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Protein separation with surfactant-coated polystyrene involving Cibacron Blue 3GA-conjugated Triton X-100

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Abstract

Through mixing of porous polystyrene particles (Amberlite XAD-4), non-ionic surfactants, and surfactant-conjugated substrates (affinity ligand) in an aqueous solution led to the formation of a novel medium (affinity admicelle) for protein separation. The ligand (CB–Triton) was synthesized by mixing a triazine dye (Cibacron Blue 3GA (CB)) and a polyoxyethylene-type non-ionic surfactant (Triton X-100) in weakly alkaline solutions. Triton X-100 and CB–Triton were competitively sorbed onto XAD-4. Albumin (bovine serum), alcohol dehydrogenase (yeast), and lysozyme (chicken egg) having specific interaction to CB were collected onto the affinity admicelle. On the other hand, the collection of ovalubmin (chicken egg white), having no binding ability to CB, was negligibly small. Lysozyme in 100 μl of chicken egg white, diluted with 900 μl of 10 mM Tris–HCl (pH 7.4), was successfully collected on 18 mg of CB–Triton admicelles and, then, it was eluted with 1 ml of aqueous solution of 100 mM phosphate (pH 7.4). The recovery based on the activity for the lysis of micrococcus and the concentration factor were 60% and 40 ($n = 3$), respectively.

Keywords: Polystyrene particles; Affinity ligands; Affinity admicelles; XAD-4; Lysozyme; Proteins; Cibacron blue

1. Introduction

Surfactant molecules can cooperatively sorb and form aggregates (namely admicelles or hemimicelles) on the surfaces of different kinds of solid materials by hydrophobic or electrostatic interactions [1–3]. Since the aggregates provide hydrophobic media, a wide range of hydrophobic compounds are efficiently incorporated into them. Since admicelles were prepared by just mixing appropriate combination of surfactant and solid materials, their potential as the media for the collection of traces of hydrophobic substances in water has been extensively studied [1–5].

We have studied the potential to use admicelles as the media for the rapid and efficient concentration of trace hydrophobic compounds prior to several instrumental analyses [6–13]. When appropriate chelating agents were incorporated into the system, passing through the admicelle-filled column also resulted in the collection of traces of heavy metal ions [10,14]. These results suggest that any specific interaction for a certain compound can be introduced by impregnating selective hydrophobic ligand molecules into the admicelles.

In the present study, a novel separation media (affinity admicelle) for protein separation was developed by mixing porous polystyrene, non-ionic surfactant, and bio-mimetic affinity ligand in aqueous buffer solutions. A polyoxyethylene-type non-ionic surfactant, Triton X-100, was employed because it had been accepted as a mild surfactant for wide range of proteins. The bio-mimetic affinity ligand was synthesized by conjugating Triton X-100 with an artificial dye (Cibacron Blue 3GA). The utility of the affinity admicelle as a novel separation medium and the feasibility for the protein purification were investigated.

2. Experimental

2.1. Reagents

Fine particles (75–140 μm) of porous polystyrene–divinylbenzene resin (Amberlite XAD-4, pore diameter 300 nm, Rohm and Haas, Paris, France) were prepared by

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crushing the resin with an 18–34-type ball mill (Mitamura Riken, Tokyo, Japan) and fractionating with stainless sieves. The particles were washed with ethanol until negligible absorption (Abs.: <0.001) at 254 nm was observed in the eluate and subsequently rinsed with water. A non-ionic surfactant, Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether for biochemistry), was obtained from Nakarai Tesque (Kyoto, Japan). Proteins, albumin (bovine serum), alcohol dehydrogenade (yeast), lysozyme (egg white), ovalbumin (egg white), and an artificial dye, Cibacron Blue 3GA (CB), were purchased from Sigma (St. Lous, USA). A buffer component, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris, for biochemistry), and other reagents were obtained from Wako Pure Chemicals (Tokyo, Japan). Water used was prepared with a Milli-Q reagent water system (Millipore, St. Louis, USA).

2.2. Preparation of affinity ligand

A bio-mimetic affinity ligand, CB-conjugated Triton (CB–Triton), was synthesized by a modified method for synthesizing CB-conjugated polyethylene glycol [15] or CB-conjugated Triton X-114 [16]. To 50 ml of aqueous mixture containing 1 g of Triton X-100, 5 g of CB, and 3.5 g of sodium chloride, was added 1 ml each of 1 M lithium hydroxide at three intervals for 180 min. After the mixture was heated at 80 °C for 60 min, it was neutralized with acetic acid. The resulting solution was dialyzed against Milli-Q water. It was applied to Sephadex G-25 column (i.d.: 20 mm, length: 200 mm) and, then, eluted with Milli-Q water for removing free CB. The contents of CB–Triton and Triton X-100 were determined based on their absorbances (A) at 603 and 288 nm, respectively. Here, the molar absorptivities [$\mathrm{l\,(mol\,cm)^{-1}}$] of CB–Triton were 6.82×10^3 at 603 nm and 1.58×10^4 at 288 nm, while those of Triton X-100 was 0 at 603 nm and 4.30×10^2 at 288 nm.

2.3. Preparation of affinity admicelles

To 1.0 ml of aqueous suspension containing 25 mg of XAD-4 was added a prescribed amount of the aqueous mixture of CB–Triton and Triton X-100. After the suspension was gently mixed for 2 h, the CB–Triton-coated polymer resins (affinity admicelles) were washed with water at six times and equilibrated with 10 mM Tris–HCl buffer (pH 7.4 at 4 °C) for 10 min.

2.4. Protein sorption

A 1.0 ml portion of the aqueous buffer (Tris–HCl, pH 7.4) solution containing 0.1 mg $\mathrm{ml^{-1}}$ of an interested protein was poured into a 1.5 ml centrifuge tube in which typically 25 mg of affinity admicelles were precedently placed. After the tube was gently mixed for 5 min, it was centrifuged at 2000 g for 30 s. All procedures were performed at 4 °C. The sorption yield of the protein was calculated based on the determination of the protein in the supernatant by a Bicinchoninate method [17].

2.5. Purification of lysozyme

Chicken egg white was diluted with 3–20 times volume of 10 mM Tris–HCl (pH 7.4). A 1 ml aliquot of the resulting solution was placed into a 1.5 ml centrifuge tube involving 18 mg of affinity admicelles. The solution was gently mixed for 5 min. After the supernatant was removed, the admicelle was washed by 1 ml of 10 mM Tris–HCl (pH 7.4) three times. Finally, the admicelles were rinsed by 1 ml of 100 mM phosphate buffer solution for 5 min for eluting lysozyme. All procedures were performed at 4 °C. Total protein was determined by Bradford method [18]. Lysozyme activity was measured based on the rate of lysis of *Micrococcus Luteus* (Sigma, St. Lous, USA) [19,20]. A 100 μl portion of sample solution was added to 2 ml of bacterial suspension that had been placed in a 1 cm quartz cell at 25 °C. One unit was defined by the decrease in the absorbance $\Delta A = 1$ at 540 nm per minute.

3. Result and discussion

3.1. Formation of affinity admicelles

Triton X-100 and CB–Triton in the aqueous solution were well sorbed onto XAD-4 resin because of their hydrophobic properties of alkylphenyl moieties. The strong sorption of ethoxylated non-ionic surfactants onto polystyrene-type resins has been reported in the areas of colloid and surface sciences [21–23]. At 4 °C, the time for their equilibrium sorption was within 2 h. Maximum sorption of Triton X-100 on 1 mg of XAD-4 was ca. 1.2 μmol. However, the sorption of Triton X-100 decreased with increasing the amount of CB–Triton sorption, probably because of their competitive sorption (Fig. 1). Indeed, total amount of the sorption (Triton X-100 + CB–Triton) was almost independent on the initial concentration of CB–Triton. This suggests that Triton X-100 and CB–Triton almost completely coat the surface of XAD-4. After the particles were washed triplicate by 1 ml each of 10 mM Tris–HCl buffer solution (pH 7.4), negligible absorption ($A < 0.002$) at 610 nm was observed in further eluates. CB–Triton, being chemically unbound affinity ligand, was stably retained on the Triton X-100-coated polystyrene.

3.2. Collection of proteins to affinity admicelles

The effect of Triton X-100 coverage on the collection of BSA was shown in Fig. 2. In the absence of Triton X-100, considerable fraction of BSA was collected to the resins. BSA is strongly sorbed onto polystyrene surfaces and, hence, it has been utilized as a coating agent for polystyrene ELISA plates in order to eliminate non-specific binding of proteins

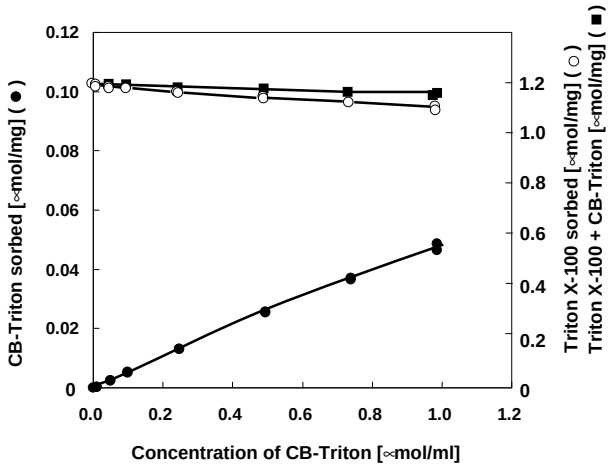

Fig. 1. Amount of Triton X-100 (○), CB–Triton (●), and the sum of Triton X-100 and CB–Triton (■) sorbed on XAD-4. Initial concentration of the sum of Triton X-100 and CB–Triton was 19.5 μmol ml^{-1}. CB–Triton concentration varied in the range of 0–0.986 μmol ml^{-1}. The mixed solution 1 ml was added to 16 mg of XAD-4.

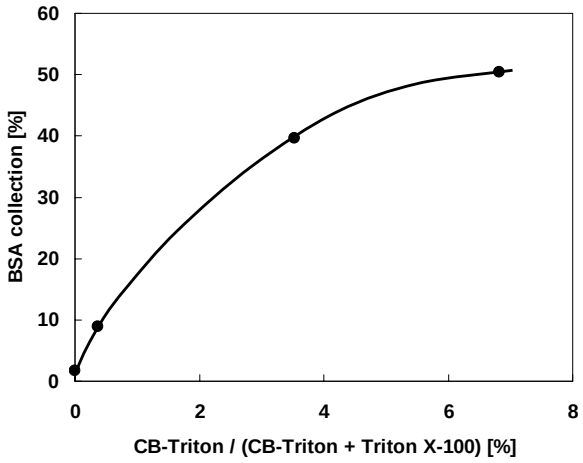

Fig. 3. Collection of BSA onto CB–Triton affinity admicelles. XAD-4: 44 mg; BSA: 0.1 mg ml^{-1}; 10 mM Tris–HCl (pH 7.4); 4 °C.

[24]. The sorption can be ascribed to the hydrophobic interaction between BSA and polystyrene surface. On the other hand, the collection of BSA decreased with increasing the sorption of Triton X-100. The surfactant molecules apparently prevent non-specific binding of the protein (Fig. 2).

Next, an affinity ligand, CB–Triton, was accompanied to the Triton X-100-coated polystyrene for providing specific interaction to certain proteins. As shown in Fig. 3, the extent of BSA collection increased with increasing CB–Triton fraction. Similar results were also obtained in the sorption of alcohol dehydrogenase (beakers yeast) and lysozyme (chicken egg) (Table 1). Constant recoveries of these proteins were obtained within 5 min. In contrast, ovalbumin (chicken egg white) having no specific interaction to CB was hardly collected by CB–Triton admicelles (Table 1). It is well-known that CB specifically interacts with a series of NAD$^+$-dependent enzymes including alcohol dehydrogenase and extensively employed as a bio-mimetic dye-affinity ligand for separating these proteins in column chromatography or aqueous two-phase separation system [25–27]. The results in the present study indicated that CB–Triton most likely acts as an affinity ligand in the surfactant-coated polystyrene media.

As listed in Table 1, lysozyme was almost quantitatively (>98%) collected to the CB–Triton admicelles. Although lysozyme has no specific binding site for NAD$^+$, its strong interaction with CB has been utilized for dye affinity-based purification method for lysozyme [28,29]. The strong interaction may be explained by the electrostatic force between negatively charged CB and positively charged lysozyme (pI = 11.0 [30]). On the other hand, low recovery of an NAD$^+$-dependent enzyme, alcohol dehydrogenase, suggests the requirement of further study concerning to the kind and/or structure of solid material, surfactant, and affinity ligand.

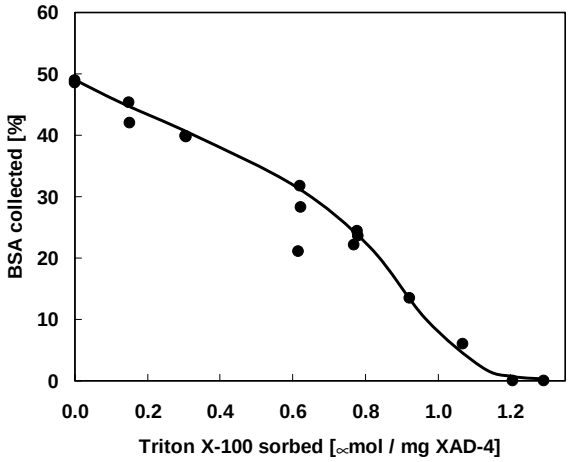

Fig. 2. Collection of BSA onto Triton X-100-coated XAD-4. XAD-4: 25 mg; BSA: 0.1 mg ml^{-1}; 10 mM Tris–HCl (pH 7.4); 4 °C.

Table 1
Collection of protein [%] to XAD-4, Triton X-100-coated XAD-4, and CB–Triton affinity admicelle

Protein	XAD-4	Triton X-100 coated XAD-4[a]	CB–Triton affinity admicelle[b]
Albumin (bovine serum)	50 ± 5 49[c]	2 ± 4 2[c]	27 ± 3 50[c]
Alcohol dehydrogenase (yeast)	48 ± 4	5 ± 3	21 ± 4
Lysozyme (chicken egg)	50 ± 6	8 ± 3	>99 >99[d]
Ovalbumin (chicken egg)	25 ± 3	3 ± 2	2 ± 2

± represents standard error (n = 3). XAD-4: 25 mg.
[a] 1.2 μmol Triton X-100 mg^{-1} of XAD-4.
[b] 1.1 μmol Triton X-100 and 0.045 μmol CB–Triton mg^{-1} of XAD-4.
[c] XAD-4: 44 mg.
[d] XAD-4: 18 mg.

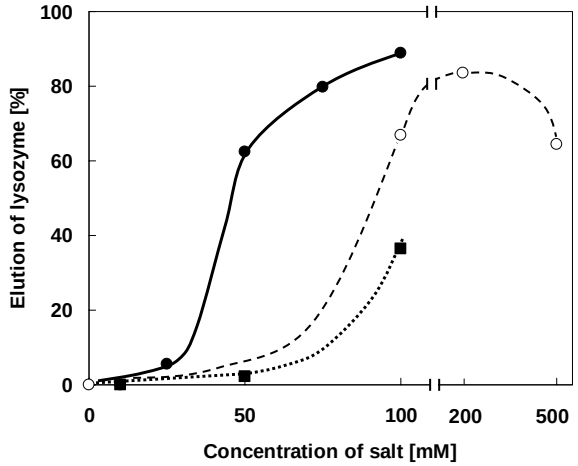

Fig. 4. Effect of salt concentration on the elution of lysozyme from CB–Triton affinity admicelles with 1 ml of aqueous buffer (pH 7.4) solution. (■) Tris–HCl; (○) NaCl with 10 mM Tris–HCl; (●) sodium phosphate.

3.3. Desorption of protein

The elution of proteins from affinity-based separation media has been often conducted by adding salts [31,32]. As depicted in Fig. 4, lysozyme was eluted with the increase in the concentration of salt or buffer components. Tris–HCl was not effective. On the other hand, lysozyme was well eluted with the aqueous solution of sodium chloride (containing 10 mM Tris–HCl; pH 7.4) or sodium phosphate (pH 7.4). Salts seem to weaken the electrostatic force between proteins and affinity ligand. Maximum elution yields were 80% for sodium chloride and 90% for sodium phosphate. In further sodium chloride concentration, the extent of the elution decreased. This may be ascribable to salt-enhanced hydrophobic interaction of lysozyme with somewhat hydrophobic Triton X-100–XAD-4 admicelles. Other proteins, albumin and alcohol dehydrogenase, were also successfully eluted with aqueous solutions of concentrated salt or buffer components.

3.4. Application to lysozyme purification

Finally, the present method was applied to the purification of lysozyme from chicken egg white. Lysozyme is commercially important enzyme as a drug, an antibacterial agent, a cell disrupting agent, or a food additive. Its potential use as an anticancer drug [33] or an inhibitor of HIV infection [34] has also been discussed. Lysozyme naturally occurs in chicken egg white composing of ca. 90% (w/w) of water, ca. 10% (w/w) of proteins, and very small amounts of lipids, sugars, and minerals. Major proteins present in chicken egg white are ovalbumin (54%), conalbumin (12%), ovomucoid (11%) and other ovo-proteins, while only ca. 3.4% (w/w) is lysozyme [35].

In the present study, chicken egg white contained 6.6 U ml^{-1} of lysozyme in 69.0 mg ml^{-1} of proteins. Specific activity of lysozyme defined by lysozyme activity containing 1 mg of proteins was 0.092 U mg^{-1}. Table 2 lists the results of purification when 1 ml of buffer solution containing egg white was treated with 18 mg of CB–Triton affinity admicelles. The first column represents the dilution of egg white with 10 mM Tris–HCl buffer (pH 7.4), being defined by: (egg white [ml] + buffer [ml])/egg white [ml]. Unless dilution, egg white was too viscous to treat for protein purification. When the extent of the dilution <three-folds, recovery of lysozyme was below 20%. In this case, total proteins containing in the solution was 23 mg. The low recovery is ascribable to the overload of lysozyme and other egg white proteins that potentially bind to the affinity ligand on admicelle. On the contrary, the recovery increased with increasing the extent of the dilution (decreasing protein amount loaded). By 10-folds of dilution, sufficient recovery (ca. 60%) was obtained. The purification factor, ca. 40, was the almost sufficient magnitude for one-step purification.

Table 2
Result of lysozyme purification from chicken egg white

Dilution[a]	Protein applied (mg ml^{-1})	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
5	13.8	3.4 ± 0.5	36 ± 5	35 ± 2
10	6.9	3.8 ± 0.7	40 ± 6	60 ± 8
15	4.6	4.5 ± 0.4	47 ± 4	81 ± 5

± represents standard error ($n = 3$).
[a] Volume ratio: (egg white [ml]+10 mM Tris–HCl [ml])/egg white [ml].

Recently, a packed bed chromatography [36] and membrane based separation methods [37] have been used for the purification of lysozyme. In those methodologies, the separation media that the affinity ligands were chemically modified were used. On the other hand, affinity admicelles or those formed on membrane-support can be prepared only by mixing surfactant-conjugated substrates (affinity ligands), surfactant molecules, and appropriate solid materials in the aqueous solution. The present method will remarkably simplify the preparation of affinity-based separation media for protein chromatography and other protein separation techniques.

4. Conclusion

A novel separation medium, affinity admicelle, was prepared just by mixing porous polystyrene, surfactant (Triton X-100), and surfactant-conjugated artificial substrate (Cibacron Blue 3GA). Collection of albumin, alcohol dehydrogenase, and lysozyme based on the specific interaction between the protein and the substrate was successfully achieved. Rinsing the affinity admicelles with concentrated salt or buffer components eluted the protein. The studies about the combination of solid support, surfactant, and affinity ligand will be fruitful for extending the present method to the purification of wide range of proteins.

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