Reduction of Nucleic Acid Content in *Candida* Yeast Cells by Bovine Pancreatic Ribonuclease A Treatment

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Yeast as a source of protein for human consumption is limited by its relatively high nucleic acid content. In this study, we developed an enzymatic method of decreasing the nucleic acid content. *Candida utilis* cells, heat-shocked at 80°C for 30 sec, were treated with bovine pancreatic ribonuclease A. Maximum leakage of nucleic acid was observed when the incubation temperature was between 55 and 65°C, the pH of the system from 6.75 to 8.0, and the enzyme-to-cell ratio 1:10,000 on a weight-by-weight basis. Other factors, such as yeast strain, age of cells, and method of propagation, did not influence the susceptibility of the yeast cells to the action of ribonuclease. Buffers and monovalent cations had no inhibiting effects. Magnesium and calcium ions at concentrations greater than 0.001 M showed marked inhibition on the rate of nucleic acid leakage. This enzymatic method reduced the nucleic acid content of yeast cells from 7.5 to 9.0% to 1.5 to 2.0% with no significant concomitant loss of protein.

The food value in terms of protein content of some single-celled organisms has been investigated repeatedly (8). Recently, a study on the effects of large amounts of yeast on blood uric acid levels was conducted (3). The results obtained suggest that, if single-cell protein is the sole protein source in human diets, the nucleic acid content must be reduced to within the safe level of 2 g per day. Maul et al. (9) developed a three-step heating process for reducing the nucleic acid content of yeast. The first step was believed to effect a denaturation of the ribosomes or to effect the activation of the endogenous ribonuclease or both. The final two steps caused a series of enzymatic reactions, resulting in the accumulation and subsequent leakage of the hydrolysis products.

Another enzymatic method in which an exogenous ribonuclease is added to a suspension of yeast cells has potential. However, contradictory reports appear in the literature. Necas (12) and DeKloet et al. (2) observed that, unless cells were first fixed, they were strongly resistant to the action of exogenous ribonuclease. Schlenk and Dainko (14), on the other hand, reported that live yeast cells treated with ribonuclease released about 80% of the ultraviolet-absorbing materials. In this study, we have developed a method of almost total nucleic acid removal from *Candida* yeast cells without significant loss of protein.

**MATERIALS AND METHODS**

**Organisms.** *C. utilis* (NRRL Y900) was used in most of the experiments. Several tests were conducted with *C. intermedia* (ATCC 6795). Stock cultures of both strains were kept on Sabouraud slants.

**Propagation methods.** *Candida* yeast culture was grown in two ways: continuously and batchwise. Continuously, culture was grown in the defined type I medium described by Miller and Johnson (10). To this medium, 6 g of separately sterilized glucose per liter was added. The dilution rate throughout the culture was kept constant at approximately 0.2 hr⁻¹ (μ_max = 0.4 hr⁻¹). The pH of the system was adjusted to and kept at 4.0. The cell concentration was approximately 4.0 to 4.5 g/liter.

Batchwise, the culture was grown by inoculating 100 ml of medium in a 500-ml Erlenmeyer flask. The medium used for cultivation was described by Miller and Johnson (10) as medium II for shake-flask studies. This medium was supplemented with 7.6 g of glucose per liter and 4.0 g of yeast extract per liter. The flask was incubated at 30°C on a rotary shaker at 200 rev/min. The cells (approximately 3.5 to 4.0 g/liter) were harvested 15 to 17 hr after inoculation.

Both batches were separated from the medium by centrifugation at 5,000 × g for 5 min. Centrifugation was done in a refrigerated centrifuge (Sorvall Superspeed RC2-B). The cells, in pellet form, were resuspended in double-distilled water. The pH of this suspension was carefully adjusted to a value within the range of pH 6.75 to 7.5 by dropwise addition of 0.1 N NaOH.
Dry weight. Dry weight was determined by using aluminum weighing dishes (6 cm in diameter by 1.5 cm in depth). A 5-ml amount of cell suspension was pipetted into the weighing dish and dried for 48 hr in a still-air oven at 80 C.

Heat treatment. The cells were heat-treated in a boiling-water bath for 5 min. A 250-ml flask containing 50 ml of cell suspension was shaken continuously during heating.

The heat-shocking step in the method developed by Maul et al. (9) was also employed. The aqueous suspension of the cells was passed through a stainless-steel tube measuring 56 cm by \( \frac{1}{32} \) inch (0.08 cm) and immersed in a vigorously stirred water bath.

Enzyme preparation. Bovine pancreatic ribonuclease A was obtained from Worthington Biochemical Corp. (Freehold, N.J.) in a solution of 0.1 m phosphate buffer containing 0.1% phenol preservative. The enzyme solutions used were prepared with double-distilled water. These solutions were stored in the frozen state and were found to be stable for several weeks.

Enzyme treatment. A 5-ml amount of the enzyme solution at a concentration of 1 \( \mu g \)/ml was added to 50 ml of the cell suspension. The cells were immediately incubated at 55 C. Throughout the incubation period, the cells were agitated on a rotary shaker at a speed of 150 to 200 rev/min. Some experiments were conducted under a suboptimal incubation temperature of 37 C to delay the reaction.

Portions (2-ml) of the samples were withdrawn at different incubation periods: 10, 30, 60, and 90 min. The samples were pipetted into small 15-ml plastic centrifuge tubes and cooled in an ice-water bath to insure reaction arrest. Samples were centrifuged at 500 \( \times \) g for 5 min. The supernatant was collected, whereas the cell pellet was stored for further analysis.

Other analyses. Protein content was determined by the biuret (15), and amino acid content was determined by a ninhydrin procedure. Analysis of the cell pellet and supernatant fluids for nucleic acid content and nucleotide composition was performed as described by Ohta et al. (13). Calculations are based upon an absorbancy of 32 ml/mg-cm for yeast RNA as determined by Ohta et al. (13).

RESULTS

Heating the cells. Preliminary experiments were performed by the method of Schlenk and Dainko (14) to determine the extent of bovine pancreatic ribonuclease action on live Candida yeast cells. An insignificant amount of ultraviolet-absorbing material was detected in the supernatant fluid after 90 min of incubation (Fig. 1). To increase permeability, cells were heat-treated before incubation with the enzyme. The cells were heated at 100 C for 5 min. There was 20 to 25% leakage of ultraviolet-absorbing material after heating. After treatment with ribonuclease, an additional 60 to 65% of the ultraviolet-absorbing material, which was believed to be nucleic acid, was observed in the suspending medium. The cells retained only approximately 10 to 25% of their initial nucleic acid content after this maximum leakage.

In the following experiments, the cells were heated before incubation with the enzyme. The influence of three parameters on reduction of nucleic acid content in yeast cells was considered in developing this method: (i) the yeast cells, (ii) the heat treatment, and (iii) the enzyme treatment. Conditions of the above parameters were regulated for optimum efficiency of the method.

Condition of yeast cells. The possibility of two different yeast cell strains showing different sensitivities to the action of ribonuclease was tested. Both C. utilis and C. intermedia were grown batch-wise. All conditions for heating and enzyme treatment were identical for both cases. The effect of bovine pancreatic ribonuclease was the same on both strains (Table 1). Approximately 80 to 90% of the total nucleic acid leaked into the suspending media of both strains.

Experiments were also conducted to determine whether the age of the culture affected the cell sensitivity to ribonuclease A. One batch of C. utilis cells was grown for 10 hr and another was grown for 17 hr. Both cases showed an almost equal release of nucleic acids.

Effects of the propagation methods were also studied. C. utilis cells grown continuously were compared to those grown batch-wise. No difference was detected in the leakage of the ultraviolet-absorbing materials between the two cell batches.

Optimization of the heat treatment. Since the temperature-time relationship used in the preliminary experiment was arbitrarily chosen, we decreased both time and temperature to reach an optimum value. The heat-shock method described by Maul et al. (9) was employed with different temperatures: 65, 70, 75, 80, and 85 C. The heat-
TABLE 1. Effect of bovine pancreatic ribonuclease on Candida utilis and C. intermedia

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Per cent nucleic acid leaked from the cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. utilis</td>
</tr>
<tr>
<td>0</td>
<td>26.0</td>
</tr>
<tr>
<td>10</td>
<td>59.0</td>
</tr>
<tr>
<td>30</td>
<td>80.0</td>
</tr>
<tr>
<td>60</td>
<td>86.0</td>
</tr>
<tr>
<td>90</td>
<td>88.0</td>
</tr>
</tbody>
</table>

* Enzyme concentration, 0.1 μg/ml; cell concentration, 4 mg/ml; pH, 6.8; incubation temperature, 55°C; cells heated for 5 min at 100°C before treatment.

Fig. 2. Effect of heat-shocking temperature on the rate of nucleic acid leakage from the cells. Conditions: Candida utilis, 4 mg/ml (dry weight), incubated with 0.1 μg of ribonuclease per ml at 55°C; pH was 6.85.

Fig. 3. Effect of incubation temperature on the rate of nucleic acid leakage from the cells. Candida utilis, 4 mg/ml, heat-shocked at 100°C for 5 min, was incubated with 0.1 μg of ribonuclease per ml; pH was 7.0.

Fig. 4. Effect of pH on the rate of nucleic acid leakage from the cells. Candida utilis cells, 4 mg/ml, were heat-shocked (100°C, 5 min), and the pH of the different samples was adjusted by using 0.1 N HCl for the acidic side and 0.1 N NaOH for the basic side. Ribonuclease concentration, 0.1 μg/ml; incubation temperature, 55°C.

**Optimization of enzyme treatment.** We used the following incubation temperatures for the enzyme treatment: 20, 30, 37, 55, 65, 75, and 85°C. As the temperature was increased, leakage rate likewise increased (Fig. 3). The highest rate occurred between 55 and 65°C. When the cells were incubated at 75 and 85°C, the decrease in leakage was marked.

Most enzymes are active only within a limited pH range, and an optimum pH is often desired. Figure 4 shows the optimum pH of ribonuclease in the reaction. The pH was adjusted with 0.1 N HCl for the acidic pH and 0.1 N NaOH for the basic pH. Maximum leakage was observed between pH 6.75 and 8.0.

We evaluated the effect of different buffer systems and ions on the action of ribonuclease. Tris-(hydroxymethyl)aminomethane, citrate, and
phosphate buffers did not interfere with the enzyme reaction. Effects of the different ions are shown in Fig. 5. In the presence of the monovalent cations Na⁺, NH₄⁺, and K⁺, leakage was faster, although not significantly, than leakage in water. In the presence of the divalent cations Mg²⁺ and Ca²⁺, leakage was much slower.

The effect of increased concentrations of calcium and magnesium ions on the ribonuclease action was tested. At the concentration level of 0.001 M, the inhibiting effect of Ca²⁺ and Mg²⁺ was not exhibited. At concentrations higher than 0.001 M, there was marked inhibition of the enzyme.

The optimum enzyme-to-cell ratio was determined by using the cell concentrations of 2.5, 5.0, 7.0, 9.0, and 15.0 mg/ml. In all cases, the enzyme concentration was 1 µg/ml, and the incubation time was 60 min. As the cell or substrate concentration was increased, the rate of nucleic acid release into the supernatant fluid likewise increased (Fig. 6). A maximum substrate concentration was obtained as the leakage rate approached a limiting value. The substrate concentration which effected a saturation of the enzyme at an enzyme concentration of 1 µg/ml within the 60-min incubation period was about 10 mg/ml or a ratio of 1:10,000. A further increase in the substrate concentration did not yield further increase in the leakage rate.

Figure 7 shows the effect of the enzyme-to-cell ratio on the process completion time. Although the ratio of 1:10,000 is not shown, we see that it takes approximately 60 min for the enzymatic reaction to finish with this ratio.

**Analysis of leakage products.** The different leakage products were determined after heat-shocking and after 90 min of incubation with the enzyme.

The leakage products are expressed in percentages based on cell dry weight as shown in Table 2. We determined that the initial amount of nucleic acid in the untreated cell is in the range of 7.5 to 9.0%. Upon heating, 2.0 to 3.0% of nucleic acid was detected in the supernatant fluid. This leakage did not increase, even when the heating was increased from 1 min to 5 min at 100 C. After incubation with ribonuclease, a threefold increase in the leakage was observed.

We found minimal leakage of proteins in treated cells relative to the total amount of protein in untreated yeast cells, which ranges from 45 to 55% based on cell dry weight.

The leakage of amino acids was also determined after heat-shocking and enzyme treatment. Nearly all leakage occurred during the heat-shocking step. A very slight increase or none at all occurred during the incubation with the enzyme.

After enzyme treatment, the supernatant fluid was further analyzed. Table 3 shows the identification and quantification of the different nucleotides that leaked. More 3'-mononucleotides than 5'-
These observed to of permeate membrane. nucleotides, struum during materials of the acids and amino acid identified and determined the intracellular nuclease further enzyme. sary step tant were mononucleotides the measurable by phosphate. guanosine phosphate; CMP, 3’-CMP 5’-GMP 3’-AMP 5’-AMP 0.88 0.23 4.0 3.27 1.38 24.2 19.65 1.31 23.0 13.20 1.2 28.0 15.72 1.59 45-55 4.0-5.5 1.0-1.2 1.0-2.0 4.0-5.5 0.3-0.5 0.2-0.4 1.0-1.2 1.0-2.0 4.0-5.5 0.3-0.5 0.2-0.4 1.0-1.2 1.0-2.0 * Heat-shocked at both conditions.

TABLE 3. Identification and determination of leaked nucleotides

<table>
<thead>
<tr>
<th>Nucleotides*</th>
<th>Absorbancyb</th>
<th>Concnc</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’-CMP</td>
<td>2</td>
<td>1.31</td>
<td>23.0</td>
</tr>
<tr>
<td>5’-AMP</td>
<td>3.27</td>
<td>0.23</td>
<td>4.0</td>
</tr>
<tr>
<td>3’-AMP</td>
<td>19.65</td>
<td>1.38</td>
<td>24.2</td>
</tr>
<tr>
<td>5’-GMP</td>
<td>0.88</td>
<td>0.07</td>
<td>1.2</td>
</tr>
<tr>
<td>3’-GMP</td>
<td>13.20</td>
<td>1.12</td>
<td>19.7</td>
</tr>
<tr>
<td>3’-UMP</td>
<td>15.72</td>
<td>1.59</td>
<td>28.0</td>
</tr>
</tbody>
</table>

* Abbreviations: AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate.

a Arbitrary unit × 5 = true unit.
b Arbitrary unit divided by molar absorptivity.

mononucleotides were identified in the supernatant fluid.

DISCUSSION

Effects of temperature on the enzymatic hydrolysis of RNA. The heat treatment was a necessary step before incubating the yeast cells with the enzyme. Thermal injury of the cells was exhibited by the measurable amounts of cell components identified and determined in the suspending medium immediately after heat treatment. In this study, nucleotides, amino acids, and proteins were observed to leak out during the heat-shocking step. These findings agree with those of Iandolo and Ordal (6) in their study of the thermal injury of Staphylococcus aureus. Their heating menstruum produced substances which were primarily nucleic acid in nature. They also identified free amino acids and proteins released from the cells. These observations indicate that a modification of the permeability barrier has occurred.

The continued leakage of ultraviolet-absorbing materials during the cell incubation with ribonuclease further indicates a modification of the cell membrane. The exogenous enzyme was able to permeate the cells and, consequently, degrade the intracellular ribonucleic acid (RNA). In another study, this cell membrane damage by heat was demonstrated in S. aureus by the intracellular penetration of an unrelated compound, 8-anilino-1-naphthalene-sulfonic acid (1).

Another salient point of the heat-shocking experiment is the limiting time-temperature relationship necessary to effect a change in the permeability of the cell membrane. The yeast cell membrane must have become completely permeable to exogenous ribonuclease at a temperature of 80 C for a residence time of 30 sec in the heating coil for maximum nucleic acid leakage. At much lower temperatures, e.g., 65 C, the relatively slow leakage of ultraviolet-absorbing material during incubation at 55 C may be due to the activated endogenous ribonuclease. Heat-shocking live cells at 65 C for 30 sec may have the same thermal activating effect on the latent enzyme as 68 C for 5 to 6 sec, the time-temperature relationship used by Maul et al. (9). A substrate change due to the thermal energy supply must also be considered. Possibly, denaturation makes the yeast RNA more available to enzyme action. Haight and Ordal (5) reported that heat caused increased instability of the ribosomes, making them more available to the ribosomal enzyme action.

The rate of RNA degradation by bovine pancreatic ribonuclease is further affected by the incubation temperature. At temperatures higher than the optimal 65 C, e.g., 75 and 85 C, a slower rate of nucleic acid leakage was observed. This marked decrease in the enzyme activity may have been due to the thermal denaturation of ribonuclease. However, there may be another explanation. Although the enzyme is reportedly very heat-stable (7), this thermal stability might occur only when the enzyme is heated in a water or buffer solution alone. When it is heated with the yeast cells to the same temperature, proteins within the cells may undergo denaturation. These denatured proteins may effect a decrease in the enzyme activity.

Effects of divalent cations on the enzymatic hy-
drolysis of RNA. In the presence of calcium and magnesium ions at concentrations greater than 0.001 M, the nucleic acid leakage was inhibited. This inhibitory effect was also observed by other investigators. Schlenk and Dainko (14) found that approximately 50% inhibition of ribonuclease activity was effected by 0.15 mM Mg\(^{2+}\) and 0.05 mM Ca\(^{2+}\). They explained that cations occupied sites on the cell membrane to which the enzyme must be attached for action, thus inhibiting the reaction. This mechanism explains inhibition even at very low ion concentrations. In our study, however, heat treatment had already damaged the cell membrane affecting enzyme entry, and inhibition by the cations as described above is unlikely. Stabilization of the ribosomes by these divalent cations causing the slow degradation of RNA is a more probable explanation.

Merrill and Reiss (11) have observed inhibition by Ca\(^{2+}\) and Mg\(^{2+}\). They proposed that before the ribonuclease can hydrolyze the specific phosphodiester linkages to form cyclic nucleotides, a nonhydrolytic alteration in the RNA structure takes place. Ca\(^{2+}\) and Mg\(^{2+}\) seem to have their inhibiting effect on this nonhydrolytic reaction by increasing the hydrogen bonding in the structure of RNA. Besides ions binding to the substrate, Eichorn et al. (4) indicated that ions binding to an active histidine site on the enzyme molecule may produce a similar effect.

Applications of the enzymatic hydrolysis of RNA. In this study, an enzymatic method of degrading RNA in yeast cells has been developed, and different factors surrounding the method have been studied. The method appears promising. Even with a slurry-type suspension, which is approximately 10 to 15 mg of cells per ml, the degradation of RNA by the added ribonuclease is feasible. This process is simpler than other methods of reducing nucleic acid content in yeast. For example, in the method developed by Maul et al. (9), the heat-shocking step is vital and critical. In our method, the heat-shocking step may be important but is not as critical. In addition, only one incubation temperature is necessary to facilitate the process in scaled-up production.

Our method, however, may be economically impractical for large-scale production. An expensive enzyme, bovine pancreatic ribonuclease A, is added, whereas the method of Maul et al. (9) requires no other substance. To lower the expense of this process, three modifications may be considered. (i) We may use crude pancreatic ribonuclease, since ribonuclease B was found to work just as well in the system. (ii) The enzyme-to-cell ratio may be increased; however, longer incubation time will be required. (iii) Microbial phosphodiesterase may be used instead of ribonuclease, producing 5'-mononucleotide flavor components and low nucleic acid single-cell protein.

LITERATURE CITED