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Affinity Purification, Crystallization, and Amino Acid Analysis of Hog Kidney Mutarotase Type II

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Hog kidney mutarotase type II was purified to polyacrylamide disc gel electrophoretic homogeneity by a five-step procedure including affinity chromatography with phloretin-linked agarose. The purified enzyme was crystallized, and the crystals were subjected to amino acid analysis. The amino acid composition of the enzyme was similar to that of bovine kidney mutarotase, except for marked differences in the contents of phenylalanine and methionine.

Keywords——mutarotase; hog kidney; affinity purification; phloretin-linked agarose; crystallization; amino acid analysis

Introduction

Mutarotase (aldose 1-epimerase, EC 5.1.3.3) is a widely distributed enzyme which catalyzes the mutarotation of D-glucose and related sugars. Although it has been postulated that the enzyme may be involved in the transport of sugars[1,2] or in D-glucose metabolism,[3-5] the function of this enzyme has not yet been established.

Fishman *et al.*[6] purified mutarotase from bovine kidney and crystallized it, but they did not report the presence of multiple forms of the enzyme. On the other hand, we reported the presence of four forms (types I—IV) of the enzyme in rat,[7] human,[8] and hog[9] kidney and purified the most abundant form (type II) to homogeneity. Therefore, the crystals of Fishman *et al.* probably contain several forms of mutarotase.

Phloretin is known to have a high affinity for mutarotase.[10] Fannin and Diedrich[11] prepared phloretin-linked agarose from cyanogen bromide-activated agarose and used it for the isolation of mutarotase as a model glucose-binding protein. Affinity adsorbents prepared from cyanogen bromide-activated agarose, however, have some serious disadvantages.[12]

In this present paper, we describe the preparation of phloretin-linked agarose from epoxy-activated agarose, as well as a purification procedure for mutarotase including phloretin-linked agarose affinity chromatography. The crystallization of hog kidney mutarotase type II was carried out, and the amino acid composition of the enzyme was determined.

Experimental

Materials——Phlorizin was purchased from Sigma Chemical Co. Phloretin was obtained by hydrolysis of phlorizin according to the method of Müller and Robertson.[13] Colorless crystals of phloretin showed a decom position point of 262°C.

Sepharose 4B was obtained from Pharmacia Fine Chemicals. *p*-Nitrobenzoyl azide was from Eastman Kodak Co. Epichlorohydrin and 5,5′-dithio-bis(2-nitrobenzoic acid)(DTNB) were from Nakarai Chemicals, Ltd. 3-(2-Aminoethyl)indole was from Tokyo Kasei Kogyo Co., Ltd. The amino acid calibration mixture for amino acid analysis was from Ajinomoto Co. All other chemicals were the same as described in our previous paper.[9]

Preparation of Phloretin-linked Agarose——Amino agarose was prepared from epoxy-activated agarose 4B by the method of Matsumoto *et al.*[12] From the amino agarose, the diazotized form of *p*-aminobenzamido-alkylagarose was prepared by the method of Cohen.[14] To 100 ml of the water-washed diazonium agarose, 200 ml of 5 mM phloretin (in ethanol) and 200 ml of 0.2 M phsophate buffer (pH 6.0) were added, and the

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whole was shaken moderately for 24 h. The colored azo gel was washed successively with 1 l of ethanol and 1 l of 50% ethanol and water, and was then stored in the cold as a suspension in water. Approximately 3 μmol of phloretin was coupled per ml of agarose as estimated from the decrease in ultraviolet (UV) absorbance of the mother liquor.

Purification of Mutarotase——Preparation of the cell-free extract from hog kidney cortex (400 g), ammonium sulfate fractionation (35—55% saturation), and gel filtration on Sephadex G-75 were performed as described previously.[9] The active fractions from the Sephadex column were pooled and applied to a column (1.6×15 cm) of phloretin-linked agarose previously equilibrated with 10 mM Tris–HCl buffer (pH 7.2). The column was washed with the same buffer until the absorbance at 280 nm of the eluate was almost zero, and then developed with 0.5 M glucose in the same buffer (Fig. 1). The mutarotase activity in fractions containing glucose was measured after dialysis. The active fractions eluted with 0.5 M glucose were pooled, concentrated to 40 ml with a YM-5 ultrafiltration membrane (Amicon Corporation), and dialyzed against 10 mM Tris–HCl buffer (pH 7.2) to remove glucose. The dialyzed sample was applied to a column of DEAE-cellulose (DE 52, Whatman Ltd.) previously equilibrated with 10 mM Tris–HCl buffer (pH 7.2). The column was washed with the same buffer and developed with a 10—120 mM Tris–HCl buffer gradient (pH 7.2) (Fig. 2). The fractions containing mutarotase type II activity were pooled and the enzyme was crystallized from them.

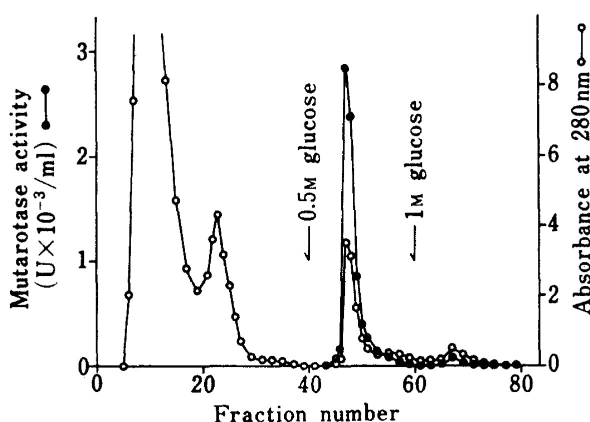

Fig. 1. Column Chromatography on Phloretin-linked Agarose

The flow rate was 20 ml/h, and 5 ml fractions were collected.

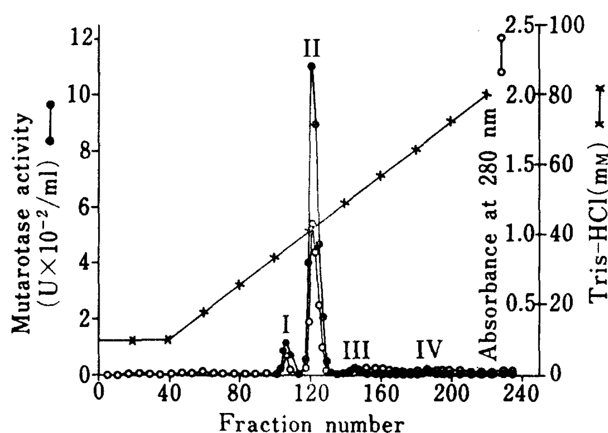

Fig. 2. DEAE-Cellulose Column Chromatography

The flow rate was 25 ml/h, and 5 ml fractions were collected.

Crystallization of Hog Kidney Mutarotase Type II——Solid ammonium sulfate was added with stirring to the pool of the active fractions from the DE-52 column to give a final 95% saturation. The solution was centrifuged, and the supernatant was discarded. The precipitate was dissolved in 5 mM ethylenediamine tetraacetic acid (EDTA) buffer (pH 7.4) containing 2 mM β-mercaptoethanol to give a protein concentration of about 3 mg/ml. Solid ammonium sulfate was added until the solution became slightly turbid. This solution was left at 4°C, and crystals began to appear within a week. Crystallization was complete within a month. By seeding with the crystal thus obtained, the period required for complete crystallization was shortened to 3 d.

Amino Acid Analysis of Hog Kidney Mutarotase Type II——Acid hydrolysis of mutarotase was carried out by the method of Simpson *et al.*[15] The enzyme crystals were dialyzed against distilled water, then lyophilized, and hydrolyzed at 115°C for 24, 48, and 72 h in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole in sealed tubes *in vacuo*. Amino acids in hydrolyzates were analyzed with a Hitachi 835 amino acid analyzer using a lithium citrate buffer system. Amino acid composition was computed from the average values of three hydrolyzates. Threonine and serine values were extrapolated to zero time of hydrolysis. The values for valine and isoleucine were taken as those of the 72 h hydrolysate. Performic acid oxidation for determining cystine and cysteine as cysteic acid was performed according to the method of Moore.[16] Sulfhydryl groups were determined by titration with DTNB in the presence of 2% sodium dodecyl sulfate at pH 8.0 according to the method of Habeeb.[17]

Other Analytical Methods——Mutarotase assay, protein determination, and polyacrylamide disc gel electrophoresis were performed as described previously.[9]

Results and Discussion

The results of a five-step purification of hog kidney mutarotase type II are summarized in Table I. The purified enzyme was disc gel electrophoretically homogeneous. We pre-

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viously purified this enzyme with a six-step procedure consisting of extraction, ammonium sulfate fractionation, and column chromatographies on Sephadex G-75, DEAE-cellulose (twice) and hydroxylapatite. In the previous procedure the enzyme (specific activity 950 U/mg) was recovered in 28% yield. The overall recovery (42%) was greatly increased by employing the present procedure, but the specific activity (953 U/mg) was not changed.

Table I. Purification of Hog Kidney Mutarotase Type II

	Step	Total activity (unit $\times 10^{-4}$)	Total protein (mg)	Specific activity (unit/mg)	Purification (-fold)	Overall recovery (%)
Cell-free extract	1	6.75	16000	4.22	1.0	100
Ammonium sulfate precipitate (35—55 %)	2	4.25	6064	7.01	1.7	63
Sephadex G-75	3	3.92	320	123	29	58
Phloretin-linked agarose	4	3.40	38.2	890	211	50
DEAE-cellulose (type II)	5	2.84	29.8	953	226	42

The point which we would like to emphasize with respect to the present purification procedure is that phloretin-linked agarose can be easily prepared and is highly effective for the purification of mutarotase. Biospecific adsorbents prepared from cyanogen bromide-activated agarose have been widely used for the affinity chromatography of various biological substances. However, the results have not always been satisfactory because of nonspecific adsorption on the gel and leakage of the ligand from the column.[12] On the other hand, the adsorbents prepared by the epoxy method using epichlorohydrin have better properties in these respects.[18,19] Fannin and Diedrich[11] purified hog kidney mutarotase by using phloretin-linked agarose prepared by the cyanogen bromide method. We also tried to purify the enzyme according to their method. A satisfactory result, however, could not be obtained because of high nonspecific adsorption on the gel. Therefore, we prepared phloretin-linked agarose from epoxy-activated agarose and used it for the purification of the enzyme. This method for preparing phloretin-linked agarose was found to be convenient and reproducible.

Hog kidney mutarotase 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.[9] The enzyme preparation obtained after column chromatography on phloretin-linked agarose was confirmed to be pure enough for use in these assays.

The crystals (needles) of hog kidney mutarotase type II are shown in Fig. 3. To the best of our knowledge, this is the first photograph of crystals of mutarotase. We failed to obtain crystals by the reverse solution technique of Jakoby,[20] which Fishman *et al.*[6] adopted for the crystallization of bovine kidney mutarotase. Crystals of hog kidney mutarotase type II, however, were easily obtained by a more usual method, as described in Experimental. We have found (unpublished data) that the bovine kidney cortex contains four types of mutarotase, as is the case in rat,[7] human,[8] and hog[9] kidney cortex. Fishman *et al.*[6] crystallized bovine kidney mutarotase from an enzyme preparation which was not separated into four forms. Therefore, their crystals probably contain several forms of mutarotase.

The amino acid composition of hog kidney mutarotase type II is shown in Table II. The results are expressed as the numbers of amino acid residues per mol of the enzyme. Amid nitrogen as ammonia was not determined. The half-cystine content (4 groups/mol) determined by the performic acid oxidation method was in good agreement with the amount (3.9 groups/mol) of sulfhydryl groups obtained by titration with DTNB, indicating that the enzyme contains 4 cysteine residues per mol but no cystine. A negative result was obtained in the phenol-sulfuric acid test for sugar on this enzyme. This result supports the validity

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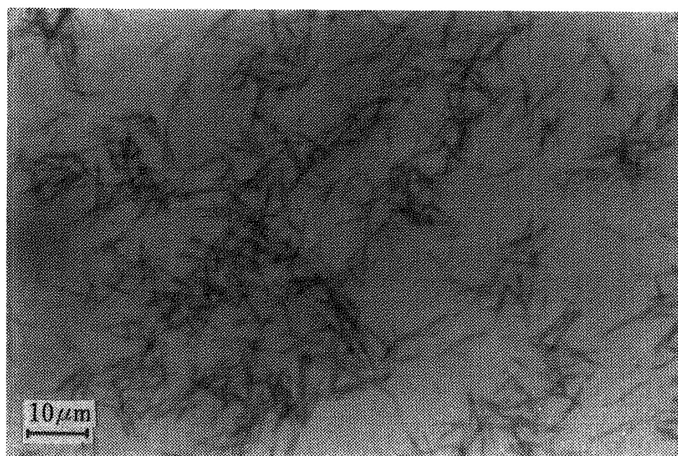

Fig. 3. Photograph of Crystals of Hog Kidney Mutarotase Type II

TABLE II. Amino Acid Compositions of Hog Kidney Mutarotase Type II and Bovine Kidney Mutarotase

Amino acid	Hog kidney mutarotase (type II)	Bovine kidney mutarotase[a]
Aspartic acid	35	36
Threonine	26	22
Serine	18	20
Glutamic acid	39	40
Proline	22	15
Glycine	39	40
Alanine	22	22
Valine	34	30
Half-cystine	4	4
Methionine	0	2
Isoleucine	13	8
Leucine	32	29
Tyrosine	13	11
Phenylalanine	22	8
Tryptophan	6	5
Lysine	19	14
Histidine	13	10
Arginine	14	12
Total residues	371	328

a) Data of Fishman *et al.* (ref. 1).

of the tryptophan content obtained by the present method (protein hydrolysis by methanesulfonic acid) as suggested by Simpson *et al.*[15] The molecular weight of the enzyme (41000) was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis against standard proteins.[9] Based on the amino acid contents, the molecular weight of the enzyme was calculated to be 40900, this value being in very good agreement with that obtained by SDS-polyacrylamide gel electrophoresis. This result also indicates that the enzyme contains no sugar.

We previously reported[21] that bovine kidney mutarotase cross-reacts with the antibody to hog kidney mutarotase type II and that spur formation is observed between a precipitin line formed with the crude extract from hog kidney and that from bovine kidney. This suggested that the enzymes from hog and beef have similar but not identical antigenic proper-

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ties. The amino acid composition of hog kidney mutarotase type II was generally similar to that of bovine kidney mutarotase reported by Fishman *et al.*,[6] except that hog mutarotase contained 22 phenylalanine residues per mol and no methionine residue, while bovine mutarotase contained 2 methionine residues per mol and only 8 phenylalanine residues per mol.

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