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AFFINITY CHROMATOGRAPHY OF *KLEBSIELLA* ARYLSULFATASE ON TYROSYL-HEXAMETHYLENEDIAMINO-β-1,3-GLUCAN AND IMMUNOADSORBENT

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Summary

A simple and convenient method for preparation of a highly purified arylsulfatase (EC 3.1.6.1) from *Klebsiella aerogenes* has been developed. Specificity of purification was achieved by using affinity chromatography on a tyrosyl-hexamethylenediamino-β-1,3-glucan or on a solid phase immunoadsorbent. By using affinity chromatography a homogeneous enzyme was obtained with high yield. It is also proposed that the beads of curdlan type polysaccharide consisting of β-1,3-glucan can be used as a good matrix for affinity chromatography.

Introduction

Arylsulfatase (EC 3.1.6.1) synthesis in bacteria is of interest because it is controlled by sulfur-containing compounds and by aromatic compounds, such as tyramine and dopamine. Most works on regulation of arylsulfatase synthesis have been done with *Klebsiella (Aerobacter) aerogenes* [1,2]. Both the *K. aerogenes* [3] and the *Pseudomonas aeruginosa* [4] enzymes have been purified using conventional techniques. However, a simple technique for purification of the enzyme to the highest degree of purity is required for the comparative biochemical and genetic studies of this enzyme in bacteria.

Affinity chromatography has introduced a powerful tool to solve these problems. Thus, tyrosine was coupled with hexamethylenediamino-CPB, since tyramine has been found to be a specific inhibitor of *Klebsiella* arylsulfatase [3]. A solid phase immunoadsorbent (IgG-CPB) specific for arylsulfatase from *Klebsiella* was also prepared. We also propose here the use of beads of curdlan type

Abbreviations: CPB; beads of curdlan type polysaccharide 13140, β-1,3-glucan, produced by *Alcaligenes faecalis* var. *myxogenes* IFO 13140 [5]; HA, hexamethylenediamine; IgG, immunoglobulin G.

polysaccharide consisting of β-1,3-glucan [5,6] as a matrix for affinity chromatography.

Materials and Methods

CPB (average particle size 80 μm) was obtained from Takeda Chemical Ind. Ltd. (Japan). *K. aerogenes* W70 used in this work and conditions for its growth have been described previously [3], except that methionine was used as a sulfur source. *p*-Nitrophenylsulfate was obtained from Sigma Chemical Co. and recrystallized from alkaline aqueous ethanol. Other chemicals were of the purest grade available commercially.

Preparation of tyrosyl-HA-CPB

50 ml of CPB (1 g) was washed with 200 ml of distilled water and then suspended in 50 ml of distilled water. 20 ml of 5% (w/v) cyanogen bromide was added to the slurry, maintained at 20—25°C, and the pH was kept at 8 for 2 min, at 9 for 2 min, at 10 for 2 min, and then finally at 11 by addition of 2 M NaOH. After 15 min at pH 11, the CPB was washed rapidly with 200 ml of distilled water to give BrCN-activated CPB. The pH was carefully controlled by using the pH-stat (type TTTI, Radiometer, Copenhagen) since preactivated CPB, unlike Sepharose 4B is soluble in aqueous alkaline solution *. The activated CPB was treated with HA as described by Cuatrescasas [7]. The carboxyl group of L-tyrosine was attached to HA-CPB using water soluble carbodiimide at pH 4.8 by the method of Cuatrecasas [7]. After 20 h at 5°C, the beads were washed with 1 mM HCl and distilled water. Fig. 1 shows the probable structure of tyrosyl-HA-CPB. Determination of unbound L-tyrosine by spectrophotometry at 280 nm showed that the amount of ligand bound was 117 μmol/g CPB, which represents a high binding capacity.

Preparation of IgG-CPB

Arylsulfatase was purified from *K. aerogenes* W70 cells (as described previously [3]. An electrophoretically homogeneous enzyme preparation with specific activity of 30 units/mg protein was obtained and used for the immunization of rabbits. Three intramuscular injections of 1 mg purified enzyme in Freund's complete adjuvant in 2 months yielded the antiserum which was monospecific for the *Klebsiella* arylsulfatase. The dialyzed 50% ammonium sulfate fraction was applied to the DEAE-cellulose column and IgG was eluted with 0.01 M potassium phosphate buffer, pH 8.0. The IgG (6.4 mg) was coupled to activated CPB (1 g) in 0.1 M $NaHCO_3$ (pH 8.0) for 20 h at 4°C. Under these conditions, over 90% of the added protein was coupled, and the IgG content was estimated to be 5.8 mg/g CPB.

Affinity chromatography.

Tyrosyl-HA-CPB or IgG-CPB was suspended in 0.02 M tris(hydroxymethyl) aminomethane (Tris) · Cl buffer (pH 7.2) and packed under gravity in a small

* Details of the preparation method and characteristics of BrCN-activated CPB will be published elsewhere by the research group of Takeda Chemical Ind. Ltd.

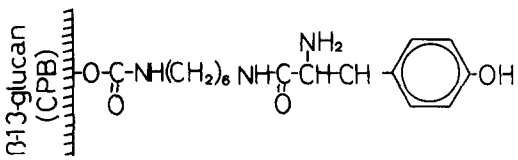

Fig. 1. Probable structure of tyrosyl-HA-CPB used for the affinity chromatography of arylsulfatase.

column. Both the gels gave approximately 20 ml bed volume/g CPB. The enzyme sample (crude extracts or DEAE-cellulose fractions) was applied onto these columns, washed and then eluted with appropriate buffer and ion concentrations at a flow rate of 0.1 ml/min. Fractions (1 or 2 ml) were collected and assayed for arylsulfatase activity and for protein by Lowry's method [8] or by absorbance at 280 nm.

Arylsulfatase assay

Arylsulfatase was measured photometrically with *p*-nitrophenyl sulfate as substrate [2]. One unit of activity was defined as the amount causing liberation of 1 μmol of *p*-nitrophenol per min at 30°C.

Gel electrophoresis

Slab gel electrophoresis with 7.5% polyacrylamide gel was carried out by Pharmacia Electrophoresis Apparatus GE4 at 50 mA in 0.05 M Tris/glycine buffer, pH 8.3. Coomassie Brilliant Blue (0.2%) in methanol/acetic acid/H_2O (5 : 1 : 5, v/v/v) was used for staining protein bands, and the gel was destained with a solution containing 7% acetic acid/5% methanol.

Results

The purification scheme is shown in Table I. The first three steps (crude enzyme preparation, ammonium sulfate precipitation, and DEAE-cellulose chromatography) were described previously [3]. All preparations were performed at 5°C.

TABLE 1

ENZYME PURIFICATION

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Crude enzyme	3890	156	0.04	100
Ammonium sulfate	810	114	0.14	73
DEAE-cellulose	32	89	2.8	57
Affinity chromatography				
Tyrosyl-HA-PS	2.2	84	38.2	54
IgG-PS	1.8	76	42.2	49

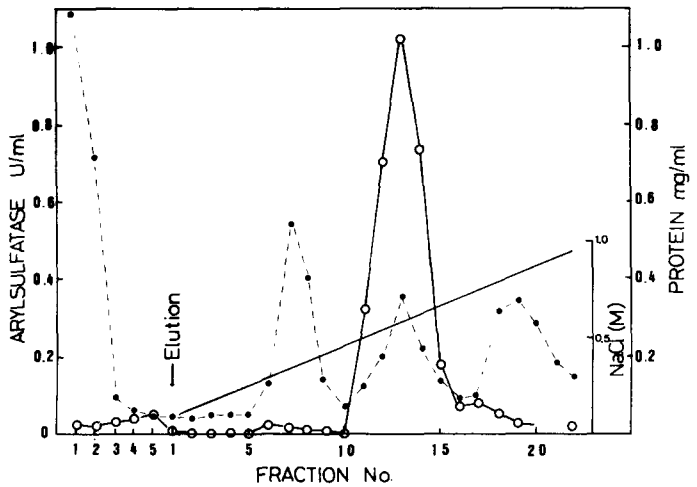

Fig. 2. Affinity chromatography of arylsulfatase on tyrosyl-HA-CPB. Dialyzed DEAE-cellulose fraction (26.4 mg protein) was applied onto a tyrosyl-HA-CPB column (0.9 × 6 cm) equilibrated with 0.02 M Tris · Cl buffer (pH 7.2). The column was washed with the same buffer containing 0.1 M NaCl, and then eluted with a linear concentration gradient of 0.1 to 0.9 M NaCl in the same buffer. Fraction size of wash and elution was 5 ml and 2 ml each, respectively. Fractions were assayed for arylsulfatase (○) and for protein (●). Fraction Nos. 11 to 14 were combined.

Purification by tyrosyl-HA-CPB

The DEAE-cellulose fraction was applied directly to a tyrosyl-HA-CPB column (0.9 × 6 cm) equilibrated with 0.02 M Tris · Cl buffer (pH 7.2). The column was washed with 20 ml of the same buffer containing 0.1 M NaCl and the elution was performed with a linear concentration gradient of 0.1 to 0.9 M

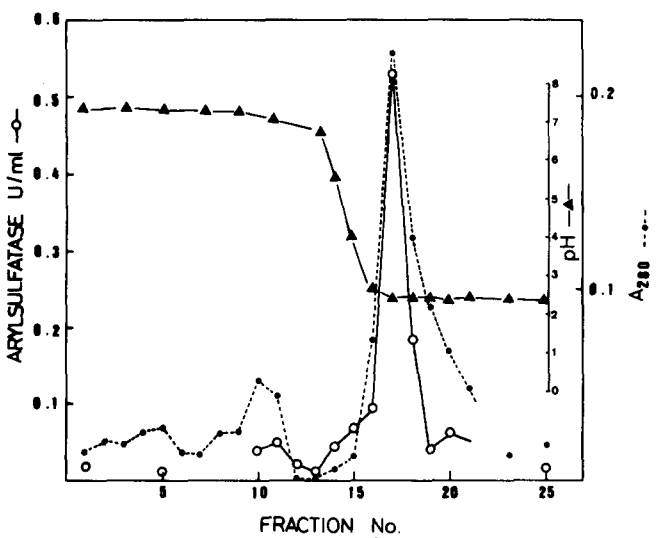

Fig. 3. Chromatography of arylsulfatase on IgG-CPB. Dialyzed DEAE-cellulose fraction (7.4 mg protein) was applied onto an IgG-CPB column (0.9 × 01 cm) equilibrated with 0.02 M Tris · Cl buffer (pH 7.2). After the column was washed with 1 M NaCl in the same buffer, the elution was performed with 0.1 M glycine/chloride buffer (pH 2.4). The fractions were immediately neutralized at pH 7.2 with 1 M glycine/NaOH buffer (pH 11.5). Fraction size was 1 ml. Protein concentration was measured by absorbance at 280 nm. Fraction Nos. 15 to 19 were combined.

NaCl (20 ml each) in the same buffer. Arylsulfatase was eluted at about 0.5 M NaCl (Fig. 2). When the column was eluted with a pH gradient of 0.2 M Tris/maleate/NaOH (pH 4.9 to 9.0) arylsulfatase was eluted at pH 5.5. Since arylsulfatase was eluted from a DEAE-cellulose column at a 0.1 M NaCl concentration [3], the enzyme was much more tightly bound to tyrosyl-HA-CPB than to DEAE-cellulose.

Purification by IgG-CPB immunoadsorbent

The sample from the DEAE-cellulose fraction was also applied to the IgG-CPB column (0.9 × 10 cm) equilibrated with 0.02 M Tris · Cl buffer (pH 7.2). The column was washed with 40 ml of 1 M NaCl in the same buffer (pH 7.2), and then eluted with 0.1 M glycine/chloride buffer (pH 2.4). Arylsulfatase was eluted at pH 2.4 (Fig. 3) and fractions were immediately neutralized at pH 7.2 with 1 M glycine/NaOH buffer (pH 11.5).

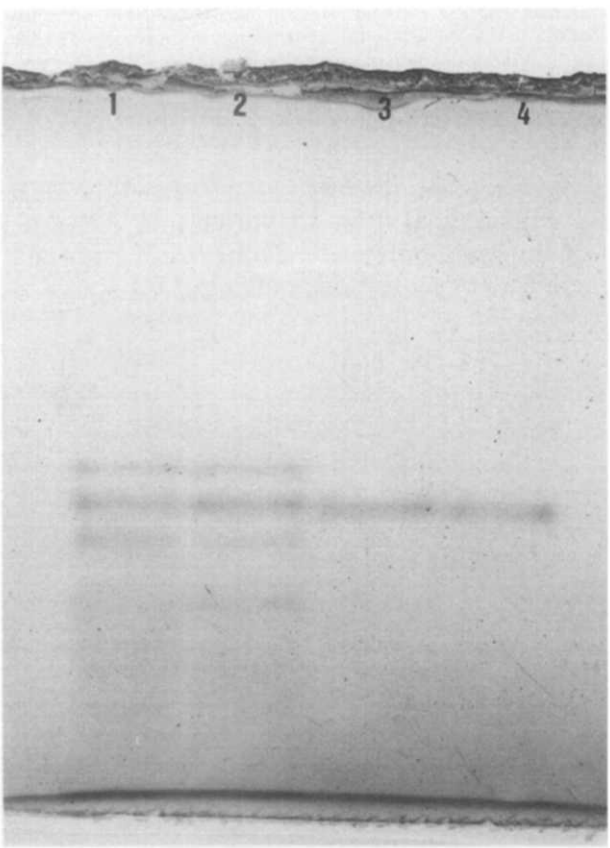

Fig. 4. Gel electrophoresis patterns of arylsulfatase purified by affinity chromatography. Crude enzyme and the DEAE-cellulose fraction were purified by tyrosyl-HA-CPB or IgG-CPB affinity chromatography. Samples (about 30 μg of protein) from affinity chromatography were subjected to slab gel electrophoresis on 7.5% polyacrylamide gel. 1, tyrosyl-HA-CPB chromatography from crude enzyme; 2, IgG-CPB chromatography from crude enzyme; 3, tyrosyl-HA-CPB chromatography from DEAE-cellulose fraction; 4, IgG-CPB chromatography from DEAE-cellulose fraction.

Purity and yield of the enzyme

A typical purification starting with 8 l bacterial culture yielded 2.2 mg enzyme with an overall recovery of 54% and 1.8 mg enzyme with a 49% recovery by using the tyrosyl-HA-CPB and IgG-CPB chromatography, respectively (Table I). The yields of the enzyme from both methods were as high as 85%, with a purification factor of about 14-fold from the DEAE-cellulose fraction. Since arylsulfatase was gradually inactivated during the elution from an IgG-CPB column with pH 2.4 buffer, tyrosyl-HA-CPB chromatography gave higher recovery than IgG-CPB chromatography. However, IgG-CPB had slightly higher specific affinity for arylsulfatase than tyrosyl-HA-CPB when crude enzyme was directly applied to both affinity columns (Fig. 4, 1 and 2). The enzyme was purified homogeneously about 1000-fold from crude extract by using both affinity chromatography methods after ammonium sulfate fractionation and DEAE-cellulose chromatography (Table I, Fig. 4, 3 and 4). The enzyme purified by affinity chromatography was electrophoretically homogeneous as obtained previously [3].

Arylsulfatase-binding capacity of the affinity columns

Arylsuflatase-binding capacity was about 0.5 μmol/g tyrosyl-HA-CPB and 0.3 μmol/g IgG-CPB, as determined with purified arylsulfatase (M_r = 47 000). Between each run, a tyrosyl-HA-CPB column was well washed with 1 M NaCl in 0.02 M Tris · Cl buffer (pH 9.0) and equilibrated in 0.02 M Tris · Cl buffer (pH 7.2). After enzyme elution was performed, an IgG-CPB column was neutralized with 0.5 M Tris · Cl buffer (pH 7.4), equilibrated in 0.02 M Tris · Cl buffer (pH 7.2) and stocked in the cold room. Little or no loss in binding capacity of the two affinity columns was noted over a 2-month period despite repeated use.

Discussion

The present purification schemes are simpler and more convenient and the overall recovery of activity is superior to those obtained using the other purification methods [3,4,9]. Purification of arylsulfatase from animal tissues has been reported by a scheme involving affinity chromatography, such as an agarose-4-hydroxy-2-nitrophenyl sulfate derivative for arylsulfatase B [10], a concanavalin A-Sepharose for arylsulfatase A [11], and a *p*-succinylaminocatechol sulfate-Sepharose for both arylsylfatases [12]. In *K. aerogenes*, arylsulfatase is a monomer with a molecular weight of 47 000 [3]. Although a few active protein bands appeared on polyacrylamide gel electrophoresis during the purification of the enzyme in some artificial conditions ([9] and unpublished results), only one gene specifies structure of arylsulfatase, and single arylsulfatase was produced [3,13]. By using these methods of affinity chromatography the nature of arylsulfatases from various organisms will be clarified. It might be noted that CPB has minimal nonspecific interaction with proteins and that ligand-bound CPB is mechanically and chemically stable during repeated affinity chromatography.

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