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Some Properties of a Yeast Cell Lytic Enzyme from *Pellicularia sasaki*, Especially Referring to Thermal Denaturation

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Abbreviation used: SDS, sodium dodecyl sulfate.

Heat stability of a yeast cell lytic enzyme, purified from the culture of *P. sasaki* No. 89, was investigated. The enzyme retained 60 % of its original activity even after the treatment at 100°C for 10 min. The complete inactivation was achieved by the treatment at 120°C for 20 min. The substrate specificity of the enzyme and the reaction products were not altered during the inactivation process. It was revealed by the SDS gel electrophoresis that the enzyme protein would undergo heat denaturation via partially inactivated intermediate which can be simply separated. According to Imahori's definition, this enzyme can be classified as "thermoresistant" enzyme.

KEY WORDS: Thermoresistant enzyme/ Lytic enzyme, on yeast cell/ β-1,3-Glucanase/

INTRODUCTION

Purification and characterization of a yeast cell lytic enzyme produced by *P. sasaki* were attempted in the preceding experiment and the results have been reported in this journal.[1,2] It has been stated in the report, that the crude enzyme preparation was inactivated by heat treatment at 70°C for 10 min., whereas a purified enzyme retained 50 % of its original activity even after the treatment at 100°C for 10 min. The enhanced stability showed by the purified enzyme may be due to the exclusion of protease which might decompose the lytic enzyme during the heat treatment and/or activity assay. Whatever the reason is, it is undoubtfull that this enzyme is remarkably stable to heat inactivation. Therefore this report deals with the detailed studies on the thermal stability of this enzyme.

MATERIALS AND METHODS

Enzyme The lytic enzyme, produced by *P. sasaki* and purified following the procedure described in the preceding paper,[1] was used as sample enzyme through out this experiment.

Activity assay The enzyme activity was assayed using viable yeast cells, yeast glucan, or laminarin as substrate. Details of the procedures have been described in a previous paper.[3]

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Chemicals All the Chemicals used were obtained commercially.

Paperchromatography Paperchromatography was conducted according to the descending method using Toyo filter paper No. 50, anilin hydrogen phthalate, and a mixture of n-butanol, ethanol and water (5:2:2).

Electrophoresis Disc electrophoresis was performed according to the method described by Ornstein[4] and Davis.[5] SDS disc electrophoresis was carried out following the procedures of Fairbanks *et al*[6] with varying concentration of SDS (0.1 %, 0.5 %, 1 %, and 2 %). Molecular weight was calculated from the movility in the SDS gel electrophoresis following the suggestion of Weber and Osborn[7] with lysozyme, egg albumin, and catalase as reference protein. SDS disc electrophoresis of dansylated enzyme was performed according to the method described by Kato *et al.*[8]

Heat treatment of enzyme Heat treatment of enzyme was conducted in a sealed tube maintained in a boiling water bath or autoclave. The sample enzymes treated under various conditions were cooled in an ice bath and subjected to further experiments.

RESULTS AND DISCUSSION

Inactivation of enzyme at high temperature As stated above, the enzyme is comparatively heat stable. Therefore, heat stability at high temperatures (100°C and 120°C) was investigated. Fig. 1 shows the remaining activity of the enzyme treated at 100°C. Even after 30 min-treatment some of the activity still remained. This enzyme, therefore, can be said to be unusually heat stable enzyme, although after 20 min-treatment at 120°C the enzyme lost all of its activity. In spite of such high heat stability, the enzyme, as stated previously,[1] has a maximum activity at 60°C and is inactive at temperatures higher than 80°C. Further, this organism is a meso-

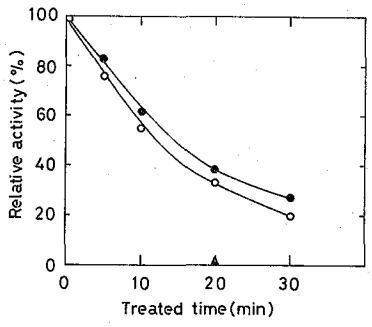

Fig. 1. Inactivation of Enzyme at High Temperature.
The enzyme was treated at 100°C (○—○, ●—●) or 120°C (▲) for various times. After rapid cooling, an aliquot was removed for remaining activity assay (1.59 μg for yeast cell lytic activity, 2.39 μg for yeast glucan hydrolysis activity). ●—●, Yeast cell lytic activity; ○—○, Yeast glucan hydrolysis activity.

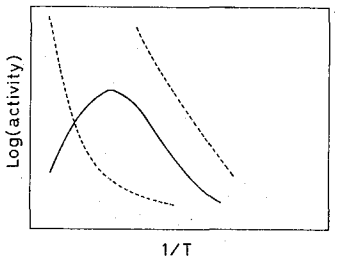

Fig. 2. Schematic Representation of Arrhenius Plot for Enzyme Activity.
Solid line, lytic enzyme from *P. sasaki*. Dotted line, thermophile's enzyme cited from Ref. 8.

phile having optimum temperature for growth at about 30°C. These facts seems to support, following Imahori's definition,[9] the "thermoresistant" nature of this enzyme but not "thermostable" nature. Further, the difference in heat stability between this enzyme and thermophile's enzyme is distinctly demonstrable by the schematic representation of Arrhenius plot (Fig. 2).

Substrate specificity of the heat treated enzyme The substrate specificity of the enzyme has not been affected by the heat treatment (Table I), though the activity has been

Table I. Substrate Specificity of Heat-treated Enzyme.

Carbohydrate	Reducing Sugar Formed (μmole per ml)	
	Native Enzyme	Heat-treated Enzyme[a]
Pramylon	0.32	0.12
Pachyman	0.34	0.17
Laminarin	0.11	0.06
Yeast glucan	0.14	0.07
Curdran	0.02	0.02
Screlotan	0.00	0.00
Lichenan	0.00	0.00
Cellulose	0.00	0.00
Luteose	0.00	0.00
Amylose	0.00	0.00
Amylopectin	0.00	0.00
Starch, soluble	0.00	0.00
Glycogen	0.00	0.00
Dextran, high MW	0.00	0.00
low MW	0.00	0.00
Yeast mannan	0.00	0.00
Dextrin	0.00	0.00
Xylan	0.00	0.00
Pullulan	0.00	0.00
Inulin	0.00	0.00

Reaction mixture containing 5 mg substrate and 6.44 μg of enzyme in 1 ml of 0.1 M sodium acetate buffer (pH 6.0) was incubated at 40°C for 2 hr and reducing sugar formed was determined by the Somogyi-Nelson method.

a) Treated at 100°C for 10 min.

lowered some extent. The reaction products of the enzyme were also not affected by the heat treatment, unlike the lytic enzyme obtained from a *Basidiomycetes* by Made *et al*,[10] as evidenced by the paperchromatographic analysis (data not shown).

Alteration of spectrum of the enzyme by heat treatment Conformational change of the enzyme by heat treatment was examined by means of difference spectrum measurement, although the measurement of the spectrum of circular dichroism is desirable for this purpose. As is shown in Fig. 3, the spectrum of the enzyme is gradualy altered by the heat treatment, that is, absorbance at near 280 nm gradualy decreased, indicating that the masking or disappearance of a chromophore proceeded during

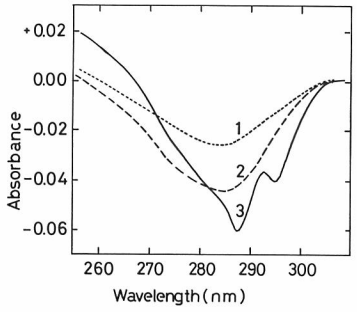

Fig. 3. Ultraviolet Difference Spectrum of Heat-treated Enzyme.
Native enzyme (1.37×10^{-5} M in 0.01 M sodium acetate buffer, pH 5.8) was placed in reference compartment of a spectrophotometer and heat-treated enzyme was placed in the sample compartment. Curve 1, 100°C, 10 min-treated enzyme; Curve 2, 100°C, 30 min-treated enzyme; Curve 3, 120°C, 20 min-treated enzyme.

the denaturation process. The sample enzyme treated at 120°C for 20 min. gave a typical spectrum for denaturated protein.

Effect of heat treatment on the primary structure of the enzyme By means of disc electrophoresis, the process of the heat denaturation of the enzyme was examined. The enzyme treated at 100°C for 10 min. gave the same one protein band as that of native enzyme, whereas that treated at 100°C for 30 min. gave two faint saterite bands in addition to the same band (data not shown). Thus the denaturation of the enzyme protein occured gradually at 100°C. The enzyme treated at 120°C for 20 min. did not give a distinct protein band but gave a diffused dull smear, suggesting complete denaturation. The fragmentation of the enzyme molecule, which was

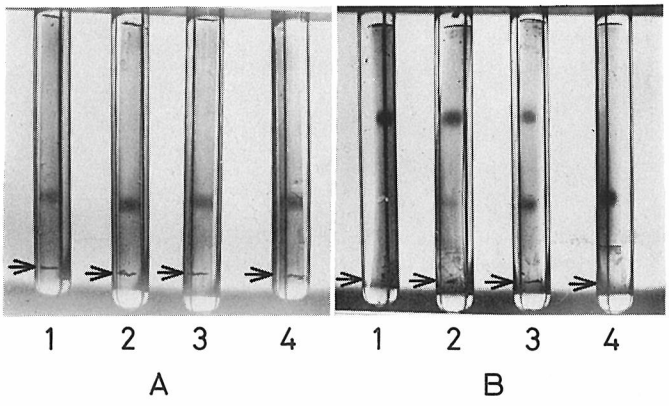

Fig. 4. SDS Gel Electrophoresis of Heat Treated Enzyme.
The sample enzymes (24.7 μg each) were respectively incubated with varying amount of SDS (0.1%, 0.5%, 1%, and 2%) in 0.05 M Tris-HCl buffer, pH 7.4 at 40°C for 2 hr. A portion of each sample, 9.88 μg, was analyzed by acrylamide (5.6%) gel electrophoresis (1.5 hr, 8 mA/tube). The arows show the position of tracking dye. A, incubated and electrophoretized under 2% SDS conc.. B, incubated and electrophoretized under 1% SDS conc.. 1, native enzyme; 2, 100°C, 10 min-treated enzyme; 3, 100°C, 30 min-treated enzyme; 4, 120°C, 20-min treated enzyme.

caused by the cleavage of peptide bonds, was denied by the result of the SDS disc electrophoresis of the dansylated samples in both 12.5 % and 7.5 % acrylamide gels (data not shown).

With an intention to reconfirm this result, determination of the molecular weight of the heat treated enzyme was conducted according to Fairbanks' SDS gel electrophoresis. As shown in Fig. 4-B, sample enzymes migrated in three different ways, presumably, according to the extent of denaturation. The native enzyme, used as control, migrated in single band with a relative mobility of 0.36 (corresponds to molecular weight of 90,000). This value differed considerably from the values obtained by the gel filtration method (25,000) and ultracentrifugation method (28,000). This abnormal movility showed by the native enzyme is enough to deduce that the native enzyme may be hard to couple with SDS under the usual conditions (1 %). Therefore, the electrophoresis was repeated under 2 % concentration of SDS. As shown in Fig. 4-A, all sample enzyme migrated in one band with a relative mobility of 0.71 which correspondes to molecular weight of 25,000.

From these observations one can conclude that heat treatment does not cleave the enzyme molecule but causes some conformational change and that a native enzyme is hard to couple with SDS but the heat treated enzyme (even if it is not inacti-

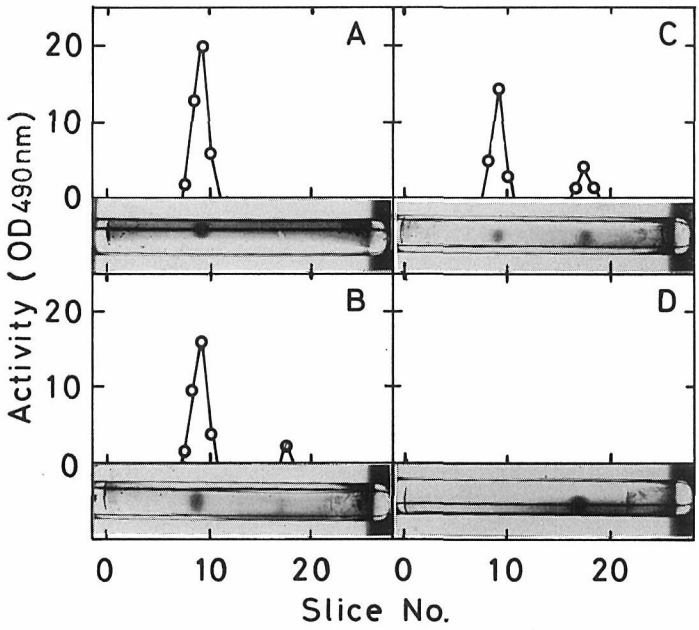

Fig. 5. SDS mediated Separation of Intermediate in Heat Denaturation Process.
The sample enzyme, 57.8 μg, was analyzed, in duplicate, by disc electrophoresis of Fairbanks *et al.*[6] A gel was sliced into 3 mm segments and each segment was incubated with 1 ml of 0.1 M sodium acetate buffer, pH 6.0, for 24 hr at 5°C. An aliquot (0.5 ml) was removed for the assay of yeast glucan depolymerization activity. The other gel was stained with Coomassie brilliant blue. A, native enzyme; B, 100°C, 10 min-treated enzyme; C, 100°C, 30-min treated enzyme; D, 120°C, 20 min-treated enzyme.

vated) brings some structural change and easily becomes complex. Possibility of the separation of denaturated enzyme from the enzyme solution in denaturation process was suggested from the latter conclusion.

SDS mediated separation of an intermediate in denaturation process On the basis of the above suggestion, the differential separation of native enzyme and denaturated enzyme was attempted by means of SDS disc electrophoresis. Before the electrophoresis, the effect of SDS on the enzyme activity was investigated. When treated the enzyme with various concentrations of SDS at 40°C for 2 hr., enzyme's activity was not affected below 3 % concentration. Fig. 5 shows the differential separation of the native enzyme (slow migrating protein) and denaturated enzyme (fast migrating protein) on acrylamide gel columns. The fast migrating proteins are considered to have received some structural change from the viewpoint of ability to couple with SDS. However, since the fast migrating protein of the 100°C treated enzymes still retained some activity, they are thought to be an intermediate between native enzyme and denaturated one. By this experiment it was revealed that the enzyme protein would undergo heat denaturation via partially inactivated intermediate which can be separated simply. The intermediate, if obtained in sufficient amounts, will be a good help for the study of the mechanism of heat stability of this enzyme.

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