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Partial Purification and some Properties of Cellulase Components from *Trichoderma koningii*

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Trichoderma koningii 로 부터 추출한 섬유소분해효소의 부분정제 및 그의 효소학적 성질

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ABSTRACT

Cellulase components, CMCase(Cx) and Avicelase(C_1), were partially purified from the culture extract of a strain of *Trichoderma koningii* by column chromatography on DEAE-Sephadex A-50 and the step of gel filtration through Sephadex G-150. Optimum *p*H of CMCase was 5.2 and Avicelase showed the highest activity at *p*H 5.6 in acetate buffer. Optimal temperatures for activities of CMCase and Avicelase were 50°C and 60°C, respectively. More than 70% of the activities of two enzymes were remained after heating at 60°C for 30 min and Avicelase is more stable than CMCase. This result shows that both of cellulase components are more stable to heat than any other fungal enzymes. The Michaelis constants, Km, of CMCase and Avicelase were 0.116% of CMC and 0.281% of avicel. And also the values of maximum velocity, Vmax, of CMCase and Avicelase were 23.20μg and 2.54μg of reducing sugar per min. Of the metal ions tested against the activities of CMCase and Avicelase, Cu^{++}, Hg^{++}, and Pb^{++} are remarkably effective inhibitors. The molecular weights of Cx and C_1 component were estimated to be about 47,000 and 61,000 by gel filtration method.

INTRODUCTION

Since the proposition of Reese *et al* (1950) for the presence of enzyme Cx and C_1 in cellulase components, that were different in their substrate specificities toward cotton fiber, a number of investigators have examined multiplicity of this enzyme in some microorganisms such as *Trichoderma viride* (Shikata *et al* 1975, Berghem *et al* 1974, 1973, Selby *et al* 1966, Li *et al* 1965), *T. koningii* (Wood *et al* 1968, 1972, Halliwell *et al* 1969, 1973), and *Thermomonospora curvata* (Stutzenberger 1972).

From the results of the fractionation studies of the culture filtrates prepared from these fungi and bacteria, it was found that cellulase contained three enzymes or classes of enzymes (Li *et al* 1965, Wood *et al* 1968, Selby *et al* 1966) which are essential for the extensive

degradation of cellulosic substrates ranging from cotton fibers to cellooligosaccharides. Two of these types of enzymes have been called C_1 and Cx and the third is a cellobiase or β-glucosidase.

According to the concept of C_1-Cx cellulase component originally proposed by Reese *et al* (1950), C_1 can attack only native cellulose of higher crystallinity and gives various fragments without further degradation, and Cx can attak only these fragments to produce reducing cellooligosaccharides including cellobiose.

On the other hand, Nisizawa *et al* (1972) and Tomita *et al* (1974) reported that C_1 and Cx components might correspond to Avicelase and CMCase and assumed that CMCase first attacks cellulose chains, mainly at amorphous regions, to produce cellulose fragments which will also serve as substrates for Avicelase. This theory strongly suggests that the C_1-Cx concept of Reese *et al*(1950) must be reconsidered in respect of precise substrate specificity.

It has often been reported that Avicelase is a true exo-enzyme (exo-glucanase) on the basis of its high production of cellobiose from crystalline cellulose (Berghem *et al* 1973, Halliwell *et al* 1973). In contrast, it was assumed by others (Tomita *et al* 1974) that Avicelase is a kind of endoglucanase with low randomness, because a small amount of glucose is always produced as well as cellobiose from any substrates.

In addition, in spite of CMCase (Cx) known as endoglucanase, a novel enzyme which has exo-CMCase property was reported recently (Shikata *et al* 1975). In this respect, the modes of action and properties of cellulase components need further investigations.

In previous paper, the isolation of cellulolytic microorganisms and induction of cellulase components were reported by the authors (Hong *et al* 1976). In this paper, the partial purification and properties of each cellulase components, CMCase and Avicelase, from *Trichoderma koningii* are investigated.

MATERIALS AND METHODS

Crude cellulase prepartation

In a previous paper, we reported *Trichoderma koningii* cellulase which was cultured in wheat bran containing 70-80% of moisture (v/v) for 6 days at 28°C. Wheat bran culture of *T. koningii* was extracted with 0.02M acetate buffer at *p*H5.4 and filtered. The filtrate was centrifuged at 10,000rpm for 20 min. the supernatant was used in this experiment as a starting cellulase preparation.

Substrates

Sodium-carboxymethyl-cellulose, CMC (ds 0.63), was supplied by Daiichi Kogyo Seiyaku Co., LTD. Avicel was a commercial product of microcrystalline cellulose powder from Hunakoshi pharmaceutical LTD.

Enzyme assays

CMCase (Cx) or Avicelase (C_1) activity was measured in the reaction mixture of 0.4ml of 1% CMC or Avicel, respectively, 0.4ml of 0.02M acetate buffer at *p*H 5.4 and 0.2ml enzyme solution. After the reaction mixture was incubated at 40°C for 30min, the activities of CMCase and Avicelase were analyzed according to the method of Somogyi-

Nelson(1944).

One unit is the activity which produces reducing power equivalent to 1.0 μg of glucose from CMC and Avicel in 30 min under the standard reaction condition.

Partial purification of cellulase components

1. *Fractionation with ammonium sulfate*

For the purification of cellulase components, fractionation of crude cellulase preparation was carried out by salting out with ammonium sulfate (20-80%). The supernatant, which had no cellulase activity, was removed by centrifugation at 7,000rpm for 20 min. After centrifugation, the precipitated was dissolved in a small amount of 0.02M acetate buffer. The solution was dialyzed against 0.02M acetate buffer at 5°C for 2 days.

2. *Column chromatography on DEAE-Sephadex A-50*

After dialysis against 0.02M acetate buffer at *p*H 5.4, 20ml of enzyme solution was applied on a DEAE-Sephadex A-50 column (2x22cm) equilibrated with 0.02M acetate buffer at *p*H 5.4. A linear gradient elution was carried out with the 0.02-0.5M of acetate buffer and 5ml eluates were collected.

3. *Rechromatography on sephadex G-150*

Enzyme preparation of Avicelase and CMCase obtained on DEAE-Sephadex A-50 was eluted on Sephadex G-150 column (2x70cm) equilibrated with 0.02 M acetate buffer, *p*H 5.4 and 5ml eluates were collected.

Determination of protein

Protein concentration was measured by the method of Lowry *et al* (1951), with bovine serum albumin as standard.

Determination of molecular weight

Following the method of Andrew (1963), molecular weights of CMCase and Avicelase were estimated by gel filtration on a Sephadex G-150 column (2x70cm), which was equilibrated with 0.02M acetate buffer at *p*H 5.4. The column was eluted with the same buffer in a cold room and 5ml eluates were collected.

RESULTS

Partial purification of cellulase components

1. *Ion-exchange column chromatography*

Twenty milliliter of the dialyzed enzyme preparation, which was fractionated with ammonium sulfate, was applied to a colum (2x22cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. Upon a linear gradient elution, as shown in Fig. 1, each 5 ml aliquot of eluent was collected in one of the tubes of a fraction collector.

Two major peaks, designated as CMCase and Avicelase, were obtained with regard to protein. However, only one of them which covered most fractions showed a strong CMCase activity containing a small two peaks of Avicelase, whereas the other, Avicelase, was practically free of the former CMCase but it was also overlapped by small amount of CMCase at the fraction numbers 100 to 120. Therefore, the corresponding fractions were pooled to obtain CMCase (fraction number's 8-38) and Avicelase (fraction number's 75-100) and they were performed rechromatography on Sephadex G-150 with the same buffer.

2. *Sephadex G-150 column chromatography*

Further purification of Avicelase on gel filtration was carried out on the

same condition. A 5ml of the enzyme solution of Avicelase, which was obtained by combining fraction number's 75 to 100 on DEAE-Sephadex A-50 column, was applied on Sephadex G-150 column(2x70cm).

Avicelase was appreciably purified through gel filtration. As shown in Fig. 2, most of cellulolytic activities of prot-

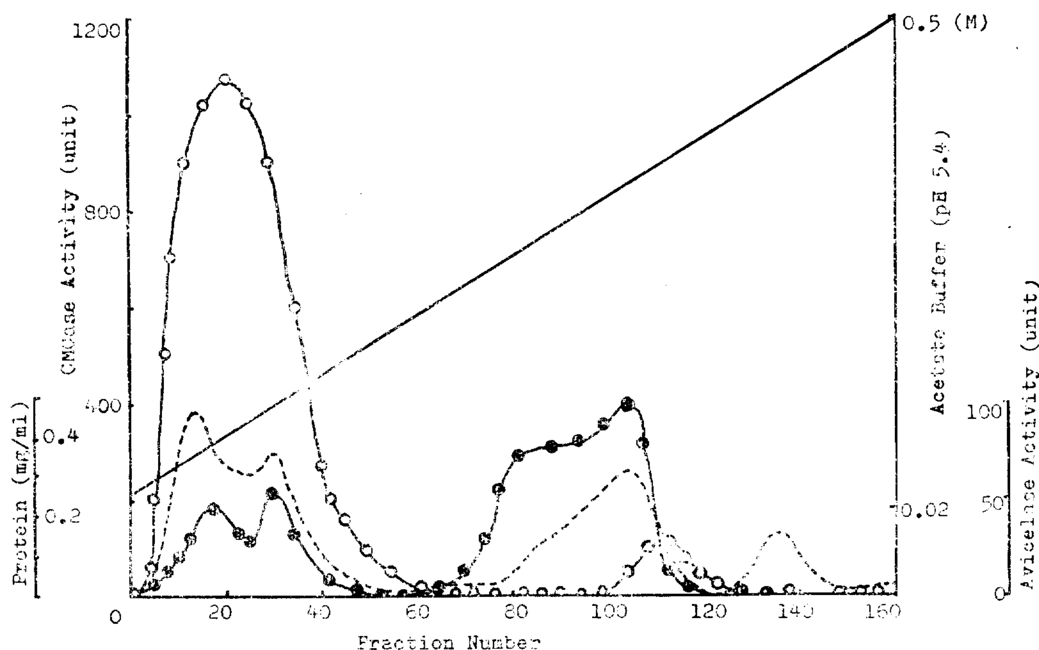

Fig. 1. Partial purification of CMCase and Avicelase on column chromatography of DEAE-Sephadex A-50. The column was eluted with a linear gradient of 0.02M to 0.5M acetate buffer at *p*H 5.4. Fraction volume was 5ml. Assays were carried out as stated in the text. •——• ; CMCase activity, ○——○; Avicelase activity, ...; protein, ——; acetate buffer (*p*H 5.4)

Table 1. Partial purification of CMCase and Avicelase by ammonium sulfate and column chromatography on DEAE-Sephadex A-50 and Sephadex G-150.

Enzyme preparation	Total protein (mg)	CMCase		Avicelase	
		Total units	Specific activity (units/mg)	Total units	Specific activity (units/mg)
20-80% sat. with					
$(NH_4)_2SO_4$	40.50	44860	1108	1436	35
Dialysis	17.01	41400	2434	1277	76
DEAE-Sephadex A-50					
CMCase(8-38)*	4.19	22500	5372		
Avicelase(75-100)*	2.92			479	164
Sephadex G-150					
CMCase(13-21)*	3.60	19710	5475		
Avicelase(10-22)*	1.00			325	325

* Corresponding fraction numbers of each cellulase component, CMCase and Avicelase.

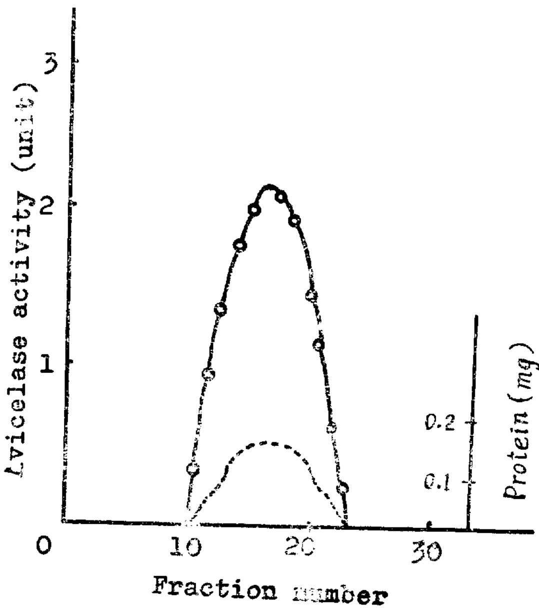

Fig. 2. Rechromatography of Avicelase on Sephadex G-150 column (2x70cm). Five milliliters of Avicelase enzyme solution obtained from DEAE-Sephadex A-50 column chromatography was applied to the column. Elution was performed with 0.02M acetate buffer at *p*H 5.4. ○——○; Avicelase activity, ……; protein.

ein component showed an approximately symmetrical bell-shaped curve.

Since all these fractions were colorless, the recovery of protein was also very high in this case. DEAE-Sepahdex A-50 column chromatography appears to be greatly useful for the decolorization of a partial purified cellulase components. The specific activities of CMCase and Avicelase in this case are summarized together with each step of the purification in Table 1. Final purification on Sephadex G-150 column chromatography shows 9.3 fold purification for Avicelase and 4.92 fold for CMCase comparing with the enzyme preparation of 20-80% saturated with ammonium sulfate.

According to the procedure described above, cellulase components, CMCase and Avicelase which were partially purified, are denoted CMCase (Cx) and Avicelase (C_1) and these two components were used in this experiment.

Effect of *p*H on cellulase activities

The *p*H dependence of each cellulase components, CMCase and Avicelase, was examined by measuring the reducing sugar formed at various *p*H values. The incubation was carried out at 40°C for 30 min on CMCase and Avicelase. As shown in Fig. 3, CMCase showed the highest activity at *p*H 5.2 and *p*H 5.6 was found to be the optimal *p*H's for Avicelase in acetate buffer.

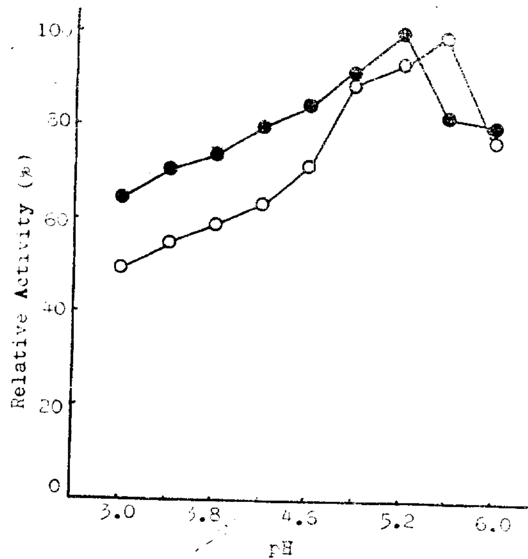

Fig. 3. Effect of *p*H on the activities of CMCase and Avicelase. ●——●; CMCase, ○——○; Avicelase

Effect of temperature on cellulase activities

The reaction mixtures were incubated with each temperature rising up every 10°C from 10°C to 70°C. The result of

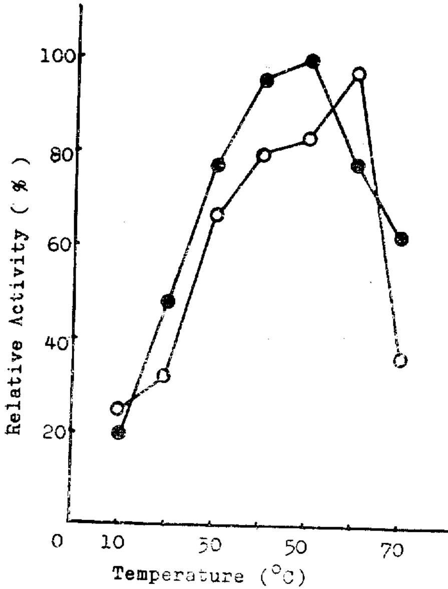

Fig. 4. Effect of temperature on the activities of CMCase and Avicelase. •——• ; CMCase, ○——○; Avicelase.

optimum temperatures for CMCase and Avicelase are shown in Fig. 4. The optimum temperature of CMCase was found to be at 50°C, while at 60°C in that of Avicelase.

Thermal stability

In order to study the thermal stability of cellulase components, CMCase or Avicelase was incubated at various temperatures for 30min in 0.02M acetate buffer at *p*H 5.4, in the absence of substrate. After preincubation and then adjusted to 40°C by cooling or warming, the residual enzyme activities were measured at 40°C in the same reaction mixture for 30 min on CMCase and Avicelase.

Fig. 5 shows the thermal stabilities of CMCase and Avicelase.

More than 70% of the activities of two enzymes were remained after heating at 60°C for 30 min and Avicelase is more

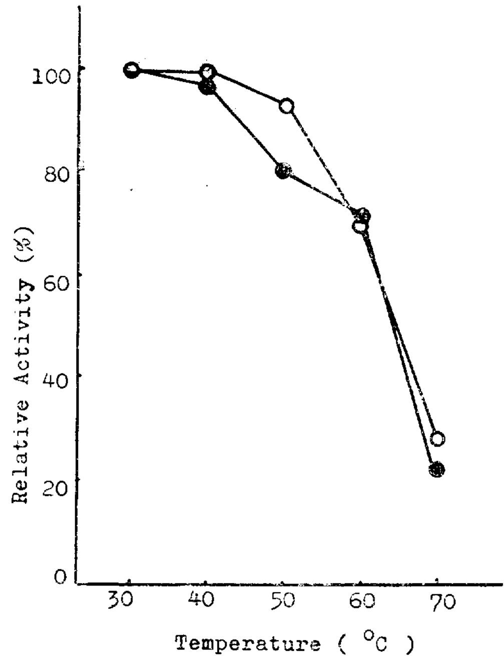

Fig. 5. Thermalstability of CMCase and Avicelase. •——• ; CMCase, ○——○ Avicelase.

stable than CMCase.

Estimation of Km and Vmax values of CMCase and Avicelase

In order to determine Michaelis constants, Km and Vmax values of CMCase and Avicelase, the degradtion extents of each substrate at stepwise increasing concentrations were examined with same enzyme amount and reaction time. Lineweaver-Burk plots of these values are shown in Figs. 6 and 7 and the values of Km and Vmax were derived from double reciprocal plots.

The Km values of CMCase or Avicelase was 0.116% (1.16mg/m*l*) of CMC or 0.281% (2.81mg/m*l*) of Avicel, respectively. Vmax values of CMCase and Avicelase were 23.2μg and 2.54μg of reducing sugar production per min.

The Km values possibly indicate the affinity of enzyme to their substrate. Therefore CMCase can attack preferably to CMC than Avicelase to Avicel.

Effect of metal ions on the activities of cellulase componts

The kinds and concentration of metal ions used in this experiment are listed in Table 2.

The effects of metal ions on the

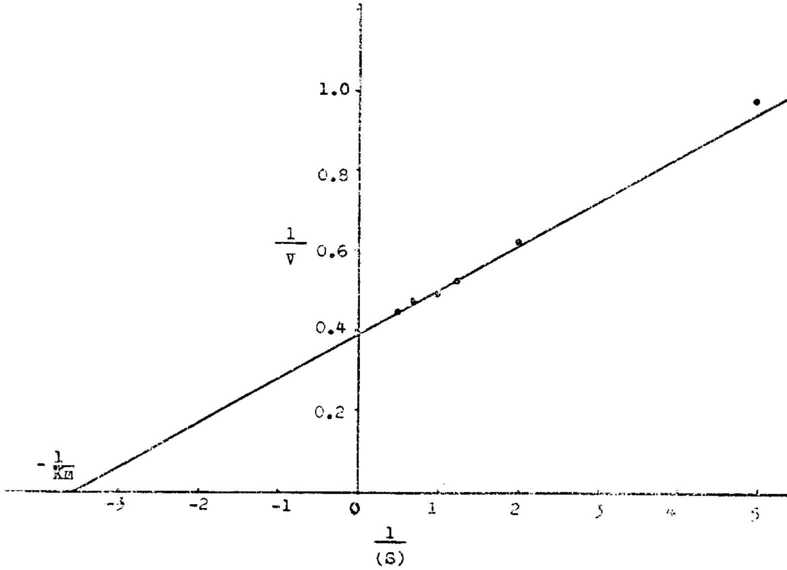

Fig. 6. Lineweaver-Burk plot for hydrolysis of CMC by CMCase(Cx). The reaction mixtures consisted of 0.4ml of various concentrations of CMC and 0.2ml of CMCase containing 0.02M acetate buffer at *p*H 5.4. (S); concentrations of CMC(%), V; velocities(μg of reducing sugar produced per min)

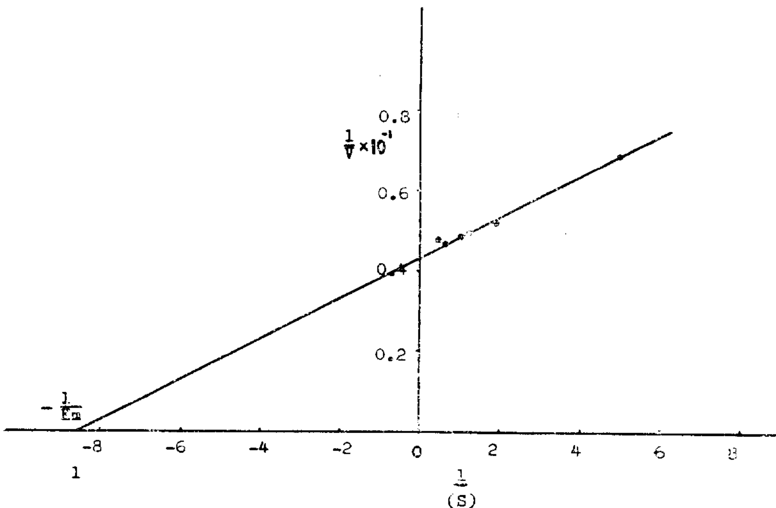

Fig. 7. Lineweaver-Burk plot for hydrolysis of Avicel by Avicelase(C_1). The reaction mixtures consisted of 0.4ml of various concentrations of Avicel and 0.2ml of Avicelase enzyme solution containing 0.02M acetate buffer at *p*H 5.4. All abbreviations are the same as in Fig. 6.

Table 2. Effects of additional various substances on CMCase and Avicelase activities.

Substances	Relative activities of cellulases (%)			
	CMCase		Avicelase	
	10^{-2}M	10^{-3}M	10^{-2}M	10^{-3}M
None	100	100	100	100
$AgNo_3$	62.0	71.4	70.8	80.2
$HgCl_2$	13.0	62.0	30.7	90.9
$ZnCl_2$	72.8	95.0	65.3	95.0
$CaCl_2$	42.8	54.0	92.3	96.1
KCl	47.1	67.4	62.3	80.8
$MgSO_4$	21.4	34.0	57.7	70.8
$CuSO_4$	11.0	32.0	60.2	76.9
$MnCl_2$	28.5	68.5	69.2	76.2
$CoCl_2$	86.0	86.0	104.0	98.0
$Pb(CH_3COO)_2 \cdot 3H_2O$	32.1	41.4	44.6	90.8

activities of cellulase components were expressed by relative activities compared with nontreated enzyme activities. As shown in Table 2, all metal ions with the exception of Cobalt ion for Avicelase showed the inhibition on the activities of cellulase components, especially the inhibition of Cu^{++}, Pb^{++}, Hg^{++} and Mn^{++} were remarkably effective. Most of metal ions did not show the role of activator for the activities of CMCase and Avicelase components.

Determination of molecular weights

Approximate molecular weights of CMCase and Avicelase were estimated by being subjected to gel filtration on Sephadex G-150 column and their elution pattern is shown in Fig. 8. Using several markers of proteins such as bovine serum albumin(67,000), pepsin(35,000), and trypsin (25,000), the molecular weights of partially purified CMCase, Cx component, and Avicelase, C_1 component, were 47,000 and 61,000, respectively.

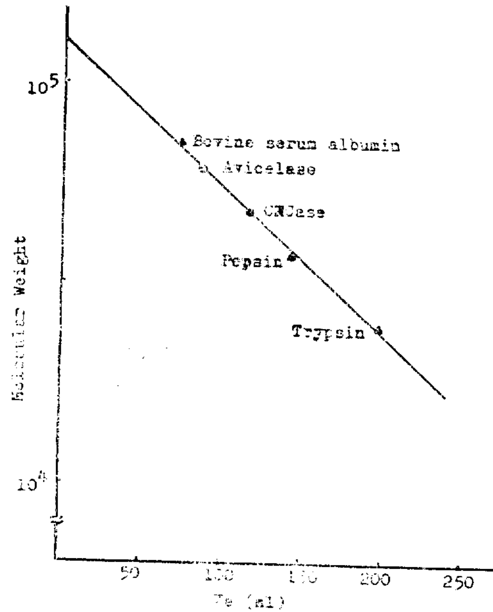

Fig. 8. Molecular weights of C_1 and Cx cellulase components on Sephdex G-150 column chromatography. Bovine serum albumin(67,000), pepsin (35,000) and trypsin (25,000) were used as standard proteins.

DISCUSSION

Although many evidences for the existence of the multiple forms of cellulolytic enzymes in particular microorganisms have been carried out by a number of investigators, the results were not coincident with each other. In our experiments, CMCase and Avicelase, which were obtained from *T. koningii* by ammonium sulfate fraction, were partially purified with column chromatography through DEAE-Sephadex A-50 and Sephadex G-150 as shown in Figs. 1 and 2. Among 3 peaks of Avicelase obtained from ion exchange chromatography, main peak was separated from CMCase and only 2 smaller peaks were associated with CMCase. And also two peaks of CMCase were obtained from the Fig. 1. The main peak was found

in fraction number's 8 to 34, the other peak is overlapped at the end of elution pattern of Avicelase. According to this result, it is assumed that cellulase components were multiple enzyme as previously reported by many investigators.

With the stepwise purification. further partial purification of CMCase and Avicelase was performed approximately 4.9 fold and 9.3 fold according to determination of the specific activities comparing with the crude enzyme preparation of 20-80% saturated ammonium sulfate. Avicelase is purified considerably on gel filtration through Sephadex G-150 column.

The effects of *p*H on the activities of cellulase components were studied. As compared with the results of Halliwell *et al* (1973) for C_1 components and those of Wood(1968) for Cx component, the good agreement could be obtained in this experiment.

The effects of temperature on the activities and thermal stabilities of cellulase components were also observed.

As shown in Fig.5, both of cellulase components were more stable than any other fungal enzymes when they were preincubated for 30 min at various temperatures.

The Km values of Avicelase was obtained to be 0.28% of Avicel, but this result is far from that of Halliwell *et al* (1973); 0.05%.

The effects of metal ions on the activities of cellulase components were studied as shown in Table 2. Most of metal ions were inhibitors on the activities of cellulase components. In addition to the Cu^{++} ions reported previously Halliwell *et al*(1973), Mg^{++}, Hg^{++}, Pb^{++} ions were effective inhibitors. From the results of Table 2, it could be found that the inhibition of metal ions were more remarkable on the activity of CMCase than that of Avicelase. Therefore it was also assumed that the property of CMCase was different from that of Avicelase.

Molecular weights of Cx and C_1 components were estimated as 47,000 and 61,000 in this experiment, but Berghem *et al* (1973) showed 46,000 molecular weight of C_1 component of *T. viride*. Molecular weght of Cx from *Fusarium solani* was reported as 37,000 by Wood (1971) and that of exo-CMCase of *T.viride* as 56,000 by Shikata *et al* (1975). Nisizawa *et al* (1972) reported the molecular weights of C_1 and Cx as 53,000 and 44,000 from *T. viride*. From the above results it is assumed that molecular weights of cellulase components are different in each species of microorganisms.

Further studies on the purification of cellulase components of *T. koningii* are to be carried out and it is also interesting to investigate whether the concept of C_1-Cx cellulases can be proved on the level of isoenzymes.

적 요

Trichoderma koningii 로 부터 추출한 섬유소 분해효소에서 DEAE-Sephadex A-50에서의 chromatography 와 Sephadex G-150에서의 rechromatography 를 통하여 부분정제된 CMCase 와 Avicelase 를 얻었다. 이 부분정제된 CMCase 와 Avicelase 의 물리화학적 성질과 kinetics 를 상호 비교한 결과 다음과 같은 사실을 얻었다.

1) CMCase의 최적 *p*H 및 온도는 *p*H 5.2와 50°C로 나타난 반면에 Avicelase의 경우 *p*H 5.6과 60°C에서 최고의 활성도를 보였다.
2) CMCase와 Avicelase 모두 열에 대한 내성이 다른 enzymes보다 훨씬 높음을 보여 주었는데 60°C에서 30분간 방치한 후의 경우 70% 이상의 활성을 계속 함유하고 있었다.
3) CMCase와 Avicelase의 Michaelis constant, Km과 최대반응속도, Vmax를 구한 결과 CMCase의 기질인 CMC에 대한 Km은 0.116%이며 Vmax는 23.2μg의 환원당/분(min)로 나타난 반면 Avicelase의 Avicel에 대한 Km은 0.281%이고 Vmax는 2.54μg의 환원당/분(min)으로 나타났다.
4) 금속이온이 CMCase와 Avicelase에 미치는 영향을 살펴본 결과 Cu^{2+}, Hg^{2+}, Pb^{2+}, Mn^{2+}, 이온이 두드러진 억제물임을 보였고 CMCase가 Avicelase보다 금속이온에 더 영향을 받는 반면 두 components 모두 activator로서 금속이온을 필요로 하지 않는다.
5) CMCase의 분자량은 47,000이고 Avicelase의 분자량은 61,000임을 보였다.

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