Enzymatic Alteration in the Shikimate Pathway during Derivation of Menaquinone-4-producing Mutants of *Flavobacterium* sp. 238-7

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Activities of seven enzymes of the shikimate pathway were compared between the wild-type strain and menaquinone-4-producing mutant strains of *Flavobacterium* sp. 238-7. Activity of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, the first regulatory enzyme in the shikimate pathway, was almost the same in these strains in the stationary phase, but the activity of a sulfonamide-resistant strain, SP0736, was 2 times higher than that of other strains in the logarithmic phase. Shikimate dehydrogenase activity was almost the same among these strains during cultivation. Other enzyme activities of mutants were 1.5–4 fold higher than those of the wild-type strain. DAHP synthase and shikimate kinase were found to be inhibited by chorismate and MK-4. Feedback inhibition for shikimate kinase by chorismate was partially removed in strain SP0736.

Menaquinone (MK)-4 productivity of *Flavobacterium* sp. 238-7 was improved by the derivation of mutants.^{1,2)} The best MK-4 producer was a sulfapyridine-resistant strain, SP0736.²⁾ MK was synthesized through the shikimate pathway, which was involved in the biosynthesis of various aromatic compounds, such as aromatic amino acids and folic acid. Sulfonamides inhibit the biosynthesis of folic acid due to inhibition of dihydropteroate synthase in competition with *p*-aminobenzoate. In this context, the shikimate pathway of strain SP0736 might be improved at the enzymatic level, since both the MK-4 productivity and the resistance to sulfapyridine were increased simultaneously.

In this paper, we describe the results of an investigation of enzymatic alteration of the shikimate pathway during the derivation of MK-4-producing mutants.

Materials and Methods

Microorganisms. The wild-type strain, an HNA-resistant mutant strain, K₃-15,¹⁾ and a sulfapyridine-resistant mutant strain, SP0736,²⁾ of *Flavobacterium* sp. 238-7 were

used in this study.

Medium and cultivation. The wild-type strain and strain K₃-15 were cultivated in 50 ml of the optimal medium constructed for MK production of strain K₃-15³⁾ in a 500-ml shaking flask at 28°C for 1–6 days. Strain SP0736 was cultivated with 35 ml of the optimal medium for strain SP0736²⁾ in a 500-ml shaking flask at 28°C for 1–6 days.

Enzyme preparation. Cells harvested at an appropriate time were washed twice with 0.2% KCl and suspended in different buffers for each enzyme assay: 100 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol (DTT) for measuring 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase and dehydroquinase synthase; 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT and 10% glycerol for dehydroquinase and shikimate dehydrogenase; 10 mM potassium phosphate buffer, pH 7.0, for shikimate kinase, 5-enolpyruvylshikimate-3-phosphate (ESP) synthase and chorismate synthase. Then cells were disrupted sonically with a Kubota insonator 201M (2 MHz for 20 min) below 4°C. After centrifugation at 18,000 × *g* for 20 min, the supernatant was used as the crude enzyme preparation.

DEAE-sephacel column chromatography. The crude enzyme preparation contained a considerable amount of activity of alkaline phosphatase. For the measurement of enzyme using phosphorylated intermediate as the substrate, the phosphatase was removed from the crude en-

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zyme preparation by DEAE-sephacel column chromatography: the enzyme solution was put on a DEAE-sephacel column (0.7×5.0 cm) equilibrated 10 mM potassium phosphate buffer, pH 7.0. After removing the phosphatase by washing with the same buffer, the enzymes adsorbed were eluted with the buffer containing 1 M NaCl. The resultant eluate was concentrated by ultrafiltration and then used for measurement of the enzyme activity.

Enzyme assay. DAHP synthase was assayed by the method described by Shiiro *et al.*⁴⁾ Dehydroquinase synthase and shikimate dehydrogenase were assayed by the method of Sugimoto and Shiiro.⁵⁾ Dehydroquinase was assayed by the method of Gollub *et al.*⁶⁾ Shikimate kinase and ESP synthase were assayed by a modification of the method of Sugimoto and Shiiro⁵⁾: the reaction mixture consisted of 100 μ mol of potassium phosphate buffer, pH 7.0, 15 μ mol of $MgCl_2$, 15 μ mol of ATP, 15 μ mol of KF, 5 μ mol of potassium shikimate and the enzyme in a total volume of 1 ml. After incubation at 30°C for an appropriate period, the decrease of shikimate was measured by Millican's method.⁷⁾

The reaction mixture for ESP synthase assay consisted of 100 μ mol of potassium phosphate buffer, pH 7.0, 10 μ mol of EDTA, 10 μ mol of KF, 4 μ mol of phosphoenolpyruvate (PEP), 2.5 μ mol of shikimate 3-phosphate and the enzyme in a total volume of 0.5 ml. After incubation at 30°C for an appropriate period, the decrease of PEP was measured by a system using lactate dehydrogenase and pyruvate kinase.

Chorismate synthase was assayed by the modification of the method of Morell *et al.*⁸⁾: the reaction mixture was the same except that nitrogen gas replacement was used instead of hydrogen gas replacement. After incubation at 30°C for an appropriate period, the increase of chorismate was determined by measuring the amount of phenylpyruvate enolate anion derived from chorismate at 320 nm.

One unit of each enzyme activity was defined as the amount catalyzing the formation or disappearance of 1 μ mol of product or substrate per min.

Measurement of chorismate. The cell-free extract used for the measurement of intracellular chorismate content was prepared by the same method as described in *Enzyme preparation*. The suspension buffer was 100 mM Tris-HCl buffer, pH 8.5. The chorismate was measured by the method described in the chorismate synthase assay.

Protein assay. Protein was measured by the method of Bradford⁹⁾ with a protein assay kit (Bio Rad Lab.).

Chemicals. DAHP, shikimate 3-phosphate and 5-enolpyruvylshikimate 3-phosphate were synthesized enzymatically with the enzyme of strain SP0736 in the same reaction mixture for the assay of each enzyme. 5-Dehydroquinase was prepared by the procedures of Haslam *et al.*¹⁰⁾ DEAE-sephacel was purchased from

Pharmacia Fine Chemicals. PEP, erythrose 4-phosphate and shikimate were purchased from Sigma Chemical Co., Ltd. Pyruvate kinase and lactate dehydrogenase were purchased from Boehringer Mannheim. All other chemicals were obtained from the usual commercial sources.

Results

Comparison of DAHP synthase activity between wild-type strain and mutant strains

In our previous study,³⁾ DAHP synthase activity was compared between the wild-type strain, an HNA-resistant mutant strain and sulfonamide-resistant mutant strains in the screening medium at the stationary phase. However, little difference was observed in the activities of these strains. In this study, therefore, the wild-type strain, strain K₃-15 and strain SP0736 were cultivated in the optimal medium for MK-4 production of each strain and the DAHP synthase activity was followed for 1–6 days (Fig. 1). Almost the same DAHP synthase activities of every strains were observed at the stationary phase, about 0.5×10^{-3} units/mg of protein. From the mid- to late-logarithmic phase, the DAHP synthase activity of strain SP0736 was about two times higher than those of other strains, and was more than 1.0×10^{-3} units/mg of protein.

Fig. 1. DAHP Synthase Activity of Wild-type Strain and Mutant Strains.

DAHP synthase activity was measured with crude enzyme prepared freshly each time from the cells of wild-type strain (A), strain K₃-15 (B) or strain SP0736 (C). Cultivation was done as described under Materials and Methods. DAHP synthase activity, ●.

Comparison of other enzyme activities in the shikimate pathway

The time courses of several enzyme activities in the shikimate pathway were examined. Table I shows the highest specific activity of each enzyme in the crude extract of the wild-type strain mutant strains.

The highest activity of dehydroquinase synthase was observed at the late logarithmic phase in all strains. The enzyme activities of mutant strains were about 3–4 fold higher than that of the wild-type strain.

The highest activity of dehydroquinase was observed at the late logarithmic phase in all strains. The enzyme activities of mutant strains were about 1.5 times higher than that of the wild-type strain.

Almost the same level of the activity of shikimate dehydrogenase was observed in all strains during the cultivation. No difference was observed in the enzyme activity among these strains.

As for shikimate kinase and ESP synthase, the highest activity was observed at the mid-logarithmic phase in all strains. Shikimate kinase and ESP synthase activities of mutant strains were about 1.5 times higher than that of the wild-type strains.

The activity of chorismate synthase could not be detected in the presence of oxygen. Therefore, the enzyme activity was measured in the nitrogen atmosphere as described under Materials and Methods. The highest activity

of chorismate synthase was observed at the mid-logarithmic phase. The enzyme activity of each mutant strain was elevated with every mutation. The enzyme activity of strain SP0736 was more than two times higher than that of the wild-type strain, and was 0.74×10^{-3} units/mg of protein.

Feedback inhibition of DAHP synthase and shikimate kinase

DAHP synthase¹¹⁾ and shikimate kinase¹²⁾ were reported to be inhibited by the intermediary and final products in the biosynthetic pathway of aromatic amino acids. Chorismate and MK-4 were found to inhibit activities of DAHP synthase and shikimate kinase of *Flavobacterium* sp. 238-7 in this study.

The release of the inhibition during the derivation of the MK-4-producing mutants was investigated (Table II). MK-4 strongly inhibited DAHP synthase activity of all strains at the concentration of 1 mM. About 10–20% of inhibition of DAHP synthase activity of all strains was observed at the chorismate

Table II. EFFECTS OF CHORISMATE AND MK-4 ON DAHP SYNTHASE AND SHIKIMATE KINASE ACTIVITIES OF MK-4-PRODUCING MUTANTS OF *Flavobacterium* SP. 238-7

Enzymes	Inhibitors conc. (mM)	Relative activities (%)		
		Wild	K ₃ -15	SP0736
DAHP synthase				
	MK-4			
	1	30.1	14.9	3.9
	0.1	84.0	91.8	97.5
	0.01	100.0	100.0	100.0
	Chorismate			
	1	78.1	89.1	78.7
	0.1	90.6	88.3	72.3
	0.01	100.0	96.4	85.6
Shikimate kinase				
	MK-4			
	1	7.7	1.9	7.7
	0.1	38.7	40.4	49.3
	0.01	67.1	70.0	94.1
	Chorismate			
	1	73.7	83.8	99.0
	0.1	76.0	85.0	98.5
	0.01	100.0	100.0	100.0

Table I. ENZYMATIC ALTERATION DURING DERIVATION OF MK-PRODUCING MUTANTS OF *Flavobacterium* SP. 238-7

Enzymes	Maximum activities (10 ⁻³ units/mg)		
	Wild	K ₃ -15	SP0736
DAHP synthase	0.64	0.60	1.22
Dehydroquinase synthase	0.18	0.64	0.52
Dehydroquinase	19.1	26.1	23.1
Shikimate dehydrogenase	3.32	3.40	3.34
Shikimate kinase	3.69	5.25	4.94
ESP synthase	4.60	6.52	5.56
Chorismate synthase	0.31	0.48	0.74

Table III. INTRACELLULAR ACCUMULATION OF CHORISMATE BY MK-4-PRODUCING MUTANTS OF *Flavobacterium* SP. 238-7

Cultivation time (hr)	Chorismate (mg/g-DCW)		
	Wild	K ₃ -15	SP0736
24	0.54	0.60	0.67
48	0.11	0.14	0.12
72	0.09	0.17	0.11
120	0.20	0.25	0.17

concentration of 1 mM. Thus, the release of the inhibition of both chorismate and MK-4 for the DAHP synthase were not observed. Shikimate kinase activities of all strains were also inhibited strongly by the MK-4 concentration of 1 mM. Chorismate caused 20–30% inhibition of the shikimate kinase activity of the wild-type strain and strain K₃-15 at the concentration 0.1–1.0 mM, the shikimate kinase activity of strain SP0736 was not inhibited even at the chorismate concentration of 1 mM. It was considered that the inhibition of shikimate kinase activity by chorismate was partially released in strain SP0736.

Intracellular accumulation of chorismate

Table III shows the intracellular chorismate concentration of each strain. The maximum chorismate content in cells was observed at the mid-logarithmic phase in all strains. Little difference was observed among these strains. Extracellular chorismate could not be detected.

Discussion

In this study, the enzymatic alteration in the shikimate pathway was investigated in the wild-type strain, an HNA-resistant mutant strain, K₃-15, and a sulfapyridine-resistant mutant strain, SP0736. In the derivation of strain K₃-15, five enzyme activities of the shikimate pathway, excepting DAHP synthase and shikimate dehydrogenase, were elevated. Furthermore, DAHP synthase and chorismate synthase activities were improved in the derivation of strain SP0736. A sulfonamide-

resistant mutant of *Brevibacterium flavum* that accumulated a large amount of L-tryptophan was found to have 2- to 3-fold increase in the DAHP synthase activity in the mutant strain compared to that of the parent strain.^{5,13} DAHP synthase activity of strain SP0736 was also improved by the acquisition of sulfonamide resistance, increasing the MK-4 productivity. Furthermore, the increase of chorismate synthase activity and the release of inhibition of shikimate kinase activity by chorismate of strain SP0736 could be also considered as a reason for the enhancement of MK-4 productivity.

A timely relationship between the MK-4 productivity and the activity of each enzyme of the mutants was not observed. However, the activities of most of enzymes in the shikimate pathway, except for DAHP synthase and shikimate dehydrogenase, of mutants were always higher than those of wild-type strain during cultivation. Thus these enzymes might contribute the enhancement of MK-4 productivity of mutants.

Sulfonamides did not inhibit the growth of *Flavobacterium* sp. 238-7 completely. In the derivation of sulfonamide-resistant mutants,²⁾ the colonies with more rapid growth were picked up. It was considered that the rapid growth of strain SP0736 in the presence of sulfonamide was due to the higher DAHP synthase activity than other strains in the early stage of cultivation.

A sulfonamide-resistant mutant of *B. flavum* producing L-tryptophan accumulated 1.5- to 2-times higher concentration of chorismate than did the parent.¹³⁾ The chorismate content of all strains of *Flavobacterium* sp. 238-7 tested in this study was almost the same level, though the abilities of enzymes of the shikimate pathway of mutants were improved. The result showed the possibility that the activity of the enzymes located next to chorismate synthase in the biosynthetic pathway of MK or other compounds might be enhanced and, consequently, the thorough use of intracellular chorismate was caused.

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