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# Immobilization of antibodies on alginate-chitosan beads

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**Abstract**

An anti-hapten IgG was covalently immobilized on glutaraldehyde-activated alginate-chitosan gel beads. The antibody immobilization efficiency was influenced by glutaraldehyde-bead reaction time, IgG concentration and pH. In addition, immobilization conditions such as glutaraldehyde and antibody concentrations influenced antibody hapten binding affinity. The immobilized IgG on the beads was stable and no reduction in the percent binding to hapten was noticed following 25 days of storage. It was concluded that antibodies could be successfully immobilized on alginate-chitosan gel beads. Such a system can be applied for the development of immunoaffinity purification and immunoassays. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Immobilization; Antibodies; Alginate-chitosan; Beads; Anti-lisinopril IgG

## 1. Introduction

Antibodies immobilized on solid surfaces have been widely used for different applications such as affinity chromatography, immunosensors and diagnostic immunoassays (Lu et al., 1996). The solid surfaces that have been utilized for immobilization of antibodies and proteins are many, such as agarose (Stewart, 1993), latex (Kondo and Teshima, 1995), dextran (Johnsson et al., 1995), sol-gel glass (Wang et al., 1993), quartz (Krapivinskaya et al., 1992), or carbon paste electrode (Fernandez and Costa, 1997).

Two polysaccharides, alginate and chitosan, were also utilized for protein immobilization. Alginate is a natural polysaccharide, non-toxic and forms insoluble gel bead due to its ion binding properties to multivalent cations such as calcium. Alginate beads were utilized to immobilize peptides non-covalently for affinity purification of antibodies and development of analytical immunoassays (Palmieri et al., 1995). The peptides were immobilized by surface adsorption on gel beads or by entrapment in gel beads. Surface adsorption proved to be more efficient for the retention of antigenic determinants.

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Protein adsorption on calcium alginate beads has shown to be affected by pH. The maximum adsorption was found at a pH slightly below the isoelectric point of the protein (Velings and Mestdagh, 1994; Dashevsky, 1998). However, the adsorption was reversible and decreased with increasing ionic strength suggesting that the interaction between proteins and alginate was electrostatic (Velings and Mestdagh, 1994). In addition, the use of calcium alginate gel in protein immobilization has been limited due to its instability upon contact with complex anions, such as phosphate, citrate and lactate, which have high affinity for calcium ions. The instability is also caused by the presence of high concentrations of non-gelling ions, such as $Na^+$ and $Mg^{+2}$ (Smidsrod and Skjak-Braek, 1990). To overcome this limitation, Martinsen et al. (1989) suggested the addition of free calcium ions to the medium while maintaining $Na^+$:$Ca^{+2}$ ratio less than 25:1 for high-glucoronate alginates and 3:1 for low-glucoronate alginates. Stabilization of calcium alginate gels by adding other multivalent ions such as $Ti^{+3}$ and $Al^{+3}$ has also been reported by Smidsrod and Skjak-Braek (1990).

Another property of calcium alginate gel that restricts its applicability to non-covalent protein immobilization is its open lattice structure that leads to protein leakage. Dashevsky (1998) reported that entrapment of the enzyme lactase into alginate beads resulted in 36% loss of the protein. Martinsen et al. (1989) found that minimum 50% of the entrapped proteins are lost from the alginate beads during the 1st h. However, this rate of diffusion decreased with increasing size of the protein. For solutes with molecular weight less than 20 000, Tanaka et al. (1984) found no reduction in diffusion coefficients into calcium alginate gel beads. For larger solutes, such as albumin, γ-globulin, and fibrinogen, however, the diffusion into, but not out of, the gel beads was retarded and depended upon the concentration of alginate and calcium chloride. It was therefore suggested that the structure of calcium alginate gels formed in the presence of large protein molecules was different from that of the gels formed in their absence (Tanaka et al., 1984).

Chitosan is a derivative of chitin, a natural polysaccharide, which can be prepared following *N*-deacetylation of chitin at an alkaline medium (Winterowd and Stanford, 1995). Chitosan was utilized for protein immobilization by covalent coupling of the enzyme β-glucosidase to glutaraldehyde-activated chitosan (Martino et al., 1996). The enzyme exhibited a considerable affinity to chitosan, giving good immobilization yields (55–85%) while maintaining enzyme activity. The immobilized enzyme was more stable than the free enzyme. However, the use of high glutaraldehyde concentration and long glutaraldehyde reaction times had adverse effects on the residual activity of the immobilized enzyme. In addition, urease was covalently immobilized on glutaraldehyde-pretreated chitosan membrane (Krajewska et al., 1990). The optimum immobilization condition was determined with respect to glutaraldehyde pretreatment of membranes and its reaction to urease enzyme. The immobilized enzyme was stable, and retained 94% of its original activity.

When alginate is mixed with chitosan, strong ionic interactions between the carboxyl residues of the alginate and the amino terminals of the chitsan occur to form a polyelectrolyte complex (Takahashi et al., 1990). This complex does not dissolve in the presence of $Ca^{+2}$ chelators or anti-gelling cations, and thus can be used to stabilize the gel and reduce porosity of the alginate beads (Smidsrod and Skjak-Braek, 1990).

In this work, an anti-drug antibody was immobilized on alginate-chitosan beads using a glutaraldehyde covalent binding method. The different parameters affecting the immobilization process, the antibody immunoreactivity and performance were investigated. This conjugation method retains the immunoreactivity of the antibody, which makes it suitable for further immunological reactions.

## 2. Experimental

### 2.1. Materials

Sodium alginate ($C_6H_7O_6Na$) type Protanal

LF120 from Protan Biopolymers of molecular weight 14 000–200 000 was used in this study. Chitosan of molecular weight 8000–12 000 was obtained from Qingdao Rich Waters Industrial Ltd, China. Lisinopril (1-[$N^2$-[(S)-1-carboxyl-3-phenylpropyl]-L-lysyl]-L-proline dihydrate) ($C_{21}H_{31}N_3O_5 \times 2H_2O$) with a molecular weight of 441.52 was supplied by Gedeon Richter, Hungary. Protein A purified anti-lisinopril rabbit antibody was supplied by the Jordanian Pharmaceutical Manufacturing Co., Jordan (JPM, 1998). Protein Micro-assay Kit for protein concentration measurement was purchased from Bio-Rad Laboratories (CA, USA). All other chemicals and reagents were of analytical grade.

### 2.2. Apparatus

A schematic diagram of the experimental setup used to prepare alginate gel beads is shown in Fig. 1. Sodium alginate solution was extruded drop-wise, through a flat-end needle, using a peristaltic pump into a gently stirred cross-linking solution (see below). The bead size was controlled by an air stream flowing coaxial to the needle tip, thus cutting the beads off at the tip of the needle. Air-flow was controlled by a pressure regulator and monitored by a gauge pressure.

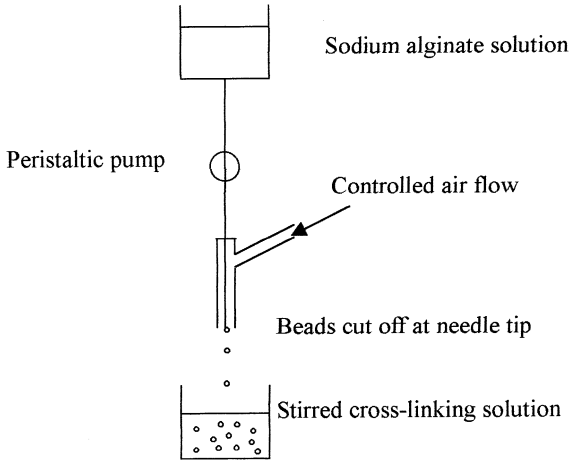


Fig. 1. Schematic diagram of bead formation apparatus.

### 2.3. Experimental procedure

#### 2.3.1. Alginate-chitosan beads formation

Alginate-Chitosan beads were prepared by dripping 1% (w/v) sodium alginate solution into a cross-linking solution composed of 0.1 M $CaCl_2$, 0.5% (w/v) chitosan and 0.5% (v/v) acetic acid. The beads were left in the cross-linking solution overnight and then washed several times with distilled water.

#### 2.3.2. Covalent immobilization of anti-lisinopril IgG on alginate-chitosan beads

Anti-lisinopril IgG was covalently immobilized on the beads as follows: 500 mg of beads were dried on a filter paper to remove excess surface water. The dried average bead mass was determined for 50 beads and found to be 0.51 mg/bead. The weighed beads were soaked in 2 ml of 0.1 M borate buffer, pH 7.2 containing 5 mM $CaCl_2$ and 0.25% (w/v) glutaraldehyde and gently mixed for 60 min. The activated beads were washed four times with 3 ml of 0.1 M borate buffer, pH 7.2 containing 5 mM $CaCl_2$ to remove excess of glutaraldehyde, and then were added to 2.5 ml of 0.2 mg/ml anti-lisinopril IgG in the same buffer. After 60 min of stirring, 0.05 ml of 1 M glycine solution was added to block unreacted glutaraldehyde on the beads (Walter et al., 1980). The beads were left mixing for 60 min. Uncoupled IgG molecules were washed out first with 0.5 M NaCl, followed by three washes with the 0.1 M borate buffer, pH 7.2. The efficiency of antibody immobilization was calculated from the relation:

$$\text{Immobilization Efficiency} = \frac{\text{Total IgG (mg) added-Washed IgG (mg)}}{\text{Total IgG (mg) added}} \times 100\% \quad (1)$$

#### 2.3.3. Lisinopril determination by ELISA

Enzyme-linked immunosorbent assay (ELISA) is used to detect and measure lisinopril concentrations. Anti-lisinopril antibody was developed in rabbits following the immunization with BSA-lisinopril conjugate. The anti-lisinopril antibody was then purified by ammonium sulfate followed by protein A. The ELISA assay for lisinopril was

performed in 96-well plates. The wells were coated with 0.1 ml/well of 12.6 μg/ml ovalbumin-lisinopril conjugate using 50 mM carbonate buffer pH 9.6 containing 0.05% $NaN_3$ at 4°C overnight. The wells then were washed three times with 50 mM Tris–HCl buffer, pH 7.2–7.4 containing 0.4% NaCl, 0.05% Tween 20 and 0.02% $NaN_3$ and blocked with the same buffer for 60 min. Lisinopril standard solutions (0.1 ml/well) were added in triplicates followed by 0.1 ml of anti-lisinopril antibody (3 μg/ml) in 50 mM borate buffer, pH 7.2–7.4 containing 1% BSA, 0.05% Tween-20 and 0.2% sodium azide, and left incubated with mixing for 120 min at room temperature. The wells were washed again followed by the addition of 0.1 ml of goat anti-rabbit alkaline phosphatase conjugate diluted in 50 mM Tris–HCl buffer pH 7.2–7.4 containing 1% BSA, 0.05% Tween-20 and 0.2% sodium azide, and left incubated for 60 min at room temperature. The wells were washed again and 0.1 ml of 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer containing 0.01% $MgCl_2$, pH 9.6–9.8, was added to each well. The substrate was left for 30 min and a yellow color was developed. The reaction was stopped by adding 0.1 ml of 2 M NaOH and the optical density was measured at 405 nm using ELISA plate reader. From the optical densities, the percent bindings were calculated and were plotted versus the corresponding standard lisinopril concentrations to create a standard calibration curve used to determine the unknowns. The minimum concentration of lisinopril (sensitivity) that can this method detect without interference is 2 ng/ml. The intra- and inter-% CV of all standard points do not exceed 8%.

#### 2.3.4. Immobilized anti-lisinopril and lisinopril binding

The binding of immobilized antibody to lisinopril was measured by incubating 500 mg IgG-bound beads with 2 ml of 0.5 μg/ml lisinopril solution in 0.1 M borate buffer, pH 7.2, for 120 min with gentle mixing at room temperature (25°C). The beads were washed four times with 2 ml borate buffer, pH 7.2, per wash. Using the competitive ELISA method presented above, the amount of bound lisinopril was calculated from the difference between the initial amount of lisinopril added in the reaction mixture and that in the wash solution.

Based on the assumption that each anti-lisinopril IgG has two binding sites for lisinopril, the efficiency of anti-lisinopril IgG binding was determined as the percentage of the number of moles IgG binding sites bound to lisinopril relative to the total moles of IgG binding sites available:

$$\text{IgG binding efficiency} = \frac{\text{Moles of bound lisinopril}}{\text{Total moles of IgG binding sites}} \times 100\% \quad (2)$$

The relative IgG-lisinopril binding was then determined as ratio of the IgG binding efficiency for the tested condition versus the reference method, which includes all the best conditions (see above) for the IgG immobilization and lisinopril binding. It has to be mentioned that when each condition was tested, the reference method was also performed. The relative anti-lisinopril IgG-lisinopril binding can be expressed mathematically as:

$$\text{Relative IgG binding} = \frac{\text{IgG binding efficiency at the condition tested}}{\text{IgG binding efficiency in reference method}} \times 100\% \quad (3)$$

### 2.4. Factors affecting antibody immobilization and binding

The following factors affecting the immobilization reaction were studied: glutaraldehyde-bead reaction time, glutaraldehyde concentration, antibody concentration and immobilization pH. In addition, the relative immobilized antibody-lisinopril binding was calculated taking into consideration the following factors: antibody-lisinopril reaction time, temperature and pH. Each experiment was performed at least three times.

Control experiments were run to check for the release of the immobilized antibody from beads. This was done by incubating the antibody-bound

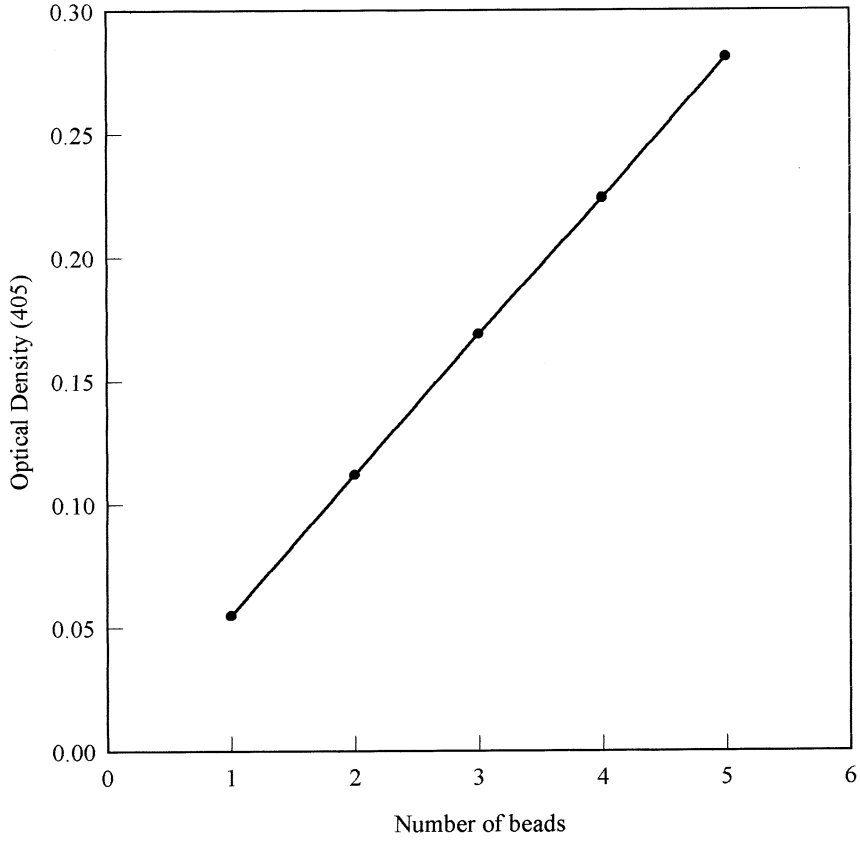


Fig. 2. The presence of rabbit IgG on beads checked by ELISA as a function of the number of beads. Each point represents the mean of triplicate readings.

beads in borate buffer, pH 7.2 for 120 min followed by assaying the buffer for any leakage of antibody by ELISA. In addition, to detect any non-specific binding of lisinopril to beads or diffusion into beads, the same procedure was followed using blank beads without antibody.

### 2.5. Statistical analysis

Statistical differences between conditions and factors affecting antibody immobilization and binding were assessed using paired $t$-test at a 99% two-tailed confidence interval (99% CI). In the $CaCl_2$ effect study, the % CV of each antibody concentration point is in the range of 1–8%, averaging 5%. Therefore, in order to reject the null hypothesis the mean difference of the % binding points should be more than 5.

## 3. Results and discussion

### 3.1. Antibody immobilization on alginate-chitosan beads

The presence of rabbit IgGs on the beads following covalent immobilization was confirmed by ELISA conducted on such beads. The results given in Fig. 2 show the optical density produced relative to the number of beads used. Blank beads as negative control were used and gave no signal. The linearity of the relationship is an evidence of the uniformity of the immobilization of rabbit IgGs on the beads (Fig. 2).

When the antibody was coupled to beads, it was observed that the beads had swollen and softened. This could be attributed to the removal of calcium from the beads to bind to the antibody

molecules due to fact that calcium ions can bind tightly to proteins (Stryer, 1987). This apparent loss of calcium caused swelling of the beads and reduction in their stability. This result is consistent with the results of Velings and Mestdagh (1994). The problem of bead stability due to immobilized antibody was overcome by adding 5 mM $CaCl_2$ in the reaction buffer. This concentration was found to be the best because it maintained bead's stability without affecting the immunoaffinity of the antibody conjugated to the beads. It was noticed that the presence of $CaCl_2$ in the assay buffer shifted the curves to the right as shown in Fig. 3. The effect was clear at concentration of 10 mM $CaCl_2$ ($P < 0.0001$, 99% CI; 11–21), while at lower concentration (5 mM), the effect was less (99% CI; 0.67–5.3). Such shift indicates reduction in antibody affinity, as given by the reciprocal of the antibody concentration at 50% binding ($P < 0.0001$). This reduction can be explained by binding of $Ca^{+2}$ to the proteins, enabling it to form cross-linking between different segments of the antibody and therefore, induce conformational changes that affected the antigen-binding site of the antibody.

### 3.2. Immobilization reaction conditions

The effect of the glutaraldehyde-bead activation time on the antibody immobilization efficiency and relative IgG-lisinopril binding affinity is shown in Fig. 4. The IgG immobilization efficiency increases as the reaction proceeds for up to 60 min. For periods longer than 60 min, the immobilization efficiency was not affected and remained constant. When the glutaraldehyde-bead activation reaction lasted for 15 min, the antibody immobilization efficiency was 70%, whilst the

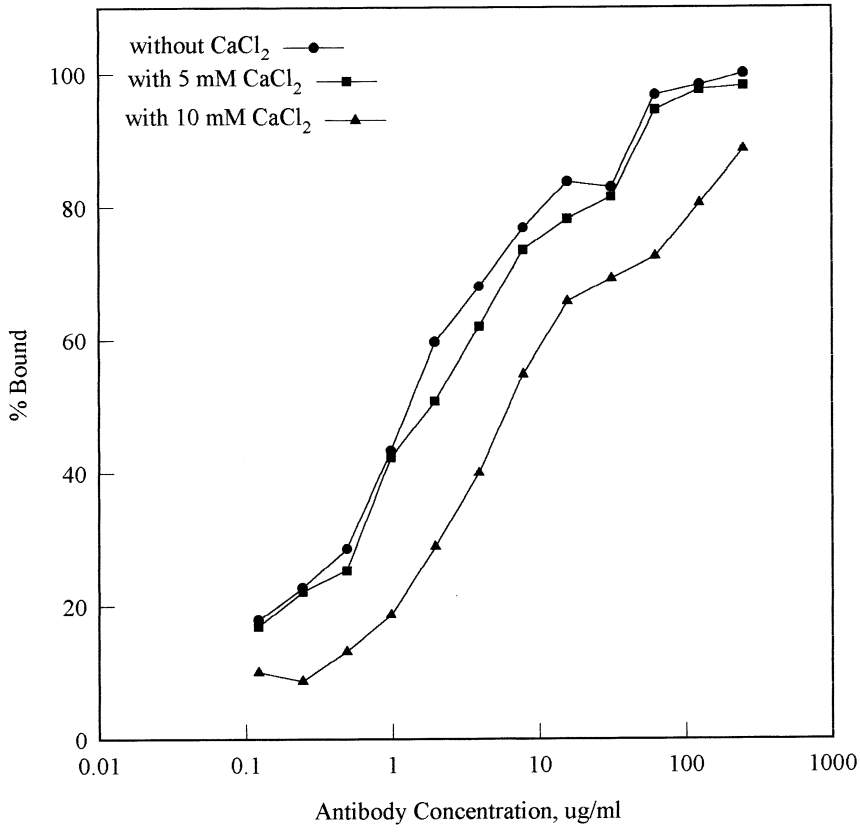


Fig. 3. Effect of $CaCl_2$ concentration on antibody dilution curves. Each point represents the mean of triplicate readings.

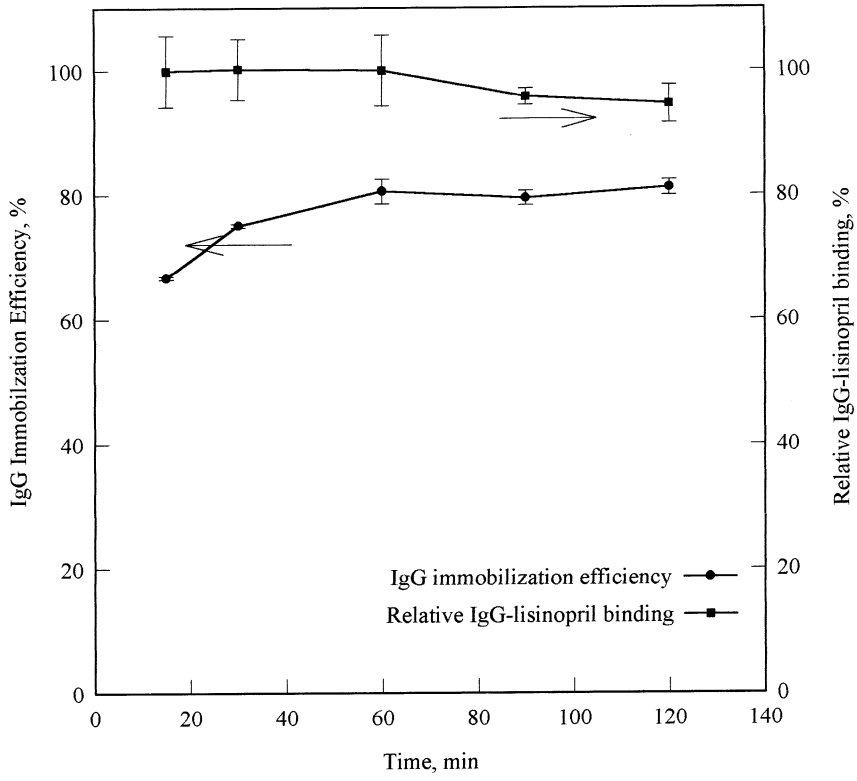


Fig. 4. Effect of glutaradehyde-bead reaction time on IgG immobilization efficiency and relative IgG-lisinopril binding. Each point represents the mean of triplicate readings ( ± S.D.).

maximum immobilization efficiency obtained was 80% after 60 min reaction time. This shows that the reaction was fast initially but then slowed down as the reaction proceeded for longer times.

As for the IgG-lisinopril binding, it was almost independent of the glutaraldehyde-bead activation time. However, the glutaraldehyde-bead reaction time did not influence the immobilized IgG-lisinopril binding affinity. This indicates that the IgG molecules were coupled in a way that did not affect their lisinopril-binding affinity.

The glutaraldehyde concentration used for bead activation did not affect the immobilization efficiency. However, the relative IgG-lisinopril binding decreased with increasing glutaraldehyde concentration (Fig. 5). Constant immobilization efficiency can be explained by considering the fixed surface area of the beads available for coupling, which was constant for all the runs. However, the relative IgG-lisinopril binding affinity was high at glutaraldehyde concentrations of 0.25% or less, while at higher concentrations, there was a significant drop in the binding affinity. As the quantity of IgG immobilized was constant, excess number of aldehyde groups available for coupling IgG molecules were available, consequently the excess aldehyde groups available to bind per IgG molecule might have resulted in deformation, unfolding or surface modification of the IgG molecules. Hence, the antigen-binding site of the IgG molecule might be inactivated, leading to reduced binding to lisinopril. Similar results were reported by Martino et al. (1996) who immobilized β-glucosidase on chitosan by glutaraldehyde. They found that increasing glutaraldehyde concentration increased the quantity of the enzyme immobilized, but with reduced activity.

The effect of antibody concentration used in the immobilization reaction is shown in Fig. 6.

IgG immobilization efficiency decreased as the concentration of IgG increased to 0.2 mg/ml, and remained almost constant at higher antibody concentrations. The relative IgG-lisinopril binding affinity was 100% at antibody concentrations at or less than 0.2 mg/ml and decreased as the antibody concentrations increased. It means that at IgG concentration up to 0.2 mg/ml, the surface area of the beads available for binding to IgG is maximum, thus becoming saturated. As the IgG concentration increased above 0.2 mg/ml, the immobilization efficiency was independent of the antibody concentration. Apparently, at high protein concentrations, protein-protein interactions occur (Deshpande, 1996), where such interactions involve weak binding forces leading to multiple IgG layers to be formed on the bead surface and increasing the quantity of immobilized IgG molecules on beads.

The immobilization efficiency increased with increasing the pH (Fig. 7). At acidic pH both amino groups of the chitosan ($pK_a = 6.3$) (Huguet et al., 1996) and those of the antibody ($pK_a \geq 8.33$) (Harlow and Lane, 1988) exist as $NH_3^+$. Since aldehyde groups are targeted to $NH_2$ and not $NH_3^+$, the number of the $NH_2$ groups on chitosan molecules available for coupling to glutaraldehyde would decrease by reducing the pH. The same thing applies to IgG molecules reacted with activated beads. Therefore, the immobilization efficiency decreased as the pH became more acidic. At pH > 7.0, immobilization efficiency was constant. Regarding the effect of immobilization pH on relative IgG-lisinopril binding affinity, the results are complicated as shown in Fig. 7. At pH below 6, the quantity of lisinopril bound to the beads was greater than the expected, which normally saturate all the binding sites of the immobi-

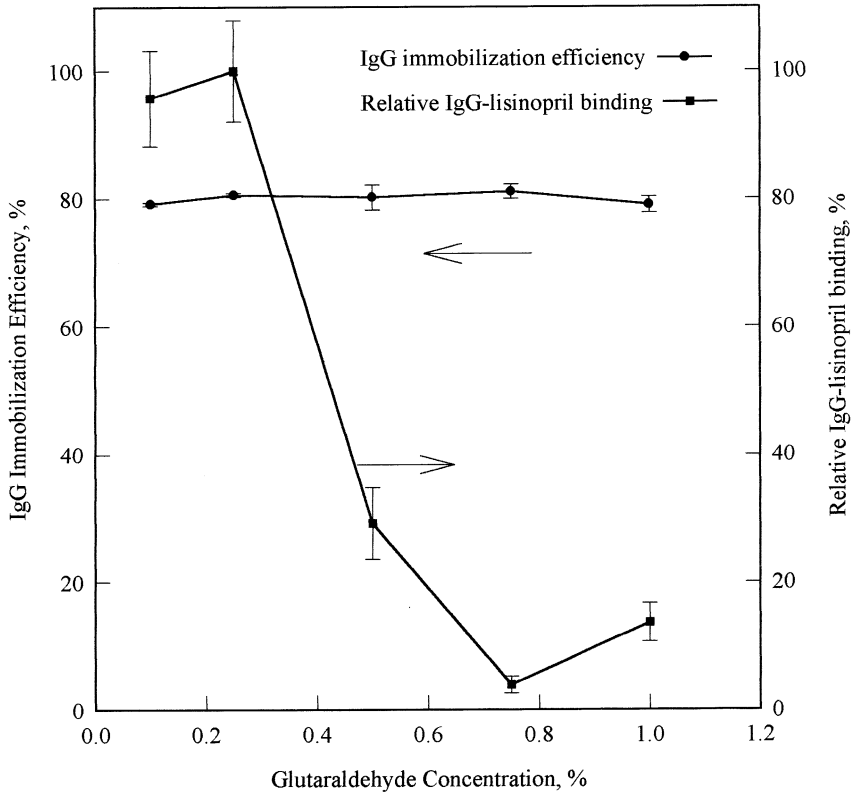


Fig. 5. Effect of glutaradehyde concentration on IgG immobilization efficiency and relative IgG-lisinopril binding. Each point represents the mean of triplicate readings ( ± S.D.).

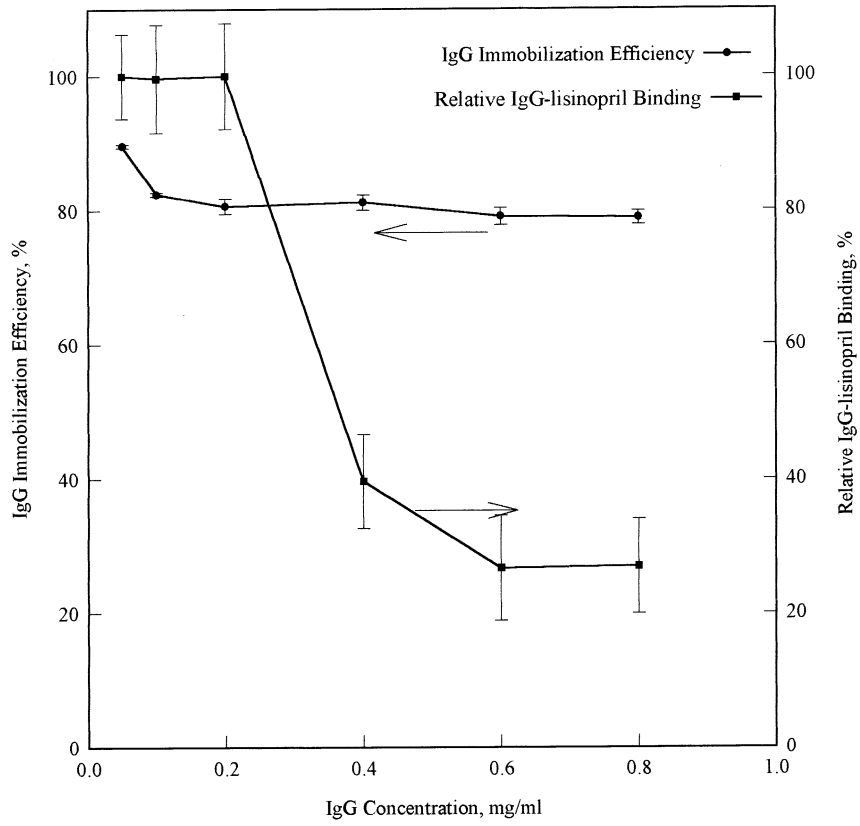


Fig. 6. Effect of antibody concentration on IgG immobilization efficiency and relative IgG-lisinopril binding. Each point represents the mean of triplicate readings ( ± S.D.).

lized IgGs. This result can be explained by considering the blocking step of the unreacted glutaraldehyde on the activated beads by glycine. Since the immobilization efficiency, and hence the quantity of immobilized IgG, was low below pH 6 as discussed earlier, some of the glutaraldehyde coupled to the beads would have their uncoupled end free for coupling. When glycine was added to block the uncoupled end of the glutaraldehyde, the amino groups of the glycine ($pK_a = 9.6$) (Harlow and Lane, 1988) were not able to react with the aldehyde due to reaction medium low pH. When lisinopril was subsequently added to the antibody-beads conjugate at assay pH 7.2, the amino groups of lisinopril ($pK_a = 10.1$) (Ip et al., 1992) was available to react with any free aldehyde group of glutaraldehyde. Thus the relative IgG-lisinopril % binding was $> 100\%$ because some of the lisinopril molecules bound to the antibody and others bound to the beads via glutaraldehyde linkage. In the pH range 6–9, however, the quantity of lisinopril bound to antibody was the same and the results showed that the best binding occurred when the immobilization was carried out at pH 7.2.

### 3.3. Stability

The immobilized IgG on the alginate-chitosan beads was tested for its stability in different conditions for up to 25 days (Fig. 8). The IgG-immobilized beads stored in borate buffer, pH 7.2 at 4°C maintained their binding to lisinopril without showing any loss of activity. However, a mild reduction in % binding to lisinopril was noticed when the antibody-immobilized beads were stored

either at 4°C in dry conditions or at 30°C in borate buffer. Moreover, storing the IgG-immobilized beads in dry conditions at 30°C markedly reduced the antibody binding to lisinopril after 20 days of storage (Fig. 8) ($P < 0.005$). Such combined conditions, dryness and high temperatures, could have destabilized and denatured the antibody, respectively, and thus reduces its binding capability.

## 4. Conclusions

IgG antibody has been covalently bound to alginate-chitosan beads using glutaraldehyde. The best conditions of the proposed immobilization procedure were determined with respect to glutaraldehyde-bead coupling and to antibody. The antibody immobilization efficiency was influenced by glutaraldehyde-bead reaction time, IgG concentration and pH. In addition, immobilization conditions such as glutaraldehyde and antibody concentrations influenced antibody hapten binding affinity. The immobilized IgG on the beads was stable and no reduction in percent binding to hapten was noticed following 25 days of storage. Since the results have shown that the antibody affinity was affected by the presence of calcium ions, it would be advisable to use a gel support that does not require calcium ions for its preparation.

The successful immobilization of antibodies on alginate-chitosan beads provides a good system for the immunoaffinity purification and development of solid-phase immunoassays. Optimizing alginate-chitosan interaction forms a good solid matrixes especially if a micron-size gel beads or thin films are used. These gel beads can be used as

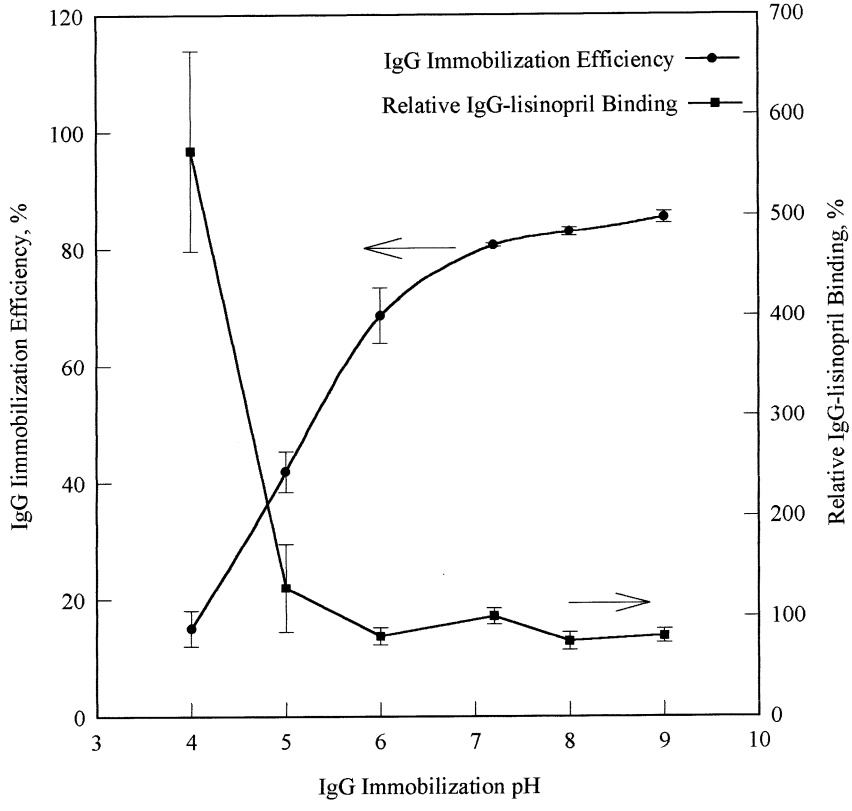


Fig. 7. Effect of pH on IgG immobilization efficiency and relative IgG-lisinopril binding. Each point represents the mean of triplicate readings ( ± S.D.).

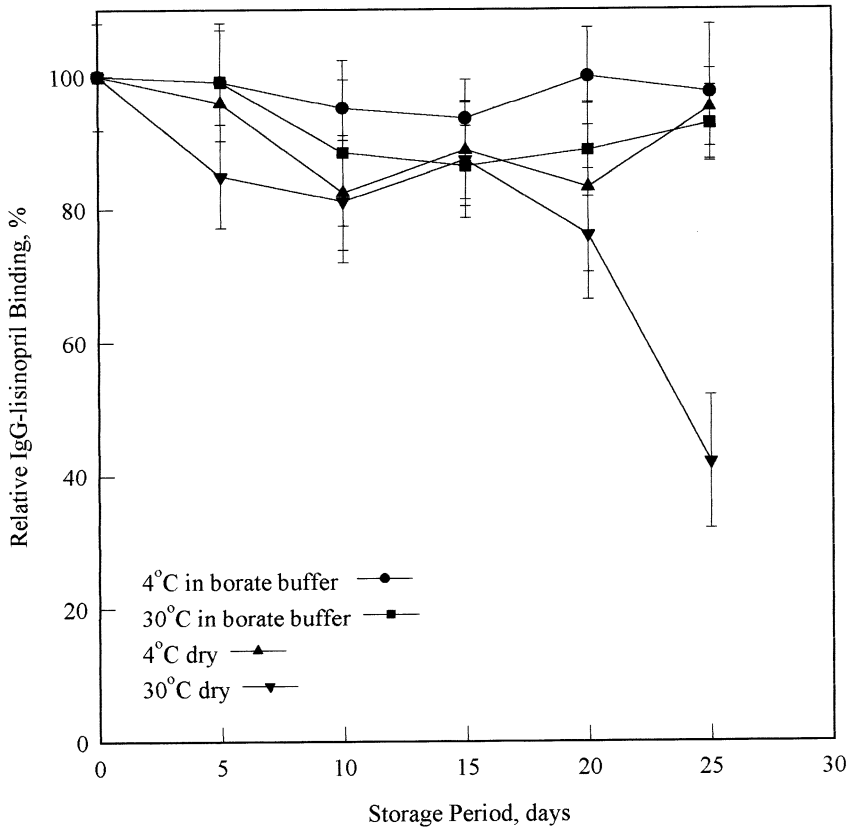


Fig. 8. Effect of storage on the relative IgG-lisinopril binding. Each point represents the mean of triplicate readings ( ± S.D.).

packed or as individual ones. Single beads, however, can be used in the development of mini-detecting systems or mini-immunosensors.

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