Characterization of a Heat-Shock Process for Reduction of the Nucleic Acid Content of *Candida utilis*

S. OHTA, S. MAUL, A. J. SINSKEY, AND S. R. TANNENBAUM

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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A process for reducing the nucleic acid content of *Candida utilis* NRRL Y900 has been developed. The optimal process consists of heating the cells suspended in spent medium initially at pH 4.0 for various times at three different temperatures. Initially a heat-shock at 68°C for 1 to 3 sec is performed followed by incubation for 1 hr at 45 to 50°C and for a 2nd hr at 52 to 55°C. The distribution of degradation products has been characterized. Initially 90% of the nucleic acids were in a polymerized form (extractable by hot perchloric acid). After 30 min, much of this material was hydrolyzed but remained within the cell (extractable by cold perchloric acid). After 2 hr, most of the hydrolysis products leaked into the surrounding medium with only a small amount of low-molecular-weight material remaining within the membrane. Predominantly 3'-mononucleotides accumulated within the cell and eventually leaked from the cell.

The necessity of reducing the nucleic acid content of single-cell protein for human consumption has been adequately summarized (5). In a previous communication, we briefly described a process for producing a dried yeast product with a low nucleic acid content from *Candida utilis* NRRL Y900 (16). The optimal process consists of heating the cells for various times at three different temperatures suspended in spent medium initially at pH 4.0. The first step is a short heat-shock, typically 1 to 3 sec, at 68°C. This is followed by incubation for 1 hr at 45 to 50°C and for a 2nd hr at 55°C.

This paper describes the reactions occurring in the various steps of the treatment and includes characterization of the reaction products and properties of the enzyme(s) in both whole cells and cell homogenates.

**MATERIALS AND METHODS**

Cell growth. *C. utilis* NRRL Y900 was grown in continuous culture on the type I medium of Miller and Johnson (16) at pH 4.0 and at a dilution rate of approximately 0.2 hr⁻¹ (µmax = 0.4 hr⁻¹). The cell concentration was about 4 to 4.5 g/liter.

Heat treatment. Heat treatment was performed by the procedure developed by Maul et al. (15). The heat-shock was done by passing the cell suspension in spent medium through a piece of stainless-steel tubing 56 cm in length and ⅜ inch in diameter, immersed in a vigorously stirred water bath. After the heat-shock, the suspension was collected in an ice bath, and the collected cell suspension was divided into 10-ml portions in Pyrex test tubes and incubated further.

**Preparation of samples for the examination of the degradation products.** The following samples were prepared for the analysis of the degradation products.

To the supernatant fraction, after the heat treatment, the cell suspension was centrifuged and washed by the same volume of water at 0°C. The pooled supernatant fluid was called "supernatant fraction."

To prepare the cold perchloric acid fraction, the precipitated cells after preparation of the supernatant fraction were suspended in the same volume of 0.5 N perchloric acid (PCA), kept at 0°C for 20 min, centrifuged, and washed with a 10-ml volume of the acid solution. The pooled supernatant fluid was called "cold PCA fraction."

To prepare the hot PCA fraction, after the extraction by cold PCA, the cells were suspended in water. Five milliliters of the suspension was mixed with an equal volume of 1 N PCA, heated at 70°C for 20 min, and centrifuged after being cooled. The supernatant fluid was called "hot PCA fraction."

To prepare total nucleic acid, 5 ml of the original cell suspension or the cell suspension washed by buffer or salt solution was mixed with an equal volume of 1 N PCA, heated at 70°C for 20 min, and centrifuged after being cooled. The supernatant fluid was called "total nucleic acid."

**Calculation of the amount of nucleic acid and the degradation products.** The amount of nucleic acid or the amount of degradation products was determined by
measuring absorbancy at 260 nm. An absorptivity of 32 ml/mg-cm for the nucleic acids was determined by chromatographic analysis of an alkaline hydrolysate of extracted ribonucleic acid (RNA) and the known spectral characteristics of the individual bases.

**Gel-permeation chromatography.** The molecular weight distribution of the degradation products was examined by gel-permeation chromatography (GPC) with Sephadex G-25 or Bio-Gel P-2 by the method described by Hohn (10) and Uziel and Cohn (29) with some modifications.

It was found that mononucleotides can be completely separated from RNA and also from nucleosides or bases when GPC was performed with Bio-Gel P-2 in phosphate buffer. The following conditions were employed: column, Bio-Gel P-2, 100 to 200 mesh, 1.5 by 147 cm; sample, 1 ml of the mixture of about 0.5 m concentration for each component; buffer, 0.033 M phosphate buffer (pH 7.0); flow rate, 30 to 35 ml/hr; fraction volume, 4 ml.

By this procedure, oligonucleotides of molecular weight greater than 1,800 can be separated from mononucleotides. Therefore, GPC was done by this new procedure under the conditions mentioned above.

Concentration of the samples was necessary because of their low absorbancy. The supernatant fraction was concentrated by lyophilization after the adjustment of pH to 7.0 by KOH solution. The cold PCA fraction was neutralized by KOH solution at 0°C, and precipitated potassium perchlorate was removed by filtration. The filtrate was concentrated by lyophilization. These dried samples were dissolved in a small amount of water, and, after filtration, 1 ml of these concentrates containing 20 to 40 absorbancy units was put on the column.

**Ion-exchange chromatography.** Gradient ion-exchange chromatography was performed by the procedure reported by Hurbert et al. (11).

When the sample was a supernatant fraction, it was directly applied to the column. In the case of a cold PCA fraction, it was concentrated by the same procedure as that for gel filtration, because PCA inhibited good separation. About 200 to 600 absorbancy units were put on the column.

The separated components were identified by this position on the chromatogram and by ultraviolet (UV) spectrometer in acid and alkaline media. The following molar absorptivities at 260 nm in acid medium were used: adenosine triphosphate (ATP) and adenosine monophosphate (AMP) = 14.2 × 10^{4}; guanosine triphosphate (GTP) and guanosine monophosphate (GMP) = 11.8 × 10^{4}; cytidine monophosphate (CMP) = 6.8 × 10^{4}; uridine monophosphate (UMP) = 9.9 × 10^{4}.

The following nomenclature is used for the degradation products as described by Hurbert et al. (11).

**Bases and nucleosides.** After addition of the sample, the column was washed by water until absorbancy was less than 0.01. The effluent and washing water were pooled and represented bases and nucleosides.

**Mononucleotides.** The part eluted by 4 N formic acid constituted mononucleotides.

**Di- and triphosphates.** The part eluted by the next three eluting solutions constituted the di- and triphosphates.

**Oligonucleotides.** The part eluted by 2 N formic acid plus 2 M ammonium formiate plus the part retained on the column constituted oligonucleotides. The latter part was calculated by the difference of the charged amount and the eluted amount.

**Other analyses.** Dry weight determinations were made by collecting cells on dried, preweighed membrane filters (Millipore Corp.) and redrying at 70°C for 18 hr. Protein was determined by the biuret procedure (25).

**Cell homogenates.** One gram of wet cells was washed once with water and ground with 4 g of powdered glass (Amend Drug and Chemical Co.) for 6 to 8 min in a chilled mortar. The paste was suspended in 30 ml of water and centrifuged at 5,000 × g for 10 min. The supernatant fluid was stored at −20°C for several months without appreciable loss of ribonuclease activity.

**Ribonuclease assay.** Reactions contained 200 μmoles of (potassium) acetate (pH 5.5 or other pH values as indicated), 1.5 mg of RNA (Sigma type VI from *Torula* yeast), other additions as indicated, and homogenate from approximately 1 mg of cells in a total of 1 ml. Reactions were conducted at 4°C for 45 min or at 52.5°C for 20 min and stopped by the addition of 1 ml of 0.4% uranyl acetate in 12% perchloric acid. After incubation for 15 min at 4°C and centrifugation for 15 min at 12,000 × g, supernatant fluids were removed, diluted (1:10) with water, and measured at 260 nm. The absorbancy change was linear with time and nearly linear with enzyme concentration to 0.6 to 0.7 absorbancy units. Blanks containing either 0.4% uranyl acetate (and stopped by addition of 12% PCA) or no enzyme were run with each assay.

**RESULTS**

**Time-temperature relationships.** The effects of the temperature and the length of time of heat-shock (step 1) on the extent of nucleic acid removal were previously shown (15) to be optimal between 62 and 68°C for a heating time of 17 sec and at 68°C for treatments of shorter duration. The effects of various times of heat-shock at 68°C followed by incubation at 50°C for 1 hr and 55°C for 1 hr are shown in Fig. 1. Other experiments showed that heat-shocking at lower temperatures for longer times or heat-shocking at higher temperatures for shorter times did not lead to an optimal process under any conditions of subsequent incubation. Omission of heat-shocking resulted in only a 10% reduction in nucleic acid compared to an 80 to 85% reduction when optimal heat-shocking is used. Heat-shock alone did not significantly alter the nucleic acid content of the cells (Table 1).

Table 1 shows the effect of different incubation temperatures for step 2, which followed heat-shock at 68°C for 5 to 6 sec (step 1) and preceded further incubation at 55°C for 1 hr (step 3). The optimum temperature was 50°C.
Figure 2 shows the effect of temperature for the third step after heat-shock (step 1) and 1 hr of incubation at 50°C (step 2). Included in Fig. 2 are the changes in the reduction of cellular hot PCA and cold PCA fractions after a reaction time of 1 hr for a range of temperatures. When step 3 was carried out in an ice bath, the final content of hot PCA-soluble nucleic acids was 4%. At 55°C, at which temperature the reduction was most extensive, the hot PCA fraction was reduced to less than 1%. This reduction was inhibited at higher temperatures.

Since the optimal temperatures for steps 2 and 3 were 50 and 55°C, respectively, steps 2 and 3 could be replaced by a one-step 2-hr incubation at a compromise temperature of 52.5°C.

After heat-shock and incubation for 2 hr at 52.5°C, the final nucleic acid content was less than 2%.

Examination of the degradation products. To determine what kinds of enzyme systems are participating in the degradation of nucleic acids in yeast during the above-described processes, it is necessary to examine the intermediate and final products of degradation. Polynucleotides and oligonucleotides have been excreted by some microorganisms during degradation of RNA (4, 7, 13, 14).

The formation of nucleoside diphosphates during RNA degradation is the result of RNA hydrolysis by polynucleotide phosphorylase (1, 12, 27, 28, 30). Most yeasts release mononucleotides during RNA degradation (9, 18, 19, 27, 31). 3',5'- or 2',3'-Cyclic mononucleotides are formed depending upon the enzymes participating in the degradation of RNA. In some cases, part of the mononucleotides can be converted to nucleosides or bases (31).

The distribution of UV-absorbing materials in the three steps is shown in Table 2. Initially 90% of the nucleic acids were in the hot PCA fraction. Immediately after heat-shocking, the distribution of nucleic acids was the same as that observed in the non-heat-shocked cells. The size of the nucleotide pool of the original cells was about 10% of the total nucleic acid. After heat treatment, more than 85% of the total nucleic acid leaked out of the cells.

GPC was employed to determine whether any oligonucleotides are present in the supernatant fluid and cold PCA fraction after 30 min of incubation at 50°C after heat-shocking, as well as in the final supernatant fluid after the second incubation at 55°C (Fig. 3).

All three fractions showed the absence of appreciable amounts of oligonucleotides, and most
Table 2. Distribution of the degradation products

| Fractions          | Nucleic acids (%) |
|--------------------|-------------------|
| Initialb           |                   |
| Total              | 100               |
| Supernatant fluid  | 2.4               |
| Cold PCA           | 8.7               |
| Hot PCA            | 90.1              |
| Recovery           | 102.2             |
| After 30 min at 50 C |                  |
| Total              | 100               |
| Supernatant fluid  | 17.2              |
| Cold PCA           | 33.8              |
| Hot PCA            | 70.2              |
| Recovery           | 101.2             |
| After 120 minb     |                   |
| Total              | 100               |
| Supernatant fluid  | 86.5              |
| Cold PCA           | 4.2               |
| Hot PCA            | 12.0              |
| Recovery           | 102.7             |

a No significant differences in the initial distribution of nucleic acids were observed immediately after heat-shocking from non-heat-shocked cells.
b For 1 hr at 50 C and 1 hr at 55 C.

of the compounds corresponded to the position of mononucleotides. After 30 min of incubation, the cold PCA fraction showed the presence of slightly higher molecular weight components than other fractions.

A more detailed examination of the nucleic acid components in the three fractions indicated in Fig. 3, as well as the initial nucleotide pool immediately after heat-shocking, was carried by ion-exchange chromatography (Table 3).

The major component of the initial cold PCA fraction was ATP. The cold PCA fraction after 30 min of incubation was a mixture of 3'-mononucleotides and 5'-mononucleotides. About 13% of the supernatant material was bases and nucleosides and 83% was mononucleotides (mainly 3'-mononucleotides). The presence of 2',3'-cyclic mononucleotides or oligonucleotides was not observed in either sample.

The final supernatant fluid contained a small amount of 5'-AMP and 5'-GMP. Their amounts correspond to 5.0 and 1.3% of the total mononucleotides, respectively. The presence of small amounts of 5'-CMP and 5'-UMP is also probable. But, as their peak positions on the chromatograms were almost the same as those of 3'-CMP and 3'-GMP, their amounts could not be calculated.

Table 4 presents the overall change of components during the heat treatment. These values were calculated from the values of Table 2, multiplied by those of Table 3. An increase in bases and nucleosides and a decrease of nucleoside polyphosphates were observed after 30 min of incubation. This suggests the presence of pyrophosphatase as well as nucleotidase or nucleosidase. It is uncertain whether the nucleotidase acts on 5'-mononucleotides or 3'-mononucleotides.

RNA degradation in cell homogenates. Cell homogenates behave differently from intact cells in two respects: no heat-shock is necessary for initiation of RNA hydrolysis and the hydrolysis occurs much more rapidly in homogenates (complete in 10 to 15 min in homogenates in contrast to 1.5 to 2 hr in whole cells).
The ribosomes sedimented from substrate, mainly because the incubation pares decreases. The primarily also degraded compounds were often the nucleosides. Bases 5'-AMP/5'-AMP were degraded to 5'-AMP at pH 5.72. The degradation products from homogenates and intact cells are listed in Table 5. The composition of degradation products from homogenates at pH 5.45 and 7.7 with those from intact cells. It is evident that at the higher pH the proportion of 5'-phosphates to 3'-phosphates increases. The ratio of 3'- to 5'-AMP from whole cells compared more favorably to the ratio of 3' to 5'-AMP of the homogenate at pH 5.45 because the incubation of the intact cells was at about this pH.

Heat-shock apparently does not function by denaturing ribosomes that are a more available substrate, since native ribosomes in homogenates from untreated cells (in the presence of 10^{-2} M magnesium ions) serve as a substrate for the ribonuclease. The ribonuclease activity from homogenates, whether heat-shocked or not, remained essentially in the supernatant fluid when sedimented at 100,000 × g for 2 hr (Table 6). The enzyme appears not to be tightly bound to ribosomes and is not limited to degradation of self-associated ribosomes.

### Table 4. Overall change of the degradation products as per cent of total cell nucleic acids

| Compounds              | Cold PCA (initial) | Cold PCA (30 min) at 30 °C | Supernatant fluid (120 min) |
|------------------------|--------------------|-----------------------------|----------------------------|
| Base and nucleoside... | 0.7                | 3.4                         | 11.5                       |
| Nucleoside monophosphate... | 2.8              | 27.3                        | 71.9                       |
| Nucleoside di-, triphosphate... | 4.3            | 2.1                         | 4.1                        |
| Oligonucleotide...     | 1.0                | 1.1                         | 0                          |
| Total                  | 8.8                | 33.9                        | 87.5                       |

* For 1 hr at 50 °C and 1 hr at 55 °C.

### Table 5. Composition of degradation products from homogenates and intact cells

| Samples                  | Homogenates | Intact cells |
|--------------------------|-------------|--------------|
| pH 5.45                  | pH 7.7      |              |
| Bases and nucleosides (%)| 21.3        | 18.7         | 12.9         |
| Nucleoside monophosphates (%)| 74.3      | 72.0         | 83.0         |
| Nucleoside di-, triphosphates (%)| 4.4      | 9.3          | 4.9          |
| Total                    | 100.0       | 100.0        | 100.8        |
| 3'-AMP/5'-AMP            | 5.7         | 2.0          | 4-5          |

Endogenous RNA in cell homogenates was also degraded at neutral and acidic conditions primarily to 3'-mononucleotides. Table 5 compares the composition of degradation products of homogenates at pH 5.45 and 7.7 with those from intact cells. It is evident that at the higher pH the proportion of 5'-phosphates to 3'-phosphates increases. The ratio of 3'- to 5'-AMP from whole cells compares more favorably to the ratio of 3' to 5'-AMP of the homogenate at pH 5.45 because the incubation of the intact cells was at about this pH.

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### Table 6. Sedimentation behavior of ribonuclease in homogenates

| Sample                          | Relative ribonuclease activity |
|---------------------------------|--------------------------------|
| Untreated homogenate            | 100                            |
| Untreated supernatant fluid     | 100                            |
| Untreated pellet                | 19                             |
| Heat-shocked supernatant fluid  | 73                             |
| Heat-shocked pellet             | 6                              |

* Homogenates were heat-shocked for 4 sec at 68 °C and sedimented at 100,000 × g for 2 hr in the presence of 10^{-2} M Mg^{2+}.

### DISCUSSION

A three-step process for removing nucleic acids from C. utilis has been developed. The function of the three steps seems to be: (i) initiation of enzymatic hydrolysis by a short heat-shock, (ii) hydrolysis of RNA by incubation at lower temperatures, and (iii) leaking of accumulated hydrolysis products into the suspending medium in an incubation above 50 °C.

The nucleic acid content is reduced from an initial value of 7 to 8% to less than 2%. The protein content of the cells increases from an initial value of approximately 45% on a dry weight basis to greater than 50%. The yeast cells remain intact as observed by light and electron microscopy.

Heat-shock (step 1) initiates enzymatic hydrolysis of nucleic acids. Several mechanisms can be postulated. Enzyme activation may occur by thermal denaturation of a ribonuclease inhibitor or by the release of ribonuclease from a subcellular compartment. Alternatively, the yeast cells may be organized in such a way that the enzyme may not attack native ribosomal RNA unless the cells are disorganized by physical or chemical treatments and require denaturation of the substrate before hydrolysis can occur. Extrapolation of the data (2) on thermal denaturation of Escherichia coli ribosomes indicates a 0.6-sec half-life at 68 °C (Table 7).

Others (17, 20–22) have reported that E. coli ribonuclease I is located between the cell wall and cell membrane, and it is released into the interior of the cell after thermal shock. This ribonuclease is reported to bind E. coli ribosomes in a latent form (6, 24, 26) and may be later activated by thermal shock. The binding, however, appears to be an artifact of isolation procedures. Neu and Heppel (20) and Haight and Ordal (8) found a ribonuclease associated with staphylococcal ribosomes which is activated by heat and which attacks the RNA closely bound within the same
ribosomes. The exact biological mechanism of heat-shocking in our process described above awaits further investigation.

For a detailed description of the enzyme systems participating in the thermally induced degradation of RNA in C. utilis, it would be necessary that these enzymes be isolated and characterized. However, it is possible from the present study of the intermediate and final products of RNA degradation to speculate which enzymes work in the present process.

The major compounds found in the intermediary and final stages of RNA degradation were 3'-mononucleotides. This fact agrees with observations of RNA degradation with other yeasts (9, 19, 23; J. Danner, Ph.D. Thesis, Brandeis Univ., Waltham, Mass., 1965).

The observations of Nakao et al. (18) are particularly interesting. They examined many species of yeasts with respect to the release of mononucleotides. It was found that 3'-mononucleotides are produced generally at acid pH, whereas at alkaline pH 5'-mononucleotides are produced. Our study substantiates theirs: heat treatment was performed at pH 4.0 and the products were 3'-mononucleotides.

In E. coli, the first product of RNA degradation by ribonuclease I is a 2', 3'-cyclic mononucleotide (24). It has not been found in RNA degradation in yeasts. In the present study, 2', 3'-cyclic mononucleotides were not detected even in the intermediary stage of RNA degradation. This suggests that, in yeasts, RNA hydrolysis by ribonuclease will proceed without producing 2', 3'-cyclic mononucleotides as the intermediate, or the hydrolysis of 2', 3'-cyclic mononucleotides will be too rapid to accumulate these compounds as intermediates.

A small amount of 5'-mononucleotides was found in the final supernatant fluid. As the amount was small, it was not clear whether it came from the initial nucleotide pool or from RNA. The analysis was made more difficult by the possible degradation of mononucleotides to nucleosides and bases.

One of the 5'-mononucleotide-forming enzymes is polynucleotide phosphorylase. This enzyme requires phosphate as substrate and Mg\(^{2+}\) ions for enzyme activity. The stimulatory effect of these compounds on RNA degradation was not observed with washed cells. Moreover, the degradation products showed the same chromatographic pattern as that in the absence of these compounds. This indicates that this enzyme does not participate in the present process with the possible exception that the enzyme attacks a specific RNA present in a small amount such as messenger RNA, as suggested by Andoh et al. (1) in E. coli.

As shown in Table 4, we observed the partial, secondary conversion of mononucleotides to bases or nucleosides. The fact that the total amount of base, nucleoside, and 5'-mononucleotide in the final supernatant fluid is greater than that of the initial nucleotide pool indicates hydrolysis of 3'-mononucleotides by a 3'-nucleotidase. The same secondary degradation was observed in other yeasts during RNA degradation (3, 31; J. Danner, Ph.D. Thesis, Brandeis Univ., Waltham, Mass., 1965).

The similarity in thermal stability of ribonuclease activities in homogenates and whole cells, in composition of the hydrolysis products, and in pH optima lead us to the conclusion that the same enzyme system is responsible for the hydrolysis of RNA both in homogenates and whole cells. If this is true, thermal shock and mechanical shock perform the same function: possibly the release of a ribonuclease from a subcellular compartment. The fact that the reaction is more rapid in homogenates (even in the presence of 10\(^{-2}\) M Mg\(^{2+}\) which partially inhibits the enzyme and stabilizes ribosomes) indicates that homogenization is more effective than heat-shock for releasing the enzyme. Another explanation for the increased rate of RNA hydrolysis in homogenates is that in homogenates a ribonuclease inhibitor is diluted out. Since the mechanism of action of the heat-shocked step of the process is not yet completely understood, it will remain the subject of further investigation.

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