Entrapment of Proteins in Poly(L-Lactide) Microspheres Using Reversed Micelle Solvent Evaporation

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INTRODUCTION

The development of delivery systems for protein drugs with high molecular weights and short biological half-lives using biodegradable polymers is of current interest (1). Low molecular weight peptides such as luteinizing hormone releasing hormone (LHRH) analogues (2) have been successfully entrapped in biodegradable microspheres with sustained-release patterns. Since methods for the preparation of such microspheres involve the preparation of w/o emulsions with vigorous agitation, they cannot be employed for most proteins, which may be susceptible to denaturation (3). Some proteins have been encapsulated by this method, but it is not clear whether protein denaturation occurred during the process (4,5).

We examined the usefulness of reversed micelle solvent evaporation as an alternative to agitation for preparing w/o emulsions. Reverse micelles have been employed for solubilizing proteins in organic solvents without denaturation (6). The present paper describes reverse micellar solubilization of model proteins, super oxide dismutase (SOD), β-galactosidase, and recombinant human tumor necrosis factor (TNF), in organic solvents and their encapsulation in poly(L-lactide) microspheres.

MATERIALS AND METHODS

Materials

Poly(L-lactide) (weight-average molecular weight, 10,000) was purchased from Taki Chemical Co. (Tokyo). Polyvinylalcohol (PVA; average molecular weight, 40,000) was supplied from Aldrich Chemical Co. (WI). Sodium dipolyoxyethylene(10)lauryl phosphate (DLP-10) and sodium 1,2-bis(2-ethylhexyloxycarbonyl)-1-ethanesulfonate (AOT) were from Nikko Chemicals Co. (Tokyo) and Wako Pure Chemicals (Osaka), respectively. Sucrose esters of fatty acids (DK-SS, DK-F160, DK-F110, and DK-F50) were supplied by Daiichi Kogyo Seiyaku Co. (Kyoto). SOD (3300 U/mg) and β-galactosidase (from *Aspergillus oryzae*) were purchased from Wako Pure Chemicals and Toyobo Co. (Osaka), respectively. TNF (2.5 U/mg) was kindly donated by Dainippon Pharmaceutical Co. (Osaka). Other chemicals were of reagent grade.

Micellar Solubilization of Proteins in Organic Solvents

Phosphate buffer solutions (PBS; 50 m*M*, pH 7.4, 20 μL) of SOD (1.7×10^5 U/mL), β-galactosidase (5.0×10^3 U/ml), and TNF (5.8×10^7 U/ml) were added to 4 mL of chloroform or methylene chloride containing 2% (w/v) AOT, DLP-10, DK-SS, and DK-F160. The solutions were briefly shaken and then allowed to sit for 5 min until clear micellar solutions were obtained. Activity losses of SOD and β-galactosidase during the solubilization process were determined by measuring the activity of the enzymes extracted from the micellar solutions. Cytochrome *c* (7) and 2-nitrophenyl-β-D-galactopyranoside (8) were used as substrate, respectively. TNF activity remaining in the micellar solution was determined by enzyme immunoassay (9). The micellar solution of TNF was added to a 1% PVA aqueous solution and the organic solvent was removed under reduced pressure. The protein activity in the aqueous solution was assayed.

Preparation of Protein-Loaded Poly(L-Lactide) Microspheres

Protein-loaded microspheres were prepared by the solvent evaporation method described previously (10). Proteins were solubilized in chloroform using 2 or 4% (w/v) surfactants. L-PLA (500 mg) was dissolved in the micellar solutions of proteins. The solution was poured into a 1% PVA aqueous solution and stirred at 250 rpm for 4 hr under reduced pressure (200 mm Hg) at 25°C to remove the solvent. Microspheres in the 25- to 45-μm sieve fraction were collected, washed with cold water, and freeze-dried. The protein content in the PVA solution was then determined by measuring its activity.

Protein entrapment was determined by measuring the activity of the protein extracted from the L-PLA microspheres. SOD- and β-galactosidase-loaded microspheres (50 mg) were dissolved in 2 mL methylene chloride and the proteins were extracted with 4 mL PBS (pH 7.4). TNF-loaded microspheres were added to 2 mL methylene chloride, and precipitated protein was collected on a 0.22-μm filter (GVHP 013, Millipore Co., Milford, MA) and dissolved in PBS.

The percentage of protein content which showed rapid release (burst release within the first hour) from the microspheres was determined. Microspheres (50 mg) were suspended in 6 mL PBS containing 0.02% Tween 80 (pH 7.4) (for SOD and β-galactosidase) or in 6 mL PBS (40 m*M*, pH 7.4) containing 0.1% bovine serum albumin and 0.1% NaN_3 (for TNF). After 1 hr of incubation at 37°C, the enzyme activity in the PBS solution was measured.

Release Study

SOD-loaded poly(L-lactide) microspheres (20 mg) were

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Table I. Activities Recovered from the Proteins Solubilized in Micellar Solutions[a]

Surfactant	Protein	Activity recovered (%)	
		$CHCl_3$	CH_2Cl_2
AOT	SOD	95.1 ± 2.0	91.2 ± 5.1
	β-Galactosidase	93.6 ± 1.1	86.7 ± 6.4
	TNF	<1	<1
LP-10	SOD	96.3 ± 1.3	50.5 ± 2.8
	β-Galactosidase	92.6 ± 0.4	39.4 ± 3.2
	TNF	4.1 ± 0.4	4.3 ± 3.2
DK-SS	SOD	90.2 ± 5.1	ND[b]
	β-Galactosidase	91.3 ± 2.2	ND
	TNF	78.2 ± 2.2	ND
DK-F160	SOD	92.7 ± 2.3	ND
	β-Galactosidase	91.7 ± 0.9	ND
	TNF	82.4 ± 1.8	ND

[a] Mean ± SD (n = 3). Surfactant concentration, 2% (w/v).
[b] Not determined.

suspended in 6 mL PBS (50 m*M*, pH 7.4) containing 0.02% Tween 80 and shaken at 37°C. At appropriate intervals the samples were taken out and centrifugated at 3000 rpm for 5 min. Enzyme activity released in the dissolution medium was assayed as described above. Enzyme activity remaining in the microspheres was also determined after extraction with PBS from the microspheres. The amount of SOD released into the medium and that remaining in the microspheres were assayed by HPLC (Model LC-10 system, Shimadzu, Kyoto) after concentration using a centrifugal concentrator (10,000 cutoff, Amicon Co.). The column was TSKgel Phenyl-5 PW RP (TOSOH Co., Tokyo) maintained at 30°C. HPLC was performed using a linear gradient from 25 to 35% acetonitrile in 0.9% NaCl containing 0.1% trifluoroacetic acid in 18 min. The flow rate was 1.0 mL/min. The variable-wavelength detector was set at 210 nm. The release medium was renewed at 14-day intervals.

RESULTS AND DISCUSSION

Micellar Solubilization of Proteins

An adequate volume of aqueous solutions of proteins could be solubilized in chloroform and methylene chloride using AOT and DLP-10 as surfactants for the preparation of the reversed micelles. Sucrose esters of fatty acids (DK-SS and DK-F160) were effective in solubilizing protein solutions in chloroform but not in methylene chloride. Table I shows the activity of the recovered proteins after solubilization in the micellar solutions and extraction with PBS.

Activity was lost to a greater extent during solubilization and/or extraction when methylene chloride was used as the organic solvent rather than chloroform. The enzyme activity of SOD and β-galactosidase recovered from the micellar chloroform solutions was greater than 90% for all surfactants studied. In contrast, TNF activity decreased significantly during solubilization, especially with AOT and DLP-10. It is suggested that TNF was inactivated on contact with chloroform. Since AOT and DLP-10 are known to provide monolayer micelles (6), the larger activity loss observed with AOT and DLP-10 may be due to the easier access of the organic solvent to the proteins.

Entrapment of Proteins in 1-PLA Microspheres

Table II shows the percentage protein activity entrapped in L-PLA microspheres and the percentage activity leaked into the PVA solution, the external dispersion phase during the solvent evaporation process. The percentage activity of the protein which was entrapped in microspheres, but was released rapidly (within 1 hr) from the microspheres, is also shown in Table II. For SOD and β-galactosidase, the activity entrapped in the microspheres prepared with AOT and DLP-10 was very low and the entrapped activity showed rapid release, even though these surfactants solubilized the enzymes without a significant loss of activity as shown in Table I.

In contrast, sucrose esters of fatty acids, DK-SS or DK-F160, gave microspheres with a higher protein entrapment and a lower extent of rapid release. Even TNF was entrapped in microspheres in its active form. For SOD and β-galactosidase, summation of the protein activity that was entrapped in the microspheres and that leaked into the external dispersion phase yielded about 100% of the theoretical activity. For TNF, however, the total amount of activity observed was about 50% of the theoretical value, indicating

Table II. Entrapment of Proteins in Poly(L-Lactide) Microspheres[a]

Surfactant	Protein	% entrapped	% leaked	% rapidly released[b]
AOT	SOD	5.1[c]	ND[d]	52.3[c]
	β-Galactosidase	6.6[c]	ND	42.3[c]
DLP-10	SOD	5.8[c]	ND	68.5[c]
	β-Galactosidase	4.0[c]	ND	72.2[c]
DK-SS	SOD	19.5 ± 9.5	75.2 ± 7.2	29.0 ± 7.8
	β-Galactosidase	35.1 ± 8.1	60.3 ± 5.3	41.0 ± 5.5
	TNF	32.2 ± 2.2	24.6 ± 3.1	16.2 ± 2.7
DK-F160	SOD	34.1 ± 4.9	62.3 ± 5.6	28.8 ± 11.0
	β-Galactosidase	29.7 ± 1.1	57.9 ± 4.6	42.9 ± 3.5
	TNF	25.8 ± 1.5	12.3 ± 7.7	42.1 ± 4.6

[a] Mean ± SD (n = 3). Surfactant concentration, 4% (w/v).
[b] Percentage rapidly released to the entrapped activity.
[c] Mean (n = 2).
[d] Not determined.

Table III. Effects of HLB and Concentration of Sucrose Esters of Fatty Acids on SOD Entrapment[a]

Surfactant	HLB	Conc. (%, w/v)	% entrapped	% rapidly released
DK-SS	20	1	24.1 ± 5.1	10.9 ± 3.0
		2	23.1 ± 5.5	17.0 ± 3.0
		4	19.5 ± 9.5	29.0 ± 7.8
DK-F160	16	2	27.5 ± 4.4	20.3 ± 2.8
		4	34.1 ± 4.9	28.8 ± 6.2
DK-F110	11	2	39.7 ± 5.5	41.2 ± 3.4
		4	35.2 ± 7.1	56.1 ± 8.2
DK-F50	6	2	53.2 ± 5.2	65.3 ± 6.1
		4	47.6 ± 8.8	80.5 ± 5.6

[a] Mean ± SD (n = 3).

some extent of inactivation during the preparation process. The microspheres prepared with sugar esters of fatty acid exhibited gradual release of proteins. The percentages of SOD, TNF, and β-galactosidase released from microspheres prepared with DK-F160 were about 70, 75, and 80% in 40 days, respectively.

The differences in protein entrapment and rapid release between the microspheres prepared with sugar esters of fatty acids and those with AOT or DK-F160 may be ascribed to the difference in micellar structure among the surfactants. It is speculated that AOT and DLP-10 gave monolayer micelles, while sugar esters of fatty acids gave multilayer micelles. Further studies are required to interpret the difference.

Effects of Hydrophile–Lipophile Balance (HLB) and Concentration of Sucrose Esters of Fatty Acids on SOD Entrapment in Microspheres

Table III shows the percentage SOD activity entrapped in microspheres prepared with several sucrose esters of fatty acids with different HLB, as a function of surfactant concentration. The percentage activity during rapid release is also shown in Table III. Both entrapment efficiency and percentage activity of rapid release increased with decreasing HLB and increasing surfactant concentration. It is suggested that the surfactants with a lower HLB tend to be entrapped largely in the microspheres, resulting in greater entrapment and a greater extent of rapid release. Further studies are required to elucidate this mechanism.

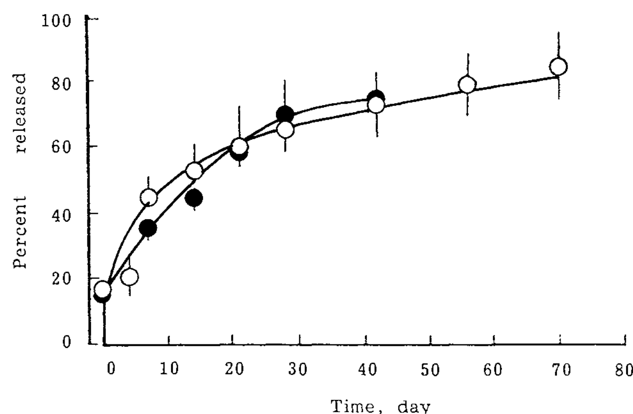

Fig. 1. SOD release from poly(L-lactide) microspheres prepared from a micellar solution containing 2% (w/v) DK-SS, as measured by enzyme activity (—○—) and protein content (—●—). Mean and SD (n = 3). Medium: 50 mM PBS (pH 7.4) containing 0.02% Tween 80.

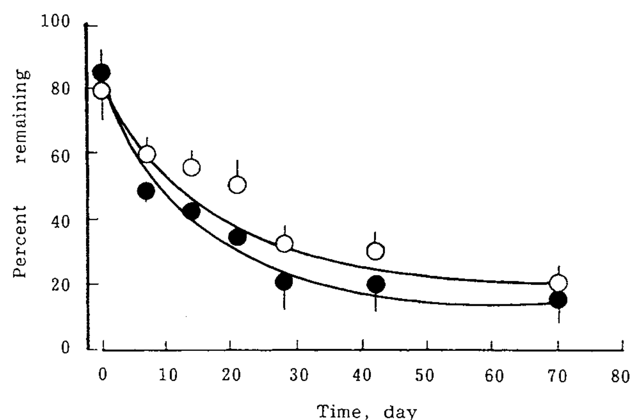

Fig. 2. SOD remaining in poly(L-lactide) microspheres prepared from a micellar solution containing 2% (w/v) DK-SS, as measured by enzyme activity (—○—) and protein content (—●—). Mean and SD (n = 3). Medium: 50 mM PBS (pH 7.4) containing 0.02% Tween 80.

In Vitro Release of SOD from Poly(L-Lactide) Microspheres

Figure 1 shows the enzyme activity and the amount of protein released from SOD-loaded poly(L-lactide) microspheres prepared from a SOD micellar solution containing 2% (w/v) DK-SS. The amount of protein was determined by HPLC. The enzyme activity and the amount of protein remaining in the microspheres during the release study are shown in Fig. 2. Following an initial burst release of about 20% within 1 hr, active SOD was gradually released over 70 days. There was no marked difference in the profiles between the activity and the amount of SOD released and remaining. The sum of the percentages of SOD released and remaining was about 100% of the initial SOD content. This indicates that no inactivation of SOD occurred during the release study.

In conclusion, the present study demonstrates that SOD, β-galactosidase, and TNF can be successfully encapsulated in L-PLA microspheres in their active form by the reversed micelle solvent evaporation technique using sucrose esters of fatty acids as surfactants. The SOD entrapped exhibited a gradual release pattern *in vitro*.

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