Chemistry of Natural Compounds, Vol. 37, No. 4, 2001

ISOLATION, PURIFICATION, AND PROPERTIES OF α-AMYLASE FROM *Bacillus subtilis*-7A

Sh. Kh. Nurmatov,[1] Z. R. Akhmedova,[1] and D. A. Rakhimov[2]

UDC 577.152.3

Cultivation of Bacillus subtilis-*7A on waste from alcohol production yielded an active extracellular enzyme α-amylase with MW 75 kDa. The enzyme was isolated from the culture medium by 60% saturated ammonium sulfate and purified until homogeneous by gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-cellulose. The optimum temperatures for the complex and purified enzyme are 30 and 50 °C, respectively. The optimum activity for both preparations occurred at pH 6.5. The substrate specificity of the isolated preparations was studied.*

Key words: *Bacillus subtilis,* α-amylase, neutral protease, complex preparation, isolation, purification, properties, amylolytic and proteolytic activity, specificity.

Amylases are one of the most widely distributed enzyme complexes encountered in animals, higher and lower plants, and microbes. They are considered important enzymes in biotechnology and are widely used in the food, medical, textile, and paper industries. Microbial amylases are replacing chemical hydrolysis for starch processing, affecting starch and similar poly- and oligosaccharides and changing the structure of the substrates [1, 2].

The wide range of applications for amylolytic enzymes (AE) requires both technical and purified and highly purified amylases with high activity and stability.

It should be noted that many sources of amylases, in particular, *Bacillus subtilis*, synthesize simultaneously proteolytic enzymes (PE) that interfere with the isolation, purification, and preparation of active homogeneous enzyme preparations [3, 4].

We found the optimum conditions for preparing active enzyme preparation of varying stability from *B. subtilis* and purified α-amylase by separating it from accompanying protease.

An extracellular preparation of amylase containing protease was obtained from the filtrate of culture medium (CM) of the strain *B. subtilis*-7A. The enzyme proteins were fractionated by various concentrations of $(NH_4)_2SO_4$. Precipitation of proteins of CM filtrate by 80% saturated $(NH_4)_2SO_4$ gives protein containing up to 75% of the initial activity whereas use of 60% saturated $(NH_4)_2SO_4$ fractionally separates both enzymes. Specifically, the precipitate contains up to 68% of the enzyme with amylolytic activity (AA) whereas the proteolytic activity (PA) is only 24% of the initial value. Furthermore, a significant portion of the ballast proteins of the CM filtrate remains in solution. This helps to increase the specific activity of AE in the precipitate (Table 1).

The AE were further purified by gel filtration over a Sephadex G-100 column equilibrated with phosphate buffer (0.01 M, pH 6.3) to give three protein fractions that differed in protein yield and enzyme activity. The first fraction had high specific AA (units/g) (141.7); the second, AA (99) and PA (13.5); the third, only PA (8.62). Thus, gel filtration of the total preparation obtained by fractionation of the CM with 60% $(NH_4)_2SO_4$ managed to separate partly α-amylase from accompanying protease. The degree of purification of α-amylase was 10.8 times; the specific activity, 141.7 units/g; the protein yield, 2.8% (Fig. 1 and Table 2).

1) Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (99871) 241 71 29, e-mail: spanta@saturn.silk.org; 2) S. Yu. Yunusov Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (99871) 120 64 75. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 309-312, July-August, 2001. Original article submitted June 11, 2001.

 0009-3130/01/3704-0364$25.00

TABLE 1. Extracellular Preparations of Amylases and Proteases from *Bacillus subtilis*-7A by $(NH_4)_2SO_4$ Fractionation

Stage	Total protein, mg	Enzyme activities								Yield, protein, %
		amylolytic				proteolytic				
		Total, units	Sp., units/mg	Purif. degree	Yield, act., %	Total, units	Sp., units/mg	Purif. degree	Yield, act., %	
CM filtrate	5250	67750	12.9	1	100	14910	2.84	1	100	100
$(NH_4)_2SO_4$ fractionation, %										
20	57.3	-	-	-	-	-	-	-	-	1.09
40	842.5	1063	1.26	0.1	1.57	180	0.21	0.07	1.2	16.05
60	1450.0	45000	31.03	2.4	66.47	3375	2.32	0.82	22.6	27.6
80	1977.0	5100	7.13	0.6	7.5	7281	3.68	1.29	48.8	37.65

TABLE 2. Isolation and Purification of α-Amylase and Neutral Protease from *Bacillus subtilis*-7A

Purif. stage	Total protein, mg	Amylolytic activity				Proteolytic activity				Yield, protein, %
		Total, units	Specific, units/mg	Purif. degree, times	Yield, act., %	Total, units	Specific, units/mg	Purif. degree, times	Yield, act., %	
CM filtrate	3700	48600	13.1	1.0	100	2450	0.66	1	100	100
$(NH_4)_2SO_4$ (60%) precipitation	2475	60210	24.3	1.85	124	879	0.35	0.53	35.9	66.89
Dialysis against tapwater	1704.5	98700	57.9	4.41	203.1	1676.5	0.98	1.48	68.4	46.1
Gel filtration on Sephadex G-100 Fractions:	684	42000	61.4	4.68	86.4	1350	1.97	3.0	55.1	18.49
1	102.4	14512	141.7	10.8	29.9	-	-	-	-	2.77
2	178.8	17700	99	7.5	36.4	2416	13.5	20.5	98.6	4.83
3	302.4	-	-	-	-	2606.4	8.62	13.1	106.4	8.17
IEC on DEAE cellulose II Fractions:										
a	51.6	-	-	-	-	1764	34.2	51.8	72	1.4
b	20.16	-	-	-	-	-	-	-	-	0.54
c	96.48	15408	159.7	12.2	31.7	-	-	-	-	2.61

The second fraction contained active enzymes α-amylase and neutral protease and was purified by ion-exchange chromatography (IEC) over DEAE-cellulose to produce three protein fractions. The first fraction had PA (34.2 units/g) and was eluted with tris-HCl buffer (0.01 M, pH 8.0). Elution with tris-HCl buffer (0.5 M, pH 8.0) gave a solution with no amylolytic or proteolytic activity. The third fraction contained only AA and was eluted with tris-HCl buffer (0.05 M) in a NaCl concentration gradient up to 0.1 M. The degree of purification of α-amylase was 12.2 times; specific activity, 159.7 units/g; protein yield, 2.61% (Fig. 2 and Table 2).

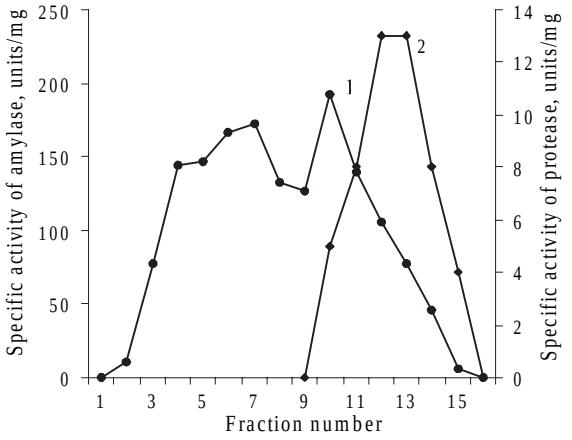

Fig. 1

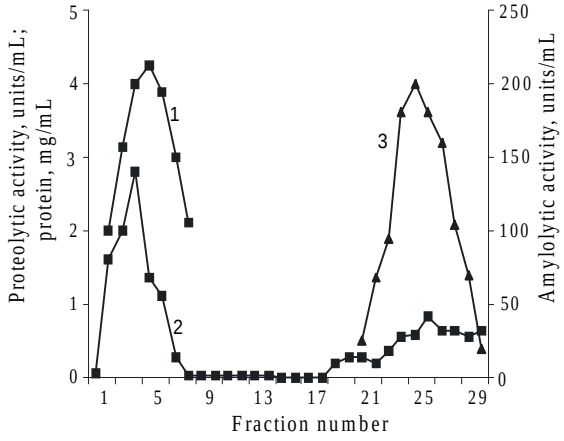

Fig. 2

Fig. 1. Gel-filtration of desalted preparation on a Sephadex G-100 column (25×450 mm) equilibrated with phosphate buffer (0.01 M, pH 6.3): amylase (1) and protease (2).

Fig. 2. Ion-exchange chromatography II of active fraction on DEAE-cellulose. Column 10×250 mm, tris-HCl buffer (0.01 M, pH 8.0). Linear gradient of NaCl (0-0.1 M). Elution rate 24 mL/h. Fraction volume, 2.4 mL: tris-HCl (0.01 M) (1-8), tris-HCl (0.05 M) (9-19), tris-HCl (0.05 M) + NaCl (0.1 M) (20-29). Protease (1), protein (2), amylase (3).

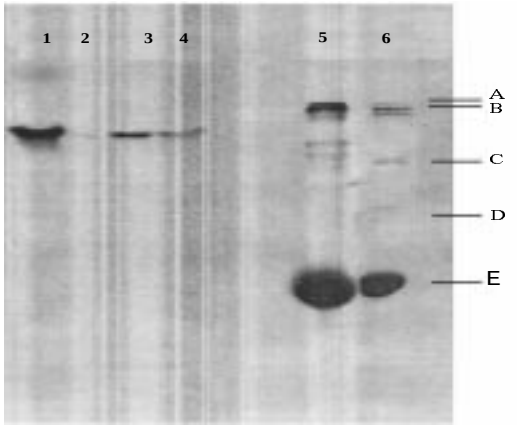

Fig. 3. Electrophoresis of purified α-amylase in 12% PAAG with 0.4% SDS: α-amylase after gel filtration (1) and after IEC (2); lyophilized preparation of α-amylase after gel filtration (3) and after IEC (4); markers (5 and 6): immunoglobulin (A, 160 kDa), alkaline phosphatase (B, 80 kDa), bovine serum albumin (C, 67 kDa), DNA-ase (D, 30 kDa), lysozyme (E, 14 kDa).

The homogeneity was measured and the molecular weight (MW) was determined for the purified α-amylase preparation using electrophoresis in 12% PAAG with SDS. It was found that the α-amylase has MW ~75 kDa and gives one protein band with R_f 0.1 (Fig. 3).

Comparison of the physicochemical and catalytic properties showed that the optimum temperature of the complex and purified preparations differed, 30 and 50°C, respectively (Fig. 4a). The optimum pH was the same, 6.5 (Fig. 4b).

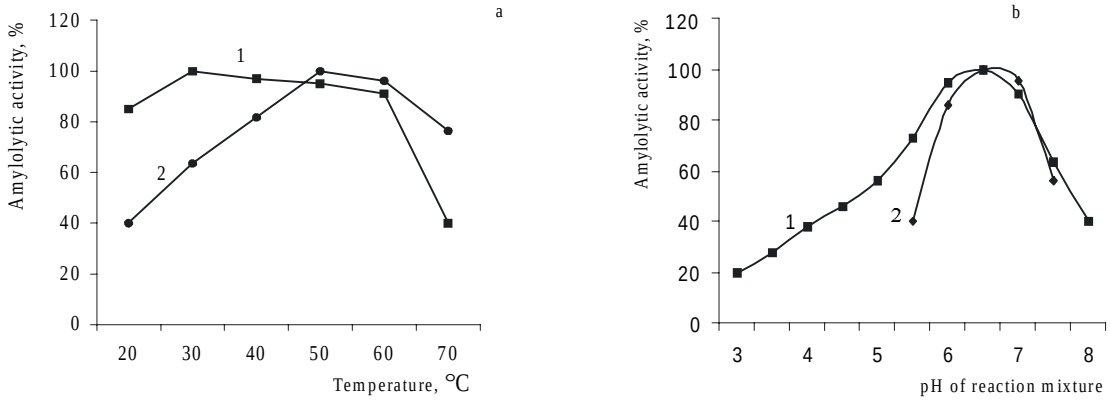

Fig. 4. Temperature (a) and pH optimum (b) activity of complex (1) and purified (2) α-amylase preparations.

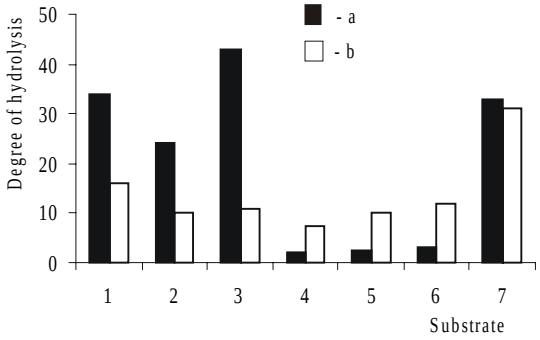

Fig. 5. Substrate specificity of complex (a) and purified (b) α-amylase preparations: mannan (1), glucomannan (2), gledan (3), inulin (4), beet pectin (5), apple pectin (6), potato starch (7).

α-Amylase is known to hydrolyze randomly endo α-1,4-glucoside bonds in amylose, amylopectin, and glycogen. However, the enzyme does not attack these bonds in branched polymers but hydrolyzes exo α-1,4-glucoside bonds, i.e., maltoses from the reducing terminus of the substrate [5, 6].

We investigated the substrate specificity of the complex and purified enzymes to determine the biochemical properties. As it turned out, the enzyme hydrolyzes polysaccharides with only endo α-(1,4)-glucoside bonds. This is characteristic of α-amylase. However, in contrast with purified enzyme, the complex preparation hydrolyzed various starchy and polysaccharide substrates at different rates and with different activities. For example, beet and apple pectin were hydrolyzed by the complex preparation, which indicates that not only α-amylase and protease but also pectinase are present in the preparation (Fig. 5).

Thus, the active enzyme α-amylase and an active amylolytic complex preparation containing amlyase, protease, and pectinase were obtained by cultivating *B. subtilis*-7A on an optimized medium. The α-amylase was isolated and purified to a homogeneous state.

EXPERIMENTAL

The hydrolytic enzyme α-amylase synthesized by *B. subtilis* was investigated. A culture of the bacterium was grown under standard conditions at 30-32°C for 72 h on optimized medium containing wheat mash, wheat flour, diammonium phosphate, urea, and $CaCO_3$.

The bacterium biomass was separated by centrifugation for 20 min at 6000 rpm. The supernatant was used to isolate AE. The AA was determined by iodometry [7]; PA, by a modified Anson method [8]; protein quantity, by the Lowry method [9].

The enzyme proteins were fractionated by salting out at 4°C for 24 h. The precipitate was separated by centrifugation at 8000 rpm for 30 min. The supernatant was desalted using dialysis first against tapwater and then distilled water for 24 h at 4°.

Gel filtration of the desalted enzyme solution was performed on a Sephadex G-100 column (25×450 mm) equilibrated with phosphate buffer (0.01 M, pH 6.3). Elution used this same buffer at flow rate 24 mL/h to give fractions of 4 mL.

IEC of the second fraction was carried out on a DEAE-cellulose column (10×250 mm) equilibrated with tris-HCl buffer (0.01 M, pH 8.0) with a linear concentration gradient of NaCl (0-0.1 M). The flow rate was 24 mL/h to give fractions of 2.4 mL.

Determination of Substrate Specificity. The substrate specificity of complex and purified enzymes was determined by viscometry in an Ostwald viscometer using 1% solutions of various polysaccharides.

The viscosity of blank substrate, i.e., the travel time in the viscometer (control), was measured. Then, enzyme solutions (1 mL) with a calculated AA of 10 units/mL were added. The mixtures were incubated at 32°C for 10 min. The viscosities were again measured after this time (experimental). The differences in the times for the incubated mixtures and the control indicated the degree of hydrolysis and specificity of the enzyme for a given polysaccharide substrate.

In all stages of purification the AE homogeneity was controlled by electrophoresis in 12% PAAG with SDS [10].

REFERENCES

1. A. Pandey, P. Nigam, C. R. Soccol, V. T. Soccol, D. Singh, and R. Mohan, *Biotechnol. Appl. Biochem.*, **31**, 135 (2000).
2. W. M. Fograty and C. T. Kelly, in: *Economic MIcrobiology, Microbial Enzymes and Bioconversions*, Academic Press, London (1980).
3. G. I. Kvesitadze, *Fungal and Bacterial Amylases* [in Russian], Metsniereba, Tbilisi (1984).
4. N. A. Kichakova, I. N. Pavlova, and I. Ya. Zakharova, *Prikl. Biokhim. Mikrobiol.*, **34**, 503 (1998).
5. P. Nigam and D. Singh, *Enzyme Microb. Technol.*, **17**, 770 (1995).
6. N. A. Zherebtsov, O. S. Korneeva, and T. N. Tertychnaya, *Prikl. Biokhim. Mikrobiol.*, **35**, 123 (1999).
7. A. P. Rukhlyadeva and G. V. Polygalina, *Methods for Determining Activity of Hydrolytic Enzymes* [in Russian], Legkaya i Pishchevaya Prom., Moscow (1981).
8. I. S. Petrova and M. M. Vintsyunaite, *Prikl. Biokhim. i Mikrobiol.*, **2**, No. 3, 322 (1966).
9. O. H. Lowry, N. G. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10. U. K. Laemmli, *Nature (London)*, **227**, 681 (1970).