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Original Communication

Rapid separation of proteins from the aqueous media using the novel gelation protocol

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ABSTRACT

Surfactants including those derived from the salts of fatty acids are often used for enhancing the solubility of proteins or conversely, for promotion of protein gelation in the aqueous media, thus applicable for separation of proteins from the biological samples. While the previously reported protein gelation protocols required the presence of highly concentrated proteins as the components of the gels, this report describes our recent attempts for capturing the proteins within the protein-independently formed surfactant-based gels which are rapidly formed after addition of surfactants and a metal-chelator to the solutions of proteins. Here, rapid isolation and removal of proteins from the liquid media using recently developed gel-forming formula with tetrasodium *N*,*N*-bis(carboxylatomethyl)-L-glutamate and sodium oleate are described. In the demonstrations, human hemoglobin, serum proteins, and mitochondrial proteins were successfully collected in the solid discs floating over the solution following centrifugation.

KEYWORDS: gel, GLDA, protein, soap

INTRODUCTION

Surfactants including those derived from the salts of fatty acids are often used for enhancing the solubility of proteins or conversely, for promotion of protein gelation in the aqueous media, thus applicable for separation of proteins from the biological samples [1]. While the previously reported protein gelation protocols required the presence of highly concentrated proteins (eg. percent order of ovalbumin and rice globulin mixed) as the components of the gels [2, 3], this report describes our recent attempts for capturing the proteins within the surfactant-based gels rapidly formed after addition of surfactants and a metal-chelator to the solutions of proteins.

Fatty acid salts such as sodium oleate (Ole-Na; Fig. 1A, left, top) and potassium laurate (Lau-K; Fig. 1A, left, bottom) form a group of the most widely used surfactants (well known as soap components), and have many uses such as ingredients of shampoos and detergents, food additives, and as components of fire-fighting foam agents [4]. Recently, from the eco-centric points of view, the use of soap components including Ole-Na and Lau-K was encouraged for replacing the other synthetic surfactants due to the relatively lower toxic impacts of soap-based detergents to aquatic organisms [5, 6]. Interestingly, during the development of novel fire-fighting foam formula, there was an unexpected episode of fatty acid salt-dependent gelation of the liquid containing a chelating agent [7]. An X-ray-based qualitative study and further HPLC-based quantitative study on the nature of newly formed gels consistently

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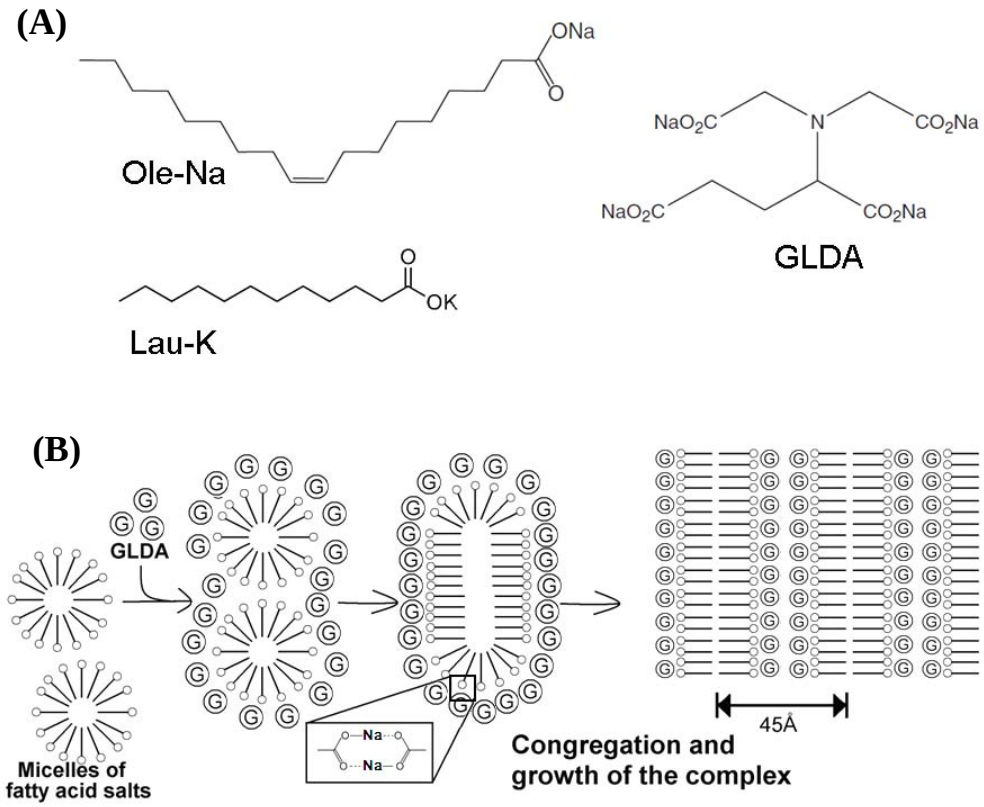

Fig. 1. Structures of gel-forming agents. (A) Structures of sodium oleate (Ole-Na), potassium laurate (Lau-K), and tetrasodium *N*,*N*-bis(carboxymethyl)-L-glutamate (GLDA). (B) The proposed model for the growth of lamellar structure from the micelles of fatty acid and chelating agent (GLDA shown as G). Note that, highlighting the orientation of Ole-Na and GLDA in the gel, their stoichiometory is not reflected in the illustration. This model was modified and adapted from [8].

suggested the stoichiometry of gel-formation in the chelator:soap mixture to be 4:1, and the likely structure of the vesicle system was recently elucidated [8] as illustrated in Fig. 1B.

Here, we report on the development of a novel protein separation protocol by making use of the above serendipitous materials, namely, Ole-Na and Lau-K as the surfactants for dissolving the proteins in the aqueous media and tetrasodium *N*,*N*-bis(carboxymethyl)-L-glutamate (GLDA) as the key chelating agent helping the formation of the gels, for the ease of separation of the proteins from the aquatic media.

MATERIALS AND METHODS

Reagents

Solution of GLDA (40%, w/w) was obtained as Chelest CMG 40 (Chelest Co., Osaka, Japan). Ole-Na and Lau-K were provided by Shabondama Soap Co. Ltd. (Kitakyushu, Japan). Human hemoglobin (Hb, lyophilized powder, tested negative for HIV and hepatitis B antigen) and lipase proteins purified from pseudomonas species (Sigma L-9518, 30 units/mg) were obtained from Sigma (St. Louis, MO, USA). Since Hb is readily auto-denatured under aerobic condition [9], the presence of methohemoglobin as the major intermediate species in the Hb solution was spectroscopically observed (data not shown). Human serum sample and rat liver mitochondrial protein sample were provided by APRO Life Science Institute Inc. (Tokushima, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Above protein samples except for lipase were dissolved in phosphate buffered saline (PBS; 137 mM NaCl, 8.1 mM $Na_2PO_4 \cdot 12\ H_2O$, 2.68 mM

KCl and 1.47 mM KH_2PO_4) prior to the separation experiments. Lipase was dissolved in 10 mM Tris-HCl (pH 8.5). Other chemicals were dissolved in distilled water.

Separation of model dye and proteins by rapid formation of gels

Model demonstrations for separating a dye or proteins from the sample solutions were performed stepwise as per the procedures illustrated in Fig. 2. Firstly, the soap-based solutions (reagent A) was added to the blank solution (PBS) or the dye/ protein sample solutions in 1.5 mL centrifugation tubes and well mixed on the vortex mixer. Then, GLDA solution (reagent B) was added to the reagent A-mixed sample solutions and well vortex-mixed to form the gel phase capturing the target dye or proteins. Then the samples were subjected to centrifugation (at 14,000 rpm, 5 min). Finally, the dye or proteins of interest are all collected in the resultant solid layer (discs) covering over the liquid media, newly formed by centrifugation-dependent solidification.

SDS-PAGE

The separation of protein by novel gel-forming protocol was evaluated by SDS-PAGE analyses of samples prepared from both the liquid phase and

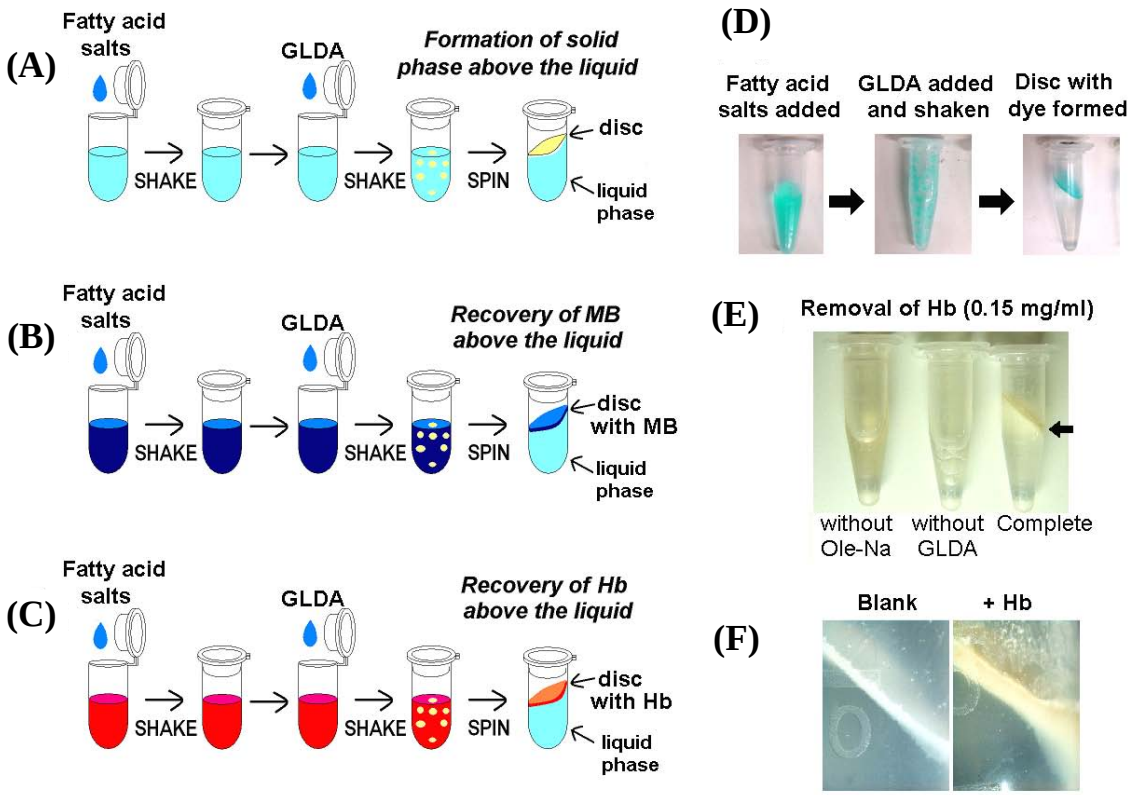

Fig. 2. Procedures for gelation and formation of solid discs from the model mixtures of fatty acid salts and GLDA. (A), gelation and gel-based disc formation in the blank solution. (B, D), Separation of methylene blue (MB) used as a model dye. Discs of MB-containing gel were formed and packed above the media in the plastic tubes. MB was immediately collected in the packed gel layer (solid disc over the media) by mixing with gel-forming reagents followed by centrifugation. (C, E) Separation of hemoglobin (Hb) used as a model pigmented protein. Hb was immediately collected in the packed gel layer (solid disc over the media) by mixing with gel-forming reagents followed by centrifugation. Arrow indicates the disc of gel recovering Hb (see also F). (F) Swelling and pigmentation of the gel-based disc by adsorbing Hb. Collection of pink pigment in the solidified layer can be observed by eyes. The concentrations of reagents used for demonstrations (D-F; in total of 1 ml, each) were 50 mM Ole-Na, 200 mM GLDA, 0.5 mM MB, and 0.15 mg/ml of Hb.

the solidified gel phase. Following the centrifugation of the samples mixed with both the reagents A and B, the resultant major layer (liquid phase) was collected in new tubes. Then, the upper layer (solid phase collected as discs) were also collected in new tubes and re-suspended in PBS for elution of proteins. The obtained sample solutions (liquid phase samples and solid phase samples) were mixed with 4X volumes of acetone (final concentration 80%) and kept at -80°C for 1 h. Following centrifugation at 14,000 rpm at 4°C for 10 min, the resultant pellets were collected and re-suspended in PBS. The obtained aliquots were analyzed on SDS-PAGE according to the method described by Laemmli [10]. The proteins were prepared in loading buffer (0.5 M Tirs-HCl, pH 6.8, 10% SDS, 0.6% 2-mercaptoethanol, 1% glycerol and 1% bromophenol blue) without boiling. The proteins were separated on 10% acrylamide gel and exposed to sensitive staining with a Silver Stain Kit II (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions.

RESULTS

Preliminary demonstrations

In the preliminary experiments, the effects of 8 different fatty acid salts (50 mM each) as the key components for gel formation in the presence of GLDA (200-500 mM) were examined, but among the fatty acid salts tested, Ole-Na and Lau-K were chosen as the best materials for rapid formation of the gels over the media (data not shown). When we mixed two transparent aqueous solutions of the reagent A (containing soap component chiefly Ole-Na) and the reagent B (containing GLDA), a white gel phase was formed when the total solute concentrations were high enough, indicating that complexation between GLDA and Ole-Na caused the gelation as reported [8]. As a preliminary run, typical demonstrations for separating a model dye or a model protein were performed (Fig. 2D-F). By using colored solutes (MB and Hb), collection of target samples in the gel-based disc formed over the media was successfully visualized.

Separation of a model enzyme from the reaction mixture

As above demonstration suggested that protein can be removed from the liquid phase by forming and separating the gels adsorbing the target proteins, removal (recovery) of commercially available lipase proteins purified from pseudomonas species (used as a model enzyme) was performed (Fig. 3). The enzyme reaction mixture containing 1 unit/ml of enzyme (6.7 ng proteins, prepared according to instruction by the vender) was subjected to separation of proteins by gel-forming protocol using 50 mM Ole-Na and 200 mM GLDA. After dissolving the proteins from the gel layer or liquid phase recovered, protein samples corresponding to x1/20 volume of enzyme reaction mixture were subjected to SDS-PAGE to examine the separation efficiency. Since the concentration of protein used for enzyme reaction was much lower than the range visualized by Coomassie brilliant blue (CBB) staining, the presence of protein on the SDS-PAGE gel was detected after silver staining (Fig. 3). As expected, two major bands corresponding to the purified lipase protein were detected only in the extracts from the gel layer suggesting that the gel-based

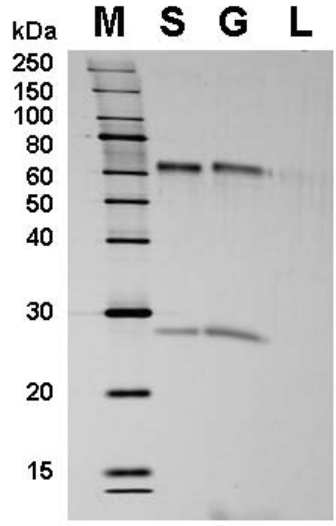

Fig. 3. Removal of model enzyme protein from the reaction mixture. Commercially available lipase proteins purified from pseudomonas species was used. Lanes: M, markers; S, standard sample without gelation-based separation; G, the sample recovered from the gel layer; L, the sample recovered from the liquid phase. Lipase used, 1 unit/mL in 200 µL (6.7 ng protein). After separation by gelation protocol as in Fig. 2, protein sample recovered in the disc of packed gel layer was dissolved in 200 µL of 10 mM Tirs-HCl. Then, 10 µL of sample (*ca.* 0.35 ng)/lane was used for SDS-PAGE analysis.

separation protocol is applicable for removal of enzyme from the media for termination of enzyme reaction or purification of the proteins.

Blending of two fatty acid salts

Further demonstrations revealed that the nature of gels formed after the mixing of sample solutions largely differed by altering the ratio of two active fatty acid salts and the concentration of GLDA used. Figure 4 shows the typical tests for determining the optimal ratio of Ole-Na and Lau-K and the optimal concentrations of GLDA for forming the gels which could be packed as solid and stable discs over the media after centrifugation. As demonstrated, single use of Ole-Na at 50 mM produced the soft and fragile gel-based discs. In contrast, single use of Lau-K at 50 mM produced hard and bulky gels and its volume could not be reduced by centrifugation. By blending two fatty acid salts at 50 mM in total, hard but less bulky discs of gels were formed. Increase in the concentrations of GLDA contributed to the formation of stable gels with minimal supplementation of Lau-K to the Ole-Na-based solution. Furthermore, the hardness of the resultant discs was largely enhanced by the presence of higher concentrations of GLDA (data not shown).

For further demonstration for separation of proteins from the crude biological samples, basically 50 mM Ole-Na and 200 mM GLDA were used, and supplementation of Lau-K was also examined.

Separation of human serum proteins

Human serum was subjected to separation of proteins by gel-forming protocol using 50 mM Ole-Na and 200 mM GLDA. After dissolving the proteins from the gel layer or liquid phase recovered, protein samples were subjected to SDS-PAGE to examine the separation efficiency (Fig. 5). By CCB staining of the SDS-PAGE gel, no protein was detected in the liquid phase of the serum (data not shown). With silver staining of the SDS-PAGE gel, both major and minor bands were visualized (Fig. 5A). Data suggested that except for the small amount of proteins corresponding to the three minor bands at *ca*. 60, 50 and 25 kDa, most of proteins in the human serum were shown to be recovered in the gel phase.

Further demonstration showed that the separation efficiency can be amended by blending Ole-Na and Lau-K at optimal ratio. While single use of neither Ole-Na (50 mM) nor Lau-K (50 mM) succeeded in elimination of proteins from the serum, addition of 6:4 (30 mM:20 mM) blending

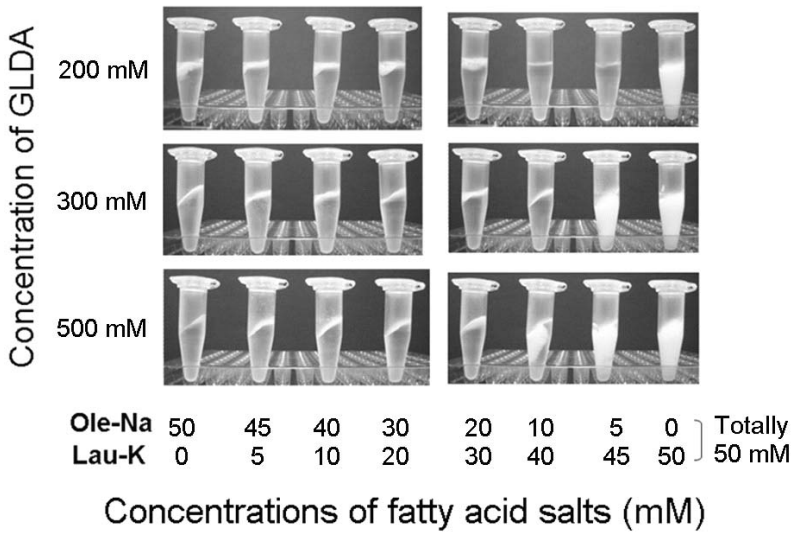

Fig. 4. Search for optimal ratio of Ole-Na over Lau-K for forming the hard layer of packed gels (discs of solid phase) collected over the media. Photographs show the gel layers formed and packed after centrifugation (14,000 rpm, 5 min).

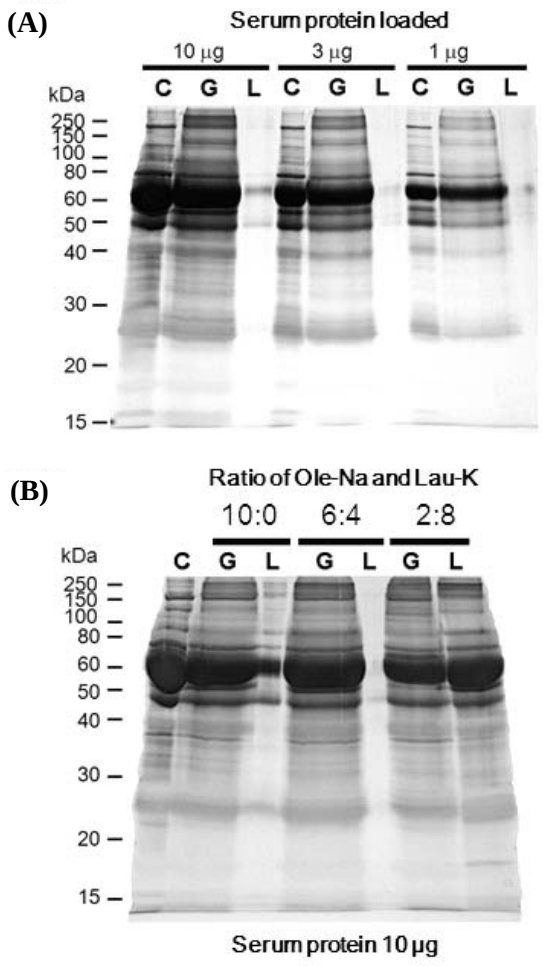

Fig. 5. Gelation-based separation of proteins from human serum using fatty acid salts and GLDA. (A) Removal of proteins from the human serum by the gel-forming reagents. Photograph shows the silver stained acrylamide gel. Ole-Na (50 mM) and GLDA (200 mM) were used. (B) Effect of Ole-Na:Lau-K blending (ratio, 3:2) on the separation efficiency of protein from the liquid phase. Lanes: C, control sample without gelation-based separation; G, samples recovered from the gel layer; L, samples recovered from the liquid phase.

of Ole-Na and Lau-K resulted in almost complete separation of protein from the serum (Fig. 5B).

Separation of mitochondorial proteins from rat liver

Mixture of mitochondrial proteins from rat liver were subjected to separation of proteins by gel-forming protocol using 50 mM Ole-Na and 200 mM GLDA. After dissolving the proteins from the gel layer or liquid phase recovered, protein samples

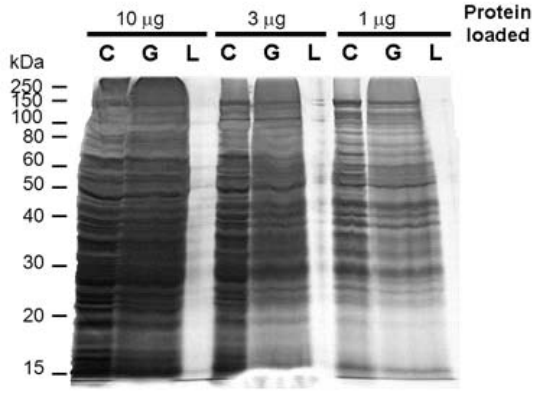

Fig. 6. Separation of proteins from the mitochondrial protein preparation from rat liver. Photograph shows the silver stained acrylamide gel. 50 mM Ole-Na and 200 mM GLDA were used. Lanes: C, control sample without gelation-based separation; G, samples recovered from the gel layer; L, samples recovered from the liquid phase.

were subjected to SDS-PAGE to examine the separation efficiency (Fig. 6). Data suggested that, most of the proteins were shown to be recovered in the gel phase, unless the proteins were overloaded.

DISCUSSION

Surfactants can self-assemble to form a variety of different micellar structures in aqueous solutions [11]. The driving force for the self-assembling process can be ascribed to the tendency of molecules to avoid unfavorable interactions between their hydrophobic parts and water (hydrophobic interactions) where the molecules aggregate together to form micelles, in which the hydrophilic head-groups (generally ionic groups) point outwards in contact with water, and the hydrophobic tails point inwards away from the water medium [8]. Recently developed several surfactant-based phase-transfer methods and extraction protocols would be the good examples of surfactant applications in chemical and biological engineering [12]. These protocols are based on the above important aspects of micellar systems using the mixture of surfactants.

To date, the phase behavior of the aqueous solution of Ole-Na has been studied by a number of researchers [13-18]. The first attempt to construct the phase diagram was reported in 1939 [19]. According to the established diagram, at low

concentration, Ole-Na is not soluble in water and forms spherical micelles. Furthermore, in the range of Ole-Na at concentrations between 25 and 50 wt %, it forms a cylindrical phase and at the concentrations over 50 wt %, a lamellar phase appears. In addition to the behavior of Ole-Na solo in the water, application of the third components to the Ole-Na/water system has been intensively studied for the purpose to control both the phase behavior and the micellar structures formed [20]. Notably, Gradzielski and his colleagues [21-23] observed the formation of a very stiff gel phase in the Ole-Na/1-octanol/water ternary system. In the presence of sufficiently high concentrations of 1-octanol, the vesicles formed are so monodisperse that they are able to form a densely packed system with long-range order and with a shear modulus that is about 100 times higher than normally found for vesicle systems. This was the first system of this type that forms a cubic-phase-like arrangement of monodisperse vesicles, and this form of gel is now referred to as ‘‘vesicle gel’’ [8]. As the use of recovered proteins for further biological studies is expected, the components used in the novel protocol must be biologically inert or less toxic. For instance, despite of gel-foaming nature, 1-octanol cannot be used due to its pharmacological action targeting the T-type calcium channels [24].

The novel pair of components for gel formation used here was originally selected for the development of fire fighting foam formulae [7]. Jeong *et al.* [8] have demonstrated that mixing of two transparent aqueous solutions of GLDA and Ole-Na resulted in formation of a white gel phase when the total solute concentrations were higher than *ca*. 2.8 wt %, suggesting that density-dependent formation of complex between GLDA and Ole-Na caused the formation of gels. This novel type of gel shows completely opaque appearance and the gel displays thixotropy. In addition, the physical state of the complex reportedly shifted from the gel state to the sol state upon gentle heating. Analysis with the optical microscopy has revealed that the observed gel consisted of very long fibers with diameters ranging from nano- to micro-meters scales. Transmission electron microscopy further revealed that the long fibers were made from many of bundled straight fibrils with a width of 200-1000 nm. The fibril consisted of lamellae parallel to the axial direction of the fibril. X-ray scattering analysis further confirmed the lamellar structure with a spacing of 4.5 nm, and the spacing was independent of the concentration and the molar ratio of GLDA and Ole-Na [8].

In the present study, rapid isolation and removal of proteins from the liquid media using novel protein-independently gelating agents namely, GLDA, Ole-Na and Lau-K are described. In the demonstrations, human hemoglobin, bacterial lipase, human serum proteins, and rat liver mitochondrial proteins were successfully collected in the solid discs floating over the solution following centrifugation. Further applications of this protocol in the various fields in protein-related science are encouraged.

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