Note

Purification and Properties of
NADPH-linked Diacetyl Reductase
(*S*-Acetoin Forming) from
Bacillus polymyxa

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In the previous paper,¹⁾ we reported on the formation of 2,3-butanediol stereoisomers (*R* and *meso*) in *Bacillus polymyxa* through the action of two kinds of enzymes. One was a previously known enzyme, *R*-butanediol dehydrogenase (EC 1.1.1.4), which catalyzes the reversible reduction of acetoin to butanediol, and the other was a new enzyme, designated¹⁾ as NADPH-linked diacetyl reductase, which irreversibly reduces diacetyl to *S*-acetoin. This paper deals with the purification and some properties of this new enzyme.

B. polymyxa IAM 1189 was cultivated, using the same procedures as those previously mentioned, under aerobic conditions.¹⁾ Preparation of a cell-free extract and assaying of the enzyme activity (in 0.2 M phosphate buffer, pH 6.0) were performed in the same ways as previously mentioned,^{1,2)} unless otherwise stated. Protein was estimated by the method of Bradford.³⁾ Purification of the enzyme was conducted as follows. All procedures were performed at 0~4°C, and 0.02 M sodium phosphate buffer (pH 7.5) was used throughout. To remove nucleic acids, the cell-free extract (53 ml) was treated with a 0.1 volume of a 2% (w/v) protamine sulfate solution, which was added dropwise with stirring over 30 min. The resulting precipitate was removed by centrifugation (14,000 × *g*) for 20 min. The enzyme solution was brought to 30% saturation with ammonium sulfate. The precipitate was removed by centrifugation, and then (NH₄)₂SO₄ was added to the supernatant to 60% saturation. The precipitate was collected, dissolved in the same buffer and then desalted on a column of Sephadex G-25. The desalted enzyme solution was applied to a DEAE-cellulose column (2.2 by 22 cm) equilibrated with the same buffer. After washing with 200 ml of the equilibrating buffer, the column was eluted at a flow rate of 50 ml/hr with a linear NaCl gradient (2 volumes of 500 ml, 0 to 1 M) in the same buffer, and 10 ml fractions were collected. Tubes containing significant diacetyl reductase activity (fractions 40 through 51) were

pooled and concentrated by pressure dialysis (UK-50, Toyo Roshi), and then the concentrate was desalted on a column of Sephadex G-25. The resultant enzyme solution was placed on a Blue Sepharose CL-6B column (1.7 by 10 cm) equilibrated with the same buffer. After washing with 70 ml of the equilibrating buffer, the column was eluted at a flow rate of 18 ml/hr with 300 ml of NaCl (0 to 1.5 M gradient) in the same buffer, and 3 ml fractions were collected. Fractions 53~68 were pooled and concentrated by pressure dialysis, and then the concentrate was used as the partially purified NADPH-linked diacetyl reductase. About 21-fold purification was achieved with an overall yield of 16%. A summary of the purification procedure is presented in Table I. The enzyme was stored at -17°C until use.

The molecular weight of the enzyme was estimated by gel filtration on a calibrated column (1.6 by 74 cm) of Sephadex G-200, according to the method of Andrews.⁴⁾ A molecular weight of 120,000 was indicated when the elution volume of the enzyme was compared with those of known protein standards ('Combithek' calibration proteins from Boehringer): *i.e.*, the partially purified enzyme preparation (1.9 mg protein in 1 ml of the buffer) was applied to the column, which had been equilibrated with 0.1 M sodium phosphate buffer (pH 7.5), and then eluted with the same buffer (flow rate, 4 ml/hr). The isoelectric point of the enzyme was found to be 6.1 on isoelectric focusing on a polyacrylamide gel according to the method of Wrigley.⁵⁾

The enzyme activity was determined in the pH range of 5.2 to 8.5 in 0.2 M Good buffer (MES, MOPS or TES). The enzyme showed maximal reactivity at pH 5.8~6.0 for the reductive reaction for diacetyl. The reverse reaction could not be confirmed, even at pH 8.6~10.8. The enzyme was stable, when incubated at 4°C for 24 hr, over the pH range of 7.5 to 8.5 in Good buffer. The thermostability of the enzyme was determined by means of 10 min preincubation of the enzyme solution at various temperatures prior to the enzyme assay. The enzyme was stable up to 35°C but rapidly inactivated over 50°C. The effects of various compounds on the enzyme activity were investigated. The enzyme was preincubated for 10 min at 25°C with each compound before the addition of the substrate under the standard assay conditions. Only the effects of cations were investigated in 0.2 M MES-(C₂H₅)₄N (pH 6.0). Thiol compounds such as 2-mercaptoethanol and L-cysteine caused 14% and 20% enhancement of the enzyme activity at a concentration of 1 mM, but at 10 mM they showed 19% and 75% inhibition, respectively. Dithiothreitol abolished 12% of the enzyme activity at 1 mM and 34% at 10 mM. As to sulfhydryl reagents, *N*-ethylmaleimide increased the activity by 31% at a concentration of 1 mM and *p*-CMB also increased it by 22% at a concentration of 0.5 mM. The chelating reagents, EDTA, 8-hydroxyquinoline and 1,10-phenanthroline, at a concentration of 1 mM caused 8%, 11% and 5% inhibition of the enzyme activity, respectively. Among the various cations tested, as chlorides,

TABLE I. PURIFICATION OF NADPH-LINKED DIACETYL REDUCTASE FROM *B. polymyxa*

Step	Total protein (mg)	Specific activity (units/mg)	Total units (U)	Yield (%)	Purification (-fold)
Crude extract	464	0.091	42	100	1
Protamine sulfate	405	0.097	39	92.9	1.07
Ammonium sulfate	107	0.136	15	35.7	1.49
DEAE-Cellulose	57	0.349	20	47.6	3.84
Blue Sepharose	3.5	1.920	6.7	16.0	21.1

The purification was carried out as described in the text, starting from 8 g (wet weight) of cells.

NH_4^+ , $(\text{CH}_3)_4\text{N}^+$, Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+} and Ca^{2+} caused no significant changes at a concentration of 5 mM, while Na^+ , K^+ , Ba^{2+} and Co^{2+} caused 12% to 20% enhancement at the same concentration. Among the organic acids examined, acetate was the most inhibitory. It abolished 35% of the activity at a concentration of 20 mM. The other acids tested (lactate, formate, pyruvate, citrate and succinate) at the same concentration showed only slight inhibition (about 5%). The substrate specificity of the enzyme was examined. Only toward 2,3-pentanedione, other than diacetyl, did it show significant activity. Even at 50 mM, no activity was detected with 2,5-hexanedione, 1,3-dihydroxyacetone, methylethylketone, 2-ketoglutaric acid, glyceraldehyde or acetaldehyde. A bincinal diketone as a substrate appears to be necessary for the enzyme. The K_m values for NADPH and diacetyl were 0.05 mM and 12.0 mM, respectively, at pH 6.0, as determined from Lineweaver-Burk plots with the standard reaction system.

Butanediol dehydrogenases generally exhibit accompanying diacetyl reductase activity,^{6,7)} and the butanediol dehydrogenase from *B. polymyxa* also catalyzes the conversion of diacetyl to acetoin.⁷⁾ However, the two enzymes mentioned above (the butanediol dehydrogenase and the diacetyl reductase of *B. polymyxa*) are distinguishable from each other in the following points, as shown in Table II, pH optimum, stability, substrate specificity, co-factor, effect of organic acids and K_m for diacetyl. Enzymes exhibiting NADPH-linked diacetyl reducing activity also exist in other bacteria (*e.g.*, *B. coagulans* ATCC 7050, *B. cereus* IAM 1072, *Proteus vulgaris* IAM 1025 and so on) besides *B. polymyxa*. This will be reported in detail elsewhere.

TABLE II. COMPARISON OF THE DIACETYL REDUCTASE AND THE BUTANEDIOL DEHYDROGENASE OF *B. polymyxa* AS TO DIACETYL REDUCING ACTIVITY

Characteristics	Diacetyl reductase	2,3-Butanediol dehydrogenase ⁷⁾
Optimum pH	pH 5.8~6.0	pH 7.0
pH stability	pH 7.5~8.5	pH 7.0~7.6
Heat stability	78% loss of activity at 50°C for 10 min	No loss of activity at 55°C for 3.5 hr
Co-factor	NADPH-linked	NADH-linked
2,3-Pentane dione	Substrate	No reaction
Effect of organic acid	Inhibited by acetate	Activated by formate or acetate
K_m for diacetyl	12 mM	87 mM

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