Epimerization of (–)-Isodihydrocarvone to (–)-Dihydrocarvone by *Pseudomonas fragi* IFO 3458†

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Received August 12, 1974

Epimerization of (–)-isodihydrocarvone (I) to (–)-dihydrocarvone (II) by *Pseudomonas fragi* IFO 3458 was studied. I was easily epimerized to II by the growing cells, the resting cells or the cell-free extracts.

An epimerase catalyzing the conversion of I to II was partially purified from the bacterial extracts about 56-fold with heat-treatment, ammonium sulfate precipitation and DEAE-Sephadex A-50 column chromatography. By the action of this epimerase, the ratio of I to II becomes about 25:75 ($K \approx 3$). It appeared that the epimerase is very stable to heat; the activity of epimerization remains 66 and 36% after treatment at 97°C for 60 and 120 min, respectively.

In the pathway of (+)-carvone metabolism by *Pseudomonas ovalis* strain 6-1¹⁾ and a strain of *Aspergillus niger*,²⁾ an initial reaction involves the hydrogenation of (+)-carvone to (–)-isodihydrocarvone (I) and the latter compound is further hydrogenated to (–)-isodihydrocarveol and (–)-neoisodihydrocarveol.

During the course of investigation on the natural distribution of carvone reducibility of various microorganisms (bacteria, yeasts and fungi), it was found that I was epimerized to (–)-dihydrocarvone (II) in the growing cells of *Pseudomonas fragi* IFO 3458, *Pseudomonas fluorescens* IFO 3081, *Aerobacter aerogenes* IFO 3319 and *Aerobacter aerogenes* IFO 12059.

In this article, we wish to report the epimerization of I to II by *Pseudomonas fragi* IFO 3458.

METHODS AND MATERIALS

Cultivation of microorganism. *Pseudomonas fragi* IFO 3458, which had been supplied by Prof. Shojiro Oomata, University of Osaka Prefecture, was grown aerobically at 30°C for 4 to 7 days in a medium consisting of either 5 g glucose or 5 ml ethanol; 1 g NH_4NO_3 ; 0.5 g KCl; 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 g dried yeast ex-

tract; 200 mg to 500 mg (+)-carvone in 1 liter of 1/30 M KH_2PO_4 - Na_2HPO_4 buffer (pH 7.2).

The metabolic products from (+)-carvone were analyzed in the following manner; after cultivation, the cultural broth (pH 7.5~8.0) was extracted with ether for 24 hr, and from the extracts a mixture of (–)-dihydrocarvone (II) and (–)-isodihydrocarvone (I) (90:10) was separated by using column chromatography over silicic acid and purified by distillation under reduced pressure; bp 92~93°C/12 mmHg, $[\alpha]_D^{22} = -11.2$ ($c=10$, ethanol), IR ν_{max} cm^{-1} 1700 (C=O), PMR $\delta_{\text{Me}_4\text{Si}}$ (in neat) 0.93 ppm (3H, d, $J=6\text{Hz}$ C_1 -methyl proton), 4.78 ppm (2H, singlet, C_9 -olefin proton). In the PMR spectrum of the mixture of I and II (10:90), the peak of I was neglected.

Preparation of compounds. (–)-Carvone, (+)-carvone, (–)-isodihydrocarvone (I), (–)-dihydrocarvone (II), (+)-neodihydrocarveol, (–)-dihydrocarveol, (–)-isodihydrocarveol and (–)-neoisodihydrocarveol were used as the substrates and the standard and authentic specimens for identification, which were prepared from the method described in the previous papers.^{1,4)} Mixtures of I and II (60:40 and 90:10 by peak area in GLC) were prepared from (+)-carvone by the action of *Pseudomonas ovalis* strain 6-1¹⁾ and used as the substrate for the assay of the epimerization. (±)-Isomenthone (isomenthone: menthone=90:10) was supplied by Prof. Hiroo Ueda, University of Osaka Prefecture.

Preparation of epimerase. The cells of *Pseudomonas fragi* IFO 3458 were harvested by centrifugation and washed five times with 1/100 M KH_2PO_4 - Na_2HPO_4 buffer (pH 7.2). Bacterial extracts were prepared from

† Biochemical Reduction of Terpenes. Part V. For the previous paper, see Ref. 3). This work was presented at the Meeting of the Kansai Division of Agricultural Chemical Society of Japan, Kyoto, May 25, 1974.

TABLE I. CONVERSION OF (–)-CARVONE AND (+)-CARVONE BY DIFFERENT MICROORGANISMS

Microorganisms	Products ^{a1} (% of the total)						Substrates (–)-Carvone (+)-Carvone
	I-A II-A	I-B II-B	I-C II-C	I-D II-D	I-E II-E	I-F II-F	
<i>Pseudomonas fragi</i> IFO 3458	19	6	3	72	0	0	0
	59	36	0	2	0	3	0
<i>Pseudomonas fluorescens</i> IFO 3081	59	12	1	1	0	0	27
	44	30	0	0	0	0	26
<i>Aerobacter aerogenes</i> IFO 3319	36	13	1	0	0	0	50
	34	24	0	0	0	0	42
<i>Aerobacter aerogenes</i> IFO 12059	33	12	0	0	0	0	54
	26	23	0	0	0	0	49
<i>Pseudomonas ovalis</i> , strain 6-1 ^{1,4)}	27	5	13	31	0	0	24
	10	59	1	2	12	15	1

^{a1} Product I-A, (+)-dihydrocarvone; I-B, (+)-isodihydrocarvone; I-C, (+)-neodihydrocarveol; I-D, (–)-dihydrocarveol; I-E, (+)-isodihydrocarveol; I-F, (+)-neoisodihydrocarveol; Products II-A~II-F are enantiomers of products I-A~I-F, respectively.

Microorganisms were cultivated aerobically at 30°C for 4 days on the medium (50 ml) described in text with either 35 mg of (–)-carvone or 46 mg of (+)-carvone except for *Pseudomonas ovalis*, strain 6-1.

Conditions for gas liquid chromatography (GLC): 10% PEG-20 M, 3 m × 3 mm, 160°C, N₂ 35 ml/min.

the washed cells (20 g from 3 liter medium) by sonic lysis (Kaijo Denki Co., Ltd., 20 Kc., 15 min, at 5~7°C) in 200 ml of the same buffer. Sonic extracts were centrifuged at 25,500 g for 30 min at –2°C. The opalescent yellow supernatant fluid had the ability to convert **I** to **II**. On the other hand, the broken cell preparation was incapable of converting **I** to **II**. The enzyme was partially purified from this supernatant fluid about 56-fold with heat-treatment (95~97°C for 10 min), ammonium sulfate precipitation (0.4~0.7 saturation) and DEAE-Sephadex A-50 column chromatography.

The reaction mixture consisted of a mixture of **I** and **II** (60:40 or 90:10), 1/30 M KH₂PO₄-Na₂HPO₄ buffer (pH 7.2) and the crude or partially purified enzyme solution. The reaction was started by the addition of the enzyme solution and stopped by the addition of ether. The ether extract was applied to analytical GLC (Shimadzu Gas Chromatograph GC-4A 10% PEG-20 M, 3 m × 3 mm, temp. 140~170°C at the rate of 1°C a min, N₂ 35 ml/min), and the epimerization was assayed by measuring the peak areas of **I** and **II** in GLC before and after the reaction.

Analytical methods. Optical rotation was measured with a Jasco Polarimeter, DIP-SL model. The IR spectra were taken on a Hitachi Infrared Spectrometer in liquid films with sodium chloride optics. The PMR spectra were recorded by a JNM-MH-60 Spectrometer at 60 MHz using tetramethylsilane as an internal standard and without solvent.

RESULTS AND DISCUSSION

When *Pseudomonas fragi* IFO 3458 was cultured with (–)-carvone and (+)-carvone, (–)-carvone was converted through (+)-dihydrocarvone rapidly to (–)-dihydrocarveol, whereas (+)-carvone was initially converted to (–)-isodihydrocarvone (**I**), which was further epimerized day by day to (–)-dihydrocarvone (**II**) rather than being hydrogenated to the corresponding dihydrocarveols (Fig. 1).

The above result suggests that there is an epimerization process between **I** and **II**. This reaction was ascertained in the incubation of either the growing cells (Fig. 2) or the resting cells (Fig. 3) of *Pseudomonas fragi* IFO 3458 with a mixture of **I** and **II** (60:40). With the cell free extracts and a partially purified preparation, the epimerization was also observed.

From the above results, it appeared that in the metabolism of (+)-carvone by *Pseudomonas fragi* IFO 3458, although the initial reaction involves the hydrogenation of (+)-carvone to (–)-isodihydrocarvone (**I**), the latter compound is epimerized to (–)-dihydrocarvone

FIG. 1. Time Course of the Conversion of (-)-Carvone and (+)-Carvone by *Pseudomonas fragi* IFO 3458.

I-C, (+)-neodihydrocarveol; I-F, (+)-neoisodihydrocarveol; III, (-)-neodihydrocarveol; IV, (+)-dihydrocarveol; V, (-)-isodihydrocarveol; VI, (-)-neoisodihydrocarveol. Conditions for GLC were the same as those described in the legend of Table I.

FIG. 2. Epimerization of (-)-Isodihydrocarvone by Growing Cells of *Pseudomonas fragi* IFO 3458.

Pseudomonas fragi IFO 3458 was cultivated in 100 ml of the growing medium described above with 15 mg of a mixture of (-)-isodihydrocarvone and (-)-dihydrocarvone (60:40) and 0.5 ml of ethanol as carbon source. Each 5 ml of the cultural broth was harvested at defined times in order to assay the epimerization reaction; observed ratio of (-)-isodihydrocarvone (●—●) and (-)-dihydrocarvone (○—○) during epimerization reaction; (-----), calculated ratio of (-)-isodihydrocarvone and (-)-dihydrocarvone, when the dihydrocarveols formed are considered as the corresponding dihydrocarvones; (×—×), blank. Conditions for GLC; 10% PEG-20M, 3 m×3 mm, temp. 140~170°C, increasing 1°C/min, N₂ 35 ml per min.

FIG. 3. Epimerization of (-)-Isodihydrocarvone by Resting Cells of *Pseudomonas fragi* IFO 3458.

I, (-)-isodihydrocarvone; II, (-)-dihydrocarvone; III, (-)-neodihydrocarvone; IV, (+)-dihydrocarvone; V, (-)-isodihydrocarvone; VI, (-)-neoisodihydrocarvone.

The reaction mixture contained 2 ml of 1/30 M -KH₂PO₄-Na₂HPO₄ buffer (pH 7.2), 5 ml of the cell suspension (35 mg as dried cell weights) and the substrate (5.0×10^{-4} M, dissolved in 0.025~0.05 ml ethanol) and incubated at 37°C.

Conditions of GLC are the same as shown in Fig. 2. Epimerization reaction of I to II by resting cells (○—○, observed ratio of I and II; -----, calculated ratio of I and II, when the dihydrocarveols formed are reversely calculated to the corresponding dihydrocarvones) and the resting cells treated at 97°C for 15 min (●—●, in this case, dihydrocarveols were not formed.).

(II) rather than being hydrogenated to either (—)-isodihydrocarveol or (—)-neoisodihydrocarveol as shown in *Pseudomonas ovalis* strain 6-1¹⁾ and a strain of *Aspergillus niger*.²⁾ In the epimerization process, I is presumably converted to II through the *enol* form as an intermediate (Fig. 4). The ratio of I to II becomes about 25:75 ($K \approx 3$).

FIG. 4. Proposed Metabolic Pathways of (+)-Carvone by *Pseudomonas fragi* IFO 3458.

I, (—)-isodihydrocarvone; II, (—)-dihydrocarvone; III, (—)-neodihydrocarveol; IV, (+)-dihydrocarveol; V, (—)-isodihydrocarveol; VI, (—)-neoisodihydrocarveol.

(±)-Isomenthone was also epimerized to menthone, though it was in a small amount (17% of isomenthone was epimerized to menthone, when 15 mg of the substrate was added to a 50 ml medium), by the growing cells of *Pseudomonas fragi* IFO 3458.

In the previous papers,^{1,2,4)} we have reported that (+)-isodihydrocarvone and (—)-dihydrocarvone (II) were always found in the microbial reduction of (—)-carvone and (+)-carvone to their corresponding major products, (+)-dihydrocarvone and (—)-isodihydrocarvone (I),

respectively. Until now, it has not been known for the reason of their small yields whether the epimerization occurs chemically or enzymatically, and whether a carvone reductase, though it is not yet substantiated, shows a low stereospecificity for carvone. However, from the present study, it is considered that at least in *Pseudomonas fragi* IFO 3458 the epimerization takes place biochemically.

TABLE II. EFFECT OF HEAT-TREATMENT ON THE EPIMERASE ACTIVITY

A mixture of 0.5 ml of 1/100 M KH_2PO_4 - Na_2HPO_4 buffer (pH 7.2) and 0.5 ml of partially purified enzyme preparation were boiled at 97°C for various times. After the heat-treatment, the mixtures were cooled with water (about 20°C). The substrate ($1.3 \times 10^{-3}\text{M}$, (—)-isodihydrocarvone: (—)-dihydrocarvone=90:10) was added to the above enzyme preparation and incubated for 3 hr at 37°C. Reaction was stopped by the addition of ether and the ether extract was applied to analytical GLC under the same conditions as described in Fig. 2.

Time (min) treated at 97°C	Epimerization (%)	Relative activity (%)
0	44	100
1	44	100
5	42	95
10	42	95
15	39	89
30	32	73
60	29	66
120	16	36
blank	0	—

On the other hand, the crude extract and the partially purified preparation were found to be very stable to heat-treatment; 66 and 36% of the epimerase activity remained after treatment at 97°C for 60 and 120 min, respectively (Table II). This epimerase is of interest in relation to the heat-stability of protein. However, it is not known at present why this epimerase preparation is stable to heat. We are now trying to purify and characterize this epimerase in order to clarify the reason of the heat-stability.

Acknowledgement. The authors wish to express their gratitude to Emeritus Prof. C. Tatsumi, Univer-

sity of Osaka Prefecture, for his constant interest and encouragement. The authors would also like to thank Prof. H. Ueda for his helpful discussions and supplying ($\pm$)-isomenthone and to Prof. S. Oomata, University of Osaka Prefecture, for supplying *Pseudomonas fragi* IFO 3458.

REFERENCES

- 1) Y. Noma, S. Nonomura, H. Ueda and C. Tatsumi, *Agr. Biol. Chem.*, **38**, 735 (1974).
- 2) Y. Noma and S. Nonomura, *ibid.*, **38**, 741 (1974).
- 3) Y. Noma, S. Nonomura and H. Sakai, *ibid.*, **38**, 1673 (1974).
- 4) Y. Noma and C. Tatsumi, *Nippon Nôgeikagaku Kaishi*, **47**, 705 (1973).

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- 1) Y. Noma, S. Nonomura, H. Ueda and C. Tatsumi,