

Removal of Bovine Serum Albumin from Cow's Milk Using Chicken Egg-yolk Antibodies Immobilized on Chitosan Gel

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Polyclonal chicken antibodies raised against bovine serum albumin (BSA) were immobilized on chitosan gel for the immunoaffinity isolation of BSA from cow's milk. Antibodies (IgY) against BSA were isolated from egg-yolk, purified and antibody reactivity to antigen was measured. IgY developed against BSA was reduced by 2-mercaptoethylamine. The reactivities of reduced and whole IgY against BSA were not significantly different. The reduced IgY was covalently linked to chitosan gel through stable covalent thioether linkages using sulfo-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) as a cross-linker. The density of antibody IgY immobilized on chitosan gel was approximately 3-5 mg per ml of chitosan gel. The ligand-binding capacity of immobilized IgY towards BSA was 0.35-0.44 mg BSA per ml of chitosan gel. A single pass of skimmed milk through the column allowed the removal of BSA from the milk sample. The milk sample was analyzed, before and after immunoaffinity separation, by SDS-PAGE. BSA was desorbed with 0.5 M-glycine-HCl buffer at pH 2.8 but the reusability of the column was limited to three cycles. Alternatively, BSA was desorbed with 0.5 M-glycine-HCl buffer containing 2 M-NaCl at pH 4.6 after longer incubation times at a slower flow rate. The low ligand-binding capacity was not an impediment to reuse of the column. The column was reused more than 20 times with minimal loss of binding capacity.

Keywords: Bovine serum albumin, cow's milk, immunoaffinity isolation, antibodies, chicken, egg-yolk, chitosan gel

INTRODUCTION

Immunoaffinity chromatography is recognized as a powerful tool in protein purification, capable of purifying up to 10 000-fold in a single step (Harlow & Lane, 1988; Labrou & Clonis, 1994). However, the use of affinity chromatography has been limited at the

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preparative scale because of two main problems: the high cost of affinity ligand and instability of the media in a multi-cycle environment.

In a process-scale operation, the most important characteristics of an affinity support are: high adsorption capacity, high mass transfer coefficient, high liquid flow rate through the adsorbent bed and low cost of packing material. Also, when immunoaffinity chromatography is considered for process-scale operations, other important aspects to consider include: the choice of ligand-coupling chemistry, ligand-coupling density, column geometry and flow rate (Subramanian *et al.*, 1994). The high cost of producing large quantities (i.e. 10–100 g) of monoclonal antibodies (MAbs) of the correct epitopic specificity and affinity has prevented their use in large-scale purification procedures (Hearn & Davis, 1990). Lack of control over the orientation and the localized density of the antibody during immobilization could give rise to functionally inaccessible antibody molecules, i.e. the FAb regions are oriented towards the matrix polymer backbone and thus effectively become buried (Velandier *et al.*, 1992), and the high density of localized antibody may cause steric hinderance, restricting access of the antigen to the antibody binding sites. To overcome some of these limitations, spacer arms have been introduced between the matrix and the ligand to enhance accessibility and binding capacity (Hermanson *et al.*, 1992). In affinity-based interactions, ligands play a very significant role in the success of the purification protocol. An ideal ligand should be specific, stable, accessible and economical.

Although rabbits, along with sheep, goat and donkey, are the principal animals used for raising polyclonal antibodies (PABs), the surge of interest in the utilization of chicken egg-yolk immunoglobulin (IgY) in recent years can be attributed to the potentials, concentration, cost and ease associated with its isolation (Shimizu *et al.*, 1988; Ebina *et al.*, 1990; Akita & Nakai, 1993; Hatta *et al.*, 1994). Chicken egg-yolk is an inexpensive source of PABs that can be tailored for many purposes. One egg can easily provide 100 mg of antibody. As a result, large quantities of antibodies can be isolated from a few hens per year.

Dietary cow's milk has been proposed as a possible environmental initiating factor for the development of insulin-dependent diabetes mellitus (IDDM) (Saukkonen *et al.*, 1994). High levels of antibodies to bovine serum albumin (BSA) in children fed cow's milk soon after birth commonly associate with the development of IDDM (Elliot & Martin, 1984; Beppu *et al.*, 1987).

Chitosan, a deacetylated chitin, can be modified chemically and shaped into highly porous, hard gels with a fast flow rate under low pressure, for a variety of biotechnological applications (Shinonaga *et al.*, 1992; Carrara & Rubiolo, 1994). These gels may be manufactured at lower cost than resins presently available since chitin is inexpensive and abundantly available (Freeman & Dror, 1994).

This paper describes the use of chitosan gel on which egg-yolk antibodies raised against BSA were immobilized in an effort to remove BSA from cow's milk without affecting the protein profile of the milk.

MATERIALS AND METHODS

Reagents

Chitosan beads, BCW 3003, bearing amine groups of particle size 300 μm average diameter, 130 $\text{m}^2 \text{g}^{-1}$ average surface area, 150 nm mean pore size and commercially known as Chitopearl, were obtained from Fuji Spinning Co. (Tokyo, Japan). Sulfo-SMCC (sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, catalog number 22322), Ellman's reagent, BCA reagent kit, 2-mercaptoethylamine and disposable 2-ml bed volume plastic columns were purchased from Pierce Chemical Co. (Rockford, IL, USA). PD-10 columns, disposable PD-10 columns, SDS-PAGE gels and molecular weight (MW) markers were obtained from Pharmacia (Quebec, Canada). BSA monomer, MW standards, cysteine hydrochloride, rabbit anti-BSA, rabbit anti-chicken IgG, rabbit anti-chicken IgG

alkaline phosphatase (AP) conjugate and *p*-nitrophenylphosphate substrate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Freund's complete (FCA) and Freund's incomplete (FIA) adjuvants were from Difco (Detroit, MI, USA). Bovine milk examples were obtained from the University of British Columbia Dairy Unit (Vancouver, Canada).

Production and Purification of Anti-BSA IgY

White Leghorn chickens were immunized with 1 ml of 2 mg ml^{-1} BSA in phosphate-buffered saline (PBS), emulsified with FCA, injected intramuscularly at four sites (250 μl at each site) using a 3-cc multi-fit interchangeable glass syringe with luer-lok tips (Becton Dickson, Franklin Lakes, NJ, USA) and 22 gauge 1-inch needles. Booster injections of BSA, emulsified with FIA, were given at 14 and 21 days after the first injections. Eggs were collected and kept at 4°C until used. High titer antibodies were isolated from eggs laid 39 days after the first injection and thereafter (Shimizu *et al.*, 1988). IgY anti-BSA was isolated from egg-yolk as follows. Egg-yolk was separated from egg-white. The yolk was rolled on to a filter paper to remove any adhering egg albumen materials. The thin yolk membrane was ruptured with a needle and the content of the yolk was collected in a volumetric cylinder. A 1:10 dilution of egg-yolk in water was made by bringing the volume close to the final volume followed by stirring for a few minutes to homogenize the solution. The pH of the solution was then adjusted to pH 5.0–5.3 with HCl and the final volume adjusted to give a 1:10 dilution of egg-yolk in acidified water. The yolk solution was left at 4°C for at least 6 h. The solution was centrifuged at 10 000 $\times g$ for 20 min. The supernatant which contained IgY and other water-soluble proteins was separated from the precipitate. To the supernatant was added 19% (w/v) of $(\text{NH}_4)_2\text{SO}_4$ and the solution was left overnight at 4°C. After centrifugation of the solution at 10 000 $\times g$ for 20 min, a precipitate containing IgY (more than 90% purity) was separated from the supernatant. The precipitate was dissolved in 0.1 M-phosphate buffer containing 0.15 M-NaCl and 5 mM-ethylene diamine tetraacetic acid (EDTA) at pH 6. The purity of the prepared IgY was demonstrated by SDS-PAGE performed on a Phastsystem (Pharmacia, Uppsala, Sweden). The activity of anti-BSA IgY against BSA was determined by ELISA (Shimizu *et al.*, 1988). IgY with the highest titer against BSA was selected for binding to the chitosan gel.

Reduction of Anti-BSA IgY

IgY was reduced using 2-mercaptoethylamine as follows. To 10 mg of IgY in 2.5 ml of 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl, 5 mM-EDTA at pH 6 was added 12 mg of 2-mercaptoethylamine and the solution incubated at 37°C for up to 90 min. The reduced antibody solution was desalted using a PD-10 column equilibrated with 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl at pH 7.2. Protein elution was monitored by measuring absorbance at 280 nm using a Shimadzu UV-60 spectrophotometer. Free sulfhydryl groups generated by the incubation of IgY with 2-mercaptoethylamine were determined using Ellman's reagent. The purified reduced antibody was analyzed by SDS-PAGE on homogeneous 20% resolving gel, under non-reducing conditions using a Phastsystem (Pharmacia). The activity of reduced IgY against antigen was measured by ELISA (Shimizu *et al.*, 1988). The reduced antibody was coupled to activated chitosan gel in 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl at pH 7.5 overnight at 4°C (Figure 1).

Preparation of Immunoaffinity Adsorbent and Coupling to Reduced IgY

Chitosan gel was activated with sulfo-SMCC to generate a terminal maleimido group for coupling to reduced IgY as shown in Figure 1. Basically, to 1 ml of chitosan gel in 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl at pH 7.2 were added 10 μmol of sulfo-SMCC. The mixture was gently mixed for at least 2 h at 4°C. The gel was washed

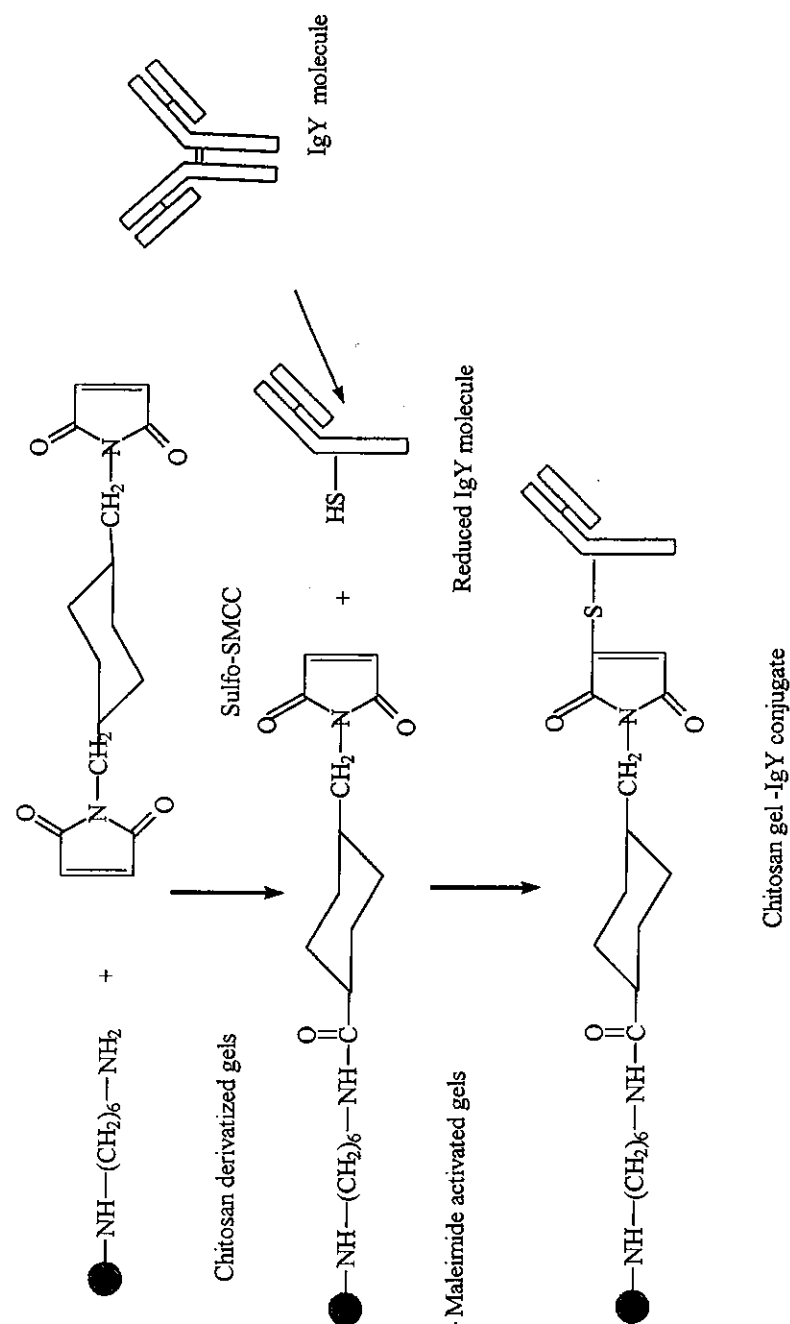


FIG. 1. Site-directed immobilization of reduced IgY molecule to chitosan beads. IgY (4–10 mg) was reduced by 2-mercaptoethylamine in 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl and 5 mM-EDTA at pH 6, at 37°C for 60 min. The reduced IgY was desalted using a PD-10 column equilibrated with 0.1 M-sodium phosphate buffer containing 0.15 M-NaCl at pH 7.5 and coupled to activated chitosan gel.

with cold coupling buffer (0.1 M-sodium phosphate buffer containing 0.15 M-NaCl at pH 7.2), cold 1 M-NaCl and again with cold coupling buffer. The gel, which now contained terminal maleimide groups, was used immediately for coupling to reduced antibody containing sulfhydryl groups as follows. Chitosan gel (1 ml) was incubated overnight at 4°C with increasing concentrations of reduced IgY in coupling buffer. The gel was decanted and washed with coupling buffer until the absorbance of the washing solution was almost zero. Then, 3 ml of BCA reagent, prepared according to the manufacturer's instructions, were added to the gel (Stich, 1990). The content of the tube was mixed gently. A blank was prepared by incubating uncoupled gel with the BCA working reagent. The tubes were incubated in a water-bath at 37°C for 30 min and cooled to room temperature. The supernatant was then decanted into another tube. The absorbance of the supernatant was read at 562 nm against the blank. The concentration of reduced IgY immobilized on chitosan gel was determined using linear regression and a standard curve constructed with BSA.

Following IgY immobilization on chitosan gel, the gel was washed with at least five volumes of coupling buffer and water, gravity packed in an empty 2 ml or 10 ml bed volume plastic column and equilibrated with 20 mM-phosphate buffer at pH 7.1.

Binding Capacity of Reduced IgY Immobilized on Chitosan Gel

Specific antigen binding capacity of antibodies immobilized on chitosan gel was determined by frontal analysis using purified BSA as antigen (Hermanson *et al.*, 1992). Samples of 1 mg ml⁻¹ BSA in 20 mM-phosphate buffer at pH 7.1 in 1 ml aliquots were applied to a 1-ml column, moving fresh tubes after each addition of solution had completely entered the gel. Sample application was stopped when the concentration of BSA in the column effluent equalled its concentration in the original solution. The column was washed with binding buffer until the absorbance at 280 nm was in the range 0.01–0.02.

Different elution buffers were evaluated for their ability to desorb BSA from the column: 0.5 M-glycine-HCl, pH 2.8; 0.5 M-glycine-HCl containing 2 M-NaCl at pH 4.6; 20 mM-sodium phosphate buffer at pH 7.1 containing 2 M-NaCl; 3 M-KCl; 2 M-MgCl₂; 1 M-NaSCN. Fractions were collected and changes in protein concentration in individual fractions were followed by measuring the absorbance at 280 nm. The protein fractions were pooled, lyophilized and reconstituted to the original volume. The amount of BSA in the bound fraction represented the binding capacity, in mg ml⁻¹ chitosan gel, of the affinity matrix constructed. The gel was equilibrated with the application buffer before being reused.

Removal of BSA from Cow's Milk

In a typical run, a 10-ml column was constructed using an empty PD-10 column. The concentration of BSA in skimmed milk samples was measured by ELISA using rabbit anti-BSA 15 as capture antibodies (Kummer *et al.*, 1992). A milk sample containing not more than the ligand-binding capacity of the antibodies immobilized on the gel was passed through the column at a flow rate of 30 ml h⁻¹. Initially, the pH of the milk was adjusted to pH 7.1 but this was found to be unnecessary. Unbound proteins were washed with the equilibration buffer until absorbance at 280 nm dropped below 0.02. The washings were combined with the original sample that passed through the column and lyophilized. The column was eluted with 0.5 M-glycine-HCl containing 2 M-NaCl at pH 4.6 at a flow rate of 20 ml h⁻¹. After reconstitution to the original volume, the concentration of BSA in the eluate was determined by ELISA using rabbit anti-BSA as capture antibodies (Kummer *et al.*, 1992). Milk fractions, before and after chromatographic separation, were analyzed on homogeneous 20% resolving gel under non-reducing conditions using a Phastsystem (Pharmacia). Following electrophoresis, the gel was stained with Coomassie brilliant blue.

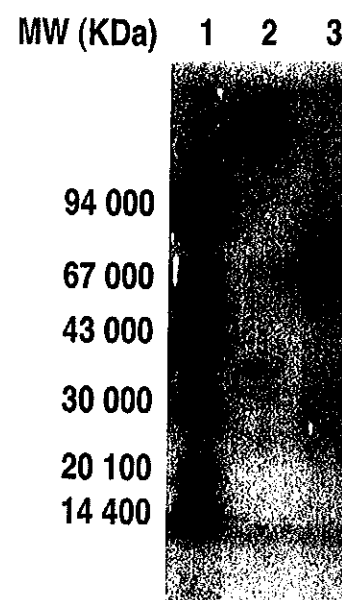


FIG. 2. Molecular weight (mw) profile of reduced IgY molecule measured by SDS-PAGE. Following reduction of IgY by 2-mercaptoethylamine (Figure 1), the MW profile of the desalted reduced IgY was determined by SDS-PAGE. Lane 1 represents molecular weight standards, lanes 2 and 3 represent the control and reduced IgY respectively.

RESULTS

Determination of Free Sulphydryl Groups in Reduced IgY

IgY anti-BSA in phosphate buffer at pH 6.0 was incubated with 2-mercaptoethylamine for up to 90 min. The concentration of free sulphydryl (SH) groups in the reduced IgY was determined using Ellman's reagent. Our repeated experiments showed that 60 min were sufficient to dissociate the IgY molecule into two fragments of equal size (Figure 2). No free sulphydryl group was detected in the native protein. Following reduction with 2-mercaptoethylamine, a maximum of two thiols per IgY was measured. The use of Ellman's reagent to measure the number of SH groups in a molecule is based on the ratio of protein absorbance at 280 nm before the addition of Ellman's reagent to protein absorbance at 412 nm after the additions of Ellman's reagent. Therefore the result obtained is not an absolute number (Hermanson *et al.*, 1992). Our results varied between 1.58 and 2.05 free SH groups per IgY molecule. Shimizu *et al.* (1992) left unanswered the possibility of a second free SH group in the IgY molecule.

Concentration of Reduced IgY Coupled to Chitosan Gel and Specific Ligand Binding Capacity

The reactivity of reduced IgY against BSA before immobilization, reported as ELISA values, was not significantly different from the reactivity of whole IgY against BSA (0.421 ± 0.12 for whole IgY versus 0.416 ± 0.10 for reduced IgY). The concentration of reduced IgY immobilized on chitosan gel, the efficiency of binding and the specific ligand binding capacity are given in Table 1. Various ligand:gel mass ratios were used to make gels with high ligand density. Total IgY concentration on chitosan gel varied between 3 and 5.5 mg of IgY per ml of gel. Binding efficiency varied between 55 and 72%. Only a fraction

TABLE 1. Concentration of IgY immobilized on chitosan gel and ligand-binding capacity

Ligand applied (mg ml ⁻¹ of gel)	Ligand immobilized (mg ml ⁻¹ of gel)	Binding efficiency (%)	Ligand binding capacity (mg ml ⁻¹ of gel)
4	2.89	72	0.43
5	3.62	72	0.35
6	3.91	65	0.35
8	5.61	70	0.39
10	5.48	55	0.44

of the total IgY immobilized on chitosan beads was antigen specific. A two-fold increase in PAb density on chitosan gel (Table 1) did not improve the ligand binding capacity. Hermanson *et al.* (1992) reported that how the target molecule 'sees' and effectively binds the ligand is often more important than the total ligand density. Reduction of IgY was performed to provide free SH groups through which the antibody could be bound to the gel, while preserving its reactivity against its antigen. In antibody immobilization, it is essential to keep the antigen binding site available for binding to an antigen. Chitosan gels were prepared by introducing a spacer containing free primary amine groups. The gels have a pore structure of 0.15 μ m which provides them with perfusive pore characteristics. The concentration of amine groups on chitosan beads was less than 30 μ mol per ml of gel as reported by the manufacturer. Sulfo-SMCC provided a stable thioether linkage between the chitosan gel and the reduced IgY molecule. If the spacer arm is too long and the surface area is small, intramolecular cross-linking can occur. As a result, ligand binding capacity can decrease significantly.

Removal of BSA from Cow's Milk and Reusability of the Column

The best elution of purified BSA was obtained using 0.5 M-glycine-HCl at pH 2.8. However, at pH 2.8, column reusability was limited to three cycles because antibodies are prone to loss of reactivity due to partial denaturation at acidic pH. At pH 2.8 the antibody-antigen complex was easily broken at a flow rate of 30 ml h⁻¹. A chaotropic agent such as 1 M-NaSCN was very effective as eluent but the column binding capacity was lost totally after one pass of NaSCN through the column. The reasons remain unknown. Desorption of BSA with 0.5 M-glycine-HCl containing 2 M-NaCl at pH 4.6 proved effective but required longer incubation times of the complex in the eluent and a flow rate of 20 ml h⁻¹.

BSA was removed from cow's milk by a single pass through a chitosan column. Skimmed milk did not require special pretreatment for passing through the column except for the adjustment of pH to 7.1 which was later on found to be unnecessary. The electrophoretic pattern of a milk sample with and without BSA is shown in Figure 3. The concentration of desorbed BSA was measured by ELISA and was over 97% of the BSA present originally in the milk sample. In a typical separation (Figure 4), a 10-ml column was prepared for BSA removal from milk as described earlier. BSA recovery was between 91 and 95% (Table 2). The large volume of washings and the reconstitution of lyophilized material into a small initial volume may account for some of the loss of desorbed BSA.

The low binding ligand capacity was not an impediment to reuse of the column several times with different milk samples (Figure 5). The ability to reuse a chromatographic column is as important as the binding capacity of the column. Sulfo-SMCC provided stable thioether linkages, between the antibody and the gel, and the column could be regenerated more than 20 times with minimal loss of affinity purification capability.

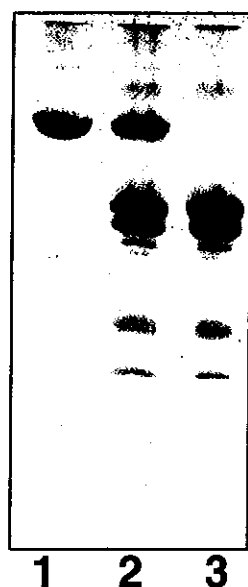


FIG. 3. SDS-PAGE pattern of standard BSA (lane 1), milk fraction before column application (lane 2) and milk fraction after one pass through the immunoaffinity column (lane 3). Electrophoresis separation was carried out on homogeneous 20% gel and the gel was stained with Coomassie blue.

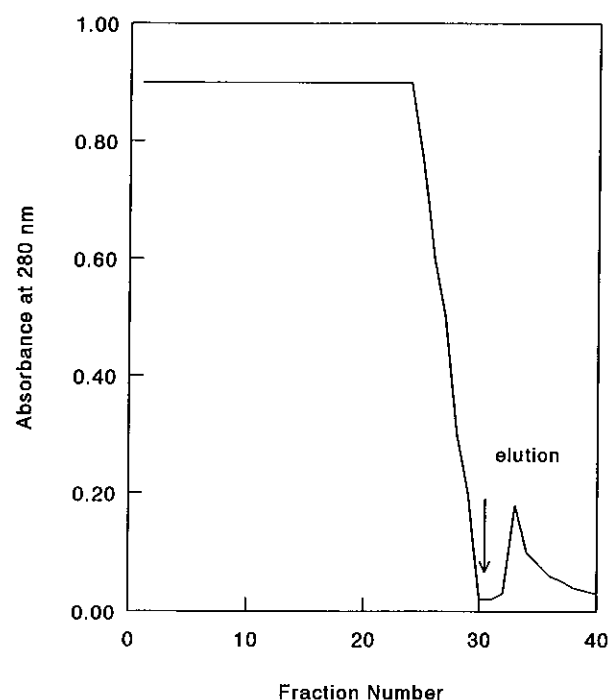


FIG. 4. Removal of BSA from cow's milk. In a typical separation, a milk sample of known BSA concentration was passed through a 10-ml column at a flow rate of 30 ml h^{-1} . The column was washed with 20 mM-phosphate buffer until absorbance at 280 nm was about 0.02. Fractions of 3 ml were collected. BSA was desorbed with 0.5 M-glycine-HCl containing 2 M-NaCl at pH 4.6 at a flow rate of 20 ml h^{-1} .

TABLE 2. Recovery of BSA from chitosan column

BSA concentration	BSA recovered	Recovery (%)
0.33	0.31	95
0.29	0.26	92
0.30	0.28	93
0.62	0.58	95
0.53	0.48	92
0.45	0.41	91

Milk sample, whose BSA concentration was determined by ELISA was adjusted to pH 7.1 and applied to chitosan column. BSA was desorbed with 0.5 M-glycine-HCl containing 2 M-NaCl at pH 4.6.

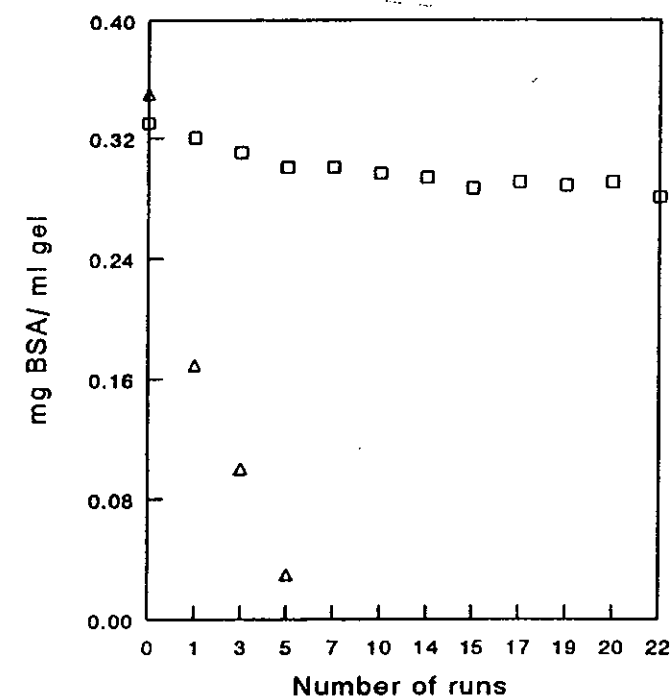


FIG. 5. Stability and reusability of the column. Milk, whose BSA concentration was determined by ELISA, was applied to the column. The column was washed with 20 mM-phosphate buffer at pH 7.1 until the absorbance at 280 nm was 0.02 or less. The column was desorbed with 0.5 M-glycine-HCl at pH 2.8 (Δ) or with 0.5 M-glycine-HCl containing 2 M-NaCl at pH 4.6 ($\square$).

CONCLUSIONS

Chicken Pabs were raised against BSA. The antibodies were isolated from egg-yolks containing higher titer, purified and reduced with 2-mercaptoethylamine. The use of 2-mercaptoethylamine did not destroy the reactivity of the antibody towards its antigen. The reduced antibodies were immobilized on chitosan gel using sulfo-SMCC. The low ligand binding capacity of the immobilized antibody was not an impediment to removal of BSA from milk in a single pass, as long as the sample contained less BSA than the column binding capacity. A column which could be reused more than 20 times was prepared.

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An Enzyme Immunoassay for Buffalo Serum Ferritin

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Buffalo ferritin has been isolated and purified from liver using conventional biochemical techniques such as thermal denaturation, ammonium sulphate fractionation, Sephacryl S-300 gel filtration and DEAE-blue gel affinity chromatography. Native gel-electrophoresis of affinity-purified ferritin followed by iron staining showed a single band corresponding to rat liver ferritin. The yield and the iron content of purified ferritin were 10.7 mg per 500 g of liver and 7% respectively. As a glycoprotein, buffalo ferritin has 12.2% neutral carbohydrate. Polyclonal antibodies raised against purified buffalo liver ferritin in rabbits were used successfully in the development of competitive indirect ELISA using alkaline phosphatase as an enzyme label. The sensitivity range of the assay was 5–30 ng with intra- and inter-assay coefficients of variation (CVs) of 3.1% and 9.2% respectively. The assay was reproducible and could be applied under field conditions for the evaluation of ferritin levels in buffalo. Using this method, the range of circulating ferritin levels in 10 normal buffalo was 1.12–3.68 $\mu\text{g ml}^{-1}$. Cross-reactivity studies by ELISA also suggest the applicability of the method to the evaluation of ferritin levels in other domestic species such as sheep and cattle.

Keywords: Buffalo ferritin, competitive indirect ELISA, cross-reactivity

INTRODUCTION

Ferritin, the iron storage protein with a molecular mass of 450 kDa, has been observed in most cell types in humans and other vertebrates, and also in invertebrates, higher plants, fungi and microorganisms (Theil, 1987). In animal species, it is present in tissues such as the spleen, liver, heart, kidney and blood. However, the concentration of ferritin in the spleen and liver is higher than in other tissues. The iron stored in this protein is an index of body iron reserves (Cook *et al.*, 1974). Ferritin has been isolated, purified and well characterized from several animal species (Page *et al.*, 1980; Farah *et al.*, 1984; Cetinkaya *et al.*, 1985). The protein is known to exist in different isomeric forms (Wagstaff *et al.*,

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