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# STUDIES ON THE REPRODUCIBILITY OF GLASS BEAD ANTIBODY AFFINITY COLUMNS

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## SUMMARY

Rabbit antibovine spleen DNase II and antihuman parotid amylase IgG were coupled to zirconium-clad (MAO, Corning) or plain (GAO, Corning) glass beads. These were challenged with DNase II or amylase (human parotid or pancreatic), respectively. Although columns of the beads bound the enzymes whereas control columns did not, recoveries from the immune adsorbents were not only not quantitative but extremely variable. The columns also shed material absorbing in the ultraviolet. The antiamylase IgG–MAO column bound amylase so firmly, yet without inactivating it, that an insoluble enzyme reactor was formed. For these experiments, amylase was labeled with $^{14}C$ *in vitro* without loss of activity.

## INTRODUCTION

Since the introduction of antibody affinity chromatography by Wofsy and Burr[1], the technique has become increasingly popular for the separation of enzymes from partially purified or crude extracts[2–4]. Immune adsorbents are prepared either by cross linking the antibody with a substance such as glutaraldehyde[5] or frequently by coupling the antibody to cellulose or Sepharose[6]. We were able to isolate hog and bovine spleen DNase II using an antibody coupled to Sepharose[7] and to draw tentative conclusions about the relatedness of the enzymes. The introduction of glass beads as a support for affinity chromatography[6] and the improved flow-rates obtainable with this substance led us to prepare antibody affinity columns from commercially available beads. Our experience and some of the difficulties observed in eluting DNase II and amylase from such columns are reported here.

## EXPERIMENTAL

### *Materials*

Bovine spleen DNase II was obtained as previously described[8]. Human parotid amylase was a gift from Dr. Robert C. Karn. A single preparation was used for all

the experiments. Antibodies to these materials were prepared in rabbits and the IgG separated[9]. This was immunoelectrophoretically homogeneous.

Antibody or normal IgG columns were prepared from IgG and GAO-3900 or MAO-3940, pore diameter 550 Å (Corning Biological Department, Mansfield, Mass., U.S.A.) using the azo coupling procedure described by Campbell *et al.*[10]. The columns were generally prepared from 0.25 g of beads poured into a 1-ml disposable plastic syringe. The washing solutions were either 0.1 *M* Tris, borate-buffered saline (BBS; 0.01 *M* borate–tetraborate buffer in 0.15 *M* NaCl[11]), or 0.04 *M* phosphate buffer, all adjusted to pH 8. All solutions were adjusted to 0.1% in sodium azide to retard bacterial decomposition. Eluting solutions were either 0.1 *M* acetic acid, pH 2.7–2.8, 4 *M* NaSCN or 6 *M* guanidinium hydrochloride.

$^{14}$C-labeled human parotid amylase was prepared from radioactive formaldehyde and parotid amylase by the method of Rice and Means[12]. Amylase (0.242 mg of protein of specific activity 1.26 units/$\mu$g) was treated with 0.005 mmole of $^{14}$C-labeled formaldehyde (specific activity 10 mCi/mmole) on ice. After 30 sec four separate additions of 10 ml each of a solution of sodium borohydride (5 mg/ml) were made. After one additional minute 50 ml of the $NaBH_4$ solution were added. The solution was applied to a 0.9 × 19 cm column of Bio-Gel P-2. The void volume of the column was 2.4 ml. The radioactive amylase (specific activity 0.95 units/$\mu$g) was recovered just after the void volume and was clearly separated from the bulk of the radioactive, non-reacted formaldehyde.

*Enzyme assays*

The DNase assays were performed as described previously[8,13]. Amylase was determined by the method of Bernfeld[14].

*Antibody affinity chromatography*

Antigens were applied to the columns and allowed to incubate at room temperature for periods ranging from 0.5 to 18 h as indicated under Results and discussion and in the tables. The variations in time were employed in attempts to improve the recovery of enzyme. The columns were washed with the appropriate buffer, pH 8, until the absorbance at 280 nm ($A_{280}$) of the eluate returned to baseline or remained steady for a number of fractions. The antigens were then eluted with 0.1 *M* acetic acid or other eluant as appropriate and the pH of the eluate adjusted to approximately neutral.

*Protein determination and $A_{280}$*

Protein was determined by the method of Lowry *et al.*[15]. $A_{280}$ was determined in a Gilford spectrophotometer and optical density units (O.D.U.) calculated as $A_{280}$ × volume (ml).

## RESULTS AND DISCUSSION

A number of chemicals were used to elute or otherwise treat the amylase prior to assay. The effect of these on enzyme activity was determined and the results are presented in Table I. It will be noted that 0.1 *M* acetic acid or 0.2 *M* ammonium hydroxide without neutralization completely destroyed the amylase activity. This was

TABLE I

EFFECT OF CHEMICALS ON HUMAN SALIVARY AMYLASE ACTIVITY

All experiments were performed at room temperature.

| *Chemical* | *Time of treatment* | *Neutralization** | *Activity remaining (%)* |
|---|---|---|---|
| 0.04 *M* phosphate, pH 6.9 | 0 | 0 | 100** |
| 0.1 *M* acetic acid | 15 min | NaOH | 89 |
| | 30 min | NaOH | 82 |
| | 15 min | $Na_3PO_4$ | 92 |
| | 30 min | $Na_3PO_4$ | 98 |
| | 2 h | 0 | 0 |
| 0.01 *M* HCl | 30 min | $Na_3PO_4$ | 0 |
| BBS 50% | 21 h | 0 | 90 |
| 75% | 21 h | 0 | 74.6 |
| 90% | 21 h | 0 | 41.6 |
| 0.2 *M* $NH_4OH$ | 2 h | 0 | ± |
| 2 *M* KI | 2 h | 0 | 0 |
| 4 *M* NaSCN | 2 h | 0 | 41.6 |
| 2 *M* NaSCN | 2 h | 0 | 66 |
| 0.1 *M* Tris, pH 8.3 | 2 h | 0 | 93.5 |

* pH was adjusted to 6.9–7.0.

** Specific activity = 1.26 units per μg protein.

also true for 2 *M* KI, whereas neutralization of acetic acid with sodium hydroxide resulted in retention of almost all and neutralization with sodium phosphate of all of the activity. The destructive effect of 0.01 *M* HCl could not be reversed by neutralization.

Several samples of bovine spleen DNase II were applied to a column of rabbit antibovine spleen DNase II IgG–GAO glass. The time of application was 0.5 h in all

TABLE II

TEST OF REPRODUCIBILITY OF RABBIT ANTIBOVINE SPLEEN DNase II IgG-GAO COLUMN

| *Run No.* | *Applied* | | *Wash* | | *Eluate* | | |
|---|---|---|---|---|---|---|---|
| | *Units* | *O.D.U.** | *Units* | *O.D.U.* | *Units* | *O.D.U.* | *%*** |
| 1 | 4.4 | 4.49 | 0.33 | 12.37 | 5.1 | 1.56 | 116 |
| 2 | 4.5 | 2.3 | 1.5 | 2.51 | 3.7 | 0.46 | 82 |
| 3 | 8.4 | 12.56 | 4.4 | 16.7 | 4.6 | 0.60 | 54 |
| 4 | 14.2 | 1.95 | 3 | 2.24 | 10.2 | 0.36 | 72 |
| 5 | 8.6 | 2.17 | 3.9 | 2.6 | 2.4 | 0.97 | 28 |
| 6*** | 34.8 | 8.47 | 4.5 | 7.19 | 23.4 | 1.16 | 67 |
| 7*** | 34.8 | 8.47 | 7.26 | 7.87 | 0.78 | 0.28 | 2 |
| 8*** | 34.8 | 8.47 | 8.8 | 6.67 | 16.4 | 0.35 | 51 |
| 9 | 6.29 | 1.08 | 1.97 | 1.95 | 2.29 | 0.13 | 35 |
| 10§ | 16.4 | 8.5 | 4.91 | 6.74 | 4.28 | 0.51 | 26 |

* O.D.U. = Optical density units, $A_{280}$ of enzyme solution × volume in ml.

** % = % of applied activity (units) eluted.

*** Portions of the same sample were used for Runs 6, 7 and 8.

§ Bovine serum albumin was added to the sample before application.

instances and the applied solution was circulated twice through the glass beads. The samples were eluted with 0.1 *M* acetic acid. The results obtained with ten different applications are shown in Table II. The recoveries ranged from 2 to 116% of the applied enzyme with an average of 53.3%. There was no obvious decrease in the capacity of the column with time and the column was used for approximately seven weeks before it was discarded. In almost every instance, the recovery (wash plus eluate) of the material absorbing at 280 nm was equal to or greater than that applied to the column. The capacity of the column varied between 0.8 and 23.4 units of DNase and there was little reproducibility when either a different sample of given specificity (Run 5) or the same sample (Runs 6, 7 and 8) were repeatedly applied. As is evident from Run 10, even the addition of bovine serum albumin to the sample failed to increase the yield.

Several control columns were prepared for the DNase II experiment using normal sheep or rabbit IgG linked to GAO glass. Results of these experiments are shown in Table III. The sheep IgG was coupled either by the azo procedure or by a Schiff-base technique using glutaraldehyde, following directions supplied by the manufacturer. In two experiments the total recovery with sheep IgG was 36.1 and 92.8% of the enzyme activity using the azo coupling procedure and 32.8% percent using the Schiff-base technique. With the rabbit IgG the recovery was 83.8% in one experiment. In the rabbit IgG experiment, which is probably the better control for the DNase experiments, all of the DNase appeared in the wash and none in the eluate fraction.

TABLE III

ANTIBODY AFFINITY CHROMATOGRAPHY OF DNase II ON NORMAL IgG–GAO COLUMNS

| | *Applied (Units)* | *Wash* | | *Eluate* | | *Total recovery (%)* |
|---|---|---|---|---|---|---|
| | | *Units* | *%* | *Units* | *%* | |
| A. Sheep IgG | | | | | | |
| 1. Azo coupling | 6.16 | 1.02 | 16.6 | 1.21 | 19.5 | 36.1 |
| | 1.33 | 1.13 | 85 | 0.17 | 7.8 | 92.8 |
| 2. Schiff-base coupling | 8.62 | 2.71 | 31.4 | 0.12 | 1.4 | 32.8 |
| B. Rabbit IgG | | | | | | |
| 1. Azo coupling | 7.08 | 3.94 | 83.8 | 0 | 0 | 83.8 |

Two columns of rabbit antihuman salivary amylase IgG–GAO were prepared by the azo coupling procedure. The results obtained with these columns are outlined in Table IV. The first column was used for one run only whereas the second column was used for a series of experiments. All these experiments were performed with the same preparation of unlabeled human parotid amylase except for Run 2c for which human pancreatic amylase was used. Runs 2e and 2f were performed with the labeled human parotid amylase described above. The application time for the enzyme was 16 to 18 h for all experiments except Runs 1a and 2a, in which 0.5 to 1 h were used. The increase in time between Runs 2a and 2b was associated with a 27% increase in recovery of amylase activity in the eluate. 0.1% sodium azide was present in all solutions

TABLE IV

AFFINITY CHROMATOGRAPHY OF HUMAN PAROTID AND PANCREATIC AMYLASE ON RABBIT ANTIHUMAN SALIVARY AMYLASE IgG-GAO COLUMNS

| *Column* | | *Applied* | | | | *Wash* | | | | *Eluate* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *No.* | *Run* | *Units* | *O.D.U.* | *μg* | *Cpm* | *Units* | *O.D.U.* | *μg* | *Cpm* | *Units* | *O.D.U.* | *μg* | *Cpm* |
| 1 | a*,*** | 312 | 0.488 | | | 133 | 1.23 | | | 40.4 | 0.538 | | |
| 2 | a*,*** | 280 | 0.488 | | | 28.6 | 2.22 | | | 48.2 | 0.829 | | |
| | b**,***,§ | 280 | 0.488 | | | 97.7 | 2.68 | | | 61.4 | 0.300 | | |
| | c**,***,§ | 75 | 6.2 | | | 5.5 | 6.8 | | | 36.9 | 0.440 | | |
| | d**,*** | 58 | 0.097 | 440 | | 0 | 3.11 | | | 23 | 0.534 | 101 | |
| | e**,*** | 36.5 | | | 2368 | | | | 17 | | | | 88 |
| | c§§ | | | | | | | | 0 | | | | 0 |
| | f** | | | | 5920 | | | | 980 | | | | 0 |
| | g**,*** | 112 | 0.194 | | 0 | 8.6 | 2.18 | | 286 | 32.4 | 0.43 | | 78 |
| | g§§§ | | | | | | | | | | | | 459 |

* On column 0.5–1 h before wash was started.
** Eluted with 0.1 *N* acetic acid.
*** On column 16–17 h before wash was started.
§ Pancreatic extract.
§§ Eluted with 4 *M* NaSCN.
§§§ Eluted with 6 *M* guanidinium hydrochloride.

to retard bacterial action. For Run 2c a crude human pancreatic extract to which the protease inhibitor α-toluenesulfonyl fluoride had been added was obtained from Dr. Jewell Ward and 75 units of the pancreatic amylase were applied to the column. Of these 36.9 units were recovered. This run was followed by another application (Run

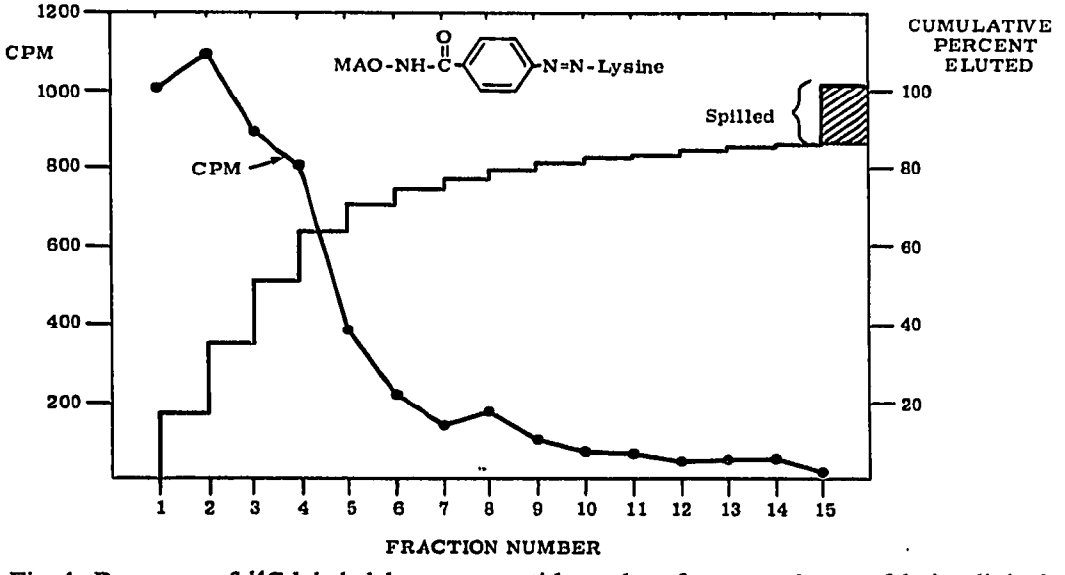


Fig. 1. Recovery of $^{14}C$-labeled human parotid amylase from a column of lysine linked to MAO-glass beads. Column size, 2.5 × 0.4 cm. Flow-rate, 1 ml/min. Eluant, 0.04 *M* phosphate buffer (pH 8).

2d) of 58 units of parotid amylase with recovery of 23 units. For all but one of the remaining runs, amylase which had been labeled *in vitro* with $^{14}C$ was applied. This could neither be washed off with phosphate buffer nor eluted with either acetic acid or 4 *M* NaSCN. Application of an additional 112 units of non-labeled amylase (Run 2g) was followed by recovery of 32.4 units in the 0.1 *M* acetic acid eluate and very little radioactive material. An additional few percent of the original radioactive counts applied was obtained on elution with 6 *M* guanidium hydrochloride.

The GAO glass columns are said to be less stable to alkali than the MAO glass, which is zirconium clad. Because the wash is performed at pH 8, a rabbit antihuman salivary amylase IgG–MAO column was prepared and challenged with human parotid amylase. A control column was prepared by linking lysine to some of the same batch of MAO glass. The control column (lysine–MAO) was challenged with unlabeled human parotid amylase, which was circulated through the column for 18 h in phosphate buffer. The column was then washed with phosphate buffer and essentially all the applied activity appeared in the first three 1-ml fractions (Fig. 2). The column was also challenged with the $^{14}C$-labeled human parotid amylase. This column leaked slightly, but the spilled material was recovered and 100% of the applied radioactivity could be accounted for —most of it within the first nine tubes (Fig. 1).

The IgG–MAO column was challenged with unlabeled human parotid amylase (Table V). We were unable to elute any of the amylase and at most 19% was recovered in the wash.

It is rather discouraging to note that the reproducibility of elution of both

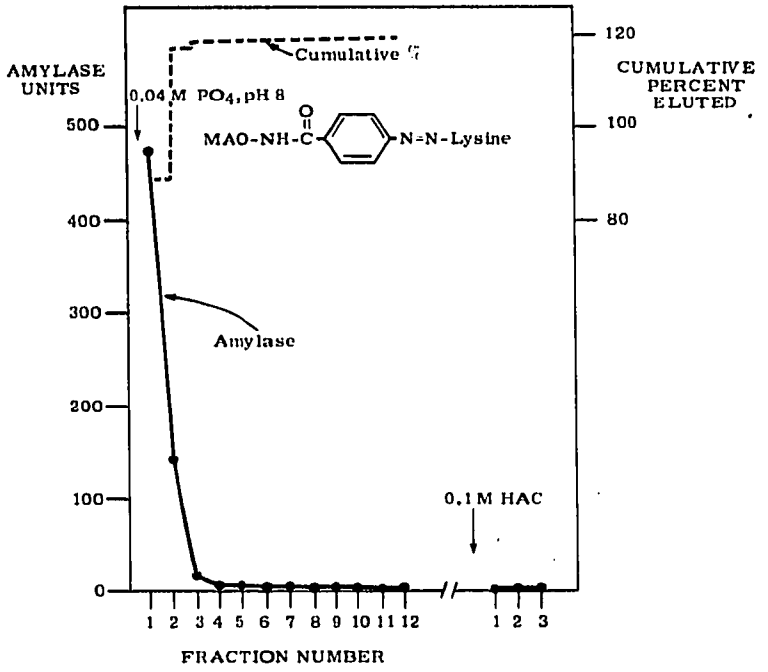


Fig. 2. Recovery of human parotid amylase activity from a column of lysine linked to MAO-glass beads. Column conditions, as in Fig. 1. HAC = acetic acid.

TABLE V

AFFINITY CHROMATOGRAPHY OF HUMAN PAROTID AMYLASE ON A RABBIT ANTI-HUMAN SALIVARY AMYLASE IgG–MAO COLUMN

| *Column* | | *Applied* | | *Wash* | | *Eluate* | |
|---|---|---|---|---|---|---|---|
| *No.* | *Run* | *Units* | *O.D.U.* | *Units* | *O.D.U.* | *Units* | *O.D.U.* |
| 1 | a*,** | 510 | 0.975 | 0 | 5.39 | 0 | 1.18 |
| | b*** | | | | | 0 | |
| 2 | a**,§ | 2125 | 4.88 | 403 | 2.15 | 0 | 0.88 |

* On column overnight before wash was started.
** Eluted with 0.1 *M* acetic acid.
*** Eluted with 2 *M* NaSCN.
§ On column 5 h before wash was started.

DNase II and amylase from GAO or MAO glass beads is so variable. In previous experiments with IgG linked to Sepharose[7] we were able to show that essentially the same amount of DNase was bound to the column following separate applications of enzyme. This is obviously not true in the current instance. The advantages of the glass beads both in physical stability and improved flow-rate makes this all the more regrettable. The beads cannot be used for either quantitative extraction or comparison of the immunological properties of enzymes from various sources in other than a qualitative way. Despite this, it is obvious that a column prepared from rabbit anti-human parotid amylase IgG is able to extract human pancreatic amylase from a crude extract —a result not unanticipated in view of the immunological identity of amylases from the two organs[16].

There is an additional difficulty to be noted with the columns. They shed an inordinate amount into the wash and eluate of substance absorbing more at 280 nm than a blank containing all the components of the solutions except the applied protein. This occurs despite repeated cycling of the column before use and constant washing between uses. It interferes with the simplest method for determination of proteins. The material eluted shows a continuously increasing absorption with decreasing wavelength. A similar increase in total $A_{280}$ recovered was obtained when amylase was passed through the lysine–MAO control column.

The experience with the MAO glass beads is puzzling. None of the amylase activity applied to the rabbit antihuman salivary amylase IgG–MAO column could be recovered in the eluate and only a fraction from one column was recovered in the wash. The amylase appeared tightly bound to the column, and a good part of the activity could be accounted for when a starch substrate was circulated through the amylase–antiamylase column. The latter then functioned as an insolubilized enzyme reactor. The enzyme might have been non-specifically adsorbed to the zirconium-clad beads, disappeared into the pores of the glass or been inactivated by time or the treatment. That this was not so is indicated by the results obtained with MAO glass beads to which lysine had been linked. In this case, both non-radioactive and $^{14}C$-labeled human salivary amylase could be recovered quantitatively after being recirculated through the column for about 18 h. We cannot account for the loss of radioactivity on the IgG–MAO column. Even the application of unlabeled amylase (Run 2g) failed to flush more than a fraction of the radioactivity from the column. It is highly un-

likely that the treatments used to make the amylase radioactive were responsible for the results as three quarters of the enzyme activity were recovered after the treatments attendant on the labeling with formaldehyde and in addition only a fraction of the unlabeled amylase applied to the column was recovered in any single experiment.

Several factors could possibly account for the variable recovery of enzyme. These include time of handling and loading. Amylase and DNase are stable for days in the test tube. Time of loading does not seem to be critical. It was kept the same for all the experiments for the DNase column and changed from 0.5–1 h to 18 h in the amylase experiments with about 25% apparent improvement in recovery. This does not account for the losses of activity. The specific activity or purity of the preparation added seems to be without effect. The DNase preparations were only semipurified. The specific activities (units/$A_{280}$) varied from 0.67 units in Run 3 of Table II to 5.8 in Run 9, whereas in Runs 5–8 solutions of the same specific activity but from two different preparations were used. In six out of the ten runs the recovery of material absorbing at 280 nm (that is, from wash plus eluate) was greater than that applied, whereas in only one case was the total enzyme activity apparently greater. The specific activity of the eluate was greater than that of the applied material in seven out of the ten runs. As the $A_{280}$ can be measured to within 1% and the DNase activity determinations are reproducible to at least 5%, errors in these measurements cannot account for the results.

The lack of reproducibility of the amylase columns cannot be accounted for either by lack of precision in measurement of the absorption of protein at 280 nm or by errors in determination of the amylase activity *per se*. For the latter the precision of determination is greater than $\pm$ 10%. It is difficult to account for the decrease in specific activity attendant upon elution from this column. Pure preparations of amylase were applied. In the case of the DNase column one might suspect residual proteolytic activity as a cause of the increased elution of material absorbing at 280 nm but this obviously would not apply in the case of amylase. Besides, the specific activity of the eluate was generally greater than that of the applied solution in the case of DNase whereas it was lower when amylase was applied to the antibody column.

It should be noted that this is the first application of the method of Rice and Means[12] to amylase. Applications of the procedure resulted in recovery of 75% of the enzyme activity and the final specific activity was 202 cpm per 0.95 units per $\mu$g protein.

There seems to be little in the literature on the use of antibody adsorbents covalently coupled to glass. Weetall[17] linked antibodies against L-asparaginase to a glass substrate similar to that used here and used it to recover enzyme. He was also able to obtain 97.3% recovery of human $\gamma$-globulin from an antihuman $\gamma$-globulin column. The efficiency of the column depended on loading. There are several differences between these experiments and those of Weetall. His glass carrier, although based on Corning glass beads, was made in his laboratory. The glass substrate was purified by heating in oxygen prior to attachment of the amine side arm. Thiophosgene was used for the antibody coupling rather than the diazotization reaction we used. Except for the determination by difference of the amount of antibody coupled to glass, $A_{280}$ was apparently not used as a measure of protein elution. Therefore we cannot know whether material absorbing at 280 nm over and above protein was eluted. The charging and elution conditions differed somewhat from those we described, but not radically enough to account for the differences in results. These differences from

Weetall's results may be the consequence of the already mentioned differences in glass and application methods, as the results we obtained with two such disparate proteins as DNase II and amylase make it unlikely that the antigens themselves are responsible. Weetall's results, contrary to those presented here, suggest that under proper circumstances, certain glass beads may be useful for quantitative antibody affinity chromatography of some proteins.

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