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Optimization and in vitro stability of legumin nanoparticles obtained by a coacervation method

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Abstract

Legumin (a storage protein from *Pisum sativum* L.) nanoparticles of about 250 nm were prepared by means of a pH-coacervation method and chemical cross-linking with glutaraldehyde. Non-stabilized nanoparticles or coacervates were obtained by mixing an aqueous solution of legumin with a buffer. The influence of some experimental parameters (pH, surfactant content and ionic strength) on the size and yield of coacervates was studied. These systems were then treated with glutaraldehyde. After 2 h, a concentration of at least 0.05 mg glutaraldehyde/mg legumin was necessary to stabilize these colloidal systems. No significant differences in size and percentage of yield were obtained between legumin nanoparticles cross-linked with different glutaraldehyde concentrations. Legumin nanoparticles were quite stable in pH conditions close to neutrality. On the other hand, nanoparticles stored under acidic conditions (pH 5.5; 37°C) showed a rapid degradation and this fact may be of interest for pharmaceutical applications like cutaneous or transdermal administration of drugs.

Keywords: Legumin; Nanoparticle; Coacervate; Coacervation; Cross-linking; Transdermal administration

1. Introduction

Nanoparticles are solid colloidal particles ranging in size form about 10 to 1000 nm (Kreuter, 1983), which have been investigated as drug carriers since the late 1970s. These carriers have been produced from synthetic and natural polymers. Natural polymers, such as proteins, have been widely employed because they can incorporate a wide variety of drugs in a relatively non-specific fashion (Kramer, 1974). Generally, the methods used to prepare small sized particles from proteins

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involved the application of emulsion techniques (Zolle et al., 1970; Gallo et al., 1984), which are simple procedures but require several washings with organic solvents to eliminate the oil used. Besides emulsions methods, coacervation or controlled desolvation methods have been developed to prepare nanoparticles from proteins (Marty et al., 1978; Bodmeier et al., 1989). It was reported that the coacervation or desolvating agents produce some modifications of the protein's tertiary structure which can be induced to give a hydrophobic material which tends to form submicronic aggregates of desolvated protein leading to a milky suspension (Marty et al., 1978). For both emulsion and coacervation techniques, a subsequent treatment by heating (Zolle et al., 1970) or by chemical cross-linking agents (Burgess and Singh, 1993; Lin et al., 1993) is necessary to harden and stabilize these nanoparticles.

In this work we describe the preparation of nanoparticles from a vegetal protein, legumin. Legumin is one of the main storage proteins in the seeds of the pea (*Pisum sativum* L.) and it belongs to the so-called 11S globulin group (Schwenke et al., 1990). Legumin possesses an oligomeric structure characterized by an arrangement of six subunits (3S components). Its molecular mass was reported to be 359 ± 25 kDa (Caer and Colas, 1993). Nanoparticulate carriers from vegetal proteins is a new approach which presents some advantages. They are less expensive than animal proteins and also possess functional groups which can be easily used either to adsorb or to covalently couple molecules capable of modifying the targeting properties of nanoparticles, i.e. antibodies (Akasaka et al., 1988) and lectins (Woodley and Naisbett, 1988).

The aim of this work was to describe the optimization and the in vitro stability of legumin nanoparticles, obtained by a coacervation method. Firstly, the influence of various processing conditions (pH, ionic strength and surfactant content) on the size and percentage of yield of legumin coacervates has been evaluated. The influence of cross-linking agents and the pH conditions of storage on the stability of legumin nanoparticles is also described.

2. Materials and methods

2.1. Materials

Synperonic PE/F 68 was purchased from I.C.I. (Kortenberg, Belgium), and glutaraldehyde grade II (25% aqueous solution) from Sigma (St. Louis, USA). Sodium hydroxide, sodium chloride and other chemicals, used to prepare phosphate buffers, were of analytical grade and obtained from Prolabo (Paris, France).

2.2. Methods

2.2.1. Legumin purification

Legumin (molecular weight 360000; isoelectric point 4.8) was purified from pea seed flour (*Pisum sativum* L., var. Amino) by a chromatographic procedure using successive ion exchange and gel filtration steps as described elsewhere (Gueguen et al., 1984; Larré and Gueguen, 1986). Briefly, the crude protein extract, prepared by stirring 10 g of flour in 100 ml sodium phosphate-citrate buffer (0.1 M, pH 7; buffer A), was fractionated on DEAE Sepharose CL 6B. A preparative column (Pharmacia K100/45) was loaded with 70 ml of crude extract and eluted at a flow rate of 40 ml/h per cm^2. The elution was performed using a stepwise gradient of 0.06 M, 0.1 M, 0.25 M and 0.5 M sodium chloride in buffer A + 0.2% sodium azide. The legumin fraction eluted at 0.25 M NaCl was then concentrated by ultrafiltration and purified by gel filtration on a column (Pharmacia K100/100) packed with Ultrogel ACA 34, previously equilibrated with buffer A + 0.2% sodium azide. Purified legumin was desalted through a Trisacryl GF05 bed, using distilled water as the eluant, and then freeze-dried.

2.2.2. Legumin coacervate and nanoparticle production

Coacervates were prepared by mixing one volume of a 0.5% w/v aqueous solution of legumin (pH 9 with NaOH 0.01 N) with two volumes of a constantly stirred 0-1% w/v solution of Synperonic PE/F 68 in phosphate buffer (pH ranging from 4.5 to 7). The ionic strength was held constant at 0.103 M, while the pH was varied be-

tween 4.5 and 7, or the pH was held constant at 6.8 while the ionic strength was adjusted with NaCl (in both cases, the Synperonic content was 0.17% w/v). Finally, the pH and ionic strength were held constant at 6.8 M and 0.103 M, respectively, while the surfactant content was varied between 0% and 0.67% w/v.

Legumin coacervates formed as described above (experimental conditions: pH 6.8; ionic strength 0.103 M; surfactant content 0.33% w/v) were cross-linked by adding different volumes of a 25% w/v aqueous solution of glutaraldehyde, to give 0–0.30 mg glutaraldehyde/mg legumin, and stirring continuously at room temperature for 0–6 h. After the cross-linking step, nanoparticles were centrifuged at 20000 rev./min for 15 min in a Beckman J2-21M/E centrifuge (UK) equipped with a J 20.1 rotor. The supernatant was removed and the pellets were resuspended in phosphate buffered saline (PBS; pH 7.4, ionic strength 0.15 M). This suspension was centrifuged again, and finally the legumin nanoparticles were kept in PBS.

2.2.3. Particle size measurements

The size of the legumin nanoparticles was determined by photon correlation spectroscopy (PCS) using a Coulter N4MD submicron particle analyzer (Coultronics, Margency, France). The size and shape of the nanoparticles were also examined using scanning electron microscopy (SEM) in a JEOL 840 instrument (Germany).

2.2.4. Determination of the percentage of coacervate and nanoparticle yield

The amount of legumin transformed into coacervates was determined as follows: 5 ml were centrifuged (20000 rev./min for 15 min) and the residue was digested with 1 N NaOH. The samples were measured in an Spectronic 601 spectrophotometer (Bioblock Scientific, Illkirch, France) at 280 nm. In preliminary experiments, absorbance at 280 nm had been shown to depend only on the concentration of the protein and to be linear up to 1 mg legumin/ml.

For nanoparticles, gravimetric determinations were performed in order to avoid any interference from glutaraldehyde residues.

2.2.5. Particle size stability study

Legumin nanoparticles were stored in four different phosphate buffer solutions (7.4, 6.8, 6.4 and 5.5; ionic strength of all buffers 0.15 M) for periods up to 6 days, in constant-temperature water-baths at 37 ± 1°C. At certain time intervals, the size of the particles was measured by PCS.

3. Results

3.1. Legumin coacervates

Fig. 1 shows the legumin coacervate size and yield obtained under different pH conditions, when the ionic strength (0.103 M) and the surfactant content (0.17% w/v) were kept constant. The optimum pH conditions, to obtain small sized coacervates or non-stabilized nanoparticles were those close to neutrality. Under these experimental conditions, the legumin coacervate production yielded less than 40% of the added protein.

The influence of ionic strength of the system on the size and yield of legumin coacervates, when the pH and surfactant content were kept constant at 6.8% and 0.17% w/v, respectively, is illustrated in Fig. 2. An increase of the ionic strength conditions produced a decrease of the size and yield of legumin coacervates.

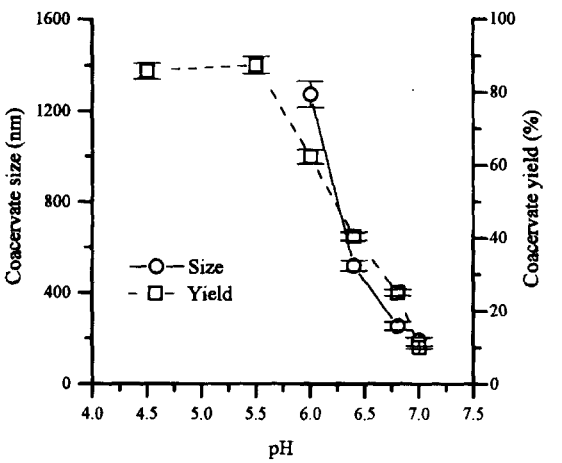

Fig. 1. Influence of pH conditions on the size and yield of legumin coacervates. Experimental conditions: ionic strength 0.103 M, surfactant concentration 0.17% w/v.

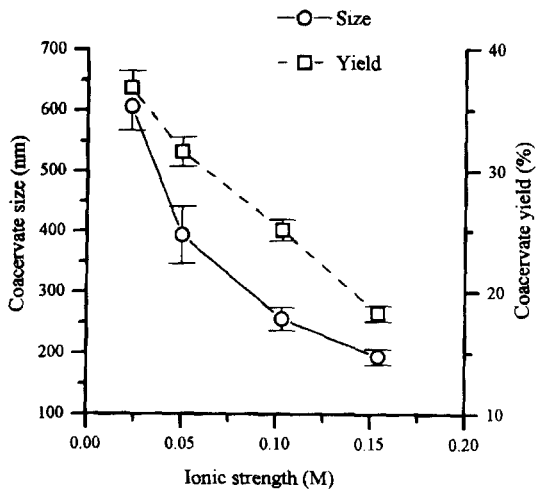

Fig. 2. Influence of ionic strength of the system on the size and yield of legumin coacervates. Experimental conditions: pH 6.8, surfactant concentration 0.17% w/v.

Finally, at pH and ionic strength conditions which enabled to obtain particles with a size close to 250 nm (pH 6.8; ionic strength 0.103 M), the influence of surfactant was tested (Fig. 3). In this case, an increase of the surfactant concentration enabled an increase of the coacervate yields to be obtained without appreciable modifications of their size.

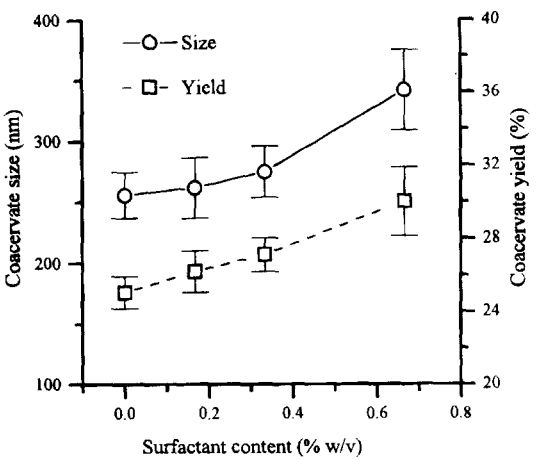

Fig. 3. Influence of surfactant content on the size and yield of legumin coacervates. Experimental conditions: pH 6.8, ionic strength 0.103 M.

3.2. Legumin nanoparticles

The second step of our work was to prepare legumin nanoparticles with a predicted size of about 250 nm. These systems were prepared at the following coacervation conditions: pH 6.8, ionic strength 0.103 M and surfactant content 0.33% w/v. These batches were then cross-linked for 2 h with 0.30 mg glutaraldehyde/mg legumin, as has been described for others protein nanoparticles (Rubino et al., 1993). The nanoparticles produced in these conditions were found to be close to the desired size with a narrow size distribution (232 $\pm$ 6 nm; polydispersity index: 0.081; $n = 6$), with a yield of 27.84 $\pm$ 1.18 (from gravimetry). On examination by SEM, small, spherical nanoparticles were observed (Fig. 4).

Monitoring the effect of cross-linking time on the size of nanoparticles (Fig. 5) revealed that at least 0.05 mg glutaraldehyde/mg legumin were necessary to stabilize these colloidal suspensions. Under this condition (0.05 mg glutaraldehyde/mg legumin), nanoparticles were stabilized after 2 h. When higher amounts of cross-linking agent were used, this step was too rapid, almost instantaneous. Glutaraldehyde concentrations less than 0.05 mg/mg legumin did not prevent the aggregation of legumin coacervates.

Aging over 6 days at 37°C, under different pH conditions (7.4, 6.8, 6.4, 5.5) did not have a significant effect on the particle size of the nanoparticles, except for systems stored at pH 5.5 (Fig. 6). These last colloidal systems increased their initial size 3-fold, after 3 h, and after 24 h they presented a filamentous precipitated aspect. By gravimetry, we could determine that about 20% of the initial legumin nanoparticle content had been solubilized.

4. Discussion

To prepare small particles, by means of a coacervation method, it is important to maintain the system at a point just before coacervation is initiated. The coacervation must be stopped as soon as the Tyndall effect of the newly formed particles turns the system turbid (Lin et al., 1993).

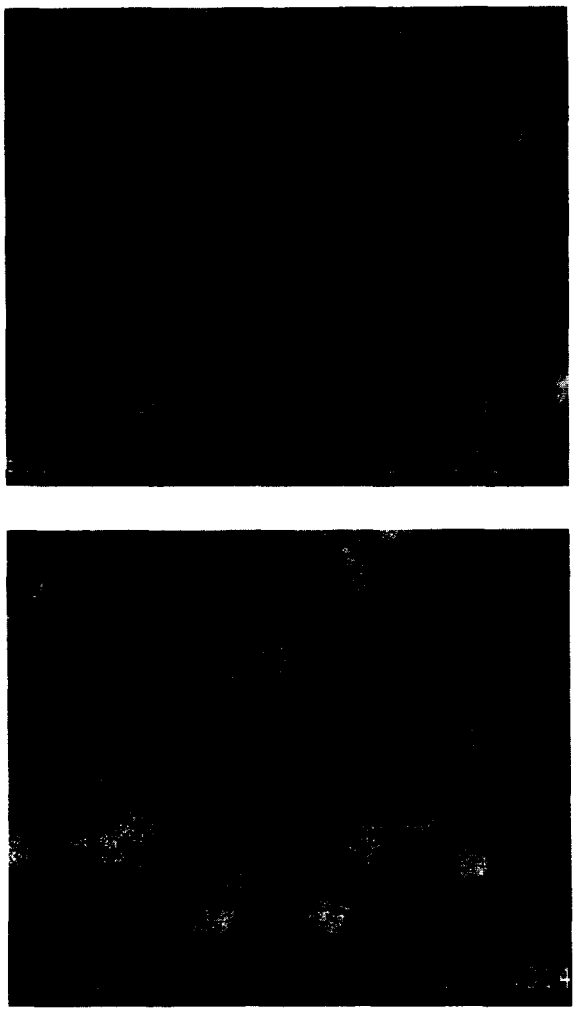

Fig. 4. SEM micrographs of legumin nanoparticles.

Legumin is a natural protein whose aqueous solubility is pH- and ionic strength dependent (Gueguen et al., 1982). In our work, it was noted that as the pH was altered within the coacervation pH range (maintaining constant ionic strength and surfactant content), the appearance of the coacervate changed markedly. The size and yield of legumin coacervates were therefore assessed over the pH range 4.5–7, at ionic strength 0.103 M and surfactant content 0.17% w/v (Fig. 1). On the one hand, at pH values ranging from 4.5 to 5.5 (close to the isoelectric point of legumin, pI = 4.8 (Derbyshire et al., 1976)) a rapid phase separation into a viscous coacervate phase (rich in

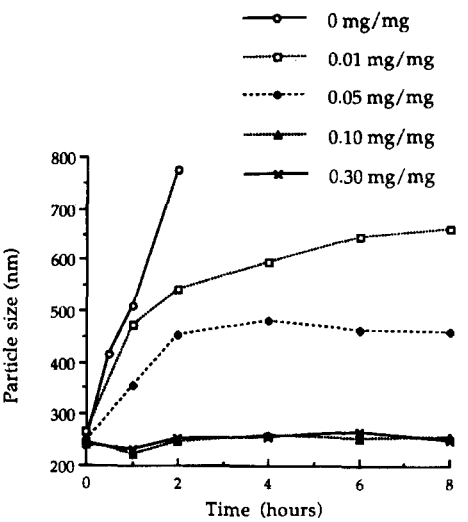

Fig. 5. Influence of glutaraldehyde concentration (mg glutaraldehyde/mg legumin) and cross-linking time on the size of legumin nanoparticles.

legumin and poor in solvent) and a relatively clear equilibrium phase (rich in solvent and poor in polymer) was produced. Under these conditions, the percentage coacervate yield was high (between 80 and 90% w/v). On the other hand, at pH values close to the neutrality, the systems became opalescent and the coacervates showed a submicronic size (Fig. 1). Unfortunately, in these conditions, the percentage of legumin coacervate yields were less than 40% of the added protein.

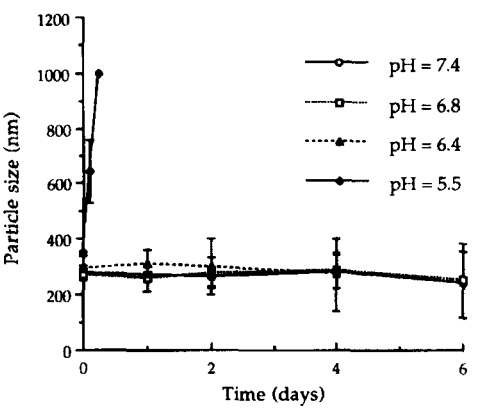

Fig. 6. Stability of legumin nanoparticles stored at different pH conditions.

The ionic strength also had an influence on the size and yield of coacervates. Under the study conditions, an increase of this parameter produced an increase in legumin solubility, and, therefore, a decrease in the particle size and yield of coacervates. Finally, increasing on the surfactant content enabled us to increase the yield of legumin coacervates.

Non cross-linked coacervates coalesced to form a separate phase. Similar results have been found for other colloidal systems obtained by means of coacervation methods (Burgess and Carless, 1985). So, glutaraldehyde was chosen to stabilize these coacervates because it is a very effective and widely used cross-linking agent for nanoparticles (Chen et al., 1987; Lin et al., 1993) and microparticles (Davis et al., 1987). It has been reported that cross-linking with glutaraldehyde only involves lysine residues and the number of modified lysines increases with glutaraldehyde concentration (Lee et al., 1981). Similarly, the degree of cross-linking affects the stability of the particles and the drug release properties (Davis et al., 1987).

Under the conditions of this study, 0.05 mg glutaraldehyde/mg legumin was the minimal concentration which enabled these coacervates to be stabilized. At higher concentrations (0.10–0.30 mg glutaraldehyde/mg legumin), it was apparent that no significant change in particle size occurred during the cross-linking step (Fig. 5).

Finally, these systems showed a good stability when they were stored under pH conditions close to neutrality (Fig. 6). The observed increase on the population standard deviations might indicate a degradation of these systems. For legumin nanoparticles stored at pH 5.5, two possible factors could explained their behaviour: firstly, the rapid increase in their particle size could have been produced by an agglomeration of particles due to a change in their particle surface characteristics, at this pH. Secondly, under these pH conditions, legumin nanoparticles could have swollen and internal hydrolysis of the protein taken place. This second explanation is more plausible, because after 24 h the nanoparticles showed a filamentous aspect and 20% of protein systems had disappeared.

In summary, legumin nanoparticles with a size of about 250–300 nm can be prepared by a coacervation method, followed by a cross-linking step with glutaraldehyde. These systems were quite stable under neutral conditions. On the other hand, they showed a rapid degradation at pH 5.5; this fact may be of interest for pharmaceutical applications like cutaneous or trans-dermal administration of drugs.

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