Development of new column for the purification of mutarotase from cattle kidney cortex based on inhibitor affinity chromatography (IAC)

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Abstract— Mutarotase (aldose-1-epimerase, EC 5.1.3.3) catalyzes the anomeric inter conversion of D (+) glucose and other aldoses. The enzyme is widely used in blood sugar determination, which is the most important routine analysis in a clinical laboratory. This analysis is used for the detection and therapeutic control of diabetes mellitus and for the diagnosis of several other metabolic diseases. The enzyme and their kits are being imported at a high cost and lot of foreign currency has been spending for this purpose. In order to produce such kit in our country, it is necessary to isolate and purify this enzyme at a low cost. Based on a previous screening study, it has been shown that cattle kidney cortex contain considerable amount of mutarotase. In order to obtain relatively pure mutarotase from crude extract with reduced processing cost, an inhibitor affinity chromatography (IAC) method has been developed. Here, we have taken advantage of the affinity of mutarotase toward phloretin and subsequent elution of the mutarotase by a specific inhibitor, whose inhibition constant is higher than that of phloretin. To accomplish this new strategy, uniform-sized agarose beads were prepared by emulsification technique. The prepared gel beads were activated using epichlorohydrin as activation reagent, after which phloretin was immobilized as an affinity ligand on the epoxy groups. The crude extract sample was applied on the column containing phloretin immobilized on agarose. After washing the column with 20 mM phosphate buffer, pH 7.4. Containing 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, mutarotase was eluted with 0.4 M sodium azide in the initial buffer. The eluted fraction after dialysis showed 40 fold purification as judged by mutarotase activity. The result indicates that newly synthesized matrix specific for mutarotase constitutes a powerful tool for its extensive purification in a single step.

Index Terms— Mutarotase, Cattle kidney cortex, Inhibitor affinity chromatography, Agarose, Epichlorohydrin, SDS-PAGE, Diagonostic kit.

1 INTRODUCTION

Mutarotase/aldose-1-epimerase, (EC 5.1.3.3) is a widely distributed enzyme, which catalyzes the mutarotation of D (+) glucose and certain sugars. Although it has been postulated that mutarotase may be involved in the transport of sugars[1, 2] or in D (+) glucose metabolism[3, 4, 5]. Mutarotase is a monomer having molecular weight of 40,000[14]. Mutarotase enzyme has a broad pH in the range of 4-8[6].

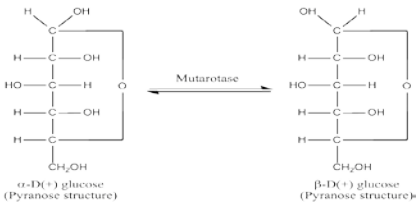

Fig.1: Mutarotation of α-D (+) glucose to β-D (+) glucose

The enzyme is involved in the determination of glucose level [7] in blood. Production and purification of mutarotase have been reported from Bovine Kidney Cortex[8] and chili peppe[9]. All the mutarotase substrates contain a reducing pyranose ring in C-1 conformation with equatorial hydroxyls at C-2 and probably C-3. The C-4 hydroxyl can be axial and various equatorial substituents are possible at C-5[10].

The effect of mutarotase on the mutarotation of a number of sugars which were available in one or the other anomeric forms, or which could be crystallized in a form containing a preponderance of one anomer, was studied. Of the 18 sugars tested, the mutarotation rate of only five was increased significantly following addition of the enzyme. These five substrates were D (+) galactose, L (-) arabinose, D (+) xylose, D (+) glucose, and D (+) fucose. The kinetics of these substrate-enzyme interactions was studied in some detail. The Km values for D (+) galactose, L (-) arabinose, D (+) xylose, D (+) glucose, and D (+) fucose were 6.5, 8.3, 13.2, 25.2 and 2.0 mM, respectively[6].Mutarotase is widely used in quality control laboratories of biochemical, pharmaceutical and clinical diagnostics. The enzyme has recently been used as an accelerator for the conversion of α-D (+) glucose to β-D (+) glucose for routine analysis of blood glucose determination in clinical laboratory. Most of the commercially

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available mutarotase comes from pig kidney, which is not generously available in Bangladesh. Therefore, enzyme and their kits are being imported at a high cost and lot of foreign currency has been spending for this purpose. In order to produce such kit in our country, it is necessary to isolate and purify this enzyme at a low cost. The objective of this study is to the development of a new technique to purify mutarotase from cattle kidney cortex in a convenient way.

2 Materials and Methods

2.1 Preparation of Crude Extract

For mutarotase 100g of fresh Cattle kidney cortex (paste) was homogenized in 500mL of 5mM EDTA buffer, pH 7.4, in an electric blender. The crude homogenate was centrifuged at 10000 rpm for 10 mins in a refrigerated centrifuge and the resultant sediment was discarded. The supernatant was collected in large beaker.

2.2 Salt Extraction of Proteins

CKC extract proteins were subjected to ammonium sulfate precipitation for the partial purification of mutarotase by the method of Bailey et al. (1969).

2.3 Determination of Mutarotase Activity

Mutarotase activity of the crude extract was assayed by measuring the changes of specific rotation with time by using an automation Polarimeter.

2.4 Preparattion of Agarose Affinity Matrix

2 g agarose was dissolved in 50 mL of distilled water and the solution was heated to a temperature above the gelation point of the agarose. The solution was designated as water phase. The mixture of liquid paraffin 100 mL and 2.5 g Tween 80 was charged into a reactor and heated to a temperature above the gelation point of the agarose and the solution was designated as oil phase. Then the heated water phase was poured into the reservoir of a spray gun and the fine droplets of water phase were sprayed over the oil phase under the pressure of dry nitrogen gas. The sized of droplet was controlled using nozzle of the spray gun and nitrogen pressure. A uniform sized emulsion was formed in the reactor. The emulsion was added into a second hydrophobic liquid cooled to a temperature below that of the gelation point of the agarose to gel the agarose droplets into beads. Finally the agarose beads were recovered from the second liquid and washed with distilled water.

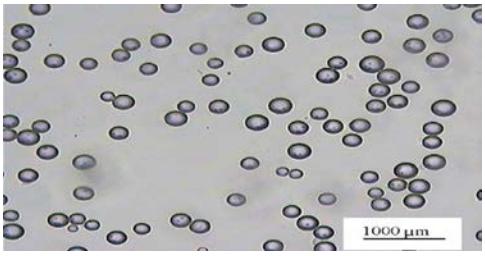

Figure 2: The optical microscopic photographs of uniform-sized agarose beads.

2.5 Cross-linking of Agarose Gel by Epichlorohydrin

About 50 mL uniform-sized agarose gels were taken in a 250 mL beaker containing 20 mL of 2 M NaOH and 5 mL of epichlorohydrin. The mixture was poured into two 50 mL falcon tube. The solutions containing gels were allowed to react for 2 hours at 40 ^{o}C with constant mixing in a homemade rotary mixture to keep the concentration uniform. After reaction, the obtained gels were washed with de-ionized distilled water until it became neutral.

2.6 Coupling of of Phloretin onto the Agarose Gel Modified with Epichlorohydrin

About 100 mL of 0.2 M phloretin solution in 1 M NaOH was added in the cross-linked agarose gels. The reaction was carried out at 40 ^{o}C for up to 16 hours. After reaction the gels were washed with 0.05 M NaOH until the gel became colourless.

2.7 Deactivation of Unreacted Epoxy Groups

For deactivation of excess epoxy groups of epichlorohydrin about 150 mL of 1 M 2-aminoethanol pH 8.0 was added to phloretin coupled gels. The reaction was carried out at 40 ^{o}C for 16 hours. After reaction the gels were washed with 500 mL of 0.05 M NaOH.

2.8 Preparattion of Agarose Affinity Matrix

To remove excess uncoupled ligand after deactivation, the gels were washed alternatively with low and high pH buffer solutions at least three times. Acetate buffer (0.1M, pH 4.0) and phosphate buffer (0.025M, pH 7.4) were used for these purposes. After washing the gels alternatively, the gels were washed with de-ionized distilled water until it become neutral.

2.9 Column Packing and Preparation

A slurry with binding buffer (20 mM Tris-HCl buffer pH 7.4 containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$) in a ratio of 75% settled medium to 25% buffer (20 mM Tris-HCl,pH 7.4) was prepared. All materials were equilibrated to the temperature at which the chromatography will be performed. The slurry medium was de-gassed. The slurry was poured into the column in one continuous motion. To minimize the introduction of air bubbles, the slurry was poured down with glass rod held against the wall of the column. Immediately the remainder of the column was filled with buffer and the adjustable top cap of the column was mounted. Finally excess buffer inside the column was discarded by decreasing the volume of the column using adjustable top cap/piston and connected to a pump. The column was washed with 2 to 3 bed volumes of buffer in order to pack the bed and to equilibrate the column with buffer. A slightly higher flow rate can be used for packing than will be used for affinity chromatography.

Fig.3: Photograph of home-made affinity column

2.10 Purification of Mutarotase by the Phloretin Affinity Agarose Matrix

6 mL of crude extract in binding buffer (20 mM Tris-HCl buffer pH 7.4 containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$) was passed through the column packed with phloretin affinity agarose matrix. The column was washed with washing buffer (20 mM Tris-HCl buffer pH 7.4 containing 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$) until the absorption at 280nm in HPLC detector become linear. The flow rate of the mobile phase was 0.25 mL/min.

2.11 Elution of Gel-bound Mutarotase from the column

Gel-bound mutarotase was removed by elution with a strong protein inhibitor, sodium azide. Five column volumes of 20 mM Tris-HCl buffer, pH 7.4 containing 0.4 M sodium azide was applied to the column at a flow rate of 0.25 mL/min.

2.12 Removing the Adhere Sodium Azide from Affinity Purified Mutarotase by Dialysis

The affinity purified Mutarotase was poured into activated dialysis bag of selectively permeable membrane (e.g. cellophane), immersed in a large volume of buffer. After some hours of stirring the buffer was replaced by fresh buffer. This was done for several times. After dialysis the purified Mutarotase was collected in a stoppard bottle.

2.13 Determination of Purity and Molecular Weight by SDS-PAGE Method

Purity of the bound fractions from the affinity columns was checked by SDS-PAGE analysis[11]. About 7 µg of total proteins were analyzed by performing the electrophoresis. Detection of the protein bands was performed with the Coomassie Brilliant Blue R-250 staining method, and purity degree was determined by electronic scanning.

Table 1: Proteins were used as markers.

Proteins	*Approximately molecular weight*
Ovalbumin	**45 KDa**
Carbonic anhydrase	**29 KDa**
Tripsin inhibitor	**20 KDa**
Lysozyme	**14.6 KDa**

The molecular weight of the affinity purified mutarotase was determined by SDS-PAGE using Ovalbumin (45 KDa), Carbonic anhydrase (29 KDa), Trypsin inhibitor (20 KDa) and Lysozyme (14.6 KDa) as reference protein.

2.14 Kinetic Study

In order to produce such kit in our country, the suitability of the affinity purified cattle kidney cortex mutarotase was verified. For this purpose, blood plasma sample is subjected to glucose analysis using glucose-oxidase-peroxidase enzyme system in presence and absence of the purified mutarotase.

3 Results & Discusion

Specific rotation of the enzyme was determined for different time intervals. As the time passed, the specific rotation decreased and anomeric form i.e. α-D –glucose concentration also decreased.

It was found that 100 mL of crude cattle kidney extract possess a mutarotase activity of 102.1 KU. In order to determine the specific activity of the enzyme, we also determined the total protein contained in 100 mL of the crude extract, which is 59 mg. Thus the specific activity is 1.73 KU/mg. It was also found that activity increases from 102.10 to 305.33 KU/100 mL and specific activity from 1.73 to 6.92 KU/mg after desalting of ammonium sulphate precipitated sample for cattle kidney cortex. But after purification, the activity increases 408.9 and the purification fold was obtained 40.00.

Purification by affinity chromatography therefore requires (a) an inhibitor that can be covalently bound to a solid support; (b) attachment of the inhibitor molecule to the matrix support by a chain of atoms long enough to enable the inhibitor to interact readily with the binding site(s) of the mutarotase that is shown in fig 5.

The central feature of this technique depends upon coupling a specific inhibitor of the mutarotase to an inert, insoluble support matrix. Under appropriate solvent conditions, chromatography of the mutarotase upon the inhibitor-matrix compound selectively retains mutarotase and thus eliminates contaminating molecules that possess no affinity for the inhibitor ligand. Subsequently, mutarotase is removed from the column by elution with another specific inhibitor whose inhibition constant (Ki) is higher than that of the inhibitor-matrix compound. In fig 6, portion (a) shows the chromatogram obtained from binding buffer that is in equilibrium with the base line. When washing buffer is passed through the column a peak was obtained which arises from contaminating molecule because inhibitor-matrix compound selectively retains mutarotase and thus eliminates contaminating molecules that possess no affinity for the inhibitor ligand (portion b). When elution buffer is passed through the column a peak of mutarotase along with azide molecule was obtained (portion c)

In figure (8), the presence of single band as shown in lane B indicating that our affinity matrix system was able to purify only one kind of protein in a single step. Thus, our affinity

matrix system was proven to be a useful tool in the purification of mutarotase in a single step.

The molecular weight was calculated from the standard curve of reference proteins which was constructed by plotting log10 molecular weight against relative mobility of the proteins on gel after electrophoresis and the molecular weight of the mutarotase was found to be 40 KDa (Figure 9).

The reaction kinetics clearly indicates that mutarotase assisted assay results in a completion of the reaction within 8 minutes for cattle kidney cortex, whereas mutarotase unassisted reaction remains incomplete even after 20 min.

Thus, mutarotase purified form cattle kidney cortex was able to reduce the time for the completion of reaction significantly. The present result clearly indicates the effectiveness of cattle kidney cortex mutarotase for its application as glucose diagnostic kit.

Table 2: Change of specific rotation with time.

Time in min	Specific Rotation in degree	Percentage of α-D(+) glucose	Percentage of β-D(+) glucose	D(+) glucose (g)	α-D(+) glucose in equilibrium mixture (mg)	β-D(+) glucose in equilibrium mixture (mg)	Conversion of α to β-anomer of glucose (μM)
0	52.48	36.00	64.00	5	1,800	3,200	0
1	46.00	29.03	70.97	5	1,451	3,548	96,774
2	40.00	22.58	77.42	5	1,129	3,870	186,379
3	33.00	15.05	84.95	5	752	4,247	290,919
4	27.00	8.60	91.40	5	430	4,569	380,525
5	23.00	4.30	95.70	5	215	4,784	440,262
6	20.00	1.08	98.92	5	53	4,946	485,065
7	19.00	0.00	100.00	5	0	5,000	500,000
8	19.00	0.00	100.00	5	0	5,000	500,000
9	19.00	0.00	100.00	5	0	5,000	500,000
10	19.00	0.00	100.00	5	0	5,000	500,000

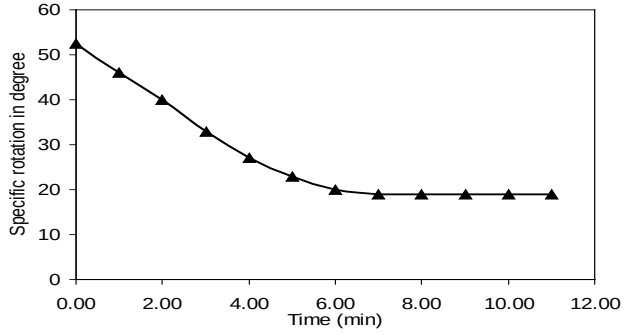

Fig.4: Polarimetric measurement of the changes in specific rotation of D (+) glucose.

Table 3: Summary of purification of mutarotase from cattle kidney cortex

Samples	Total mutarotase activity (KU/100 mL)	Total protein content (mg/100 mL)	Specific activity (KU/mg)	Purification fold
Crude homoge-nate	102.10	59.00	1.73	1.00
Crude extract	305.33	44.12	6.92	4.00
Affinity purified	408.19	5.9	69.18	40.00

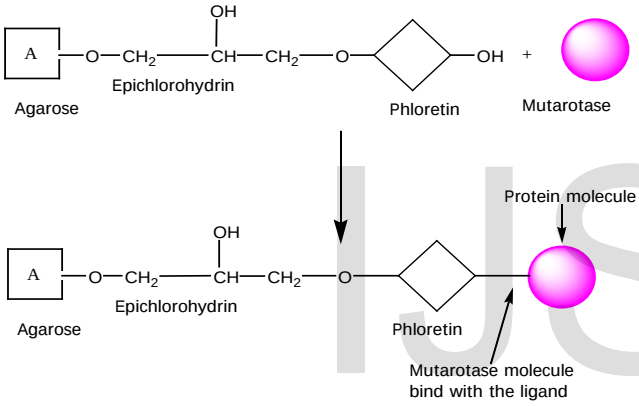

Fig.5: Binding of Mutarotase with ligand

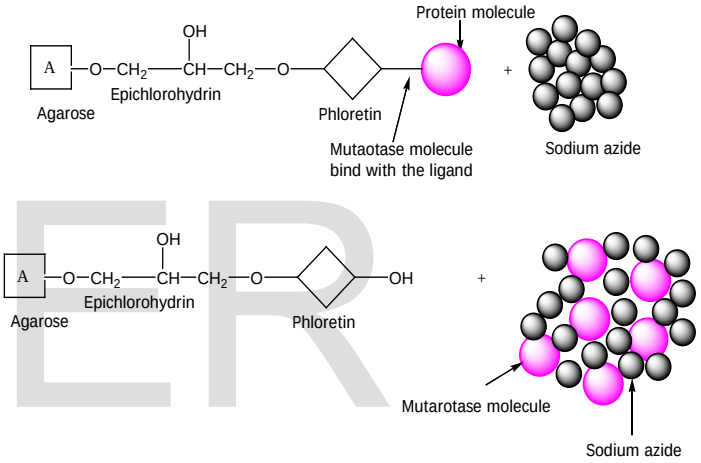

Fig 7: Separation of Mutarotase molecule from Ligand by sodium azide

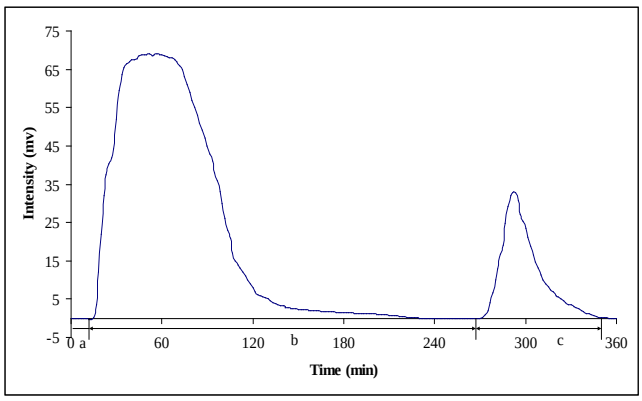

Fig.6: Chromatogram of affinity purification, (a) Equilibrate with 20 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$; (b) Injection of 6 mL crude extract and washing with 20 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$; (c) Elution of mutarotase enzyme with 20 mM Tris-HCl, pH 7.4 containing 0.4 M sodium azide.

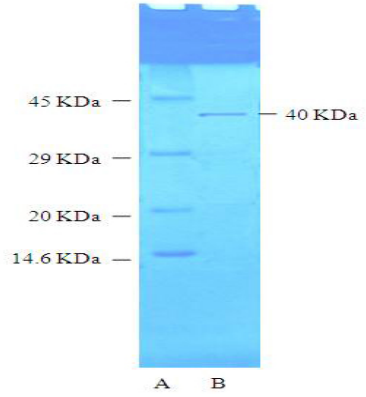

Fig.8: Purity test by SDS-PAGE method (A= Marker protein, B =Affinity purified protein)

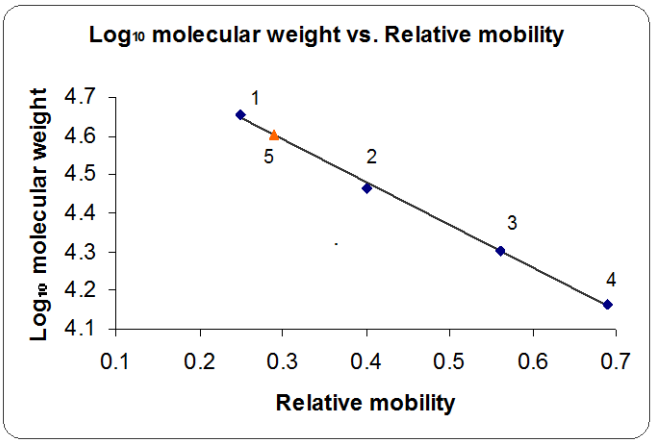

Fig.9: Estimation of the molecular weight of the purified mutarotase derived from cattle kidney cortex

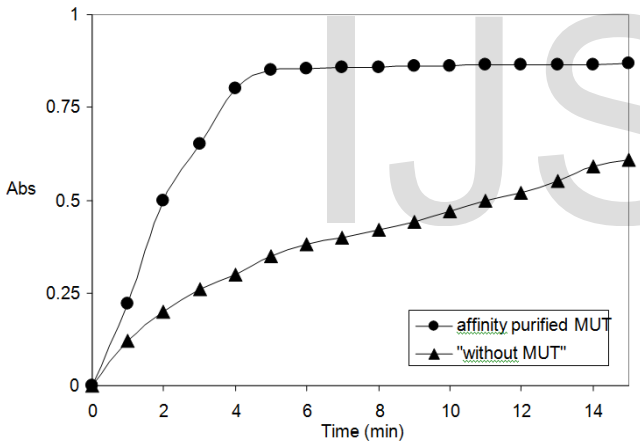

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Fig.10: Plot of absorbance against time for the mutarotase-assisted and unassisted reaction kinetics. 100 µl mutarotase from cattle kidney cortex (▲) having OD_{280nm} 0.1 were mixed with 100 µl glucose oxidase, 100 µl peroxidase, 10 µl dye and 1.69 mL 25 mM phosphate buffer, pH 7.0 containing 10g/100mL glucose.

4 ACKNOWLEDGEMENT

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