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Maleate *cis-trans* Isomerase from *Arthrobacter* sp. TPU 5446

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A gram-positive aerobic bacterium, *Arthrobacter* sp. strain TPU 5446 with a high maleate *cis-trans* isomerase (MAI) activity, was isolated from soil. Enzyme activity was induced when the strain was cultivated in the presence of maleic acid and yeast extract as the carbon and nitrogen sources, respectively. From a crude extract of the cells, the enzyme was partially purified by ammonium sulfate fractionation and DEAE-Toyopearl column chromatography. The enzyme acted specifically on maleic acid and produced fumaric acid stoichiometrically.

[**Key words:** maleate *cis-trans* isomerase, enzymatic isomerization, *Arthrobacter* sp.]

There is a group of isomerases that catalyze the *cis-trans* isomerization reaction of conjugated double bonds under mild conditions (1). In the course of our studies on the enzymatic synthesis of optically active compounds from unsaturated acids (2–4), we focused our attention on maleate *cis-trans* isomerase (MAI, EC 5.2.1.1) (1), an enzyme which is known to be distributed only in two genera of gram-negative aerobes, *Pseudomonas* (5, 6) and *Alcaligenes* (7). Although the enzyme catalyzes the isomerization reaction between maleic acid and fumaric acid, the simplest geometric isomers with a carbon-carbon double bond, little else is known about the enzyme, such as cofactor requirements, the mechanisms of the enzyme reaction, stabilizing factors, etc. In order to elucidate the differences in enzymological properties of MAI from different species, we isolated *Arthrobacter* sp. TPU 5446 having high MAI activity from soil and describe the partial purification of the enzyme from the strain.

A number of maleic acid-assimilating microorganisms were isolated from soil samples using an enrichment culture technique with a medium containing 10 g yeast extract, 10 g maleic acid, 2 g K_2HPO_4, 1 g NaCl, and 0.2 g $MgSO_4 \cdot 7H_2O$ in 1 *l* of tap water (pH 7.0). Soil samples collected in Toyama Prefecture were added to 10 ml of the screening medium in a test tube and cultivated with or without shaking at 30°C. A loopful of the culture broth was transferred to new medium every two days. After two transfers, the culture broths were spread onto plates containing the screening medium (2% agar). Colonies which formed were isolated in the usual manner. Cell-free extracts of the strains were prepared and the enzyme activity contained therein was assayed by measuring the maleic acid consumed or fumaric acid produced using HPLC (Table 1).

From 564 strains of maleic acid-assimilating bacteria, 5 strains that were able to isomerize maleic acid to fumaric acid were obtained. They were identified as *Alcaligenes faecalis* strain TPU 5338, *A. denitrificans* strain TPU 5343, *Proteus vulgaris* strain TPU 7010, *Pseudomonas alcaligenes* strain TPU 7150, and *Arthrobacter* sp. strain TPU 5446 from their morphological and physiological characteristics using the Minitek™ identification system or general identification methods (8). Table 1 presents the enzyme activities of the cell-free extracts of the strains isolated. We believe this is the first time that it has been demonstrated that MAI producers are also present among gram-positive bacteria (*Arthrobacter*) and in *Enterobacteriaceae* (*Proteus*). Although van der Werf *et al.* (9) suggested the possible existence of MAI activity in microorganisms (*e.g. Bacillus, Corynebacterium, Enterobacter, Pseudomonas, Serratia, Rhodotorula, Saccharomyces, Trichosporiella, Trichosporon, Mortiella*) during their screening for maleate hydratase, an enzyme which hydrates maleic acid to form D-malic acid, they never measured MAI activity in the strains. It is interesting that *P. vulgaris* TPU 7010, which was isolated without shaking, exhibited enzyme activity only when the strain was grown statically. This feature seems to be similar to the 3-methylaspartate ammonia-lyase expression system in *Enterobacteriaceae* (4). Among the isolated strains, *Arthrobacter* sp. TPU 5446 was selected as the most likely source of MAI because of its high productivity of the enzyme. Another maleic acid-assimilating *Arthrobacter* sp., strain MCI 12612, exhibited maleate hydratase activity (2) without MAI activity.

The cultivation conditions required for the production of MAI activity by *Arthrobacter* sp. TPU 5446 were investigated. It was found that both maleic acid and yeast extract were required for production of the enzyme. As

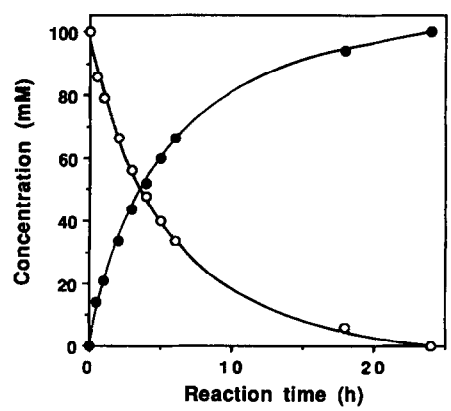

FIG. 1. Time course of maleic acid isomerization by the partially purified enzyme from *Arthrobacter* sp. TPU 5446. The reaction mixture (1 ml) contained 0.2 units of the partially purified enzyme (10 μl), 100 μmol of disodium maleate, 200 μmol of Tris-HCl (pH 8.0), 1 μmol of dithiothreitol, and 1 μmol of 2-mercaptoethanol. The concentrations of maleic (○) and fumaric (●) acids were determined by HPLC.

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TABLE 1. Maleate *cis-trans* isomerase activity in bacteria

Strain			Conditions[a]	Growth (OD_{660})	Total activity (U/l culture)	Total protein (mg/l culture)	Specific activity (U/mg)
Alcaligenes	*faecalis*	TPU 5338	A	10.9	136	937	0.145
	faecalis	IAM 1473	A	10.1	154	958	0.161
	denitrificans	TPU 5343	A	11.3	92	981	0.0938
	denitrificans	IAM 12598	A	10.1	34	886	0.0384
	xylosoxidans	IAM 12686	A	9.63	50	852	0.0587
Pseudomonas	*alcaligenes*	TPU 7150	A	8.38	86	1100	0.0781
	fluorescens	TPU 7102	A	6.18	158	989	0.160
Proteus	*vulgaris*	TPU 7010	A	5.16	0	747	0
			B	1.65	8	189	0.0423
Arthrobacter	sp.	TPU 5446	A	17.3	204	1170	0.174
	globiformis	IAM 12102	A	15.3	142	975	0.146
	pascens	IAM 12343	A	15.4	104	953	0.109
	ureafaciens	IAM 12140	A	16.9	87	1040	0.0837

[a] Cultivation was done with (A) or without (B) shaking.

Strains were grown in 5 ml of the screening medium in an 18-mm test tube with (250 strokes/min) or without shaking at 30°C for 24 h. The cells were harvested by centrifugation. The collected cells were suspended in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol and 1 mM dithiothreitol, and were disrupted at 4°C for 15 min using a Cosmo Bioruptor UCD-200T ultrasonic oscillator. The supernatant solutions obtained by centrifugation were used as cell-free extracts. The enzyme reaction was done in a reaction mixture (0.5 ml) containing 50 μmol of Tris-HCl (pH 8.0), 25 μmol of disodium maleate, 0.5 μmol of dithiothreitol, and enzyme solution for 1 to 24 h at 30°C. The amounts of maleic and fumaric acids were determined by HPLC (Shimadzu) at 240 nm with a SequeTag™ column (0.5 × 25 cm; MilliGen/Biosearch, USA) at a flow rate of 0.5 ml/min using an elution buffer containing 5 mM of KH_2PO_4 and 5 mM of H_3PO_4 in 35% methanol. One unit of MAI activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of fumaric acid from maleic acid per min.

shown in Table 2, the strain demonstrated high MAI activity when grown in medium containing 1.0% each of maleic acid and yeast extract as carbon and nitrogen sources, respectively. No MAI activity was observed with other carbon sources such as glucose, glycerol, sucrose, fumaric acid, or succinic acid. It has been reported (10) that maleic acid isomerization activity is markedly induced by malonic acid in *A. faecalis* IB-14. However, *Arthrobacter* sp. TPU 5446 did not significantly exhibit enzyme activity in medium containing malonic acid and yeast extract at any concentrations (0.2–2.0%) when used as carbon and nitrogen sources, respectively.

The enzyme was partially purified from the cell crude extract. All enzyme purification procedures were carried out at 4°C. The enzyme lost its activity upon sonic oscillation and dialysis. It was reported that MAIs from *Pseudomonas* (5) and *Alcaligenes* (7) are also unstable and are protected from inactivation by reducing agents. Inactivation of the enzyme could be slightly prevented by 2-mercaptoethanol and dithiothreitol. Potassium phosphate buffer (pH 7.0) containing 5 mM of 2-mercaptoethanol and 1 mM of dithiothreitol was used throughout the purification procedure. Cell-free extract of *Arthrobacter* sp. TPU 5446 from 40 l of culture was fractionated with ammonium sulfate (40–80% saturation). The active precipitate was dissolved in 0.01 M buffer and dialyzed against the same buffer. The enzyme solution was applied to a DEAE-Toyopearl 650M column (Tosoh Corp., Tokyo) and eluted with 0.1 M buffer containing 0.1 M NaCl. The active fractions were collected and used as the partially purified enzyme solution (0.33 U/mg protein). During the purification, total enzyme activity decreased from 5,875 (units) to 27.5 (units). The addition of several agents such as metal ions, chelating agents, glycerol, organic solvents, reducing reagents, detergents, organic acids, and sulfhydryl compounds, and the variation of pH and temperature did not prevent inactivation. Otsuka (6) reported that inactivated MAI from *Pseudomonas* sp. can be reactivated with low concentrations of glutathione and cysteine, however, the *Arthrobacter* enzyme could not be protected from inactivation by these compounds at any concentration (0.1–50 mM).

The substrate specificity of the enzyme was examined

TABLE 2. Effect of maleic acid and yeast extract on the formation of maleate *cis-trans* isomerase

Conc. of maleic acid (%)	Nitrogen[a] source (%)	Conc. of nitrogen source (%)	Growth OD_{660}	Total activity (U/l culture)	Total protein (mg/l culture)	Specific activity (U/mg)
0.2	Yeast extract	1.0	13.2	66.7	975	0.0684
0.5	Yeast extract	1.0	16.4	168	1060	0.159
1.0	Yeast extract	1.0	17.3	200	1170	0.171
2.0	Yeast extract	1.0	16.7	88.7	1030	0.0859
1.0	Yeast extract	0.5	9.01	71.3	775	0.0920
1.0	Peptone	1.0	12.3	57.5	869	0.0662
1.0	Meat extract	1.0	11.0	69.9	776	0.0901
1.0	$(NH_4)_2SO_4$	0.5	4.56	57.1	424	0.135

[a] The strains also showed no or low activity less than 1 U/l culture when they were grown in a medium containing 1.0% maleic acid and 0.5–1.0% of the following nitrogen sources; $NaNO_3$, urea, casamino acid, beef extract, malt extract, corn steep liquor, and soybean meal.

Basal medium contained 0.2% K_2HPO_4, 0.1% NaCl, and 0.02% $MgSO_4 \cdot 7H_2O$, pH 7.0. The strains were grown at 30°C for 24 h with shaking (250 strokes/min). The enzyme activity in a cell-free extract was measured as described in Table 1.

with several unsaturated compounds. Like MAIs from *Pseudomonas* (5) and *Alcaligenes* (7), the enzyme was also specific for the conversion of maleic acid to fumaric acid. No other compounds tested underwent *cis-trans* isomerization in the presence of a high concentration of the enzyme. These include: fumaric acid, citraconic acid, mesaconic acid, 2-chloromaleic acid, 2-chlorofumaric acid, 2-bromomaleic acid, 2,3-dimethylmaleic acid, *cis*-3-chloroacrylic acid, *trans*-3-chloroacrylic acid, *trans*-2-pentenoic acid, and tiglic acid.

The stoichiometry of the isomerization reaction was examined using partially purified *Arthrobacter* MAI. As shown in Fig. 1, it was found that 1 mol of fumaric acid was produced per mol of maleic acid consumed. The enzyme was shown to catalyze the stoichiometrical isomerization reaction of maleic acid to fumaric acid, as in the case with MAIs from *Pseudomonas* (5) and *Alcaligenes* (7).

The enzyme from *Arthrobacter* showed similar enzymological properties, such as substrate specificity and stoichiometry, to known MAIs from *Pseudomonas* and *Alcaligenes*. However, the enzyme was not induced by malonic acid and lost most of its activity during purification, even in the presence of reducing reagents.

REFERENCES

1. **Seltzer, S.:** The enzymes, 3rd ed., p. 381–406. Academic Press, New York, NY (1972).
2. **Asano, Y., Ueda, M., and Yamada, H.:** Microbial production of D-malate from maleate. Appl. Environ. Microbiol., **59**, 1110–1113 (1993).
3. **Ueda, M., Sashida, R., Yamada, H., and Asano, Y.:** Production of (*S*)-(+)-citramalic acid from itaconic acid by resting cells of *Alcaligenes denitrificans* strain MCI 2775. Appl. Microbiol. Biotechnol., **40**, 466–469 (1993).
4. **Asano, Y. and Kato, Y.:** Occurrence of 3-methylaspartate ammonia-lyase in facultative anaerobes and their application to synthesis of 3-substituted (*S*)-aspartic acids. Biosci. Biotech. Biochem., **58**, 223–224 (1994).
5. **Scher, W. and Jakoby, W. B.:** Maleate isomerase. J. Biol. Chem., **244**, 1878–1882 (1969).
6. **Otsuka, K.:** *cis-trans* isomerase; isomerization from maleic acid to fumaric acid. Agric. Biol. Chem., **25**, 726–730 (1961).
7. **Takamura, Y., Takamura, T., Soejima, M., and Uemura, T.:** Studies on the induced synthesis of maleate *cis-trans* isomerase by Malonate (III). Purification and properties of maleate *cis-trans* isomerase induced by malonate. Agric. Biol. Chem., **33**, 718–728 (1969).
8. **Barrow, G. I. and Feltham, R. K. A.:** Cowan and Steel's manual for the identification of medical bacteria, 3rd ed. Cambridge University Press, UK (1993).
9. **van der Werf, M. J., van den Tweel, W. J. J., and Hartmans, S.:** Screening for microorganisms producing D-malate from maleate. Appl. Environ. Microbiol., **58**, 2854–2860 (1992).
10. **Takamura, Y., Nakatani, T., Soejima, M., and Aoyama, T.:** Studies on the induced synthesis of maleate *cis-trans* isomerase by Malonate (II). The induction by malonate analogues and the repression by various carbon sources. Agric. Biol. Chem., **32**, 88–93 (1968).