Note

Chromatographic Purification of Ribose 5-Phosphate Ketol Isomerase from *Candida utilis* on *p*-Mercuribenzoate-6-aminoethyl-Sepharose 4B Adsorbent

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Candida utilis ribose 5-phosphate ketol isomerase has already been purified from cell-free extracts of *C. utilis* by the authors¹⁾ according to conventional procedures composed of ammonium sulfate precipitation, DEAE-cellulose (DE-23) column chromatography, Bio-Gel P-200 gel filtration and repeated preparative polyacrylamide gel electrophoresis.

But, by this procedure, the enzyme yield was very low. So, in order to get a good yield of the enzyme preparation, purification procedure using affinity chromatography was tested. As an adsorbent for affinity chromatography, Sepharose 4B having *p*-mercuribenzoate (*p*-MB) as a ligand and 6-amino-hexylamine as a spacer was used.

Preparation of *p*-MB-6-aminoethyl-Sepharose 4B²⁾

Sepharose 4B (25 ml) suspended in distilled water (25 ml) was activated by addition of cyanogen bromide (6.25 g) at pH 11~11.5. Cyanogen bromide-activated Sepharose 4B gel was swollen and washed successively with water (one liter), 0.1 M NaHCO₃ (one liter) and finally with water (one liter). To the cyanogen bromide-activated Sepharose 4B (25 ml), 0.05 M 6-amino-hexylamine (25 ml) was added and adjusted pH at 10. After stirring at 4°C for 16 hr, it was filtered on a glass filter and successively washed with 0.1 M NaHCO₃ and water. A half gram of *p*-MB to be coupled with 6-aminoethyl-Sepharose 4B was dissolved in 45 ml of 40% dimethylformamide.

To the mixture, 6-aminoethyl-Sepharose 4B (25 ml) and 4 mm 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide were added. After being adjusted pH to 6.0 with 1 N HCl, the mixture was kept at room temperature for 16 hr.

After then, it was filtered with a glass filter and washed successively with 0.1 M NaHCO₃ (one liter), 40% dimethylformamide (500 ml) and finally with water (one liter). Next, the column was washed successively with 0.1% bovine serum albumin (250 ml), 8 M urea in 0.1 M citrate buffer (pH 5.5, 250 ml) to remove any trace of uncoupled *p*-MB, and then washed successively with 8 M urea in 0.1 M citrate buffer containing 50 mM 2-mercaptoethanol (250 ml), water (250 ml), 40% dimethylformamide (250 ml) and water (250 ml).

Purification of *C. utilis* ribose 5-phosphate ketol isomerase by affinity chromatography on a *p*-MB-6-aminoethyl-Sepharose 4B was carried out in the following procedure. The adsorbent was equilibrated with 0.01 M Tris-HCl buffer (pH 7.3, hereafter referred as column buffer), and then 15.0 mg of partially purified ribose 5-phosphate ketol isomerase preparation (DE-23 eluate) dissolved in 70 ml of the column buffer, was applied to the column. After washing the column successively with the column buffer and the column buffer containing 0.1 M NaCl, the column buffer containing 0.2 M NaCl was used to elute ribose 5-phosphate ketol isomerase from the column as shown in Fig. 1. Ribose 5-phosphate ketol isomerase (3.4 mg) was recovered by this procedure. The purified enzyme preparation by affinity chromatographic method showed homogeneity on a polyacrylamide disc electrophoresis as shown in Fig. 2. The results of purification of ribose 5-phosphate ketol isomerase by the conventional procedure and by this improved procedure, are shown in Table I. As shown in Table I, the latter method is much better

FIG. 1. Elution Pattern of Ribose 5-Phosphate Ketol Isomerase on *p*-MB-6-Aminoethyl-Sepharose 4B.

After the column (1.0 × 7.0 cm) was equilibrated with 0.01 M Tris-HCl (pH 7.3), 15 mg of partially purified ribose 5-phosphate ketol isomerase dissolved in 70 ml of the column buffer was applied. After washing successively with the column buffer and the column buffer containing 0.1 M NaCl, the column was eluted with the column buffer containing 0.2 M NaCl. The flow rate was 2 drops/sec., and 5 ml of each fraction was collected. ○—○, ribose 5-phosphate ketol isomerase activity; -----, absorbance at 280 nm.

FIG. 2. Polyacrylamide Disc Electrophoresis of Ribose 5-Phosphate Ketol Isomerase Purified by *p*-MB-6-Aminohexyl-Sepharose 4B Affinity Chromatography.

Protein (150 μ g) was applied to the gel. Electrophoresis was carried out at 2.0 mA/tube at 4°C. The gel was stained with Amido Black dye.

than the former one in respects of the enzyme yield and also of advantage in preparation time. For the enzyme yield, the yield at the final purification by Domagk³⁾ is 27% from the crude enzyme solution, that value is higher than 19.3% of ours, but when the yield is estimated as that of DEAE-eluate taken as 100%, 63% of ours is higher than 36.5% of Domagk's. For this point, a problem remains that 30.4% of yield at DEAE-stage of ours is lower than 70.4% of Domagk's at the same stage.

REFERENCES

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TABLE I. COMPARISON OF TWO PURIFICATION PROCEDURES FOR RIBOSE 5-PHOSPHATE KETOL ISOMERASE

The measurements for the enzyme activity and protein determination were carried out as described in the previous paper.¹⁾

The following results represent values obtained from 15.0 mg protein of D-23 eluate

Method	Conventional method ^a	Affinity chromatographic method ^b
Purification step	Preparative polyacrylamide	PMB-C ₆ -Sepharose 4B
Activity		
Total activity (units)	0.8	45.6
Specific activity (units/mg of protein)	11.6	13.4
Yield ^c (%)	1.1	63.3

^a Conventional method included the following purification procedures: Ammonium sulfate precipitation, DE-23 column chromatography, Bio-Gel P-200 gel filtration, and preparative polyacrylamide gel electrophoresis.

^b Affinity chromatographic method included the following purification procedures: Ammonium sulfate precipitation, DE-23-, and *p*-MB-6-aminohexyl-Sepharose 4B column chromatographies.

^c Yield is expressed as percents of the enzyme activity in the indicated step against that of the DE-23 eluate activity.

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