PURIFICATION, CRYSTALLIZATION, AND PROPERTIES OF THE α-AMYLASE OF *PSEUDOMONAS SACCHAROPHILA**

by

ALVIN MARKOVITZ, HAROLD P. KLEIN AND EDMOND H. FISCHER

Departments of Microbiology and Biochemistry, University of Washington School of Medicine, Seattle, Washington (U.S.A.)

The present paper reports the purification and crystallization of the extracellular α-amylase of *Pseudomonas saccharophila*. Some of the properties of the crystalline enzyme are described and compared with crystalline α-amylases from other sources.

MATERIALS AND METHODS

Details regarding the organism used, its cultivation, and methods for the determination of amylase activity have been presented[1]. In the present study, maltose at a final concentration of 0.2 % was used as the sole carbon source for the growth of these bacteria. Submerged cultures were grown in 20 l carboys containing 18–19 l of medium. The cultures were vigorously aerated by passing air into the medium through an "extra rough" sintered glass gas disperser.

Carbon determinations. Oxidation of the amylase to CO_2, and the collection of this gas as $BaCO_3$ were accomplished by using the reagents and apparatus described by CALVIN *et al.*[2]. This procedure was modified in two ways. Potassium iodate was omitted from the oxidizing mixture, and the samples for wet oxidation were routinely heated for 15 min., cooled 15 min., and again heated for 5 min. This heating procedure was adopted to prevent loss of radioactivity resulting from incomplete combustion. EVANS AND HOUSTON[3] have shown that the ^{12}C–^{14}C bond is more difficult to split than the ^{12}C–^{12}C bond.

Chemicals. Merck soluble starch was used for the starch-iodine assay and for enzymic digest preparations, while "Noredux" soluble starch** was employed for measuring increase in reducing groups. Baker's "Potato starch for iodometry" and Celite 525*** were used for the column purification of the extracellular α-amylase.

Electrophoretic and ultracentrifugal analyses. The Perkin-Elmer electrophoresis apparatus and the Spinco analytical ultracentrifuge were used throughout this work.

RESULTS

Purification and crystallization of the alpha-amylase

The initial steps in the purification of the extracellular alpha-amylase were carried out according to the procedure developed by THAYER[4]. His method involves adsorption of the enzyme on a starch-celite column after the organisms have been removed by centrifugation. The enzyme is then eluted using a 0.5% or 1.0% soluble starch solution, or by an enzymic digest of soluble starch[4]. This latter method was utilized here

* Supported in part by State of Washington funds for medical and biological research. The work reported here was taken in part from a thesis submitted to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy by ALVIN MARKOVITZ, March 1955.

** Ziegfried A.G., Zofingen, Switzerland.

*** Johns-Manville Co., San Francisco, California.

References p. 273.

as it allows a free flow of the eluant through the column. The procedure finally adopted for the purification and crystallization of the extracellular α-amylase of *P. saccharophila* is as follows. The organisms are grown in 20 l carboys, and after they have depleted the maltose supplied (about 24 h), the cells are removed from the culture medium by means of the Sharples supercentrifuge. If necessary, the culture is centrifuged in this manner a second time. The supernatant fluid, designated as (E_1)* (see Table I) is cooled overnight in the cold room and then passed through a column of starch-celite (1:2) to remove the enzyme from the culture medium, which can then be discarded, The columns for the adsorption are 10 cm in diameter and are filled to a height of 9 to 15 cm with the starch-celite mixture. The amylase is eluted according to the gradient elution technique described by THAYER[4], using an enzymic digest of a 1% starch solution as eluant. The identification of fractions of high enzymic activity is facilitated by employing the semi-quantitative spotplate test described by THAYER[4]. The eluate (approximately 875 ml, E_2) is lyophilized and the dry residue taken up in a small volume of distilled water (approximately 23 ml), after which this solution is centrifuged in the Spinco Model L centrifuge for one hour at 40,000 r.p.m.

TABLE I

PURIFICATION AND CRYSTALLIZATION OF THE α-AMYLASE OF *Pseudomonas saccharophila**

Enzyme fraction	*Units/ml*	*Volume (ml)*	*per cent original activity***
E_1	68	66,735	100
E_2	3,200	875	62
E_3	96,000	22.7	49
E_4	—	—	—
E_5	400,000	4.8	45
E_6	—	—	—
E_7	380,000	2.0	22
E_8	18,000	1.9	1
E_9	360,000	2.0	21

* Enzyme units measured by the starch-iodine method. For a description of enzyme fractions, see text.

** Corrected for enzyme removed.

The supernatant fluid (E_3) is treated with a saturated solution of ammonium sulfate (pH 5.3) to 75% saturation; the resulting precipitate, after centrifugation, is dissolved in water and dialyzed in the cold room for about 20 h against 0.001 *M* NH_4OH and 0.001 *M* $CaCl_2$, adjusted to pH 7.5. The dialyzed protein solution (E_4) is reprecipitated with ammonium sulfate at 75% saturation at pH 5.3 and the new precipitate is dissolved in approximately 5 ml of distilled water, (E_5). Solid sodium acetate is added to (E_5) to a final concentration of 0.05 *M* and the solution is fractionated with acetone** at —10°C between 40 and 75%. The white acetone precipitate (E_6) is dissolved in a small volume of water, and from this solution a precipitate is obtained by adding a a saturated solution of ammonium sulfate at pH 5.3 to 75% saturation. This precipitate, after centrifugation, is dissolved in a minimum amount of distilled water. Left

* Henceforth, in this paper the various enzyme fractions will be designated in parentheses.

** The acetone used was purified by $KMnO_4$–Na_2CO_3 treatment and then redistilled.

References p. 273.

in the cold room (4°C) in this state, the enzyme crystallizes, the quantity of crystals increasing substantially in a period of a week. The suspension of crystals is designated as (E_7), the supernatant above the crystals as (E_8) and the crystalline pellet, taken up in the same volume of water, as (E_9).

In one such purification, shown in Table I, the recovery of the enzyme in the form of crystals represented 21% of that present in the starting material. It is interesting to note that 95% of the enzymic activity of fraction (E_7) was found in the crystals. Fig. 1 shows a photomicrograph of crystalline α-amylase obtained by the method described.

Fig. 1. Crystalline extracellular α-amylase of *Pseudomonas saccharophila*. 450 ×

Criteria of purity for the alpha-amylase

The purification of the enzyme was followed by the determination of the specific activity of the various fractions, as well as by electrophoresis and ultracentrifugation at various pH values between 5.5 and 8.0. The electrophoretic and analytical ultracentrifuge analyses usually showed that fraction (E_5) was homogeneous with respect to molecular charge and size. Sometimes, at this stage, small amounts of contaminating material could be detected, but these were eliminated in the final steps of the purification. Electrophoretic and ultracentrifugal patterns of a preparation at the (E_5) stage are shown in Fig. 2 and 3 respectively.

Using our best preparations, we obtained specific enzymic acitivities of 1600–1700 mg maltose produced per mg carbon and 5700–6000 mg maltose produced per mg nitrogen, using the reducing sugar method[1] to assay the enzymic activity.

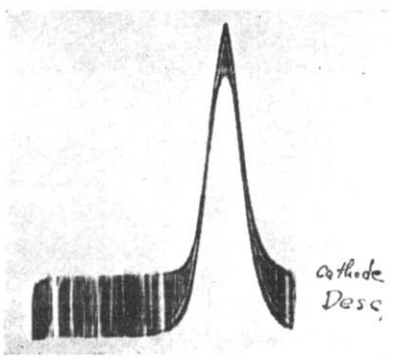

Fig. 2. Electrophoretic pattern of the α-amylase of *Pseudomonas saccharophila*. Ionic strength = 0.1 in phosphate buffer pH 7.0. Time of photograph = 78 min; mobility = $1.0 \cdot 10^{-5}$ cm²/sec.

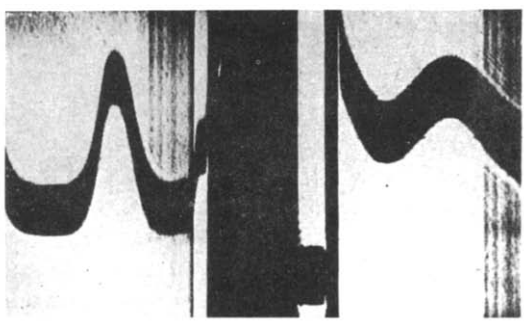

Fig. 3. Ultracentrifugal pattern of the α-amylase of *Pseudomonas saccharophila*. Ionic strength = 0.1 in phosphate buffer pH 7.0. Picture 1 at 16 min; picture 2 at 80 min. $S_{20,w}$ = 4.66. 260,000 *Xg*.

References p. 273.

Properties of the alpha-amylase

All of the following studies were carried out using a crystalline preparation of the enzyme. *Activity as a function of pH*: For these experiments, the enzyme was dissolved in *M*/30 phosphate-*M*/30 succinate buffer adjusted to the desired pH. Fig. 4 illustrates the relationship between activity and pH, and shows the enzyme to be active between pH 3 and 7.7, with a range of optimal activity between 5.25 and 5.75. *Stability as a function of pH:* In these studies, enzyme solutions were incubated at room temperature at various pH values. *M*/30 phosphate-*M*/30 succinate buffers were used between pH 3 and 7, while *M*/30 glycine buffer served between pH 8 and 11. After the desired length of time at a particular pH, the enzyme solutions were diluted 10-fold with *M*/15 acetate buffer at pH 5.5 prior to enzyme assays. Table II contains data demonstrating that the enzyme is stable between pH 4.5 and 8. *Activity as a function of temperature*: Enzymic assays for these experiments were carried out at temperatures between 0° and 40° C, at intervals of approximately 5° C, the exact temperature of any assay being determined in a duplicate tube containing the same volume of water.

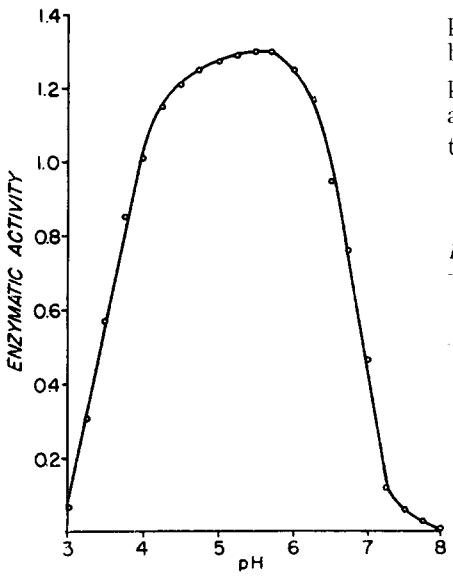

Fig. 4. Activity of the α-amylase of *Pseudomonas saccharophila* as a function of pH. Activity is given in terms of the reducing sugar method[1].

TABLE II

STABILITY OF THE α-AMYLASE OF *Pseudomonas saccharophila* AS A FUNCTION OF PH*

pH	Enzymic activity after:		
	1 hour	4 hours	20 hours
3.0	0.48	0.13	0.05
4.0	1.35	0.95	0.67
4.5	1.25	1.27	1.25
5.0	1.37	1.21	1.29
6.0	1.15	1.15	1.37
7.0	1.32	1.21	1.29
8.0	1.18	1.21	1.24
9.0	1.10	0.91	0.91
10.0	1.01	0.85	0.68
11.0	0.89	0.63	0.37

* Activity is given in terms of the reducing sugar method[1].

Furthermore, the time for each assay varied from 1 to 30 min., depending on the temperature of incubation, in order to obtain approximately the same reducing values at the end of the tests. As a result of these determinations, it was found that this enzyme is maximally active at 40° C. A plot of the logarithm of the velocity as a function of the reciprocal of the absolute temperature is given in Fig. 5. Using Arrhenius' equation, the energy of activation of the reaction was calculated and found to be 14,400 calories from 0° to 15°C, and 8,500 calories from 15° to 40°C. The coefficient of temperature (Q_{10}) is 2.7 between 0 and 10 C; 2.0 between 10 and 20 C; 1.7 between 20 and 30 C; and 1.6 between 30 and 40 C. *Activity as a function of the substrate concentration, and determination of the Michaelis constant* (K_m): These enzyme assays were performed at different substrate concentrations, varying from 0.625 to 50.0 mg per

References p. 273.

ml. A plot* of the velocity of the reaction versus the ratio, velocity/starch concentration is given in Fig. 6. From the slope of the curve, K_m is estimated to be 0.6 g of

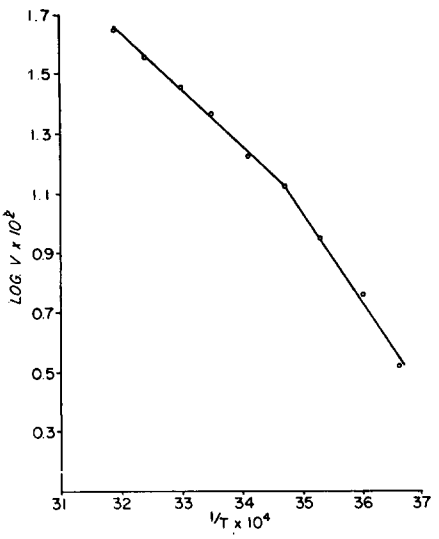

Fig. 5. Arrhenius plots of the activity of the α-amylase of *Pseudomonas saccharophila* measured at different temperatures.

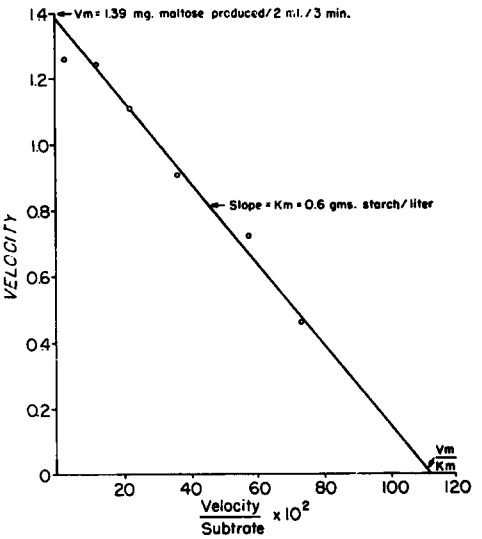

Fig. 6. Graphical determination of the Michaelis Constant (K_m) for the α-amylase of *Pseudomonas saccharophila*, using starch a the substrate.

starch per l. *Ultraviolet absorption spectrum:* A sample of the dissolved crystals was diluted in *M*/30 phosphate buffer at pH 7, and the ultraviolet absorption spectrum of the resulting solution was determined with the aid of a Beckman spectrophotometer. Fig. 7 presents the spectrum obtained and indicates a maximum of absorption at 280 mμ, with an inflection point around 290 mμ, which is common for α-amylases[5–8].

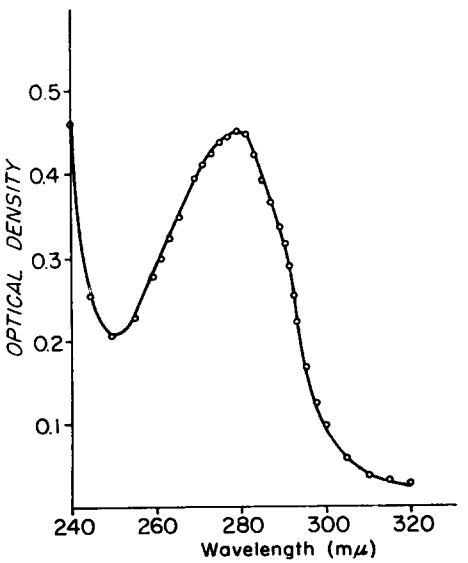

Fig. 7. Ultraviolet absorption spectrum of the α-amylase of *Pseudomonas saccharophila*.

DISCUSSION

Determinations of various physical and chemical properties of crystalline α-amylase of *P. saccharophilla* have been performed in order to ascertain the purity of the crystalline material, to characterize the enzyme, and to compare its properties with those of crystalline α-amylases of different origin. Unfortunately, under optimal conditions, the total quantity of extracellular α-amylase produced by *P. saccharophilla* in 18–19 l

* The advantages of such a plot over others used to determine K_m are discussed by HOFSTEE[9].

References p. 273.

of culture medium is of the order of 40 mg. With a total yield of 20% for the purification, this represents 8 mg of crystalline material per carboy. These small amounts precluded a more extensive study of the chemical and physical properties of the enzyme.

The optimum pH for the α-amylase was determined to be between pH 5.25 and 5.75 and the pH range of maximum stability from 4.5 to 8.0. In comparison, the α-amylase of *Bacillus subtilis* (strain Takamine) has an optimum pH range of activity between 5.3 and 6.8[10], which is much broader than that determined for the alpha-amylase of *P. saccharophila*. Similarly, the pH stability range of the *B. subtilis* enzyme is far greater than that of the *P. saccharophila* enzyme.

The energy of activation of the α-amylase of *P. saccharophila* was found to be 14,400 calories per mole between 0° and 15° C and 8,500 calories per mole between 15° and 40° C. The α-amylase of *B. subtilis* is the only other α-amylase so far studied that has this characteristic of double energy of activation. For the enzyme of *B. subtilis* the energy of activation is 15,000 calories per mole between 0° and 12° C and 9,180 calories per mole between 12° and 55° C[10]. The α-amylases of malt, *Aspergillus oryzae*, swine pancreas, human saliva and human pancreas show a single energy of activation between 0° and 40° C although the value for the animal enzymes differs from that of the vegetable and microbial enzymes[8].

In a determination of the Michaelis constant the value of 0.6 g starch per l was found using one set of experimental conditions in which only the starch concentration was varied. In an extensive study of crystalline alpha-amylases of swine pancreas and human saliva, Fischer (unpublished observations) found values of 0.6 ± 0.2 g starch per l for the Michaelis constants.

While comparisons of the properties of enzymes from different sources, such as have been made above, are interesting, such comparisons may be misleading. The procedures by which a particular enzyme is purified may affect the properties of the preparation, so that a given enzyme may exhibit variations in many properties depending on its previous treatment. For example, STEIN AND FISCHER (unpublished results) have studied the alpha-amylase of *Bacillus subtilis* and consider it to be a type of metallo-protein containing large amounts of extremely firmly bound calcium. The metal stabilizes the enzyme, protecting it very effectively against heat denaturation, extremes of pH, and proteolytic degradation. Depending on the conditions used during cultivation of these organisms, the procedures employed in handling the enzyme during its purification and crystallization, final preparations may be obtained which vary qualitatively as well as quantitatively with respect to their metal content. These variations in turn may affect, in an unpredictable manner, the enzymic properties of the protein.

ACKNOWLEDGEMENT

We are pleased to acknowledge the assistance of Mr. R. WADE in obtaining electrophoretic and ultracentrifugal data.

References p. 273.

SUMMARY

A method for the purification and crystallization of the alpha-amylase of *Pseudomonas saccharophila* has been described. Using crystalline preparations, some properties of this enzyme have been determined.

RÉSUMÉ

Une méthode de purification et de cristallisation de l'alpha-amylase de *Pseudomonas saccharophila* a été décrite. Quelques-unes des propriétés de l'enzyme ont été déterminées sur des préparations cristallisées.

ZUSAMMENFASSUNG

Eine Reinigungs- und Kristallisationsmethode der Alpha-Amylase von *Pseudomonas saccharophila* wurde beschrieben. Auf Grund von kristallisierten Präparaten werden einige Eigenschaften dieses Enzyms beschrieben.

REFERENCES

1 A. MARKOVITZ AND H. P. KLEIN, *J. Bacteriol.* (1955), in press.
2 M. CALVIN, C. HEIDELBERGER, J. C. REID, B. M. TOLBERT AND P. F. YANKWICH, *Isotopic Carbon, Techniques in its Measurement and Chemical Manipulation*, John Wiley and Sons, Inc., New York (1949).
3 E. A. EVANS AND J. L. HOUSTON, *J. Chem. Physics*, 19 (1951) 1214.
4 P. S. THAYER, *J. Bacteriol.*, 66 (1953) 656.
5 P. BERNFELD, A. STAUB AND ED. H. FISCHER, *Helv. Chim. Acta*, 31 (1948) 2165.
6 ED. H. FISCHER AND P. BERNFELD, *Helv. Chim. Acta*, 31 (1948) 1832.
7 S. SCHWIMMER AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 1063.
8 ED. H. FISCHER AND R. DE MONTMOLLIN, *Helv Chim. Acta*, 34 (1951) 1944.
9 B. H. J. HOFSTEE, *Science*, 116 (1952) 329.
10 R. MENZI AND ED. H. FISCHER, *Helv. Chim. Acta* (1955), in press.

Received July 20th, 1955