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EXPERIMENTAL ARTICLES

Some Secretion Characteristics of Bacterial Ribonuclease

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Abstract—The investigation of the effect of some components of the medium on the distribution of the secretory guanyl-specific ribonuclease of *Bacillus intermedius* (EC 3.1.4.23) among various cell fractions and culture liquid showed that the amount of this enzyme in the culture liquid does not depend on the concentration of calcium ions in the medium (within 1–5 mM). The study of the effect of the amino acid substitutions Trp34Asn and Trp70Asn in the ribonuclease molecule showed that the secretion of ribonuclease depends on the formation rate of its secondary structure. The amino acid substitution Trp34Asn completely inhibits ribonuclease secretion.

Key words: guanyl-specific ribonuclease, localization, secretion, immunoblotting, *Bacillus intermedius*, recombinant strains.

The gram-positive sporogenous bacterium *Bacillus intermedius* secretes various hydrolytic enzymes (proteinases, phosphatases, and RNases) into the medium. These extracellular enzymes are dominated by guanyl-specific ribonuclease, as is evident from the fact that 99% of this enzyme occurs in the medium, whereas the fraction of cell walls contains less than 1% of active RNase [1]. Other extracellular enzymes, subtilisin (EC 3.4.21.62) and glutamyl endopeptidase (EC 3.4.21.19), are found in an active form not only in the culture liquid but also in the membrane fraction (up to 10%) and not in the fraction of cell walls [2]. The alkaline phosphatase of *B. intermedius* (EC 3.1.3.1) localizes mainly in the membrane fraction (up to 70%), the rest being distributed between the cell walls and the culture liquid [3]. Our earlier studies showed that some bivalent metal ions play a key role in the release of proteinases and phosphatase from membranes into the medium [2, 3].

The molecular mass of guanyl-specific ribonuclease (12.3 kDa) is less than those of phosphatase (46 kDa), subtilisin (32.5 kDa), and glutamyl endopeptidase (29 kDa). The ribonuclease molecule has a high content of aromatic amino acids (which are involved in the formation of the secondary structure of the enzyme molecule) [4, 5] but lacks sulfur-containing amino acid residues [5, 6]. The folding of the enzyme molecule to the native conformation is governed by the formation rate of its secondary structure at physiological pH values and does not depend on the concentration of metal ions in the medium [5]. The in vitro experiments of Hartley [7] showed that the completely denatured molecule of *Bacillus amyloliquefaciens* barnase (a structural analogue of *B. intermedius* binase) can spontaneously fold to the native conformation within 5–10 s.

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This work was undertaken to study the effect of the substitution of the amino acid residues involved in the formation of the secondary structure of *B. intermedius* ribonuclease on the secretion of this enzyme by recombinant *Escherichia coli* strains.

MATERIALS AND METHODS

The strain *Bacillus intermedius* 3-19 (Str 500) used in this study is a streptomycin-resistant mutant of the wild-type strain *B. intermedius* 7P from the Culture Collection at Kazan State University. The *Escherichia coli* strains SURE and XLI Blue were used as recipients for plasmids bearing mutant RNase genes. Plasmid pML5 carrying the whole gene of *B. intermedius* ribonuclease was a generous gift from E.B. Chernokal'skaya (United States).

Basal medium for the cultivation of *B. intermedius* 3-19 contained (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.001; $MgSO_4 \cdot 7H_2O$, 0.015; NaCl, 0.2; and $MnSO_4$, 0.005 (pH 8.5). The medium was sterilized at 1 atm. Solutions of glucose, lactose, inorganic phosphate, and $CaCl_2$ were sterilized separately at 0.5 atm and added aseptically immediately before inoculation to final concentrations of 1, 1, and 0.01% and 1–5 mM, respectively. Streptomycin sulfate was also added to the medium before inoculation in an amount of 500 μg/ml.

The recombinant *E. coli* strains bearing genes of the original and mutant ribonucleases of *B. intermedius* were grown in the medium described earlier [8]. Ampicillin was added to the medium before inoculation in an amount of 70 μg/ml.

Bacterial growth was monitored by measuring culture turbidity at 590 nm using a KFK-2 photoelectrocolorimeter.

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RNase activity was assayed by measuring the amount of products of RNA hydrolysis soluble in 4% $HClO_4$. The products were assayed with 12% uranyl acetate [9]. One unit (U) of RNase activity was defined as the amount of enzyme that increased the extinction E_{260} of the reaction mixture by one optical density unit per hour. Specific RNase activity in the culture liquid and cell fractions was defined as the ratio of RNase activity to the biomass and expressed in U/g cells. The transformed strains were screened for RNase activity by using agar plates containing 2 mg/ml RNA and 0.001% toluidine blue [9].

B. intermedius protoplasts were prepared and lysed as described elsewhere [1]. To obtain membranes, the lysed protoplasts were supplemented with 1 mg/ml DNase (Serva, Germany), incubated at 30°C for 30 min, and centrifuged at 30000 *g* for 1 h. RNase activity was assayed in the culture liquid, the fraction of cell walls (which was prepared by removing protoplasts by centrifugation at 10000 *g* for 10 min), the membrane fraction, and the cytoplasm (which was obtained from the fraction of lysed protoplasts by the precipitation of nucleic acids with streptomycin sulfate).

To prepare the spheroplasts of *E. coli* cells, the cells were harvested by centrifugation; resuspended in 0.1 M Tris–HCl buffer (pH 8.0) containing 20% sucrose, 10 mM EDTA, and 0.5 mg/ml lysozyme (Serva); and incubated at room temperature for 30 min. The spheroplasts were collected by centrifugation at 10000 *g* for 40 min and lysed in hypotonic 5 mM Tris–HCl buffer (pH 8.0). To prepare the fraction of cytoplasm, the mixture of lysed spheroplasts was supplemented with streptomycin sulfate to a concentration of 7% and centrifuged at 15000 *g* for 60 min. To prepare the whole spheroplast lysate (the sum of membranes and cytoplasm), the spheroplasts were lysed in 10 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 5% β-mercaptoethanol. Experiments were carried out with the culture liquid, the fraction of soluble periplasmic proteins (which were prepared by removing the spheroplasts by centrifugation), the whole spheroplast lysate, and the fraction of soluble cytoplasmic proteins (which were prepared by removing DNA and cytoplasmic membranes from the fraction of lysed spheroplasts). Cell fractions were assayed for the concentration of chromosomal DNA by electrophoresis in 1% agarose gel, followed by gel staining with ethidium bromide. A comparison of DNA concentrations in the fractions of periplasm and cytoplasm showed that the lysis of spheroplasts during the preparation of the periplasmic fraction did not exceed $5 \pm 2\%$.

Trp34Asn and Trp70Asn mutations were induced in the RNase gene cloned on plasmid pML5 by PCR-based site-directed mutagenesis [10]. The PCR products containing *Hin*dIII and *Xba*I restriction sites were purified by electrophoresis in 12% PAAG [11] and cloned by using the Bluescript KS+ vector. The presence of the mutations in the gene was confirmed by DNA sequencing [12]. The plasmid was isolated and *E. coli* cells were transformed by routine methods [11].

PCR was performed by using *Taq* DNA polymerase and other reagents purchased from USB (United States). The electrophoresis of the PCR fragments of DNA was carried out with reagents purchased from Bio-Rad (United States).

Rabbit antiserum to RNase was prepared and purified on a column with DEAE-cellulose as described by Leshchinskaya *et al.* [9]. The immunoblotting of RNase was carried out by the Burnette method [13] using rabbit antibodies to RNase and PhotoBlot reagents (Promega, United States).

The results were statistically processed using Student's *t*-test statistics for the significance level $P \le 0.05$. Calculations were carried out with the aid of the Microsoft Excel program and were considered to be confident when the standard deviation σ was equal to or less than 15%.

RESULTS AND DISCUSSION

According to earlier observations [1, 8], the concentration of RNase in the culture liquid of *B. intermedius* is maximal in medium lacking inorganic phosphate and peaks in the growth retardation phase.

The assay of ribonuclease in the culture liquid and various cell fractions (cell walls, membranes, and cytoplasm) of *B. intermedius* grown under different conditions (glucose or lactate as carbon sources, the presence of Ca^{2+} ions in the medium at concentrations of 1–5 mM, the presence or absence of inorganic phosphate (P_i) at a concentration of 0.1%) showed that, in all these cases, 99% of the enzyme localized in the culture liquid, whereas its content in the cell fractions was less than 1% (table). The cellular enzyme localized mainly in the fraction of cell walls and in the fraction that remained after the treatment of bacterial cells with lysozyme in the presence of an osmotic stabilizer and the removal of protoplasts. Under all the cultivation conditions studied, active RNase was not detected in the membrane fraction and was almost absent in the cytoplasm (300 U/g cells as compared to 50×10^6 U/g cells in the culture liquid).

Similar data were obtained by Merchante *et al.* [14] for the alkaline ribonuclease of the bacterium *Bacillus subtilis*, which secreted RNase into the medium in response to phosphate starvation. Most of the *B. subtilis* RNase (more than 75%) was detected in the culture liquid and about 10% in the fraction of cell walls, whereas the membranes and cytoplasm did not contain RNase activity at all.

The addition of P_i to the cultivation medium of *B. intermedius* diminished the activity of RNase in the culture liquid and the cell walls by 90 and 46%, respectively. These data suggest that P_i augments the relative amount of RNase occurring in the cell walls and, consequently, affects the process of enzyme secretion.

The distribution of RNase activity between the culture liquid and the cell fractions of *B. intermedius*

Essential component of the medium	Culture liquid	Cell walls	Membranes	Cytoplasm
Control (basal medium)	13.0×10^6	2.0×10^3	0	230
Glucose, 1%	49.8×10^6	7.9×10^3	0	580
Lactate, 1%	45.0×10^6	7.3×10^3	0	400
$CaCl_2$, 1 mM	13.0×10^6	1.9×10^3	0	210
$CaCl_2$, 2 mM	13.5×10^6	1.8×10^3	0	300
$CaCl_2$, 5 mM	12.8×10^6	2.0×10^3	0	350
Na_2HPO_4, 0.01%	0.8×10^6	4.0×10^3	0	200

Note: RNase activity is expressed in arbitrary units (see Materials and Methods). The standard deviation did not exceed 10%.

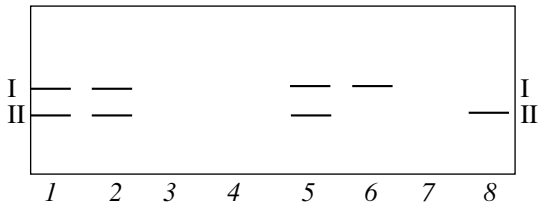
Varying the concentration of Ca^{2+} ions in the medium did not influence the distribution of *B. intermedius* RNase among the culture liquid and the cell fractions (table), indicating that Ca^{2+} ions do not seem to affect the secretion of this enzyme.

Experiments with the recombinant *E. coli* SURE and XLI Blue strains were conducted to study the effect of tryptophan residues (which are known to be involved in the formation of the secondary structure of the RNase molecule [5, 6, 15]) on the secretion of RNase. The processes of protein secretion by gram-positive and gram-negative bacteria differ only at the stage of protein transfer through the outer layer of the cytoplasmic membrane, whereas the mechanisms of protein translocation through the cytoplasmic membrane of these two types of bacteria are universal [16].

The screening of agar plates with toluidine blue incubated for 48 h showed the presence of transparent zones around the colonies of *E. coli* cells bearing plasmid with the original RNase gene but not around the colonies of *E. coli* cells bearing the mutant RNase genes with the Trp34Asn and Trp70Asn substitutions. The results of the screening experiments were confirmed by assaying RNase activity in the culture liquids and in the cell fractions of *E. coli* strains carrying the original and mutant (Trp34Asn and Trp70Asn) RNase genes. Namely, the culture liquid and the periplasmic fraction of the strain with the wild-type RNase gene exhibited RNase activity equal to, respectively, 6200 ± 200 and 4800 ± 200 U/ml, whereas the culture liquid and the cell fractions of the mutant strains had no RNase activity. These data suggest that the altered structure of mutant proteins impairs their secretion or folding after their release from the cytoplasmic membrane.

Immunoblot analysis showed that the original extracellular RNase of *E. coli* exists as two isoforms, I and II (figure, lanes *1* and *2*), whereas the extracellular RNase of *E. coli* has only one isoform (figure, lane *8*). These data show that the RNases of the recombinant *E. coli* strains are processed with the involvement of proteolytic enzymes other than in the case of bacilli. The whole lysates of the spheroplasts of the recombinant *E. coli* strain bearing the original RNase gene also showed the presence of two RNase isoforms (figure, lane *5*).

The culture liquids of the recombinant *E. coli* strains bearing the RNase genes with the Trp34Asn and Trp70Asn substitutions did not contain RNase (figure, lanes *3* and *4*). At the same time, the whole lysate of the spheroplasts of the recombinant *E. coli* strain bearing the RNase gene with the Trp34Asn substitution showed the presence of RNase (figure, lane *5*). These data suggest that bacterial strains with the Trp34Asn-RNase gene produce an intracellular enzyme precursor alone. This precursor was not detected in the periplasmic and cytoplasmic fractions, indicating that the secretion of the modified enzyme is blocked and it obviously remains bound to the cytoplasmic membrane. The efficiency of RNase secretion is likely to be dependent on the formation rate of the secondary structure of the



The immunoblot analysis of the culture liquid and the cell fractions of the recombinant *E. coli* strains with antibodies to the *B. intermedius* RNase. Lanes: (*1, 2*), the culture liquid of the *E. coli* strain bearing plasmid with the original RNase gene; (*3, 4*) the culture liquid of the *E. coli* strains bearing plasmids with the mutant Trp34Asn and Trp70Asn-RNase genes, respectively; (*5*) the whole lysate of the spheroplasts of the *E. coli* strain bearing plasmid with the original RNase gene; (*6*) the whole lysate of the spheroplasts of the *E. coli* strain bearing plasmid with the mutant Trp34Asn-RNase gene; (*7*) the whole lysate of the spheroplasts of the *E. coli* strain bearing plasmid with the mutant Trp70Asn-RNase gene; (*8*) extracellular RNase purified from the culture liquid of *B. intermedius* to an apparent homogeneity. I and II mark, respectively, the precursor and the mature form of the secretory RNase of *B. intermedius.*

enzyme molecule. The substitution of the amino acid residue Trp-34, which is involved in the formation of the *N*-terminal α-helix of the enzyme [5], blocks its secretion and makes its translocation through the cytoplasmic membrane impossible.

Enzyme assay showed that neither the culture liquid nor the lysate of the spheroplasts of the recombinant *E. coli* strain bearing the Trp70Asn-RNase gene contains this mutant enzyme (figure, lane *7*). This finding was confirmed by immunoblot analysis, which showed the absence of the mutant protein with the Trp70Asn substitution in the whole spheroplast lysate (figure, lane *7*), periplasm, and cytoplasm of the recombinant *E. coli* strain. These data indicated that the modified enzyme precursor is not translocated through the cytoplasmic membrane and is presumably subjected to intracellular proteolysis. It can be suggested that the Trp70 residue plays a key part in the formation of the hydrophobic core of the RNase molecule [4–6, 15].

The secondary structure of protein molecules is known to be responsible for the efficiency of their incorporation into membranes [17]. Due to the low molecular mass (12 kDa) and the high content of aromatic amino acids (including three tryptophan residues) in the RNase molecule, its translocation through the cytoplasmic membrane may occur by means of inversion through the lipid moiety of the membrane at the expense of the partial formation of the secondary structure elements (similarly to the mechanism observed for the low-molecular-weight gpVIII protein of phage M13 [18]).

These speculations are in agreement with the experimental data of Chen and Nagarajan [19], who showed that the overexpression of heat shock proteins in the recombinant *E. coli* strain bearing the *B. amyloliquifaciens* RNase (barnase) gene blocks the secretion of other secretory polypeptides since all the potential secretion sites in the membrane become occupied. At the same time, barnase was successfully secreted under such conditions, forming the mature enzyme [19].

Thus, the comparative analysis of the extracellular RNase, phosphatase, and proteinases of *B. intermedius* showed that the RNase is secreted more efficiently than the other enzymes, its relative content reaching 85–92% of the total extracellular hydrolases [1]. The secretion of RNase in *B. intermedius* is distinguished by the absence of active membrane-bound forms. At the same time, phosphatase, subtilisin, and glutamyl endopeptidase are secreted by means of formation of membrane-bound enzyme forms, which are released from the membrane with the involvement of metal ions [2, 3]. The efficiency of RNase secretion depends on the formation rate of the secondary structure of the enzyme molecule. In other words, the structural features of protein molecules play an important part in their release from cells into the medium.

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