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PURIFICATION OF EXTRACELLULAR α-AMYLASE AND PROTEINASE FROM *Bacillus subtilis* SKB 256

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The enzymes α-amylase and proteinase with specific activity 310 and 68-72 units/mg, respectively, were isolated from Bacillus subtilis SKB 256 culture medium. The enzyme preparations were purified and α-amylase and proteinase were separated using biospecific chromatography. Gel filtration of α-amylase was performed over a column (20 × 600 mm) of TSK HW-55F gel (Toyopearl, Japan).

Keywords: *Bacillus subtilis* SKB 256, α-amylase, proteinase, biospecific chromatography.

The enzymes amylase and proteinase are widely used in scientific research, medicine, and pharmaceutics. Because of this, ultrapure preparations of these enzymes are currently in high demand.

Our goal was to develop conditions for preparing high-purity enzyme preparations of α-amylase and proteinase from *Bacillus subtilis* SKB 256, a producer of these enzymes.

Various classical and modified methods of protein chemistry involving the specifics and properties of the enzymes to be purified were employed in order to develop the most effective and reproducible methods for preparing highly active enzyme preparations. In the first stages, enzymes isolates were purified of a large quantity of ballast proteins, free amino acids, carbohydrates, and other substances contained in the culture medium (CM) filtrate of the producer. Protein fractions with the highest activities were isolated using fractional precipitation of proteins by ammonium sulfate, isopropanol (i-PrOH), and acetone. Enzyme preparations with the highest activity were obtained by precipitation of enzymes from CM using i-PrOH.

α-Amylase was adsorbed using a modified literature method [1]. After the enzyme was adsorbed on starch, α-amylase was separated from it by incubation at 40°C for 60 min. Under these conditions, the starch was hydrolyzed and bound α-amylase was released into the solution. Non-specifically bound proteinase and other proteins were simultaneously released.

Thus, biospecific adsorption on starch produced α-amylase preparation with specific activity 153.7 units/mg that was partially purified from associated protease and ballast proteins.

α-Amylase was further purified by gel filtration of this fraction over a column of TSK HW-55F gel at flow rate 48 mL/h. Fractions of 4 mL were collected. An α-amylase fraction with specific activity 310 units/mg was obtained. Disk electrophoresis in PAAG gel showed that this enzyme was homogeneous.

Proteinase is known to have a rather high affinity for protein substrates such as hemoglobin or cytochrome C. The binding strength with them is much greater than with other proteins or synthetic peptides (although the catalytic step may be slower). Therefore, we hypothesized that biospecific sorbents could be fabricated based on these substrates using porous supports impregnated with the proteins.

The synthetic sorbent sorsilen, a copolymer of terephthalic acid and ethyleneglycol [2], was selected based on results of preliminary tests of several sorbents (polyamides, basalt fibers, inorganic polymers, Millipore filtration media, etc.). Commercial sorsilen has pore volume 2–3 cm^3/g and specific surface area 50–120 m^2/g of polymer. Complexes with proteins are stable over a wide pH range, from 3.0 to 8.5, in neutral salt solutions (2 M).

Preliminary studies found that adsorbed hemoglobin and cytochrome C underwent enzymatic hydrolysis upon incubation in solutions containing proteinase. Furthermore, EtOH inhibited this process. The enzymatic process in media with >20% EtOH was practically stopped although the presence of EtOH had little effect on binding of the enzyme to hemoglobin.

A study of the effect of monoatomic alcohols on the activity of proteinase from *B. subtilis* SKB 256 showed that the enzyme specific activity was reduced in the presence of EtOH.

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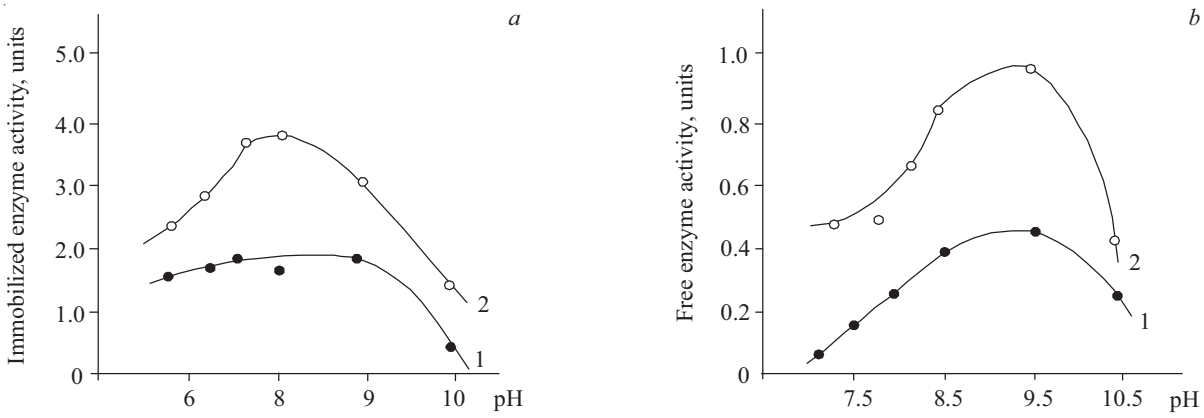

Fig. 1. Effect of pH on adsorption (a) and desorption (b) of proteinase from *B. subtilis* SKB 256 in the presence of NaCl (0.1 M): sorsilen impregnated with hemoglobin (1) and sorsilen impregnated with cytochrome C (2).

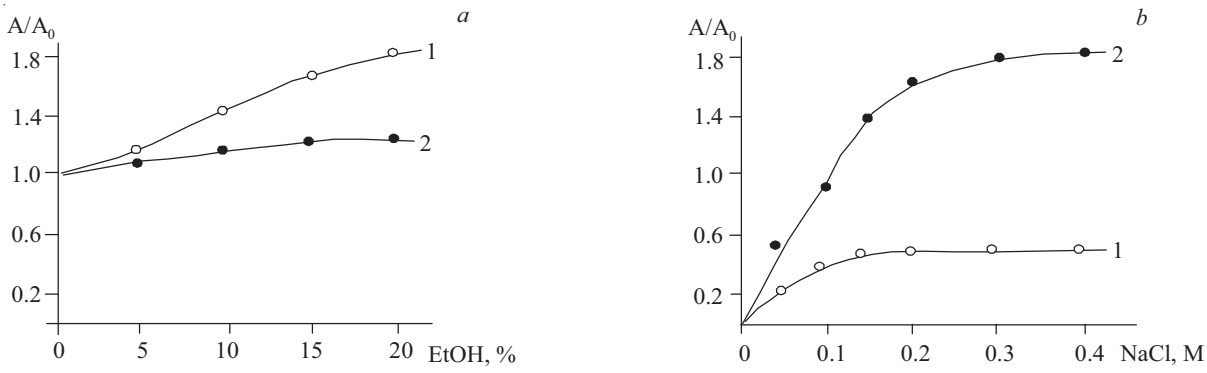

Fig. 2. Effect of eluent composition on desorption of proteinase from *B. subtilis* SKB 256: EtOH in NaCl solution (0.1 M) (a), NaCl (0.1 M) in EtOH (20%) (b); sorsilen–hemoglobin (1), sorsilen–cytochrome C (2). A, activity of enzyme desorbed in the presence of EtOH; A_0, activity of enzyme desorbed without EtOH.

The pH value of the aqueous EtOH had a dramatic effect on the binding of proteinase from *B. subtilis* SKB 256 to the biospecific sorbent (Fig. 1).

The binding of proteinase to the protein substrates after sorsilen was impregnated with them was greatest at pH values 7.0–8.0 (Fig. 1a). Under these conditions, sorsilen (1 g) impregnated with hemoglobin bound 2.8-3.2 activity units of enzyme; support (1 g) impregnated with cytochrome C, 1.6–1.8 activity units. A large amount of the sorbed enzyme can be desorbed upon elution with NaCl (1 M) at pH 8.5-9.0 (Fig. 1b). The activity yield for both sorbents under these conditions was 35–38% of the total adsorbed enzyme activity.

Next, we studied the effect of the NaCl concentration in EtOH eluent on desorption of proteinase from *B. subtilis* SKB 256 from the biospecific sorbent (Fig. 2). Proteinase can be desorbed upon elution with NaCl (0.1 M). This desorbed from sorsilen–cytochrome C A_0 = 65 enzyme act. units; from sorsilen–hemoglobin, 105 enzyme act. units; or about 35 and 38% of proteinase adsorbed on the biospecific sorbent. Figure 2a shows that enzyme desorption with 20% EtOH in the eluent increased from sorsilen–hemoglobin by 1.8 times; from sorsilen–cytochrome C, by 1.2 times.

The solution ionic strength also had a significant effect on enzyme desorption. Figure 2b shows that rinsing the sorbent with 20% EtOH without NaCl did not desorb proteinase although greater than 70% of ballast proteins were removed. Increasing the ionic strength facilitated desorption of proteinase at [NaCl] = 0.2–0.3 M. The desorption curve reached a plateau for both the sorsilen–hemoglobin and sorsilen–cytochrome C systems. The activity yield was typically much greater for the sorsilen–hemoglobin sorbent. The best eluent for this sorbent was 20% EtOH containing NaCl (0.4 M); for sorsilen–cytochrome C, 20% EtOH containing NaCl (0.15 M).

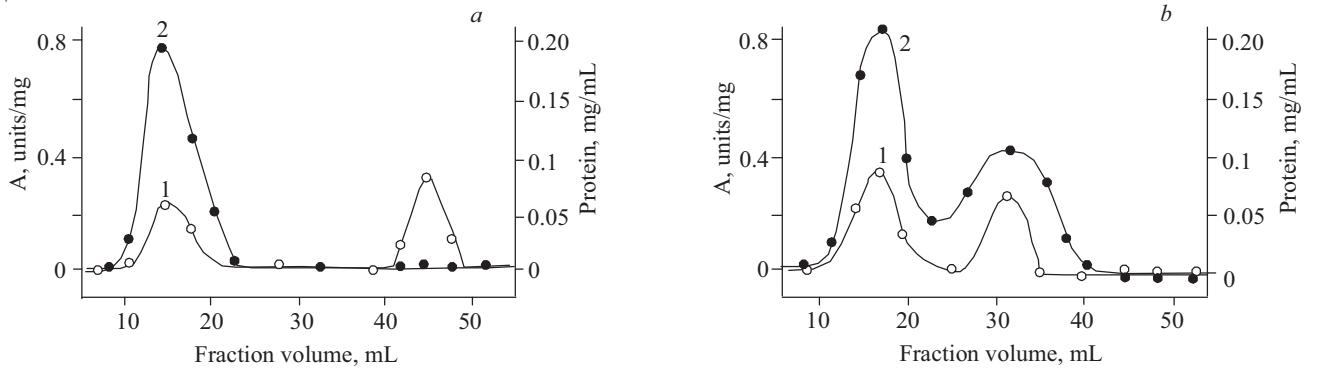

Fig. 3. Gel filtration over Sephadex G-100 of the fraction eluted from biospecific sorbent by water (a) and aqueous EtOH (b); protein content (1) and activity of proteinase from *B. subtilis* SKB 256 (2).

Proteinase from *B. subtilis* SKB 256 obtained after biospecific processing was rather pure but not homogeneous (Fig. 3) because two peaks were eluted by gel filtration over Sephadex G-100. The protein peak and activity peak coincided. These results indicated that *B. subtilis* SKB 256 synthesizes molecular forms of proteinase that differ in molecular weight but have the same affinity for the biospecific sorbent. Carrying out the desorption with 20% EtOH had a positive effect on the enzyme purity. The amount of impurities observed in the effluent was much lower for purification with EtOH (Fig. 3b).

Thus, biospecific sorption on starch and biospecific sorbents prepared by impregnating sorsilen with proteins can be used to purify α-amylase and proteinase from *B. subtilis* SKB 256.

EXPERIMENTAL

We used strain *Bacillus subtilis* SKB 256 from the microorganism collection of the Microbiology Institute of the Academy of Sciences of the Republic of Uzbekistan.

Strain *B. subtilis* SKB 256 is known to be an active antagonist of phytopathogenic fungi and bacteria. It is also known that this culture synthesizes proteolytic enzymes [3].

The culture was grown in liquid nutrient medium containing (g/L) cotton meal extract 10.0, molasses 30.0, K_2HPO_4 7.0, $MgSO_4 \cdot 7H_2O$ 0.1, NH_4NO_3 1.0, $Na_3C_6H_5O_7 \cdot 3H_2O$ 0.5, corn extract 2.5, tapwater 1 L. The culture was grown for 2 d in Ehrlenmeyer flasks on a rocker (200 rpm, UVMT-12-250, NPOtipko Plant Elion, Russia) at 32–36°C.

The amylolytic activity was determined by the literature method [4] using soluble starch as the substrate. Iodine reagent (I_2 + KI) was used as the dye.

The proteolytic activity was determined by a modified Anson method [5]. The unit of activity was the ability of the enzyme to convert casein solution (1%) into a condition that was not precipitated by trifluoroacetic acid in an amount corresponding to tyrosine (1 μmol) in 1 min at 30°C and pH 8.5.

The amount of protein was determined by the Lowry method [6].

The biospecific adsorption of α-amylase on starch was measured by a modified literature method [1]. The resulting protein fraction was used for further investigations as a partially purified α-amylase preparation.

Gel filtration of α-amylase was performed over a column (20 × 600 mm) of TSK HW-55F gel (Toyopearl, Japan) equilibrated with potassium-phosphate buffer (0.05 M) containing KCl (0.1 M) at pH 6.5.

The biospecific sorbent for purifying proteinase was prepared by impregnating sorsilen with hemoglobin (Reanal, Hungary) or cytochrome C (Biomed, Poland). We have previously reported the impregnation conditions [7]. Sorsilen was prepared at the Higher Technical School (Prague, Czech Rep.). We used Sephadex from Pharmacia (Sweden). Bound proteinase was desorbed from the support using NaCl (0.5 M) containing EtOH (20%). Then the eluted fraction was purified by gel filtration by passage over a column (1.4 × 30 cm) of Sephadex G-100. Proteinase was eluted from the column by distilled water. Proteinase of overall activity 68–72 act. units (40 mg) was placed on a column (1.4 × 30 cm) in distilled water (2 mL). Protein content in the effluents was determined by the Lowry method. Proteinase activity was measured by a modified literature method [8].

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