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EXPERIMENTAL ARTICLES

Preparations of *Bacillus pumilus* Secreted RNase: One Enzyme or Two?

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Abstract—Immunochemical analysis of the following purified preparations of *Bacillus pumilus* RNase (binase) was carried out: industrially manufactured enzyme (Institute of Organic Synthesis, Riga, Latvia) and the enzymes isolated from the culture liquid of the native *B. pumilus* producer and from the *Escherichia coli* BL21 recombinant strain bearing the pGEMGX1/ent/Bi plasmid. Electrophoresis of all three samples of purified binase revealed two protein fractions with ribonuclease activity possessing molecular masses of ~12 and 25 kDa. The possible presence of binase II, a second secreted RNase, was ruled out. Both high- and low-molecular mass proteins interacted with binase-specific antibodies in the immunoblotting reaction, which indicated their antigenic identity. The difference in molecular mass between these proteins indicated the possible presence of two forms of binase in solution, a monomer and a dimer.

Keywords: Bacillus pumilus, RNase, binase, binase II, immunodiffusion, immunoblotting

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Microbial ribonucleases (RNases) are widely used in genetic engineering, biochemistry, and medicine. Their biological properties associated with the regulation of gene expression, cell growth and differentiation, protection from pathogens, and apoptosis induction are of special interest. The cytotoxic effects of such microbial RNases as binase, secreted RNase of *Bacillus pumilus* (previously *B. intermedius* [1]), barnase of *B. amyloliquefaciens* [2], RNase of *Streptomyces aureofaciens* CCM 3239 [3], and α-sarcin, a ribotoxin from fungi of the genus *Aspergillus* [4], against cancer cells make microbial RNases promising antitumor agents [5–9]. Binase was recently shown to exhibit an antiviral effect [10], as well as to possess mutagenic [11, 12] and antimutagenic properties [13]. Production of a homogenous, highly purified protein is a prerequisite for development of binase-based preparations.

The regulation of binase biosynthesis in both native and recombinant strains was studied [14–17]. Secretion of the enzyme into the environment was shown to occur at the growth retardation phase. Expression of the binase gene increases under depletion of inorganic phosphate in the environment due to the interaction of the PhoP transcription regulator, which controls the specific response of bacilli to phosphate starvation, with the RNase gene promoter [14]. These data were used to develop the procedures for the isolation of this enzyme [18]. *B. pumilus* is known, however, to possess another RNase, which may be secreted into the medium. This ribonuclease, binase II with molecular mass of 29638 Da (compared to 12212 Da for binase), activated by Mg^{2+}, is also an alkaline protein with the activity optimum at pH 8.5, which has been less extensively studied [19]. The issue of homogeneity of binase preparations is therefore of importance.

The goal of the present work was to assess the homogeneity of RNase preparations obtained from the culture liquid of *B. pumilus* and of a recombinant strain *Escherichia coli* BL21 bearing the pGEMGX1/ent/Bi plasmid, in comparison to the industrially produced binase preparation (Institute of Organic Synthesis, Riga, Latvia).

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in the work were obtained from the collection of Department of Microbiology, Kazan Federal University. For binase production, *B. pumilus* strain 7P was grown in the medium containing the following (%): peptone, 2.0; glucose, 1.0; $CaCl_2$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; $MnSO_4$, 0.01; pH 8.5. The cultivation was carried out at 37°C until the late exponential phase [20]. For binase II production, *B. subtilis* strain 3922 with the pJF28 plasmid, bearing the ribonuclease gene under its own promoter [21], was grown under the same conditions. The recombinant strain *E. coli* BL21 pGEMGX1/ent/Bi was cultivated as described previously [22]. This strain bears the binase structural gene on the plasmid pGEMGX1/ent/Bi; its expression is regulated by the T7 promoter, and the signal peptide of

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PhoA alkaline phosphatase is involved in secretion of the protein into the medium.

RNase preparations. The three binase samples investigated were (1) produced in the Institute of Organic Synthesis (Riga, Latvia), (2) isolated from the culture liquid of the recombinant strain *E. coli* BL21 pGEMGX1/ent/Bi, and (3) isolated from the culture liquid of *B. pumilus* 7P. RNases were isolated and purified as described in [23]. Catalytic activity of the preparations was confirmed using high-polymer yeast RNA (Vector, Berdsk, Russia) [12]. An increase in OD_{260} of the acid-soluble products of RNA hydrolysis by 1 U after 1 min at 37°C was accepted as an activity unit.

The binase chemical dimer obtained by covalent cross-linking of the protein with the help of dimethyl suberimidate [24] was kindly provided by N.V. Kalacheva (Kazan Federal University).

Binase II was obtained from the culture liquid of *B. subtilis* 3922 pJF28 according to the procedure described previously [21].

Electrophoretic analysis. Protein electrophoresis in PAG in the presence of 0.1% sodium dodecyl sulfate (SDS) was carried out according to Laemmli [25]. Ribonuclease activity was revealed by zymography. For this purpose, 7 mg/mL high-polymer RNA of the *Torula* yeasts (Sigma, United States) was added to the electrophoresis gel. After electrophoresis, SDS was removed by washing the gel with the buffer containing 10 mM Tris-HCl and 20% isopropanol, pH 7.5. Renaturation of the proteins was carried out by sequential incubation of the gel for 10 min in 10 mM Tris-HCl, pH 7.5 and in 100 mM Tris-HCl, pH 7.5. The zones of RNA hydrolysis were revealed by staining the gel with 0.2% toluidine blue (Sigma, United States) for 10 min.

Polyclonal serum. The polyclonal serum to binase was obtained by immunizing rabbits with the commercial enzyme preparation. Immunization was carried out as a course of four subcutaneous injections (with 10–12-day intervals) of increasing amounts of protein (1, 2, 4, and 8 mg) with complete (first and second injections) or incomplete (third and fourth injections) Freund's adjuvant. For revaccination, 10 mg of soluble protein was injected subcutaneously after 30 days. The serum was obtained 10–12 days after revaccination and was used for the isolation of specific antibodies.

Immunochemical analysis of the serum was carried out by precipitation in agar gel [26]. For this reaction, 1% agar (Difco) in physiological saline was used. Precipitation lines indicated the presence of antibodies in the serum.

Specific antibodies. Specific antibodies to binase were isolated from the polyclonal serum using CNBr-activated Sepharose 4B (Sigma, United States). The sorbent was washed on a glass filter with 0.1 N HCl, 0.1 M $NaHCO_3$ buffer (pH 8.3), and 0.5 M NaCl. Washed sepharose was mixed with binase (1) solution (2 mg/mL) and incubated for 2 h at room temperature. The unbound enzyme was washed out on a glass filter with 0.5 M NaCl under optical density control. The rabbit immune serum containing antibodies to binase was mixed with an equal volume of 0.2 M Tris-HCl buffer (pH 7.5) and incubated with the affine sorbent for 3 h at room temperature. The bound specific antibodies were eluted with 0.05 M glycine–HCl buffer (pH 2.8), and the eluate was neutralized with NaOH. The antibody-containing fractions were analyzed by two-dimensional immunodiffusion. Immunologically active fractions were combined and used for immunoblotting.

Immunoblotting. The proteins fractionated in 15% PAG were transferred onto a nitrocellulose membrane by semi-dry electrophoresis. The remaining binding centers were blocked with 1% skim milk in Tris–saline buffer (pH 7.4), and specific primary antibodies to binase were added. Unbound antibodies were washed off the membrane, and it was incubated for 1.5 h in the solution of secondary antibodies. Antirabbit antibodies conjugated with horseradish peroxidase (Sigma, United States) were used. The immune complexes were detected by chemiluminescence. The membrane was visualized using the ChemiDoc gel documentation system (Bio-Rad, United States).

High-efficiency liquid chromatography and mass spectrometry (HPLC-MS). The proteins corresponding to two fractions were excised from the gel stained with Coomassie R250. To remove the stain, the fragments were washed with 1 : 1 mixture (vol/vol) of acetonitrile and 200 mM NH_4HCO_3. The proteins were cleaved with trypsin (Promega, United States) overnight at 37°C. The resultant peptides were extracted with 0.1% trifluoroacetic acid and dried in a vacuum concentrator. The peptides were identified using an HPLC-MS system (Bruker, Germany).

Modeling and structure comparison. The primary structures of binase I (GenBank P00649.3) and binase II (GenBank CAA66713.1) were compared using the Muscle algorithm (http://www.ebi.ac.uk/Tools/msa/muscle/). The sequences were analyzed using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). A three-dimensional model of binase II was constructed on the I-Tasser server [27]. The model was visualized using the Jmol software (http://www.jmol.org/). Three-dimensional structures of binase I (PDB 1buj) and II (the model obtained) were compared using the FATCAT software package [28].

RESULTS AND DISCUSSION

Electrophoresis under denaturing conditions revealed two bands in all three studied binase samples: the industrially produced preparation (1), those isolated from the culture liquid of recombinant *E. coli* BL21 with the plasmid pGEMGX1/ent/Bi (2), and those of the original producer strain *B. pumilus* 7P (3) (Fig. 1a). One fraction had molecular mass of

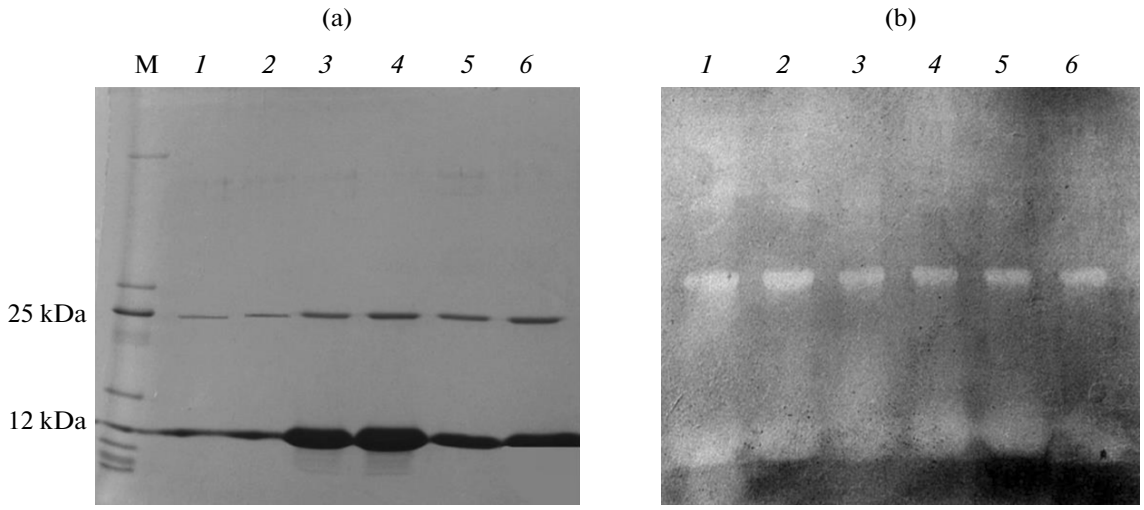

Fig. 1. Electrophoregram (a) and Zymogram (b) of purified binase: marker proteins (M); binase from *E. coli* recombinant strain (2), 5 μg per sample (*1, 2*); commercial binase preparation (1) 15 μg per sample (*3, 4*); and binase from the culture liquid of the original producer *B. pumilus* (3), 10 μg per sample (*5, 6*). Protein amount on the zymogram (b) is 10 μg

~12000 Da (which agrees with the known binase Mr = 12213 Da), while Mr of the second fraction was 25000 Da. Zymography confirmed ribonuclease activity in both fractions: both the high- and the low-molecular mass fractions from all three binase samples yielded hydrolysis zones visible as transparent bands (Fig. 1b). Thus, the presence of two proteins possessing RNase activity could be expected in the preparations.

B. pumilus is known to produce two types of extracellular ribonucleases, binase I containing 109 amino acid residues [1] and binase II (263 amino acid residues) [25]. The guanyl-preferring binase I cleaves RNA to 3'-phosphate mono- and oligonucleotides in two stages with formation of a 2'-3'-cyclic intermediate. The nonspecific binase II hydrolyzes the substrate to 5'-phosphate derivatives in one stage. While activity of both enzymes peaks at 37°C and pH 8.5, binase II is activated by Mg^{2+} ions. Both enzymes are synthesized during the growth retardation phase in response to phosphate starvation [1, 25]. The level of binase II synthesis is probably low, since its activity in the culture liquid is almost 20 times lower than that of binase I. Moreover, in spite of the similar secondary structures of the signal peptides responsible for successful translocation, secretion of binase II is probably less efficient than that of binase I. The signal sequences of both RNases consisting of 29 amino acid residues direct the proteins into the environment via the Sec pathway, as was determined using the PRED-TAT algorithm (http://www.compgen.org/tools/PRED-TAT/submit). The presence of binase II in the preparation of purified binase I is therefore theoretically possible.

Immunochemical analysis was carried out in order to determine whether the high-molecular mass fraction was represented by binase II. The presence of specific antibodies to binase I was confirmed by precipitation bands in two-dimensional immunodiffusion (Fig. 3a). However, since the polyvalent serum was obtained by immunizing rabbits with the commercial binase preparation (1), containing both the low- and the high-molecular mass proteins, it was essential to exclude the possible antibody (Ab) interaction with binase II.

The primary sequences of binases I and II exhibit low similarity, with identity of amino acid composition slightly exceeding 10% (Fig. 2c). Three-dimensional structure is, however, important for the functioning of enzymes. For binase I it was experimentally determined in the crystal (PDB 1gou) and in solution (PDB 1buj). The three-dimensional structure of binase II has not been known and was modelled in the present work (Fig. 2b), using the spatial structures of the extracellular endonucleases of vibrios (PDB 2pu3, 1oup, 2g7f), which most closely resemble binase II in their secondary structure, as templates. Comparison of the three-dimensional structures of binase I and binase II revealed 32 equivalent positions with the standard deviation of atomic positions $C\alpha = 3.75$ Å. The structures were, however, not generally similar (*P* value exceeded 0.05 and was 0.88).

Binase II was isolated from the culture liquid of the recombinant strain *B. subtilis* 3922 pJF28 and was analyzed by two-dimensional immunodiffusion. Two-dimensional immunodiffusion of binase II and three samples of binase I revealed no immune reaction between binase II and Ab to binase I, while all binase I

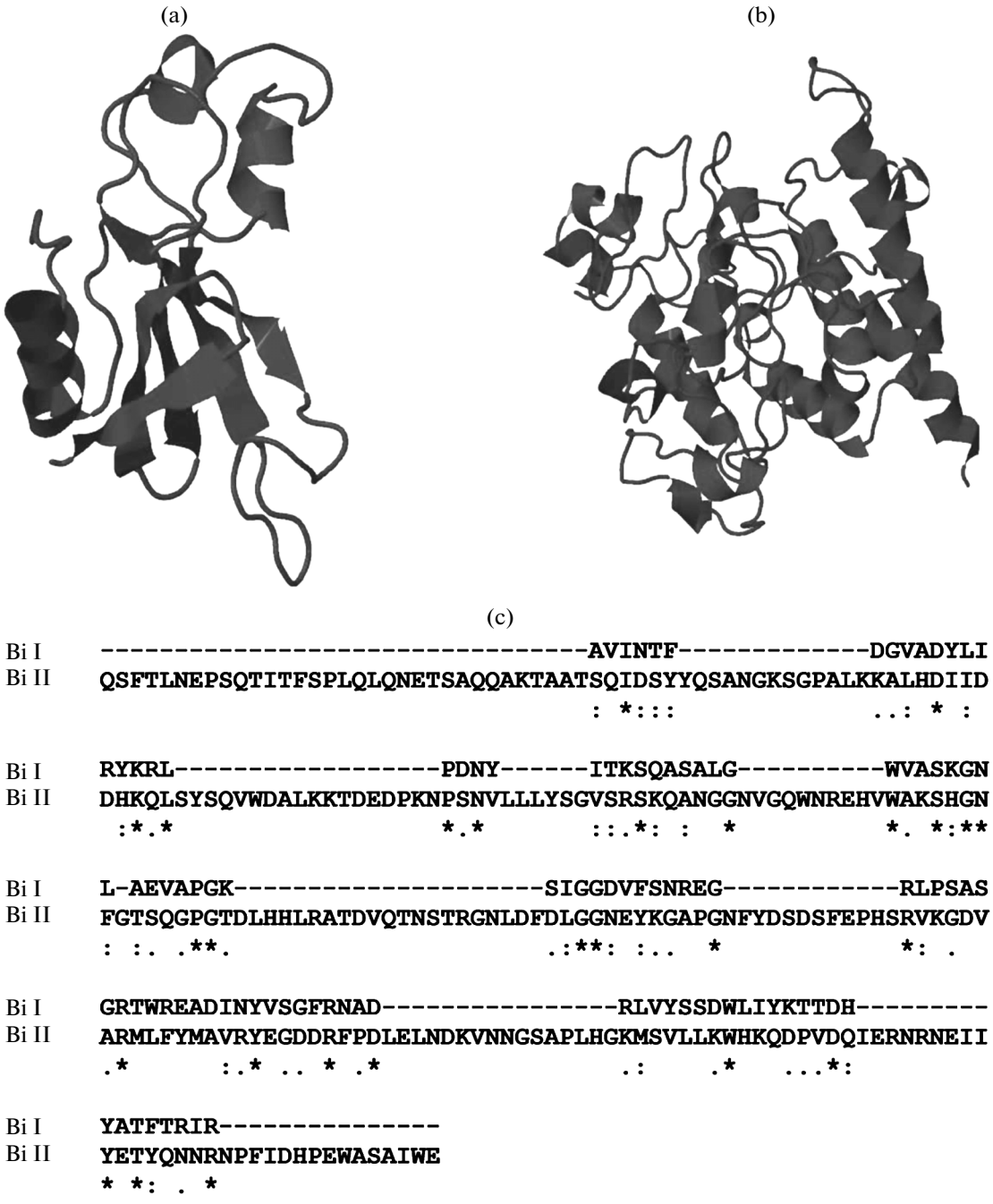

Fig. 2. Secreted ribonucleases of *B. pumilus*: three-dimensional structure of binase I (PDB 1buj) (a); three-dimensional model of binase II (C-score = –1.56, TM-Score = 0.52 ± 0.15, RMSD = 9.4 ± 4.6) (b); and aligned primary sequences of the ribonucleases (c). Identical amino acids and conservative replacements are marked by asterisks and colons and dots, respectively.

preparations exhibited serological activity (Fig. 3c). Thus, binase II is antigenically unrelated to binase I.

Immunoblotting analysis of the high-molecular mass fraction of binase preparations was carried out. For this purpose, specific antibodies were isolated from the polyvalent serum to binase I using the "CNBr-activated sepharose 4B—binase" affine sorbent and combining the immunologically active eluted fractions. Fractions 1 and 2 were especially active (Fig. 3b) and were subsequently used as a combined preparation of specific antibodies to binase. Three samples of purified binase and its chemical dimer obtained by covalent cross-linking with dimethyl suberimidate were used in immunoblot assay. The results are presented on Fig. 4. Immunoblot of the chemical dimer was similar to immunoblots of all

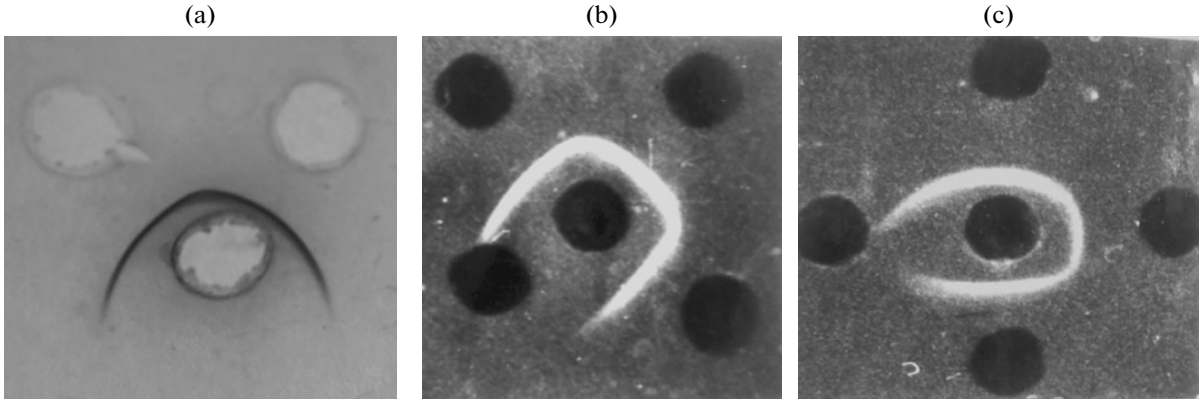

Fig. 3. Two-dimensional immunodiffusion of: binase I (1, upper row) with polyvalent serum (PS) to binase (lower row) (a); binase I (1) and (2), (upper row), binase (3) (lower row), and binase II (lower row) with PS (central well) (b); binase (1) (central well) with the eluent fractions containing specific antibodies to binase (clockwise from the upper row: PS, fraction 1, fraction 2, and fraction 3) (c).

three binase samples, confirming the possibility of binase dimerization. Results of the immunological reaction indicated that both protein fractions (with Mr = 12000 Da and with Mr = 25000 Da) interacted with specific antibodies to binase, i.e., they were antigenically identical. In order to obtain additional confirmation of identity of the two fractions, they were analyzed on an HPLC-MS system. Both fractions were found to contain the peptides corresponding to the mature form of *B. pumilus* ribonuclease (Fig. 5). The calculated molecular mass of the proteins was the same, 12204 Da, pI 9.52. The difference in molecular masses of the fractions on electrophoregrams indicates that in solutions binase may exist in two forms, as a monomer and a dimer.

Some ribonucleases, such as RNase L and BS-RNase, were shown to be natural dimers [29, 30], with dimerization necessary for their functioning. Thus, RNase L, a component of the cell response to viral infection, is activated by dimerization [29, 31]. BS-RNase, which is not susceptible to the cytosolic RNase inhibitor [30], forms two types of dimers, which occur in a 2 : 1 ratio [32]. The predominant noncovalently bound dimer is formed by exchange of the N-terminal domains between monomers, while the other dimer type is formed of two monomers bound by two intramolecular disulfide bonds. The noncovalently bound dimer is more stable and is responsible for the cytotoxic properties of BS-RNase [33].

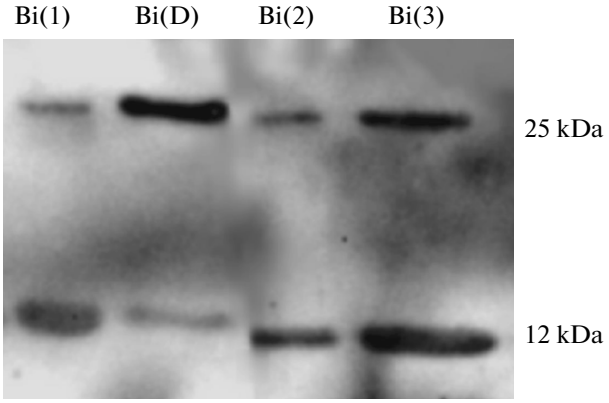

Fig. 4. Immunoblotting of binase preparations (Bi 1–3) and binase chemical dimer (Bi D). Protein amount per sample is 10 μg.

Existence of two molecular forms of binase, a monomeric and a dimeric one, was considered by Polyakov et al. [34], Konovalova et al. [35], Shirshikov et al. [36], and Ermakova [37]. In these works, however, existence of dimeric structures formed due to hydrogen bonds and hydrophobic interactions was theoretically proposed in binase crystals, while their formation in solutions was considered hypothetical [34, 36]. Formation of dimeric and aggregated forms of binase on the membrane surface, which may enhance the penetration of this enzyme into the cell, is also probable [35]. Our results indicate the absence of the high-molecular mass RNase binase II from the preparations of purified binase, while binase itself exists in two forms, a monomer and a dimer. Further research is required in order to establish whether binase is secreted by *B. pumilus* as a native dimer.

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(a)

AVINTFDGVA DYLIR**YK**RLP DNYITKSQAS ALGWVASKGN LAEVAPGKSI
GGDVFSNREG R**LPSASGR**TW READINYVSG FRNADRLVYS SDWLIYKTTD
HYATFTR**IR**

(b)

AVINTFDGVA DYLIR**YKR**LP DNYITKSQAS ALGWVASKGN LAEVAPGKSI
GGDVFSNR**EG** **RLPSASGR**TW READINYVSG FRNADRLVYS SDWLIYKTTD
HYATFTR**IR**

Fig. 5. Peptide fragments obtained by trypsinolysis of the protein fractions with molecular masses 12 (a) and 24 kDa (b) analyzed in an HPLC-MS system and superimposed over the sequence of *B. pumilus* ribonuclease. Non-coinciding amino acids are marked by boldface.

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