Proteolytic Enzyme from *Oerskova* sp. CK Lysing Viable Yeast Cells'

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*Oerskova* sp. CK produced three types of yeast glucan hydrolases, one of them, F-L lysing viable yeast cells had proteolytic activity and considerable difference was observed between lytic and proteolytic activities in thermal stability. F-L was considered to be a single protein from following results. Both activities were influenced in parallel by acid treatment and inhibitors. The reaction rates of two substrates being added to the enzyme system were less than the sum of the rates of reactions measured separately. Both activities were detected in the same fraction by isoelectric focusing.

On the evidence that F-L lost lytic activity by the heat treatment at 60°C for 15 min, we speculated that the enzyme underwent autolysis during heat treatment and binding site for polysaccharide was released, subsequently lost the lytic activity, since active site of F-L remained unaffectedly. These speculations were based on the following results. Difference in specific activity between native and treated F-L was not observed. The specific activity of heat treated F-L purified by gel filtration was 1.6 times more active than that of native F-L. Native F-L had a affinity for dextran but treated F-L lost it. F-L being treated in the presence of DFP was eluted at the same fraction where native F-L was eluted. Based on these results, a model for the yeast cell wall was proposed.

In the previous paper,1-3 we reported that *Oerskova* sp. CK produced three types of yeast glucan hydrolases designated as F-L, F-0 and F-2 according to the purification with ECTEOLA-, CM-cellulose chromatographies and Biogel P-100, P-60 filtrations and these enzymes appeared to be homogeneous by disc electrophoresis. F-L showed very high lytic activity toward viable yeast cells and had little yeast glucan hydrolase activity but other enzymes exhibited weak or no lytic activities and had strong hydrolase activities.

Furthermore, high synergistic effects4) on lysing viable yeast cells were observed between F-L and either F-0 or F-2 and the latters showed high lytic activities toward yeast cells pretreated with small amounts of F-L which could not lysed the cells.

Nagasaki et al.5) reported the lysis of viable yeast cells with phosphomannanase and β-1, 3-glucanase but phosphomannanase alone could not lyse the cells. From these data, F-L would be a valuable tool in the structural analysis of yeast cell walls.

The present paper described further studies on properties of F-L and a model of yeast cell wall.

MATERIALS AND METHODS

Preparation of enzymes. F-L and F-0 were obtained from a culture filtrate of *Oerskova* sp. CK and purified according to the procedure described in the previous report.6) Trypsin and α-chimotrypsin were purchased from Miles-Seravac and Pronase E from Kaken Kagaku.

Substrates. Kyokai 7 used as substrate for lytic activity was cultivated in the medium consisting of glucose 2%, yeast extract 0.5%, polypeptone 0.5%, KH₂PO₄ 0.1%, MgSO₄•7H₂O 0.04% at 30°C for 8 hr shaking. The cells were washed twice centrifugically with distilled water and suspended in 0.1 M phosphate buffer (pH 7.5) at optical density of 1.4 (660 nm). Yeast cell walls were prepared from Kyokai 7 grown in the cultural conditions mentioned above except that incubation time was 30 hr, cells were mechanically disrupted in Braun cell homogenizer and throughly washed with 10% NaCl solution, distilled water and 95% ethanol by centrifugation. The purified walls were lyophilized and stored in the desiccator until

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required. Milk casein from Merk, bovine serum albumin and edestin from Wako pure chemical, gluten from Tokyo kasei, hemoglobin (2 x cryst.) from Sigma and ovalbumin from Miyazaki chemical were used as substrates for proteolytic activity. Hemoglobin was denaturated by urea and other substrates were denaturated by heating.

**Lytic activity.** In standard assay for lytic activity, a mixtures of 3 ml of yeast cell suspension and 1 ml of enzyme solution were incubated at 30°C for 1 hr with gentle shaking. Then the optical density of the mixture was measured at 660 nm. Reference cell contained the same mixture except 1 ml of water instead of the enzyme solution. Lytic activity was calculated from the following equation:

\[
\text{Lytic activity} = \frac{(OD_{660} \text{ nm of reference} - OD_{660} \text{ nm of reaction mixture}) \times 10^6 \text{ dilution rate/initial OD}_{660} \text{ nm of reference}}
\]

When yeast cell walls were used as substrate for lytic activity, the reaction mixture consisted of 2 ml of yeast cell suspension (2 mg dry wt./ml), 1.5 ml of 0.1 M phosphate buffer (pH 7.5) and 0.5 ml of enzyme solution. Lytic activity was determined under standard assay conditions.

**Proteolytic activity.** The reaction mixture containing 1.5 ml of 25% Hammarsten milk casein solution, 1 ml of 0.1 M phosphate buffer (pH 7.5) and 0.5 ml enzyme solution were incubated at 30°C for 30 min. The reaction was stopped by addition of 3 ml of 0.4 M trichloroacetic acid solution, followed by filtration. The filtrate (0.6 ml) was mixed with 3 ml of 0.4 M sodium carbonate solution and then 0.3 ml of phenol reagent was added. The optical density of the color developed was measured at 500 nm. One proteinase unit was defined as the amount of enzyme which liberates 1 µg tyrosine in the reaction mixture in 30 min under the assay condition and the specific activity was expressed as units per µg enzyme protein.

**Measurement of soluble carbohydrate.** Soluble carbohydrate was determined by the phenol-H₂SO₄ method using glucose as a standard.

**Measurement of protein.** Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard.

**Isoelectric focusing.** Experiments were carried out in a sorbitol density gradient using a 110 ml LKB column at 15°C. The pH gradients from 9 to 11 were produced with ampholytes. A potential of 700 V was maintained across the column for 15 hr followed by 1000 V for 24 hr. Fractions (2.5 ml each) were collected for analysis.

**Heat treatment of F-L.** F-L suspended in 0.01 M phosphate buffer (pH 7.5) was incubated at 60°C for 15 min in a water bath and immediately cooled in an ice water bath.

**Chemicals.** DFP (Diisopropyl fluorophosphate) was purchased from Sigma and trypsin inhibitor (from soy bean) from Miles Laboratories. Potato inhibitor was prepared according to Matsushima.

### RESULTS

**Hydrolysis of various substrates by F-L**

When F-L was reacted with milk casein and bovine serum albumine, it was found that F-L could hydrolyze both substrates. As shown in Table I, the specific activity of F-L was almost half of other enzymes.

**TABLE I. PROTEOLYTIC ACTIVITIES**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Casein</th>
<th>Bovine albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.A. a)</td>
<td>S.A.R. b)</td>
<td>S.A. a)</td>
</tr>
<tr>
<td>F-L</td>
<td>10.2</td>
<td>1.0</td>
</tr>
<tr>
<td>a-Chymotrypsin</td>
<td>21.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Trypsin</td>
<td>18.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Pronase-E</td>
<td>14.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

a) Specific activity (U/Protein µg).
b) Specific activity ratio.

**Effect of pH**

Effects of pH on both lytic and proteolytic activities of F-L were approximately the same as shown in Fig. 1. The optimum pH values for both activities were approximately 9.0.

![Fig. 1. Effect of pH on the Enzyme.](image)

Proteolytic and lytic activities were determined under the standard assay conditions. Buffers used were 0.1 M phosphate (---) and 0.1 M Tris-HCl (-----). ○, proteolytic activity; ●, lytic activity.
Effect of temperature on the stability

Figure 2 shows the thermal inactivation curves of the lytic and the proteolytic activities. The lytic activity decreased by the treatment at 40°C and was lost by heating at 50°C for 15 min, on the other hand, the proteolytic activity was stable up to 60°C but completely lost by heating at 70°C for 15 min. Difference between the two activities was observed in the thermal stability.

Lysis of viable yeast cells by F-0 and heat treated F-L

After the preincubation of F-L at various temperature for 15 min, the synergistic effects between F-0 and F-L were investigated. As shown in Fig. 3, these effects were not observed at 50°C treatment and coincided with heat stability curve of F-L lytic activity.

Effect of acid treatment

The enzyme was preincubated with various pH solutions at 30°C for 15 min. Both activities were considerably stable over pH 2.0 as shown in Fig. 4 and the tendency of inactivation by acid treatment was closely related.

Effect of inhibitors

Effects of EDTA, monoiodoacetate, DFP,
potato and trypsin inhibitors on the enzyme activity were investigated. DFP and potato inhibitor inhibited both activities remarkably as shown in Figs. 5 and 6 but other reagents did not inhibit up to $10^{-3}$ M. Inhibition patterns of both inhibitors were similar to each other and proteolytic activity was more strongly inhibited than lytic activity.

**Hydrolysis of isolated yeast cell walls**

F-L, $\alpha$-chymotrypsin, trypsin and pronase E were allowed to react with isolated yeast cell walls and their lytic activities were measured. As shown in Fig. 7 F-L lysed cell walls rapidly but other enzymes could not lyse at all.

The property of products released from cell walls by F-L was investigated by gel filtration.

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**Hydrolysis of two substrates presented in the reaction system**

Hydrolitic activity was estimated when the two substrates were present in the reaction system, though F-L decomposed yeast cell...
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The reaction mixtures consisted of 1.5 ml buffer solution (pH 7.5) containing either 3 mg isolated cell walls or 30 mg milk casein, 1.5 ml buffer solution and 1.0 ml enzyme solution containing various concentration indicated in the figure. In the case of two substrates used together, the reaction mixture contained the same mixture but with 1.5 ml of another substrate solution instead of buffer solution. The activities were determined under the standard assay conditions except that reaction time of lytic activity was 30 min.

walls and milk casein. As shown in Fig. 9, activities were approximately in proportion to the enzyme concentration within a range of 12–36 μg. When each of the substrates was added separately to the reaction system, each activity was determined to be 47% and 280 U, respectively, in the presence of 36 μg of F–L. When the two substrates were present in the reaction system, the lytic and proteolytic activities were reduced to 37.5% and 150 U, respectively, at the same enzyme concentration.

Isoelectric focusing

Since the F–L did not migrate in usual electrophoresis at pH 8.9, this enzyme is considered to be a basic protein. Isoelectric focusing was performed and the result is shown in Fig. 10. The PI value was approximately 9.35 and both lytic and proteolytic activities were observed in the same fractions.

Hydrolysis of various substrates by heat treated F–L

F–L and heat treated F–L were allowed to react with various substrates. Difference between the two activities was not observed as shown in Table II.

TABLE II. HYDROLYSIS OF VARIOUS SUBSTRATES BY F–L AND HEAT TREATED F–L

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U/Protein μg) F–L</th>
<th>Heat treated F–L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk casein</td>
<td>10.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>11.9</td>
<td>11.9</td>
</tr>
<tr>
<td>Edestin</td>
<td>10.7</td>
<td>10.6</td>
</tr>
<tr>
<td>Gluten</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>9.7</td>
<td>9.6</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>8.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Gel filtration of heat treated F–L on Biogel P–100

Heat treated F–L and native F–L were separately loaded on a column of Biogel P–100 previously equilibrated with 0.01 M phosphate buffer (pH 7.5) and eluted with the same buffer. As shown in Fig. 11, heat treated F–L was eluted at the 19th fraction in which the proteolytic activity was assayed and the successive elution of protein was detected. On the other hand, native F–L was eluted at the 16th fraction in which both the lytic and
proteolytic activities were observed. F-L was heated in the presence of $6 \times 10^{-4}$ M DFP at the final concentration and the same chromatography was performed. Treated F-L was eluted at the 16th fraction and appeared the same position with native F-L.

**Hydrolysis of milk casein by F-L purified on Biogel P-100 after heat treatment**

Heat treated F-L was purified on Biogel P-100 and activity was measured with milk casein as substrate and the results is shown in Fig. 12 together with the activity of native F-L. The proteolytic activities of F-L and heat treated F-L were 19.7 U and 32.1 U, respectively and the latter was about 1.6 times more active than the former, when 5 $\mu$g of each enzyme was separately added to the reaction mixture.

**Gel filtration of heat treated F-L on Sephadex G-75**

Heat treated F-L and native F-L were separately applied on a column of Sephadex G-75 previously equilibrated with 0.01 M phosphate buffer (pH 7.5) and eluted with the same buffer. As shown in Fig. 13 (below), heat treated F-L was eluted at the 20th fraction in which the proteolytic activity was detected. On the other hand, native F-L was
eluted at the 54th fraction in which both the lytic and proteolytic activities were detected (Fig. 13, above).

**DISCUSSION**

As reported previously, F-L was an enzyme essential for the lysis of viable yeast cells among enzymes produced by *Oerskovia* sp. CK and high synergistic effect on lysing the yeast cell between F-L and β-1, 3-glucanase was observed. But F-L had little yeast glucan hydrolase activity.

When F-L was subjected to react with various proteinase substrates, the enzyme showed the proteolytic activity and specific activity was about half compared with those of commercial enzymes. Yeast cell wall glucan extracted by the method of Peat et al. contained about 0.6% nitrogen compounds, but F-L was considered to be a kind of β-1, 3-glucanase in previous report, because F-L reacted with yeast glucan.

The effect of pH on the lytic activity of F-L was similar to that observed with it’s proteolytic activity, but distinct difference between them was observed in the thermostability. The lytic activity was lost by the treatment at 60°C for 15 min but the proteolytic activity remained unaffected. This evidence might suggests that F-L might consist of two kinds of enzymes, however, F-L is considered to be a single protein based on the following experimental results. (1) Both activities of F-L were lost in nearly parallel on acid treatment. (2) Both activities were inhibited in parallel by DFP and potato inhibitor. (3) When the enzyme was reacted with two kinds of substrate (milk casein and isolated yeast cell walls) under the saturated condition, the total rate of reaction was less than the sum of the individual reaction. (4) Carbohydrates released from yeast cell walls with F-L were considered to be a large molecular weights, because F-L was not contaminated with polysaccharide-hydrolase. (5) Both activities were detected in the same fraction by the isoelectric focusing.

During the heat treatment, the enzyme might have undergone autolysis and the binding site for polysaccharide might be released, on the other hand, active site for proteolytic

**Fig. 14. A Model for Yeast Cell Wall.**

A: a model presented by us, B: a model presented by Kidby *et al.*
activity might have remained unchange. The change of enzyme activity of F-L by this treatment was speculated from the following phenomena. (1) When native F-L and heat treated F-L were reacted with various substrates, their specific activity was not changed. (2) Heat treated F-L was eluted after native F-L in gel filtration on Biogel P-100 and the successive elution of protein was detected. (3) When heat treated F-L was purified with gel filtration, specific activity of purified enzyme was about 1.6 times that of native F-L. (4) F-L was eluted at 54th fraction applied on a column of Sephadex and the distribution coefficient of F-L (Kd) was much larger than 1.0. These facts suggest that native F-L had an affinity for dextran. On the other hand, heat treated F-L was eluted at the 20th fraction and its distribution coefficient was less than 1.0. (5) Heat treated F-L in the presence of DFP was applied on a column of Biogel P-100 and the enzyme was eluted at the same fraction with that of native F-L.

It is well known that proteolytic enzyme undergo autolysis during purification and heat treatment. Byrne et al. reported that digestion of bovine hepatic fructose 1,6-diphosphatase with nagarse (commercial proteolytic enzyme) resulted in a 2 to 3-fold increase in enzyme activity at pH 9.0 and a simultaneous loss of activity at pH 6.5 and column chromatography of the trichloroacetic acid-soluble material after nagarse digestion revealed a large number of ninhydrin-positive peaks.

The model of yeast cell wall has been proposed by Lampen, Kidby et al. and Ouchi et al. as shown in Fig. 14 (right) but according to our experimental results, protein are considered to be located sporadically as binding materials of yeast glucan network as shown in Fig. 14 (left). It is considered that F-L reacted with proteins on the surface layer of yeast cell wall and decomposed them, then the lysis of yeast occurs. For the further elucidation of yeast cell wall, analysis of products released from the wall by F-L is in progress.

REFERENCES
5) S. Nagasaki and S. Yamamoto, Research Reports of The Kochi University, 17, 93 (1968).