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Factors affecting protein release behavior from surfactant–protein complexes under physiological conditions

Hiromu Yoshiura [a], Masakazu Hashida [a], Noriho Kamiya [a,b,*], Masahiro Goto [a,b,*]

[a] *Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan*
[b] *Center for Future Chemistry, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan*

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Abstract

Protein release behavior from its complex with edible surfactants was investigated under physiological conditions using hen egg lysozyme and *Aspergillus niger* glucose oxidase as model proteins. It revealed that protein release rates could be controlled by hydrophobicity of surfactants and the molar ratio of proteins to surfactants in the preparation of the complexes. Evaluation of functional integrity of a protein on the basis of specific activity of an enzyme released from the complex suggested that lower hydrophobicity of surfactants led to higher retention of catalytic activity. In addition, it was found that protein release rates from the complexes were correlated with the aqueous droplet size of water-in-oil emulsions in the preparation of the complexes. The results suggest the potential of surfactant–protein complexes in pharmaceutical formulations for mucosal delivery of therapeutic proteins.

Keywords: Drug delivery; Emulsions; Peptide drug; Protein drug; Protein release; Protein stability; Surfactant–protein complex

1. Introduction

A wide variety of synthetic peptides and recombinant proteins have been potential candidates in pharmaceutical applications (Talmadge, 1993; Playford et al., 2004). With respect to the systemic delivery of peptides and proteins, injection or implant are major routes due to the low bioavailability through the gastrointestinal (GI) tract, where hydrophilic macromolecular substances suffer from enzymatic degradation and low absorption at the mucous site (Hovgaard et al., 1996). However, several disadvantages, such as low patient compliance, possibility of infection and pain during repeated administration by injection or implant, have prompted the discovery of an alternative way to administrate pharmaceutical macromolecular substances.

As for insulin, the molecular weight of which is in the boundary between peptides and proteins, a prototype formulation for pulmonary administration has recently been demonstrated (Courrier et al., 2004). However, pulmonary delivery also has its problems, such as damage to the nasal mucosa, due to repeated administration. Therefore, efforts for the development of oral delivery systems of peptide and protein drugs have been intensified because peroral routes offer obvious advantages, such as ease of administration and greater patient compliance and acceptability (Sood and Panchagnula, 2001). With respect to peptide drugs, a number of oral delivery systems have been investigated with, for instance, liposomes (Zhang et al., 2005), transmucosal macromolecular capsules (Prego et al., 2005), chemical modifications (Calceti et al., 2004), enhancing additives (Lee and Sinko, 2004) and emulsions (Onuki et al., 2000). Among these systems, emulsion is one of the most suitable formulations for protein drugs. Because emulsions are flexible systems, in which release properties can be adjusted by several methods, such as volume fraction of the dispersal phase, droplet size and osmotic gradient, and are relatively easier to prepare than other dosage forms (Jørgensen et al., 2003). In addition, emulsions can be used as depot formulations enabling the controlled release of protein drugs after administration. We thus selected emulsions for the development of an oral protein delivery system. However, water-in-oil-in-water (W/O/W) emulsions, a basic carrier for peptides and proteins, have some

* Corresponding authors at: Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan. Tel.: +81 92 802 2806; fax: +81 92 802 2810.
E-mail addresses: noritcm@mbox.nc.kyushu-u.ac.jp (N. Kamiya), mgototcm@mbox.nc.kyushu-u.ac.jp (M. Goto).

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problems, such as difficulty in controlling the size of oil droplets, and possible protein denaturation at the water–oil interface (Graham and Phillips, 1979).

To overcome these intrinsic problems associated with W/O/W emulsions, we have recently prepared a new type of emulsion for oral insulin delivery, solid-in-oil-in-water (S/O/W) emulsions, in which a surfactant–insulin complex is directly dispersed in the oil phase (Toorisaka et al., 2003, 2005). It reveals that solubilization of insulin into the oil phase enhances the bioavailability upon oral administration to rats, which may be attributed to the suppression of proteolytic degradation in the GI tract, and enhancement of permeation through the intestinal mucosa, by surface modification of insulin with lipophilic surfactants. The results support the idea that modification of hydrophilic macromolecules with lipophilic surfactants is a powerful strategy to create oil-based formulations. In the present study, we investigated in detail factors affecting functional integrity of proteins in the first step of preparation of an oil-based formulation, the complex formation of proteins with edible surfactants. In vitro protein release experiments provided us with practical information on either the retention of biological activity of proteins upon the complex formation, or the release behavior of proteins from their complexes under physiological conditions.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme, glucose oxidase (GOx) and horseradish peroxidase were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sucrose fatty acid esters [sucrose laurate (L-195), sucrose oleate (O-170) and sucrose erucate (ER-290)] used as surfactants were kindly provided by Mitsubishi–Kagaku Foods (Tokyo, Japan). *Micrococcus lysodeikticus* dry cells were purchased from Sigma–Aldrich (USA). All other reagents used were of analytical grade.

2.2. Preparation of surfactant–protein complexes

Surfactant–protein complexes were prepared as follows: a 3.3 ml aqueous solution of protein (1.0 mg/ml) and a 6.6 ml hexane solution of each surfactant at different concentrations (1.0, 5.0 and 10.0 wt%) were poured into a round-bottom flask (100 ml), and mixed with a homogenizer at 26,000 rpm for 2 min to form water-in-oil (W/O) emulsions. The resulting emulsions were frozen rapidly in liquid nitrogen, and lyophilized using a freeze–drying machine (EYELA-FD5N; Japan) for 24 h. The resulting viscous solid materials were employed as surfactant–protein complexes.

2.3. Quantification of proteins released from surfactant–protein complexes

The release of a protein from its surfactant–protein complex was monitored as follows. The complexes were placed in phosphate-buffered saline (PBS; 17 ml). The mixture was gently agitated at 35 rpm and 37 °C. Aliquots (0.5 ml) were withdrawn at predetermined time points (1, 2, 3 and 24 h). After centrifugation, enzymatic activity and protein concentration of the samples were determined separately. The protein concentration was measured by bicinchoninic acid (BCA) protein assay kit (Sigma, USA). The enzymatic activity of lysozyme in the release medium was determined by measuring turbidity change of a *M. lysodeikticus* bacterial cell suspension, as previously reported (Sellak et al., 1992). The enzymatic activity of GOx in the release medium was determined by measuring the increase in the absorbance at 460 nm, resulting from oxidation of dianisidine through a peroxidase-coupled system (Swoboda and Massey, 1965). Specific activity of enzymes was calculated by dividing catalytic activity by the protein concentration.

2.4. Determination of aqueous droplet size of W/O emulsions

The average diameter of aqueous droplets in W/O emulsions was determined by the dynamic laser scattering (DLS) method, using a SALD-200V ER laser light-scattering instrument (Shimadzu, Japan) equipped with a semiconductor laser with vertically polarized incident light at a wavelength of 670 nm. Coal oil was employed to disperse the W/O emulsions. After measurement of particle size for each sample, the wet adapter was cleaned thoroughly and dried with acetone to avoid any cross contamination.

2.5. Determination of the size of surfactant–protein complex

The average diameter of the surfactant–protein complex prepared with different types of surfactants at different concentrations was determined by DLS (Zetasizer Nano-ZS; Malvern Instruments, UK) equipped with a He–Ne laser ($\lambda = 633$ nm). Toluene was used to disperse the surfactant–protein complexes.

3. Results and discussion

3.1. Basic characterization of surfactant–protein complexes

The size of the surfactant–protein complexes was determined (Fig. 1). GOx and ER-290 were used as a protein and a surfactant, respectively. On the basis of DLS measurements, the average diameters of native GOx in PBS and the ER-290–GOx complex in toluene were determined to be around 8.0 and 280 nm, respectively. Assuming that GOx dimers formed spheres in PBS, the volume occupied by the GOx dimer was calculated to be $\sim 2.0 \times 10^{-24}\ m^3$. On the other hand, the volume occupied by the surfactant–protein complex was calculated to be $\sim 8.8 \times 10^{-20}\ m^3$, from the data of Fig. 1. The results indicate that one surfactant–protein complex consists of $\sim 4.4 \times 10^4$ GOx molecules.

We also calculated the theoretical value of the number of GOx molecules in a surfactant–protein complex, based on the data of the size of W/O emulsions in the preparation of surfactant–protein complexes (Table 1). In this case, the volume occupied by one aqueous droplet of W/O emulsion is calculated

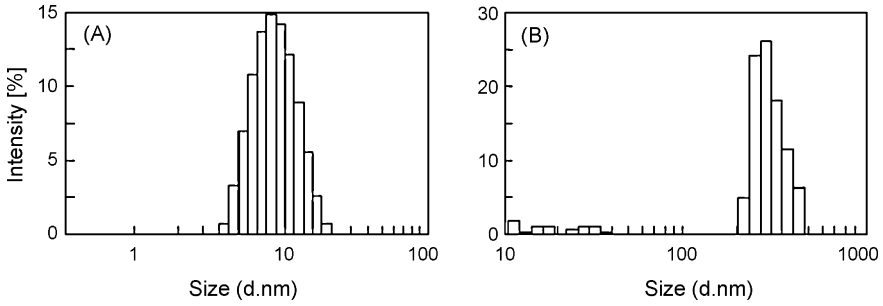

Fig. 1. Size distribution of an intact GOx sample dissolved in PBS (A) and surfactant–GOx complex solubilized in toluene (B) at 25 °C. The surfactant employed for complex formation was ER-290, and the concentration in the preparation step was 5.0 wt%.

Table 1
Effect of the type of surfactant and its concentration on the inner aqueous droplet size of W/O emulsions in the preparation of surfactant–enzyme complexes

Enzyme	Surfactant	Surfactant concentration (wt%)	Average diameter of water droplets of W/O emulsions (μm)
Lysozyme	L-195	1.0	7.43 ± 0.09
	O-170	1.0	3.41 ± 0.21
		5.0	0.98 ± 0.17
		10	0.87 ± 0.25
	ER-290	1.0	1.09 ± 0.19
GOx	L-195	1.0	7.62 ± 0.14
	O-170	1.0	2.77 ± 0.21
		5.0	0.73 ± 0.16
		10	1.16 ± 0.22
	ER-290	1.0	1.14 ± 0.19
		5.0	0.99 ± 0.17
		10	1.27 ± 0.21

to be $\sim 4.0 \times 10^{-18}$ m^3 when the surfactant concentration is set at 5.0 wt% against hexane in the preparation step. Considering that the concentration of GOx in the aqueous phase was 1.0 mg/ml, the number of GOx molecules in one aqueous droplet of W/O emulsions was estimated to be $\sim 3.0 \times 10^4$. Based on the calculation results, the number of GOx molecules in one surfactant–protein complex showed fairly good agreement with that in one aqueous droplet of W/O emulsion. Therefore, it is likely that one surfactant–protein complex should be formed from one droplet of W/O emulsion by freeze–drying.

3.2. Protein release behavior from surfactant–protein complexes

The protein release kinetics from the surfactant–protein complexes were studied. Lysozyme and GOx, which were used as model proteins in this study, are highly water-soluble, therefore, enzymatic activity in the supernatant of the release medium (PBS) should reflect the release of functionally active proteins from the complexes. Fig. 2 shows the active protein release from surfactant–enzyme complexes prepared from three sucrose ester surfactants with different lengths of alkyl chains (L-195, O-170 and ER-290). As shown in Fig. 2, the rate of protein release followed the order of the surfactant alkyl chain length with which the complexes were formulated: L-195 (C12) > O-170 (C18:1) > ER-290 (C22:1). The foregoing observations can be explained by the hypothesis that if the surfactant alkyl chain length is sufficiently long, the hydrophobic interaction between surfactant molecules will strengthen the resultant complex, and hence will afford slower release kinetics, compared to those with the surfactants with shorter alkyl chains. The results suggest that the hydrophobicity of the surfactant in the surfactant–protein complexes is a key parameter for governing the rate of protein release.

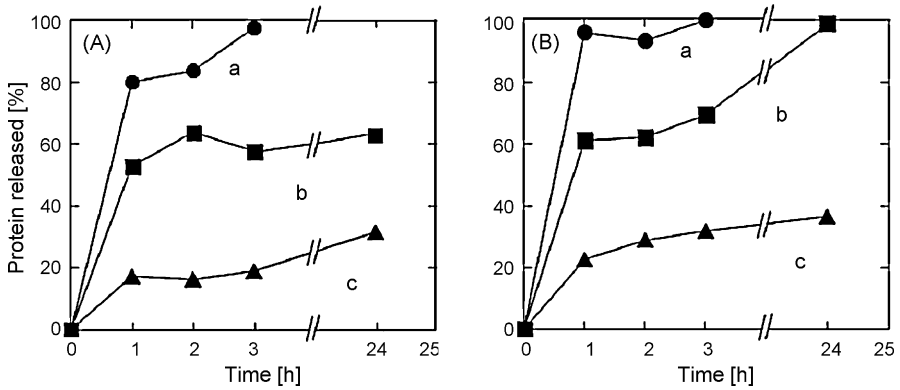

Fig. 2. Time course of active enzyme release from the complexes prepared with different surfactants. Enzymes employed were lysozyme (A) and GOx (B). Surfactants employed for the complex formation were L-195 (a), O-170 (b) and ER-290 (c), and the concentration in the preparation step was kept at 1.0 wt%.

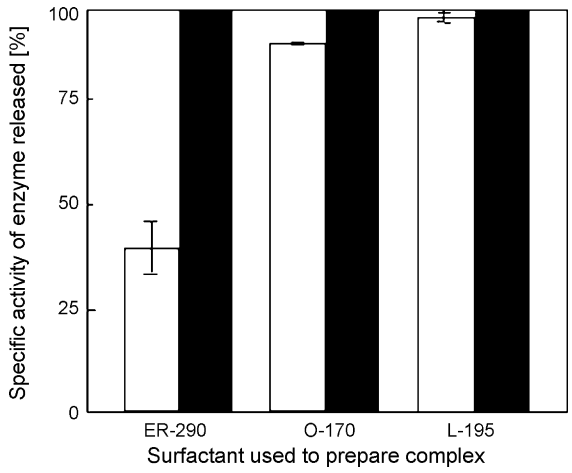

Fig. 3. Relative specific activity of enzymes released from the complexes prepared with different surfactants after 24 h incubation. White and black bars show the results of lysozyme and GOx, respectively.

3.3. Effect of surfactants on the functional integrity of enzymes

Fig. 3 shows the specific activity of enzymes released from their complexes after 24 h incubation. The results show that lysozyme gradually lost its enzymatic activity as the hydrophobicity of the surfactants increased, and lost ∼40% of its initial activity when ER-290 was employed. There may be the interaction between the protein and surfactant after the release of protein from the surfactant–protein complex, which is one reason for the inactivation of released proteins. To verify the possibility, we conducted a confirmatory experiment. In this experiment, the same amount of surfactant as in the surfactant–protein complexes used in Fig. 3 was added to the PBS (17 ml), in which lysozyme (3.3 mg) was dissolved. The mixture was incubated for 24 h in the same conditions as in Fig. 3. As a result, we confirmed that there was no change in the specific activity of lysozyme. The result shows that inactivation of lysozyme observed in Fig. 3 is caused by the formulation process of surfactant–lysozyme complex.

By contrast, GOx fully retained its activity in all cases. This is because of the difference in characteristics between lysozyme and GOx. Lysozyme is a small basic protein with a molecular weight of ∼14 kDa, while GOx is a dimeric glycoprotein with a molecular weight of ∼80 kDa in its dimeric form. Furthermore, their isoelectric points (p*I*s) and structural characteristics are quite different. We considered the following two points in an attempt to understand the results. The first is the difference in p*I*, at which lysozyme and GOx are positively and negatively charged, respectively, in the complex formation process. The second is that GOx is a glycoprotein with a large number of sugar chains (14.2 wt% of sugar) (Pazur et al., 1965). Since ER-290 is a non-ionic surfactant, electrostatic interaction between the head group of the surfactant molecules and proteins may not be dominant. On the other hand, the long alkyl chains of ER-290 could interact with the hydrophobic part of lysozyme, while the large number of sugar chains of GOx could reduce direct interaction with the protein surface, leading to the retention of enzymatic activity. To improve oral absorption of protein drugs, retention of the biological activity of a target protein, by keeping the hydrophobicity of the formulations, is important to enhance mucosal epithelium permeability. Therefore, O-170 and ER-290 were chosen for coating lysozyme and GOx, respectively, in the subsequent experiments.

3.4. Effect of the aqueous droplet size of W/O emulsions in the preparation of surfactant–protein complexes

In order to presurmise the protein release rate from its complex, the correlation between the protein release rate and the aqueous droplet size of W/O emulsions in the formulation step of surfactant–protein complexes was investigated. Table 1 summarizes the effect of surfactant type and its concentration on the aqueous droplets of W/O emulsions during the preparation of surfactant–protein complexes. The aqueous droplet size of W/O emulsions was independent of the type of proteins, but was significantly influenced by the type of surfactant. For instance, L-195 gave ∼7.5 μm aqueous droplets, while ∼1.1 μm aqueous droplets were formed for both proteins with ER-290 at the same surfactant concentration (1.0 wt%). On the basis of the data in Table 1 and Fig. 2, one can see the correlation between the protein release rates from its complex and the aqueous droplet size of W/O emulsion. In the case of GOx, in which specific activity of the released protein was fully retained and independent of the type of surfactant, the smaller the inner aqueous droplet size of W/O emulsions (i.e. L-195 > O-170 > ER-290), the slower the release rates (Fig. 2B). Although ER-290 caused partial inactivation of lysozyme after prolonged incubation (Fig. 3), the same trend was also observed in the protein release from the complexes with lysozyme (Fig. 2A). To gain further insights into the relationship, we investigated how the aqueous droplet size affected the protein release rates from the complexes prepared with the same surfactant, O-170.

Fig. 4 depicts the GOx release behavior from O-170–GOx complexes prepared with a different surfactant concentration. When the concentration of O-170 was raised from 1.0 to

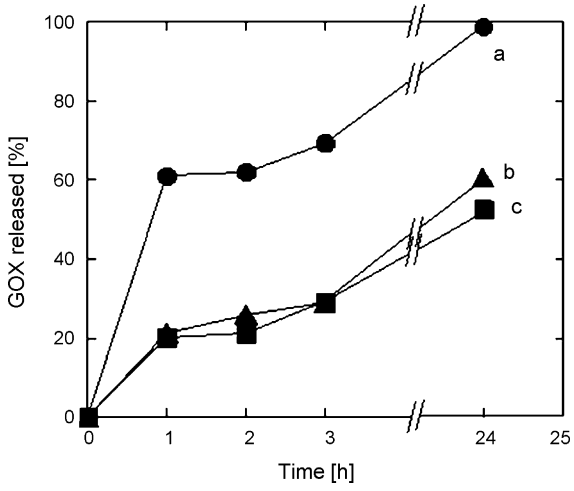

Fig. 4. Time course of active GOx release from the complexes prepared with 1.0 (a), 5.0 (b) and 10 wt% (c) of O-170.

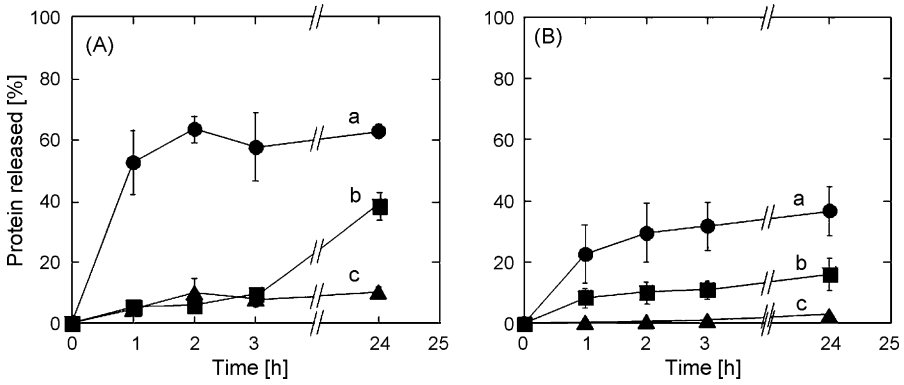

Fig. 5. Time course of active enzyme release from O-170–lysozyme complexes (A) or ER-290–GOx complexes (B). Surfactant concentration in the preparation of complexes was 1.0 (a), 5.0 (b) and 10 wt% (c).

5.0 wt%, the aqueous droplet size of W/O emulsions decreased to ∼1 μm, and a further increase in the surfactant concentration to 10 wt% showed little change in the diameter (Table 1). Accordingly, the resultant complexes exhibited a similar saturation profile in the GOx release rates from 5.0 to 10 wt%. This result proves that the release rate of protein from its complex depends on the aqueous droplet size of W/O emulsions. It was found that the rate of protein release became slower in proportion to the decrease in droplet size of W/O emulsions. One may hypothesize that when the droplet size of W/O emulsions is large, the emulsion is generally unstable. Low stability of the emulsion destabilizes the resultant surfactant–protein complexes, which could result in the initial burst of protein from its complex.

3.5. Effect of the surfactant concentration in the formulation on protein release rates

Finally, we investigated the control of protein release rates by changing the concentration of surfactants. Lysozyme release behavior from O-170–lysozyme complexes and GOx release behavior from ER-290–GOx complexes prepared with the different surfactant concentrations were investigated. As shown in Fig. 5, the initial burst in protein release was substantially suppressed by simply changing the concentration of surfactant in the complex formulation step. In this case, protein release behavior somewhat differed from that observed in Fig. 4. With respect to ER-290–GOx complexes, analysis of the data in Table 1 and Fig. 5 reveals that the aqueous droplet size of W/O emulsions was independent of the surfactant concentration. However, the protein release rates differed drastically, and were further controlled by the concentration of surfactants. The results suggest that the higher the surfactant's concentration, the firmer are the resultant surfactant–protein complexes. It should be noted that specific activity of proteins released was perfectly retained in each protein, indicating the possibility of controlling the protein release rates by choosing a proper surfactant.

4. Conclusion

In the present study, we characterized a new type of pharmaceutical protein formulation, surfactant–protein complexes, from the viewpoint of controlling the rate of protein release from its complex with edible surfactants under physiological conditions. With proper combination of surfactant and protein, the protein release rate is controllable, while retaining the functional integrity illustrated by perfect retention of its enzymatic activity. We also found that there is a correlation between the aqueous droplet size of W/O emulsions, which is a parameter of stability of emulsions in the formulation step, and protein release rates. This finding could be useful for choosing and/or designing a suitable surfactant for complex formulation. Results obtained suggest that surfactant–protein complexes have the potential to act as a unit of pharmaceutical protein formulation.

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