FEMS Microbiology Letters 122 (1994) 211–216

Published by Elsevier

FEMSLE 06170

Thermolabile ribonucleases from antarctic psychrotrophic bacteria: detection of the enzyme in various bacteria and purification from *Pseudomonas fluorescens*

G.S.N. Reddy [a], G. Rajagopalan [b] and S. Shivaji *,[a]

[a] *Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India*
[b] *Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560 012, India*

(Received 27 June 1994; revision received 18 July 1994; accepted 20 July 1994)

Abstract: Thirteen terrestrial psychrotrophic bacteria from Antarctica were screened for the presence of a thermolabile ribonuclease (RNAase-HL). The enzyme was detected in three isolates of *Pseudomonas fluorescens* and one isolate of *Pseudomonas syringae*. It was purified from one *P. fluorescens* isolate and the molecular mass of the enzyme as determined by SDS-PAGE was 16 kDa. RNAase-HL exhibited optimum activity around 40°C at pH 7.4. It could hydrolyse *Escherichia coli* RNA and the synthetic substrates poly(A), poly(C), poly(U) and poly(A-U). Unlike the crude RNAase from mesophilic *P. fluorescens* and pure bovine pancreatic RNAase A which were active even at 65°C, RNAase-HL was totally and irreversibly inactivated at 65°C.

Key words: Thermolabile ribonuclease; RNAase; Antarctic psychrotrophic bacteria; *Pseudomonas fluorescens; Pseudomonas syringae*

Introduction

Enzymes play a crucial role in life of a micro-organism and hence it is that they are stable and function optimally in the range of temperature in which the micro-organism survives and grows. Though much is known about the enzymes from mesophilic and thermophilic bacteria [1,2] with respect to their optimal activity and thermal stability properties, comparatively little is known about the cold-adapted enzymes from psychrotrophic bacteria. Most of these cold-adapted enzymes have a low K_m at the environmental temperature, are cold-active, exhibit optimal activity at lower temperatures (10–30°C) and are heat-labile [3–8].

Most of the information available so far on the functional traits of cold-adapted enzymes is from micro-organisms isolated from aquatic environments [3–5]. Hence, in the present investigation attempts were made to identify cold-active heat-labile ribonucleases (RNAse) from 13 terrestrial

* Corresponding author. Tel: +91 (40) 852241-50; Fax: +91 (40) 851195; email: shivas%ccmb@uunet.in.

SSDI 0378-1097(94)00322-X

psychrotrophic bacteria from Antarctica belonging to the genera *Pseudomonas* [9], *Arthrobacter* [10] and *Micrococcus* [11]. Further, a cold-active heat-labile ribonuclease (RNAase-HL) was also purified to homogeneity from a psychrotrophic *Pseudomonas fluorescens* (10CW) and the enzyme was characterised with respect to its activity and stability at various temperatures and compared with that of bovine pancreatic RNAase A and the crude enzyme from the cell-free extract of mesophilic *P. fluorescens*.

Materials and Methods

Bacterial strains and growth conditions

Thirteen psychrotrophic bacteria (Table 1) isolated from soil and water samples collected at Schirmacher Oasis, Antarctica [9–11] were maintained in ABM medium as described earlier. Mesophilic *P. fluorescens* (ATCC 8251) and *Escherichia coli* strain D10 were grown in broth containing peptone (0.5%, w/v), sodium chloride (1%, w/v) and yeast extract (0.2%, w/v).

Purification of RNAse-HL

Exponential phase cultures of *P. fluorescens* were harvested by centrifugation at $6000 \times g$, for 10 min at 4°C, washed with TE buffer (10 mM Tris and 1 mM EDTA; pH 7.4), suspended in 40 ml of TE buffer and sonicated at 4°C till all the cells were fragmented. The sonicated suspension was centrifuged at $115\,000 \times g$ at 4°C for 90 min and the supernatant was collected, reduced to 6 ml by lyophilisation and loaded on a Bio-Gel P60 column (120 cm × 1.8 cm). The column was eluted with TE buffer and the active fractions were pooled and chromatographed on a DNA-cellulose affinity chromatography column (30 cm × 1.8 cm) prepared according to the method of Litmann [12]. The active fractions were pooled, dialysed for 24 h against TE (four changes), lyophilised and further purified by FPLC. The FPLC active peak (Peak 8) was dried by lyophilisation, dialysed and loaded on a μBondapak C-18 reverse phase HPLC column (30 cm × 3.9 cm) of pore size 10 μm. The column was eluted with a linear gradient of 0–60% acetonitrile in solvent A using a Hewlett and Packard 1090 HPLC unit.

Gel electrophoresis

Sodium dodedcyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and agarose gel electrophoresis were performed according to standard methods [13].

RNAase assay by the gel method

RNAase was assayed in 10 ml of 10 mM Tris/HCl buffer (pH 7.4) containing magnesium chloride (0.5 mM), EDTA (1 mM), sodium chloride (150 mM), *E. coli* RNA (1 μg) [prepared as described earlier, 14] or a synthetic substrate such as poly(U), poly(C), poly(A) or poly(G) or poly (A-U) (10 μg) and minimal amounts of enzyme. Incubations were carried out at the temperature specified for 30 min. The reaction was stopped by the addition of an equal volume of sample buffer

Table 1
Purification of RNAase-HL from psychrotopic *P. fluorescens* (10 CW)

Purification step	Protein loaded (mg)	Active protein obtained (mg)	Total activity (units)	Specific activity * (units/mg)	Recovery (%)	Purification fold
Bio-gel P60	350.0	150.0	510.0	3.4	100.0	1.0
DNA-Cellulose	150.0	6.0	360.0	60.0	70.5	17.6
FPLC (peak 8)	6.0	1.5	124.05	82.7	24.3	24.3
FPLC (Peak 1) **		3.3	10.56	3.2		
HPLC	1.5	0.6	75.0	125.0	14.7	36.8

* One unit of activity is equivalent to an increase of 1 unit of absorbance at 260 nm due to the hydrolysis of the substrate (for details see Materials and Methods); ** Peak 1 of FPLC was not used for further purification.

(pH 7.4) containing Tris (10 mM), EDTA (1 mM), glycerol (10%) and bromophenol blue (0.1%) and directly electrophoresed in 1% agarose gels. The enzyme was considered to be active if the *E. coli* total RNA consisting of 23S, 16S and 5S RNA or the band indicative of polyribonucleotides which were used as the substrates were hydrolysed partially or completely so as to yield a smear on the gel or a prominent band in the 4–5 S region, respectively. The results were also quantitated by densitometric scans of the gels but in such cases poly(U) was used as the substrate. Poly(U) in the absence of RNAase resolved into a single broad band. The intensity of this band was considered equivalent to 100% unhydrolysed RNA. Hydrolysis reduces the intensity of the poly(U) band and the decrease in intensity was taken to be proportional to the % RNA hydrolysed.

RNAase assay by the spectrophotometric method

The nucleolytic activity of RNAase-HL was also measured by the increase in absorbance at 260 nm by the method of Kalnitsky et al. [15] using poly(U) as the substrate. An increase in absorbance by 1 unit at 260 nm was taken to be equivalent to 1 unit of enzyme activity. The concentration of proteins was determined by the method of Lowry et al. [16] using bovine serum albumin as the standard.

Results and Discussion

Very few enzymes have been purified from psychrotrophic bacteria and established to be cold-adapted. The enzymes reported so far are a lactate dehydrogenase from *Vibrio marinus* [6], an alkaline phosphatase from an unidentified Antarctic bacterium [5], a lipase from a psychrotrophic *Moraxella* [4] and an α-amylase from *Alteromonas haloplanctis* A23 from Antarctica [3]. In the present study cell-free extracts of 13 isolates of psychrotrophic bacteria exhibited ribonuclease activity. But, when the extracts were heated to 65°C for 30 min and then immediately assayed for activity the activity persisted in the majority of the bacteria except in three isolates of *P. fluorescens* (10CW, 39W and 51W) and one isolate of *P. syringae* (Lz4W) indicating that in these four isolates the RNAase was heat labile. The absence of an heat labile RNAase in some of the isolates of *P. fluorecens, P. putida, A. protophormiae* and *M. roseus* is intriguing and difficult to explain based on the present data.

Attempts were made to purify the heat labile RNAase from 10CW an isolate of *P. fluorescens* using cell free extracts of the bacteria. It was not possible to use the osmotic shock method of Heppel [17] since no RNAase activity was detected in the supernatant. This indicated that it is a cytosolic enzyme and not a periplasmic enzyme, because if it was present in the periplasm then osmotic shock should have released the enzyme.

Purification of RNAase-HL

The cell-free extract of *P. fluorescens* (Table 1) following chromatography on Bio-Gel P60 resolved into two peaks (I and II respectively). The RNAase activity was confined to peak I (150 mg of protein) which following affinity chromatography on a DNA-cellulose column resolved into one major peak (I) which exhibited RNAase activity and a minor peak (II) which was devoid of activity. The active fractions eluted at a concentration of 0.5 M NaCl. FPLC of this fraction on a Mono S column further fractionated the proteins into two major and seven minor peaks with maximum activity in peak 8 which eluted at a concentration of 0.5 M NaCl with a retention time of 23 min. HPLC of the active peak 8 on a μBondapak C-18 reverse phase column resolved the proteins into two peaks I and II (Fig. 1). Peak I with a retention time of 31.68 min exhibited RNAase activity and following SDS-PAGE and staining with coomassie blue it resolved into a single band (Fig. 1).

In the present investigation cold-active RNAase-HL from *P. fluorescens* was purified 37-fold and the recovery of RNAase HL was about 15%. This low recovery is due to the fact that following FPLC though the activity was associated with the unadsorbed peak (peak 1) and three other eluted peaks (6, 7 and 8), only peak 8 which exhibited maximum activity was processed for further purification by HPLC thus resulting in a drop in recovery.

Properties of RNAase-HL

The molecular mass of RNAase-HL as determined by SDS-PAGE both in the presence and absence of β-mercaptoethanol was 16 kDa (Fig. 2). Under the assay conditions described in Materials and Methods the activity of RNAase-HL was linear up to 30 min. It hydrolysed both *E. coli* RNA and poly rU over a pH range of 4 to 9 with maximum activity (95% hydrolysis) at pH 7.4. The enzyme was active over a broad range of temperature (5°C to 60°C) and exhibited about 45% activity at 5°C, maximum activity at 40°C (100%), reduced activity at 50°C (20%) and total absence of activity at 65°C. The enzyme was capable of hydrolysing poly(A) (90%), poly(C) (100%), poly(U) (95%), poly(A-U) (50%) and *E. coli* total RNA (95%) but did not hydrolyse poly(G) and DNA from *E. coli* and calf thymus. Thus it appears that RNAase-HL is neither pyrimidine nor purine specific with respect to its substrate specificity. It also did not hydrolyse DNA indicating that it is not a phosphodiesterase. Sulphydryl group blockers such as iodoacetate, PCMB and Hg^{2+} inhibited the activity of the enzyme. Thus, this enzyme differed from pancreatic RNAase A with respect to its substrate specificity since unlike RNAase A which is pyrimidine specific RNAase-HL does not exhibit any substrate specificity. However, RNAase-HL and bovine pancreatic RNAase A did not show significant differences with respect to temperature and pH optima and their sensitivity to various metal ions (such as Ag^{+}, K^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+} and inhibitors such as spermidine, urea, diethyl pyrocarbonate, vanadyl ribonucleoside complex and polyvinyl sulphate.

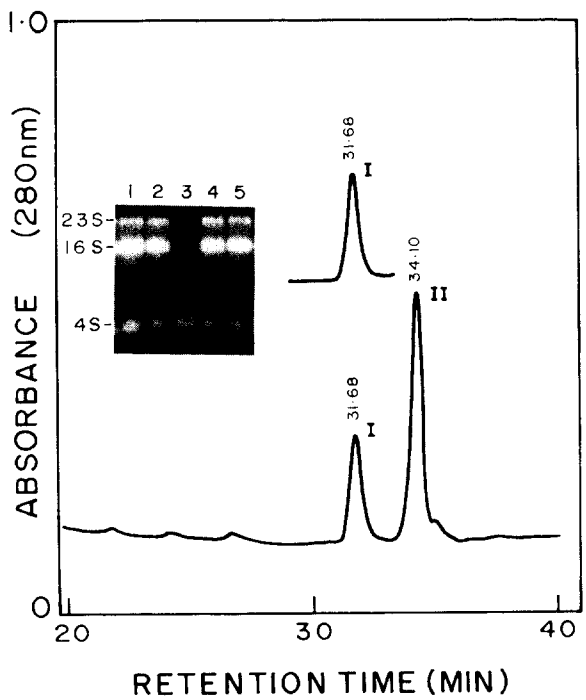

Fig. 1. Purification of RNAase-HL by HPLC using peak 8 of FPLC which exhibited maximum activity. Peak 8 was fractionated by HPLC using a μBondapak C-18 reverse phase column. The absorbance of the proteins was monitored at 280 nm. RNAase activity was associated only with peak 1 with a retention time of 31.68 min. Inset shows *E. coli* total RNA (lane 1), activity of peak I after heat inactivation at 65°C for 30 minuts (lane 2), before heat inactivation (lane 3) and peak II exhibiting no RNAase activity (lanes 4 and 5) before and after heat inactivation.

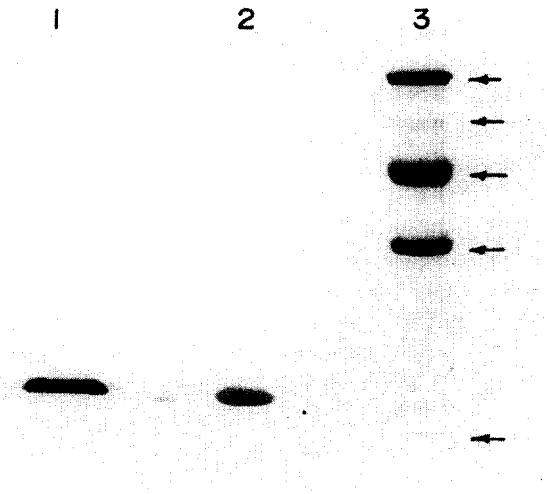

Fig. 2. SDS-PAGE pattern of RNAase-HL from *P. fluorescens* (10CW) following electrophoresis on a 10% gel in the presence (lane 1) and absence (lane 2) of β-mercaptoethanol. The protein loaded was the HPLC peak I with a retention time of 31.68 minutes. The mol. wt. markers (lane 3; shown by arrows) were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and α-lactalbumin (14.4 kDa). (The other two visible bands one below bovine serum albumin and the other above α-lactalbumin are degradation products of bovine serum albumin and were observed when BSA was run separately).

Stability and heat inactivation of RNAase-HL

The RNAase activity in the cell-free extracts of mesophilic *P. fluorescens* extract was not inhibited following heat treatment for 30 minutes at 65°C but was only inhibited following heat treatment at 95°C. In contrast the cell-free extract from the psychrotrophic *P. fluorescens* was totally devoid of RNAase activity following treatment either at 65°C or 95°C for 30 min. Further, it was also observed that heating of the cell-free extracts at 95°C for 30 min and subsequent cooling to room temperature gradually also did not restore the activity of the mesophilic and psychrotrophic *P. fluorescens*. Bovine pancreatic RNAase-A was also irreversibly inactivated under similar conditions. Pure RNAase-HL from *P. fluorescens* (10CW) was also inactivated totally when heated at 65°C for 30 min irrespective of whether the enzyme was assayed for activity immediately following heat treatment or cooled to room temperature after heating prior to assay for the activity. Thus RNAase-HL is unique in that it is heat-labile at 65°C unlike the RNAase from mesophilic *P. fluorescens* and bovine pancreatic RNAase A. The pure RNAase-HL retained its activity even after a couple of months at −20°C, after 12 h at 4°C and even after 4 h at 25°C.

Earlier studies had indicated the presence of heat-labile RNAases both in prokaryotes [18,19] and eukaryotes [20,21]. In *E. coli* the enzyme RNAase II is an extremely unstable enzyme and is totally inactivated after 20 min at 45°C (19). In *Bacillus subtilis* RNAase was completely inactivated by heating at 60°C for 10 min [18]. The present report is to our knowledge the first report on a heat-labile RNAase from a psychrotrophic bacterium.

Earlier studies on heat-labile RNAases did not indicate whether heat inactivation of the respective enzymes was a reversible or an irreversible effect. Irreversible thermal-inactivation of bovine pancreatic RNAase A at 90°C and between pH 4 to 8 has been shown to be due to hydrolysis of peptide bonds at aspartic acid residues, deamidation of aspargine and glutamine residues and elimination of cystine residues [22]. But, total inactivation at lower temperatures (at 65°C) as observed for RNAase-HL may not be due to these changes. In fact Ramnath and Vithyathil [23] observed that RNAase A is not totally inactivated at 70°C. Hence, the irreversible inactivation of RNAase-HL at 65°C, as compared to 90°C required for pancreatic RNAase, is likely to be due to a different mechanism and would be worth investigating.

Acknowledgements

Our thanks to Dr. M.K. Ray for his useful suggestions.

References

1 Herbert, R.A. (1992) A perspective on the biotechnological potential of extremophiles. *TIBTECH*. 10, 395–402.
2 Jaenicke, R. and Zavodszky, P. (1990) Proteins under extreme physical conditions. *FEBS Lett*. 268, 344–349.
3 Feller, G., Lonhienne, T, Deroanne, C., Libioulle, C., Van Beeurmen, J. and Gerday, C. (1992) Purification, characterization and nucleotide sequence of the thermolabile α-amylase from the Antarctic psychrotroph *Alteromonas halophanctis* A23. *J. Biol. Chem*. 267, 5217–5221.
4 Feller, G., Thiry, M., Arpigny, J.L., Mergeay, M. and Gerday, C. (1990) Lipases from psychrotrophic antarctic bacteria. *FEMS Microbiol. Lett*. 66, 239–244.
5 Kobori, H., Sullivan, C.W. and Shizuya, H. (1984). Heat labile alkaline phosphatase from Antarctic bacteria. Rapid 5' end-labelling of nucleic acids. *Proc. Natl. Acad. Sci. (USA)* 81, 6691–6695.
6 Mitchell, P., Yew, H.C. and Mathemeier, P.F. (1985) Properties of lactate dehydrogenase in a psychrophilic marine bacterium. *Appl. Environ. Microbiol*. 49, 1332–1334.
7 Ray, M.K., Uma Devi, K., Seshu Kumar G. and Shivaji, S. (1992) Extracellular protease from the Antarctic yeast *Candida humicola*. *Appl. Environ. Microbiol*. 58, 1918–1923.
8 Shivaji, S., Chattopadhyay, M.K. and Ray, M.K. (1994). Bacteria and yeasts of Schirmacher Oasis, Antarctica: Taxonomy, biochemistry and molecular biology. *Proc. NIPR Symp. Polar Biol*. 7, 174–185.
9 Shivaji S., Rao, N.S., Saisree, L., Sheth, V., Reddy, G.S.N. and Bhargava, P.M. (1988) Isolation and identification of *Micrococcus roseus* and *Planococcus* sp. from Schirmacher Oasis, Antarctica. *Appl. Environ. Microbiol*. 55, 767–770.
10 Shivaji, S., Rao, N.S., Saisree, L., Reddy, G.S.N., Seshu Kumar, G. and Bhargava, P.M. (1989b) Isolation of *Arthrobacter* from the soils of Schirmacher Oasis, Antarctica. *Polar Biol*. 10, 225–229.
11 Shivaji, S., Rao, N.S., Saisree, L., Sheth, V., Reddy, G.S.N. and Bhargava, P.M. (1988) Isolation and identification of *Micrococcus roseus* and *Planococcus* sp. from Schirmacher Oasis, Antarctica, *J. Biosci*. 13, 409–414.

12 Litmann, R.M. (1968) A deoxyribonucleic acid polymerase from *Micrococcus luteus (Micrococcus lysodeikticus)* isolated on deoxyribonucleic acid-cellulose. *J. Biol. Chem.* 243, 6222–6233.
13 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
14 Shivaji, S. and Bhargava, P.M. (1984) Ribonucleolytic activity of bovine spermatozoa plasma and acrosomal membranes. *Biosci. Rep.* 4, 71–81.
15 Kalnitsky, G., Hemmel, J.P. and Dierks, C. (1959) Some factors which affect the enzymatic digestion of ribonucleic acid. *J. Biol. Chem.* 234, 1512–1516.
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
17 Heppel, L.A. (1967) Selective release of enzymes from bacteria. *Science*, 156, 1451–1455.
18 Nakai, M., Minani, Z., Yamazaki, T., and Tsugita, A. (1965) Studies on the nucleases of a strain of *Bacillus subtilis.* 57, 95–99.
19 Spahr, P.F. (1964) Purification and properties of ribonuclease II from *Escherichia coli. J. Biol. Chem.* 239, 3716–3726.
20 Asima, T., Uchida, T. and Egami, F. (1968) Studies on extracellular ribonuclease of *Ustilago sphaerogena. Biochem. J.* 106, 601–607.
21 Felling, J. and Wiley, C.E. (1960) Ribonuclease of *Euglena gracilis. Science* 132, 1835–1836.
22 Zale, S.E. and Klibanov, A.M. (1986) Why does ribonuclease irreversibly inactivatge at high temperatures? *Biochemistry* 25, 5432–5444.
23 Ramnath, S. and Vithayathil, P.J. (1981) Irreversible thermal denaturation of bovine pancreatic ribonuclease A. *Int. J. Peptide Prot. Res.* 17, 107–117.