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Isolation of an Urate-Binding Protein by Affinity Chromatography

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Abstract

This paper describes an affinity chromatography procedure to purify an urate binding protein from human serum. The specific ligand was 8-amino-2,6-dihydroxypurine bound to Sepharose through the amino group. The specific elution was obtained with an uric acid or allopurinol solution. Electrophoretic analysis of the eluted protein shows a single sharp band with an α_2-globulin mobility. Molecular weight, determined by gel filtration, is approximately 70,000 daltons.

Index Entries: Binding protein, for urate; affinity chromatography, of a urate-binding protein; protein, isolation of a urate-binding.

Introduction

Since urate binding to serum proteins may influence the deposition in joints and the renal excretion of urate, several authors have studied the interaction between urate and serum proteins (*1–7*). However, the existence in humans of one or more serum proteins that specifically bind uric acid is still debated. There is no agreement on the nature of the binding protein identified as the serum albumin or as a specific globulin, nor on the extent of the bound quote (*3, 8, 9*). Several in-vitro techniques have been used, including ultrafiltration (*9*), ion exchange chromatography, gel filtration, and equilibrium dialysis (*10, 11*).

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We have attempted a new approach by the use of affinity chromatography (*12*). In this paper, we describe the use of a specific ligand (8-aminoxanthine) coupled to Sepharose to purify an urate binding protein from human serum (13).

Materials

Lyophilized fraction III, according to Cohn, was purchased from Biagini (Italy).

8-Amino-2,6-dihydroxypurine was prepared according to Jones and Robins (*14*); 2-amino-6,8-dihydroxypurine and 6-amino-2,8-dihydroxypurine were purchased from Sigma Chemical (USA).

Uric acid was obtained from Carlo-Erba (Italy) and allopurinol from Shoum (Italy). ^{14}C-uric acid was purchased from Amersham/Searle Corp. (England). Bio-Gel P 60 and Bio-Rad protein assay were purchased from Bio-Rad Corp. (USA). All other chemicals were reagent grade.

Specific Adsorbent Synthesis

Sepharose adipic acid hydrazide was obtained by treating cyanogen bromide-activated Sepharose 4B with adipic acid hydrazide, according to Wilchek and Lamed (*15*). Acid azide groups were generated as follows: the hydrazide derivative was suspended in 1.0*N* HCl. The suspension was cooled to 0°C, and one-tenth its volume of cold 1.0*M* sodium nitrite was added. After 2–3 min, the gel was washed with 0.2*M* NaCl at 0°C. The gel was added to a solution of the appropriate substituted purine (300 mg) dissolved in 0.1*M* borate buffer, pH 9.0; coupling was allowed to proceed for up to 24 h, after which the resin was exhaustively washed with the 0.1*M* borate buffer and borate buffer plus 0.5–1*M* NaCl until no absorption was detected at 260 mμ. Unreacted azide groups were treated with 1*M* ethanolamine for several hours. In this way we prepared three different resins: 8-amino-2,6-dihydroxypurine–Sepharose, 6-amino-2,8-dihydroxypurine–Sepharose, 2-amino-6,8-dihydroxypurine–Sepharose, as in Fig. 1. Subsequently the resin was transferred to a column (volume 25 mL) and was equilibrated with 20m*M* Tris buffer, pH 7.4.

Affinity Chromatography Procedure

A solution of lyophilized fraction III, which is albumin free, was prepared as follows: 1 g of this fraction was added to 20 m*M* Tris buffer, pH 7.4, bringing the volume up to 200 mL; then this solution was centrifugated at 10,000 rpm for 15 min (Beckman J-21 B centrifuge). The supernatant was pooled, dialyzed against 20 m*M* Tris buffer, pH 7.4, containing 0.02% sodium azide for 48 h, and subseqeuntly filtered through the specific adsorbent. Then the resin was washed with 20 m*M* Tris buffer, pH 7.4, until the optical density of the affluent (280 mμ) approached zero. At this time the elution of the specific proteins was performed

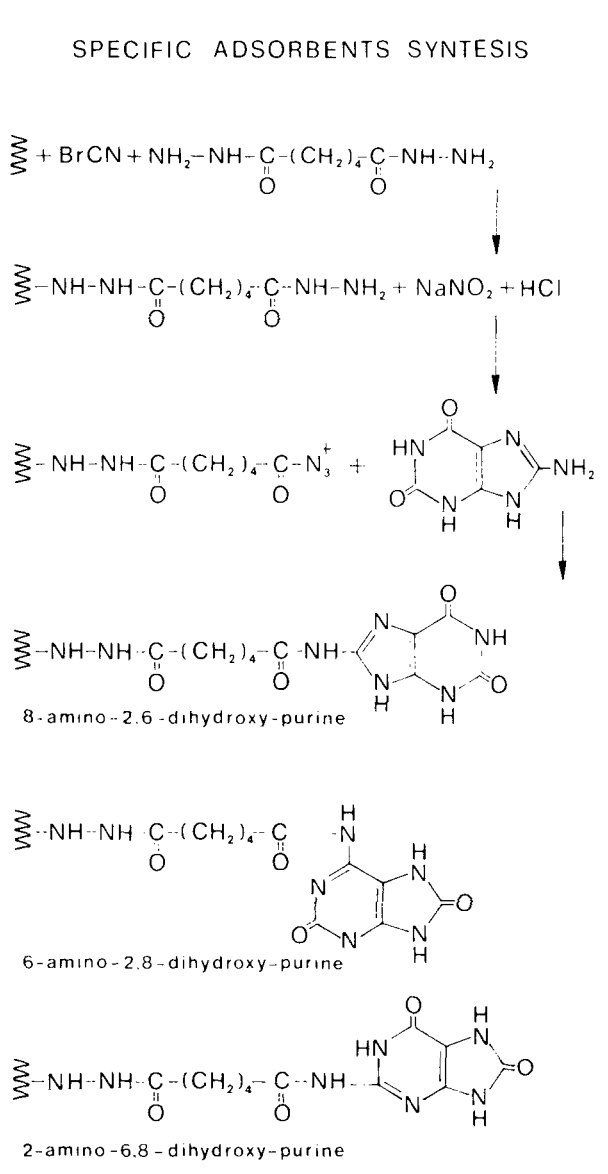

Fig. 1. Synthesis of specific absorbents.

with 20 m*M* Tris buffer, pH 7.4, containing 50 mg% of uric acid or 70 mg% of allopurinol. The 8-aminoxanthine–Sepharose showed the highest binding capacity and therefore this resin was used in the further studies.

A control experiment was carried out to demonstrate the specificity of the column. The fraction III solution was chromatographed on a column of hydrazide–Sepharose and the elution procedure was repeated. No proteins were eluted by the specific buffer containing urate or allopurinol.

The specific eluate from the 8-aminoxanthine–Sepharose column was exhaustively dialyzed against 10 m*M* ammonium acetate and then lyophilized with a Minifast mod. 1700 (Edwards). The lyophilized proteins were analyzed by

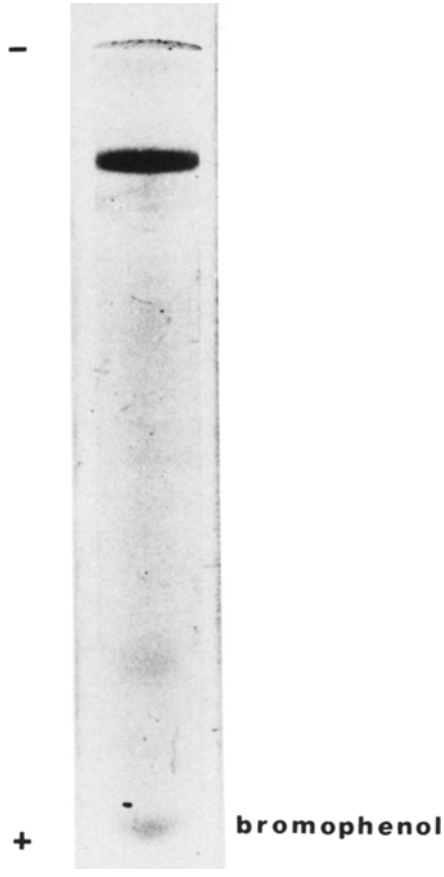

Fig. 2. Disk gel electrophoresis of the lyophilized protein.

polyacrylamide disk gel electrophoresis using 7.5% acrylamide according to the method described by Davis (*16*). Only a single sharp band, as shown in Fig. 2, is present and moves as an α_2-globulin. Electrophoresis in SDS–urea shows only a band, suggesting that this urate binding protein is apparently homogeneous. This band was also stained by periodic acid-Schiff reagent indicating the presence of carbohydrate in the protein.

The molecular weight of this purified urate binding α_2-globulin was determined as shown in Fig. 3.

Gel filtration was carried out on a 2.5 × 90 cm column of Bio-Gel P 60 and the fractions were analyzed for specific uric acid binding. The uric acid binding assay was carried out as follows: ^{14}C-uric acid (26.5 μCi/mM) solution was added to the solutions of the eluate from gel filtration to obtain the final concentration of 15 μ*M* in total volume of 200 μL. The solution was at pH 8.0. After incubation at 22°C for 45 min, 0.5 mL of gamma-globulin solution (1 mg/mL) and 2 mL of ammonium

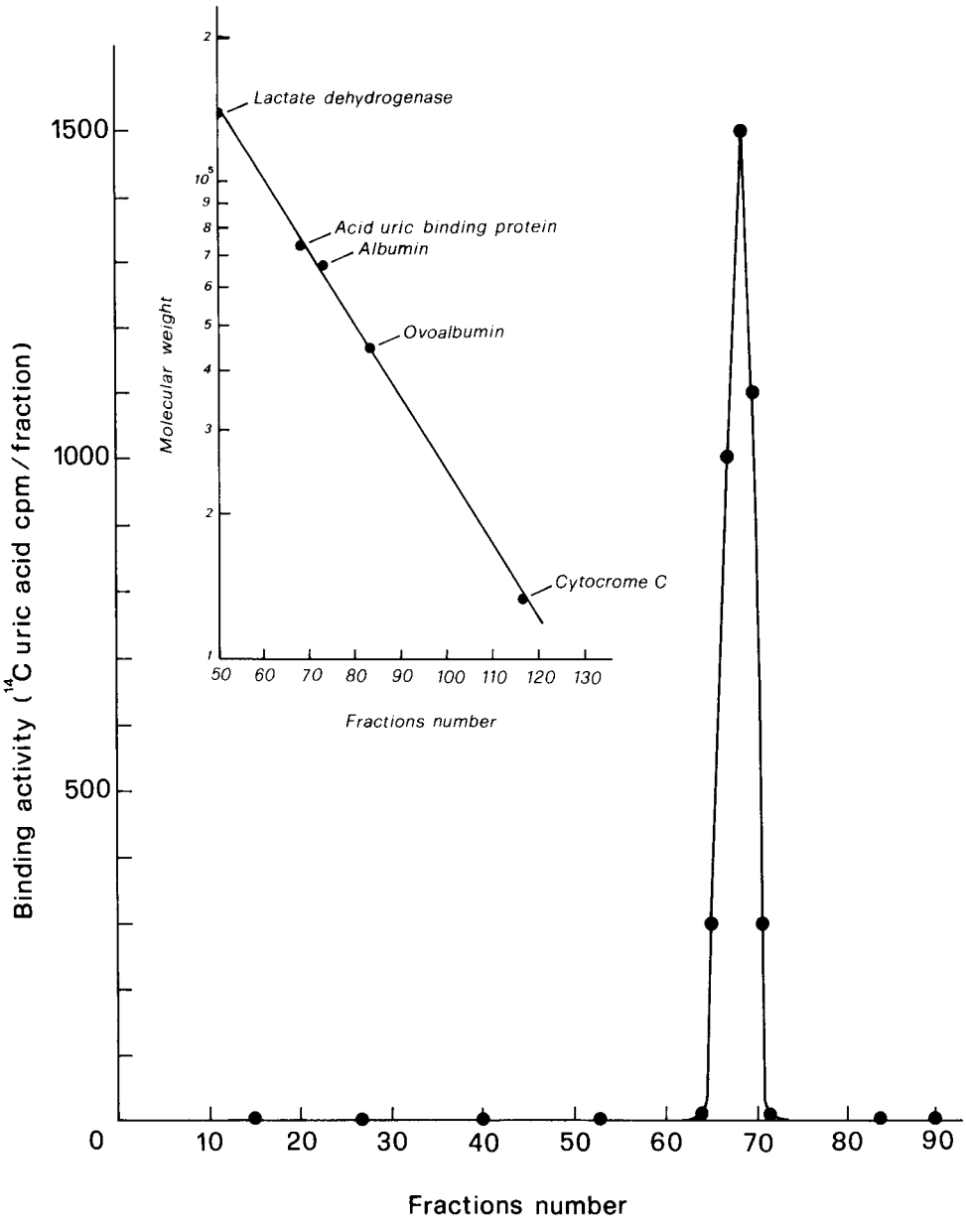

Fig. 3. Gel filtration ofthe urate-binding protein on Bio Gel P 60. In the inset, the estimation of the molecular weight is shown.

sulfate (80%) were added and then filtered under low vacuum through HAMK filters (0.45 Millipore, USA). After washing with 2 mL of ammonium sulfate (60%) the filters were placed in vials containing 10 mL of scintillation liquid (Acquasure, NEN, USA) and counted. Specific binding is defined as total ^{14}C-uric acid binding minus binding obtained in presence of 1 m*M* uric acid. The elution peak was compared with the peak of proteins with known molecular weight. In this way a molecular weight of 70,000 ± 4,000 daltons was determined.

These data indicate that affinity chromatography is an useful tool for the study of urate binding proteins and that an α_2-globulin is present in the fraction of human serum that specifically binds uric acid.

Studies are now in progress to best characterize this protein.

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