Purification and Properties of NADP-Dependent Shikimate Dehydrogenase from *Gluconobacter oxydans* IFO 3244 and Its Application to Enzymatic Shikimate Production

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NADP-Dependent shikimate dehydrogenase (SKDH, EC 1.1.1.25) was purified from *Gluconobacter oxydans* IFO 3244. SKDH showed a single protein band on native-PAGE accompanying enzyme activity. It required NADP exclusively and catalyzed only the shuttle reaction between shikimate and 3-dehydroshikimate. The optimum pH for shikimate oxidation and 3-dehydroshikimate reduction was found at pH 10 and 7 respectively. SKDH proved to be a useful catalyst for shikimate production from 3-dehydroshikimate.

**Key words:** enzymatic shikimate production; *Gluconobacter oxydans*; oseltamivir; shikimate dehydrogenase

In spite of the warning from WHO advocating prevention with an increased stock of medicine against a global pandemic flu infection, there are still insufficient measures for the stock of oseltamivir. The direct reason comes from the technical difficulties of shikimate (SKA) production, making it the Achilles’ heel in oseltamivir synthesis. The metabolic location of SKA is remote from D-glucose and two different metabolic pathways, the glycolysis and the pentose phosphate pathway, yielding phosphoenolpyruvate and erythro-4-phosphate respectively, should be combined to form 3-deoxy-arabino-heptulosonate 7-phosphate before reaching 3-dehydroquinate. Thus, even given the highly armed microbial biotechnology so far developed, it appears difficult to lead the metabolic flow to SKA production. Organic synthesis of SKA is still impractical, since SKA involves three asymmetric carbons in the molecule. SKA production from plant sources is also limited.

A series of investigations of oxidative fermentation with acetic acid bacteria have allowed us to find a novel route producing SKA from quinate in a culture medium of growing cells or in a reaction mixture involving resting cells or dried cells. Membrane-bound quino-protein quinate dehydrogenase and 3-dehydroquinate dehydratase function on the outer surface of the cytoplasmic membrane of organisms catalyzing quinate oxidation to 3-dehydroshikimate (DSA) via 3-dehydroquinate (DQA). In the cytoplasmic fraction, SKDH catalyzes the reduction of DSA to SKA leading the metabolic flow to SKA in the SKA pathway. Recently, we developed a better strategy for high SKA production allowing the two separately localized enzymes occurring outside and inside the cells of acetic acid bacteria to work sequentially. After quinate was converted to DSA by the membrane enzymes, DSA was further converted to SKA by SKDH in the presence of NADP-dependent D-glucose dehydrogenase (GDH, EC 1.1.1.47) and excess D-glucose as an NADPH regenerating system. SKDH continued to produce SKA until DSA added initially in the reaction mixture was completely converted to SKA. Thus our preliminary demonstration of enzymatic high SKA production was successful. However, purification and characterization of SKDH from acetic acid bacteria remained to be examined, although useful information about SKDH from other sources is available. In this paper, purification of SKDH from *G. oxydans* IFO 3244 was examined and the catalytic properties of SKDH in relation to SKA production were determined to strengthen the basic aspects of enzymatic production of SKA.

*G. oxydans* IFO 3244 was grown in a quinate medium, as described previously. Cell pastes (300 g wet wt.) were suspended in 1-liter of 2 mM potassium phosphate buffer, pH 7.2, containing 5 mM β-mercaptoethanol (KPB). After the cells were broken, the cell homogenate was centrifuged at 68,000 × g for 90 min to separate the cell-free extract from the membrane fraction. Cell-free extract (0.07 units/mg) was applied to a DEAE-cellulose column (5 × 30 cm) equilibrated with KPB. After the column was washed with KPB containing 100 mM KCl, SKDH was eluted with KPB containing 200 mM KCl. The KCl concentration in the
pooled SKDH fraction (0.20 units/mg) was diluted to 100 mM with 5 mM β-mercaptoethanol and applied to a DEAE-Sephadex A-50 column (2.5 × 20 cm) equilibrated with KPB containing 100 mM KCl. Elution of SKDH was done stepwise with KPB containing 175 and 200 mM KCl. The pooled SKDH fraction (2.88 units/mg) was diluted four times with 5 mM β-mercaptoethanol, yielding the KCl concentration less than 100 mM, and applied to a Blue-Dextran Sepharose 4B column (1.5 × 20 cm) equilibrated with KPB containing 100 mM KCl. SKDH was eluted with KPB containing 300 mM KCl. The pooled SKDH (166.7 units/mg) was precipitated with ammonium sulfate, and the precipitate was dissolved in a minimum volume of KPB and applied to a Sephadex G-75 column (1.5 × 125 cm) equilibrated with KPB. When fractionated with 35 drops (about 1.2 ml/tube), SKDH appeared with a sharp elution pattern, of which the peak fraction was found at the fraction number of 92. The pooled SKDH contained 2.8 mg of protein showing the specific activity of 178.5 units/mg. Through the above procedures, SKDH was purified with a magnification of 2,550 and 23% recovery indicating that SKDH extended to about 0.04% in the cell-free extract of G. oxydans IFO 3244. In the case of E. coli SKDH, the enzyme was purified 20,000-fold from the crude extract, with a final yield of 21%. In the SKDH purification described above, SKA oxidation was measured in 1 ml of reaction mixture containing SKDH, 0.25 μmol of NADP, 10 μmol of SKA, and 50 mM of glycine–NaOH, pH 10.0. One unit of SKDH activity was defined as the enzyme catalyzing formation of 1 μmol of NADPH. For the protein concentration measurement, an optical absorption of 1% protein solution at 280 nm was assumed tentatively to be 10.0.

Upon native polyacrylamide gel electrophoresis (PAGE), SKDH showed a single band accompanying SKDH activity (Fig. 1). The molecular mass of SKDH was estimated to be 60 kDa, because SKDH came out from Sephadex G-100 gel filtration chromatography (1.2 × 125 cm) immediately after bovine serum albumin (68 kDa, Sigma, St. Louis, MO) and far from the position of carbonic anhydrase (30 kDa, Sigma). However, two stained bands, at 60 kDa and 30 kDa, were observed irrespective to the absence or presence (up to 20%/v/v) of β-mercaptoethanol during heat treatment of SKDH before SDS–PAGE (Fig. 1). Unlike SKDH from E. coli, which always occurs as a monomer of 30 kDa, judging from the stained bands in SDS–PAGE, more than 50% of the SKDH purified in this study was occupied with a dimer composed of two identical subunits of 30 kDa. At present, it is unknown whether SKDH still contains another species of SKDH of 60 kDa in SDS–PAGE as an undissociable isozyme or as an artifact. The total figure of SKDH from G. oxydans IFO 3244 will be reported in a future study.

SKDH was highly specific to SKA and DSA. Quinate, DQA, and protocatechu ate were not available as substrates for SKDH. Since DSA and DQA are commercially unavailable, both substrates used were prepared according to the previous study. Apparent K_m values for SKA and DSA were measured to be 0.5 mM and 0.2 mM in SKA oxidation and DSA reduction, respectively. It is worthwhile to note that the SKDH purified in this study was very stable and that almost 100% of the enzyme activity was kept safe as a solution in a refrigerator at 4°C for several months. As seen in Fig. 2A, the optimum pH for SKA oxidation was found at pH 10, because the reaction rate of SKA oxidation above pH 10 declined significantly (data not shown). DSA reduction was optimum at pH 7. GDH activity under various pH conditions was also plotted as a reference. The results described above suggest that the reaction at neutral pH is the pH optimum for the coupling reaction of SKDH with GDH for SKA production. In order to demonstrate that the coupling reaction was driven forward to SKA formation, NADP was given instead of NADPH. The other criteria were almost the same as described previously. SKDH (0.1 U of DSA reduction activity) was reacted with 20 μmol of DSA in the presence of 0.25 μmol of NADP, 100 units of GDH, and 250 μmol of D-glucose in 1.0 ml in an Eppendorf plastic tube. The reaction was terminated after 180 min of incubation by the addition of 0.1 ml of 60% TCA. The SKA formed was measured with

![Fig. 1. Native PAGE and SDS–PAGE of SKDH.](Image)

Native PAGE was done with two columns of disc gel by loading 20 μg of SKDH. A, protein staining with Commassie brilliant blue. B, enzyme activity staining by incubating the developed gel in a reaction mixture containing 1 mM of SKA, 50 μM of NADP, 0.04 mg per ml of nitroblue tetrazolium, 0.14 mg per ml of phenazine methosulfate, and 50 mM glycine–NaOH, pH 10.0. C, SDS–PAGE. Left lane, prestained SDS–PAGE standard marker proteins including phosphorylase B, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.1 kDa, and lysozyme, 14.4 kDa. Right lane, SKDH (5 μg) was applied.
the supernatant and plotted as shown in Fig. 2B. As expected, the optimum pH of the coupling reaction was observed at pH 7.0, with the highest SKA production (about 20 μmol) in a stoichiometric manner. Thus, a pH of 7.0 for SKA production, set in the previous report, has been found to be reasonable.3)

Different amounts of DSA were added to examine the proportionality of the coupling reaction in SKA production. As expected, the coupling reaction was entirely proportional to the different amounts of DSA added implying that NADPH regeneration was guaranteed by GDH and excess D-glucose during the reaction (Fig. 3). The coupling reaction was also proportional to the SKDH added in the reaction mixture (data not shown). When the deproteinized reaction mixture was developed with a Dowex column (1 × 4, acetate form) up to 25 mM NaCl of linear gradient, only one sharp elution peak of SKA appeared, at about 15 mM NaCl, but no DSA was detected in the chromatogram, though the authentic DSA came from the column at 20 mM NaCl under these conditions. These observations indicate that the DSA initially added in the reaction mixtures for SKA production was completely converted to SKA within the expected incubation time.

In this study, SKA production was examined with highly purified SKDH and GDH. As found previously,2) DSA was produced from quinate by growing cells, resting cells, dried cells, and cell membrane. Thus, DSA need not be purified before SKA production, and any solutions containing DSA as the major product can be used directly for SKA production, because no other NADP-dependent enzymes from the organism, if they existed in the same reaction mixture, would ever disturb the coupling reaction and, furthermore, the SKA produced can finally be purified by column chromatography, as exemplified in this study.

Fig. 2. Effect of pH on SKA Oxidation, DSA Reduction, GDH, and a Coupling Reaction for SKA Production.

A, SKDH (0.05 units) was reacted with SKA (○) and with DSA (●) under the standard assay conditions, except that the buffer solutions used were varied. McIlvaine buffer solutions were used for the assay from pH 5.0 to 8.0. The enzyme activity was assayed in 50 mM glycine–NaOH at pH 9.0 and 10.0. NADP-Dependent GDH (0.05 units) was assayed under similar conditions (△). B, the reaction rate of the coupling reaction was measured by assaying SKA formed under various pH conditions. SKDH (0.1 U) was reacted with 20 μmol of DSA in the presence of 0.25 μmol of NADP, 100 units of GDH, and 250 μmol of D-glucose. After the reaction was terminated by the addition of TCA, the SKA formed was measured with the supernatant. McIlvaine buffer solutions were used for the reaction from pH 4.0 to 8.0. SKA production was done in 50 mM glycine–NaOH at pH 9.0 and 10.0.

Fig. 3. Time Course of SKA Production under Different Amounts of DSA.

The reaction mixture (3 ml) consisted of SKDH (1 reductase unit), GDH (1,000 units), D-glucose (3 mM), NADP (2.5 μmol), and 30 mM potassium phosphate buffer, pH 7.0, containing 5 mM β-mercaptoethanol and 5 mM EDTA. Different amounts of DSA were added: ○, 25 μmol; ●, 50 μmol; ■, 100 μmol; ■, 200 μmol. Incubation was carried out at 25 °C by standing the reaction mixture. Part of the reaction mixture (0.1 ml) was taken out periodically as indicated and mixed with 10 μl of 60% TCA to terminate the reaction. The SKA formed was measured with the supernatant. The conversion ratio was expressed as SKA formed to DSA initially added.
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