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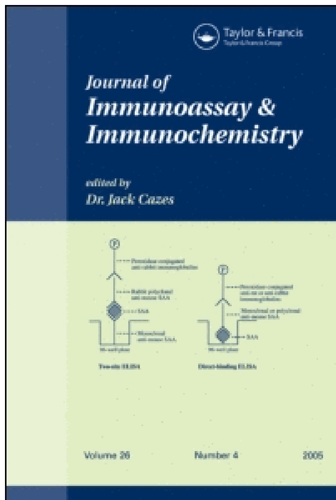

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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To cite this Article Cuvelier, Antoine , Bourguignon, Jeannette , Muir, Jean-François , Martin, Jean-Pierre and Sesboüé, Richard(1996) 'Substitution of Carbonate by Acetate Buffer for IGG Coating in Sandwich Elisa', Journal of Immunoassay and Immunochemistry, 17: 4, 371 — 382

To link to this Article: DOI: 10.1080/01971529608005799

URL: http://dx.doi.org/10.1080/01971529608005799

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JOURNAL OF IMMUNOASSAY, 17(4), 371-382 (1996)

SUBSTITUTION OF CARBONATE BY ACETATE BUFFER FOR IGG COATING IN SANDWICH ELISA

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ABSTRACT

The first step of sandwich ELISA, namely adsorption of antibodies to plastic microtiter plates, was studied as a function of the pH of the coating buffer. Coating efficiency was assessed in terms of maximum signal (absorbance) observed in ELISA and also estimated by measuring the amount of functional antibodies adsorbed to the plate. While goat antibodies displayed better results after coating with acetate pH 5 buffer, rabbit IgGs generally worked well at pH 7.4. On average, the classical carbonate pH 9.6 buffer was only 50% as efficient.
(KEY WORDS: sandwich ELISA, pH, coating buffer, protease inhibitors).

INTRODUCTION

Since the pioneer work of Engvall et al. (1), Enzyme-Linked ImmunoSorbent Assay (ELISA) has been one of the most widely used techniques for the study of low-level proteins in biological fluids and is often preferred to radioimmunoassay. Although many variations have been introduced in the original

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method with respect to the number of stages or the nature of the product under study (antigen or antibody), these assays always include a preliminary step corresponding to the immobilization of the first reagent. If this solid-phase coating stage has been well studied when an antigen is used, little attention has been paid to the factors which may influence the immobilization of antibodies. While devising ELISAs for the study of two protease inhibitors, we noticed significant variations in the amounts of immunoglobulins bound to microtiter plates with respect to their origin, i.e. animal species, and/or their behavior according to the pH used. Optimization of these parameters led to a significant enhancement of our assays in terms of linearity and sensitivity.

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MATERIALS AND METHODS

Reagents

Polystyrene microtiter plates were obtained from Nunc (Maxi-Sorp). Horseradish peroxidase (HRP) EIA grade, 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate(6)] (ABTS) were from Boehringer. 45% liquid gelatin (Teleostean gelatin G-7735) was from Sigma. Polyclonal rabbit anti-inter-α-trypsin inhibitor immunoglobulins were from Dako. All chemicals used were of analytical grade.

Antisera, immunoglobulins and antibodies

Polyclonal antisera directed against α-1-antitrypsin (AAT) and inter-α-trypsin inhibitor (ITI) were produced in goat and/or rabbit; they were regularly absorbed on corresponding protein-depleted serum immobilized onto Sepharose as previously described (2). Immunoglobulins (IgGs) were purified by caprylic acid

and ammonium sulfate precipitation according to McKinney and Parkinson (3); they are referred to as crude IgGs. The pH 2.5 treatment described by Ishikawa et al. (4) was used to obtain acidified IgGs. Purified antibodies obtained by absorption-elution on purified or enriched protein immobilized onto Sepharose (2) were coupled to HRP following the modified periodate method (5).

Protein Standards

Purified AAT and ITI were obtained as previously described (6-7). AAT, ITI and IgG concentrations were estimated by spectrophotometric titration (respective extinction coefficients : $E_{280\ nm}$ = 0.5, 0.71 and 1.4 cm^{-1} mg^{-1} mL) or protein levels were measured with the Bradford's method adapted to microtiter plates (8). Pooled normal human serum was obtained from healthy blood donors; its AAT and ITI content was determined by rocket immuno-electrophoresis (9).

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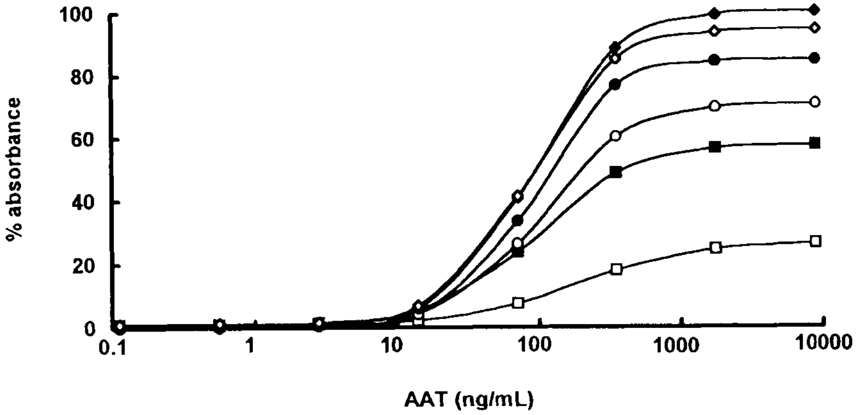
ELISA Procedure

The general procedure followed was that already described (10) except that IgG coating was performed with IgGs diluted in three 0.1 M buffers with different pHs: the 'classical' carbonate (pH 9.5), a neutral phosphate (pH 7.4) and an acidic acetate (pH 5.0). Crude or acidified IgG stock solutions (≈ 10 mg/mL) were diluted to the desired concentration and dispensed as 100 µL aliquots in microtiter plate wells. After a 2 hr incubation at 37°C, the plates were further incubated at 4°C for 16 hrs. After each further incubation step, wells were emptied and washed three times with 0.01 M phosphate buffer NaCl 0.15 M pH 7.4 containing 0.1%



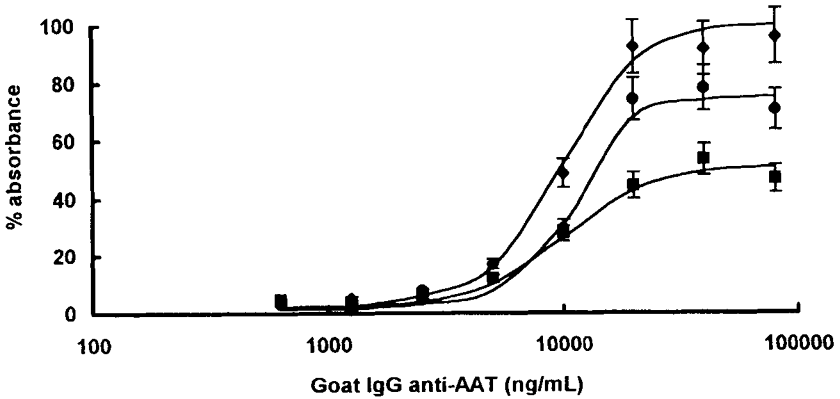


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FIGURE 1. **Effect of IgG pretreatment and influence of the buffer used in the coating phase in sandwich ELISA with goat anti-AAT.** Upper: Standard curves obtained with goat anti-AAT; full symbols: IgGs pretreated at pH 2.5; open symbols: crude IgGs; (■/□): carbonate buffer 0.1 M pH 9.6, (●/○): phosphate buffer 0.1 M pH 7.4, (◆/◇): acetate buffer 0.1 M pH 5.0; for the sake of clarity, only calculated curves are given (corresponding to at least two replicates). Lower: curves fitted to values obtained with dilutions of pretreated IgGs with respect to pH and a single dose of antigen corresponding to 90 ng/mL of AAT. Abscissas: protein levels in logarithmic scale; ordinates: % of maximum absorbance (405 nm).

(v/v) Tween 20 (PST). Standards and samples serially diluted in the same buffer supplemented with 0.5% (w/v) gelatin (PSTG) were dispensed into the wells and after mixing, the plates were incubated for 3 hrs at 37°C. Peroxidase labeled antibodies diluted in PSTG were then dispensed in the wells and the plates incubated for 2 hrs at 37°C. After washing, the ABTS substrate (7.5 mg / 10 mL) solubilized in citrate buffer 0.1 M, pH 4.5, containing 0.01% H_2O_2 was dispensed as 100 μL amounts per well. Absorbance was then read every 5 min at 405 nm with a BioRad 3550 spectrophotometer linked to an IBM compatible microcomputer. The results were analyzed with the Microplate Manager Software (BioRad, France), and standard curves were obtained with the four parameter logistic regression method.

In order to estimate the amount of functional IgGs adsorbed on microtiter plates, experiments were performed with serial dilutions of IgGs in varying buffers for the coating step. Thereafter a constant amount of protein (or diluted serum) was dispensed in the wells and the antigen was detected by peroxidase labeled antibodies and ABTS.

RESULTS

As a first step in the design of our immunoassays, the possible influence of pH during the coating phase was investigated, together with the effect of acid pre-treatment of IgGs. As shown in Figure 1 (upper) for goat anti-AAT, incubation of IgGs for 10 min at pH 2.5 clearly enhanced the observed signal with respect to that obtained with crude, untreated IgGs, thereby allowing a maximum two-fold amplification. Furthermore, the use of pH 5 or pH 7.4 buffer for IgG coating gave

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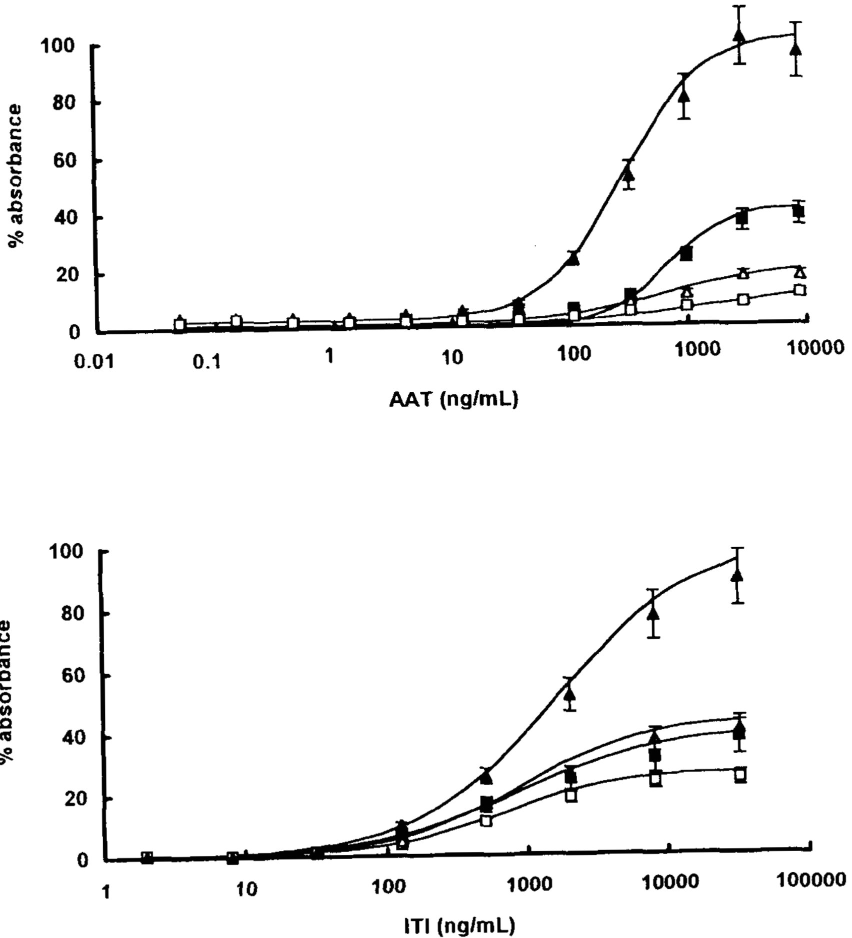

FIGURE 2. **Effect of IgG pretreatment and influence of the buffer used in the coating phase in sandwich ELISA with rabbit anti-AAT (upper) and rabbit anti-ITI (lower).** Full symbols: IgGs pretreated at pH 2.5; open symbols: crude IgGs; (■/□): carbonate buffer 0.1 M pH 9.6, (▲/△): phosphate (pH 7.4) or acetate (pH 5.0) 0.1 M buffer. Curves were fitted to observed values corresponding to at least two replicates. Abscissas: protein levels in logarithmic scale; ordinates: % of maximum absorbance (405 nm). Since phosphate and acetate buffers gave identical results, only one curve was depicted for the sake of clarity.

better results than pH 9.6 buffer with a maximum 1.7-fold amplification factor. When the level of acidified IgGs was varied while using a single dose of antigen (Figure 1, lower), acetate buffer allowed the adsorption of lower amounts of IgGs displaying a better functional activity.

When rabbit IgGs directed against AAT or ITI were used (Figure 2), acetate and phosphate buffers gave identical responses averaging two-times those observed with carbonate buffer. Again, pH 2.5 IgG pretreatment enhanced the observed signals 2- to 5-fold.

With commercial rabbit anti-ITI IgGs (Figure 3, upper), dose-response curves ranged from phosphate (lower) to acetate (higher). The corresponding amounts of functional IgGs coated on microtiter plates with respect to pH (Figure 3, lower) were close to each other with no appreciable variation in maximum values observed. However, acetate buffer allowed a slight (30-40%) reduction of the quantity of IgGs used with no alteration of the maximum signal observed.

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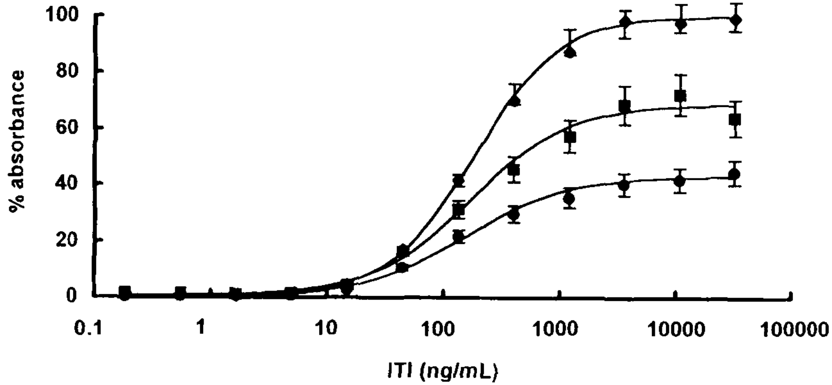
DISCUSSION

Many studies have been devoted to the quantification of protein adsorption on various polymers and the numerous factors involved in this phenomenon: nature of protein (antigen or antibody), type of the solid phase (plastic, silica, glass, agarose, polyacrylamide) and mechanism concerned (passive adsorption, use of a spacer, chemical treatment of the matrix). However, only a few studies addressed the possible influence of these conditions on the main characteristic of IgGs, i.e. their antibody function. In order to avoid possible interference with other factors, we used well-established procedures: i) enrichment of IgG fractions with caprylic acid, a highly efficient technique in terms of antibody performance (11), and ii)



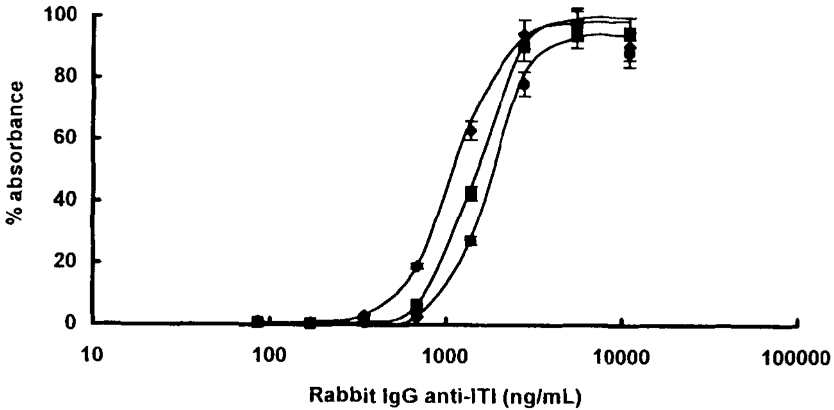


FIGURE 3. **Influence of the buffer used in the coating phase in sandwich ELISA with commercial rabbit anti-ITI.** Upper: Standard curves obtained with rabbit anti-ITI. Lower: calculated curves fitted to values obtained with dilutions of IgGs with respect to pH and a single dose of antigen corresponding to 150 ng/mL of ITI. (■): carbonate buffer 0.1 M pH 9.6, (●): phosphate buffer 0.1 M pH 7.4, (◆): acetate buffer 0.1 M pH 5.0. Abscissas: protein levels in logarithmic scale; ordinates: % of maximum absorbance (405 nm).

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washing of the plates with detergent to avoid a possibly detrimental blocking step (12).

Ishikawa et al. (4) were the first to notice that exposure of IgGs to pH 2.5 prior to their adsorption on plastic led to a 3- to 6-fold enhancement in ELISA activity. This observation, based on the fact that antibodies purified by adsorption-elution on insolubilized matrices displayed a higher activity than IgGs, was clearly confirmed in the present work. Such a procedure has been shown to induce variations in the conformation of IgGs with an increase of hydrophobicity (13-15).

The most widely used buffer for plate sensitization is carbonate pH 9.6, based on the original work of Catt and Tregear (16). However, pH 7.4 buffer is also frequently used and showed a slightly better (≈ 20%) coating efficiency than carbonate in a study where IgG protein amounts and not their antibody activity were assessed (17). The main factor involved in pH-dependent adsorption of IgGs on polystyrene is obviously the charge of the molecules, i.e. their isoelectric point (18). In the present work, four different polyclonal IgG specimens showed varying behavior with respect to pH. That goat and rabbit IgGs displayed maximum efficiency and adsorption at different pH may easily be explained by their peculiar molecular stability (19), as well as species-related difference in the isoelectric points of our antibodies (data not shown). While only little variation was evidenced in the amount of functional rabbit IgGs coated with respect to pH (Fig. 3), goat IgGs i) were better adsorbed and ii) gave a higher ELISA signal at pH 5 (Fig. 1). Such a different behavior of goat and rabbit IgGs was not related to the antigens studied since similar results were obtained when AAT and ITI levels were both determined with rabbit IgGs. The latter displayed better coating efficiency at

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neutral pH, but with varying degrees according to the antibody source (i.e. commercial or available in the laboratory). On average, our procedure led to a minimum 3-fold enhancement of the observed signal and a 2-3 fold reduction in the amount of IgGs needed.

In their extensive work on the IgG modification induced by polystyrene adsorption, Butler et al. (20) pointed out that passive adsorption at pH 9.6 not only led to poor coating efficiency but should be considered "a method of protein denaturation". The loss of functional activity was particularly noticeable for monoclonal antibodies while polyclonal species displayed a monoclonal behavior after adsorption. These authors developed alternatives based either on the protein-avidin-biotin-capture (PABC) system, or immobilization of IgG through a previously adsorbed antiglobulin or covalent attachment to the plates. As demonstrated here, none of these complicated or supplementary steps may be necessary as long as the pH of the coating buffer is matched to the characteristics of the antibody used. Our observation may be of peculiar importance when working with monoclonal antibodies.

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ACKNOWLEDGMENTS

The skillful technical assistance of F. Bonnet-Muschio and B. Dautréaux is gratefully acknowledged. We thank J. Grassi for helpful comments and J.C. Barré for reading of the manuscript.

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