A lytic activity on cell wall of Rhizopus species which was shown by culture filtrate of Bacillus R-4 was separated into two fractions (Fraction I and II) with the lytic activity by means of SP-Sephadex column chromatography.

The lytic activity of the Fraction II was twice as strong as that of the Fraction I and the lytic action pattern on Rhizopus cell wall of the mixture of these fractions was similar to that of the crude enzyme preparation.

The Fraction I was recognized as a kind of proteolytic enzyme and was purified as an ultracentrifugally and electrophoretically homogeneous preparation.

On the other hand, the Fraction II was a kind of carbohydrolase which acted on glycol chitosan and liberated hexosamine from Rhizopus cell wall accompanying the lysis.

In the previous paper, it was reported that Bacillus R-4 produced the lytic enzyme which degraded Rhizopus cell wall specifically. During the course of the investigation, it was suggested that the crude lytic enzyme preparation was composed of at least two enzymes, a protease and an unidentified carbohydrolase.

The present paper deals with the purification and some properties of these enzymes. The roles of these enzymes on the lysis of Rhizopus cell wall are also described.

MATERIALS AND METHODS

Cultivation of the Bacillus R-4 and preparation of crude enzyme

A cultivation medium of the Bacillus R-4 was composed of 5% of peptone, 3% of dextrin, 0.1% of NaNO₃, 0.1% of KH₂PO₄ and 0.05% of MgSO₄·7H₂O and was adjusted to pH 6.0. Cultivation was carried out in a 30-liter jar fermentor containing 12 liters of the medium. Inoculation was made by the addition of 300 ml of the seed culture broth obtained by reciprocal shaking in the same medium as above in Sakaguchi flask at 28°C for 24 hr. The jar fermentor was operated for 48 hr at 28°C under the following conditions; aeration was 3 liters per min and agitation was 200 rpm. After the cultivation, a crude enzyme preparation was prepared by salting out from the culture broth with ammonium sulfate and further desalting by dialysis against deionized water as described previously.11

Preparation of Rhizopus cell wall. Rhizopus cell wall used as a substrate of the lytic enzyme was prepared from the pulpy mycelia of Rhizopus delemar according to the method described previously.11

Assay of the lytic activity. Assay of the lytic activity was carried out according to the method described previously. One unit of the lytic activity was defined as an enzyme quantity which reduced 1% of the initial optical density (at 660 nm) of the Rhizopus cell wall suspension per 10 min.

Assay of some enzyme activities. Protease activity was measured according to the method described by Hagihara.11 One unit of the protease activity was defined as an enzyme quantity which liberated 1 μmole of tyrosine from Hammarsten milk casein per min.

Glucanase and chitinase activities were measured by the method described previously.11

Chitosanase activity was measured as follows: 1 ml of enzyme solution was added to 1 ml of substrate solution containing 20 mg of glycol chitosan. After 10 min incubation at 40°C, glucosamine liberated was determined by the method of Elson-Morgan.11 Glycosidase activity was measured as follows: 1 ml enzyme solution was added to 1 ml of substrate solution containing 0.1 mg of maltose or salicin. After 20 hr incubation at 40°C, reducing sugar liberated was determined by the method of Somogyi-Nelson.41

Studies on Lytic Enzyme on Fungal Cell Wall. Part II. See Reference 1.)
All reactions were carried out in 0.05 M Tris-HCl buffer (pH 7.6), unless otherwise stated.

Determination of protein. Protein content of an enzyme preparation was estimated by the measurement of the absorbancy at 280 nm by using milk casein as a standard. The specific activity of an enzyme was expressed as the enzyme units per mg of protein.

Determination of sugars and ninhydrin positive substances. Various sugars and ninhydrin positive substances liberated from the cell wall by the action of the lytic enzyme were determined as follows: An aliquot of the reaction mixture was withdrawn and heated for 5 min in a boiling water bath. The mixture was then cooled in an ice water bath and precipitate was removed by centrifugation. Total sugar, reducing sugar, total hexosamine and ninhydrin positive substances in the supernatant solution were determined by the method of Phenol-sulfuric acid, Somogyi-Nelson, Elson-Morgan and Moore et al., respectively.

Chemicals. Crystalline protease from Bacillus subtilis was kindly donated from Prof. D. Tsuru of Nagasaki University and Bioprase (Nagase Co.), Milezyme (Miles Lab.) and Pronase (Kaken Chemical.) were commercial products. Pulluran and colloidal chitin were prepared from culture filtrate of Pullularia pullulans and commercial chitin by the method described by S. Ueda et al. and D. M. Reynolds et al., respectively. SP-Sephadex C-50, Sephadex G-75 and G-100 were the products of Pharmacia Co., Ltd., Uppsala.

RESULTS

1. Separation of two fractions with lytic activity from the crude enzyme preparation by SP-Sephadex column chromatography

The crude enzyme preparation was dialyzed against 0.003 M phosphate buffer (pH 7.6) at 8°C for 50 hr. The dialyzed solution (2320 ml) was loaded on a column of SP-Sephadex C-50 (2.8 × 85 cm) equilibrated with 0.003 M phosphate buffer (pH 7.6). The column was washed thoroughly with the same buffer and then eluted by a linear gradient concentration of NaCl from zero to 0.5 M at a flow rate of 30 ml per hr. The effluent was fractionated into 12 ml portions.

As shown in Fig. 1, the lytic activity was separated into two fractions (Fraction I and II). In the Fraction I, the elution pattern of a protease activity was similar to that of the lytic activity.

2. Lysis of Rhizopus cell wall by Fraction I and II

The Fraction I (fractions No. 50 to 70) and II (fractions No. 85 to 110) obtained by SP-Sephadex column chromatography were concentrated in a collodion bag under reduced pressure, respectively.

In order to examine the action patterns of these two fractions on Rhizopus cell wall, the attempt was made to determine time-course of decrease in turbidity of each reaction mixture.

As shown in Fig. 2, the optical density

![Graph](image_url)

**Fig. 1.** Column Chromatogram of the Crude Enzyme Preparation on SP-Sephadex C-50.

The experimental details are described in the text. 
- O-O, Lytic activity; - - - , Protease activity; ---, O.D. at 280 nm; ------, NaCl concentration.
Lytic Enzyme on Rhizopus Cell Wall

FIG. 2. Lysis of Rhizopus Cell Wall by Fraction I and II.

One milliliter of each enzyme preparation was added to 4 ml of 0.2 M Tris-HCl buffer (pH 7.6) containing 5 mg of Rhizopus cell wall, and incubated at 40°C. Crude enzyme preparation; Fraction I; Fraction II; Mixture of the Fraction I and II. Decreased about 60% by the action of Fraction II, while only 30% decrease was observed by the action of Fraction I. When the mixed solution of Fraction I and II was allowed to react on Rhizopus cell wall suspension, decrease in turbidity of the reaction mixture reached to about 80% after long period incubation. This result was almost the same as that which was given by the crude enzyme preparation. The upper limits of turbidity decrease curves shown in Fig. 2 had no relation to the enzyme concentrations.

3. Products from Rhizopus cell wall by the action of Fraction I and II

In order to characterize the enzymes in the Fraction I and II participating in the decomposition of Rhizopus cell wall, ninhydrin positive substances and some sorts of sugars released from the cell wall by the actions of both fractions were examined.

As can be seen in Fig. 3, a remarkable liberation of ninhydrin positive substances was observed by the action of the Fraction I and the amount of them increased as the lysis of the cell wall proceeded. On the other hand, very small amount of ninhydrin positive substances were observed in the reaction mixture of the Fraction II, in spite of the fact that the lytic action of the Fraction II was twice as strong as that of the Fraction I.

FIG. 3. Ninhydrin Positive Substances from Rhizopus Cell Wall by the Actions of the Fraction I and II.

One milliliter of each enzyme fraction was added to 4 ml of 0.1 M phosphate buffer (pH 7.6) containing 5 mg of Rhizopus cell wall, and incubated at 40°C. Determination of ninhydrin positive substances in the reaction mixture was carried out by the method described in MATERIALS AND METHODS. Fraction I; Fraction II.

FIG. 4. Various Sugars from Rhizopus Cell Wall by the Actions of Fraction I and II.

Ten milliliters of each enzyme fraction were added to 10 ml of 0.2 M Tris-HCl buffer (pH 7.6) containing 200 mg of Rhizopus cell wall, and incubated at 40°C. Fraction I (Reducing sugar; Total hexosamine; Total sugar) and Fraction II (Reducing sugar; Total hexosamine; Total sugar).
As shown in Fig. 4, concerning to the sugars, the Fraction II liberated remarkable amounts of reducing sugar and hexosamine from the cell wall, while the Fraction I hardly liberated any kind of sugar. By the action of the Fraction II, the amount of hexosamine in the reaction mixture increased in parallel with that of reducing sugar during the incubation. After 6 hr incubation, the amount of hexosamine corresponded to about 80% of reducing sugar. On the other hand, the amount of total sugar determined by the method of phenol-sulfuric acid increased at early stage of the reaction and did not increase in further incubation. The amount of total sugar was less than 40% of reducing sugar after 6 hr incubation.

4. Activities of enzymes in Fraction I and II
From the results of above experiment, it was assumed that a proteolytic enzyme participated in the lytic action of the Fraction I and a carbohydrolase or carbohydrolases participated in that of the Fraction II. Therefore, several enzyme activities on the substrates indicated in Table I were examined.

As shown in the table, a proteolytic activity was detected in Fraction I and a carbohydrolase activity which degraded only glycol chitosan was demonstrated in Fraction II.

5. Purification of the lytic enzyme in Fraction I
Further purification of the lytic enzyme of the Fraction I was attempted. The starting material was the concentrated preparation of Fraction I obtained by SP-Sephadex C-50 column chromatography. In each step of the purification, both lytic and proteolytic activities were checked as the guides. Unless otherwise indicated, all procedures were carried out at 8°C.

Gel filtration on Sephadex G–75. The con-

TABLE I. ACTIVITIES OF SOME ENZYMES IN FRACTION I AND II

Assay of each enzyme activity was carried out by the method described in MATERIALS AND METHODS.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fraction I</th>
<th>Fraction II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease</td>
<td>Milk casein</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Soluble starch</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>–</td>
</tr>
<tr>
<td>Glucanase</td>
<td>Pullulan</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Laminaran</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CMC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>Glucosidase</td>
<td>Maltose</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Salicin</td>
<td>–</td>
</tr>
<tr>
<td>Chitinase</td>
<td>Colloidal chitin</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Glycol chitin</td>
<td>–</td>
</tr>
<tr>
<td>Chitosanase</td>
<td>Glycol chitosan</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carboxy methyl cellulose.
Ultracentrifugation was carried out at concentration of 0.96% purified enzyme in 0.01 M Tris-HCl buffer (pH 7.6) containing 0.002 M calcium acetate, with a Hitachi model UCA-1 analytical ultracentrifuge at 20°C. The photographs were taken from 30 min to 50 min after reaching the maximum speed (55430 rpm).

A typical chromatographic pattern obtained by the gel filtration is presented in Fig. 5. The lytic and proteolytic activities showed the same elution patterns.

The active fractions (No. 30 to 40) were combined and concentrated to 10 ml in a collodion bag under reduced pressure.

**Gel filtration on Sephadex G-100.** The concentrated solution was loaded on a column of Sephadex G-100 (2.8 × 125 cm) equilibrated with the same buffer as described above. Elution was made with the same buffer at a flow rate of 15 ml per hr and the effluent was fractionated into 12 ml portions.

As shown in Fig. 6, a symmetric peak in which the lytic and proteolytic activities completely overlapped was obtained.

The main fractions (No. 40 to 50) were combined and concentrated to 10 ml in a collodion bag under reduced pressure.

**Homogeneity of the purified preparation.** Homogeneity of the purified preparation of the Fraction I was tested by the methods of ultracentrifugal sedimentation and disk electrophoresis on polyacrylamide gels. From the results shown in Figs. 7 and 8, the preparation was considered to be homogeneous.

The overall procedures of purification and the results are summarized in Tables II and III. Regarding to the protease activity, the purification results in a 430-fold increase in...
TABLE II. PURIFICATION PROCEDURE OF THE FRACTION I

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>Fractionated with ammonium sulfate (0.75 saturation)</td>
</tr>
<tr>
<td>Precipitate</td>
<td>Dissolved in deionized water and dialyzed against 0.003 M phosphate buffer (pH 7.6)</td>
</tr>
<tr>
<td>Dialyzed solution (Crude enzyme)</td>
<td>Chromatographed on SP-Sephadex C-50 column equilibrated with 0.003 M phosphate buffer (pH 7.6) and eluted by NaCl</td>
</tr>
<tr>
<td>Eluted solution (Fraction I)</td>
<td>Dialyzed against 0.01 M Tris-HCl buffer (pH 7.6) containing 0.002 M calcium acetate and chromatographed on Sephadex G-75 column equilibrated with the same buffer</td>
</tr>
<tr>
<td>Eluted solution</td>
<td>Chromatographed on Sephadex G-100 column equilibrated with 0.01 M Tris-HCl buffer (pH 7.6) containing 0.002 M calcium acetate</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>Concentrated by means of a colloidion bag under reduced pressure</td>
</tr>
</tbody>
</table>

specific activity with a recovery of 11.8% of the original activity.

6. Enzyme properties of Fraction I

Figures 9 and 10 show the effects of pH on the activity and on the stability of the purified preparation of Fraction I, respectively. The Fraction I was most active at pH values between 7.0 and 7.8, and stable in the range of pH 7.0 to 9.0 at 30°C for 20 hr.

As shown in Fig. 11, when the purified preparation was incubated at 50°C, both lytic activity and protease activity were rapidly inactivated and lowered to about 50% of the initial activities after 10 min incubation.

7. Comparison of lytic activity of various protease

As described above, it was confirmed that the lytic enzyme of the Fraction I was a neutral protease. Then the lytic actions of several microbial proteases which are most active at neutral or alkaline pH were examined.

As shown in Table IV, no protease tested, other than Fraction I, could decompose the

TABLE III. LYTIC ACTIVITY AND PROTEASE ACTIVITY IN THE COURSE OF PURIFICATION OF THE FRACTION I

<table>
<thead>
<tr>
<th>Fraction and step</th>
<th>Lytic activity</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td></td>
<td>(U)</td>
<td>(U/mg)</td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>2.68 x 10⁶</td>
<td>9</td>
</tr>
<tr>
<td>Dialyzed solution</td>
<td>1.32 x 10⁶</td>
<td>123</td>
</tr>
<tr>
<td>SP-Sephadex C-50 chromatography</td>
<td>1.52 x 10⁶</td>
<td>615</td>
</tr>
<tr>
<td>(3.40 x 10⁶)¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-75 chromatography</td>
<td>1.20 x 10⁶</td>
<td>764</td>
</tr>
<tr>
<td>Sephadex G-100 chromatography</td>
<td>7.80 x 10⁴</td>
<td>948</td>
</tr>
</tbody>
</table>

¹ Lytic activity of the Fraction II.
Lytic Enzyme on Rhizopus Cell Wall

FIG. 10. Effect of pH on the Stability of Fraction I.
Fraction I was preincubated at pH values indicated at 30°C for 20 hr and the remaining activities were measured.
- - - Lytic activity; --- Protease activity.

FIG. 11. Effect of Temperature on the Stability of Fraction I.
Fraction I, in 0.2M Tris-HCl buffer (pH 7.6), was preincubated at 50°C during each period indicated, and the remaining activities were measured.
- - - Lytic activity; --- Protease activity.

Rhizopus cell wall.

DISCUSSION

In the previous paper, the authors presumed that the crude enzyme preparation of Bacillus R-4 would be composed of a protease and an unidentified carbohydrolase participating in the lysis of Rhizopus cell wall.

The present study was carried out to clarify the natures of these enzymes. First of all, two fractions, Fraction I and II, which had lytic activity were obtained from the crude enzyme preparation by means of SP-Sephadex column chromatography. Fraction II showed a lytic activity twice as strong as that of Fraction I. As shown in Fig. 2, the lytic pattern of the mixture of two fractions on Rhizopus cell wall was very close with that of the crude enzyme preparation. From this fact, it was confirmed that the decomposition of Rhizopus cell wall by the crude enzyme preparation was carried out by the cooperative action of the enzymes contained in both fractions.

In order to characterize enzymes in these fractions which participate in the lysis of Rhizopus cell wall, the detection of enzyme activities were carried out. From the result shown in Fig. 3, it was presumed that the enzyme of Fraction I was a protease and that of Fraction II was a sort of carbohydrolase which degraded glycol chitosan specifically.

The protease was purified to a homogeneous state. The purified preparation also showed the lytic activity. Therefore, it was confirmed that the protease of Fraction I was responsible for the lysis of the Rhizopus cell wall.

In comparison with various microbial proteases tested, Fraction I seemed to be a novel type of protease from a view point of a lytic action on Rhizopus cell wall (Table IV).

With regard to carbohydrolase, it has been
pointed out by many authors that $\beta$-1,3-glucanase and chitinase were essential factors of the lysis of fungal cell walls.9\textsuperscript{~}~11\textsuperscript{~} However, no activity of glucanases and chitinase was detected in Fraction II. As described above, the decomposition of Rhizopus cell wall may be caused by the action of the other type of carbohydrolase, tentatively named chitosanase. Concerning to enzymes which act on chitosan or its derivatives, there have been few reports\textsuperscript{12} and their properties and action pattern on the substrate still remaine obscure. Moreover, it has never been reported that these enzymes are responsible for the lysis of fungal cell walls. Therefore, the enzyme found in Frac

From the present study, it was suggested that the structure of polysaccharides which constitute Rhizopus cell wall is different from that of the other fungal cell walls.

Acknowledgement. The authors are grateful to Prof. D. Tsuru of Nagasaki University for the generous supply of crystalline protease from Bacillus subtilis.

REFERENCES