Instability of Bovine Insulin in Poly(lactide-co-glycolide) (PLGA) Microspheres

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Biodegradable poly(lactide-co-glycolide) (PLGA; 50/50) microspheres containing bovine insulin as a model protein were prepared by an oil-in-oil (o/o) emulsion solvent evaporation process. When aluminum tristearate (0.15% (w/v)) was employed as a dispersing agent, the loading efficiency of insulin was almost 100% and the yield was over 80%. The average diameter of the PLGA microspheres always ranged between 100 and 200 μm. Morphology study using a scanning electron micrograph showed smooth, spherical, fairly monodispersed PLGA microspheres containing insulin. In relation to release profile, the very low release rate of insulin was demonstrated (only 1% of insulin released after 7 d release test in pH 7.4 Tris buffer) for the PLGA microspheres. Nevertheless, the degradation of bovine insulin in PLGA microspheres was confirmed by high performance liquid chromatography. This degradation seemed to be caused by an acidic condition caused by poly(lactide-co-glycolide) polymer.

Key words bovine insulin; poly(lactide-co-glicolide); protein delivery; degradation

Polypeptides and proteins require parenteral delivery. Most exhibit low oral bioavailability due to denaturation or degradation in the gastrointestinal tract. A number of polymeric systems were developed to sustain the release of peptides or proteins from delivery systems, among which biodegradable poly(lactide-co-glycolide) (PLGA) microspheres[1-3] seemed advantageous. Nevertheless, PLGA has carboxyl group and causes an acidic condition. In addition, the transfer of protons from bulk solution to the surface of microspheres was also reported.[4] Degradation of those active compounds which are not stable in an acidic condition, or the electric interaction of the loaded drug with the PLGA polymer may become a serious problem in application of this biodegradable delivery system.

In the present study, therefore, we selected bovine insulin which is not stable[5] under an acidic condition as a model drug. The microencapsulation of biodegradable PLGA microspheres containing insulin was performed previously using an o/o solvent evaporation method,[6,7] and the obtained microspheres were characterized. The stability of bovine insulin in PLGA microspheres was examined using high performance liquid chromatography (HPLC), and its possibility as a long-acting protein delivery system was evaluated.

Experimental

Chemicals PLGA50/50 (Mw=53000) were purchased from Medisorb Technologies (Cincinnati, OH, U.S.A.). Bovine insulin (powder sizes were under 20 μm) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other reagents were all of special grades.

Preparation of PLGA Microspheres The microspheres containing bovine insulin were prepared as follows: 1.0 g of PLGA 50/50 was weighed and dissolved in 10 ml of acetonitrile. Bovine insulin powder was dispersed in the polymer solution. This dispersion was emulsified using three propellers (250 rpm) in a continuous phase consisting of 200 ml of mineral oil containing 0.15% (w/v) of aluminum tristearate (Nakalai Tesque Inc., Kyoto, Japan) at 10 °C. The temperature of this emulsification system was gradually raised to 30 °C and it was stirried (250 rpm) for another 7.5 h. The hardened PLGA microspheres were filtered, washed three times with *n*-hexane to remove mineral oil and dried for at least 24 h under reduced pressure at room temperature. The microsphere yield was determined as a weight percentage of the recovered microspheres after drying divided by the initial amount of polymer and drug employed. Morphology study was performed using a scanning electron microscope (SEM) (Akashi WS 250, Tokyo, Japan).

Insulin Loading The actual insulin loading percentage in PLGA microspheres was determined in the following way: about 10 mg of microspheres were precisely weighed and dissolved in 2 ml of acetonitrile for another dissolution in PLGA 50/50 polymer in glass of vial. The polymer solution containing suspended insulin was centrifuged at 3000 rpm for 5 min. The acetonitrile was decanted and replaced with fresh acetonitrile, this procedure was repeated three times. The remaining pellet was dissolved in 5 ml of pH 7.4 Tris buffer containing 0.1% (w/w) TFA (trifluoro acetic acid). The concentration of the solution was determined using HPLC. Twenty microlitres was injected onto a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10AV), an integrator (Shimadzu C-R6A) and reversed phase column (Asahipak ODP-50 6D, 6.0 × 150 mm, Asahi Chemical Industry Co, Ltd., Tokyo, Japan). The mobile phase was a mixture of acetonitrile: 0.3% (v/v) ethanol amine solution (adjusted to pH 2.0 by adding a phosphoric acid) = 28 : 72. The flow rate was 2.0 ml/min; the wavelength was set at 220 nm and the column was operated at 40 °C. Loading was calculated from the weight of the initial microspheres and the amount of drug incorporated.

***In Vitro* Insulin Release Test** The *in vitro* release profile of insulin from PLGA microspheres was determined as follows: Microspheres corresponding to 100 mg of insulin were suspended in 5 ml of Tris buffer (pH 7.4) containing 0.02% (w/v) Tween 80, and shaken horizontally at 75 rpm at 37 °C. At predetermined intervals, 200 μl of the suspension was taken as a sample, centrifuged (3000 rpm, 5 min) and the concentration of the supernatant was analysed by HPLC as described above.

Stability of Insulin in PLGA Microspheres The stability of bovine insulin in PLGA microspheres was determined as follows: after release test (0, 1, 4 and 7 d), bovine insulin remaining in PLGA matrix was extracted using the method as described in the "Insulin Loading" in the Experimental section.

Results and Discussion

PLGA microspheres containing different loads of insulin were prepared as shown in Table 1. The loading efficiency was almost 100% in every batch, and the yields were always over 80%. Morphology study using SEM showed a comparatively smooth surface of the PLGA microspheres containing bovine insulin, and the mean diameters were about 200 μm (Fig. 1).

The release rate of bovine insulin from PLGA microspheres was very small in all batches. For example, in the case of 20% (w/w) insulin loaded PLGA microspheres, only 1% of total bovine insulin has been released into pH 7.4 Tris buffer at 37 °C after 7 d. Figure 2 shows the HPLC

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Table 1. Loading Efficiency of PLGA Microspheres with Different Insulin Loading

Insulin loading (%)	Loading efficiency (%)
20	98.85 (11.34)
10	97.23 (5.44)
5	100.41 (10.58)

Each value represents the mean (S.D.) of three experiments. 0.15% (w/v) of aluminum tristearate was used as a dispersing agent in every batch in the manufacturing process.

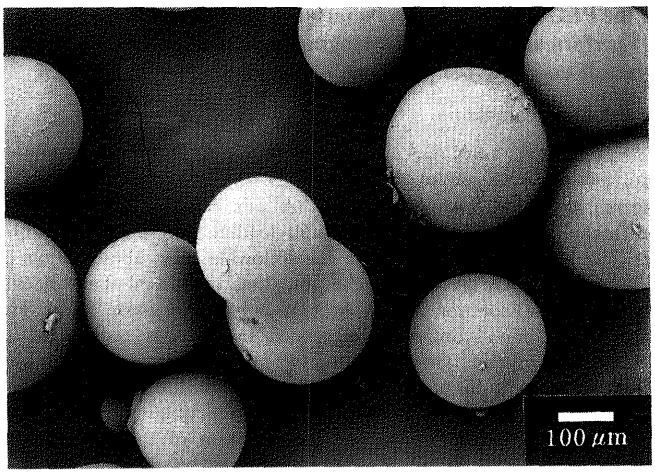

Fig. 1. Scanning Electron Micrograph of PLGA Microspheres Containing 20% (w/w) of Bovine Insulin

chromatogram for extracted native bovine insulin or degradation product of insulin in PLGA microspheres. The data suggests that over 10% of bovine insulin in PLGA matrix was converted to a degradation product after a 7 d release test in pH 7.4 Tris buffer. A previous article reported[5] that bovine insulin in solution was unstable under an acidic condition. In fact, our brief stability study using native bovine insulin solution also suggested that the degradation rate of the insulin was accelerated at 2.2 or pH 3.0 (data not shown). The retention time for HPLC peak corresponding to the native insulin or the degradation product of the insulin in our brief stability test well coincided with the retention time

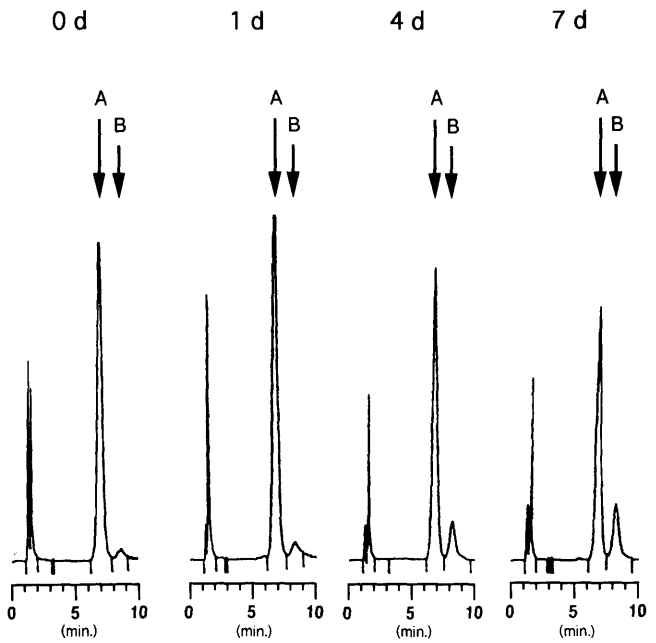

Fig. 2. Chromatograms of Bovine Insulin and the Degradation Product Extracted from PLGA Microspheres

A and B represent the peak corresponding to the bovine insulin and the degradation product, respectively.

of the insulin or the degradation product extracted from PLGA microspheres, respectively. This phenomenon suggests that PLGA microspheres formed an acidic condition inside the microspheres owing to the acidic characteristics of PLGA itself and thereby accelerated the degradation of bovine insulin, even though we did not determine the precise assignment of the degradation product of bovine insulin.

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