Formation and Isolation of Spherical Fine Protein Microparticles Through Lyophilization of Protein-Poly(ethylene glycol) Aqueous Mixture

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Purpose. Preparation of spherical fine protein microparticles by the lyophilization of a protein-poly(ethylene glycol) (PEG) aqueous mixture was investigated. The main objective was to establish a method for preparing protein microparticles suitable for pharmaceutical production.

Methods. Aqueous solutions containing bovine serum albumin (BSA) and PEG at various mixing ratios were freeze-dried. The lyophilizates were dispersed in methylene chloride and subjected to particle size analysis. Analogous studies were performed using several model proteins. A phase diagram of the PEG–BSA aqueous system was obtained by the titration method.

Results. The particle size of BSA decreased as the PEG–BSA ratio increased. A bending point was observed in this relationship, at which the PEG–BSA ratio coincided with that of the critical point on the phase diagram of the PEG–BSA system. These results were explained by the freezing-induced condensation, followed by phase separation in the PEG–BSA system.

Conclusions. Spherical fine protein microparticles were successfully obtained at high yield and without any activity loss under optimum conditions. This new technology could be applicable to proteins with a wide range of molecular weights, and is expected to be developed for dry powder inhalations or long-term sustained release microsphere formulations.

KEY WORDS: protein microparticles; lyophilization; poly(ethylene glycol); phase separation; amphiphilic polymer; critical point.

INTRODUCTION

With the recent remarkable progress in gene-related technologies, increasing numbers of bioactive recombinant protein drugs, such as human growth hormone, erythropoietin, and interferons, have been offered for clinical use (1,2). Most of these drugs are generally formulated as injection forms because of their extremely poor oral bioavailability (3). Injections, however, are inevitably more painful than other dosage forms, which affects 'patient compliance.' For patients suffering from chronic diseases who require long-term treatment, this problem is especially serious. Therefore, an alternative dosage form for protein drugs has been desired from the viewpoint of 'improved pharmacotherapy' and the 'quality of life' of patients.

Over the past few decades, much work has focused on developing alternative dosage forms, including noninvasive drug delivery systems such as the dry powder inhalation system (4,5), or long-term sustained release injection including a once-monthly formulation (6). Some new formulations have already been marketed while others are under clinical development (7).

When developing protein delivery systems, one must minimize the denaturation of protein drugs during preparation. Protein drugs are often applied as solid particles: In dry powder inhalation for instance, the most effective diameter for inhaled particles to reach the main absorption site—the alveoli—is about 2–3 μm (4). As another example, the preparation of protein-loaded microspheres of biodegradable poly(lactide-co-glycolide) (PLGA) by a solid-in-oil-in-water (S/O/W) emulsion method has recently been reported (8). This method might be superior to the classical water-in-oil-in-water emulsion method because solid-state proteins retain their activities in organic solvents, while protein molecules in a soluble state are easily denatured at the water-organic solvent interface (9). In S/O/W emulsion, micronization of protein drugs is indispensable for attaining high entrapment efficiency (8).

The above examples suggest that particle design is a key to the development of protein delivery systems. Spray-drying or jet-milling has been frequently used in pharmaceutical productions to prepare fine protein particles with single micron-ordered sizes (4,5). With these methods, however, the denaturation of proteins by heat stress or various mechanical stresses can be critical problems. The low yield of the required size fraction and considerable loss of product are also shortcomings. Therefore, the development of a new micronization technology for protein drugs, which does not weaken their biological activities and which elicits a high yield, is desired. Furthermore, commercially acceptable manufacturing processes must be established for actual production.

Recently, phase separation during lyophilization of various aqueous polymeric mixtures has been reported (10–12). We also found that spherical protein microdomains formed spontaneously during the freeze-drying of an aqueous mixture of protein and a certain amount of poly(ethylene glycol) (PEG). Concerning this phenomenon, we have taken notice of the amphiphilic nature of PEG, which would enable isolation of protein microparticles from the lyophilizate. This implies the possibility of a new technology for the preparation of microparticles of protein drugs.

Our final goal is to establish a method for preparing protein microparticles suitable for pharmaceutical production. The objectives of the present study are to determine the optimum operating conditions for preparing 'spherical' protein microparticles and to examine the applicability of this technique to a wide range of protein drugs. Bovine serum albumin (BSA) and PEG 6000 are mainly used as a model protein and a phase-separation inducer, respectively. The mechanism behind the formation of spherical particles is also discussed on the basis of the PEG–protein phase-separation principle.

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MATERIALS AND METHODS

Materials

Proteins

Bovine serum albumin (BSA), superoxide dismutase (SOD), soybean trypsin inhibitor (STI), and Bowman–Birk inhibitor (BBI) were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP) was obtained from Wako Pure Chemical (Osaka, Japan). Gelatins (type A and D, average MW: 7,000 and 3,000, respectively) were provided by Nippi Co. (Tokyo, Japan).

Reagents

Poly(ethylene glycol)s (PEG 6000 and 70000), 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and SOD Test Wako were supplied by Wako Pure Chemical (Osaka, Japan). PEG 2000, PEG 4000, and PEG 20000 were obtained from Katayama Chemical (Tokyo, Japan). Micro-BCA protein assay reagent was obtained from PIERCE Chemical (Rockford, IL). All other reagents and solvents used were of reagent grade.

Methods

Lyophilization

A series of mixed solutions (1 mL) containing 4 mg of BSA and an amount (0–36 mg) of PEG 6000, prepared in glass test tubes (16 mm in diameter), were frozen onto a pre-cooled shelf of a freeze dryer (RLE-52ES, Kyowa Vacuum, Japan) at –50°C. Freeze-drying was performed by maintaining the shelf temperature at –20°C for 3 hr and 20°C for 12 hr under a pressure of about 0.02 torr.

For the preparation of the microparticles of various proteins, 25 mL of the aqueous mixture containing 100 mg of protein and a prescribed amount of PEG was lyophilized in the same manner.

Isolation of Protein Microparticles

The obtained lyophilizate was dispersed in an appropriate amount of methylene chloride under sonication. After centrifugation at 12,000 rpm for 5 min, the precipitates were collected and redispersed in fresh methylene chloride. This operation was repeated three times to remove the remaining PEG, and the final precipitates were dried under vacuum until they came to have a constant weight.

Phase Diagrams

The phase diagram of the PEG–protein aqueous mixture was determined by the conventional continuous titration method (13). Stock solutions of 20%(w/v) PEG 6000 and 10%(w/v) BSA were prepared, and allowed to equilibrate at 20°C. The PEG solution was added dropwise (10 μL each) to 300 μL of BSA solution until the solution became turbid, and the quantity of PEG solution required to reach the turbid point was recorded. After distilled water was added to the mixture, the PEG solution was added again, and the turbid point was recorded. These operations were repeated to obtain the necessary data set, and the phase diagram (binodal) for the PEG/BSA aqueous system was established. An identical experiment was performed at 0°C.

Composition Change of PEG/BSA Aqueous Mixture During Freezing

Ten milliliters of solution containing PEG and BSA was placed in a freezer at –20°C. At predetermined intervals, 1 mL of unfrozen fraction was withdrawn. The BSA concentration was determined by a Micro-BCA protein assay and PEG content was calculated from the dry weight.

Characterization of Protein Microparticles

Particle Size Analysis. The freeze-dried samples or isolated microparticles were dispersed with 1 mL of methylene chloride under sonication, and the resulting suspension was subjected to particle size analysis by a laser diffraction particle size analyzer (SALD-1100, Hitachi, Japan). Ethanol was used as a dispersing solvent.

Quantitative Analysis of Protein Microparticles. The purity of the protein microparticles was determined by the Micro-BCA protein assay against each raw material. Enzymatic activities of SOD and HRP microparticles were analyzed by a photometric assay using SOD Test Wako and ABTS as a substrate.

Scanning Electron Microscopic Study. The morphologies of the freeze-dried samples and isolated microparticles were observed by scanning electron microscopy (Model S-2250, Hitachi, Japan). Samples were mounted on metal stubs with a double-sided carbon tape, and coated with Pt and Pb film at a current of 20 mA for 3 min.

RESULTS

Figure 1 outlines the basic procedure applied in this study, which consists of two major steps: lyophilization of the PEG/protein aqueous mixture, and isolation of protein particles from the lyophilizate. PEG is used as the lyophilization additive for two reasons. Firstly, PEG is an amphiphilic polymer, which enables the co-lyophilization with protein in an

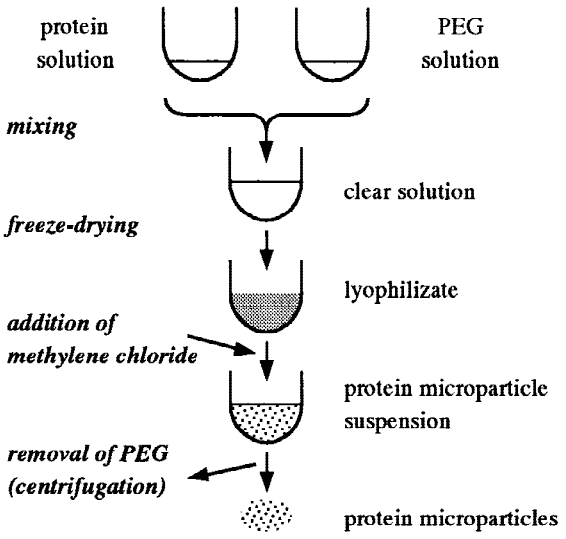

Fig. 1. The basic procedure for the preparation of protein microparticles.

aqueous media, as well as subsequent selective isolation of protein from the lyophilizate by soaking with an appropriate organic solvent, such as methylene chloride. Secondly, PEG is a well-known phase separation inducer. In our preliminary study, the protein microparticles obtained by this method were a "true sphere," with a smooth surface and a diameter of about 2–3 μm. This implies that a liquid–liquid phase separation is involved in the formation of protein microparticles.

Influence of PEG–Protein Ratio

The effect of the mixing ratio with PEG 6000 on the size of protein particles was examined using BSA as the model protein. A series of PEG–BSA aqueous mixtures containing fixed amounts of BSA (4 mg) and amounts of PEG from 0 to 36 mg were freeze-dried. After the addition of methylene chloride, the resulting suspensions were subjected to particle size analysis. Average diameters were plotted against the PEG–BSA weight ratio in Fig. 2. The average sizes of BSA particles decreased with an increase in the PEG–BSA ratio. In this profile, there seems to be an apparent bending point around the mixing ratio of 0.25 (PEG:BSA = 1:4), which implies that the formation mechanism of particles would be different above and below this bending point. When the PEG–BSA weight ratio was adjusted to more than 4 (PEG:BSA = 4:1), extremely stable suspension of submicron-sized BSA particles was formed.

Figure 3 gives a comparison of size distribution profiles between four typical batches of BSA particles, prepared at different PEG–BSA ratios. At a ratio of 9 (PEG:BSA = 9:1), the microparticles showed a comparatively narrow size distribution pattern ranging from 0.3 to 3 μm (Fig. 3a). At a ratio of 1 (PEG:BSA = 1:1), the size distribution became somehow broader (Fig. 3b). The apparent two peak profiles suggested that this results from partial aggregation between protein particles formed during the freezing process. Meanwhile, the distribution pattern at the ratio of 0.1 (PEG:BSA = 1:10) (Fig. 3c) was similar to that of BSA powder lyophilized without PEG (Fig. 3d). This means that the addition of such a small amount of PEG has essentially no effect on the particle size.

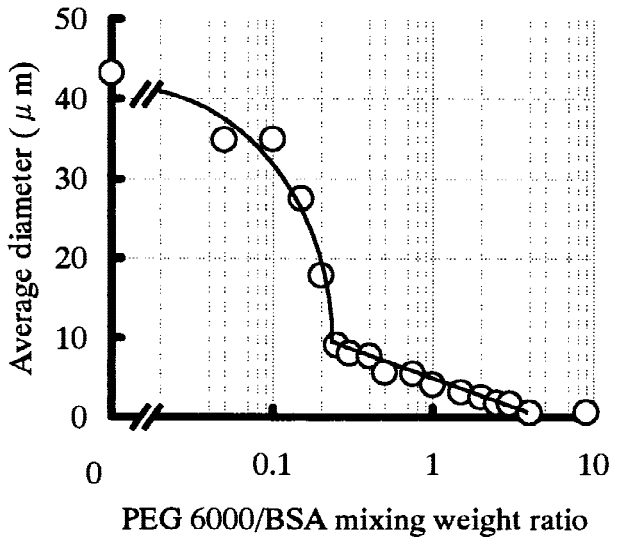

Fig. 2. The relationship between the PEG–BSA mixing weight ratio and average diameters of BSA microparticles.

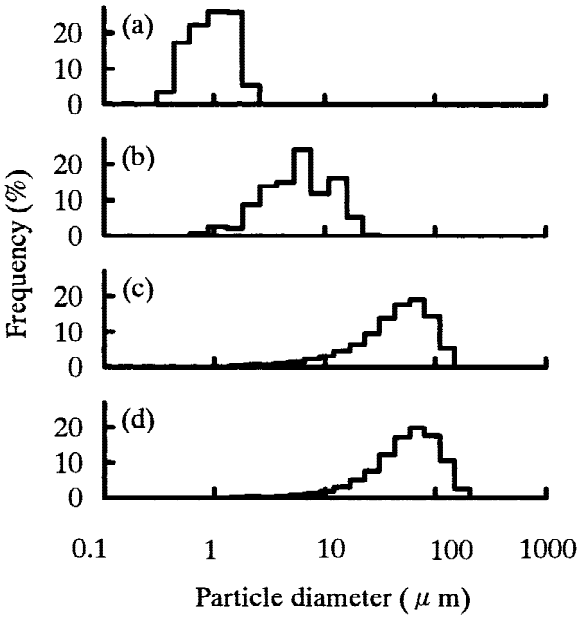

Fig. 3. The typical size distributions of BSA microparticles after suspending the lyophilizates of the PEG–BSA aqueous mixture with methylene chloride. Each symbol shows the case for the mixing ratio of 9 (a), 1 (b), 0.1 (c), and 0 (BSA only) (d).

Figure 4 shows the scanning electron micrographs (SEM) of the co-lyophilizates of PEG and BSA at the various mixing ratios, and the morphologies of the BSA particles after removal of PEG. At the mixing ratio of 1 (Fig. 4a) and 9 (Fig. 4c), the micron-ordered spherical domains were distinctly observed all over the lyophilizate. After the removal of PEG with methylene chloride, a large number of small spherical particles could be collected (Fig. 4d and 4f). The apparent diameter of each microparticle at the ratio of 9 was slightly smaller than that at the ratio of 1. This SEM observation suggests that the lyophilizate obtained under this condition consists of the continuous phase of PEG and dispersed BSA microparticles. At the mixing ratio of 0.1, on the other hand, the lyophilizate was merely observed as larger fragments (Fig. 4b). Furthermore, after the removal of PEG, many circular holes were newly created on the lyophilizate (Fig. 4e). This means that the lyophilizate obtained under this condition consists of the continuous phase of BSA dispersing the circular domains of PEG.

Influence of PEG Grade

To examine the influence of molecular weights of PEG on the formation of BSA microparticles, the same experiment was performed using various commercial grades of PEG. In the case of PEG 20000 and 70000, nearly similar results as with PEG 6000 (see Fig. 2) were obtained (data not shown). However, PEG 2000 and PEG 4000 provided slightly different patterns in the correlation between mean sizes of BSA microparticles and PEG–BSA mixing ratio (data not shown). Namely, the bending point was observed around the mixing ratio of 1 and 0.8 for PEG 2000 and PEG 4000, respectively.

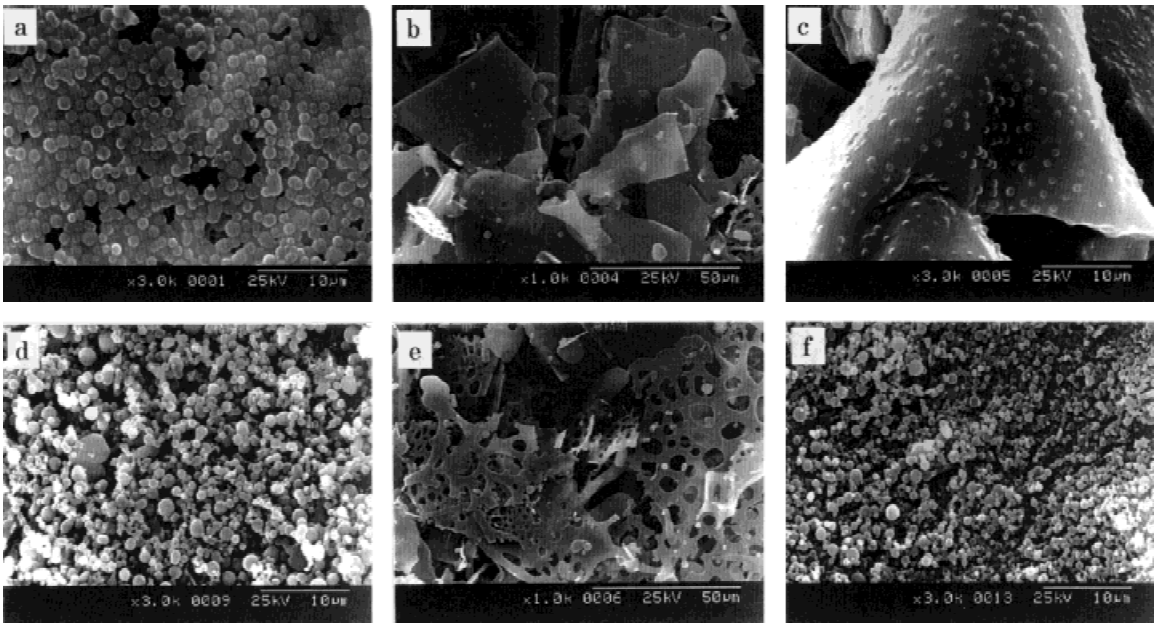

Fig. 4. SEM photographs of the lyophilizates of the PEG–BSA aqueous mixture and BSA microparticles after removal of PEG from the lyophilizates. PEG/BSA lyophilizate at the mixing ratio of 1 (a), 0.1 (b), and 9 (c). BSA particles isolated from the lyophilizate at the mixing ratio of 1 (d), 0.1 (e), and 9 (f).

Influence of Molecular Weight of Proteins

Various proteins with different molecular weights (listed in Table 1) were also applied to the proposed method using PEG 6000. Even though the data are not shown here, the size of the microparticles decreased with the increase of the PEG–protein mixing ratio in all proteins. A distinctive bending point was also observed in each profile, as in the case of BSA (see Fig. 2). In Table 1, the approximate bending point and typical characteristics (average size, purity, and recovery efficiency) of the yielded protein microparticles are summarized. From this table, we can observe the following. Firstly, proteins with smaller molecular weights were inclined to have a bending point at a higher mixing ratio. Secondly, when the PEG–protein ratio exceeded the bending point, the particle diameter was less than 10 μm. Thirdly, an extremely high recovery of more than 70% and high purity of almost 100% were attained in all cases. Moreover, as with SOD and HRP, enzymatic activities of microparticles were nearly equivalent to those of the corresponding raw material.

Phase Diagram of the PEG–BSA Aqueous System

The formation of microparticles should be closely related to the phase separation occurring in the lyophilization process. A phase diagram study was then performed. Figure 5 shows the phase diagram of the PEG–BSA aqueous mixture at 20°C (solid line) and 0°C (dotted line). The system had three different states, clear (region A); turbid (liquid–liquid phase separation, region B); or precipitated (liquid–solid

Table 1. The Characteristics of Micronization of Various Kinds of Protein

			Typical characteristics of isolated protein microparticles after the micronization treatment				
Protein	MW	PEG/protein ratio at bending point[a]	PEG Ratio	Average particle diameter (μm)	Purity[b] (%)	Recovery[c] (%)	Activity (%)
BSA	67,000	0.2–0.25	1	4.2	100.1	96.1	—
HRP	40,000	0.25–0.3	1	3.1	100.3	94.3	99.8
SOD	32,000	0.3–0.4	1	4.3	100.3	92.9	109.7
STI	20,100	0.6–0.8	1	12.0	105.4	83.1	ND[d]
			3	6.0	ND[d]	ND[d]	ND[d]
BBI	8,000	1–2	1	12.2	107.5	83.1	ND[d]
			3	8.7	ND[d]	ND[d]	ND[d]
Gelatin A	7,000	1–2	1	17.2	94.4	68.6	—
			5	5.0	96.7	74.5	—
Gelatin D	3,000	1–2	1	5.2	84.4	93.7	—

[a] Data are shown as the range of PEG–protein mixing ratio within which the bending point was observed.
[b] Purities were determined by Micro-BCA protein assay against the raw material.
[c] Recovery percentages from 100 mg protein loading were determined.
[d] Not determined.

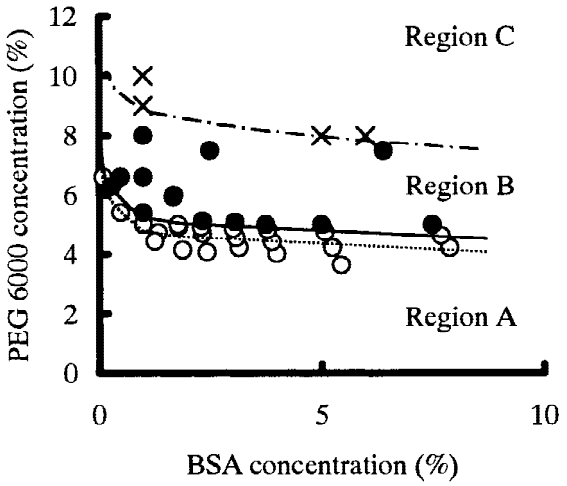

Fig. 5. Phase diagram of the PEG–BSA aqueous mixture system. The solid line represents the binodal at 20°C, and the dotted line the binodal at 0°C. The dashed line represents the border between region B and region C. Each symbol shows the state of the system at 20°C; clear (open circle), turbid (liquid–liquid phase separated) (closed circle), and precipitated (liquid–solid phase separated) (cross).

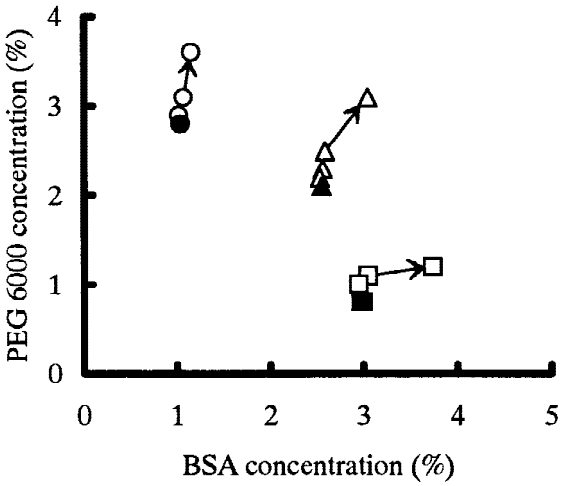

Fig. 6. Composition changes in the unfrozen fraction of PEG–BSA aqueous mixture during freezing. The closed symbols represent the initial composition. The open symbols represent the sequential change of the composition in the unfrozen fraction.

phase separation, region C); in accordance with the given concentration of both polymers. The asymmetric shape of the phase diagram suggested that the phase transition would be more influenced by PEG than BSA. In fact, the existence of PEG at 5–8% resulted in liquid–liquid phase separation over a wide concentration range of BSA. When the PEG concentration exceeded 8%, the BSA phase partially aggregated before finally precipitating as a solid phase (14). At 0°C, the A-to-B transition line (binodal curve) slightly shifted toward the lower concentration region, suggesting less temperature dependence. Further, unlike other aqueous polymeric solution systems, a very stable water-in-water emulsion was given in region B; that is, once the BSA (or PEG) droplets were formed, they were not easily agglomerated even after sitting for a long time.

Freezing-Condensation of the PEG–BSA Aqueous System

Figure 6 shows the sequential changes in composition in the unfrozen fraction of typical PEG–BSA aqueous mixtures during the freezing process. From the starting composition shown by the closed symbol, the unfrozen solution was found to be concentrated while maintaining the relative composition, accompanied by the freezing of free water.

These findings are quite useful when discussing the formation of BSA particles during lyophilization.

DISCUSSION

Characteristics of PEG–BSA Phase Separation

It was reported in the 1960s that when two different kinds of hydrophilic polymers co-exist at a high concentration in aqueous media, the system eventually separates into two distinctive phases by coacervation (15). This phenomenon is currently utilized mainly in the biochemical field, as an aqueous two-phase partitioning system, for the isolation or purification of macromolecules, such as cellular proteins and nucleic acids (16). Among many systems, the PEG–dextran aqueous system is most commonly applied for this purpose. In the pharmaceutical field, an application of phase separation using the PEG–dextran system to the preparation of dextran microspheres as a drug carrier has been recently reported (17). In this type of phase separation, the volume of each phase is decided by the composition of both polymers. An intrinsic critical point—at which both aqueous phases are separated with equal composition and equal volume—should exist on the binodal curve of each system, which is considered to be an important parameter representing the system. This theoretical consideration should be true of the PEG–protein system discussed in this study (18). Although the critical point of the PEG 6000–BSA system is not shown in Fig. 5, it was reported at 16.7% of BSA and 3.7% of PEG (19).

Generally, the freezing of free water causes the condensation of the unfrozen fraction (10), as was shown in Fig. 6. This situation is schematically represented in the phase diagram in Fig. 7. The initial point in region A (given as a clear solution) moves to the right on the proportional line passing through the origin (dotted line), and finally enters region B above the binodal, resulting in the liquid–liquid phase separation. When the PEG–BSA mixing ratio—expressed as the slope of the line—exceeds that of the critical point (as shown by the point 'X'), PEG will form the continuous phase in region B. If the line runs below the critical point (as shown by the point 'Y'), PEG will form the dispersed phase. Therefore, the critical point can be an index to predict which polymeric component will be distributed in the dispersed or continuous phase after the A-to-B transition. In other words, the critical point might mean the point of 'phase transposition' between two reversible water-in-water emulsions, *i.e.* PEG-in-protein emulsion and protein-in-PEG emulsion. The PEG 6000–BSA ratio at the critical point is estimated to be about 0.22 by simple calculation (3.7%/16.7%). It was noted that this value was very close to the PEG–BSA ratio of the bending point obtained experimentally in Fig. 2 (around 0.2–0.25), suggesting that the spherical fine particles of BSA can be obtained only when the PEG 6000–BSA ratio is over 0.22. This consideration might be applicable to the other proteins shown in Table 1. In short, the observed bending point in each system might represent a PEG–protein ratio at the critical point.

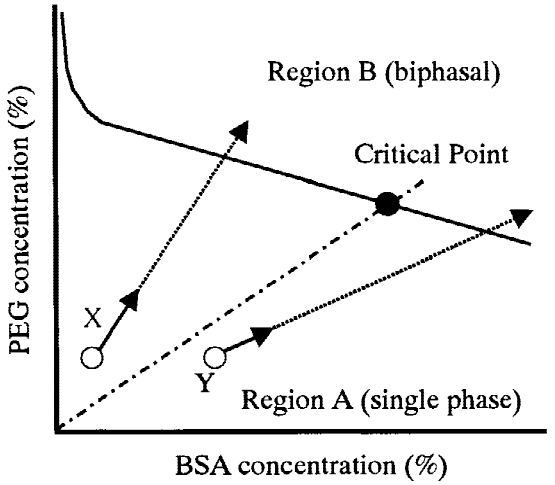

Fig. 7. Schematic representation of the transition in polymeric composition in the PEG–BSA aqueous system induced by condensation during the freezing process. The closed circle shows the critical point of the PEG–BSA aqueous system. Point 'X' indicates the initial solution containing 0.5% PEG and 0.5% BSA. Point 'Y' indicates the initial solution containing 0.5% PEG and 4% BSA.

Mechanism of Spherical Microparticle Formation

From the experimental results demonstrated in the previous section, the mechanism for the formation of spherical fine particles can be speculated, taking into consideration the phase separation behavior during the freezing process. Figure 8 is a schematic representation of the particle formation process in two different polymer compositions, *i.e.* X and Y in Fig. 7.

In the case of solution X (PEG–BSA ratio > 0.22), the BSA-in-PEG emulsion is formed in region B when the solution is concentrated until the A-to-B transition. Further freezing and drying finally produced a lyophilizate of PEG containing BSA microparticles (Fig. 4a and 4c). When PEG was dissolved by methylene chloride, spherical fine microparticles of BSA were obtained (Fig. 4d and 4f). Meanwhile, in the case of solution Y (PEG–BSA ratio < 0.22), the PEG-in-BSA emulsion is formed in region B. Consequently, the obtained lyophilizate consists of the continuous phase of BSA and the dispersed phase of PEG, which finally produces large fragments of BSA after wash-out treatment (Fig. 4e).

A Possible New Method for the Preparation of Protein Microparticles

Even though some factors involved in the particle formation, such as freezing rate (20), remain to be studied, the proposed method is useful for several reasons.

Firstly, the entire process is simple and practical (Fig. 1), utilizing only currently available technology. Although methylene chloride was used for removing PEG in this study, any other organic solvents, acting as the 'solvent' for PEG and as the 'nonsolvent' for protein, can be used for the same purpose (21).

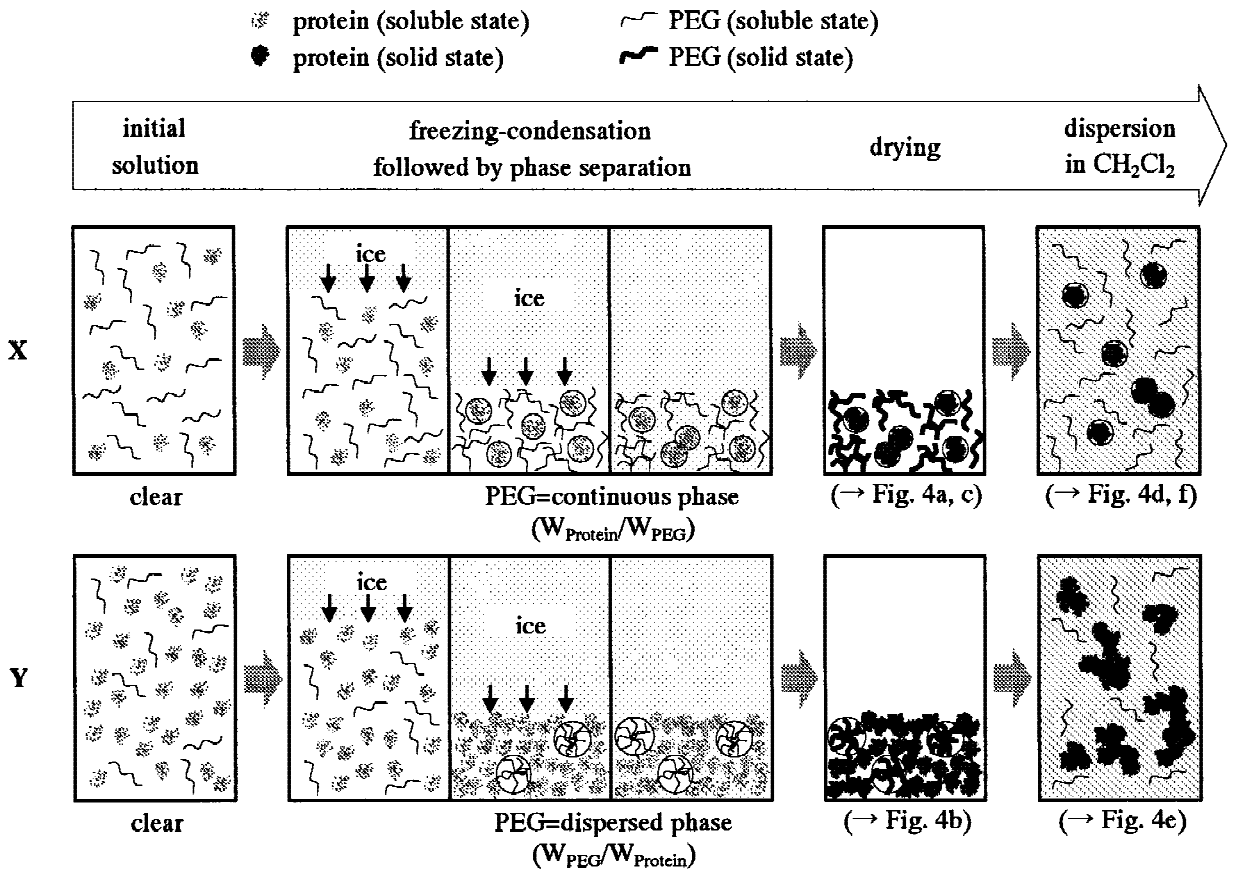

Fig. 8. Schematic representation of the formation of protein microparticles. X indicates a PEG–protein mixing ratio higher than that of the critical point. Y indicates a PEG–protein mixing ratio lower than that of the critical point.

Secondly, the particles attained are small enough to be applied to various DDS formulations, and the size can be controlled from a single-micron order to a sub-micron order by applying suitable operating conditions (Fig. 2 and Table 1). For example, with this technology, the dry powder formulation can be developed to enable the delivery of fine protein microparticles directly to the alveoli by inhalation. As another example, the protein microparticles can be incorporated more efficiently into biodegradable microspheres by the S/O/W emulsion method (8).

Thirdly, the purity of the protein microparticles is extremely high (Table 1), implying that the proposed method will always provide high quality products. This may be due to the characteristics of the phase separation of the PEG–protein system, as suggested from Fig. 5. In the two-phase separation system, each phase generally comprises one major polymeric component with a small amount of another coexisting polymeric component, and both phases are thermodynamically equilibrated via complicated interactions between polymers and water (15,16). The composition of each phase can be estimated from the binodal curve. In fact, the binodal in Fig. 5 suggests that a very small amount of PEG can coexist in the BSA phase and vice versa. This might be one major reason why the high purity of protein microparticles is realized with this method.

Furthermore, this method can achieve a high yield of products (at least 70% even on a small scale: see Table 1), compared with the conventional spray-drying process (less than 50% according to our recent small-scale experiment, data not shown).

It is noted that the biological activities were completely retained after the lyophilization of SOD and HRP. In general, proteins or enzymes with high molecular weights become unstable under the various physicochemical stresses in the preparation process, which may cause a loss of biological or enzymatic activity (22,23). In fact, in the study of freeze-dry formulations of unstable protein drugs, the suppression of loss of activity during the lyophilization process has been one of the most crucial issues. The effects of cryoprotectants—which signify pharmaceutical additives including PEG for the prevention of protein denaturation during freeze-drying—have been reported for the stabilization of some enzymes, *i.e* lactate dehydrogenase and beta-galactosidase (24,25). Therefore, for more unstable proteins, more consideration for the application with the proposed method would be necessary.

CONCLUSIONS

In the present study, it was confirmed that co-lyophilization with PEG facilitates the formation of spherical fine particles of protein through a process based principally on the aqueous phase separation. Because PEG functions not only as a phase-separation inducer but as an amphiphilic polymer, protein particles can be effectively isolated from the PEG–protein lyophilizate by washing out the PEG with an appropriate organic solvent. The data suggested the possibility of establishing a new method for preparing spherical fine microparticles of protein drugs of high purity, with high yield, and without any loss of biological activities.

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