Preparation and Characterization of a Composite PLGA and Poly(Acryloyl Hydroxyethyl Starch) Microsphere System for Protein Delivery

Byung H. Woo,[1] Ge Jiang,[1] Yeong W. Jo,[2] and Patrick P. DeLuca[1,3]

Received June 6, 2001; accepted August 3, 2001

Purpose. To prepare and characterize a novel composite microsphere system based on poly(D,L-lactide-co-glycolide) (PLGA) and poly(acryloyl hydroxyethyl starch) (acHES) hydrogel for controlled protein delivery.

Methods. Model proteins, bovine serum albumin, and horseradish peroxidase were encapsulated in the acHES hydrogel, and then the protein-containing acHES hydrogel particles were fabricated in the PLGA matrix by a solvent extraction or evaporation method. The protein-loaded PLGA-acHES composite microspheres were characterized for protein loading efficiency, particle size, and *in vitro* protein release. Protein stability was examined by size-exclusion chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and monitoring the enzymatic activity.

Results. Scanning electron microscopy showed discrete PLGA microspheres containing many acHES particles. The composite microspheres were spherical and smooth in size range of 39–93 μm. The drug loading efficiency ranged from 51 to 101%. The composite microspheres showed more favorable *in vitro* release than conventional PLGA microspheres. The composite microspheres showed 20% less initial with a gradual sustained release compared to high burst (~60%) followed by a very slow release with the conventional PLGA microspheres. The composite microspheres also stabilized encapsulated proteins from the loss of activity during the microsphere preparation and release. Proteins extracted from the composite microspheres showed good stability without protein degradation products and structural integrity changes in the size-exclusion chromatography and SDS-PAGE analyses. Horseradish peroxidase extracted from microspheres retained more than 81% enzymatic activity.

Conclusion. The PLGA-acHES composite microsphere system could be useful for the controlled delivery of protein drugs.

KEY WORDS: poly(lactide-co-glycolide); hydroxyethyl starch; microsphere; protein; controlled release.

INTRODUCTION

Biodegradable microspheres have been extensively investigated as delivery systems for biologically active peptides and proteins (1–5). Sustained release characteristics of microspheres reduce the need for frequent administrations and enhance patient compliance by maintaining *in vivo* drug levels in the therapeutic range (3,4). Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) are the most widely used and well characterized polymers for biodegradable microspheres (6–8).

Nevertheless, there are problems associated with the use of these systems for protein delivery. Protein instability has been observed during the preparation of protein-loaded microspheres (9–12). Usually, an aqueous protein solution is dispersed in an organic polymer solution by using a homogenizer or sonicator to create a water-in-oil (w/o) emulsion. The exposure of protein to organic solvent or aqueous/organic interface might have adverse effects on the stability of the proteins. During drug release, the adsorption of protein on the hydrophobic polymer matrices and a low pH generated during the polymer degradation process could cause degradation of the entrapped protein (13,14). An initial burst release of protein drugs from the microspheres presents still another problem. The fast diffusion of protein drugs located on the surface of internal pores and channels formed by the evaporation of solvent and water during the microsphere preparation, particularly in a water-in-oil-in-water (w/o/w) emulsion technique, contributes to the burst release (15). One approach to overcome this problem is chemical modification or physical blending of PLGA with hydrophilic monomers and polymers such as polyethylene glycol (16), poly(ethylene-co-vinyl acetate) (17), and polyvinyl alcohol (PVA) (18). Another approach is physical encapsulation of protein-loaded hydrophilic particles or hydrogels into a PLGA matrix. For example, heterogeneous structured microspheres were prepared by fabrication of PLGA with hydrophilic particles such as agarose hydrogels (19), PVA (20), or gelatin nanoparticles (21). These heterogeneous composite systems were designed to stabilize entrapped protein drugs and to improve drug release characteristics. However, in the preparation processes of these heterogeneously combined microspheres, protein drugs were exposed to large amounts of organic solvent and multiple freezing-thawing or heating-cooling processes during protein loading on the primary hydrophilic particles. In addition, little information is available on biologic activity changes of encapsulated protein drugs in these composite microspheres.

In this study, hydrophilic starch-based hydrogel particles containing model proteins were prepared by a simple swelling procedure. The protein-loaded hydrogel particles were then encapsulated in the PLGA microspheres to form the hydrogel-PLGA combined composite microspheres using a solvent extraction or evaporation method. Bovine serum albumin (BSA) and horseradish peroxidase (HRP) were used as model protein drugs. Physicochemical characteristics and *in vitro* protein release of microspheres were studied to establish poly(acryloyl hydroxyethyl starch)-PLGA (acHES-PLGA) composite microspheres as a novel protein delivery system.

METHODS

Materials

Poly (D,L-lactide-co-glycolide) (PLGA), copolymer ratio of 50:50 (lactic/glycolic; MW 28,000) and Resomer® RG503H were supplied by Boehringer Ingelheim (Ingelheim, Germany). Hydroxyethyl starch [Hetastarch (HES)] was obtained from Dupont Pharmaceuticals (Wilmington, DE). Acryloyl chloride was purchased from Aldrich Chemicals Com-

[1] Faculty of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Rose Street, Lexington, Kentucky 40536.

[2] Dong-A Pharmaceutical Co., 47-5 Sanggal-ri, Kiheung-up, Yonginsi, Kyunggi-do 449-900, Korea.

[3] To whom correspondence should be addressed. (e-mail: ppdelu1@uky.edu)

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pany, Inc. (Milwaukee, WI). BSA and polyvinyl alcohol (PVA, MW 30,000–70,000) were obtained from Sigma Chemical Co. (St. Louis, MO). HRP, 1-Step™ Slow TMB-ELISA and micro-BCA protein assay kit were obtained from Pierce (Rockford, IL).

Preparation of acHES Hydrogel Particles

Vinyl Derivatization of HES Polymer

Acrylic acid ester of hydroxyethyl starch (acHES) was prepared as described previously (22). Briefly, 20 g of HES was dissolved in 60 mL dimethyl acetamide and an appropriate amount (2–10 mL) of distilled acryloyl chloride, based on desired degree of derivatization (DD, number of vinyl groups introduced on the hydroxyethyl group in every unit of HES polymer chain), and an equimolar amount of triethylamine were added slowly to the HES solution. The reaction mixture was precipitated by adding 200 mL of precooled acetone, and the precipitate was dissolved in 50 mL deionized water. The solution was transferred to a dialysis tubing with a molecular weight cutoff of 14,000 and dialyzed against deionized water with frequent change of water for 48 h. The dialyzed solution was freeze-dried. The DD was determined by proton-NMR spectroscopy (22).

Preparation of acHES Hydrogel Particles

AcHES polymer (7:3 mixture of DD = 0.14 and 0.25) was dissolved in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) to make a 30% (w/v) solution, and ammonium peroxidisulfate was added to the solution to form a dispersed phase. The dispersed phase was added to 50-mL mineral oil containing 0.3% of Sorbitan Sesquioleate while stirring to form a w/o emulsion. N,N,N′,N′,tetramethylethylenediamine (300 μL) was added to the emulsion to initiate the polymerization reaction followed by continuous stirring at room temperature for 1 h. The suspension containing polymerized droplets was poured into precooled hexane while sonicating. The acHES hydrogel particles were collected by centrifugation at 1000 rpm for 5 min, washed twice with hexane and ethanol, rinsed with deionized water several times, and freeze-dried.

Preparation of PLGA-acHES Composite Microspheres

The PLGA-acHES composite and conventional PLGA microspheres were prepared by a modified solvent extraction or evaporation method with 5–10% target loading of BSA and 5% for HRP. In brief, 25–50 mg of BSA and 25 mg HRP were dissolved in 0.25 mL of 0.1 M PBS (pH 7.4). The protein solutions were added to acHES particles (10% of total polymer weight), and the particles were allowed to swell for 5 min with vortex mixing at room temperature. Twenty percent (w/w) PLGA (90% of total polymer weight) in methylene chloride was added to the swollen acHES particles and vortexed 3 min at room temperature to form a (protein in hydrogel)/(polymer in solvent) dispersion. This primary dispersion was then added to precooled 100 mL 6% PVA solution and stirred by a Silverson mixer (Silverson, Chesham Bucks, England) at 5000 rpm for 1 min. The resulting secondary suspension was transferred to 1 L deionized water and stirred gently for 3 h at room temperature to remove the organic solvent and solidify the polymer. The microspheres were washed with water and freeze-dried. For the conventional PLGA microspheres, a primary emulsion was prepared by mixing the protein solutions with 20% PLGA solution, and then the emulsion was added to 6% PVA solution while stirring at 5000 rpm. The resulting suspension was transferred to 1 L deionized water and stirred gently for 3 h at room temperature to remove the organic solvent and fabricate the polymer. The microspheres were washed with water and freeze-dried.

Particle Characterization

Particle Size Measurement

PLGA-acHES microspheres (10 mg) were dispersed in 10 mL 0.1% Tween 80 solution. The particles were sized by laser diffractometry by using a Malvern 2600 laser sizer (PC6300; Malvern Instruments, Worcestershire, England). The average particle size was expressed as the volume mean diameter in micrometers.

Morphology of Microspheres

The surface morphology and internal structure of fractured microspheres were examined by scanning electron microscopy (SEM) (model S800; Hitachi, Tokyo, Japan) after palladium-gold coating of the microsphere samples on a aluminum stub.

Drug Loading Efficiency

Ten-milligram protein-loaded PLGA-acHES microspheres were hydrolyzed in a mixture of 0.9 mL of 1 M NaOH and 0.1 mL PBS with vigorous shaking at room temperature for 1h. protein standard solutions (0.1 mL) were also hydrolyzed by adding 0.9 mL 1 M NaOH with same procedures. After hydrolysis, 1 mL 0.9 M HCl was added to neutralize the sample solutions. Protein concentrations were determined by micro-BCA total protein assay method. The loading efficiency was calculated by the actual protein loading to the theoretical loading of protein in PLGA-acHES microspheres based on the amount used in the microsphere preparation.

In Vitro Protein Release

Microspheres were weighed and placed in 15-mL centrifuge tubes containing PBS with 0.02% sodium azide as a preservative. The tubes were incubated at 37°C with occasional shaking. At designated times, samples were collected, and the release medium was replaced with fresh PBS. The samples were assayed by a micro-BCA method or by using a fluorescence spectrophotometer (model F2000; Hitachi) at excitation and emission wavelengths of 280 and 350 nm. The two assay methods showed comparable results for *in vitro* release samples.

Protein Stability

The structural integrity of proteins extracted from microspheres was characterized by size-exclusion chromatography (SEC) and sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) as described previously (23). Ten-milligram microspheres were dissolved in 0.1 mL CH_2Cl_2. Proteins were extracted from the polymer solution by addition of 1 mL 0.1 M PBS followed by agitation for 1 h.

SEC was performed by using a Biosep SEC-S2000 column (4.6 × 300 mm; Phenomenex, Torrance, CA). The mobile phase was 0.1 M phosphate buffer (pH 7.0) containing 0.02% sodium azide. The flow rate was 0.5 mL/min, and the detection wavelength was UV 280 nm. The injection volume was 20 μL.

SDS-PAGE was carried out in the presence of 0.1% SDS using a 9% slab gel prepared by a gel casting and electrophoresis unit (Mini-Protean® H electrophoresis system; Bio-Rad, Hercules, CA). Protein samples and standards were treated with SDS-PAGE sample buffer containing SDS and dithiothreitol for 3 min at 95°C, and electrophoresis was performed at a constant voltage of 200 V. Protein bands on the gel were stained with Coomassie Brilliant Blue.

The enzymatic activity of HRP was determined by using a substrate solution, 1-Step™ Slow TMB-ELISA. 5 μL of HRP standard solutions (1–10 μg/mL), and samples were mixed with 0.4 mL of the substrate solution and incubated at room temperature for 2 min. The absorbance at 450 nm was measured, and the specific activity of samples was calculated by using an activity calibration curve obtained from standard HRP solutions.

RESULTS AND DISCUSSION

Characterization of Microspheres

The acHES particles for protein entrapment possess a submicrometer particle size of average 0.14 μm, a low bulk density of 0.05 g/cc, and high specific surface area as shown in Table I. In addition, the acHES hydrogel particles showed fast and good swelling property. The hydrated particles showed about an 11-fold larger particle diameter and were 10.5-fold heavier than the dry particles. This finding suggests that the hydrophilic starch-based hydrogel could absorb a large amount of aqueous drug solution inside and protect the drugs from degradation due to solvent and polymer interactions during the microsphere preparation and drug release.

SEMs of BSA-loaded acHES-PLGA composite microspheres are shown in Figs. 1a and b. Figure 1a shows spherical shape and smooth surface of the PLGA-acHES composite microspheres. Figure 1b is a SEM photomicrograph of a fractured PLGA-acHES microsphere showing its interior structure and the distribution of acHES particles inside microsphere. One composite microsphere contains many acHES hydrogel particles in which target protein was entrapped before preparing the PLGA matrix. The procedure prevented the protein drugs from contacting the organic solvent during the microsphere preparation. Figure 2a shows the conventional PLGA microspheres, which are spherical in shape and have small pores on a smooth surface. Compared with the composite microspheres, the conventional PLGA microspheres have a very porous honey comblike interior (Fig. 2b).

Table I. Characterization of acHES Hydrogel Particles

Characteristics	Measured
Particle size	0.14 μm (dry particle) 1.56 μm (swelled in water)
Maximum hydration in water ($W_{hydrated}/W_{dry}$)	10.5
Bulk density	0.05 g/cc
Specific surface area	0.98 ± 0.26 sq.m./g
Degradation in 0.1 mg/mL α-amylase in 24 h	25.3 ± 0.1%

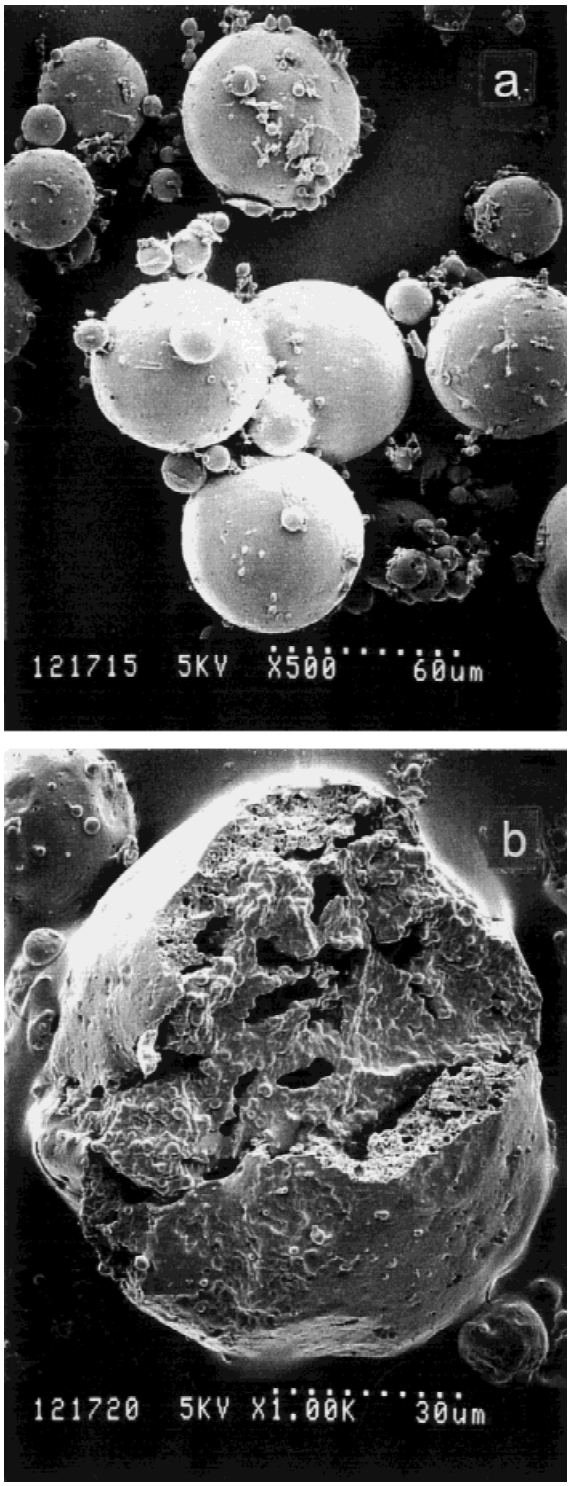

Fig. 1. SEM micrographs of BSA-loaded PLGA-acHES composite microspheres (a) and interior structure of a fractured microsphere (b).

As shown in Table II, the average particle size of the composite microsphere ranged from 39.1 to 93.1 μm. A similar mean particle size was observed from different protein

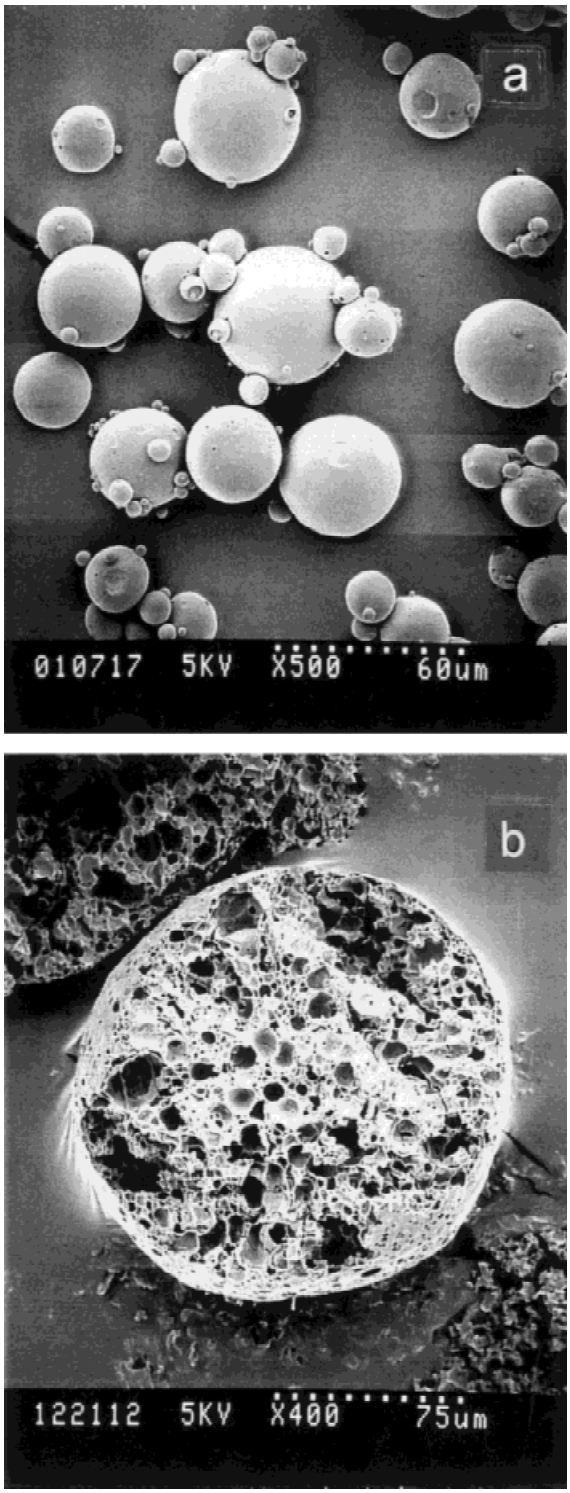

Fig. 2. SEM micrographs of BSA-loaded PLGA microspheres (a) and interior structure a fractured microsphere (b).

contents of BSA-loaded microspheres that showed the particle size ranged from 39 to 52 μm. HRP-loaded microspheres were larger than BSA-loaded microspheres prepared by the same target drug load. The particle size of PLGA-acHES composite microspheres was mainly affected by two preparation parameters: concentration of PLGA in the solvent and the mixing speed of the primary suspension in the continuous phase. The size of particles increased with increase in polymer concentration and decrease in stirring rate.

Drug Encapsulation Efficiency

As shown in Table II, BSA was encapsulated successfully in the composite and PLGA microspheres with 88–101% drug-loading efficiency. The composite microspheres prepared with 5% target BSA load showed a little higher encapsulation efficiency than 10% target load. For the PLGA microspheres, a similar drug encapsulation efficiency was obtained from the different target BSA load. However, there was no significant difference in drug encapsulation efficiency with different target drug loads. HRP-loaded microspheres showed lower loading efficiencies of 40.5–50.9% compared to BSA-loaded microspheres. The drug encapsulation efficiency increased with increasing PLGA polymer concentration in the disperse phase and PVA in the continuous phase (data not shown). Higher viscosity achieved by increasing polymer and PVA concentrations could minimize diffusion of protein from the disperse phase to continuous phase during the fabrication of microspheres and may also have resulted in more condensed PLGA matrices around entrapped aqueous protein droplets. The drug encapsulation efficiency was not improved by increasing the ratio of hydrogel to PLGA.

In Vitro Release

As shown in Fig. 3a, BSA- and HRP-loaded composite microspheres showed about 40% initial protein release in 24 h followed by slow release for 21 days. An accelerated and nearly linear release was observed between 21 and 42 days. This three-stage release pattern could be explained as follows. The initial release is due to diffusion of proteins from the acHES hydrogel particles located near the microsphere surface, interchannels and inner pores formed by solvent evaporation during the microsphere solidification process. The second stationary phase observed between the initial release and the third phase in which release was increased by erosion of polymer matrices. Compared to the composite microspheres, as shown in Fig. 3b, the conventional PLGA microspheres showed about 20% more initial release followed by a very slow release for 42 days. This findings suggests that the PLGA-acHES composite structure could suppress the initial burst release of encapsulated protein compared to PLGA microspheres. The cross-linked starch-based polymer structure of acHES could retard the diffusion of protein drugs from the inside of hydrogel particles through the PLGA matrix. In addition, the swelled hydrogel particles could prevent the penetration of release media into the channels and pores in the PLGA matrices. At the third phase of erosion-controlled release, PLGA microspheres showed very slow release for 42 days compared to gradual release of the composite microspheres (Fig. 3). As the PLGA polymer degrades by hydrolysis in the aqueous release media, the properties of the microspheres change, the molecular weight of polymer decreases, acid number of degraded polymer increases, and hydration increases. The spherical microsphere particles stick together and turn into a sticky gel-like cake, and finally, the hydrated polymer becomes completely soluble. The swelling, hydration, and gelling of PLGA matrices may block the diffusion channels in the microspheres and, consequently, de-

Table II. Characterization of Protein Loaded Microspheres

Microsphere	Protein	Target load (%)	Drug content (%)	Encapsulation efficiency (%)	Average particle size (μm)	Specific activity (%)
Composite	BSA	10	9.1 ± 1.0	91.4 ± 9.7	39.1	—
	BSA	5	5.0 ± 0.1	100.5 ± 2.3	51.5	—
	HRP	5	2.5 ± 0.2	50.9 ± 4.2	93.1	80.9 ± 0.7 (93.4)[a]
PLGA	BSA	10	9.2 ± 0.7	92.4 ± 6.8	29.5	—
	BSA	5	4.4 ± 0.3	87.5 ± 0.6	not done	—
	HRP	5	2.0 ± 0.1	40.5 ± 2.8	103.5	61.5 ± 18.5 (91.3)[a]

[a] Specific activity of HRP released in 0.1 M PBS within 24 h.

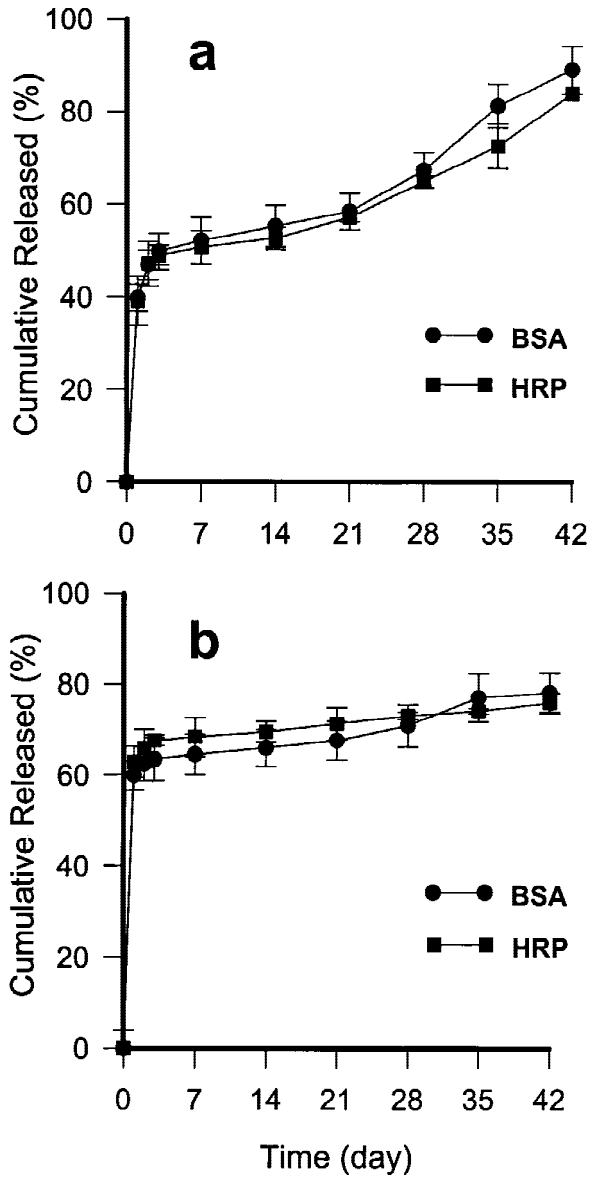

Fig. 3. *In vitro* protein release profiles of PLGA-acHES composite (a) and PLGA (b) microspheres.

crease the release of relatively large protein molecules located in the core of the microspheres. In addition, an increase of acid number of the polymer causes more protein binding to the polymer, and the protein-polymer interaction may be another reason for slow release from PLGA microspheres. Compared to PLGA microspheres, for the composite microspheres, dissolution of the PLGA domains could expose the entrapped drug-containing acHES hydrogel particles to the release media, and the exposed hydrogel could release more drug molecules with little or no interaction with the PLGA polymer. As a result, the composite microspheres showed more favorable *in vitro* release than the conventional PLGA microspheres for protein drug delivery.

Protein Stability and Activity

As shown by SDS-PAGE analysis in Fig. 4, the proteins extracted from the PLGA-acHES composite microspheres showed good stability without structural integrity changes. Figure 5 shows SEC chromatograms of standard HRP (A) and protein extracted from HRP loaded composite microspheres (B). The protein extracted showed same retention time of native HRP without the trace of protein aggregates and degradation products. These results suggest that the

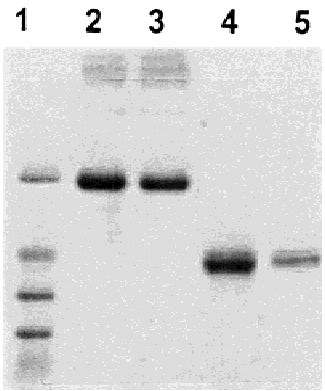

Fig. 4. SDS-PAGE of proteins extracted from the PLGA-acHES composite microspheres for the assessment of protein stability. (Lane 1) Molecular weight standard markers, BSA (MW 66,000), ovalbumin (MW 45,000), glyceraldehyde-3-phosphate dehydrogenase (MW 36,000), and carbonyl anhydrase (MW 29,000); (2) BSA standard; (3) BSA extracted from microspheres; (4) HRP standard; and (5) HRP extracted from microspheres.

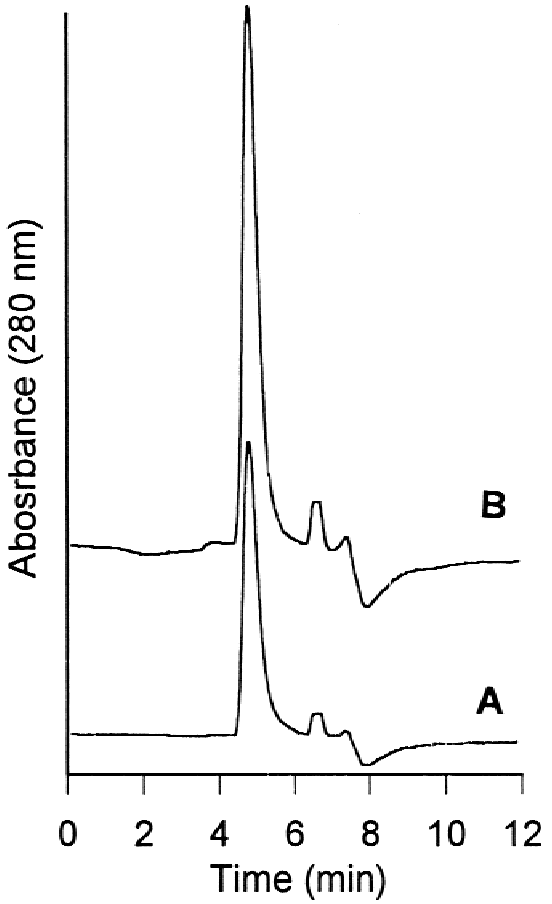

Fig. 5. SEC chromatograms of the standard HRP(A) and HRP extracted from HES-PLGA composite microspheres (B).

preparation process of the composite microspheres did not affect the structural integrity of proteins. The enzymatic activity changes of HRP were also examined to determine the activity loss of the proteins during the microsphere preparation process and *in vitro* drug release. As shown in Table II, HRP was more stable in the composite microspheres than PLGA microspheres. HRP extracted from the composite and PLGA microspheres showed 80.9 and 61.5% specific activities, respectively. The different activity between HRP extracted and released suggests that the loss of activity mainly occurred during the extraction of protein from the microspheres. However, in addition to stabilizing the protein during the preparation process, the composite microspheres protected entrapped HRP during *in vitro* release. As shown in Fig. 6, HRP from the composite microspheres showed much higher enzymatic activity ($P < 0.05$) than HRP from PLGA microspheres after 7 days of incubation in the release medium. The results suggest that the starch-based hydrogel particles in the composite microspheres could stabilize protein drugs from the degradation, aggregation, and loss of activity not only during the microspheres preparation process but also during the release.

CONCLUSIONS

A novel biodegradable microsphere system has been developed for controlled protein delivery. The composite microspheres of a starch-based polymer and PLGA have been successfully formulated with spherical morphology, suitable particle size, high protein incorporation efficiency, and good protein stability. The system possesses sustained protein release and protein stabilization characteristics. This novel delivery system might be useful for the sustained delivery of protein drugs.

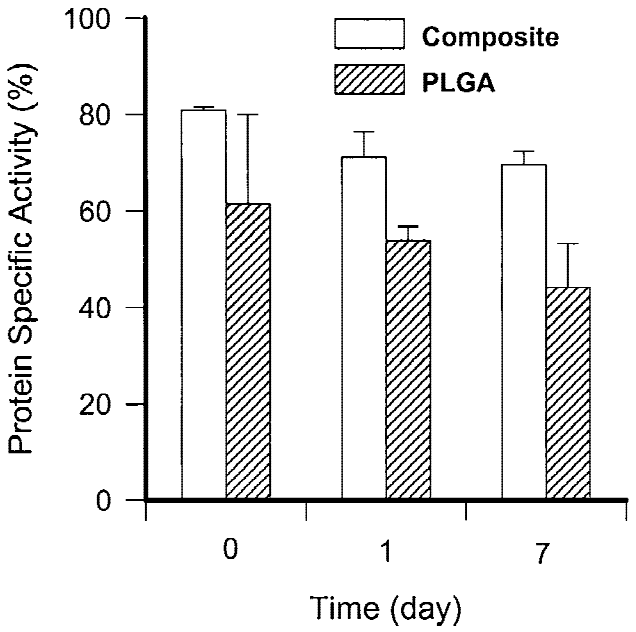

Fig. 6. Remaining HRP activity in the PLGA-acHES composite and PLGA microspheres as a function of incubation time in PBS at 37°C.

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