Chemistry of Natural Compounds, Vol. 43, No. 4, 2007

ISOLATION AND CHARACTERISTICS OF HIGHLY ACTIVE α-AMYLASE FROM *Bacillus subtilis*-150

K. T. Normurodova, Sh. Kh. Nurmatov, B. Kh. Alimova, O. M. Pulatova, Z. R. Akhmedova, and A. A. Makhsumkhanov

UDC 577.152.3

Partially purified enzyme preparation with specific activities of 153.7 U/mg for α-amylase and 0.15 U/mg for protease was produced by selective adsorption on starch. Enzymes were purified until homogeneous electrophoretically by gel-filtration over HW-55 TSK-gel with specific activities of 245 U/mg for α-amylase and 1.44 U/mg for protease. The optimum temperature and pH for purified α-amylase activity are 40-50°C and pH 6.0. The effects of various metal ions on the activity and stability of the enzyme were studied.

Key words: *Bacillus subtilis*-150, α-amylase, protease, enzyme preparation, purification, properties, enzyme activity.

We have studied the local bacterial strain *Bacillus subtilis*-150, which produces active α-amylase and neutral protease and the conditions for active fermentation of the strain cultivated on the optimal nutrient medium [1, 2]. The strain *B. subtilis*-150 differed from the previously studied strain *B. subtilis*-7A in certain cultural signatures and α-amylase activity [3].

Various methods for isolating highly purified and homogeneous forms of bacterial amylase have been reported [4, 5]. Fractional precipitation by $(NH_4)_2SO_4$ for which 60% saturation by $(NH_4)_2SO_4$ produced an enzyme preparation with specific activity 31 U/mg, where the yield by activity was 66.5% [3], was used earlier to prepare α-amylase preparation from *B. subtilis*-7A.

Herein we report the isolation and investigation of the physical chemical and catalytic properties of purified α-amylase from *B. subtilis*-150. We precipitated the protein fraction from culture liquid (CL) using acetone, ethanol, and isopropanol. A high yield by activity (82%) was obtained for precipitation of proteins by isopropanol in a 1:1 ratio. α-Amylase was less sensitive to the action of isopropanol and precipitated almost completely whereas the less stable protease was partially precipitated. The resulting enzyme preparation had specific activity 285.3 U/mg (Table 1).

Successful use of various natural polysaccharides for purification of amylolytic enzymes has been reported [6, 7]. The most suitable sorbent for specific binding of α-amylase was soluble starch, which is very cheap and available. α-Amylase was sorbed on soluble potato starch at -20°C in the presence of ethanol and calcium acetate for 30 min. After the sorption of enzymes on starch was complete, the enzyme—substrate complex was split by increasing the temperature to 40°C and incubating for 60 min to hydrolyze the starch. The α-amylase was released and transferred into buffer with a certain amount of associated protease. We showed previously that α-amylase and protease of this strain have different thermal stabilities [1]. Therefore, the α-amylase activity was retained during incubation for 30 min at 60°C whereas that of protease under the same conditions decreased by more than 80%. Thus, biospecific chromatography over starch was able to produce partially purified α-amylase preparation with a specific activity of 153.7 U/mg.

Gel-filtration of partially purified α-amylase preparation was performed over a column (20 × 600 mm) packed with HW-55 TSK-gel (Toyopearl, Japan). The results showed that the eluted protein fractions 13-18 contained active proteins with both amylase and protease activity where α-amylase was the major elution peak and protease the minor one. α-Amylase was eluted in 20 mL (fractions 12-16) whereas protease was eluted after it in 12 mL (fractions 17-19). Fractions corresponding to peak maxima were used to analyze the purity of proteins. Associated proteins in CL were eluted in six fractions containing various concentrations of proteins.

Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan, 100128, Tashkent, ul. A. Kadyri, 7b, fax (+99871) 144 82 25, e-mail: amakhsum@mail.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 374-376, July-August, 2007. Original article submitted June 15, 2007.

 0009-3130/07/4304-0454 ©2007 Springer Science+Business Media, Inc.

TABLE 1. α-Amylase and Protease Preparations from *Bacillus subtilis*-150 and Their Purification

Preparation and purification step	Protein, mg	Purification degree	α-Amylase activity				Protease activity			
			U/mL	specific U/mL	total, U	yield, %	U/mL	specific U/mL	total, U	yield, %
CL supernatant	800	1	330.0	35.1	28080	100	63.7	6.8	5440	100
Precipitation (isopropanol, 1:1)	80.5	10	4605.8	285.3	22966.7	82	187	11.6	933.8	17.2
Preparation*	61	13	937.5	153.7	9375.7	33.4	0.91	0.15	9.2	0.2
Gel-filtration over HW-55 TSK-gel:										
α-amylase fraction	5	160	128.6	245	1225	4.3	-	-	-	-
protease fraction	0.5	1600	-	-	-	-	0.18	1.44	0.7	0.01

*Prepared after adsorption on starch with subsequent incubation at 60°C.

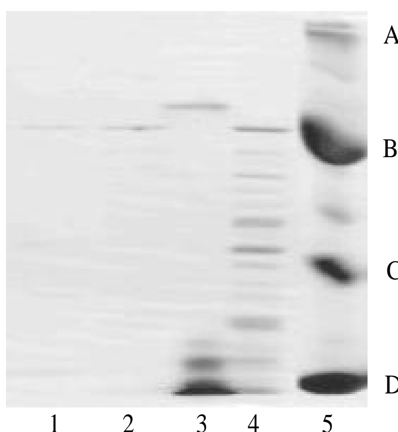

Fig. 1. Electrophoresis (PAAG-SDS, 12%) of α-amylase preparations from *B. subtilis*-150: purified α-amylase after gel-filtration over TSK-gel (HW-55) (1), affinity chromatography of α-amylase on starch (2), fraction from CL precipitation by isopropanol (3), CL of *B. subtilis*-150 (4), markers: phosphorylase B (104 kDa, A), BSA (67 kDa, B), DNA-ase (30 kDa, C), lysozyme (14 kDa, D) (5).

Thus, the aforementioned methods were used to prepare electrophoretically homogeneous fractions of α-amylase and associated protease with rather high activities of 245 U/mg for α-amylase and 1.44 U/mg for protease with a protein yield of 4.3% (for α-amylase) (Table 1).

The homogeneity and molecular weight of purified α-amylase was determined by electrophoresis in PAAG (12%) with SDS [8]. It was found that α-amylase had a molecular weight of about 69-70 kDa (Fig. 1).

The experimental molecular weight was greater than that of α-amylase isolated previously from this bacterium. In particular, the molecular weight of α-amylase from *B. stearothermophilis* culture was 75 kDa [9].

The physical chemical and catalytic properties, optimum temperature and pH, and stability and effect of various metal ions on the enzyme activity were studied to characterize and compare the purified α-amylase preparation produced by us.

The effect of pH on enzyme activity and stability was examined in the pH range 3.5-11.5. Figure 2 shows the optimum pH for purified α-amylase from *B. subtilis*-150 and its stability at various pH values. It can be seen that α-amylase is more stable at pH values 5.5-9.5. The enzyme had the highest activity at pH 6.0 whereas it retained 25-30% of the activity at pH 10.5-11.5.

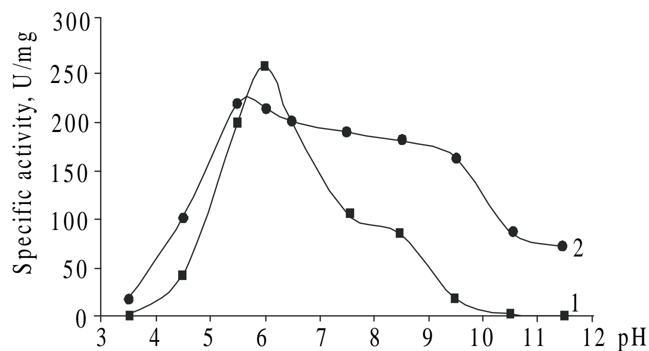

Fig. 2.

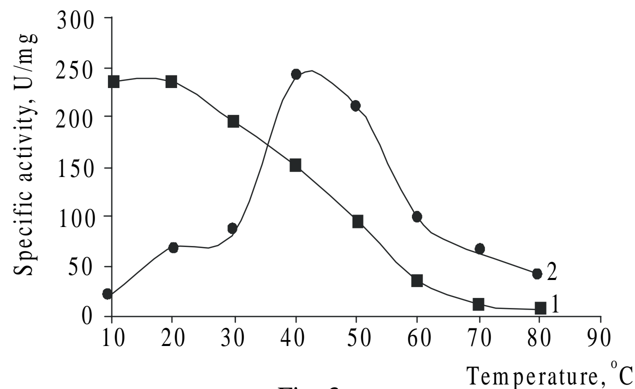

Fig. 3.

Fig. 2. Effect of pH on activity and stability of purified α-amylase from *B. subtilis*-150: optimum (1), stability (2).
Fig. 3. Effect of temperature on activity and stability of purified α-amylase from *B. subtilis*-150 (legend the same as in Fig. 2).

The activity of α-amylase from *B. subtilis*-150 was studied as a function of temperature in the range 10-80°C (Fig. 3). Figure 3 shows the optimum temperature for activity and stability of purified α-amylase from *B. subtilis*-150. The enzyme had the highest activity at 40-45°C for 10 min. At 50°C, the enzymatic activity was 90% of the maximum whereas at 60°C, it was about 50% of the initial activity. After incubation of α-amylase for 30 min at 40 and 50°C, the enzyme retained 68 and 44% of its activity, respectively. The enzyme retained only 3-6% of its maximum activity after incubation at higher (70-80°C) temperatures. Thus, the thermal stability of α-amylase from *B. subtilis*-150 that was studied by us was slightly inferior to several bacterial amylases [10, 11].

As noted previously, amylolytic enzymes are metalloenzymes with up to six Ca^{2+} atoms at the active site [12, 13]. Therefore, we studied the effects of various concentrations and salts of Ca^{2+} and mono- and divalent metals on the activity of *B. subtilis*-150 α-amylase. As it turned out, ions of certain metals at final concentrations of 1 mM had positive effects on the amylase activity. For example, Ca^{2+} increased the enzyme activity by 45%; Mg^{2+} and Na^{+}, 35; Mn^{2+}, 30; K^{+}, 20; and Co^{2+}, 10; whereas Li^{+} (6), Fe^{2+} (24), Cu^{2+} (12), Ni^{2+} (6), and Ba^{2+} (10) inhibited the activity from 6 to 24%.

Thus, a purified enzyme preparation of α-amylase from *B. subtilis*-150 was prepared by sequential use of precipitation by an organic solvent, selective adsorption on starch, and chromatography over HW-55 TSK-gel.

EXPERIMENTAL

Culture of *B. subtilis*-150 was grown in two stages. First, the strain was innoculated in liquid nutrient medium containing tryptone and yeast extract and cultivated for 14-15 h at 30°C on a rocker rotating at 150 rpm. Second, an innoculation (0.5%) of the nutrient medium was transferred to optimized fermentation medium [4] and grown for 84 h at 30°C on a rocker rotating at 150 rpm.

Bacterial cells were separated from CL by centrifugation at 8000 rpm for 20 min. CL was precipitated using isopropanol in a 1:1 ratio.

Gel-filtration of the active protein fraction after precipitation of CL by isopropanol was performed on a column (20 × 600 mm) packed with HW-55 TSK-gel (Toyopearl, Japan) equilibrated with potassium phosphate buffer (0.05 M) containing KCl (0.1 M) at pH 6.5 with elution rate 48-50 mL/h and fraction volumes 3.5-4.0 mL.

The optimum pH and pH stability of the studied enzyme toward soluble starch was determined in phosphate buffer (0.05 M) at pH 3-10 and 40°C. Enzyme without substrate at different pH values of phosphate buffer (0.05 M) was incubated for 30 min after which the activity toward soluble starch was determined.

The optimum temperature of the enzyme was established by incubating the enzyme reaction mixture with substrate for 10 min at 10-80°C in phosphate buffer (0.05 M) at pH 6.0. The thermal stability of the enzyme was determined by incubating enzyme solution without substrate for 30 min in phosphate buffer (0.05 M) at pH 6.0 and 50-90°C.

The effects of various metal ions on enzyme activity were determined in phosphate buffer (0.05 M) at pH 6.0 and metal-ion concentrations of 1 mM.

The homogeneity of amylolytic enzymes was monitored at all purification stages by electrophoresis in PAAG (12%) with SDS using the Laemmli method [8].

REFERENCES

1. K. T. Normurodova, K. D. Davranov, Sh. Kh. Nurmatov, A. A. Makhsumkhanov, B. Kh. Alimova, and O. M. Pulatova, in: *Materials of the Third Moscow International Congress "Biotechnology: State and Prospects,"* part. 1, Moscow (2005), p. 354.
2. K. T. Normurodova, *Uzb. Biol. Zh.*, No. 1-2, 6 (2006).
3. Sh. Kh. Nurmatov, Z. R. Akhmedova, and D. A. Rakhimov, *Khim. Prir. Soedin.*, 309 (2001).
4. A. Linden, F. Niehaus, and G. Antranikian, *J. Chromatogr. B: Biomed. Sci. Appl.*, **737**, 253 (2000).
5. D. R. Nagarajan, G. Rajagopalan, and C. Krishnan, *Appl. Microbiol. Biotechnol.*, **73**, 591 (2006).
6. M. N. Moseley and L. Keay, *Biotehnol. Bioeng.*, **12**, 251 (1970).
7. O. V. Kislukhina, *Mikrobiol. Prom. Ref. Sb.*, No. 3, 3 (1971).
8. U. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
9. D. O. Kang, I. K. Hwang, B. Y. Kim, S. C. Ahn, T. I. Mheen, J. S. Ahn, and S. M. Bvun, *J. Biol. Chem.*, 270 (1995).
10. K. Ratanakhanokchai, J. Kaneko, Y. Kamio, and K. Izaki, *Appl. Environ. Microbiol.*, **58**, 2490 (1992).
11. M. E. Farezvidal, A. Fernandezvivas, F. Gonzales, and L. M. Arias, *J. Appl. Bacteriol.*, **78**, 14 (1995).
12. N. A. Zherebtsov, O. S. Korneeva, and T. N. Tertychnaya, *Prikl. Biokhim. Mikrobiol.*, **35**, 123 (1999).
13. W. M. Fogarty, *Microbial Enzymes and Biotechnology*, Appl. Sci. Publ., London (1983).