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Novel Extracellular Ribonuclease from *Bacillus intermedius*—Binase II: Purification and Some Properties of the Enzyme

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Abstract—The recombinant enzyme binase II was isolated from the culture liquid of *Bacillus subtilis* 3922 transformed with the pJF28 plasmid bearing the *birB* gene. The procedure of the enzyme purification included precipitation by polyethylene glycol with subsequent chromatography on DEAE-cellulose, heparin-Sepharose, and Toyopearl TSK-gel. The enzyme was purified 142-fold yielding a preparation with specific activity 1633 U/mg. The molecular weight of binase II is 30 kD. The enzyme is activated by Mg^{2+} and virtually completely inhibited by EDTA. The pH optimum for the reaction of RNA hydrolysis is 8.5. The properties of the enzyme are close to those of RNase *Bsn* from *B. subtilis.* The character of cleaving of synthetic single- and double-stranded polyribonucleotides by binase II suggests that the enzyme binds the substrate in the helix conformation, and its catalytic mechanism is close to that of RNase VI from cobra venom.

Key words: *Bacillus intermedius*, *Bacillus subtilis*, binase II, RNase *Bsn*, biosynthesis, enzyme purification

Extracellular ribonucleases (RNases) produced by virtually all species of bacilli exhibit a wide spectrum of biological activity. The tandem of genes encoding the RNase from *Bacillus amyloliquefaciens* (barnase) and its intracellular inhibitor (barstar) is used in the molecular selection of hybrid seeds of monoecious plants and for obtaining *Phytophthora*-resistant varieties of potatoes [1-3]. Ribonucleases from bacilli possess an antiviral effect [4] and can be used in small doses as stimulators of growth and the physiological activity of plants and microorganisms [5]. Finding of RNase with new properties will allow creation of new preparations exhibiting different kinds of biological activities.

The most studied RNases produced by bacilli are guanyl-specific enzymes producing cyclic 2′,3′-phosphodiesters that are hydrolyzed then to nucleoside-3′-phosphate derivatives. These RNases consist of about 110 amino acids and exhibit 85-100% similarities in their primary structure. They are stable over a wide range of pH values (3-10), exhibiting the highest activity at pH 8.5, and do not require metal ions for their activity. The genes for a number of the guanyl-specific ribonucleases have been cloned [6-9].

Bacillus subtilis bears no genes homologous to the genes of the guanyl-specific RNases, but it produces the ribonuclease *Bsn* encoded by the *bsn* gene. This RNase consists of 241 amino acid residues and hydrolyzes RNA non-specifically yielding oligonucleoside-5′-phosphates [10].

The fragment of the chromosome of *B. intermedius* 7P bearing the gene of a newly identified secreted ribonuclease was cloned in *Bacillus subtilis* by researchers of Kazan State University [11]. The subsequent procedures of subcloning and sequencing, as well as further investigation of the fragment were performed in collaboration with the Gissen Institute of Microbiology and Molecular Biology (Germany). These studies showed that the ribonuclease encoded by the *birB* gene and named binase II is a homolog of the 5′-phosphate-producing *Bsn* ribonuclease secreted by *B. subtilis*. Coding regions of the *bsn* and *birB* genes exhibited 65% identity in the nucleotide composition and 72% identity in the encoded amino acids [12].

In the present work the new recombinant ribonuclease from *B. intermedius* (binase II) was isolated for the first time and some of its properties were characterized.

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MATERIALS AND METHODS

A strain of *B. subtilis* 3922 (hisB2 trpC2 leuB) with a low endogenous RNase activity obtained from the Collection of Microorganisms of the Laboratory of Biosynthesis and Bioengineering of Enzymes of Kazan State University was used as a host for production of binase II. To obtain recombinant producing strains, the pJF28 plasmid bearing a fragment of the chromosome of *B. intermedius* (4.9 kb) was used. The fragment included the *birB* gene and the gene providing the resistance to erythromycin.

To obtain the recombinant protein, the culture was grown in a shaker at 37°C and 200 rpm for 22 h in a medium of the following composition (g/liter): peptone, 20.0; glucose, 14.0; $MgSO_4$, 0.3; $MnSO_4$, 0.1; NaCl, 3.0; $CaCl_2$, 0.1. The media were sterilized at 1 atm for 20 min, the pH being adjusted to 8.5 with 40% NaOH before the sterilization. Glucose solution was prepared and sterilized separately.

Chemicals for preparation of media and buffer solutions were from Reakhim (Russia); inorganic phosphate-deficient peptone was from Semipalatinsk Meat Plant (P_i content did not exceed 1.5 mg/g dry weight); erythromycin was from OAO Biokhimik (Saransk, Russia). For purification of the enzyme we used polyethylene glycol from Serva (Germany); DEAE-cellulose and CM-cellulose from Reanal (Hungary); Toyopearl TSK-gel from Toyo Soda Kogio (Japan); Sepharose CL-4B from Pharmacia (Sweden); heparin from Sigma (USA). Enzymatic activity was assayed using yeast high-molecular-weight RNA (NIKTI BAV, Novosibirsk, Russia) and also poly(A), poly(A)poly(U), and poly(I)poly(C) (Reanal).

The ribonuclease activity was determined by the content of the acid-soluble products of the RNA hydrolysis as described earlier [12]. $MgSO_4$ (1 mM) was added in the incubation mixture as the activator. The unit of the activity was defined as the amount of the enzyme resulting in an increase in absorption at 260 nm by 1 optical density unity in 1 ml of the solution for 1 h of incubation at 37°C. The specific activity of the enzyme characterizing the productivity of the culture in terms of RNase synthesis was defined as the ratio of the RNase activity in the culture liquid to the biomass of the culture.

To isolate the ribonuclease, the culture of *B. subtilis* 3922 containing the pJF28 plasmid was grown in 1 liter of the optimized medium for 22 h, then the biomass was centrifuged at 4000 rpm, and polyethylene glycol was added to the supernatant to achieve 30% saturation. The suspension was centrifuged, and the pellet was dissolved in 35 ml of buffer A (0.01 M CH_3COOLi, 1 mM $MgSO_4$, pH 8.0). After dialysis against the same buffer, the solution was applied to a DEAE-cellulose column (40 × 93 mm) and eluted with a linear gradient (0.01-0.6 M) of buffer A. The active fractions were pooled (at lithium acetate concentration of about 0.4 M) and dialyzed against buffer A. The resulting solution was applied to a heparin-Sepharose column (18 × 80 mm), and the protein was eluted with a linear gradient (0.01-0.3 M) of buffer A. The active fractions (21 ml) were pooled (lithium acetate concentration, ~0.15 M) and dialyzed against buffer A. Then the protein fraction was concentrated on a DEAE-cellulose column (11 × 89 mm). In the final step of the purification, the proteins were separated according to their molecular weights using a Toyopearl TSK-gel column (15 × 280 mm).

At the different purification stages the enzyme purity was monitored using SDS-electrophoresis according to Laemmli [13].

The rate of transformation of the synthetic substrates was calculated using the equation: $v = \Delta A/t$, where ΔA is the change in absorption (optical units), t is the time (sec). The bimolecular rate constant was calculated according to the equation: $k_{cat}/K_m = v/[S][E]$, where [E] is the concentration of the free enzyme, [S] is the concentration of the free substrate, and v is the rate of the substrate transformation.

To analyze data, standard methods of statistical analysis were used: standard deviations of the mean values and 95% confidence intervals were calculated [14].

RESULTS AND DISCUSSION

The ability of *B. intermedius* to secrete the second RNase (binase II) was revealed when the fragment of the bacterial chromosome was cloned in *B. subtilis* [11, 12]. Binase II was isolated from the culture liquid of the *B. subtilis* 3922 strain transformed with the plasmid containing the *birB* gene and grown on the optimized nutrient medium (composition of the medium is given in "Materials and Methods"). The activity of the endogenous RNase *Bsn* of the producing strain was no more than 200 U/h per ml, which was 20-fold less than the activity of the recombinant enzyme (binase II).

The procedure of isolation of the extracellular ribonuclease *Bsn* that is homologous to the binase II from *B. subtilis* using ammonium sulfate precipitation with subsequent chromatography on S-Sepharose, heparin-Toyopearl, and hydroxyapatite was described by Nakamura et al. [10]. However, the described procedure resulted in only 2.8-fold purification of the enzyme (2% yield in terms of activity), and the most significant losses occurred in the first stages of the purification. We assumed that the low yield of the RNase *Bsn* was due to the removal of Mg^{2+} from the active site of the enzyme while saturating the culture liquid with ammonium sulfate (the enzyme requires Mg^{2+} for its catalytic activity). A method for isolation of binase II different from that described for the RNase *Bsn* was developed. First, pH stability of the binase II in the culture liquid was investigated. It was shown that, being stable in the range of pH 7-9, the enzyme lost its activity irreversibly at extreme pH values. Thus, acidification of the culture liquid at first stages of isolation of the

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                    +         -         +      ++   +-   --++         -
BinII    1 TSAQQAKTAATSQIDSYYQSANGKSGPALKKALHDIIDDHKQLSYSQVWD   50
Bsn      1 TSS--AVITPSADTETYYKEASGKSGTALKSALHRIISGHTKLSYSQVWN   48

              ++ --- +                + +              +-+    + +
BinII   51 ALKKTDEDPKNPSNVLLLYSGVSRSKQANGGNVGQWNREHVWAKSHGNFG  100
Bsn     49 ALKETDEDPANPSNVILLYTQESRAKSKNGGSVGDWNREHVWAKSHGNFG   98

                     - ++ +  -       +    - -     - +         - - -
BinII  101 TSQGPGTDLHHLRATDVQTNSTRGNLDFDLGGNEYKGAPGNFYDSDSFEP  150
Bsn     99 TAAGPGTDIHHLRPADVQVNSARGNMDFDNGGSEYPKAPGNYYDGDSWEP  148

               + + + -   +         + - - +- - -   -+          + +     +
BinII  151 HSRVKGDVARMLFYMAVRYEGDGREPDLELNDKVNNGSAPLHGKMSVLLK  200
Bsn    149 RDEVKGDVARMLFYMAVRYEGGDGYPDLELNDKTGNGSAPYMGKLSVLLK  198

              ++ -   -   -+ + -    -      +     -+ -        +
BinII  201 WHKQDPVDQIERNRNEIIYETYQNNRNPFIDHPEWASAIWE   241
Bsn    299 WNKQDPVDSKEKRRNEIIYEDYQHNRNPFIDHPEWADEIW   239
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Fig. 1. Primary structure of binase II of *B. intermedius* compared to that of the extracellular RNase *Bsn* of *B. subtilis*. Nonequivalent amino acid changes are shown bold. Amino acid charges are shown for binase II.

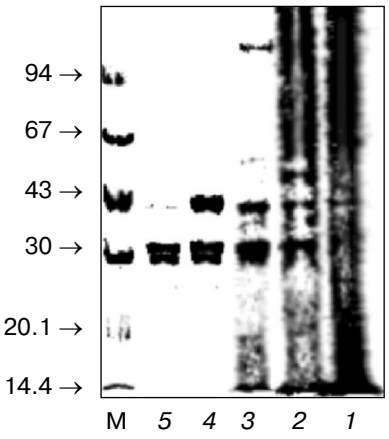

Fig. 2. SDS-PAGE of the enzyme preparations obtained at different stages of purification: *1*) culture liquid; *2*) polyethylene glycol precipitation; *3*) DEAE-cellulose chromatography; *4*) heparin-Sepharose chromatography; *5*) Toyopearl TSK-gel gel filtration; M) protein markers (molecular weights are given in kD on the left): phosphorylase *b* (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carboanhydrase (30 kD), soybean trypsin inhibitor (20.1), α-lactalbumin (14.4).

binase II (unlike binase I) was unacceptable [6]. During the analysis of the primary structure of the binase II, the probable charge of the protein at different pH values was estimated, assuming that the position of the N-terminal amino acid was analogous to that of RNase *Bsn* (Fig. 1). Considering the protein instability in acid medium as well as the data on the primary structure of binase II, heparin-Sepharose, DEAE-cellulose, and Toyopearl TSK-gel were chosen for purification of the enzyme. The suggested method resulted in 141.2-fold purification of the enzyme (Table 1) with 1.7% yield.

The molecular weight of the enzyme was determined using SDS PAGE. As seen from Fig. 2, we failed to obtain the homogeneous preparation, but the data indicated an increase in the intensity of the protein band corresponding to 30 kD with simultaneous decrease in the intensity of other bands during purification. Thus, the molecular weight of the enzyme was ~30 kD. The molecular weight of the RNase *Bsn* from *B. subtilis* determined by the same method was 32 kD [10], and in both cases the proteins were revealed as two associated bands.

Study of the enzymatic properties of binase II showed that the enzyme was activated by Mg^{2+} and virtually completely inhibited on incubation with EDTA (Table 2). Investigation of the pH dependence of the activity of binase II towards high-molecular-weight yeast RNA detected by the accumulation of low-molecular-weight soluble products showed that the activity was maximal at pH 8.5 (Fig. 3). However, the activity towards poly(A)poly(U) was virtually constant in the range of pH 7.2-8.7. Kinetic parameters for the hydrolysis of some single- or double-stranded polyribonucleotides are given in Table 3. The differences in the rates of cleaving of single- and double-stranded RNA can be characterized by the K_m and k_{cat} values. This suggests that binase II binds the substrate in the helix conformation. The same conclusion was made earlier in our work on investigation of the RNase VI from cobra venom [15].

Thus, we first isolated a secreted ribonuclease, binase II, that differs in its properties from the RNase of this bacterial species described earlier. Until now, *B. intermedius* has been known as the only species of bacilli secreting two types of RNases: low-molecular-weight guanyl-specific binase I and high-molecular-weight binase II, but the activity of binase II cannot be revealed in the culture liquid of the natural producing strain because of a high level of the binase I production.

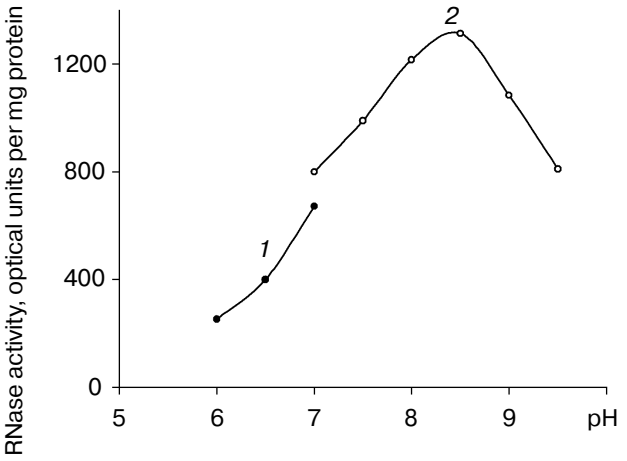

Fig. 3. Dependence of the ribonuclease activity on pH of the reaction medium: *1*) phosphate buffer; *2*) Tris-HCl buffer.

The characteristics of binase II studied in the present work (pH optimum, activation by Mg^{2+}, and inhibition by EDTA) demonstrated its similarity with the RNase *Bsn* of *B. subtilis* encoded by the gene having 72% of homology with the gene of binase II.

Table 1. Isolation of binase II

Purification stage	Total RNase activity	Specific activity, U/mg	Purification extent	Yield, %
Culture liquid	324000	11.57	1	100
Precipitation by polyethylene glycol	93959	98.2	8.5	29.0
DEAE-cellulose chromatography	41195	185.6	16.0	12.7
Heparin-Sepharose chromatography	38184	586.5	50.7	12.0
Concentrating on DEAE-cellulose	18733	567.6	49.0	6
Gel filtration on Toyopearl TSK-gel	5433	1633	141.2	1.7

Table 2. Effect of Mg^{2+} and EDTA on the activity of binase II in the culture liquid and in the purified preparation (activity of binase II on incubation in 0.1 M Tris-HCl, pH 8.5 (control sample), was taken as 100%)

Material assayed	RNase activity, %		
	control	Mg^{2+}, 1 mM	EDTA, 10 mM
Culture liquid	100	137.0	49.8
Purified enzyme	100	288.0	2.9

Table 3. Kinetic parameters of the reaction of cleaving of synthetic polynucleotides by binase II from *B. intermedius*

Substrate	K_m, μM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$
poly(I) poly(C)	110	59	$5.4 \cdot 10^6$
poly(A) poly(U)	270	50	$1.9 \cdot 10^6$
poly(A)	210	400	$5.2 \cdot 10^5$

Note: The parameters were determined at pH 7.5; the k_{cat} values were calculated taking the molar absorption coefficient (ε_{280}) for the binase II as 47,500 $M^{-1}\cdot cm^{-1}$.

At the same time, the pH optimum of RNA hydrolysis catalyzed by binase II coincides with the optimal conditions for the substrate cleaving by the main ribonuclease secreted by *B. intermedius*—binase I. The data on cleaving of the synthetic homopolynucleotides presented above indicate that the catalytic mechanism of binase II is similar to that of RNase VI from cobra venom.

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