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Purification and Properties of Hog Kidney Mutarotase

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Hog kidney mutarotase was separated into four forms (types I—IV) by DEAE-cellulose column chromatography. The most abundant form (type II) was purified to homogeneity as judged by polyacrylamide disc gel electrophoresis. The physico-chemical properties of the pure type II enzyme were as follows: molecular weight, 41000; isoelectric point, pH 5.48; K_m for α-D-glucose at pH 7.4 and at 25°, 19 mM; optimum pH, 6.5—7.5; optimum temperature, 30°. The enzyme activity was greatly reduced at acid pH below 6.0. The enzyme lost little activity on storage for at least 130 days at 4°, whereas about 12% of the activity was lost during the same period at −20°. When the enzyme was heated for 10 min at 59°, the activity was completely lost.

Keywords——mutarotase; hog kidney; purification; multiple forms; stability

Introduction

Mutarotase (EC 5.1.3.3) is an enzyme which catalyzes the anomeric interconversion of D-glucose and other sugars. The enzyme has recently been used as an accelerator of mutarotation of D-glucose in the assay of D-glucose and its anomers using glucose oxidase (EC 1.1.3.4) or glucose dehydrogenase (EC 1.1.1.47) in both clinical and laboratory work.[1]

In this paper, we describe the first observation of the presence of four forms of mutarotase from hog kidney, the purification of the most abundant form to polyacrylamide disc gel electrophoretic homogeneity, and the properties of the purified mutarotase.

Experimental

Materials——Dithiothreitol, bovine serum albumin (fraction V), chicken egg albumin (crystallized), α-chymotrypsinogen A (6×crystallized), and myoglobin (sperm whale skeletal muscle) were obtained from Sigma Chemical Co., St. Louis, U.S.A. Sephadex G-75 and G-100 were from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE 52) was from Whatman Ltd., Springfield, UK. Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, U.S.A. Cellulose powder (type A) was from Toyo Kagaku Sangyo, Tokyo, Japan. Glucose oxidase was kindly supplied by Dr. K. Kusai of Nagase & Co. Ltd., Osaka, Japan and partially purified as described previously.[2] All other chemicals were of reagent grade.

Assay of Mutarotase Activity——Mutarotase activity was determined by the method of Miwa and Okuda[3] using glucose oxidase and a polarographic oxygen electrode (Beckman Instruments Inc., Fullerton, U.S.A.). One unit of the enzyme was defined as the amount which converts 1.0 μmol of α-D-glucose to β-D-glucose per min under the assay conditions used.

Determination of Molecular Weight of Mutarotase——Molecular weight determinations in the presence of sodium dodecyl sulfate (SDS) were performed by the method of Weber and Osborn[4] using bovine serum albumin (MW 68000), chicken egg albumin (MW 45000), α-chymotrypsinogen A (MW 25500), and myoglobin (MW 16800) as standards. Gel filtration on Sephadex G-100 for determination of molecular weight was carried out by the method of Andrews[5] using the same standards as used for SDS polyacrylamide disc gel electrophoresis.

Determination of K_m Value of Mutarotase——The K_m value of mutarotase for α-D-glucose was determined by the polarimetric method of Bailey *et al.*[6] because the maximal concentration (around 1.5 mM) of α-D-glucose applicable to our assay method for mutarotase is not high.

Polyacrylamide Disc Gel Electrophoresis——Electrophoresis on 7.5% polyacrylamide gels was carried out with a Tris-glycine buffer (pH 8.3) as described by Davis.[7] Gels were stained for protein with a solution of 0.25% Coomassie brilliant blue and destained in 7% acetic acid.

Isoelectric Focusing——Isoelectric focusing was performed according to the directions of the manufacturer (LKB Instruments Inc., Bromma, Sweden) with pH 4 to 6 Ampholine at a final concentration of 1%.

Protein Determination——Protein determinations were made by the method of Lowry *et al.*[8]

Purification of Mutarotase——All steps were performed at 4° unless otherwise specified. The pH of buffers was measured at room temperature (around 25°).

Step 1. Preparation of Cell-free Extract: The hog kidney cortex (400 g) was homogenized with a mixture of 400 ml of 0.1 M Tris-HCl buffer (pH 7.2) and 160 ml of chloroform in a Waring blender for 3 min at room temperature. The homogenate was centrifuged at $10000 \times g$ for 30 min and the resultant sediment was discarded.

Step 2. Ammonium Sulfate Treatment: The precipitate sedimenting at 35—55% saturation of $(NH_4)_2SO_4$ was suspended in 10 mM Tris-HCl buffer (pH 7.2) and dialyzed twice (10 hr each) against 3 l of the same buffer.

Step 3. Gel Filtration: The dialyzed enzyme preparation was divided in half, and each half (46 ml each) was applied to a column (4.5×70 cm) of Sephadex G-75, previously equilibrated and subsequently eluted with 10 mM Tris-HCl buffer (pH 7.2). The flow rate was 30 ml/hr. The active fractions from both columns were pooled and concentrated to 35 ml with a YM-5 ultrafiltration membrane (Amicon Corporation, Lexington, U.S.A.).

Step 4. First DEAE-cellulose Chromatography: The concentrated sample was applied to a column (2.5×25 cm) of DE 52 previously equilibrated with 10 mM TriHs-Cl buffer (pH 7.2). The column was washed with the same buffer until the absorbance at 280 nm became almost zero and then developed with a 10—120 mM linear Tris-HCl buffer gradient (pH 7.2) (Fig. 1). The fractions containing mutarotase type II activity were pooled, concentrated to 10 ml by ultrafiltration, and dialyzed three times (4 hr each) against 500 ml of 5 mM phosphate buffer (pH 7.2).

Step 5. Second DEAE-cellulose Chromatography: The same chromatography as performed in step 4 was carried out except for the use of phosphate buffer instead of Tris-HCl buffer and the use of a 5—25 mM linear buffer gradient. The active fractions were pooled, concentrated to 10 ml, and dialyzed against 5 mM phosphate buffer (pH 7.2).

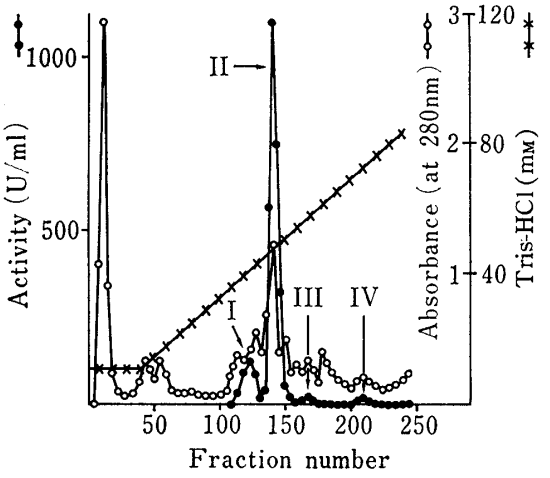

Fig. 1. First DEAE-cellulose Column Chromatography

The flow rate was 21 ml/hr and 5 ml fractions were collected.

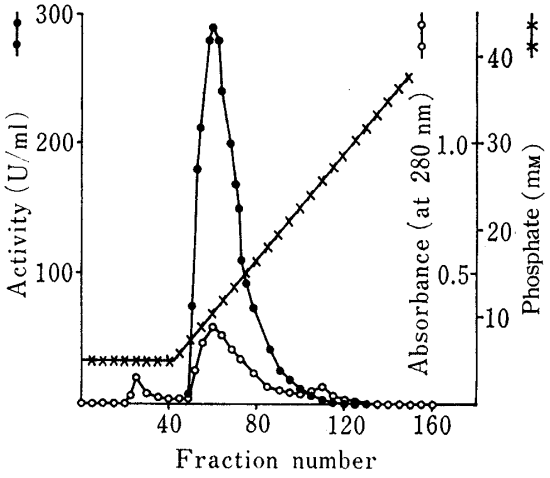

Fig. 2. Hydroxylapatite Column Chromatography

The flow rate was 21 ml/hr and 5 ml fractions were collected.

Step 6. Hydroxylapatite Chromatography: The dialyzed sample was placed on a column (2.5×20 cm) of a mixture (2: 1, w/w) of hydroxylapatite and cellulose powder, which was equilibrated with 5 mM phosphate buffer (pH 7.2). The column was washed with the same buffer and developed with a 5—70 mM linear phosphate buffer gradient (pH 7.2) (Fig. 2). The fractions containing activity were pooled and ultrafiltered until the protein concentration was 2 mg/ml or greater. The purified enzyme was stored at 4°.

Results

Purification of Mutarotase Type II

The results of 224-fold purification of mutarotase type II are shown in Table I. Four forms (designated as types I, II, III, and IV in order of elution) of mutarotase were separated

Table I. Purification of Hog Kidney Mutarotase Type II

	Step	Total activity ($\times 10^{-4}$ units)	Total protein (mg)	Specific activity (units/mg)	Purification (−fold)	Overall recovery (%)
Cell-free extract	1	6.83	16100	4.24	1.0	100
Ammonium sulfate precipitate (after dialysis)	2	4.10	5380	7.62	1.8	60
Sephadex G-75 eluate	3	3.68	308	119	28	54
First DEAE-cellulose column eluate (Type II)	4	2.69	43.8	614	145	39
Second DEAE-cellulose column eluate (Type II)	5	2.44	32.5	751	177	36
Hydroxylapatite column eluate (Type II)	6	1.90	20.0	950	224	28

by the first DEAE-cellulose chromatography (Fig. 1). The possibility of the formation of artificial multiplicity during purification was checked by the same method as described previously[9] and ruled out. Only the most abundant form (type II) was further purified. The purified type II enzyme (specific activity 950 U/mg) was recovered in 28% yield after hydroxylapatite column chromatography (Fig. 2). The enzyme was ascertained to be homogeneous by polyacrylamide disc gel electrophoresis (Fig. 3).

Physico-chemical Properties of Mutarotase Type II

The molecular weight of mutarotase type II was determined to be 41000 by SDS polyacrylamide gel electrophoresis and to be 40000 by gel filtration analysis on Sephadex G-100. These

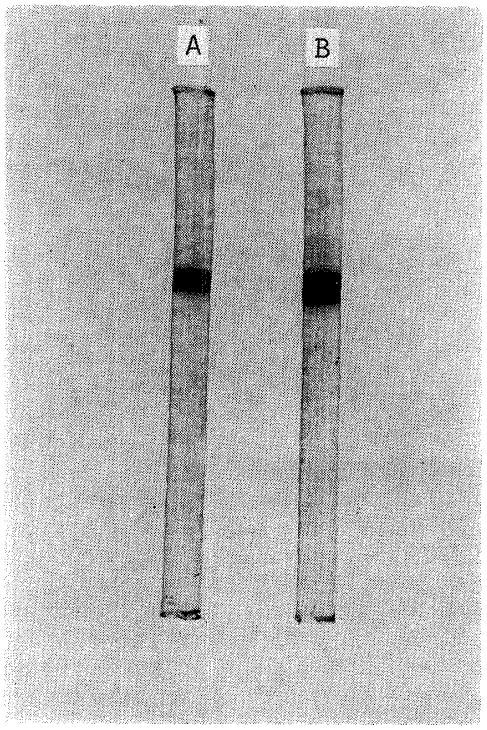

Fig. 3. Polyacrylamide Disc Gel Electrophoresis

Gel A contained 20 μg of pure mutarotase type II obtained after hydroxylapatite column chromatography and gel B, 60 μg of the enzyme. Electrophoresis was performed at a constant current of 3 mA per column (6 × 60 mm) for 90 min at room temperature.

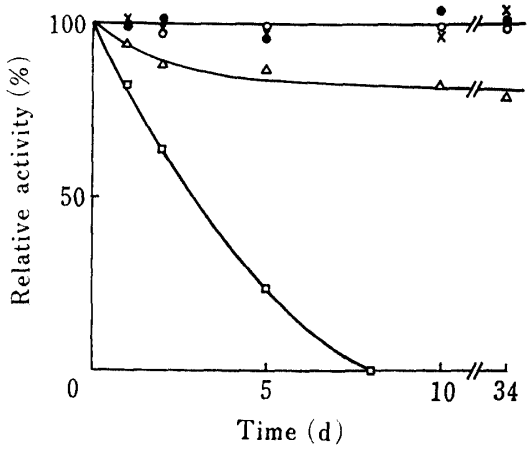

Fig. 4. Effect of pH on the Stability of Hog Kidney Mutarotase Type II

Enzyme solutions (1.4 mg/ml) in 10 mM EDTA buffer at various pH values were maintained at 4° and the enzyme activity was measured at the indicated times.

×, pH 9.0; ●, pH 7.4; ○, pH 6.5; △, pH 6.0; □, pH 5.0.

results showed that the enzyme was a monomeric protein. The isoelectric point of the enzyme was pH 5.48. The K_m value for α-D-glucose was 19 mM at pH 7.4 and at 25°. The dependence of the enzyme activity on pH was investigated at 10 pH values from 4 to 10 with 10 mM EDTA buffer, and a bell-shaped pH-activity curve showing the optimum pH at 6.5 to 7.5 was obtained. The effect of temperature (15° to 40°) on the enzyme activity was determined in 10 mM EDTA buffer (pH 7.4) and the optimum temperature was found to be 30°.

Stability Properties of Mutarotase Type II

Effect of pH——The effect of pH on the stability of mutarotase type II was studied at 4°. The enzyme could be stored for at least 1 month at pH 6.5, 7.4, or 9.0 with no loss of activity (Fig. 4). However, about 20% of the enzyme activity was lost within 1 month at pH 6.0 and all of the activity was lost within 8 days at pH 5.0.

Effect of Protein Concentration——The enzyme was stable for at least 1 month when stored at 4° at concentrations of 1.4 and 0.14 mg protein/ml, but considerable loss of activity was observed at a concentration of 14 μg/ml (Fig. 5). The presence of 2 mg/ml bovine serum albumin in an enzyme solution (14 μg/ml) completely protected the enzyme from loss of activity.

Effect of Temperature——The enzyme solutions (1.4 mg/ml) in 10 mM EDTA buffer (pH 7.4) were maintained at 4° and −20° for 130 days. Only a few percent of the enzyme activity was lost during that period at 4° and about 12% of the activity at −20°.

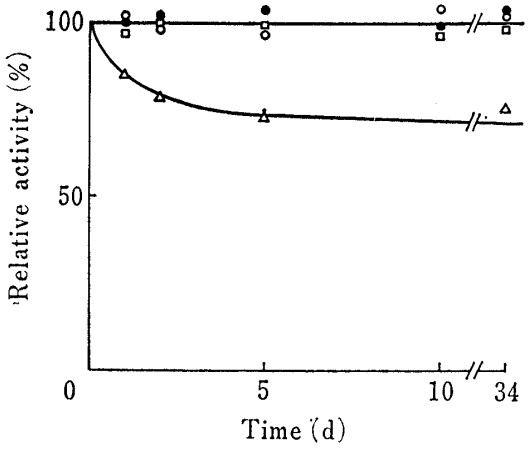

Fig. 5. Effect of Protein Concentration on the Stability of Hog Kidney Mutarotase Type II

Enzyme solutions of various protein concentrations in 10 mM EDTA buffer (pH 7.4) were maintained at 4° and the enzyme activity was measured at the indicated times.
○, mutarotase 1.4 mg/ml; □, mutarotase 0.14 mg/ml; △, mutarotase 14 μg/ml; ●, mutarotase 14 μg/ml+bovine serum albumin 2 mg/ml.

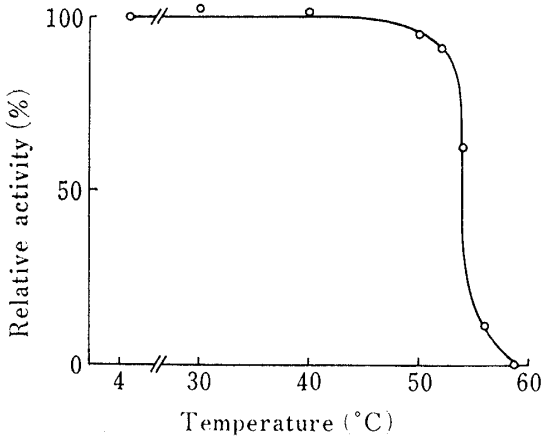

Fig. 6. Heat Stability of Hog Kidney Mutarotase Type II

The enzyme (1.4 mg/ml) in 10 mM EDTA buffer (pH 7.4) was incubated for 10 min at each temperature, then the enzyme activity was measured.

Heat Stability——The heat stability of the enzyme was studied by incubating the enzyme for 10 min at various temperatures. The enzyme was quite stable up to 50° (Fig. 6).

Effect of Buffer Concentration——The enzyme was completely stable in 10 mM phosphate and Tris-HCl buffers (pH 7.4) for 1 month at 4° (Fig. 7). However, the enzyme gradually lost its activity in buffers of 0.1 and 0.2 M: about 10% of the activity was lost during 1 month in 0.1 and 0.2 M Tris-HCl buffers (pH 7.4), and about 30% in 0.1 and 0.2 M phosphate buffers (pH 7.4).

Effect of Freezing at −20°——The enzyme was slightly less stable at −20° than at 4° in 10 mM and 0.1 M Tris-HCl buffers (pH 7.4), and in 10 mM phosphate buffer (pH 7.4) (Fig. 8). However, all of the enzyme activity was lost within a week in 0.1 M phosphate buffer (pH 7.4)

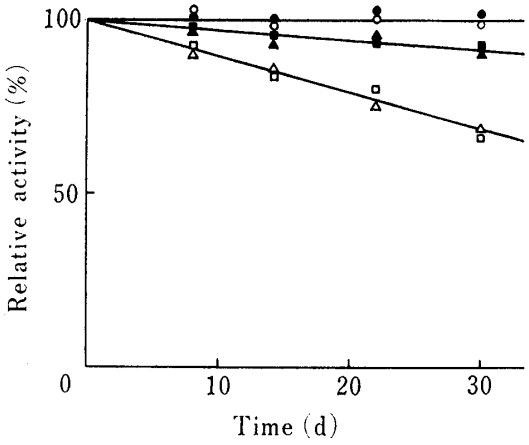

Fig. 7. Effect of Buffer Concentration on the Stability of Hog Kidney Mutarotase Type II

Enzyme solutions (1.4 mg/ml) in phosphate and Tris-HCl buffers (pH 7.4) of various concentrations were maintained at 4° and the enzyme activity was measured at the indicated times.
○, 10 mM phosphate; □, 0.1 M phosphate; △, 0.2 M phosphate; ●, 10 mM Tris; ■, 0.1 M Tris; ▲, 0.2 M Tris.

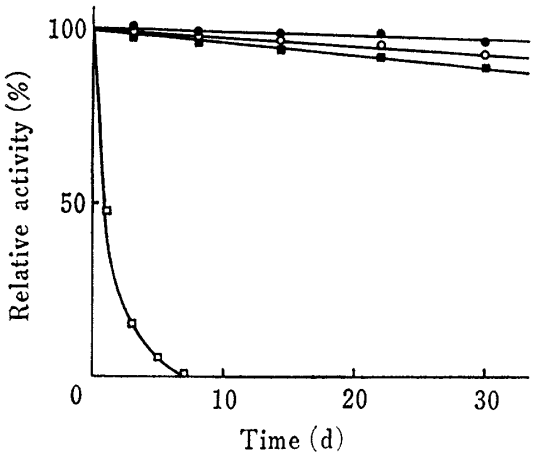

Fig. 8. Effect of Freezing at −20° on the Stability of Hog Kidney Mutarotase Type II

Enzyme solutions (1.4 mg/ml) in phosphate and Tris-HCl buffers (pH 7.4) were maintained at −20° and the enzyme activity was measured at the indicated times.
○, 10 mM phosphate; □, 0.1 M phosphate; ●, 10 mM Tris; ■, 0.1 M Tris.

at −20°. The stabilities of the enzyme in 10 mM and 0.1 M EDTA buffers (pH 7.4) were almost the same as those in 10 mM and 0.1 M Tris-HCl buffers, respectively (data not shown).

Discussion

We have previously reported the presence of multiple forms of rat kidney mutarotase[9] and of human kidney mutarotase,[10] but no one who has purified mutarotase from hog kidney has described the occurrence of multiplicity in the enzyme.[11] This paper is thus the first report on the presence of multiple forms (types I, II, III, and IV) of hog kidney mutarotase. Because of the small quantities of types I, III, and IV only type II (the most abundant form) could be purified to disc gel electrophoretic homogeneity. We first determined the isoelectric point and the optimum temperature of hog kidney mutarotase. Other properties (molecular weight, K_m value, and optimum pH) of purified type II were similar to those reported by other investigators.[11] This means either that the enzyme preparations obtained by other investigators contained exclusively the type II enzyme or that the four types of the enzyme are very similar in physico-chemical properties.

Several kinds of commercial preparations of hog kidney mutarotase are now available. However, there has been little information on the stability properties of the enzyme, which must be known for the proper use of the enzyme as a reagent. Thus, we investigated the stability properties of our homogeneous enzyme preparation (type II). The stability of the enzyme was found to be impaired by lowering the pH (Fig. 4) and by decreasing the protein concentration (Fig. 5). The enzyme lost all of its activity when heated at 59° for 10 min (Fig. 6), but it fully retained activity when incubated at 30° for 24 hr (data not shown).

Since phosphate or Tris-HCl buffer of relatively high concentration is used in almost all kinds of commercially available reagent kits containing mutarotase, we studied the stability of mutarotase at 4° in buffers (pH 7.4) of 0.1 and 0.2 M in comparison with that in 10 mM buffers. Although mutarotase was more stable in 10 mM buffers than in 0.1 and 0.2 M buffers, the enzyme could be stored without marked loss of activity in 0.1 and 0.2 M buffers for at least 2 weeks (Fig. 7). The enzyme was a little more stable at 4° than at −20°. The enzyme completely lost its activity within a week in 0.1 M phosphate buffer (pH 7.4) at −20°, while the loss of

activity was very slow in 10 mM phosphate buffer (pH 7.4). This rapid loss of activity in 0.1 M phosphate buffer (pH 7.4) seemed to be a result of separate localization of $H_2PO_4^-$ and HPO_4^{2-} caused by freezing and of the resultant lowering of pH around $H_2PO_4^-$ (Fig. 8).[12] The enzyme in 10 mM EDTA buffer (pH 7.4) was scarcely affected by frequent freezing (at −20°) and thawing (data not shown).

These studies on the properties of mutarotase should be useful in connection with routine handling of the enzyme in the laboratory.

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