KOR. JOUR. MICROBIOL. March, 1985, p. 25-33 Vol. 23, No. 1

Fractionation of Extracellular Cellulase Produced by *Cellulomonas* and Reaction Mechanisms of the Isolated Enzymes

Kim, Byung-Hong and J.W.T. Wimpenny

Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA WALES.

*Cellulomonas*가 생산하는 균체외 Cellulase의 분리 및 분리된 효소의 작용기작

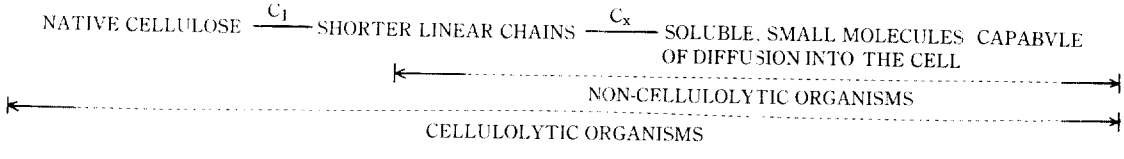
金炳弘 · J. W. T. Wimpenny

(英国, Cardiff, University College, Cardiff 微生物学科)

The cell-free cellulolytic enzyme was separated into 3 different enzyme proteins by gel-filtration and ion-exchange chromatography. These fractions were named enzyme A, enzyme B and enzyme C. The mode of action of each of the separated enzymes on crystalline cellulose was examined using infra-red spectroscopy and X-ray crystallography.

It was concluded that enzyme B is of the C_1-type and reduces the crystallinity of the substrate by generating an unstable glucopyranose ring structure, whilst enzymes A and C are of the C_x-type and hydrolyse the reaction product of enzyme B to constituent sugars. A reaction scheme for this cellulase system is proposed and discussed.

Little is known about the mode of action of the complex of enzymes called "cellulase" from any microbial species and this applies even more to the bacterial cellulase enzymes. The observation that some organisms can digest native cellulose whilst others can only hydrolyse the modified non-crystalline form of the substrate led to the suggestion that the fomer possess an activity (called the C_1 enzyme) in addition to the hydrolytic activity (the C_x enzyme) which is present in both types of organism. The following reaction mechanism has been oroposed (Reese *et al* 1950):



The C_1 enxyme has been isolated and has been reported to be ineffective either on amorphous cellulose or on crystalline cellulose on its own, but to have a synergistic effect with the C_x activity on crystalline cellulose (Mandels and Reese 1964 Selby and Maitland 1967).

Several workers have reported that the C_1 enzyme is an β-1.4-glucan cellobiohydrolase which produces cellobiose from non-reducing free ends of the cellulose molecule (Eriksson and Pettersson

*Present Address; Microbiology Laboratory, Korea Advanced Institute of Science & Technology, P.O. Box 131, Dongdaemun, Seoul, Korea

1975, Pettersson 1975, Wood 1972). These authors argued that the β-1.4-glucan cellobiohydrolase cannot initiate the hydrolysis of native cellulose because there are few free ends that the enzyme can attack, and they proposed a new mechanism as follows:

$$\text{NATIVE CELLULOSE} \xrightarrow{C_x} \text{REACTIVE CELLULOSE} \xrightarrow{C_1} \text{CELLOBIOSE}$$

Reese (1975) has questioned the new hypothesis critically, and argued that the C_1 enzymes used in these experiments are not a pure enzyme but a mixture of C_1 and β-1.4-glucan cellobiohydrolase. To support his argument he demonstrated changes in the physical characteristics of cellulose treated with *T. viride* cellulase in the presence of a selective C_x enzyme-inhibitor, Methocel.

Eriksson *et al* (1974) observed that the presence of oxygen in the reaction mixture increased the cellulolytic activities of several fungal cellulases including the enzyme from *T. viride* and suggested that an oxidation step was important in cellulolysis.

Cellobiohydrolases were purified from fungal origin (Nummi *et al* 1983, Chanzy *et al* 1983) and from bacterial cellulase (Creuzet *et al* 1983, Nakamura and Kitamura 1983). All the purified enzyme were found to have activities on amorphous as well as on crystalline substrates.

MATERIALS AND METHODS

The organism and its maintenance

Cellulomonas flavigena KIST 321 (Bae and Kim 1974) was maintained on a slope of potato-cellulose agar (potato extract 5.0g, yeast extract 0.2g, $NaNO_3$ 2.0g, $MgSO_4 \cdot 7H_2O$ 0.5g, KH_2PO_4 0.2g, K_2HPO_4 1.0g, $FeSO_4 \cdot 7H_2O$ 0.01g, and microcrystalline cellulose Avicel PH-101 5.0g in 1 litre of distilled water). The culture was grown at 30 °C for 48 hours before storing at 4 °C.

Enzyme Production

A loopful of culture was transferred from a slope to a flask containing 50ml of potato-cellulose broth (the same composition as potato-cellulose agar without the agar). The flask was incubated at 30 °C on a gyrotary shaker (120 rpm) for 48 hours before using it as an inoculum.

The main culture was grown in a 1.0 l fermenter constructed according to the design of Evans *et al* (1970) with temperature controller, pH controller and oxygen electrode. The culture was grown at a temperature of 30 °C and a pH of 6.8-7.2 for 45 hours in a mineral salts medium ($(NH_4)_2SO_4$ 4.0g, NaCl 3.0g, K_2HPO_4 0.5g KH_2PO_4 0.5g, $MgSO_4 \cdot 7H_2O$ 0.1g, $CaCl_2 \cdot 2H_2$ 0.1g, in 1 liter of distilled water) to which was added 20g, 1^{-1} Whatman No. 1 filter paper. A 5% inoculum was used to initiate growth. A silicone based antifoam agent (DG-525, Dow Corning Ltd., Barry, Wales) was used to control foaming during growth. The fermenter was operated at volumetric oxygen transfer coefficient (K_La) values between 50-100 hr^{-1}

Enzyme purification

The enzyme protein in the culture filtrate was concentrated first by salt precipitation. The fraction precipitated between 20-50% saturated ammonium sulphate contained almost all of the activity and this was redissolved in 1/20 of the original supernatant volume of 0.02 *M* phosphate buffer, pH 7.0. After undissolved matter was removed by centrifugation at 38,000 x g for 10 minutes at 4 °C, the redissolved solution was used as the starting material for cellulase purification.

1. *Gel filtration* Low molecular weight proteins in the sample were separated by gel filtration chromatography on a 2.5 x 40.0cm columan packed with Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden). A similar procedure to Wood (1968) was employed using 0.02 *M* phosphate buffer, pH 7.0.

The fractions containing cellulolytic activity were pooled, and concentrated by ultra-filtration on an Amicon PM-10 ultra-filter mounted in an Amicon stirred filter cell Model-12 (Amicon Ltd., High Wycombe, England).

2. *Ion-exchange chromatography* The concentrated main protein peak was further separated by ion-exchange chromatography on DEAE

Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.05 *M* phosphate buffer, pH 7.0, and packed in a 2.5x 30.0cm, column. The buffer molarity of the sample was adjusted to 0.05 *M* with phosphate buffer, pH 7.0 before being applied to the column. The column was eluted by lineary increasing the strength of phosphate buffer from 0.05 *M* to 0.25 *M* at a constant pH.

Assays for cellulase activity

Cellulase activities on filter paper and carboxymethyl cellulose (CMC) were measured by modified methods of Mandels and Weber (1969). 10 pieces of $1cm^2$ Whatman No.1 filter paper (about 0.2g) were incubated in 10ml of 0.02 *M* phosphate buffer, pH 7.0 at 41 – °C for about 10 minutes before 10ml of enzyme solution were added. The reaction mixture was incubated for 3 hours in shaking water bath at the same temperature and the reducing sugar produced was then measured by Somogyi-Nelson's method as described by Hodge and Hofreiter (1962) aginst glucose standared.

A high viscosity carboxy-methyl cellulose (CMC)-sodium salt with a degree of substitution of 0.7-0.8 was used as substrate for activity measurements on a noncrystalline cellulose derivative. 0.5ml aliquots of 1% w/v CMC solution in 0.02 *M* phosphate buffer, pH 7.0 were preincubated in a 40 °C water bath in screw capped test tubes before adding 0.5ml of enzyme. The reaction mixture was incubated at the same temperature for 30 minutes without shaking before determining the reducing sugar released by Somogyi-Nelson's method.

A unit of enzyme was defined as 1 μ mole reducing sugar equivalents released per hour. In the enzyme assay experiments a control was always run with heat denatured enzyme to correct for reducing sugar in the enzyme solution.

X-ray diffraction

The aqueous suspension of enzyme treated Avicel PH-101 was cooled in an ice-bath, and washed with ice cold 0.1 *N* NaOH, distilled water, 0.1 *N* HCl and finally with distilled water on a grade 3 sintered glass filter (Maximum pore sixe 20-30 μm, Corning Lte., Stone, England) until the effluent was free from Cl^- tested for with silver nitrate. The residue was then freeze-dried. The control sample was prepared in the same way using distilled water in the place of the enzyme solution.

A conventional transmittance diffraction pattern of the washed sample was obtained on a flat film in a Warhus camera with Ni-filtered copper Kα irradiation. The circular diffraction patterns were scanned across the centre to give a quantitative measurement of the peak height.

Infra-red spectroscopy

Samples treated as described above were used to obtain infra-red spectra. KBr pellets were prepared according to the method of Hurtubise and Kräsig (1960) using 4mg of sample and 200mg of KBr. the spectra were read with a Perkin-Elmer IR Spectrometer Model 257 (Perkin-Elmer Ltd., Beaconsfield, England) against a KBr blank.

RESULTS

Cellulase production

It was found that the cell-free and cellulose-free cellulolytic activity reaches to its maximum at 45 hours after inoculation. The activities were measured as 1.2 units· ml^{-1} on Whatman No.1 filter paper and 15.1 units· ml^{-1} on CMC.

Fractionation of the cellulolytic enzyme system

The cellulolytic activity present in the culture supernatant of *C. flavigena* was precipitated between 20-50% saturated ammonium sulphate, and the precipitate was fractionated by gel-filtration after dissolving in 0.02 *M* phosphate buffer, pH 7.0. Enzyme activity in each fraction was measured using CMC and filter paper as substrates (Figure 1).

A low molecular weight protein was separated from the main peak which appeared to be a mixture of more than one protein. Enzyme activity measurements showed that the main protein peak contained most of the activity on filter paper, but has no clear selective specificity for either of the substrates. For these reasons it was decided to purify the main peak further. The low melecular

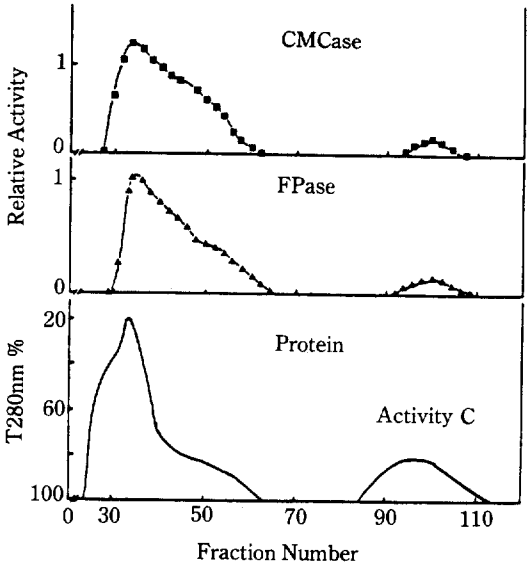


Fig.1. Gel filtration of *C. flavigena* cellulase on Sephadex G-75.
The protein was precipitated from the culture supernatant between 20-50% saturation ammonium sulphate. The precipitate was redissolved in 0.02 *M* phosphate buffer, pH 7.0 and 15ml of the solution was applied to a 2.5 x 40.0cm column packed with Sephadex G-75. The flow rate and the fraction size were 60ml hour^{-1} and 4ml, respectively. The protein was measured by recording the transmittance of the efflunet at 280 nm, and the enzyme activities on Whatman No 1 filter paper (FPase) and on CMC (CMCase) were determined in each fraction.

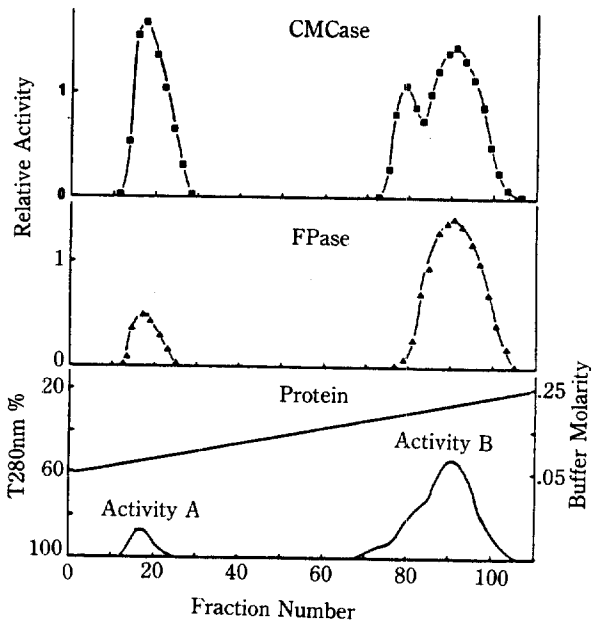


Fig.2. Ion-exchange chromatography of the high molecular weight protein fraction prepared by gel filtration of material from a *C. flavigena* culture supernatant.
The sample obtained by gel filtration was concentrated by ultra filtration and applied to a 2.5 x 30.0cm DEAE Sephadex A-50 column.
The column was eluted by linearly increasing the ionic strength of pH 7.0 phosphate buffer from 0.05 to 0.25 *M*.and 4 ml of effluent were collected in each fraction. The enzyme activity of each fraction was measured using CMC and whatman No 1 filter paper. The first enzyme protein was named A, and the second enzyme B.

weight protein was desigated enzyme C.

Fractions from 30 to 60 from the gel filtration step were pooled and concentrated using an ultrafilter PM-10. Two protein peaks were obtained by ion-exchange chromatography (Figure 2). The first peak was named enzyme A and the second enzyme B. The protein trace showed that enzyme B is mixture of more than one enzyme protein. Enzyme A appeared to have much higher activity on CMC than on filter paper whilst enzyme B showed a relatively higher activity on filter paper.

Substrate activity ratio (SAR) comparing enzyme activities on carboxymethyl cellulose (CMC) with activity on filter paper.

All fractions of each enzyme were pooled, and their activities were measured on CMC, a non-crystalline substrate, and on Whatman No. 1 filter paper which contains a high proportion of crystalline cellulose. From the measured rates, SAR values were calculated by dividing the activity on CMC by the activity on filter paper (Table 1). Enzymes A and C gave much higher values than enzyme B. In other words enzyme A and enzyme C can hydrolyse non-crys talline substrate extremely well, and are probably therefore Cx-type enzymes which cannot attack crystalline cellulose On the other hand enzyme B seems to be a C_1-type enzyme which can attack crystalline cellulose but has relatively little hydrolytic activity. This result indicates that though the purities of the separated enzymes are not high they have sufficiently different activities to warrant further research on their mode of action.

Table 1. SAR[1] values for the separated enzyme.

Enzyme	Ratio
A	234.2
B	8.6
C	36.6
Crude	12.6

1. SAR; Ratio of enzyme activity on CMC to that on Whatman No. 1 filter paper.

Enzymes A and B were separated by ion-exchange chromatography on a DEAE Sephadex A-50 column from the main peak obtained by gel filtration on a Sephadex G-75 column where enzyme C was separated. Activities of each enzyme were measured using both CMC and Whatman No. 1 filter paper as substrates, and the SAR values were calculated from their activities.

Activities of individual and recombined enzymes on the microcrystalline cellulose, Avicel PH-101

The activities of the separated enzymes alone or recombined were measured using microcrystalline cellulose Avicel PH-101. The reducing sugar released by the enzymes was measured after 3 hours incubation at 40 °C and at pH 7.0 (Table 2). Significant synergistic effects were observed between enzyme B and enzyme A, and enzyme B and enzyme C, but not between enzyme A and enzyme C.

Table 2. Activities of separated enzymes on microcrystalline cellulose Avicel pH-101.

Enzyme	Relative Activity (%)
A	8.55
B	20.30
C	nd[1]
A + B	39.50
A + C	8.55
B + C	24.57
A + B + C	48.08
Control[2]	100.00

1. not detected
2. The control value was for material precipitated from the culture supernatant by 20-50% saturated ammonium sulphate.

Enzyme fractions were incubated with Avicel PH-101 in 0.02*M* phosphate buffer, pH 7.0 for 3 hour at 40℃. The reducing sugar in the reaction mixture was measured by Somogyi-Nelson's method.

The activity recovery was 48.1% of the original starting material. The loss is believed to take place during fractionation especially in the ultrafiltration step.

X-ray diffraction patterns of enzyme-treated Avicel PH-101

The undigested substrates from the previous experiment were used for X-ray diffraction analysis (Figures 3 and 4). Much lower crystallinity was observed in the samples treated with enzyme B, enzyme B + C and enzyme A + B than in the control or the sample treated with the complete recombined enzyme A + B + C. No significant changes in the crystallinity were measured in the samples treated with enzyme A or enzyme C.

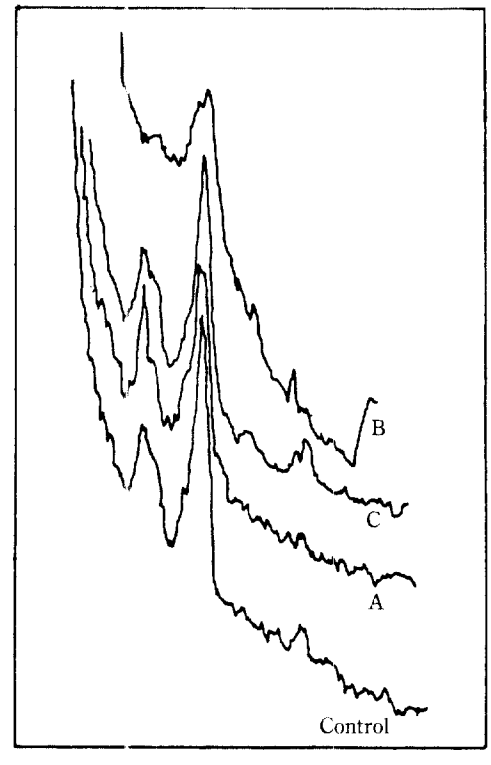


Fig.3. X-ray diffraction patterns of Avicel PH-101 treated with separated cellulolytic enzymes from culture supernatant of *C. flavigena*.
Samles were incubated with the enzyme fractions at 40 °C for 3 hours before washing with 0.1 *N* HCl and water, and freeze-drying. The diffraction patterns were scanned across the centre to give a quantitative measurement of the peak height. The control was prepared in the same way with water in the place of enzyme.

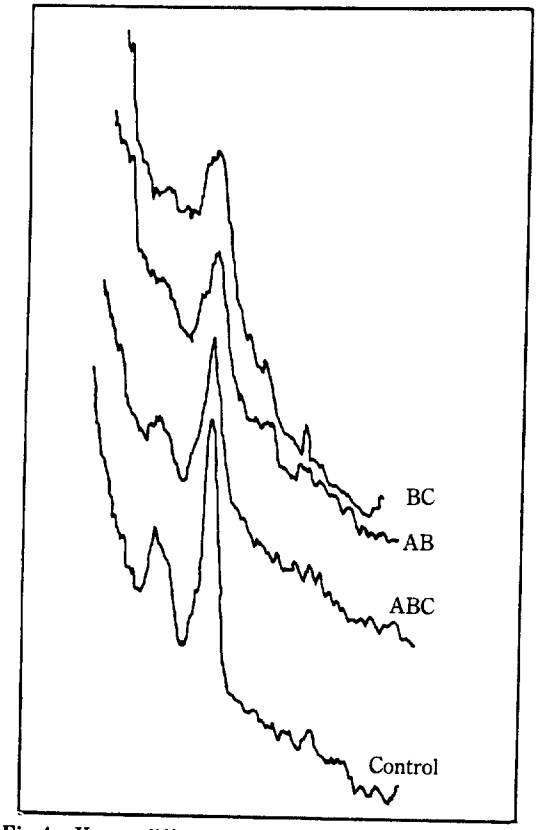


Fig.4. X-ray diffraction patterns of Avicel PH-101 treated with with the recombined enzymes. The experimental details are shown in the caption to Figure.3.

Infra-red spectra of enzyme-treated Avical PH-101

Similar samples to those employed in the previous experiment were used to take infra-red spectra. Some of them are shown in Figures 5. Samples treated with the C_1-type enzyme B alone or in combination with other enzymes including enzyme A + B + C give indication of a new infra-red absorption at a wave number of 800cm^{-1}, otherwise there are no significant differences between the spectra. The band the at the wave number 800cm^{-1} is assigned to ring breathing in an unmodified cellulose (O'Connor 1971). This indicates an unstable glucopyranose ring structure within the cellulose molecule generating an absorption band at a wave number of 800cm^{-1}.

Determination of the crystallinity of enzyme treated Avical PH-101 from IR spectroscopic data

The IR spectra shown above were used to determine the crystallinities of enzyme treated Avicel PH-101 according to the method of Nelson and O'Connor (1974). This technique compares the ratios of the extinctions at different wave numbers with those obtained from standard materials to assess the relative crystallinity (Table 3).

The extinction ratios of the samples at 1429/897cm^{-1} showed that the samples have a cellulose I type of crystal lattice, since the ratio values are greater than any for cellulose II. It was found that the samples treated with enzyme B and enzyme B + C are significantly less crystalline than the control, and that all other samples including the one treated with all recombined enzyme A + B + C are more crystalline than the control.

DISCUSSION

The three fractions produced by gel and ion-exchange chromatography were considered sufficiently pure to allow an investigation of the mode of action of the *Cellulomonas* cellulase system. It was shown that fractions A and C have high substrate activity ratios on CMC compared to filter paper. Fraction B had a much lower value. Using Avicel as substrate fraction B released the most reducing sugar on its own. Fraction A produced 40% of this value whilst C produced virtually none. On recombining these fractions no synergism was observed between A and C but synergistic activities were found in any of the combinations containing fraction B. This result

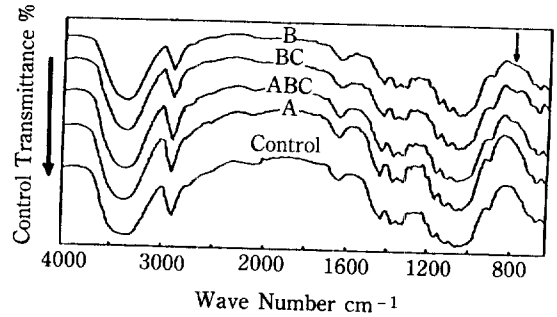


Fig.5. Infra-red spectra of Avicel PH-101 treated with cellulolytic enzyme fractions. Experimental details for sample preparation are shown in the caption to Figure 3. Each spectrum was taken from a KBr pellet containing 4mg of sample and 200mg of KBr.

Table 3. Crystallinity of Avicel PH-101 treated with partially purified cellulolytic enzymes from *C. flavigena*, assessed from infra-red extinction data.

Sample	Infra-red Extinction Ratio E 1429/897cm^{-1}
Standard	
Cellulose I	
Crystalliine	4.30
Amorphous	0.78
Cellulose II	
Crystalline	0.34
Amorphous	0.67
Treated with enzyme fraction	
A	2.50
B	2.12
C	2.42
A+B	2.42
B+C	1.62
A+B+C	2.71
Control	2.37

Crystallinity was measured as described by Nelson and O' Connor (1964), and the values for the standard were taken from the same reference. The spectra shown in Figures 5.

shows that the cellulolytic system of the organism has C_1-activity defined by Reese *et al* (1950).

Infra-red spectroscopy give indications of a new absorption absorption band at 800cm^{-1} in the samples treated with fraction B and any combination of fractions containing B. This has been attributed to ring breathing due to unstable glucopyranose ring structures in the native cellulose (O'Connor, 1971). Infrared extinction ratios at 1429/897cm^{-1} indicate a reduction in crystallinity in Avical treated with fraction B alone compared with either of the other two fractions alone. Of the combinations, B + C show the lowest and A + B + C show the highest crystallinity. This suggests that fractions B + C are together capable of reducing crystallinity but not of completing hydrolysis in non crystalline regions whilst the inclusion of fraction A allows the removel of all the glucose molecules in non-crystalline regions leaving a highly crystalline residue.

These concludions are further substantiated by X-ray diffractions studies, which demonstrate a reduction in crystallinity in Avicel treated with B but not with C or A and in substrate treated with any combination containing B though of the latter the sample treated with B + C shows the least and that with A + B + C the greatest crystallinity.

It is suggested on the basis of all the above evidence that fraction B is a C_1-enzyme and fractions A and C are two different but necessary enzymes of the C_x variety. These activities operate synergistically to cause the total degradation of cellulose as follows:

$$\text{CRYSTALLINE CELLULOSE} \xrightarrow[\text{Generation of unstable glycopyranose ring}]{C_1\ (\text{Fraction B})} \text{REACTIVE CELLULOSE} \xrightarrow[\text{Hydrolysis}]{C_x\ (\text{Fraction A,C})} \text{REACTIVE SOLUBLE}$$

Since partially purified enzymes were used in this study it is difficult to decide whether the C_1-type enzyme produces reducing sugar as proposed by several workers (Eriksson and Pettersson 1975, Pettersson 1975, Wood 1972) or not (Mandels and Reese 1964, Selby and Maitland 1967, Reese 1975).

Infra-red spectroscopic studies showed that the enzyme treated samples do not produce any band at a wave number of 1750 cm^{-1} where C=O stretching gives a band (O'Connor 1971). This suggests that oxidation of the substrate does not take place to a significant degree or at all over the course of the enzyme reaction.

요 약

Cellulomonas flavigena KIST 321이 생산하는 균체외 cellulose분해효소를 gel filtration법과 ion-exchange chromatography법으로 3종의 다른 효소를 분리하여 이들을 A, B, 및 C효소로 명명하였다. 분리된 각 효소를 결정성 기질에 처리하여 일어나는 기질의 구조변화를 적외선 분광법과 X-ray crystallography법으로 측정하여 다음

결론을 얻었다.

B효소는 결정성 cellulose의 구성단위인 glucopyranose의 불안정화로 그 결정도를 감소시키는 C_1-형의 효소이며 A 및 C효소는 C_x형의 효소로 B효소의 반응생성물에 작용하여 glucose를 생산하였다. 이들 각 효소의 작용에서 본균의 cellulase의 작용기작을 고찰하였다.

Acknowledgements

The authors wish to thank the Korea Institute of Science and Technology for supplying the bacterial strain. BHK gratefully acknowledges the receipt of a grant from the British Council.

REFERENCES

1. Bae, M., B.H. Kim, 1974. Isolation and identification of cellulose utilizing bacteria. *Korean J. Appl. Microbiol. Bioeng.* **2:**1-7
2. Chanzy, H., B. Henrissat, R. Vuong and M. Schulein, 1983. The action of 1.4-¼-glucan cellobiohydrolase on *Valonia* cellulose microcrystals. An electron microscopic study *FEBS Lett.*, **153,** 113-118.
3. Creuzet, N., J.F. Berenger and C. Erixon, 1983. Characterization of exoglucanase and synergistic hydrolysis of cellulose in *Clostridium stercoratium, FEMS Microbiol. Lett.*, **20,** 347-350.
4. Eriksson, K.R., B. Pettersson, 1975. Extracellular enzyme system utilised by the fungus *Sporotrichum pulverulentum* for the breakdown of cellulose. Part 3: Purification and physico-chemical caracterisation of an exo-1, 4-¼-glucanase. *Eur. J. Biochem.*, **51,** 213-218.
5. Eriksson, K.E., B. Pettersson and U. Westermark, 1974. Oxidation: An important enzyme reaction in fungal degradation of cellulose. *FEBS Ltee.* **49,** 282-285.
6. Evans, C.G., D. Herbert and D. Tempest, 1970. The continuous cultivation of microorganisms. 2. Construction of chemostat. In: *Methods in Microbiology* Vol 2 (J.R. Norris, D.W. Ribbon, eds). pp 177-327. London: Academic Press.
7. Hodge, J.E. and B.T. Hofreiter, 1962. Determination of reducing sugars and carbohydrates. In: *Methods in Carbohydrate Chemistry* Vol 1 (R.L. Whistler, M.L. Wolfrom, eds), pp 380-394. New York: Academic Press.
8. Hurtubise, F.G. and H. Krasig, 1960. Classification of fine structural characteristics in cellulose by infra-red spectroscopy. *Anal. Chem.* **32,** 177-181.
9. Mandels, M. and E.T. Reese, 1964. Fungal cellulase and the microbial decomposition of cellulosic fabric. *Develop. Indst. Microbiol.* **5,** 5-2.
10. Mandels, M. and J. Wever, 1969. The production of cellulases. In: Cellulases and their application, *Advanced in Clemistry Series,* **95,** (R.F. Gould, ed) pp 391-414. Washington, D.C.: American Chemical Society.
11. Nelson, M.L. and R.T. O'Connor, 1964. Relation of certain IR bands to cellulose crystallinity and crystal lattice type. *J. Appl. Polym. Sci.*, **8,** 1325-1341.
12. Nakamura, K, and K. Kitamura, 1983. Purification and some properties of a cellulase active on strystalline cellulose from *Cellulomonas uda, J. Ferment. Technol.*, **61,** 379-382.
13. Nummi, M., M.L. Mike-Raavola, A. Lappalaien, T.M. Enari and V. Raunio. 1983. Cellobiohydrolase from *Trichoderma reesei, Biochem. J.*, **215** 677-683.
14. O'Connor, R.T., 1971. Infra-red spectroscopy: Analysis of chemically modified cotton. In :*Cellulose and cellulose derivatives,* part IV (N.M. Bikales, L. Segal, eds) pp 51-87. New York: Wiley-Interscience.
15. Pettersson, L.G., 1975. The mechanism of enzymic hydrolysis of cellulose by *T. viride.* In: *Symposium on enzymic hydrolysis of cellulose* (M. Bailey, T.M., Enari, M. Linko, eds) pp 255-262. Helsinki: Technical Research Center of Finland.
16. Reese, E.T., 1975. Polysaccharides and the hydrolysis of insoluble substrates.

In:*Biological transformation of wood by microorganisms* (W. Liese, ed) pp 156-181. Berlin. Spring-Verlag.

17. Reese, E.T., R.G.H. Siu and H.S. Levison, 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *J. Bacteriol.* **59,** 485-497.
18. Selby, K. and C.C. Maitland, 1967. The cellulase of *T. viride.* Separation of the components. *Biochem. J.,* **104,** 716-724.
19. Wood, T.M., 1968. Cellulolytic enzyme system of *Trichoderma koningi. Biochem. J.*. **109,** 217-227.
20. Wood, T.M., 1972. The C_1 component of the cellulase complex. In: *Fermentation technology today* (G. Terui, ed) pp 711-718, Osaka, Japan, Society for fermentation technology.

(Received, February 15, 1985)