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리파아제가 함입된 락타이드-글리콜라이드 공중합체 나노입자의 제조 및 특성

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Formulation and Characterization of Lipase Loaded Poly(D,L- lactide-*co*-glycolide) Nanoparticles

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초록 : 제조과정에서 단백질 약물의 생물학적 활성의 보존은 약물의 성공적인 전달에 있어 여전히 중요한 과제이다. 이중에멀션 유기용매 증발법을 사용하여 나노입자를 제조하였고, 입자의 형태, 크기, 함입률 그리고 방출속도와 방출되는 효소의 활성을 살펴보았다. 입자의 크기는 고분자인 락타이드 글리콜라이드 공중합체의 농도가 증가할수록 커졌으며, 유화제의 농도에는 큰 차이가 없었으나, 4% PVA의 사용에서 가장 좁은 입자분포를 얻을 수 있었다. 최적의 조건에서 72.6%의 단백질 함입률과 198.3±13.8 nm 크기의 나노입자를 얻었다. 입자로부터 효소의 방출은 첫 방출시기에 매우 빠르게 일어났으며 12일 내에 83%가 방출되었다. 이에 따른 방출되는 효소의 활성은 6일째까지 증가되었다.

Abstract : The preservation of biological activity of protein drugs in formulation is still a major challenge for successful drug delivery. Lipase was encapsulated in poly(D,L-lactide-*co*-glycolide) PLGA nanoparticles using a w/o/w solvent evaporation technique. The lipase-containing PLGA/poly(vinyl alcohol) (PVA) nanoparticles were characterized with regard to morphology, size, size distribution, lipase-loading efficiency, *in vitro* lipase release, and stability of lipase activity. The size of nanoparticles increased as polymer concentration was increased. The size of particles was not significantly affected by the PVA concentration; on the other hand, the particle size distribution was the narrowest when 4% of PVA was used. In optimum conditions, we possessed nanoparticles that characterized 72.6% of encapsulation efficiency, 198.3±13.8 nm size diameter. During the initial burst phase, the *in vitro* release rate was very fast, reaching 83% within 12 days. Until days 6, enzyme activity increased as the amount of lipase released was increased.

Keywords : PLGA, nanoparticles, lipase, activity, protein.

Introduction

Patients with cystic fibros(CF) have exocrine pancreatic insufficiency(PI)[1] and require the regular intake of pancreatic enzyme replacement therapy to improve the digestion of dietary fat, protein, and other nutrients. But, the protein drugs are often fragile or unstable in comparison with conventional synthetic drugs. Therefore, lipase drugs are easily denatured or easily lose their biological activity.

Poly(D,L-lactide-*co*-glycolide) (PLGA) was particularly suitable for use in drug delivery application. After 30 years of commercial use of PLGA in medical suturing,[2] its biocompatibility, bioabsorbability, changeable biodegradability, and good mechanical properties have been well recognized.[3,4] PLGA chains are cleaved by hydrolysis into natural metabolites(lactic and glycolic acids), which are eliminated from the body by the citric acid cycle.[5-9] Because of these beneficial properties, PLGA polymers such as biodegradable structures, implantable screws, pin, drug delivery devices and tissue engineering scaffolds are already approved by the US Food and Drug Administration(FDA).[10-14] PLGA nano- and microparticles are also currently being extensively investigated for sustained delivery of therapeutic agents, including DNA, proteins, and low molecular weight agents.[15-19]

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Although encapsulation shields the protein from proteolytic attack and can prolong the period of release, instability of the entrapped protein can affect the integrity of protein due to an unfavorable microenvironment within the nanoparticles. Thus, stabilization of lipase is a major issue impeding successful therapies for patients.

In this study, we used the lipase enzyme to examine the release rate and stability of the activity in the PLGA/poly(vinyl alcohol) (PVA) nanoparticles.

Experimental

Materials. Lipase (EC 3.1.1.3) from porcine pancreas and *p*-nitrophenyl butyrate was purchased from Sigma(St. Louis, USA). PLGA(50:50 ; MW 40000 g/mole), PVA(MW 30000~70000 g/mole), chloroform, and other reagents were also purchased from Sigma (St. Louis, USA).

Preparation of Nanoparticles. Lipase-loaded PLGA nanoparticles were prepared by a w/o/w emulsion solvent evaporation technique.[20] In brief, 200 μL of protein solution containing 40 mg of lipase in phosphate buffered saline (pH 7.4, 10 mM) was emulsified with 2 mL chloroform solution containing from 20 to 100 mg PLGA to produce a primary emulsion. The emulsification was carried out by probe sonicator(Sonicator® XL, Misonix, Farmingdale, NY, USA) for 30 sec at 50 W in an ice-bath. The above emulsion was further emulsified in an aqueous PVA solution(12 mL, concentration of PVA was varied from 1% to 5% w/v depending upon the protocol) to form a multiple water-in-oil-in-water(w/o/w) emulsion. The emulsification was carried out using a microtip probe sonicator for 5 min at 50 W in an ice-bath. The emulsion was stirred overnight on a magnetic stir plate to allow chloroform to evaporate. Nanoparticles were recovered by ultra-centrifugation at 35000 rpm for 20 min at 4 ℃(Beckman Optima TM LE-80 K, Beckman Instruments, Inc., Palo Alto, CA, USA) and were washed two times with distilled water to remove PVA and un-entrapped lipase before freeze-drying.

Particle Size and Zetapotential Measurement. Particle size and polydispersity were determined by the light scattering method(DLS-8000, Otsuka Electronics, Japan). For measurement, 0.2 mL of the nanoparticle suspension was dispersed in 3 mL of distilled water. The analyses were performed at a scattering angle of 90° and temperature of 25 ℃. Each sample was measured in triplicate and an average particle size was expressed as the mean diameter. The zeta-potential of nanoparticles was measured by using an electrophoretic light-scattering spectrophotometer(ELS-8000, Otsuka Electronics, Japan).

Determination of Lipase-Loading Efficiency. The amount of lipase encapsulated in nanoparticles was determined by analyzing the protein content in the washings from the nanoparticle formulation step. Protein content was measured using a BCA protein assay kit(Pierce, Rockford, IL, USA). The percentage of encapsulation efficiency was calculated as follows:

$$\text{Encapsulation efficiency}(\%) = \frac{\text{Actual protein loading}}{\text{Theoretical protein loading}} \times 100\%$$

Morphology Observation. The appearance and shape of nanoparticle surfaces were analyzed by SEM. Samples were prepared by finely spreading concentrated nanoparticle dispersions over slabs and drying them under vacuum. The samples were then coated with a fine gold layer and observed by scanning electron microscope(SEM) using a JSM-6400 (JEOL, Tokyo, Japan).

***In vitro* Protein Release from Nanoparticles.** 10 mg of freeze-dried lipase-loaded nanoparticles were resuspended in 50 mM of potassium phosphate buffer(pH 8.0). For each of the resuspended nanoparticle solutions, 1 mL was aliquoted in a new tube. The temperature of incubation medium was maintained at 37 ℃. Agitation was provided using a shaking water bath. Samples were centrifuged at appropriate intervals(20 min, 35000 rpm, 4 ℃). The supernatant was assayed for protein release and enzymatic activity. The amount of lipase in the release medium was determined using a BCA protein assay kit.

Activity of Released Lipase. Lipase activity was measured using *p*-nitrophenyl butyrate(pNPB) as substrate. In brief, after a predetermined interval, samples were centrifuged and supernatant was collected. *p*NPB was first solubilized with dimethyl sulfoxide in one-tenth of the final volume, then diluted to 0.5 mM with 50 mM potassium phosphate buffer(pH 7.0) containing 0.5% Triton X-100. The supernatant was then reacted with pNPB and mixing solution was measured by determining the absorbance at 410 nm using a UV spectrophotometer(Cary-3E, Varian, Australia).

Results and Discussion

In order to study the influence of PLGA concentration on the size of nanaparticles, some batches were prepared by using PLGA at different concentrations. All samples used 4% of PVA in the aqueous phase. PLGA was varied at levels between 20 and 100 mg(1-5%), and the influence of the initial mass of polymer on the particle size was studied. The effect of the PLGA concentration on particle size is

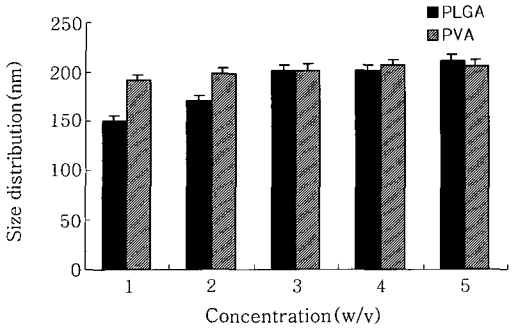

Figure 1. Effect of PLGA and PVA concentration on the size of particles. Data are shown as mean±S.D. (n=3).

shown in Figure 1. When 20 mg of PLGA were used, the particle diameter was 150.6 nm. When 40, 60, 80, and 100 mg of PLGA were used, the particle diameters were also increased to 170.1, 199.7, 200.7, and 211.4 nm, respectively. The increase in particle size with an increasing polymer concentration was observed by other authors for PLA and PLGA polymers.[21,22] The polymer concentration in the internal phase of the emulsion was an important factor, since the size of nanoparticles increased as polymer concentration was increased. This was probably caused by the increasing viscosity of the dispersed phase, resulting in a poorer dispensability of the PLGA solution into aqueous phase.

PVA concentration in the external water phase is known to be a factor that influences the size of microspheres.[23] The effect of the PVA concentration in the aqueous phase on the yield of nanoparticles is shown in Figure 1. Even though it was not the optimum condition, a 3% PLGA solution was purposely used to detect the difference in the yield in this experiment. Data indicate the use of PVA at concentrations within a range of about 1–5%. When between 1% and 5% of PVA concentrations were used, the particle size was not significantly affected by the PVA concentration. On the other hand, when 4% of PVA in the aqueous phase was used, a narrower size distribution was observed irrespective of the particle size(data not shown). In these results, we observed that 4% PVA concentration was the optimum conditions for the preparation of lipase-loaded nanoparticles.

Encapsulation efficiency was dependent on various formulation parameters. In order to optimize the lipase encapsulation in this method, concentrations of PLGA and PVA were examined. Figure 2 shows that the encapsulation efficiency of lipase increased with increasing polymer concentration, and also showed that the encapsulation efficiency of lipase decreased with increasing PVA concentration. Indeed, encapsulation efficiency was increased from 62.3% to 72.6% when the PLGA concentration was increased from 1% to 3%. On the other hand, the encapsulation efficiency of lipase was not affected by a concentration of PLGA in the range of 3–5%. Additionally, the encapsulation efficiency of lipase was not significantly affected by a concentration of PVA in the range of 1–4%. On the other hand, encapsulation efficiency was decreased from 72.6% to 62.6% when the PVA concentration was increased from 4% to 5%.

Figure 3 shows the nanoparticle morphology as observed by SEM. The PLGA nanoparticles were obtained using 3% PLGA and 4% PVA solution. The average diameter of the particles was around 198 nm, and we observed that the nanoparticles had a spherical shape. In order to experiment with release activities, nanoparticles were prepared using 3% PLGA and 4% PVA, and characterization of the particles is shown in Table 1.

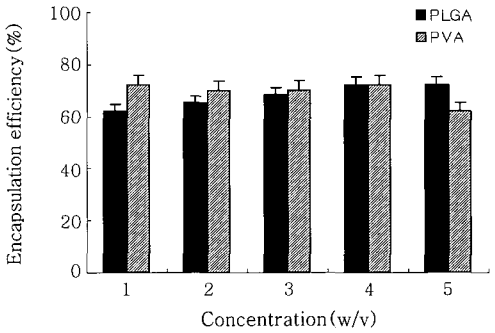

Figure 2. Effects of PLGA and PVA concentration on encapsulation efficiency of lipase. Data are shown as mean ± S.D. (n=3). In PLGA concentration experiment, 4% of PVA solutions were used. In PVA concentration experiment, 3% of PLGA were used.

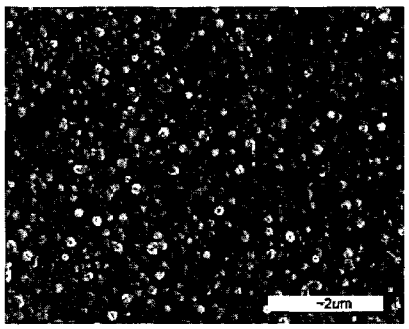

Figure 3. Lipase-loaded PLGA nanoparticles observed by SEM.

Table 1. Characterizations of Lipase-Loaded PLGA/PVA Nanoparticles

Nanoparticles size±S.D.(nm)	198.3±13.8
Polydispersity±S.D.	0.16±0.04
Zetapotential±S.D. (mV)	−23.4±6.2
EE(%)±S.D.	72.6±1.8

The amount of lipase released was studied as a function of time. A double-phasic release pattern was observed. During the initial burst phase(0-3 days), the release rate was very fast, reaching 56%. During the second phase(3-12 days), the releasing rate was a little slower. Within 12 days, 83% of the lipase had been released. The protein released during the initial release phase was mainly due to desorption and diffusion of protein from the surface and small pores on the surface of particles. The intermediate phase is typical of polymers because a relatively long degradation time is required.[24] The results shown in Figure 5 indicate that the percentages of active enzyme recovered during incubation are consistent with the percentage of total protein released over time(Figure 4). Nanoparticles released an important amount of the active enzyme for up to 6 days. This could be due to the fact that the free enzyme is not stable in the release medium for periods longer than 3 days, as shown in Figure 5. The comparison of the activity data with the results of protein released (83% after 12 days of incubation; data in Figure 4) leads to the conclusion that 50% of the lipase released was still active after a 12 days incubation period. It can be concluded that nanoparticles prepared by PLGA/PVA might be potential protein drug carriers.

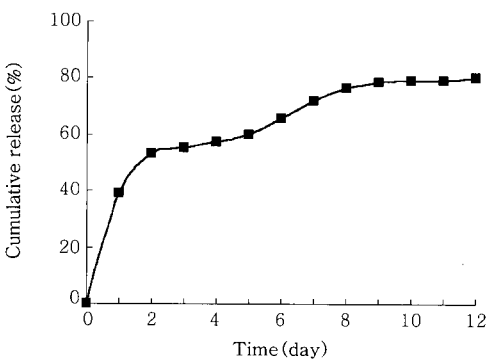

Figure 4. *In vitro* release of lipase from PLGA/PVA nanoparticles during incubation at 37 ℃(drug loading 72.6%, size 198.3±13.8; each point is the mean value of two sets of samples).

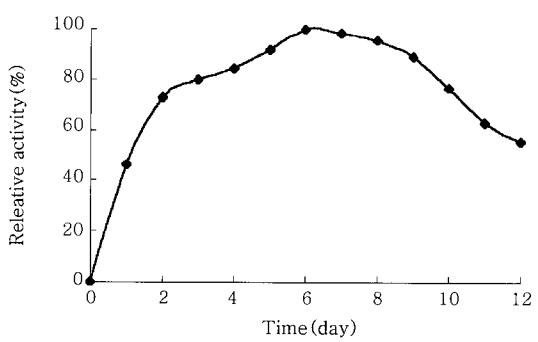

Figure 5. Relative activity of released lipase from PLGA/PVA nanoparticles.

Conclusions

The lipase-loaded nanoparticles were encapsuled using the w/o/w double emulsion solvent evaporation method. The PLGA(3%)/PVA(4%) composite nanoparticles possessed a spherical shape and narrower size distribution. Lipase can be efficiently encapsulated within PLGA nanoparticles and be continuously released while still preserving its enzymatic activity. These data should be given particular consideration in the design of carrier systems for the treatment of patients with CF.

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