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Stabilization of protein encapsulated in poly(lactide-*co*-glycolide) microspheres by novel viscous S/W/O/W method

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Abstract

In stabilizing proteins during microsphere fabrication, the viscous solid-in-water-in-oil-in-water (S/W/O/W) method was compared to the conventional multi-emulsion W/O/W and S/O/W method. Solid proteins lyophilized with cyclodextrin derivatives and polyethylene glycol (PEG) pass through an organic solvent phase and are then embedded in aqueous microdroplets of first emulsion. Proteins were stabilized at the water/organic solvent interface by an internal aqueous phase containing viscous polysaccharides, and then can be safely encapsulated without degradation. In addition, these microspheres showed a long-term protein release followed by nearly zero-order kinetics with minimal initial burst. This means that the viscous S/W/O/W method provides a safe strategy for microsphere fabrication and has promising properties, involving the preservation of protein bioactivity, the inhibition of protein denaturation or agglomeration, and long-term protein release.
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Keywords: S/W/O/W; Viscous polysaccharide; Cyclodextrin derivatives; Poly(lactide-*co*-glycolide); Microsphere

1. Introduction

Proteins and peptides are frequently administered due to their short circulation time and instability in plasma. Such medication imposes discomfort and inconvenience on patients, particularly patients requiring a long-term treatment (Shinha and Trehan, 2003). In a prominent example of growth hormone-deficient children with short stature, five to seven injections every week for a period of 1–2 years are indispensable. It addresses long-acting protein formulations that do not need daily repetitive administration (Tracy et al., 1999; Hahn et al., 2004).

In recent decades, various approaches mentioned in the scientific and patent literature have been aimed at improving protein duration time in the body. For instance, PEGylations and subgroup modification of protein for shielding of antigenic and immunogenic epitopes, and for avoiding uptake by the reticuloendothelial system, showed long-term activity in plasma and enhanced circulation time (Francis et al., 1998; Abuchowski et al., 1984). Microcapules or microspheres have been extensively pursued in attempts to avoid the peaks and valleys of drug plasma levels associated with conventional administration and for sustained release (Seyrek et al., 2003; Bezemer et al., 2000; Morlock et al., 1998).

In particular, microspheres fabricated with FDA-approved poly(ester), poly(lactide-*co*-glycolide) (PLGA), have presented well-defined protein encapsulation and long-acting behavior (Freiberg and Zhu, 2004; Bilati et al., 2005; Rahman and Mathiowitz, 2004; Yeo and Park, 2004).

However, despite successful application of PLGA to low molecular synthetic drug formulations like Risperdal Consta®, several problems, such as protein instability during microsphere fabrication, protein denaturation during release and incomplete release (Shinha and Trehan, 2003; Freiberg and Zhu, 2004; Bilati et al., 2005; Rahman and Mathiowitz, 2004; Yeo and Park, 2004; Chi et al., 2003; Aubert-Pouessel et al., 2004; Whitaker et al., 2005), all of which have been encountered in protein delivery by PLGA microspheres, have interrupted developments for commercialization.

Organic solvent used for the preparation of microspheres denatures proteins. Metabolites and acids liberated by degrading poly(ester)s degrade proteins, which makes insoluble protein agglomeration and leads to incomplete protein release (Shinha

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and Trehan, 2003; Chi et al., 2003; Aubert-Pouessel et al., 2004; Whitaker et al., 2005).

The Costantino group thus utilized the spray freeze drying method to encapsulate protein into micrsopheres without physical denaturation and chemical degradation. Solid proteins mixed with organic solvent, were sprayed into liquid nitrogen (Costantino et al., 2002). It seems, however, that the long duration of solid proteins in organic solvent is unstable, as described in the literature (Castellanos et al., 2006).

Recently, stabilization of proteins using surfactants and cyclodextrins has been attempted to prevent protein denaturation at the water/organic solvent interface. Physical interaction with proteins and shielding proteins from degrading environments were considered to mechanism of stabilization. However, their stabilizing effects on labile proteins were not consistent among various proteins (Kang et al., 2002; Perez-Rodriguez et al., 2003; De Rosa et al., 2005).

It is interesting to note that macromolecule, poly(ethylene glycol)-*b*-poly(L-histidine) (PEG-PH) diblock copolymer, used as a "temporal and reversible molecular shield" (Kim et al., 2005), and magnesium hydroxide (Zhu et al., 2000) were introduced to neutralize acids liberated by degrading poly(ester)s during long-time release, exploiting their acid-chelating properties. However, it is apparent that this research does not entirely solve issues relevant to protein stability and delivery.

In this study, we have focused on maintaining protein stability during protein encapsulation and protein release, to fulfill the requirements mentioned above. In the emulsion step, fine solid proteins lyophilized with cyclodextrin derivatives and PEG were incorporated into aqueous microdroplets comprising viscous polysaccharides solution, without the influence of organic solvent and then were coated with a mixture of PLGA. Polysaccharides such as starch and hyaluronate formulate an internal highly viscous aqueous solution that may delay certain interactions between proteins and organic solvent. In addition, viscous polysaccharides may have a favorable influence on protein stability after microspheres hydration, reducing the passive diffusion of the metabolites and the acids liberated by PLGA, and minimizing initial burst.

2. Materials and methods

2.1. Materials

Lysozyme (from Chicken egg white, 50,000 EU/mg), soluble starch, potato starch, sodium hyaluronate (HA), 2-hydroxypropyl-β-cyclodextrin (HP-CD), 2,6-di-*O*-methyl-β-cyclodextrin (DM-CD), 6-*O*-maltosyl-β-cyclodextrin (OM-CD), polyethylene glycol (M_n 2000) (PEG2K), sorbitan monooleate 80 (SM80), sodium bicarbonate, sodium azide, Tween 80, KBr, sodium chloride, rhodamine isothiocyanate (RITC), urea, trinitrobenzene sulfonic acid (TNBS), coomassie blue, polyvinyl alcohol (PVA) (M_n 13,000–23,000) and micrococcus lysodeikticus were obtained from Sigma (St. Louis, MO, USA). Dichloromethane (DCM) was bought from J.T. Baker (Deventer, The Netherlands). Sulfobutyl ether β-cyclodextrin 7 sodium salt (Captisol®) (SBE-CD) was kindly provided from CyDex Corp. (Calabasas, USA). Poly(lactide-*co*-glycolide)s, RG 502H and RG 503H were purchased from Boehringer-Ingelheim (Petersburg, USA). Human Leutenizing hormone releasing hormone (hLHRH, gonadorelin) and leuprolide acetate (Leu) were obtained from Cytoshop (Rehovot, Israel). Recombinant human growth hormone (rhGH) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BCA protein assay Kit was purchased from Pierce (Milwaukee, USA).

2.2. Methods

2.2.1. Preparation of protein loaded microspheres

Proteins was dissolved with PEG2K and cyclodextrin derivative (HP-CD, DM-CD, OM-CD, and SBE-CD) in a deionized water and lyophilized by freeze-drying for two days. Before the experiment, protein powders were ground. RG 502H (or RG 503H) with/without SM80 as a drug-release-modifier was dissolved in a DCM solution and a viscous aqueous solution containing polysaccharides (such as soluble starch, potato starch, and hyaluronate) was prepared. The two solutions were emulsified through vortexing for 1 min. Ground proteins were mixed with the first emulsion solution for 5–15 s and then were injected into 0.5 wt.% PVA and 0.9 wt.% NaCl aqueous solution for secondary emulsion formation. The emulsification was continued for 4 min by a homogenizer (manufactured by Tokushu Kika Kogyo Corp.) at 4000 rpm (Scheme 1). The microspheres formed by this method were hardened during mild stirring for 40 min and were collected by centrifugation at 3000 rpm for 2 min. The obtained microspheres were washed three times with 0.9 wt.% NaCl aqueous solution and freeze-dried for 3 days (Tables 1 and 2).

The solid-in-oil-in-water (S/O/W) (Castellanos et al., 2006) and water-in-oil-in-water (W/O/W) multi-emulsions (De Rosa et al., 2005) were involved in preparing the control samples. In the S/O/W method, solid proteins lyophilized with/without excipients (PEG2K, SBE-CD, starch), were dispersed in a DCM solution and then injected to 0.5 wt.% PVA and 0.9 wt.% NaCl aqueous solution for secondary emulsion formation (Table 3). In the W/O/W method, proteins dissolved in a phosphate buffer saline (PBS, pH 5.1) or 2.5 wt.% starch aqueous solution, with/without excipients (PEG2K, SBE-CD) were incorporated into PLGA microspheres, which then underwent the conventional procedure of the W/O/W methods (Table 4). Their hardening and purification steps are described above.

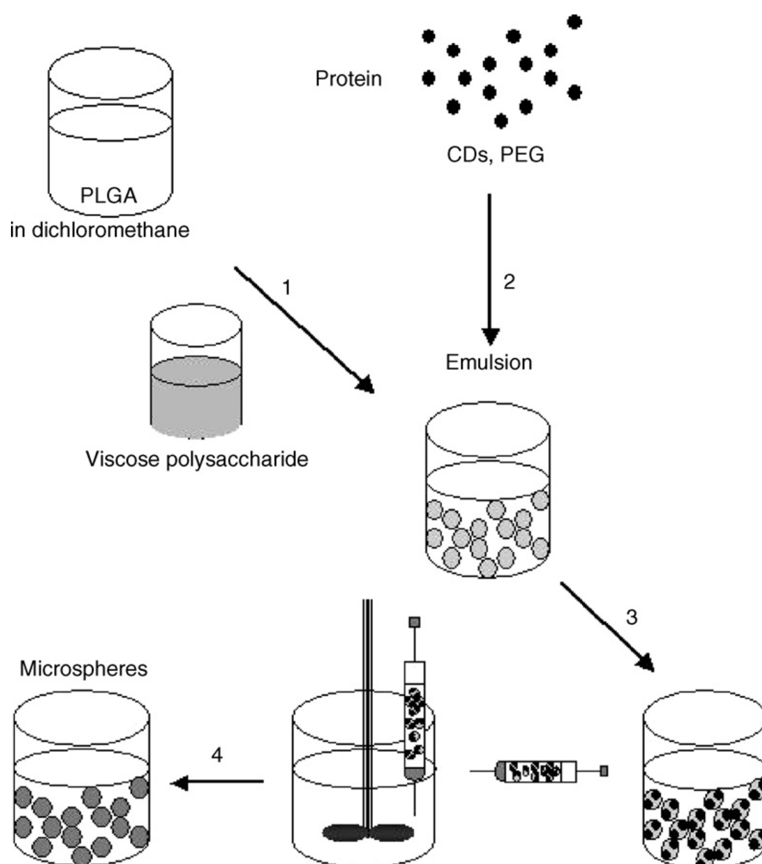
2.2.2. Protein loading of microsphere

The actual protein loading content in microspheres was determined by the TNBS method (Bezemer et al., 2000). Briefly, microspheres (20 mg) or protein standard (0–30 mg) in 6 M HCl (1 ml) were incubated for 24 h at 37 °C and then were mixed with 1 M NaOH (6 ml) for another 24 h. The sample (50 μl) was reacted with 0.5 wt.% TNBS solution (50 μl) and 4 wt.% sodium bicarbonate (pH 9.0) (125 μl). After 2 h incubation, the absorbance of each sample was read on a microplate reader using a test wavelength of 450 nm. The absorbance at 450 nm is pro-

Table 1
Compositions used for constituting lysozyme-loaded microspheres

Code	Lysozyme (mg)	Cyclodextrin		PEG2K (mg)	Internal aqueous phase			Polymer		DCM (ml)	SM80 (ml)	Protein %[a]	Size[b] (μm)	IPA (%)[c]
		Species	mg		Species	Conc. (%)[d]	ml	Species	mg					
SR01	100	–	–	–	Starch[e]	2.5	0.5	RG502H	500	3	0	9.3 ± 0.6	23.3 ± 1.2	4.3 ± 0.6
SR02	100	–	–	25	Starch[e]	2.5	0.5	RG502H	500	3	0	9.6 ± 0.3	25.1 ± 1.5	4.2 ± 0.7
SR03	100	SBE-CD	100	–	PBS[f]	–	0.5	RG502H	500	3	0	6.0 ± 0.8	21.6 ± 2.3	12.1 ± 0.6
SR04	100	SBE-CD	100	25	Starch[e]	2.5	0.5	RG502H	500	3	0	11.4 ± 0.5	23.7 ± 1.6	0.8 ± 0.2
SR05	100	SBE-CD	100	25	Starch[e]	1.0	0.5	RG502H	500	3	0	8.5 ± 0.6	20.1 ± 3.1	5.1 ± 0.3
SR06	100	SBE-CD	25	25	Starch[e]	2.5	0.5	RG502H	500	3	0	10.6 ± 0.8	21.5 ± 2.5	3.2 ± 0.2
SR07	100	HP-CD	100	25	Starch[e]	2.5	0.5	RG502H	500	3	0	10.5 ± 0.7	22.5 ± 1.9	1.4 ± 0.2
SR08	100	DM-CD	100	25	Starch[e]	2.5	0.5	RG502H	500	3	0	9.03 ± 0.5	20.6 ± 0.9	0.7 ± 0.1
SR09	100	OM-CD	100	25	Starch[e]	2.5	0.5	RG502H	500	3	0	9.9 ± 0.5	18.9 ± 1.3	1.0 ± 0.3
SR10	100	SBE-CD	100	25	HA	1.0	0.5	RG502H	500	3	0	10.3 ± 0.6	23.5 ± 1.2	1.0 ± 0.4
SR11	100	SBE-CD	100	25	Gelatin	1.5	0.5	RG502H	500	3	0	12.3 ± 0.3	18.9 ± 2.1	2.4 ± 0.5
SR12	100	SBE-CD	100	25	Potato starch	2.5	0.5	RG502H	500	3	0	10.5 ± 0.4	23.8 ± 2.3	3.6 ± 0.6
SR13	100	SBE-CD	100	25	Starch[e]	2.5	0.5	RG502H	500	3	0.25	9.4 ± 0.6	19.6 ± 2.2	0.7 ± 0.2
SR14	100	SBE-CD	100	25	Starch[e]	2.5	0.5	RG502H	500	3	0.10	10.6 ± 0.7	25.9 ± 1.6	0.8 ± 0.4

[a] Actual protein content (wt.%) in the microspheres.
[b] Mean particle size of microsphere.
[c] Mean weight percentage of insoluble protein aggregation to total protein amount in microsphere ($n = 3$).
[d] Concentration.
[e] Soluble starch.
[f] PBS pH 5.1.



Scheme 1. Procedure of microsphere fabrication by the novel viscous S/W/O/W method.

portional to the protein concentration. Here, the actual protein content was calculated by a weight percentage ratio of protein encapsulated in microsphere to total amount of microsphere.

2.2.3. Morphology and particle size distribution

The morphology of microspheres was confirmed with scanning electron microscopy (SEM, Hitachi S-3000 N).

A Fiber-optics particle analyzer (Photal FPAR-100, otsuka electronics Co., Ltd.) was employed to confirm the particle size distribution of microspheres. The sample was prepared by suspending dry microspheres (15 mg) in 2 wt.% Tween 80 solution (15 ml) and then sonicating water bath for 3 min to prevent aggregation between microspheres (Wang et al., 2002).

2.2.4. Protein distribution in microsphere

For visualizing the protein distribution in microspheres, lysozyme was labeled with RITC (Takano et al., 2004). Lysozyme (300 mg) and RITC (30 mg) in a pH 9.0 borate buffer were incubated for 1 h at room temperature. After the reaction, pH in the solution was down to 7.4 with 0.1 M boric acid. The solution was transferred to a pre-swollen dialysis membrane tube (Spectra/Por; MWCO 10,000) and lyophilized. The obtained RITC-labeled lysozyme was incorporated into microspheres by the protocol described in Section 2.2.1 (Table 2). To observe the morphology of a cross-sectioned part, microspheres were embedded in gelatin/glycerin gel and cross-sectioned by ultra microtome (Kim et al., 2005). Protein distribution of cross-sectioned microspheres was examined by a confocal microscope (Leica TCS NT, Leica, Germany).

2.2.5. In vitro stability studies

The formation of insoluble protein aggregate (IPA) during microsphere preparation was determined from the lysozyme extracted from the microspheres (Perez- Rodriguez et al., 2002). The solution of microspheres (40 mg) dissolved in DCM (1 ml) was stirred for 30 min. This solution was centrifuged for 20 min at 5000 rpm and precipitates were collected for a next step. After adding 10 mM PBS (pH 5.1) to the precipitates, insoluble precipitate was separated by centrifugation and then dissolved in 1 ml of 6 M urea. The content of insoluble lysozyme aggregate in 6 M urea was measured by the TNBS method described earlier.

Furthermore, for evaluating the molecular weight change of proteins, proteins (10 μg) recovered from microspheres and standard protein (control) were examined using 15% SDS-PAGE (Bio-rad electrophoresis system, 16 cm × 16 cm with 0.75-mm thickness). The gels after electrophoresis were stained with coomassie blue.

2.2.6. In vitro protein release

Protein release in PBS (pH 7.4, 0.01% sodium azide, 0.02% Tween 80) was monitored by a BCA protein assay kit. The microsphere samples were immersed in PBS and incubated under mild stirring at 37 °C. The release medium was taken at each time point and analyzed with a microplate reader at 562 nm.

Table 2
Compositions used for constituting microspheres encapsulated with different proteins

Code	Protein		Cyclodextrin		PEG2K (mg)	Internal aqueous phase			Polymer		DCM (ml)	SM80 (ml)	Protein %[a]	Size[b] (μm)	IPA (%)[c]
	Species	mg	Species	mg		Species	Conc. (%)[d]	ml	Species	mg					
PA01	HLHRH[e]	10	SBE-CD	10	5	Starch[f]	2.5	0.5	RG503H	100	0.1	0	7.8 ± 0.3	28.1 ± 2.3	–
PA 02	Leu	10	SBE-CD	10	5	Starch[f]	2.5	0.5	RG503H	100	0.1	0	6.8 ± 0.5	30.2 ± 1.9	–
PA 03	rhGH	10	SBE-CD	10	5	Starch[f]	2.5	0.5	RG502H	100	0.1	0	7.6 ± 0.6	28.0 ± 2.6	–
PA 04	HLHRH[e]	10	SBE-CD	10	5	Starch[f]	2.5	0.5	RG503H	100	0.1	0.05	8.1 ± 0.4	27.5 ± 2.9	–
PA 05	Leu	10	SBE-CD	10	5	Starch[f]	2.5	0.5	RG503H	100	0.1	0.05	7.3 ± 0.3	28.3 ± 1.5	–
PA 06	rhGH	10	SBE-CD	10	5	Starch[f]	2.5	0.5	RG502H	100	0.1	0.05	7.9 ± 0.4	29.7 ± 1.3	–
PA07	RITC-Lys[g]	100	SBE-CD	100	25	Starch[f]	2.5	0.5	RG502H	500	3	0	10.7 ± 0.2	24.5 ± 2.7	–

[a] Actual protein content (wt.%) in microspheres.
[b] Mean particle size of microspheres.
[c] Mean weight percentage of insoluble protein aggregation to total protein amount in microsphere ($n = 3$).
[d] Concentration.
[e] Gonadorelin.
[f] Soluble starch.
[g] RITC-labeled lysozyme.

Table 3
Compositions used for constituting microspheres by the S/O/W method

Code	Lysozyme (mg)	Cyclodextrin		PEG2K (mg)	Starch (mg)[a]	Polymer		DCM (ml)	SM80 (ml)	Protein %[b]	Size[c] (μm)	IPA (%)[d]
		Species	mg			Species	mg					
SW01	100	–	0	0	0	RG502H	500	3	0	6.5 ± 0.3	32.6 ± 1.9	15.5 ± 0.7
SW02	100	–	0	0	12.5	RG502H	500	3	0	8.9 ± 0.2	30.4 ± 2.9	13.3 ± 0.6
SW03	100	–	0	25	12.5	RG502H	500	3	0	8.3 ± 0.3	35.1 ± 1.3	13.5 ± 0.8
SW04	100	SBE-CD	100	25	12.5	RG502H	500	3	0	8.6 ± 0.4	28.5 ± 2.2	9.3 ± 1.2

[a] Soluble starch.
[b] Actual protein content (wt.%) in microspheres.
[c] Mean particle size of microspheres.
[d] Mean weight percentage of insoluble protein aggregation to total protein amount in microsphere ($n = 3$).

Table 4
Compositions used for constituting microspheres by the W/O/W method

Code	Internal aqueous phase						Polymer		DCM (ml)	SM80 (ml)	Protein %[a]	Size[b] (μm)	IPA (%)[c]
	Lysozyme (mg)	SBE-CD (mg)	PEG2K (mg)	Species	Conc. (%)[d]	ml	Species	mg					
WW01	100	0	0	PBS[e]	–	0.5	RG502H	500	3	0	5.9 ± 0.3	18.3 ± 1.1	23.5 ± 0.5
WW02	100	100	0	PBS[e]	–	0.5	RG502H	500	3	0	5.6 ± 0.2	13.9 ± 0.9	16.6 ± 0.3
WW03	100	100	25	PBS[e]	–	0.5	RG502H	500	3	0	5.3 ± 0.4	15.6 ± 2.1	16.5 ± 0.4
WW04	100	0	0	Starch[f]	2.5	0.5	RG502H	500	3	0	9.3 ± 0.6	26.3 ± 2.3	13.9 ± 0.2
WW05	100	100	25	Starch[f]	2.5	0.5	RG502H	500	3	0	9.6 ± 0.4	25.9 ± 2.1	12.5 ± 0.3

[a] Actual protein content (wt.%) in microspheres.
[b] Mean particle size of microspheres.
[c] Mean weight percentage of insoluble protein aggregation to total protein amount in microsphere ($n = 3$).
[d] Concentration.
[e] PBS pH 5.1.
[f] Soluble starch.

2.2.7. Long-term bioactivity of proteins

The micrococcus lysodeikticus (ATCC 4698) cell suspension (0.2 mg/ml, 66 mM PBS 6.2) (1.3 ml) was incubated with lysozyme aqueous solution (20 μl) obtained from microspheres during *in vitro* protein release tests, or with lysozyme standard solution (1–100 μl). The decrease in absorbance intensity at 450 nm with time was associated with bioactivity of lysozyme against micrococcus lysodeikticus cells. The relative bioactivity of lysozyme was calculated from the slope of the linear part of the curve (absorbance versus time) according to a technique described by van de Weert et al. (2000).

3. Results

3.1. Morphology and drug loading of microspheres

The shape of microspheres was regular and spherical as visualized in the SEM photograph (Fig. 1(a)). The size of microspheres measured by a fiber-optics particle analyzer was in the range of 13–36 μm (Tables 1–4) based on the intensity-average diameter with a unimodal distribution (Fig. 1(b)). The distribution of protein drug on the microsphere was observed by confocal microscopy. The cross-sectioned sample showed a broad distribution of RITC-labeled lysozyme on the microspheres (Fig. 1(c)).

The drug loading of the microspheres was in the range of 5–12% (Tables 1–4). Particularly, the drug content in the microspheres with viscous polysaccharides was approximately 1.3–2.0 times higher than that without viscous polysaccharides (Tables 1, 3 and 4). This difference may be attribute to the suppression of leakage of proteins due to high viscosity in the internal aqueous phase, in the step of secondary emulsion formation.

3.2. Stabilization of proteins

Tables 1, 3 and 4 present the protein-stabilizing effects from each manufacturing method derived from various compositions used for preparing microspheres. Under an equivalence composition, insoluble aggregation of proteins by denaturation was up to 13.3 ± 0.6% by the S/O/W method (Table 3) and 13.9 ± 0.2% by the W/O/W (Table 4), but only 4.3 ± 0.6% by the viscous S/W/O/W method (Table 1).

With SBE-CD known as a protein-stabilizer, insoluble aggregation of proteins by denaturation was up to 9.3 ± 1.2% by the S/O/W method (Table 3) and 12.5 ± 0.3% by the W/O/W (Table 4), but only 0.8 ± 0.2% by the viscous S/W/O/W method (Table 1).

In addition, the protein stabilizing effect by PEG2K was negligible, as shown in SR01–SR02. However, in the emulsification step, solid proteins were broken to pieces because the PEG2K was dissolved in DCM, which seems to facilitate embedment of solid protein into the aqueous microdroplets of emulsion.

At SR04–SR06 in Table 1, the microspheres exhibited less stability of proteins by decreasing the excipient's concentration. The stability of proteins dwindled to 5.1 ± 0.3% or 3.2 ± 0.2%,

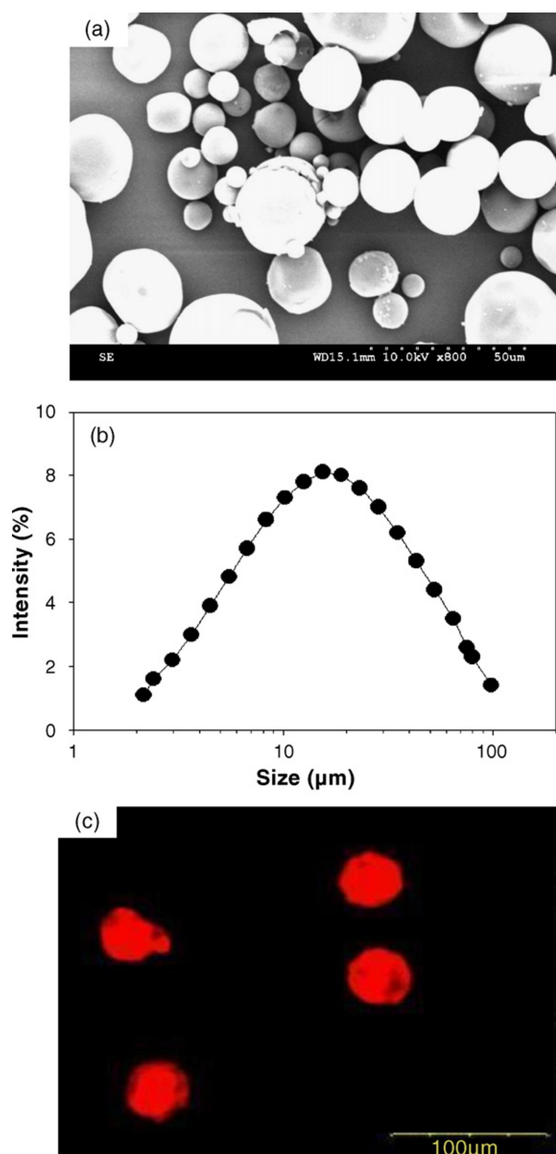


Fig. 1. The particle size distribution of SR04 microspheres determined by (a) SEM and by (b) fiber-optics particle analyzer. The distribution of RITC-labeled lysozyme in PA07 microspheres was visualized by (c) confocal microscopy.

in the cases of the viscous S/W/O/W method containing 1.0 wt.% starch solution or SBE-CD 25 mg, respectively.

There is little difference in the degree of insoluble protein aggregation with species of cyclodextrin derivatives (SR04, SR07–SR09) and SM80 content (SR13–SR14).

The insoluble lysozyme aggregates produced by the viscous S/W/O/W method with viscous 2.5 wt.% soluble starch, 1.0 wt.% HA, 1.5 wt.% gelatin, and 2.5 wt.% potato starch were in the range of 0.8–3.6% (Table 1).

3.3. In vitro release of protein in microspheres

The cumulative protein release from microspheres was plotted and compared to evaluate release profiles of microspheres obtained from various manufacturing methods. The microspheres fabricated by the viscous S/W/O/W method followed

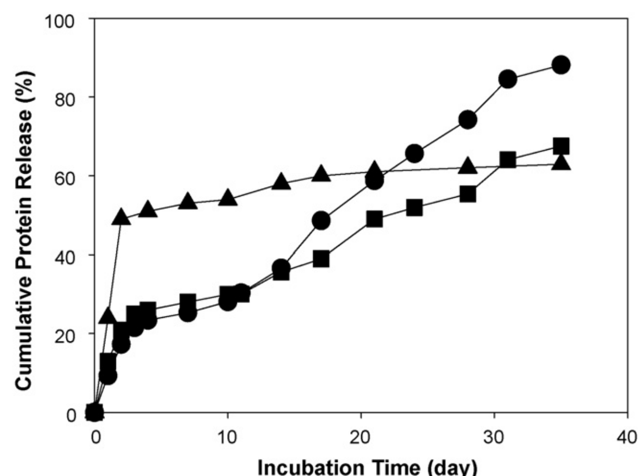


Fig. 2. The cumulative lysozyme release (wt.%) from SR04 (●), SW04 (■), WW05 (▲) microspheres prepared by different manufacturing methods. The average value from triplicate experiment was plotted.

nearly zero-order kinetics (Fig. 2), having a plateau region for 3–11 days. The release profile of microspheres fabricated by the S/O/W method was similar to that by the viscous S/W/O/W, but showed decreased total drug release about 20%. In addition, the W/O/W method induced protein burst of about 50% for 2 days.

When other polysaccharides (such as potato starch, HA, and gelatin) were embedded in microspheres (Fig. 3), potato starch (SR12) showed gradual protein release, but gelatin (SR11) and HA (SR10) were not compatible with protein release. Total drug release in SR10 and SR11 was confined to less than 40 wt.%.

In Fig. 4, with SM80 (used as a protein-release-modifier), gonadorelin (11.82 kDa) and leuprolide acetate (12.69 kDa), were released following nearly zero-order kinetics (Fig. 4(b)), but having little release for 2–10 days, and almost reached a plateau in 30 days. In addition, about 10–15 wt.% of the protein loaded in the microspheres was released for 1 day, thus presenting minimal initial burst.

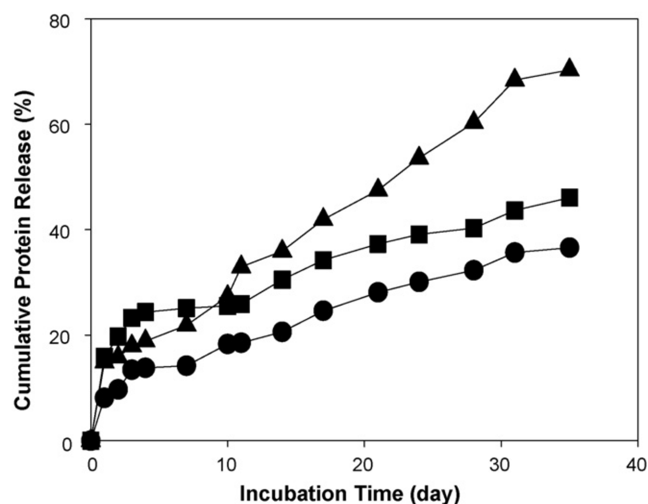


Fig. 3. The cumulative lysozyme release (wt.%) from the microspheres containing different polysaccharides; SR10 (●), SR11 (■), SR12 (▲). The average value from triplicate experiment was plotted.

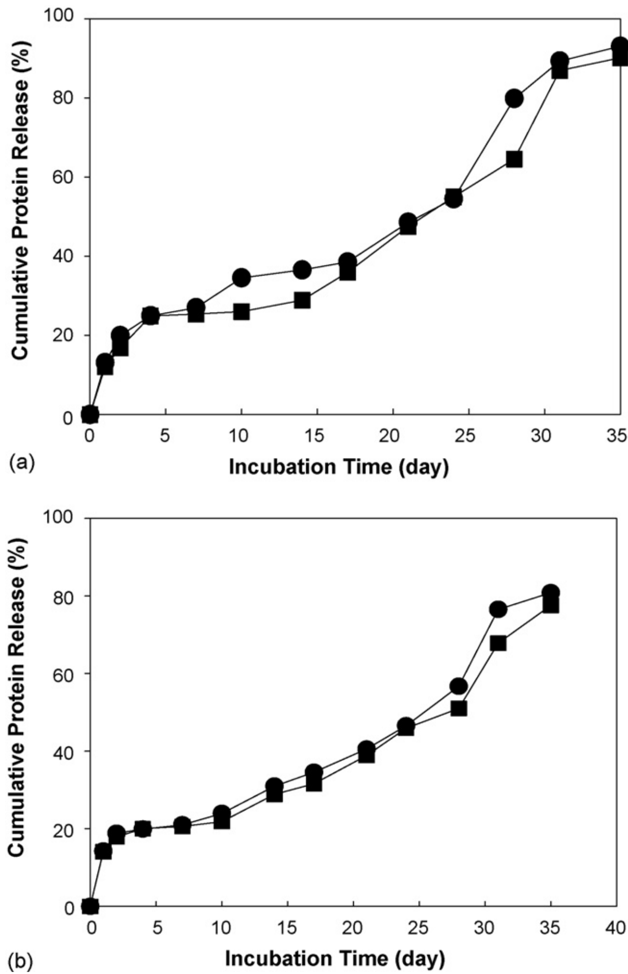


Fig. 4. The cumulative peptide release (wt.%) from the microspheres (a) without SM80 and with (b) SM80 as shown in Table 2: gonadorelin (●) and leuprolide acetate (■). The average value from triplicate experiment was plotted.

The rhGHs were encapsulated in microspheres using a safe method. Fig. 5 shows SDS-PAGE analysis of rhGH standard (for control) and rhGH extracted from the microspheres prepared by the viscous S/W/O/W method (PA03 and PA06 in Table 2). The major band was located at ca. 22 kDa but the other band was not detected in the SDS-PAGE analysis.

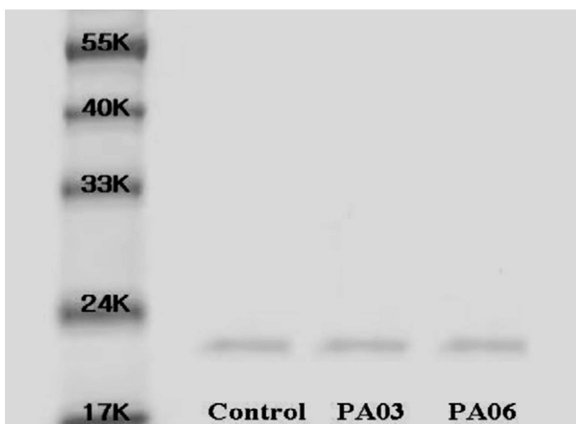


Fig. 5. The SDS-PAGE for control standard rhGH and rhGH extracted from PA03 and PA06 microspheres.

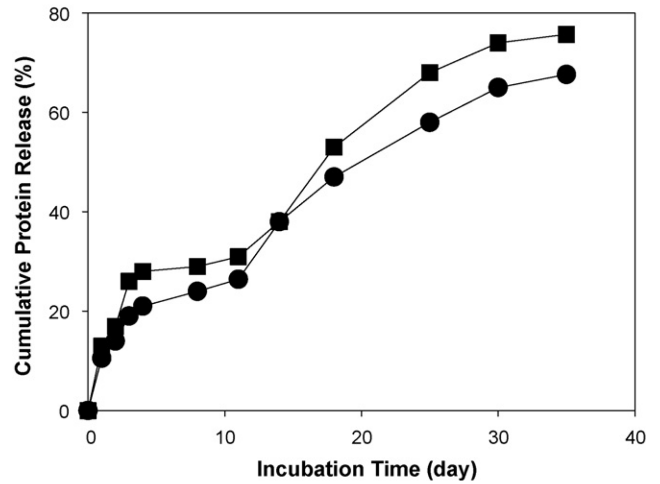


Fig. 6. The cumulative rhGH release (wt.%) from PA03 (●) and PA06 (■) microspheres. The average value from triplicate experiment was plotted.

The release of rhGH from the microspheres was accelerated after 11 days through a first plateau region (3–11 days) and reached a second plateau in 30 days. The addition of release-modifier (SM80) improved protein release within a narrow range (Fig. 6).

3.4. In vitro bioactivity of proteins during release

Fig. 7 shows long-term stability of proteins. After 3 days, the difference between the viscous S/W/O/W method (SR01) and the non-viscous S/W/O/W (SR03) became significant. In addition, the viscous S/W/O/W method with SBE-CD (SR04) showed only a slightly better stabilizing effect when compared to the viscous S/W/O/W method without SBE-CD (SR01). Overall, more than 95% of the protein (SR01 and SR04) fraction released for 1 month was preserved in the bioactive form, which sug-

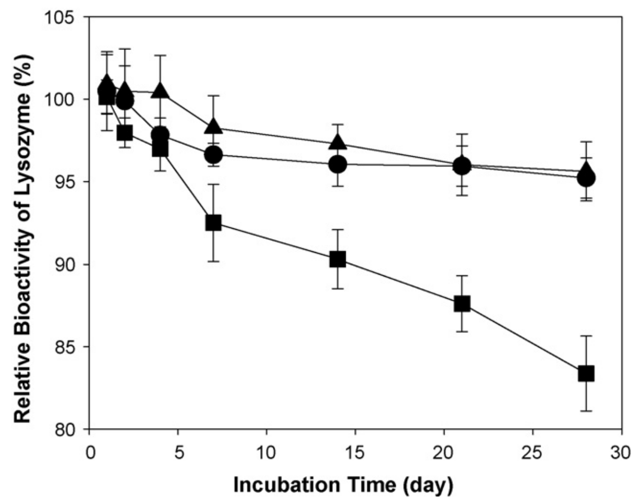


Fig. 7. The relative bioactivity of lysozyme against micrococcus lysodeikticus cells. Lysozymes were released from SR01 (●), SR03 (■), SR04 (▲) microspheres with incubation time ($n = 3$).

gests long-term stability of proteins and continuous therapeutic effect.

4. Discussion

As protein's instability at the water/organic solvent interface was associated with protein denaturation such as insoluble aggregation (Shinha and Trehan, 2003; Freiberg and Zhu, 2004; Bilati et al., 2005; Rahman and Mathiowitz, 2004; Yeo and Park, 2004; Chi et al., 2003; Aubert-Pouessel et al., 2004; Whitaker et al., 2005), fragile proteins need to be secured from degrading conditions.

For the purpose of protecting denaturation of proteins by organic solvent, our viscous S/W/O/W method is distinguished from the conventional W/O/W and S/O/W methods. In first preparing an emulsion like W/O, solid proteins were instantly incorporated into aqueous microdroplets of pre-prepared emulsion, which minimize the time required for forming emulsion with proteins. This approach is based on the premise that the aqueous microdroplets surrounding by firm viscous interface will protect the proteins from degrading environment formed by the water/organic solvent interface. As shown in Tables 1, 3 and 4, the protein-stabilizing effects of the viscous S/W/O/W method were well established. In the S/O/W method, meanwhile, proteins exposed directly to organic solvent underwent an indispensable denaturation (Castellanos et al., 2006). In the W/O/W method, emulsifying with protein aqueous solution and organic solvent invokes complicated mixing between the water phase and the organic solvent, probably endowing the mixture with the strong influence of the organic solvent, which can result in a potentially degrading environment for proteins. Herein, it is not surprising that the W/O/W method with viscous internal aqueous phase (starch solution) improved protein stability when compared with the conventional W/O/W method with a PBS solution (Table 4). In addition, viscous polysaccharides helped to increase drug loading of microspheres, blocking leakage of proteins out of microspheres (Tables 1–4).

Furthermore, the viscous S/W/O/W method and SBE-CD were combined to decrease protein denaturation (SR04–SR14 in Table 1). Coupling cyclodextrin derivatives with the viscous S/W/O/W method led to a slight decrease of insoluble protein aggregation, up to 0.8%, although their individual activities on stabilizing proteins were not perfect. Here, the enhanced stability of proteins from organic solvent was probably due to protein shielding formed by physical interaction between the hydrophobic part of the proteins and of the cyclodextrin derivatives (De Rosa et al., 2005).

At SR04–SR06, the microspheres exhibited less protein stability, a result of the decrease in the stabilizing excipient's concentration. This coincides with the increasing influence of the organic solvent. Thus, enhanced protein stability can be expected when stabilizing excipient concentration is elevated.

However, such contribution is presumably not relevant to the success designation of injectable sustained protein formulation. The SBE-CD content of more than 100 mg made for porous microspheres (data not shown), which may induce initial protein burst during release. The starch concentration of more than 2.5 wt.% comprised a high viscous internal phase that hardly mixed with proteins. These facts suggest remarkably optimized conditions for constituting long-acting formulations containing bioactive proteins.

Furthermore, in constructing microspheres with bioactive proteins, no noticeable difference in four kinds (SR04 and SR07–SR09) of cyclodextrin derivatives was observed. However, considering that the intramuscular irritation of *M. vastus lateralis* of rabbits was elevated in order DM-CD > HP-CD > OM-CD > SBE-CD (Irie and Uekamax, 1997), DM-CD is estimated to be incompatible for constituting injectable microspheres. Species of viscous polysaccharides and the addition of SM80 were not significant contributor to reduced insoluble protein aggregation.

These findings have interpreted the viscous S/W/O/W method as a safe method for preparing protein-loaded microspheres.

Protein release profiles and kinetics from protein-encapsulated microspheres were evaluated under *in vitro* condition and presented in Figs. 2–4 and 6. In Fig. 2, the microspheres fabricated by the viscous S/W/O/W method followed nearly zero-order kinetics, having a broad protein drug distribution (Fig. 1(c)) in the microspheres as visualized by confocal microscopy. However, the S/O/W method showed decreased total drug release, probably due to exacerbated release by protein denaturation during microsphere fabrication and protein degradation during incubation at 37 °C (Abgar et al., 2001). The W/O/W method induced drug burst release of about 50% for 2 days. It is thought that in the W/O/W method, much (hydrophilic) protein was incorporated to the surface area of microspheres, which is ascribed as a typical cause of initial burst (Shinha and Trehan, 2003; Tracy et al., 1999; Bilati et al., 2005; Yeo and Park, 2004).

In Fig. 3, viscous solution comprising soluble starch and potato starch facilitated sustained protein drug release, but HA and gelatin were not compatible with protein release. Furthermore, gonadorelin and leuprolide acetate (Fig. 4(a)) embedded in microspheres by the viscous S/W/O/W method were released following nearly zero-order kinetics, having a duration time about 4–17 days. It appeared that the addition of SM80 decreased duration time to 2–10 days (Fig. 4(b)).

In the case of rhGH, SDS-PAGE analysis of rhGH extracted from the microspheres showed that rhGH remained intact during microsphere fabrication (Fig. 5). The release profile of stable rhGH was similar to that shown in Fig. 4, having an initial plateau region (3–11 days) and a second plateau in 30 days (Fig. 6). This consequence, combined with the stabilizing effect of the viscous S/W/O/W method, resulted in constant release of proteins with therapeutic activity.

It is known that the acidic microenvironment (Shinha and Trehan, 2003; Zhu et al., 2000) and reactive adducts created by PLGA degradates such as lactic and glycolic acids (Shinha and Trehan, 2003; Freiberg and Zhu, 2004; Bilati et al., 2005; Rahman and Mathiowitz, 2004; Yeo and Park, 2004; Chi et al., 2003; Aubert-Pouessel et al., 2004; Whitaker et al., 2005; Murty et al., 2003), byproducts of degradation formed by acid-catalyzed hydrolysis, are associated with physical denaturation and chemical degradation of proteins. Heparin, polysrobate 20,

matose, sucrose, dextran 40, as a stabilizing excipieint, were utilized as stabilizing excipients to maintain protein bioactivity, but their stabilizing effects were limited (Shinha and Trehan, 2003; Freiberg and Zhu, 2004; Bilati et al., 2005; De Rosa et al., 2000; Blanco and Alonso, 1998).

As shown in Fig. 7, more than 95% of the protein (SR01 and SR04) fraction released for 1 month was preserved in the bioactive form. Proteins surrounded by viscous polysaccharides were relatively stable, while proteins without viscous polysaccharides underwent gradual degradation due to indispensable physical denaturation during incubation at 37 °C (Murty et al., 2003) and degrading conditions built up by PLGA's degradates (Shinha and Trehan, 2003; Freiberg and Zhu, 2004; Bilati et al., 2005; Rahman and Mathiowitz, 2004; Yeo and Park, 2004; Chi et al., 2003; Aubert-Pouessel et al., 2004; Whitaker et al., 2005; Murty et al., 2003). These results support the idea that the viscous S/W/O/W method truly facilitates bioactive protein release from PLGA microspheres and enhanced sustained protein release. Furthermore, we hypothesized that the shielding of protein with viscous polysaccharides delays degrading interaction of proteins and acids liberated by PLGA's degradates and a viscous pathway constitutes sustained protein release. This hypothesis requires further investigation for proof.

5. Conclusion

The viscous S/W/O/W method was composed of solid proteins lyophilized with cyclodextrin derivatives and PEG, a viscous internal aqueous phase for protein stabilization, PLGA, and a drug-release-modifier. In the procedure step of the viscous S/W/O/W, solid proteins pass through an organic solvent phase in a short time and are incorporated in the aqueous microdroplets stabilized with viscous polysaccharides solution. In *in vitro* protein stability test, this method showed significantly less denaturation at the water/organic solvent interface, and demonstrated continuous bioactivity over time. In addition, it endowed release profiles following nearly zero-order kinetics and a minimal initial burst. Encapsulation of proteins into PLGA microspheres by this method can therefore be a promising strategy for developing the injectable sustained release formulation of protein.

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