Short Communications

The preparation of crystalline β-methylaspartase

The enzyme β-methylaspartase, which catalyzes the reversible conversion of β-methylaspartate to mesaconate and ammonia, has been isolated and purified some 45-fold from *Clostridium tetanomorphum* by BARKER *et al.*[1]. As the preliminary step in a study of the stereochemistry and mechanism of the reaction catalyzed by this enzyme, β-methylaspartase has been further purified and crystallized in this laboratory. The method of purification and assay of BARKER *et al.*[1] was used, except that DEAE-cellulose chromatography and subsequent crystallization were substituted for the calcium phosphate gel step.

DEAE-cellulose (Brown Company, Berlin, New Hampshire) was stirred into water and allowed to settle. The light particles which floated were decanted and the process repeated until none remained. The DEAE-cellulose was filtered off, stirred thoroughly into satd. KCl, filtered again and stirred into a fresh KCl solution. The DEAE-cellulose was freed of solution on a Buchner funnel. This material was added to a solution of 0.01 *M* potassium phosphate buffer, pH 7.85, and packed under N_2 at 5 lb./in.^{2} to a height of 30 cm in a 2 × 60 cm column which was jacketed for iced water. The column was washed with 200 ml 0.01 *M* potassium phosphate buffer, pH 7.85.

40 ml of a solution of β-methylaspartase in 0.01 *M* potassium phosphate buffer, pH 7.85 (containing 127 mg of protein of specific activity 200) from the ethanol-fractionation step of BARKER *et al.*[1] were placed on the column. The enzyme was eluted with KCl in 0.01 *M* potassium phosphate buffer, pH 7.85. The concentration of KCl was changed in a stepwise manner and 5-ml fractions were collected at the rate of 2 ml/min. The enzyme is displaced by 0.1 *M* KCl under these conditions (see Fig. 1).

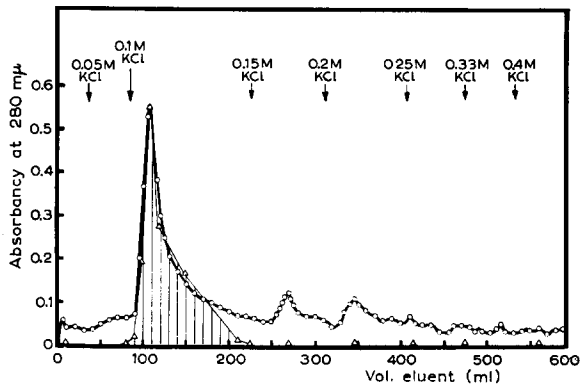

Fig. 1. Chromatography of β-methylaspartase. O—O, u.v. absorption at 280 mμ. △—△, enzyme activity in arbitrary units. For conditions, see text.

Abbreviations: DEAE-cellulose, diethylaminoethyl-cellulose; EDTA, ethylenediaminetetraacetate.

All active fractions in the peak were combined (140 ml total) and added to 10 ml 1.0 *M* potassium phosphate buffer, pH 7.85. 84 g of ammonium sulfate were then added and the precipitated protein centrifuged off at 18,000 × *g*, at 15°. The precipitate was dissolved in 2 ml 0.01 *M* potassium phosphate buffer, pH 7.85. Satd. ammonium sulfate solution, pH 7.85, was then added slowly until there was incipient cloudiness. The solution was left in the refrigerator overnight after which time small, needle-like crystals could be seen in the microscope. More saturated ammonium sulfate solution was added until no more crystals appeared. The enzyme was once recrystallized. The 2.5-fold purification of β-methylaspartase is shown in Table I.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Fraction	*Volume*	*Protein*	*Activity*		*Specific activity*
			Units	*Yield*	
	ml	*mg*	× 10^3	%	*units/mg*
1. Ethanol fraction (see ref. 1)	40	127	25.4	60	200
2. Combined fractions from DEAE-cellulose	140	39	19.1	45	490
3. Product of first crystallization	—	32	15.8	37	494
4. Product of second crystallization	—	26.3	13.1	31	498

The crystals are small and are seen most readily with phase-contrast microscopy (see Fig. 2). They are very stable, retaining full activity at 4° after 1 year. A 1-% solution of the crystals containing 0.10 *M* potassium phosphate buffer, pH 7.05, and 0.2 *M* KCl shows a single and symmetrical peak in the ultracentrifuge corresponding to a sedimentation coefficient of 5 S (see Fig. 3).

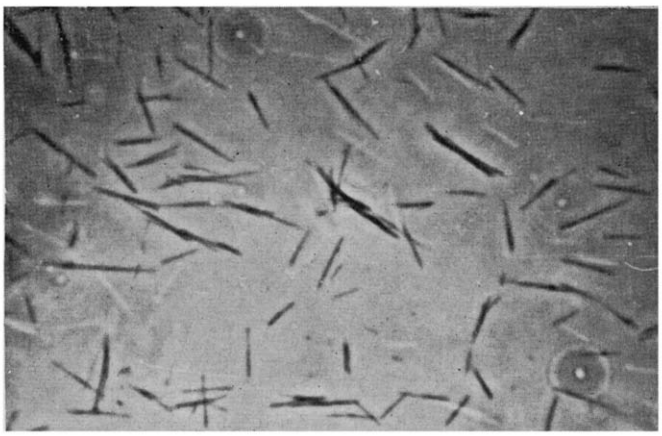

Fig. 2. Crystals of β-methylaspartase (once recrystallized), × 750.

After dialysis against 0.005 *M* EDTA in 0.005 *M* potassium phosphate buffer, pH 7.0, the divalent metal ion requirement of the crystalline enzyme is satisfied by the following under the conditions of the assay:

$$Mg^{++} > Mn^{++} > Co^{++} > Ni^{++}$$

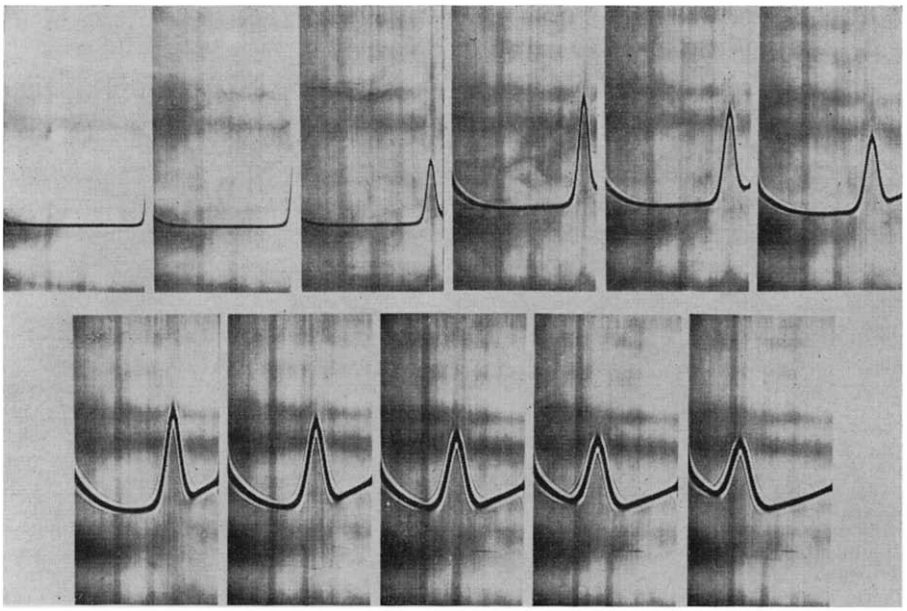

Fig. 3. Sedimentation pattern of β-methylaspartase (1 % by weight, once recrystallized) in 0.10 *M* potassium phosphate buffer, pH 7.05, containing 0.2 *M* KCl obtained in the Spinco Model E Ultracentrifuge. Temp. 13.7°, rotor speed 59,780 rev./min. Exposure times 0, 8, 16, 24, 32, 48, 64, 80, 96, 112 and 128 min. Angle of Schlieren diaphragm 80°, 80°, 80°, 70°, 70°, 70°, 60°, 60°, 60°, 60°, and 60°.

Less active preparations are activated only by Mg^{++} (see ref. 1). Also, under conditions where less purified preparations of β-methylaspartase were shown to produce a mixture of β-methylaspartate isomers from mesaconate and ammonia[1], only the *threo* isomer could be detected when the crystalline enzyme was used to catalyze the reaction between mesaconate and ammonia. Paper electrophoresis[1] and nuclear-magnetic-resonance spectroscopy** were used to distinguish the isomers.

However, it has not yet been possible to determine unequivocally whether the crystalline enzyme eliminates ammonia from the *erythro* isomer.

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Department of Biochemistry, University of California, Davis, Calif. (U.S.A.)

HAROLD J. BRIGHT
LLOYD L. INGRAHAM

[1] H. A. BARKER, R. D. SMYTH, R. M. WILSON AND H. WEISSBACH, *J. Biol. Chem.*, 234 (1959) 320.
[2] L. BENOITON, S. M. BIRNBAUM, M. WINITZ AND J. P. GREENSTEIN, *Arch. Biochem. Biophys.*, 81 (1959) 434.

* BARKER *et al.* tentatively assigned the α-L-*threo* configuration to the isomer of β-methylaspartate which is more readily used by β-methylaspartase. BENOITON *et al.* confirmed the α-L-assignment by the action of a stereospecific acylase[2]. We have since proved the *threo* configuration of this isomer by nuclear-magnetic-resonance spectroscopy, the details and implications of which will be published shortly elsewhere.

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