Reaction Kinetics and Absorption Property of Low Molecular Weight Endo-glucanase Component of Cellulase

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(Received February 22, 1980)

Cellulase 성분중 Endo-gluanasec 의 반응 및 흡착특성에 관한 연구

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(1980년 2월 22일 수리)

Abstract

Low molecular weight endo-glucanase was partially purified from cellulase complex using Sephadex G-100 gel chromatography. Biochemical properties of the purified component was investigated. Optimum pH and temperature determined were 6.0 and 50°C, respectively. Enzymatic hydrolysis of four cellulosic substrates having varying crystallinity was evaluated. It was found that hydrolysis of amorphous region was followed by the hydrolysis of crystalline region. In order to examine the effect of adsorption of the enzyme onto the cellulosic substrates on the hydrolysis kinetics, adsorption studies were carried out. Time course of adsorption of low molecular weight endo-glucanase onto various cellulostances was observed for 25 min. The rate and amount of adsorption to amorphous cellulose was greater than those to the crystalline cellulose. This result suggested that the role of endo-glucanase was more important to the hydrolysis of amorphous cellulose than to the crystalline region of the cellulose.

Introduction

As one of the earth's most abundant renewable resources, cellulose is recognized as an increasingly important resource. Since half of the most municipal wastes is cellulosic material, utilization of cellulose can help solving the serious global problems, energy shortage and pollution.

Since Reese[1] proposed the C_1-C_x concept, the mechanism of cellulose degradation was extensively investigated, although still imcompletely unders-tood.[2~8] In these mechanism studies, the sequence of enzymatic attack on cellulose has been the subject.

Cellulosic substance have the unique physical properties that affect the hydrolysis. These include substrate multiplicity, porosity, heterogeneity, crystallinity, and adsorptivity. By focusing on these physical properties including substrate multiplicity, the detailed mechanism of cellulose degradation could be understood. Since the adsorption of the enzyme on cellulosic surface is a prerequisite for the hydrolysis, detailed studies on the adsorp-

tion may lead to a better understanding of the mechanism of enzymatic degradation processes.

Some workers[9~12)] have studied the adsorption of the cellulase on several cellulosic substances. Adsorption of some purified components on cellulose was also studied.[9)] But low molecular weight endo-glucanase was excluded because its significance to cellulose hydrolysis kinetics were neglected

It this research, low molecular weight endoglucanase from *T. reesei* QM 9414 was purified and its biochemical characteristics were studied. And the adsorption charactistics of the purified cellulase component on various cellulosic substances having different degree of crystallinity were examined and discussed in light of its role on hydrolysis kinetics.

Materials and Methods

Materials

Cellulase from *Trichoderma reesei* QM 9414 was kindle provided by U. S. Army Natick Laboratories. Microcrystalline cellulose (Avicel) and carboxymethyl cellulose (CMC) used for assay standards were also provided by U. S. Army Natick Laboratories. p-Nitrophenyl β-D-glucoside was purchased from Calbiochem. 3, 5–Dinitrosalicylic acid was obtained from Sigma. Chromatography media, Sephadex G–100 and Bio-gel P–4 were purchased from Pharmacia and Bio-Rad Laboratories, respectively. All other reagents used were of analytical grade.

Analytical methods

(a) Avicel hydrolyzing activity

The reaction mixture contained 2. 0 ml of 1% Avicel in 0. 05 M sodium acetate buffer (pH5. 0)) and 0. 2 ml of enzyme solution in a total volume of 2. 2 ml. After incubation with continuous shaking at 30°C for 2 hr, the mixture was centrifuged at 1,000×g for 10 min, and the supernatant was analyzed for reducing sugar by the method of Somogyi and Nelson[13]. The unit of enzyme activity was defined as the amount of enzyme needed to liberate reducing sugars equivalent to 1μmole/min from Avicel under the assay condition described

above.

(b) Carboxymethyl cellulose hydrolyzing activity

The reaction mixture consisted of 2. 0 ml of 1% solution of carboxymethyl cellulose in 0. 025 M citrate buffer (pH 6. 0) and 0. 1 ml of enzyme solution. After incubation of the mixture at 50°C for 10 min. It was analyzed fore reducing sugars according to Miller et al[41)]. The unit of enzyme activity was defined as the amount of enzyme needed to liberate reducing sugars equivalent to 1 μmole/min from CMC under the assay condition.

(c) β–glucosid ase activity

The substrate for the determination of β–glucosidase activity was p-nitrophenyl-β-D-glucoside. The assay mixture contained 1. 0 ml of 1 mM p-nitrophenyl-β-D-glucoside in 0. 05 M sodium acetate buffer (pH5. 0)[8)] and 0. 1ml of enzyme solution. After incubation at 40°C for 10 min. 2. 0 ml of 1 M Na_2CO_3 was added to the mixture. The mixture was diluted with 10. 0 ml distilled water and the p-nitrophenol liberated was determined by the absorbance at 400 nm. One unit enzyme activity is equivalent to 1μmole p-nitrophenol/min liberated under the assay conditions.

(d) Determination of total sugar

Total sugar produced was analyzed by the method of phenol/H_2SO_4 method[15)].

Enzyme fractionation

Cellulase from *Trichoderma reesei* QM 9414 was fractionated on Sephadex G–100 column (2. 5×40 cm) (pH5. 6). 600 mg of crude enzyme was dissolved in 10 ml of 0. 025 M citrate buffer. The solution was then centrifuged (in a refrigerated centrifuge) at 10,000×g for 20 min to remove insoluble materials. The column was equilibrated same buffer and the experiment was run in the same buffer. The flow rate and the fraction volume were 15 ml/ hr and 5 ml, respectively. Peak I and peak II were pooled, desalted on Bio-Gel P–4, lyophilized, and the fractions were stored in a refrigerator. This fractionated enzyme by Sephadex G–100 gel filation was used for all experiments except for the hydrolysis experiment.

Adsorption study

Three different cellulosic substrates having varying crystallinity and physical properties were employed for the adsorption study. Adsorption experiments were performed at 5°C. Reaction mixture was constantly stirred by the magnetic stirrer. After the adsorption, the reaction mixture was sedimented for about 10min at 0°C to prevent further adsorption. The enzyme activity of supernatant was measured using 1.5 unit of the low molecular weight endo-glucanase in each tube.

Results and Discussion

The cellulase complex contains endo-β-1,4-glucanase, exo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase (cellobiose). Depending on microorganisms as the source of enzyme, each component has a different isozyme system. It is known that all cellulase producing microorganisms produce a low molecular weight endo-glucanase (about 12,000).[5,6,16]

Low molecular weight endo-glucanase was purified from a cellulase complex on a Sephadex G-100 column (Fig. 1). High molecular weight components (about 50,000) were eluted first and subsequently low molecular weight component was eluted. Endo-glucanase activity (CMCase) was found at each peak, and the cellulase from *T. reesei* QM 9414 appear to have two or more endo-
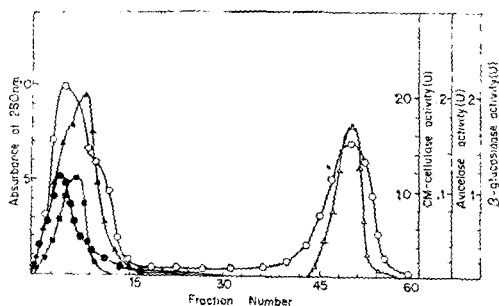

Fig 1. Elution Profile of Cellulase on Sephadex G-100.

Protein determination by absorbance at 280nm (○), CMC cellulase activity (▲), Avicel cellulase activity(■), and β-glucosidase activity (●).

glucanase components. Each fraction of the peak was pooled and desalted on Bio-gel P-4 column. The desalted enzyme fractions were lyophilized and stored at 4°C until used.

To examine the hydrolysis pattern of cellulosic substances having different crystallinity, four cellulosic substrates having decreasing order of crystallinity, cotton, Avicel, filter paper, and Walseth cellulose were subjected to hydrolysis with cellulase complex for 48 hrs at 50°C (Fig. 2).
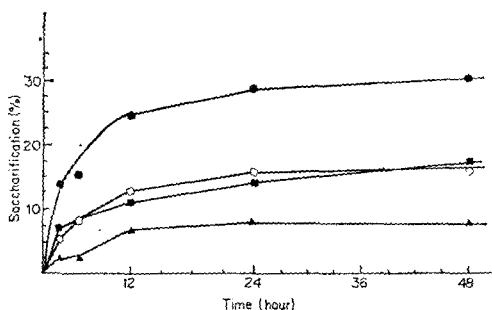

Fig 2. Hydrolysis Pattern of Cellulosic Substrates. Walseth cellulose (●), Filter paper (○), Avicel (■), and dewaxed cotton (▲).

Hydrolysis curve of cotton, filter paper and Avicel was leveled off at its final conversion 7%, 16% and 17%, respectively. Hydrolysis of Walseth cellulose continued after 48 hrs. Amorphous cellulose was rapidly hydrolyzed and the rate of hydrolysis decreased greatly as the crystalline portion of the cellulose were being attacked. From the observation of hydrolysis pattern, two hydrolysis kinetic regime was found: first the hydrolysis of amorphous region followed by crystalline region hydrolysis.

Effect of pH on activity and stability of the low molecular endoglucanase was obtained (Fig. 3). The stability was tested by preincubating the enzyme at the indicated pH for 20 min.[17] Optimum pH was 6.0 and stability was maintained in the range of pH 5.0–pH 7.0. The decline in activity between pH 5.0 and 6.0 and between pH 6.0 and 7.0 can be ascribed to the effect of pH on the ionizable groups of the active site or substrate. The decline in activity above pH 6.5 and below pH 5.5 can be ascribed in part to irreversible denaturation of the enzyme. The optimum pH of
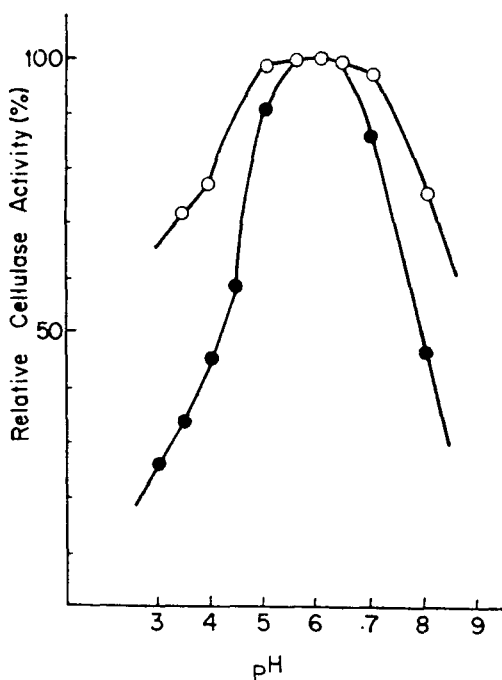
Fig. 3. Endo-glucanase Activity and pH Relation-
ship

　Relative endo-glucanase activity (●), st-
ability of endo-glucanase (○). For stability
test Endo-glucanase samples were preincu-
bated for 20 mln. at a given pH, followed
by the activity assay.

the low molecular weight endo-glucanase was slig-
htly higher than that of the other cellulase com-
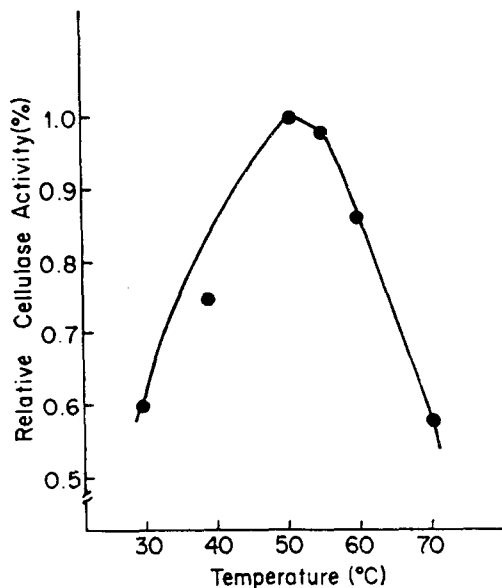
Fig. 4. Endo-glucanase Activity and Temperature
Relationship.

　Optimum temperature was determined with the
10–min assay. Optimum temperature of the low
molecular weight endo-glucanase was 50°C (Fig.
4). This result is in good agreement with the
optimum saccarification temperature of cellulase
complex. The activation energy of the enzyme-
catalyzed reaction was obtained from the Arrhenius
plot, V_m vs. inverse value of reaction temperature.
The activation energy for CMC hydrolysis was
10. 8 Kcal/mole.

　Kinetic constant of low molecular weight endo-
glucanase for CMC was obtained from Lineweaver-
Burk plot (Fig. 5). K_m and V_{max} values are 0. 39
% CMC and 0. 57 mM/min.
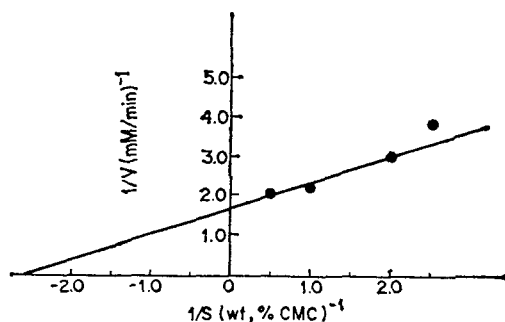
Fig. 5. Determination of Kinetic Constants for the
Endo–glucanase.

　Adsorption of the enzyme on cellulosic surfaces
is a prerequisite and the initial step for the hy-
drolysis of cellulose because cellulose substrate is
solid material. Employing three different cellulosic
substances having various crystallinity, adsorption
characteristics was studied. To compare the adsor-
ption characteristics, the cellulose concentration at
which the adsorption site for the enzyme was not
limited must be used. The cellulose concentration
at which the adsorption site was not limited for
1. 5 unit of the enzyme to be adsorbed was deter-
mined as 2 %. (Fig. 6)

　Time course of adsorption of the low molecular
weight endo-glucanase onto cellulosic substance was
evaluated(Fig. 7). Adsorption curves were leveled
off after 2–5 min. Maximum adsorption was 55 %,
45.%, and 22 % for the Walseth cellulose, Avicel,
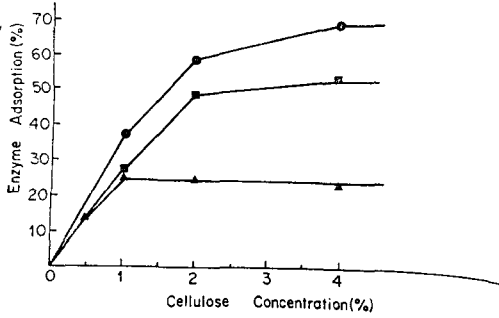and cotton, respectively. The enzyme was more

Fig. 6. Effect of Cellulose Concentration on the Adsorption of Endo-glucanase Celluloses.
Walseth cellulose (●), Avicel (■), and dewaxed cotton (▲), Adsorption period 15 min.
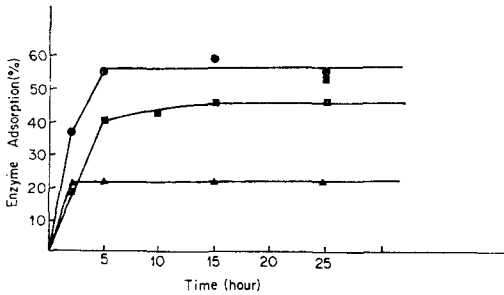

Fig. 7. Time Profile of Endo-glucanase Adsorption onto Celluloses (2% each).
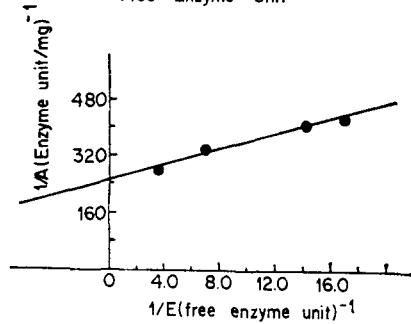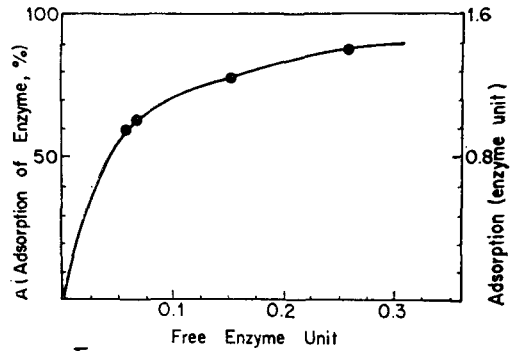Walseth cellulose (●), Avicel (■), and dewaxed cotton (▲).

Fig. 8. Adsorption Isotherm (Fig. 8-a) Determination of Adsorption Coefficient (b) and the Maximum Amount of Enzyme Adsorbed (Am) by the Double Reciprocal Plot (Fig. 8-b).

rapidly adsorbed to amorphous cellulose were greater than that to crystalline cellulose. This result suggested that the role of the low molecular weight endo-glucanase was more important to the hydrolysis of amorphous cellulose than to the crystalline region of the cellulose. Due to the relatively large pore size of the amorphous cellulose, the enzyme should be more readily diffusable to the amorphous cellulose than to the crystatalline cellulose.

The pattern in which the low molecular weight endo-glucanase is adsorbed to cellulosic substance was investigated (Fig. 8). It was found that the adsorption pattern is similar to Langmuir adsorption pattern is similar to Langmuir adsorption isotherm. This result was in good agreement with others.[11]

$$\frac{1}{A} = \frac{1}{Am\ b}\ \frac{1}{E} + \frac{1}{Am}$$

Adsorption coefficient (b) and maximum amount

of enzyme adsorbed per mg of cellulose (Am) was determined from the double reciprocal plot of Langmuir adsorption isotherm (Fig. 8). The values of b and Am determined are 23.4 unit^{-1} and 4×10^{-3} unit/mg, respectively.

요 약

저분자량의 효소인 셀루라아제가 Sephadex G-100 gel chromatography를 사용하여 정제되었다. 정제된 성분의 생화학적 성질들이 조사되었는데 최적 pH와 온도가 각각 6.0과 50°C이였다. 서로 다른 결정도(crystallinity)를 갖고 있는 4가지 섬유소 기질의, 효소에 의한 가수분해가 측정되었다. 무정형(amorphous) 부분의 가수분해가 일어난 후에 결정화되어 있는 부분의 가수분해가 뒤따라 온다는 것을 알수 있었다. 효소가 섬유소 기질에 흡착되는 정도가 가수분해 반응에 미치는 영향을 보기 위해 흡착에 대한 연구가 수행되었다. 시간에 따라서 소분자량의 endo-glucanase가 여러가지 섬유소 기질에 흡착되는 정도의 변화가 25분간 측정되었다. 무정형의 섬유소에 흡착되는 속도와 정

도가 결정형 ˙섬유소에 [대한 그것들 보다 더욱 크게 나타났다. 이러한 결과는 endo-glucanase 가 섬유소의 결정화 부분의 가수분해 보다는 무정형 부분의 가수분해에 대해서 더욱 중요한 역할을 한다는 것을 시사해 준다.

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