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Preparation and In Vitro Characteristics of Lactoferrin-loaded Chitosan Microparticles

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ABSTRACT In order to achieve the delivery and controlled release of lactoferrin (LF), a biologically multifunctional protein, chitosan microparticles loaded with LF were prepared. Several types of chitosan microparticles containing LF were prepared by the w/o emulsification-solvent evaporation method, and the particle characteristics and release properties in JP 2nd fluid, pH 6.8, were examined. All kinds of microparticles were obtained at a yield of more than 75% (w/w). LF-loaded microparticles prepared by nonsonication and nonaddition of sulfate, named Ch-LF(N), showed high drug content, small particle size and spherical particle shape. Also, for release properties, Ch-LF(N) exhibited gradual drug release over 7 hr with less remaining in the microparticles. Considering the mucoadhesive properties of chitosan microparticles, Ch-LF(N) are suggested to be useful for gradual supply to topical diseased sites or for effective delivery to intestinal areas with abundant LF receptors.

KEYWORDS Chitosan microparticles, Lactoferrin, Particle characteristics, Drug release

INTRODUCTION

Recently, the number of patients suffering from refractory and chronic illnesses such as viral diseases and immune abnormality including immunosuppression has been increasing. Although many highly potent drugs are being developed against such diseases, their use is often limited due to toxic side effects and problems such as drug resistance. Lactoferrin (LF) is an iron-binding glycoprotein, expressed abundantly in milk, saliva, tears, other exocrine secretions, and neutrophil granules, etc. (Orsi, 2004; Suzuki et al., 2005). As LF exhibits various biological functions including antibacterial, antiviral, antitumor, antiinflammatory and immunomodulatory effects (Weinberg, 1974; Arnold et al., 1977, 1980; Iigo et al., 1999; Cirioni et al., 2000; Samaranayake et al., 2001; Legrand et al., 2004; Jenssen, 2005), and displays no toxic side effects, it has attracted significant attention as a possible novel agent for host defense and the treatment of various refractory diseases including severe infections by bacteria or viruses. These functions are based on various mechanisms, the sequestration of iron essential for the growth of bacteria, binding with the surface of pathogens, binding with the surface of host cells, immunomodulation by interaction with host cells etc. In vitro studies on bacteriostatic and/or bactericidal activities of LF revealed

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the minimal inhibitory concentration (MIC) for effectiveness (Wakabayashi et al., 1998; Kuipers et al., 2002; Lee et al., 2004). In order to achieve such a direct action of LF at diseased sites, the concentration of LF around the diseased sites has to be considered in the design of the formulations. In addition to such direct action of LF, it has been demonstrated that oral administration of LF is very effective against diseases developing at remote sites, for example, oral candidiasis and urinary tract infection (Haversen et al., 2000; Takakura et al., 2003). In formulation studies, as LF is not completely stable in the stomach, enteric coating is useful to efficiently deliver to the intestine where LF receptors are mainly distributed (Shimizu, 2004). Transfer of LF into the blood has been detected using enteric-coated tablets (Shimizu, 2004). Also, oral pretreatment using LF-containing liposomes was more effective than LF alone for antiinflammatory effects, indicating that liposomes might assist in LF function (Ishikado et al., 2005). Furthermore, liposomes could control LF retention at the administration site, which was considered important for local effectiveness (Trif et al., 2001). Thus, the characteristics of LF formulations appear to be critically associated with effectiveness. Polymeric microparticles are useful to control the behavior of drugs because the particle size, surface charge, drug release, etc. importantly related to the drug action, or effectiveness, can be changed (Chen et al., 1987; Tabata & Ikada, 1988; Hu et al., 2002). As far as we know, few studies on polymeric microencapsulation of LF have been reported. In this study, we attempted to prepare microparticulate formulations of LF using chitosan as a carrier because chitosan is a biodegradable and biocompatible polymer (Mi et al., 2002; Yoshino et al., 2003; Hejazi & Amiji, 2003), and the potential of the obtained microparticles was evaluated in vitro. Several types of microparticles were prepared using emulsification and solvent evaporation, and were evaluated based on particle characteristics and in vitro drug release.

MATERIALS AND METHODS

Materials

Daichitosan VL (81% deactylation degree and extra low viscosity grade) was supplied by Dainichiseika Color & Chemicals Mfg. Co., Ltd. (Tokyo, Japan), and used as chitosan (Ch) throughout the experiment. Bovine lactoferrin (LF) was donated by NRL Pharma Inc. (Kawasaki, Japan). Sesame oil (Japanese pharmacopoeia grade) was purchased from Miyazawa Yakuhin Co., Ltd. (Tokyo, Japan). Sorbitan sesquioleate (SO-15) was obtained from Nikko Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

Preparation of Microparticles

Ch (50 mg or 100 mg) and LF (20 mg) were dissolved in 8 mL of 1% (v/v) acetic acid aqueous solution. The resultant solution was added to 100 mL of sesame oil containing SO-15 at 1% (w/v), which was stirred at 1000 rpm with a 4-blade propeller. The mixture was stirred at the same speed for one hour. The resultant emulsion was treated as follows to obtain several types of microparticles (Table 1): 1) Ch-LF microparticles prepared without sonication, named Ch-LF(N), were produced by evaporation of water and acetic acid from the emulsion at 45°C with a rotary evaporator; 2) for Ch-LF microparticles prepared with sonication, abbreviated to Ch-LF(S), the emulsion was sonicated at 28 Hz (100 W) for 10 min, and water and acetic acid were evaporated in the same way as above; 3) Ch-sulfate complex microparticles containing LF (Ch/SO_4-LF(S)) were prepared by sonication, mixing with sodium sulfate aqueous solution and evaporation

TABLE 1 **Formulations and Particle Characteristics of LF-loaded Chitosan Microparticles**

Formulation	Ch (mg)	$NaSO_4$ (mg)	LF (mg)	LF content[a] (%, w/w)	Particle diameter[b] (μm)
Ch-LF(N)	100	–	20	20.0 ± 1.2	4.9 ± 2.4
Ch-LF(S)	100	–	20	21.7 ± 0.7	12.2 ± 4.5
Ch/SO_4(25)-LF(S)	50	25	20	16.9 ± 0.7	81.9 ± 40.8
Ch/SO_4(50)-LF(S)	50	50	20	12.2 ± 0.6	34.4 ± 14.5

[a]The results are expressed as the mean ± S.D. (n=3).
[b]The Green diameter was measured for 200 microparticles.

of the aqueous phase, by referring to the literature (Hejazi & Amiji, 2002; Hori et al., 2005; Alsarra et al., 2005). Namely, after the emulsion was sonicated at 28 Hz (100 W) for 10 min, 1 mL of water containing sodium sulfate (25 mg and 50 mg) was added, and the mixture was stirred at 500 rpm for 10 min, and then evaporation was conducted in the same manner as above to yield Ch/SO_4(25)-LF(S) and Ch/SO_4(50)-LF(S), respectively. All microparticles were separated by centrifugation at 3000 rpm for 10 min, washed with hexane, and dried in air. Each resultant powder was used in the following experiments after agitation in the glass bottle with a spatula.

Stability of UV Absorption Properties of LF

JP 14 1st fluid (aqueous solution containing 0.2% (w/v) NaCl, pH adjusted to 1.2 with HCl) and JP14 2nd fluid (aqueous solution containing 0.05*M* KH_2PO_4 and 0.0236*M* NaOH, pH 6.8) were used for the measurement of UV absorption of LF. The stability of UV absorption of LF was examined as follows. LF was incubated in JP14 1st or 2nd fluid at a concentration of 0.5 mg/mL at 37°C for 24 hr ($n = 1$). At appropriate time points, a sample was taken and measured for UV absorption at 278 nm and UV profiles in order to confirm the stability of the UV absorption properties of LF. The determination of LF was done by the calibration curve method. A Beckman DU-640 spectrophotometer was used for the UV absorption studies throughout the studies.

Particle Size and Shape of Microparticles

The microparticles were coated with approximately 10 nm platinum, and observed by scanning electron microscopy (SEM) using a JEOL JSM-5600LV scanning electron microscope to investigate their shape and size. For each particle on the scanning electron micrograph, parallel lines touching its opposite sides were made in the same direction, and the distance between the parallel lines, called Green diameter, corresponding to Feret diameter shown by The Association of Powder Process Industry and Engineering, Japan, was measured as the particle size. Two hundred particles, chosen at random from the scanning electron micrographs, were measured for the Green diameter, and the mean value and standard deviation were calculated.

In Vitro Release and Drug Content

The in vitro release test was performed using JP14 2nd fluid as a release medium. To 10 mL of release medium, 5 mg of microparticles were added, and the resultant mixture was shaken horizontally at 100 strokes/min at 37°C. At appropriate time points, 3 mL of the sample was taken and centrifuged at 3000 rpm for 10min, and the supernatant was measured spectrophotometrically at 278 nm. The supernatant and residue were returned to the incubation medium, and the test was continued.

After the release test, the microparticles were dissolved in JP14 1st fluid by vigorous shaking, and the solution was measured spectrophotometrically at 278 nm to obtain the amount of LF remaining in the microparticles. The drug content was calculated by the summation of the amount of LF released in the in vitro release test and the amount of LF remaining in the microparticles.

RESULTS AND DISCUSSION

Particle Characteristics

The yield of the microparticles, calculated by the total recovery (w/w) of Ch and LF, was more than 75% (w/w) for all kinds of microparticles. Particle size and shape were calculated based on scanning electron micrographs (Fig. 1). The particle size distribution

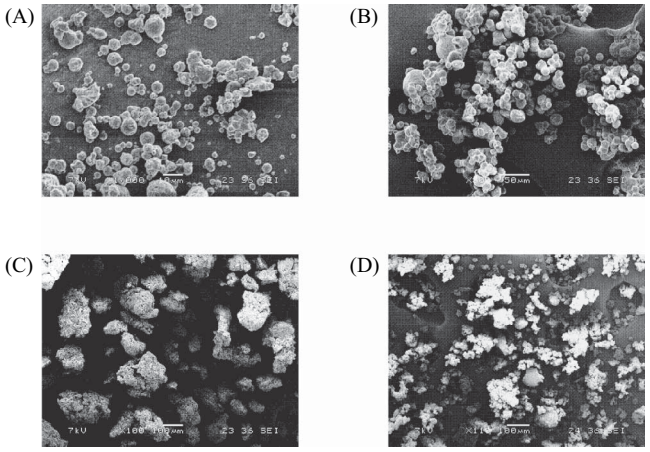

FIGURE 1 **Scanning Electron Micrographs of Microparticles. A, Ch-LF(N); B, Ch-LF(S); C, Ch/SO_4(25)-LF(S); D, Ch/SO_4(50)-LF(S). The Scale is Shown in Each Photo as a White Bar.**

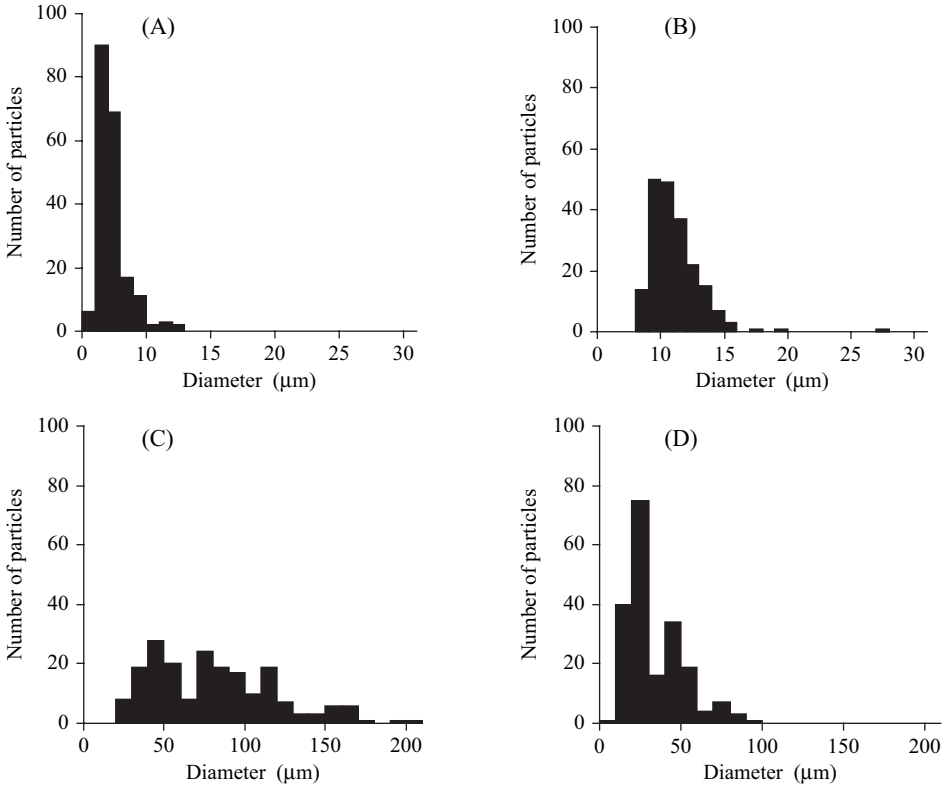

FIGURE 2 **Particle Size Distribution of Microparticles. A, Ch-LF(N); B, Ch-LF(S); C, Ch/SO_4(25)-LF(S); D, Ch/SO_4(50)-LF(S). The Green Diameter was Measured for 200 Microparticles Chosen at Random.**

using 200 particles from each formulation are shown in Fig. 2 and particle characteristics for the obtained formulations are summarized in Table 1.

Ch-LF(N), prepared without sonication or sulfate, possessed the smallest size (mean size 4.9 μm), and most of the particles were less than 10 μm in diameter. Ch-LF(S), obtained with sonication but no sulfate, was larger (mean size 12.2 μm) with a distribution of 8–16 μm. Both formulations displayed a single lobe of size distribution and spherical particle shape. On the other hand, chitosan-sulfate complex microparticles, Ch/SO_4(25)-LF(S) and Ch/SO_4(50)-LF(S) had a mean size of 81.9 and 34.4 μm, respectively, much bigger than Ch-LF(N) and Ch-LF(S). Each size distribution was broad, in particular, the size of Ch/SO_4(25)-LF(S) was distributed between 40–170 μm. Both complex microparticles showed irregular shapes.

The UV absorption method was available for the determination of LF. It was confirmed by the stability test of the UV absorption. As shown in Fig. 3, the UV absorption value (peak wavelength 278 nm) and UV profile of LF were stable in both JP 1st and 2nd fluid for at least 24 hr at 37°C, which allowed us to determine the amount of LF in both media by the UV absorption method.

The drug content was better in Ch-LF than in Ch/SO_4-LF. For Ch-LF, the drug content was greater than the ideal drug content (16.7%, w/w), which might be due to the preparation conditions including separation and washing of the microparticles. By contrast, for Ch/SO_4-LF, the drug content was lower than the ideal drug content. Ch-sulfate complex appeared to be disadvantageous for the inclusion of LF in the microparticles.

In Vitro Release

From the stable characteristics of LF UV absorption in both JP 1st and 2nd fluid as stated above (Fig. 3), the amount given at a 100% release, corresponding to the drug content, could be calculated by summation of the amount of LF released in the release test and the amount of LF remaining in the microparticles after the release test.

The release of LF was investigated using JP14 2nd fluid, which could reflect the environment of oral

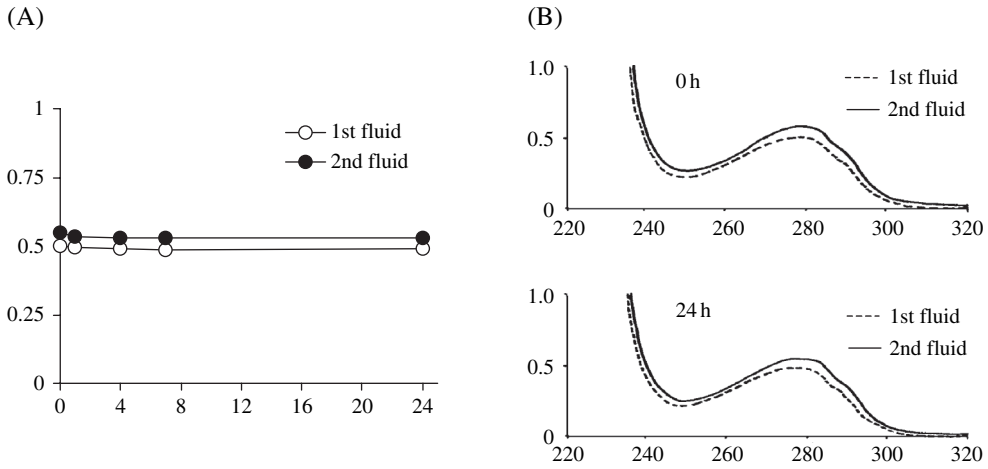

FIGURE 3 **Change in UV Absorption of LF at 278 nm in JP 1st and 2nd Fluids (A) and UV Absorption Profiles in JP 1st and 2nd Fluids after Incubation for 0 and 24 hr at 37°C (B).**

cavity or intestines. The release profiles of LF from the microparticles are shown in Fig. 4. Approximately 20% was released as an initial burst for all types of microparticles. This was considered to be due to the rapid release of LF around the surface of the microparticles. As the microparticles were washed only with hexane, LF around the surface was thought to remain without being washed out. LF associated with the initial burst might be removed by washing with aqueous solution. Also, the initial burst was considered to lead to a high concentration at the administration site, which would have to be made clear in vivo. Ch-LF(N) released LF gradually for 7 hr, and more than 70% was released during the incubation for 24 hr. The other types of microparticles exhibited a slower release. The difference in particle size and/or sonication in the preparation appeared to be associated with the differences in the release profiles between Ch-LF(N) and Ch-LF(S). Ch-sulfate complex microparticles also showed suppressed release as compared with Ch-LF(N). Complex microparticles and Ch-LF(S) displayed a gradual release over 24 hr, while Ch-LF(N) exhibited very slow release from 7–24 hr. Ch-LF(N) showed the gradual release of a greater amount of LF over the first 7 hr, and the remaining ratio at 24 hr was the least. In order to predict the direct action of LF at the diseased site, LF generally needs to be supplied around the site at a concentration over a minimal inhibitory concentration (MIC) (Kuipers et al., 2002). Fairly fast gradual release from Ch-LF(N) may be adequate for achieving such direct action of LF against topical diseases like oral candidal infection; the localization of Ch-LF(N) at the diseased sites may enhance and prolong the topical action. In addition to LF concentration around the diseased sites, the systemic action of LF is very important for efficacy. The in vivo analysis for the dose and administration schedule will be needed to evaluate Ch-LF(N) more exactly. LF displays various actions in the intestine, such as regulating the balance among intestinal bacteria and immunomodulatory function via intestinal LF receptors (Shimizu, 2004; Legrand et al., 2004; Suzuki et al., 2005). LF undergoes degradation in the stomach (Shimizu, 2004; Suzuki et al., 2005; Ishikado et al., 2005), and its solution may be inadequate for efficient delivery to target sites in the intestine. Ch-LF(N) are

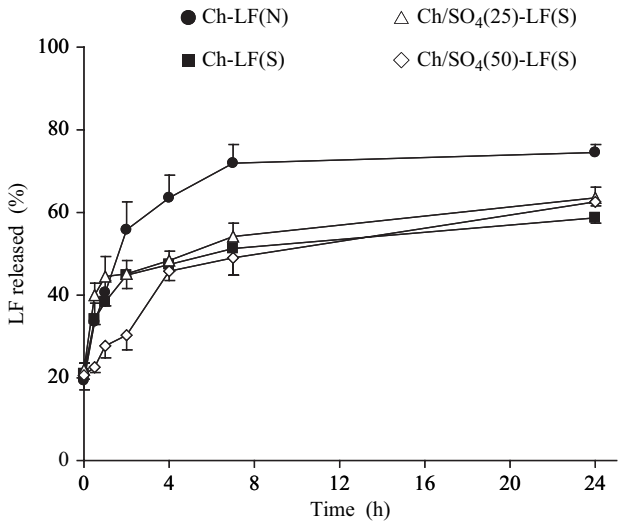

FIGURE 4 **Release of LF from Microparticles in JP 2nd Fluid at 37°C. The Results Are Expressed as the Mean ± SD (*n*=3).**

considered to be efficiently delivered into the intestine by protection from exposure in the stomach with enteric coating, etc. Ch-LF(N) are expected to be retained longer in the intestine because of mucoadhesive properties (Shimoda et al., 2001; Kaur & Smitha, 2002; Nafee et al., 2004), resulting in more efficient functions of LF.

Thus, from the viewpoint of particle characteristics and release profiles, Ch-LF(N) have excellent particle characteristics and release profiles, and are suggested to be possibly useful for topical and/or intestinal delivery of LF. As for their practical use, bioadhesive patches including Ch-LF(N) may be useful for topical application against the diseases like oral candidiasis, and also, microparticles treated with an enteric coating or enteric-coated capsules may be suitable for the efficient delivery of LF for intestinal localization and systemic immunity.

CONCLUSION

Chitosan microparticles loaded with LF were prepared in several different conditions using the emulsification and solvent evaporation method. Microparticles produced with nonsonication and nonaddition of sulfate, Ch-LF(N), showed good particle characteristics as compared with other formulations obtained in this study. Namely, Ch-LF(N) had an average particle diameters of approximately 5 μm, and a spherical shape, and showed a fairly high drug content; further, Ch-LF(N) showed superior gradual release. Ch-LF(N) are suggested to be possibly useful for topical and/or intestinal delivery of LF, although further optimization or refinement of particle characteristics and release profiles may be needed for practical use.

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