become a convenient option for protein purification. A major problem with immobilized antibodies is the high affinity of the antigen–antibody complex. Harsh conditions are often used to elute the bound protein from the immobilized antibody. To avoid such destructive conditions, antibodies to contaminating proteins can be immobilized to a matrix allowing selective removal of such contaminants (*2*). Our study utilizes this method of "reverse immunoadsorption" to further purify sulfhydryl oxidase (*3*) and γ-glutamyltransferase (*4*), from contaminating bovine IgG.

Controlled pore glass was used as the immobilization matrix because of its mechanical strength and ease of manipulation and cleaning (*5–8*). Nonaqueous thionyl chloride was used to activate succinamidopropyl-glass (*9,10*) and thus provide a surface reactive with amino and thiol groups.

MATERIALS AND METHODS

Synthesis of Thionyl Chloride-Activated Succinamidopropyl-Glass Beads

Thionyl chloride-activated beads were prepared according to the modified procedure of DuVal et al. (*10*). Controlled-pore glass (CPG) beads (3000 Å pore diameter, 120/200 mesh, Sigma Chemical Co., St. Louis, MO) were cleaned by immersion in two vol of reagent grade concentrated nitric acid in a boiling water bath. After one hour, the beads were washed with distilled water until the pH of the wash was neutral. Finally, the beads were washed several times with acetone to remove any excess water (*11*).

Using the organic method of DuVal et al. (*10*), aminopropyl-glass was synthesized by placing clean beads in a 10% solution of 3-aminopropyltriethoxysilane in toluene for 4 h with occasional stirring in an 80°C water bath. Beads were then washed with fresh toluene and finally with acetone until they appeared white. 2,4,6-Trinitrobenzenesulfonate (TNBS) (0.1% in saturated sodium borate; Sigma) was added to an aliquot of the washed beads to qualitatively detect the appearance of amino groups (*12*). Beads should turn yellow and remain yellow after several washes with distilled water signifying completion of the derivatization of the 3-aminopropyl functionality to the glass bead surface. The remaining aminopropyl glass beads were then succinylated by treatment for 10–20 min in acetone containing 10% (w/v) succinic anhydride (Sigma) and 1% trethylamine (Sigma). After washing the beads with acetone, the TNBS procedure was used to test an aliquot for completion of the succinylation reaction; if all the amino groups were derivatized, no yellow color should develop.

The beads were placed in a round bottom flask and washed by swirling with methylene chloride (Sigma). After decanting the methylene chloride, enough thionyl chloride (Eastman Kodak Company, Rochester, NY) was added to cover the beads and the mixture was allowed to react in a heating mantle for 1 h at 60°C. Finally, the beads were washed several times with methylene chloride until the smell of thionyl chloride (and/or the yellowish color of beads) was removed. Beads were dried (110°C) and stored desiccated at room temperature.

Synthesis of Anti-IgG Glass

Thionyl chloride-activated succinamidopropyl-glass beads (3000 Å) were rehydrated with phosphate-buffered saline (PBS, 0.02*M* sodium phosphate, 0.15*M* NaCl, pH 7.3) and degassed. Two grams of beads were poured into a column and washed with a small volume of PBS (pH 7.3) and then allowed to drain dry before adding immunoglobulin. Affinity isolated rabbit anti-bovine IgG (whole molecule) (Sigma, Lot #114F-8805) in PBS (pH 7.3) were recirculated through the column overnight at 4°C (flow rate = 0.2 mL/min). The protein concentration should be at least 1 mg/mL. The total protein reaction volume should be 1.5–4.5 mL/mL of glass beads, but a minimum of 0.5 mL of solution/mL of beads is acceptable. Following IgG immobilization, 1.0*M* glycine methyl ester (Pierce) in PBS (pH 7.3) was recirculated for about 2 h to block unreacted thionyl chloride-activated sites, after which the column was washed with 2*M* urea in PBS (pH 7.3) and finally with 0.2*M* NaCl in 0.02*M* phosphate buffer (pH 7.3). Column buffer (PBS, pH 7.3) was used for routine washes.

Analysis of Ligand Binding by Analytical Affinity Chromatography

The binding efficiency of the anti-IgG column was characterized by continuous elution analytical chromatography (*13*) in PBS (pH 7.3) on a 3 mm × 50 mm column packed with 0.353 mL of anti-IgG immobilized to succinamidopropyl-glass (3000 Å). Bovine IgG was applied to the column at various concentrations in sequence resulting in characteristic elution curves, each with a unique breakthrough point or first moment. Each individual inflection point (first moment) can be determined using the equation:

$$V = na - a\Sigma P_i / P_0 \quad (1)$$

where n is number of fractions collected, a is volume of each fraction, P_0 is the plateau concentration of ligand, and P_i is concentration of ligand at a given elution volume. Once the inflection point is known for each ligand concentration, both the total number of accessible binding sites for the column and the dissociation constant (K_d) for the bound ligand (anti-IgG) can be determined by the equation:

Table 1
List of Tested Desorption Eluants

0.5 g Triton X-100/L of 0.9% NaCl
0.1*M* acetic acid; 0.5 g Triton X-100/L of 0.9% NaCl
0.1*M* acetic acid pH 2.8
0.02*M* HCl
0.02*M* HCl, 10% ethylene glycol
Glycine-HCl, pH 2.5
Glycine-HCl, pH 2.5, 10% ethylene glycol
Glycine-HCl, pH 2.5, 10% dioxane
Sodium citrate, pH 2.5
8*M* urea

$$1 / (V - V_o) = K_d / M_t + [L] / M_t \quad (2)$$

where M_t is total number of accessible binding sites, [L] is the plateau concentration of applied IgG, and V_0 is the breakthrough volume of an unretarded ligand (e.g., BzTyrOEt; *N*-benzoyl-L-tyrosine ethyl ester).

Examination of Binding and Elution Conditions

Bovine IgG (Calbiochem Corp., San Diego, CA) was radiolabelled with ^{125}I (New England Nuclear, Boston, MA) using Iodo-Gen iodination reagent from Pierce (Rockford, IL) according to the manufacturer's instructions. Following iodination, roughly 90% of the radioactivity was precipitable with anti-bovine IgG.

Anti-IgG matrix (~70 μL) was saturated with ^{125}I-IgG by recirculation (0.2 mL/min) of radiolabeled IgG for 4 h at 4°C. The radioactivity (counts/min) bound to the matrix was determined and the effectiveness of the eluants listed in Table 1 was measured.

Anti-IgG Affinity Chromatography

Sulfhydryl oxidase isolated from bovine milk and covalently isolated on cysteinylsuccinamidopropyl-glass (*7*) and γ-glutamyltransferase isolated from bovine milk (*4,14*) or kidney (*14,15*) were further purified by recirculating (0.2 mL/min) for 4 h at 4°C through a column of anti-bovine IgG immobilized on thionyl chloride-activated succinamidopropyl-glass (3000 Å CPG).

Enzyme-Linked Immunosorbent Assay (ELISA)

Antigen preparations were adsorbed overnight at 4°C to Nunc transferable solid phase (TSP) plates (Vangard Int., Neptune, NJ). Plates were

washed three times with 10 m*M* PBS, pH 7.5, blocked 30 min with 1% ovalbumin (OVA) in 20 m*M* PBS, pH 7.3, and then incubated 1 h at 37°C with rabbit anti-bovine IgG (whole molecule; Sigma) diluted in 0.7% OVA in 20 m*M* PBS, pH 7.3. Plates were then washed three times with wash solution (0.02% Tween 20; Kirkegaard and Perry, Gaithersburg, MD) and incubated 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L; Kirkegaard and Perry) diluted in 0.7% OVA in 20 m*M* PBS, pH 7.3. Finally, plates were washed four times with wash solution and incubated 30 min with substrate [0.4 mg *p*-nitrophenyl-phosphate (Calbiochem, San Diego, CA)/mL in 10% diethanolamine (Sigma) pH 9.8]. TSP plates were removed from the 96-well plates and the color reaction was measured at 405 nm.

Immunoblot Analysis

Antigens were spotted onto nitrocellulose (0.45 μm; Schleicher and Schuell, Keene, NH) in 1-μL aliquots and allowed to dry. Membranes were soaked briefly in 10 m*M* Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), blocked 30 min at room temperature in 1% OVA in TBS, and incubated 1 h at 37°C with rabbit anti-bovine IgG (whole molecule) diluted in 1% OVA in TBS.

Immunoblots were washed three times with TBST and incubated 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) diluted in 0.7% OVA in 20 m*M* PBS, pH 7.3. Finally, blots were washed three more times with TBST and then transferred to color development solution [6.6 μL nitroblue tetrazolium (50 mg/mL in dimethylformamide) and 3.3 μL 5-bromo-4-chloro-3-indoylphosphate (50 mg/mL in dimethylformamide)/mL alkaline phosphatase buffer (100 m*M* Tris-HCl pH 9.5, 100 m*M* NaCl, 5 m*M* MgCl)].

RESULTS AND DISCUSSION

It was independently determined that 11 mg trypsin/g beads could be effectively immobilized to the thionyl chloride-activated beads. In addition, after 15 mo in a desiccator at room temperature, the activated beads had not lost any of their original binding capacity as measured by the capability of immobilizing trypsin activity (data not shown).

The affinity and capacity of the anti-IgG-glass to bind bovine IgG was characterized by continuous elution analytical chromatography. Continuous elution profiles for elution of various concentrations of bovine IgG are shown in Fig. 1. The first moments of these profiles are plotted according to Eq. (2) in Fig. 2, yielding values of $M_T = 1.97$ nmoles and $K_d = 9.45 \times 10^{-7} M$. The value of M_T corresponds to a binding capacity of 0.85 g/L of matrix.

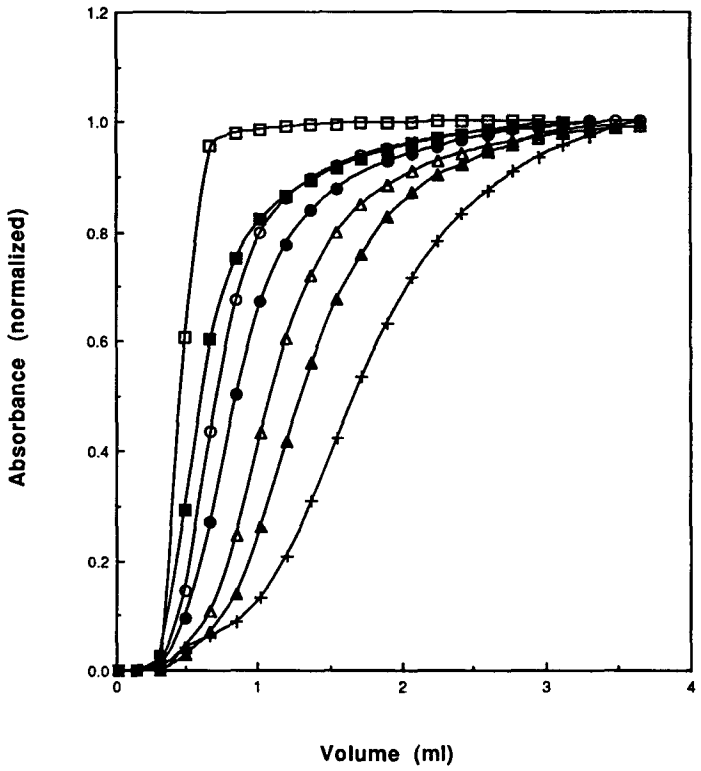

Fig. 1. Elution profiles of various concentrations of bovine IgG passed through anti-IgG immobilized CPG (3000 A). Isocratic elution conditions were PBS (pH 7.3) at room temperature and a flow rate of 0.33 mL/min. Symbols: □ = BzTyrOEt; ■ = 1.02 mg IgG/mL; ○ = 0.60 mg IgG/mL; ● = 0.40 mg IgG/mL; △ = 0.30 mg IgG/mL; ▲ = 0.20 mg IgG/mL; + = 0.15 mg IgG/mL.

Regeneration by various eluting solvents was examined with ^{125}I-bovine IgG. At saturation, 223,470 counts/min were bound to roughly 70 μL of matrix. Washing with 0.1*M* acetic acid solution, pH 2.8, removed 85% of the radioactivity. This eluant was more effective or equally effective when compared to the other eluants tested (Table 1). The column was regenerated repeatedly in these studies using these conditions without any detectable loss of binding capacity.

Preparative application of this matrix for removal of contaminating bovine IgG was examined using preparations of bovine sulfhydryl oxidase and γ-glutamyltransferase. Data obtained using ELISA to detect contaminating IgG in these preparations from bovine milk are presented in Fig. 3, which shows that the immunoglobulins were effectively removed from both sulfhydryl oxidase and γ-glutamyltransferase preparations by passage through the anti-IgG-glass column. The effectiveness of this matrix in the elimination of IgG from γ-glutamyltransferase preparations

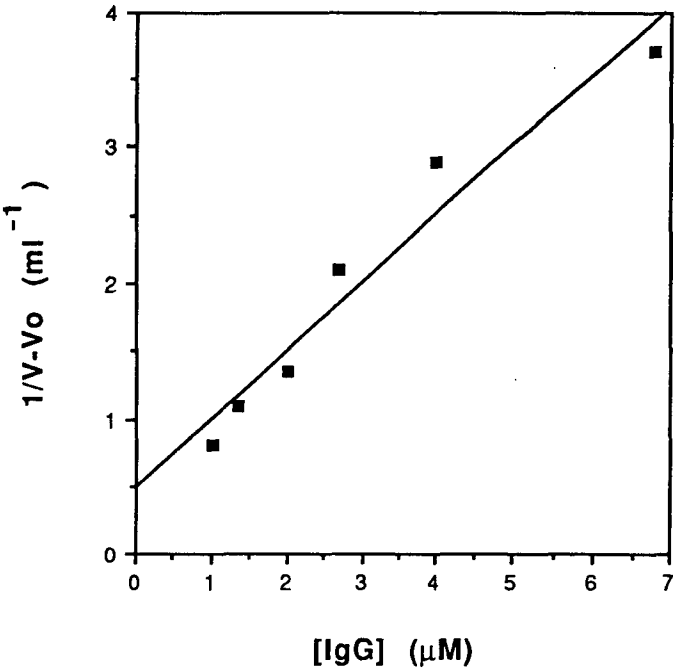

Fig. 2. Linear plot of the reciprocal relationship between the change in first moment and the concentration of ligand (IgG) applied to the immobilized anti-IgG affinity matrix.

from either bovine milk or kidney was also examined. Analysis of contaminating IgG by dot immunoblots, Fig. 4, indicate that no detectable IgG was present in the matrix-treated preparations.

Purification of bovine milk sulfhydryl oxidase or γ-glutamyltransferase has been hampered by the close association of these proteins with contaminating IgG (*7,16*). The reason for the difficulty in removing the remaining small amounts of IgG in these preparations is not known but it should be noted that 85% of milk IgG_2 and 90% of IgG, occur in the whey fraction from which these preparations are obtained (*17*). Furthermore, an interaction between IgG and sulfhydryl oxidase has been suggested based on other evidence although the nature of the interaction is presently unknown (*16*). Hence a mild treatment such as passage through a column containing an anti-IgG matrix that effectively removes the IgG represents a significant improvement in the isolation. Typical data obtained with two sulfhydryl oxidase preparations listed in Table 2 indicate a threefold increase in specific activity with very little loss of total activity.

Another advantage of using the anti-IgG-glass matrix is the capability and ease of regeneration with 0.1*M* acetic acid. The column described was used repeatedly for more than 1 y without significant loss of binding ability.

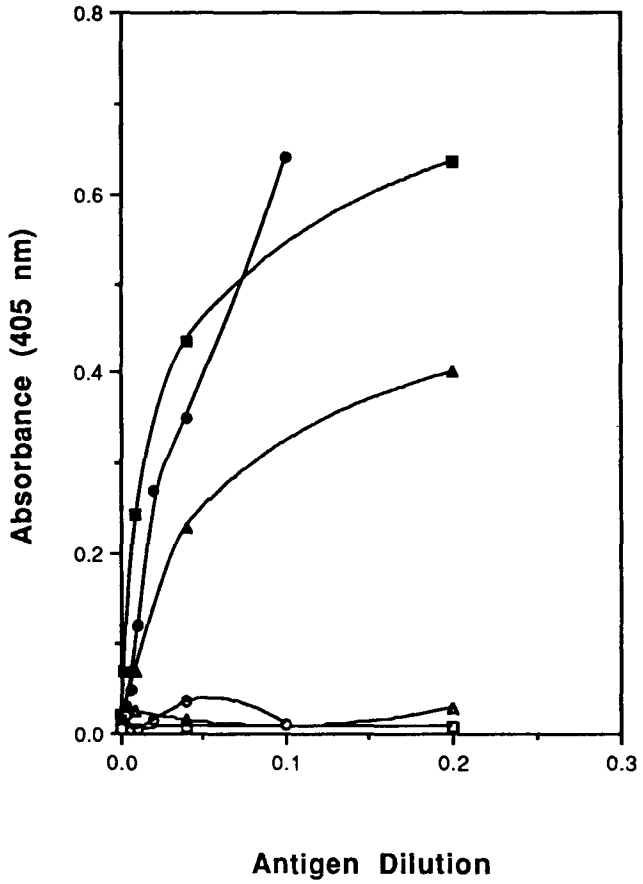

Fig. 3. ELISA determination of the relative amount of contaminating IgG present in cysteinylsuccinamidopropyl-glass purified SOX and purified GGT *before* and *after* treatment with the anti-bovine IgG 3000 Å CPG column. Symbols: ■ = positive control bovine IgG (1 μg/well) before recirculation; □ = positive control IgG after recirculation; ▲, ● = IgG present in SOX and GGT, respectively, before recirculation; △, ○ = amount of IgG in SOX and GGT, respectively, after recirculation through the anti-IgG column. The dilution represents a volume of sample divided by the final volume of its dilution.

These studies further illustrate the utility of thionyl chloride-activation of succinamidopropyl glass or silica matrices for immobilization of proteins or enzymes. We have used this technique both for the preparation of immobilized enzymes (*9,10*) and for affinity columns (*18,19*). Furthermore, the present results reveal that the activated matrix, which was shown previously to be reactive with sulfhydryl or primary amino groups (*9,10*), remains stable over long periods of storage. Immobilization is accomplished simply by adding the protein to the activated matrix in a physiological buffer.

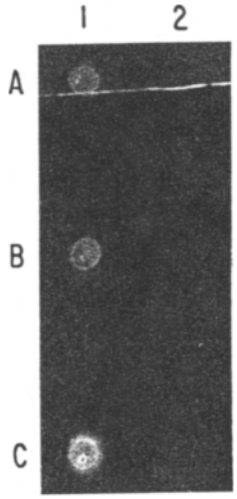

Fig. 4. Dot immunoblot of milk and kidney GGT samples *before* and *after* treatment with the anti-bovine IgG affinity column. Milk GGT before, A1; milk GGT after, A2; kidney GGT before B1; kidney GGT after B2; positive control (bovine IgG), C1; negative control (no antigen), C2. The immobilized proteins were treated with rabbit anti-bovine IgG.

Table 2
Purification of Bovine Skim Milk Sulfhydryl Oxidase Activity by Recirculation Through an Anti-Bovine IgG 3000 Å CPG Matrix (2 Separate Experiments)*

Protein	Treatment with anti-IgG column	Protein concentration, μg/mL	SOX activity, U	SOX specific activity, U/mg	% recovery of activity
Control, IgG	No	7.10	NA[a]	NA	NA
Control, IgG	Yes	0.00	NA	NA	NA
SOX	No	13.90	1.80	10.60	—
SOX	Yes	3.74	1.44	31.28	80
SOX	No	5.00	1.08	18.00	—
SOX	Yes	1.25	0.96	64.00	89

[a] NA = not applicable.
* Control = 7 μg/mL bovine IgG.

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