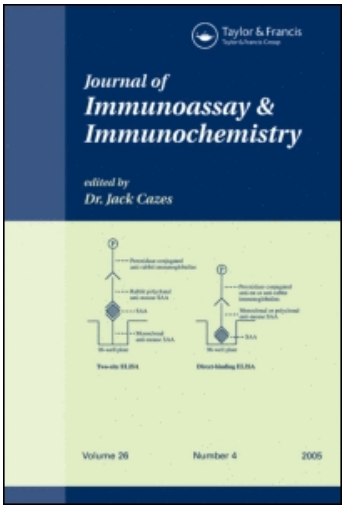

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Kosmotropes Enhance the Yield of Antibody Purified by Affinity Chromatography using Immobilized Bacterial Immunoglobulin Binding Proteins

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Abstract: The yield of antibody purified using affinity chromatography on immobilized Protein A or Protein G was increased up to 5-fold (500%) by including kosmotropic salts in the binding buffer. The binding buffer is used to equilibrate the affinity column before applying a sample to the column and also to dilute the sample prior to loading onto the affinity column to optimize conditions for a maximal binding of antibodies to affinity gels. In this study, the kosmotropic salts that were effective in greatly increasing antibody binding to Protein A included both inorganic and organic salts of ammonium; sodium; or potassium sulfate, phosphate, polycarboxylates; for example, succinate, citrate, isocitrate, N-(2-hydroxyethylene diamine triacetate (HEDTA), ethylene diamine tetraacetate (EDTA), and ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′N′-tetra acetate(EGTA). On an equal-molar basis, the greater the number of carboxylic groups within the polycarboxylate molecule, the greater the increase in the yield of the purified antibody that was observed. The data show that kosmotropes can be used as effective additives to enhance the binding of immunoglobulins to Protein A or Protein G gels with a resultant increase in the yield of the purified antibodies. Thus, it appears that strongly hydrated anions (citrate, sulfate, and phosphate) and weakly hydrated cations (ammonium, potassium) increase the yield of antibody purified on either Protein A or Protein G affinity gels.

Keywords: Antibodies, IgG, Protein A, Protein G, Affinity chromatography

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INTRODUCTION

Bacterial Fc receptors, such as Protein A and Protein G, have been widely used as affinity ligands in affinity gels for the purification of immunoglobulins, as well as for universal immunoglobulin binding conjugates in immunological analysis.[1–8] These bacterial Fc receptors predominantly bind the constant Fc portion of mammalian immunoglobulin from various species, albeit with varying degrees of affinity. For example, IgG from serum of human, rabbit, guinea pig, pig, monkey, and dog bind to Protein A-Sepharose with the strongest affinity. On the other hand, the IgG from cow, mouse, and horse bind with only moderate affinity. Lastly, the IgG from sheep, goat, and rat and IgY from chicken only weakly bind to Protein A.[1–7]

The widespread use of Protein A or Protein G affinity gels for antibody purification stems from the commercial availability of ready for use gels. Alternatively, the affinity gels can also be easily prepared by coupling protein A or Protein G to activated solid supports. The ease and simplicity of using these affinity gels for purifying antibodies also contribute to their popular uses. In general, the purification procedure can be described as follows. Serum is first diluted with a buffer solution and, then, the diluted serum is applied onto a pre-equilibrated column of affinity gel. This is then followed by washing the gel with the same binding buffer solution and, finally, the bound immunoglobulins are eluted with an appropriate eluant.

The quantity of immunoglobulins purified by using these affinity gels depends on

a. the class, subclass, and the species of immunoglobulins to be isolated;
b. the binding affinity of these immunoglobulins to the affinity gel;
c. the adsorption conditions; and
d. the elution conditions.

The first two properties are invariant for a given system. Thus, only the last two conditions can be modified to improve the yield of the affinity purified antibodies. In this communication, we present a general method for significantly improving the yield of immunoglobulins purified using either Protein A or Protein G gel by adding readily available kosmotropic salts to the binding buffer in the antibody adsorption process. Kosmotropes or kosmotropic salts (order makers) denote salts that interact strongly with water molecules. They tend to increase the viscosity of water and to stabilize the structures of proteins.[8–10] Neutral salts play important roles in the chromatographic behavior of proteins. In hydrophobic interaction chromatography, protein binding to the immobilized hydrophobic ligands is promoted in solutions having high concentrations of neutral salts and is especially promoted by kosmotropic salts.[11–13] Conversely, the elution of proteins bound to a hydrophobic matrix is promoted by chaotropes.[14] Chaotropes are ions that have weaker interactions with water than water itself, and are

known as water-breakers.[8] Kosmotropes, such as sulfate and phosphate, have been shown to mediate affinity chromatography of dehydrogenases of halophilic bacteria and proteins of *E. coli*.[15,16] This communication describes the effects of neutral salts on the affinity purification of antibodies on Protein A or Protein G affinity matrices and demonstrates dramatic enhancing effects of kosmotropic salts on the yield of immunoglobulins purified using Protein A or Protein G affinity gels.

EXPERIMENTAL

Chemicals

Immobilized Protein A gel, Protein A Avid Gel, was from BioProbe International Inc (Tustin, CA), Affi-gel Protein A was from BioRad Labs, Inc. (Richmond, CA), Protein G gels were from Sigma (St. Louis, MO), animal sera were from Pel-Freez Biological (Rogers, AK), and normal human serum was from in-house volunteers. All other chemicals were from Sigma (St. Louis, MO).

Monoclonal Antibody

Mouse monoclonal antibody to rat Kappa chain from ascites fluid was obtained as follows. BALB/c mice were immunized by injection of purified rat Kappa chain emulsified with Freund's complete adjuvant. Spleens from immunized mice were removed aseptically and minced with tweezers. Mouse myeloma RG 11–15 cells were fused with spleen cells from immunized mice by using 50% poly(ethylene glycol) solution. After the selection in HAT medium and screening for the hybridoma clones that secrete antibody, the hybridoma cells were injected intraperitoneally into BALB/c mice that were irradiated with 500 rad gamma-rays. The harvested ascites fluid was clarified by centrifugation and was the source of the monoclonal antibodies.

Affinity Purification of Antibody on Protein A or Protein G Affinity Gel

A typical affinity chromatographic procedure for purification of IgG is as follows. To a 3 mL polyethylene column, was added 1 mL of immobilized Protein A or Protein G. The gel was allowed to settle by gravity. The column was then equilibrated with 10 mL buffer solution (0.1 M glycine, 0.1 M sodium phosphate, 0.1 M sodium borate, pH varies from 7.5 to 9.1, containing various kosmotropic salts). Serum or ascites fluid, diluted one- to six-

fold with this buffer, was then applied to the column as indicated for each experiment. The column was then washed with the same buffer (10 mL). The bound immunoglobulin was finally eluted from the column with an acid elution buffer (either 0.1 M glycine-HCl, pH 2.8 or 0.1 M sodium acetate-acetic acid, pH 3.5). The concentration of the eluted immunoglobulins was determined spectrophotometrically from absorbance measurements at 280 nm.

RESULTS AND DISCUSSION

The effects of kosmotropic inorganic salts, such as ammonium phosphate, ammonium sulfate, and sodium sulfate, on the yield of human IgG isolated from affinity Protein A gel have been reported in the patent literature.[17–19] Figure 1 shows a quantitative relationship between the concentration of kosmotropic inorganic salt in the binding buffer and the increase in the yield of isolated IgG. All data summarized and presented in the figures and tables below are averages of three trials; the CVs of the averages are less than 20%.

With the exception at salt concentration of 0.5 M, there is a general trend that increasing the concentration of kosmotropic salts linearly increases the yield of the isolated immunoglobulins. For human serum IgG, an increase of up to1.6-fold in the yield of purified IgG was achieved by the addition of 1 M sodium sulfate to the binding buffer at pH 7.5 (closed squares in

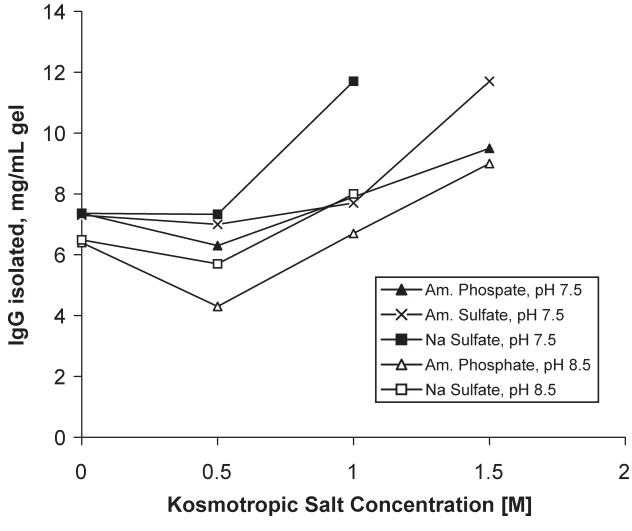

Figure 1. The effects of ammonium phosphate, ammonium sulfate, and sodium sulfate (the kosmotropic salts) on the yield of IgG purified from human serum using Protein A affinity gel.

Fig. 1). At pH 8.5, the yield of purified antibodies was enhanced only 1.2-fold (open squares in Fig. 1). Similarly, ammonium phosphate salt most effectively increases the yield of human IgG purified using Protein A gel at pH 7.5 rather than 8.5 (Fig. 1, open and closed triangles). Figure 1 also shows that ammonium sulfate at pH 7.5 was effective in enhancing the yield of antibodies purified with Protein A gel (the crosses in Fig. 1). For mouse monoclonal antibodies, the increased yield of the purified antibodies was much more pronounced (Fig. 2). A 500% increase in the yield of purified mouse monoclonal IgG was observed when the ascites fluid, diluted with 1.75 M potassium phosphate, was applied onto a Protein A column pre-equilibrated with the same binding buffer containing 1.75 M potassium phosphate. As the concentration of potassium phosphate increases beyond 0.5 M in the diluent buffer, there is an almost linear increase in the yield of the isolated IgG. The increase in the yield of human IgG purified on immobilized Protein A or Protein G is less pronounced than that of IgG from other sera (Fig. 1). This is because, intrinsically, human IgG already binds Protein A with the highest affinity as compared with IgG from other species.[1,2,4,20] Similarly, potassium phosphate also causes a dramatic increase in the yield of mouse antibodies from mouse serum isolated on a Protein A column. By adding the mouse serum with 1.2 M potassium phosphate binding buffer, the yield of mouse IgG isolated using Protein A column increases by 240%.

In addition to inorganic kosmotropic salts, certain organic salts, such as potassium citrate, and other polycarboxylates, were also effective in enhancing the yield of antibodies isolated by Protein A or Protein G affinity chromatography. Figure 3 shows that increasing the concentration of potassium citrate salts in the binding buffer, at the optimal concentration of 0.8 M, increases the yield of isolated IgG by almost 400%. Beyond this

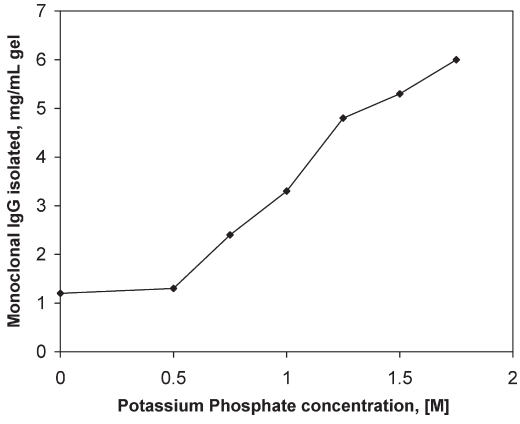

Figure 2. The effects of increasing concentrations of potassium phosphate on the yield of mouse monoclonal antibodies purified on a Protein A affinity gel.

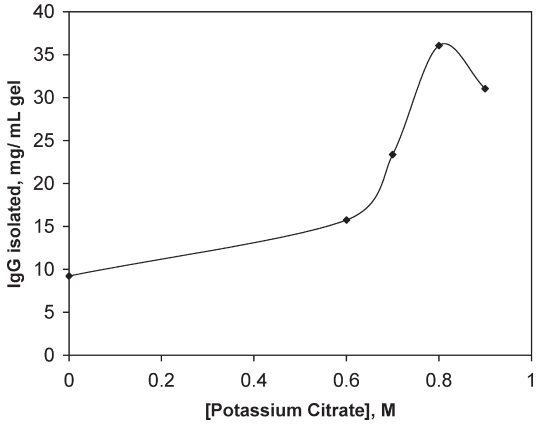

Figure 3. The effects of increasing concentrations of potassium citrate on the yield of IgG purified on a Protein A affinity gel.

optimal concentration of 0.8 M, the enhancing effect of potassium citrate decreases, perhaps caused by starting of precipitation of the antibodies.

The effects of pH of the binding buffer on the yield of IgG isolated were studied using 0.7 M potassium citrate in the binding buffer. The results showed that the optimal pH was 8 (Figure 4). Table 1 lists the effects of various organic salts (at 0.67 M) in binding buffers on enhancing the yield of IgG when isolated using Protein A affinity chromatography. These results showed that only organic salts containing 2 or more carboxylate functional groups are capable of increasing the antibody yield. On an equal molar basis, organic salts with the highest number of carboxylic groups were most

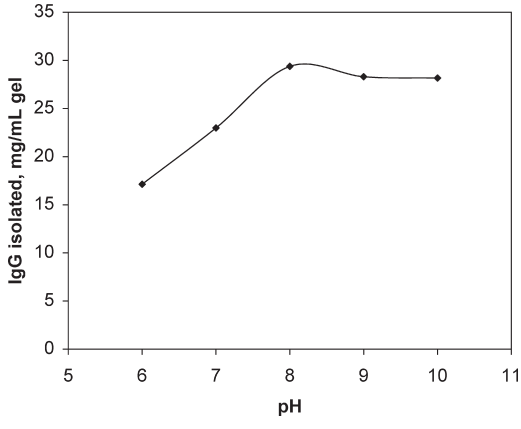

Figure 4. The effects of pH of the binding buffer on the yield of antibodies purified on a Protein A affinity gel.

Table 1. Effects of the number of carboxylic groups of an organic acid on enhancing the yield of antibodies purified using Protein A affinity chromatography

CARBOXYLIC ACID				IgG isolated (mg/mL gel)
Mono-carboxylate	Di-carboxylate	Tri-carboxylate	Tetra-carboxylate	
Acetate				10.9
Glycine				10.3
	Aspartate			13.7
	Glutamate			12.8
	Malate			14.1
	Glutarate			13.2
	Succinate			16.7
	Tartrate			13.4
	Ketoglutarate			16.9
		N-(2-Hydroxy ethylene diamine triacetate (HEDTA)		17.4
		Isocitrate		**21.5**
		Citrate		**23.31**
			Ethylene diamine tetra acetate (EDTA)	**22.5**
			Ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′N′-tetra acetate(EGTA)	**20.5**

effective in promoting the binding of IgG to Protein A column. Indeed, a plot of the number of carboxylic groupings of various organic salts used in the binding buffer versus the average quantity of IgG isolated showed a remarkably linear relationship with a correlation coefficient of 93% (Fig. 5). The enhancing effects of salts of polycarboxylate in the binding buffer was also demonstrated in IgG purification using Protein G affinity chromatography (Fig. 6). Sodium citrate at 0.65 M and sodium EDTA at 0.55 M, respectively, enhance the yield of IgG purified by Protein G affinity chromatography by 68% and 52%. The enhancing effects of sodium salts of citrate and EDTA on the yield of affinity purified IgG are additive, which is a characteristics of a kosmotrope.[25] For example, a combination of one-half the concentration previously used sodium citrate (0.325 M) and sodium EDTA (0.275 M) in the

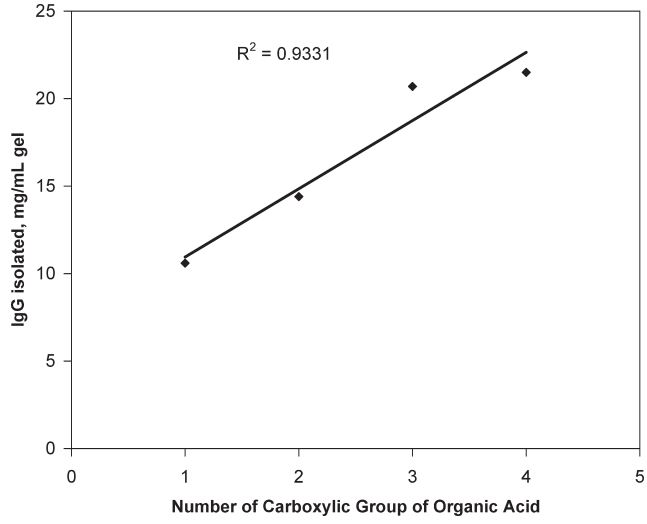

Figure 5. Dependency of the number of carboxylic groups of an organic salt used in the binding buffer to enhance the yield of antibody purified using Protein A affinity gel.

binding buffer enhances the yield of affinity purified IgG by 60%, which is the average percentage of full strength sodium citrate (0.65 M) and sodium EDTA (0.55 M) used alone (average of 68% and 52% = 60%).

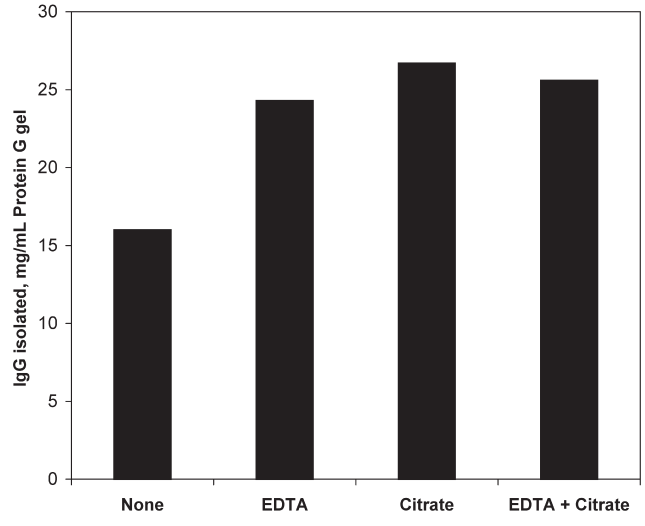

Figure 6. The additive effects of polycarboxylic salts on the increased yield of IgG isolated on a Protein G affinity gel.

Although current data clearly demonstrate that the use of kosmotropes result in a significant increase in the yield of antibodies purified using Protein A or Protein G affinity chromatography, the precise mechanism for such effects cannot yet be firmly established. It is, however, well documented that several of the kosmotropic salts used in this study have been extensively used in non-specific hydrophobic interaction chromatographic separations of biomolecules,[11–14] in thiophilic (biomimetic) chromatographic purification of IgG,[21–22] and in aza-arenophilic chromatographic purification of IgG.[23–24] In this study, we found that anions that enhance the yield of IgG purified with Protein A affinity chromatography included phosphates, sulfates, and polycarboxylates. These are the major intracellular anions and are all kosmotropes.[10] On the other hand, the major intracellular monovalent cations are chaotropes, which include potassium ion, lysyl ε-ammonium groups, arginyl guanidium group, and the imidazolium group of histidine. Salts of these kosmotropes and chaotropes are highly soluble.[10] A useful way for investigating the interactions between water molecules, salt ions, and proteins is the use of viscosity coefficient, β, of the Jones-Dole equation.[26] The viscosity coefficient (β) is a thermodynamic parameter that measures the extent of an increase or decrease in the viscosity of water caused by the addition of ions. The β values for ions that decrease the viscosity of water have negative values. Such ions are known as chaotropes. The interaction between chaotropes and water is weaker than water with itself.[9] In contrast, the β values for ions that increase the viscosity of water are positive and are known as kosmotropes. The interaction between kosmotropes and water is stronger than water with itself.[9] The anions such as phosphates, sulfates and polycarboxylates, capable of enhancing the yield of IgG purified with Protein A affinity chromatography, all have positive β values. In addition to their effects in mediating chromatographic separation of proteins,[15,16] kosmotropes such as sulfate and phosphate have previously been shown to greatly activate lyophilized enzymes in organic solvents.[27]

CONCLUSIONS

Kosmotropes such as phosphates, sulfates, and polycarboxylates added to the binding buffer enhance the yield of IgG purified on Protein A or Protein G affinity gels.

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