Action Pattern of a Yeast Cell Lytic Enzyme from *Pellicularia sasaki*

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General action pattern of the enzyme, which is isolated from the culture medium of *Pellicularia sasaki* No. 89 and disrupts the living yeast cells, was established through the hydrolysis kinetics and the quantification of the hydrolysis products from β-1,3-glucans, to be a higher oligosaccharide producing type of endo β-1, 3-glucanase. The mechanism on the binding site is proposed to explain the action pattern and specificity of the enzyme.

From the results of pH dependence of kinetic parameters and of the chemical modification of carboxyl residues with Woodward's reagent K, it is indicated that carboxyl and amino groups were probably involved in the catalytic site.

INTROUDCTION

The purification and characteristics of an endo β-1,3-glucanase from *Pellicularia sasaki* No. 89 have been reported in the previous paper.[1] The action pattern of the enzyme has not been elucidated. The present paper deals with the study on the nature of the active site by chemical modification of amino acid residues with structure-function selective reagents, and on the action pattern of the enzyme.

MATERIALS AND METHODS

Enzyme — The endo β-1,3-glucanase was prepared by the procedure described in the previous paper.[1] The $E_{1cm}^{1\%}$ at 280 nm of 15.75 was used for the determination of the enzyme concentration.

Chemicals — To identify various states of amino acid residues in the tertiary structure of protein in term of their reactivities, the following structure-function selective reagent was used. N-Ethyl-5-phenyl isoxazolium-3'-sulfonate (Woodward's reagent K) was purchased from Fluka Ag, Chemische Fabrik and recrystallized three times from 1N HCl with acetone before use. ^{3}H-reduced laminaran was prepared from laminaran by the reduction with NaB^3H_4. All other chemicals were obtained commercially and were recrystallized or redistilled when necessary.

Enzyme assay and analytical procedures — Enzyme assay, analytical methods and chromatographic procedures were the same as have been reported in the previous

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paper.[2~8]

Photooxidation of histidine residue — The histidine residue in the enzyme was photooxidized at 10°C in the presence of 0.05 % Rose bengal under the conditions similar to those reported by Westhead.[9]

Modification of carboxyl group — The enzyme was treated with Woodward's reagent K under the conditions similar to those reported by Brake and Weber.[10]

Measurement of radioactivity — Aliquots of radioactive solutions were added to vials containing 10 ml of toluene scintillation fluid and counted in a Packard Model 3303 scintillation spectrometer. Correction for quenching was made by the external standard method.

RESULTS AND DISCUSSION

Identification of Functional Groups

pH dependence of kinetic parameters — Before the determination of kinetic paraters, the effect of pH on the activity of the enzyme was reexamined precisely. The enzyme was stable in the pH range of 4 to 7 when treated for 10 minutes at 80°C. Beyond this range, it became markedly unstable. However, it is possible to obtain the useful data of the initial velocity within the pH range of 3 to 8 by monitoring the time course of the reaction and by extrapolating the initial velocity.

At low concentrations of substrate, the initial velocity of hydrolysis of yeast glucan followed simple and hyperbolical Michaelis-Menten kinetics. At higher concentrations of substrate, the reaction velocity decreased. Therefore, the Michaelis parameters were determined at low concentrations of substrate on the assumption that the intercept on the base line gave a value for K_m.

The Michaelis parameters of the enzyme varied with pH as shown in Fig. 1. Following the interpretations of Dixon plot by Dixon[11] and Cleland,[12] there appear to be two groups (pK values close to 2.8 and 7.5) in the enzyme. These groups
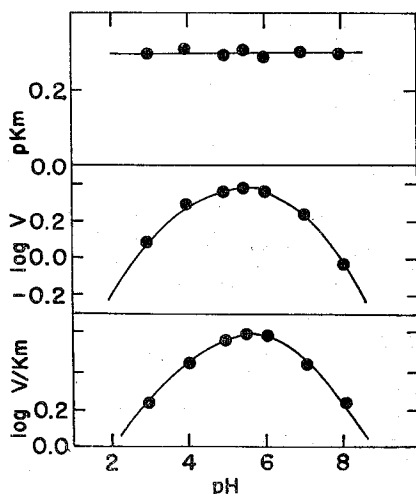

Fig. 1. Dependency of Rate Parameters on pH for the Enzyme.

are probably carboxyl group with a pK of 2.8 and the protonated imidazole ring of histidine residue or amino group with a pK of 7.5.

Thermodynamic parameter (standard enthalpy change) for the two groups in the enzyme as determined by the van't Hoff analysis were +1.5 Kcal per mole for the group with a pK of 2.8 and +1.1 Kcal per mole for the group with a pK of 7.5, suggesting these groups are carboxyl group and amino group, respectively.

These observations make it likely that a carboxyl and amino groups are parts of the catalytic mechanism.

Chemical modification of the enzyme — As a histidine residue was presumed to be one of catalytic groups that is titrated at pH 7.5, the effect of dye-activated photooxidation on the enzyme was investigated with Rose bengal. Photooxidation at pH 6.0 caused no loss of the activity of the enzyme, indicating no histidine residue in the enzyme is essential for the activity of the enzyme. An attempt through the use of specific reagent was made to confirm this result. For the carbethoxydation of all accessible histidine residues in the enzyme, the enzyme was treated with 0.5 mM diethylpyrocarbonate at pH 6.5. The treatment caused no inhibition even after 60 minutes treatment. Difference spectra made between treated and untreated enzyme during the course of the reaction indicated that one histidine residue was carbethoxydated. Because the enzyme contains one histidine residue as described in the previous paper,[1] these experiments rule out any possibility that the histidine residue is involved in the catalysis.

In an effort to ascertain what role, if any, carboxyl group plays in the catalytic action of the enzyme, experiments employing Woodward's reagent K were attempted. The enzyme rapidly inactivated with Woodward's reagent K (Fig. 2). About 80 % inhibition could be attained by increasing the molar ratio of the reagent to the enzyme to 2,000:1. However, this resulted in considerable precipitation of the protein. The rate of inactivation obeied psudo-first order kinetics for about 3 minutes, after which loss of reagent due to a base-catalyzed rearrengement became significant.
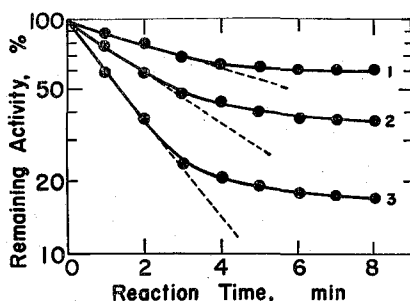

Fig. 2. Effect of Woodward's reagent K on the Enzyme Activity. The modification of the enzyme (112.1μg) was carried out in the presence of varying concentrations of Woodward's reagent K in pH 6.0 at 20°C.
1, Woodward's reagent K, 1.99×10^{-3} M.
2, Woodward's reagent K, 4.97×10^{-3} M.
3, Woodward's reagent K, 9.95×10^{-3} M.

Determination of Action Pattern

Action of enzyme on linear β-1,3-glucan (Pachyman) — Pachyman in the form of an insoluble dispersion was incubated with the enzyme. The main product produced over the whole course of hydrolysis, was laminarapentaose on the basis of the results of paper chromatography and gel filtration on Bio-Gel P-2 as shown in Fig. 3.
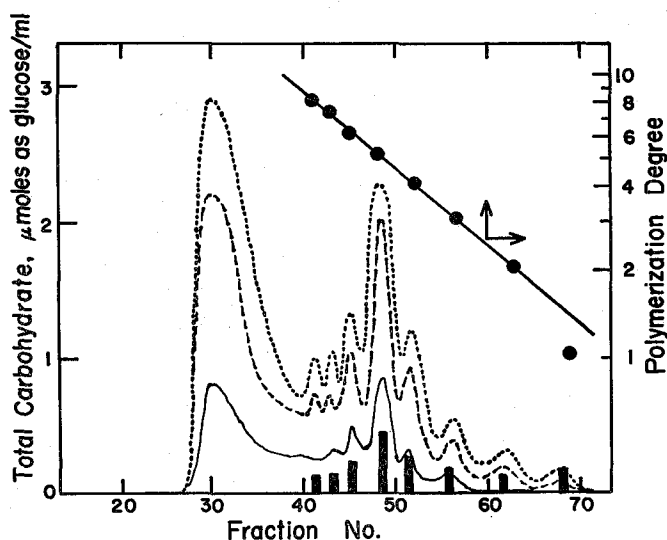

Fig. 3. Hydrolysis of Pachyman
    A reaction mixture containing 550 mg of pachyman and 500 μg of the enzyme in a total volume of 100 ml of 0.05 M sodium acetate buffer, pH 6.0 was incubated at 40°C with gentle shaking. Sample of 5 ml was removed at prescribed time. After the concentration of the sample, sample was fractionated on Bio-Gel P-2 column (0.9 × 150 cm). The flow rate was 5 ml per hour. Fractions of 1 ml were collected.
    ————: 1 hour. — — — —: 9 hours. - - - - -: 27 hours.
    Stakes represent molar ratio of oligosaccharide

Action of enzyme on β-1,3-linked chain interrupted by β-1,6-linkages (Laminaran) — Laminaran was hydrolyzed by the enzyme to give mainly higher oligosaccharide along with a small amount of glucose and lower oligosaccharides. As described before, the reaction velocity fell off at high concentration of substrate. To characterize the phenomenon, the higher oligosaccharide isolated from the reaction mixture by Bio-Gel P-2 gel filtration was hydrolyzed with the enzyme. The degree of polymerization of the higher oligosaccharide isolated was determined to be about 10 on the basis of the relationship between molecular weight and elution volume. After the enzymatic hydrolysis of the higher oligosaccharide, gel filtration showed that the products were a series of saccharides from glucose to heptaoligosaccharide in an equimolar ratio (Fig. 4). There was no demonstrable attack on β-1,3-oligosaccharides (from trisaccharide to heptasaccharide) as examined by paper chromatography. The observations suggest that the enzyme requires a β-1,3-long chain of consecutive glycosyl residues for full activity. The inference that a series of glycosyl
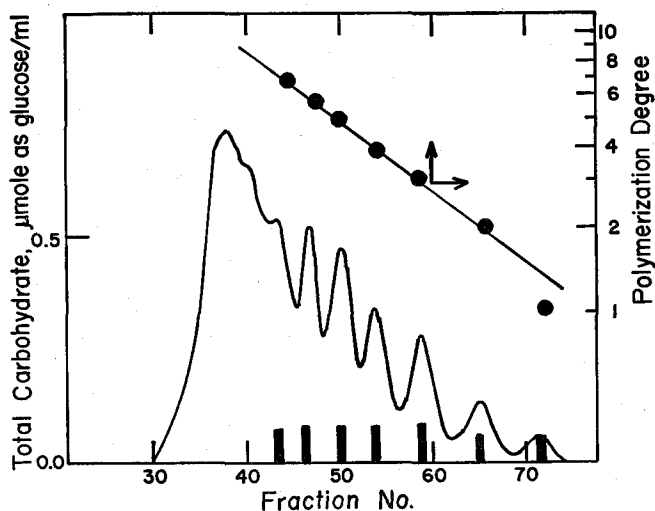
Fig. 4. Gel Filtration of the Hydrolyzate of the Higher Oligosaccharide
Isolated from the Digeste of Laminaran.

A reaction mixture containing 10 μmoles as glucose of the higher
oligosccharide and 5 μg of the enzyme was incubated at 40°C for
12 hours with gentle shaking. After the termination of the reaction,
the reaction mixture was concentrated to 1 ml and fractionated
on Bio-Gel P-2 column as described in Fig. 3. Stakes represent
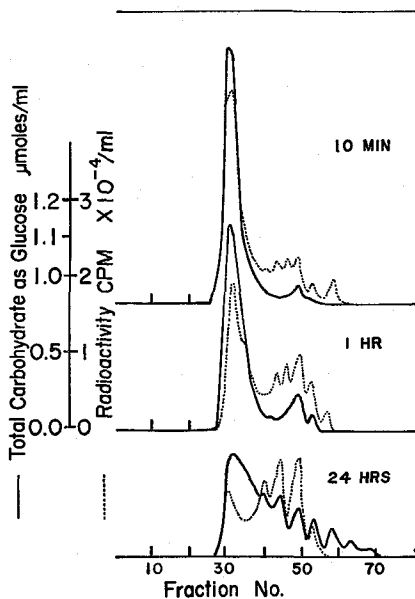molar ratio of oligosaccharide.

Fig. 5. Hydrolysis of ^{3}H-reduced Laminaran.

A reaction mixture containing 20 mg of ^{3}H-reduced laminaran and
100 μg of the enzyme in 4 ml of 0.05 M sodium acetate buffer,
pH 6.0 was incubated at 40°C with gentle shaking. Sample of 1 ml
was removed at prescribed time and fractionated on Bio-Gel P-2
column (0.9 × 150 cm). The flow rate was 5 ml per hour.

residues are bound at the active site has led to a subsite of the enzyme.

To check further on the action pattern of the enzyme on laminaran, ^{3}H-reduced laminaran was hydrolyzed. The reaction mixture was gel-filtrated on Bio-Gel P-2 column at appropriate intervals, and the radioactivity and total carbohydrate in the eluate were estimated (Fig. 5). At the early stage of the hydrolysis, little amounts of the radioactivity were found in the mono- and di-saccharide fractions, indicating these sugars did no arise as primary product from the reducing end of laminaran, and are probably a secondary products. The radioactivity of the tri-saccharide fractions once increased, and decreased. This phenomenon may be explained by transglucanosylation occurred concomitantly with hydrolysis.

After the reaction for 24 hours, the reaction mixture was divided into two portions; one was analyzed by Bio-Gel P-2 gel filtration, and the other was reduced
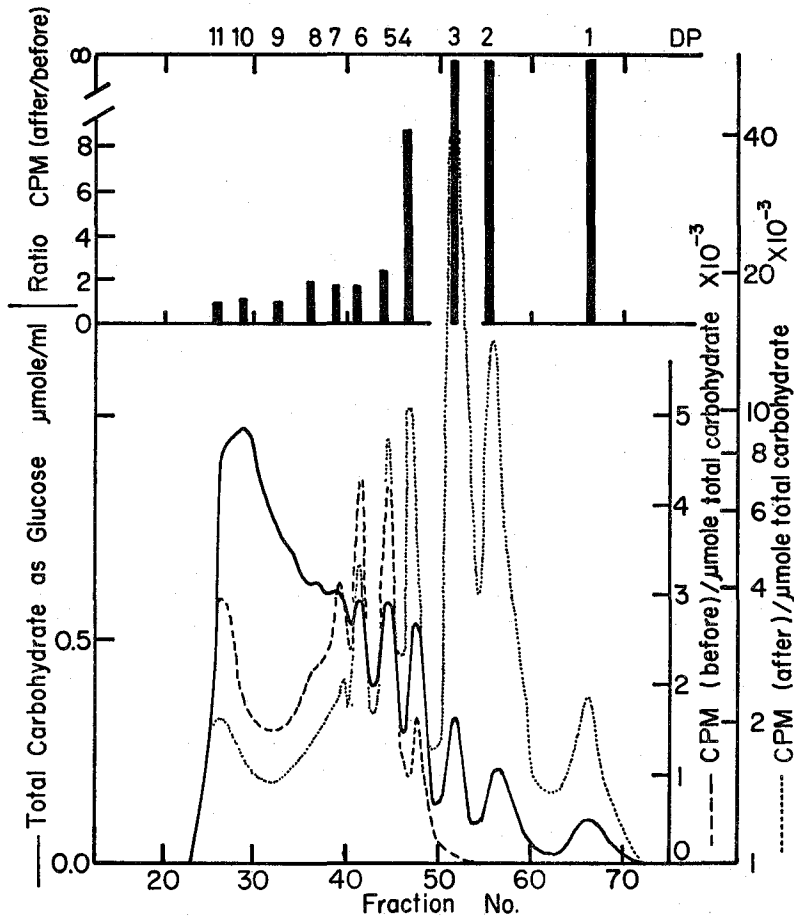

Fig. 6. Gel Filtration of ^{3}H-reduced Laminaran Hydrolyzate on Bio-Gel P-2.
A reaction mixture containing 10 mg of ^{3}H-reduced laminaran and 50 μg of the enzyme in 2 ml of 0.05 M sodium acetate buffer, pH 6.0 was incubated at 40°C for 24 hours. Sample was divided into two portions and treated as described in the Text. Bio-Gel P-2 fractionation was the same as described in Fig. 3.

with NaB³H₄ and followed by the gel filtration. The increment of radioactivity in a corresponding sugar fraction before and after the reduction with NaB^3H_4 is directly proportional to the amount of the newly formed reducing end in the sugar fraction. The two elution profiles, monitored by the measurement of total carbohydrate, are identical as shown in Fig. 6. For the lower oligosaccharides, such as mono-, di-, tri- and tetra-saccharide, much increment was determined, indicating these lower oligosaccharides are secondary products from primary product having the poly-merization degree of about 10.

Action of enzyme on mixed-linked and highly-branched β-1,3-glucan (Yeast glucan) —
Yeast glucan was incubated in the form of an insoluble dispersion under the standard conditions with the enzyme. Hydrolysis kinetics was followed by measuring the release of reducing sugar and the solubilization of total carbohydrate. With rapid decrease in absorbance at 610 nm, there was an increase in the solubilization of
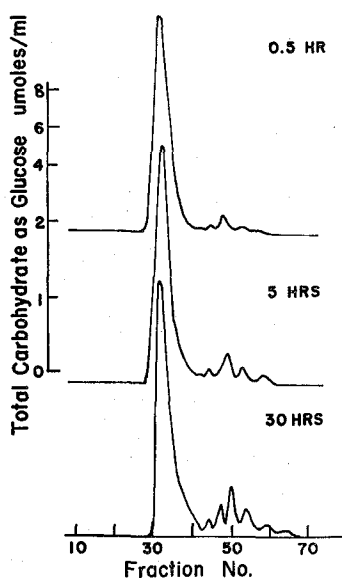

Fig. 7. Hydrolysis of Yeast Glucan.
A reaction mixture containing 500 mg of yeast glucan and 700 μg
of the enzyme in 100 ml of 0.05 M sodium acetate buffer, pH 6.0
was incubated at 40°C with gentle shaking. Sample of 5 ml was
removed at intervals, concentrated to 1 ml under reduced pressure
at 40°C, and fractionated on Bio-Gel P-2 as described in Fig. 3 to 6.

total carbohydrate. Even after exhaustive hydrolysis of yeast glucan, no produc-tion of glucose was observed. Decrease of apparent polymerization degree (total carbohydrate solubilized per reducing sugar released) was noted with the progress of the reaction, indicating a sizable portion of the yeast glucan, although in solu-tion, was hydrolyzed further into lower reducing oligosaccharides. The enzyme produced a series of β-1,3-oligosaccharides, mainly laminarapentaose, as reaction products of the action on yeast glucan Fig. 7).

Transglucanosylation — The fact that the radioactivity of the trisaccharide produced from ³H-reduced laminaran decreased with the progress of the reaction, together with the low rate of the enzyme action on yeast glucan, laminaran and pachyman at high concentrations, suggested that transglucanosylation occurred concomitantly with hydrolysis. To check on the possibility of the transfer of glucanosyl residues, 10 mᴍ laminaran was hydrolyzed in the presence of 200 mᴍ p-nitrophenyl-β-glucoside. The reaction mixture was chromatographyed at appropriate intervals, and the chromatograms were detected under ultraviolet light. The enzyme did not catalyze the hydrolysis of p-nitrophenyl-β-glucoside at any observable rate. The transglucanosylation to enzymatically synthesize p-nitrophenyl-β-oligosaccharides (slower moving compounds than p-nitrophenyl-β-glucoside) occurred in addition to hydrolysis of laminaran.

ACKNOWLEDGMENT

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