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Nucleases in Yeast Acting on the 3'-Terminus of Transfer Ribonucleic Acid*

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Two enzymes capable of hydrolytic removal of 3'-terminal adenosine from specifically labeled tRNA were identified in yeast. One of them was highly purified by chromatography on phosphocellulose. It acts on the 3'-terminus of tRNA by removing free adenosine, followed by 2'- (or 3'-) cytidylic acid; it also hydrolyses dinucleoside monophosphates, viral and ribosomal RNA, but it is inactive against either native or denatured DNA. Hence, the enzyme is a ribonucleate 3'-nucleotidohydrolase, and may be identical to previously described »ribosomal« ribonuclease of yeast. The other enzyme is a labile acidic protein, with M. W. around 31000. It removes the 3'-terminal adenosine of tRNA as adenosine-5'-monophosphate in a reaction with a pH optimum of 7.2, which is not inhibited by Mg^{2+}. Chemical and photochemical modification of tRNA increases its susceptibility to the enzyme. In conjunction with tRNA adenylyl (cytidylyl) transferase, the enzyme may be responsible for the turnover of 3'-terminal adenylic acid in yeast.

INTRODUCTION

It has been known for a long time that the 3'-terminal AMP of tRNA turns over in exponentially growing yeast[1], *Escherichia coli*[2] and rat liver[3]. In fact, even crude extracts from sources ranging from bacteria and yeast to a brine shrimp and rat liver, are capable of catalyzing such a turnover in vitro[4]. Evidence has been presented that the turnover is a result of two enzymatic reactions[4], i. e. the removal of terminal AMP by a nuclease, followed by incorporation of AMP from ATP by tRNA-nucleotidyltransferase [tRNA adenylyl (cytidylyl) transferase (*EC* 2.7.7.25)]. The mixtures of the two enzymes, and, for that matter, even crude enzyme preparations, can be used to introduce radioactive AMP from ATP into the 3'-terminal position of tRNA[4]. Using such specific substrate, we attempted to isolate from yeast the nuclease participating in the exchange reaction. Though we were not successful in reaching this goal, we would like to report in the present paper the occurence of two enzyme activities in yeast acting on the 3'-terminus of tRNA by two different types of cleavage, and to describe some properties of these enzymes as revealed by

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experiments with crude enzyme preparations. A possible biological significance of the reactions, catalyzed by these enzymes, will be discussed.

MATERIALS AND METHODS

Materials. [8 — ^{14}C]-adenosine-5'-triphosphate, ammonium salt, 50 Ci · mol^{-1}, was a product of Amersham, England. DEAE-cellulose (Selectacel Ion Exchange Cellulose DEAE Typ 20, 0.84 meq · g^{-1}) was from Carl Schleicher und Schuell; phosphocellulose was Whatmann Column Chromedia Pll Cellulose Phosphate, medium, 7.4 meq · g^{-1}; carboxymethylcellulose, Servacel CM 32, 0.6 meq · g^{-1}, was from Serva Feinbiochemica, Heidelberg; Sephadex and DEAE-Sephadex A-50 were from Pharmacia Fine Chemicals AB, Uppsala. All the ion exchangers were washed and converted into suitable ionic forms according to the recommendations of the manufacturers. Streptomycin sulfate was from Pliva, Zagreb; other reagents were of standard laboratory quality.

Assay of the enzyme activity was based on the removal of radioactive AMP or adenosine from the 3'-terminus of tRNA, i. e. tRNApCpCp*A. This substrate was prepared by an earlier method[4], using unfractionated yeast tRNA instead of $tRNA^{Tyr}$, and 2 mM [^{14}C]ATP, specific activity 5 Ci · mol^{-1}. The assay mixtures contained, in 125 mm^3, 1 nmol of tRNApCpCp*A, up to 0.1 mg of enzyme protein, 3.75 μmol Tris-acetate, pH 7.2; 1.87 μmol magnesium acetate and 0.62 μmol EDTA. After 20 min incubation at 37 °C, the reaction was terminated by pipeting 100 mm^3-samples onto Whatman 3 MM filter paper disks (25 mm diameter) and immersing them into cold 10% trichloroacetic acid. After 10 min the disks were washed with a 1 : 1 mixture of ethanol and ether, and then ether alone (5 to 7 min each). After drying under an infrared lamp, the disks were immersed into 5 cm^3 of scintilation fluid and their radioactivity measured. One enzyme unit was defined as the amount of enzyme which, under the above conditions, removed 1 μmol of 3'-terminal AMP per minute.

Autolysis of yeast was performed essentially by the method of Cramer[5]. 1 kg of fresh commercial yeast (Pliva, Zagreb) was warmed to 37 °C and mixed with 320 cm^3 of toluene, preheated to the same temperature, until a smooth mixture was obtained. After 15 min at 37 °C 300 cm^3 of 1 M tris (hydroxymethyl)aminomethane was added and the mixture stirred for 2.5 h at room temperature. It was then cooled to +4 °C and centrifuged for 45 min at 10000 *g*. The aqueous phase of the supernatant (»crude extract«) contained 5 to 8 g protein and 0.4 to 1.1 E. U. of nuclease in ca 600 cm^3; it was used for further fractionation.

Further fractionation of the enzyme. All centrifugations were for 15 min at 15000 *g*. All operations were done at +4 °C. To the crude extract, 1 M $BaCl_2$ was added dropwise, with constant stirring, to the final concentration of 0.1 mol dm^{-3}, continuing the stirring for 15 more min. After centrifugation, enough streptomycin sulfate (0.4 g · cm^{-3}) was added dropwise to reach the concentration of 20 g · dm^{-3}, and stirring continued for 15 more min. The precipitate was removed by centrifugation, and $(NH_4)_2SO_4$ added, gradually and with constant stirring, to reach 40% saturation. After additional stirring for 15 min, the precipitate was centrifuged off, and the supernatant brought to 70% saturation by the addition of $(NH_4)_2SO_4$ as above. After at least 30 min or overnight stirring, the precipitate was collected by centrifugation, dissolved in the smallest possible volume of 10 mM Tris · HCl, pH 7.4, 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, mixed with the same volume of glycerol and kept at —20 °C.

RESULTS

In crude extracts of commercial yeast we identified two enzyme activities capable of removing acid-soluble products from tRNA, labelled by ^{14}C in the 3'-terminal adenosine.

The first of these enzymes is a very potent nuclease. It was purified from crude cell extracts by removal of nucleic acids with streptomycin sulfate, ammonium sulfate fractionation (50% to 70% saturation), complete removal of nucleic acids by DEAE-cellulose chromatography, and finally by chromato-

graphy on phosphocellulose. Highly purified nuclease was eluted with ca 0.4 M KCl in 0.05 M Tris · HCl, pH 7.5, 1 mM EDTA (Figure 1). The enzyme was not homogeneous, as judged from the SDS-gel electrophoresis. It did not hydrolyze bis(*p*-nitrophonyl)phosphate and either native or denatured calf thymus DNA, but was very active against bulk yeast RNA, RNA of bacteriophage MS2, and purified yeast $tRNA^{Tyr}$. The latter substrate was in part degraded endonucleolytically, as indicated by chromatography of the digest on a column of Sephadex G-100 at 60 °C. The radioactive breakdown product of ^{14}C-Tyrosyl-$tRNA^{Tyr}$ was identical to the breakdown product of the same

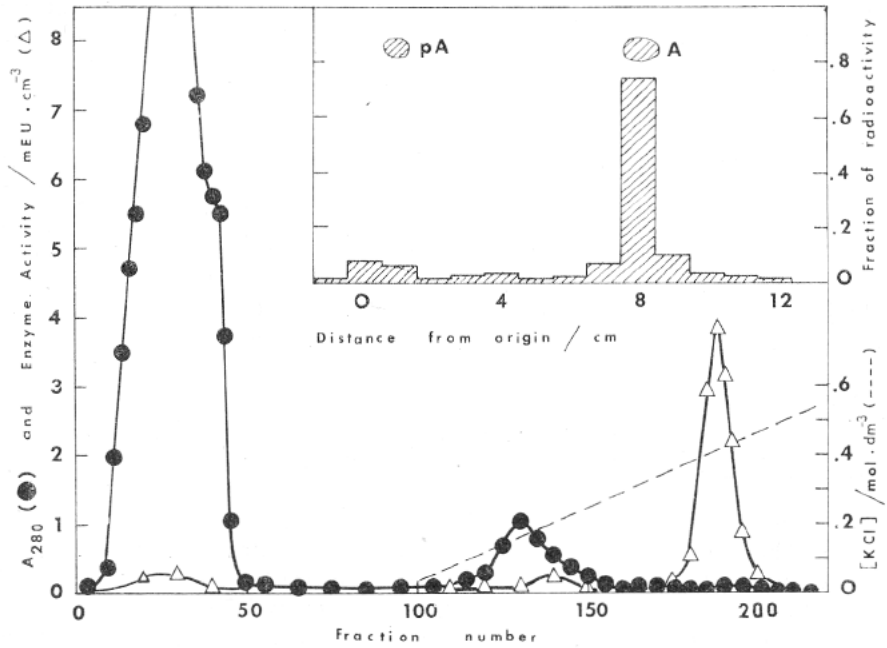


Figure 1. Purification of the major ribonuclease of yeast by chromatography on phosphocellulose. Inset: Identification of adenosine as the main breakdown product of terminally-labeled tRNA. Radioactivity was determined in 1 cm-strips of the paper chromatogram. Positions of two relevant markers are also shown.

substrate by pancreatic ribonuclease, i. e. 2'(3')-tyrosyl-adenosine. Yeast tRNA, terminally labeled in adenosine, gave adenosine, and not 5'-AMP, as the breakdown product (Figure 1, inset). With tRNA, terminally labelled by [α — ^{32}P] ATP, i. e. tRNApCpC*pA, as the substrate, the major radioactive breakdown product was cytidylic acid. Synthetic dinucleoside monophosphate, UpC, was degraded to uridylic acid and cytidine. All these data indicated that the enzyme catalyzed the hydrolysis of phosphodiester bond in RNA and oligoribonucleotides in such a manner that 3'-phosphates and 5'-hydroxyls were produced. It should be classified, therefore as ribonucleate 3'-nucleotidohydrolase. The enzyme of such a specificity could not be responsible for the turnover of terminal AMP in tRNA, and its further studies were abandoned.

The finding that crude enzyme preparations, obtained after the precipitation of ribosomes with $BaCl_2$, catalyzed the removal of AMP rather than

adenosine from terminally-labelled tRNA, suggested that the predominant, 3'-phosphate-producing ribonuclease had co-precipitated in such a treatment. This prompted our search for another enzyme, i. e. ribonucleate 5'-nucleotidohydrolase, in the $BaCl_2$-supernatant. This was somewhat facilitated by the observation that crude extracts, obtained by prolonged autolysis of yeast at 37 °C, retained reasonable levels of the desired activity. As shown in Table I, subsequent $BaCl_2$-precipitation caused very little, if any, loss of total apparent acitivity; probably, the ribosome-bound enzyme, present before $BaCl_2$-precipitation, was in a »latent« form, escaping the detection. Further fractionation with streptomycin sulfate and ammonium sulfate (Table I) led to apparent 2.5-fold purification and complete recovery of enzyme acticity. This preparation could be kept for at least one year at —20 °C in 50% glycerol without appreciable loss of activity.

TABLE I

Partial purification of ribonuclease from 1 kg *of yeast*

Step	Total protein	Total activity*	Specific activity	Purification ratio
	g	E. U.	E. U. g^{-1}	
I Crude extract	6.05	0.98	0.162	1
II $BaCl_2$-supernatant	6.22	0.98	0.158	0.97
III Streptomycin sulfate	5.62	1.05	0.186	1.15
IV Ammonium sulfate	2.47	1.00	0.405	2.50

* Enzyme assays in the first two steps. *i. e.* prior to removal of endogenous nucleic acids by streptomycin sulfate, lead unevitably to underestimates of enzyme activity because of the dilution of radioactive substrate.

We have made considerable effort towards further purification of this nuclease. The enzyme did not bind to phosphocellulose or carboxymethylcellulose, but did bind to DEAE-cellulose and DEAE-Sephadex, suggesting acidic nature of the protein. However, elution from DEAE columns usually gave broad peaks, overlaping with main protein peaks, and was always accompanied by major losses of enzyme activity. Hence, neither the above mentioned chromatographic methods, nor their combinations, could be used for further purification of the nuclease. Although the reason for these losses is not clear, a co-purification of a proteolytic activity, detectable in our preparations, might have been at least partially responsible.

The enzyme purified through the ammonium sulfate step catalyzed specifically the removal of AMP from terminally-labeled tRNA (Figure 2). Some adenosine was detected only after prolonged incubations, and probably was the product of contaminating phosphatase action on AMP, rather than the action of another enzyme on the original substrate. The reaction showed an optimum at pH 7.2, the activity being roughly the same between 6.8 and 8.0 (Figure 2, inset). Mg^{++} ions, known to stabilize the conformation of tRNA in solution, even at 0.05 mol · dm^{-3} did not prevent the enzyme from acting on the terminal AMP. Tris · HCl, pH 7.2, and especially KCl, are inhibitory at lower concentrations (Figure 3). EDTA, though not inhibitory up to 0.01 mol · dm^{-3}, seems to facilitate enzyme inactivation during prolonged storage, if present at concentrations higher than 2 mmol · dm^{-3}. On the contrary, glycerol

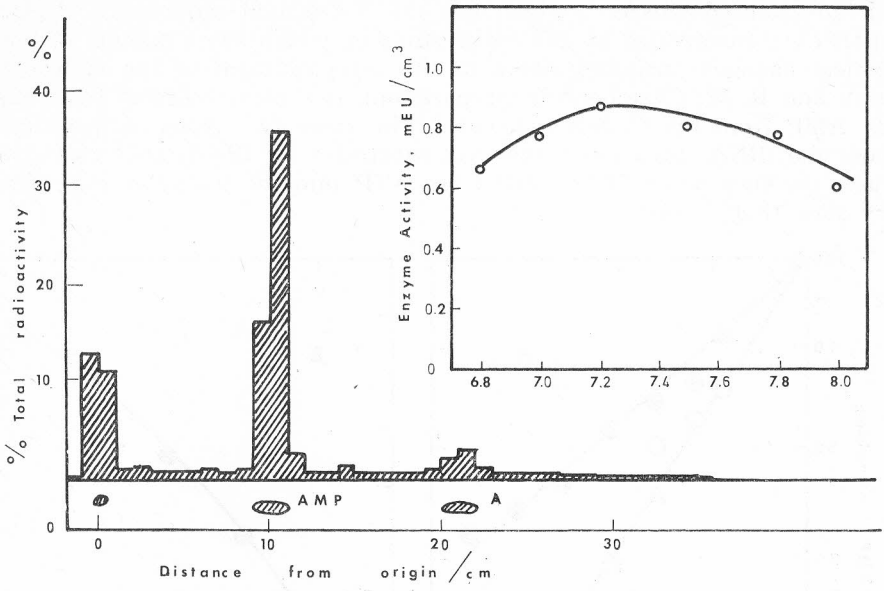
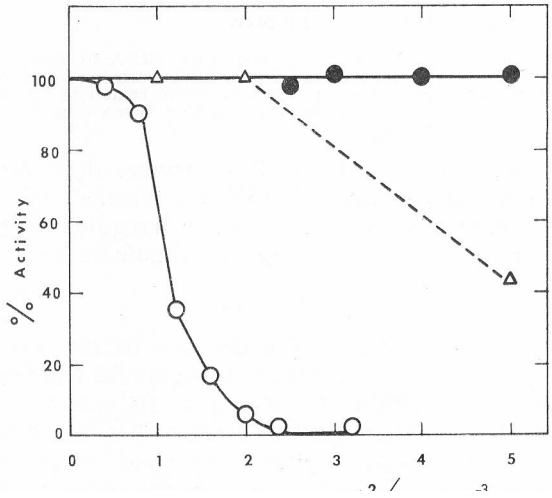


Figure 2. Identification of AMP as the major breakdown product of terminally-labeled tRNA incubated with second yeast nuclease. Inset: Dependence of enzyme activity on pH.

stabilizes the enzyme. Even under otherwise optimal conditions for stability, moderately high temperatures cause rapid inactivation of the enzyme. Thus, 50% activity is lost in 10 min at 46 °C and in 30 min at 30 °C.

tRNA molecules of altered structure, produced by chemical or photochemical modifications of $tRNA^{Tyr}$, were better substrates for the enzyme than the unmodified molecules. Figure 4A shows that the 3'-terminal AMP in both



bisulfite-modified $tRNA^{Tyr}$, which has the 3'-terminal sequence ... UpUpA[6], and $tRNA^{Tyr}$ inactivated by 254 nm-irradiation, which has a number of photochemical changes, including some in the acceptor stem of the molecule (I. Kućan and R. W. Chambers, in preparation), is removed almost twice faster than AMP from the control molecules. The same two types of modification render the tRNA molecules rather bad substrates for tRNA-nucleotidyl transferase: the incorporation of AMP from ATP into the modified molecules is very slow (Figure 4B).

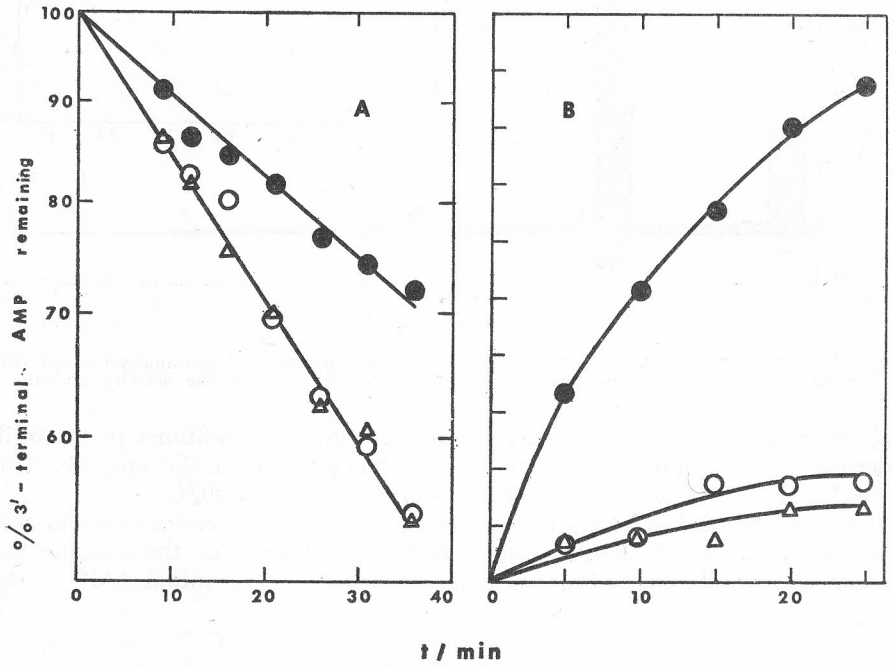


Figure 4. Kinetics of the removal of 3'-terminal AMP from tRNA by crude enzyme preparation (A), and exchange of 3'-terminal AMP, measured by incorporation of AMP from radioactive ATP into non-radioactive tRNA (B). The substrates were untreated tRNA (●), tRNA irradiated at 254 nm (○), and bisulfite-treated tRNA (△).

Approximate molecular weight of the enzyme, determined by chromatography on a column of Sephadex G-150, was around 31000. However, the distribution of the enzyme activity was rather irregular, suggesting that the enzyme was partially present in the form of oligomers and/or agregates.

DISCUSSION

Two distinct enzymes, capable of hydrolysis of the 3'-termini of tRNA, were identified in yeast cells. One of them is a general ribonuclease, while the other may be specific for tRNA. According to its mode of action the first enzyme is a ribonucleate 3'-nucleotidohydrolase, i. e. an enzyme specific for RNA, producing nucleoside-3'-phosphates as final reaction products. The enzyme seems to adsorb to ribosomes, so in crude extracts it is present in a »masked« or »latent« form. We found no evidence for its base specificity. These and other properties indicate that this enzyme might be identical to

the previously described »ribosomal« ribonuclease of yeast[7–10]. Due to the enzyme's reasonable stability, and to the fact that it bound phosphocellulose very strongly, we were able to reach considerable purification (ca. 300-fold in the phosphocellulose step alone); nevertheless, the enzyme was not yet homogeneous. The biological role of this enzyme in yeast is not clear, since the reaction products, ribonucleoside 3'-(or 2'-)phosphates, cannot be directly used in biosynthesis. Interestingly enough, a similar enzyme is present in wild type *Escherichia coli* (»ribonuclease I«[11,12]), but strains lacking the enzyme, e. g. *E. coli* MRE600[13] appear normal in every respect.

The other enzyme, which, unfortunately, resisted our attempts of purification, can be tentatively classified as a ribonucleate 5'-nucleotidohydrolase. Only upon further purification will it be possible to assess the true specificity to the enzyme, and to distinguish between a general phosphodiesterase, an unspecific ribonuclease (like *E. coli* ribonuclease II, *EC* 3.1.4.20), or a true tRNA-specific 5'-nucleotidohydrolase. It should be re-called that two ribonucleases, identified by Bock and coworkers[9,10] in post-ribosomal supernatant of yeast, also produce 5'-phosphates as the reaction products. However, unlike our enzyme, the final products are dinucleotides and trinucleotides. This makes us believe that we have detected a new ribonuclease in yeast, possibly an enzyme specific for the 3'-terminus of tRNA.

There is no doubt that the enzyme participates in the exchange of terminal AMP in the extracts of yeast. The sequence of reactions is:

$$\text{tRNApCpCpA}_{\text{OH}} + \text{H}_2\text{O} \rightarrow \text{tRNApCpC}_{\text{OH}} + \text{AMP} \quad (1)$$

$$\text{tRNApCpC}_{\text{OH}} + \text{ATP} \rightarrow \text{tRNApCpCpA}_{\text{OH}} + \text{PPi} \quad (2)$$

An earlier finding[4] that inorganic pyrophosphate is not required for the removal of terminal AMP, proves that the turnover is not catalyzed by nucleotidyl transferase alone, in which case the hydrolysis step (1) would be replaced by a pyrophosphorolysis reaction, i. e. the reversal of reaction (2). We would like to suggest that the same sequence of reactions takes place *in vivo*. Why does the exchange of terminal AMP in tRNA occur at all? We can propose two possible reasons, which do not mutually exclude each other:

(1) The nuclease removing AMP from the 3'-terminus of tRNA, described in this paper, is in fact a tRNA-maturation enzyme, removing primarily the nucleotides extending beyond the universal 3'-terminal sequence ... CpCpA; the enzyme could be analogous to ribonuclease PIII of *E. coli*[14]. If the final point of cleavage by this enzyme is determined primarily by the length of the single-stranded stretch of four nucleotides protruding from the double-helical acceptor stem, the enzyme may, because of the flexibility of the terminal tetranucleotide, remove occassionally the next nucleotide, i. e. the terminal AMP. Such damage is easily repaired by the action of nucleotidyl transferase. Our finding that the tRNA with altered 3'-terminal sequence is a much better substrate for the nuclease, certainly speaks in favor of this possibility. Further purification of the enzyme, and experiments with tRNA-precursors, are needed for further elucidation of this question.

(2) The reactions have a regulatory role in cell metabolism. At low cellular concentrations of ATP, e. g. when the carbon source is being exhausted, tRNA molecules would gradually lose terminal AMP. Hence, the last supplies of ATP would not be wasted for the synthesis of aminoacyl-tRNA, which would

require additional GTP molecules to get incorporated into polypeptide chains. In agreement with this notion is the well-known fact that dormant bacterial spores and stationary phase yeast cells contain tRNA lacking most of its 3'-terminal AMP[16,17].

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SAŽETAK

Djelovanje nukleaza u kvascu na 3'-kraj transfer ribonukleinske kiseline

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U stanicama kvasca identificirana su dva enzima, koji mogu hidrolitički odstraniti 3'-terminalni adenozin iz specifično-označene tRNA. Jedan je od njih znatno pročišćen kromatografijom na fosfocelulozi. On djeluje na 3'-kraj tRNA odstranjujući prvo slobodni adenozin, a zatim 2'- (ili 3'-) citidilnu kiselinu; on također hidrolizira dinukleozid-monofosfate, virusnu i ribosomsku RNA, ali je neaktivan prema nativnoj i denaturiranoj DNA. Prema tome, taj enzim je ribonukleat-3'-nukleotidohidrolaza, i možda je identičan s ranije opisanom kvaščevom »ribosomskom« ribonukleazom. Drugi je enzim labilni kiseli protein relativne molekulske mase oko 31 000. On odstranjuje 3'-terminalni adenozin iz tRNA kao adenozin-5'-monofosfat, u reakciji s pH-optimumom pri pH = 7,2, koju ne inhibira Mg^{2+}. Kemijska i fotokemijska modifikacija tRNA povećava njezinu osjetljivost prema enzimu. U zajednici s adenilil(citidilil)transferazom, taj bi enzim mogao biti odgovoran za izmjenu 3'-terminalne adenilne kiseline u stanicama kvasca.

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