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Gene Cloning and Characterization of Maleate *cis-trans* Isomerase from *Alcaligenes faecalis*

Kazuhisa Hatakeyama, Yoko Asai, Yasukazu Uchida, Miki Kobayashi, Masato Terasawa, and Hideaki Yukawa
Tsukuba Research Center, Mitsubishi Chemical Corporation, 8-3-1, Chuo, Ami, Inashiki, Ibaraki, 300-03, Japan

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Maleate *cis-trans* isomerase, which catalyses the conversion of maleate to fumarate, was purified and characterized from *Alcaligenes faecalis* IFO13111. The molecular weight of maleate isomerase was estimated as 60 kDa, consisting of a 28 kDa dimer as shown by gel-filtration chromatography and SDS-PAGE analysis. Kinetic studies showed that the Michaelis constant for maleate was 4.0×10^{-5} M. The reverse reaction (fumarate to maleate) activity of the enzyme was detected even though it was quite weak. The maleate isomerase gene (*maiA*) was cloned by hybridization using the oligonucleotide DNA probes designed on the basis of the determined N-terminal amino acid sequences of the purified enzyme. The determined DNA sequence of the *maiA* gene contains an open reading frame which encodes a 254-amino-acid sequence. The amino acid sequence of the *maiA* gene product shows no significant homology to any amino acid sequences in the protein data base.

Maleate *cis-trans* isomerase (EC 5.2.1.1), catalyzes geometric isomerization of maleate to fumarate without bond migration. Maleate isomerase is known to be distributed to several maleate assimilating bacteria, *Pseudomonas, Alcaligenes, Serratia, Proteus* and *Arthrobacter* [1,2,3]. Although the enzyme catalyzes the simple geometric isomerization of maleate to fumarate, there are quite a few report about the further characterization of the enzyme or the mechanism of *cis-trans* isomerization reaction [4], because the primary structure of the enzymes have still not been determined.

Here we report the characterization of maleate isomerase from *Alcaligenes faecalis* IFO13111. Enzymatic characterization of this enzyme revealed the presence of the weak reverse reaction and some other properties. Cloning and sequencing of maleate isomerase gene revealed the primary structure of the enzyme. This is the first report of the gene cloning and sequencing of *cis-trans* isomerase and these results will promote the analysis of *cis-trans* isomerase of carbon-carbon double bonds.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

A. faecalis IFO13111 was obtained from Institute for Fermentation, Osaka. The composition of the maleate medium used was as follows (per liter): 10 g of maleic acid, 10 g of meat extract, 10 g of peptone, 5 g of NaCl (pH adjusted to 7.0 with sodium hydroxide). *A. faecalis* was cultivated with shaking at 30°C. Strains of *Escherichia coli* were grown in LB medium [5]. Cultures of *E. coli* were supplemented with antibiotics at following concentrations: Ampicillin, 50 μg/ml; Kanamycin, 50 μg/ml.

Assay of the Maleate cis-trans Isomerase

Maleate *cis-trans* isomerase was assayed by the following two methods.

Method 1. In the purification process, the enzyme activity was assayed by measuring the decrease in absorbance at 240 nm in the presence of swine heart fumarase (Boehringer, Manheim, Germany) in according to the method of Otsuka *et al.* [6].

Method 2. Although method 1 has very good sensitivity for the enzyme purification, the addition of fumarase to the reaction system was sometimes not adequate for the enzyme characterization. In the case of the investigation of the purified enzyme properties, we adopted the method of measuring the increased absorbance at 290nm due to the formation of fumarate from maleate in accordance to Scher and Jakoby [4].

One unit of maleate *cis-trans* isomerase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of fumaric acid from maleic acid per min.

Purification of Maleate Isomerase from A. faecalis

The activity of maleate isomerase from *A. faecalis* IFO13111 was induced by growing cells in the presence of 1% (w/v) maleate or malonate. The highest activity was measured in cell lysate from cultures in late exponential phase of growth (data not shown). Cells were harvested in late exponential phase and washed once with buffer A (20 mM potassium phosphate buffer [pH 7.2], 0.5 mM dithiothreitol (DTT)). The cells were resuspended in buffer A.

All subsequent protein purification procedures were carried out at 4°C. The microbial cells were disrupted by sonification (Sonifier 250,

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Branson) and cellular debris was removed by centrifugation at 10,000 × g for 30 min. After proteins in the supernatant were treated with 15% (wt/vol) streptomycin sulfate, they were fractionated with ammonium sulfate precipitation. The proteins precipitated in the 50-to-70% fraction were collected by centrifugation and dissolved in buffer A. This protein solution was desalted by dialysis with buffer A. The desalted solution was then loaded onto a DEAE-Sephacel (Pharmacia Biotech, Inc., Piscataway, N.Y.) equilibrated with buffer A and eluted by increasing the buffer B (20 mM potassium phosphate buffer [pH 7.2], 0.5 mM DTT, 0.2 M KCl) concentration gradually in a linear gradient of 0% buffer B (40 min), 0 to 100% buffer B (100 min), and 100% buffer B (200 min) at a flow rate of 2.0 ml/min. The active fractions were collected and then loaded onto a Sephacryl S-200 (Pharmacia), and the proteins were eluted in a 2× volume of buffer A at a flow rate 0.5 ml/min. And the active fractions were collected, pooled, and concentrated by ultrafiltration through a Ultra Filter UP-20 membrane (Advantec, Japan). The concentrated protein was loaded onto a Poros HQ/M (PerSeptive Biosystems, Cambridge, MA) equilibrated with buffer C (50 mM Tris-HCl buffer [pH 7.5], 0.5 mM DTT) and eluted by increasing the buffer D (50 mM Tris-HCl buffer [pH 7.5], 0.5 mM DTT, 0.5 M NaCl) concentration in a linear gradient of 0% buffer D (10 min), 0 to 100% buffer D (30 min), and 100% buffer D (40 min) at a flow rate of 10.0 ml/min.

The homogeneity of the purified maleate cis-trans isomerase was confirmed by SDS-[4-to-20%(wt/vol) gradient] polyacrylamide gels electrophoresis (PAGE) and then by Coomassie blue R-250 staining [7]. Protein concentrations were determined by the dye-binding method described by Bradford [8].

The purified enzyme was stored at 4°C and approximately 90% of enzyme activity remained by storing at 4°C for one month in buffer A.

Design of Oligonucleotide DNA Probes for Cloning of maiA

The N-terminal amino acid sequence of purified maleate isomerase was determined by a model 477A protein sequencer (Applied Biosystems, Foster City, CA) with an on-line phenylthiohydantoin analyzer (Applied Biosystems). The first 15 amino acids provided sufficient information to deduce the sequence of oligonucleotide DNA probes as follows:

AF1: 5′-ATG-AAR-ACI-TAY-CGI-ATY-GGI-CAR-ATY-GTI-CC-3′

and

AF2: 5′-CAR-ACI-ACI-ATG-GAR-ACI-GAR-ATY-CCI-GCI-ATG-3′.

Southern Hybridization

Chromosomal DNA was isolated from *A. faecalis* as described by DiLella and Woo [9] and was digested with appropriate restriction enzymes, separated on a 0.8% (wt/vol) agarose gel, and blotted onto a nylon membrane via the alkaline transfer method [5]. Southern hybridization were done at 45°C with oligonucleotide DNA labeled at 5′ end with [γ-^{32}P] ATP using MEGALABEL kit (Takara, Kyoto, Japan) as per manufacture's instructions. The membrane was subsequently washed twice with 2× SSC (1×SSC:0.15 M NaCl/0.015 M Na_3·citrate [pH 7.6]) - 0.1% (wt/vol) SDS for 15 min and 1× SSC-0.1% (wt/vol) SDS for 15 min.

Construction and Screening of a A. faecalis IFO13111 Phage Library

A. faecalis chromosomal DNA was partially digested with *Xho*I so as to yield high molecular weight DNA fragments. The digested DNA fragments were ligated to the *Xho*I site of the λ FIXII phage vector (Stratagene, La Jolla, CA). The ligation mixture was packed using Gigapack II Gold (Stratagene) as per the manufacturer's instructions. The library was amplified once in *E. coli* P2392. λ phage plaque immobilization on nylon membranes (Amersham, Arlington Heights, Ill) and plaque hybridization were performed as described by Sambrook *et al* [5]. *E. coli* JM109 [5] was used as a host for the subcloning of maleate *cis-trans* isomerase gene.

DNA Sequencing

Specific restriction fragments were cloned into pBluescript II (Stratagene) and sequenced by dideoxy chain termination [5] using a 373A DNA sequencer (Applied Biosystems). Nucleotide sequences were determined on both strands. DNA sequence data were analysed by the Genetyx (Software Development, Tokyo, Japan).

RESULTS

Properties of the Purified Maleate cis-trans Isomerase

The enzyme purification process was described in methods. The purified enzyme showed specific activity of 40 units per mg protein. Properties of the purified enzyme were shown as follows.

(a) Molecular weight. The purified enzyme gave a single band by SDS-PAGE and had a molecular weight of approximately 28,000. By gelfiltration chromatography, the molecular weight of the holoenzyme was estimated to be approximately 60,000 (Fig. 1). Therefore, it can be assumed that the enzyme consists of two subunits with identical sizes. At the highest specific activity, 40 units per mg protein, the turnover number was 2,200 moles of product per mole dimer enzyme per min.

(b) Effect of pH and temperature on activity of the enzyme. Enzymatic activity in 1 mM sodium maleate was determined with pH range from 6.5 to 10. The enzyme activity was assayed for 30 mM substrate in 50 mM buffers as follows; phosphate-borate buffer, from pH 6.5-7.5; Tris-chloride buffer, from pH 7.5 to 9.0; glycine buffer, from pH 9.0 to 10.0. The enzyme showed maximal activity at pH 8.0; in the pH range from 7.0 to 9.0, more than 75% of the maximal activity was found. At pH 6.5 and 9.3, about 50% of the maximal activity was found. The temperature optimum of the enzyme determined by the initial velocity for 15 min was 42-45°C. At 35 and 50°C, the enzyme was exhibited about 75% of the maximum activity.

(c) Effect of temperature on the stability of the enzyme. To determine the effect of temperature on stability of the enzyme, purified enzyme was incubated in 50 mM Tris-chloride buffer (pH 8.0) at the temperature range from 40°C to 60°C for 30 min and the remaining activity was measured. The maleate *cis-trans* isomerase was stable below 45°C and almost lost its activity above 53°C for 30 min.

(d) Kinetics of the enzyme. As shown in Fig. 2, the Km value for sodium maleate was determined from a Lineweaver-Berk plot [10] as 0.04 mM, which was

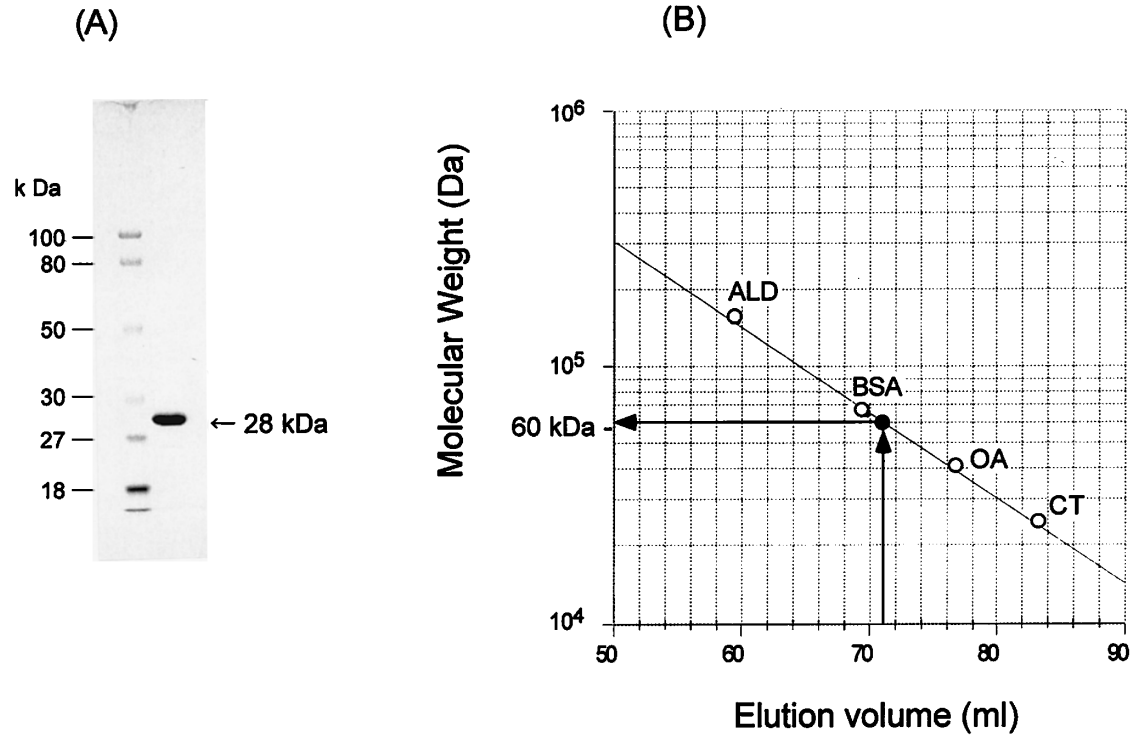

FIG. 1. Estimation of the molecular mass of the purified enzyme. (A) PAGE of the purified enzyme. Lanes:1, SDS-PAGE of marker proteins; 2, SDS-PAGE of the enzyme. (B) Sephacryl S200 gel filtration of the purified enzyme. Standard protein used were: CT, chymotrypsin (25 kDa); OA, ovalbumin (43 kDa); BSA, bovine serum albumin (67 kDa); ALD, aldorase (158 kDa).

approximately 8 times lower than *Pseudomonas fluorescens* maleate *cis-trans* isomerase [4].

(e) Stoichiometry and reversibility. The stoichiometry of the isomerization reaction was examined using purified maleate *cis-trans* isomerase from *A. faecalis* IFO13111. The reaction solution after the reaction was centrifuged to obtain supernatant which was subjected to HPLC analysis (System Gold, Beckman, Fullerton,CA) by using column for organic acid analysis (SCR-101H column, Shimadzu, Kyoto, Japan) and a UV detector (210 nm). As a result, a peak of fumaric acid and maleic acid were detected separately and it was confirmed one mol fumaric acid was produced per mol maleic acid consumed (data not shown). The enzyme was shown to catalyze the stoichiometrical isomerization reaction of maleic acid to fumaric acid, as in the case with maleate *cis-trans* isomerase from *Pseudomonas* [4], *Arthrobacter* [3] and so on. The reverse reaction was not detected previously [4] because of the detection limits of the assay. In this study, fumarate was detected as the product from maleate by the HPLC analysis of the reaction solution of the purified enzyme. At equilibrium point, 0.2 mM maleic acid was detected from the reaction solution of which initial substrate concentration was 100 mM sodium maleate or 100 mM sodium fumarate (pH 8.0).

(f) Inhibition and activation. The isomerase activity was inhibited by various sulfhydryl reagents such as *p*-chloromercuriphenylsulfonate and iodoacetamide and oxidizing agents such as hydrogen peroxide and *N*-chlorosucciimid, and activated by reducing agents such as DTT and 2-mercaptoethanol.

Cloning and Nucleotide Sequence of the A. faecalis maiA Gene

The N-terminal sequence of the purified maleate *cis-trans* isomerase from *A. faecalis* IFO13111 was determined. The sequence of the first 25 amino acids was Met-Lys-Thr-Tyr-Arg-Ile-Gly-Glu-Ile-Val-Pro-Ser-Ser-Asn-Thr-Thr-Met-Glu-Thr-Glu-Ile-Pro-Ala-Met-Leu. On the basis of N-terminal sequence of maleate *cis-trans* isomerase, hybridization probes (AF1 and AF2) were synthesized and used for a southern hybridization. As a result of a southern blot of *A. faecalis* IFO13111 chromosomal DNA cleaved with various restriction enzymes, strain IFO1311 revealed to have a single *maiA* gene and the several restriction enzyme treated fragments were suitable for subcloning of the *maiA* gene (Fig. 3). Positive phage clones were isolated by plaque hybridization as described in methods. One of the positive phage clones has the positive 2.2-kb *Xho*I fragment, which was subcloned to pBluescript II KS+

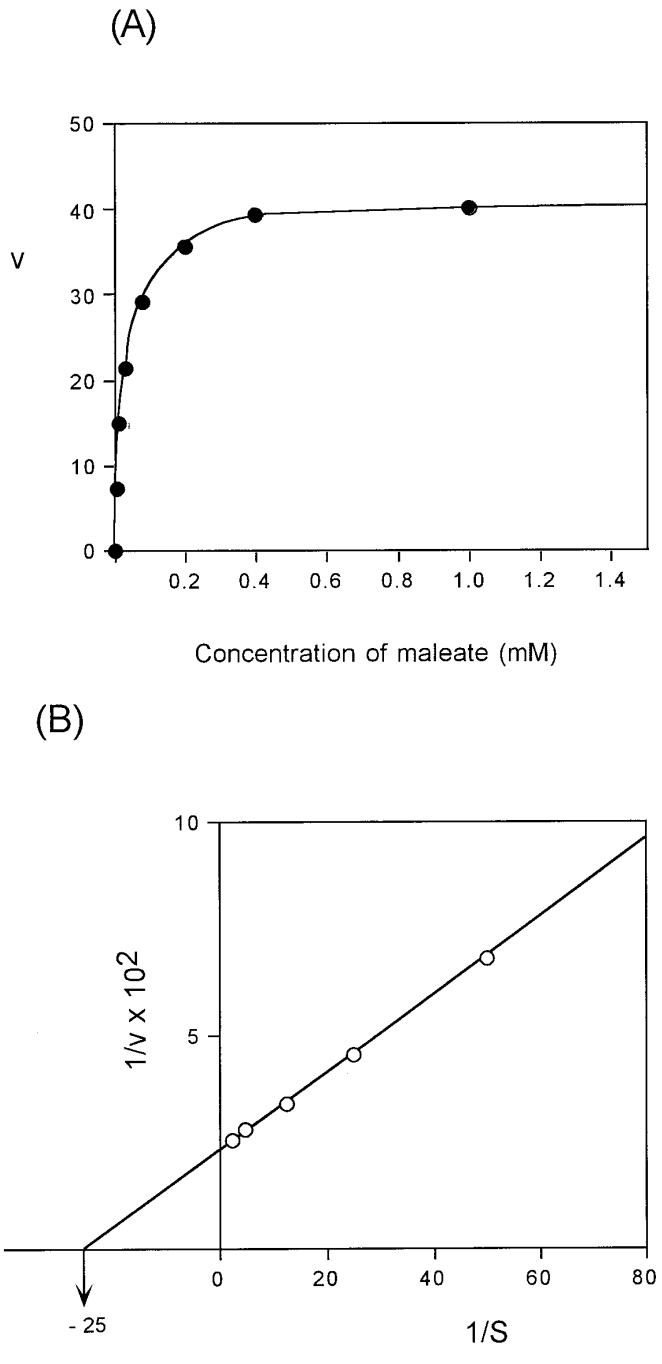

FIG. 2. Effect of substrate concentration on maleate *cis-trans* isomerase (A) Lineweaver-Berk plots of maleate *cis-trans* isomerase (B) The enzyme activity was assayed by the method 2 described in materials and methods. Velosity (v) was expressed as units per mg protein at 30°C, pH 8.0, and concentration (S) of maleate as substrate as mmole per liter.

(Stratagene) and sequenced. The nucleotide and deduced amino acid sequences of *maiA* are shown in Fig. 4. An open reading frame of 762 bp encoding a protein of 254 amino acid residues with estimated molecular mass of 27,971 Da was identified. The first 25 amino acids from the deduced sequence was identical to the N-terminal sequence derived from the purified native maleate *cis-trans* isomerase. The amino acid sequence of the maleate *cis-trans* isomerase deduced from the *maiA* gene from *A. faecalis* IFO13111 does not show a significant homology with any amino acid sequences in SWISS-PLOT protein data bank. This result suggests the maleate *cis-trans* isomerase belongs to a new family of enzymes, because this might be a first report of the nucleotide sequence of *cis-trans* isomerase of carbon-carbon double bond.

DISCUSSION

Maleate *cis-trans* isomerase from *A. faecalis* IFO13111 was purified to a single band on SDS-PAGE and the properties of the enzyme were characterized. Maleate *cis-trans* isomerase from *A. faecalis* IFO13111 does not require any co-factor for its function like other known maleate *cis-trans* isomerase from such as *P. fluorescence* [4], and *Arthrobacter* sp. [3]. The maleate

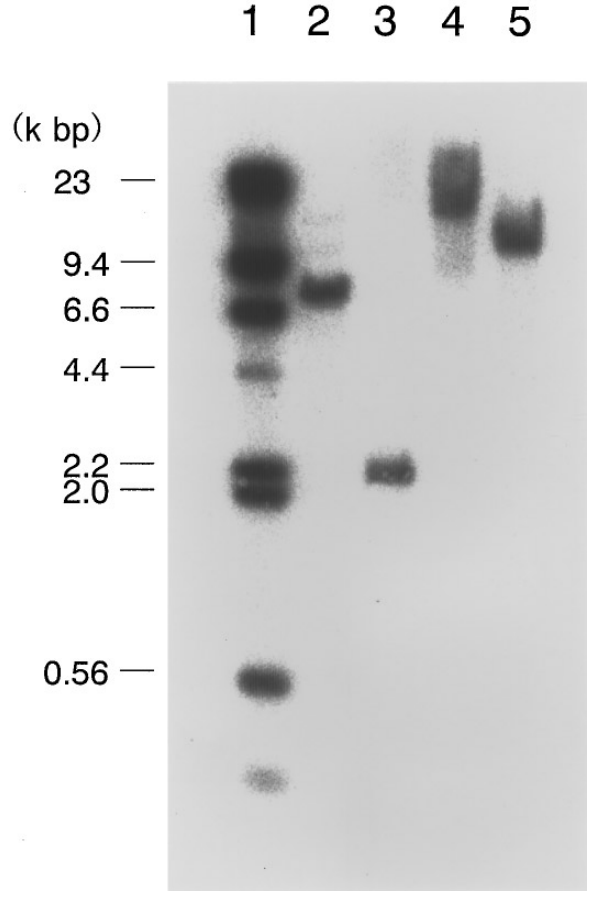

FIG. 3. *maiA*-specific restriction fragments detected by Southern blotting. Lane 1, *Hin*dIII molecular weight marker; 2, *Pst*I digestion of *A. faecalis* IF013111 chromosomal DNA; 3, *Xho*I digestion; 4, *Bam*HI digestion; 5, *Hin*dIII digestion.

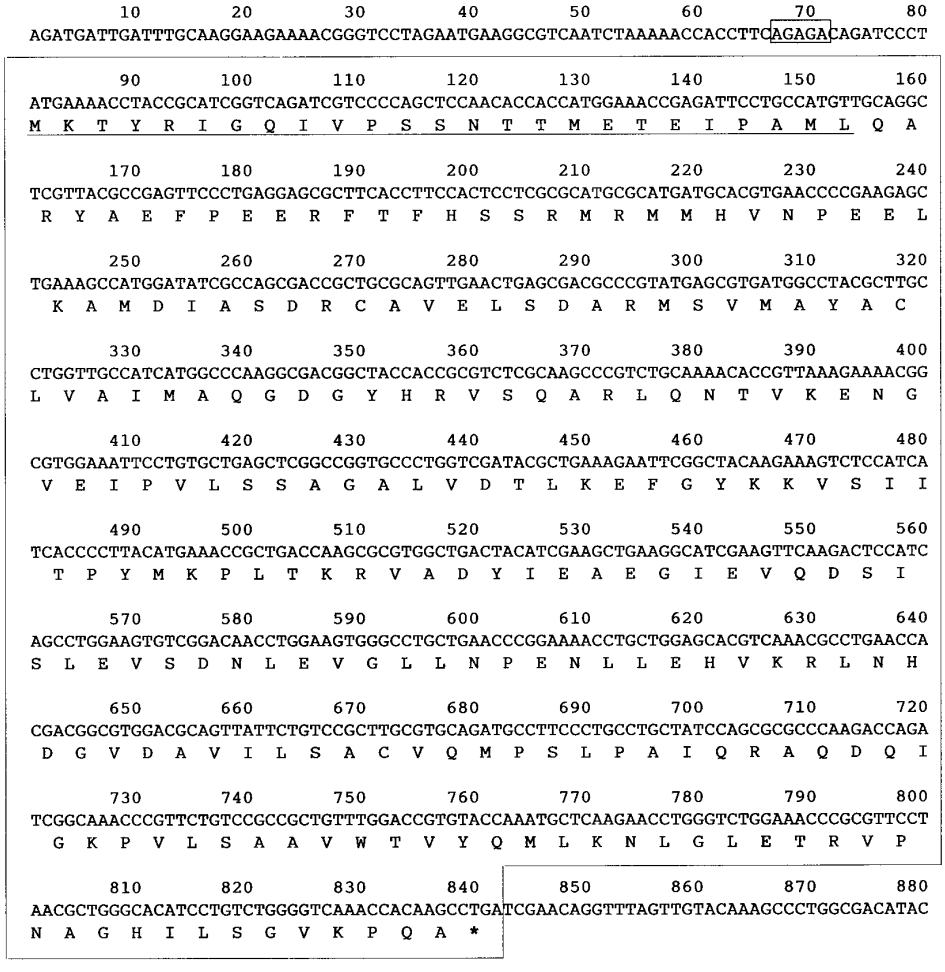

FIG. 4. The nucleotide and deduced amino acid sequences of *A. faecalis maiA* gene. The N-terminal amino acid sequence, which was chemically determined, is shown by a solid underline. Putative ribosome binding site is enclosed by open box. The data will appear in the DDBJ, EMBL, GenBank nucleotide sequence databases under the accession number AB005051.

cis-trans isomerase activity of *A. faecalis* IFO13111 was inhibited by various sulfhydryl reagents and oxidizing agents and activated by DTT or 2-mercaptoethanol. These reducing agents seems to be effective to activate sulfhydryl group of enzyme which is involved in an active site of this enzyme.

The *maiA* gene from *A. faecalis* was cloned by hybridization using the oligonucleotide DNA designed on the base of the N-termial amino acid sequences of the purified maleate *cis-trans* isomerase. Its deduced product has a molecular weight (28 kDa) identical to the enzyme subunit's molecular weight determined by SDS-PAGE analysis. The nucleotide sequencing of *maiA* gene revealed maleate *cis-trans* isomerase from *A. faecalis* IFO13111 has three cysteine residues per one subunit of the enzyme. In some case of other *cis-trans* isomerase, sulfhydryl group is furnished as a coenzyme [11]. In the case of maleate *cis-trans* isomerase from *A. faecalis* IFO13111, the enzyme does not require any sulfydryl group as a coenzyme for its activity and it is inhibited by various sulfhydryl reagents and oxidizing agents and activated by reducing agents. These results suggest that at least one of three cysteine residues take a part of enzyme activity and now we are investigating the function of these residues by site directed mutagenesis of *maiA* gene.

Cis-trans isomerization is an intramolecular rearrangement reaction without changes in empirical formula. The product is usually the thermodynamically more stable isomer, *trans* form. The reverse reaction (formation of maleic acid from fumaric acid) could be detected for the first time on maleate *cis-trans* isomerase. Equilibrium constant for maleate *cis-trans* isomerase from *A. faecalis* IFO13111, which is calculated from the experimental results, was estimated as approximately 500. Webb estimated the isomerization at pH 8.4 involving the dianions, maleate and fumarate, to have an equilibrium constant of 175 [12], which is almost matched to our results.

Maleate *cis-trans* isomerase has been paid attention

as industrial catalyst because fumarate is a substrate for the formation of aspartic acid by aspartase (L-aspartate ammonia-lyase [EC 4.3.1.1]) and L-malic acid by fumarase (fumarate hydratase [EC 4.2.1.2]). For example, the production of L-aspartic acid [13] and L-malic acid [14] from fumaric acid by the coryneform bacterium *Brevibacterium flavum* MJ233 was previously reported. Maleate *cis-trans* isomerase is observed to be the promising enzyme for the production of fumarate from maleate because this enzyme has a broad pH range for its activity, a relative low Km value, and a high equilibrium constant. The cloning and sequencing of *maiA* gene will be available for the breeding of catalyzing strains and the *maiA* overexpressed transformants would be useful for the process for the production of fumarate and its derivatives such as L-aspartate or L-malate.

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