Purification and Characteristics of a Mitochondrial Endonuclease from the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: *Saccharomyces cerevisiae* contains a membrane-bound mitochondrial nuclease. The enzyme was purified nearly 500-fold from sphaeroplasts of the organism by differential centrifugation, differential solubilization, heparin–agarose chromatography, and gel filtration. A final specific activity of 98 μmol min^{-1} (mg of protein)$^{-1}$ was obtained. The enzyme requires further purification to achieve homogeneity. Two peaks of activity were obtained after gel filtration with apparent molecular weights of 140000 and 57000. Otherwise, these two components have nearly identical characteristics. Without detergent the enzyme is insoluble and has very low activity. Zwittergent 3-14 or Triton X-100 in the presence of KCl could be used to solubilize and activate the enzyme. A number of other detergents were much less effective in solubilizing or activating the nuclease. The enzyme requires Mg^{2+} for activity, and this can be replaced to some degree by Mn^{2+} but not by Ca^{2+} or Zn^{2+}. It is most active at pH 6.5–7.0 and degrades the substrate to small oligonucleotides with 5′-phosphate ends. The relative rates of hydrolysis were 100 for poly(A), 31 for ssDNA, 19 for RNA, 2.1 for dsDNA, and ≤0.2 for poly(C). Under the assay conditions used the enzyme appears to constitute about 90% of the total nuclease activity of the cell. The enzyme is unstable, especially at neutral and alkaline pH.

Yeasts have become increasingly popular for research dealing with the physiology and molecular biology of the cell and, in particular, for research related to the biogenesis and the origin of mitochondria (Rosamond, 1982; Gray & Doolittle, 1982). However, much remains to be learned about transcription in yeast nuclei and mitochondria and about the enzymes involved in the processing of nucleic acids.

A number of different nucleic acid degrading enzymes can be distinguished in yeasts. In many cases neither the intracellular location nor the function of the enzymes has been determined (Piñon, 1970; Piñon & Leney, 1975; Imada et al., 1975; Shetty et al., 1980). An endonuclease requiring Mg^{2+} or Mn^{2+} for activity was partially purified by Nakao et al. (1968) and Lee et al. (1968) from the supernatant of an extract of a *Saccharomyces* species. Two clearly distinct enzymes have been found to be associated with the ribosomal fraction. A Mg^{2+}-independent RNase was described by Ohtaka et al. (1963) and Nakao et al. (1968), and a Mg^{2+}-dependent exonuclease degrading RNA from the 5′ end was characterized by Stevens (1978, 1979, 1980). Nuclear enzymes also have been reported in yeasts, namely, two RNase H's (Wyers et al., 1976a,b) and a DNase (Bryant & Haynes, 1978). The DNase, however, was present at such low activity that it may have arisen from contaminating membrane material, including mitochondrial membranes. So far, nuclear RNases which might be involved in the processing of RNA in the nucleus have not been found in yeasts.

A number of reports have dealt with nucleic acid degrading enzymes from mitochondria of *Saccharomyces cerevisiae* and *Neurospora crassa*. Linn & Lehman (1966) were the first to report the mitochondrial nature of an endonuclease in *N. crassa*, and Piñon (1970) and Paoletti et al. (1972) subsequently investigated a similar enzyme in *S. cerevisiae*. Some earlier reports did not recognize that at least a major part of the enzyme might be membrane bound, and Piñon (1970) and Martin & Wagner (1975) were the first to use detergents in their investigation. Martin & Wagner (1975) concluded that about half of the mitochondrial nuclease activity in *N. crassa* was membrane bound. More recent investigations with *S. cerevisiae* all recognized the necessity of using detergents to solubilize and purify the mitochondrial enzyme(s) (Jacquemin-Sablon et al., 1979; Morosoli & Lusena, 1980; Rosamond, 1981). It is difficult to determine whether these reports deal with the same enzyme or whether yeast mitochondria contain a number of closely related nucleic acid degrading enzymes. During the purification of the mitochondrial endonucleases several components (peaks) with nuclease activity have been observed (Nakao et al., 1968; Jacquemin-Sablon et al., 1979; Morosoli & Lusena, 1980). In addition, the isolated nucleases seem to differ in some of their properties, notably the molecular weight. Consequently, it appeared that the mitochondria contain a number of different endonucleases. However, in some cases the actual amount of enzyme involved was extremely small (Jacquemin-Sablon et al., 1979; Rosamond, 1981), and therefore, it could have arisen from other sources or it could have been a minor fraction of the mitochondrial enzymes.

The apparent hetrogeneity of the mitochondrial enzymes and the instability problems that seemed to prevent further purification made it clear that additional work was required to characterize these nucleolytic enzymes in yeast. This report deals with a Mg^{2+}-dependent endonuclease from *S cerevisiae*. It is a mitochondrial enzyme and appears to be a major nuclease in yeast. A purification procedure was devised to obtain the enzyme in good yield. Some of its characteristics and its relationship to other nucleolytic enzymes in yeast were determined.

Experimental Procedures

Materials

Poly(A),[1] poly(C), and calf thymus DNA were obtained from Sigma Chemical Co. The DNA was used as a source of double-stranded DNA (dsDNA). The single-stranded DNA (ssDNA) was prepared by heating dsDNA at 100 °C for 10 min and then cooling it rapidly. Highly polymerized yeast

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[1] Abbreviations: poly(A), poly(adenylic acid); poly(C), poly(cytidylic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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RNA, the Zwittergents, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Calbiochem. The other detergents, snake venom phosphodiesterase (phosphodiesterase I), spleen phosphodiesterase (phosphodiesterase II), DEAE-cellulose, and heparin–agarose were also obtained from Sigma Chemical Co. The alkaline phosphatase from *Escherichia coli* was a product of Worthington Biochemical Corp. while Sephacryl S-200 was purchased from Pharmacia Fine Chemicals. Zymolyase 60 000 was obtained from the Kirin Brewery Co. Ltd.

Methods

Growth of the Organisms and Preparation of Sphaeroplasts. The yeasts were grown aerobically at 30 °C on a rotary shaker in 2% yeast extract with 2% ethanol or 1% glucose at pH 4.5. Most experiments were carried out with *Saccharomyces cerevisiae* SK-1 grown with ethanol as the carbon source.

So that sphaeroplasts could be obtained, 1.5 g of wet cells was diluted to 10 mL with 1.3 M sorbitol and 0.1 M potassium phosphate, pH 7.5, and 0.025 mL of β-mercaptoethanol and 0.15 mL of Zymolyase 60 000 (10 mg/mL) were added. Incubation was carried out at 30 °C with occasional agitation. The formation of sphaeroplasts was followed by phase contrast microscopy and by measuring the decrease in the OD_{600} of the cell suspension after dilution into H_2O. It was usually complete within 30–35 min.

Isolation of Mitochondria. Sphaeroplasts were used to isolate mitochondria as described by Linnane & Lukins (1975) except the final centrifugation was carried out at 22 000 rpm for 18 h in a Beckman SW 40 rotor with a linear gradient prepared from 50% and 80% sorbitol in 1 mM EGTA, pH 7.0.

Preparation of Respiratory-Deficient (Petite) Mutants. A culture of *S. cerevisiae* SK-1 was grown in 2% yeast extract and 1% glucose, pH 4.5, to about 9×10^6 cells/mL. Ethidium bromide was added to a final concentration of 20 μg/mL, and after further incubation at 30 °C for 2 h the culture was diluted and plated for the isolation of colonies of respiratory-deficient organisms.

Enzyme Assays. Nucleic acid degrading enzymes were assayed as described earlier (von Tigerstrom, 1972) except 50 μL of enzyme preparation diluted with 50 mM Tris-HCl, 10 mM KCl, 5 mM $MgCl_2$, and 0.2% Zwittergent 3-14, pH 7.0, was added to 150 μL of the assay solution described below. The amounts of perchloric acid soluble nucleotides formed were determined at different time intervals.

Total nuclease activity was routinely determined in 90 mM Tris-HCl, 25 mM potassium phosphate, 200 mM KCl, 5 mM $MgCl_2$, and 1 mg/mL poly(A), pH 7.0. Mg^{2+}-independent nuclease activity was determined with the same assay solution except $MgCl_2$ was replaced by 20 mM EDTA. The Mg^{2+}-dependent nuclease activity was calculated by subtracting the Mg^{2+}-independent nuclease activity from the total nuclease activity. One unit of enzyme is the amount which produces 1 μmol of acid-soluble products from the respective nucleic acid substrate per min at 37 °C.

Fumarase was assayed according to the method of Hill & Bradshaw (1969). Cytochrome *c* oxidase activity was determined by measuring the oxidation of reduced cytochrome *c* spectrophotometrically (Errede et al., 1978). Cytochrome *c* was dissolved in 0.1 M Hepes/Tris buffer, pH 7.0, and it was reduced with $Na_2S_2O_4$. Zwittergent 3-14 at 0.05% was present during the reaction. The conditions used for the phosphodiesterase I, phosphodiesterase II, and alkaline phosphatase reactions have been described (von Tigerstrom, 1972).

Column Chromatography. Heparin–agarose chromatography was carried out on a preparative scale by using a 2.5 cm i.d. × 15 cm column. It was equilibrated with 50 mM Tris-HCl, 75 mM KCl, 25 mM potassium phosphate, 5 mM $MgCl_2$, and 0.2% Zwittergent 3-14, pH 6.0 (buffer 1). After application of 86.5 mL of the dialyzed enzyme solution, the column was eluted with 150 mL of buffer 1 and then with a linear gradient prepared from 250 mL of buffer 1 and 250 mL of 50 mM Tris-HCl, 1 M KCl, 25 mM potassium phosphate, 5 mM $MgCl_2$, and 0.2% Zwittergent 3-14, pH 6.0 (buffer 2). Fractions of 8.1 mL were collected at a flow rate of 100 mL/h. Heparain–agarose chromatography was also carried out on a smaller scale (1.2 cm i.d. × 20 cm) with a gradient prepared from 80 mL of buffer 1 and 80 mL of buffer 2. In this case 3-mL fractions were collected at a rate of 30 mL/h.

A Sephacryl S-200 superfine column (2.6 cm i.d. × 96 cm) was used for gel filtration. It was equilibrated with buffer 2; 4–5 mL of enzyme was usually applied and eluted at 40 mL/h collecting 3.3-mL fractions. The column was calibrated with the molecular weight markers γ-globulin (M_r 150 000), *Escherichia coli* alkaline phosphatase (M_r 84 000), ovalbumin (M_r 43 000), and RNase A (M_r 13 700). The partition coefficients (K_{av}) for these proteins were 0.14, 0.25, 0.33, and 0.55, respectively. All column chromatography was carried out at 8 °C.

Analysis of Nucleic Acid Degradation Products. Fifteen milliliters of assay solution containing 15 mg of poly(A) was incubated with 5 units of enzyme for 60 min at 37 °C. After 20 min the poly(A) was completely degraded to acid-soluble products. The solution was diluted to 190 mL with cold H_2O to reduce the ionic strength, and the pH was adjusted to 8.2 before separation of the products by DEAE-cellulose chromatography (Marushige et al., 1969). The degradation products were eluted in two peaks. These were concentrated and analyzed for total and alkaline phosphatase labile phosphate and by paper chromatography before and further treatment with phosphodiesterase I and with 0.25 M KOH at 37 °C for 18 h. The solvent systems isobutyric acid/1 M NH_4OH (5:3), 0.1 M sodium phosphate (pH 6.8)/$(NH_4)_2SO_4$/*n*-propanol (100:60g:2), and 2-propanol/concentrated NH_4OH/H_2O (7:1:2) were used for paper chromatography.

Single-stranded DNA (22.5 mg) was degraded similarly with an excess of purified enzyme for 90 min. Ninety-three percent of the products were eluted from DEAE-cellulose between 0.21 and 0.35 M NH_4HCO_3, and they were analyzed by paper chromatography before and after degradation with phosphodiesterases I and II.

Other Methods. Polyacrylamide gel electrophoresis was carried out by a published procedure (Maizel, 1971). Protein concentrations were determined by the method of Bradford (1976) and by absorption at 280 nm (Warburg & Christian, 1941). Total phosphate and inorganic phosphate concentrations were obtained by the method of Ames (1966).

Results

Assay Conditions and Levels of Nuclease Activities in Different Yeasts. Our preliminary investigations and results by Nakao et al. (1968) indicated that yeasts contain two major nucleic acid degrading activities, one active only in the presence of Mg^{2+} and the other also active in the presence of EDTA (Ohtaka et al., 1963; Nakao et al., 1968). The pH optimum for both was about pH 7, and poly(A) was chosen as the substrate since both types of enzymes were more active with poly(A) than with RNA or DNA. Thus total activity was measured in the presence of Mg^{2+} and Mg^{2+}-independent activity in the presence of EDTA. The Mg^{2+}-dependent activity was estimated by subtraction. Although some of the

Table I: Mg^{2+}-Dependent and Mg^{2+}-Independent Nuclease Activities in *S. cerevisiae*[a]

organism	carbon source	units/mg of protein: Mg^{2+} dependent	Mg^{2+} independent
SK-1	ethanol	0.20	0.027
SK-1	glucose	0.22	0.014
SK-1 petite	glucose	0.13	0.028

[a] *S. cerevisiae* SK-1 or a petite mutant derived from this organism was grown with the carbon source indicated, and the nuclease activities were determined in sphaeroplast lysates as described under Methods.

Table II: Activation of the Mg^{2+}-Dependent Nuclease by Different Detergents

detergents	act.[a] (%)
none	11
Zwittergent 3-08	12
Zwittergent 3-10	14
Zwittergent 3-12	61
Zwittergent 3-14	100
Zwittergent 3-16	109
Triton X-100	87
deoxycholate	7
deoxycholate + 3-14	80
Tween 80	21
cholate	14
octyl glucopyranoside	18
CHAPS	20

[a] The 400–20000*g* particulate fraction was prepared from a sphaeroplast lysate. It was diluted into 50 mM Tris-HCl, 10 mM KCl, and 5 mM $MgCl_2$, pH 7.0, containing 0.2% of the detergent indicated, and the nuclease activity was determined as described under Methods. The activity obtained with Zwittergent 3-14 was assigned a value of 100.

minor nucleolytic enzymes in yeast cannot be detected under these conditions, this method could be used to estimate Mg^{2+}-dependent and Mg^{2+}-independent nuclease in sphaeroplast extracts and during the early stages of purification of the Mg^{2+}-dependent nuclease.

Table I shows the specific activities in extracts of *S. cerevisiae* SK-1 grown with ethanol or glucose as the carbon source and of a petite mutant, derived from this organism, grown in glucose medium. In *S. cerevisiae* SK-1 the specific activity of the Mg^{2+}-dependent enzyme(s) was about 10 times higher than the specific activity of the Mg^{2+}-independent enzyme(s), and similar levels of activity were found when the organism was grown on ethanol or on glucose. The respiratory-deficient (petite) mutant produced somewhat less Mg^{2+}-dependent activity.

Solubilization and Activation. If the Mg^{2+}-dependent nuclease was assayed without a suitable detergent, only about 10% of the potential activity was expressed. The extent to which various detergents activate the enzyme is shown in Table II. Good activation was obtained with the Zwittergents 3-14 and 3-16 and with Triton X-100. Other detergents tested had little effect. Figure 1 shows the activation obtained with different concentrations of Zwittergent 3-14 and Triton X-100.

As will be shown below, the Mg^{2+}-dependent enzyme was associated with particulate material. It could be solubilized by some detergents in the presence of KCl. This is shown in Table III. Mild sonication of the preparations in the presence of Zwittergent 3-14 or Triton X-100 and KCl resulted in essentially complete solubilization.

It is interesting to note that deoxycholate is a relatively good agent for solubilization but does not activate the nuclease.

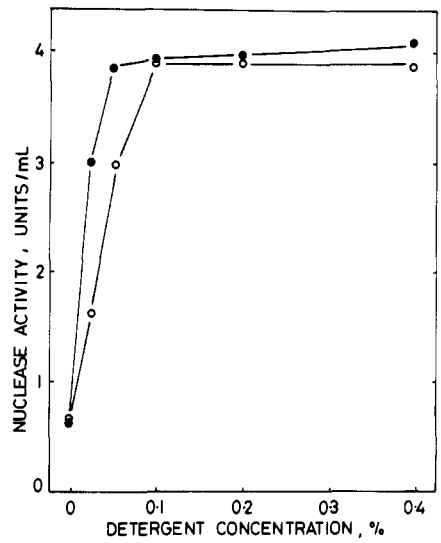

FIGURE 1: Effect of different Zwittergent 3-14 (●) and Triton X-100 (○) concentrations on the Mg^{2+}-dependent nuclease activity. The 400–20000*g* particulate fraction of a sphaeroplast lysate was diluted 35-fold with 50 mM Tris-HCl, 10 mM KCl, and 5 mM $MgCl_2$, pH 7.2, containing the detergent concentrations indicated. The nuclease activity was determined as described under Methods.

Table III: Solubilization of Mg^{2+}-Dependent Nuclease[a]

detergent	KCl	act. (%)
none	–	1
none	+	2
Zwittergent 3-14	–	13
Zwittergent 3-14	+	77
deoxycholate	+	64
Triton X-100	+	26
Tween 80	+	3
cholate	+	8
octyl glucopyranoside	+	4
CHAPS	+	10

[a] A 400–20000*g* particulate fraction of a sphaeroplast lysate was suspended in 50 mM Tris-HCl, 10 mM KCl, and 5 mM $MgCl_2$, pH 7.2, and where indicated, detergents were added to a final concentration of 0.2% and KCl was added to give a 1 M concentration. After frequent mixing at 0 °C over a 30-min period, the preparations were centrifuged at 105000*g* for 30 min. The supernatants were diluted 40-fold and assayed for nuclease activity as described under Methods. A sample which was not centrifuged served as the 100% control.

Thus, activation and solubilization appear to be different phenomena. Zwittergent 3-14 was used throughout the work to activate and solubilize the enzyme. If the salt concentration in a solubilized preparation was reduced to about 0.15 M KCl, the enzyme started to come out of solution. Thus, high ionic strength in combination with the detergent is required to keep the enzyme in solution.

Particulate Nature of the Mg^{2+}-Dependent Nuclease. The Mg^{2+}-dependent and Mg^{2+}-independent enzymes can be readily separated by centrifugation. For example, when a sphaeroplast lysate is sonicated briefly and centrifuged at 20000*g* for 20 min, greater than 90% of the Mg^{2+}-dependent enzyme is sedimented and must, therefore, be associated with relatively large particulate material. Most of the Mg^{2+}-independent enzyme, which is probably a ribosomal RNase (Nakao et al., 1968), remains in the supernatant.

Figure 2A shows the results of chromatography on heparin–agarose of an unfractionated sphaeroplast lysate. This is compared in Figure 2B to results obtained with the particulate fraction of the sphaeroplast lysate. In each case the enzymes from about 0.4 g of yeast cells were solubilized and dialyzed

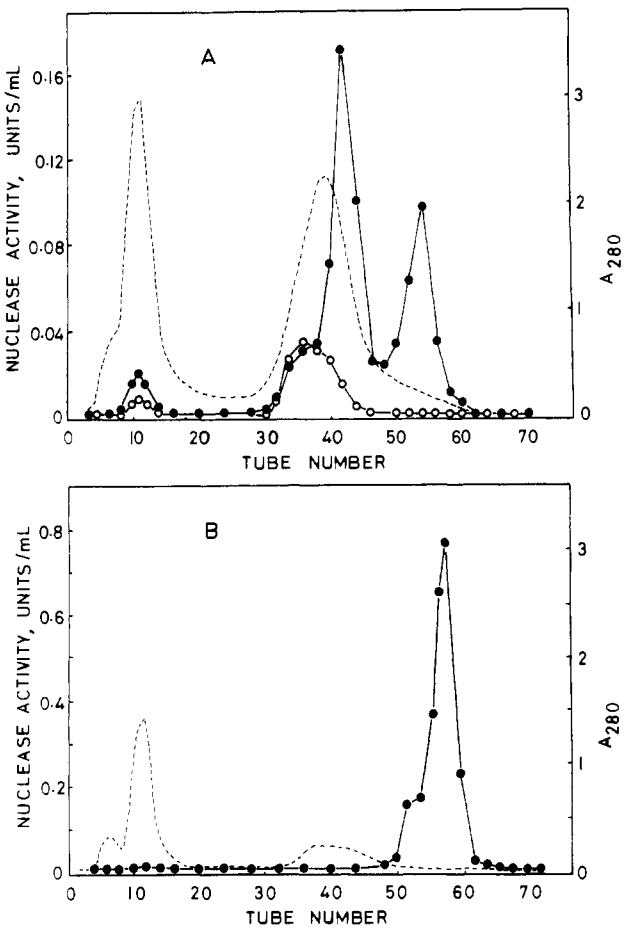

FIGURE 2: Separation of Mg^{2+}-dependent and Mg^{2+}-independent nuclease activity on a 1.2 × 20 cm column of heparin–agarose. A sphaeroplast lysate (A) and the 400–20000*g* particulate fraction of a sphaeroplast lysate (B), each from 0.4 g of cells, were solubilized in the presence of 1 M KCl and 0.2% Zwittergent by mild sonication. Any remaining particulate matter was removed by centrifugation. After dialysis the preparations were applied to the column and eluted as described under Methods. The fractions were analyzed for A_{280} (---) and nuclease activity with poly(A) in the presence of Mg^{2+} (●), poly(A) in the presence of EDTA (○), and RNA and ssDNA in the presence of Mg^{2+} (not shown). The Mg^{2+}-independent activity in part B was very low and is not shown. The activities obtained with ssDNA and RNA were proportional to the activities with poly(A) with Mg^{2+} except the Mg^{2+}-independent enzyme did not hydrolyze DNA.

before chromatography. The column fractions were assayed with poly(A), ssDNA, and RNA in the presence of Mg^{2+} and, to detect the Mg^{2+}-independent RNase, also with poly(A) in the presence of EDTA.

In Figure 2A three peaks of activity can be seen. The first or run-off peak contained a large amount of protein and, probably, a mixture of minor nucleolytic enzymes. No attempt was made to identify the enzymes in this peak. The second and third peaks were both active with poly(A), ssDNA, and RNA in the presence of Mg^{2+}. The shoulder of the second peak contained Mg^{2+}-independent RNase.

The preparation used for the separation in Figure 2B contained much less protein and Mg^{2+}-independent RNase but almost all of the Mg^{2+}-dependent nuclease originally found in the sphaeroplasts. In contrast to Figure 2A this activity was eluted in one major peak at a relatively high KCl concentration. Since there was good recovery of enzyme activities from both columns, it can be concluded that the activity found in the second peak in Figure 2A was not absent in the particulate fraction but had shifted to a later point of elution and was eluted with activity in the third peak. The elution of the Mg^{2+}-dependent enzyme in two peaks in Figure 2A is likely due to an association of part of the enzyme with other proteins in the unfractionated sphaeroplasts. This comparison shows that the Mg^{2+}-dependent nuclease is largely particulate and that most of it is probably due to one enzyme.

Purification of the Mg^{2+}-Dependent Nuclease. A number of different purification schemes have been attempted. The one given below appears to be the most suitable with respect to the yield and purification achieved. It involves selective solubilization of the particulate materials from a sphaeroplast lysate. The following describes the purification of the enzyme from cells of a 6-L culture. All manipulations were carried out at 0–4 °C except where indicated.

Step I. Sphaeroplasts were prepared from 46.2 g of wet cells as described under Methods. Sphaeroplasting was usually complete within 35 min. No changes of enzyme levels were detected even if sphaeroplasting was continued for 90 min.

Step II. The sphaeroplasts were centrifuged at 20000*g* for 20 min. The supernatant was discarded, and the pellet was suspended with a Dounce homogenizer in 640 mL of TKE buffer (50 mM Tris-HCl, 10 mM KCl, and 1 mM EDTA, pH 7.2) and centrifuged as above. The resulting pellet was suspended in 320 mL of TKE buffer and recentifuged at 20000*g* for 20 min. The pellet was then suspended in 300 mL of TKM buffer (50 mM Tris-HCl, 10 mM KCl, and 5 mM $MgCl_2$, pH 7.2) and sonicated (100 mL lots 4 × 30 s). The resulting suspension was centrifuged at 400*g* for 5 min to remove cells, ghosts, and large debris. The supernatant was retained, and the pellet was washed 2 times with TKM buffer by resuspension and centrifugation at 400*g*. The supernatants were combined to yield 400 mL of the 400–20000*g* particulate fraction.

Step III. To the particulate fraction obtained above was added Zwittergent 3-14 to 0.2% final concentration. After brief stirring this was centrifuged at 105000*g* for 30 min. The supernatant was discarded, and the pellets were dissolved in 45 mL of solubilization buffer (50 mM Tris-HCl, 1 M KCl, 5 mM $MgCl_2$, and 0.2% Zwittergent 3-14, pH 7.2) and stirred for 17 h. Small amounts of particulate material were removed by centrifugation to give 47.6 mL of solubilized nuclease preparation.

Step IV. the solubilized enzyme was diluted 2-fold and dialyzed with buffer 1. The particulate material that appeared during dialysis was removed by centrifugation. About 20% of the enzyme was lost during dialysis and centrifugation. The supernatant was then used for heparin–agarose chromatography as described under Methods. The elution of the enzyme is shown in Figure 3 and resulted in a 22-fold purification. Fractions 68–75 were pooled and concentrated by ultrafiltration.

Step V. The enzyme obtained in step IV was used for gel filtration with Sephacryl S-200 as described under Methods and in Figure 4. Two nuclease peaks (I and II) were obtained with apparent molecular weights of about 140000 and 57000. Each peak was pooled and concentrated by ultrafiltration. The proportion of the activity found in each peak was relatively unaffected by the KCl concentration and pH, but for reasons not understood, it varied from one purification to another. Rechromatography of the peaks did not alter the results significantly. However, as discussed below, the activities in the two peaks are thought to be due to the same enzyme. The purification of the nuclease is summarized in Table IV.

Polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$ of the enzyme from peaks I and II (Figure 4) showed several protein bands, indicating that the enzyme requires

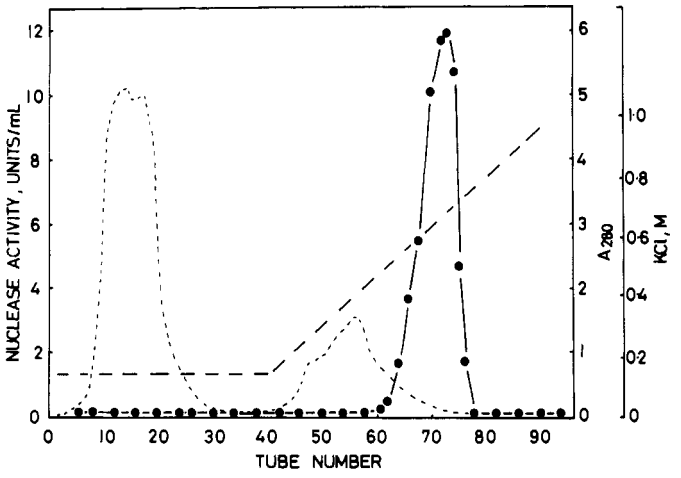

FIGURE 3: Purification of Mg^{2+}-dependent nuclease by heparin–agarose column chromatography. The enzyme from step III of the purification was applied to a 2.5 × 15 cm column and eluted as described under Methods. The fractions were analyzed for A_{280} (---), nuclease activity (●), and KCl concentration (– –).

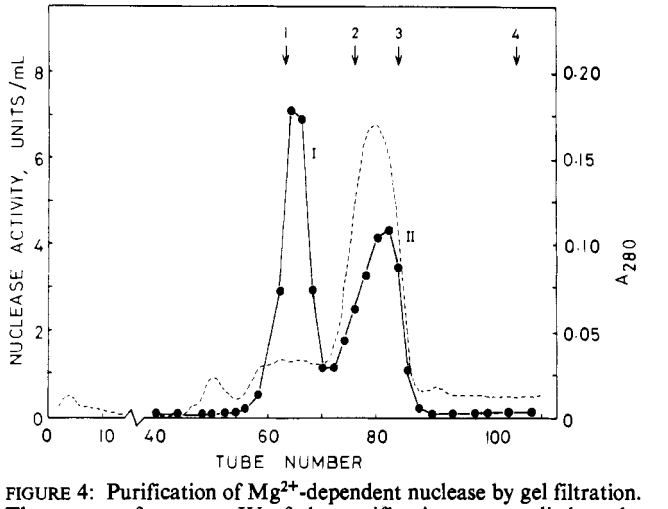

FIGURE 4: Purification of Mg^{2+}-dependent nuclease by gel filtration. The enzyme from step IV of the purification was applied to the Sephacryl S-200 column and eluted as described under Methods. A_{280} (---) and nuclease activities (●) were determined in the fractions. The positions 1, 2, 3, and 4 indicate where γ-globulin, alkaline phosphatase, ovalbumin, and RNase A were eluted.

further purification. This was not unexpected judging by the elution profile from Sephacryl S-200. However, the preparations seem to be free of other nucleolytic enzymes. As described below, the instability and chemical nature of the enzyme have prevented the identification of any of the bands with nuclease activity.

Intracellular Location. Although the enzyme was first noted in nuclear fractions, an analysis of Ficoll density gradients clearly indicated that the enzyme was present primarily in smaller components. Subsequently it was found that most of the nuclease activity was associated with mitochondria. Figure 5 shows the result of a sorbitol density gradient centrifugation of a crude mitochondrial preparation and that the nuclease cosedimented with cytochrome *c* oxidase and fumarase. The remaining activity was found primarily in the supernatants obtained during the purification of the mitochondria. These supernatants also contained significant amounts of the mitochondrial marker enzymes. This would indicate that a very large proportion, or perhaps all, of the nuclease might be located in the mitochondria.

A purification of the enzyme from isolated mitochondria has also been carried out, but a significantly lower yield was

Table IV: Purification of Mg^{2+}-Dependent Nuclease

steps	volume[a] (mL)	protein (mg)	nuclease units	sp act. (units/mg)
(I) sphaeroplast lysate	310	6603	1404	0.21
(II) differential centrifugation	400	1160	1184	1.01
(III) solubilization	48	390	1010	2.59
(IV) heparin–agarose	68	10	539	56.3
(V) Sephacryl S-200				
peak I	41	1.8	177	98
peak II	57	5.4	226	42

[a] The volumes have been corrected for samples removed at each stage to carry out assays and protein determinations.

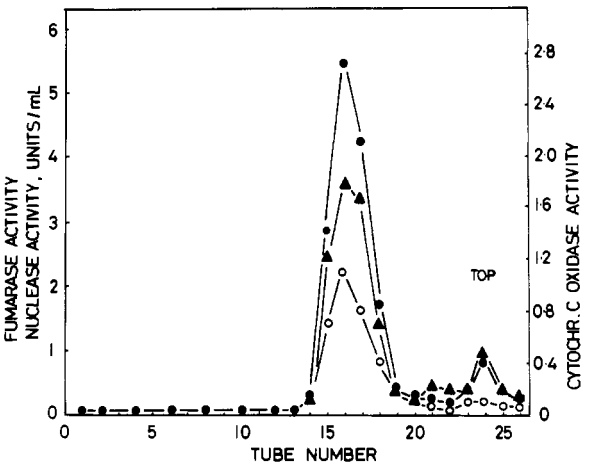

FIGURE 5: Density gradient centrifugation of mitochondria. Yeast mitochondria were prepared and centrifuged in a linear sorbitol gradient as described under Methods. The nuclease (●), cytochrome *c* oxidase (▲), and fumarase (○) activities were determined. Protein concentrations (not shown) were proportional to the nuclease and cytochrome *c* oxidase activities.

obtained without improvement of the final specific activity. The nuclease purified from isolated mitochondria also eluted in two peaks from Sephacryl S-200.

Stability. The purified enzyme is rapidly inactivated at 40 °C and pH 7.2 but is more stable at pH 6.0. Some instability was even noted with the purified protein under assay conditions, but this could be overcome by diluting the enzyme into solutions containing serum albumin.

The enzyme carries a net $^{+}$ve charge at least below pH 7.5. However, the requirement for a detergent plus a relatively high KCl concentration for solubility of the enzyme and the instability of the enzyme made it impossible to carry out meaningful electrophoresis under nondenaturing conditions. Extensive attempts to renature the enzyme after exposure to denaturing conditions required for polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (Maizel, 1971) or isoelectric focusing of membrane proteins (O'Farrell, 1975) were unsuccessful.

Optimum pH. As shown in Figure 6 the enzyme from peaks I and II after the gel filtration step has a maximum activity at pH 6.5–7.0. The same pH optimum was obtained for preparations at other steps in the purification. However, the instability of the enzyme at higher pH values became noticeable after steps IV and V, and it was essential to dilute the enzyme with a solution containing at least 0.2 mg/mL bovine serum albumin. Otherwise a lower apparent pH optimum was obtained.

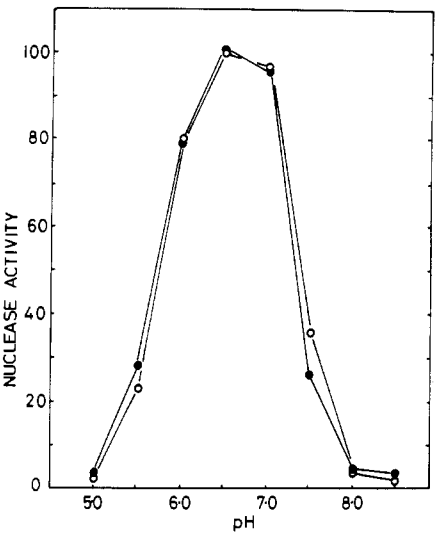

FIGURE 6: Mg^{2+}-dependent nuclease activity at different pH values. The purified enzyme from peaks I (●) and II (○) after gel filtration (Figure 4) was diluted into 50 mM Tris-HCl, 10 mM KCl, 5 mM $MgCl_2$, 0.2% Zwittergent 3-14, and 0.2 mg/mL bovine serum albumin, pH 7.0. The nuclease activities were determined with the assay solution described under Methods adjusted to the different pH values with acetic acid and KOH.

Table V: Substrate Specificity of Mg^{2+}-Dependent Nuclease[a]

substrate	rates of hydrolysis	
	peak I	peak II
poly(A)	100	100
ssDNA	31	30
RNA	19	18
dsDNA	2.1	2.7
poly(C)	≤0.2	≤0.2

[a] The enzyme isolated in peak I and peak II from Sephacryl S-200 was assayed as described under Methods with the substrates indicated to determine the rates of hydrolysis. The rate obtained with poly(A) was given a value of 100.

Metal Ion Requirement. The nuclease is essentially inactive in the presence of EDTA. This inhibition, however, is completely reversed by the addition of Mg^{2+}. Mg^{2+} can be replaced to some degree by Mn^{2+} but not by Ca^{2+} or Zn^{2+}. Normally the reaction is carried out with 5 mM $MgCl_2$. In addition to this divalent metal ion requirement, K^+ appears to stimulate the reaction. Replacement of KCl and potassium phosphate in the assay solution with the respective Na salts resulted in a 50% reduction of the activity. Furthermore, Tris-HCl appears to have some stimulatory effect. For this reason Tris-HCl is one of the components in the assay solution although it has little or no buffering capacity at pH 7.0.

Substrate Specificity and Mode of Action. The enzyme's substrate specificity is shown in Table V. Almost identical results were obtained with preparations from peaks I and II (Figure 4). Poly(A) is hydrolyzed at a much faster rate than ssDNA and RNA. dsDNA and poly(C) are very poor substrates. The rates of hydrolysis obtained with dsDNA at pH 7.0 or pH 5.6 in the presence or in the absence of KCl were also very low.

The nuclease was assayed in duplicate with poly(A), and the products soluble in 4% perchloric acid or perchloric acid containing 0.15% uranyl acetate were determined. The apparent rate of hydrolysis was faster when 4% perchloric acid was the precipitating agent. Thus, the enzyme appeared to be an endonuclease (Berry & Campbell, 1967). This was confirmed when the products of poly(A) and ssDNA degradation were analyzed. The products from extensive degradation of poly(A) were eluted from DEAE-cellulose in two peaks; 72% of the material eluted at 0.29 M (peak 1) and 28% at 0.33 M NH_4HCO_3 (peak 2). The ratios of total phosphate to alkaline phosphatase labile phosphate were 2.1 and 2.85 for the products in peak 1 and peak 2, respectively. The oligonucleotides from both peaks were readily hydrolyzed by phosphodiesterase I yielding only one product which could be cochromatographed with 5′-AMP in three different systems. Alkaline hydrolysis, however, yielded adenosine as a major product as well as other compounds which were probably adenosine 3′,5′-diphosphate and 3′-AMP. These results show that the nuclease degraded poly(A) almost exclusively to pApA and pApApA. Extensive hydrolysis of ssDNA by the purified endonuclease also yielded primarily di- and trinucleotides. These could be readily converted to the four nucleoside monophosphates by phosphodiesterase I. Therefore, the enzyme is an endonuclease degrading the substrate to small oligonucleotides with 5′-phosphate ends.

Discussion

A Mg^{2+}-dependent endonuclease from yeast has been purified nearly 500-fold to a specific activity of 98 μmol min^{-1} mg^{-1}. Although the final purification step yielded two peaks of activity, it is likely that they are due to the same enzyme since a number of other characteristics such as substrate specificity, pH optimum, stability, and general physical and chemical properties are very similar, if not identical. The enzyme degrades nucleic acids to short oligonucleotides with 5′-phosphate ends. Of the substrates tested the enzyme is most active with poly(A). The nuclease is an integral membrane protein associated with mitochondria. With the assay conditions used here it could be shown that the enzyme is the most active nuclease not only in the mitochondrion but also in the whole yeast cell whether poly(A), ssDNA, or RNA is used as the substrate.

Three publications which have appeared recently also dealt with membrane-bound mitochondrial DNases (Jacquemin-Sablon et al., 1979) or nucleases (Morosoli & Lusena, 1980; Rosamond, 1981). The emphasis and the approach taken in each of these investigations, the conditions used for assaying the enzyme, and some of the results obtained were sufficiently different that it is difficult to conclude whether they deal with the same enzyme or with different enzymes. A comparison of the enzymes to those reported by Jacquemin-Sablon et al. (1979) is difficult since these workers were concerned primarily with the ethidium bromide activated enzyme. They did, however, mention two other activities and one of these was a DNase acting on ssDNA. The following points suggest that, despite apparent major differences, the enzyme reported by Morosoli & Lusena (1980) and that reported by Rosamond (1981) are the same as the one described in this paper. All reports agree on characteristics such as the association with the mitochondrial membrane, solubilization with detergent in the presence of KCl, metal ion requirement, and endonucleolytic nature of attack to generate 5′-phosphate-ended oligonucleotides after extensive hydrolysis of the substrate. The pH optimum of the nuclease reaction varies somewhat being 7.6 according to Rosamond (1981), pH 7.0 according to Morosoli & Lusena (1980), and pH 6.5–7.0 according to this report, but these differences might be due to differences in the assay conditions used.

All reports state that the enzyme is more active with ssDNA than dsDNA. Unfortunately, little information is available with regard to the activity with RNA. Poly(A) was not used in the other studies and only Morosoli & Lusena (1980) re-

ported that the enzyme also degraded RNA, which is in agreement with our results. Jacquemin-Sablon et al. (1979) only established that the enzyme preparation was free of RNase when measured with poly(C) as the substrate. It was shown here (Table V) that the enzyme is active with RNA but not with poly(C). An earlier study (Rosamond, 1981) noted that the purified enzyme had increased activity with dsDNA at acid pH. This was not confirmed here either in the presence or in the absence of KCl. It should be noted that the specific activity of their enzyme was lower by a factor of about 500–1000, indicating a less purified preparation. Thus, the activity toward dsDNA might be due to a contamination, probably by one of the enzymes described by Jacquemin-Sablon et al. (1979). Similarly, the fact that Morosoli & Lusena (1980) found that mononucleotides were part of the degradation products might be due to a contaminating enzyme or due to the much longer incubation period used for the hydrolysis of ssDNA. The amounts of di- and trinucleotides formed under their conditions, however, are in good agreement with our analysis.

Major differences exist between the molecular weights reported for the nuclease: 14000 by Morosoli & Lusena (1980), 105000 by Rosamond (1981), and 57000 and 140000 for peaks I and II from gel filtration in this study. These differences are difficult to explain. The molecular weight of 14000 was established after $NaDodSO_4$–polyacrylamide gel electrophoresis and activity staining of the gel. The enzyme preparation was heated before electrophoresis although the sensitivity of the enzyme to heat had been established (Morosoli & Lusena, 1980). We also have found that the enzyme is quickly inactivated by heat, especially at high pH and in the presence of $NaDodSO_4$, but we have not been able to reverse this inactivation. With regard to the other molecular weight determinations, it must be pointed out that the amount of detergent bound to the enzyme has not been taken into consideration. However, it is possible that the two different molecular weights given here (57000 and 140000) and the one determined by Rosamond (1981), 105000, might be due, in part, to aggregation of the enzyme. Clearly, the enzyme is not contained in micelles.

More work is definitely required with a homogeneous enzyme preparation so that the molecular weight of the enzyme can be determined more precisely under denaturing and nondenaturing conditions. In addition, this would probably resolve with greater certainty whether yeast mitochondria contain one major endonuclease or two closely related endonucleases acting on ssDNA and RNA.

Although considerable effort has gone into describing this enzyme, little is known about its function. So far it was assumed that its function might be in mitochondrial DNA metabolism (Morosoli & Lusena, 1980; Rosamond, 1981). However, a role for the enzyme in the processing of newly synthesized RNA within the mitochondrion should be considered. Endonucleolytic activity is clearly required for the processing of RNA (Abelson, 1979). Since it has now been established that at least some mRNAs in yeast mitochondria are derived from larger transcripts (Lazowska et al., 1980; Gray & Doolittle, 1982), it is conceivable that the endonuclease may be involved in the processing of this RNA or in the degradation of excised intervening sequences. However, only future work with suitable mutants and the effect of their lesions on the nucleic acid metabolism in mitochondria may establish the function of this interesting enzyme.

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