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Thymosin-loaded enteric microspheres for oral administration: Preparation and in vitro release studies

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Abstract

Thymosin, a water-soluble polypeptide compound, was encapsulated within enteric microspheres of acrylic acid resin II by modified oil in oil (o/o) emulsion solvent evaporation method. The mixture emulsifier composed of lecithin and Span 80 was critical to the formation of sphere-shaped thymosin microparticles. Optimizing process parameters, such as the volume ratio of organic solvent to water, initial drug feed and polymer concentration, resulted in high drug encapsulation efficiency of 89.7% (6% polymer concentration and 0.5% initial drug feed). In vitro release studies suggested that thymosin release from microspheres exhibited pH dependent profiles. For formulation with 6% polymer concentration and 0.5% initial drug feed, 68.7% thymosin was released within 4 h in pH 6.8 PBS buffer, while only 6.5% was observed in acid medium.

Keywords: Thymosin; Acrylic acid resin; Microspheres; Emulsion solvent evaporation method

1. Introduction

Microparticles used as drug carriers for controlled delivery of bioactive compounds have received immense attention in recent years (Okada, 1997; Couvreur et al., 1997). There are several techniques to the preparation of microparticles, including the solvent evaporation, phase separation and spraying-drying method. As for water-soluble compounds, conventional oil in water (o/w) solvent evaporation method will result in low loading efficiency, due to rapid partitioning of the drug from the organic phase into the aqueous phase (O'donnell and McGinity, 1997). Several methods such as oil in oil (o/o), water in oil in water (w/o/w) and water in oil in oil in oil (w/o/o/o) solvent evaporation method have been reported to circumvent this problem (Iwata, 1992).

The oil in oil (o/o) solvent evaporation method has been used extensively to prepare microparticles, which were composed of polylactide (PLA), poly (lactic-co-glycolic acid) (PLGA) and synthetic biodegradable polymers (Viswanathan et al., 1999; Yeh et al., 2001; Chaw et al., 2003). Other polymers including ethyl cel-

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lulose (Lin and Wu, 1999), cellulose acetate phthalate and anionic acrylic resin (Amorim and Ferreira, 2001) were also employed in these studies. In o/o emulsion system, the drug was first dispersed in the polymer organic solution to form an inner oil phase. Then this obtained oil phase is emulsified into an outer oil phase, which is immiscible with inner oil phase, to produce oil in oil emulsion. Then the organic solvent of the inner oil phase of the o/o emulsion is evaporated. After solidification, microparticles are harvested by centrifugation or filtration. This anhydrous process can often enhance the entrapment efficiency of the hydrophilic drug significantly, since the diffusion of the drug from the inner oil phase into the outer oil phase is depressed due to the poor solubility of hydrophilic drug in outer oil phase.

Thymosin, consisting of biologically active polypeptide components, has been approved to be an immune-potentiating agent. At present, the dosage forms of Thymosin product used in clinic refer to injection, aseptic lyophiliazed injection and capsule or tablet for oral administration. With the aim to improve the bioavailability of thymosin through oral administration, we developed thymosin-loaded microspheres for enteric administration using acrylic acid resin II as matrix in this study. As a great deal of research papers reported, the microspheres with good dispersity can enhance the absorption of polypeptide via gastrointestinal tract (Labhasetwar et al., 1997; Lavelle et al., 1995). On the other hand, acrylic acid resin II is gastro-resistant synthetic polymer, which is soluble in buffer solution with pH > 6. Accordingly, entrapment of thymosin into microparticles of acrylic acid resin II can protect thymosin from degrading and inactivating in the stomach due to the extremely low pH condition. At the small intestine, however, thymosin can be released from the microparticles as a result of the dissociation of acrylic acid resin II in the relatively alkali condition. Enteric drug delivery can be thus achieved.

Considering the high water solubility of Thymosin, the acrylic acid resin II microparticles were prepared using o/o solvent evaporation method. The process variables including emulsifier type and concentration, the initial amount of drug, the volume ratio of the inner and outer oil phase and the polymer concentration were evaluated. In vitro drug release tests were performed in buffers with pH at 1.0, 5.8 and 6.8, to investigate the pH dependent release profiles.

2. Materials and methods

2.1. Materials

Acrylic acid resin II (Ac II) was purchased from Zhanwang Chemical and Pharmaceutical Limited Corporation (Huzhou, China). Thymosin was kindly supplied by Xilong Biochemical Technical Limited Corporation (Jilin, China), and protein content in thymosin was about 31.3% (w/w) as determined by Lowry method in our laboratory. Lecithin was obtained from Chemical Factory of Eastern China Normal University (Shanghai, China). Span 80 and Span 85 were purchased from Shanghai Chemical Corporation (Shanghai, China). All the other agents were commercially available and were used as received.

2.2. Preparation of thymosin-loaded microspheres

Thymosin-loaded microspheres based on Ac II were prepared by modified oil in oil (o_1/o_2) emulsion solvent evaporation technique. In brief, the inner oil phase (o_1) was obtained by mixing ethanol solution containing dissolved Ac II and aqueous thymosin solution with mechanical stirring. This obtained organic phase (o_1) was emulsified into outer oil phase (o_2) of liquid paraffin containing appropriate amount of emulsifier by mechanical agitation at 500 rpm. This oil in oil (o_1/o_2) emulsion was stirred magnetically at 25 °C for 14 h to evaporate the organic solvent of inner oil phase. The microspheres were collected by centrifugation at 3000 rpm for 5 min, washed three times with petroleum ether and then dried under vacuum.

2.3. Microspheres characterization

2.3.1. Determination of encapsulation efficiency

The amount of thymosin in the microparticles was determined according to the literature (Hora et al., 1990). Accurately weighted 20 mg dried microspheres were dissolved in 5 ml PBS (pH 7.4) at 37 °C for 2 h. After filtration through a 0.22 μm filter, the thymosin concentration in the filtrate was quantified by Lowry–Peterson procedure. The entrapment efficiency was expressed as the ratio of the actual thymosin to the theoretical thymosin content.

2.3.2. Morphology of microspheres

Microparticle shape was observed using optical microscope (Olympus CKX41, Japan). Surface morphology and internal structure of microspheres were characterized using scanning electron microscopy (SEM) (S-260, Cambridge, England). An appropriate sample of microspheres was mounted on metal stubs, using double-sided adhesive tape. The morphologies were observed after a gold–palladium layer was sputtered by E-1020 ion sputter for 120 s.

2.3.3. Size distribution of microspheres

The microspheres were dispersed in water. Average particle size was determined by laser light scattering method (Coulter LS-230, Miami, USA).

2.4. In vitro release studies

Thymosin-loaded microspheres were placed in 5 ml release medium with pH 1.0, 5.8 or 6.8. The test was performed in a 37 °C incubator-shaker set at 60 rpm. At appropriate intervals, 1 ml of release medium was removed from the supernatant after the samples were centrifuged at 3000 rpm, for 5 min. Thymosin concentration was measured by Lowry–Peterson method.

3. Results and discussion

3.1. Effect of the emulsifier

Since the formation of the microparticles is dramatically influenced by the stability of the primary oil in oil emulsion, it is important to select the optimal emulsifier to prepare well-defined microparticles with high drug entrapment and high microparticles yield. Accordingly, the effect of emulsifier such as Span 80, Span 85 and lecithin on the microsphere formation was evaluated. When the liquid paraffin containing less than 5% (w/v) Span 80 or Span 85 was adopted as outer oil phase, the formed o_1/o_2 emulsion was unstable, which resulted in the particle aggregation during the solvent evaporation stage, and hence no microparticles with good dispersity was obtained. However, when the concentration of Span 80 or Span 85 was enhanced to above 5%, Ac II was precipitated from the inner oil phase immediately after adding the inner organic phase into the outer oil phase of liquid paraffin containing emulsifier. This may be due to the fact that the solubility of Ac II in ethanol was decreased dramatically when the emulsifier concentration was so high, since both Span 80 and Span 85 is highly soluble in ethanol.

Table 1
The effect of mixture emulsifier on the microparticle preparation[a]

Mixture emulsifier concentration		Product characteristics
Lecithin (%, w/v)	Span 80 (%, w/v)	
0.25	1.00	Irregular particles with few microspheres
0.25	0.50	Spherical particles with few aggregates
0.25	0.25	Spherical particles with few aggregates
0.10	0.35	Irregular particles
0.15	0.35	Aggregates with some spherical particles
0.20	0.35	Aggregates with some spherical particles
0.25	0.35	Spherical particles with few aggregates

[a] The amount of thymosin was kept as 6.26 mg; the polymer concentration was 5%; the volume ratio of polymer solution to liquid paraffin was kept as 1:10; the volume ratio of water to organic phase 1:20.

Lecithin, due to its excellent emulsification capacity, is also a frequently used emulsifier by many researchers in the o/o solvent evaporation technique (Yeh et al., 2001). The effect of lecithin was also investigated in this study. It was found that sphere-shaped blank microparticles were prepared successfully when lecithin concentration was higher than 0.25% (w/v) (Fig. 1a). However, when the thymosin was introduced into the inner oil phase, irregular particles with few sphere-shaped microparticles were obtained, which can be seen from Fig. 1b. It seemed that introduction of aqueous thymosin solution into the inner oil phase changed the interface tension between two oil phases, which in turn resulted in the destabilization of the preformed o_1/o_2 emulsion. Therefore the microspheres yield was very low when lecithin alone was adopted as emulsifier.

Interestingly, sphere-shaped microparticles were achieved when emulsifier mixture was used in the outer oil phase (Fig. 1c). The composition of the outer phase was studied to optimize the preparation process. As presented in Table 1, keeping lecithin as constant as 0.25% (w/v) in this case, the microsphere yield was decreased when the Span 80 concentration was increased from

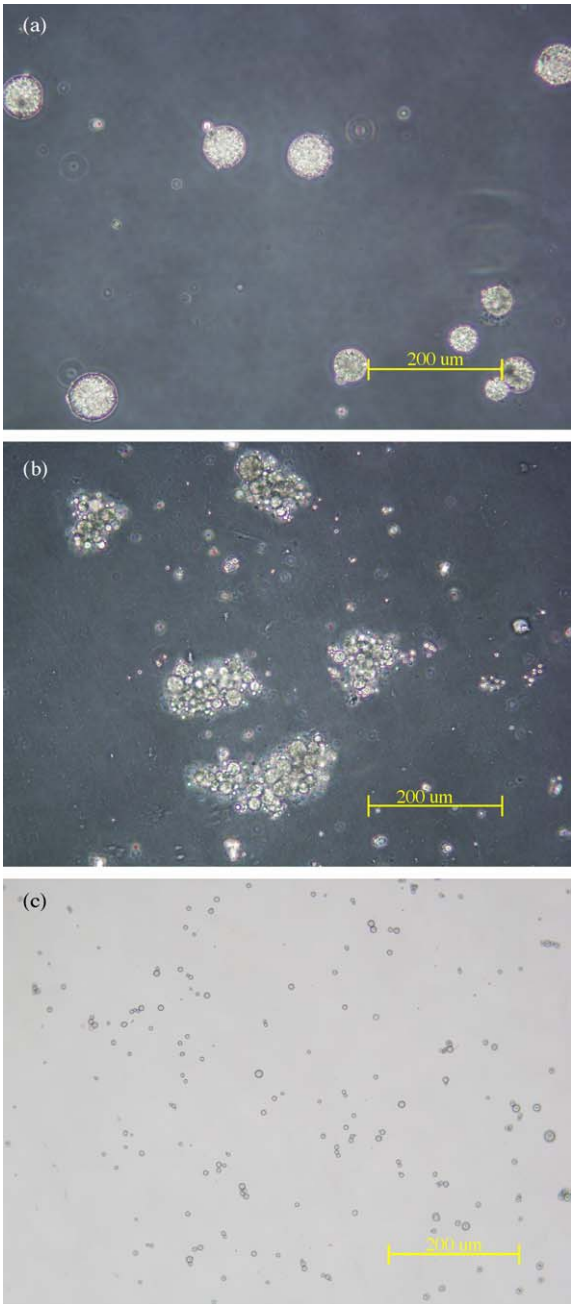

Fig. 1. Opital microscope photographs of microparticles: (a) blank microspheres prepared with lecithin as emulsifier alone; (b) thymosin-loaded microparticles prepared with lecithin as emulsifier alone; (c) thymosin-loaded microspheres prepared with 0.25% (w/v) lecithin and 0.35% (w/v) Span 80 as mixture emulsifier.

Table 2
The influence of polymer solution concentration on the entrapment efficiency[a]

Polymer concertration (%, w/v)	Entrapment efficiency (%)
1.5	44.0
3.0	61.0
5.0	75.2
6.0	84.8

[a] The amount of thymosin was kept as 6.26 mg; the volume ratio of polymer solution to drug solution was 20:1; the volume ratio of polymer solution to liquid paraffine was 1:10; the mixture emulsifier was composed of 0.35% Span 80 and 0.25% lecithin.

0.5 to 1.0%. On the other hand, as Span 80 concentration was set as 0.35%, decreasing lecithin concentration from 0.25 to 0.1% resulted in the increase of irregular particles in the final product. These results suggested that enough lecithin concentration was the prerequisite for the successful preparation of well-defined microspheres with high yield, while higher Span 80 was undesirable.

3.2. Optimization of thymosin entrapment efficiency

By using mixture emulsifier, drug-loaded microspheres with high yield were prepared successfully. Thymosin entrapment efficiency, however, was not high enough to perform the following studies. This might be due to the migration of drug in water-organic solvent mixture towards the outer skins of microspheres during the early stage of the fabrication process, which in turn resulted in drug loss into the outer oil phase, which was in accordance with the result reported by Chaw et al. (2003). Process parameters including the volume ratio of organic solvent to water, initial drug feed and polymer concentration, were therefore optimized to prepare microspheres with high drug loading.

The effect of polymer concentration on thymosin entrapment efficiency is listed in Table 2. The significant enhancement in drug entrapment was observed when the polymer concentration increased. For instance, the thymosin entrapment efficiency was increased from 44 to 84.8% as polymer concentration was increased from 1.5 to 6%. As reported by Shukla and Price (1991), the drug particles dispersed in the droplets would diffuse from the inner to the surface of the droplets during the emulsification and subsequent

Table 3
The influence of initial amount drug on the entrapment efficiency[a]

Polymer concentration (%, w/v)	The initial amount of drug (%, w/w)	Actual weight of drug (mg)	Entrapment efficiency (%)
3	1	3.13	86.1
	2	6.26	78.4
	4	12.52	74.3
6	0.5	3.13	89.7
	1	6.26	80.4
	2	12.52	77.9

[a] The volume ratio of polymer solution to drug solution was 20:1; the volume ratio of polymer solution to liquid paraffin was 1:5; the mixture emulsifier was composed of 0.35% Span 80 and 0.25% lecithin.

Table 4
The influence of the volume ratio of polymer solution and drug solution on the entrapment efficiency[a]

Polymer concentration (%, w/v)	The volume ratio of polymer solution and drug solution	Entrapment efficiency (%)	Mean particle size (μm)
3	20:1	86.1	15.5
	40:1	90.3	22.7
	60:1	83.4	18.4
6	20:1	89.7	65.4
	40:1	89.2	57.8
	60:1	86.9	60.5

[a] The amount of thymosin was 3.13 mg; the volume ratio of polymer solution to liquid paraffin was 1:5; the mixture emulsifier was composed of 0.35% Span 80 and 0.25% lecithin.

solvent evaporation process. Increasing the polymer solution concentration would enhance the viscosity of the inner oil phase, and hence would lower the diffusion rate of drug towards the droplets surface. On the other hand, the higher viscosity of droplets synchronously resulted in droplets with larger size (Scully, 1976), which consequently lengthened the diffusion distance and also lowered the specific area. Considering these two synergic factors, when the stirring rate was kept constant, the higher polymer concentration was propitious to improve the drug entrapment efficiency as a result (Zhu et al., 2003).

It was reported by many researchers that the initial drug feed influenced the entrapment efficiency dramatically when microspheres were prepared by o_1/o_2 emulsion technique (Zhu et al., 2003). As showed in Table 3, for formulations with 3% polymer solution, the drug entrapment efficiency was 86.1, 78.4 and 74.3% for microparticles produced with 1, 2 and 4% of initial drug loading respectively. In the case of formulations with 6% of polymer solution, the entrapment efficiency was 89.7, 80.4 and 77.9% for microparticles with 0.5, 1 and 2% of initial drug loading, respectively. It is clear that decreasing the initial amount of drug did increase the entrapment efficiency of thymosin into microparticles.

Table 4 reflects the effect of the volume ratio of organic solvent to water in inner oil phase on the entrapment efficiency. For both formulations with 3 and 6% polymer solution, varying the volume ratio of organic solvent to water led to the slight change of entrapment efficiency. As reported by Viswanathan et al. (1999), proteins could be entrapped into polymer matrix successfully using w/o/o method only when proteins had been precipitated within water-acetonitrile phase. If proteins were not fully precipitated, the entrapment efficiency was low. When water and ethanol phase were mixed and merged into a single phase, precipitation of thymosin occurred. At 20:1 of the volume ratio of organic solvent to water, thymosin had been precipitated completely from the aqueous solution. Consequently, the entrapment efficiency did not significantly improved when the volume ratio of organic to water was increased from 20:1 to 60:1.

3.3. Release studies

To evaluate the pH-dependent release profiles of thymosin from microparticles, in vitro release tests were performed in buffers with different pH. Since Ac II is insoluble in release media with pH 1.0, microparticles were only slightly swollen and remained intact in this case. As clearly shown in Figs. 3 and 4, a slow thymosin release behavior was observed for microparticles at pH 1.0. After 240 min, about 38% of the total drug was released from microparticles prepared with 3% polymer concentration, while only 6.53% drug was released in the case of particles for 6% polymer concentration. Fig. 2 manifests the SEM photographs of thymosin-loaded microspheres. It is found that there was no significant difference either in surface morphology or the internal cross-sectional structure between microparticles derived from 3 and 6% polymer concentration. Consequently, higher release rate in the case of microparticles based on 3% polymer concentration may be attributed to the higher surface area, since

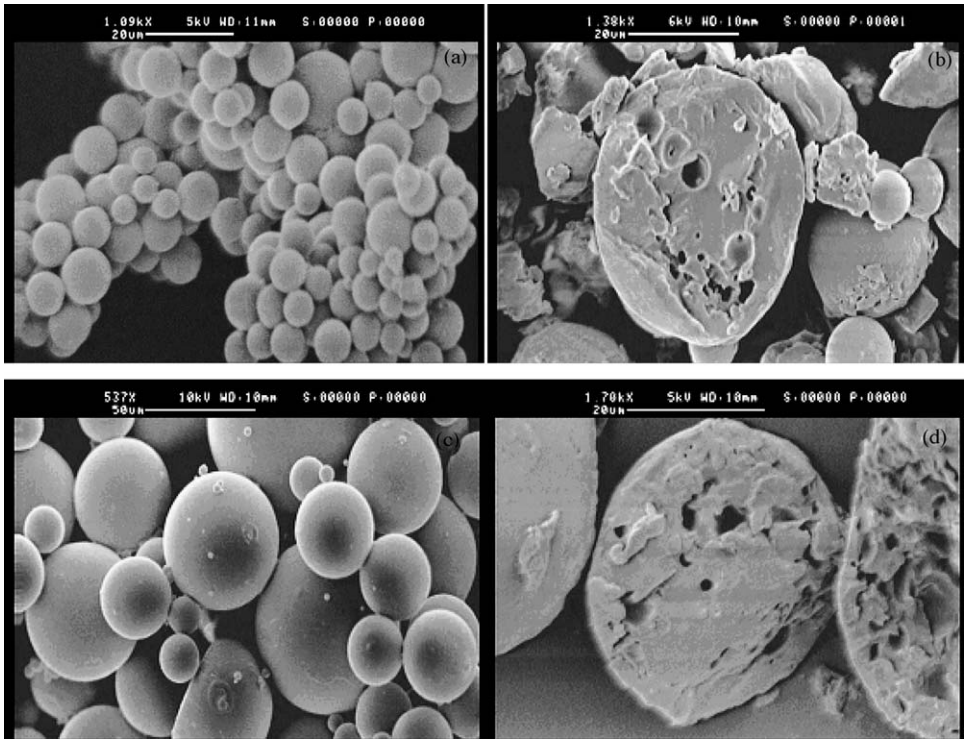

Fig. 2. SEM photographs of thymosin-loaded microspheres: (a and c) are microspheres prepared with 3 and 6% polymer concentration respectively, while (b and d) are corresponding cross-sectional views.

small particle size was observed for these microparticles (Table 4).

On the other hand, Ac II is thoroughly swollen at pH 5.8, and even dissociation may be expected for some carboxyl under this condition. Therefore, as can be seen from Figs. 3 and 4, the faster thymosin release was observed for microparticles in release media with pH 5.8 compared with that in pH 1.0 release media. About 55 and 26% thymosin was released within 240 min in pH 5.8 from microparticles of 3 and 6% polymer concentration respectively. This effect was further demonstrated by release tests in the pH 6.8 buffer (Figs. 3 and 4). Acrylic Ac II can be completely dissolved in aqueous media of pH 6.8, and therefore faster drug release occurred due to the quick destruction of microsphere morphology. In addition, slower release rate was found for microspheres derived from 6% polymer concentration compared with that for microparticles from 3% polymer concentration regardless of pH 5.8 or 6.8, and surface area effect might be also responsible for these results.

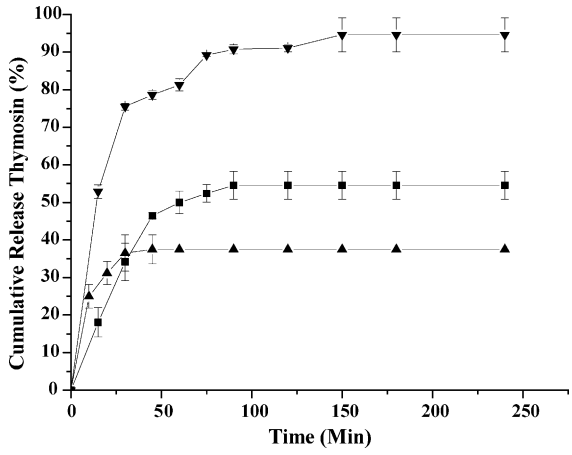

Fig. 3. The percentage of thymosin released from microspheres prepared with 3% polymer concentration at: (▲) pH 1.0; (■) pH 5.8; (▼) pH 6.8.

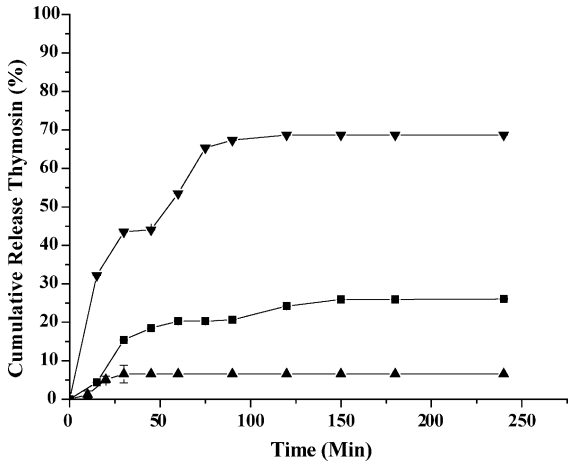

Fig. 4. The percentage of thymosin released from microspheres prepared with 6% polymer concerntration at: (▲) pH 1.0; (■) pH 5.8; (▼) pH 6.8.

4. Conclusions

Modified o_1/o_2 emulsion solvent evaporation method was adopted to encapsulate water-soluble polypeptide components, thymosin, into microspheres based on Ac II as polymer carrier and lecithin/Span 80 as mixture emulsifier. It is suggested that proper lecithin and Span 80 concentration was the prerequisite for the successful preparation of well-defined microspheres with high yield. Process parameters, such as the amount of drug, the volume ratio of inner to outer oil phase as well as polymer concentration, were optimized to produce microspheres with higher drug entrapment efficiency. In vitro release studies revealed that thymosin release from the microspheres exhibited distinct enteric profiles regardless of microspheres prepared with 3 or 6% polymer concentration. In vivo studies are undergoing in our laboratory to evaluate the bioactivity of the entrapped thymosin.

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