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Methodology Report

Comparison of Methods for the Purification of Alpha-1 Acid Glycoprotein from Human Plasma

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Alpha-1 acid glycoprotein (AGP) is a highly glycosylated, negatively charged plasma protein suggested to have anti-inflammatory and/or immunomodulatory activities. Purification of AGP could be simplified if methods that exploit its high solubility under chemically harsh conditions could be demonstrated to leave the protein in its native conformation. Procedures involving exposure of AGP to hot phenol or sulphosalicylic acid (SSA) were compared to solely chromatographic methods. Hot phenol-purified AGP was more rapidly cleared from mice in vivo following intravenous injection than chromatographically purified AGP. In contrast, SSA-purified AGP demonstrated an identical in vivo clearance profile and circular dichroism spectrum to chromatographically purified AGP. Similarly, no differences in susceptibility to enzymatic deglycosylation or reactivity with *Sambucus nigra* lectin were detected between AGP purified via the two methods. Incorporation of the SSA step in the purification scheme for AGP eliminated the need for a large (4 mL resin/mL of plasma) initial chromatographic step and simplified its purification without causing any detectable distortion in the conformation of the protein. Confirmation that this procedure is nondenaturing will simplify AGP purification and investigation of its possible biological roles in laboratory animals.

1. Introduction

Alpha-1 acid glycoprotein (AGP; also known as orosomucoid) is one of the most heavily glycosylated proteins in human plasma, with approximately 45% of its 44 kDa molecular weight being contributed by N-linked glycans [1]. These glycans cluster on the N-terminal half of the 183 amino acid AGP polypeptide chain and are attached to Asn 15, 38, 54, 75, and 85 [2]. AGP exhibits an unusually high degree of glycan branching, being one of the few plasma proteins possessing not only di- and tri-antennary, but also tetra-antennary glycans [3]. This degree of branching indirectly results in a high sialic acid composition, which contributes significantly to the unusually low isoelectric point (pI) of the protein, which ranges from 2.8 to 3.8 due to the existence of multiple isoforms [4]. Recently, a high-resolution crystal structure of recombinant, nonglycosylated AGP revealed the AGP polypeptide to be folded into an eight-stranded β-barrel, characteristic of members of the lipocalin protein family [5].

The physiological role of AGP is unclear. Indirect immunomodulatory effects of AGP on different classes of leukocytes have been reported [6–10], in addition to potential roles in promoting endothelial barrier function [11–13], and in directly binding both lipopolysaccharide [14] and neutral and basic drugs [5]. During the acute phase response, circulating levels of human AGP rise form two- to sevenfold [15]. If this increase is part of an anti-inflammatory response by the host, then mimicking it pharmacologically could provide a therapeutic advantage. In this regard, several laboratories have reported the ability of exogenous bovine or human AGP to either dampen inflammatory responses or

decrease mortality in rodent models of hypovolemia, septic shock, or sepsis, at doses in the 0.2–0.4 g intravenous AGP per kg body weight range [16–20].

Because AGP is neither an enzyme nor an enzyme inhibitor, optimizing its stepwise purification from plasma is challenging and cannot rely on traditional measurements of stepwise changes in specific enzymatic activity. Many protocols exploit the low pI and/or extremely high solubility of AGP to achieve its enrichment [21–26]. However, such procedures rely at least in part on relatively harsh conditions that could disrupt the natural conformation of the protein and/or its glycan chains. To extend our preliminary findings of anti-inflammatory effects of AGP administration in a mouse model of early sepsis as a possible prelude to translating the approach to humans, we sought to improve the ease with which AGP could be purified from human plasma. We avoided commercial sources of AGP because on a high mg to g scale, their cost is prohibitive for research. Here we compare several procedures for the purification of AGP from human plasma and report that brief exposure of AGP to sulphosalicylic acid facilitated the isolation of purified AGP indistinguishable in either a biological or biophysical assay from that obtained in a longer procedure employing a more cumbersome chromatographic step.

2. Materials and Methods

2.1. Materials. Affi-Gel Blue resin (150–300 μm particle size), Bio-Gel HT hydroxyapatite resin, CHT Ceramic hydroxyapatite resin, and Bio-Beads SM-2 adsorbent were from Bio-Rad Laboratories (Hercules, CA). HiTrap Blue prepacked columns (volume 5 mL) were purchased from GE Healthcare (Piscataway, NJ). Sulphosalicylic acid, goat antihuman AGP antiserum, and purified human AGP were from Sigma (St. Louis, MO). Horseradish peroxidase-(HRP-) conjugated rabbit antihuman AGP IgG was from Rockland Immunochemicals, Inc. (Gilbertsville, PA). The PyroGene endotoxin assay was from Lonza (formerly Cambrex) BioScience (Walkersville, MD). New Zealand White rabbits and C57/Bl6 mice were purchased from Charles River Laboratories (St. Constant, QC).

2.2. Plasma. Human plasma was prepared from whole blood collected into 1/7 volume of citrate-phosphate-dextrose anticoagulant. Blood donors were healthy laboratory staff volunteers whose blood was obtained with informed consent under the terms of a protocol approved by the Research Ethics Board of Hamilton Health Sciences Corporation. Plasma from 23 donors was pooled and aliquoted prior to freezing at −80°C. All AGP preparations in this study therefore were derived from the same source material.

2.3. AGP ELISA. AGP was quantified in solutions ranging in complexity from plasma to purified AGP, using a sandwich enzyme-linked immunosorbent assay (ELISA). Goat antihuman AGP antiserum diluted to 2.5 μg/mL in 0.05 M sodium carbonate pH 9.6 was immobilized onto microtiter plate wells overnight at 4°C. Wells were then blocked with 1% (w/vol) BSA in phosphate-buffered saline (PBS), washed with PBS supplemented with 0.1% (vol/vol) Tween 20 (PBS-T), and incubated either with samples or commercial purified AGP standards, both diluted in 1% (w/vol) nonfat skim milk powder in PBS-T (MPBS-T). Following PBS-T washes, plates were exposed to HRP-conjugated rabbit antihuman AGP IgG diluted 1 : 5000 in MPBS-T and were developed using o-phenylenediamine dihydrochloride as a chromogenic substrate. Colour development was stopped by addition of 1/3 volume of 2.5 M H_2SO_4 and quantified as the absorbance at 490 nm, using a BIO-TEK EL808 plate reader (Winooski, VT). All antibody reactions took place at room temperature for 90 minutes. The OD_{490} correlated best ($r^2 > 0.95$) with AGP dilutions between 10 and 100 ng/mL.

2.4. Protein Analysis. Total protein concentration was determined by the method of Bradford [27], using either BSA or AGP as a standard. Polypeptide composition of proteinaceous solutions was examined electrophoretically using SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean II electrophoresis system as directed by its manufacturer (Bio-Rad). Immunoblotting was carried out as described [28] using the same HRP-conjugated anti-AGP IgG as in the ELISA. *Sambucus nigra* agglutinin (SNA) reactive proteins were detected on nitrocellulose using a DIG Glycan Differentiation Kit (Roche Diagnostics, Mannheim, Germany). In some experiments, terminal sialic acid residues were removed from AGP glycans using neuraminidase; in others the purified protein was enzymatically deglycosylated using Peptide: N-Glycosidase F (PNGase F) under denaturing conditions. Neuraminidase and PNGase F reactions were carried out following the manufacturer's directions (both from New England Biolabs, Ipswich, MA). Band intensities on Coomassie-stained gels or antibody- or lectin-decorated immunoblots were quantified using Quantity One software following image capture on a Gel Doc system (Bio-Rad Laboratories, Mississauga, ON).

2.5. Open Column Chromatographic Purification of Human AGP. AGP was initially purified from human plasma using the method of Herve et al. [29]. Except where indicated, all buffers and solutions used in this study contained 0.02% (w/vol) sodium azide as a bacteriostatic agent. Briefly, 50 mL of plasma was dialyzed against 10 mM sodium phosphate pH 5.0 and then applied to a 200 mL bed volume Affi-Gel Blue (agarose-coupled Cibacron Blue F3G-A dye) column equilibrated with the same buffer. The flow-through was dialyzed against 10 mM sodium phosphate pH 7.0, 100 mM NaCl, and applied to a 30 mL bed volume Bio-Gel HT hydroxyapatite column. After a 3-column volume wash, the column was eluted with 20 mM sodium phosphate, 100 mM NaCl. For brevity this procedure is referred to as Method 1.

2.6. Hot Phenol Purification of AGP. In some experiments, AGP was purified from plasma using the method of Chan and Yu [23]. An equal volume of plasma was mixed with 25 mL 10 mM Tris-Cl pH 7.4 and combined with 50 mL of phenol saturated with sterile water. The phenol/plasma

mixture was heated to 70°C for 20 minutes with stirring, then placed on ice for 10 minutes and centrifuged for 10 minutes at 20,000× g. The supernatant was then subjected to the same Affi-Gel Blue step described in the previous section. For brevity, this procedure is designated Method 2.

2.7. Incorporation of ÄKTA Prime into Open Chromatographic Purification of AGP. Twenty-five mL of plasma was chromatographed on Affi-Gel Blue exactly as described in Section 2.3, except that a 100 mL bed volume was packed into a column of 3.0 cm internal diameter (I.D.). The flow-through was then dialyzed into 1 mM sodium phosphate pH 7.0, 50 mM NaCl, and loaded onto a CHT ceramic hydroxyapatite column (16 mm I.D., 5 mL bed volume) connected to an ÄKTA-Prime automated liquid chromatography system (GE Healthcare) at a flow rate of 2 mL/min. The column was washed with load buffer, eluted stepwise with 50 mM NaCl containing either 5, 20, or 40 mM sodium phosphate pH 7.0, and purged with 500 mM sodium phosphate pH 7.0, 500 mM NaCl. Fractions from the middle steps were pooled and concentrated to complete the procedure. For brevity, this procedure is identified as Method 3 in this paper.

2.8. Sulphosalicylic Acid Preparation of AGP. AGP was first enriched by sulphosalicylic acid (SSA precipitation, as previously described [24, 25]). Twenty-five mL of human plasma was precipitated using 8.3 mL of ice-cold 12% (vol/vol) SSA. The precipitate was pelleted by centrifugation at 4300× g for 15 minutes at 4°C. The pH of the supernatant was adjusted to 7.0 via the dropwise addition of 5.0 M NaOH and dialyzed at 4°C against 1 mM sodium phosphate pH 7.0, 50 mM NaCl. It was loaded onto CHT ceramic hydroxyapatite using the ÄKTA Prime system exactly as described in Section 2.5. The eluate was dialyzed against 10 mM sodium phosphate pH 5.0 and applied to a prepacked 5.0 mL HiTrap Blue column on the ÄKTA Prime work station at 2 mL/min. The flow-through was collected and concentrated by cycles of ultrafiltration and dilution using disposable centrifugal concentration devices (Millipore, Billerica, MA) to change the buffer to 20 mM sodium phosphate pH 7.0, 50 mM NaCl, as in Method 3. For brevity, this procedure is identified as Method 4 in this paper.

2.9. Endotoxin Removal. Purified AGP preparations were made 1% (v/v) in Triton X-114 and incubated, with rocking, for 30 minutes at 4°C. Following incubation in a 37°C water bath for 10 minutes, phase separation was achieved by centrifugation at 20,000× g for 10 minutes. The upper aqueous AGP-containing phase was drawn off. Any Triton X-114 that carried through was removed via adsorption to SM-2 Bio-Beads, added to the aspirate at 0.3 g/mL, and stirred for an hour at 4°C. Endotoxin levels before and after this procedure were tested using the PyroGene assay kit following the manufacturer's instructions.

2.10. Circular Dichroism. The circular dichroism spectrum of purified AGP was determined using a J-600 spectropolarimeter (Jasco Analytical Instruments, Easton MD). AGP was adjusted to 1.0 mg/mL in 20 mM sodium phosphate pH 7.0, 50 mM NaCl and analyzed using a 0.1 cm path length in a cylindrical quartz cuvette. Spectra were acquired in 0.2 nm increments from 199 to 250 nm, with a scanning speed of 50 nm/min and were reported as the mean of 8 scans.

2.11. Animal Studies. Purified AGP preparations were radiolabeled using ^{125}I-labeled sodium iodide by the Iodogen method [30]. Rabbits weighing 2.3–3 kg were injected via the marginal ear vein with 4×10^6 dpm/kg of ^{125}I-labeled azide-free protein. Mice weighing 20–30 g were injected with $1.1–3.8 \times 10^7$ dpm/kg of ^{125}I-labeled protein via the tail vein. Blood sampling and determination of acid-precipitable radioactivity in plasma was carried out as previously reported by this laboratory for both species [31, 32]. Protein-bound radioactivity in the pellet was quantified using a Packard Minaxi γ counter. Results were presented on semilogarithmic clearance curves, either of the percentage of the injected dose remaining in the animal over time or of the percentage of acid-precipitable radioactivity remaining in the plasma, normalized to take the first, five-minute postinjection plasma sample, as 100%. The former analysis was employed in mice to probe recovery differences, and the latter in rabbits. In the former instance, radioactivity detected in the sampled plasma was extrapolated to whole-body radioactivity assuming a murine blood volume equal to 8% of body weight and a mean haematocrit of 40%. Animal experiments employed procedures approved by the Animal Research Ethics Board of the McMaster University Faculty of Health Sciences.

2.12. Statistical Analysis. Data was analyzed using InStat version 3.01 statistical analysis software (GraphPad, San Diego, CA). A two-tailed Student's t-test was employed for parametric comparison and a Mann-Whitney test for nonparametric comparison, taking $P < .05$ to indicate significance. Data is reported as the mean ± the standard deviation.

3. Results

A previous preliminary report from this laboratory suggested an anti-inflammatory role of transfused purified human AGP in a mouse model of early sepsis, using a dose of 0.13 g/kg body weight [33]. Further investigation employing such relatively high doses required us to find a more efficient method of purifying AGP from human plasma.

Initially, we compared two published methods of AGP purification: one involving two chromatographic steps, including a large (4 mL resin/mL of plasma) column (Method 1) [29] and a simplified procedure involving a hot phenol extraction (Method 2) [23]. As shown in Figure 1(a), in our hands the hot phenol method did not purify AGP to homogeneity in a single step; instead both a 45 kDa protein immunoreactive with anti-AGP antibodies (data not shown) and multiple polypeptides of lesser mobility were detected on SDS-PAGE. In contrast, and as expected, in Method 1, chromatography on a Cibracron Blue dye column yielded

a mixture of AGP and primarily one other protein, and a second hydroxyapatite chromatography step completed the purification to homogeneity. Adding an additional Cibacron Blue dye chromatography step to the hot phenol Method 2 procedure rendered the two protocols equivalent in terms of yielding electrophoretically pure AGP. Immunoblotting of the two preparations gave an antibody decoration profile identical to the Coomassie-stained gel shown in Figure 1 (data not shown).

The in vivo behaviour of the two AGP preparations following injection in mice was next examined following radioiodination. As shown in Figures 1(b) and 1(c), AGP purified by the hot phenol Method 2 left the murine circulation more rapidly than that purified by the solely chromatographic Method 1, as indicated by its lesser abundance in sampled blood plasma at all time points that were investigated, both in the initial equilibration (Figure 1(b)) and subsequent terminal (Figure 1(c)) phases of its clearance. For instance, the percentage of the injected dose found in the blood at the first time point (5 minutes post-injection) was 67 ± 18% for chromatographically purified AGP versus 34 ± 17% (mean ± SD, $n = 6$, $P = .0079$) for hot phenol-purified AGP. Twenty-four hours later, this difference remained significant, at 19 ± 6% versus 8.1 ± 2.7% (mean ± SD, $n = 6$, $P = .0017$). The area under the mean observed clearance curve, a single measure capturing the entire clearance profile, was also reduced, from 730%-hours for Method 1 AGP, to 324%-hours for Method 2 AGP.

Hypothesizing that the chemically harsh phenol treatment could have resulted in loss of sialic acid residues, we fully desialylated Method 1 AGP by neuraminidase treatment (Figure 2(a), left). This procedure increased the mobility of AGP, and, more importantly, eliminated its binding by SNA lectin, which reacts with terminal sialic acid residues linked to galactose (Figure 2(a), right). We next compared the binding of Method 1 and Method 2 AGP preparations, at three different dilutions, of either SNA lectin or anti-AGP antibodies. As shown in Figure 2(b), the ratio of SNA lectin binding to anti-AGP binding varied at different dilutions of AGP but was always higher for Method 2 than Method 1. These results indicated that loss of sialic acid residues did not correlate with the more rapid in vivo clearance of Method 2 (hot phenol) AGP compared to Method 1 AGP.

It was nevertheless clear that some undesirable modification, conformational or otherwise, correlated with the use of AGP Method 1, which involved a chemically harsh hot phenol treatment. We sought to replace this purification step with a nonchromatographic procedure that also rapidly enriched the preparation in AGP. As shown in Figure 3(b), SSA precipitation enriched AGP from being a minor component of plasma to being the most abundant of 4 principal polypeptides in the SSA supernatant, similar to the way in which flowing through Cibacron Blue dye columns enriched AGP in the alternative protocol. Note that to facilitate comparison between the two methods, the two procedures were adapted to use the AKTA Prime automated liquid chromatography system, which necessitated using forms of hydroxyapatite and immobilized Cibacron Blue dye capable of withstanding elevated pressure. Using the form

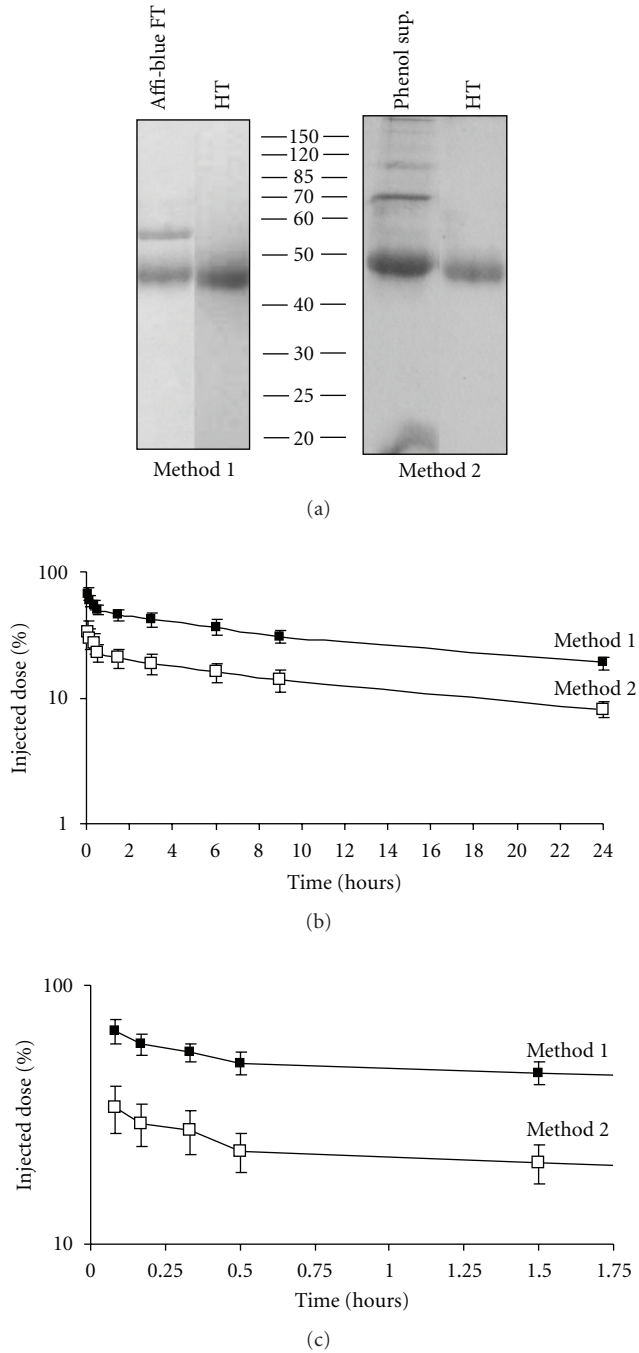

Figure 1: Comparison of AGP purified by solely chromatographic Method 1 or hot phenol-incorporating Method 2. (a) shows Coomassie-stained SDS reducing gels of equivalent (5 μg total protein/lane) samples of AGP preparations after initial (Cibacron Blue dye column flow-through, Affi-Blue FT, or hot phenol aqueous phase, Phenol sup.) or secondary hydroxyapatite chromatography (HT), with the Method number given beneath, and the position of molecular mass markers indicated between (in kDa) the gel images. (b) shows the percentage of the injected dose remaining in mice following injection of radioiodinated AGP purified by either Method 1 or Method 2 ($n = 6$, mean ± SD) versus time. (c) is identical to (b), except that the first hour post-injection is expanded to allow visualization of initial time points.

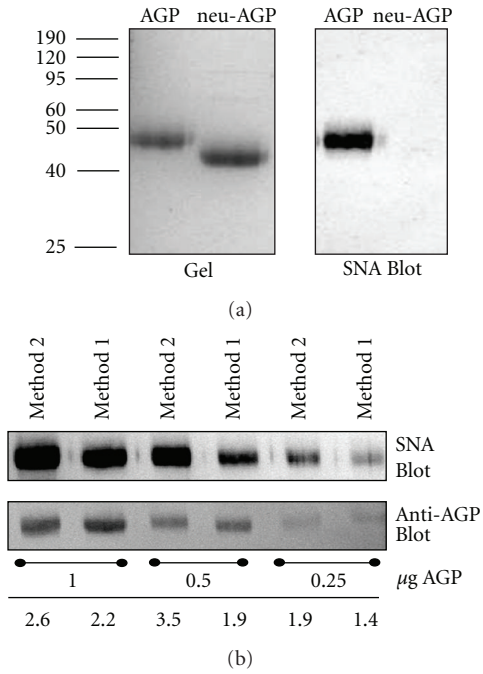

Figure 2: Reaction of AGP with neuraminidase and reaction of Method 1 versus Method 2 AGP with lectins and antibodies. AGP purified by Method 2 (AGP; 2 μg total protein/lane) was treated with or without neuraminidase (neu-AGP) and examined on SDS reducing gels or replica SNA blots (a). Molecular mass markers (in kDa) are shown at left. (b) shows 0.25–1.0 μg AGP from Methods 1 or 2 on blots probed either with Sambucus nigra agglutinin (SNA Blot) or with Anti-AGP antibodies. The SNA: anti-AGP ratio of the band intensities is shown beneath the lanes.

of hydroxyapatite optimized for high pressure (the so-called "ceramic" hydroxyapatite) yielded slightly different results from ordinary hydroxyapatite, which necessitated final polishing on a high pressure form of immobilized Cibacron Blue in Method 3, as opposed to the highly similar Method 1. The only step differing between the three-step Methods 3 and 4 was therefore reduced to the initial passage through a large immobilized Cibacron Blue dye column in Method 3, versus SSA precipitation in Method 4. As shown in Figure 3, this outcome was reproducible; three separate preparations from each method yielded electrophoretically indistinguishable preparations (Figures 3(a) and 3(b)), which reacted both with anti-AGP antibodies and SNA lectin (Figure 3(c)).

The qualitative assessment of the efficacy of Methods 3 and 4 shown in Figure 3 was consistent with the more quantitative data shown in Table 1, which used ELISA to quantify AGP levels in the protein mixtures at each stage of purification. The methods yielded similar levels of enrichment, of 90- to 110-fold, and similar yields, of 324 to 372 mg/L of plasma. Although Method 4 appeared to give slightly lower yields than Method 3, this difference may have arisen due to uncertainty in the ELISA or total protein determinations, since both methods yielded electrophoretically pure material, but differed slightly in specific activity (0.89 mg AGP/mg total protein versus the theoretically impossible 1.06 mg AGP/mg total protein).

The clearance of AGP purified by Method 3 and Method 4 was compared in vivo following injection into rabbits. Recoveries of the two forms of AGP did not differ significantly (49.8 ± 5.4% versus 44.8 ± 1.7%, $P = .262$, NS), allowing us to compare normalized plasma clearance curves. As shown in Figure 4(a), these were essentially superimposable. Where the mean curves diverged slightly (at days 1, 4, and 7), the mean residual AGP plasma concentration did not differ significantly. For instance, one day post-injection, residual Method 3 AGP was 23.8 ± 1.9% versus 21.2 ± 1.6 for Method 4 AGP (mean ± SD, $P = .092$, NS). Similarly, when circular dichroism, a conformation-sensitive biophysical method [34], was used to assess AGP purified by either of the methods, the spectra were similarly indistinguishable (Figure 4(b)). Finally, AGP purified by both methods yielded a single 20 kDa species when enzymatically deglycosylated with PNGase F, as was also the case with commercially obtained AGP (Figure 4(c)).

No difference was observed between AGP preparations in terms of the efficacy of endotoxin removal. Preparations contained significant levels of endotoxin (130–230 EU/mL) prior to being subjected to depyrogenation via Triton X-114 extraction, which reduced this load to 0.01–0.03 EU/mL. However, recovery varied widely, from 10.4 to 43.2% of initially extracted AGP, and appeared to correlate not with method of purification, but with initial AGP concentration.

4. Discussion

Purification of plasma proteins is often complicated by the abundance of serum albumin, which contributes up to 50% of the protein content of plasma, and the difficulty in completely separating this abundant component from the protein of interest [35]. Although methods of plasma fractionation have been established for many decades, frequently these methods are not appropriate for small research laboratories that lack access to industrial quantities of plasma. In this context, albumin removal can seriously constrain the time and complexity of plasma protein purification.

Flow-through chromatography on Cibacron Blue dye columns [35, 36] is an effective but limiting step in AGP purification, one that requires large columns (e.g., 4 mL of packed beads per mL of plasma) [29]. Initially, we sought to optimize AGP purification by substituting this step with a hot phenol extraction previously reported to purify AGP to homogeneity in a single step [23]. Although we did not obtain full purification in this single step, the enrichment was sufficient to allow purification to homogeneity with the addition of a hydroxyapatite polishing step. However, AGP purified by this procedure was more rapidly cleared from the murine circulation than chromatographically purified AGP not exposed to hot phenol. The clearance curves were most easily explained by their being two populations of AGP in the preparation: one, at least in part denatured and the other native in conformation [37]. This interpretation would predict that the first population was rapidly removed from

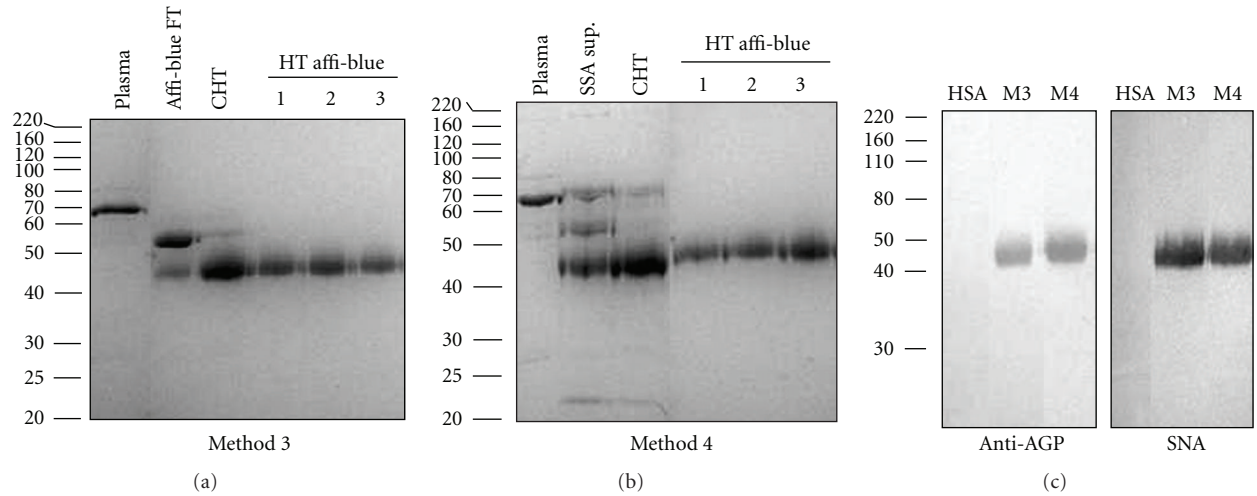

Figure 3: Comparison of AGP purified by solely chromatographic Method 3 or sulphosalicylic acid-incorporating Method 4. (a) and (b) show Coomassie-stained SDS reducing gels of equivalent (5 μg total protein/lane) samples of AGP preparations at intermediate steps of either Method of AGP purification: Affi-Blue FT, Cibacron Blue dye column flow-through, CHT, ceramic hydroxyapatite peak; HT Affi-Blue, HiTrap Cibacron Blue dye column flow-through; SSA Sup., supernatant following sulphosalicylic acid precipitation and neutralization. (c) depicts nitrocellulose blots of replicated gels containing 1.0 μg of purified human serum albumin (HSA) or of AGP purified by either Method 3 (M3) or Method 4 (M4) and probed either with Anti-AGP antibodies (at left) or Sambucus nigra agglutinin (SNA lectin) at left. The position of molecular mass markers and their mass in kDa is shown at left of each panel.

Table 1: Comparison of stepwise purity and yield of AGP purification methods.

	Total protein (mg)	AGP (mg)	AGP/total protein	Fold enrichment versus starting material
Method 3				
Plasma	1786	17.1	0.0096	1.0
Affi-Gel Blue FT	86.6	17.8	0.21	21
CHT Eluate	16.3	9.1	0.56	58
HiTrap Blue FT	8.7	9.3	1.06	110
Method 4				
Plasma	1786	17.7	0.0099	1.0
SSA Supernatant	43.3	13.3	0.31	31
CHT Eluate	20.8	7.8	0.38	38
HiTrap Blue FT	9.19	8.1	0.89	89

the circulation on initial passages prior to the first blood sampling, whereas the second was identical to chromatographically purified AGP. The parallel nature of the clearance curves supports this interpretation. Indeed, early investigators preconditioned radiolabeled protein by injection into a first animal, sampling of the blood, and its injection into a second animal to filter out denatured radiolabeled material [37]. Similarly, the presence of denatured protein has been deduced in clearance studies by competition with agents blocking reticuloendothelial uptake [38].

A reasonable hypothesis to account for the rapid clearance of the phenol-purified AGP was that it had lost terminal sialic acid residues and had therefore become a ligand for the high-affinity asialoglycoprotein, found predominantly in the liver [39]. However, phenol-purified AGP reacted as well, if not more avidly with Sambucus nigra agglutin in, a lectin specific for glycans terminating in sialic acid linked to galactose, than AGP purified under less harsh conditions. While it remains possible that a few sialic acid residues were lost per AGP molecule, a situation likely below the limits of detection of the technique we employed, our results in this regard eliminated the possibility of extensive desialylation.

Having established that the hot phenol procedure likely distorted AGP conformation to an unacceptable extent, we sought an alternative procedure that was rapid but nondenaturing. Like the hot phenol procedure, SSA treatment had been reported to precipitate all other plasma proteins save AGP, with the soluble, acidified AGP retaining the ability to bind ligands such as Auramine O [24, 25]. Perhaps due to improved electrophoretic methods, we detected additional polypeptides in the SSA supernatant, which were nonetheless removed in simpler, more rapid chromatographic steps than the large Cibacron Blue dye column. That this procedure (i.e., Method 4) was not only less limiting than the one involving the large column, but also produced a similarly native conformation of AGP was suggested not only by

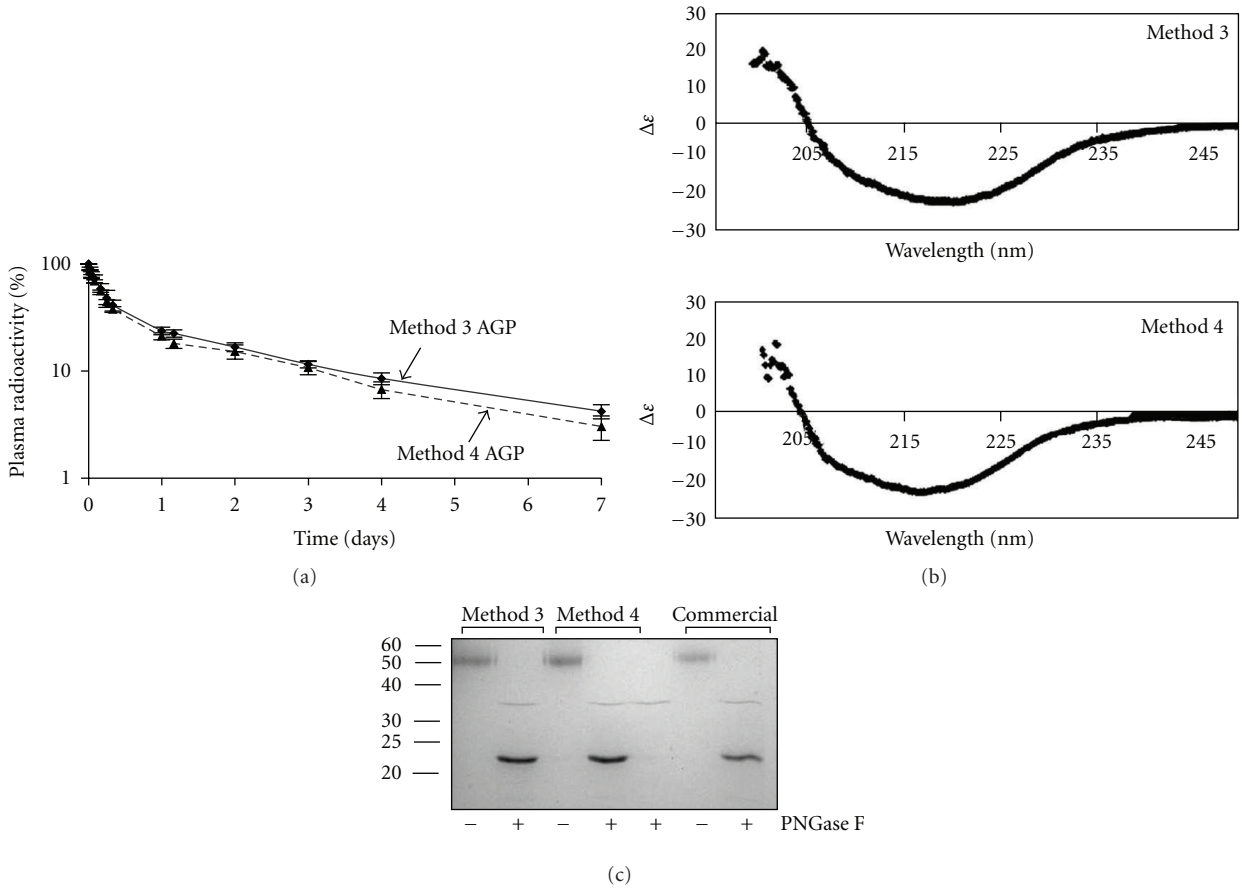

Figure 4: In vivo clearance, circular dichroism spectra, and deglycosylation of AGP purified by Methods 3 or 4. (a) shows residual, normalized acid-precipitable radioactivity in rabbit plasma following injection of radioiodinated AGP purified as indicated by arrowed text. (b) depicts circular dichroism spectra of AGP purified by Method 3 (upper) and Method 4 (lower); $\Delta\varepsilon$ represents the difference between the absorption by AGP of left-handed and right-handed circularly polarized light at different wavelengths. (c) shows samples of AGP purified by either method, with (+) or without (−) reaction with PNGase F; reaction of commercial AGP (Sigma) is also shown. Molecular mass markers are shown at left in kDa.

the identical in vivo clearance curves of radioiodinated preparations of AGP prepared by the two methods, but also by the identical circular dichroism spectra of the two preparations. The latter method is exquisitely sensitive to changes in protein secondary structure [34]. Not only did the two CD spectra match each other, they also closely resemble previously published AGP spectra, in particular with respect to the negative peak observed between 205 and 240 nm, and taking into account differences in CD methodology [40].

Methods 3 and 4 yielded similar quantities of AGP per mL of plasma, but the SSA step in Method 4 eliminated the large Cibacron Blue dye column without provoking denaturation of the glycoprotein. Other procedures involving acid conditions have been suggested to alter AGP conformation, but these involved much longer exposure of the protein to low pH (e.g., pH 3.85 for 16 hours) or exposure of AGP to not only acid but also oxidizing agents (e.g., perchloric acid) [21–26].

AGP in the circulation of most individuals is a combination of two isoforms produced by ORM1 and ORM2 genes, which produce AGP proteins of identical polypeptide length with similar glycosylation profiles, but which differ at up to 23 amino acid positions within the chains [41]. Although procedures have been described to separate these products, they require the use of a large Cibacron Blue dye column and buffers of pH less than 5.0 [42]. We did not address isoform separation in this study, both because of its reported use of a chromatographic step similar to the one we were trying to remove from the purification protocol, and because isoform separation has not been carried out in studies of AGP's putative anti-inflammatory properties in vivo.

Our results suggest that incorporation of the SSA step into an AGP purification protocol simplified the procedure while conserving the native conformation of the protein, as indicated by biophysical and in vivo approaches. This method should simplify the isolation of quantities of AGP sufficient to explore its putative anti-inflammatory properties in small animal models, without concerns that the native conformation of the protein has been altered. It should be noted that this study has limitations, especially with respect to biophysical methods. Our use of circular dichroism to compare the conformation of AGP preparations purified

using Methods 3 or 4 at best yielded a rough estimate that the conformations were similar. Others have performed much more rigorous studies of AGP structure using vacuum-ultraviolet CD in the presence of liposomes [40], monitored organogold complex binding by AGP using CD [43], and employed Raman and Fourier-transform infrared spectroscopy [44–46] to probe AGP structure.

In spite of this limitation, and of the lack of an enzymatic assay capable of reporting AGP conformation, our use of in vivo clearance to deduce a native conformation is a novel and relevant way of validating the SSA-incorporating purification protocol for future studies of AGP administration in animal models.

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