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Enzymatic Properties of a Cellulase from *Ganoderma lucidum*

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不老草가 生産하는 Cellulase의 酵素學的 性質

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Abstract: A cellulose-degrading enzyme from *Ganoderma lucidum* was partially purified by ammonium sulfate precipitation and its enzymatic properties were studied. The enzyme had an optimum pH for activity at 4.0, and its stability range was pH 4.0~7.0. The optimum temperature was 55°C and the enzyme retained 80% original activity after heated at 50°C for 60 min. The activation energy of the enzyme for CMC degradation was calculated and found to be 6.2 Kcal/mole. The enzyme was activated by the addition of Co^{++}, Mn^{++}, but slightly inactivated by Hg^{++}. Various enzyme inhibitors and chemical reagents did not affect the enzyme activity. The enzyme acted on native cellulose as well as CMC. The Michaelis constant for CMC was calculated to be 2.4 mg glucose eq/ml.

Keywords: Cellulose degrading enzyme, *Ganoderma lucidum*, *Basidiomycetes*, Michaelis constant.

Cellulose is the most abundant organic substance occurring in nature, many industrial and agricultural wastes such as rice straw, bagasse, corn cobs and pineapple cobs. The biological degradation of cellulose has been of paramount importance in the activities of the living system.

Cellulase is one of the most important enzymes as a potential means of obtaining energy, chemicals and single-cell protein from cellulose. Since Reese *et al.* proposed C_1-C_x concept, the mechanism and kinetic study of cellulose degradation of the fungus *Trichoderma* have been extensively investigated (Berghem *et al.*, 1976; Maguire, 1977; Mandels, 1981). Many studies on cellulolytic enzymes have been reported (Ikeda *et al.*, 1973; Robson *et al.*, 1984; Okada, 1985; MacKenzie *et al.*, 1985; Lee *et al.*, 1985). Cellulolytic enzymes from species of the *Thermomonospora* (Stutzenberger, 1979), *Thermoactinomycetes* (Hägerdal *et al.*, 1978), *Bacteroides* (Saddler *et al.*, 1979) and *Cellulomonas* (Stoppok *et al.*, 1982) are capable of hydrolyzing crystalline forms of cellulose. And also many reports have appeared on *Trichoderma reesei* mutants capable of hyperproduction of cellulase (Kawamori *et al.*, 1985; Morikawa *et al.*, 1985).

Kawai (1973) reported the study on the productivity and distribution of various carbohydrolases produced by various *Basidiomycetes*, by this result C_2 enzyme activity produced by *Ganoderma* sp. was very low, but C_x enzyme showed very high activity. But there were few studies on cellulose degrading enzyme from *Ganoderma* species. *Ganoderma lucidum* is a medicinal *Basidiomycete* which can produce a fruit body in its life cycle.

We found that a strain of *Ganoderma lucidum* accumulates a large amount of cellulolytic enzyme

in the medium. This paper describes the properties of the extracellular cellulolytic enzyme obtained from the solid culture of *Ganoderma lucidum*.

Materials and Methods

Microorganism and Culture Condition

Ganoderma lucidum was provided by Chungang Agriculture Enterprise Co., and the strain was maintained on potato-dextrose agar medium. The medium for cellulase production was sawdust solid medium (Table I) and the cultivation was carried out in 1000 ml flask containing 700 g of the medium at 26°C for 20 days.

Table I. Composition of media for stock culture and enzyme production.

Medium for stock culture	
Potato dextrose agar (Difco) pH	5.2
Medium for enzyme production	
Sawdust of oak	70%
Sawdust of poplar	10%
Rice bran	20%
$CaCO_3$	0.5%
Tap water	67 ml

Chemicals

Carboxymethyl cellulose disodium salt, α-cellulose, CM cellulose and 3, 5-dinitrosalicylic acid were purchased from Sigma Chemical Company St. Louis, Mo, U.S.A. Avicel was obtained from Wako Pure Chemical Industries, Ltd, Japan and all other chemicals used were of analytical-reagent grade.

Preparation of Enzyme Solution

After cultured medium was kneaded with same volume of McIlvaine buffer (pH 5.0), the medium was stored at 4°C for 15 hours. And then it was centrifugated at 4°C for 30 minutes. The supernatant solution was saturated with ammonium sulfate, subsequently stored at 4°C for 18 hours. The precipitate was collected by centrifugation (5,000 rpm, 30 min,) and dissolved in McIlvaine buffer solution (pH 5.0), then dialyzed against the same buffer solution at 4°C for 24 hours. After filtration, it was diluted suitably and used as crude enzyme solution.

Assay of Cellulase Activity

Cellulase activity was determined by measuring reducing sugar released from carboxymethyl cellulose disodium salt (CMC-2Na). A 0.5 ml of McIlvaine buffer solution (pH 5.0), 0.4 ml of 1.0% CMC-2Na dissolved in same buffer solution and 0.1 ml of enzyme solution were incubated at 40°C for 60 minutes. The reducing sugar released was measured as glucose by DNS method of Colowick *et al.* (1955). One unit of cellulolytic activity was defined as 1 μmole of glucose released/ml/min using CMC-2Na as substrate.

Activation Energy

The effect of temperature on the initial velocity of CMC-2Na hydrolysis was examined over the range of 22°C to 50°C with 10 degree increments, and was plotted in the conventional Arrhenius manner (log k versus 1/T, wherein k is the initial rate of hydrolysis and T is the absolute temperature). Activation energy (Ea) for the enzyme was calculated from the equation $2.303 \log k_1/k_2 = Ea/R\ (1/T_2 - 1/T_1)$.

Km Value

The affinity of CMC-2Na to the enzyme was determined by the enzymatic hydrolysis of CMC-2Na. Initial velocity of substrate hydrolysis were plotted against the various concentrations in Lineweaver-Burk plot (Lineweaver and Burk, 1934) to obtain apparent Km value.

Results and Discussion

Effect of pH on Enzyme Activity

The effect of pH on the hydrolysis of CMC-2Na was measured with the use of McIlvaine

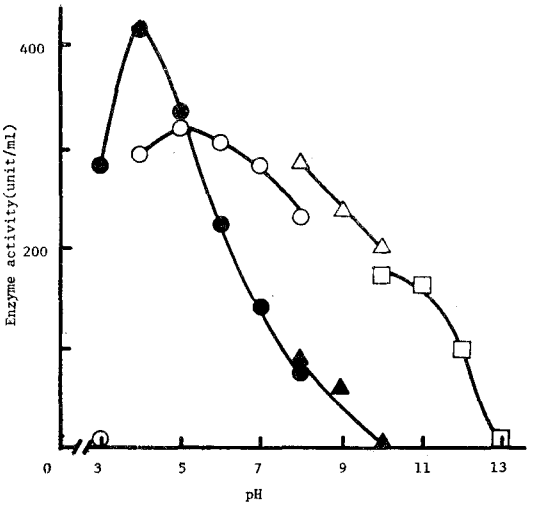

Fig. 1. Effect of pH on enzyme activity. Enzyme activity(●, ▲) was determined at various pH value, and remaining activity(○, △, □) was measured after treatment at 40°C for 1hr at various pH value. McIlvaine buffer(pH 3~8), Clark and Lubs buffer (pH 8~13).

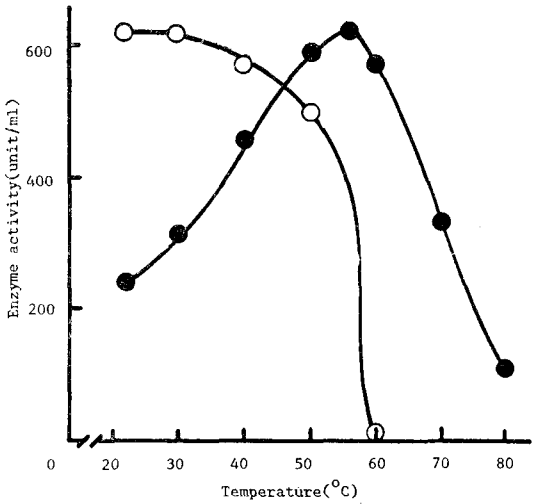

Fig. 2. Effect of temperature on enzyme activity. Enzyme activity(●) was determined at various temperature and remaining activity(○) was measured after treatment at various temperature for 10 min.

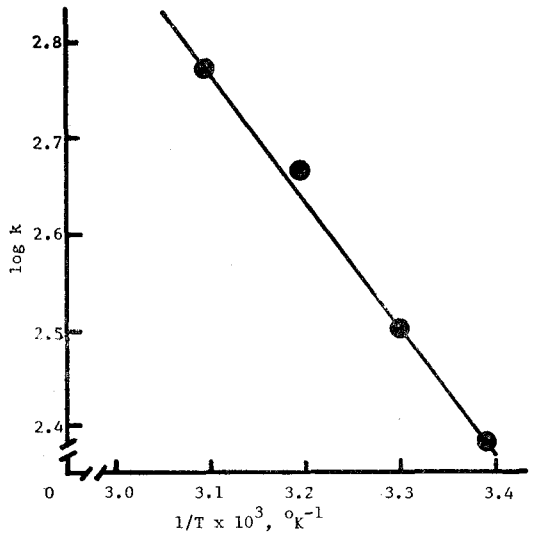

Fig. 3. Arrhenius plot for the cellulolytic reaction of the enzyme.

buffer(pH 3.0~8.0) and Clark and Lubs buffer solution (pH 8.0~13.0). The enzyme exhibited optimal activity at pH 4.0. The enzyme was stable in solution at pH 4.0 to 7.0 when treated at 40°C for 1 hour. Rapid inactivation of the enzyme occurred at pH values above 8.0 and below 4.0 (Fig. 1).

The optimum pH on the enzyme activity was similar to the results reported for that of *Aspergillus niger*(Okada, 1985; Hurst *et al.*, 1977). But it showed slightly higher value than that of acid cellulase from *Aspergillus niger*(Ikeda *et al.*, 1973), and showed slightly lower value than those of cellulases from *Clostridium* sp. (Lee and Blackburn, 1975), *Cellulomonas* sp. (Han and Srinivasan, 1968) and *Humicola insolens* YH-8 (Hayashida and Yoshioka, 1980). And also the pH range for stability was more narrow than that of the cellulases from *Aspergillus niger* (Ikeda *et al.*, 1973; Hurst *et al.*, 1977).

Effect of Temperature on Enzyme Activity

To determine the optimal temperature, the enzyme activity was measured in range of temperature between 22°C and 80°C at pH 5.0, and the results were shown in Fig. 2. Maximum activity was observed at 55°C. This enzyme was extremely sensitive to temperature above 50°C, and enzyme activity was completely lost by heat

treatment at 60°C for 1 hour. The optimum temperature of the enzyme reaction was lower than that of cellulases from *Clostridium* sp. (Lee and Blackburn, 1975) and *Bacillus* sp. (Robson and Chambliss, 1984). But it was slightly higher than that of cellulases from *Aspergillus niger* (Hurst *et al.*, 1977; Okada, 1985). Thermal stability was lower than value of cellulases from *Humicola insolens* YH-8 (Hayashida and Yoshioka, 1980) and *Aspergillus niger* (Ikeda *et al.*, 1973; Hurst *et al.*, 1977; Okada, 1985).

By the means of Arrhenius' equation, the activation energy of the hydrolysis of CMC-2Na was calculated from the slope of Fig. 3 and found to be 6.2 Kcal/mole. Li *et al.* (1965) reported that the energy of activation was 5.1 Kcal/mole for amorphous cellulose, but 6.4 Kcal/mole from 36° to 60°C and 16.7 Kcal/mole below 37°C for CMC.

Effects of Metal Ions and Chemical Reagents on the Enzyme Activity

In order to investigate the effects of various metal ions and chemical reagents on the cellulase activity, the mixtures containing 0.4 ml of buffer solution (pH 4.0), 0.1 ml of 10 mM metal ion or chemical reagent and 0.1 ml of enzyme solution were preincubated at 30°C for 10 minutes. Then 0.4 ml of 1.0% CMC-2Na solution was added to the mixture, and incubated at 55°C for 60 minutes. After incubation, the remaining activity of the enzyme was determined as described previously. The final concentration of metal ions and chemical reagents in the reaction mixture were 1 mM, and the results were shown in Table Ⅱ and Ⅲ.

The enzyme was activated by the addition of Mn^{++} and Co^{++} but inhibited by Hg^{++}. All chemical reagents did not affect the enzyme activity except slight inhibition by sodium dodecyl sulfate (Table Ⅲ). Cellulase from *Aspergillus niger* (Okada, 1985) was inhibited by Ag^{+}, Hg^{++} and

Table II. Effect of metal salt on enzyme activity.

Metal salt	Activity (unit/ml)	Relative activity (%)
None	441	100
$CuSO_4$	472	107
$CaCl_2$	426	97
$AlCl_3$	444	101
$CoCl_2$	509	115
$MnCl_2$	593	134
$ZnSO_4$	444	101
$FeSO_4$	417	95
$BaCl_2$	448	102
$LiSO_4$	441	100
$MgSO_4$	413	94
$Pb(NO_3)_2$	413	94
$AgNO_3$	407	92
$HgCl_2$	306	69

The final concentration of metal salt in reaction mixture was 1 mM.

Table III. Effect of chemical reagent on enzyme activity.

Chemical reagent	Activity (unit/ml)	Relative activity (%)
None	406	100
Thiourea	426	105
Sodium azide	459	113
SDS*	296	73
MIA**	455	112
EDTA-2Na***	414	102
ε-Amino-n-caproic acd	430	106
Sodium arsenate	414	102
o-Phenanthroline	390	96
p-CMB****	353	87
2,4-DNP*****	353	87
Urea	398	98
L-Cystine	422	104
Sodium fluoride	398	98

* Sodium dodecyl sulfate
** Monoiodoacetic acid
*** Ethylenediaminetetraacetic acid disodium salt
**** *p*-Chloromercuribenzoic acid
***** 2,4-Dinitrophenol

The final concentration of chemical reagent in the reaction mixture was 1 mM. The enzyme solution with various chemical reagents was preincubated at 30°C for 10 min.

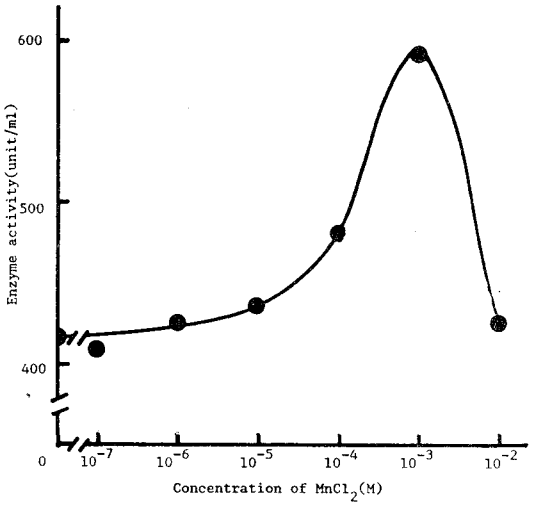

Fig. 4. Effect of $MnCl_2$ concentration on enzyme activity.

Fe^{++}, corresponding to about 75, 67 and 55% respectively. But EDTA and sulfhydryl reagents did not affect enzyme activity. And acid-cellulase produced by *Aspergillus niger* (Ikeda *et al.*, 1973) was inhibited by Mg^{++} and Mn^{++}, and also inhibited by pCMB, SDS, sodium azide, EDTA, N-bromosuccinimide and iodine. Cellulase from *Bacillus* sp. was also inhibited by Hg^{++}. The effect of enzyme activity at various concentration of Mn^{++} were shown as Fig. 4.

Substrate Specificity and Km Value

The enzyme activity was examined with various cellulosic substances, and the results were summarized in Table Ⅳ. This enzyme was able to hydrolyze CMC-2Na and native cellulose except insoluble carboxymethyl cellulose (Sigma Chem. Co., for ion exchange chromatography).

Hydrolysis of CMC-2Na by the enzyme followed the zero-order kinetics over the portion of the curve through 50% hydrolysis. With CMC-2Na as a substrate, a typical Michaelis-Menten relationship was obtained between the substrate concentration and the initial velocity of the reaction. The Lineweaver-Burk plot yielded a straight line, from which a Km value of 2.4 mg/ml was obtained with CMC-2Na as a substrate (Fig. 5). This value was much lower than the value obtained for cellulase from *Aspergillus niger* (Okada, 1985).

Table IV. Substrate specificity of enzyme activity.

Substrate	Activity (unit/ml)	Relative activity(%)
CMC-Na	241	100
Avicel	46	19
α-Cellulose	42	17
DEAE-cellulose	51	21
CM-cellulose	0	0
Filter paper	42	17
Xylan(from larchwood)	328	136

All substrates were added to the reaction mixture with the concentration of 0.5%.

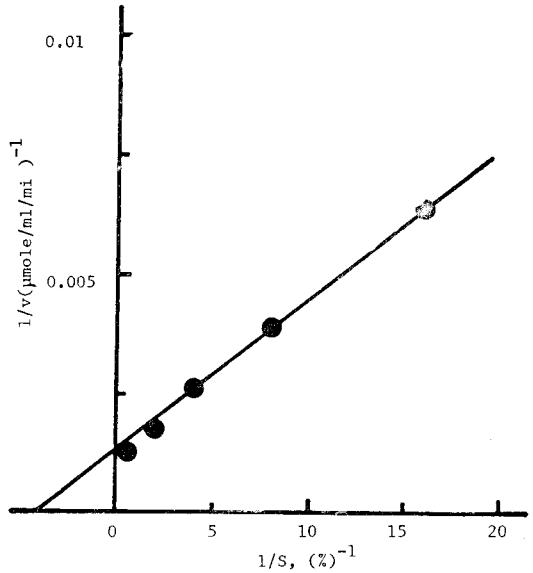

Fig. 5. Lineweaver-Burk plot of CMC hydrolysis by the enzyme. The enzyme reaction was carried out at pH 4.0 and 50°C. After the reaction, liberated reducing sugars in the reaction mixture were measured by DNS method.

摘 要

*Ganoderma lucidum*의 個體培養物로 부터 얻은 cellulase를 ammonium sulfate fractionation으로 組精製한 後 이 酵素의 基本的인 特性을 調査하였다.

이 酵素의 最適作用 pH와 溫度는 각각 pH 4.0, 55°C 였으며 pH 4.0~7.0사이와 50°C 以下의 溫度에서는 比較的 安定하였다. CMC-2Na分解에 대한 activation energy는 6.2 Kcal/mole이었다. Mn^{++}, Co^{++}에 의해서 酵素活性이 增加되었으나 Hg^{++}에 의해서는 沮害되었다. 한편 本 酵素活性은 SDS에 의해서 약 27%沮害된 것을 除外하고는 여러가지 chemical reagents에 의해서는 아무런 影響을 받지 않았다. 本 酵素의 CMC-2Na에 대한 Km値는 2.4 mg/ml 였으며 CMC-2Na 以外에 天然 cellulose에도 作用을 하였다.

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