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Characterization of peroxidase:anti-peroxidase immune complexes by capillary zone electrophoresis and high-performance size-exclusion chromatography

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Abstract

Determination of the molecular constituents of commercial peroxidase:anti-peroxidase (PAP) preparations is necessary for the proper interpretation of PAP applications based on competitive binding assay. Capillary zone electrophoresis with field 300 V/cm, 40 cm capillary length (20 cm effective length), and high-performance size exclusion chromatography equipped with Superose12 HR10/30 column revealed that a PAP preparation used for Fcγ receptor studies contained multiple sizes of immune complexes, an excess amount of free peroxidase, and little or no free anti-peroxidase antibody. The antibody:antigen ratios of the three major immune complex components were 2:2, 1:2, and 1:1. These techniques provide useful methods of qualitative, as well as quantitative analysis of PAP preparations.

Keywords: Enzymes; Peroxidase:anti-peroxidase

1. Introduction

Peroxidase:anti-peroxidase (PAP) immune complexes were first developed about 25 years ago as an adjunct for histochemical staining [1]. These immune complexes contain one or more peroxidase molecules which can be used with a variety of substrate for tissue staining or colorimetric analysis. For this reason, PAP has been used extensively as a signal amplification reagent in the immunohistochemical staining of tissues and cells [2–6]. PAP has several attractive features including its stability as an immune complex, lack of radioactivity, low background staining, and inexpensive cost [1,2]. However, detailed information about PAP preparations such as molecular composition and relative concentrations of complexed or free antibody and antigen have not previously been reported. Neither commercial distributors nor investigators have documented the stoichiometry of components in PAP preparation. However, for many applications, the relative concentrations of the component molecules may alter the functional properties of PAP.

To detect Fcγ receptors (type II and III) on canine monocytes and endothelial cells [7,8], PAP immune complexes have been selected as Fc-specific receptor binding ligands. PAP has also been used as a potential bridging molecule in the mediation of

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leukocyte cytotoxicity toward endothelial cells in vitro. Since the presence of free immunoglobulin G (IgG) may interfere with the binding of immune complexes to Fcγ receptors, a detailed analysis of the PAP preparation was required to determine the relative concentrations of immune complexes and free antigen or antibody.

As complementary methods, both capillary zone electrophoresis (CZE) and high-performance size-exclusion chromatography (HPSEC) were selected to characterize the content of PAP preparation.

CZE has been applied successfully in the analysis of biological macromolecules including proteins, enzymes, antibodies and their complexes [9–13]. The simplicity, rapidity, high efficiency, low reagent consumption and small sample volume of CZE systems make the technique a very powerful separation tool. The basic mechanism of the capillary zone electrophoretic separation of macromolecules is based on the charge to size ratio [14]. However, the net charges of supramacromolecule complexes, such as PAP may also vary with quaternary structure. Sometimes, the lack of this kind of information may cause difficulty in fully characterizing the molecule of interest.

In contrast, HPSEC is a liquid chromatography technique that separates molecules according to stokes' radius. One major advantage of HPSEC is its excellent correlation of molecular size with the retention time [15].

Together, the two methods are complementary in determining the size and relative composition of different molecular species.

2. Experimental

2.1. Chemicals and reagents

Analytical grade protein standards including cytochrome C, bovine serum albumin (BSA), Goat anti-rabbit IgG, and apoferritin were obtained from Sigma (St. Louis, MO, USA). Rabbit PAP, purified peroxidase, and rabbit anti-peroxidase antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Poly-(ethyleneimine)hydroxyethylated aqueous solution was obtained from Polysciences (Warrington, PA, USA). All other reagents were of analytical grade. Samples were dissolved in 50 m*M* phosphate buffered saline (PBS) buffer (pH 7.2) and stored at 4°C between analyses. Buffer solutions were prepared fresh daily with double deionized water filtered through a Nylon-66 (0.2-μm) membrane filter (Woburn, MA, USA) and deaerated via a vacuum pump for 15 min prior to use.

2.2. Capillary electrophoresis

A CZE system was built in-house, consisting of a Spellman CZE 1000R power supply (Spellman, Plainview, NY, USA) and a Isco CV^4 UV–VIS absorbance detector (Isco, Lincoln, NE, USA). NI-DAQ software from National Instruments (Austin, TX, USA) was used for data acquisition. Fused silica capillaries of 75 μm I.D. and 360 μm O.D. from Polymicro Technologies (Phoenix, AZ, USA) were coated with poly(ethyleneimine) according to Ref. [16].

CZE analysis of PAP was performed at room temperature. The total length of the capillaries used for analysis was 40 cm (20 cm effective length). A detection window was opened at the center by removing a segment of polyimide coating approximately 5 mm long. The running buffer was 10 m*M* PBS (pH 7.0). Samples (1 mg/ml of PAP, 1 mg/ml of peroxidase, and 1.3 mg/ml of anti-peroxidase) were individually injected hydrodynamically in 5 s at the anode end. The capillary was then subjected to 12 kV voltage (300 V/cm) producing a current of 30 μA. Eluted proteins were detected in the capillary by UV-absorbance at 200 nm.

2.3. High-performance size-exclusion chromatography

HPSEC separations of PAP were achieved using a Hewlett-Packard model 1090 liquid chromatographic pumping system (Andover, MA, USA) with a Lambda-Max model 481 UV detector from Waters (Milford, MA, USA). A Superose 12 HR10/30 size-exclusion column (Pharmacia, Piscataway, NJ, USA) with a calibration range between 10 and 2000 kDa, was equilibrated with 50 m*M* PBS (pH 7.2) corresponding to ten times column volume (25 ml) at a

flow-rate of 0.3 ml/min before sample loading. The injection volumes for PAP (0.4 mg/ml), peroxidase (0.2 mg/ml) and anti-peroxidase (0.1 mg/ml) were 30 μl per sample. Eluted proteins were detected by UV absorption at 280 nm. Each preparation was analyzed at least three times to check reproducibility.

2.4. Statistical analysis of the experimental data

Statistical analysis of the data was performed using correlation and non-linear regression analysis. The generated non-linear regression equation was used to estimate M_r of immune complex components in PAP preparation. P values <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. PAP characterization by CZE

The results in Fig. 1A show that at least two components are present in the PAP preparation. The corresponding migration time for the peaks were 6.1, and 7.5 min, respectively. Fig. 1B represents the CZE profile of purified peroxidase which elutes as a single peak at 7.5 min. As shown in Fig. 1C, purified anti-peroxidase antibody is detected at 7.4 min. As a result of comparing the CZE profiles in Figs. 1A, B and C, it can be determined that the commercial PAP preparation may contain an excess amount of free peroxidase (represented by the peak eluted at 7.5 min). Since the migration time for peroxidase is very close to the one for free anti-peroxidase antibody, we can not rule out the possibility that a small amount of free anti-peroxidase antibody is also present in the PAP preparation. The molecular nature of the components eluted at 6.1 min is hard to determine. Since the manufacture of PAP involves only the addition of free peroxidase and anti-peroxidase antibody, this peak should represent complexed PAP species. However, the ratio of antibody to antigen in these species can not be determined by the present CZE methodology.

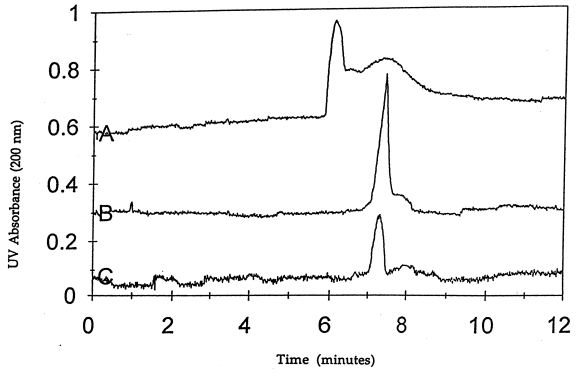

Fig. 1. Electropherogram of PAP, peroxidase and anti-peroxidase. Conditions: field 300 V/cm; current, 30 μA; 10 m*M* PBS (pH 7.0); capillary length, 40 cm; effective length, 20 cm; capillary I.D., 75 μm; room temperature; detection, 200 nm. A. Profile of PAP preparation (1 mg/ml). B. Profile of peroxidase (1 mg/ml). C. Profile of anti-peroxidase (1.3 mg/ml).

3.2. PAP characterization by HPSEC

Several peaks can be identified in the HPSEC separation profile for PAP (Fig. 2A). The first major peak appeared at retention time of 26 min. Since the protein complex with the highest M_r should elute first, this peak represents the protein or protein complex with highest molecular mass. A second peak with a retention time at about 30.5 min was broad, implying that more than one component was present in this peak. A third small peak eluted at retention time of about 33 min. The last peak was observed at a retention time of 43 min, which corresponds to that of purified free peroxidase (Fig. 2B). This finding suggested that an excess amount of free peroxidase was present in the PAP preparation. The peak representing purified anti-peroxidase antibody eluted at a retention time of 36 min (Fig. 2C). However, no distinct peak is found in the PAP profiles at this position, which suggested little or no free anti-peroxidase antibody is present in the PAP preparation.

Based on the information provided from manufactures, the M_r of PAP and peroxidase are around 420 and 40 kDa, respectively. Five purified standard proteins (M_r range from 12.4 to 443 kDa) were selected to determine the M_r of each component in the PAP preparation. The retention time in HPSEC and the M_r of each standard protein is listed in Table 1.

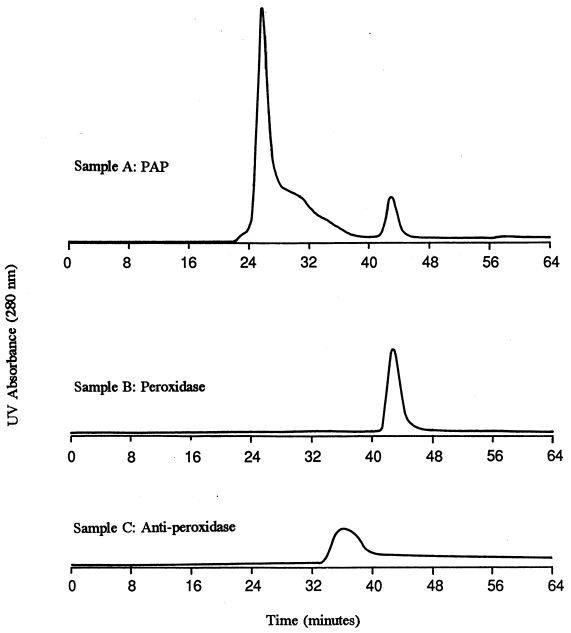

Fig. 2. Characterization of PAP preparation by HPSEC: the Superose12 HR10/30 column was equilibrated with 50 m*M* PBS (pH 7.2) for 2 h at a flow-rate of 0.3 ml/min before loading samples. PAP (0.4 mg/ml), peroxidase (0.2 mg/ml), and anti-peroxidase (0.1 mg/ml) were subsequently injected into the HPLC column 2 h apart. The eluted proteins were detected by an on-line UV absorbance detector at 280 nm. A. Profile of PAP preparation. B. Profile of peroxidase. C. Profile of anti-peroxidase.

3.3. Statistics of data obtained from HPSEC

Correlation and non-linear regression were used to analyze the relationship between the M_r versus the retention time for the five protein standards, yielding a correlation coefficient (R) of 0.999 ($R^2=0.994$, $P<0.01$).

Based on the generated regression equation, the M_r of each PAP component was estimated (Table 2). The theoretical M_r of possible antigen/antibody combinations suggested that the major immune complex species (peak 1) might consist of two antibody (anti-peroxidase) and two antigen (peroxidase) molecules. Relatively fewer immune complexes consisted of one antibody with two peroxidase molecules (peak 2) or one antibody with one peroxidase molecules (peak 3).

Table 1
Summary of standard protein M_r and retention times in HPSEC

Standard protein	Retention time (min)	M_r (kDa)
Cytochrome C	49.5	12 400
Rabbit-peroxidase	43	40 000
BSA	39.5	66 000
Goat-anti-rabbit (IgG)	36	150 000
Apoferrtin	25	443 000

3.4. Immune complex separation

The molecular characterization of immune complexes such as PAP is complicated by the presence of multiple protein species in combination, particularly when a polyclonal antibody preparation is used. Moreover, the conformation of immune complexes changes somewhat with experimental conditions, such as the alteration of pH. Since PAP preparations are commonly used at or near their physiological conditions, it is desirable to have the best knowledge of their conformation under similar conditions. Unfortunately, the separation of the immune complexes with unmodified fused-silica capillaries at near neutral pH is unavoidably accompanied by the irreversible adsorption of the proteins onto the capillary wall. Many methods have been developed to overcome this adsorption problem [17]. In this study, a poly(ethyleneimine) hydroxylated aqueous solution was adsorbed along the capillary inner surface which was further stabilized by cross linking. The treated capillary was very hydrophilic and effective in preventing the proteins from absorbing onto the capillary wall as compared to the untreated capillary.

Analysis by both CZE and HPSEC demonstrate the heterogeneity of PAP immune complexes. Since PAP is generated from a polyclonal rabbit antibody, molecules of similar charge and size may be formed which recognize different epitopes with different three dimensional conformations. Based on the calculation of peak area in the HPSEC chromatogram, about 70% of the immune complexes (first peak) were formed at a ratio of two antibodies to two antigens.

3.5. Use of immune complex to detect Fcγ receptors

Fcγ receptors are membrane bound glycoproteins which specifically bind to the Fc portion of IgG.

Table 2
M_r estimation of immune complex components in PAP preparation

Sample PAP	X=Retention time (min)	Y=EXP(15.95−0.1179X) Y=Estimated M_r (kDa)
Peak 1	26	394 194 (380 000)[a]
Peak 2	30.5	231 885 (230 000)[b]
Peak 3	33	172 715 (190 000)[c]
Peak 4	43	53 103 (40 000)[d]

[a] Molecular mass (theoretic value) of immune complexes formed by two antibodies with two antigens.
[b] Molecular mass (theoretic value) of immune complexes formed by one antibody with two antigens.
[c] Molecular weight (theoretic value) of immune complexes formed by one antibody with one antigen.
[d] Molecular weight (theoretic value) of peroxidase.

They serve as a bridge between the cellular and humoral immune system and mediate many immune functions [18–25]. In species for which monoclonal antibodies are not yet available, the gene which encodes for Fcγ receptors has not yet been cloned, the alternative methods of demonstrating Fcγ receptors are dependant on a variety of ligand-based binding assays. These commonly used ligands include antibody-coated sheep erythrocytes [26], radio- or fluorescein-labeled monomeric immunoglobulin [27,28], and heat-aggregated complexes of immunoglobulins [29]. However, the sensitivity varies greatly from method to method. PAP immune complexes have several unique characteristics as compared to other ligands. PAP is a classic immune complex which contains an intrinsic enzyme as the component antigen, which with a suitable substrate, allows for quantitative detection of receptor binding. In addition, many different species of PAP are commercially available at a reasonable cost. However, the presence of excess free IgG in the PAP preparation may potentially interfere with immune complex binding to Fcγ receptors. Fortunately, the amount of free IgG in the PAP preparation was almost negligible based on both the HPSEC and CZE results.

3.6. Molecular considerations on the use of PAP preparations in the study of immune complexes disease

Traditionally, the study of the roles of immune complexes in the pathogenesis of autoimmune disease depended largely on the use of serum collected from the patients with immune complex-mediated disease [30]. In addition to immune complexes, the serum also contained other factors such as cytokines, growth factors, and unrelated immunoglobulin species. These unrelated factors undoubtedly caused confounding effects. Even when the serum was subjected to ultra centrifugation, it was still impossible to completely purify immune complexes. An alternative method to detect the presence of immune complexes in the serum of immune complex-mediated disease would be to subject the patient's serum to a Protein A or G column. Recovery of immunoglobulin components from the column could then be further examined by HPSEC to separate immunoglobulin and immune complexes.

PAP molecules provide a pre-formed, well-characterized immune complex molecule which can also be used in experimental models of immune complex-mediated autoimmune disease. Even in the presence of free peroxidase, PAP should prove superior to the use of patient serum for in vitro as well as in vivo studies.

3.7. The use of PAP as a possible bridging molecule to link inflammatory cells and endothelial cells in the cytotoxicity assay

Most naturally formed immune complexes are polyvalent with at least two free Fc portions within the molecule. PAP complexes have the similar composition, with more than 70% (area under the curve of first peak) of immune complexes contained two antibody molecules. This structure suggests that the majority of PAP molecules are capable of binding two Fcγ receptors through their two Fc portions. Therefore, it would be theoretically possible to use PAP as a bridge to mediate interactions between

leukocytes and endothelial cells. However, the presence of antigen: antibody species with only one antibody may block some Fcγ receptor sites to exclude functional bridging molecules.

4. Conclusion

Peroxidase:anti-peroxidase immune complex PAP from a commercial preparation was characterized by using CZE and HPSEC techniques. The results indicated that at least three immune complex components as well as an excess amount of free peroxidase were present in the PAP preparations. The M_r of the three major immune complex components was determined to be 380, 230, and 190 kDa which would correspond to an antibody:antigen molecular composition of 2:2, 1:2 and 1:1 ratio, respectively. The characterization of PAP molecules not only provides information on the use of PAP as a ligand to detect Fcγ receptors, but also better defines a molecular model for the study of immune complex-mediated disease in vitro or in vivo.

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