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Communication

High Shikimate Production from Quinate with Two Enzymatic Systems of Acetic Acid Bacteria

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3-Dehydroshikimate was formed with a yield of 57–77% from quinate *via* 3-dehydroquinate by two successive enzyme reactions, quinoprotein quinate dehydrogenase (QDH) and 3-dehydroquinate dehydratase, in the cytoplasmic membranes of acetic acid bacteria. 3-Dehydroshikimate was then reduced to shikimate (SKA) with NADP-dependent SKA dehydrogenase (SKDH) from the same organism. When SKDH was coupled with NADP-dependent D-glucose dehydrogenase (GDH) in the presence of excess D-glucose as an NADPH re-generating system, SKDH continued to produce SKA until 3-dehydroshikimate added initially in the reaction mixture was completely converted to SKA. Based on the data presented, a strategy for high SKA production was proposed.

Key words: *Gluconobacter oxydans*; NADP-shikimate dehydrogenase; oxidative fermentation; quinate oxidation; shikimate production

SKA is a key intermediate for aromatic amino acids as well as for large numbers of antibiotics, alkaloids, and herbicides. Recently, another immediate impact has been given to SKA as a precursor for oseltamivir synthesis protecting people from pandemic flu infection. In spite of global warnings from WHO about pandemic flu, including avian influenza, there are insufficient measures for oseltamivir over the world. One reason is the technical difficulties in preparing SKA, because two different metabolic pathways, the glycolysis and the pentose phosphate pathway, must be combined in forming 3-deoxy-*arabino*-heputulosonate 7-phosphate before reaching SKA. Furthermore, the metabolic location of SKA is far from D-glucose and it is difficult to lead the metabolic flow to SKA production by classic fermentation technology or by modern molecular biotechnology.[1,2] Although resources are limited, SKA is also produced by extraction from plants like *Illicium anisatum* or *I. verum*.[3] Total synthesis of SKA by organic chemistry has not been practical. Nevertheless, it is necessary to develop a novel method for more effective and convenient SKA production contributing to oseltamivir synthesis.

QDH and 3-dehydroquinate dehydratase are localized predominantly on the outer surface of the cytoplasmic membranes of some species of *Gluconobacter* strains, and quinate is oxidized to 3-dehydroshikimate *via* 3-dehydroquinate in a sequential manner.[4–6] In the cytoplasm, SKDH (EC 1.1.1.25) catalyzes a reversible reaction of SKA oxidation to 3-dehydroshikimate and 3-dehydroshikimate reduction to SKA. In the previous papers, we proposed SKA production by a single cellular system of acetic acid bacteria,[4,5,7,8] but many times of trial and error combining the two separately localized enzymatic systems taking place outside and inside the cells together have given insufficient production of SKA. As a positive response to global needs for oseltamivir, we have developed a better strategy for high SKA production allowing the two separately localized enzymatic systems to work sequentially. Dried cells or the membrane fraction involving QDH and 3-dehydroquinate dehydratase can be used for 3-dehydroshikimate production in the first reaction (enzymatic system 1 in Fig. 1). The second reaction is a coupling reaction composed of two cytosolic enzymes, SKDH and GDH (EC 1.1.1.47) as an NADPH re-generating enzyme (enzymatic system 2 in Fig. 1). The coupling reaction of the two cytosolic enzymes in the presence of excess D-glucose worked well, as expected, until 3-dehydroshikimate added initially was converted completely to SKA. The overall reaction carried out for SKA production by the two different enzymatic systems is depicted in Fig. 1.

Dried cells and membrane fraction were prepared from *Gluconobacter oxydans* IFO 3244, as described previously.[4] As will be explained elsewhere, a highly purified SKDH having 60 units/mg of SKA oxidation activity at pH 10.0 and 25 units/mg of 3-dehydroshikimate reduction activity at pH 6–7 was prepared. According to the previous paper,[9] GDH having 120 units/mg at pH 9 was purified from the same organisms. For enzymatic determination of SKA and 3-dehydro-

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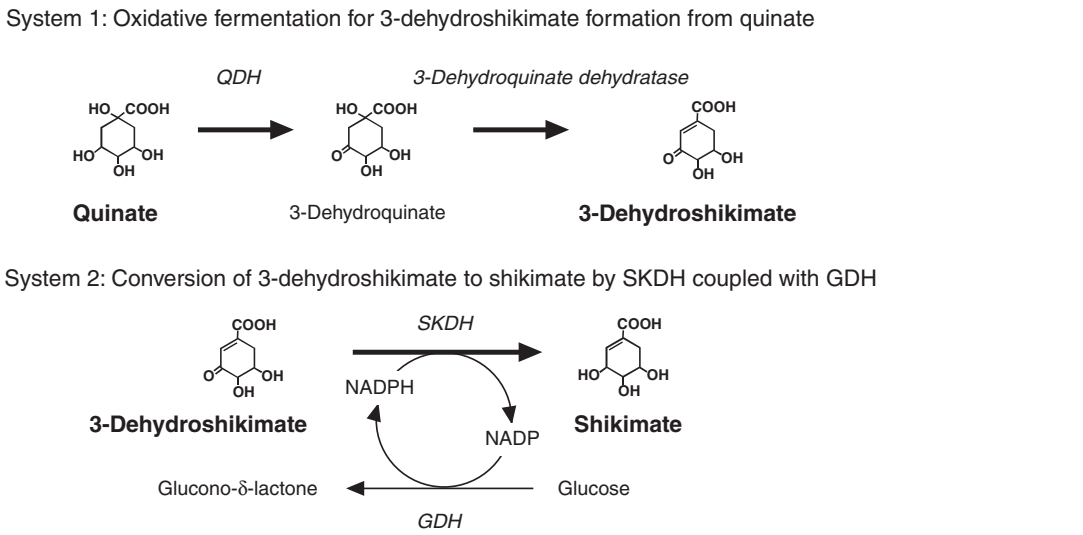

Fig. 1. Schematic Drawings of Overall Reaction for SKA Production.

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shikimate, SKDH has been used extensively because it is highly reactive only with SKA and 3-dehydroshikimate. On the other hand, quinate, 3-dehydroquinate, 3-dehydroshikimate, and SKA were detected or identified by paper chromatography, as described by Yoshida and Hasegawa.[10] Since 3-dehydroquinate and 3-dehydroshikimate are not commercially available, they were prepared after chromatographic separation from the reaction products of quinate oxidation by dried cells, as described previously.[4]

For enzymatic system 1, the dried cells or the membrane fraction were incubated with quinate under different pHs (pH 3–10), and the 3-dehydroshikimate formed was measured using the deproteinized supernatants. In most cases, 1 g of quinate was incubated with 2.5 g of dried cells or 5 g of dried membrane fraction in 100 ml of McIlvaine buffer, pH 3.0–8.0, in a 500-ml Erlenmeyer flask. For the reactions at pH 9.0 and 10.0, 50 mM glycine–NaOH buffers were used. Under the conditions above, the initial quinate concentration corresponded to 52 mM. Incubation was carried out at 30 °C under shaking. Under these conditions, QDH oxidized quinate transferring electrons generated to ubiquinone-10 in the cytoplasmic membrane and then to the terminal oxidase functioning as ubiquinol oxidase, in which molecular oxygen is reduced to water. The cells or the membrane fraction was removed by centrifugation after the addition of trichloroacetic acid (TCA) to 5.5%. After 50 times dilution of the supernatant with 0.1 M acetate buffer, pH 6.0, 3-dehydroshikimate reduction activity was measured at 25 °C with SKDH by incubating an aliquot of the diluted supernatant with 0.25 µmol of NADPH and the enzyme (1 unit of 3-dehydroshikimate reduction activity) in 0.1 M acetate buffer, pH 6.0, in a total volume of 1.0 ml. Since SKDH was highly specific to 3-dehydroshikimate reduction and SKA oxidation, either the rate assay or the

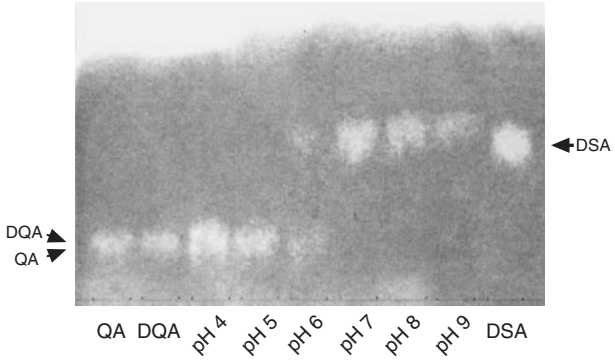

Fig. 2. Paper Chromatogram of 3-Dehydroshikimate Production.
Dried cells or dried membrane preparation was incubated with quinate (0.1 M) under various pHs. Reaction mixtures obtained after 20 h incubation were developed by paper chromatography. Standard quinate, 3-dehydroquinate, and 3-dehydroshikimate were also developed, as indicated. QA, DQA, and DSA mean quinate, 3-dehydroquinate, and 3-dehydroshikimate respectively.

end point measurement was valid to estimate the concentration of the two substrates by reading the absorbance change at 340 nm. 3-Dehydroshikimate was detected as a brilliant yellow spot on a paper chromatogram, although the efficiency and reaction rate for 3-dehydroshikimate production varied under the conditions employed. Under the conditions above, incubation at pH 7.0–9.0 for 20 h gave the fastest conversion rate of quinate to 3-dehydroshikimate (Fig. 2). The apparent concentration of 3-dehydroshikimate reached 30–40 mM, indicating 57–77% conversion efficiency from quinate. At pH 6.0, about 50% of quinate oxidation appeared, while no positive 3-dehydroshikimate production was observed at pH 4.0 or 5.0, although the quinate initially added was almost completely converted to 3-dehydroquinate, judging from the developed color spots. Quinate and 3-dehydroquinate gave almost the same *Rf*-values on

the paper chromatogram under the conditions employed, but they were identified with each other because of different colors developed. Quinate gave a pink spot while 3-dehydroquinate was colored yellow, as noted in the previous paper.[4] Under prolonged incubation with dried cells at pH 7.0 to 9.0 for over 50 h, the 3-dehydroshikimate produced was readily converted to protocatechuate, an unfavorable byproduct for 3-dehydroshikimate production. More stable 3-dehydroshikimate production was observed when quinate oxidation was conducted for 2 d at rather acidic pH, pH 4 to 6 (data not shown). With more minute control of quinate oxidation, higher yields of 3-dehydroshikimate are expected in future. The fractions containing 3-dehydroshikimate from Dowex column chromatography were neutralized with alkali and freeze-dried to concentrate the compound. The dried material was dissolved in a small volume of water and insoluble materials were removed by centrifugation. The 3-dehydroshikimate thus prepared was determined to be 2.1 M by assay of 3-dehydroshikimate reduction activity, and was used for SKA production.

The reaction mixture for enzymatic system 2 consisted of 250 mg of 3-dehydroshikimate (about 1.4 mmol), NADP (5 µmol), SKDH (20 units of 3-dehydroshikimate reduction activity), GDH (1,000 units), and excess D-glucose (5 mmol). The total volume was adjusted to 20 ml with 30 mM potassium phosphate buffer, pH 7.0, containing 5 mM β-mercaptoethanol and 5 mM EDTA. Incubation was carried out at 25 °C by standing the reaction mixture. Part of the reaction mixture (0.1 ml) was taken out periodically and mixed with 10 µl of 60% TCA to terminate the reaction. After removal of precipitate, an aliquot of the reaction mixtures (5 µl) was developed by paper chromatography, and two different coloring reagents were sprayed under the conditions employed previously.[4] A clear red spot of SKA appeared, as shown in the upper frame of Fig. 3. Under the conditions above, the reduction of 3-dehydroshikimate to SKA was complete within 75 min. As expected, the spot of 3-dehydroshikimate almost disappeared from the reaction mixture before 90 min incubation. The SKA formed was also measured with an aliquot of the reaction mixture by end point measurement with SKDH (1 oxidation unit) in 50 mM glycine–NaOH, pH 10.0, in the presence of 0.25 µmol of NADP. The conversion rate of 3-dehydroshikimate to SKA by the coupled enzyme reaction was almost 100% (lower frame in Fig. 3) and showed a good coincidence with the results from paper chromatography.

Since SKDH showed strong reactivity with 3-dehydroshikimate even in highly diluted solution, the reaction system for SKA production can be scaled up successfully with some increased SKDH and GDH. It may not be necessary to increase NADP or NADPH if NADPH re-generation is guaranteed with excess D-glucose. The above experiment allowed us to finish within the expected reaction period. Thus SKA production should continue until the given 3-dehydroshikimate is totally converted to SKA in the same way as exemplified in this study. There should be no negative factors against SKA production when the two different enzymatic systems are employed in a sequential manner. Thus other forms of SKDH and GDH such as immobilized enzyme catalysts should also be successful for SKA production, as suggested in the previous report.[4] In order to make these results practical for industrial applications like other oxidative fermentation, we must confirm that the system developed here is valid even in increased substrate concentrations. Since abundant quinate can be supplied readily from industrial processing of coffee wastes,[11–13] scaling up of this enzymatic system for higher SKA production should be possible to provide the direct precursor for oseltamivir, contributing to prevent unexpected pandemic flu.

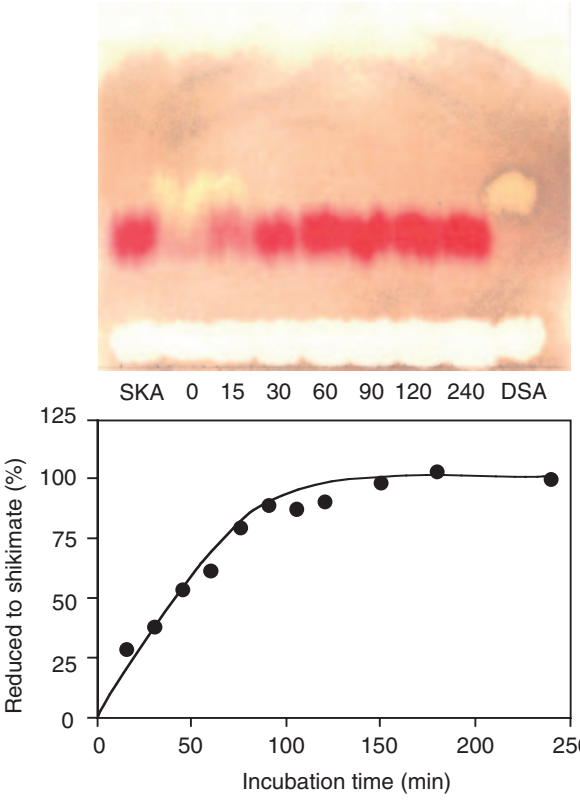

Fig. 3. Conversion of 3-Dehydroshikimate to SKA by SKDH Coupled with GDH.

Upper frame: The coupled enzymatic reaction by two NADP-dependent enzymes was conducted in the presence of 3-dehydroshikimate and NADP as described in the text. Part of the reaction mixture was taken out periodically, as indicated, and mixed with TCA to terminate the reaction. An aliquot of the reaction mixture (5 µl) was spotted and developed by paper chromatography. Lower frame: An aliquot of the terminated reaction mixture (25 µl) was diluted 50 times with 50 mM of glycine–NaOH, pH 10.0. SKDH activity was measured in 50 mM of glycine–NaOH, pH 10.0, with 30 µl of the diluted samples by the end point measurement of SKA oxidation. After 1 h incubation, the optical density was measured at 340 nm and the conversion rate of 3-dehydroshikimate to SKA was determined as plotted.

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