Cellulases of a Marine Mollusc, *Dolabella* sp.

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1. A crude cellulase extract was prepared from the hepatopancreas of a marine mollusc, *Dolabella* sp., and partially purified by ammonium sulphate fractionation. 2. The optimum pH values of the partially purified preparation were 6.5 and 8.0 for Walseth cellulose and CM-cellulose respectively. It was most stable at pH 6.0 and showed moderate thermostability. 3. The partially purified preparation was subjected to starch-zone electrophoresis, and incompletely resolved into several fractions that contained one or more cellulase components of different substrate specificity. 4. Some of these cellulase fractions showed practically no aryl β -glucosidase activity and hydrolysed aryl β -cellobioside with difficulty. From substrates such as higher cello-oligosaccharides, cellodextrin, CM-cellulose, Walseth cellulose and cotton fibre, they produced cellobiose as the major and cellotriose as the minor end products, both of which were resistant to further attack by cellulase. 5. From the slope of the curves of viscosity-reducing power for CM-cellulose, the cellulase components from *Dolabella* were presumed to be of a 'more-random' or a 'less-random' type in the mode of action. 6. In the hepatopancreas of this mollusc, β -glucosidases were also present, which hydrolysed cellobiose as well as aryl β -glucosides. The optimum pH values of these enzymes were about 5.5.

The specificity of cellulases from fungi and bacteria has been widely investigated (Gascoigne & Gascoigne, 1960). Studies on their purification have advanced to such an extent that a purified cellulase preparation, which behaved as a single component on electrophoresis and ultracentrifugation, was obtained from a culture filtrate of *Myrothecium verrucaria* (Whitaker, 1953; Whitaker, Colvin & Cook, 1954). In addition, crystalline preparations have been obtained from culture filtrates of *Irpex lacteus* and *Aspergillus saitoi* respectively (Nisizawa, 1955; Matsumura & Maejima, 1963). The presence of various isoenzymic entities of cellulase of different specificities from certain micro-organisms has been reported (Reese, Siu & Levinson, 1950; Nisizawa & Hashimoto, 1959; Li, Flora & King, 1963; Pettersson, Cowling & Porath, 1963; Pettersson & Porath, 1963; Wakabayashi & Nisizawa, 1964).

Very little information is available about cellulolytic enzymes of animal origin, however, apart from a series of early studies on *Helix pomatia* carried out by Karrer, Schubert & Wehrli (1925) and Karrer & Schubert (1926, 1928), and the recent work of Myers & Northcote (1959) on a partially purified cellulase from the digestive tract of this mollusc. Yokoe (1960) has demonstrated the presence of cellulolytic enzymes in the crayfish, *Procambarus clarkii*.

In the present work, the specificities of cellulolytic enzymes in the hepatopancreas of a Japanese marine mollusc, *Dolabella* sp., have been examined and compared with those of cellulases from micro-organisms.

MATERIALS AND METHODS

Acetone-dried powder. Fresh hepatopancreas from individual marine molluscs of the *Dolabella* sp. was washed with tap water, and cut into small pieces in a beaker containing at least 5 vol. of acetone cooled at -20° . The acetone treatment was repeated twice and the tissue finally extracted with similarly cooled ether. The dark-green defatted material was dried *in vacuo* over CaCl_2 , ground in a mortar to a fine acetone-dried powder and stored in a desiccator at room temperature.

Crude enzyme solution. A sample (43 g.) of the acetone-dried powder was extracted with 700 ml. of water with continuous stirring at 1° for 4 hr. During extraction, the pH was adjusted to 7.0 by the addition of 5% (v/v) acetic acid. Insoluble material in the extract was removed by centrifugation at 5000g for 20 min. The resulting supernatant solution (670 ml.) was fractionated at 1° with $(\text{NH}_4)_2\text{SO}_4$ between 40 and 70% saturation. The precipitate formed was collected on the centrifuge at 11000g for 20 min., taken up in a small volume of water and then dialysed in an animal membrane against running tap water at 10° for 3 days. A crude enzyme solution (140 ml.) was thus obtained, which contained 13.4 mg. of protein/ml.

Starch-zone electrophoresis. Before electrophoresis part of the crude enzyme preparation was dialysed against

veronal buffer, pH 8.3 and I 0.05, overnight at 1° with continuous stirring. A plastic tray (5 cm. $\times$ 2 cm. $\times$ 45 cm.) was packed with potato starch, as described by Miller & Blum (1956). A block of starch (5 cm. $\times$ 2 cm. $\times$ 1 cm.) was removed from the tray at a distance of about 20 cm. from the cathode and replaced by another small amount of dry starch mixed with 1.5 ml. of the buffered enzyme solution to make a sludge. The enzyme solution applied contained 22.1 mg. of protein (measured as bovine serum albumin). Electrophoresis runs were carried out at 1° for 28 hr. under a constant current (22 ma) and varying field strengths (260–270 v). After the run, the starch in the tray was cut crosswise into 1 cm. widths and each block was extracted in a test tube with 8 ml. of McIlvaine (1921) buffer, pH 6.0 (0.05 M), at 5° for 1 hr. After centrifugation at 1500g, the extracts were tested for protein concentration and for the activities of cellulase and related enzymes.

Enzyme assays. (1) β -Glucosidase. (a) With *p*-nitrophenyl β -D-glucoside as substrate. The reaction mixture contained 0.5 ml. of 13.6 mm-*p*-nitrophenyl β -D-glucoside, 1 ml. of McIlvaine buffer, pH 5.5 (0.2 M), and 0.5 ml. of enzyme solution. After incubation at 30° for 2 hr., 0.5 ml. of the mixture was placed in a test tube containing 10 ml. of 0.1 M- Na_2CO_3 . The amount of *p*-nitrophenol liberated was then calculated from the extinction at 420 m μ obtained with a Hitachi model FPW-4 photoelectric photometer.

(b) With cellobiose as substrate. The reaction mixture contained 0.5 ml. of 13.6 mm-cellobiose, 1 ml. of McIlvaine buffer, pH 5.5 (0.2 M), and 0.5 ml. of enzyme solution. After incubation at 30° for 68 hr., the increase in reducing power was determined for each 0.5 ml. of the mixture by the method of Shaffer & Somogyi (1933).

(2) Cellulase. (a) CM-cellulose-saccharifying activity (release of reducing groups). CM-cellulose of degree of substitution 0.650 (manufactured by Daiichi Industrial Pharmaceutical Co. Ltd., Shimokyo-ku, Kyoto, Japan) was employed. The reaction mixture contained 0.5 ml. of 1% CM-cellulose solution, 1 ml. of McIlvaine buffer, pH 8.0 (0.2 M), and 0.5 ml. of enzyme solution. After incubation at 30° for 3.5 hr., 1 ml. of the mixture was added to 1 ml. of alkaline copper reagent (Somogyi, 1952), heated for 10 min. in a boiling-water bath and then cooled rapidly to room temperature before the addition of 0.5 ml. of arsenomolybdate reagent (Nelson, 1944) and water to give a total volume of 12.5 ml. The extinction of the resulting coloured solution was measured at 660 m μ with a Hitachi model FPW-4 photoelectric photometer.

(b) CM-cellulose-liquefying activity (decrease in viscosity). The reaction mixture contained 1.5 ml. of 1% CM-cellulose solution, 3 ml. of McIlvaine buffer, pH 8.0 (0.2 M), 0.5 ml. of enzyme solution and 1 ml. of water. The mixture was incubated in an Ostwald viscometer at 30° and the decrease in viscosity was measured at intervals. The cellulase activity was expressed in terms of the specific fluidity, ϕ_{sp} . (i.e. $1/\eta_{sp}$).

(c) Walseth-cellulose-saccharifying activity. The reaction mixture contained 0.5 ml. of 1% Walseth cellulose suspension, 1 ml. of McIlvaine buffer, pH 6.5 (0.2 M), and 0.5 ml. of enzyme solution. After incubation at 30° for 18 hr., the mixture was centrifuged at 1500g for 10 min., and 1 ml. of the supernatant solution was analysed for reducing sugar in the same way as with CM-cellulose.

(d) Avicel-saccharifying activity. The reaction mixture contained 0.5 ml. of a 1% suspension of Avicel (a micro-

crystalline form of cellulose manufactured by the American Viscose Co., Marcus Hook, Pa., U.S.A.), 1 ml. of McIlvaine buffer, pH 6.5 (0.2 M), and 0.5 ml. of enzyme solution. L-shaped tubes containing the mixture were shaken by a Monod culture apparatus at 30° for 58 hr. The mixture was centrifuged at 1500g for 10 min., and 1 ml. of the supernatant was analysed for reducing sugar in the same way as with CM-cellulose.

(e) Cotton-fibre-saccharifying activity. The reaction mixture contained 10 mg. of absorbent cotton fibre, 1 ml. of McIlvaine buffer, pH 6.5 (0.2 M), and 1 ml. of enzyme solution. After incubation at 30° for 8 days, 1 ml. of the mixture was analysed for reducing sugar in the same way as with CM-cellulose.

(3) Swelling-factor activity for cotton fibre. A 60 mg. sample of absorbent cotton fibre was placed into a test tube containing 10 ml. of McIlvaine buffer, pH 7.0 (0.5 M), and 1 ml. of enzyme solution. The mixture was incubated at 30° for 20 hr., and the cotton fibre taken out with forceps, washed with water, pressed lightly in several layers of filter paper to remove superficial water and then rapidly placed into another test tube containing 10 ml. of 18% (w/v) NaOH solution. After 15 min. at 30° , the cotton fibre was transferred to a glass filter in a centrifuge tube with a cover of aluminium foil and centrifuged at 1200g for 30 min. The slightly wet cotton fibre was immediately weighed. The control experiments were done in the same way but with water in place of the enzyme solution. The weight of the wet cotton fibre in the controls was practically constant in each experiment, fluctuating only within $\pm 2.5\%$.

Total protein in enzyme solution. Total protein of crude enzyme preparation and enzyme fractions obtained from starch-zone electrophoresis was estimated by the extinction at 750 m μ (Lowry, Rosebrough, Farr & Randall, 1951), obtained with a Hitachi model FPW-4 photoelectric photometer. An extinction value of 0.1 corresponded to 53 μ g. of bovine serum albumin.

Paper chromatography. Small samples of incubation mixtures and the solutions of standard substances were repeatedly spotted on Toyoroshi no. 50 filter paper, and developed by the descending technique with the solvent system butan-1-ol-pyridine-water (6:4:3, by vol.) at room temperature for 20 or 40 hr. Products were detected by the acetone- AgNO_3 method (Trevelyan, Procter & Harrison, 1950; Smith, 1954), where the paper was first dipped in a dilute aqueous solution of acetone containing AgNO_3 , then in aqueous ethanol containing NaOH (0.5 N). In some cases a benzidine-acetic acid spray reagent (Bacon & Edelman, 1951) was used and the paper heated at 80° for 10 min.

RESULTS

Optimum pH values of cellulases and β -glucosidase.

Samples of the crude enzyme preparation were diluted to a suitable concentration by adding McIlvaine (1921) buffers at pH values from 2.2 to 8.0 (prepared from 0.1 M-citric acid and 0.2 M-disodium hydrogen phosphate), and also by adding Menzel (1921) buffers at pH values 9.0 and 10.0 (prepared by mixing 0.05 M-sodium carbonate and 0.1 M-sodium hydrogen carbonate). Each enzyme solution thus diluted was incubated with various

substrates at 30°. The incubation times were 2 hr., 3 hr. and 1.5 hr. for CM-cellulose, Walseth cellulose and *p*-nitrophenyl β -D-glucoside respectively. Each enzyme activity was determined as described above. As shown in Fig. 1, the maximum activities of the crude enzyme preparation for CM-cellulose, Walseth cellulose and *p*-nitrophenyl β -D-glucoside were at pH 8.0, 6.5 and 5.5 respectively. Thus distinct differences were observed among the three pH-activity curves. It has been reported that optimum pH values of the cellulases from *Trichoderma viride* (Niwa, Kawamura & Nisizawa, 1965) and *Aspergillus niger* (King & Smibert, 1963) for CM-cellulose were 1 pH unit lower than for insoluble cellulosic substrates. In contrast, the optimum pH of *Dolabella* cellulase for CM-cellulose was rather higher than for the insoluble substrate Walseth cellulose.

Stabilities of crude enzyme preparation towards pH and temperature. In two sets of experiments, nine incubation mixtures each containing an equal amount of crude enzyme preparation were individually adjusted to pH values from 2.2 to 10.0 by adding the same kinds of buffer as used above. After one set had been kept at 1° for 24 hr., and another set at 45° for 2 hr., both series of buffered enzyme solutions were diluted to an appropriate concentration with McIlvaine buffer, pH 8.0 (0.2 M). Samples were incubated with 1% CM-cellulose solution at 30° for 2 hr., and the remaining cellulase activities were determined by the Nelson-Somogyi method. The results are indicated in Fig. 2. The

buffered solutions of the crude enzyme preparation were stable over the range pH 4.0–9.0 at 1°, but comparatively unstable at 45° except at pH 6–7. Even at favourable pH values the enzyme lost nearly 30% of its activity after 2 hr. at 45°. This cellulase therefore appeared to be rather thermolabile.

Starch-zone electrophoresis of crude enzyme preparation. The enzyme activities of fractions obtained by starch-zone electrophoresis were measured with various substrates. Fig. 3 shows the patterns of these activities and of total protein. When the movement caused by electroendosmosis is taken into account, all enzyme activities tested migrated towards the anode. All cellulase activities were incompletely resolved in this experiment, but showed a tendency to separate into at least seven components. Each showed a characteristic pattern of hydrolytic activities towards Walseth cellulose and Avicel in addition to CM-cellulose. In contrast, the activities for *p*-nitrophenyl β -D-glucoside and cellobiose had a main peak near the anode. These two activities therefore seemed to be due to the same enzyme protein, although the relative cellobiase activity was far higher. On the other hand, the activity patterns towards Walseth cellulose and Avicel resembled each other more than the pattern towards CM-cellulose, although the former activities were much lower than the latter. Since at least four cellulase components, fraction nos. 18, 20, 22 and 25, which migrated slower in this order were practically free of aryl β -glucosidase activity, they were used as cellulase samples for the observation of their substrate specificities.

Fig. 1. pH-activity curves of cellulase and β -glucosidase for the crude extract of *Dolabella* sp. $\circ$, CM-cellulose-saccharifying activity; $\blacktriangle$, Walseth-cellulose-saccharifying activity; $\bullet$, β -glucosidase activity measured by *p*-nitrophenol liberated from *p*-nitrophenyl β -D-glucoside. Experimental details are given in the text.

Fig. 2. Stability of CM-cellulose-saccharifying activity towards pH and temperature. $\circ$, Treatment at 1° for 24 hr. at each pH value; $\blacktriangle$, treatment at 45° for 2 hr. at each pH value. Experimental details are given in the text.

Fig. 3. Zone-electrophoretic patterns of cellulases and β -glucosidase. They are not corrected for electroendosmosis. A 22.1 mg. sample was applied at the position indicated by the arrow. ●, CM-cellulose-saccharifying activity; ▲, Walseth-cellulose-saccharifying activity; ■, Avicel-saccharifying activity; ○, cellobiase activity; △, β -glucosidase activity measured by *p*-nitrophenol liberated from *p*-nitrophenyl β -D-glucoside; □, total protein. Experimental details are given in the text.

Table 1. *Hydrolytic activities of several fractions separated by starch-zone electrophoresis towards cellobiose and p-nitrophenyl β -D-glucoside*

Experimental details are given in the text.

Fraction no.	Percentage hydrolysis at 30° for 68 hr.		Activity ratio (a/b)
	(a) With 3.4 mm-cellobiose	(b) With 3.4 mm- <i>p</i> -nitrophenyl β -D-glucoside	
17	16.3	2.7	6.0
23	35.9	5.1	7.0
29	88.2	41.2	2.1
30	98.0	47.8	2.1
31	68.6	35.7	1.9

Table 2. *Swelling-factor activity for cotton fibre and the production of reducing sugar from it by fractions resolved by starch-zone electrophoresis*

Experimental details are given in the text.

Fraction no.	Swelling factor (mg. of 18% NaOH absorbed)	Reducing sugar, as glucose (μ g./ml.)
7	0.4	0
17	—	6
18	13.6	—
19	11.1	9.5
20	12.9	—
21	11.6	13.5
22	11.4	—
24	9.9	16
25	8.8	—
26	8.4	—
28	14.6	8.5
30	13.3	15

Specificities of cellobiase and aryl β -glucosidase. To confirm the possible identity of cellobiase activity with that of aryl β -glucosidase, 0.25 ml. each of 13.6 mm-cellobiose and 13.6 mm-*p*-nitrophenyl β -D-glucoside was independently added to 0.5 ml. of McIlvaine buffer, pH 5.5 (0.2M), in a test tube and incubated with 0.25 ml. of fraction nos. 17, 23, 29, 30 and 31 at 30° for 68 hr., all of which showed cellobiase and aryl β -glucosidase activities. A

sample (0.5 ml.) of the mixtures was analysed for the reducing sugar and *p*-nitrophenol liberated. The percentage hydrolysis is summarized in Table 1. The ratio of cellobiase activity (a) to aryl β -glucosidase activity (b) ranged from 2 to 7. The result might simply be explained by assuming that both β -glycosidases are different; a more probable

interpretation is that a few components or iso-enzymes of a β -glucosidase capable of hydrolysing both cellobiose and *p*-nitrophenyl β -D-glucoside, but different in their specificities, are present. However, the interpretation of the ratios becomes more complicated if the cellulases of *Dolabella* could split cellobiose at different rates, as might be presumed from the results in Tables 3-5.

Swelling-factor and saccharifying activities for cotton fibre. Swelling-factor activity towards cotton fibre, and the release of reducing sugar from it by most fractions obtained by starch-zone electrophoresis, were determined (Table 2).

Any fractions tested that had cellulolytic activity also showed some swelling-factor activity and they also produced reducing sugar, but fractions without

cellulolytic activity showed no such swelling activity. Consequently, swelling-factor activity must be one of the cellulolytic activities of cellulase.

Since reducing sugar was formed from absorbent cotton fibre by these enzyme fractions, it is evident that native cellulose must be also attacked by these cellulases.

Hydrolysis products from various substrates. Hydrolysis products from various substrates were identified by paper chromatography after treatment with cellulase fraction nos. 18, 20, 22, 25 and 30, the first two having much lower activities towards cellobiose and *p*-nitrophenyl β -D-glucoside, and the last two having appreciably higher activities in particular towards cellobiose. A sample of each enzyme fraction was incubated at 30° with the

Table 3. *Hydrolysis products from various substrates after treatment with fraction no. 18*

The relative amounts of the products on paper chromatograms are indicated by: (+), trace; +, light-coloured; ++, medium-coloured; +++, heavy-coloured. G₁, G₂, G₃ and G₄ represent glucose, cellobiose, cellotriose and cellotetraose respectively; g₂ and g₃ are unidentified oligosaccharides, and seemed to be biose and triose respectively; D.P., degree of polymerization. Experimental details are given in the text.

Substrate	Incubation time (hr.)	Products					
		G ₁	g ₂	G ₂	g ₃	G ₃	G ₄
Cellobiose	5		(+)				
Cellotriose	5				(+)		
Cellotetraose	5			+			
Cellopentaose	5	+		+			
Cellohexaose	5	++	(+)	+++	(+)		
	24	++++	+	+++++	+	+++	+++
Vanillin cellobioside	24	(+)					
Cellodextrin (D.P. 25)	5			+			
CM-cellulose	1			+		(+)	
	3	+		++		+	
	5			(+)			
Walseth cellulose	24			+			

Table 4. *Hydrolysis products from various substrates after treatment with fraction no. 20*

The relative amounts of the products on paper chromatograms are indicated as in Table 3. Abbreviations are also the same as in Table 3.

Substrate	Incubation time (hr.)	Products					
		G ₁	g ₂	G ₂	g ₃	G ₃	G ₄
Cellobiose	5		(+)				
Cellotriose	5				(+)		
Cellotetraose	5			+			
Cellopentaose	5	+		+			
Cellohexaose	5	++	(+)	+++	(+)		
	24	++++	+	+++++	+	+++	+++
Vanillin cellobioside	24	(+)					
Cellodextrin (D.P. 25)	5	+		+			
CM-cellulose	1			+		(+)	
	3	(+)		++		+	
	5			(+)			
Walseth cellulose	24			+			

same amount of each 1% substrate solution or suspension in McIlvaine buffer, pH 8.0 (0.01M). After an appropriate period of incubation, a portion of the reaction mixture was boiled for 10min., centrifuged, if necessary, and examined by paper chromatography for 40hr. at room temperature. The acetone-silver nitrate reagent was used as detector, except when vanillin β -cellobioside was the substrate, when development was conducted for 20hr. and the benzidine spray reagent was used. The relative amounts of hydrolysis products were estimated by the colour intensity and size of the spots. The results are shown in Tables 3-7.

Cellulase fractions containing the higher cellobiase activity produced glucose in addition to cellobiose from CM-cellulose, Walseth cellulose and higher cello-oligosaccharides, whereas the fractions containing the lower cellobiase activity formed

cellobiose from these substrates as a predominant product, and no glucose. Therefore the cellulase itself does not readily attack cellobiose. In contrast with tetra-, penta- and hexa-cello-oligosaccharides, cellotriose and aryl β -cellobioside were also attacked with difficulty. However, two unidentified spots with R_{Gl} values close to that of cellobiose and cellotriose were formed from some cello-oligosaccharides, and from cellobiose, by the various cellulase fractions, including that fraction which was practically free of cellobiase activity. It is possible that these unknown oligosaccharides, designated g_2 and g_3 , arise by transglycosylation from cellobiose catalysed by a contaminating β -glucosidase.

The cellulase fractions having the lower cellobiase activity attacked vanillin β -cellobioside with difficulty, whereas those fractions having the

Table 5. *Hydrolysis products from various substrates after treatment with fraction no. 22*

The relative amounts of the products on paper chromatograms are indicated as in Table 3. Abbreviations are also the same as in Table 3.

Substrate	Incubation time (hr.)	Products					
		G ₁	g ₂	G ₂	g ₃	G ₃	G ₄
Cellobiose	5		(+)				
Cellotriose	5	(+)		(+)	(+)		
Cellotetraose	5	(+)		+			
Cellopentaose	5	+		++			
Cellohexaose	5	++	(+)	+++	(+)		
	24	++++	+	+++++	(+)	+++	+++
Vanillin cellobioside	24	+					
Cellodextrin (D.P. 25)	5	+		+			
CM-cellulose	1			+		+	
	3	+		++		+	
	5						
Walseth cellulose	24	(+)		(+)			

Table 6. *Hydrolysis products from various substrates after treatment with fraction no. 25*

The relative amounts of the products on paper chromatograms are indicated as in Table 3. Abbreviations are also the same as in Table 3.

Substrate	Incubation time (hr.)	Products					
		G ₁	g ₃	G ₂	g ₃	G ₃	G ₄
Cellobiose	5	(+)	(+)				
Cellotriose	5	(+)		(+)	(+)		
Cellotetraose	5	(+)		+			
Cellopentaose	5	++		+			
Cellohexaose	5	++	(+)	++	(+)		
	24	++++	+	+++++	(+)	+++	+++
Vanillin cellobioside	24	+					
Cellodextrin (D.P. 25)	5	+		+			
CM-cellulose	1			(+)		(+)	
	3	+		+		(+)	
	5						
Walseth cellulose	24	(+)		(+)			

Table 7. *Hydrolysis products from various substrates after treatment with fraction no. 30*

The relative amounts of the products on paper chromatograms are indicated as in Table 3. Abbreviations are also the same as in Table 3.

Substrate	Incubation time (hr.)	Products					
		G ₁	g ₂	G ₂	g ₃	G ₃	G ₄
Cellobiose	3	+	(+)				
Cellotriose	3	+		+	(+)		
Cellotetraose	3	++		++		++	
Cellopentaose	3	++		+++		++	++
Cellohexaose	3	+++	+	++++	(+)	++	+++
Cellodextrin (D.P. 25)	3	+		+

Fig. 4. Relation of fluidity to reducing power on the hydrolysis of CM-cellulose by several fractions obtained by starch-zone electrophoresis. The reaction mixture for the measurement of fluidity or reducing power is composed of 1% CM-cellulose (degree of substitution 0.650), McIlvaine buffer, pH 8.0 (0.2M), and enzyme solution in the proportions 1:2:1. ●, Fraction no. 18; ■, fraction no. 20; ▲, fraction no. 22; ○, fraction no. 25; □, N-HCl.

higher cellobiase activity produced glucose, though only a little. The cellobiase or β -glucosidase must therefore hydrolyse this cellobioside.

Relation between decrease in viscosity and release of reducing sugar during hydrolysis of CM-cellulose by various cellulase fractions. The cellulase fractions that were practically free of aryl β -glucosidase (nos. 18, 20, 22 and 25) were incubated with 0.25% CM-cellulose solution. Every 10 min. the decrease in the viscosity of the mixture and the production of reducing sugar were measured, by using a whole incubation mixture and a 1 ml. sample respectively. For comparison, the hydrolysis of 0.25% CM-cellulose solution by N-hydrochloric acid was performed. The results are shown in Fig. 4.

A curve of different slope was obtained for each cellulase fraction, the curve for fraction no. 22 being nearest to and the curve for fraction no. 18

being farthest from that for N-hydrochloric acid. The cellulase component fraction in no. 22 therefore may be of the 'more-random' type and that in fraction no. 18 may be of the 'less-random' type (Nisizawa, Hashimoto & Shibata, 1963).

DISCUSSION

An extract from the hepatopancreas of *Dolabella* sp. was partially purified and fractionated by starch-zone electrophoresis. All the proteins of the enzyme solution migrated towards the anode. The major peak of the protein showed only a slight cellulase activity. Most of the activity was found in the protein peak sloping towards the cathode. Several protein fractions obtained from this part hydrolysed not only CM-cellulose, Walseth cellulose and Avicel but also cotton fibre, and each showed a characteristic pattern of substrate specificity. In addition, all of the fractions showed some swelling-factor activity towards cotton fibre. On the basis of this fact, it was assumed that the swelling-factor activity is an associated cellulolytic activity of cellulase. If the swelling factor is different from ordinary cellulase and exists independently in each cellulase fraction, the presence of several components must be also assumed for this factor as with cellulase. However, results from studies on fungal cellulases (Niwa *et al.* 1965) support the former view.

In contrast with higher cello-oligosaccharides, lower oligosaccharides such as cellobiose, cellotriose and aryl β -cellobioside were not readily hydrolysed by the cellulase fractions free from aryl β -glucosidase. It therefore appears that cellulases of the hepatopancreas of *Dolabella* sp. resemble more closely those of *Pseudomonas fluorescens* than some fungal cellulases (Nisizawa *et al.* 1963) in this respect. Moreover, cellobiose was predominant among the hydrolysis products from higher cello-oligosaccharides, cellodextrin (degree of polymerization 25), CM-cellulose and Walseth cellulose after treatment with *Dolabella* cellulases. Possibly they specifically release a cellobiose residue from cello-

oligosaccharides that had been produced more randomly from long-chain substrates.

In the studies of Karrer *et al.* (1925) and Karrer & Schubert (1926, 1928), an exo-type removal of cellobiosyl residues from insoluble substrates was postulated for the mode of action of the cellulases from *Helix pomatia*. On the basis only of the end products, a similar mechanism might also be suggested for the hydrolysis by *Dolabella* cellulases. However, since the cellulase fractions rapidly decreased the viscosity of CM-cellulose with incubation time, the mode of action of the cellulases is not solely of the exo-type. The assumption by Karrer *et al.* (1925) and Karrer & Schubert (1926, 1928) appears to be due to the lack of data from the hydrolysis of higher cello-oligosaccharides as well as CM-cellulose.

From a comparison of the fluidity-reducing power curves of several cellulase fractions obtained by electrophoresis, it is suggested that a crude extract from *Dolabella* is a mixture of the components of a 'more-random' type and a 'less-random' type. The mode of hydrolysis of the 'less-random' type should resemble the mechanism of an exo-type. The presence of both types of cellulase in animal sources is noteworthy, since a similar fact was reported by Nisizawa *et al.* (1963) for some cellulolytic fungi and bacteria. In other words, most cellulases in Nature appear to be of a 'random' type irrespective of their origin.

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