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Purification of the Amylase of *Bacillus macerans*

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As reported previously,[1] *Bacillus macerans*, when grown on a suitable medium, elaborates an enzyme which converts starch to the non-reducing, crystalline, so-called Schardinger dextrins. This new type of amylase was present in large amounts only in old cultures (two to four weeks old), after the period of active growth and fermentation had passed. Its presence was detected by the iodine test for crystalline dextrins,[1,2] which distinguishes *B. macerans* from other closely related bacteria. Improved cultural conditions, which are favorable to the development of the *macerans* amylase, and the conditions of temperature and hydrogen ion concentration for its optimal activity, have been described elsewhere.[2] The present report concerns the purification of the enzyme preliminary to its use for the chemical study of the components of starch, which is in progress in this Laboratory.

The crude *macerans* enzyme, as prepared by filtering the fluid portion of the culture through a Seitz pad or a Berkefeld candle to remove the bacterial cells, was concentrated ten-fold and at the same time separated from 90 to 95% of the accompanying material by precipitation with an equal volume of acetone. Aqueous solutions of the acetone precipitates contained 0.15 to 0.3 mg. of solids to each unit of enzyme activity, as compared with 3 to 6 mg. per unit in the culture filtrates; further reduction of this figure to 0.03 mg. per unit could be accomplished by adsorption on aluminum hydroxide at *p*H 4.8 and elution with 0.01 *M* phosphate buffer of *p*H 7.6, the phosphate being removed subsequently by dialysis. Although the enzyme was not stable to prolonged dialysis, there was little loss of activity if the procedure was carried out rapidly at low temperature. Concentration of the eluate by further acetone precipitation also increased the stability of the enzyme to dialysis. The loss of activity during dialysis was not due to passage of the enzyme through the viscose membrane used, since in an ultrafiltration experiment with this membrane the filtrate was without activity. The pore size of the membrane proved to be 5 millimicrons. Ultrafiltration experiments with graded collodion membranes, prepared according to the technique of Bauer and Hughes,[3] showed that the enzyme was completely held back by a membrane of 40 millimicrons, though passing quantitatively through one of 60 mμ.

In the earlier experiments, in which adsorption was carried out at *p*H 5.8 to 6.1, which represents the middle of the optimal range for activity of the enzyme,[2] excellent recovery was secured, but the products had a solid content of 0.05 to 0.06 mg. per unit of activity. By adsorption at *p*H 4.8, just above the acid range which inactivates the enzyme,[2] it was possible to obtain satisfactory recovery (90 to 95%) with considerable increase in purity. The final conditions yielded preparations showing 1 unit of activity in 0.029 to 0.034 mg. of solids. The best preparation that was obtained had been purified 140-fold from the crude culture filtrate, and it digested one thousand times its weight of starch in thirty minutes at 40°.

The adsorption procedure was applicable only to acetone-purified enzyme solutions, not to the crude culture filtrates.

Experimental

Culture Medium.—Cultures were grown in 1-liter lots of medium in Fernbach flasks (capacity 2800 ml.). Five per cent. rolled oats in 2% calcium carbonate proved to be a satisfactory medium for large-scale enzyme production. The ingredients were mixed and left in the cold room overnight before sterilization in order to prevent foaming in the autoclave. The medium was autoclaved thirty minutes at 120°. The maintenance of stock cultures and tests for purity have been described elsewhere.[2] Five milliliters of stock culture was used to inoculate each liter of medium.

Measurement of the Activity of the Enzyme Solutions.—Three weeks after inoculation the enzyme content of the cultures was usually 2–3 units per ml., that is, 0.5 ml. of the clear supernatant culture fluid converted 1 ml. of 3% starch (30 mg.) in twenty to thirty minutes at 40° to the "brown violet stage" with iodine.[2] The iodine test for crystalline dextrins proved satisfactory for quantitative estimation of enzyme content throughout the purification procedures. It was unnecessary to use buffers in testing the solutions, except in the case of the eluates from adsorption, the *p*H of which was above the optimum for *macerans* enzyme.

Filtration.—A large Seitz filter[4] ("E. K. Size 14"), capacity 2 liters, was the most convenient means of removing

(1) Tilden and Hudson, THIS JOURNAL, **61**, 2900 (1939).

(2) Tilden and Hudson, *J. Bact.*, **43**, 527 (1942).

(3) Bauer and Hughes, *J. Gen. Physiol.*, **18**, 143 (1934).

(4) American Seitz Filter Corp., 480 Lexington Ave., New York, N. Y.

the bacteria from large amounts of culture fluid. If the bacteria were not removed, the solution of the acetone precipitate showed a significantly higher content of total solids.

Acetone Precipitation.—To each liter of ice-cold filtrate was added an equal volume of cold acetone. Precipitation occurred almost at once. Addition of a few grams of "Filter-cel" favored rapid settling of the precipitate, which was filtered on a pad of "Filter-cel" on a Büchner funnel and washed with 60% (by volume) of aqueous acetone. The pad of "Filter-cel" and precipitate, which was readily detached from the funnel, was transferred to a beaker and thoroughly extracted with water, then filtered through a fresh pad on a smaller funnel. This technique was desirable because it permitted thorough extraction of the enzyme from the gummy precipitate. Water of one-tenth the volume of the original culture filtrate was usually ample for quantitative recovery of the enzyme, and sometimes less water was sufficient. When a large volume of culture filtrate was to be purified, the amount of water necessary to extract all the enzyme was determined by a preliminary purification on a portion of it. The preliminary experiment was desirable also because the amount of acetone necessary to precipitate all of the enzyme was sometimes less than the equal volume; in the case of filtrates of cultures younger than the average, and containing correspondingly less impurities, three-fourths of an equal volume was often sufficient.

Adsorption on Aluminum Hydroxide.—The suspension of aluminum hydroxide was prepared according to the technique described by Sherman, Caldwell and Adams.[5] The aluminum oxide content was determined by evaporation to dryness and ignition to constant weight. The stock suspension was diluted to contain 3 g. of aluminum oxide in 100 ml. and was further diluted as required. The acetone-purified enzyme was pooled in lots of 300 ml. or more, and preliminary tests of small portions were made to determine the optimal *p*H for adsorption and the quantity of aluminum hydroxide needed. The suspension was an exceedingly potent adsorbent for *macerans* enzyme, 0.4 ml. of a 0.25% suspension being sufficient, in the presence of 0.1 *N* acetate buffer of *p*H 4.8, to remove 93% of the enzyme from 5 ml. of a solution containing 20 units. The suspension showed a slight loss of potency for adsorption after standing several months.

The mixture of enzyme, aluminum hydroxide, and normal acetate buffer was diluted sufficiently to make the final concentration of buffer 0.1 *N*. The flocculent aluminum hydroxide was allowed to settle somewhat, then packed by centrifugation and the supernatant fluid decanted. The sediment was washed with water in the centrifuge, the washings discarded, and the enzyme extracted from the sediment by vigorous stirring with a volume of 0.01 *M* phosphate buffer of *p*H 7.6 equal to the volume of enzyme solution used for adsorption. As soon as settling was evident, the extract was separated by centrifugation. A second extraction with half a volume of the phosphate buffer solution was necessary for recovery of all the enzyme from the aluminum hydroxide. The eluate was concentrated by acetone precipitation. Two volumes of acetone were required, and it was necessary to add a small amount of sodium chloride to obtain complete precipitation. During the entire procedure (adsorption, washing, elution, precipitation with acetone) a low temperature was maintained by the use of crushed ice around the centrifuge tubes.

Dialysis.—The concentrated eluate was dialyzed in viscose tubing in bottles of ice-cold distilled water. The bottles were placed in a shaking machine and rocked gently, the cold water being renewed every fifteen minutes for a period of three and one-half hours.

Stability of the Enzyme Solutions.—The purified enzyme solutions retained their activity for many months if kept cold (5°) and protected from growth of molds and bacteria. They were sterilized by Berkefeld or Seitz filtration and stored in sterile stoppered flasks. The preservative usually used was thymol, 0.5 g. for each 100 ml. of enzyme solution, but toluene (10 ml. per 100 ml.) and "Dowicide A"[6] (50 mg. per 100 ml.) were also satisfactory preservatives.

The dried enzyme was less stable. Samples of acetone precipitates dried in a desiccator over phosphorus pentoxide in the cold room (5°), and kept cold, had lost about 35% of their activity after seventy-nine days.

Summary

A method is described for purifying the amylase of *Bacillus macerans*, the enzyme which converts starch to crystalline dextrins. The steps involved were precipitation by acetone, adsorption on aluminum hydroxide in the presence of acetate buffer at *p*H 4.8, elution with phosphate buffer at *p*H 7.6, concentration with acetone, and dialysis. A product was obtained which had an activity 140 times that of the initial enzyme solution and was capable of converting 1000 times its weight of starch in thirty minutes at 40°.

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(5) Sherman, Caldwell and Adams, *J. Biol. Chem.*, **88**, 295 (1930).

(6) Made by the Dow Chemical Company, 30 Rockefeller Plaza, New York, N. Y.