A rapid method for the isolation of ribonuclease from yeast (*Saccharomyces carlsbergensis*)

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A ribonuclease (RNAase; EC 3.1.14.1) from brewer's yeast was purified 90-fold. Crude RNAase was initially separated from other proteins by precipitation at pH 4.0 after incubation of the mechanically disrupted yeast cells at pH 6.0 and 52°C for 30 min. The RNAase was purified from the supernatant by ultrafiltration with a PM-30 membrane and adsorption chromatography on hydroxyapatite. The RNAase preparation was free of phosphatase, deoxyribonuclease and phosphodiesterase activities. It showed maximum activity at pH 6.0 and a temperature optimum of 52°C with yeast RNA as substrate. This RNAase hydrolysed yeast RNA to nucleoside 3'-phosphates and showed no evidence of base specificity.

Ribonucleases (RNAases) are widely distributed in animal and plant tissues. In general, RNAases from animal tissues show specificity for the phosphodiester bonds of the cytosine-containing and uracil-containing nucleotides of RNA (Anfinsen & White, 1961). Plant RNAases have uniform pH optima (5.0) and specificities. RNAases from plants degrade RNA completely to mononucleotides, either 3'-nucleotides or cyclic phosphate esters (Anfinsen & White, 1961). RNAases with specificities different from pancreatic RNAase have been demonstrated in micro-organisms and it was suggested that these enzymes might be useful for studies on the sequencing of RNA. Almost all the microbial species investigated to date possess intracellular RNAase. The two most studied microbial RNAases, RNAases T₁ and T₂ from *Aspergillus oryzae*, showed considerable differences in their base specificities for the hydrolysis of phosphodiester bonds (Egami & Nakamura, 1969). Although microbial RNAases are considered valuable materials for studies in protein and nucleic acid chemistry, very little is known about the properties and potential uses of RNAases from yeast; they may have practical applications in the hydrolysis of RNA during preparation of yeast enzymes or proteins. We developed a rapid method for the isolation of RNAase from brewer's yeast (*Saccharomyces carlsbergensis*) and studied its specificity.

Abbreviation used: RNAase, ribonuclease.

Materials and methods

Materials

Brewer's yeast, *Saccharomyces carlsbergensis*, obtained from Genesee Brewing Co., Rochester, NY, U.S.A., was washed three times with distilled water and was disrupted by the procedure described by Shetty & Kinsella (1979). The disrupted yeast was then lyophilized and stored at -10°C. Yeast RNA and DNA, *o*-carboxyphenylphosphate, calcium bis(*p*-nitrophenyl)phosphate, phenylmethanesulphonyl fluoride, EDTA, *N*-bromosuccinimide, *N*-ethylmaleimide, dithiothreitol, hydroxyapatite and the 3'- and 5'-forms of AMP, GMP, CMP and UMP were purchased from Sigma. Carbazole was obtained from Eastman Organic Chemicals, New York, NY, U.S.A.

Isolation and purification of RNAase from disintegrated yeast cells

A 5% (w/v) disintegrated yeast cell suspension was prepared in 10 mM-citrate/phosphate buffer, pH 6.0, containing phenylmethanesulphonyl fluoride (0.1 mM), EDTA (1 mM), NaN₃ (0.02%) and NaCl (0.5 M). The slurry was stirred for 15 min and then incubated at 52°C for 30 min with constant stirring. The incubated sample was cooled and the pH was decreased to 4.0 by the addition of 1 M-HCl. The sample was then centrifuged at 28 000 *g* for 45 min at 5°C. The supernatant was further concentrated by ultrafiltration with a PM-30 membrane in an

Amicon ultrafiltration apparatus. The concentrate was then lyophilized. The RNAase was separated from other proteins by hydroxyapatite chromatography. A column (4 cm × 20 cm) was packed with hydroxyapatite and equilibrated with 10 mM-phosphate buffer, pH 7.0, containing 0.5 M-NaCl. The crude RNAase (ultrafiltration concentrate) was dissolved in the same buffer (10 mg of protein/5 ml) and loaded on the column. The column was then developed by increasing the phosphate concentrations in steps. For each elution step, 1–1.5 column volumes of the buffer were used. Fractions (3 ml) were collected and their A_{280} and A_{260} were measured.

Enzyme assay

The activity of yeast RNAase was determined by a modification of the procedure of Takahashi (1962). The reaction mixture, unless otherwise stated, contained 0.75 ml of a 0.6% (w/v) solution of purified yeast RNA in 10 mM-citrate/phosphate buffer (pH 6.0)/0.5 M-NaCl and an appropriate amount of enzyme solution in 0.25 ml of the same buffer. After incubation at 52°C for 20 min, 0.5 ml of 0.75% uranyl acetate in 12.5% (w/v) HClO₄ was added. The mixture was allowed to stand for 30 min at 5°C and then the precipitate was removed by centrifugation. The A_{260} of the supernatant was measured after appropriate dilution with water. A unit of yeast RNAase activity is defined as the amount of enzyme that, under the above conditions, gave an increase in absorbance of 0.1. The specific activity is expressed in units/mg of protein.

Acid phosphatase was assayed according to the procedure described by Spahr & Hollingworth (1961) using *o*-carboxyphenylphosphate as the substrate. Phosphodiesterase activity was assayed by using calcium bis(*p*-nitrophenyl)phosphate as described by Frisch-Niggemayer & Reddi (1957). Deoxyribonuclease activity was determined as described for the RNAase assay, but the substrate was replaced by 0.6% (w/v) DNA.

Hydrolysis of yeast RNA and identification of nucleotides

Purified yeast RNA (30 mg) in 5 ml of 10 mM-citrate/phosphate buffer (pH 6.0) was digested by 500 µg of purified yeast RNAase at 52°C. After complete hydrolysis of RNA [aliquots were tested with 0.4% uranyl acetate in 7.5% (w/v) HClO₄] the nucleotides in the digested sample were separated by ion-exchange chromatography on a column (1 cm × 25 cm) of Dowex 1 (X8; formate form; 200–400 mesh) by the procedure described by Ohtaka *et al.* (1963). Elution was with a gradient composed of 30 ml of 1 M-formic acid and 120 ml of 4 M-formic acid. Fractions (3 ml) were collected and their A_{260} was measured. The separated nucleotides were then analysed to differentiate pentose 5'-phosphates (greenish-blue) from other pentose phosphates (purple) by using the carbazole colour reaction (Dische & Landsberg, 1957). The identity of individual nucleotides was further confirmed by u.v. absorption spectroscopy (Cary 219 spectrophotometer) and paper chromatography (Markham & Smith, 1951) with standard nucleotides for comparison.

Other measurements

Nitrogen was determined by the micro-Kjeldahl method (Association of Official Agricultural Chemists, 1970) and protein by the method of Lowry *et al.* (1951).

Results and discussion

Isolation of RNAase from disrupted yeast cells

The scheme for isolation of RNAase from disrupted yeast cells is given in Table 1. The disrupted yeast cells were incubated at 52°C in 10 mM-citrate/phosphate buffer, pH 6.0, containing phenylmethanesulphonyl fluoride (0.1 mM), EDTA (1 mM) and NaCl (0.5 M). Under these conditions, the cellular RNAase was released and hydrolysed the yeast RNA into low-molecular-weight products.

Table 1. *Purification of yeast RNAase*

Treatment	Protein yield [mg/g of dry yeast (N × 6.25)]	Total RNAase activity (units/g of dry yeast)	Enzyme recovery (%)	Specific activity (units/mg of protein)	Purification (-fold)
Incubation at 52°C and pH 6.0 for 30 min	229	27 000	100	118	1
pH 4.0 supernatant	174	24 000	88.9	138	1.17
Ultrafiltration concentrate (PM-30 membrane)	4.09	20 760	76.9	5080	43.0
Hydroxyapatite chromatography	1.26	13 440	49.8	10 670	90.4

Purification of enzymes from microbial extracts is complicated by the presence of nucleic acids that interfere with salt fractionation or chromatographic procedures (Pringle, 1975; Snoke & Klein, 1977). The maximum hydrolysis of yeast nucleic acids by endogenous RNAase occurred at pH 6.0 and 52°C (Sinskey & Tannenbaum, 1975; Lindblom, 1977). Danner & Morgan (1963) reported that yeast RNAase was associated with ribosomes in the latent form and that the latency was broken by treatment with 0.5M-NaCl at pH 5.0. It was also found that 0.1mM-phenylmethanesulphonyl fluoride and 1mM-EDTA inhibited intracellular proteolytic enzymes during the incubation of disrupted yeast cells for RNA hydrolysis (Woods & Kinsella, 1980). However, phenylmethanesulphonyl fluoride and EDTA have no inhibitory effect on yeast RNAase activity. Hence the artifacts caused by the intracellular proteolytic enzymes of yeast during the isolation of RNAase were controlled by the addition of phenylmethanesulphonyl fluoride and EDTA. This also greatly increased the yield of RNAase. In addition to the hydrolysis of RNA during the incubation procedure, more than 55% of the yeast protein spontaneously coagulated. This further simplified the

purification of RNAase. Subsequently decreasing the pH to 4.0 caused the precipitation of another 25% of the total protein, leaving RNAase in the supernatant. NaCl (0.5M) enhanced the precipitation of protein at pH 4.0 and also protected yeast (*Saccharomyces cerevisiae*) RNAase from heat denaturation during incubation (Lindblom, 1977).

Purification of yeast RNAase

To achieve purification of RNAase from the supernatant of pH 4.0 incubated yeast cells, several ultrafiltration membranes were initially tested. Membranes (Amicon) XM-100 A and XM-50, with molecular weight cutoffs of 100 000 and 50 000 respectively, failed to separate RNAase from the other proteins present. However, no RNAase activity was detected in the filtrate from PM-30 and UM-20 membranes. PM-30 membrane was selected in preference to the UM-20 membrane because of its higher molecular weight cutoff property (greater than 30 000) and since more of the undesirable components were removed. However, a good separation of RNAase from other contaminating proteins was achieved by using hydroxyapatite adsorption chromatography. The unadsorbed frac-

Fig. 1. Column chromatographic separation of the mononucleotides formed during the hydrolysis of RNA by yeast RNAase

The reaction mixture [30mg of yeast RNA and 500µg of purified RNAase in 5 ml of 10mM-citrate/phosphate buffer (pH 6.0)] was incubated at 52°C for 3–4 h and then was applied to a column (1cm × 25 cm) of Dowex 1 (X8; formate form; 200–400 mesh). Elution was with a gradient composed of 30ml of 1M-formic acid and 120ml of 4M-formic acid. O, A₂₆₀; ●, [formic acid].

tion that was initially eluted from the column contained mostly nucleic acids, as shown by the A_{260} being higher than the A_{280} . The fraction that was eluted at 100mM-phosphate contained RNAase activity. The purification was 90-fold with a recovery of 49% (Table 1).

The purified yeast RNAase was free of acid phosphatase, deoxyribonuclease and phosphodiesterase activities and exhibited maximum activity at pH 6.0 with a temperature optimum of approx. 55°C. RNAase isolated from *Saccharomyces cerevisiae* showed a temperature optimum of approx. 55°C and a pH optimum of approx. 5.8 (Lindblom, 1977).

Specificity of yeast RNAase

Four discrete fractions (Fig. 1) were obtained after the hydrolysis of yeast RNA by the isolated RNAase. All the fractions from the column gave a purple colour with the carbazole reagent, indicating that the fractions contained pentose 2'- or 3'-phosphates (Dische & Landsberg, 1957). The nucleotides were confirmed to be 3'-CMP, 3'-AMP, 3'-GMP and 3'-UMP by their u.v. absorption spectra and by paper chromatography with standard 3'- and 5'-mononucleotides. Thus RNAase from brewer's yeast showed a pattern of hydrolysis of RNA similar to that of the RNAase isolated from baker's yeast (Ohtaka *et al.*, 1963). Brewer's yeast RNAase did not show any specificity for particular bases, and yeast RNA was quantitatively digested to nucleoside 3'-phosphates.

Conclusion

A simple three-step procedure for the purification in good yield of RNAase from *Saccharomyces*

carlsbergensis was developed. The enzyme was not base-specific and yeast RNA was hydrolysed to nucleoside 3'-phosphates.

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