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EXPERIMENTAL ARTICLES

Control of the Synthesis of Plasmin-like and Plasminogen-activating Proteinases in Marine Bacteria

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Abstract—The biosynthesis of proteinases with various substrate specificities was studied in *Bacillus firmus* 44b and *Bacillus oligonitrophilus* 21p as influenced by the growth conditions and growth phases of the bacteria. The period of the maximum synthesis of plasmin-like enzymes was observed 6 h later than the period of the maximum growth rate of *B. firmus* 44b, and 3 h later than the maximum growth rate period of *B. oligonitrophilus* 21p. The periods of the maximum accumulation of activating enzymes were delayed 9 and 12 h, respectively, as compared to the periods of rapid growth of these two bacteria. Catabolite repression of proteinase synthesis and stimulation of the latter with substrate proteins were insignificant. The production of both plasmin-like and plasminogen-activating enzymes was most sensitive to repression by nitrogen deficiency. The production of plasminogen-activating proteinases was less dependent on the carbon source than the production of plasmin-like enzymes.

Key words: marine bacteria, plasmin-like and plasminogen-activating proteinases, control of proteinase synthesis

Microorganisms are more promising producers of enzyme preparations than animals and plants due to such properties as a high growth rate, the possibility of their cultivation on inedible raw material, the high activity and specificity of their enzyme systems, and because the genetic and physiological approaches are developed to influence their regulatory systems.

Recently, the microbial proteases were shown to be capable of limited proteolysis of such human blood proteins as fibrin, plasminogen, and protein C, which offers opportunities for preventive correction of human hemostasis, diagnostics and treatment of atherosclerosis, and thrombogenesis [1–3].

For several years, we performed a detailed screening of microbial culture collections and microorganisms that we isolated from various natural biocenoses to reveal proteolytic enzymes with plasmin-like and tissue-type plasminogen-activating properties. As a result, the active producers were chosen, in particular, *Bacillus firmus* 44b isolated from the Black Sea detritus of mineral origin and *Bacillus oligonitrophilus* 21p isolated from the intestine of a Black Sea ruff [4, 5]. In this work, we studied how the biosynthesis of proteinases is controlled in these producers and whether the preferential accumulation of certain enzymes in the culture liquid is possible.

MATERIALS AND METHODS

In our experiments, we used *B. firmus* 44b and *B. oligonitrophilus* 21p from the collection of the Microbiology Department of the Moscow State University. Proteinase producers were maintained on fish–peptone agar. To obtain inoculum, the microorganisms were grown in 750 ml flasks containing 100 ml of fish–peptone broth on a rotary shaker (200 rpm) at 28°C for 24 h. The experimental media were inoculated with this material (5 % v/v). The enzyme production was studied in the batch culture grown for 48 h in synthetic under the above conditions medium (1% glucose; 0.07% NH_4NO_3; 0.25% KH_2PO_4; 0.05% $MgSO_4$; and 0,5% NaCl) [4]. The culture growth was followed nephelometrically (540 nm), and every 3–6 h, the biomass contained in the sampled material was weighed; the pH value was determined with a potentiometer.

Plasmin-like activity was determined by the Astrup and Mullertz method on heated fibrin plates, whereas the magnitude of tissue-specific plasminogen activation was measured by the Lassen method on unheated fibrin plates and expressed in arbitrary units per 1 ml of culture liquid. The amount of enzyme that induced the formation of a 10-mm^2 zone of fibrinolysis was defined as one unit of activity. Fibrin plates with applied samples were incubated at 37°C for 3 h [5].

To determine the effect of proteins and peptide substrates on proteinase synthesis, we used casein by Hammersten, fibrin, and gelatin (Olaine), bovine serum

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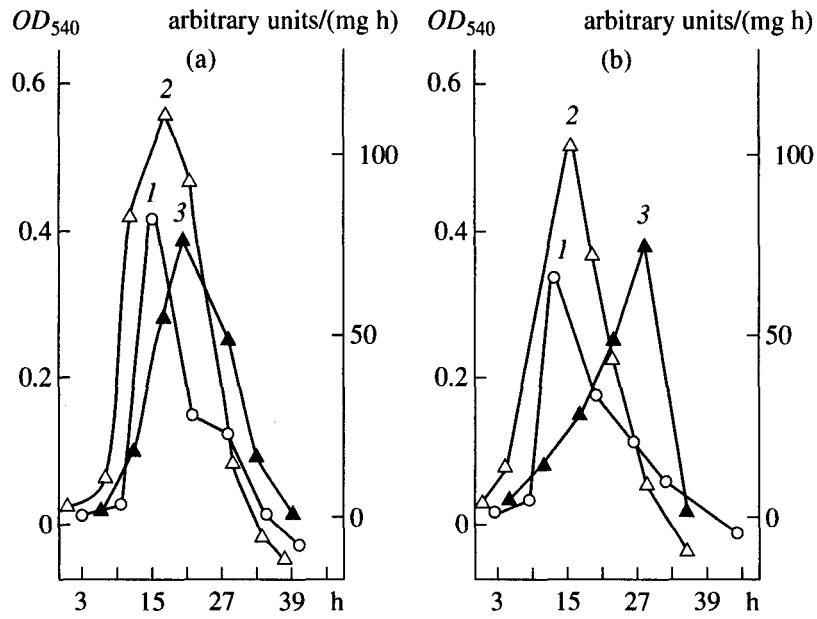

Fig. 1. Dynamics of growth and synthesis of proteinases in *B. firmus* 44b (a) and *B. oligonitrophilus* 21p (b): (*1*) specific growth rate, h^{-1}; (2) specific rate of synthesis of plasmin-like enzymes, arbitrary units/(mg h); (*3*) specific rate of synthesis of plasminogen-activating enzymes, arbitrary units/(mg h).

albumin (Spofa), and enzymatic peptone (Semipalatinsk). The proteins were added to the synthetic medium in the amount of 1%, except for peptone, added at 0.5% (w/v). The protein and peptone concentrations were predetermined to be optimal for proteinase production.

In short-term experiments, the microbial cells were collected at the end of the logarithmic phase of growth on synthetic medium, washed three times with sterile tap water under aseptic conditions, and transferred into fresh complete medium or medium deficient in a carbon and/or nitrogen source to make up 3 mg biomass per 1 ml (as judged from the dry-cell weight). Incubation was conducted at 28°C for 12 h on a shaker (200 rpm) in 250 ml flasks containing 30 ml of medium. The material was sampled every 2 h.

The specific growth rate (μ) and specific rate of enzyme synthesis (*E*) were calculated from the formulae $\mu = dxdt^{-1}x^{-1}$ and $E = dEdt^{-1}x^{-1}$, where *x* is biomass (g/l), *dx* is the biomass increment (g/l), and *dE* is activity increment (activity unit/l) for the time interval *dt* (h).

RESULTS AND DISCUSSION

Microbial cell metabolism determined by the versatile control of enzyme synthesis and activity enables microorganisms to inhabit various environments and complete with other representatives of the microbial world [6].

The effective molecular mechanisms that regulate synthesis of physiologically active compounds should be elucidated to make possible an increase in the biosynthetic capacity of a producer, the prolongation of the synthetic phase in its life cycle, and changes in the ratio of the produced components [7–11].

Inhibitor analysis of the substrate specificity characteristic of the extracellular proteases showed that *B. firmus* 44b synthesized proteinases of the serine class typical of bacilli. *B. oligonitrophilus* 21p produced a more complex spectrum of enzymes that, in addition to the enzyme of the serine class, also contained metalloproteinases and, probably, thiol proteinases or thiol-dependent subtilisin enzymes [5]. We, therefore, studied the external effects not only on the intensity of proteolytic enzyme biosynthesis, but also on the proportion of the proteinase complex components produced by the bacteria.

Comparison of dynamics of growth and secretion of various proteinases in the cultures of *B. firmus* 44b and *B. oligonitrophilus* 21p (Fig. 1) showed that the plasmin-like enzyme production reached the highest level when the growth rate of the producer decelerated. The most active proteinase synthesis was determined at the end of the logarithmic and the beginning of the stationary growth phases. The highest rate of activating enzyme synthesis was observed at the end of the stationary phase and the beginning of autolysis, thus correlating with the cell differentiation processes, namely with total sporulation.

Thus, in *B. firmus* 44b (Fig. 1a), the highest growth rate (0.41 h^{-1}) was observed from 15 to 21 h of cultivation, whereas the maximum production of the plasmin-like enzymes (115 arbitrary units/(mg h)) occurred over 21–27 h of growth; that of the activating enzymes

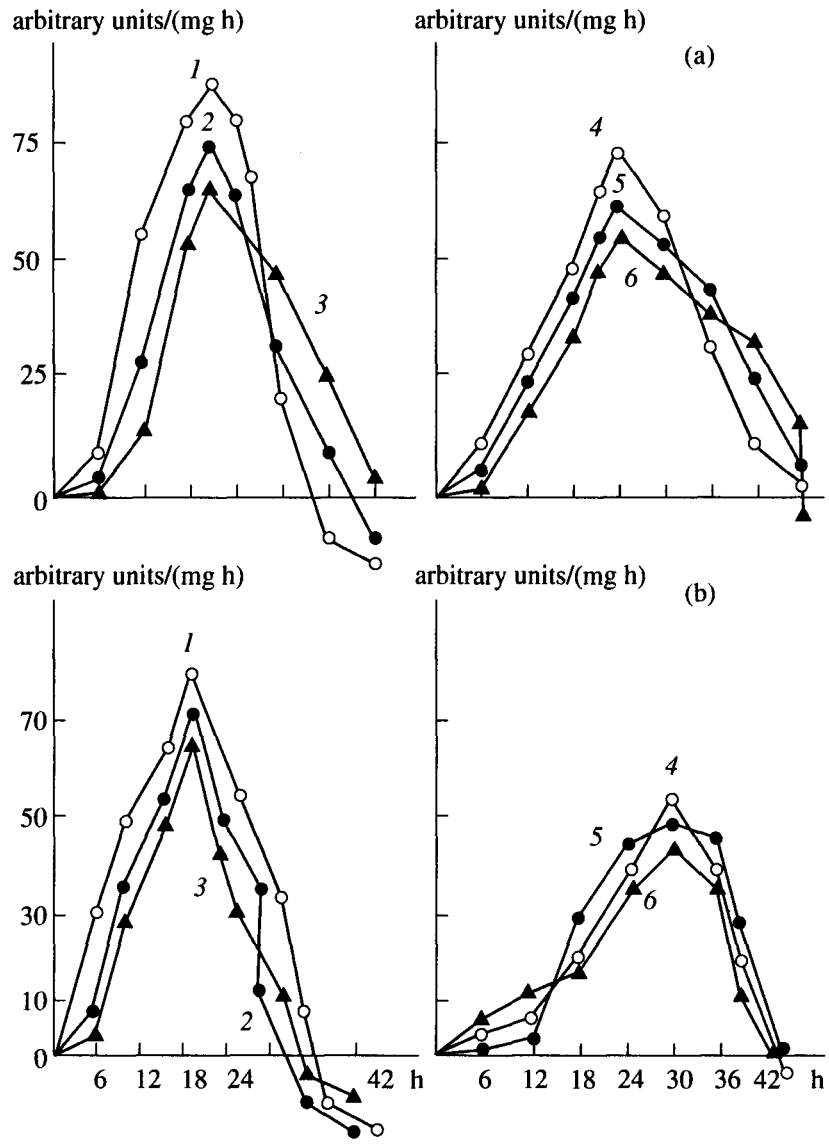

Fig. 2. Effect of glucose concentration in medium on the proteinase synthesis in *B. firmus* 44b (a) and *B. oligonitrophilus* 21p (b): (*1–3*) specific rate of synthesis of plasmin-like proteinases, arbitrary units/(mg h); (*4–6*) specific rate of synthesis of plasminogen-activating proteinases, arbitrary units/(mg h); glucose content in the medium: (*1, 4*) 1%; (*2, 5*) 2%; (*3, 6*) 4%.

(75 arbitrary units/(mg h)) occurred over 24–30 h of growth.

B. oligonitrophilus 21p displayed less extensive growth (maximum growth rate was 0.34 h^{-1}) and production of the plasmin-like proteinases (105 arbitrary units/(mg h)) that earlier achieved the maximum (by 18–24 h of cultivation) (Fig. 1b). The level of enzyme production was the same as with *B. firmus* 44b (75 arbitrary units/(mg h)) but achieved the maximum only by 30–36 h and not by 24–30 h, as was the case with *B. firmus* 44b.

Thus, in both microorganisms studied, the active synthesis of plasmin-like proteinases occurred when their growth decelerated and, therefore, a 6-h interval was observed between the period of maximum accumulation of these enzymes and the maximum growth rate of *B. firmus* 44b; a 3-h interval was observed between these parameters in *B. oligonitrophilus* 21p. The intervals between the maximum growth rate periods of these two microorganisms and the maximum accumulation of the plasminogen-activating proteinases was 9 and 12 h, respectively.

Thus, it is possible to determine the efficiency of different biosynthetic stages and obtain the preparations containing the prevalent amounts of a particular proteolytic enzyme.

It is known that proteinase synthesis in bacteria may be regulated through catabolite repression. An increase in the glucose concentration in the medium leads to a considerable increase in the growth rate of the producer and in the biomass yield, whereas the proteolytic enzyme synthesis is repressed partially or com-

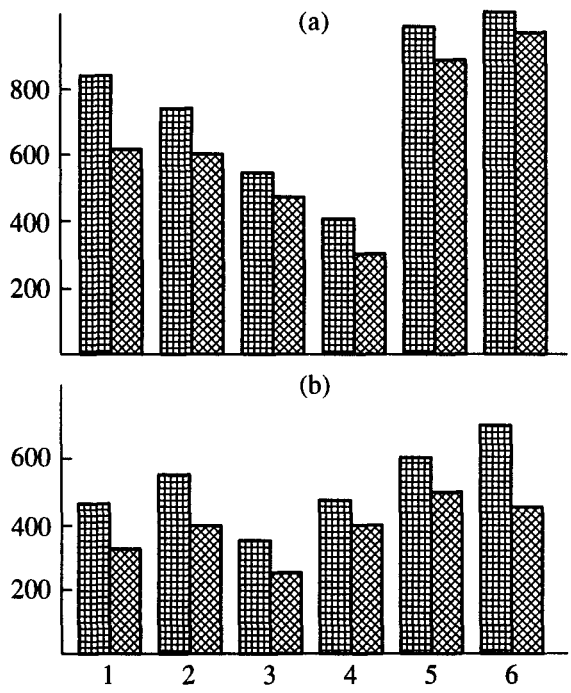

Fig. 3. Effect of protein and peptide substrates, added to the medium as a single carbon and nitrogen source, on proteinase synthesis in *B. firmus* 44b (a) and *B. oligonitrophilus* 21p (b): the ordinate shows the production of (▦) plasmin-like and (▩) plasminogen-activating enzymes, arbitrary units/mg of biomass: (1) control (glucose and ammonium nitrate); (2) casein; (3) fibrin; (4) gelatin; (5) bovine serum albumin; (6) enzymatic peptone.

pletely and not restored until the glucose is utilized. However, the behavior of both marine bacilli studied in our experiments was different (Fig. 2). Thus, with the increase in the glucose content in the medium from 1 to 4%, the biomass yield increased insignificantly, and the specific rates of synthesis of both plasmin-like (Fig. 2a, curves *1–3*) and plasminogen-activating (Fig. 2a, curves *4–6*) enzymes became slightly reduced in *B. firmus* 44b, whereas in *B. oligonitrophilus* 21p, they remained almost the same as in the control. Glucose concentration of 7 or 10% strongly inhibited both the growth of the studied organisms and their biosynthetic processes. This may be due to the adaptation of the marine bacteria to low contents of nutrient substances in their environs.

Induction of proteinase biosynthesis by peptides and proteins also appeared to be ineffective in the studied marine bacteria. When casein, fibrin, or gelatin served the only carbon and nitrogen source in the growth medium of *B. firmus* 44b, the amount of proteinases synthesized per one unit of biomass was reduced (Fig. 3a). The production of both enzyme groups slightly increased when bovine serum albumin or peptone were added to the growth medium. The same was observed with *B. oligonitrophilus* 21p, although the reaction to the proteins and peptides introduced was still less expressed. Note that the ratio of plasmin-like to plasminogen-activating enzymes within a proteinase complex synthesized was, as a rule, similar to that observed in the control when the bacteria were grown on the synthetic medium containing glucose and ammonium nitrate.

In our further experiments, we found that the repression–derepression, in particular, the repression by nitrogen deficiency, was the most efficient and rapid mechanism that regulated proteinase biosynthesis in the studied marine bacteria (Fig. 4). In short-term experiments with washed cells collected at the period of maximal production of proteolytic enzymes and incubated in medium under nitrogen or carbon source deficiency, the maximum activity of plasmin-like and plasminogen-activating enzymes was found in both producers after the repression by nitrogen deficiency was released. In this case, the lag-period preceding the secretion of proteolytic enzymes was insignificant, and the maximum level of the enzymes was rapidly reached. However, in *B. firmus* 44b, the accumulation of the plasminogen-activating enzymes was the highest after 4 h of incubation, whereas the maximum activity of plasmin-like enzymes was observed after 6 h of incubation. In *B. oligonitrophilus* 21p, the plasmin-like enzymes were secreted earlier than the plasminogen-activating enzymes (the maximum levels of these enzymes were reached after 4 and 8 h of incubation, respectively). In the studied microorganisms, both enzyme groups displayed a poor response to the elimination of the repression by carbon deficiency (Fig. 4), and their activity was revealed in the culture medium at the same time in the control and in the experiment.

Thus, our results indicate that the maximum growth rate of the marine bacilli and the highest synthesis of their proteinases do not coincide in time. The secretion of both plasmin-like and plasminogen-activating enzymes occurred during the post-exponential phase of microorganism development, and the activating enzymes reached the maximum production rate much later than the plasmin-like enzymes.

Proteinase synthesis in *B. firmus* 44b and *B. oligonitrophilus* 21p was adequately controlled by repression–derepression rather than by the induction mechanism, which is a specific feature of these bacteria. The production of proteolytic enzymes was poorly stimulated by the protein substrates and catabolite repression. The studied organisms are likely adapted to the environment with a low content of nutrient substances.

Repression by nitrogen deficiency was the most efficient control of proteinase synthesis in the studied organisms. Note that although the sensitivity of plasmin-like and plasminogen-activating enzymes to repression by nitrogen deficiency was similar, they differed in their reaction to the repression by carbon deficiency. The production of activating proteinases depended on the carbon source to a lesser extent than the production of plasmin-like enzymes. This opens up possibilities for obtaining the predominant synthesis of

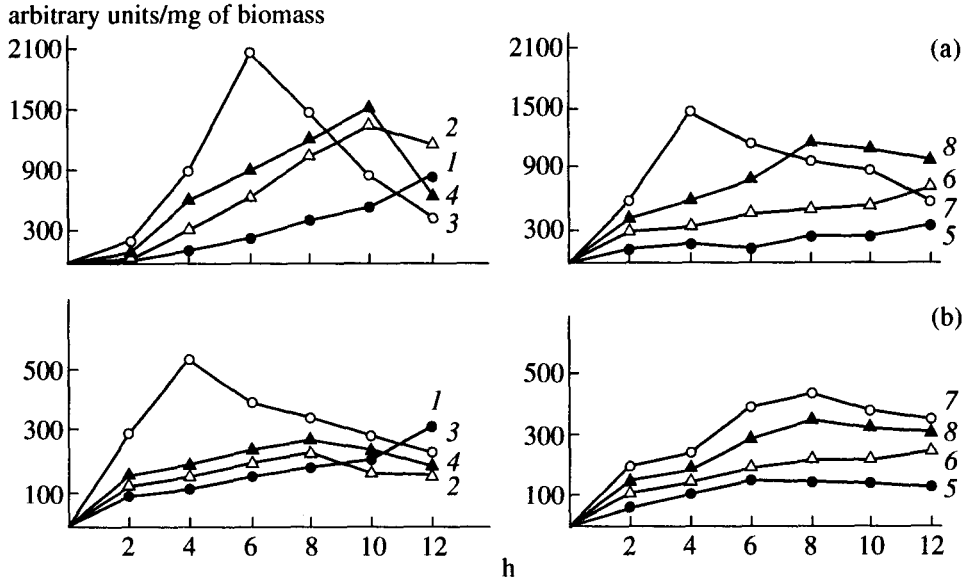

Fig. 4. Dynamics of proteinase synthesis by washed cells of *B. firmus* 44b (a) and *B. oligonitrophilus* 21p (b) after the repression by nitrogen or carbon deficiency was eliminated: (*1–4*) plasmin-like activity, arbitrary units/mg of biomass; (*5–8*) plasminogen-activating activity, arbitrary units/mg of biomass; (*1, 5*) control (synthetic medium containing glucose and ammonium nitrate as carbon and nitrogen sources); (*2, 6*) control without the carbon source; (*3, 7*) control without the nitrogen source; (*4, 8*) control (with neither the carbon nor the nitrogen source).

a required enzyme using a proper carbohydrate component and/or varying the proportion of carbon and nitrogen sources in culture medium.

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