PARTIAL PURIFICATION AND CHARACTERISATION OF AN RNASE FROM A FACULTATIVE THERMOPHILIC BACTERIUM

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Abstract

An RNase of molecular weight 30000 was isolated and partially purified from a facultative thermophilic bacterium, JB-1. The maximal activity was measured at pH 5,5 and 318 K for RNA digestion. The time dependence of thermal inactivation was determined at different temperatures and the activation energy of thermal inactivation calculated to be 254 kJ/mole. Ag^{+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and K^{+} ions inhibited the RNase activity. Mg^{2+} ions had no effect on the enzyme activity. In the presence of Ca^{2+} ions an activation of RNase was observed. Phenyl-methyl-sulphonyl-fluoride and EDTA inhibited the catalytic activity. The iodoacetamide after a slight activation — also caused an inhibition of RNase activity.

Introduction

The ability of microorganisms to growth and thrive at high temperature has been a focal point of investigation. FARELL and CAMPBELL (1969) devided thermophilic bacteria into three categories: obligate or strict thermophiles with optimal growth temperatures ranging from 338 to 348 K but showing no growth below 313 to 315 K; facultative thermophiles with maximal growth temperatures between 323 and 338 K, but also capable of reproducing at temperatures below 303 K; thermotolerant bacteria with growth maxima at 318 and 323 K and showing growth below 303 K.

The question arose what is the chemical basis of the thermostability of the thermophilic or thermotolerant bacteria, i.e. how can flow the metabolism by a normal rate at higher temperature, which features result the higher thermostability of these enzymes in such microorganisms.

There are data in the literature which indicate that the lipid composition is different and as a consequence of this the membrane structures have higher melting points in thermophilic microorganisms. HEILBRUNN and BELEHRADEK (1937) pointed out that organisms growing at elevated temperatures contain lipids, with higher melting points and proposed that growth at different temperatures was dependent upon the melting point of the cellular lipids. BROCK (1967) in reviewing thermophilic growth proposed that the increase observed in percentage of saturated and branched—chain fatty acids with an increase in growth temperatures could enhance membrane stability and that the integrity of the cytoplasmic membrane may be the limiting factor in the growth of thermophiles.

Other experiments show that some individual enzymes of such bacteria are of higher conformational stability. In comparative studies, AMELUNXEN and LINS (1968

reported that nine of eleven enzymes from B. stearothermophilus were significantly more thermostable than their counterparts from the mesophile, *Bacillus cereus*.

BROCK (1967) speculated that the thermostability of thermophilic proteins might be due to a rigid, inflexibile conformation. CAMPBELL (1955) reported that the α-amylase from a facultative thermophile (*Bacillus* sp.) was thermostable if the cells were grown at 328 K, but was thermolabile from cells grown at 310 K.

BARNES and STELLWAGEN (1973) compared two thermophilic and two mesophilic enolases and found a significant relationship between an increase in thermostability and a decrease in the number of residues they considered capable of forming H-bonds. CASS and STELLWAGEN (1975) did not find any difference in the H-bonding potential comparing the phosphofructokinase of one thermophilic and two mesophilic bacteria.

In some cases the removal of Ca^{2+} ions from the enzyme resulted in loss of thermostability [FEDER (1971), VOORDOUW (1975)]. Thermophilic extracellular enzymes such as thermostable neutral protease, alkaline protease and α-amylase of Bacillus stearothermophilus, which lack disulfide bonds are stabilized by the presence of calcium. It has been suggested by POLLOCK (1962) that the calcium may take place of disulfide bonds in stabilising proteins without disulfide cross linkages.

In the frame of the comparative investigations of the conformational stability of enzymes, we have isolated a new facultative thermophilic bacterium (JB-1) and studied the characteristics some of its individual enzymes.

The present paper is concerned with some physicochemical properties of RNase from a facultative thermophilic bacterium, isolated in our department.

Materials and Methods

Growth of bacteria

JB-1 bacteria were grown up in the growth medium described by JÓNÁS (1980) with vigorous aeration at 323 K. The average yield of bacterial paste was about 3–5g wet wt. /litre of culture. The cells were harvested in the late logarithmic phase and collected by centrifugation with 6000 g for 60 min and stored as a frozen paste at 253 K.

Preparation of extract and partial purification of RNase

Bacterial paste was thawed and resuspended in 0,01 M acetate buffer, pH 5,5 and broken by treatment with lysozyme HACHIMORI (1970) or by French press. Cell debris was centrifuged off and the supernatant was saturated with ammoniumsulphate up to 60% of saturation. The insoluble proteins were removed by centrifugation and discarded. The supernatant was dialysed overnight against 0,01 M acetate buffer, pH 5,5. The dialysed supernatant was applied to a column of DEAE cellulose (15×1.4 cm), equilibrated with 0.01 M acetate buffer pH 5.5 and washed with the same buffer. Bound proteins were eluted from the column by a linear gradient of 0–0.5 M NaCl in 0.01 M acetate buffer at a flow rate of 30 ml/h. The total volume of the gradient was 100 ml and fractions of 5 ml were collected. Fractions, containing RNase activity were combined and stored at 253 K with no detectable loss of enzyme activity within 2 months.

Protein content was determined by the method of LOWRY et al. (1951) with bovine serum albumin as a standard or by measuring the absorbance at 280 nm, using an approximate extinction coeffecient of $A^{0,1\%}_{280nm}=1.0$.

Estimation of molecular weight

The molecular weight for RNase was determined by gelfiltration chromatography and sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis. The gel chromatography was

performed on Sephadex G 50 column (2.5×40 cm), equilibrated with 0.1 M acetate buffer pH 5.5 using trypsin, cytochrome c, lysozyme, soybean trypsin inhibitor and myoglobin as molecular weight markers.

The SDS-polyacrylamide gel electrophoresis was performed according to WEBER and OSBORN (1969).

Enzyme assay

The RNase activity was determined by the method of BERNARDI (1966) with some modifications. The reaction mixture contained 250 mM of acetate buffer, pH 5.5, 0.5 mg of yeast RNA and enzyme preparation in a final volume 0.5 ml. The reaction was carried out at 318 K for 20 minutes and terminated by adding 2.5 ml of 2.5% ice cold TCA solution containing 0.3 % $La(NO_3)_2$ (UDVARDY, 1973). After centrifugation the absorbancy of the supernatant was measured at 260 nm and the acid soluble digestion products were determined. One unit of RNase activity was defined as the amount of the enzyme, producing a hydrolysate having 1.0 absorbance at 260 nm in one hour reaction time.

The assay conditions were the same, when the effects of different cations, EDTA, phenyl--methyl-sulphonyl-fluoride and iodoacetamide were investigated.

Chemicals and biochemicals were reagent grade and purchased from REANAL (Budapest). Markers used for gel electrophoresis and phenyl-methyl-sulphonyl-fluoride were obtained from SERVA. Sephadex G 50 was a product of Pharmacia, Uppsala, Sweden.

Results and discussion

Partial purification of the RNase

The RNase was purified with ammonium-sulfate saturation and on DEAE cellulose column. During 60% ammonium-sulfate saturation 3 fold purification was achieved and the enzyme remained in the supernatant.

The dialysed supernatant of 60% ammonium sulfate saturation was applied to a DEAE cellulose column (Fig. 1). The RNase was not bound to the cellulose however many proteins and nucleic acids were tightly bound and were eluted only with a gradient of NaCl. During this chromatography the specific activity of enzyme

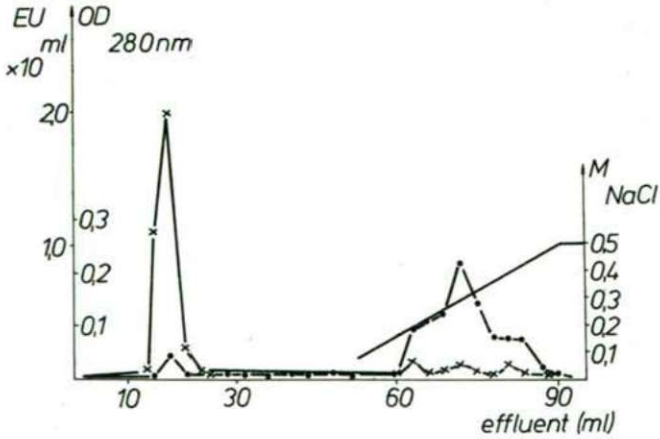

Fig. 1. DEAE cellulose chromatography of RNase. The dialysed supernatant of 60% saturation of $(NH_4)_2SO_4$ was applied to the column and eluted as described in the text. (· — ·) protein (x —x) RNase activity.

increased by 80 fold with a 79% recovery of activity. All assays, described below were carried out with this partially purified enzyme preparations. A summary of the purification procedure is given in Table 1.

Table 1. Summary of the properties of the fractions obtained during purification

		RNase activity (total units)	Protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract		950	1453	0.653	100	1
Supernatant of 60% saturation with $(NH_4)_2SO_4$		915	468	1.95	96	3
DEAE cellulose chromatography	eluted protein fractions	57	453	0.125	6	—
	unbound protein fractions	750	14.3	52.3	79	80

The effect of temperature and pH on the partially purified RNase activity

The temperature dependence of the enzyme activity was determined between 300 and 340 K (Fig. 2). According to our results, the enzyme activity was maximal between 313 and 323 K and was assayed as a routine at 318 K. At this temperature

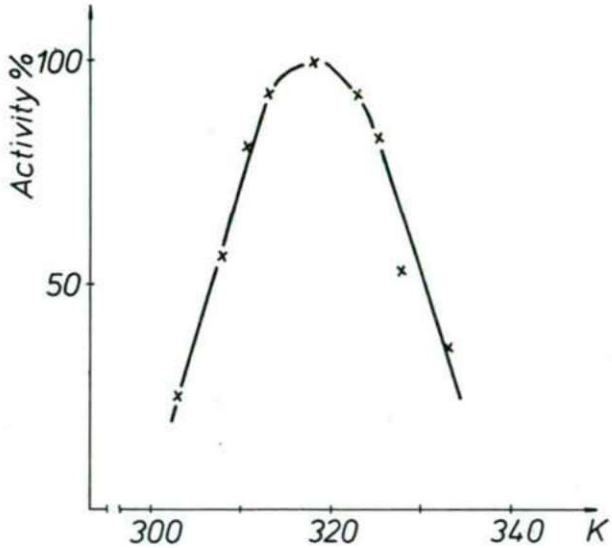

Fig. 2. Effect of temperature on the enzyme activity. In the assays 1.5 unit of RNase was used. Reaction time was 20 minutes.

the activity was proportional to the time of incubation for 15–20 minutes and was proportional to the amount enzyme.

The effect of pH on the RNase activity was evaluated in sodium acetate and tris/HCl buffers (Fig. 3). We have found, that RNase has a sharp maximum at pH 5.5.

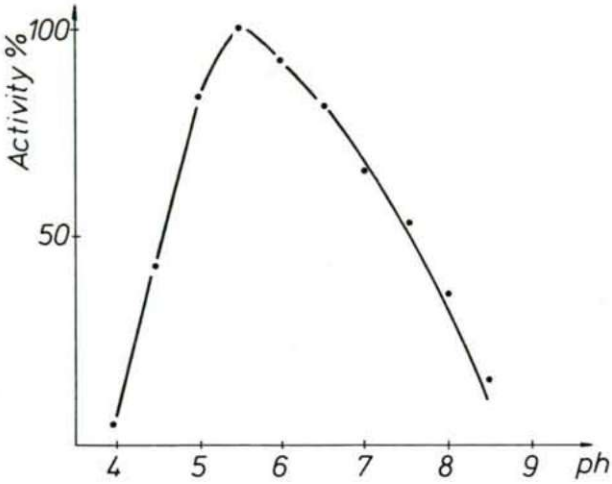

Fig. 3. Effect of pH on the activity. The assays were carried out under standard conditions, except pH which was changed as specified in the figure. The buffers used were 50 mM sodium acetate and 50 mM tris/HCl. In the assays 1.5 unit of RNase was used. Reaction time was 20 minutes.

The effect of temperature on the stability

The spontaneous inactivation of the enzyme was assayed by following the remaining activity at different temperatures at pH 5.5 (Fig. 4a). The enzyme inactivation did not follow a first order kinetic, it seemed to be composed of two parts: a fast reaction which leads to about 50% loss of the activity and a second, very slow further inactivation process. The fast period of inactivation showed a linear relationship in semilogarithmic plot (Fig. 4b). The rate constants of the denaturation process was determined from the initial slope. The logarithmus of the rate constants were plotted against the reciprocal of the absolute temperature (Fig. 5). From the linear Arrhenius plot the activation energy of the inactivation was calculated as 254.93 kJ/mole.

There are few quantitative data in the literature about the thermal stability of various RNase preparations. The kinetics of spontaneous inactivation of RNase we could not compare with mesophile ones or other thermophilic RNases, because of the lack of publications, dealing with kinetic analysis. It seems that RNases are relatively stable enzymes. According to Arima (1968) RNase T_2 is stable for 5 minutes at pH 6.0, 353 K, while RNase T_1 is active for 10 minutes at pH 6.0, 373 K. The heat resistance of these enzymes higher at lower pH, than in alkaline medium.

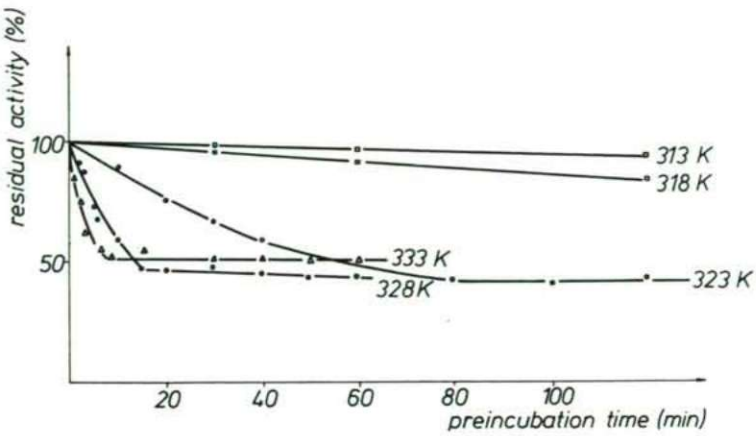

Fig. 4a. The thermal inactivation of the bacterial RNase. Enzyme solutions (0.1 mg/ml) were incubated at different temperatures. At appropriate intervals an aliquote of the enzyme solutions was taken and the reamining activity was measured.

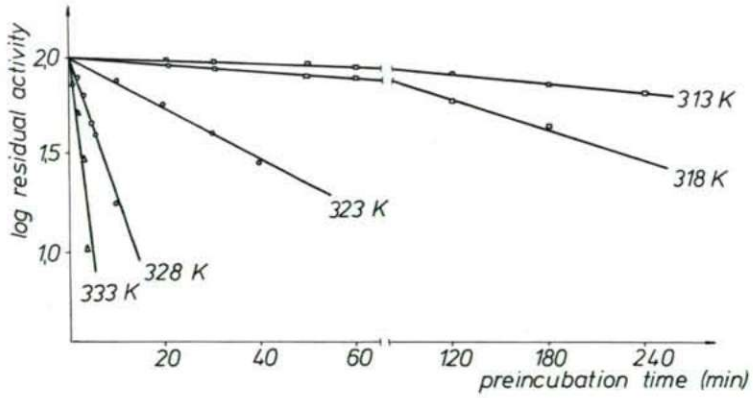

Fig. 4b. Temperature dependence of log remaining activity. Further details see text.

The thermal denaturation of RNase T_1 was determined by MOTOHISA (1979) and found $\Delta H = 564$ kJ/M. Comparing with these, the activation energy of RNase, investigated in our laboratory, seems to be not extremely high, although it was isolated from a facultative thermophilic bacterial strain.

Effect of mono and divalent cations on the activity

Generally RNases require no mono or divalent metal ions for activity. It is well known, that mono or divalent cations can considerably modify the structure of RNA molecule, which can affect the accessibility of substrate bounds to the RNase or the release of products from cleaved RNA molecules.

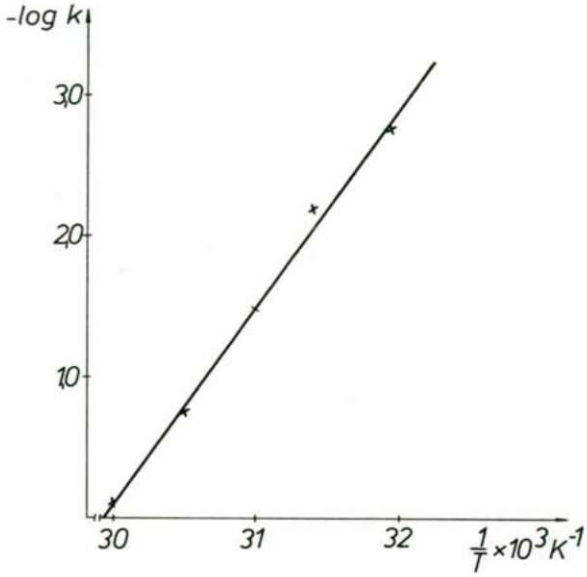

Fig. 5. Temperature dependence of the rate constants of thermal inactivation of RNase. The enzyme solutions were incubated in 50 mM; pH 5.5 of acetate buffer.

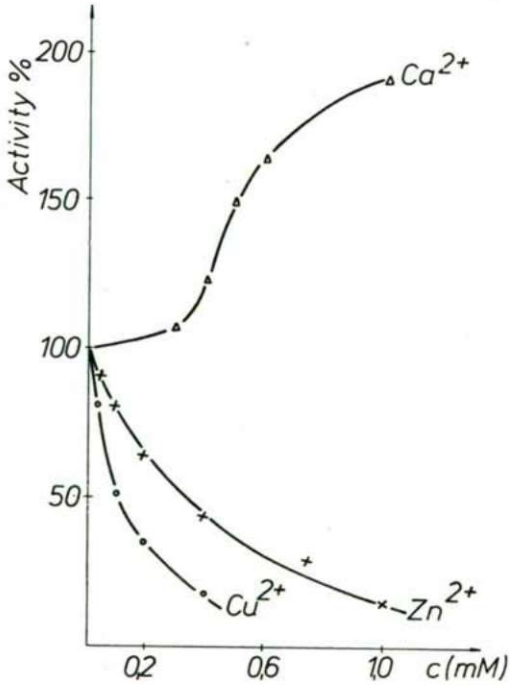

Fig. 6. Effects of Zn^{2+}, Cu^{2+} and Ca^{2+} on the activity of bacterial RNase. Enzyme solution contained 0.2 mg/ml of proteins.

Table 2. Effect of mono and divalent cations on the enzyme activity. Enzyme solutions (0.2 mg/ml containing different concentrations of various cations were incubated at 318 K, at pH 5.5 for 20 minutes and the remaining activity was measured.

Metal	Concentration mM	Inhibition (%)
Mn^{2+}	0.1	19.6
Co^{2+}	0.02	16.5
	0.75	24.4
	1.0	35.9
Mg^{2+}	0.1	no inhibition
	0.2	no inhibition
Ni^{2+}	0.2	23.4
	0.4	43.4
K^{+}	0.04	0.0
	0.1	0.0
	0.2	9.8
	0.4	19.2
Ag^{+}	0.2	17.1
	0.4	39.3

The effect of various cations on RNase activity are presented in Table 2, and Fig. 6. Almost in all cases inhibition of activity was observed at concentrations below 1 mM. Mg^{2+} ions did not effect the reaction rate. In the presence of Ca^{2+} ions the enzyme activity increased. Enhanced activity was found below 1 mM concentration. Further increase of the Ca^{2+} concentration had no further activating effect (not shown on the figure).

Molecular weight

The molecular weight of RNase was 30900 as estimated by Sephadex gel filtration. The measurements, using SDS polyacrylamide gel electrophoresis gave similar results a molecular weight of 30000.

The molecular weight of microbial RNases varying from 10000 to 40000. Molecular weight of RNase of Bacillus cereus was found 30000. The RNases isolated from Aspergillus oryzae and Ustilago sphaerogena had a molecular weight of 10000.

Effect of specific chemical reagents on RNase

The carboxamidomethylation of RNase at pH 5.5 — after a slight activation — measured at about 10^{-8} M concentration of reagent — caused inhibition of activity. The carboxamido-methylated residue might have been essential for the catalytic activity (Fig. 7).

In some fungal RNases activation was observed after pretreatment with iodoacetic acid or iodoacetamide (ARIMA, 1968). The effect of phenyl-methyl-sulphonyl-

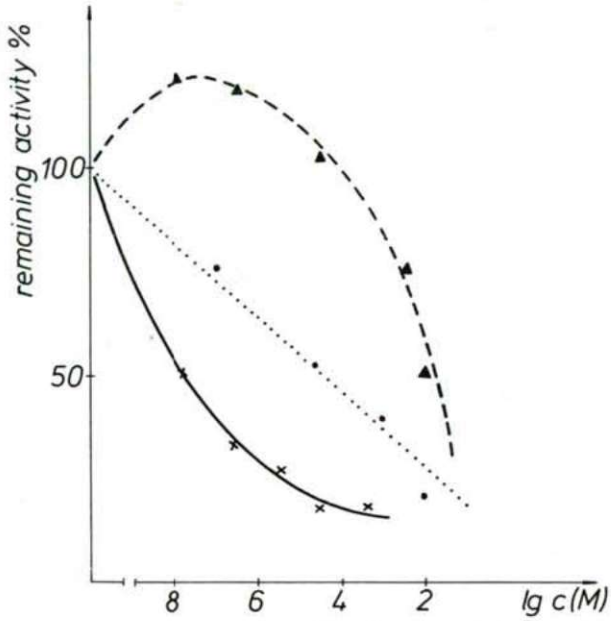

Fig. 7. Effect of phenyl-methyl-sulphonyl-fluoride (PMSF), iodoacetamide and EDTA on RNase activity. Assay conditions were those described in the Methods section. (x—x PMSF; △——△ iodoacetamide; ○·····○ EDTA).

fluoride was determined at concentration from 10^{-8} to 10^{-2} M. The phenyl--methyl-sulphonyl-fluoride inhibited the enzyme activity, which suggest that seryl side chains may have important role in the mechanism of the catalysis.

The RNase activity was totally abolished by the addition of EDTA. This indicate, that this enzyme is an RNase of metalloprotein type.

The data presented show that the facultative thermophilic bacterial RNase has properties similar to B. cereus RNase in respect to inactivation by EDTA and molecular weight RUSHIZKY (1964) and Ca^{2+} activation SHIIO (1966).

The properties of enzyme were also similar to RNase of Rhizopus in respect to stability and inhibition by mono or divalent cations, however this latter enzyme was not inhibited by EDTA (TOMOYEDA et al. 1969).

As above detailed experimental results show our RNase preparation of JB-1 bacteria behaves "uniformly" towards the inhibitors, i.e. all inhibitors caused practically complete loss of enzyme activity and no heterogenity could have been indicated. The apparent heterogeneous behaviour of the enzyme preparation in the heat inactivation experiments (Fig. 4) might reflect the existence of two form of different stability of one enzyme, or the existence of two enzymes with similar active site but with different conformational stability. If only one enzyme exists in the bacteria, either this enzyme could occur in two conformational (or quaterner structural) forms of different stability and different catalytic activity or the enzyme occurs under normal conditions in a more active but less stabile form and the heat treatment causes its transformation to a less active but more stabile form. The further investigation of the problem is in progress in our laboratory.

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