Purification and Characterization of an Exo β -1,3-Glucanase from a Fungi Imperfecti

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An exo β -1,3-glucanase has been purified from the culture fluid of a Fungi Imperfecti which had been used for the production of a yeast cell lytic enzyme. Method for the purification of the enzyme involved ammonium sulfate fractionation, column chromatography on DEAE-Sephadex A-50, BioGel P-150 and SE-Sephadex C-50, and affinity chromatography using powdered pachyman prepared from a Chinese medicine "Bukuryo," *Poria cocos* Wolf.

The molecular weight of the enzyme was determined to be 43,000 by the molecular sieve method using BioGel P-60. The enzyme removes single glucosyl residues successively from the non reducing terminus of β -1,3-glucan with inversion of configuration. The enzyme has comparatively narrow pH optima with an optimum pH of 6.0 and is most active at 50°C. The K_m values of the enzyme for laminarin, laminarihexaose, laminaripentaose, and laminaritetraose are 0.066, 0.83, 2.30, and 18.60 ($\times 10^{-3}$ M), respectively. Some other properties of the enzyme are also described.

β -1,3-Glucanase is an ubiquitous enzyme distributed in various kinds of organisms. Microorganisms have been specially indicated to be a good source of the enzyme by many workers.^{1~15)} In its mode of action two types of β -1,3-glucanase, exo and endo types, are known to exist. Further, in the latter type the existence of a "larger oligosaccharides-producing" type and a "smaller oligosaccharides-producing" one has recently been suggested by some workers.^{16,17)}

The use of the enzymes as a tool for research in structural carbohydrate chemistry is one of skillful application among many expected applications. The exo β -1,3-glucanase and/or β -glucosidase are considered to be useful particularly for this purpose. Many workers have done research on this kind of glucanase. Among them, Nelson's study on *Basidiomycetes* exo β -1,3-glucanase¹⁸⁾ is one of the most comprehensive studies.

In our previous paper¹⁶⁾ endo β -1,3-glucanase of "larger oligosaccharides-producing" type obtained from a Fungi Imperfecti, which was isolated as an able organism to decompose

viable yeast cells, was studied from an enzymological view point. The organism, in addition to this type of β -1,3-glucanase, produces a "smaller oligosaccharides-producing" β -1,3-glucanase and an exo β -1,3-glucanase in good amount. This report deals with the purification and properties of the exo β -1,3-glucanase.

MATERIALS AND METHODS

Microorganism and enzyme production. For the production of the β -1,3-glucanase one loopful mycelia had been transferred from a stock culture of the organism¹⁶⁾ into 500 ml shaking flasks containing 100 ml of a medium composed of 2% glucose, 1% NH_4NO_3 , 0.25% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.02% FeCl_2 , pH 5.0. After two days incubation at 30°C with shaking, 50 ml aliquots of the culture were seeded into 2 liter shaking flasks containing 500 ml of a medium consisting of 4% baker's yeast, 0.3% K_2HPO_4 , and 0.05% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, pH 3.5 (before autoclave). The flasks were set on a shaker for 4 days at 30°C. The identification of the organism has been retarded since it did not form spore in any of the culture media so far tested.

Substrates. Yeast glucan, BH_4 -reduced* yeast glucan, IO_4 -oxidized** laminarin, BH_4 -reduced laminarin, IO_4 -oxidized and BH_4 -reduced laminarin, IO_4 -

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* Sodium borohydride-reduced.

** Sodium periodate-oxidized.

oxidized, BH_4 -reduced and mild-acid hydrolyzed laminarin, and methylated laminarin were prepared as described in the previous paper.¹⁶⁾ Laminarioligosaccharides were prepared following the method described by Whelan.¹⁹⁾ *p*-Nitrophenyl- β -glucoside was prepared from glucose following the usual method.²⁰⁾ Phosphomannan, coffee mannan and luteose were prepared respectively from *Hansenula holstii* IFO 0986, coffee beans (Santos), and *Penicillium luteum* Zukal IFO 6345, according to the method described by Rogovin *et al.*,²¹⁾ Hashimoto and Fukumoto,²²⁾ and Anderson and Raistrick,²³⁾ respectively. Sclerotia glucan, Sclerotan, was prepared following the procedure described by Kitahara and Takeuchi²⁴⁾ from the sclerotia of *Corticium rolfsii* IFO 4878 cultured on solid Wickerham medium. The other substrates used in this study were obtained commercially.

Enzyme assay. The assay of β -1,3-glucanase was based on the measurement of glucose cleaved from laminarin. The reaction was conducted, otherwise noted, at 40°C for 10 min with 0.5 ml of a suitably diluted enzyme solution and 0.5 ml of laminarin solution (10 mg/ml in 0.1 M Na-succinate buffer, pH 5.8). The amount of enzyme which yields 1 μ mole of glucose under this condition was defined as 1 unit.

Determination of anomeric configuration of glucose. The determination of anomeric configuration of the glucose produced from laminarin by the enzyme was made with a gas chromatograph, Shimadzu gas chromatograph Model GC-4PTF (Shimadzu Seisakusho Ltd., Kyoto), fitted with a hydrogen flame ionization detector. A sample solution withdrawn from the laminarin solution being depolymerized by the enzyme was lyophilized as soon as possible to minimize mutarotation, trimethylsilylated, and then chromatographed under the following conditions. A stainless steel column, 0.3 $\times$ 2000 cm, packed with 60~80 mesh Chromosorb W coated with 1.5% methyl phenyl silicone (SE-52) was used, and as a reference sugar α - and β -glucoses were used.

Chemical analyses. Reducing sugar was determined by the 3,5-dinitrosalicylic acid (DNSA) method²⁵⁾ or the Somogyi-Nelson method.²⁶⁾ Carbohydrate was determined by the phenol-sulfuric acid method.²⁷⁾ Glucose was measured by the glucose oxidase method using Glucostat (Worthington Biochemicals, USA) or, alternatively, by the chemical method, stated above, based on the reducing action. Protein was determined by the Lowry method²⁸⁾ or ultraviolet extinction method.

Miscellaneous. Disc electrophoresis was conducted according to the standard method described by Ornstein and Davis.²⁹⁾ Paper chromatography was per-

formed according to the descending method using No. 50 filter paper (Toyo Roshi, Japan) and *n*-butanol: ethanol: water (5: 2: 2 v/v) as solvent. Ultracentrifugation analysis was performed in a Hitachi Model 282 analytical ultracentrifuge equipped with Schlieren optics and photoelectric scanner.

RESULTS AND DISCUSSION

1. Purification of the enzyme

The culture fluid obtained as stated in the foregoing section, was filtered through cheese-cloth and centrifuged with a continuous centrifuge machine at 13,000 $\times g$. The clear crude enzyme solution was submitted to ammonium sulfate fractionation. The precipitate formed between 20% and 80% saturation of ammonium sulfate was collected by centrifugation at 18,000 $\times g$. The precipitate was dissolved in an appropriate volume of 0.01 M Tris-HCl buffer, pH 7.0, and dialyzed against the same buffer overnight. The dialyzed solution was poured on the top of a DEAE-Sephadex A-50 column previously equilibrated with 0.01 M Tris-HCl buffer, pH 7.0, and the column was washed with the same buffer. The breakthrough and the washing containing β -1,3-glucanase were combined and concentrated to approximately 1/10 of the initial volume in a rotary evaporator.

To the concentrated solution solid ammonium sulfate was added to give 80% saturation. The precipitate formed was collected by centrifugation, dissolved in an appropriate volume of 0.01 M Na-acetate buffer, pH 5.0, and chromatographed on a BioGel P-150 column. The active fractions from the column were pooled, concentrated, dialyzed against 0.01 M Na-acetate buffer, pH 5.0, and applied to a SE-Sephadex C-50 column previously equilibrated with 0.01 M Na-acetate buffer, pH 5.0. The column was washed with the same buffer and then the enzyme was eluted with 0.01 M K-phosphate buffer, pH 7.0. The active fractions were pooled in a dialysis tubing and concentrated to about 1/3 of the initial volume by placing it in dry Sephadex powder as a desiccant. The concentrated solution was poured on the top of a column, 1 $\times$ 10 cm,

TABLE I. SUMMARY OF THE PURIFICATION PROCEDURES OF THE EXO β -1,3-GLUCANASE

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture filtrate	15,300	58,524	39,688	7	(100)
Ammonium sulfate fractionation	410	5,111	23,019	45	58
DEAE-Sephadex chromatography	18.2	430	3,433	80	8.7
BioGel P-150 gel filtration	47.0	220	2,884	131	7.3
SE-Sephadex chromatography	3.3	13.4	2,138	1596	5.4
Affinity chromatography	9.6	6.4	1,084	1694	2.7

packed with powdered pachyman prepared from a chinese medicine "Bukuryo," *Poria cocos* Wolf fungus, previously washed thoroughly with 0.1 M Na-succinate buffer, pH 5.8, and the column was washed with the same buffer. The protein peak which came out last from the column was the β -1,3-glucanase. By this affinity chromatography the enzyme was separated from inactive protein. Table I shows the summary of the purification procedures of the enzyme.

2. Physical properties of the enzyme

The enzyme preparation obtained through the above mentioned procedures was judged to be homogeneous by the criterion of ultracentrifugation analysis, disc electrophoresis on polyacrylamide gel, and the specificity test which will be shown in Table III. The molecular weight of the enzyme determined by the gel filtration chromatography using BioGel P-60 was 43,000.

3. Effect of pH on the activity and stability of the enzyme

In Fig. 1 the enzyme activities measured at various pH values (○—○) are plotted. When the enzyme was heated at 40°C for 10 min in buffer solutions of various pH values, the pH-stability curve (●—●) shown in the figure was obtained. The enzyme showed relatively narrow pH-optima and pH-stability, and was most active and stable at pH 6.0.

FIG. 1. Effect of pH on the Activity and Stability of the Enzyme.

Each of the reaction mixtures contained 1.06 μ g of the enzyme and 5 mg of laminarin in 1 ml of the following 0.01 M buffer solution: pH 4.0~7.0, Na-acetate buffer; pH 6.0~9.0, K-phosphate buffer. ○—○, pH-activity curve; ●—●, pH-stability curve; the enzyme solutions adjusted to various pH values with the above buffer solutions were preincubated at 40°C for 10 min. After rapid cooling with ice water the remaining activity was assayed under the standard assay conditions. The maximum values observed in respective series of the experiments were arbitrarily taken as 100.

4. Effect of temperature on the activity and stability of the enzyme

Figure 2 shows the temperature-activity curve and temperature-stability curve of the enzyme. In the purified form it is rather unstable, but egg-albumin was an effective

FIG. 2. Effect of Temperature on the Activity and Stability of the Enzyme.

○—○, temperature-activity curve. ●—●, temperature-stability curve; enzyme solutions containing 1.06 μg of the enzyme in 1 ml of $M/10$ Na-acetate buffer, pH 6.0, were preincubated for 10 min at various temperatures and after rapid cooling with ice water the remaining activity was assayed under the standard assay condition. The maximum values observed in respective series of the experiments were arbitrarily taken as 100.

stabilizing agent at a concentration of 100 $\mu\text{g}/\text{ml}$.

5. Effect of metal ions and inhibitors on the enzyme activity

When enzyme activity was measured in the presence of $10^{-4} M$ of metal ion, Hg^{2+} , Zn^{2+} , Cu^{2+} , and Co^{2+} , ions inhibited the enzyme activity as shown in Table II. Among the inhibitors tested N-bromosuccinimide (90% inhibition) and *p*-chloromercuric benzoate (75~80% inhibition) showed remarkable inhibiting effect at a concentration of $10^{-5} M$, suggesting that the SH-group contributed to the activity of the enzyme.

6. Effect of sugars and sugar derivatives on the enzyme activity

Among the sugars and sugar derivatives tested; mannose, glucose, fructose, sorbose, arabinose, xylose, ribose, sorbitol, mannitol, erythritol, inositol, rhamnose, N-acetylglucosamine, gluconic acid, and δ -gluconolactone,

TABLE II. EFFECT OF METALS ON THE ENZYME ACTIVITY

The enzyme, 0.5 μg , had been incubated previously for one hour in 0.5 ml of $10^{-4} M$ various metal solutions in 0.01 M sodium succinate buffer, pH 5.8, at 5°C, and then the glucanase activity in the solution was assayed under the standard assay conditions.

Metal	Relative activity	Metal	Relative activity
Without metal	100	NiSO_4	62
LiSO_4	76	CuSO_4	60
MgSO_4	92	ZnSO_4	55
$\text{Al}_2(\text{SO}_4)_3$	76	Na_2HAsO_4	55
CaCl_2	98	AgNO_3	115
MnSO_4	115	Ag_2SO_4	62
MnCl_2	63	Na_2WO_4	89
FeSO_4	100	HgCl_2	6
CoSO_4	59	$\text{Pb}(\text{NO}_3)_2$	106

only δ -gluconolactone, a typical inhibitor on glycosidase, inhibited 30% of the enzyme activity at a concentration of $10^{-5} M$, which is more dilute than the concentration which gave 50% inhibition of Emulsin β -glucosidase ($2 \times 10^{-5} M$)³⁰⁾ and Limpet β -glucosidase ($8 \times 10^{-5} M$).³⁰⁾ In this respect, it seemed that the enzyme has a similar property to β -glucosidase.

7. Substrate specificity of the enzyme

As is shown in Table III, the enzyme de-

FIG. 3. Effect of Substrate Concentration on the Reaction of the Enzyme with Laminari-oligosaccharides.

Reaction mixture contained 0.99 μg of the enzyme and varying amounts of substrate in 1 ml of 0.01 M Na-acetate buffer, pH 6.0. The reaction was carried out at 50°C for 20 min. Glucose was assayed by the glucose oxidase method. ●—●, laminaritetraose; ◐—◐, laminaripentaose; ○—○, laminarihexaose.

TABLE III. SUBSTRATE SPECIFICITY OF THE ENZYME

Substrates, 2.5 mg/ml, were incubated at 40°C and pH 5.0 (0.05 M Na-acetate buffer) for 10 min in the presence of the enzyme, 2.12 μ g/ml, and then the reducing sugar formed was measured by the Somogyi-Nelson method.

Substrate	Reducing sugar (μ moles/ml)	Substrate	Reducing sugar (μ moles/ml)
Amylose	0.00	Luteose	0.00
Amylopectin	0.00	Lichenan	0.00
Glycogen	0.00	Laminarin	2.49
Starch	0.00	Yeast glucan	0.39
Dextran	0.00	Pachyman	0.28
(MW 70,000)	0.00		
(MW 197,000)	0.00	Maltose	0.00
Dextrin	0.00	Melibiose	0.00
Xylan	0.00	Trehalose	0.00
Cellulose	0.00	Raffinose	0.00
Succinoglucan	0.00	Cellobiose	0.00
Phosphomannan	0.00	<i>p</i> -NP- β -Glucoside	0.00
Coffee mannan	0.00	<i>P</i> - α -Glucoside	0.00

composed glucans consisted mainly of β -1,3-glucosidic linkages, such as yeast glucan, sclerotia glucan, pachyman, and laminarin. The enzyme decomposed, though very slow, laminarioligosaccharides from laminaritetraose to laminarihexaose with an increasing decomposition rate in this order (Fig. 3). The degradation of laminaribiose and laminaritriose, though the rates were not determined, proceeded very slowly. From these and that the enzyme lacks ability to decompose *p*-NP- β -glucoside, the enzyme can be referred to as an exo β -1,3-glucanase.

8. Kinetics of the enzyme

The K_m values of the enzyme for laminarin (Fig. 4), laminarihexaose, laminaripentaose, and laminaritetraose (Fig. 5) were determined the Lineweaver-Burk plots being prepared to be 0.066×10^{-3} M, 0.83×10^{-3} M, 2.30×10^{-3} M and 18.60×10^{-3} M, respectively. Since the affinity of an enzyme for its substrate is usually considered to be the reciprocal of K_m , in the scope of the present investigation it may be said that the affinity of the enzyme for its substrate increases with the increasing chain length of the substrate.

The activation energy of the enzyme for

FIG. 4. Effect of Laminarin Concentration on the Enzyme Activity (A), and Lineweaver-Burk Plot (B). The reaction was carried out at 50°C using 1.06 μ g/ml of the enzyme and a varying amount of laminarin from 0.2 mg/ml to 1.0 mg/ml. Velocity was expressed as μ moles of reducing sugar, assayed by the Somogyi-Nelson method, formed per 10 min.

FIG. 5. Lineweaver-Burk Plot for the Enzyme Action on Laminari-oligosaccharides.

The data were taken from the experiment shown in Fig. 3. The velocity was expressed as μ mole of glucose formed per 10 min. ●—●, laminaritetraose; ◐—◐, laminaripentaose; ○—○, laminarihexaose.

laminarin hydrolysis was determined to be 6.2×10^3 cal/mole, the Arrhenius plot being prepared on the basis of the data shown in Fig. 2.

The above mentioned properties are consistent with the general properties of exo glucanase.

9. Mode of action of the enzyme

Action on laminarin. Action of the enzyme

FIG. 6. Hydrolysis Curve of Laminarin Being Depolymerized by the Enzyme.

The reaction mixture contained 250 mg of laminarin and 33.15 μ g of the enzyme in 50 ml of M/10 Na-acetate buffer, pH 5.8, and the reaction was carried out at 40°C. ○—○, reducing sugar measured by the Somogyi-Nelson method; ●—●, glucose measured by the glucose oxidase method.

on laminarin was followed by measuring the reducing capacity and the amount of glucose formed. As is shown in Fig. 6, the reducing capacity measured was almost in accordance with the amount of glucose, suggesting that the hydrolysis product is predominantly glucose. The suggestion was proved to be true by the paper chromatographic analysis on the product. A small amounts of gentiobiose and unidentified sugar were also detected on the paper. Anomeric configuration of the glucose formed was examined by means of gas chromatography. The results are shown in Fig. 7. The observed ratio of α -glucose to β -glucose in the reaction products was 56.5:43.5, however, when allowed to advance mutarotation the ratio changed to 43.5:56.5. Whereas the formation of β -glucose due to mutarotation of a 0.06% solution (1/2 concentration of the glucose enzymatically produced) of α -glucose in water at 40°C was found to be 48.6% in 10 min. On the basis of these experimental results and available data on the mutarotation of glucose anomers (α : β in the equilibrium state is 36:64), the anomeric configuration of the glucose formed by the enzyme was supposed to be α anomer.

FIG. 7. Analysis of Glucose Anomers by Means of Gas Chromatography.

Reaction mixture containing 5 mg of laminarin and 6.6 μ g of the enzyme in 2 ml of deionized water was incubated at 40°C. After 10 min incubation 0.5 ml aliquots of the mixture were withdrawn for analysis (see MATERIALS AND METHODS). Sample 1, lyophilized directly after the withdrawal (α : β =56.5:43.5). Sample 2, after the withdrawal heated in boiling water for 5 min, left at room temp. for 2 hr and then lyophilized (α : β =43.5:56.5). Control, 0.06% solution of α -glucose that had been incubated for 10 min at 40°C before lyophilization (α : β =51.4:48.6).

Action on chemically modified laminarins. When the enzyme was tested on chemically modified laminarins, the hydrolysis rates shown in Table IV were obtained. Although both periodate oxidation and the subsequent reduction alter both the reducing and non reducing terminals, the altered non reducing terminal can be quantitatively removed by mild acid hydrolysis without affecting reducing terminal. Therefore, it can be no doubt that the decrease in hydrolysis rate shown in the table are due solely to structural alterations at the non reducing terminal. Addition of the derivatives which showed the rate decrease (IO_4 -oxidized lamina-

TABLE IV. HYDROLYSIS OF CHEMICALLY MODIFIED LAMINARIN BY THE ENZYME

Chemically modified laminarin of 0.05% solution was incubated with 6.63 $\mu\text{g/ml}$ of the enzyme at 40°C for 10 min and then the glucose formed was measured by the glucose oxidase method.

Laminarin derivatives	Relative hydrolysis rate
Control (laminarin)	100
IO ₄ -oxidized laminarin	3.36
IO ₄ -oxidized and BH ₄ -reduced laminarin	0.00
IO ₄ -oxidized, BH ₄ -reduced, and mild acid-hydrolyzed laminarin	76.17
Methylated laminarin	0.00
BH ₄ -reduced laminarin	125.84

rin and IO₄-oxidized and BH₄-reduced laminarin) to the laminarin solution being hydrolyzed by the enzyme, caused 80% and 38% inhibition of the enzyme activity, respectively. The inhibition pattern of IO₄-oxidized and BH₄-reduced laminarin as examined using the Lineweaver-Burk plot was a typical competitive one as is shown in Fig. 8. This suggests that the rate decrease observed with the non reducing terminus-modified laminarin is due, not to loss

FIG. 8. Double Reciprocal Plot for the Laminarin Hydrolysis by the Enzyme in the Presence of IO₄-Oxidized and BH₄-Reduced Laminarin.

The reaction was carried out using 1.06 μg of the enzyme under the standard assay conditions except that the concentration of laminarin was varied and IO₄-oxidized and BH₄-reduced laminarin was added to the reaction mixture. ●—●, 0%; ○—○, 0.01%; □—□, 0.03%; △—△, 0.05% IO₄-oxidized and BH₄-reduced laminarin.

of affinity but, to a difficulty encountered by the enzyme in removing these altered terminal residues.

Action on β -glucans with mixed linkages. According to Johnson *et al.*³²⁾ and Kitahara and Takeuchi³³⁾ a glucan from the sclerotinia of *Corticium rolfsii* has such structure that every third glucose residue in β -1,3-linked glucose residues has another β -glucose residue attached at position 6. The glucan was decomposed by this enzyme to produce glucose and gentiobiose in likewise as by the exo β -1,3-glucanase from *Basidiomycetes* QM 806.¹⁸⁾

From lichenin (0.51 $\mu\text{mole/ml}$), a linear polymer composed of β -cellobiose units joined through 1,3-linkages,^{3,4)} the enzyme liberated a small quantity of glucose (0.31 $\mu\text{mole/ml}$) and the action stopped thereafter, whereas β -glucosidase from sweet almond used for comparative experiment did not act appreciably on this glucan. On paper chromatographic analysis no product other than glucose was detected. Therefore the liberated glucose is supposed to originate only from the 1,3-linkages happened to exist at the non reducing terminus of the lichenin molecule.

10. Effect of alcohols on the enzyme activity

The hydrolysis rate of laminarin by the enzyme was reduced by the presence of alcohol, such as methanol, ethanol, or *n*-propanol as is shown in Fig. 9. The data for each of the alcohols, although showing a little scattering, appear to fit a straight line drawn in the figure. Paper chromatographic analysis on the reaction products ruled out the possibility of transglycosylation reaction by which non-reducing alkyl glucoside causing apparent reduction in hydrolysis rate is formed. The inhibition of the enzymatic action by ethanol is reversible as is shown in Table V and hence is not an irreversible denaturation of the enzyme protein. The mechanism of the inhibition was further analyzed by means of the double reciprocal plot (Fig. 10) and was revealed to be a non competitive inhibition with constant *K_m*. Therefore, the observed reduc-

FIG. 9. Effect of Alcohols on the Enzyme Activity. The reaction mixture contained 5 mg of laminarin, 1.06 μ g of the enzyme, and varying amounts of alcohol in 1.0 ml of 0.01 M Na-acetate buffer, pH 5.8. The reaction was carried out at 40°C for 10 min and the reducing sugar formed was measured by the DNSA method. The activities have been expressed as the relative activity to the control enzyme to which alcohol has not been added. $\odot$ — $\odot$, ethyl alcohol; $\circ$ — $\circ$, methyl alcohol; $\bullet$ — $\bullet$, propyl alcohol.

TABLE V. STABILITY OF THE ENZYME IN THE PRESENCE OF ETHANOL

The enzyme, 1.06 μ g/ml, was incubated at 0°C for 30 min in ethanol solution of various concentrations, after which time the enzyme solutions had been dialyzed against M/10 Na-acetate buffer, pH 6.0, overnight at 5°C to remove alcohol. The laminarin degrading activity in the solutions was assayed under the standard assay condition using the DNSA method. A comparison of the results and those taken from Fig. 10 (activity assayed in the presence of ethanol) is given in the table.

Concentration of ethanol (%)	Relative activity	
	In the presence of ethanol	After dialysis
0	100	100
30	54	105
50	20	107
80	0	71

tion in the hydrolytic rate of laminarin is believed to be due, not to a loss of affinity but, to the reduction of k_{+2} in the equation,

$$E + S \xrightleftharpoons[k_{-1}]{k_{+1}} ES \xrightleftharpoons[H_2O]{k_{+2}} E + P,$$
 suggesting that water is the rate-limiting factor.

FIG. 10. The Double Reciprocal Plot for the Laminarin Hydrolysis by the Enzyme in the Presence of Ethanol.

In the presence of ethanol, varying amounts of laminarin were digested by 1.06 μ g of the enzyme. The velocity was expressed as μ moles of glucose, measured by the DNSA method, formed per 10 min at 50°C. The concentrations of ethanol were 0% ($\bullet$ — $\bullet$), 10% ($\circ$ — $\circ$), 20% ($\odot$ — $\odot$), 25% ($\circ$ — $\circ$), and 30% ($\odot$ — $\odot$).

To study the effect of water on the enzyme activity, glycerol was chosen as a solute according to Nelson's idea.¹⁸⁾ Glycerol has a dielectric constant similar to water even at high glycerol concentration (50%) and often used to protect enzyme from denaturation. As is

FIG. 11. Effect of the Molarity of Water on the Hydrolytic Rate.

The reaction mixture contained 5 mg of laminarin and 1.06 μ g of the enzyme in 1.0 ml of 0.01 M Na-acetate buffer, pH 5.8 containing various concentrations of glycerol. The reaction was performed at 40°C for 10 min and then glucose formed in the mixture was assayed by the glucose oxidase method. The observed values have been expressed by the relative values to the value for the system without glycerol. $\bullet$ — $\bullet$, glycerol; $\circ$ — $\circ$, ethanol (data taken from Fig. 10).

shown in Fig. 11, the velocity is linearly related to the molarity of water. The enzymatic activities in the ethanol system, when plotted against the molarity of water, also showed the linear relationship with the slope similar to that of glycerol. These results seem to support that water is involved in the rate limiting step of the reaction. The mechanism of how the water limits the rate of the enzymatic action remain to be solved.

According to the hypothesis that glycosidases and glycanases are distinguished from each other in that the former act with retention of configuration of the glucosyl bond and prefer as substrate the saccharides with shorter chains, whereas the latter cause inversion thereof and prefer the saccharides with longer chains, it is clear that the present enzyme is an exo β -1,3-glucanase since it acts with inversion of configuration of the glucosyl bond and prefers the saccharides with the longer chains.

The fungus employed for the production of the enzyme produced a flavor characteristic to mushroom in culture media, but was excluded from the genus *Basidiomycetes* in that the fungus has no basidium and clamp connection and did not form fruiting body in the cultivation on logs of tree, *Quercus serrata* Thunb. The fungus was tentatively placed in Fungi Imperfecti group, since it did not form spore in any of the culture media so far tested.

When compared the properties of the present enzyme with those of known fungal exo β -1,3-glucanases, such as *Basidiomycetes* QM 806,¹⁸⁾ *Rhizopus niveus*,¹²⁾ *Sclerotium libertiana*,⁵⁾ and *Corticium rolfsii*¹³⁾ glucanases, relatively high initial velocity of hydrolysis, narrow pH optima and stability, and inhibition by Hg^{2+} , Ca^{2+} and Zn^{2+} ions were seemed to be characteristic of this enzyme. As stated in the foregoing sections the present enzyme removes single glucosyl residues successively from the nonreducing terminus of β -1,3-glucan until almost 100% decomposition is obtained. In case gentiobiose units are mixed in the majority of β -1,3-linkages the enzyme bypasses the 1,6-linkages, leaving gentiobiose behind.

The enzyme, having such action pattern, is expected to be a very useful tool for the structural analysis of β -glucans.

REFERENCES

- 1) R. A. Aitkin, B. P. Eddy, M. Ingram and C. Weurman, *Biochem. J.*, **64**, 63 (1956).
- 2) B. A. Stone, *ibid.*, **66**, 1 p (1957).
- 3) E. T. Reese and M. Mandels, *Can. J. Microbiol.*, **5**, 173 (1959).
- 4) D. J. Manners and G. Wilson, *Biochem. J.*, **135**, 11 (1973).
- 5) J. Ebata and Y. Satomura, *Agric. Biol. Chem.*, **27**, 478 (1963).
- 6) C. G. C. Chester and A. T. Bull, *Biochem. J.*, **86**, 28, 31, 38, (1963).
- 7) K. Horikoshi, H. Koffler and K. Arima, *Biochim. Biophys. Acta*, **73**, 267 (1963); H. Tanaka and H. J. Phaff, *J. Bacteriol.*, **89**, 1570 (1965); Y. Kobayashi, H. Tanaka and N. Ogasawara, *Agric. Biol. Chem.*, **38**, 959 (1974); *idem*, *ibid.*, **38**, 973 (1974); H. Tanaka, Y. Kobayashi and N. Ogasawara, *ibid.*, **38**, 967 (1974).
- 8) K. Nishizawa, I. Morimoto, N. Hanada and Y. Hashimoto, *Arch. Biochem. Biophys.*, **96**, 152 (1962).
- 9) K. Horikoshi and Y. Atsukawa, *Agric. Biol. Chem.*, **37**, 1449 (1973).
- 10) J. Monreal, F. D. E. Uruburu and J. R. Villanueva, *J. Bacteriol.*, **94**, 241 (1967).
- 11) A. T. H. Abd-El-Al and H. J. Phaff, *Biochem. J.*, **109**, 347 (1968).
- 12) H. Horitsu, T. Satake and M. Tomoyeda, *Agric. Biol. Chem.*, **37**, 1007 (1973).
- 13) A. Kaji, T. Ohsaki and O. Yoshihara, *Nippon Nôgeikagaku Kaishi*, **45**, 278 (1974).
- 14) H. Ohashi and H. Iizuka, *ibid.*, **45**, 461 (1971).
- 15) M. Kawai, *ibid.*, **47**, 473 (1973).
- 16) S. Yamamoto, J. Fukuyama and S. Nagasaki, *Agric. Biol. Chem.*, **38**, 329 (1974); S. Nagasaki, J. Fukuyama, S. Yamamoto and R. Kobayashi, *ibid.*, **38**, 349 (1974).
- 17) J. S. D. Bacon, Abstracts of papers, Third International Symposium on Yeast Protoplasts, Salamanca, Spain, October, 1972, p. 29.
- 18) F. I. Huotari, T. E. Nelson, F. Smith and S. Kirkwood, *J. Biol. Chem.*, **243**, 952 (1968); T. E. Nelson, J. Johnson, Jr., E. Jantzen and S. Kirkwood, *ibid.*, **244**, 5972 (1969); T. E. Nelson, *ibid.*, **245**, 869 (1970).
- 19) W. J. Whelan, "Methods in Carbohydrate Chemistry," Vol. I, ed. by R. L. Whistler, Academic Press, New York and London, 1962, p. 330.
- 20) Y. Urushihara and H. Arakawa, "Gatterman-Wieland Yukikagaku Jikkensho," Kyoritsu Shup-

- pan Co., Tokyo, 1959, p. 356.
- 21) S. P. Rogovin, V. E. Sohns and E. L. Griffin, Jr., *Ind. Eng. Chem.*, **53**, 37 (1961).
- 22) Y. Hashimoto and J. Fukumoto, *Nippon Nôgeikagaku Kaishi*, **43**, 317 (1969).
- 23) C. G. Anderson and H. Raistrick, *Biochem. J.*, **30**, 16 (1936).
- 24) M. Kitahara and Y. Takeuchi, *Res. Bull. Fac. Agr. Gifu Univ.*, **8**, 100 (1957).
- 25) J. B. Sumner, *J. Biol. Chem.*, **47**, 5 (1921); *idem*, *ibid.*, **65**, 393 (1925).
- 26) N. Nelson, *ibid.*, **153**, 375 (1944).
- 27) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Robers and F. Smith, *Anal. Chem.*, **193**, 265 (1951).
- 28) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 29) L. Ornstein, *Ann. New York Acad. Sci.*, **121**, Art. 2, 321 (1964); B. J. Davis, *ibid.*, **121**, Art. 2, 404 (1964).
- 30) G. A. Levvy and S. M. Snaith, "Advances in Enzymology," Vol. 36, ed. by A. Meister, Interscience Publishers, New York, 1972, p. 173.
- 31) S. M. Snaith and G. A. Levvy, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 28, ed. by R. S. Tipson and D. Horton, Academic Press, New York and London, 1973, p. 402.
- 32) J. Johnson, S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti and F. Smith, *Chem. Ind.*, 820 (1963).
- 33) M. M. Kitahara and Y. Takeuchi, *Nippon Nôgeikagaku Kaishi*, **35**, 468 (1961); *idem*, *ibid.*, **35**, 474 (1961).
- 34) N. B. Chanda, E. L. Hirst and D. J. Manners, *J. Chem. Soc.*, **1957**, 1951; S. Peat, W. J. Whelan and J. G. Roberts, *ibid.*, **1957**, 3916.
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