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Phosphotransacetylase of *Lactobacillus fermenti*, properties of the enzyme and determination of coenzyme A

Phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalyzes the reversible transfer of acetyl group from acetyl-CoA to phosphate as shown in Reaction (I), and was first discovered by STADTMAN and co-workers[1-3] from *Clostridium kluyverii* extract. In 1963, BERGMEYER[4] isolated this enzyme in crystalline form from *C. kluyverii*. Many other workers also found this enzyme in several bacteria such as *Escherichia coli*[2,5,6], and *Bacillus megaterium*[7], *etc.*

$$CH_3COOPO_3H_2 + CoASH \rightleftharpoons CH_3COSCoA + H_3PO_4 \qquad (1)$$

In the field of biochemical analysis several methods have been devised for the determination of CoASH (ref. 8). Among these, the phosphotransacetylase method has widely been accepted because of its high specificity for CoASH. However, *C. kluyverii*, the source of the enzyme, is very troublesome to cultivate.

This communication is concerned with the presence of a high level of phosphotransacetylase in the extract of *Lactobacillus fermenti* strain 36 (IFO No. 3071, ATCC No. 9338); a study on the application of partially purified enzyme to the spectrophotometric estimation of CoASH is also included. *L. fermenti*, one of heterofermentative lactic acid bacteria, has been widely used for the microbiological assay of thiamine, and since these organisms are easily cultivated, it seems that an obstacle of cultivation as seen in *C. kluyverii* is overcome.

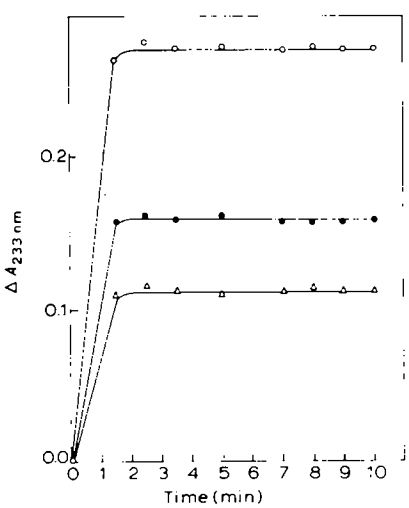

Fig. 1. Change of absorption at 233 nm of reaction mixture under the various concentrations of CoASH. Each cuvette contained 10 μmoles of acetylphosphate, 230 μmoles of Tris-HCl (pH 7.7) and 33.5 μg (0.67 unit) of phosphotransacetylase (Fraction E-3-D) in a final volume of 3.0 ml (○—○) with 0.183 μmole of CoASH; (—●—) with 0.105 μmole of CoASH; and (△—△) with 0.0737 μmole of CoASH, respectively. Temperature, 30°. Quartz cells, light path 1.0 cm. Reaction was initiated in all cases by addition of enzyme at zero time. In Assay method II, the reaction mixture contained the same components as described above, except that the concentration of CoASH was fixed at 0.2 μmole per 3 ml.

L. fermenti strain 36 was obtained from the Institute for Fermentation, Osaka. Organisms used for the preparation of the enzyme were grown in the medium containing 1% Bacto-peptone (Difco), 0.5% Bacto-yeast extract (Difco), 1% Bacto-beef extract (Difco) and 1.0% D-glucose (pH 6.5). Incubation after inoculation of organisms was carried out at 37° for 18 h without shaking.

Phosphotransacetylase activity was determined by the method of STADTMAN[9] (Assay Method I). The activity was also followed by an increase in absorption at 233 nm that had been described by BERGMEYER[8], except that glutathione was omitted (Assay Method II, see Fig. 1).

Twenty grams of deep frozen cell paste were suspended in 160 ml of Tris–HCl (pH 7.7). The cell suspension was then sonically disrupted (Kubota, type KMS-200) for 20 min, and the sonicate was centrifuged at 15 000 × *g* for 40 min. The supernatant (Fraction E-1) was added with 16.4 ml of 2% streptomycin sulfate (pH 7.0). After standing for 20 min, it was centrifuged at 7000 × *g* for 20 min. The supernatant (Fraction E-2) was fractionated with $(NH_4)_2SO_4$. The fractions precipitating at 30–65% saturation were recovered by centrifugation at 27 000 × *g* for 30 min. The precipitate was dissolved in 20 ml of 0.1 M Tris–HCl (pH 7.7) (Fraction E-3), and dialysed for 18 h at 5° against 4 l of the same buffer solution. The diffusate (Fraction E-3-D) obtained did not show any loss of activity during dialysis. As shown in Table I, almost 98% of enzyme activity was recovered in Fraction E-3-D. It seems that this crude enzyme preparation is very stable through purification procedures including freezing and dialysis. Fraction E-3-D was used in all experiments described in this communication, and this fraction maintained full activity for at least a month when stored at −10° to −15°. 62-fold dilution of this fraction could satisfactorily be used for the CoASH assay system (Assay Method II), and 0.1 ml of this diluted

TABLE I

PARTIAL PURIFICATION OF PHOSPHOTRANSACETYLASE FROM *lactobacillus fermenti*

All purification procedures were carried out at 3–5°. Enzyme activity was measured according to Assay method I (see ref. 9), and protein was estimated by the $A_{280\ nm}$ method except when mentioned otherwise.

Fraction		*Volume (ml)*	$A_{280\ nm}$	*Enzyme units*			
				Total	*Per ml*	*Per* $A_{280\ nm}$	*Yield (%)*
E-1	Cell-free extract	164		54 100	330		100.0
E-2	Streptomycin sulfate supernatant	172	2064	56 553	329	27.4	104.5
E-3	30–65% $(NH_4)_2SO_4$ satd. ppt.	20	750	50 175 12 735*	2509 637*	66.9 17.0**	92.7
E-3-D	Diffusate of fraction E-3	32.8	755 805***	13 515*	412*	17.9** 16.8†	98.4

* Determined by Assay method II (see Fig. 1).
** Determined by Assay method II and $A_{280\ nm}$ method.
*** Protein (mg) determined by the method of LOWRY *et al.*[10].
† Determined by Assay method II and the method of LOWRY *et al.*[10].

solution in 3 ml of reaction mixture was equivalent to about 0.03 of the spectrophotometric scale of absorbance at 233 nm.

Fig. 1 illustrates the typical results of acetyl-CoA formation in the presence of *L. fermenti* enzyme. The increase in absorbance at 233 nm was directly proportional to the amounts of CoASH added. The forward reaction of Reaction (I) was completed during the first 3 min of the reaction. On the basis of the fact, it is concluded that the partially purified enzyme preparation is applicable to the quantitative determination of CoASH.

The optimal pH was shown to be pH 7.7 in Tris-HCl.

Neither CoASSCoA, 4′-phosphopantetheine, 4′-phosphopantethine, nor CoASS-glutathione served as substrates in the phosphotransacetylase reaction.

On the other hand, it was found that 3′-dephosphoCoASH also reacted with acetylphosphate in the presence of *L. fermenti* enzyme, but the reaction velocity for 3′-dephosphoCoASH was much slower than that for CoASH as described by BERGMEYER[8] in the case of Clostridium enzyme. The catalytic activity of 3′-dephosphoCoASH was observed to be one tenth that of CoASH. As seen in Fig. 2, the increase in absorbance observed in the reaction mixture containing both CoASH and 3′-dephosphoCoASH was rapid and steep within the initial 2 min, representing the acetylation of CoASH in the reaction mixture. Moreover it continued to increase for about a further 8 min after CoASH had been completely acetylated. The rate of continuing increase in absorbance was the same as that of 3′-dephosphoCoASH alone. On the basis of this fact, it was concluded that the CoASH contents in the reaction mixture containing 3′-dephosphoCoASH could be calculated at 2 to 5 min after the initiation of reaction by the method that was illustrated in Fig. 2.

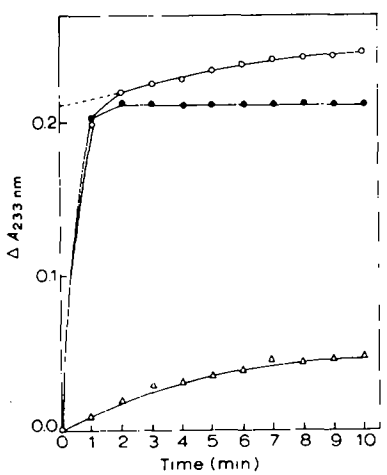

Fig. 2. Differential determination of CoASH contents in the sample contaminated with 3′-dephosphoCoASH. Each cuvette contained 10 μmoles of acetylphosphate, 240 μmoles of Tris-HCl (pH 7.7) and 20 μg of enzyme protein (Fraction E-3-D) in a final volume of 3.0 ml (△—△) with 0.18 μmole of 3′-dephosphoCoASH, (—●—) with 0.18 μmole of CoASH, and (—○—) with both 0.18 μmole of CoASH and 3′-dephosphoCoASH, respectively. Other conditions were those given in Fig. 1. Equation for curve a (△—△), calculated from 0 to 5 min, was $A_a = 0.0027 + 0.0711\,t$; for curve b (●—●), calculated from 2 to 5 min, was $A_b = 0.213 - 0.000167\,t$; and curve c (○—○), calculated from 2 to 5 min, was $A_c = 0.212 + 0.045\,t$, respectively. The ordinate intersect of curve b agreed with that of curve c.

TABLE II

EFFECT OF CATIONS AND ANION ON PHOSPHOTRANSACETYLASE

Reaction mixture contained the same components as that of Assay method II (see Fig. 1), except that cations were added in the chloride form and anion was added as sodium salt. CoASH added was from Sigma. The protein concentration of the mixture was 1.675 μg (0.02 unit) per 3 ml.

Addition	*Concn.*	*Activity recovered (%)*
None	—	100
NH_4^+	10	212
NH_4^+	33	155
K^+	10	177
Na^+	20	92
Li^+	10*	72
Rb^+	10	141
Ca^{2+}	10	52
Ba^{2+}	10	25
Mg^{2+}	10	58
Cs^+	10	59
SO_4^{2-}	10	78**

* As the reaction mixture contained acetyl phosphate dilithium salt, the total Li^+ concn. should be corrected to 17 mM.

** Compared with the reaction mixture contained equimolar NaCl.

As shown in Table II, K^+, NH_4^+, and Rb^+ were required for its maximum activity, while Ca^{2+}, Mg^{2+}, Ba^{2+}, Cs^+, and SO_4^{2-} were inhibitory.

Propionylphosphate could also serve as substrate in the phosphotransacetylase reaction, though the rate of reaction was about one third that which was observed in the case of acetylphosphate.

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