Isotopic Determination and Optimum Reaction Conditions of Pantothenic Acid Synthetase

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Partial purification and properties of pantothenic acid synthetase (D-pantoate: β -alanine ligase (AMP), E.C. 6.3.2.1) which catalyzes the condensation of D-pantoic acid and β -alanine in the presence of ATP have been reported by Mass,¹⁾ Mass and Novelli²⁾ and Pfeleiderer *et al.*^{3~6)} with the enzyme preparations from *Escherichia coli*. The former group employed a micro-bioassay technique with a pantothenate-auxotrophic mutant of *E. coli*⁷⁾ for the determination of the enzyme activity while the latter a spectrometric method³⁾ in a complicated system of enzyme reactions, in which AMP formed during the condensation was converted into ADP (myokinase) and then into ATP (pyruvate kinase) and the liberated pyruvate was allowed to oxidize NADH in the presence of lactate dehydrogenase. These methods, though elaborated, lack satisfactory simplicity, speed and other conveniences for studying this enzyme.

In the experiments reported here, which constitute a part of detailed studies on the biosynthesis of pantothenic acid in this laboratory, we introduced an isotopic method of determining the activity of pantothenic acid synthetase by the use of β -alanine-1-¹⁴C, and the radioactive pantothenic acid formed was determined after separation by ion-exchanger column chromatography by measuring its radioactivity. By using this technique, simpler and speedier than the reported ones, we studied on some properties of this enzyme in a 35-fold purified preparation from *E. coli* B and revised the optimum pH and temperature of the enzyme reaction, which had been reported previously. Apparent *K_m* values for the substrates under these conditions are presented also.

Materials. Pantothenic acid synthetase was isolated from the acetone powder of *E. coli* B (IFO 13168), which had been cultured at 30°C for 20~24 hr with aeration in a minimum synthetic medium⁸⁾ at pH 7.0, by homogenizing with glass homogenizer in 10 volumes of tris-HCl buffer (0.01 M, pH 7.2), centrifuging at 5,000×*g* for 15 min, and dialyzing the supernatant against distilled water. This crude preparation was

purified by treatment with 0.2~0.7 saturation of (NH₄)₂SO₄, Sephadex G-100 chromatography, DEAE-Sephadex (A-50) chromatography, Sephadex G-150 chromatography, DEAE-Sephadex (A-50) rechromatography and treatment with 0.4~0.6 saturation of (NH₄)₂SO₄, in succession, to obtain a 35-fold purified enzyme preparation.

β -Alanine-1-¹⁴C was a product of International Chemical and Nuclear Co. (0.25 mCi/4.7 mg). Other chemicals were supplied from Nakarai Chemicals Ltd., Kyoto.

Enzyme reaction. The reaction mixture contained 5 μ M ATP, 20 μ M potassium D-pantoate, 10 μ M MgSO₄·7H₂O, 50 μ M glycine buffer (pH 10.0), 0.1 μ Ci β -alanine-1-¹⁴C (for isotopic determination) or 20 μ M cold β -alanine (for bioassay) and 1~400 μ g of the enzyme protein, and distilled water was added to make the total volume 1 cc. The reaction mixture was incubated at 30°C for 15~60 min with shaking, and then the reaction was stopped by heating at 100°C for 5 min after adjusting pH below 5 with N HCl.

Assays. In the isotopic assay the reaction mixture was charged onto a Dowex-50 (H⁺ form) column (0.6×6 cm) and the synthesized, radioactive pantothenic acid was eluted with distilled water (5 cc). The radioactivity of the eluate was measured with a gas-flow counter (Aloka, model TDC 2). The radioactive product of the enzyme reaction was identified to be pantothenic acid by paper chromatographic examination of the eluate.

Microbial assay was performed by the use of a pantothenic acid-requiring mutant⁹⁾ of *E. coli* (No. 229) which had been cultured in the Davis medium¹⁰⁾ containing 1 mg of pantothenic acid per 1 liter. This assay could determine 0.5~500 μ g of pantothenic acid.

Comparison of the isotopic and bioassay methods. Both methods were compared to each other in assessing the effect of ATP concentration on the activity of pantothenic acid synthetase (Table I). Similar

TABLE I. EFFECT OF ATP CONCENTRATION ON THE ENZYME ACTIVITY AS DETERMINED BY THE ISOTOPIC AND BIOASSAY METHODS

The activities are given relative to the one observed with 5.0 μ M ATP in each method.

ATP Concn. (μ M)	Relative enzyme activity	
	Isotopic method	Bioassay method
0.1	22	—
0.5	55	52
1.0	72	70
2.0	85	84
5.0	100	100

TABLE II. K_m VALUES (M) FOR SUBSTRATES HERE FOUND AND REPORTED PREVIOUSLY

β -Alanine	Pantoic acid	Reaction conditions	Ref.
5.6×10^{-5}	1.2×10^{-4}	pH 10.0 (glycine); 30°C	
$3.8 \sim 7.4 \times 10^{-4}$	$1.5 \sim 3.5 \times 10^{-3}$	pH 8.5 (tris-HCl); 25°C	1)
9.5×10^{-4}	6.6×10^{-4}	pH 8.5 (tris-HCl); 25°C	3)

results were obtained by both methods of determination, but the amount of enzyme protein here used was 0.5 μ g in the isotopic method while it was 3 μ g in the bioassay method. In addition, the former method was so sensitive as able to determine the effect of 0.1 μ M of ATP in this experiment while the latter was sensitive only down to 0.5 μ M.

Figure 1 shows the time course of the enzyme reaction with different enzyme concentrations as determined by the isotopic method. The formation of labeled pantothenic acid is linearly proportional to the reaction time up to 30 min and to the enzyme concentration at 0.5~1.5 μ g/tube. With the crude extract the linearity existed up to 400 μ g enzyme protein/tube for 1 hr.

FIG. 1. Time Course of Enzyme Reaction at Different Enzyme Concentrations.

○—○ 0.5, △—△ 1.0, and □—□ 1.5 μ g enzyme protein/tube.

Optimum pH and temperature. By using this isotopic method, the effects of pH and temperature on the reaction of pantothenic acid synthetase were studied. Figure 2 shows the effect of pH. The optimum pH was found at 10.0 in glycine-KOH-KCl buffer (50 mM). This value is considerably different from pH 8.50 (Tris-HCl buffer, 100 mM) as reported to be optimum by Mass,¹⁾ at which only 55% of the enzyme activity at the optimum pH (and optimum temperature) here found was observed. The enzyme

FIG. 2. Effect of pH.

Reactions were conducted at 30°C for 30 min with shaking (120 strokes/min); 1 μ g enzyme protein was contained per tube.

Buffers: △—△ Potassium phosphate, ●—● Tris-HCl, ○—○ Glycine-KOH-KCl.

was most active at 30°C. Against the activity at this optimum temperature, the enzyme was 90% active at 37°C and 60% active at 45°C. Mass¹⁾ and Novelli¹¹⁾ reported the optimum temperature of this enzyme to be 25°C.

This enzyme preparation required a divalent ion, Mg^{2+} or Mn^{2+} , and a monovalent ion, K^+ or NH_4^+ , as activators, in agreement with the reported results.¹⁾

K_m Values. Under the optimum conditions here found for the reaction of pantothenic acid synthetase, the apparent K_m values for both substrates were calculated and are given in Table II together with the reported values for the purpose of comparison. The data indicate that the newly found conditions afford the enzyme with higher affinities to the substrates.

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