(c) Measurement of the activities of β-glucosidase and cellobiase: The reaction mixture was composed of one volume of 4.8 mM. solution of substrate, two volumes of 0.02 M sodium citrate buffer, pH 5.1, and one volume of enzyme solution. After the suitable incubation time at 30°C, aliquots of the reaction mixture (usually 0.5 ml for cellobiase and 0.2 ml for β-glucosidase) were removed, and the reducing power was measured by Nelson method and *p*-nitrophenol liberated was determined by the absorbancy at 400 mμ in 0.1 M sodium carbonate.

Results

1. Disintegration assay The results of tests for filter paper-disintegration by various bacteria are shown in Table 1. Of the bacteria tested, *Pseudomonas fluorescens* var. *cellulosa* was found to have the strongest activity for the disintegration of filter paper.

Table 1. Comparison between the cellulolytic activities of some mesophilic aerobic bacteria.

Microorganism	Duration of cultivation (days)					
	3	4	5	6	7	10
Pseudomonas fluorescens var. *cellulosa*	+					
Sporocytophaga,						
red colony	−	+				
yellow colony	−	+				
white colony	−	−	+			
black colony	−	−	+			
orange colony	−	−	−	−	+	
Unidenfied,						
No. 4	−	−	−	+		
No. 8	−	−	−	+		
No. 11	−	−	−	−	−	+

+: Residual filter paper was disintegrated by mild shake of the culture.
−: Residual filter paper was not disintegrated by moderate shake of the culture.

2. Starch-zone electrophoresis of crude extracellular cellulase preparation A starch block (5×2×45 cm) in a plastic tray was prepared according to Miller *et al*[33]. Crude extracellular cellulase was dissolved in veronal buffer, pH

* The 5000 units of No. 27 fraction, for example, corresponds to one unit calculated according to the Report of the Commission of Enzymes of the International Union of Biochemistry, based on the reducing power as glucose.

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8.6, and μ 0.05, to give a 1.2 % final concentraion, and dialyzed against the same buffer overnight at 0°C. A sludgy mass of starch containing about 5 ml of the cellulase solution was packed into an about 1 cm crosswise slit in the starch block at a distance of 15–16 cm from cathode. After electrophoresis, the starch block was cut crosswise into 1 cm pieces and each was transferred to a test tube containing 10 ml of 0.85 % saline solution. The mixture in the tube was fully shaken and decanted after standing overnight at room temperature. Each extract was assayed for protein concentration[34,35] β-glucosidase activity, and CMC-cellulose-saccharifying and-liquefying activities. The results are shown in Fig. 1. The cellulase activity was

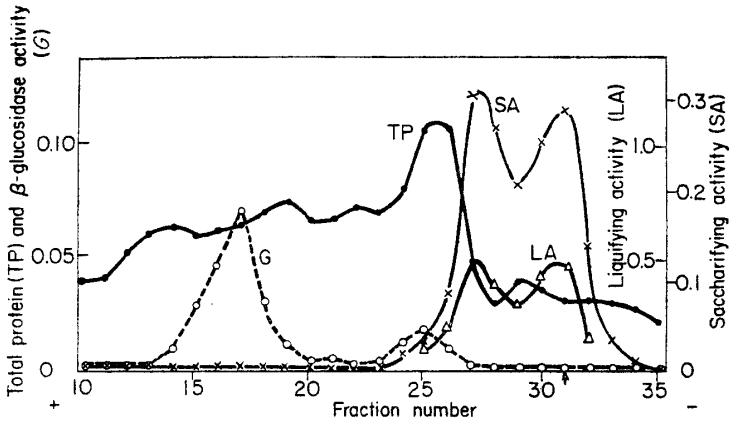

Fig. 1. Resolution of β-glucosidase and cellulase components of *Pseudomnas* culture filtrate by starch zone electrophoresis. Conditions; veronal buffer at pH 8.6, μ 0.05, at 5°C, for 24 hours, 2.4 mA/cm^2 and 6-8 volts/cm. The original position (↑) is not corrected for electroendosmosis. TP; Total Lowry protein measured by the extinction at 750 mμ, G; β-glucosidase activity measured by the extinction at 400 mμ, SA; saccharifying activity for CM-cellulose measured by the extinction at 500 mμ, LA; liquefying activity for CM-cellulose expressed by specific fluidity.

separated into two peakes, though they were partly overlapped each other, and the saccharifying activity was in accordance with the liquefying one. Therefore, the eluate in fraction No. 27 or 30, especially latter, was used for the experiments as purified cellulase preparation.

The β-glucosidase was completely separated into two components with different activity. The major component migrates toward anode much faster than the minor one, but both of them did not overlap the cellulase activities, The major part of protein was separated from any of the cellulase components, and the specific activities of the cellulases were considerably enhanced; 0.14 and 0.23 units of CM-cellulose liquefying activity per μg of protein for No. 27 and No. 30, respectively.

3. Effect of pH on cellulase activities The hydrolysis rate was measured at different pH values from 4.8 to 7.3, with 0.02 M sodium citrate buffer. The results are shown in Fig. 2. A maximum of the activity-pH-curves is at pH 6.8, being

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in good agreement with the values reported by Asai and Okamoto[10] for their cellulase preparation obtained by an entirely different procedure.

4. Time course of hydrolysis and effect of enzyme concentration upon the hydrolysis of CM-cellulose and hydrocellulose

The rates of initial hydrolysis of CM-cellulose by purified extracellular cellulase were measured by both reductmetry and viscometry as a function of the amount of enzyme. The results are shown in Fig's 3 and 4, respec tively. The hydrolysis rates are followed by the zeroorder kinetics in all cases.

Relationships between the rate of hydrolysis of hydrocellulose at various periods of time and enzyme concentration were investigated. The results are shown in Fig. 5 and Table 2.

As clearly seen from the bottom line of the table, the increment of the logarithms of ratios between the amounts of reducing sugars produced and the roots of reaction time were practically parallel with the logarithms of the

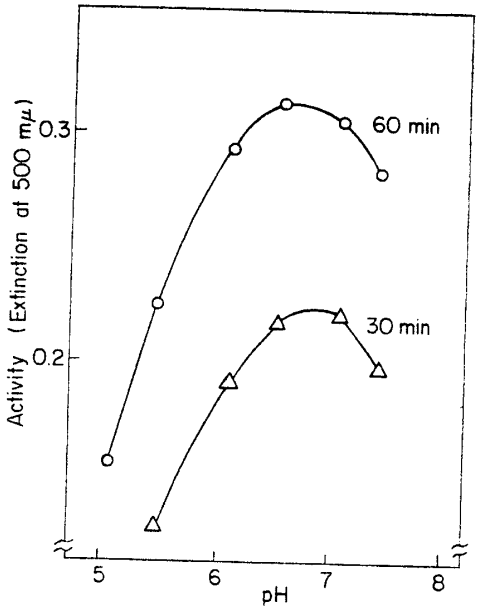

Fig. 2. Effect of pH on the saccharifying activity of purified extracellular cellulase for CM-cellulose. CM-cellulose was dissolved at 0.5 per cent final concentration in 0.02 M sodium citrate buffer. Reaction mixture was incubated at 30°C. for the time indicated.

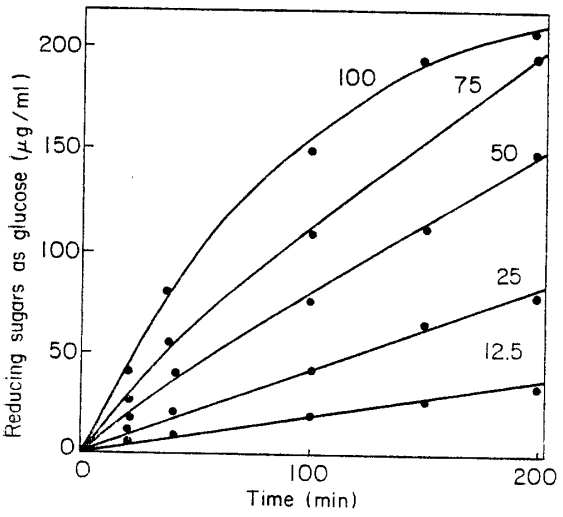

Fig. 3. The formation of reducing sugar during the hydrolysis of CM-cellulose by purified extracellular cellulase of different concentration. Numbers in the figure represent μg protein per ml of the cellulase preparation, and the substrate was dissolved at 0.5 per cent final concentration in 0.02 M sodium citrate buffer, pH 6.1.

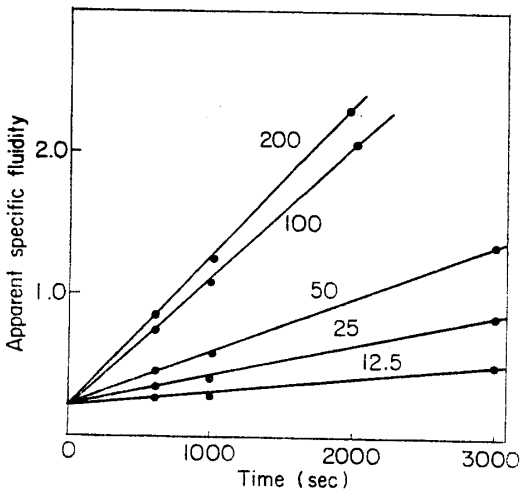

Fig. 4. The time course of increase in apparent specific fluidity of CM-cellulose during the hydrolysis by purified extracellular cellulase of different concentration. Numbers in the figure represent μg protein per ml of the cellulase preparation, and the substrate was dissolved at 0.5 per cent final concentration in 0.02 M sodium citrate buffer, pH 6.1.

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enzyme concentrations and the proportional constant of 0.43 was obtained. Thus, these results can be expressed by the following equation;

$$P = 11.7\, E^{0.43}\, t^{0.5}$$

where P is the amount of reducing sugars produced (mg as glucose) E is the relative enzyme concentration, and t is reaction time in minute.

5. Relationships between specific fluidity (LA) **and the amounts of reducing sugars** (RA) **in the hydrolysis of CM-cellulose** Using CM-cellulose as substrate, relationships between increase in the specific fluidity and increase in the reducing power caused by extracellular and cell-bound cellulases were investigated to compare the mode of their action. A tangent

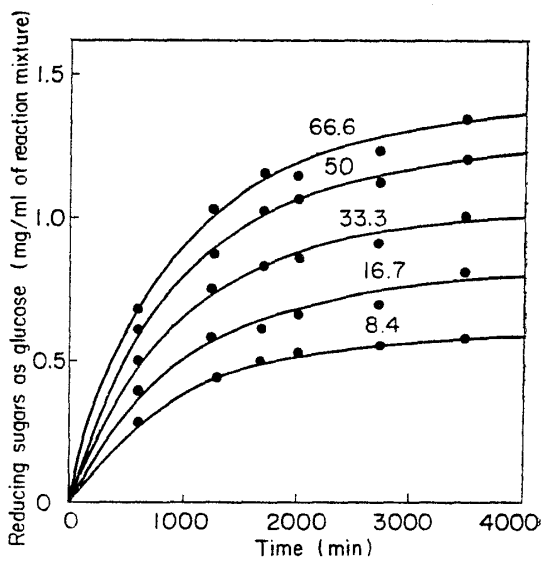

Fig. 5. Hydrolysis of hydrocellulose by purified extracellular cellulase of different concentration.
Numbers in the figure represent μg protein per ml of the cellulase preparation, and the substrate was suspended in 50 mM. sodium citrate buffer, pH 6.1 at 0.5 per cent final concentration.

Table 2. Relationships between the relative concentration of purified extracellular cellulase and increase in reducing sugars produced from hydrocellulose.

Relative concentration of enzyme (E)	1	2	4	6	8
$t^{1/2}$	$p/t^{1/2}$				
24.5	1.14	1.59	2.0	2.41	2.74
31.6	1.20	1.55	2.0	2.47	2.81
44.7	1.18	1.52	1.97	2.41	2.69
Mean values of $p/t^{1/2}$	1.17	1.55	1.99	2.45	2.75
Log $p/t^{1/2}$	0.069	0.191	0.300	0.385	0.438
δLog $p/t^{1/2}$	0	0.122	0.231	0.361	0.359
Log E	0	0.301	0.602	0.778	0.903
Log $E/\delta \log p/t^{1/2}$	–	2.47	2.61	2.46	2.45

Data were cited from Fig. 5. P; reducing sugars as glucose in mg, t; incubation time in minute.

(LA/RA) obtained with 1 N-hydrochloric acid was shown for comparison. The results are shown in Fig. 6.

Tangents for all cellulase preparations indicate that the mode of hydrolysis of CM-cellulose by all these cellulases were of a random type, although more or less differences were found in randomness.

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6. Hydrolysis products from various substrates Hydrolysis products from various substrates by purified extracellular cellulase were identified by paper chromatography. The standard reaction mixture (0.2 ml) contained 2.4 μ moles of substrate, except CM-cellulose, native cellulose and cellulose and cellodextrins, 1 μ mole of citrate buffer, pH 6.1, and 0.4 unit of cellulase. The final concentrations of CM-cellulose, native cellulose and cellodextrins were 0.5, 1.0, and 2.5 per cent, respectively. After the suitable periods of incubation at 30°C., 20 μl aliquots of the reaction mixture were removed and analyzed for hydrolysis products by paper chromatography.

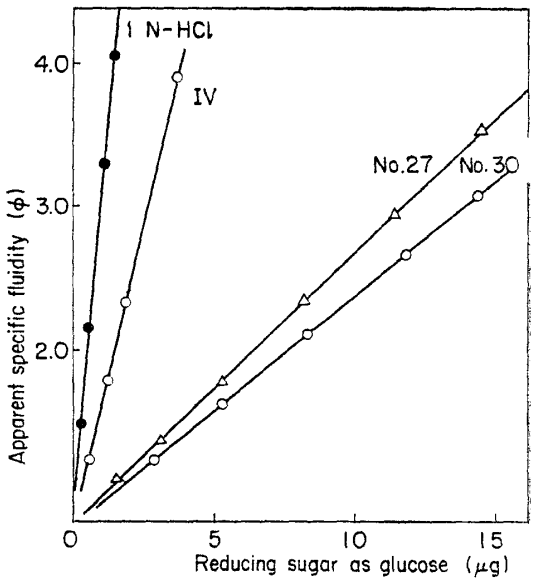

Fig. 6. Fluidity vs. reducing suger in the hydrolysis of CM-cellulose by acid and three cellulase components from *Pseudomonas* (No's 27, 30 and IV).

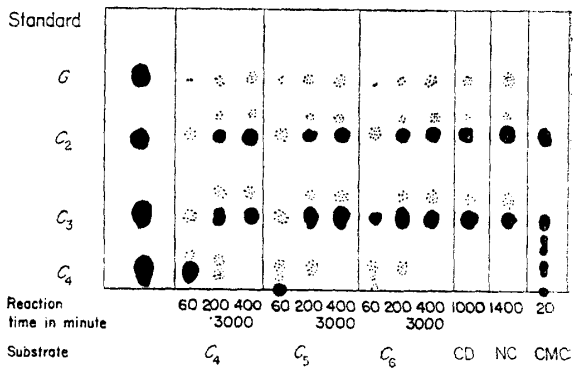

Fig. 7. Diagram showing the paper chromatograms of hydrolysis products from various substrates by purified extracellular cellulase. G; glucose, c_2; cellobiose, c_3; cellotriose, c_4; cellotetraose, c_5; cellopentaose, c_6; cellohexaose, CD; cellodextrin, NC; native cellulose, CMC; GM-cellulose. Solvent system; buthanol/pyridin/water (6:4:3). descending tedhnique. Detection; alkaline silver nitrate method.

Cellobiose, cellotriose, *p*-nitrophenyl β-glucoside, vanillin β-glucoside, methyl β-glucoside, and methyl β-cellobioside were not hydrolyzed by any of the extracellular cellulase, except for very low activity for vanillin β-cellobioside. As shown in Fig. 7, cellotriose, cellobiose, and glucose were produced from cellotetraose, cellopentaose, cellohexaose, cellodextrins and cellulose powder, in an approximate ratio of 10:10:1. Cellooligosaccharides which seemed to be cellotetraose or cellopentaose and other higher oligosaccharides with lower *Rg* were also found at earlier stages of hydrolysis of cellohexaose, cellodextrins, and other longer chain substrates. Oligosaccharides of lower *Rg* values were also found at early stages of hydrolysis of CM-cellulose.

The existance of such higher oligosaccharides in the hydrolysis products from the longer chain substrates suggests that the hydrolysis of cellulosic substrates by *Pseudomonas* cellulases is effected by a random mechanism.

7. Transglycosylation reaction by cellulases Since the presence of oligosac-

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charides of unknown *Rg* seemed to be due to the enzymic transglycosylation reaction, the possible transglycosylation activity of purified extracellular cellulase was examined in a reaction mixture of 25 mM substrates and methanol as an acceptor at around 7 per cent final concentration. However, no significant difference was found in the reducing power of reaction mixtures with or without methanol. Analysis of the reaction mixtures by paper chromatography also failed to detect any trace of methyl β-glucoside or cellobioside and any other glycosides different from the products in the absence of methanol.

On the other hand, several spots of unknown sugars which seem to be transglycosylation products were detected in the paper chromatograms of the hydrolysis products in Fig. 7. These oligosaccharides were possibly di- and trisaccharides judging from their *Rg* values. Since no cellobiase and β-glucosidase activities were found in the purified extracellular cellulase preparation used in this experiment and a β-glucosidase preparation separated from the crude cellulase preparation produced gentiobiose as a transfer product*, the formation of these unknown sugars in the paper chromatograms may be due to the transglycosylation activity of cellulase. To estimate the approximate molar ratios of these products, they were extracted from the filter paper and determined colorimetrically. Table 3 shows the results as expressed by the relative values. The di- and trisaccharides seeming to be the transglycosylation products are in considerably large amounts.

Table 3. Approximate molar ratios of hydrolysis and transglycosylation products from several substrates by purified extracellular cellulase.

Product / substrate	c_3	g_3	c_2	g_2	G
c_4	1.0	0.15	1.0	0.1	0.05
c_5	1.0	0.3	1.0	0.25	0.1
c_6	1.0	0.4	1.0	0.2	0.1
Native cellulose	1.25	0.2	1.0	0.05	0.15

The molar amount for cellobiose was taken as unity. G, C_2, C_3, C_4, C_5, C_6, represent glucose, cellobiose, cellotriose, cellotetraose, cellopentase,and cellohexaose, respectively. Transglycosylation products that seemed to be di- and trisaccharides are represented as g_2 and g_3, respectively.

To confirm the possible transglycosylation activity of cellulase, ^{14}C-glucose was added at 5 per cent final concentration to a reaction mixture which contained cellotetraose or cellodextrin as substrate, and the hydrolysis products were analyzed chromatographically and radioautographically in the routine procedure. The results are shown in Fig. 8. The radioactivities were detected mainly in the spots of unknown saccharides, but low activities were found in the spots of cellotriose and cellobiose. Therefore, it may first be suggested that cellulase would transfer cellobiosyl

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residue of the substrates to ^{14}C-glucose to form unknown ^{14}C-trisaccharide and even ^{14}C-cellotriose. On the other hand, unknown ^{14}C-disaccharide and ^{14}C-cellobiose might be secondarily produced from higher cellooligosyl ^{14}C-glucose which had been possibly formed by the transglycosylation reaction.

One of the unknown ^{14}C-saccharides seemed to be ^{14}C-trisaccharide (g_3) was eluted from paper chromatogram and partially hydrolyzed with N-HCl for 1 hour. The products obtained were then identified by paper chromatography. The results are indicated in Fig. 9. Partial acid hydrolyzate of g_3 contained radioactive glucose, cold cellobiose, and a small amount of active laminaribiose, and unhydrolyzed g_3. The fact suggests that the possible structure of g_3 may be 3-*o*-cellobiosyl glucose. The g_3, however, has been slightly contaminated with active gentiobiose before hydrolysis, and this disaccharide was also found in the hydrolyzate of g_3.

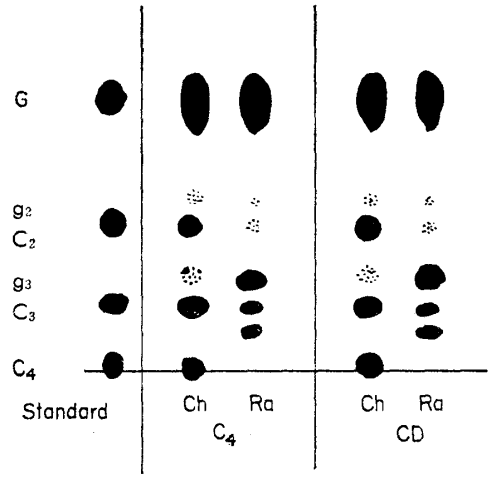

Fig. 8. Diagram showing the paper chromatograms (Ch) and radioautograms (Ra) of the hydrolysis and transglycosylation products by purified cellulase from cellotetraose (C_4) and cellodextrin (CD) incubated with ^{14}c-glucose as acceptor.

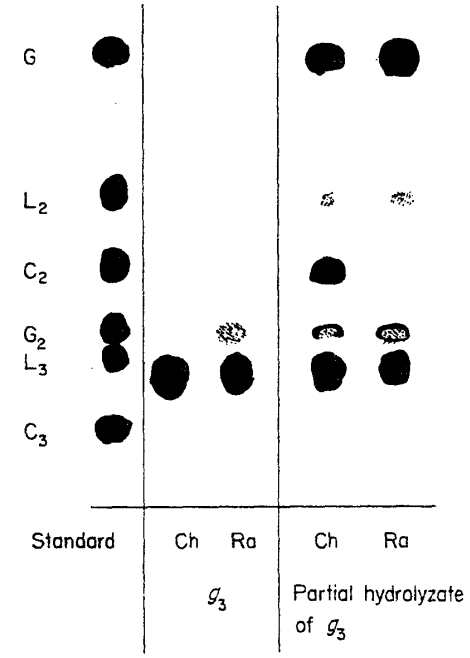

Fig. 9. Diagram showing paper chromatograms (Ch) and radioautograms (Ra) of an unknown trisaccharide (g_3) and those of the partial acid hydrolyzate from it. L_2; laminaribiose, L_3; laminaritriose, G_2; gentiobiose.

Discussion

In the present work, two cellulase components were obtained from the culture filtrate of a cellulolytic pseudomonad in a purified state. They were entirely free from cellobiase and β-glucosidase. In addition, a cellulase preparation that seems to represent the cell-bound cellulase was also obtained, although in a crude state. A characteristic feature of the extracellular cellulases is their wide substrate specificity. They hydrolyzed not only native cellulose but also various cellooligosaccharides down to cellotetraose. Cellotriose and cellobiose were not hydrolyzed. Predominant products found in the digests by the extracellular cellulase, therefore, are cellotriose and cellobiose but small amount of glucose. This substrate specificity is inconsistent with those of a cellulase of *Myrothecium verrucaria*[3,4-a], and also any of the cellulase components of *Irpex*

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lacteus (5,6). In this respect, *Pseudomonas*-extracellular cellulases resemble the cellulase components of *Trichoderma viride* (8). Putting aside its specificity toward lower cellooligosaccharides, stress should be layed on the fact that the extracellular cellulase of the *Pseudomonas* hydrolyze a variety of substrates from native cellulose to higher cellooligosaccharides. Possibly they must have a nature of both C_l and C_x cellulase components, according to the concept of Reese[36]. Although the cellulase preparations in this work were purified by electrophoresis, they might be contaminated by so-called C_1-component as has recently been reported by Selby and Maitland[37] and Wood[38] on its presence in the culture filtrate of *Trichoderma viride*.

Purified extracellular *Pseudomonas* cellulases showed similar kinetics to the cellulases of other origin[39~43]. The reactions by these cellulases seem to proceed according to the equation;

$$P=k\cdot t^m\cdot E^n$$

where k is proportional constant and p is hydrolysis extent effected by the enzyme amount E after the reaction time t. In the equation proposed by Schütz[44], the value of m was 0.5. The same value was obtained with *Pseudomonas* cellulase on the hydrolysis of colloidal hydrocellulose. *Irpex lacteus* and *Aspergillus niger* cellulases have been reported[39] to hydrolyze the hydrocellulose with the values of 0.5 and 0.33 for m, respectively. The values of n for *Pseudomonas* cellulase, however, were different from those for *Irpex* (0.5) and *Aspergillus* (0.33) cellulases; 1.0 for CM-cellulose and 0.4 for hydrocellulose when k was 11.7. These differences in the rates of hydrolysis of the colloidal hydrocellulose may be explained, at least in part, by the differences in the diffusion velocity of cellulases and/or products to and from the heterogeneus phase of this substrate, although no advanced kinetic study was performed in the present work.

Another important problem implied in the present work is concerned with the mechanism of action in the hydrolysis of CM-cellulose. All three cellulases of *Pseudomonas* were shown to be of a typical random type, although the degree of randomness seemed to be different. In general, the following consideration on the cellulolytic process may account for the differences in randomness or "more" random hydrolysis. In the cellulolysis sequence, long chain substrates $\xrightarrow{k_1}$ intermediates $\xrightarrow{k_2}$ end products where k_1 and k_2 represent velocity constants, if k_1 is much greater than k_2, then the "more random" mechanism would be predominant. However, if k_2/k_1 ratio is in reverse and lower molecular saccharides are abundant in the end products, an apparent "less random" mechanism would prevail. Similar relationships have observed most recently by Toda *et al.* [45] on fungal cellulase. The cellulase components from *T. Viride*, which have higher affinity for lower cellooligosaccharides hydrolyzed CM-cellulose less randomly, while the other components of lower affinity showed more random characteristic.

With cellotetraose as well as other cellulosic materials as donor substrates, the

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purified *Pseudomonas* cellulase was found to catalyze glycosyl transfer to ^{14}C-glucose giving rise to at least four labeled sugars, two 1,4-linked ^{14}C-oligosaccharides and at least two not fully identified ^{14}C-sugars, presumably ^{14}C-laminaribiose and 3-0-β-cellobiosyl ^{14}C-glucose. The former (^{14}C-cellooligosaccharides) may be the hydrolysis product of the cellooligosyl ^{14}C-glucose which might be formed by transglycosylation activity of this cellulase. These resalts are particularly noticeable, because the fact is one of few instances of transglycosylation to sugar catalyzed by polysaccharidases of an endotype when the intermediary enzyme-glycosyl complex is relatively stable as seen with lysozyme[46,47] and a certain α-amylase[48].

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