

PURIFICATION OF ALPHA-AMYLASE FROM *BACILLUS SUBTILIS* LINE # 1024 WITH ATCC 465

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ABSTRACT

Alpha-amylase E.C.3.2.1.1. (1,4 glucan, 4 glucanohydrolase) can be obtained from salivary glands, pancreas and microorganisms such as *Pseudomonas* and *Asperigillus*, as well as muscles and ovarian tubes.^{1,2} Alpha-amylase from *Bacillus subtilis* #1024(ATCC 465)* was purified with a highest degree of purity in our laboratory (31.59 U/mg).

The extracellular alpha-amylase was subjected to different purification techniques such as anionic and cationic exchange chromatography and preparative electrophoresis.

The final fold of purification was equivalent to 38, which was higher than the previous reported values, polyacrylamide gel electrophoresis with sodium dodecyl sulphate(PAGESDS), Laemmli method, gave a molecular weight of 82000 Daltons.

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INTRODUCTION

The study of the biosynthesis and excretion of extra-cellular proteins by bacteria has long been a concern of investigators. The enzyme obtained from different microorganism classes has played an important role in the enzyme industry.

Alpha-amylase is an endo-enzyme which obtained its name from its hydrolyzing effect on starch with the production of alpha-glucose, a metalloenzyme which binds at least one atom of calcium for each enzyme molecule, the Ca^{2+} ion being apparently

From the mechanistic point of view, alpha-amylase can hydrolyze the alpha(1→4) bonds in polyglucosans, amylose and glycogen.³

Because of chemicoorganotrophic characteristics and the ease with which *Bacillus subtilis* culture can be pro-

duced, we undertook the purification of alpha-amylase from this microbial source,⁴ with the hope that through the elaboration of the method, we could prepare the purified enzyme in high yields.

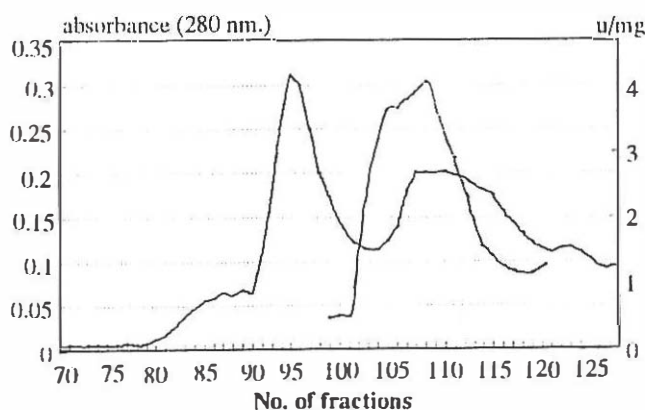


Fig. 1. Purification of alpha amylase by anion exchanger chromatography on D.E. cell.

*ATCC: American Type Culture Collection.

Purification of Alpha-Amylase From *B. Subtilis*

MATERIALS AND METHODS

Bacillus subtilis line #1024 with ATCC 465 was obtained from the Iranian Research Organization for Science and Technology. The cells were separated from the culture medium and were kept at 4°C for further use. The cells were separated by centrifugation at $3500 \times g$ for half an hour.

To 883 ml of supernatant, ammonium sulphate was added gradually while stirring, the final ammonium sulphate concentration was 62% saturation under cold conditions with the final pH= 7.0.⁵ The suspension was centrifuged at $10,000 \times g$ for 15 minutes at 4°C. The precipitate obtained was dissolved in 50 mL of 10 mM of tris buffer containing 2 mM of calcium acetate, pH= 8.4. The solution was dialyzed against the same buffer overnight. The dialyate was concentrated five times by concentration dialysis and 10 mL was applied to a column of DEAE-cellulose* (2.5 × 24 cm) which was equilibrated with the same buffer. A linear NaCl gradient (0-1 M) was used for the process of elution.⁵ 130 fractions (2.5 mL) were collected, and the absorptions were read at 280 nm.

Three distinct peaks were obtained from which the peak obtained from fractions 104-117 had the enzyme activity (Fig. 1).^{6,7} These fractions were pooled and concentrated to 10 mL by concentration dialysis (membrane; exclusion MW, 12000 or less).

The concentrated specimen was dialyzed overnight against 2 mM tris buffer containing calcium acetate (2 mM), PH= 6.4. The dialyate was applied to a cM cellulose* column (2.5 × 23 cm) preequilibrated with the same buffer.

The fractions were collected and assayed for activity and protein content. The maximum activity occurred at fractions 57-70. The protein content was maximal in fractions 50-60 (Fig. 2).

Concentration dialysis was performed on the fractions possessing the highest activities (57-70). The final volume of the concentrated pooled fractions was 5 mL. One milliliter of this dialysate was applied to a preparative electrophoresis column.^{8,9} An alpha-amylase specimen 130 U/mg (Merck) was used as standard (Fig. 3). Our preparation showed a main single band at the same position as the standard alpha-amylase, with a specific activity of 31.59 U/mg. A minor band appeared at fractions 40-42 which did not exhibit any activity and was hence disregarded (Fig. 4).

Analytical electrophoresis performed on the purified sample obtained from CM-cellulose column, showed the main single band when the gel was stained for protein content or amylase activity.^{6,10,12}

*Diethyl-amino ethyl-cellulose

*Carboxy methyl-cellulose

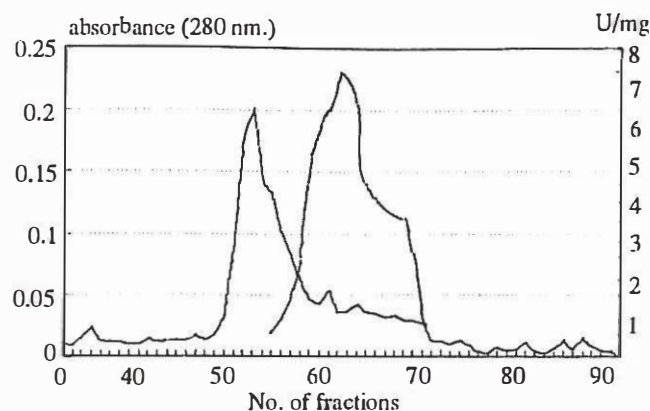


Fig. 2. Purification of alpha amylase by cation exchanger chromatography on C.M. cell.

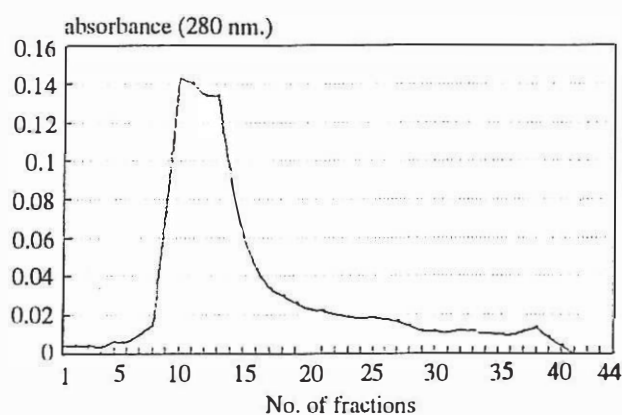


Fig. 3. Preparative electrophoresis, alpha amylase (standard).

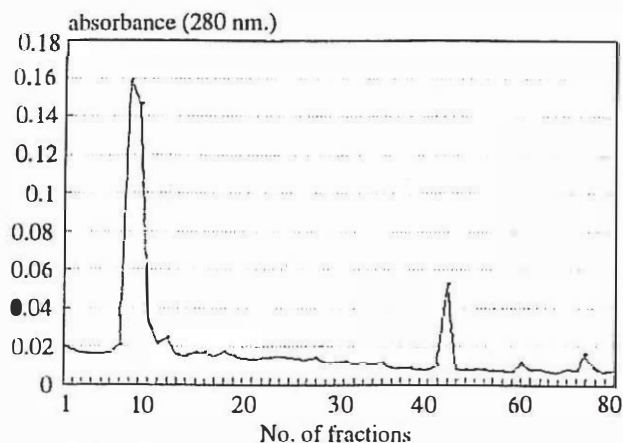


Fig. 4. Preparative electrophoresis, alpha amylase.

Molecular weight determination was carried out using S.D.S. PAGE* method of Laemmli Sigma's kit(MW-SDS-70). Thus the molecular weight of 82000 was obtained for our purified amylase (Fig. 5).^{13,14}

*sodium dodecyl sulphate-PAGE

Table I. Steps in purification of alpha-amylase from *Bacillus subtilis*

Fraction	Total Volume(ml)	Total Protein (mg)	Protein(mg/l)	Total Activity(U)	Specific act.(U/mg)	Purification factor(fold)
Supernatant	883	1236.2	1400	1026.729	0.830	1
Ammonium sulphate precipitate	100	86	860	454.195	5.281	6.4
DEAE-Cellulose Column Chromatography	10	2.66	266	43.903	16.504	19.88
CM-Cellulose Column Chromatography	5	0.145	29	4.238	29.227	35.21
Preparative Electrophoresis	1	0.0113	11.3	0.357	31.59	38.06

RESULTS AND DISCUSSION

The results from purification processes are summarized in Table I.

The final product from preparative electrophoresis showed an activity of 31.59 U/mg with a purification fold of 38.

The use of Laemmli method in estimating the molecu-

lar weight of the purified enzyme showed a single main band with the molecular weight of 82,000 D which assigned to a single unit of amylase.

The previously reported values for alpha-amylase were in the range of 60-90 KD. The variations in molecular weights could be assigned to the line of microbial species used.

The degree of purification was further substantiated by

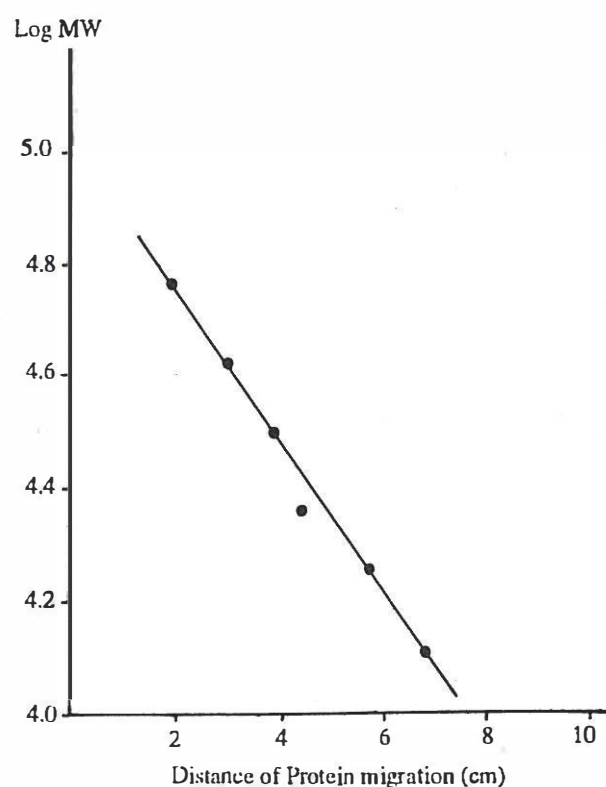


Fig. 5. Standard curve for MW determination of proteins obtained using Laemmli method.

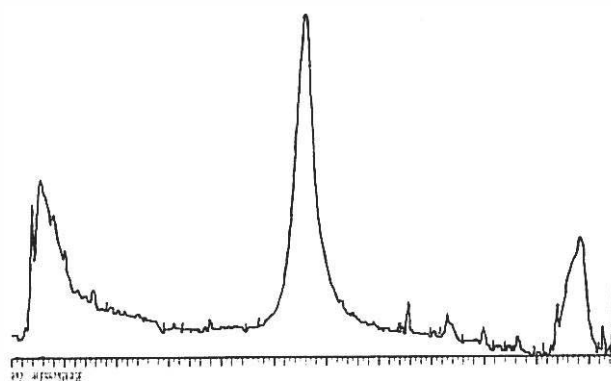


Fig. 6. Alpha-amylase purified from *Bacillus subtilis* (stained for protein by coomassie brilliant-blue staining method).

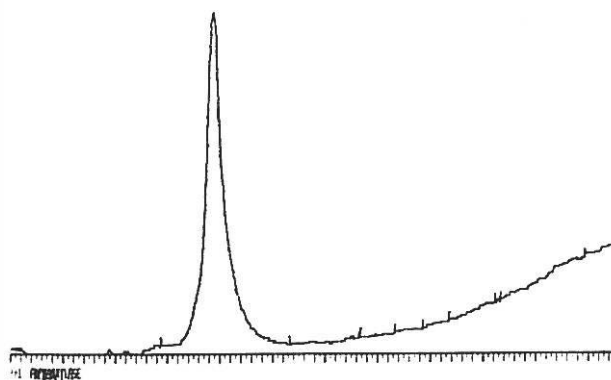


Fig. 7. Standard alpha-amylase from "Merck".

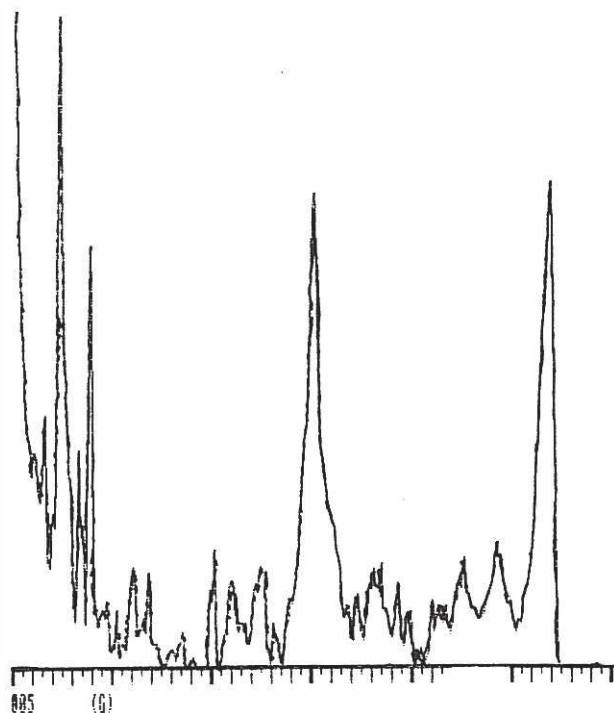


Fig. 8. Alpha-amylase purified from *Bacillus subtilis*.

the use of "PAGE" according to the method of Davis(Figs. 6,7,8).

The membrane-bound alpha-amylase was extracted by the same method as explained for extracellular amylases,⁵ and the two preparations were compared quantitatively. It was found that the extracellular enzyme could have higher yields. Although the line of *Bacillus subtilis* we used was from a common preparation obtained from the Iranian Research Organization for Science and Technology and was not expected to give high yields, we suggest that in using the wild mutant lines, large quantities could be obtained.

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