Isolation and Biochemical Characterization of α-2-Opsonic Glycoprotein from Rat Serum*

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The importance of a circulating α-2-globulin as a determinant of hepatic Kupffer cell phagocytosis of nonbacterial particulate matter has been well documented. This protein which manifests opsonic activity exerts a humoral control on hepatic reticuloendothelial phagocytic activity and deficiencies of its serum level exist with advanced malignant diseases or acutely following traumatic injury. The present study was designed to isolate this α-2-opsonic protein from rat serum by using a combination of gel filtration in 6 M urea on cross-linked Sepharose 6B with prior sequential steps of ammonium sulfate fractionation, high voltage preparative free flow electrophoresis, and conventional Sepharose 4B gel filtration. Immunochemical analysis utilizing nonabsorbed antiserum to the rat opsonic protein as well as polyacrylamide gel electrophoresis was used to evaluate the level of purification, which was in excess of 99%. The purified protein was selectively immunoreactive with non-specific antisera and its serum concentration was quantified by electroimmunoassay. The protein consists of two subunits held together by disulfide bonds and the minimum molecular weight as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 1.75 × 10⁶. Amino acid analysis revealed no detectable methionine and a high concentration of hydrophilic amino acid residues. Amino sugar analysis confirmed its classification as a glycoprotein with approximately 1.8% glucosamine and no galactosamine. The purification and biochemical analysis of this α-2-glycoprotein may provide insight as to its mechanism of action with respect to reticuloendothelial function.

The reticuloendothelial system (RES)† has been shown to

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† The abbreviations used are: RES, reticuloendothelial system, be involved in both specific and nonspecific aspects of host resistance to infection (1), neoplasia (2), and traumatic injury (3). The basis of this involvement in host defense centers around the recognition and phagocytic ability of RE cells. While the RES is diffuse in distribution, the major components are sessile RE cells in the liver and spleen which monitor the vascular compartment and remove circulating foreign and abnormal particulate matter. Among the substances removed from the blood by the RES, especially the hepatic Kupffer cells, are injured platelets and effete red blood cells, denatured proteins and fibrin aggregates, and a variety of inert and metabolizable test colloidal materials (4-7). Recently, several studies have indicated that RES phagocytic clearance of nonbacterial colloidal substances is conditioned, in part, by the opsonic ability of serum or plasma to support hepatic Kupffer cell phagocytic function (8-11). Depression of this clearance mechanism exists during advanced tumor growth (12, 13) and following traumatic as well as burn shock (3, 14) and is associated with an opsonic deficiency as detected by bioassay.

An understanding of the serum factor(s) that conditions RE cell phagocytic function may be an important step in the elucidation of the mechanism whereby the phagocytic system recognizes "self" from "nonself" or "self" from "altered self." While this recognition or discrimination mechanism with respect to bacterial phagocytosis has been shown to be related to complement as well as the immunoglobulin fraction of serum (15, 16), the recognition of circulating nonbacterial particulate and colloidal materials and their associated clearance appears modulated by a serum α-2-globulin (11, 13, 18). This α-2-globulin stimulates Kupffer cell colloid phagocytosis in vivo and modulates in vivo RES capacity especially during RES colloid blockade (6, 8, 17, 18).

Recently, we reported a high degree of purification of this rat serum α-2-globulin with retention of biological activity (18). Subsequent studies by McLain et al. (19) and Molnar et al. (20) have confirmed its existence with additional documentation that it is unrelated to the complement system. The present study was designed to achieve purification of this α-2-opsonic protein to antigenic purity from rat serum so that chemical and structural analysis with respect to molecular weight and amino acid composition could be accomplished.
MATERIALS AND METHODS

**Serum Collection** — Blood from normal rats was drawn via inferior vena cava from unfasted ether-anesthetized male Sprague-Dawley rats (200 to 300 g) using plastic syringes rinsed with 0.1 M EDTA to prevent clotting. The blood was centrifuged at 8000 x g at room temperature and then centrifuged at 5000 x g for 20 min at 4°C. Serum was obtained and maintained at 4°C for up to 2 weeks following fractionation (18).

**Ammonium Sulfate Precipitation** — The pooled serum was diluted with 1.6 volumes of ice-chilled 0.016 M phosphate in buffered 0.154 M NaCl, pH 7.4 (PBS), and 2-mercaptoethanol was added to a final concentration of 0.01% (v/v). Addition of 2-mercaptoethanol increased the yield of active protein isolated (18). At 4°C, 1.4 volumes of 100% saturated (NH₄)₂SO₄ (Schwarz/Mann, ultrapure) in PBS was added dropwise with stirring to the diluted chilled serum yielding a final 8% (NH₄)₂SO₄ saturation of 35%. After equilibration for 1.0 h at 4°C, the suspension was centrifuged at 8000 x g for 20 min at 4°C. Following decantation, the sediment was dissolved in cold distilled water containing 2-mercaptoethanol at a concentration of 0.01% (v/v) to a volume equal to the original volume of serum, and dialyzed overnight against 30 to 40 volumes of 0.053 M Tris/citrate buffer (0.01% 2-mercaptoethanol), pH 8.4, at 4°C.

**High Voltage Flow Electrophoresis** — The dialyzed opsonically active fraction was further fractionated using high voltage flow electrophoresis (Brinkmann Instruments, FF-1) as previously described (11, 17, 18). Electrophoresis was conducted at a range of 120 to 140 mA and 2600 V. The buffer flow rate was 115 ml/h and sample application rate was 0.5 ml/min. Following plotting of the elution profile, the α-2 globulin peak fractions were pooled and dialyzed against 25 volumes of 55% saturated (NH₄)₂SO₄ in PBS containing 0.01% 2-mercaptoethanol at 4°C for 6.5 h. The suspension was then centrifuged at 8000 x g for 20 min and the sedimented protein stored at −78°C under N₂.

**Gel Filtration on Sepharose 4B in 6 M Urea** — The combined (NH₄)₂SO₄-treated FF-1 fraction was dissolved in 6.0 ml of phosphate buffered saline containing 0.01% 2-mercaptoethanol. An aliquot was taken and diluted with phosphate-buffered saline to test for activity, and the rest was subjected to gel filtration and applied to a Sepharose 4B (Pharmacia) column (72 x 2 cm) previously equilibrated with phosphate-buffered saline (0.01% 2-mercaptoethanol, 0.01% NaCl). Dialysis was done by pumping at a rate of 15 ml/h and fractions of 4.4 ml were collected. Protein content was monitored by measuring the absorbance at 280 nm.

**Cystine-Cysteine Analysis** — Three equal aliquots of the major protein peaks were tested for opsonic activity and the active peak was dialyzed against 55% saturated (NH₄)₂SO₄ in PBS (0.01% 2-mercaptoethanol) for 6.5 h. The suspension was then centrifuged at 8000 x g for 20 min at 0°C. The supernatant was discarded and the precipitate was stored at −78°C. The α-2-globulin fraction was then carried through a manner identical with chromatography of the whole fraction to yield a relatively homogeneous peak with a purification level of 150-fold (18). The center of this active peak was virtually homogeneous as tested by gradient gel electrophoresis and used to immunize the rabbits in order to obtain antisera for the electromunnoassay (21).

**Gel Filtration on Sepharose 4B in 6 M Urea** — In order to achieve further purification of the biologically active protein fraction obtained by gel filtration on Sepharose 4B, the protein was precipitated by dialysis against 55% saturated (NH₄)₂SO₄ in PBS at 4°C for 6.5 h. The precipitate was dissolved in 2.0 ml of 6 M urea (ultrapure, Schwarz/Mann) in PBS. The sample was then applied to a column (1.5 x 100 cm) of Sepharose 4B-CL (cross-linked) which had been extensively equilibrated in 6 M urea at 25°C. The column was eluted by gravity flow at a rate of 0.8 ml/h under a pressure head of 30 cm of 6 M urea. Every two fractions (4.0 ml/fraction) were pooled and dialyzed at 4°C against PBS for 24 h with four changes of buffer to remove the urea from the samples. Aliquots of the dialyzed fractions were then tested for immunoreactive α-2-opsonic protein by electroimmunnoassay as previously described (21, 22) using absorbed monospecific antisera to the opsonic protein. The fractions were stored in plastic culture tubes at −20°C until analysis. Outlined in Fig. 1 is a composite presentation of the isolation procedure utilized in purification of the protein. The technique includes several specific and sensitive techniques and it is outlined in Fig. 1 in order to facilitate reproduction of these studies.

**SDS-Polyacrylamide Gel Electrophoresis** — SDS-polyacrylamide electrophoresis on 5.6% gels was used to determine minimum molecular weight in accordance with the technique developed by Fairbanks et al. (23). Gel electrophoresis on 3.3% gels was done following the procedure of Weber and Osborn (24). The experimental samples and molecular weight standards for electrophoresis on the 5.6% gels were handled in an identical manner. A 20-μl sample of the purified protein containing approximately 20 μg of protein (25) was added to 80 μl of reducing buffer made up of 1% SDS, 8 M urea, and 0.11 M 2-mercaptoethanol in Tris/acetate electrophoresis buffer (23). The samples were allowed to stand at room temperature overnight. Thereafter, each sample was supplemented with 10 μl of acrylonitrile and allowed to equilibrate at room temperature for 4 h. The treatment with acrylonitrile blocked any free sulfhydryl groups that were generated during the overnight reduction, thus preventing the reformation of disulfide bonds (26). Samples of opsonic protein not treated with 2-mercaptoethanol or acrylonitrile were also electrophoresed in order to detect any differences in mobility.

Since the opsonic protein solution was too dilute (0.154 mg/ml) for direct electrophoresis on 3.3% gels, it was necessary to concentrate the protein so that 10 to 15 μg could be applied to each well. Thus, the lyophilized opsonic protein is partially insoluble, SDS was added to the protein solution before lyophilization. In this procedure, each 1.5 ml of aliquot of the protein solutions (opsonin solutions and molecular weight standards) in plastic 1.5-ml microcentrifuge tubes (Walter Sarstedt, Inc., Princeton, N. J.) was supplemented with 5 μl of 20% (w/v) SDS. All samples were then frozen with a large surface area exposed as possible and lyophilized. The samples were dissolved in 50 μl of sample preparation solution containing 0.1 M sodium phosphate buffer at pH 7.2 which was 50% glycerol by volume and 0.05% (w/v) bromphenol blue (Bio-Rad Laboratories, Richmond, Calif.). To those samples which were to be reduced, 5 μl of 2-mercaptoethanol were added. When the lyophilized SDS-protein residue in each sample tube was completely dissolved, the samples were incubated for 6 to 30 min in a boiling water bath. To prevent the tops of the tubes from "popping off" during incubation, 25-gauge needles were pushed through the tops of the tubes to act as pressure valves. After the proper incubation time, the samples were removed from the hot water bath, allowed to cool, and applied to the gel using Pasteur pipettes. All of the analytical polyacrylamide gels were stained in a similar manner using the method developed by Fairbanks et al. (23). Gels were scanned with a model R-112 densitometer (Beckman Instruments).

**Amino Acid Analysis** — Amino acid analyses were carried out on a Beckman Model 121-A rapid amino acid analyzer modified by Adams (32) using a Hewlett Packard 5830 A gas chromatograph.
Fig. 1. Flow chart for sequential steps relative to the isolation and purification of \( \alpha-2 \)-opsonic protein from rat serum. The protein as isolated is called either opsonic protein, aspecific opsonin, or recognition factor.
Tryptophan Analysis—Due to its lability during acid hydrolysis, tryptophan was analyzed by a fluorometric method developed by Sasaki et al. (33).

A 0.400-ml aliquot of the opsonic protein in 0.072 m triethylammonium acetate, pH 8.0, was placed in a large capacity centrifuge tube and immersed in boiling water for 5 min to denature the protein. An identical volume of the buffer was treated in the same manner to act as a blank. When the solutions had cooled, 10 μl of a freshly prepared solution, containing 0.05% (w/v) each of chymotrypsin and pronase, were added to each tube. The mixtures were allowed to digest overnight at room temperature. The solutions were then diluted with 3.0 ml of 6 M urea in 0.072 m triethylammonium acetate buffer, pH 9.2, and 3.0 ml of these diluted solutions were transferred to quartz cells for fluorescence measurements.

Fluorescence measurements were made with an Amino-Bowman spectrofluorometer (American Instrument Co., Silver Spring, Md.). The excitation wavelength was 228 nm and the emission vs &s read sequentially in the solution in the cell, the solution mixed, and the fluorescence intensity read again after each 3-nmol addition. The amount of tryptophan in the opsonic protein was calculated by subtracting the X axis extrapolation from that extrapolated for the fluorescence intensity read again after each 3-nmol addition. The active opsonic fraction was isolated to antigenic purity by this immunochemical technique, while the antigenically heterogeneous fractions (Rockets 4 to 7) were retarded fractions eluting from the column. The possibility that the major rocket to the opsonic protein masks precipitin rockets of impurities is remote since the concentration of any contaminant and antibody to such contaminants would have to be exactly the same ratios as that of the opsonin and antipsonin, respectively. Additionally, any significant molecular species contaminating the opsonic protein should show a discrete band on SDS gel electrophoresis (Fig. 4) in the unredated state unless the contaminant also demonstrated heterogeneity in the absence of mercaptoethanol. "Rocket" electroimmunoassay was used rather than immunoelectrophoresis since the classic technique of immunoelectrophoresis was not as sensitive an index of immunological purity.

The homogenous 2nd and 3rd fractions were pooled, protein concentration determination, and confirmed to contain the opsonic protein by electroimmunoassay utilizing monospecific antiserum to the protein (21). The standard curve obtained with this protein was used to determine its concentration in normal serum and in aliquots used for amino acid analysis (Fig. 3). The relationship of rocket height (millimeters) to opsonic protein concentration is curvilinear and fits a rectangular hyperbola (21). A double reciprocal plot of the data points (1/rocket height versus 1/μg of opsonic protein) forms a straight line and was used as the standard curve in accordance with the electroimmunoassay method (21, 34). The cross-reactivity of the protein purified in 6 M urea on Sepharose 6B with the monospecific antiserum to this α-2-globulin identifies this protein as the rat serum opsonic protein and individual rockets were observed with both the isolated protein and with diluted (10%) normal rat serum. From the standard curve obtained with normal serum (1/rocket height versus 1/μg serum), the concentration of the protein in normal serum was determined to be 650 to 750 μg/ml.

With the demonstration that the opsonic protein was homogeneous, several modifications were made to the method of molecular weight was determined (Fig. 4). The active protein as isolated by gel filtration at 4°C has a molecular weight of approximately 800,000, which was confirmed by gradient polyacrylamide gel electrophoresis at the same temperature (18). As presented in Fig. 4, SDS gel electrophoresis of the opsonic protein identified in position 5 revealed the presence of a lower molecular weight contaminant, which was less than 1% (0.8%) of the total protein (Fig. 5) as detected by scanning the stained gels. Parallel electrophoresis of molecular weight standards is also presented in Fig. 4, positions 1 to 4. Position 1 shows the electrophoresis of IgG and it is evident that very little of this nonreduced protein entered the gel. Position 2 depicts the IgG standard. In positions 3 and 4,
the electrophoresis of bovine serum albumin is shown. Position 3 was overloaded in order to visualize the albumin trimer and tetramer to provide additional molecular weight standards.

Presented in Fig. 6 is the plot of the logarithm of molecular weight against relative electrophoretic migration of both the molecular weight standards and the opsonic protein samples in the 3.3% gel. The high molecular weight α-2-globulin protein is indicated by (×₁), while the lower molecular weight contaminant (1%) is indicated by (×₂) on the plot. The minimum molecular weight of the subunit of the opsonic protein molecule as calculated by linear regression is 227,000 to 228,000. The molecular weight of the contaminant, which could be a breakdown product of the major protein is approximately 120,000.

When the reduced S-cyanoethylated opsonic protein was electrophoresed in the presence of SDS on 5.6% acrylamide gels at pH 8.5 using a modification of the Fairbanks (23) procedure, results similar to the results on 3.3% gels were achieved, as indicated in Fig. 7. These data are not as valid since the molecular weight of the opsonic protein does not fall on the line between the standard proteins; however, when used for the calculation of the molecular weight, an estimate of 232,000 was calculated, which is within 5% of the results obtained on 3.3% acrylamide gels at pH 7.2. Therefore, the minimum molecular weight of the subunits of the opsonic protein molecule as approximated by these two systems was calculated to be approximately 229,000.

Since by gradient polyacrylamide gel electrophoresis, SDS gel electrophoresis, as well as electroimmunoassay, the opsonic protein was judged to be at least 99% homogeneous, an amino acid analysis of the opsonic protein was performed for further characterization. The complete amino acid analysis of

![Fig. 5. Results of the quantitative scan of the SDS gel electrophoresis of purified opsonic protein. The scan illustrates the relative concentration of the opsonic protein (migration distance = 3.3 cm) and the contaminant (migration distance = 11.8 cm). The scanning wavelength was 550 nm. Approximate molecular weights are indicated on the figure.](http://www.jbc.org/)

![Fig. 6. The relationship between molecular weight and relative migration of proteins with known molecular weights (×₁). The migration of the purified opsonic protein (×₂) and the smaller molecular weight contaminant (×₃) is shown. Gel concentration was 3.3% at pH 7.2.](http://www.jbc.org/)
this protein is presented in Table I in terms of nanomoles per fraction as a function of hydrolysis time and residues per M. = 229,000 subunit. The most striking features are its low methionine content and high concentration of hydrophilic amino acid residues.

The presence of 1.8% glucosamine identifies the opsonic protein as a glycoprotein. It appears that the linkage of asparagine to glucosamine since no galactosamine was detectable on the chromatogram.

**DISCUSSION**

Recently extensive documentation has appeared with respect to the role of a plasma α-2-globulin in both animals and humans as a determinant of hepatic Kupffer cell phagocytosis of nonbacterial particulate matter (12-14, 17, 18, 21). Previous studies resulting in the partial purification of this protein revealed that the biological activity of this α-2-globulin in terms of *in vitro* Kupffer cell phagocytosis is dependent on heparin and that the protein is of larger molecular weight (approximately 800,000 at 4°C) as determined by gel filtration. However, at 37°C, the activity elutes at a molecular weight of approximately 400,000, indicating that the 4°C species is an aggregated complex of the 37°C species (18, 20). It has been reported that the conversion from the 4°C complex to the biologically active 37°C species is not associated with a measurable change in conformation as detected by optical rotary dispersion (20).

The fact that most α-2-macroglobulins of serum are classically associated with serum proteinase inhibition or manifest some relationship to the complement system (or both) emphasizes the uniqueness of this α-2-opsonic protein, since it clearly lacks both of these characteristics. For example, recent studies by McLain et al. (19) indicate that this rat serum α-2-protein is independent of C₃, C₄, and C₅ components of the complement system and is not associated with serum proteinase inhibition (19). Additionally, it also appears by immunological techniques that the protein from rat serum is not related to the rat plasma α-2-macroglobulin acute phase protein (19, 20). While one might suggest that this protein resembles the human α-2-macroglobulin with respect to method of isolation, molecular weight, and stability, purification of the analogous human opsonic protein has recently demonstrated that there is no cross-reactivity with monospecific antisera to the human α-2-macroglobulin (36). Additionally, the serum levels of human α-2-macroglobulin are nearly 10-fold greater than the concentration of α-2-opsonic protein in both rat serum and human serum (21-36). Although the α-2-macroglobulin and the presently isolated α-2-opsonic protein have similar molecular weights at 4°C, the molecular weight of the α-2-opsonic protein at physiological temperatures is 400,000 (19, 21), which is approximately 50% of the molecular weight of the classical α-2-macroglobulin at comparable temperatures. Thus, these two proteins are distinct entities in both rat and man. Functionally, the dependence on heparin has been demonstrated to be involved in the ability of the opsonic protein to bind to particulates, although some evidence suggests that heparin may be involved in augmenting the direct phagocytic event (6, 17, 18, 20, 37, 38).

The present study demonstrates a high degree of purification of the rat α-2-opsonic protein as tested by immunochemical techniques. Excessive treatment of the protein with SDS and the reducing agent 2-mercaptoethanol failed to produce a significant quantity of a molecular species smaller than approximately 229,000 daltons, indicating either that the native subunit of this protein is a single polypeptide chain or that there are intermolecular disulfide bonds highly resistant to reduction by the methods used. The fact that relatively gentle treatment of the protein with SDS and 2-mercaptoethanol produced a sharp band on SDS gels, whereas the unreduced protein treated with SDS consistently yielded a diffuse migration band suggests the production of a heterogenous population. While the inclusion of a low concentration of 2-mercaptoethanol in all steps during the purification procedure helps to maintain the yield of biologically active protein, the
mechanism of this protection can only be speculated. This suggests the involvement of free sulfhydryl groups in the biological activity of this molecule.

The complete amino acid analysis of the purified α-2-opsonic protein reveals a protein with no detectable methionine, a fairly high hydroxy amino acid content, and a strikingly low lysine but fairly high arginine content. The presence of glucosamine in the analysis of the opsonic protein confirms its classification as a glycoprotein, but whether its biological activity is dependent on the carbohydrate moiety is still unknown. The fact that we were unable to produce a molecular species less than approximately 229,000 indicates that the active 400,000- to 450,000-dalton species is probably made up of two of the monomeric polypeptide subunits held together by disulfide bonds. The tryptophan content expressed in mole per cent of bovine serum albumin as reported by Peters and Hawn (39) is nearly 50% of the amount found in the opsonic protein, while the tyrosine content is approximately the same as in bovine serum albumin. This explains the difference in protein yields as measured by A280 measurements (tryptophan and tyrosine) as compared to the Lowry (25) method (tyrosine) based on albumin as a standard in both methods. Although there appears some similarity between the amino acid composition of the protein and human α-2-macroglobulin, these proteins are different as judged by immunological and molecular weight studies. Moreover, the methionine content of the classical α-2-macroglobulin is much greater than of the opsonic protein. Using the homogeneous protein as a standard for determining α-2-opsonic protein levels by electrophor- nous assay, we observed that in normal rat serum its concentration ranges between 650 and 750 μg/ml, which is distinctly less than the serum concentration of α-2-macroglobulin and also much greater than the acute phase reactant α-2-protein in normal serum.

The large molecular weight α-2-opsonic protein as isolated in this study appears to be a previously uncharacterized rat serum protein from a biochemical and structural standpoint. Purification of the analogous human serum protein by the techniques described in the present study has led to an analogous protein, which is nearly homogeneous as revealed by gradient polyacrylamide gel electrophoresis and manifests high biological activity as measured in vitro (36). Further biochemical characterization of the human opsonic protein will be needed to determine whether this protein has been described but has not yet been assigned a function.

With respect to functional activities, the findings to date emphasize an important role in nonspecific host defense, especially with respect to macrophage function during tumor growth (12, 13) and following traumatic shock (14, 40). Its blood level as detected by bioassay appears to be related to Kupffer cell phagocytic activity and depletion or exhaustion (or both) of this protein appears most pronounced during advanced malignant disease as well as acutely after trauma in humans (3, 13, 14, 40). The isolation of the analogous human protein (36) and the development of the electroneu- nosassay for the human protein will greatly facilitate the investigation of its biological significance and participation in disease processes.

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