A STUDY OF TWO YEAST PROTEINASES*

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Autolysis is a procedure frequently employed in the isolation of materials from yeast. Although the intracellular yeast proteinases play an important rôle in autolysis, these enzymes have not been characterized by using up to date methods of analysis, and the existing literature concerning yeast proteinases presents conflicting results. In 1917, Dernby (1) reported that yeast contains two proteinases, a “pepsin” and a “trypsin.” The presence of two proteinases was indicated mainly by the fact that a yeast autolysate showed two pH optima for gelatin liquefaction; namely, at pH 4.5 and pH 7.0. In 1926–28, Grassmann, Willstätter, and coworkers published a series of papers on the proteolytic activity of yeast (2–4). These authors concluded that there is only one proteinase in yeast. This enzyme was reputed to be of the papain type with an optimal pH of 5.0 as measured by an alcoholic KOH titration of gelatin hydrolysis (4). However, in 1936, Hecht and Civin (5) claimed that yeast contains a “pepsin” which displays optimal activity at pH 1.8.

In the present paper, the separation and partial characterization of two individual yeast proteinases are described. One has an optimal pH of about 3.7 and is rapidly denatured by urea. The other has optimal activity at pH 6.2 and may be activated by concentrated urea solutions.

EXPERIMENTAL

Most of the analyses for proteinase activity were made according to the method of Anson (6) with slight modification. Both acid-denatured hemoglobin and alkaline urea-denatured hemoglobin substrates were employed. The former substrates contained 2 per cent hemoglobin with 0.07 N citrate as a buffer. The latter contained 2 per cent hemoglobin, 6.6 M urea, 0.07 N acetate, 0.07 N phosphate, and 0.07 N borate as a universal buffer. 1 ml. of enzyme solution was added to 5 ml. of substrate and the mixture was incubated for 10 or 20 minutes at 30°. 10 ml. of 0.3 N trichloroacetic acid were added and the digestion products in the filtrate were measured either

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by reaction with the Folin-Ciocalteu reagent or by reading the absorption of the filtrate at 280 mμ. Both of these procedures for measuring the digestion products were employed in this investigation and gave similar results. Aliquots of crude yeast preparations were adjusted to the pH of the substrate immediately before analysis.

Protein concentrations were measured by the method of Robinson and Hogden (7), twice recrystallized pepsin being used as a reference protein.

Results

Autolysis and Purification—The procedure employed for extraction and partial purification of the yeast proteinases is outlined in Table I. The best yields of these enzymes were obtained when the initial autolysis was carried out at pH 7.0 to 7.6. Toluene, dioxane, and glycerol were also used to plasmolyze the yeast, but the subsequent autolysate showed little or no proteinase activity. Ethyl acetate autolysates yielded only about 50 per cent of the activity obtained with chloroform or ether. The majority of proteinase A present in the whole autolysate is in an insoluble condition and does not appear in the supernatant liquid (Step 2).

The pH-activity curve of the supernatant liquid obtained in Step 2 can be seen in the lower curves of Fig. 1. Curve A is the pH-activity curve obtained by using hemoglobin substrates containing no urea. Curve B represents the activity of the autolysate against urea-denatured hemoglobin. Evidence presented below indicates that Curves A and B are primarily manifestations of two different proteinases which, for the sake of brevity, will be referred to as proteinases A and B, respectively.

There are several peculiarities about Curves A and B which should be noted. (1) It was found that proteinase A is almost instantaneously destroyed by 5.5 M urea at pH 2.0 to 4.0. Therefore, this enzyme has little or no effect on the urea-containing substrates in this pH region; (2) the decline of Curve A above pH 3.8 is caused by the fact that the hemoglobin is not in a denatured form in the absence of urea in the higher pH range (8). Although there is considerable yeast proteinase activity against denatured hemoglobin in the region of pH 6 to 9, native hemoglobin is resistant to proteolysis in this range; (3) the peak at pH 5 in Curve B is caused by a rapid activation of proteinase B by the 5.5 M urea present in the hydrolysis mixture during analysis. In the following section, the fact that urea activates proteinase B at pH 5, but not at pH 4 or 6, is illustrated.

Similar pH-activity curves were displayed by autolysates of four different strains of Saccharomyces cerevisiae: two of bakers’ yeast grown in pure culture1 and a top and a bottom brewers’ yeast. Similar curves were also

1 Kindly supplied by Dr. A. S. Schultz of these Laboratories.
TABLE I

Purification of Yeast Proteinases

800 gm. of compressed Fleischmann's bakers' yeast were plasmolyzed with 400 ml. of chloroform or ether. After 30 to 60 minutes, 1.2 liters of water were added and the pH was adjusted to 7.0 with NaOH. (No proteinase activity was detectable at this stage.) After autolysis for 18 hours at 25°, the suspension was centrifuged and the supernatant solution adjusted to pH 5.0 and allowed to stand under toluene for 18 hours at 25°. 2 volumes of 95 per cent ethanol were added gradually at about 10°, and the resulting precipitate was dried in vacuo over CaCl₂. The activities are expressed in terms of the micrograms of "tyrosine" produced per 5 ml. of trichloroacetic acid filtrate after a 10 minute digestion period at 30°. Proteinase A was measured by using acid-denatured hemoglobin at pH 3.5; proteinase B was measured by using urea-hemoglobin substrates at pH 6.2.

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Proteinase A activity</th>
<th>Proteinase B activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per ml.</td>
<td>per mg. protein</td>
</tr>
<tr>
<td>1</td>
<td>Entire chloroform autolysate...</td>
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</tr>
<tr>
<td>2</td>
<td>Supernatant liquid from autolysate...</td>
<td>176</td>
</tr>
<tr>
<td>3</td>
<td>Supernatant after incubation at pH 5</td>
<td>350</td>
</tr>
<tr>
<td>4</td>
<td>63% ethanol ppt...</td>
<td>365</td>
</tr>
</tbody>
</table>

Fig. 1. Change in the pH-activity curves of yeast proteinases which occurred when supernatant liquid of a crude bakers' yeast autolysate was incubated at pH 5 for 18 hours at 25°. The substrates employed for Curves A contained no urea, whereas those used for Curves B contained 6.6 M urea. The lower curves (A and B) represent the activity of the autolysate before incubation; the upper curves (A' and B') represent the activity of the same autolysate after incubation.
obtained with an autolysate of a strain of *Saccharomyces mangini*. The remainder of the data reported here was obtained by using autolysates of Fleischmann's compressed yeast for bakers.

In Table I, when the supernatant liquid from the autolysate was adjusted to pH 4.5 to 5.0, a heavy precipitate formed and much of the proteinase activity was found in the precipitate. The enzymes are merely adsorbed on the inert precipitate, since purified proteinase preparations are completely soluble at pH 4.5 to 5.0. This precipitate is not removed and usually disappears gradually during incubation at pH 5. During overnight incubation at pH 5, a remarkable self-purification of the proteinases takes place. An activation occurs at this pH and the total amount of proteinases A and B activity increases about 3- to 4-fold. In addition, it has been found that the proteinases digest the inert proteins of the autolysate most rapidly at pH 5. Thus the activity per mg. of protein increases 10- to 20-fold. Further purification has been obtained by dialysis and by alcohol, acetone, or ammonium sulfate precipitation of the activated autolysate. The proteinases may be precipitated from the supernatant liquid of the autolysate with 63 per cent ethanol before activation to obtain a stable dry preparation which may be activated subsequently by incubation at pH 5.

The specific activity of proteinase B in the alcohol precipitate (Table I) is about 5 per cent of that of crystalline trypsin. The amount of activated proteinase B which is obtainable from 1 gm. of compressed bakers' yeast is equivalent to about 0.1 mg. of trypsin.

**Activation of Yeast Proteinases**—The top curves of Fig. 1 illustrate the pH-activity curves of proteinases A and B after the activation at pH 5 mentioned above. The optimal pH for activated proteinase B is now approximately pH 6.2. The peak at pH 5 disappeared because this enzyme was activated and the urea in the pH 5 substrate could not cause additional activation.

The rate of activation of the yeast proteinases depended upon the pH at which the autolysate was incubated. The effect of pH on the activation of proteinases A and B is illustrated in Fig. 2. The optimal pH for the activation of proteinase A was about 4.9, whereas the optimal pH for the activation of proteinase B was about pH 4.4. At pH 3.8 to 4.3, proteinase B could be activated without any increase in the amount of proteinase A activity.

The rate of activation of proteinase B was increased by the presence of calcium ions. In an experiment in which an unactivated preparation was incubated at pH 5, the presence of 1 per cent CaCl₂ not only increased the activation rate but also increased greatly the total amount of proteinase B formed. Calcium ions are known to have a similar effect on the conversion
of trypsinogen to trypsin (9). The rate of activation of proteinase B was not increased by 0.1 m cyanide or 0.0004 m cysteine.

**Fig. 2.** The extent of activation of the yeast proteinases as a function of pH. Four aliquots of an unactivated autolysate were incubated at four different pH values at 30°. After 60 and after 127 minutes, each aliquot was analyzed for proteinase A and proteinase B activity, acid-denatured hemoglobin at pH 3.5 being used as substrate for proteinase A, and urea-denatured hemoglobin at pH 6.2 as substrate for proteinase B.

**Fig. 3.** The effect of 3 m urea on the proteinase B activity per ml. of an unactivated yeast autolysate. Aliquots of the autolysate were incubated at 30° and the proteinase B activity was measured at intervals by withdrawing 1 ml. aliquots and incubating for 10 minutes with 5 ml. of the urea-hemoglobin substrate at pH 6.2.

It was found that concentrated urea solutions greatly accelerated the rate of activation of proteinase B at pH 5 (Fig. 3). The maximal activation in a 20 minute period was obtained with 3 m urea at pH 4.8. Proteinase B was also rapidly activated at pH 4.4 by 3 m urea. However, the
destruction by urea was faster at this pH and prevented the attainment of greater activation. At pH 6.2 there was no apparent activation, only a gradual destruction by urea. Urea concentrations from 1.0 M to 6.0 M were found to activate proteinase B in 30 minutes at pH 5, the maximal activation occurring in 3 M urea. These results explain the sharp peak at pH 5 in the pH-activity curve of an unactivated proteinase B preparation.

Enzyme Calibration Curves—When activated proteinase B was exposed to 5.5 M urea at pH 5.0, 6.2, and 7.5, the enzyme was found to be inactivated appreciably in 20 minutes at 30°. Since the proteinase B substrates contain 5.5 M urea during digestion, it was therefore expected that the enzyme

![Fig. 4. Calibration curves of activated yeast proteinases at various pH values. An alcohol precipitate, similar to that in Table I, Step 4, of an activated autolysate was used. The activity of proteinase A was measured with acid-denatured hemoglobin at pH 3.5. For proteinase B, urea-denatured hemoglobin at pH 5.0, 6.2, and 7.5 was employed.](image)

would be destroyed gradually during incubation with the substrate; in fact, digestion rate curves showed that the rate of hemoglobin decomposition at pH 5.0, 6.2, and 7.5 decreased gradually during a 20 minute digestion period.

In order to measure accurately proteinase activities under these conditions, it was necessary to employ enzyme calibration curves. Fig. 4 shows the relationship between the amount of enzyme employed and the digestion products from the action of proteinase A at pH 3.5 and proteinase B at pH 5.0, 6.2, and 7.5. The extent of digestion was proportional to the enzyme concentration only at the lower enzyme levels. Therefore, in order to obtain a true measure of the amount of proteinase in a given preparation, calibration curves were employed throughout this work in the manner described by Anson (6).

Effect of Sulfhydryl Reagents on Yeast Proteinases—p-Chloromercuribenzoate (pCMB), which is a specific reagent for sulfhydryl groups (10),
has been found to inactivate yeast proteinase B, but to have no effect upon proteinase A (Fig. 5). Curves similar to those in Fig. 5 were also obtained when higher levels of pCMB were employed, and when the exposure to this reagent was carried out at other pH values between pH 4.5 and 7.0. The residual activity against urea-containing substrates between pH 5 and 7 may be attributable to proteinase A, which may be more resistant to the urea in the substrates in this pH range, although it was inactivated immediately by urea at lower pH values. A preparation of proteinase B containing no proteinase A was found to be inactivated completely by pCMB. The activity removed by this reagent may be restored by the addition of NaCN.

![Fig. 5. The effect of p-chloromercuribenzoate on yeast proteinases. The solid lines represent the pH-activity curves of an activated preparation of proteinases A and B. After incubation in a solution containing 0.001 M p-chloromercuribenzoate at pH 5 for 60 minutes at 25°, the preparation showed pH-activity curves represented by the broken lines.](image)

Although proteinase B appears to contain essential SH groups, these groups differ from those of papain. Cyanide or cysteine must be present; otherwise papain cannot attack hemoglobin (6). These compounds were without effect on the splitting of hemoglobin by yeast proteinases. In addition, iodoacetate, which inactivates papain, had no effect on the yeast proteinases.

It was found that 0.15 mg. per ml. of purified ovomucoid (Worthington Biochemical Laboratories) had no effect on proteinase A, but caused a 40 per cent inhibition of proteinase B. Crude soy bean extracts which were active against trypsin had no effect on either yeast proteinase.

**Thermostability of Yeast Proteinases**—The effect of pH on the stability of yeast proteinases at 50° was studied. Distinct pH-stability curves were obtained; proteinase A was most stable at pH 4.8, while proteinase B was most stable around pH 5.7. Fig. 6 represents the effect of heating aliquots
of an activated preparation at pH 4.0 and at pH 7.1 on the pH-activity curves of the yeast proteinases. The aliquot heated at pH 7.1 was entirely freed of proteinase A, while retaining about half of proteinase B. When this heated aliquot was treated with pCMB, it was completely inactivated. The aliquot heated at pH 4.0 retained most of its proteinase A activity and a small residual activity against urea-hemoglobin substrates at pH 5 to 7. The pH-activity curve of this aliquot was unaffected by pCMB. Thus, the effect of heating at pH 4 was similar to the effect of pCMB on the yeast proteinases.

**Action on Other Proteins**—pH-activity curves for the splitting of other proteins by unactivated and by activated preparations of yeast proteinases were determined. The methods employed were identical to those used with hemoglobin. The pH-activity curves of unactivated and activated preparations with urea-denatured ovalbumin or pumpkin-seed globulin (Difco) as substrate were similar to the curves obtained with hemoglobin (Fig. 1). The splitting of twice crystallized ovalbumin by unactivated yeast proteinases showed an additional peak at pH 7.8 (Fig. 7). It is possible that this peak represents the activity of a third yeast proteinase. Native ovalbumin was not attacked at any pH between 2 and 9, except for a slight hydrolysis in the region of pH 3.0, presumably attributable to proteinase A.

When urea-denatured lysozyme, fibrinogen (Armour), or casein was employed as substrate, unactivated yeast preparations did not show any peak at pH 7 to 8 in the pH-activity curves (Fig. 7). A curve (not shown) was determined by using fibrinogen substrates containing no urea. The activ-

![Figure 6. pH-activity curves of an activated yeast proteinase preparation before and after heating aliquots at pH 4.0 and at pH 7.1 for 20 minutes at 50°C. ●, activity against acid-denatured hemoglobin; ○, activity against urea-denatured hemoglobin.](http://www.jbc.org/content/233/5/926/F6.large.jpg)
ity increased steadily from pH 4 to 9, reflecting only the increased denaturation and vulnerability of the fibrinogen at higher pH values.

Fig. 7. Broken line, ovalbumin hydrolysis by an unactivated yeast proteinase preparation as a function of pH. The substrates were similar to the urea-hemoglobin substrates, containing 2 per cent recrystallized ovalbumin (11) and 6.6 M urea. Solid lines, hydrolysis of urea-denatured lysozyme by a yeast autolysate as a function of pH. The lower curve was obtained with an unactivated preparation and the upper curve with the same preparation after partial activation. The substrates contained 6.6 M urea and 2 per cent lysozyme prepared by adsorption on bentonite (12).

Fig. 8. The hydrolysis of casein by an activated yeast proteinase preparation as a function of pH. These substrates contained 2 per cent Difco isoelectric casein and no urea. Varying amounts of casein were insoluble in the range of pH 2.0 to 5.6.

When casein substrates containing no urea were employed, the pH-activity curve in Fig. 8 was obtained. Although this curve applies to an activated preparation, it is similar to the pH-activity curves for an unactivated preparation with hemoglobin substrates (Fig. 1). When the enzyme
solution used in Fig. 8 was treated with pCMB, the activity below pH 4 was unaffected, while the activity above pH 5 was practically abolished. The shape of the curve in Fig. 8 has a limited significance, however, because the casein digestibility is probably partially influenced by the effect of pH on the degree of solubility of the casein. When casein substrates were used, the peaks at pH 2.7 and 5.5 increased greatly during the activation process.

**DISCUSSION**

Proteinases A and B probably represent the two proteinases described by Dernby (1) in 1917. A preparation made by Dernby's procedure gave a pH-activity curve similar to the lower curves of Fig. 1, and this preparation underwent activation during the subsequent dialysis step employed by Dernby.

Grassmann, Willstätter, and coworkers stated that yeast contains only one proteinase, a papain-like enzyme (2-4). Using gelatin as a substrate, they reported an optimal pH of 5.0, optimal activation at pH 7, rapid activation by cyanide, and relative instability at pH 5.0. None of these properties agrees with the findings reported here. Grassmann et al. used an alcoholic KOH titration procedure to measure the gelatin hydrolysis; this method is a measure of the combined activity of proteinases and peptidases. It appears likely that the preparations used by Grassmann et al. contained peptidase activity, and some of the properties attributed to proteinase action were actually manifestations of peptidase activity. These authors state that they had freed their proteinase of known peptidases. However, additional peptidases have since been found in yeast (13).

As pointed out by Northrop and Kunitz (14), the alcoholic KOH method of proteinase analysis is insensitive as well as non-specific. Because of this insensitivity, Grassmann et al. employed a 24 or 48 hour digestion period at 40° in the analysis of their samples. We have found rapid inactivation of proteinases A and B below pH 3.5 and above pH 6.5 at 40°. Therefore, the optimal pH found under these conditions is primarily a reflection of the optimal pH for stability.

Hecht and Civin (5), employing Anson's pepsin substrate, claimed the discovery of a yeast "pepsin" with optimal activity at pH 1.8. These authors added a concentrated yeast autolysate to the unbuffered hemoglobin-HCl solution at pH 1.8. When this procedure was repeated here, it was found that the actual pH during hydrolysis was pH 3.0 to 4.0, which is approximately optimal for proteinase A activity. When the pH during digestion was controlled carefully, autolysates prepared by the method of Hecht and Civin displayed normal pH-activity curves similar to those in Fig. 1. They also reported that dilution or dialysis of an autolysate inactivated yeast "pepsin." These findings are explained by the fact that diluted
or dialyzed autolysates have less effect on the pH of the pepsin substrate; the actual pH during hydrolysis would be closer to pH 1.8 and therefore less proteinase A activity would be manifested.

None of the substrates employed in this investigation yielded continuous pH-activity curves which represented a valid measure of the effect of pH on hydrolysis rate. Native proteins are unsatisfactory substrates because of the effect of pH on the degree of insolubility or denaturation. Urea-containing substrates are also unsatisfactory because of the marked effect of urea in the destruction of proteinase A at pH values below 4.0 and the activation of proteinase B at pH 5.0. Nevertheless, all of the urea-denatured proteins employed as substrates showed approximately the same optimal pH for activated proteinase B. Since the isoelectric points of these proteins varied from 4.6 to 10.5, the optimal pH appears to depend on the nature of the enzyme rather than on the charge on the substrate molecule (15).

The effect of concentrated urea solutions on the activation of proteinase B is an unusual phenomenon. It may be caused by acceleration of the conversion of a zymogen to the active enzyme or by denaturation of an inhibitor present before activation. A similar activation, the conversion of prototyrosinase to tyrosinase by 2 M to 7 M urea, has been reported by Bodine and Allen (16).

The author wishes to express his appreciation to Dr. Sutton Redfern for the helpful interest shown in this investigation.

**SUMMARY**

Two different proteinases have been obtained from bakers' yeast and have been partially characterized. Proteinase A is extremely labile in urea solutions and has an optimal pH of 3.7 for the splitting of acid-denatured hemoglobin. Proteinase B exhibits an optimal pH of 6.2, urea-denatured proteins being used as substrates. Proteinase B contains essential sulfhydryl groups, whereas proteinase A does not.

These enzymes are liberated simultaneously from the yeast cell by chloroform or ether autolysis. When the supernatant solution from this autolysate is incubated overnight at pH 5, the activity per ml. of proteinases A and B increases 300 to 400 per cent. During this process, these proteinases digest 70 to 80 per cent of the other proteins in the autolysate. At pH 4.3 proteinase B may be activated independently of proteinase A. The rate of activation of proteinase B is increased greatly by concentrated urea at pH 5. These proteinases have different pH-stability curves, so that they may be inactivated selectively by heat treatment.

The activity of the yeast proteinases in hydrolyzing various native and
denatured proteins was measured as a function of pH. Four different strains of *Saccharomyces cerevisiae* were examined and all were found to contain these two proteinases. These findings are compared and correlated with those of previous investigators.

**BIBLIOGRAPHY**

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