Soluble Complex Formation of Bovine Immunoglobulin G_2 with Staphylococcal Protein A Studied by Gel Filtration Chromatography

KEI-ICHI SHIMAZAKI
Dairy Chemistry Research Laboratory
Department of Animal Science
Obihiro University of Agriculture
and Veterinary Medicine
Obihiro, 080, Japan

ABSTRACT

The binding property of bovine IgG_2 to staphylococcal Protein A was investigated by the methods of gel filtration chromatography and affinity chromatography. High performance gel filtration chromatography was carried out using TSK gel G3000SW and G2000SW columns, and immobilized Protein A column was used for affinity chromatography. Although bovine IgG_2 did not form any precipitin lines with Protein A by double diffusion method on agar gel, IgG_2 could bind to immobilized Protein A column. Moreover, by gel filtration chromatography, peaks of the complex between bovine IgG_2 and Protein A were observed in addition to the IgG_2 monomer peak. Thus, it is concluded that bovine IgG_2 interacts with staphylococcal Protein A and forms "soluble complexes". Carbethoxylated IgG_2 lost its affinity to Protein A indicating that histidyl residues in IgG_2 is essential for the binding to Protein A.

INTRODUCTION

Staphylococcal Protein A interacts with the Fc part of Ig of most animals (7, 12, 20). For Ig of human (IgG_1, IgG_2, IgG_4, and IgA), mouse (IgG_2 and IgG_3), and guinea pig (IgG_1 and IgG_2), such interaction results in the formation of precipitate with Protein A (20). However, rabbit IgG_2 forms a soluble complex with Protein A (10, 15), and the interaction of ruminant Ig with Protein A is weak (12, 13). In the case of bovine Ig and sheep Ig, IgG_2 is adsorbed on immobilized Protein A but very little IgG_1 is adsorbed (8). The authors suggested previously that bovine IgG_2 formed soluble complex but not insoluble complex with Protein A (16, 17). The purpose of this study is to obtain evidence of the formation of the soluble complex and to investigate further about bovine IgG_2 interaction with Protein A using high performance gel filtration chromatography. Also, chemical modification of histidyl residues in bovine IgG_2 was performed to examine the contribution of the residues in the interaction with Protein A.

MATERIALS AND METHODS

Preparation of Immunoglobulins

Bovine IgG_1 and IgG_2 were purified from colostrum and serum, respectively, as follows. Immunoglobulin fraction was precipitated from whey or serum with 33% saturated ammonium sulfate. After dialysis with .005 *M* sodium phosphate buffer, pH 8.0, the Ig fraction was subjected to ion exchange chromatography on DEAE-Toyopearl 650M column of 2.4-cm diameter and 100 ml of bed volume using a stepwise elution. The IgG_2 fraction, which is the fall-through fraction from DEAE-Toyopearl column equilibrated with .005 *M* sodium phosphate buffer, pH 8.0, was purified further by a CM-Toyopearl 650M column (2.6 cm diameter, 127 ml bed volume) equilibrated with .01 *M* sodium acetate buffer, pH 5.0. The IgG_1 fraction was eluted from the DEAE-Toyopearl column by an equilibration buffer containing .15 *M* NaCl. The fraction thus obtained was purified further by rechromatography. Ion exchange Toyopearl gels were the products of Tosoh Co. Ltd. (Tokyo). Identification of each IgG was performed by immunoelectrophoresis and double diffusion with antibovine Ig serum

Received November 19, 1987.
Accepted September 15, 1988.

according to Butler and Maxwell (5). Antisera to bovine Ig and to bovine whey proteins were prepared by immunization of rabbits. Commercial rabbit antisera to bovine IgG_1 and to bovine IgG_2 (Miles Laboratories, Inc., Elkhart, IN) were also used.

Protein A-Binding Experiments

The binding properties of Ig to Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) were studied by one of the following procedures: 1) by the stepwise method, using .1 *M* sodium phosphate buffer, pH 7.0 for adsorption, and 1 *M* acetic acid solution for elution; 2) by the gradient method using a two-chambered gradient mixer, with sodium phosphate-citrate buffer (McIlveine buffer), pH 7.0 as the starting buffer, and .1 *M* citric acid, pH 2.2 as the final buffer. The total volume for the gradient elution was 20 ml. The bed volume of Protein A-Sepharose column of 8 mm diameter was 5 ml and the flow rate was 15 to 20 ml/h. The eluate was monitored by UV absorption at 280 nm.

Gel Filtration Chromatography

High performance liquid chromatography was carried out with the HLC-803D equipment using gel filtration columns of TSK gel G3000SW plus G2000SW (7.5 mm diameter × 600 mm) combined with TSK guard column SW (7.5 mm diameter × 75 mm). The HPLC system was from Tosoh Co. Ltd. (Tokyo). The effluent was .1 *M* sodium phosphate buffer, pH 7.0, and HPLC was performed at a flow rate of 1.0 ml/min at 19 ± 2°C. The eluent was monitored by UV absorption at 280 nm. Sample volume injected was .005 to .05 ml. Stock solutions of Ig and Protein A (Sigma Chemical Company, St. Louis, MO) were filtered through .45 μm filter unit (Millipore Ltd., Bedford, MA). The two were then mixed in various ratios and adjusted to a constant final volume by the addition of the solvent. Molecular weight estimation was performed using the molecular weight standard proteins: horse cytochrome c, bovine chymotrypsinogen A, ovalbumin, bovine serum albumin, rabbit aldolase, bovine catalase, and horse ferritin (products of Boehringer Mannheim GmbH, Mannheim, FRG).

Protein Concentration Determination

Protein concentration was determined by UV absorption at 280 nm using the extinction coefficient of 13.7 for Ig (4) and 1.40 for Protein A (20). Absorbance was measured by a double-beam spectrophotometer UVIDEC 430 (JASCO, Tokyo).

Chemical Modification of Immunoglobulin G_2

Carbethoxylation of histidyl residues was carried out as reported by Miles (14). Two milliliters of IgG_2 (1.0 mg/ml) in .1 *M* sodium phosphate buffer, pH 6.0, were reacted with .01 ml of 10 m*M* diethylpyrocarbonate (DEP, Aldrich Chemical Company Inc., Milwaukee, WI) in acetonitrile. The reaction was performed at room temperature for 60 min. To remove the carbethoxyl groups, 1 ml of carbethoxylated IgG_2 solution was incubated with1 ml of 1 *M* hydroxylamine, pH 7.0, for 20 h at room temperature and dialyzed for 6 h against four changes of .1 *M* potassium phosphate buffer, pH 7.8, at 6°C. The value of 152,000 was used for the molecular weight of IgG_2 (3).

RESULTS

Purification of Immunoglobulin G_2 by CM-Toyopearl

To remove impurities contained in the IgG_2 preparation eluted from the DEAE-Toyopearl 650M column (fall-through component as in Figure 1A), the IgG_2 fraction was subjected to cation exchange chromatography. It was separated into two fractions by the CM-Toyopearl 650M column as in Figure 1B. Both fractions, designated CM-1 and CM-2, reacted with anti-IgG_2 serum, but neither fraction formed any precipitin line with anti-IgG_1 serum by double diffusion method on agar plate. Affinity properties to staphylococcal Protein A of these two fractions were examined using immobilized Protein A column. The CM-1 fraction showed only bound component to Protein A-Sepharose column whereas CM-2 fraction showed both bound and unbound components to the column (Figure 2). Thus, CM-1 fraction was used for subsequent experiments.

Gel Filtration Chromatography of Immunoglobulin G and Protein A Mixture

The complex formation between IgG_2 and Protein A was shown by the gel filtration

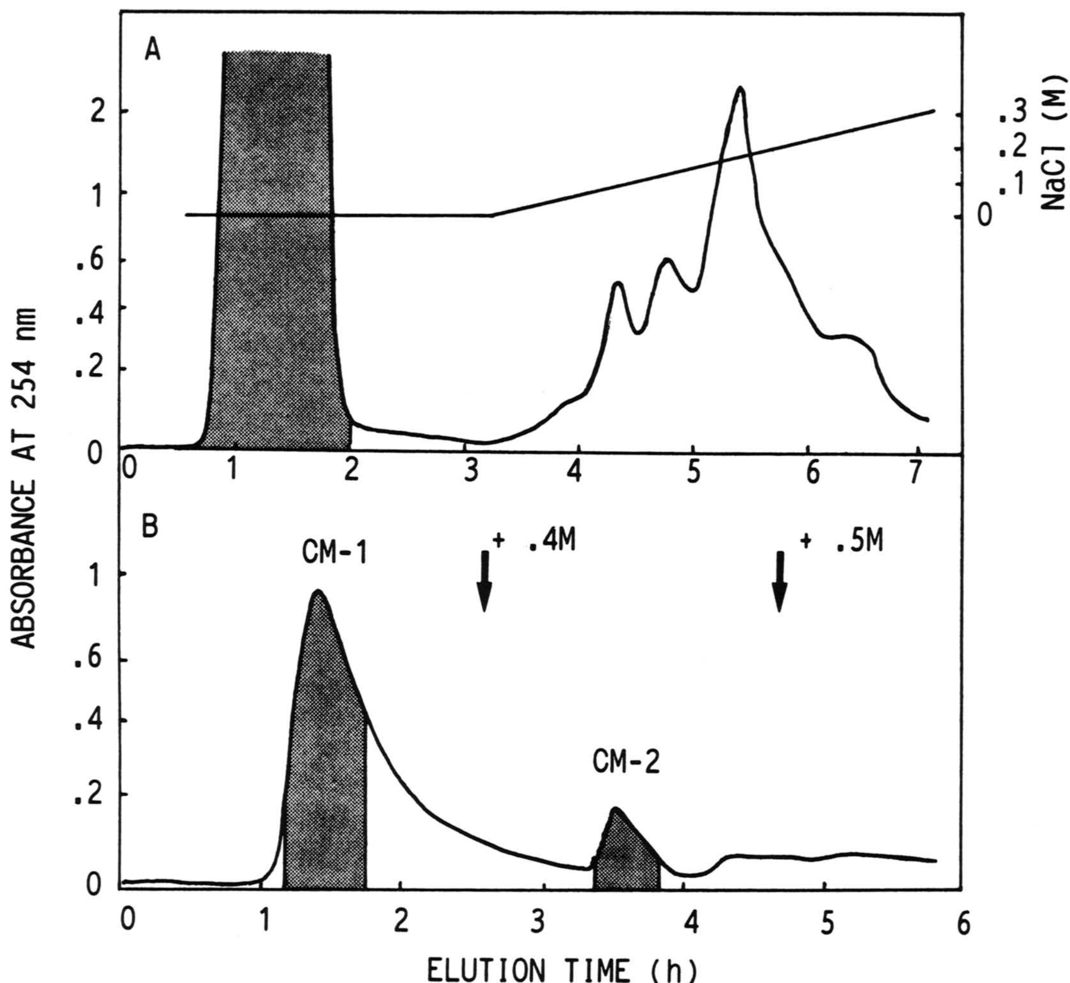

Figure 1. Purification of IgG_2 by ion exchange chromatography. A) The DEAE-Toyopearl 650M chromatography of crude Ig preparation from bovine serum: Column bed volume, 100 ml (2.4 cm diameter); initial buffer, .005 *M* sodium phosphate buffer, pH 8.0; flow rate, 110 ml/h; sample amount, ca. 1.3 g. Elution was carried out by linear gradient method with increasing NaCl concentration from 0 to .3 *M* in the initial buffer. Eluent was monitored by UV detector at 254 nm. B) The CM-Toyopearl 650M chromatography of the IgG_2 fraction from A (dotted area in A, fall-through fraction). Column bed volume, 127 ml (2.6 cm diameter); initial buffer, .01 *M* sodium acetate-acetic acid buffer, pH 5.0; sample amounts, 87 mg; flow rate, 110 ml/h. Elution was done by stepwise method with increasing NaCl concentration in the initial buffer. Eluent was monitored by UV detector at 254 nm.

analysis as in Figure 3. No aggregates of IgG_2 molecules could be observed when pure IgG_2 was analyzed by gel filtration chromatography. Changes in chromatographic patterns of the IgG_2 and Protein A mixture occurred largely within 2 h of mixing. Peaks of larger molecular weight components appeared in front of the eluting position of IgG_2 peak. As shown in Figure 4, which is the plot of the peak area ratio versus incubation time, the first peak (P-1 in Figure 3) that appeared at the position of the void volume of the column showed the presence of large molecular weight complex. The eluting position of the last peak, P-3, was the same as that of monomer IgG_2. The molecular weight of the intermediate complex, P-2, was estimated to be 488,000 daltons. Figure 5 shows the plots of the chromatographic peak heights

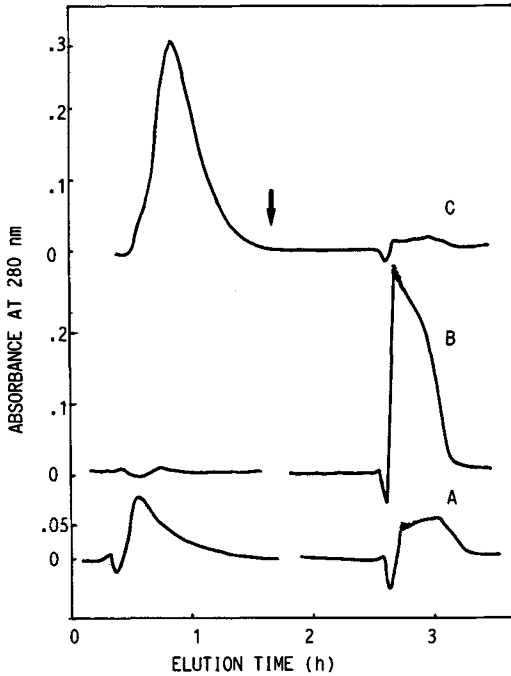

Figure 2. Protein A-Sepharose CL-4B chromatography of bovine IgG_2 fractions from serum and of IgG_1 fraction from colostrum: Column bed volume, 5 ml (8 mm diameter); flow rate, 15 to 20 ml/h. Initially, .1 *M* sodium phosphate buffer, pH 7.0, was used for binding, then the eluant was changed to .1 *M* acetic acid solution for elution at the time marked by arrow. Samples were A) IgG_2 (CM-2) fraction, B) IgG_2 (CM-1) fraction; and C) IgG_1 fraction. Eluent was monitored by UV detector at 280 nm.

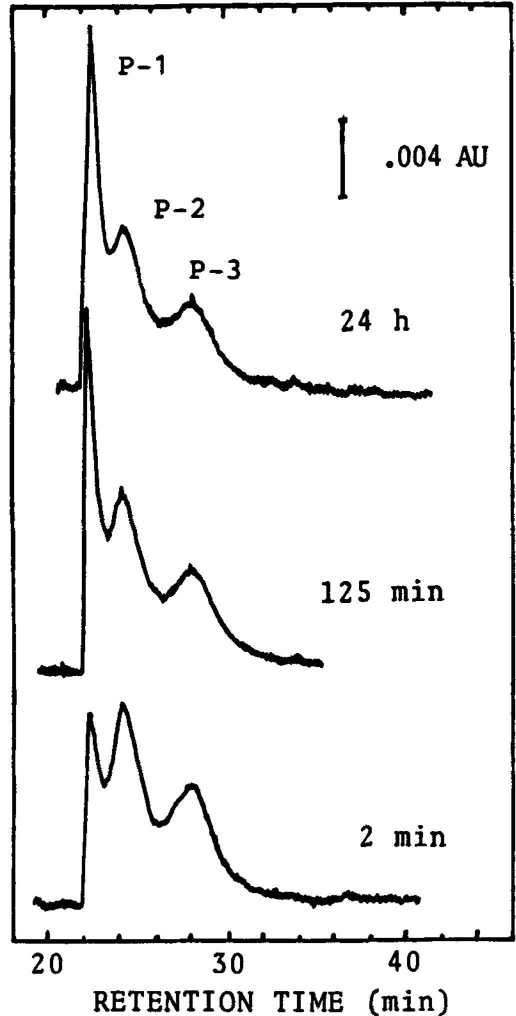

Figure 3. Gel filtration chromatographic profiles of IgG_2 and Protein A mixture at various times (2, 125, and 1440 min) after mixing. Column, TSK gel G3000SW + G2000SW + guard columns; sample volume, .025 ml; IgG_2 concentration, 1.92 mg/ml; IgG_2 : Protein A ratio (wt/wt), 4.8 (mole ratio = 1.4); effluent, .1 *M* sodium phosphate buffer, pH 7.0; flow rate, 1.0 ml/min. Eluent was monitored by UV detector at 280 nm.

when IgG_2 was mixed with Protein A in various mixing ratios. The mole ratio (IgG_2:Protein A) was changed from 1.4 to 7.0. As the absorbance value at 280 nm of Protein A is only one-tenth that of IgG_2, the contribution of Protein A in the absorbance of the chromatographic profiles could be neglected. The binding experiments of IgG_1 to Protein A-Sepharose column showed that IgG_1 was eluted from the column under the same binding conditions (Figure 2C). Also the gel chromatographic pattern of IgG_1 was not altered after mixing with Protein A (data not shown). Therefore, bovine IgG_1 does not form any molecular complexes with Protein A.

Binding Property of Chemically Modified Immunoglobulin G_2

To examine the contribution of histidyl residues in IgG_2 to Protein A binding, carbethoxylation of the residues by DEP was performed. The number of histidyl residues modified

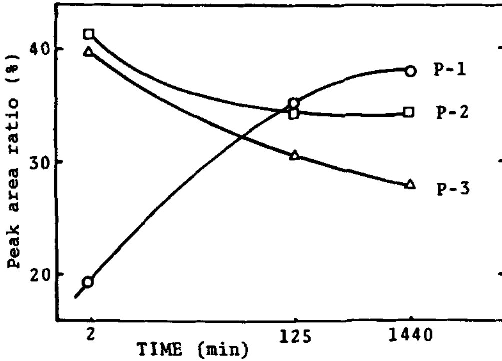

Figure 4. Plot of incubation time versus peak area ratio (%) calculated from Figure 3. Time is shown in logarithmic scale.

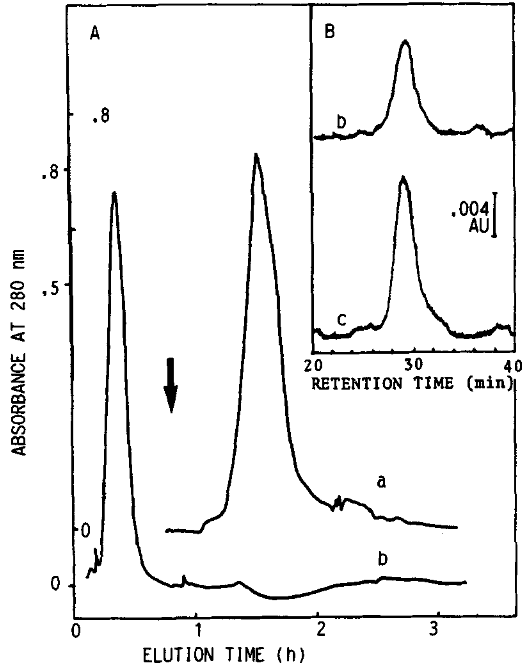

Figure 6. Protein A-Sepharose CL-4B chromatography (A) and gel filtration chromatography (B) of carbethoxylated IgG_2. A) Samples, IgG_2 (a) and carbethoxylated IgG_2 (b); binding buffer, McIlveine buffer, pH 7.0; dissociating buffer, .1 *M* citric acid; flow rate, 15 to 20 ml/h. Arrow denotes the starting point of the gradient elution. Eluent was monitored by UV detector at 280 nm. B) Samples, carbethoxylated IgG_2 (b), and carbethoxylated IgG_2 mixed with Protein A (c); sample volume, .05 ml; IgG_2: Protein A ratio, 1.2. Analysis was carried out at 1 h after mixing. Experimental conditions for the gel filtration chromatography were the same as in Figure 3.

by DEP was estimated to be 8.3 mol/mol of IgG_2. The binding ability of the modified IgG_2 to Protein A-Sepharose column was lost completely as shown in Figure 6A. Also, complex formation with soluble Protein A could not be detected by gel filtration chromatography (in Figure 6B). However, the interacting ability with Protein A was recovered after the modified IgG_2 was subjected to decarbethoxylation. Such behavior of histidyl residues is very similar to that of human IgG (9).

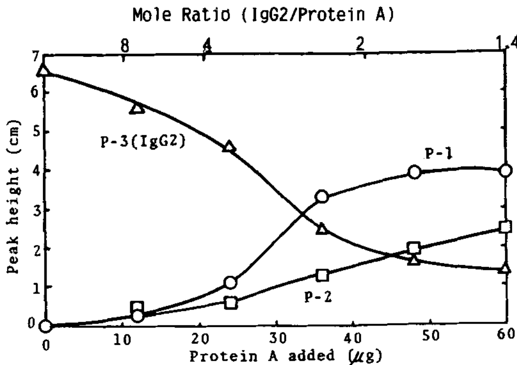

Figure 5. Relationships between the amounts of Protein A added (or IgG_2:Protein A mole ratio) and the height of each peak appeared in the gel chromatographic profiles. The amount of IgG_2 analyzed was 2.9 mg and that of Protein A was from 0 to .06 mg.

DISCUSSION

Results of gel filtration and affinity chromatography showed the soluble complex formation of bovine IgG_2 and Protein A. The apparent molecular weight of one of the complexes was estimated to be 488,000 daltons. By simple summation it could be estimated that the complex is formed with three IgG_2 and one Protein A or with two IgG_2 and four Protein A. The molecular weight of 42,000 daltons for Protein A (18) was used for this estimation. Considering that Protein A has four binding sites for IgG and that IgG has two binding sites for Protein A (11), the IgG_2 :Protein A ratio described cannot be derived, and such ratio has not been reported with IgG-Protein A complexes in any animal

species. Such inconsistency could arise from the fact that Protein A molecule has an extended shape [the frictional ratio is almost 2 (11)], and larger molecular weight values are estimated by hydrodynamic methods.

On the chromatographic patterns of the mixture of IgG_2 and Protein A, a peak eluted at the void volume (P-1 in Figure 3) should be a high molecular weight component but not large enough to precipitate. This may explain why ruminant Ig show weak interaction with Protein A (13, 20). In this case, weak interaction does not necessarily mean that the interacting force is weak but may indicate that IgG-Protein A complex can form only oligomers but not large matrices. In other words, the number of cross-linkages between IgG_2 and Protein A molecules is small. The soluble complex between Protein A and rabbit IgG was reported in detail (10, 11, 15), but not about bovine IgG_2. Precipitin line between bovine IgG_2 and Protein A was observed with counterimmunoelectrophoresis in the presence of polyethyleneglycol (19). However, polyethyleneglycol is one of the most useful protein salting-out agents (1), and molecular interaction in the presence of such additive should be somewhat altered compared to that in native state (6). This observation does not deny the conclusion of the soluble complex formation. The explanation by the electrostatic potential map on the association-dissociation reaction between human IgG and Protein A was proposed recently (2). The molecular orientation may explain the difference between the weak interaction in ruminant's Ig and the strong precipitating interaction in other animals' Ig.

ACKNOWLEDGMENTS

The author thank to K. Sukegawa and Y. Kanamaru for their helpful discussions.

REFERENCES

1 Arakawa, T., and S. N. Timasheff. 1985. Mechanism of poly(ethylene glycol) interaction with proteins. Biochemistry 24:6756.
2 Arata, Y. 1986. Structure and function of immunoglobulins. Tanpakusitsu Kakusan Kouso 31:782. (In Japanese.)
3 Brunner, J. R. 1981. Cow milk proteins: twenty-five years of progress. J. Dairy Sci. 64:1038.
4 Butler, J. E. 1971. Physicochemical and immunochemical studies on bovine IgA and glycoprotein-a. Biochem. Biophys. Acta 251:435.
5 Butler, J. E., and C. F. Maxwell. 1972. Preparation of bovine immunoglobulins and free secretory component and their specific antisera. J. Dairy Sci. 55:151.
6 Cooper, K. M., and M. Moore. 1983. Critical aspects of immune complex assays employing polyethylene glycol. J. Immunol. Methods 60:289.
7 Forsgren, A., and J. Sjöquist. 1966. Protein A from *S. aureus*. I. Pseudo-immune reaction with human gamma-globulin. J. Immunol. 97:822.
8 Goudswaard, J., J. A. van der Donk, A. Noordzij, R. H. van Dam, and J.-P. Vaerman. 1978. Protein A reactivity of various mammalian immunoglobulins. Scand. J. Immunol. 8:21.
9 Haake, D. A., E. C. Franklin, and B. Frangione. 1982. The modification of human immunoglobulin binding to staphylococcal Protein A using diethylpyrocarbonate. J. Immunol. 129:190.
10 Hanson, D. C., M. L. Phillips, and V. N. Schumaker. 1984. Electron microscopic and hydrodynamic studies of protein A-immunoglobulin G soluble complexes. J. Immunol. 132:1386.
11 Hanson, D. C., and V. N. Schumaker. 1984. A model for the formation and interconversion of protein A-immunoglobulin G soluble complexes. J. Immunol. 132:1397.
12 Kronvall, G., U. S. Seal, J. Finstad, and R. C. Williams, Jr. 1970. Phylogenetic insight into evolution of mammalian Fc fragment of gamma G globulin using staphylococcal Protein A. J. Immunol. 104:140.
13 Lind, I., I. Live, and B. Mansa. 1970. Variation in staphylococcal Protein A reactivity with gamma G-globulins of different species. Acta Pathol. Microbiol. Scand. B78:673.
14 Miles, E. W. 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. Page 431 *in* Methods in enzymology. Vol. 47. C.H.W. Hirs and S. N. Timasheff, ed. Academic Press Inc., New York, NY.
15 Mota, G., V. Ghetie, and J. Sjöquist. 1978. Characterization of the soluble complex formed by reacting rabbit IgG with protein A of *S. aureus*. Immunochemistry 15:639.
16 Shimazaki, K., and K. Sukegawa. 1981. Comparison of binding amounts of bovine immunoglobulin G_1 and G_2 to *Staphylococcus aureus*. Nippon Chikusan Gakkaiho 52:425. (In Japanese.)
17 Shimazaki, K., and K. Sukegawa. 1983. Binding properties of bovine milk immunoglobulins with staphylococci. Page 653 *in* Proc. Vth World Conf. Anim. Prod., Tokyo, Jpn.
18 Sjöquist, J., B. Meloun, and H. Hjelm. 1972. Protein A isolated from Staphylococcus aureus after digestion with lysostaphin. Eur. J. Biochem. 29:572.
19 Slone, G. J., and J. E. Butler. 1978. Incomplete inhibition of the immunoglobulin G-binding behavior of nitrated Protein A. Infect. Immun. 21: 659.
20 Surolia, A., D. Pain, and M. I. Khan. 1982. Protein A: nature's universal anti-antibody. Trend Biochem. Sci. 7:74.