Physicochemical, Chemical and Enzymatic Properties of a Crystalline Yeast Cell Lytic Enzyme from a *Rhizopus* Mold†

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A kind of β -1,3-glucanase which degrades the cells of living yeasts has been crystallized from a culture filtrate of a strain resembling *Rhizopus chinensis* and has been studied from physicochemical, chemical and enzymological perspectives. The physicochemical and chemical properties revealed by the studies are as follows: molecular weight, 22,000~24,000; molecular extinction coefficient, $2.335 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$; $s_{20,w}^0$, 2.47S; $D_{20,w}^0$, 9.46×10^{-7} ; isoelectric point, pH 5.2; Stokes radius, 23 Å; intrinsic viscosity, 0.0336 dl.g^{-1} ; axial ratio, 3.9; amino acid composition, 205 residues of 17 amino acids; N-terminal amino acid, valine; C-terminal amino acid sequence, -Asp-Thr-Ala-Gly-Ser; optimum pH 5.5; optimum temperature, 40°C; stability stable at pH 4.5~6.0 or below 80°C. Though the exact bond split by the enzyme could not be identified, within the scope of this paper the enzyme's mode of action suggested it to be either a "more random" or a "debranching" type of β -1,3-glucan-glucanohydrolase.

A number of papers showing significant advances in the understanding of enzymic disruption of the cells of living yeasts (or enzymic formation of the protoplasts) have appeared,^{1~41)} but only a few researchers^{32~41)} have succeeded in attempts to purify the enzyme responsible for such action. The authors have crystallized β -1,3-glucanase having such action from a culture filtrate of a *Rhizopus* sp.^{35,36)} and a species of Fungi Imperfecti^{40,41)} respectively. Independently, Mada *et al.*,³⁴⁾ Kitamura *et al.*,^{33,37)} and Doi *et al.*^{32,39)} also have isolated β -1,3-glucanases, which degraded living yeast cells, from culture filtrates of *Irpex lac-teus* M and *Arthrobacter luteus*.

It has been recognized by some researchers^{19,23)} that general β -1,3-glucanase evidences little activity against the cells of living yeast. In this respect, the above mentioned β -1,3-glucanases seems to have unique properties when

compared with the general β -1,3-glucanase wide spread in microorganisms. However, with the exception of the Fungi Imperfecti^{40,41)} and *Arthrobacter*³⁹⁾ glucanases, the enzymatic and physicochemical properties of those glucanases have not yet been fully elucidated.

The present paper describes physicochemical, chemical and enzymic properties of the *Rhizopus* glucanase, of which purification, crystallization and substrate specificity have been tentatively reported in a previous paper.³⁶⁾

MATERIALS AND METHODS

Microorganism. The strain described in a previous paper³⁶⁾ served for production of the yeast cell-lytic enzyme. The organism was readily recognized by its characteristic rhizoid formation to belong to the genus *Rhizopus*. The morphological, cultural and physiological characteristics of the organism were systematically investigated according to Iizuka's classification method⁴²⁾ of the genus *Rhizopus*.

Production of the lytic enzyme. After extensive investigation the following conditions were selected for the production of the lytic enzyme: medium: 5% baker's yeast and 0.1% $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 6.0; culture conditions: 30°C for 72 hr with shaking.

Enzymes used. Purification and crystallization of the sample enzyme were conducted as described in a

† Studies on Fungal Enzymes Active in Hydrolysing Yeast Cell Wall, Part III. Part II; see Reference 41). This work was supported partly by the grant, No.856056 from the Ministry of Education, Japanese Government. A part of this report was presented at the 272nd Meeting of the Kansai branch of the Agr. Chem. Soc. of Japan, held at Kyoto, May 22, 1971 and at the annual Meeting of the Soc. of Ferment. Technol., Japan, held at Osaka, November 17~20, 1971.

previous paper³⁶⁾; crystals of the enzyme have also been shown in the same paper. To compare the mode of action of the sample enzyme to other β -glucanases, endo β -1,3-glucanase and endo β -1,6-glucanase were prepared from the same organism. Production, purification procedures and properties of those enzymes will be reported later.

Assay of enzymes. Assay of the lytic enzyme involved a measurement by the phenol-sulfuric acid method or Somogyi-Nelson method of the liberation of reducing sugar from yeast glucan or laminarin or a measurement of turbidity change due to the disruption of living yeast cells. Details of the procedures have been described in a previous paper.⁴³⁾ Assay of β -1,3-glucanase and β -1,6-glucanase involved a measurement by the 3,5-dinitrosalicylic acid (DNSA) method of the liberation of reducing sugar from laminarin and luteose respectively.

Ultracentrifugation analysis. Sedimentation velocity, sedimentation equilibrium, and diffusion experiments were performed in a Hitachi Model 282 analytical ultracentrifuge equipped with Schlieren optics, Rayleigh interference optics and photoelectric scanner. Prior to centrifugation an enzyme solution was dialyzed against M/100 acetate buffer, pH 5.0, containing M/10 potassium chloride for 72 hr at 5°C with several changes of the buffer. The final dialyzate was used as a reference solution for all runs. All measurements were made at 20°C.

Disc electrophoresis. Disc electrophoresis on polyacrilamide gel was performed according to the procedures of Davis,⁴⁴⁾ Ornstein⁴⁴⁾ or Weber and Osborn.⁴⁸⁾

Viscosity measurements. Viscosity was measured in a micro Ubbelohde type capillary viscometer with flow times for water of 150 sec at $20.00^\circ \pm 0.01^\circ\text{C}$. All measurements were made at $20.00^\circ \pm 0.01^\circ\text{C}$.

Amino acid analysis. Quantitative analysis of amino acids of the enzyme protein was achieved by the conventional ion exchange chromatography using Yanagimoto amino acid analyzer Model LC-2 (Yanagimoto Co., Ltd., Kyoto) equipped with a 15 cm and a 150 cm column of Amberlite CG 120 Type 3. In addition to this, the following methods were employed for respective amino acids; the method of Scoffen⁴⁷⁾ was used for the assay of tryptophan, the method of Goodwin and Morton⁴⁸⁾ was used for the assay of tyrosine and tryptophan, cysteine was determined as cysteic acid and S-carboxymethylcysteine according to the method of Crestfield *et al.*⁴⁹⁾ and Moore *et al.*⁵⁰⁾ respectively, and methionine was estimated from a number of component peptides which separated in a acrylamide gel column with disc electrophoresis after the cyanogen bromide treatment described by Steers *et al.*⁵¹⁾

Analyses for N-terminal amino acid and C-terminal amino acid. N-Terminal amino acid of the enzyme was determined by the dinitrophenyl (DNP) method of Sanger,⁵²⁾ improved phenylthiohydantoin (PTH) method of Erikson and Sjöquist⁵⁴⁾ and Dansyl (DNS) method of Hartley.⁵⁵⁾ The dinitrophenylation was done on a native enzyme, performate-oxidized enzyme, S-carboxymethylated enzyme, and sodium borohydride-reduced and S-carboxymethylated enzyme. Quantitative estimation of the DNP-amino acid was carried out following the method described by Levy,⁵⁶⁾ and identification of the PTH-amino acid and the DNS-amino acid were performed by the paper chromatography of Sjöquist⁵⁷⁾ and Boulton and Bush⁵⁸⁾ respectively.

C-Terminal amino acid was determined by the hydrazinolysis method of Akabori,⁵⁹⁾ acid-catalyzed hydrazinolysis method⁶⁰⁾ and -carboxypeptidase A method.⁶¹⁾ Amino acids released from the sample protein by the action of the carboxypeptidase A were analyzed with the amino acid autoanalyzer.

RESULTS

1. Physicochemical properties

Absorption spectrum. The absorption spectrum of the crystalline lytic enzyme was taken by a Shimadzu recording spectrophotometer Model MPS-50L (Fig. 1). In the investigated wave length range the enzyme exhibited maximum absorption at 278 nm. Assuming the molecular weight of the enzyme to be 23,000,

FIG. 1. Ultraviolet Absorption Spectra of the Crystalline *Rhizopus* Glucanase.

The enzyme protein, 4 mg, was dissolved in 10 ml of M/100 acetate buffer, pH 7.0, or of N/10 NaOH, and the absorption spectrum of the solution was recorded on a Shimadzu recording spectrophotometer model MPS 50-L. Solid line, spectrum in buffer; dotted line, spectrum in NaOH solution.

the number of tryptophan and tyrosine residues per mole of enzyme were estimated from the curve to be 2 and 9 respectively, and the molecular extinction coefficient (ϵ) was calculated to be $2.335 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Ultracentrifugation analysis. (1) The sedimentation pattern of the enzyme is shown in Fig. 2. The boundary of the enzyme moved as a single symmetrical peak, as was expected for homogeneous materials.

FIG. 2. Plots of $s_{20,w}$ and $D_{20,w}$ vs. Concentration of Protein, and Sedimentation Pattern of the Crystalline *Rhizopus* Glucanase.

The experimental conditions are given in the text. The photograph was taken (protein conc., 3.83 mg/ml) 88 min after the rotor reached a speed of 56,000 rpm. Sedimentation is proceeding from right to left. ○—○, sedimentation velocity; ●—●, diffusion coefficient.

(2) The sedimentation coefficient of the enzyme was measured at various protein concentrations. There appeared to be a slight concentration dependence in the values over the protein concentration range of 0.35 to 10.18 mg/ml studied (Fig. 2). The concentration dependence is represented by the equation

$$s_{20,w} = s_{20,w}^0 (1 - 0.0445 C)$$

With the extrapolation of $s_{20,w}$ to zero protein concentration the value of 2.47 was determined for $s_{20,w}^0$.

(3) The diffusion coefficient of the enzyme was calculated, using the maximum ordinate-area method, from the Schlieren patterns of the enzyme solutions. As is shown in Fig. 2, the diffusion coefficient ($D_{20,w}$) increased linear-

ly with increasing concentration. On extrapolating the straight line to zero protein concentration the value of 9.46 was obtained for $D_{20,w}^0$. Rate constant of the line was calculated from the slope to be $D_{20,w}^0 \times 0.0132$, and therefore the equation

$$D_{20,w} = D_{20,w}^0 (1 + 0.0132 C)$$

represents empirical value for $D_{20,w}$.

(4) The enzyme was subjected to equilibrium ultracentrifugation in aqueous and heavy water buffers according to the method described by Edelstein and Schachman.⁶²⁾ Figure 3 shows the plot of the natural logarithm of

FIG. 3. Simultaneous Determination of Partial Specific Volume and Molecular Weight of the Enzyme.

The experiment was performed according to the method described by Edelstein and Schachman.⁶²⁾ For this experiment the speed was 20,000 rpm, multi-channel cell was used, and protein concentration was 30 $\mu\text{g}/0.1 \text{ ml}$. ○—○, sedimentation equilibrium in H_2O ; ●—●, sedimentation equilibrium in D_2O .

absorbance at 280 nm against the radius square. The graphs are both linear over the entire range, with the slope in D_2O equal to 0.7894 that of the slope in H_2O . The partial specific volume of the enzyme was calculated to be 0.736 according to the method of Edelstein and Schachman, and it checks very well with the value calculated from the amino acid composition of the enzyme protein according to the method of McMeekin and Marshall.⁶³⁾

Determination of molecular weight. (1) The molecular weight of the enzyme was calculated to be 23,000 by substituting the empirical values

for $D_{20,w}^0$ and $s_{20,w}^0$ in the Svedberg equation.

(2) The molecular weight of the enzyme was determined to be 23,400 according to the sedimentation equilibrium method of Van Hold and Baldwin.⁶⁴⁾ This value is in essential agreement with that obtained above.

(3) On comparing the mobility of the enzyme on polyacrilamide gel containing sodium dodecyl sulfate with that of reference proteins, a molecular weight of 22,000 was estimated for the denatured enzyme.

Isoelectric point. The isoelectric point of the enzyme was determined to be around pH 5.2 by paper electrophoresis using Miller and Gordner's buffer⁶⁵⁾ and Toyo Roshi No. 51 filter paper as supporting material.

Stokes radius. The Stokes radius of the enzyme was obtained by molecular sieve chromatography using Sephadex G-100 according to the procedures of Acker.⁶⁶⁾ The pore radius of the column was determined with catalase, ovalbumin, chymotrypsinogen A, myoglobin and cytochrome C as standards. On the basis of the elution characteristics on the calibrated column, Stokes radius of the enzyme was calculated to be 23 Å, corresponding to a molecular weight of 24,000. On substituting this value for the Stokes radius in the Stokes-Einstein equation, the diffusion coefficient of the enzyme was calculated to be 9.01×10^{-7} cm²sec⁻¹. This value is very similar to that obtained above with the method of Ehrenberg.

Intrinsic viscosity and molecular shape. To obtain information on molecular shape, viscosity studies were conducted with an enzyme solution in M/100 acetate buffer, pH 5.0, containing M/10 potassium chloride (Fig. 4). Reduced viscosity, η_{sp}/C , was plotted against protein concentration, C . The data show that the reduced viscosity is independent of the enzyme concentration, and a value of 3.36 ml/g was obtained for the intrinsic viscosity. On substituting the observed values, 0.0336, 0.736 and 23,000 respectively, for η , $\bar{v}$ and M in the Scheraga-Manderkern equation,⁶⁷⁾ a value, 2.25×10^6 , was obtained for β . According to

FIG. 4. Plots of the Reduced Viscosity vs. Concentration of Protein.

Enzyme solution of various concentrations in M/100 acetate buffer, pH 5.0, containing M/100 KCl was subjected to viscosity measurement in a Ubbelohde type capillary viscometer. Further details are described in the text.

the Scheraga's data,⁶⁸⁾ the value means that the shape of the enzyme molecule may be a prolate spheroid with an axial ratio of 5, which is roughly in agreement with the value, 3.93, calculated from $[\eta]$ and $\bar{v}$ taking the Kuhn-Kuhn equation.⁶⁹⁾

2. Chemical properties

Amino acid composition. Table I gives the amino acid composition of the enzyme protein. From the data the molecular weight and the partial specific volume of the enzyme were calculated to be 22,400 and 0.736 respectively. The values are consistent with those obtained with other methods. After being separated into component peptides by cyanogen bromide treatment, a value of 3 for the methionine residue of the enzyme was further confirmed by disc gel electrophoresis (Fig. 5).

Subunit structure. In order to break it down into its component peptides the enzyme protein was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea, respectively. In either case enzyme protein migrated in a single band and was shown to be composed of a single polypeptide.

N-Terminal amino acid of the enzyme protein. On hydrolysis of the dinitrophenylates of native enzyme, performate-oxidized enzyme, S-

TABLE I. AMINO ACID COMPOSITION OF THE ENZYME PROTEIN

Amino acid	Number of / Mole residue / protein
Tryptophan	2 ^{a)}
Lysine	15
Histidine	4
Arginine	3
Aspartic acid	32
Threonine	14
Serine	7
Glutamic acid	13
Proline	11
Glycine	24
Alanine	21
Cysteine	0
Valine	19
Methionine	3 ^{b)}
Isoleucine	7
Leucine	8
Tyrosine	13
Phenylalanine	9

^{a)} Determined by the method of Matsubara and Sasaki.⁵²⁾ The value agreed with that obtained by the method of Scoffen⁴⁷⁾ or of Goodwin and Morton.⁴⁸⁾

^{b)} The value was confirmed with the method of Steers *et al.*⁵¹⁾

carboxymethylated enzyme, and reduced and S-carboxymethylated enzyme, 1.10, 0.82, 1.21 and 0.63 mole of DNP-valines were found per mole of respective enzyme preparations. The N-terminal residue valine was further confirmed by the phenylthiohydantoin method and Dansyl method.

C-Terminal amino acid of the enzyme protein.

On hydrazinolysis of the enzyme, about 0.68 mole of serine was released per mole of enzyme along with small amounts of glycine and alanine. On hydrazinolysis in the presence of hydrazine sulfate as catalyst, 0.77 mole of serine was released per mole of enzyme. These results support the data, from which -Asp-Thr-Ala-Gly-Ser was estimated for the C-terminal amino acid sequence, obtained with the carboxipeptidase A method (Fig. 6).

3. Enzymatic properties

Effect of pH on enzyme activity. Enzyme activity was measured at various pH from 2.0

FIG. 5. Scanning Densitogram of the Acrylamide Gel Columns on Which Native or BrCN-Treated Enzyme had been Electrophoretized.

The BrCN treatment was performed on 13.7 mg of the enzyme according to the method of Steers *et al.*⁵¹⁾ Electrophoresis was carried out following the method of Davis⁴⁴⁾ employing approximately 60 μ g (native enzyme) or 80 μ g (BrCN-treated enzyme) of enzyme, and the distribution of protein in the stained gels was recorded with the aid of a densitometer, Toyo densitorol DM-6, at a wave-length of 622 nm and with a slit of 0.22 mm.

to 8.0. As is shown in Fig. 7, maximum activity was obtained at around pH 5.5.

Effect of temperature on the enzyme activity.

Enzyme activity was measured at various temperatures from 20°C to 60°C, as shown in Fig. 7, maximum activity being obtained at about 40°C.

Effect of pH on the stability of the enzyme.

Enzyme solutions adjusted to various pH were treated at 45°C for 10 min and subsequently assayed for remaining activity under the stan-

FIG. 6. Rate of Release of Amino Acids from the Lytic Enzyme being Digested by Carboxypeptidase A.

A reaction mixture containing crystalline enzyme* (1.8 mg/ml) and diisopropylphosphofluoridate-treated carboxypeptidase A (22 μ g/ml) was incubated at 30°C and pH 8.0. Aliquots of the reaction mixture were removed at intervals indicated, heated in boiling water for 5 min, and the amino acids in the solution were assayed using an automatic amino acid analyzer. ●—●, Serine; ○—○, glycine; ×—×, alanine; ▲—▲, threonine; △—△, aspartic acid.

*Since native enzyme was not digested by the carboxypeptidase A, heat denatured enzyme dissolved in KOH solution was employed.

dard assay conditions. As is shown in Fig. 7, the enzyme was stable at pH 4.5~6.0.

Effect of temperature on the stability of the enzyme. As is shown in Fig. 7, the enzyme retained 70% of its original activity even after treatment at 80°C. When stored at 3°C the enzyme retained 97% of its activity even after 158 hr, whereas at 35°C its activity dropped to 80% of its initial activity after 24 hr.

Action on yeast glucan. After digestion of yeast glucan by the enzyme, turbidity change and release of glucose, carbohydrates, and reducing sugars were measured. As is shown in Fig. 8, the turbidity of a glucan suspension lowered rapidly with the release of carbohydrates and comparatively small amount of reducing sugar, but glucose was scarcely released during the digestion. The results of Sephadex G-15 column chromatography of the hydrolysis products are shown in Fig. 9. A large molecule fraction separated from the reaction mixtures at an initial stage of the diges-

FIG. 7. Effect of pH and Temperature on the Enzyme Activity.

In the temperature-stability experiment, enzyme solutions in M/100 acetate buffer, pH 5.0, were respectively heated for 10 min at various temperatures indicated and the remaining activities were assayed under the standard assay conditions. Further experimental conditions are described in the text. ○—○, lytic activity on living yeast cells; ●—●, yeast glucan depolymerization activity; pH 4~6, acetate buffer; pH 6~7.5, phosphate buffer.

tion was demonstrated by ultracentrifugation analysis to be composed primarily of a polysaccharide with a molecular weight of 7,500, $\overline{DP}$ 42 (Fig. 10). The polysaccharide had been digested by the crystalline *Rhizopus* glucanase, β -1,3-glucanase and β -1,6-glucanase respectively with oligosaccharides as products, as demonstrated in Fig. 11.

Reduction by sodium borohydride and oxidation by periodate of yeast glucan did not affect enzyme activity, whereas β -1,3-glucanase treatment of yeast glucan affected its activity, as is shown in Table II.

Action on laminarin. The lytic enzyme differs clearly from general endo β -1,3-glucanase in its action on laminarin; that is, endo β -1,3-glucanase obtained from the same organism decomposed laminarin almost completely, with G_1 to G_3 oligosaccharides as products,

FIG. 8. Action of the Crystalline Enzyme on Yeast Glucan as Revealed by Turbidity Change and Carbohydrate Release.

A reaction mixture containing 500 mg of yeast glucan and 0.5 mg of the enzyme in 100 ml of M/20 acetate buffer, pH 5.0, was incubated at 40°C with gentle shaking, and at indicated times an aliquote of the mixture was withdrawn for the measurements. Assay methods were as follows: glucose, the glucose oxidase method; released carbohydrate, the phenol-H₂SO₄ method; reducing sugar, the Somogyi-Nelson method. ●—●, Turbidity; ×—×, released carbohydrate; ○—○, reducing sugar; △—△, glucose.

FIG. 9. Separation on Sephadex G-15 of Products of Digestion of Yeast Glucan by the Crystalline *Rhizopus* Glucanase.

Sample was obtained from the experiment of Fig. 8. After 40 hr incubation, the reaction mixture was centrifuged to eliminate insoluble matter and concentrated. One ml of the concentrated solution was applied to a Sephadex G-15 column, 15×800 mm. Flow rate was 1 ml/5 min and 2 ml of eluate were collected in each tube. Carbohydrate was assayed by the phenol-H₂SO₄ method, (○—○); glucose was assayed by the glucose oxidase method, (●—●).

FIG. 10. Determination of Molecular Weight and Sedimentation Coefficient of an Oligoglucan Solubilized by the Enzyme from Yeast Glucan.

A reaction mixture, of which composition was the same as that described in the legend to Fig. 8, was incubated for 20 min at 40°C, heated for 5 min, centrifuged (10,000 rpm, 10 min), and the supernatant was concentrated, dialyzed vs. 0.1 M KCl and used for the experiment. Determination of $s_{20,w}^0$; 4% sample, synthetic boundary cell, 60,000 rpm, 20°C: determination of molecular weight; 0.1, 0.2 and 0.4% sample, multichannel cell, 20,000 rpm, 20 hr, 20°C.

TABLE II. EFFECT ON ENZYME ACTIVITY OF CHEMICAL AND ENZYMIC MODIFICATION ON YEAST GLUCAN

The β -1,3- and the β -1,6-glucanase treated yeast glucan were prepared from the insoluble residues of yeast glucan which was subjected to the digestion with respective glucanases. The other glucans were prepared following the procedures described by Misaki *et al.*⁷¹⁾ For the experimental conditions, see the previous paper.⁴⁰⁾

Substrate	Relative activity (%)
Native glucan	100
NaBH ₄ -Reduced glucan	102
IO ₄ -Oxidized, NaBH ₄ -reduced and mild acid-hydrolysed glucan	101
β -1,3-Glucanase treated glucan	22
β -1,6-Glucanase treated glucan	78

while the lytic enzyme did not produce oligosaccharide from laminarin in a readily recognizable amount and hence the gel filtration of laminarin treated with the lytic enzyme on a Sephadex G-15 column showed the same elution pattern as that of control laminarin.

FIG. 11. Paper Chromatogram of the Digests of Enzymatically Solubilized Polysaccharide.

The polysaccharide was obtained by precipitation with alcohol from the reaction mixture described in the legend to Fig. 10. Reaction mixtures containing 20 mg of the polysaccharide and 0.375 mg of the lytic enzyme, 0.848 mg of β -1,3-glucanase or 1.18 mg of β -1,6-glucanase in 4.0 ml of M/20 acetate buffer, pH 5.5, were incubated at 40°C. After 2 hr incubation the mixtures were heated and chromatographed. Identification of the sugar spots was done following the French and Wild's method.⁷⁰⁾ A, Control; B, incubated with the lytic enzyme; C, incubated with β -1,3-glucanase (G_1 , $G_{1-3}G$, $G_{1-6}G_{1-3}G$, $G_{1-6}G_{1-6}G$ or $G_{1-3}G_{1-3}G_{1-6}G$); D, incubated with β -1,6-glucanase (G_1 , $G_{1-3}G$, $G_{1-6}G$, $G_{1-3}G_{1-3}G_{1-3}G$, $G_{1-6}G_{1-6}G$ or $G_{1-3}G_{1-3}G_{1-6}G$); E, laminari oligosaccharides; F, glucose and gentiobiose.

A series of laminari oligosaccharides ranging from G_3 to G_8 was tested for substrate for lytic enzyme, but none of them was digested.

Action on living yeast. In a previous paper the lytic activity of the enzyme on *S. cerevisiae* LK2 G12 was studied, but its activity on the other yeasts remained to be studied. Table III shows susceptibility of yeasts to the enzyme.

Immunological studies of the lytic enzyme. Serological differentiation among some yeast

TABLE III. SUSCEPTIBILITY OF YEASTS TO THE CRYSTALLINE ENZYME

	Relative rate of lysis ^{a)}	
	Log-phase cells	Stationary-phase cells
<i>Candida utilis</i> IFO 0396	67	15
<i>albicans</i> IFO 0197	0	0
<i>macedoniensis</i> IFO 0706	50	25
<i>pseudotropicalis</i> IFO 0617	80	75
<i>krusei</i> IFO 0013	50	85
<i>guilliermondii</i> IFO 0566	93	100
<i>Cryptococcus laurentii</i> IFO 0609	0	20
<i>Endomyces hordei</i> IFO 0104	0	0
<i>Hansenura Jadini</i> IFO 0987	25	20
<i>octospora</i> IFO 0145	60	40
<i>bimundalis</i> IFO 1366	50	40
<i>Kluyveromyces polysporus</i> IFO 0996	75	60
<i>Kloeckera apiculata</i> IFO 0154	70	27
<i>Pichia polymorpha</i> IFO 0195	33	28
<i>Saccharomyces ellipsoideus</i>	10	20
<i>delbrueckii</i> IFO 0285	86	38
<i>cerevisiae</i>	80	75
<i>carlsbergensis</i> IFO 0641	104	102
<i>oviformis</i> IFO 0262	91	83
<i>cerevisiae</i> LK2 G12	100	20
<i>Sporobolomyces salmonicolor</i> IFO 0374	0	0
<i>Torulopsis candida</i> IFO 0768	0	0
<i>Tricosporon cutaneum</i> IFO 0174	0	0
<i>margaritiferum</i> IFO 1208	0	0

^{a)} The assay was conducted, using 10 μ g/tube of enzyme, under the standard assay conditions, and the susceptibility of *S. cerevisiae* LK2 G12 was assigned to 100. Under these conditions nearly complete disruption was obtained for *S. cerevisiae* LK2 G12 (Δ O.D. at 610 nm=0.25).

cell lytic enzymes, such as present lytic enzyme, lytic enzyme from a Fungi Imperfecti,^{40, 41)} crude lytic enzyme from *Flavobacterium dormitator* var. *glucanolyticae*⁴³⁾ and snail gut juice, using Ouchterlony's immunodiffusion tests was attempted. A clear band appeared only between the antiserum and the present lytic enzyme (antigen).

DISCUSSION

From the cultural characteristics of the organism, especially maximum growth temperature, it was considered to belong in the

Chinensis section of the genus *Rhizopus*. In this section two species, *Rhizopus chinensis* and *Rhizopus pseudochinensis*, are included; they are distinguished from each other only by the Voges-Proskauer test. The suspended organism was negative for the test and, hence, identified as *Rhizopus chinensis*. The morphological, cultural and physiological characteristics of the organism were the same as those of *Rhizopus chinensis* IFO 1078, which was used as a reference organism, except the ability to form yeast cell-lytic enzyme. Therefore it has been designated as *Rhizopus chinensis* R-69.

From a culture filtrate of the strain a kind of endo β -1,3-glucanase which lyses the cells of living yeast (or forms protoplasts from log phase yeast) was crystallized. The enzyme was homogeneous according to various criteria and its molecular weight, tentatively given as 19,000 in a previous paper,³⁶⁾ was determined to be 22,000–24,000 by the various means described above.

The enzyme has been separated from β -1,3-glucanase and β -1,6-glucanase according to the criteria of the DNSA method, but it is still active on yeast glucan consisting of β -1,3- and β -1,6-glucosidic linkages. The data given in Fig. 7 show that the glucanolytic activity and the living yeast cell lytic activity originate from the same enzyme protein. In a previous paper the enzyme was supposed to be β -glucanase of a debranching type, inasmuch as it liberated from yeast glucan the oligosaccharides which correspond in degree of polymerization to side chains of the yeast glucan. An attempt to prove the disappearance of the branching point from laminarin and yeast glucan to be a result of enzyme action by methylation analysis combined with gas liquid chromatography was unsuccessful because of the difficulty of methylation. As stated in "RESULTS" in this paper, laminarin which contains linear β -1,3-linked chains of D-glucose units with occasional β -1,6-links has not been depolymerized by the enzyme to a readily distinguishable extent. On the other hand, yeast glucan, which is a highly polymerized and branched β -1,3-glucan containing significant amount of β -1,6-glucosidic

linkage, was cleaved rapidly by the enzyme, releasing high molecular weight carbohydrate and a comparatively little amount of reducing sugar. These observations suggest that the enzyme is more specific for yeast glucan than for laminarin. Considering the difference in the structural features of yeast glucan and laminarin, the action of the enzyme seems to be related to branching points; however, that the enzyme may be a "more random" type of glucanase, such as the C₁ component of cellulase, also seems reasonable, inasmuch as the increase in reducing sugars in Fig. 8 may be attributed to the second order cleavage of released carbohydrate.

Recently Doi *et al.*³⁹⁾ have reported the presence of "laminaripentaose cut out type" glucanase in an *Arthrobacter* bacteria. Our enzyme reported here differs from their glucanase in its mode of action on yeast glucan, especially at the initial stage of the reaction, and also from the "more random" type glucanase of Fungi Imperfecti reported previously^{40, 41)} in its mode of action on laminarin. However, as the accurate bond split by the enzyme could not be identified within the scope of this paper, our next paper will focus on that subject.

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