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Immunoaffinity chromatography of recombinant *Amb a* I in the presence of a denaturing agent

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ABSTRACT

Recombinant proteins expressed in *E. coli* are often sequestered into inclusion bodies and require the use of denaturing agents in order to solubilize them. The recombinant form of *Amb a* I, the major allergen from short ragweed pollen, is one such protein. In some cases solubility can be maintained after the removal of the denaturing agent, particularly if the protein can be folded into its native conformation. However, not all proteins refold readily and after the removal of the denaturing agent the proteins will reaggregate and/or precipitate. In the case of *Amb a* I, the recombinant protein stays in solution at low concentrations but aggregates with itself and other proteins. The recombinant *Amb a* I is not expressed at high levels and may be toxic to *E. coli*. Therefore, isolation from a complex mixture of *E. coli* proteins was necessary. Monoclonal antibodies which recognize the denatured form of *Amb a* I were available, allowing for immunoaffinity purification. However, because the protein was not monomeric, this chromatographic technique did not provide an improvement in the purity level when run in normal buffer solutions. Analysis of one monoclonal antibody's stability to urea indicated it could tolerate the presence of 2 *M* urea and recover full activity. Use of this antibody as an immunoaffinity reagent in a column run in 2 *M* urea, which minimized aggregation of the *E. coli* produced proteins, gave a high degree of purification of recombinant *Amb a* I in one step. This illustrates the potential for the use of denaturing and other solubilizing agents in immunoaffinity chromatography of recombinant proteins.

INTRODUCTION

Short ragweed pollen is a major cause of hayfever in North America. The major allergen in this pollen was identified as an M_r 38 000 amino-terminally blocked protein and termed antigen E in 1964 [1] and later renamed *Amb a* I according to standardized nomenclature [2]. Recently, the cDNAs encoding *Amb a* I were cloned and the protein shown to be composed of a mixture derived from a homologous multigene family [3]. The relative level of expression of the different gene products in ragweed pollen is unknown, however, the predominant protein sequence derived from biochemically purified *Amb a* I corresponds to the cDNA designated *Amb a* I.1 [3]. However, when three different cDNAs, *Amb a* I.1, I.2, and I.3, were expressed as recombinant proteins in *E. coli* lysates, they all bound human IgE and stimulated the proliferation of T cells from ragweed allergic patients in *in vitro* cultures [4]. In T cell assays, the antigen is taken up by presenting cells, processed to peptide fragments, and recognized by T cells as linear epitopes when bound to surface expressed major histocompatability complexes [5,6]. Therefore, it is not always necessary to refold the recombinant protein for use in such a system.

Purification of these recombinant proteins was required in order to further study their relative importance as allergens. Recombinant *Amb a* Is were produced at relatively low levels in *E. coli*. The proteins formed inclusion bodies which required the use of denaturants in order to solubilize them. When the denaturants were removed the proteins

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tended to aggregate and at high concentrations they precipitated, as is often observed with recombinant proteins.

Immunoaffinity chromatography is a very selective protein purification technique due to the specificity of the interaction between monoclonal antibodies and their cognate antigens. Therfore, it is particularly useful for purifying low-abundance proteins, such as recombinant *Amb a* I proteins, from complex mixtures such as *E. coli* lysates. Elution of an antigen from an immunoaffinity column often requires harsh conditions which can denature the antibody and render the column inactive. Therefore, the running and elution conditions should be optimized for each immunoaffinity column in order for it to be reusable [7]. Monoclonal antibodies which bind to denatured, pollen-derived *Amb a* I had been previously produced and characterized [8]. Here we describe the use of the monoclonal antibody, JB4E3-3, as an immunoaffinity reagent in low denaturant concentrations under which the antibody is stable and the recombinant protein is less aggregated.

MATERIALS AND METHODS

Recombinant Amb a I protein expression

Amb a I.1 cDNA [3] was cloned into the vector pTrc99A [9] and recombinant *Amb a* I.1 (r*Amb a* I.1) expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) in the JM109 strain of *E. coli*. The protein formed inclusion bodies which were pelleted after cell lysis and solubilized with 8 *M* urea as has been described previously [4].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses

The SDS-PAGE and transfer procedures were performed as described elsewhere [10]. Gel electrophoresis was performed on 12% acrylamide gels under reducing conditions. For Western blot analysis proteins were transferred from the gels to nitrocellulose (0.1 μm, Schleicher & Schuell, Keene, NH, USA) in a Hoefer apparatus (San Francisco, CA, USA). The nitrocellulose blots were blocked with 1% defatted milk, stained with India ink and probed with JB4E3-3 at 1 ng/ml. The JB4E3-3 was detected with biotinylated rat anti-mouse antibody (Kirkegaard and Perry, Gaithersberg, MD, USA) and ^{125}I-streptavidin. Films were developed by autoradiography at −80°C with an intensifying screen.

Enzyme-linked immunosorbent assay (ELISA) detection of recombinant Amb a I.1

A qualitative ELISA assay for the detection of the protein in fractions from chromatographic separations was developed. Immulon II 96-well plates (Dynatech, Chantilly, VA, USA) were coated with JB4E3-3 (0.1 μg/well) overnight at 4°C. Plates were washed between steps with phosphate-buffered saline (PBS). Blocking was done with 0.5% gelatin. Blocking and subsequent steps were performed for 1 h at room temperature. Aliquots of chromatographic fractions were incubated in the wells to allow capture of the antigen. Plates were incubated with rabbit anti-denatured *Amb a* I antisera at a 1:500 dilution. The polyclonal antisera was used in this assay to give a greater probability that proteolytic fragments, as well as intact r*Amb a* I.1, would be detected. Rabbit antibodies were detected with biotinylated goat anti-rabbit antibody (1:5000 dilution, Southern Biotechnology, Birmingham, AL, USA). Plates were incubated with streptavidin-conjugated horseradish peroxidase. Color was developed with the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard and Perry) and the development stopped with the addition of 1 *M* phosphoric acid. Positive fractions were identified by absorbance at 450 nm read on a Bio-Tek Model 310 plate reader (Winooski, VT, USA).

Gel filtration chromatography

Chromatograms were run on an FPLC system (Pharmacia, Piscataway, NJ, USA) with a Superose 12 (HR 10/30, 30 × 1 cm) column in 200 m*M* sodium phosphate (pH 7) at 0.5 ml/min. The detectors were set at 0.5 and 0.05 AUFS for 214 and 280 nm, respectively. Urea-solubilized cell pellets were either dialyzed against PBS or used directly. Samples were filtered and 100 μl was injected onto the column. Fractions of 0.5 ml were collected and analyzed by ELISA.

ELISA analysis of JB4E3-3 stability

A direct ELISA was used to optimize the conditions for the use of JB4E3-3 as an immunoaffinity

reagent. Short ragweed (*Ambrosia artemisiifolia*) pollen (Greer Labs, Lenoir, NC, USA) was extracted with PBS at 10 g/l overnight at 4°C. The extract was clarified by centrifugation and filtering and denatured by boiling. This was used to coat the wells of Immulon II 96-well plates (Dynatech, Chantilly, VA, USA) at 1 ng *Amb a* I/well overnight at 4°C. Plates were washed between steps with PBS. Subsequent steps were performed at room temperature for 1 h each. Plates were blocked with 1% bovine serum albumin in PBS. JB4E3-3 was added at 0.1 μg/well with pretreatment or treatment on the plate with various reagents described in the text. The JB4E3-3 was detected with biotinylated rat anti-mouse antibody (Kirkegaard and Perry). The plates were developed as described above.

Immunoaffinity chromatography

The immunoaffinity agent, murine monoclonal antibody JB4E3-3, was raised against denatured *Amb a* I derived from ragweed pollen [8]. The antibody was produced in ascites and purified by precipitation with ammonium sulfate at 45% of saturation. The antibody was coupled to cyanogen bromide-activated Sepharose (Pharmacia, Piscataway, NJ, USA) at 5 mg/ml according to the manufacturer's protocol and a 7-ml open column was prepared. The column was equilibrated with PBS containing 2 *M* urea by gravity flow. The *E. coli* cell pellet was obtained and solubilized with 8 *M* urea as previously described [4]. The solubilized pellet was diluted 1:4 with PBS, filtered, and loaded onto the column. The column was washed with approximately 50 ml of PBS containing 1 *M* NaCl and 2 *M* urea. The column was then eluted with 10 m*M* cyclohexylaminopropanesulfonic acid (CAPS) containing 2 *M* urea (pH 10.5). Fractions of 100 drops were collected and analyzed by absorbance at 280 nm and by SDS-PAGE.

RESULTS AND DISCUSSION

Amb a I can be purified from ragweed pollen by a series of biochemical purification steps including two batch ion-exchange steps, three ammonium sulfate precipitations, gel filtration chromatography and finally an ion-exchange chromatographic step [11]. Due to the proteolytic sensitivity of the protein, it is degraded during this procedure and in our laboratory approximately 5 mg of intact pure protein is recovered from 100 g of pollen. The cDNAs encoding several related *Amb a* I proteins have been expressed in *E. coli* in order to provide large quantities of unambiguous gene products [3]. However, the r*Amb a* I proteins are not expressed at high levels, producing only 0.5–3 mg per liter of culture grown in shaker flasks. This represented approximately 1–4% of the cellular protein. Efforts aimed at increasing expression levels by modifying or changing the expression vector or *E. coli* strain used have yielded increased levels of mRNA but not of protein.

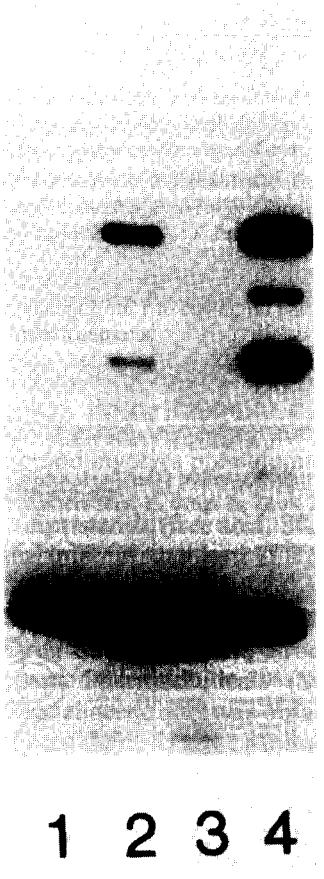

Fig. 1. JB4E3-3-probed Western blot of r*Amb a* I.1 expressed in *E. coli*. An SDS-PAGE immunoblot was generated with the following samples: lane 1, JM109 negative control *E. coli* lysate; lane 2, *E. coli* lysate containing expressed r*Amb a* I.1; lane 3, supernatant after centrifugation of *E. coli* lysate containing expressed r*Amb a* I.1; lane 4, urea-solubilized pellet after centrifugation of *E. coli* lysate containing expressed r*Amb a* I.1.

In order to purify a low-abundance protein it is preferable to use a high-resolution chromatographic technique such as affinity chromatography. A panel of monoclonal antibodies which recognize denatured *Amb a* I derived from pollen had been raised [8]. Some of these monoclonal antibodies were available for use as potential immunoaffinity reagents. Here we describe the characterization of the monoclonal antibody JB4E3-3. This antibody was tested by Western blot analysis to determine if it recognized r*Amb a* I.1. As shown in Fig. 1, the antibody specifically recognized the expressed r*Amb a* I.1 at an apparent molecular mass close to the predicted molecular mass of 42 000, as well as three proteolysis products. The molecular mass of the recombinant protein is higher than that of the native protein by approximately 4000 due to the presence of a leader sequence. Proteolysis of this recombinant protein had been previously detected on a Western blot probed with a rabbit anti-*Amb a* I antiserum [4]. JB4E3-3 also bound an *E. coli* protein of M_r approximately 14 000 seen in the *E. coli* control cell lysate in Fig. 1, lane 1. As seen in lanes 3 and 4, containing the cell supernatant and the cell pellet, respectively, this lower-molecular-mass protein is soluble. It was possible to remove this protein by washing the cell pellet (see Fig. 4, panel B, lanes 1 and 2).

A direct-binding ELISA assay was developed to determine the optimal elution conditions for use of JB4E3-3 as an immunoaffinity reagent (Table I). A number of potential solutions for elution of the column were tested in this assay including low-pH and high-pH buffers, as well as high-salt, and chaotropic solutions. In the pretreated samples, the antibody solution was diluted 1:100 with the test solution, then dialyzed against PBS. It was then used in the binding assay and detected with biotinylated rat anti-mouse antibody in order to determine if the JB4E3-3 had been permanently inactivated. The activity of the antibody was decreased by pretreatment with a buffer at pH 12.5 and by solutions containing very high salt. In addition, the ability of the different solutions to dissociate the antibody–antigen (Ab–Ag) complex was assayed by incubating the antibody (diluted 1:100 with PBS) in the coated wells, washing, adding the test solutions for a brief period of time, removing the solution, and continuing the assay to detect the amount of JB4E3-3 still bound to the *Amb a* I on the plate. Phosphate buffer at pH 11.0 proved to be the most effective dissociating reagent of those that did not denature the anti-

TABLE I

ABSORBANCE READINGS FROM THE DIRECT BINDING ELISA TO DETERMINE THE OPTIMAL ELUTION CONDITIONS FOR AFFINITY CHROMATOGRAPHY

Pretreated Ab, JB4E3-3, was diluted with the solutions indicated, dialyzed, and used in the binding assay. Complexes of JB4E3-3 and denatured *Amb a* I (Ab–Ag complex) were formed on the plate and then treated with the solutions indicated.

Treatment	Absorbance at 450 nm of the Ab–Ag complex treated	Absorbance at 450 nm of the pretreated Ab
0.1 *M* Glycine (pH 2.5)	0.934	1.037
0.1 *M* Glycine and 0.5 *M* NaCl	1.065	1.062
0.1 *M* Phosphate (pH 10.0)	1.021	1.175
0.1 *M* Phosphate (pH 11.0)	0.171	1.124
0.1 *M* Phosphate (pH 12.5)	0.133	0.283
3 *M* $MgCl_2$	1.059	0.992
5 *M* $MgCl_2$	0.317	0.491
5 *M* LiCl	1.134	1.069
10 *M* LiCl	0.180	0.271
1 *M* NH_4SCN	1.118	1.198
2 *M* NH_4SCN	1.030	1.182
3*M* NH_4SCN	1.000	0.491
10 m*M* CAPS and 2 *M* Urea	0.013	0.861

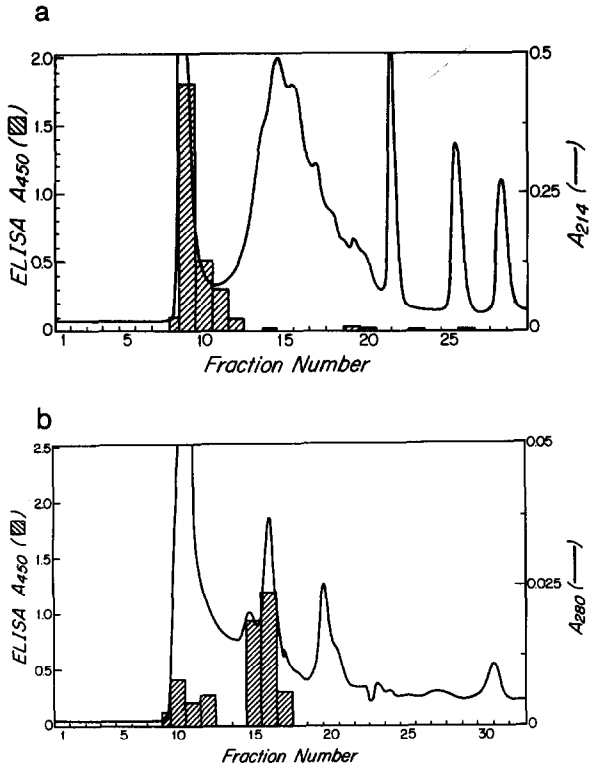

Fig. 2. Gel filtration chromatography of urea-solubilized pellet from *E. coli* lysate containing expressed r*Amb a* I.1 (a) after dialysis into PBS and (b) without dialysis. The capture ELISA data detecting r*Amb a* I.1, shown in shaded areas, is overlaid on the chromatograms.

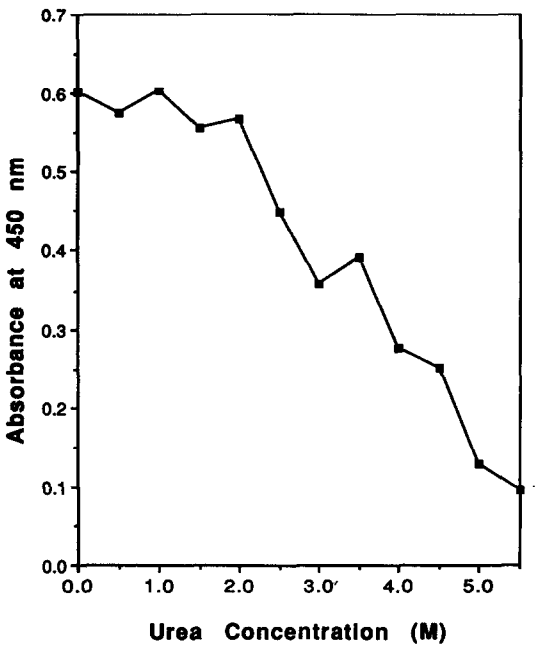

Fig. 3. Direct binding ELISA to determine the effect of urea on JB4E3-3 binding to *Amb a* I in denatured pollen extract. Absorbances are plotted as a function of the urea concentration.

body. In a later assay a CAPS buffer at pH 10.5 was also shown to be an effective eluting agent (Table I) and that buffer was preferred for use in the purification protocol because the lower pH would provide a less hostile environment for the antibody.

A JB4E3-3 Sepharose column was prepared for purification of r*Amb a* I.1 and run essentially as described in Materials and Methods except that the elution buffer was a pH 11 phosphate buffer and urea was not included. The protein which was eluted from the column showed a large number of bands and an insignificant increase in the level of purity of r*Amb a* I.1 when analyzed by SDS-PAGE (data not shown). In order to understand this result, gel filtration chromatograms were run on the urea-solubilized cell pellet both with and without prior removal of urea by dialysis. The r*Amb a* I.1 was detected by the capture ELISA described in Materials and Methods. Fig. 2a shows that dialysis of the solubilized pellet caused the protein to aggregate to a high-molecular-mass form with essentially all of the r*Amb a* I.1 activity eluting at the column void (> 300 000 dalton). In contrast, the undialyzed recombinant protein, shown in Fig. 2b, eluted predominantly at the approximate molecular mass of a monomer, although some higher-molecular-mass aggregates were detected probably due to the fact that the mobile phase in the column did not contain urea. These results suggest that when the dialyzed r*Amb a* I.1 was loaded onto the immunoaffinity column it was aggregated with other proteins as well as with itself and therefore its relative abundance was not increased significantly by the chromatography. Previously, ion-exchange chromatography and hydrophobic interaction chromatography had been used in an attempt to purify the protein but no activity was recovered from those columns. Aggregation of the proteins could have contributed to those results in addition to the hydrophobic nature of the unfolded protein.

To prevent protein aggregation, detergents are

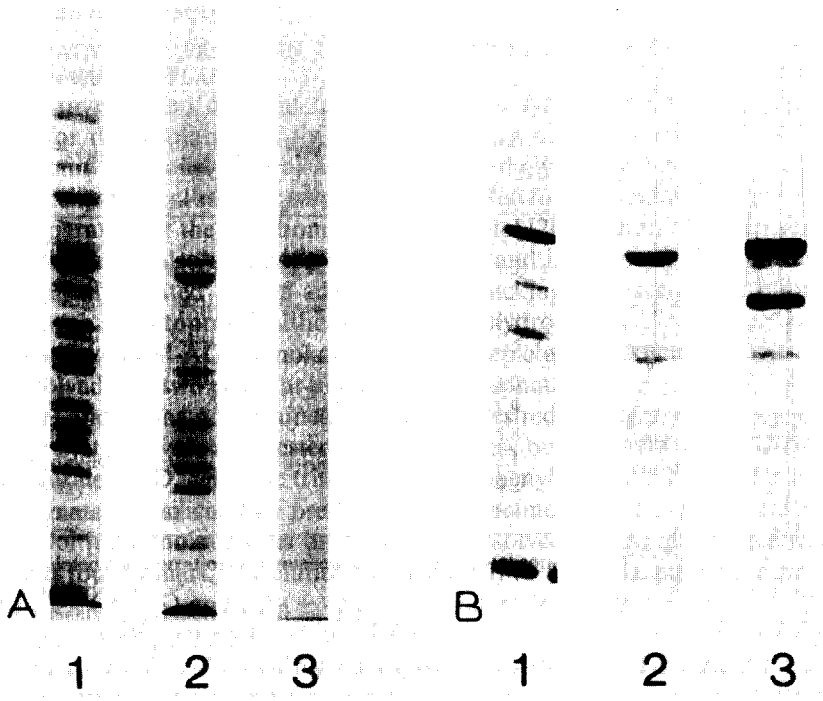

Fig. 4. SDS-PAGE and Western blot analysis of r*Amb a* I.1 purified in the presence of 2 *M* urea. (A) Coomassie blue-stained gel analysis; (B) analogous lanes from a duplicate gel which was blotted and probed with JB4E3-3. Lanes: 1 = whole cell lysate of *E. coli* expressing r*Amb a* I.1; 2 = urea-solubilized washed cell pellet of these cells; 3 = JB4E3-3 Sepharose-purified r*Amb a* I.1.

often used. However, the r*Amb a* I.1 was to be used in cellular assays where detergents could potentially adversely affect cell viability. Another approach to preventing reaggregation of solubilized inclusion body proteins is to refold the proteins to their native conformation [12]. Determining the conditions for refolding of a protein often requires testing a number of variables and is generally monitored by an activity assay. In the case of r*Amb a* I.1, its only known biological activity is as an allergen and in the denatured form it is capable of stimulating T cells from allergic individuals and binding IgE, although IgE binding is reduced [4,13]. Consequently, development of an assay for refolding would be very difficult. Therefore, we considered diluting the urea and the protein and loading immediately onto the immunoaffinity column with the expectation that the kinetics of protein aggregation would be slow in low denaturant concentrations. In order for this approach to be effective, the antibody must tolerate exposure to some level of urea. Urea is sometimes used as an elution reagent in immunoaffinity chromatography at either 2 or 8 *M*, and columns can sometimes be regenerated after treatment with 2 *M* urea [7]. Therefore, it is not unreasonable to expect that a monoclonal antibody could tolerate low denaturant concentrations. The stability of JB4E3-3 in the presence of urea was assayed in a direct binding ELISA, the results of which are shown in Fig. 3. The antibody showed no decrease in binding activity at concentrations of urea up to 2 *M*. An additional direct ELISA was performed which demonstrated that JB4E3-3 was not irreversibly denatured by the combination of high pH (10.5) and 2 *M* urea to be used during elution (Table I).

Immunoaffinity purification of r*Amb a* I.1 was done with 2 *M* urea present throughout the procedure. Fig. 4 shows the result of this purification step analyzed on duplicate 12% acrylamide SDS-PAGE gels, one Coomassie Blue stained (panel A) and the other analyzed by a Western blot probed with JB4E3-3 (panel B). Approximately a twenty-fold in-

crease in purity of the recombinant protein as compared to the whole cell lysate was achieved as determined by densitometric analysis of the Coomassie Blue stained gel shown (panel A, lanes 1 and 3). The intact *Amb a* I.1 and its proteolytic fragments, identified by Western blot analysis (panel B, lane 3), made up approximately 80% of the protein recovered from the column (panel A, lane 3). The JB4E3-3 Sepharose column was run four times in 2 *M* urea with no detectable loss of activity. Changes in the column capacity were not assessed.

We have demonstrated that it is possible to use an antibody as an immunoaffinity reagent in the presence of a denaturing agent. Although it is preferable to run immunoaffinity columns under the mildest possible conditions in order to maximize their lifespan, the use of harsh reagents may be necessary in order to solve particularly difficult purification problems. Protein aggregation is a problem often encountered with recombinant proteins solubilized from inclusion bodies. However, if an antibody can be chosen as an immunoaffinity reagent based on its stability in the presence of harsh reagents such as denaturants or detergents, protocols can be designed which allow purification of the protein of interest and preserve the activity of the immunoaffinity column.

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