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An Integrated Process for Separation of Major and Minor Proteins From Goat Serum

Sulakshana Jain and M. N. Gupta*

Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India, E-mail: munishwar48@yahoo.co.uk

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Abstract

Analysis of minor proteins in animal sera is of considerable clinical significance. To be able to detect these proteins, depletion of major proteins (albumin and immunoglobulin G [IgG]) is necessary. Many of these proteins are also required in pure form for a variety of biochemical applications. The present work uses goat serum as the system and describes the separation and purification of both major and several minor proteins. This was carried out by judicious adaptation and combination of separation technologies such as immobilized metal ion affinity chromatography (on a somewhat novel matrix), dye affinity chromatography, and lectin affinity chromatography. Albumin, IgG, α_2-macroglobulin, α_1-proteinase inhibitor, and transferrin were obtained from the serum. The purified preparations were found to be homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Index Entries: Albumin; immobilized metal ion affinity chromatography; IgG; α_1-proteinase inhibitor; α_2-macroglobulin; serum proteins.

Introduction

Analysis of proteins present in blood plasma/serum is reported to be one of the highest priorities of the Human Proteome Organization in view of clinical significance of such data *(1)*. One major difficulty in this analysis has been the wide range of concentrations in which various proteins are present. Human serum albumin constitutes 57 to 71% of total serum proteins and immunoglobulin G (IgG) is present in a range of 8 to 26%. Thus, protocols for removal of abundant proteins are necessary before one can

*Author to whom all correspondence and reprint requests should be addressed.

look at minor and low-abundance proteins. Such protocols should be specific and show promise for working across species. The great potential (for diagnostics) of such a protocol has led to many attempts at developing it (*[2–4]*; see also www.appliedbiosystems.com). The low-abundance proteins are very difficult to see on traditional two-dimensional electrophoretic gels unless abundant proteins are removed *(1)*.

The present work uses goat serum as a model and integrates various available approaches for purification of individual serum proteins (from sources other than goat serum) to evolve a process for both recovery and purification of individual serum proteins. Thus, it is not restricted to merely analysis but actual access to various serum proteins in reasonably purified form. This process recovers and purifies albumin, IgG, α_1-proteinase inhibitor, α_2-macroglobulin, and transferrin. The techniques used are immobilized metal ion affinity chromatography (IMAC) (without using a chelating ligand) *(5)*, dye affinity chromatography *(6)*, and lectin affinity chromatography *(7)*. The purity of the individual protein is assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods

Chemicals

Sodium alginate (low viscosity; cat. no. A-2158), epichlorohydrin, and Con A Sepharose were purchased from Sigma (St. Louis, MO). Sephadex G-200 was from Amersham Pharmacia Biotech AB (Sweden). Goat serum, chicken antigoat IgG peroxidase conjugate, tetramethylbenzidine–hydrogen peroxide, and pure IgG (from goat serum) were from Bangalore Genie (Bangalore, India). Seralose 4B was from Sisco Research Laboratory (Mumbai, India). All other chemicals were of analytical grade.

Estimation of IgG

IgG was estimated by its capacity to bind the antigoat IgG in an enzyme-linked immunosorbent assay (ELISA) using tetramethylbenzidine–hydrogen peroxide as a substrate *(8)*.

Estimation of Trypsin Inhibitory Activity

Trypsin inhibitory activity of α-protease inhibitor was measured as a degree of inhibition of trypsin activity. The latter was assayed according to the method of Erlanger et al. *(9)*. One unit of inhibitor activity is taken as the amount of inhibitor required to completely inhibit the activity of 1 μg of trypsin *(10)*.

Estimation of Protein

Protein was estimated by the dye-binding method using bovine serum albumin as the standard protein *(11)*.

Preparation of Copper (II)-Charged Crosslinked Alginate Beads

Beads were prepared by a procedure outlined by Jain and Gupta *(5)*.

Polyacrylamide Gel Electrophoresis

SDS-PAGE of the samples using 12% gel was performed according to Laemmli *(12)* on a Genie gel electrophoresis unit (Bangalore Genie).

Immobilized Metal Ion Affinity Chromatography

Buffer A (0.05 *M* Tris-HCl buffer, pH 7.0) was pumped through a bed of Cu-alginate beads (bed volume of 10.0 mL). The height of the sedimented adsorbent bed was 10.0 cm. Goat serum diluted in buffer A containing 6.8 mg of proteins was applied onto the column. The column was washed with buffer A and eluted with an EDTA gradient (0–0.3 *M*) in buffer A.

Dye Affinity Chromatography for Purification of Albumin

Buffer B (phosphate-buffered saline) was pumped through a bed of Cibacron blue-Seralose (bed volume of 10.0 mL) at a flow rate of 0.4 mL/min. Three milliliters of peak 3 containing 5.2 mg of protein was applied onto the column. The column was washed with buffer B and eluted with 1 *M* NaCl in buffer B. All elutions were carried out at a flow rate of 0.4 mL/min. Fractions of 4 mL were collected.

Con A Affinity Chromatography for Purification of α_1-Proteinase Inhibitor

Chromatography was performed on a 1.1 × 4 cm glass column (bed volume of 2 mL) using buffer C (0.02 *M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl) as equilibration buffer. Peak 1 (0.5 mL) containing 0.64 mg of protein was applied onto the column at a flow rate of 0.3 mL/min. The column was washed with buffer C and eluted with a mannose gradient (0–0.5 *M*) in buffer C. All elutions were carried out at a flow rate of 0.3 mL/min. Fractions of 3 mL were collected.

Dye Affinity Chromatography for Purification of Transferrin

Chromatography was performed on a 1.1 × 4 cm glass column (bed volume of 2 mL) using buffer D (1.54 m*M* sodium azide, pH 7.1) as equilibration buffer. From peak 1 (Fig. 5) 0.5 mL containing 0.3 mg of protein was applied on the cibacron blue seralose column at a flow rate of 0.3 mL/min. The column was washed with buffer D and eluted with 0.75 *M* KH_2PO_4/Na_2HPO_4, pH 7.1, in buffer D. All elutions were carried out at a flow rate of 0.3 mL/min. Fractions of 3 mL were collected.

Gel Filtration Chromatography for Purification of α_2-Proteinase Inhibitor

Chromatography was performed on a 2 × 50 cm glass column using 0.02 *M* Tris-HCl, pH 7.0. Peak 1 (0.5 mL containing 0.4 mg of protein) was

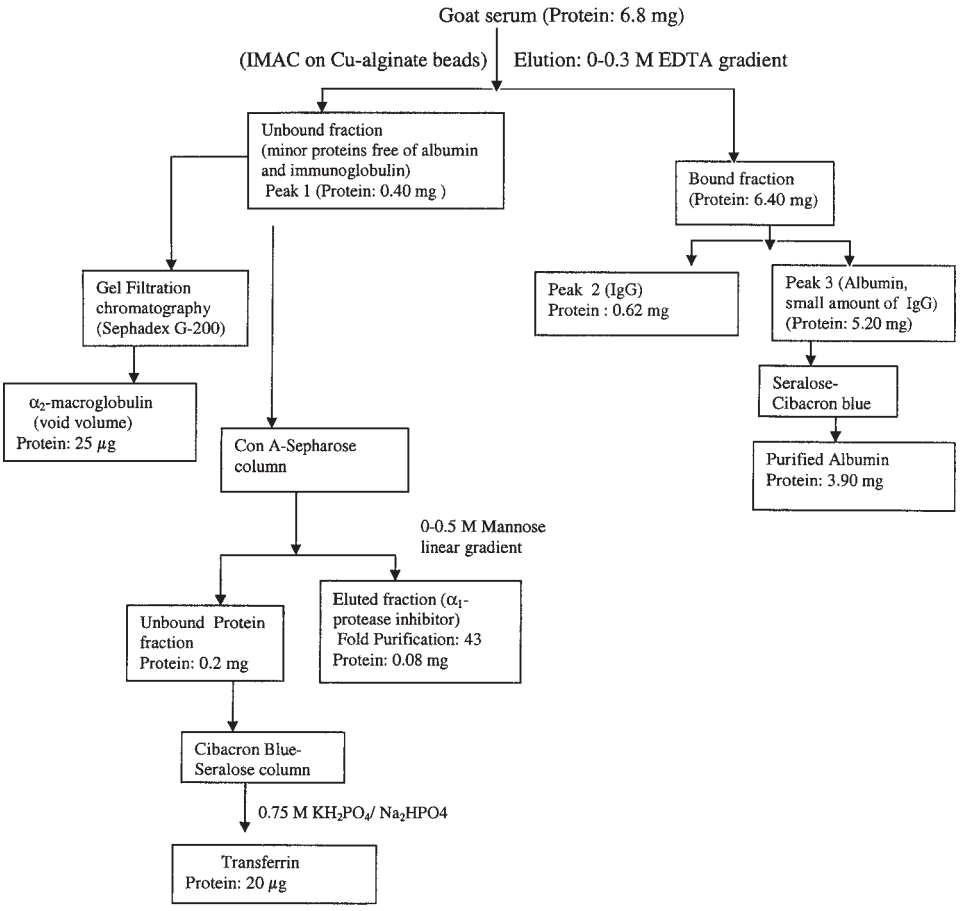

Fig. 1. Flow sheet of fractionation and purification of protein from goat serum.

applied on a Sephadex G-200 column (bed volume of 140 mL) at a flow rate of 0.275 mL/min. Fractions of 2.75 mL were collected.

Results and Discussion

Figure 1 presents a flow sheet of strategies used for the recovery and purification of major and minor proteins present in blood serum. The first step was the purification of IgG. IMAC of diluted goat serum on copper-charged crosslinked alginate beads (Fig. 2) eluted IgG in pure form (peak 2). The method used was a modified form of one reported recently *(5)* in which crosslinked alginate beads with 94 μmol/mL of Cu^{2+} (bound to alginate) were used. Here, crosslinked alginate beads were charged with 188 μmol/mL of Cu^{2+}. This charging to a higher concentration of Cu^{2+} ensured that the albumin in the sample bound quantitatively. The fold purification was 6.8 (as estimated by ELISA using chicken antigoat IgG peroxidase conjugate) with 62.4% recovery. Albumin was purified from peak 3 (Fig. 2) using a

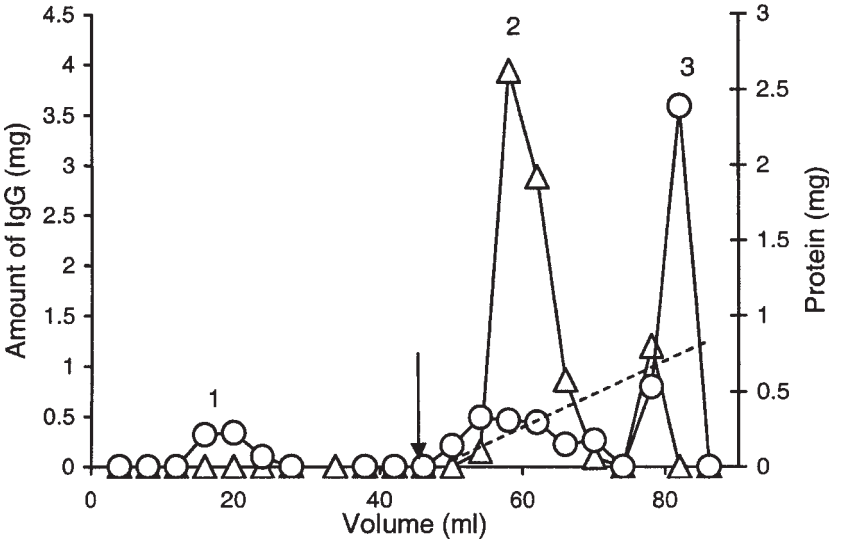

Fig. 2. Chromatography of goat serum on copper-charged crosslinked alginate beads. Chromatography was performed as described in Materials and Methods. The arrow indicates the start of the eluent. The dashed line represents the linear gradient (0–0.3 *M* EDTA) of the eluent. (△) Amount of IgG; (○) protein.

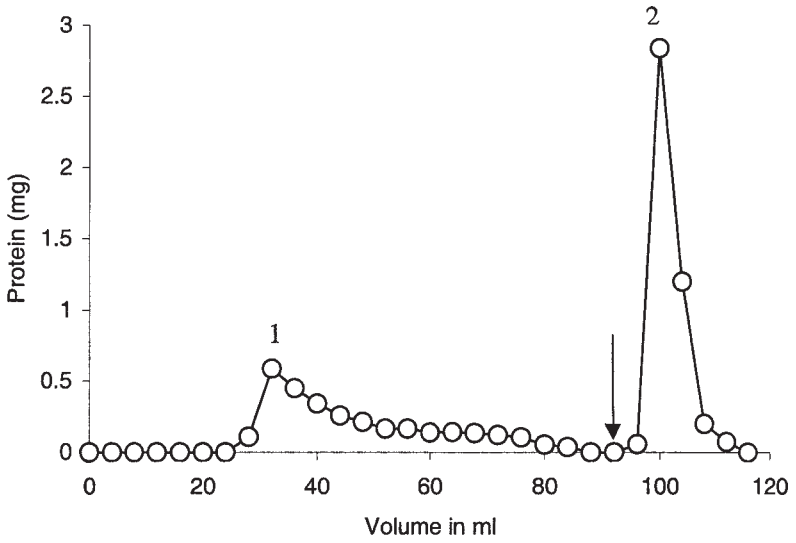

Fig. 3. Chromatography of peak 3 (Fig. 2) on Cibacron blue-Seralose 4B column. Chromatography was performed as described in Materials and Methods. The arrow indicates the start of the eluent.

cibacron blue-agarose column *(2,13)* (Fig. 3). Mahany et al. *(13)* found that relative affinities of albumin for the dye affinity column (blue-Sepharose) were human = baboon > guinea pig = rat. Travis et al. *(2)* reported that a cibacron blue–Sepharose column removed about 98% of the albumin from

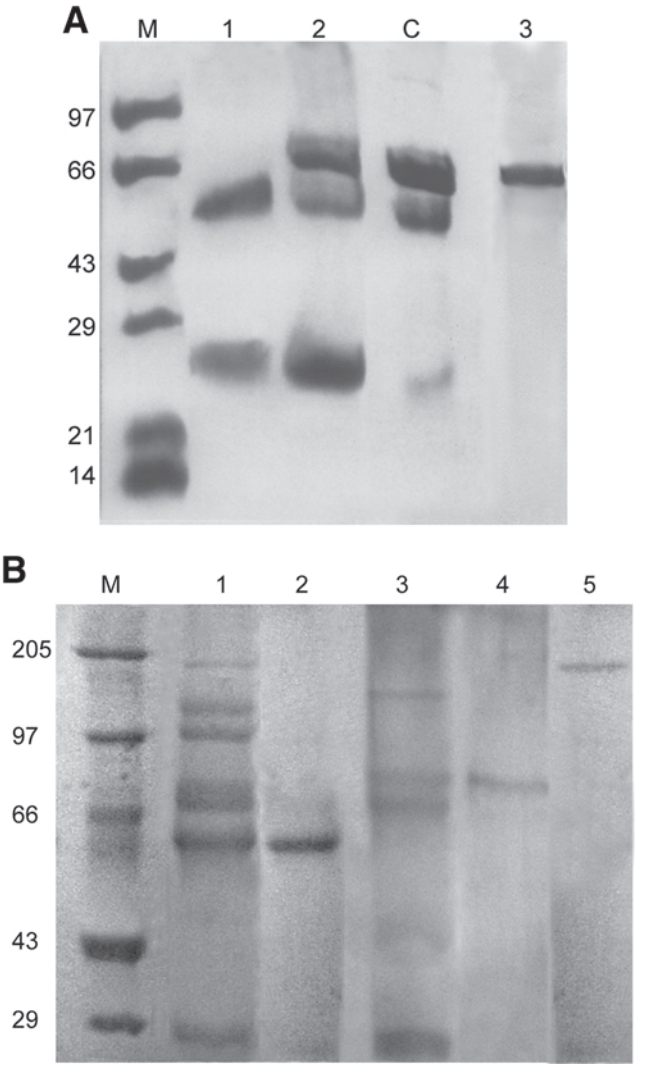

Fig. 4. **(A)** SDS-PAGE analysis. Electrophoresis was carried out using a 12% cross-linked polyacrylamide gel. Lane M, marker proteins; lane C, crude serum; lane 1, peak 2 (Fig. 2); lane 2, peak 3 (Fig. 2); lane 3, peak 2 (Fig. 3). The same amount of protein (25 µg) was applied in each lane. **(B)** Electrophoresis was carried out using a 7% cross-linked polyacrylamide gel. Lane M, marker proteins; lane 1, peak 1 (Fig. 2); lane 2, peak 2 (Fig. 5); lane 3, peak 1 (Fig. 5); lane 4, peak 2 (Fig. 6); lane 5, peak 1 (Fig. 7). The same amount of protein (5 µg) was applied in each lane.

the whole human serum. In the present study, 99% of the albumin present in peak 3 bound to the Cibacron blue-agarose column. In addition, the elution was also of a higher level (~95%). IgG purified as just discussed gave two bands on SDS-PAGE (Fig. 4A) corresponding to a molecular weight of light and heavy chains *(5)*. The purified albumin gave a single band on SDS-PAGE (Fig. 4A) corresponding to a mol wt of 67 kDa, which

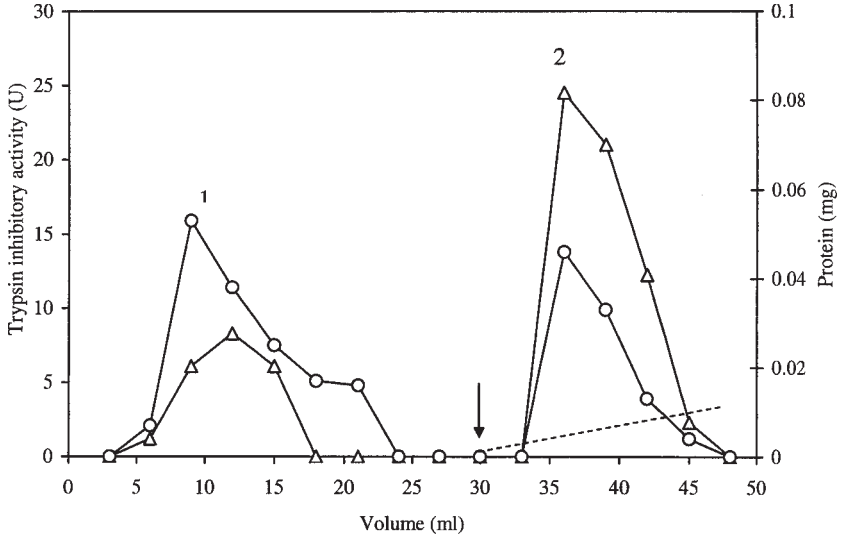

Fig. 5. Chromatography of peak 1 (Fig. 3) on Con A–Sepharose column. Chromatography was performed as described in Materials and Methods. The arrow indicates the start of the eluent. The dashed line represents the linear gradient of the eluent. (△) Trypsin inhibitory activity; (○) protein.

agrees with the earlier reported molecular weight (see www.applied biosystems.com).

The small amount of protein (peak 1 in Fig. 2) that did not bind to the IMAC column contained other minor proteins that were visible on SDS-PAGE after removal ("depletion") of the two major proteins (Fig. 4B).

The peak 1 (Fig. 2) material containing minor proteins was chromatographed on Con A–Sepharose columns. α_1-Proteinase inhibitor is known to be a glycoprotein and is reported to bind to a Con A affinity column *(10)*. Figure 5 shows that peak 1 material resolved into unbound and bound fractions. The latter eluted with a mannose gradient gave α_1-proteinase inhibitor. Table 1 shows that the inhibitor could be purified 43.3-fold with a recovery of 50.9%. Elution with a higher concentration of mannose or α-methyl mannoside (up to 1 *M*) failed to elute any more protein. It has also been reported that the recovery of this inhibitor from the serum as a stand-alone purification target has been much less *(10)*.

The unbound fraction of Con A–Sepharose column (Fig. 5) was next subjected to dye agarose affinity chromatography (Fig. 6) to obtain pure transferrin (Fig. 4B, lane 4).

One minor protein that was not recovered in the above protocol was α_2-macroglobulin. Presumably, this glycoprotein *(14)* bound to the Con A column too tightly and was lost since it could not be eluted. However, this high molecular weight protein could be recovered from peak 1 material of the IMAC column (Fig. 2) by gel filtration on Sephadex G-200 in void volume (Fig. 7). SDS-PAGE analysis of this oligomeric protein gave a subunit

Table 1
Purification of α_1-Proteinase Inhibitor From Peak 1 (Fig. 1)
on Con A–Sepharose Column

Step	Trypsin inhibitory activity (U)	Protein (mg)	Activity yield (%)	Specific activity (U/mg)	Fold purification
Crude serum	222	6.8	100	32.6	1
IMAC column peak 1	162	0.4	73	405	12.4
ConA–Sepharose column (peak 2)	113	0.08	50.9	1412.5	43.3

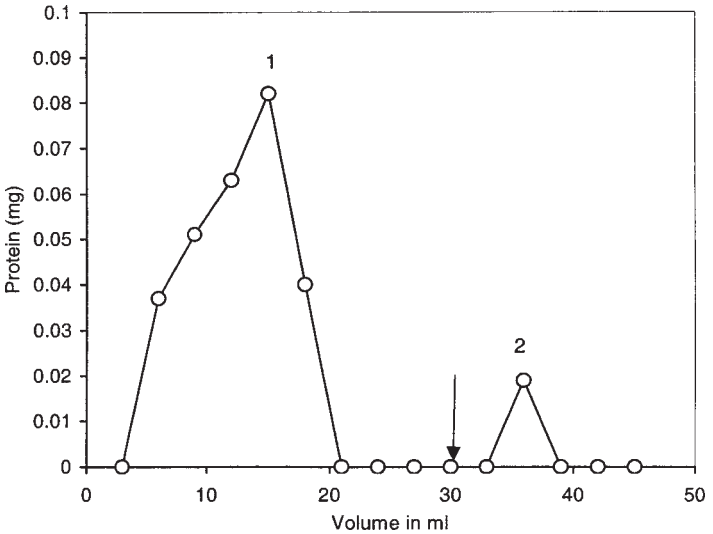

Fig. 6. Chromatography of peak 1 (Fig. 5) on Cibacron blue-Seralose 4B. Chromatography was performed as described in Materials and Methods. The arrow indicates the start of the eluent.

mol wt of 180 kDa, which is in agreement with the earlier reported value *(14)*. The single band observed on SDS-PAGE reflected the successful purification of α-macroglobulin. In principle, it should be possible to separate this protein out by this step before going ahead with isolation and purification of other minor proteins. The other peaks obtained during the gel filtration (Fig. 7) showed considerable impurity. For example, transferrin was present in peak 4 but was contaminated with α-proteinase inhibitor and an approx 110-kDa protein. Similarly, α-proteinase inhibitor was present in peak 5 but was contaminated with a 50-kDa protein. These data were based on SDS-PAGE analysis of peak 4 and 5 material (data not shown). These results show that by judicious combination of various puri-

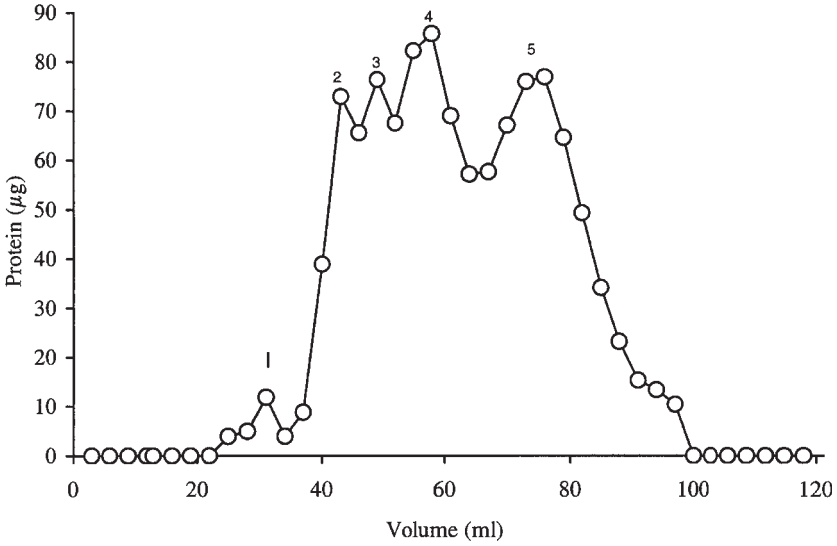

Fig. 7. Chromatography of peak 1 (Fig. 2) on Sephadex G-200 column.

fication steps, albumin and IgG (major proteins) and some minor proteins, such as α_2-macroglobulin, α_1-proteinase inhibitor, and transferrin, could be recovered.

At present, the analysis and detection of serum proteins (after albumin depletion) is being actively pursued (*[3–5,15]*; *see also* www.applied biosystems.com). Immunodepletion techniques are now available commercially (*[16]*; see also www.appliedbiosystems.com). If a minor protein is to be used as a marker (for a given situation), eventually its characterization would be extremely useful. The present work shows that it should be possible to do so by strategic combination of techniques. All the techniques used in the present work are chromatographic techniques. Hence, it would be possible to use such strategy in high-performance liquid chromatography format and with a robotic platform. Finally, it should be emphasized that simultaneous purification of these proteins has well-known applications. Serum albumin is a major therapeutic product in the case of human serum albumin *(17)*. α_2-Macroglobulin is important in the inhibition and removal of potentially harmful proteases of both endogenous and exogenous origin *(14)*. IgG, of course, finds applications in diagnostics *(18)*, drug targeting *(19)*, enzyme immobilization *(20)*, and biosensor design *(21)*. Finally, as discussed by Zolg and Langen *(22)*, such protein separation protocols may be valuable for obtaining diagnostic markers and biomarkers for the diagnostic and pharmaceutical industries.

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