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A NOVEL PREPARATION OF IMMUNOADSORBENTS

CONTROLLED COUPLING OF PROTEINS TO SEPHAROSE-GELATIN BY HETEROBIFUNCTIONAL REAGENT

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Summary

The heterobifunctional reagent, *N*-succinimidyl 3-(2-pyridyldithio)propionate, was utilized for controlled coupling of lysozyme and bovine serum albumin to Sepharose-gelatin. Initially the protein (lysozyme or bovine serum albumin) was reacted with N-succinimidyl 3-(2-pyridyldithio)propionate at the free amino groups to give 3-(2-pyridyldithio)propionyl-protein. The latter was reduced to thiopropionyl-protein and was conjugated to 3-(2-pyridyldithio)-propionyl-Sepharose-gelatin through sulfhydryl-disulfide exchange. Sepharose-gelatin-lysozyme and Sepharose-gelatin-albumin were prepared in this manner. They were capable of binding their respective antibody and the eluted antibody was found to be pure on electrophoresis in SDS-polyacrylamide gels and to show heterogeneity by isoelectric focusing. Antibodies bound to Sepharose-gelatin-albumin were found to be less tightly bound to the immunoadsorbent than in the case of Sepharose-albumin, as more antibodies were eluted on the former immunoadsorbent with 0.1 M glycine-HCl (pH 3) than on the latter. The new method permits controlled coupling of proteins to an insoluble matrix (Sepharose-gelatin), and the bond through which reaction occurs is known with precision.

Abbreviations: SDS, sodium dodecyl sulfate; Bis, *N,N'*-methylene bisacrylamide; Temed, *N,N,N,N'*-tetramethylethylenediamine; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; PDP, 3-(2-pyridyldithio)-propionyl.

Introduction

The coupling of soluble ligands and proteins to an insoluble matrix has been a useful technique for the purification of biologically active proteins, e.g. enzymes, hormones, antibodies and antigens. The development of CNBr-activated Sepharose by Axen et al. [1] for coupling proteins and peptides through free amino groups has been the method of choice. Activation of Sepharose by cyanogen bromide was dependent on the conditions of activation, e.g. pH, temperature and concentration of CNBr, and led to the formation of cyanate ester groups [2,3] which were determined colorimetrically by pyridine and barbituric acid [3]. Cyanate esters, which represented 70% of the incorporated nitrogen in freshly activated Sepharose, decreased on storage, with 50% loss after 15 days at 4°C [3]. The reaction between the amino groups of the ligand and activated Sepharose occurred through the cyanate ester to form N-substituted isourea groups [3—5]. Despite the importance of the cyanate esters as intermediates in the coupling of the ligand to activated Sepharose, there was no simple correlation between the concentration of cyanate ester groups and the coupling capacity towards a given ligand [3]. Owing to the difficulty of predicting the coupling capacity of an activated Sepharose preparation for a given ligand, an alternative procedure for coupling proteins to an insoluble matrix which was more controllable was developed. The procedure depends on the use of the heterobifunctional reagent SPDP developed by Carlsson et al. [6]. Lysozyme or bovine serum albumin is reacted with SPDP to form PDP derivatives. On reduction with dithiothreitol, thiopropionyl derivatives of proteins are formed which react with PDP-Sepharose gelatin through sulfhydryl-disulfide exchange to form the immunoadsorbent. Sepharose gelatin is used as a versatile insoluble matrix for the attachment of proteins. Immunoadsorbents prepared with lysozyme or bovine serum albumin are capable of binding antibodies from their respective antisera and the antibodies eluted are free of contaminating serum proteins.

Materials and Methods

Chicken egg-white lysozyme, 3X crystallized, crystallized bovine serum albumin, Triton X-100 and dithiothreitol were obtained from Sigma; Sepharose 4B and SPDP from Pharmacia; lysozyme substrate from Difco Laboratories; gelatin USP from J.T. Baker Chemical Co.; trinitrobenzenesulfonic acid from Nutritional Biochemicals Corporation; Ampholine, pH 3—10, 40% solution from LKB; acrylamide, Bis, Temed, urea and Coomassie Brilliant Blue R-250 were from Bio-Rad.

Reaction of lysozyme, bovine serum albumin and Sepharose-gelatin with SPDP

Lysozyme (3.5 μmol) was dissolved in 10 ml 0.1 M sodium phosphate buffer containing 0.1 M NaCl (pH 7.2) and was reacted with 5 and 10 M excesses of SPDP heterobifunctional reagent in 0.25 ml dioxane. The solution was stirred at room temperature for 30 min, and dialyzed against water followed by phosphate-buffered saline (pH 7.3) (0.01 M phosphate/0.15 M NaCl). Bovine serum albumin (1 μmol) was dissolved in 14 ml 0.1 M sodium phosphate/0.1 M NaCl

(pH 7.2) and then reacted with 5 and 17 M excesses of SPDP in 0.25 ml dioxane at room temperature for 30 min. After completion of the reaction, the solution was dialyzed against water and then phosphate-buffered saline (pH 7.3). Sepharose-gelatin (60 ml packed volume) was diluted to 100 ml with 0.1 M sodium phopshate/0.1 M NaCl buffer (pH 7.2). The suspension was reacted with 20 mg SPDP/0.5 ml dioxane for 30 min and then washed with water and phosphate-buffered saline containing 0.02% sodium azide to remove all soluble reaction products.

Coupling of lysozyme, bovine serum albumin and gelatin to cyanogen-bromide-activated Sepharose 4B

Sepharose 4B was activated by an acetonitrile solution of CNBr in 1 M Na_2CO_3, pH 11.6, for 2.5 min [7]. To 30 ml (5 mg/ml) lysozyme in 0.1 M sodium phosphate (pH 8) was added 40 g wet, activated Sepharose 4B and the suspension was shaken at 4°C for 18 h. The suspension was filtered and the cake was resuspended in 30 ml 0.5 M glycine/0.1 M sodium phosphate buffer, pH 8, and reacted at 4°C for 3 h. After reaction, the Sepharose conjugate was washed with 0.1 M sodium acetate/0.5 M NaCl (pH 4); 2 M urea containing 0.5 M NaCl; 0.4 M glycine-HCl (pH 2); water; phosphate-buffered saline (pH 7.3)/0.02% sodium azide. Sepharose-albumin was similarly prepared by reacting 16 g activated Sepharose 4B with 20 ml (5 mg/ml) bovine serum albumin in 0.1 M phosphate buffer (pH 8). The amount of conjugated lysozyme and bovine serum albumin was determined from the difference in concentration before and after reaction with activated Sepharose calculated from the absorbance at 280 nm. Sepharose gelatin was prepared by reacting 50 g wet, activated Sepharose with 40 ml (5 mg/ml) gelatin in 0.1 M phosphate buffer (pH 8) at 4°C for 18 h. This was followed by filtration, reaction with 0.5 M glycine and washing as described above. The amount of gelatin conjugated was determined from the difference in concentration of gelatin before and after reaction with activated Sepharose as determined by trinitrobenzenesulfonic acid [8].

Reduction of PDP-lysozyme or PDP-bovine serum albumin

The conversion of PDP-proteins to thiopropionyl derivatives was achieved by reaction with dithiothreitol at 0.025 M concentration at pH 4.5 [6]. However, in the present work it was found that the dithiothreitol concentration was high and it required a longer time of dialysis to remove from the reaction mixture. Therefore, the feasibility of reduction of PDP-proteins (without affecting cystine disulfide bonds) at higher pH (pH 6 or 7) using dithiothreitol at 0.007 M final concentration was investigated. Lysozyme and bovine serum albumin were reduced with 0.007 M dithiothreitol at pH 6 or 7 for 30 min, and the free sulfhydryl groups were quantitated by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) as described in detail [16]. The results showed that at pH 6, 0.11 and 0.4 disulfide bond was reduced with dithiothreitol in lysozyme and bovine serum albumin, respectively, and that the values increased to 0.74 and 5.0 at pH 7, respectively. Therefore, a pH 6 and a concentration of 0.007 M dithiothreitol were suitable for reduction of PDP-lysozyme and bovine serum albumin.

10 ml (5 mg/ml) PDP-lysozyme in phosphate-buffered saline was brought to pH 6 with 0.3 M NaH_2PO_4, and an aliquot of 0.1 M dithiothreitol to give a final

concentration of 0.007 M was added. The solution was stirred for 20 min, then dialyzed against fresh amounts of water every 2 h until the dialyzate was free of dithiothreitol as shown by a negative reaction with 5,5'-dithiobis(2-nitrobenzoic acid). Reduction of PDP-albumin was performed at pH 6 on 14 ml (5 mg/ml) PDP-albumin as described above. The solution was dialyzed against water until free of dithiothreitol and then used immediately for conjugation with PDP-Sepharose gelatin.

Conjugation of thiopropionyl-lysozyme and thiopropionyl-bovine serum albumin to PDP-Sepharose-gelatin

PDP-Sepharose-gelatin (30 ml packed volume) was washed with 0.1 M sodium phosphate containing 0.1 M NaCl and was dried by suction on a filter. To the cake was added 10 ml thiopropionyl-lysozyme or 14 ml thiopropionyl-albumin and 10 ml 0.1 M phosphate/0.1 M NaCl (pH 7.2). The suspension was mixed for 30 min at room temperature. The amount of protein conjugated was determined from the concentration of protein before and after conjugation by the Folin phenol method [9]. After conjugation the suspension was filtered and washed with 300 ml phosphate-buffered saline (pH 7.3), 200 ml each of 0.1 M glycine-HCl (pH 2), water, then phosphate-buffered saline/0.02% sodium azide.

Determination of the number of PDP groups coupled covalently either to lysozyme or bovine serum albumin

To 1 ml aliquot of PDP-lysozyme or PDP-albumin in 0.1 M phosphate buffer/0.1 M NaCl (pH 7.2) of known protein concentration (determined by the method of Lowry et al. [9]) was added 0.1 ml 0.1 M dithiothreitol and the absorbance was read at 343 nm. The number of PDP groups was calculated by using a molar extinction coefficient at 343 nm equal to $8.08 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [10] for the liberated pyridine-2-thione.

Analytical methods

Polyacrylamide gel electrophoresis was performed in the dissociating system of SDS-sodium phosphate as described by Maizel [11] and electrophoresis was conducted at 6 mA/tube for 2.5 h. The gels were stained with 0.03% Coomassie Brilliant Blue R 250 in 25% isopropanol and 10% acetic acid for 18 h followed by destaining with 10% isopropanol and 10% acetic acid [12]. Isoelectric focusing was carried out in polyacrylamide gels at 4°C cast in a glass cylinder (0.5 × 10 cm). The aqueous gel solution contained 6.8% acrylamide/0.18% Bis/3.6 M urea/0.08% Temed/1.26% Ampholine, pH 3—10, with 0.03% ammonium persulfate as catalyst. The protein sample was mixed with 1.5 ml of the aqueous gel solution, poured into a glass cylinder and allowed to gel. The upper anode contained 5% phosphoric acid and the lower cathode contained 5% ethylenediamine. The gels were run at 2.5 mA/tube for 2 h and were stained with 0.2% bromophenol blue [13]. The enzyme activity of lysozyme and PDP-lysozyme was based on the rate of lysis of *Micrococcus lysodeikticus* in 0.06 M phosphate buffer (pH 6.2)/0.09% NaCl and was determined by a modification of the method used by Prasad and Litwack [14].

Immunochemical methods

Antisera to lysozyme were from the first bleeding of an immunized goat designated G528-1, from the fourth bleeding of pig 1 designated pig 1—4 and from the sixth bleeding of pig 2 designated pig 2—6. Antisera to bovine serum albumin designated R-HP was obtained from pooled sera of three rabbits [15] and R173-2 was from the second bleeding of rabbit 173. Quantitative precipitin reaction for lysozyme, PDP-lysozyme, bovine serum albumin and PDP-albumin was carried out by adding 0.2 ml of antiserum in phosphate-buffered saline to increasing amounts of the antigen in 0.2 ml volume as described [15].

Isolation of pure antibodies on immunoadsorbents

The immunoadsorbent was packed in a 1 × 4 cm column (Sepharose lysozyme; Sepharose-gelatin lysozyme; Sepharose-albumin and Sepharose-gelatin-albumin). The homologous sera were applied to the columns, mixed and incubated overnight at 4°C. The unabsorbed material was run out and then the column was connected to a fraction collector. The column was washed with phosphate-saline (collecting fractions of 3.6 ml) until absorbance at 280 nm reached 0.01. The absorbed antibody was sequentially eluted (in tubes containing 0.5 ml 1 M Tris-HCl, pH 8.5) with 0.1 M glycine-HCl (pH 3), then with the same buffer at pH 2.5 and pH 2.0. The pooled antibody peaks were dialyzed against water, and then phosphate-buffered saline. The amount of antibody eluted with each eluants was calculated from the volume of the pool and the concentration of the protein determined by the method of Lowry et al. [9].

Results

Reaction of SPDP with lysozyme and bovine serum albumin

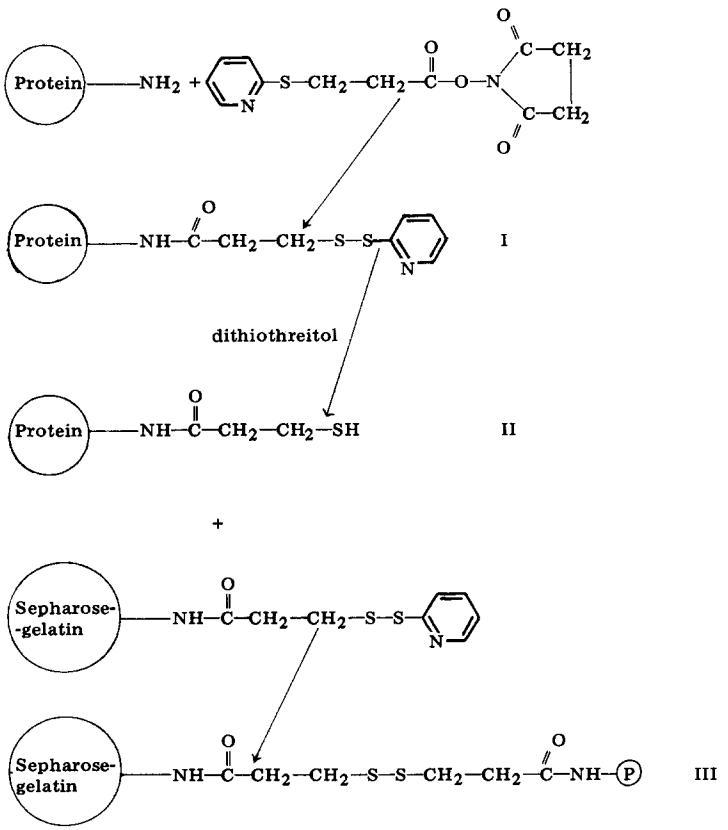
Reaction of SPDP with the free amino groups of lysozyme at 5 M excess introduced three PDP groups. The introduced groups contributed to the absorbance at 280 nm and, therefore, the concentration of PDP-lysozyme was determined by the method of Lowry et al. [9] using a standard curve of lysozyme. A decrease in the solubility accompanied the modification as shown by appearance of turbidity. PDP-lysozyme was solubilized by mixing 50 μl of the turbid protein solution with 50 μl 5% SDS or 50 μl 1% Triton X-100 and diluting with 1 ml phosphate-buffered saline. The solubilization with SDS resulted in complete loss of enzymic activity of PDP-lysozyme, whereas solubilization with Triton X-100 resulted in 100% retention of the enzymic activity when compared to native lysozyme. Therefore, the native conformation was maintained on modification. Moreover, the immunochemical reactivity of PDP-lysozyme was closely similar to lysozyme in that at equivalence 0.146 mg antibody precipitated with PDP-lysozyme, compared to 0.142 mg antibody precipitated with lysozyme. Reaction of SPDP with lysozyme at 10 M excess resulted in modification of five amino groups and was accompanied by low solubility properties, it was partially soluble in 5% SDS, 1% Triton X-100 and 8 M urea.

Reaction of SPDP at 5 molar excess with bovine serum albumin resulted in modification of five amino groups. PDP-albumin had good solubility in contrast to PDP-lysozyme. The immunochemical reactivity of PDP-albumin with

anti-bovine serum albumin serum was not altered significantly from that of native albumin. At equivalence, 0.16 mg antibody precipitated with PDP-albumin compared to 0.15 mg antibody precipitated with bovine serum albumin. Therefore, attachment of five PDP groups to bovine serum albumin resulted in retention of immunochemical reactivity due to maintenance of the conformation at the antigenic determinants. Reaction of SPDP with bovine serum albumin at 17 M excess resulted in modification of thirteen amino groups and retention of its good solubility properties.

Coupling of lysozyme and bovine serum albumin to Sepharose-gelatin

It is to be noted that the coupling was performed on a protein derivative which had similar enzymic and immunochemical properties as the native protein and it proceeded as followes: 1. Reaction of the protein with SPDP; 2. reduction of PDP-protein to thiopropionyl-protein; 3. conjugation of thiopropionyl-protein to PDP-Sepharose gelatin. The scheme of the reaction is shown below:



It is shown that the protein is separated from the Sepharose-gelatin by an arm which may afford good properties to the immunoadsorbent. Coupling was done on two derivatives each of lysozyme and bovine serum albumin which had different degrees of substitution. Table I shows the amount of protein conjugated on the immunoadsorbent.

TABLE I

CHARACTERISTICS OF THE IMMUNOADSORBENTS

Protein used	Protein coupled (%)	Protein coupled (mg/ml packed absorbent)
Sepharose 4B		
Lysozyme	99	3.0
Bovine serum albumin	81	4.05
Gelatin	84	2.7
PDP-Sepharose-gelatin		
Lysozyme-3 *	82	2.4
Lysozyme-5 *	n.d. **	
Bovine serum albumin-5 *	48	1.77
Bovine serum albumin-13 *	34	1.4

* Designates the protein that was conjugated to PDP-Sepharose gelatin as thiopropionyl protein. The number after the protein refers to the number of amino groups in the protein modified by SPDP.
** n.d., not determined.

Isolation of antibodies to lysozyme on the immunoadsorbents

Three antisera to lysozyme were fractionated on three different immunoadsorbents (Sepharose-lysozyme, Sepharose-gelatin-lysozyme-3 and Sepharose-gelatin-lysozyme-5) (Fig. 1; Table II). The elution pattern was similar for Sepharose-lysozyme and Sepharose-gelatin-lysozyme-3. Most of the antibodies were eluted with glycine-HCl, pH 3, and the relative proportion of antibodies (for a given antiserum) eluted with the three eluants was similar for Sepharose-lysozyme and Sepharose-gelatin-lysozyme-3. However, the three antisera showed variations in the relative proportion of the antibodies eluted with the three eluants, indicative of the heterogeneity of the antibody. Pig antilysozyme sera contained only non-precipitating antibodies [17]. Sepharose-gelatin-lysozyme-5, where lysozyme was substituted at five amino groups, was still an efficient immunoadsorbent despite the greater insolubility of the derivatized lysozyme. Polyacrylamide gel electrophoresis in SDS of the eluted antibody from pig 1—4 antiserum on Sepharose-lysozyme, Sepharose-gelatin-lysozyme-3 and Sepharose-gelatin-lysozyme-5 were identical (Fig. 2). In addition to the main band of IgG there were two minor bands of high molecular weight. With G528-1 antiserum the gel electrophoresis pattern of the antibody eluted from

TABLE II

RECOVERY OF ANTIBODY TO LYSOZYME FROM IMMUNOADSORBENT COLUMNS

Serum	% Antibody eluted at a given pH compared to total								
	Sepharose-lysozyme			Sepharose-gelatin-lysozyme-3			Sepharose-gelatin-lysozyme-5		
	pH 3	pH 2.5	pH 2	pH 3	pH 2.5	pH 2	pH 3	pH 2.5	pH 2
Pig 1-4	54.7	29.0	16.3	56.8	26.4	16.8	61.5	19.8	18.7
Pig 2-6	69.8	19.2	11.0	74.0	15.4	10.6	n.d. *	n.d.	n.d.
G528-1	76.0	19.0	5.0	72.5	22.3	5.2	65.1	26.4	8.5

* n.d., not determined.

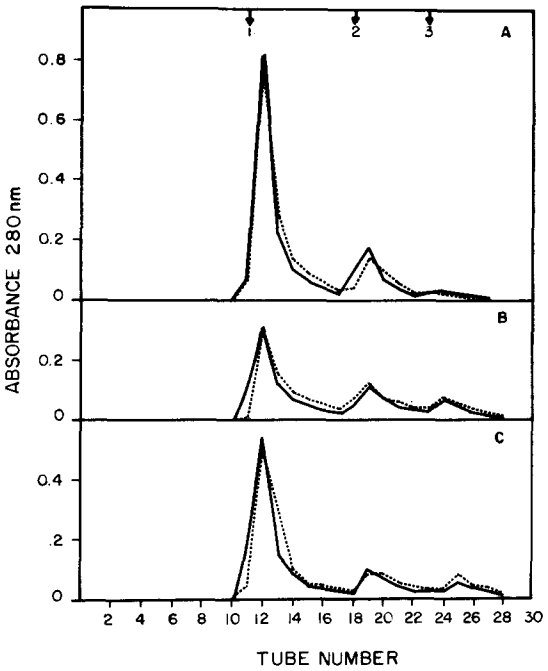


Fig. 1. Elution pattern of antilysozyme sera on immunoadsorbents; - - - - - -, Sepharose-lysozyme; ———, Sepharose-gelatin-lysozyme-3, and elution with 0.1 M glycine-HCl, pH 3, at arrow 1, then changed to pH 2.5 at arrow 2 and finally to pH 2 at arrow 3. Antisera were: A, G528-1; B, pig 1—4; and C, pig 2—6.

the three immunoadsorbents was similar. In addition to the major band of IgG there was a band of a lower molecular weight, indicating cleavage of the IgG molecule and the recovery of an antigen-binding fragment of the IgG. Isoelectric focusing of the antibodies (Fig. 3) isolated from pig 1—4 on the three immunoadsorbents showed a similar pattern, characterized by the appearance of several components. The pattern of the antibodies derived from serum G528-1 with the three immunoadsorbents was similar and would indicate that the immunoadsorbents prepared by the new procedure were effective.

Isolation of antibodies to bovine serum albumin on the immunoadsorbents

Two antisera (R-HP and R173-2) were fractionated on three immunoadsorbents (Sepharose-albumin, Sepharose-gelatin-albumin-5 and Sepharose-gelatin-albumin-13) shown in Figs. 4 and 5 and Table III. More antibody was eluted at pH 3 on Sepharose-gelatin-albumin-5 than on Sepharose-albumin, indicating possibly a favorable conformation of the antigen-antibody which will permit dissociation at a lower pH or the lack of nonspecific adsorption on the matrix. The two antisera showed differences in the capacity of the constituent antibodies to dissociate from the immunoadsorbent at a given pH, e.g. with R173-2 serum all the antibodies were dissociated at pH 2.5, whereas with R-HP serum about 3—5.6% required a pH of 2.0 to dissociate. Disc electrophoresis in SDS of antibodies eluted at pH 3, 2.5 and 2.0 from antiserum R-HP on Sepharose-gelatin-albumin-5 showed a major band of IgG, and possibly two additional high molecular weight components. There was no apparent difference in

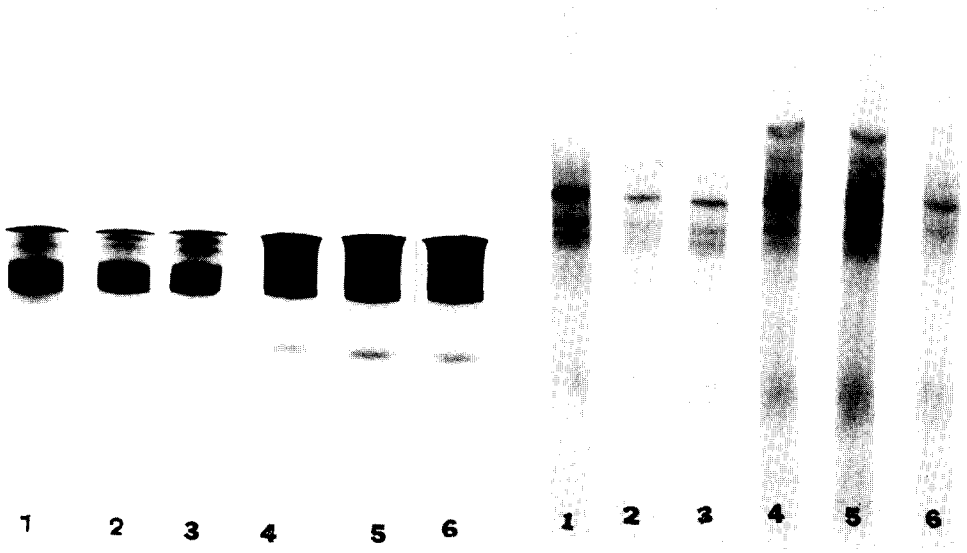


Fig. 2. Gel electrophoresis in SDS of: antibody eluted at pH 2.0 from pig 1—4 antilysozyme antiserum on immunoadsorbent columns is shown in tubes 1—3. Tube 1, antibody eluted from Sepharose-lysozyme; tube 2, antibody eluted from Sepharose-gelatin-lysozyme-3, and tube 3, antibody eluted from Sepharose-gelatin-lysozyme-5. Antibody eluted at pH 2.0 from G528-1 antilysozyme antiserum is shown in tubes 4—6. Tube 4, antibody eluted from Sepharose-lysozyme; tube 5, antibody eluted from Sepharose-gelatin-lysozyme-3, and tube 6, antibody eluted from Sepharose-gelatin-lysozyme-5.

Fig. 3. Isoelectric focusing pattern of antibody eluted at pH 2 from pig 1—4 and G528-1, antilysozyme sera applied on immunoadsorbent columns. Antibody eluted from pig 1—4 antilysozyme antiserum is shown in tubes 1—3. Tube 1, antibody eluted from Sepharose-lysozyme; tube 2, antibody eluted from Sepharose-lysozyme-3 and tube 3, antibody eluted from Sepharose-gelatin-lysozyme-5. Antibody eluted at pH 2 from G528-1 antilysozyme antiserum is shown in tubes 4—6. Tube 4, antibody eluted from Sepharose-lysozyme; tube 5, antibody eluted from Sepharose-gelatin-lysozyme-3 and tube 6, antibody eluted from Sepharose-gelatin-lysozyme-5.

the antibody eluted at pH 3, 2.5 and 2 as similar patterns on gel electrophoresis were obtained. Similarly, the antibody isolated from R173-2 antiserum (on the three immunoadsorbents) showed a major IgG band and two minor components. Isoelectric focusing pattern of antibody from R-HP antiserum eluted

TABLE III

RECOVERY OF ANTIBODY TO BOVINE SERUM ALBUMIN FROM IMMUNOADSORBENT COLUMNS

Serum	% Antibody eluted at a given pH compared to total								
	Sepharose-albumin			Sepharose-gelatin-albumin-5			Sepharose-gelatin-albumin-13		
	pH 3	pH 2.5	pH 2	pH 3	pH 2.5	pH 2	pH 3	pH 2.5	pH 2
R-HP	77.4	17.2	5.6	89.0	8.0	3.0	n.d. *	n.d.	n.d.
R173-2	71.4	28.6	0	79.0	21.0	0	72.0	28.0	0

* n.d., not determined.

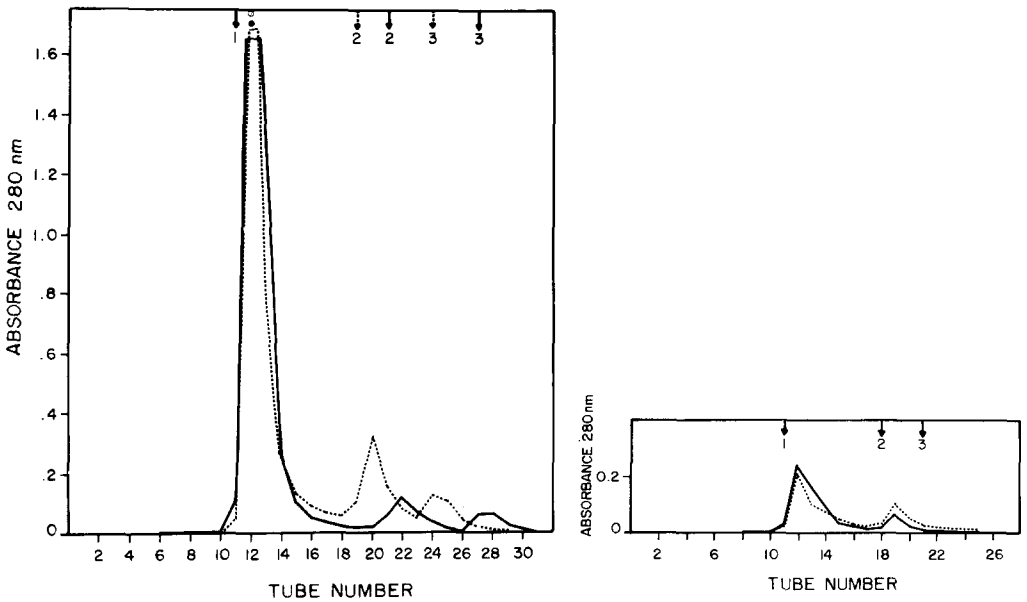


Fig. 4. Elution pattern of anti-bovine serum albumin R-HP on: ·····, Sepharose-albumin with 0.1 M glycine-HCl, pH 3, at arrow 1, then changed to pH 2.5 at dashed arrow 2 and finally to pH 2 at dashed arrow 3. Elution on: ———, Sepharose-gelatin-albumin with 0.1 M glycine-HCl, pH 3, at arrow 1, then changed to pH 2.5 at solid arrow 2 and finally to pH 2 at solid arrow 3.

Fig. 5. Elution pattern of antibovine serum albumin R173-2 on: ------, Sepharose-albumin; ———, Sepharose-gelatin-albumin and elution with 0.1 M glycine, pH 3, at arrow 1, then changed to pH 2.5 at arrow 2, then finally to pH 2 at arrow 3.

with pH 3 showed a very heterogeneous population. Some of the components eluted at pH 2.5 had similar isoelectric points as those eluted at pH 3. Moreover, a large number of antibodies eluted from R173-2 had similar isoelectric points as those obtained from R-HP.

Discussion

Cyanogen-bromide-activated Sepharose [1] has been widely used as an insoluble matrix for attaching various ligands covalently for affinity chromatography. Despite its popularity, there is no simple correlation between the number of cyanate ester groups on activated Sepharose and its coupling ability for ligands [3]. Moreover, there is a lack of agreement on the allowable extent of binding of ligands on activated Sepharose which is compatible with high efficiency. Whereas it was reported [18] that a low degree of substitution was necessary to prevent hydrophobic interactions with nonspecific proteins, Kumel et al. [19] found that a low degree of binding of ligand resulted in a tight binding with the specific protein and a difficulty of desorption of the specific protein from the coupled ligand. Therefore, there was a need to develop a method which permitted controlled coupling of a protein. The present study showed that this was feasible by the use of the heterobifunctional reagent SPDP which was developed by Carlsson et al. [6] and used to form

protein-protein conjugate. This work is different from covalent chromatography developed by Brocklehurst et al. [20] where the 2-pyridyl disulfide group attached to Sepharose 4B formed covalent bonds with sulfhydryl-containing proteins and was used for the isolation and purification of various sulfhydryl-containing proteins [20—22]. Their work did not address itself to the development of immunoadsorbents. In the present work, the insoluble matrix consisted of Sepharose gelatin which was prepared by conjugating gelatin to CNBr-activated Sepharose 4B. By virtue of its poor antigenicity [23], gelatin was chosen since it offered a versatile means for conjugating various proteins to it. The active form of Sepharose-gelatin was generated by reaction with SPDP to introduce PDP groups on gelatin. These groups are very stable as shown by the absence of any decrease in their 2-pyridyl disulfide content at pH 7.5 and 4°C for 3 months [6]. Therefore, it is possible to keep the PDP derivative of Sepharose gelatin in stock at 4°C for the coupling of various proteins or other ligands. As the protein to be coupled is reacted with SPDP, not only is the extent of reaction controlled and quantitated with precision but also the immunochemical and biological activity of the modified protein is assessed for any changes. Reaction of lysozyme with SPDP resulted in two derivatives having three and five substitutions, respectively. The former retained the enzymic and the immunochemical reactivity of native lysozyme. Similarly, the immunochemical reactivity of bovine serum albumin in SPDP-albumin was preserved. The modification may be accompanied by decreased solubility which may be bothersome as was found with lysozyme and which was most likely due to changes in the net charge of the molecule due to substitution of the positively charged ammonium group by an uncharged function. On the other hand, with bovine serum albumin, no change in solubility was apparent. Similar observation was reported by Carlsson et al. [6] for decreased solubility of modified proteins. PDP-lysozyme and bovine serum albumin were reduced conveniently at pH 6 and 0.007 M dithiothreitol concentration.

Coupling of 3-thiopropionyl lysozyme or thiopropionyl-albumin to PDP-Sepharose-gelatin resulted in the preparation of immunoadsorbents which were useful in isolating pure antibodies from the respective antisera. There does not seem to be any advantage for the extensive modification of proteins with SPDP. Lysozyme and bovine serum albumin which were modified at three and five amino groups by SPDP, respectively, served well as ligands for coupling to PDP-Sepharose-gelatin, and effective immunoadsorbents were prepared. Although the binding ability of antibody to Sepharose-lysozyme and Sepharose-gelatin-lysozyme may be similar (by virtue of similarity in elution at pH 3, 2.5 and 2) there was indication that with one anti-bovine serum albumin, the binding on Sepharose-gelatin-albumin-5 was less firm than on Sepharose-albumin by virtue of the elution of 89% antibody on the former immunoadsorbent compared to 77.4% on the latter. Antibodies eluted on Sepharose-lysozyme and Sepharose-gelatin-lysozyme as well as on Sepharose-albumin and Sepharose-gelatin-albumin were similar, as shown by polyacrylamide gel electrophoresis and isoelectric focusing.

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