Microbiology, Vol. 70, No. 1, 2001, pp. 18–21. Translated from Mikrobiologiya, Vol. 70, No. 1, 2001, pp. 24–28.

EXPERIMENTAL ARTICLES

The Effect of Thermal Treatment on the Catalytic Activity and Biological Properties of *Bacillus intermedius* Ribonuclease

A. I. Kolpakov, O. N. Il'inskaya, O. B. Ivanchenko, and N. S. Karamova

Department of Microbiology, Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia

Received February 22, 2000; in final form, April 28, 2000

Abstract—*Bacillus intermedius* ribonuclease (binase), which is known to exert a growth-stimulating effect at low concentrations and a genotoxic effect at high concentrations, loses these abilities completely after exposure to 100°C for 10 min, but retains approximately 95% of its catalytic activity and structural integrity. Other types of modification, such as photoinactivation and site-specific mutagenesis, gave rise to enzyme forms with unaltered structure but reduced (sometimes to trace amounts) catalytic activity. Genotoxicity was always proportional to the catalytic activity of the native enzyme, while a notable growth-stimulating effect could be exerted by enzymes with low activity. The loss of biological activity of thermoinactivated binase was related to the increase in the number of negatively charged groups on the enzyme surface, which led to a substantial decline in the adhesive properties of the enzyme.

Key words: *Bacillus intermedius* ribonuclease, thermoinactivation, catalytic activity, growth stimulation, genotoxicity, adsorption.

The response of cells to an exogenous effector varies with its concentration in the medium and can be recorded by evaluating the induced biological effects. Earlier, we showed that not only catalytically active RNases (pancreatic RNase, *Bacillus intermedius* RNase (binase) and *Bacillus amyloliquefaciens* RNase (barnase)), but also catalytically inactive (photoinactivated) binase are able to stimulate the growth of the yeast *Candida tropicalis* [1]. Similar experiments with ribonucleases modified by site-specific mutagenesis showed that RNases with reduced catalytic activity can also stimulate the growth of *Escherichia coli* and *Bacillus subtilis.* In this case, the growth-stimulating effect depended on the catalytic activity of the enzymes, although a certain growth-stimulating effect was also noted with a virtually inactive mutant binase in which histidine was replaced by the glutamine group in the active center [2, 3]. The ability of mutant RNases to induce genotoxic effects in microbial cells was also proportional to their catalytic activity [4]. The estimation of the contributions of the catalytic activity and the structure of RNases to their biological effects showed that the growth-stimulating effect strongly depends on the enzyme structure, which determines the nonspecific interaction of RNase with the cell surface [5]. Genotoxic effects depend primarily on the catalytic activity of the enzymes. The study of the growth-stimulating activity of pancreatic DNase showed that it completely loses both catalytic and growth-stimulating activities after exposure to 100°C for 10 min [6]. At the same time, photoinactivated RNase retained 0.1% of its activity [5], and mutant binase with an amino acid substituted in the active center retained 1.5–2.9% of its original activity [7]. In general, binase is a highly thermostable enzyme capable of refolding [8], but it remains unknown to what extent thermal treatment affects its structural properties, catalytic activity, and ability to induce biological (growth-stimulating and genotoxic) effects. The goal of the present work was to study the growth-stimulating effect of low concentrations of thermoinactivated binase and the mutagenic effect of its high concentrations.

MATERIALS AND METHODS

Experiments were carried out with the extracellular alkaline RNase of *B. intermedius* (binase) (EC 3.1.4.23) purified to apparent homogeneity. The molecular weight of the RNase was 12300 Da, and the specific activity was 1 million units/mg protein. Its cata-

Table 1. The effect of heat treatment (100°C, 10 min) on the activity of 0.1% RNase solution in distilled water (pH 5.6)

RNase activity	Time span between RNase cooling and its assay			
	RNase activity before heating	30 min	60 min	90 min
Units	856000	790000	823000	820000
%	100	92.28	96.14	95.79

0026-2617/01/7001-0018$25.00

Table 2. Effect of native and boiled RNase on the *S. cerevisiae* culture at a growth-stimulating concentration of 0.001 μg/ml

RNase added	Culture density, million cells/ml (% increase)				
	0	1 h	3 h	5 h	7 h
Control (without RNase)	12.0	14.0	30.0	50.0	55.8
Native RNase	12.0	21.5(22.8%)	41.5(38.3%)	68.8(37.6%)	70.4(26.2%)
Boiled RNase (100°C, 10 min)	12.0	18.0(2.8%)	32.5(8.3%)	54.4(8.8%)	59.2(6.9%)

lytic, physical, and chemical characteristics were described in greater detail by Balaban *et al.* [9]. The effect of the exogenously added bacterial RNase on the growth of the yeast *Saccharomyces cerevisiae* race 823, which was obtained from the Collection of Microorganisms of the Research Institute of Food Biotechnology, was studied, taking into account the growth phase of the yeast culture at the instance of enzyme addition. In the control experiments, equivalent amounts of distilled water were added to the culture in place of the enzyme. The RNase genotoxicity was determined by the degree of reversion of the histidine auxotrophs *Salmonella typhimurium* TA1538, TA98, TA1535, and TA100, to prototrophy [10]. The induction of the SOS response of *E. coli* PQ37 cells was estimated by measuring the activity of β-galactosidase, the operon of which is controlled by the *sfiA* gene of the SOS regulon in these cells [11]. The catalytic activity of RNase was estimated by measuring the amount of acid-soluble products of RNA hydrolysis [12]. All experiments were replicated at least five times. The statistical significance of the differences between the control and the experimental values was estimated for $P < 0.05$ using Microsoft Excel.

RESULTS AND DISCUSSION

The experiments showed that the exposure of RNase to 100°C for 10 min affected its catalytic activity only slightly (Table 1). This finding is in agreement with the data of Protasevich *et al.*, who showed that RNase without disulfide bonds in the molecule is capable of renaturation, and retains its structural integrity over a wide range of pH values, from 2.5 to 9.0 [13]. At temperatures above 60°C, the randomness of RNase molecules began to increase, while the percentage of β-sheets decreases, suggesting the irreversible denaturation of RNase. It is known that the half-life of RNase in 1% solution at 80°C is approximately 1 h [14]. That is, after a 1-h incubation period under the conditions described, the content of the completely denatured molecules of RNase in 1% solution will comprise 50% of the protein. Taking into account that our experiments were carried out with a one order of magnitude higher dilution of RNase solution, in which the denaturation rate must be much lower, and the time of thermal treatment at 100°C was as low as 10 min, we roughly estimated the content of denatured RNase molecules to be 8% after such treatment. Interestingly, this estimate is close to the percent loss of catalytic activity of the heat-treated enzyme (Table 1). Therefore, we can assume that the structure and catalytic activity of the heat-treated RNase in our experiments were altered by no more than 8%.

As shown earlier [1, 15], the growth-stimulating effect of low RNase doses is most pronounced with respect to exponential-phase cells. With this knowledge, the effects of native and heat-treated forms of *B. intermedius* RNase were studied by adding the enzyme preparations at a growth-stimulating concentration (0.001 μg/ml) to the mid-exponential yeast culture (4 h after inoculation). As shown in Table 2, native RNase stimulated a 22.8–38.3% increase in growth of the yeast culture within the study period, as compared to the control. The effect of heat-modified RNase was much lower (2.8–8.8%).

Similar data were obtained in experiments on the genotoxic (mutagenic) effect of high concentrations of RNase. As shown in Table 3, the enzyme at a concentration of 1 mg/ml exerted a mutagenic effect that exceeded the spontaneous mutation level by 2.4 times, whereas the genotoxic effect of heat-treated RNase on *S. typhimurium* TA1535, TA1538, and TA98 was statistically insignificant.

The SOS response-inducing effect of the native *B. intermedius* RNase, which was estimated by the SOS chromotest, was 3.2-fold, again indicating the genotoxicity of binase. As for the heat-treated enzyme, its ability to induce the SOS-response of bacterial cells was fully lost.

Table 3. The effect of boiling on the genotoxicity of *B. intermedius* RNase

Test	Native RNase, 1 mg/ml	Boiled RNase (1 mg/ml; 100°C, 10 min)
Ames test (degree of induction of mutagenesis)		
S. tiphimurium TA1535	2.7	1.15
S. tiphimurium TA1538	2.4	1.3
S. tiphimurium TA98	2.4	0.8
S. tiphimurium TA100	1.9	1.4
SOS chromotest (degree of induction of SOS response)	3.2	0.99

Table 4. The effect of boiling on the adsorption of RNase by yeast cells

Incubation time, min	RNase activity, units/ml			Degree of adsorption, %
	before incubation with cells	after incubation with cells	RNase adsorption*	
		Native RNase		
0	920000 ± 15000	914300 ± 14500		
15	917000 ± 13100	779450 ± 13400	137550	15.0
30	918500 ± 14300	712756 ± 11890	205744	22.4
60	918000 ± 15500	705024 ± 12300	212976	23.2
		Boiled RNase		
0	890000 ± 11000	885000 ± 10900		
15	888000 ± 12200	855070 ± 11200	32930	3.7
30	885600 ± 12080	841320 ± 11900	44280	5.0
60	886900 ± 12100	846990 ± 12340	39910	4.5

* RNase adsorption is expressed as the difference between the activities of RNase solution before and after incubation with yeast cells. The degree of adsorption is expressed as the percent decrease in RNase activity (RNase activity before incubation with cells was taken to be 100%).

The results suggest that the inability of heat-treated RNase to induce the respective biological effects (growth stimulation and genotoxic effect) is not directly related to the loss of its catalytic activity or structural integrity.

Indeed, heat treatment decreased the last two parameters by about 8%, and completely destroyed the biological activity of RNase. It is known that protein denaturation is often accompanied by a change in pH of the heat-treated protein solution due to the exposure of ionizable groups on the exterior side of the protein globule [16]. In our experiments, the heat treatment of 1 mg/ml RNase solution increased its pH from 5.44 to 5.88, which is indicative of an increase in the number of negatively charged groups on the surface of the enzyme globule. As a result, heat-treated RNase probably loses the ability to attach to the cell membrane (and hence to exert the respective biological effects) because of the repulsion of negatively charged groups present on its surface and on the surface of the microbial cell.

To prove this hypothesis, we examined the ability of *S. cerevisiae* cells to take up RNase from the medium by measuring the RNase activity of enzyme solution before and after incubation with the cells (Table 4). After a 30-min incubation period of yeast cells in the solution of native binase, RNase activity decreased by 22.4%, whereas the activity of boiled binase in solution decreased by only 5%. These data suggest that yeast cells virtually cannot adsorb boiled RNase.

In conclusion, the data obtained in this study show that the biological activity of RNase is strongly related to its ability to attach to the cell surface, which is determined by the electrostatic interaction of ionized groups on the surface of the enzyme and the cells. Heat-treated RNase, which retains its catalytic activity, is unable to attach to cells and completely loses its biological activity.

At the same time, the biological activity of mutant RNases is proportional to their catalytic activity [4], most likely because single amino acid substitutions in the active center of such RNases do not substantially affect their ability to attach to cells.

ACKNOWLEDGMENTS

This work was supported by project no. 97-0-10.0-190 from the Coordination Center of Basic Research at St. Petersburg University and by grant 015.11.01.13 in the scope of the Russian Universities Basic Research program.

REFERENCES

1. Kolpakov, A.I. and Kupriyanova, F.G., Effect of Exogenously Added RNases on the Reproduction of *Candida tropicalis*, *Mikrobiologiya*, 1992, vol. 61, no. 6, pp. 969–974.
2. Kipenskaya, L.V., Kupriyanova-Ashina, F.G., Il'inskaya, O.N., Kolpakov, A.I., and Leshchinskaya, I.B., Effect of *Bacillus intermedius* RNase Preparations with Different Catalytic Activities on the Reproduction of *Escherichia coli* and *Bacillus subtilis*, *Mikrobiologiya*, 1998, vol. 67, no. 2, pp. 165–168.
3. Leshchinskaya, I.B., Kupriyanova, F.G., Yakovlev, G.I., Kipenskaya, L.V., and Ilinskaya, O.N., Molecular Mechanisms of Growth Stimulating Effects of *Bacillus intermedius* RNase, *Karadeniz J. Med. Sci.*, 1995, vol. 8, no. 4, pp. 218–219.
4. Ilinskaya, O.N., Ivanchenko, O.N., Karamova, N.S., and Kipenskaya, L.V., SOS-inducing Ability of Native and Mutant Microbial Ribonucleases, *Mutat. Res.*, 1996, vol. 354, pp. 203–209.
5. Kurinenko, B.M., Golubenko, I.A., Bulgakova, R.Sh., Davydov, R.E., Nekhoroshkova, Z.M., Valiullina, S.F., and Shlyapnikov, S.V., Photooxidation of *Bacillus inter-*

medius RNase and Formation of Photoinactivated Product, *Bioorg. Khimiya*, 1986, vol. 12, no. 4, pp. 457–466.

6. Kupriyanova-Ashina, F.G., Dmitriev, V.V., Egorov, S.Yu., and Kolpakov, A.I., Penetration of Pancreatic DNase into *Candida tropicalis* Cells, *Mikrobiologiya*, 1992, vol. 61, no. 6, pp. 975–980.
7. Yakovlev, G.I., Moiseyev, G.P., Struminskaya, N.K., Borzykh, O.A., Kipenskaya, L.V., Znamenskaya, L.V., Leshchinskaya, I.B., Chernokalskaya, E.V., and Hartley, R.W., Mutation Analysis of Active Site of RNase *Bacillus intermedius* (Binase), *FEBS Lett.*, 1995, vol. 354, pp. 305–306.
8. Randzhbar, B., Protasevich, I.I., Shul'ga, A.A., Kurbanov, F.T., Lobachev, V.M., Kirpichnikov, M.P., and Makarov, A.A., Barnase, Binase, and Their Hybrids: Differences in Conformation and Heat Denaturation Parameters, *Mol. Biol.* (Moscow), 1997, vol. 31, no. 3, pp. 492–499.
9. Balaban, N.P., Sharipova, F.R., Golubenko, I.A., *et al.*, Physico chemical Characteristics of Ribonuclease *Bacillus intermedius*, Proc. 1st Int. Meeting "Structure and Chemistry of Ribonucleases," Moscow, 1989, pp. 349–352.
10. Maron, M. and Ames, B.N., Revised Methods for the *Salmonella* Mutagenicity Test, *Mutat. Res.*, 1983, vol. 113, pp. 173–215.
11. Qillardet, P. and Hofnung, M., The SOS Chromotest: A Review, *Mutat. Res.*, 1993, vol. 297, pp. 235–279.
12. Kolpakov, A.I. and Il'inskaya, O.N., Optimized Assay of Ribonucleases with High-Polymeric RNA, *Klin. Lab. Diagn.*, 1999, no. 5, pp. 14–16.
13. Protasevich, I.I., Platonov, A.L., Pavlovsky, A.G., and Esipova, N.G., Distribution of Charges in *Bacillus intermedius* 7P Ribonuclease Determines the Number of Cooperatively Melting Regions of the Globule, *J. Biomol. Struct. Dyn.*, 1987, vol. 4, no. 5, pp. 885–893.
14. Faizullin, D.A. and Stupishina, E.A., Effect of Conformational Factors on the Reversibility of the Heat Denaturation of Binase, in *Fermenty mikroorganizmov* (Microbial Enzymes), Kazan, 1998, pp. 109–115.
15. Kupriyanova-Ashina, F.G., Lutskaya, A.Yu., Kolpakov, A.I., Kepecheva, R.M., and Leshchinskaya, I.B., Effect of *Bacillus intermedius* RNase on the Growth of *Saccharomyces cerevisiae*, *Prikl. Biokhim. Mikrobiol.*, 1996, no. 2, pp. 254–259.
16. Joly, M., *A Physico-Chemical Approach to the Denaturation of Proteins*, London: Academic, 1965. Translated under the title *Fizicheskaya khimiya denaturatsii belkov*, Moscow: Mir, 1968.