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SEMI-AUTOMATIC ANALYSIS OF PROTEINS AND PROTEIN COMPLEXES BY AUTOMATED ENZYME IMMUNO ASSAY AFTER SEPARATION BY HIGH-PERFORMANCE GEL-PERMEATION CHROMATOGRAPHY

SIZE DISTRIBUTION OF C3–IgG COMPLEXES

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SUMMARY

Enzyme immunoassay (ELISA) was applied in the establishment of an immunological method for the characterization of column effluents. Serum samples, containing aggregated immunoglobulin (IgG), were separated by high-performance gel-permeation chromatography on a TSK column and fractions were collected directly in 96-well ELISA microplates. IgG was determined on anti-IgG-coated plates, followed by development with biotin-labelled anti-IgG and enzyme-labelled avidin. Complement factor C3 was determined on anti-C3-coated plates, developed with biotin-labelled anti-C3 and enzyme-labelled avidin. Complexes between IgG and complement factor C3 were determined on anti-IgG-coated plates, developed with biotin labelled anti-C3 antibody and enzyme-labelled avidin. Complex formation between C3 and IgG after incubation of serum with aggregated IgG was demonstrated. The methodology described is generally applicable to the analysis of biological components and is uniquely useful for the analysis of complexes between different components.

INTRODUCTION

Recent technical developments have extended the use of high-performance liquid chromatography (HPLC) to the analysis and purification of proteins[1,2]. The development of methods for automated post-column analysis of fractionated samples is important in the exploitation of the capabilities of HPLC. Enzyme immuno assay (or ELISA for "enzyme-linked immunosorbent assay"), carried out in 96-well microplates[3], is well-suited to mechanization. We wish to report on the practicability of combining HPLC with ELISA in order to introduce characterization of column effluents by immunological techniques.

Our primary aim was the establishment of methods for the characterization and quantification of humoral immune complexes[4], but the methods developed are applicable to any substance for which antibody can be raised.

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MATERIALS AND METHODS

Reagents

Phosphate-buffered saline (PBS): 0.14 *M* sodium chloride, 10 m*M* phosphate, pH 7.4. PBS–Tween: 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, Merck-Schuchardt, F.R.G.) in PBS. Gelatin buffer: 0.5% (w/v) gelatin (Bacto Gelatin, 0143-01, Difco, Detroit, MI, U.S.A.), 0.5 *M* sodium chloride, 10 m*M* phosphate, 1 m*M* magnesium chloride, pH 7.4[5]. Phosphate–citrate buffer: 0.1 *M* phosphate, 0.2 *M* citrate, pH 5.0. Ethylenediaminetetraacetic acid (EDTA) at 0.4 *M*, pH 7.4.

Pepsin (no. P-7012) and bovine serum albumin (BSA, no. A-7906) were obtained from Sigma (St. Louis, MO, U.S.A.). Avidin-peroxidase from Sigma (freeze-dried, code A 3151, lot 72F-3930) was solubilized at 0.5 mg/ml of PBS, containing 1% (w/v) BSA and 0.01% (w/v) merthiolate, and kept at 4°C. Serum was kept frozen in aliquots at −70°C. The cell-free hemolymph from locally collected *Helix pomatia* was the source of hemocyanin. Poliovirus virions were obtained from Grace Glikmann, Statens Serum Institut, Copenhagen.

Antibodies

Rabbit anti-human C3c (code A 062, lot 109E), normal rabbit IgG (code x903, lot 0488) and rabbit anti-human Fcγ (code A 089, lot 069D) were obtained as the IgG fractions, purified from the corresponding antisera by Dakopatts (Copenhagen, Denmark).

Pepsin digestion

The $F(ab')_2$ fragments of the different rabbit IgG preparations were attached to the solid phase in ELISA. The fragments were prepared by pepsin digestion at pH 4.5[6] and Sephacryl S-300 gel chromatography.

Biotin labelling of antibodies

Specific rabbit immunoglobulins (1–2 mg/ml) were dialysed against PBS, adjusted to pH 8.5 with 5% (w/v) sodium carbonate. Biotin-N-hydroxysuccinimid ester (BNHS) (no. H 1759, Sigma) was dissolved at 1 mg/ml in dimethylsulphoxide and added to the protein solution in the porportion of 1 mg of BNHS per 6 mg of protein. The mixture was incubated for 4 h at room temperature and dialysed against PBS. The method is a modification of previously published methods[7,8].

Chromatography

All samples were centrifuged in 300-μl polyethylene micro test tubes (Milian Instruments, Geneva, Switzerland) for 3 min at 10^4 *g* in a Beckman microfuge (Beckman-Spinco, Palo Alto, CA, U.S.A.) before inserting the test tubes in Waters sample processor holders. Generally, 20 μl of sample (serum, final dilution 1:20) was injected with a Waters Intelligent Sample Processor (WISP™, Waters Assoc., Milford, MA, U.S.A.) and the samples were pumped by means of a Waters 6000 A solvent delivery system through a precolumn (75 × 7.5 mm I.D.) and a size separation column (TSK G 6000 PW 600 × 7.5 mm I.D., Toyo Soda, Tonda, Japan) at a rate of 1 ml/min. This column is stated to be capable of separating molecules of an M_r below 3×10^7 (Technical Data Booklet, Toyo Soda). The buffer was 100 m*M* sodium sulphate, 20

m*M* phosphate, 7.5 m*M* sodium azide, pH 6.8. Chromatography was monitored at 280 nm on a spectrophotometer (Cecil, Cambridge, U.K.), equipped with a 10-mm, 8-μl flow cell set at $E_{max} = 0.1$ with automatic switch to $E_{max} = 1.0$ when maximum deflection is reached.

Fractions of 250 μl were collected directly on antibody-coated microplates, (with 96 wells of 300 μl, see the ELISA section below) which were placed in a specially constructed microplate fraction collector (Skatron A/S, Lier, Norway). Before use, each well received 25 μl of PBS, containing 1% Tween and 5.0% gelatin. Alternatively, when the experimental design called for the analysis of each fraction in various assays (sample, 20 μl of serum diluted 1:2), they were collected on polystyrene microplates (Nunc, Kamstrup, Denmark), which were pretreated, to minimize protein adsorption, by incubation with non-ionic detergent (PBS–Tween) for 2 h followed by three washings with PBS–Tween. A twelve-channel dispensing–suction device designed for the harvesting of cells from microplates was used for the washing (Cell-harvester, Skatron, Lier, Norway). Instruments for automatic washing are available (Dynatech or Skatron).

Heat-aggregated gammaglobulin

A solution containing 10 mg of human IgG (Kabi, Sweden) per ml of PBS was incubated for 30 min at 63°C, and after centrifugation for 15 min at 2000 *g*, the supernatant was used. The protein concentration was determined from $E^{1\%}_{280nm} = 13.5$ after diluting a sample with 9 volumes of 1% (w/v) sodium dodecyl sulphate (SDS), in order to avoid interference from light scattering.

Complement-mediated solubilization

A 500-μl volume of aggregated IgG at 1 mg/ml of PBS was mixed with 500 μl of serum, or as a control, with 500 μl of serum containing 10% (v/v) 0.4 *M* EDTA to inhibit complement activation. After incubation for 30 min at 37°C, 50 μl of 0.4 *M* EDTA were added to the first sample in order to stop the complement-mediated solubilization. As a further control, 500 μl of the same serum were diluted with 500 μl of PBS without aggregated IgG and incubated for 30 min at 37°C, after which 50 μl of 0.4 *M* EDTA were added.

ELISA

Preparation of antibody-coated microplates: aliquots of 200 μl 0.1 *M* sodium carbonate buffer, pH 9.6, containing the antibody, were dispensed into the 96 wells of a specially treated polystyrene microplate (Immunoplate II, Nunc). To avoid interactions with rheuma factors, the $F(ab')_2$ fragment of rabbit antibody was used. Preliminary experiments showed 2.5 μg of $F(ab')_2$ per ml to be suitable. Pipetting was done with an eight-channel micropipette (Flow Laboratories, Irvine, U.K.). Automatic 96-channel pipetting machines are available (Costar, Dynatech or Skatron). After incubation overnight at room temperature in a humidified box, the wells were emptied by inversion and washed three times with PBS–Tween. The plate was now ready for assay, but it could be stored with PBS in the cold or dried and stored in a dessicated atmosphere. The coated plates were either used directly for fraction collection or, for multiple assays of a single fractionation, each well received 180 μl of gelatin buffer and then 20 μl of each fraction of the serum, separated by HPLC.

The plates were now incubated overnight at room temperature in a humidified atmosphere. The next day, the plates were washed three times with PBS–Tween, and 200 μl of biotin-labelled antibody was added. Based on preliminary experiments, the biotinylated antibodies were used at the following dilutions in PBS–Tween: anti-Fc, 1;4000 and anti-C3c, 1:1000. The plates were incubated for 2 h at room temperature followed by three washings with PBS–Tween. The plates were then incubated for 1 h at room temperature with 200 μl of avidin-peroxidase, diluted 1:2000 in PBS–Tween. After washing the enzyme activity remaining in the wells was measured by adding 200 μl of *o*-phenylenediamine at 0.4 mg/ml of phosphate–citrate buffer and then 0.4 μl of 35% hydrogen peroxide per ml. The absorbance of the 96 wells at 405 nm was read by means of a multichannel spectrophotometer (Titertek, Flow Laboratories) after incubation of the plates for 60 min at 37°C.

In summary, the HPLC–ELISA procedure involves the following steps: Coating of microplates with antibody; washing and storage; collection of HPLC fractions; incubation; washing; incubation with biotin-labelled antibody; washing; incubation with enzyme-labelled avidin; washing; addition of substrate; development; reading of absorption.

The assays performed as described have a dynamic range of about 1–100 ng of protein per ml. However, the range may be adjusted as required by altering the concentrations of the various reagents. The total time for the analysis may be reduced to about 4 h if a high sensitivity is not required. The possible carcinogenicity of peroxidase substrates may be avoided by using conjugates with alkaline phosphatase or β-galactosidase.

Microplate fraction collector

Essential for the effectual combination of HPLC and ELISA was the construction of a suitable fraction collector. The fraction collector, now manufactured by Skatron A/S (Lier, Norway), accommodates 4 microplates, each with 96 wells of 300 μl, or 4 × 96 test tubes of about 1 ml, placed in the microplate pattern. It is provided with a rapid switch mechanism (50 msec) to meet the requirements posed by the high flow-rates of HPLC.

As nozzle, a PTFE tube is used (0.1 mm I.D., 1.0 mm O.D.), drawn out into a fine tip by mechanical stretching over a hot-air blower. This ensures the delivery of the effluent in a steady stream and thus collection of fractions of identical volume.

The effluent passes through a three-way solenoid valve (PSV-3, Pharmacia) which allows easy switching from collecting system to waste. The dead volume of the switch was reduced from about 20 μl to less than 5 μl by passing the PTFE tubing right to the core of the switch. To do this the tubing must be slightly drawn out, a piece of a silicone tubing being used as a washer.

RESULTS

Separation characteristics of the high-performance gel-permeation chromatography (HPLC–GPC) column

The separation characteristics of the TSK G 6000 PW column were determined with the following markers (mol. wt. in parenthesis): hemocyanin ($8.9 \cdot 10^6$)[9], poliovirus virions ($5.5 \cdot 10^6$)[10], bovine thyroglobulin ($6.7 \cdot 10^5$)[11], human IgG ($1.5 \cdot 10^5$),

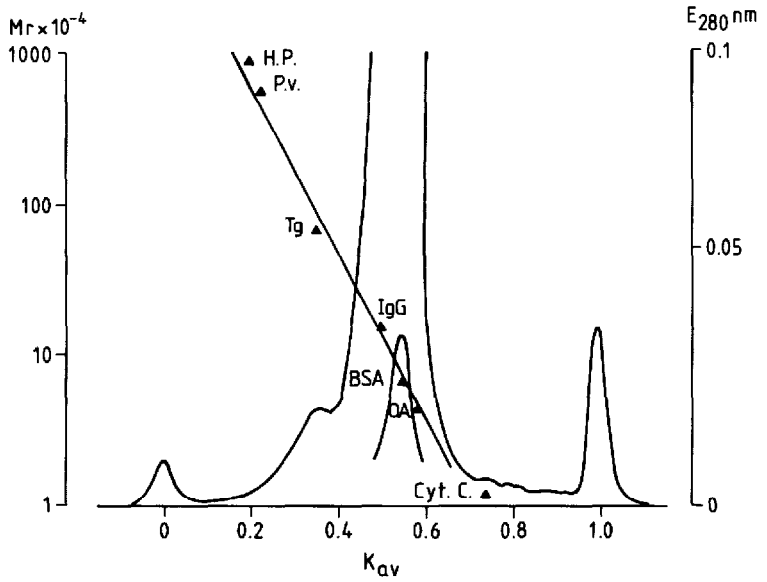

Fig. 1. Calibration of the TSK G 6000 PW column. The elution profile of serum proteins (10 μl of serum) is shown together with the V_e for *Helix pomatia* hemocyanin (H.P.), poliovirus virions (P.v.), thyroglobulin (Tg), IgG, albumin (BSA), ovalbumin (OA) and cytochrome *c* (Cyt c).

bovine serum albumin ($6.7 \cdot 10^4$), ovalbumin ($4.4 \cdot 10^4$), cytochrome C ($1.2 \cdot 10^4$). The proteins and virus were eluted as single, symmetrical peaks. Plots of log M_r against K_{av} fit reasonably well with a straight line (Fig. 1).

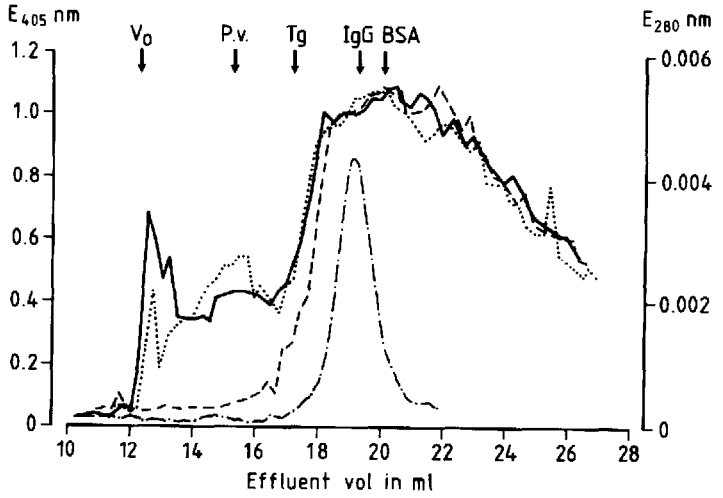

Fig. 2. Estimation by ELISA of IgG in serum fractionated by HPLC. (—) IgG in serum without added aggregated IgG; (...) IgG in serum to which was added aggregated IgG and EDTA before incubation at 37°C and fractionation; (——) IgG in serum incubated with aggregated IgG before fractionation. Also shown is the elution of 5 μg purified monomeric IgG estimated by E_{280} (—.—).

HPLC–GPC combined with ELISA for IgG

Fractions were collected on microplates coated with anti-IgG antibody, followed by development with biotinylated anti-IgG.

The elution pattern of IgG in serum with or without pre-incubation with aggregated IgG is shown in Fig. 2. No high-molecular-weight IgG is seen when serum without aggregated IgG was analysed.

When aggregated IgG was added to serum, high-molecular-weight IgG appeared with a broad distribution from the V_0 (void volume) to the position of monomeric IgG. Incubation in serum without EDTA, *i.e.* under complement-activating conditions, induced a shift in the distribution of aggregated IgG towards the V_0.

Also shown is the elution of purified IgG, as measured by monitoring at 280 nm. The difference between this almost symmetrical peak and the distribution of IgG in serum estimated by ELISA is technical, since the ELISA is performed under conditions of extreme overload of the detection system in the region where the monomeric serum IgG is eluted. The bulk of the peak, therefore, cannot be visualized, and only the intial rise and the tail of the response are pictured. The asymmetry of the peak is caused by the tendency of the IgG (possibly only of a subpopulation of the IgG) to be adsorbed on the column material. After the elution of V_t, the IgG measured is still significantly above background.

A background without discernible peaks (similar to the dotted and broken lines in Fig. 4) was seen when plates coated with normal rabbit $F(ab')_2$ were used instead of antibody-coated plates. To obtain a low background the use of buffer with high salt concentration and gelatin was essential. This buffer was a modification of that used by Kato *et al.*[5], omitting BSA to avoid interference from the frequently occurring anti-BSA in human sera[4].

HPLC–GPC combined with ELISA for C3c

Fractions were collected on microplates coated with anti-C3c antibody, followed by development with biotinylated anti-C3c.

During complement activation, the C3 molecule is cleaved at several positions, resulting in various cleavage products, such as C3a, C3b, C3c and C3d. Some of the products may become covalently attached to immune aggregates or to cells. Antibody raised against C3c reacts with C3, C3b, C3c and complexes containing these components.

When serum without added aggregated IgG was fractionated, all the molecules with C3c determinants were eluted in one peak at about an M_r of $2 \cdot 10^5$, corresponding to monomeric C3 (Fig. 3). When aggregated IgG had been added to serum with the complement system inactivated by EDTA, the same elution pattern was seen, whereas a peak containing molecules with C3c determinants was found at V_0 when aggregated IgG was incubated in serum with intact complement (Fig. 3). Also, part of the molecules with C3c determinants is eluted only slightly earlier than the monomeric C3 in the two previous fractionations. This could be explained by the independent observation of attachment of a significant proportion of C3 components to monomeric IgG after activation by aggregated IgG[12,13].

HPLC–GPC, combined with ELISA, for C3c–IgG complexes

Fractions were collected on microplates coated with anti-IgG antibody, followed by development with biotinylated anti-C3c.

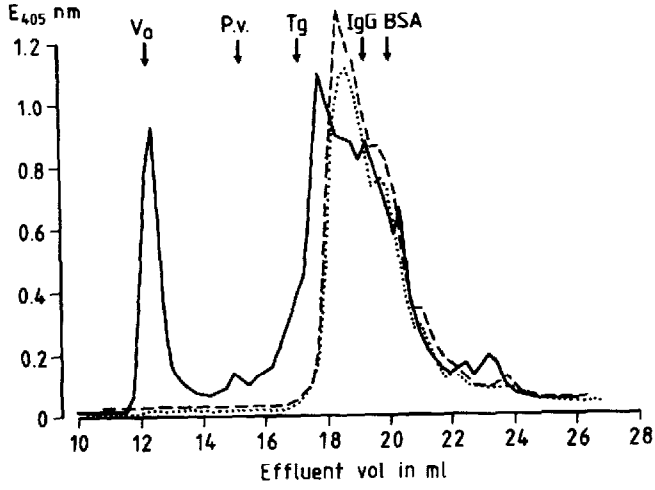

Fig. 3. Estimation by ELISA of C3 in serum fractionated by HPLC. (---) C3 in serum without added aggregated IgG; (...) C3 in serum to which was added aggregated IgG and EDTA before incubation at 37°C and fractionation; (——) C3 in serum incubated with aggregated IgG before fractionation.

The elution pattern of C3–IgG composite molecules is seen in Fig. 4. In serum without added aggregated IgG no peaks are seen. The same was found when serum and aggregated IgG had been incubated in the presence of EDTA. When aggregated IgG was added to serum with a functioning complement system, a peak of IgG–C3 composite molecules was eluted at V_0 with a prolonged tail. A second peak is seen at about an M_r of $4 \cdot 10^5$.

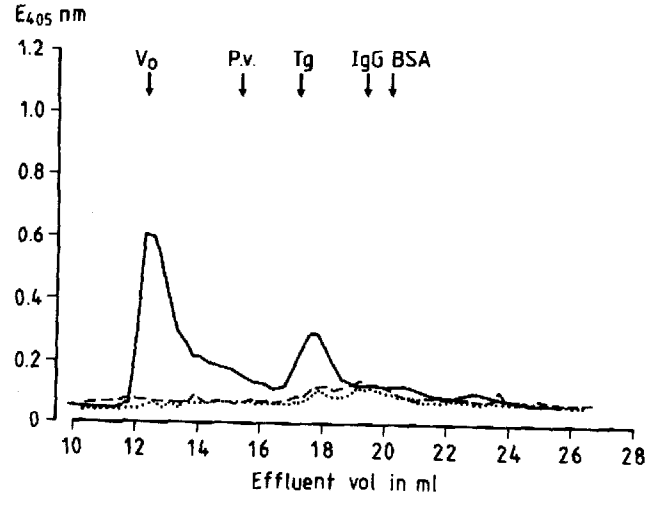

Fig. 4. Estimation by ELISA of C3–IgG complexes in serum fractionated by HPLC. (---) C3–IgG complexes in serum without added aggregated IgG; (...) C3–IgG complexes in serum to which was added aggregated IgG and EDTA before incubation at 37°C and fractionation; (——) C3–IgG complexes in serum incubated with aggregated IgG before fractionation.

DISCUSSION

The solid-phase enzyme immunoassays (ELISA) were invented ten years ago[14], and became applicable to routine use with the marketing some years ago of instruments for the automatic spectrophotometry of microplates. The availability of various other devices for the automation of procedures involving microplates makes these multi-well containers attractive in many laboratory procedures for handling small volumes. HPLC technology has lately developed rapidly in the field of protein fractionation. Routine analysis of, for example, serum proteins is now possible, given adequately specific detection systems. The apparently ideal combination of HPLC and ELISA, has now been attempted and we believe that our results demonstrate the viability of this idea.

We were interested in the analysis of the size and composition of circulating immune complexes, *i.e.* high-molecular-weight protein aggregates. With the development of the TSK series of columns, such analysis by the HPLC technique became feasible. The total volume of the standard columns (300 × 7.5 mm or 600 × 7.5 mm) is conveniently contained within the 96 wells of 300 μl each, provided by a conventional microplate. Consequently, a suitable fraction collector for microplates was constructed.

The ELISA methods applied were essentially as described by Voller[3], except for the use of the $F(ab')_2$ fragments of the antibodies rather than intact antibody for coating of the microplate and the use of the biotin–avidin system[7,8,15] for the attachment of the enzyme to the second "sandwhich" layer of antibody. Both modifications are measures for maximizing the signal-to-noise ratio. Coating with $F(ab')_2$ minimizes interference by rheuma factors, which are commonly occurring anti-IgG antibodies. Biotin labelling of the second antibody and attachment of the enzyme through coupling to avidin, an eggwhite protein with high affinity ($K_{ass} = 10^{15}\ M^{-1}$) for biotin[16], ensures gentle treatment of the antibody without risk of polymerization. It may, moreover, have an enhancing effect, since the coupling of a number of biotin molecules per antibody molecule may allow the attachment of several avidin–enzyme conjugates[15]. Another advantage is that biotinylation is a very simple procedure and that the same enzyme conjugate may be used for all the assays performed.

The ELISA results in this paper are presented simply as the optical densities, read by the multichannel spectrophotometer. For quantitative estimations, standard dilutions must be applied in one or two rows of the microplate. The standard curve is roughly logarithmic (not shown) and the ordinates of the figures given might therefore be read as approximate log concentration in arbitrary units.

No attempt was made to estimate the protein recovery from the column. However, in a previous paper[4] a recovery greater than 90% was observed when radiolabelled BSA, free or complexed to anti-BSA, was analysed.

The separation characteristics of the TSK G 6000 PW column are well-suited to the analysis of large proteins. Even *Helix pomatia* hemocyanin with an M_r of 8.9×10^6 is eluted after V_0. The heat-aggregated IgG was eluted partly at V_0 and partly between V_0 and the position of monomeric IgG. Surprisingly, some of the included aggregated IgG appeared to be moved to V_0 after reaction with complement, a process usually referred to as "complement-mediated solubilization". This process is characterized by the solubilization of immune precipitates procured by the action of

complement component C3 in particular[17]. It should, however, be noted that interactions between heat-aggregated IgG and complement may only crudely reflect the process of immune complex solubilization.

ELISA was found to be useful for the estimation of individual components in HPLC effluents. A special advantage of the solid-phase ELISA method is its capability of estimating complexes between two different molecular species. This was exemplified by the detection of C3–IgG complexes by means of microplates, coated with anti-IgG antibody, followed by development with anti-C3 antibody. This application may be extended to many other systems of interacting molecules.

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