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Affinity separation by protein conjugated IgG in aqueous two-phase systems using horseradish peroxidase as a ligand carrier

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Abstract

A novel affinity separation method in an aqueous two-phase system (ATPS) is suggested, using protein conjugated IgG as a ligand. For verification of the proposed approach, horseradish peroxidase (HRP) and human IgG was used as a ligand carrier and affinity ligand, respectively. The partition of the affinity ligand, human IgG, was controlled by the conjugation of HRP. Two ATPSs, one consisting of potassium phosphate (15%, w/w) and polyethylene glycol (PEG, M.W. 1450, 10%, w/w) and the other of dextran T500 (5%, w/w) and PEG (M.W. 8000, 5%, w/w), were used. The conjugated human IgG-HRP favored a PEG-rich top phase, whereas human IgG, rabbit anti-human IgG and goat anti-mouse IgG preferred a salt or dextran-rich bottom phase. Using the conjugated human IgG-HRP, rabbit anti-human IgG was successfully separated into a PEG-rich top phase from the mixture with goat anti-mouse IgG. The appropriate molar ratio between human IgG-HRP and rabbit anti-human IgG was around 3:1 and 1:1 for the salt and dextran-based ATPS, respectively. The dextran-based ATPS showed a better recovery yield and purity than the salt-based ATPS for the range of test conditions employed in this experiment. The yield and purity of the recovered rabbit anti-human IgG were 90.8 and 87.7%, respectively, in the dextran-based ATPS, while those in the salt-based ATPS were 78.2 and 73.2%.

Keywords: Affinity separation; Aqueous two-phase system; Protein conjugated IgG; Ligand carrier protein

1. Introduction

ATPS is a well-known and useful technology for the separation of biomolecules, offering biocompatibility, diversity, ease of scale-up, etc. Various molecules have been used for the construction of ATPSs, including salt, dextran, polyethylene glycol (PEG), polysaccharide, and block copolymers. Electrostatic repulsion between the phase-forming molecules, which are dissolved together above critical concentrations, constructs two immiscible phases. Since 65–90% of the ATPS is generally water, it provides favorable conditions regarding the stability of biomolecules [1,2]. The partition coefficient (K) presents the favored phase of a target molecule in ATPS [3]. It is affected by the surface properties of the molecules [4], the molecular weight and concentration of polymers [5–7], etc. In addition, the additional salt in the ATPS affects the hydrophobicity of the ATPS and the molecules, and thus the target molecules inside the ATPS can be separated more easily [8,9]. However, a high concentration of salt in the ATPS can damage the target molecule, resulting in lowered activity of the recovered molecules. Therefore, partitioning of impurities is also affected by the increment of the additional salt concentration. Consequently, lower purity of the recovered target molecule could be obtained [10,11].

Affinity partitioning (AP) based on the interaction between a molecule and its affinity ligand is a function of the number of ligands per molecule, the concentration of ligands, and their binding characteristics [12]. The affinity between the molecule and its ligand has been used for the improvement of selectivity in ATPSs. PEG and poly-*N*-isopropylacrylamide (poly(NIPAM)) have been used as ligands for affinity separation in an ATPS [13–16]. Ling et al. [17] selectively recovered mono-methoxy-poly(ethylene glycol) (MPEG) modified *Streptococcus* B1 in an ATPS composed of PEG (M.W. 6000, 5%, w/w) and dextran T250 (7.5%, w/w). This technique was also applied for the

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quantification of bacterial cells. Protein A was separated from a crude extract of *E. coli* DH1 suspension using a human IgG and Eudragit bound magnetic ferro-fluid [18]. The separation of alcohol dehydrogenase was demonstrated using the dye Procion red from a bovine liver crude extract in ATPS-based countercurrent chromatography [19]. Soybean peroxidase was purified by metal affinity partitioning in an ATPS [20]. The affinity ligand was PEG-IDA-Cu^{2+} in salt (Na_2SO_4 or potassium phosphate) and a PEG (M.W. 4000)-based ATPS. The bioaffinity between an enzyme and substrate was also used for the separation of cutinase with a fatty acid substrate in an ATPS [21].

Despite such extensive work in this area, to our knowledge there has been no report on the use of large molecular weight protein as a carrier to change the partition of the affinity ligand. Protein is a good candidate for controlling the partition, because its relatively high molecular weight enhances uneven distribution in an ATPS. Proteins typically have higher biocompatibility than metals or dyes. Furthermore, an appropriate carrier enzyme could be directly used for the analysis of the concentration of molecules, as performed in the experiment reported here. In this study, horseradish peroxidase was selected as a model protein to serve as a carrier for the control of partition of an affinity ligand, human IgG. Human IgG was used as an affinity ligand for selective binding with a target molecule, rabbit anti-human IgG. Goat anti-mouse IgG was used as a model impurity molecule for the separation.

2. Experimental

2.1. Aqueous two-phase systems

Two ATPSs were tested in this study. One was composed of 10% (w/w) PEG (M.W. 1450) (Sigma–Aldrich Co., USA) and 15% (w/w) potassium phosphate (Samchun Co., Korea). The other consisted of 5% (w/w) PEG (M.W. 8000) (Sigma–Aldrich Co.) and 5% (w/w) dextran T500 (M.W. 500,000) (GE Healthcare Co., Sweden). These systems were constructed from concentrated stock solutions of 20% (w/w) PEG 1450, 10% (w/w) PEG 8000, and 10% (w/w) dextran T500 in 10 mM potassium phosphate buffer (pH 7.0). The concentrated stock solution of potassium phosphate (30%, w/w) for phase-separation was dissolved in distilled water. Each ATPS was constructed by mixing of stock and buffer solutions at an appropriate ratio to make a total amount of 1 g, including IgG solutions. The total amount of IgGs added in each ATPS was fixed at 10 mg in all cases except the experiment on the effect of molar ratio on partitioning. Total 20 mg of IgGs was added when the effect of molar ratio on partitioning was surveyed to assure the accuracy by using more than 2.0 μl of solutions, especially for 1:10 and 10:1 ratio. The volume of added IgG stock solutions in ATPS was decided considering the concentration and molecular weight of IgGs in the solutions. The averaged molecular weight of rabbit anti-human IgG and goat anti-mouse IgG is 140 kDa while that of human IgG-HRP is 180 kDa (supplied by manufacturer). The mixed ATPSs were centrifuged at 3000 rpm for 15 min and kept for 1 h to complete phase-separation after adding antibody solutions.

2.2. Antibodies

The three commercially available antibodies were used without further purification treatment. HRP conjugated human IgG (Pierce Co., USA) were used as a ligand for affinity separation of rabbit anti-human IgG (Pierce Co.). Goat anti-mouse IgG was purchased from Pierce Co. The concentrations of IgGs in stock solutions were 0.6, 0.6 and 1.0 g/l for rabbit anti-human IgG, goat anti-mouse IgG and human IgG-HRP, respectively. The concentrations of antibodies were analyzed by enzyme-linked immunosorbent assay (ELISA). Using alkaline phosphatase (AP) conjugated goat anti-rabbit IgG and AP conjugated rabbit anti-goat IgG, the concentration of rabbit anti-human IgG and goat anti-mouse IgG, respectively, was determined.

2.3. Analysis

The concentrations of IgGs were determined using a 96-well plate and an ELISA reader (Biorad Co., USA). After the IgGs were coated on the well using a 50 mM sodium carbonate buffer solution (pH 9.6, Samchun Co.), the concentration of human IgG-HRP was directly measured using the ELISA reader at 405 nm with 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS, KPL Co., USA) as a substrate. The concentration of rabbit anti-human IgG and goat anti-mouse IgG was determined by AP activity using *p*-nitrophenyl phosphate (PNPP, Pierce Co.) as a substrate. Since polymers and salts affect the analysis of IgGs, the standard equations for the calculation of each IgG were individually obtained using same polymer and salt solutions in ATPSs (data not shown).

2.4. Partition coefficient

Partition coefficient of the IgG was defined as the ratio of the concentration of IgG in the top and bottom phases. After measuring the antibody concentration in each phase, the logged partition coefficient ($\log K$) of the antibody was calculated. The $\log K$ becomes 0 when IgG is equally distributed in both phases.

3. Results and discussion

3.1. Partition of IgGs

Partition of individual HRP conjugated human IgG, rabbit anti-human IgG, and goat anti-mouse IgG was surveyed. As an affinity ligand, the opposite partition of human IgG-HRP from rabbit anti-human IgG and goat anti-mouse IgG is essential for the separation. Potassium phosphate and dextran T500 were selected as a salt and polymer, respectively, in a mixture with PEG, based on previous reports on the partition of IgGs and HRP [8,22–25]. The partition coefficients of human IgG-HRP, rabbit anti-human IgG, and goat anti-mouse IgG in salt or dextran-based ATPSs are shown in Fig. 1. All IgGs including HRP conjugated human IgG show the same trend in both the salt and dextran-based ATPSs. The partition coefficient of HRP conjugated human IgG was positive, whereas the coefficients of the other two IgGs were negative. It is thought that the surface

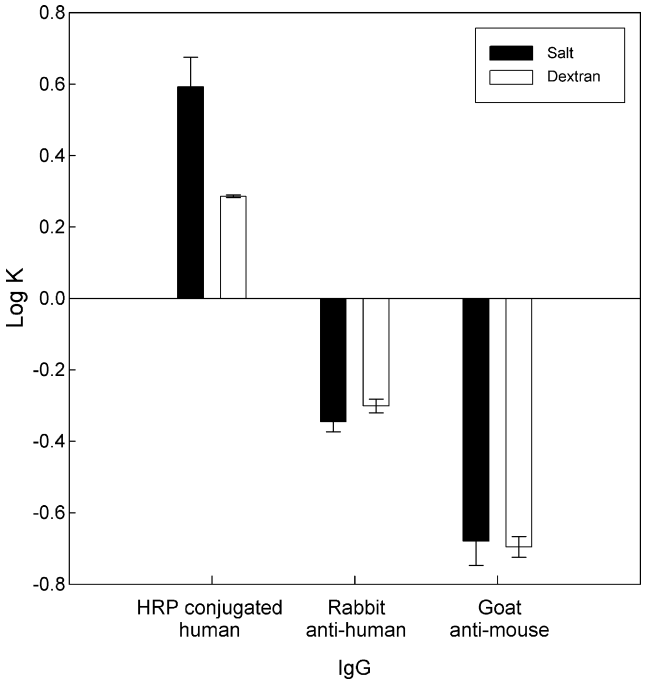

Fig. 1. Partition coefficient of IgGs in salt or dextran-based ATPS composed with PEG.

properties of human IgG-HRP were changed by conjugation. Further investigation on the effect of conjugation on the surface properties and partition of human IgG-HRP is required. The dextran-based ATPS has lower selectivity on human IgG-HRP than the salt-based system, because the differences of the partition coefficients are smaller. The partition coefficient of human IgG-HRP in the dextran-based ATPS was around half that of the salt-based ATPS, while the remaining two IgGs show similar partition coefficients in both ATPSs.

3.2. *Effect of molar ratio of IgGs on partitioning*

The partition coefficients of human IgG-HRP, rabbit anti-human IgG, and goat anti-mouse IgG in ATPSs with varying molar ratio are shown in Fig. 2. The partition coefficient of human IgG-HRP was lower than the original values (in Fig. 1), 0.6 and 0.3, for salt and dextran-based ATPSs, respectively, when the ratio between human IgG-HRP and rabbit anti-human IgG was 1:1, 1:5 or 1:10 (see lines with black circles in Fig. 2). The increment of the fraction of human IgG-HRP induced an increased partition of rabbit anti-human IgG in both the salt and dextran-based ATPSs (see lines with white circles in Fig. 2). On the contrary, the partition coefficient of human IgG-HRP was decreased as the fraction of rabbit anti-human IgG was increased, because rabbit anti-human IgG preferred the salt or dextran-rich bottom phase, as described in section 3.1. Thus, rabbit anti-human IgG can be partitioned more into the PEG-rich top phase following the human IgG-HRP, and vice versa. As shown in Fig. 2a, the partition of rabbit anti-human IgG is maximal in the salt-based ATPS when the ratio of human IgG-HRP to rabbit anti-human IgG is 10:1; however, it was drastically decreased with an decrease of the ratio of human IgG-HRP. The partition coefficient of rabbit anti-human IgG was lower than human IgG-HRP in the salt-based ATPS when the ratio of rabbit anti-human IgG was larger than 1:1 (e.g. 1:1, 1:5 and 1:10). Since the partition coefficient of rabbit anti-human IgG was higher than human IgG-HRP, at 10:1 and 5:1, theoretically more than one rabbit anti-human IgG should bind on a human IgG-HRP. Considering that the molecular weight of HRP and IgG is roughly 40 and 150 kDa, respectively, it is thought that the active site of rabbit anti-human IgG would mainly affect the partition of rabbit anti-human IgG, as suggested by Wingren et al. [25]. The

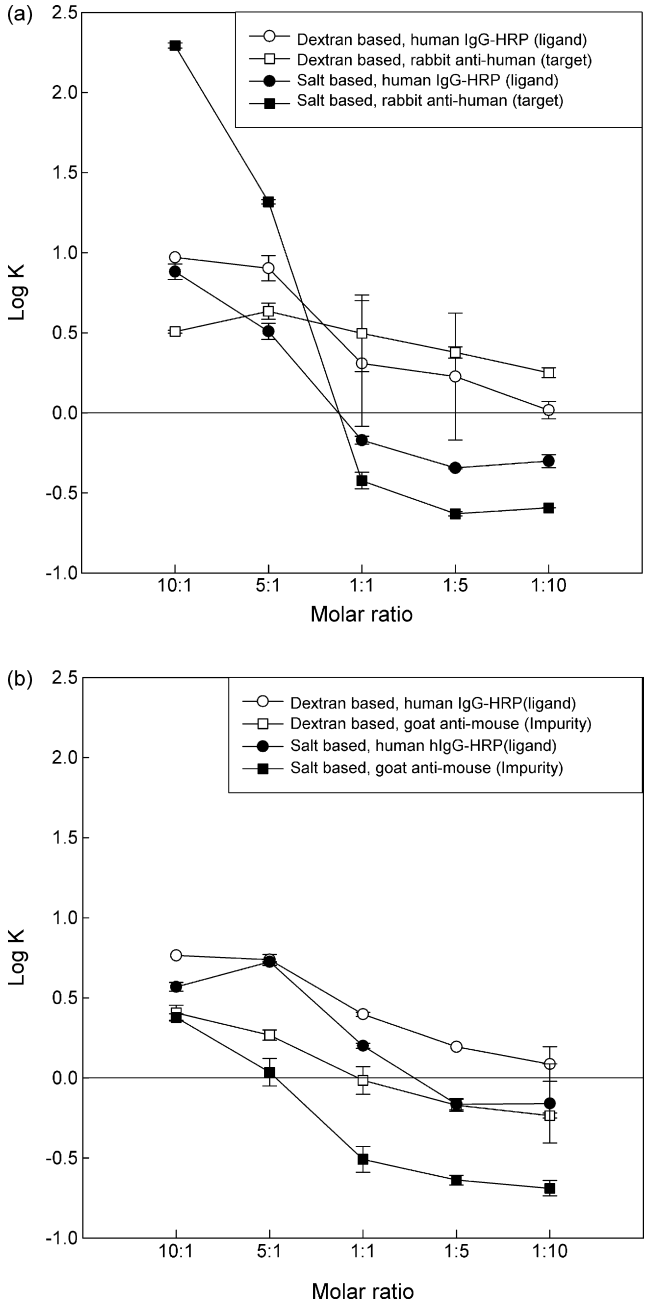

Fig. 2. Effect of molar ratio between human IgG-HRP and rabbit anti-human IgG or goat anti-mouse IgG on partitioning of IgGs in salt or dextran-based ATPS. (a) From the mixture of human IgG-HRP (ligand) and rabbit anti-human IgG (target) and (b) from the mixture of human IgG-HRP (ligand) and goat anti-mouse IgG (impurity).

active site of rabbit anti-human IgG would not be exposed to the solution after binding with the human IgG-HRP.

The partition coefficient shows a different trend in the dextran-based ATPS. While the ratio of human IgG-HRP is higher than rabbit anti-human IgG (e.g. 10:1 and 5:1), the coefficient of human IgG-HRP is higher than rabbit anti-human IgG. In addition, the complex of human IgG-HRP and rabbit anti-human IgG would favor a PEG-rich top phase in the dextran-based ATPS more than in the salt-based ATPS, because the partition coefficient of human IgG-HRP and rabbit anti-human IgG is larger than 0, even with a molar ratio of rabbit anti-human IgG that is higher than 1:1 (e.g. 1:1, 1:5 and 1:10). Human IgG-HRP and rabbit anti-human IgG were partitioned in the PEG-rich top phase in all molar ratio ranges in the dextran–PEG ATPS, even at a 1:10 ratio. Again, it can be concluded that a multiple number of rabbit anti-human IgGs can bind on a human IgG-HRP and the complex prefers the PEG-rich top phase.

Goat anti-mouse IgG was used as a model impurity for the affinity separation, and thus the mixture with human IgG-HRP showed similar trends with the rabbit anti-human IgG, as shown in Fig. 2b. The amount of partitioned human IgG-HRP was inversely proportional to the ratio of goat anti-mouse IgG. However, the difference in the partition coefficients by the increment of goat anti-mouse IgG was relatively smaller than that of rabbit anti-human IgG. The partition coefficient of human IgG-HRP with goat anti-mouse IgG was higher than 0, even when the molar ratio of goat anti-mouse IgG was greater than 1:1. Considering the partition coefficient of human IgG-HRP (lower than 0) with rabbit anti-human IgG in the dextran-based ATPS (Fig. 2a), these observations can be interpreted by the weaker binding force of cross-reactivity between goat anti-mouse IgG and human IgG-HRP relative to the affinity of goat anti-mouse IgG with rabbit anti-human IgG. No inversion of the partition coefficient of human IgG-HRP was found by the molar ratio of goat anti-mouse IgG, in contrast with the case of rabbit anti-human IgG. Even if the partition coefficient of human IgG-HRP and goat anti-mouse IgG were affected by their cross-reactivity, it would be smaller when a mixture of rabbit anti-human IgG and goat anti-mouse IgG were used, because human IgG-HRP has a much stronger binding force with rabbit anti-human IgG than with goat anti-mouse IgG.

3.3. Affinity separation in ATPSs

The affinity separation of rabbit anti-human IgG from a model mixture that contained rabbit anti-human IgG and goat anti-mouse IgG was conducted. The appropriate molar ratio of human IgG-HRP, rabbit anti-human IgG, and goat anti-mouse IgG for affinity separation was selected as 3:1:1 and 1:1:1 for salt and dextran-based ATPSs, respectively. The ATPS conditions were chosen under consideration of partition coefficients of the ligand, target and impurity IgGs (Fig. 3) for higher purity than recovery yield. Consequently, the partition coefficients were slightly changed when a mixture of target (rabbit anti-human IgG) and impurity (goat anti-mouse IgG) molecules, instead of a single molecule, was used. The competition between affinity binding and cross-reactivity is expected to affect the

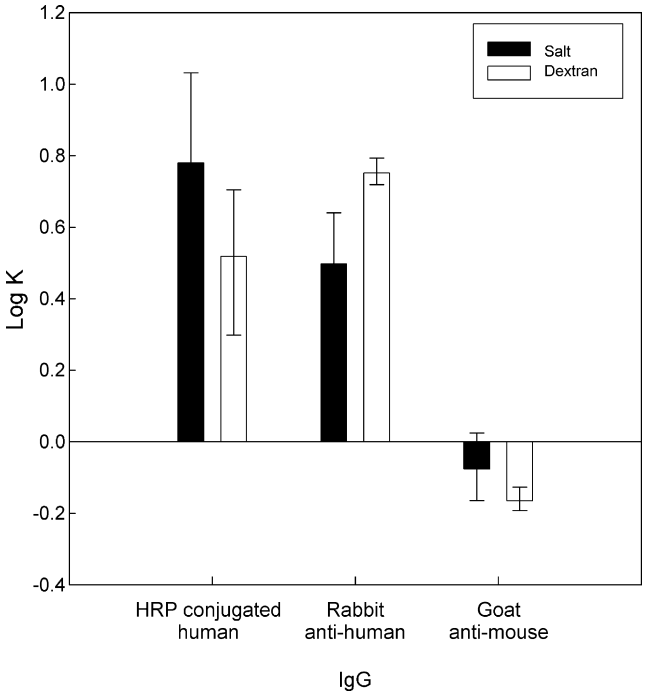

Fig. 3. Partition coefficient of IgGs after affinity separation by human IgG-HRP from a mixture of rabbit anti-human IgG and goat anti-mouse IgG in salt or dextran-based ATPS.

binding of molecules. Further experiment on this assumption is needed. The partition coefficient of human IgG-HRP was increased from 0.59 to 0.78 and from 0.29 to 0.52 in salt and dextran-based ATPSs, respectively, compared with the partition coefficient when only human IgG-HRP was dissolved in the ATPS (Fig. 1). Rabbit anti-human IgG was moved to the salt or dextran-rich bottom phase when it was added alone, but was lifted to the PEG-rich top phase from the mixture with human IgG-HRP, even when goat anti-mouse IgG was mixed together. The target IgG was successfully separated from the mixture by the affinity separation method. The partition coefficient of rabbit anti-human IgG was inverted by human IgG-HRP from −0.34 to 0.50 and from −0.30 to 0.75 in the salt and dextran-based ATPSs, respectively. The model impurity molecule, goat anti-mouse IgG, still partitioned more in the salt or dextran-rich bottom phase, but the partition coefficient was increased from −0.68 to −0.08 and from −0.7 to −0.17 in the salt and dextran-based ATPSs, respectively. The dextran-based ATPS showed better recovery yield and purity than the salt-based system. The recovery yield and purity of rabbit anti-human IgG from the mixture were 78.2 and 73.2%, respectively, in the salt-based ATPS. In the case of the dextran-based ATPS, the yield and purity of rabbit anti-human IgG were 90.8 and 87.7%, respectively. These results demonstrate that the proposed method of affinity separation by protein conjugated IgG in an ATPS is a promising technique for the separation of biomolecules.

4. Conclusion

A novel affinity separation method in an ATPS using protein conjugated IgG is suggested. Rabbit anti-human IgG was

successfully recovered from a mixture with goat anti-mouse IgG using human IgG-HRP conjugate as affinity ligand. The separation technique using protein as a carrier molecule of an affinity ligand described here is very promising for the separation of biomolecules, since proteins have biocompatibility, diversity and uneven distribution in ATPS, etc. In addition, some of the ligand carrier proteins conjugated with IgG, such as HRP, alkaline phosphatase and β-galactosidase, can be directly used for the quantitation of IgGs. Further investigations using various proteins as carrier molecule for the control of the partition of the affinity ligand and application of developed techniques on real samples are needed.

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