Hydrophobic interaction chromatography of human serum α₁-antitrypsin and α₁-acid glycoprotein

Sándor Oláh, Tibor Kremmer*, Mariann Boldizsár

National Institute of Oncology, Department of Biochemistry, P.O. Box 21, Ráth Gy. u. 7–9, H-1122 Budapest, Hungary

Abstract

The relative hydrophobicity of human serum α_1-antitrypsin (AAT) and α_1-acid glycoprotein (AGP) in comparison to various reference proteins was determined by hydrophobic interaction chromatography (HIC). Apolar character of glycoproteins was generated using three different cosmotropic salts, ammonium sulfate, sodium sulfate, sodium citrate and isocratic, or reversed linear gradient elution techniques. Human serum AAT and AGP showed different apolar properties on Fractogel EMD phenyl and propyl columns modulated either by the type and concentration of cosmotropic salts, or by the pH of the mobile phase. According to its higher carbohydrate content AGP proved to be more polar than AAT. Human serum AAT and AGP were pre-separated by Cibacron Blue F3G-A dye ligand affinity chromatography and based on their different hydrophobicity were fractionated and purified by HIC. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Biocompatibility and effectivity of hydrophobic interaction chromatography (HIC) in the isolation, purification and characterization of various biopolymers has recently been approved [1–6]. Separation mechanisms based on the solvophobic theory and salting-out effects were explained [7–13], and several stationary phases, matrices and ligands (hydroxypropyl, propyl, benzyl, isopropyl, phenyl, pentyl, octyl) with various hydrophobicity were developed [3,4,17–19]. In spite of some similarities to reversed-phase high-performance liquid chromatography (HPLC) [2], the generation of the hydrophobicity of proteins and peptides by relatively high concentration of cosmotropic salts, the mode of elution using negative concentration gradient, and the advantage of handling polar macromolecules under mild but apolar conditions make HIC more characteristic and versatile among the chromatographic techniques. Effects of cosmotrophic salts, mainly ammonium sulfate, pH of the mobile phase and temperature were also investigated in detail [2,7,12,14–17,21], and retention parameters were often related to ovalbumin as reference protein [20]. Regarding the nature of biopolymers to be separated the hydrophobic behavior of structurally and functionally important complex proteins with high carbohydrate content attracted particular attention [29,30]. It has been recognized that the relative hydrophobicity of many serum proteins, mainly albumin, and some glycoproteins plays determinant role in the binding and transport of metabolites and drugs of apolar character [36,40].

*Corresponding author. Fax: +36-1-2248-620.

In the present work the hydrophobic characteristics of two human serum sialoglycoproteins of pathophysiological and pharmacological importance, α_1-antitrypsin (AAT) and α_1-acid glycoprotein (AGP) were determined by HIC. AAT and AGP are characteristic acute phase human serum proteins applied widely as biochemical markers in the laboratory diagnosis of inflammation and malignant diseases [25,28]. AGP is a typical sialoglycoprotein of M_r 40 000 with extremely acidic nature ($pI \cong 2$–3) and solubility, and its high carbohydrate content (45%) is formulated in five Asn-linked, mostly biantennary oligosaccharide chains [23–25]. AAT is a M_r 52 000 proteinase inhibitor of human serum containing 12% carbohydrate in three antennary structures [26,27]. Concerning their hydrophobicity only very few data are available on the HIC parameters of AAT [22], while AGP has not been investigated yet. Several methods comprising precipitation, pre-separation and subsequent chromatographic (affinity, ion-exchange, desalting) techniques have been reported for the isolation and purification of human serum AAT and AGP [24,27,36,41–43]. In spite of their simplicity and specificity the one-step immuno-affinity methods have the main disadvantages of short life-time and high price of the stationary phases and the low yield as well [44]. In general, a proper sample preparation providing the specificity is the basic demand of the various separation techniques. In our studies, Cibacron Blue F3G-A dye ligand affinity chromatography proved to be a simple and useful technique for the simultaneous pre-separation of human serum AAT and AGP [31,41]. In a consecutive step AAT and AGP could be separated and purified by HIC based on their different hydrophobic parameters determined by us.

2. Experimental

2.1. Chemicals

Ammonium sulfate, sodium sulfate and trisodium citrate of analytical grade (Reanal, Budapest, Hungary) and Suprapur sodium dihydrogenphosphate from Merck (Darmstadt, Germany) were purchased. Salts were solved in 20 mM phosphate buffer and adjusted to the pH desired. In all solutions HPLC-grade distilled water was used.

Reference proteins (cytochrome c, CYT; myoglobin, MYO; ovalbumin, OVA; α-chymotrypsinogen A, CHY; α_1-acid glycoprotein, AGP; albumin, ALB; α_1-antitrypsin, AAT) were obtained from Sigma (St. Louis, MO, USA). Human serum AGP was also isolated and purified according to our method [24]. AAT either of commercial origin, or isolated from pooled human serum was purified further in our laboratory by Cibacron Blue F3G-A dye ligand affinity chromatography on a Fractogel TSK AF-Blue column (30×1 cm, I.D., 32–63 μm, Merck) [31]. Fractogel EMD propyl and Fractogel EMD phenyl stationary phases (20–40 μm, Merck) were packed into Pharmacia-LKB HR 5/5 columns (5× 0.5 cm I.D.).

2.2. Instrumentation

Dye ligand affinity separation of human serum proteins was performed using a fast protein liquid chromatography (FPLC) system (Pharmacia) consisting of two P-500 pumps, an LCC-500 integrator unit and a UV-1 detector at 278 nm.

In HIC experiments a Merck–Hitachi LiChro-Graph HPLC system equipped with an L-6200A Intelligent Pump, an AS-2000A Autosampler, an L-4250 UV–Vis detector (278 nm) with a D-2500 Chromato-Integrator, and/or an L-7450 diode array detection (DAD) system (220–400 nm, fixed 278 nm) with a D-7000 Interface Module and a D-7000 HPLC System Manager was used.

Identification and purity of proteins originating from the various chromatographic procedures was checked either by immunoelectrophoresis according to Laurell [32], or using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis with a Bio-Rad Mini Protean II vertical slab equipment. Electropherograms were stained either for proteins with Coomassie Brillant Blue G-250, or for carbohydrates with periodate-Schiff (PAS) reagent [33].

2.3. Hydrophobic interaction chromatography

Capacity factors of reference proteins, as well as of AAT and AGP were determined on Fractogel EMD propyl and Fractogel EMD phenyl apolar

stationary phases with isocratic elution at pH 7.2 using ammonium sulfate, sodium sulfate and tri-sodium citrate in ranges 0–2 *M*, 0–1.3 *M* and 0–1 *M*, respectively. Mean retention values were calculated at least from two consecutive experiments. Retention times of proteins were measured in a wide range of pH (3–8) applying ammonium sulfate from 1.8 to 0 *M* for 7 min in a linear gradient elution program. Relative retention times (RRTs) to ovalbumin were determined at pH 7.2 using the above elution program.

2.4. Isolation of human serum AAT and AGP

Human serum AAT and AGP were isolated using Cibacron Blue F3G-A dye ligand affinity chromatography according to Birkenmeier and Kopperschläger [31] slightly modified by us as follows: 2 ml of human serum was injected directly onto the Fractogel TSK AF Blue column equilibrated with 10 m*M* phosphate buffer at pH 6.6, and a fraction eluted with the void volume was collected. Other constituents of serum (mostly albumin) were washed down with 2 *M* NaCl. Pre-separated AAT and AGP were further fractionated on a Fractogel EMD propyl column (Superformance 10×1 cm, I.D., Merck) at pH 7.2 using isocratic elution with 1.1 *M* ammonium sulfate for 3 min, then a linear gradient from 1.1 to 0 *M* for 10 min (see Fig. 5).

3. Results and discussion

Recently, the influence of the relative hydrophobicity of carbohydrate-rich complex proteins on their structural and functional role has been extensively investigated [34–39]. HIC proved to be suitable for the characterization as well as for the separation of a number of biopolymers including natural glycoconjugates based on their apolar interactions under the experimental conditions applied [20]. In order to investigate the hydrophobic properties of AAT and AGP retention times were measured on two apolar stationary phases at pH 7.2 using isocratic elution with various concentrations of three different cosmotrophic salts. Figs. 1 and 2 show the retention profiles obtained on Fractogel EMD propyl and phenyl columns, respectively, and demonstrate the
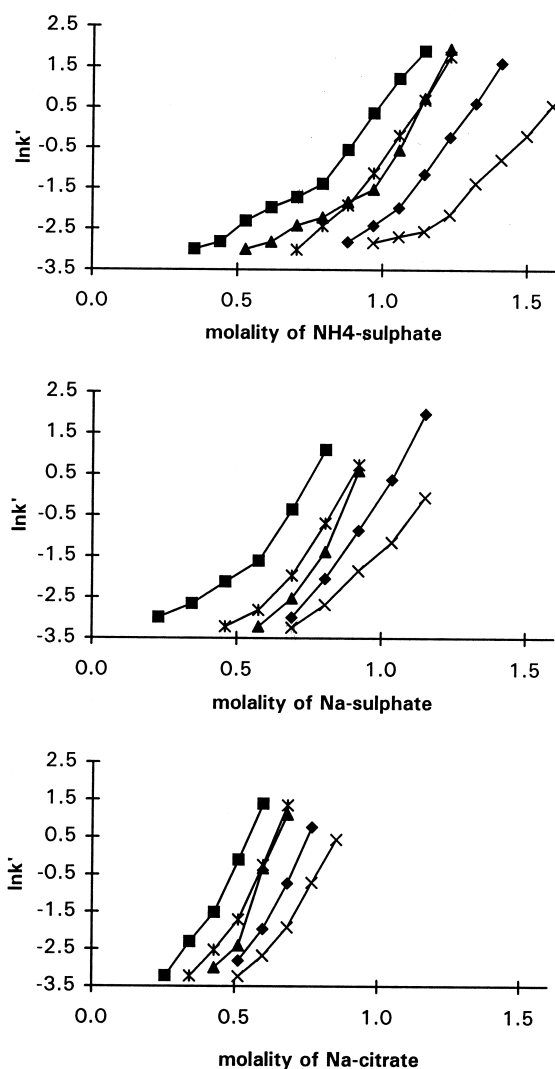

Fig. 1. Retention profiles of reference proteins on Fractogel-propyl column. Symbols: ◆, AGP; ■, AAT; ▲, ALB; ✳, MIO; ✳, OVA.

different hydrophobicity of AAT, AGP and some reference proteins as well on these stationary phases. The more apolar phenyl column resulted in higher retention of proteins at the same salt concentration than the propyl phase due to the stronger apolar interactions. On the other hand, the various cosmotrophic salts modulated the selectivity of HIC in a different way, depending either on the apolarity of stationary phases, and/or the hydrophobicity of the proteins investigated.
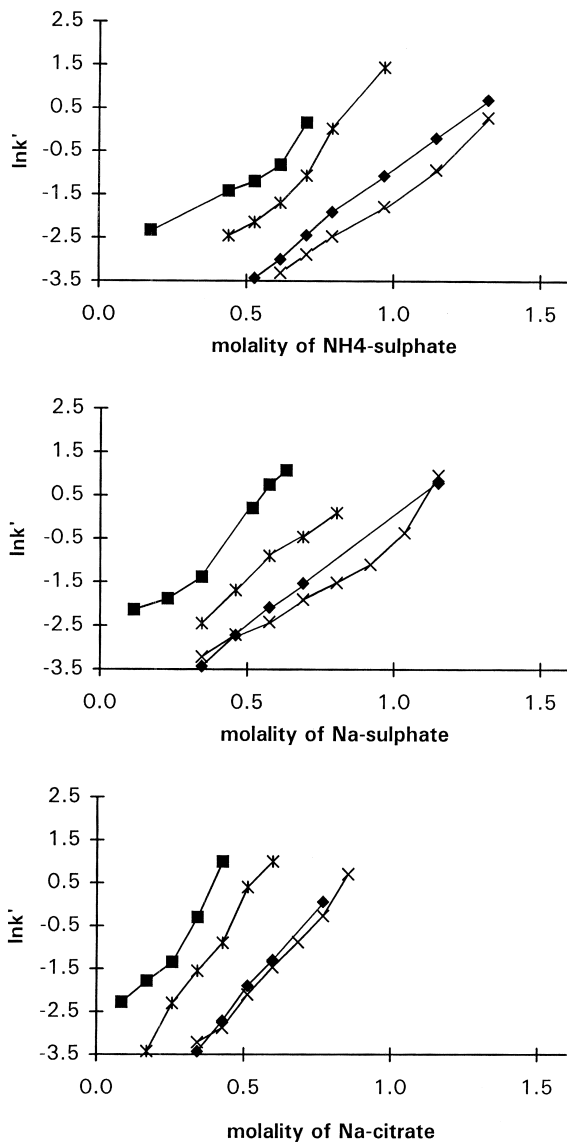
Fig. 2. Retention profiles of reference proteins on Fractogel-phenyl column. Symbols: ◆, AGP; ■, AAT; ✕, MIO; ✳, OVA.

Data of Table 1 demonstrate that a series of reference proteins were eluted from Fractogel EMD propyl and phenyl columns according to their hydrophobicity [20]. Definite apolar properties of AAT and AGP were also indicated by their retention times related to ovalbumin (RRTs). Presumably, as a consequence of its relatively higher carbohydrate content AGP exerted more polar character than AAT. Considering the relative apolarities of proteins the more polar propyl column proved to be more useful for the elimination of albumin from AAT or AGP, as well as for the separation of AAT from AGP. Selectivity differences calculated for the critical pairs of AAT/AGP, AAT/ALB and AGP/ALB are shown in Fig. 3. From these results it can be stated that all the three cosmotrophic salts were suitable in an optimal initial concentration for the separation of AAT from AGP. However, for the comparability of our results to the data of literature ammonium sulfate was thoroughly used in the further studies.

Fig. 4 demonstrates the pH dependence of retention times of AAT and AGP on Fractogel EMD phenyl and propyl columns, respectively, using gradient elution with ammonium sulfate in a concentration range from 1.8 to 0 M for 7 min. In general, it can be stated that the retention times of serum glycoproteins were increasing gradually by decreasing the pH of the mobile phase. However, the retention of AAT was resistant to the changes of pH on the phenyl column due to the strong apolar interactions. Obviously, the effect of pH on the retention of glycoproteins is depending on the polarity differences either between AAT and AGP, and/or the propyl and phenyl columns as well. Considering the sensitivity and lability of sialoglycoproteins to acidic pH, and the optimal differences in retention times for the separation of AGP and AAT, the nearly physiological pH 7.2 was selected for our experi-

Table 1
Relative retention times (RRTs) of reference proteins in relation to ovalbumin on Fractogel-propyl and Fractogel-phenyl columns[a]

Protein	CYT	MYO	AGP	ALB	OVA	AAT	CHY
Fractogel-propyl	0.20	0.55	0.87	0.99	1.00	1.17	1.34
Fractogel-phenyl	0.11	0.66	0.80	0.99	1.00	1.09	1.24

[a] Results are means of three consecutive independent measurements.
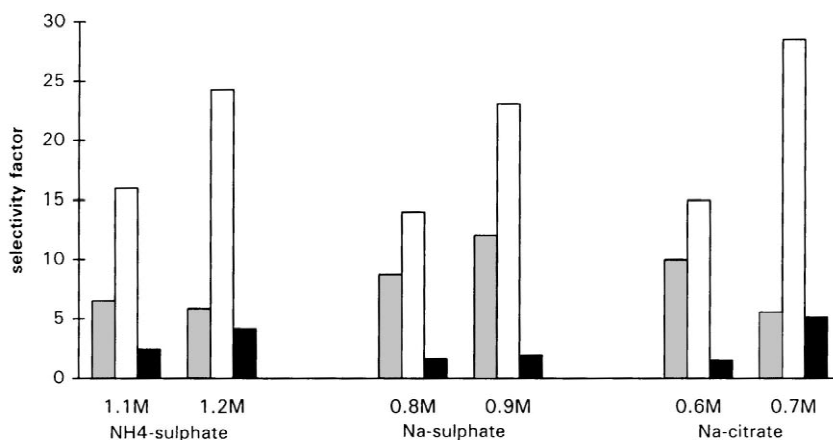
Fig. 3. Effect of cosmotrophic salts on the selectivity of AAT–AGP–ALB pairs using a Fractogel EMD propyl column. Isocratic elution at pH 7.2. Symbols: ▨, AAT/ALB; ☐, AAT/AGP; ■, ALB/AGP.

ments and recommended as a standard HIC condition.

Differences in the hydrophobic characteristics of human serum AAT and AGP presented here led to
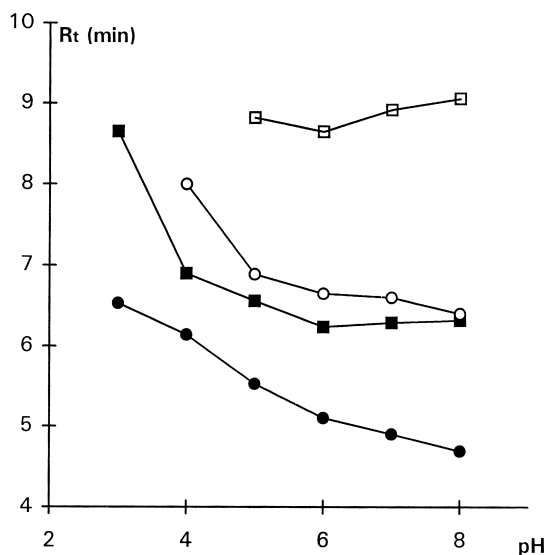

Fig. 4. The pH dependence of the retention time of human serum α₁-acid glycoprotein (AGP) and α₁-antitrypsin (AAT) on Fractogel-propyl and Fractogel-phenyl columns. Symbols: —●—, Propyl/AGP; —■—, Propyl/AAT; —○—, Phenyl/AGP; —☐—, Phenyl/AAT.

the practical conclusion that they can be separated from each other by HIC. However, for the effectivity of HIC a proper sample preparation by eliminating the complex matrix of the human serum was required. Recently, Cibacron Blue F3G-A dye ligand affinity chromatography proved to be successful for the simultaneous pre-separation of human serum AAT and AGP [31,41]. An improvement of this method allowed one to directly apply the whole serum on the Fractogel TSK AF-Blue column. SDS–polyacrylamide gel electrophoresis demonstrated that the unbound AAT and AGP eluted together from the Cibacron Blue F3G-A column with 0.01 M phosphate buffer at pH 6.6 were contaminated with some minor serum proteins only. In a consecutive step this fraction was applied for HIC. Fig. 5 shows that AAT and AGP could be separated and purified on a Fractogel EMD propyl column using ammonium sulfate gradient elution at pH 7.2. The purity of AAT and AGP separated was verified by gel electrophoresis and only AGP showed a contamination of albumin of less than 5%. According to the immuno-electrophoretic studies, yields for AAT and AGP compared to the original serum contents were 65 and 78% on average, respectively. Analytical columns (5×0.5 cm I.D.) used in our experiments had a capacity of 5–10 mg glycoproteins. Increasing the dimensions of the preparative columns (10×1 cm I.D.) allowed the separation of AAT and AGP each
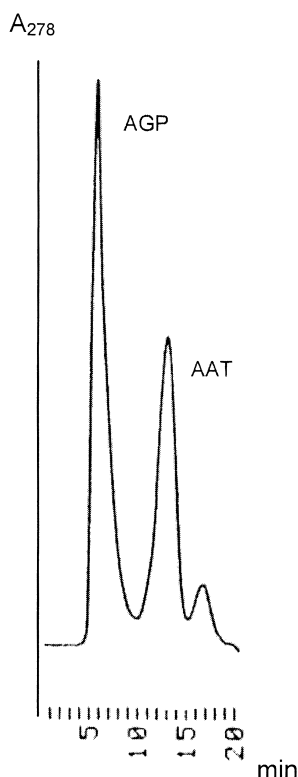
A$_{278}$

Fig. 5. Hydrophobic interaction chromatography of human serum AAT and AGP on Fractogel EMD propyl column (10×1 cm I.D., 20–40 μm). Eluent A: eluent B containing 1.1 *M* NH$_4$-sulfate. Eluent B: 20 m*M* Na-phosphate buffer, pH 7.2. Elution program: 100% eluent A for 3 min, then linear gradient with eluent B 0–100% for 10 min. Sample: 1 ml AF-Blue void volume fraction. Flow-rate: 1 ml/min. Detection: UV at 278 nm.

in an amount of 50 mg without the loss of resolution and purity.

4. Conclusions

HIC under the experimental conditions applied revealed the marked apolar properties of human serum AAT and AGP on Fractogel EMD phenyl and propyl columns. In comparison to various reference proteins the hydrophobic characteristics of AAT and AGP were determined by investigating the cosmotrophic effects of ammonium sulfate, sodium sulfate, sodium citrate and pH of the mobile phase on the retention profiles. Based on these observations experimental conditions for the HIC separation of AAT

and AGP were optimized. For the application of HIC to the isolation and purification of these glycoproteins AAT and AGP were pre-separated from human serum by Cibacron Blue F3G-A dye ligand affinity chromatography. Using linear ammonium sulfate gradient elution at pH 7.2 AAT and AGP were fractionated on the Fractogel EMD propyl column resulting in glycoproteins of appropriate purity and yield. From the results presented here it can be concluded that a consecutive application of Cibacron Blue dye ligand affinity and HIC provides a simple, fast and efficient method for the simultaneous isolation and purification of human serum AAT and AGP.

Acknowledgements

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