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Development of Drug Delivery Systems from Vegetal Proteins: Legumin Nanoparticles

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ABSTRACT

Legumin (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. This preparative method enabled to avoid the use of organic solvents but only yielded about 27% of protein added as nanoparticles. No significant differences in size, percentage yield, and surface charge were obtained between legumin nanoparticles cross-linked with different glutaraldehyde concentrations. Legumin nanoparticles were quite stable in phosphate-buffered saline (PBS). They follow a zero-order degradation, and by increasing glutaraldehyde concentration, a longer half-life (t_{50}) was obtained. The amount of methylene blue (MB), used as a model of hydrophilic drug, loaded was about 6.2% of the initial dye. Its release from the nanoparticles consisted of a rapid initial phase followed by a slower second period. The rates in this second phase were inversely related to the degree of cross-linking.

INTRODUCTION

Colloidal carriers, in the form of microparticles and nanoparticles, have the potential to achieve sustained drug release and to deliver drugs to specific target sites. They possess advantages over soluble carriers in terms of drug-loading capacity and stability (1). These carriers have been produced from natural and synthetic polymers. Natural polymers, such as proteins, are metabolizable and can incorporate a great variety of drugs in a relatively nonspecific fashion (2).

Several methods have been reported in the literature for the preparation of micro- and nanoparticles from protein raw materials. Most methods involve the application of emulsion technologies (3–5), but these manufacturing procedures require large amounts of oil which need to be eliminated by several washings with organic solvents (6). Besides emulsion methods, coacervation or controlled desolvation methods have been developed to prepare particles from proteins (i.e., albumin) using ethanol or sodium sulfate as the coacervation agent (7,8), or by changing the pH conditions (9). For all techniques (emulsion and coacervation methods), a subsequent treatment either by heating or by chemical cross-linking agent is necessary to stabilize the particles.

The goal of our work was to prepare and evaluate biodegradable nanoparticles from a vegetal protein: legumin. Legumin is one of the main storage proteins in the seeds of the pea (*Pisum sativum* L.). It belongs to the group of so-called 11S globulins, with sedimentation coefficients between 11S and 14S, and molecular masses between 300 and 400 kDa (10). The isoelectric point of pea legumin was reported to be 4.8 (11), and this protein is rich in acidic amino acids and arginine (12).

We report here the main physicochemical characteristics of legumin nanoparticles, their in vitro stability, and the release profiles of loaded methylene blue (used as a model of a hydrophilic drug).

MATERIALS AND METHODS

Materials

Materials used included glutaraldehyde grade II from Sigma Chemical Co. (St. Louis, USA); Synperonic PE/F 68 was purchased from ICI (Kortenberg, Belgium), and methylene blue from COOPER (Melun, France). Sodium hydroxide, sodium chloride, and other chemicals used to prepare phosphate buffers were of analytical grade and obtained from Prolabo (Paris, France).

Methods

Legumin Purification

Legumin (molecular weight 360,000; isoelectric point 4.8) was extracted and 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 (12,13). 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/hr cm^2. The elution was performed using a stepwise gradient 0.06 M, 0.1 M, 0.25 M, and 0.5 M sodium chloride in buffer A + 0.2% sodium azide. 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 eluant, and freeze-dried.

The purity of the extracted protein was calculated to be close to 95% by means of ultracentrifugation in density gradient and HPLC techniques described elsewhere (12).

Legumin Nanoparticle Production

Empty and MB-loaded legumin nanoparticles were prepared using a pH-coacervation method and chemical cross-linking with glutaraldehyde.

Typically, 125 mg of legumin were dissolved in 25 ml of water at pH 9 with NaOH 0.01 N. This solution was then poured into 50 ml of a constantly magnetic stirred (250 rpm) phosphate buffer phase (pH 6.8, ionic strength 0.15 M with NaCl) containing 0.2% w/v Synperonic PE/F 68 as stabilizer. The legumin coacervates thus formed were cross-linked by adding different volumes of a 25% w/v aqueous solution of glutaraldehyde (to give 0.10–0.30 mg glutaraldehyde/mg legumin) and stirring continuously (250 rpm, magnetic stirred) at room temperature for 2 hr. After the cross-linking step, legumin nanoparticles were centrifuged at 20,000 rpm for 15 min in a Beckman J2-21 M/E centrifuge (U.K.) equipped with a J20.1 rotor. The supernatant was removed and the pellets were resuspended in phosphate-buffered saline solution (PBS; pH 7.4, ionic strength 0.15 M). This suspension was centrifuged again, and

finally the nanoparticles were either kept in PBS or dried under vacuum at room temperature and stored in a dessiccator at 4°C.

Finally, for methylene blue (MB)-loaded legumin nanoparticles, the dye was directly added to the Synperonic PE/F 68 phase at a 0.04% w/v concentration (initial dye available: 160 μg MB/mg legumin), and the colloidal systems were prepared as described above.

Physicochemical Characterization of Legumin Nanoparticles

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 examinated using scanning electron microscopy (SEM; JEOL 840, Germany). The surface properties of the legumin nanoparticles were analyzed by determining their zeta potential on a Malvern Zetasizer 4 (Malvern Instruments, France) in saline solution (NaCl 0.9% w/v).

The amount of legumin transformed into nanoparticles was determined as follows: 5 ml of a nonstabilized suspension was centrifuged (20,000 rpm for 15 min) and the residue was digested with NaOH 1 N for 30 min at room temperature. The samples were measured in a spectrophotometer Spectronic 601 (Bioblock Scientific, Illkirch, France) at 280 nm. Absorbance at 280 nm had previously been shown to depend solely upon the concentration of the protein and to be linear up to 1 mg legumin/ml.

Moreover, gravimetric determinations were made with cross-linked coacervates (nanoparticles), and to validate spectrophotometric results, six different coacervation yield experiments were tested by spectrophotometry and gravimetry, as the method of reference.

In Vitro Degradation Studies

Empty legumin nanoparticles were suspended in PBS (pH 7.4; ionic strength 0.15 M; 0.2% sodium azide as preservative). The final concentration of the nanoparticle suspension was 1 mg/ml. Vials containing these systems were placed into a vertical shaking water bath and incubated (operating conditions: 30 strokes per min; 37°C). At certain time intervals, the particles were separated by centrifuging at 20,000 rpm for 15 min. The supernatants were decanted and replaced with PBS. After recentrifugation, the supernatants were discarded and the particles were digested in 3.0 ml of NaOH 1 N for 30 min at room temperature. The degradation of legumin nanoparticles was estimated by comparing UV absorbance at 280 nm of the digested nanoparticle suspensions with the absorbance obtained after digesting initial nanoparticle controls.

Determination of Methylene Blue Content in the Nanoparticles

Supernatants obtained from the two centrifugation steps (during the preparation of nanoparticles) were collected and MB was determined spectrophotometrically at 663 nm using a calibration curve which was linear in the concentration range of 2–32 μg/ml. The payload was calculated as the quotient between the amount of MB loaded in legumin nanoparticles (difference between the theoretical amount added and the amount of MB recovered from the two washings steps) and the legumin nanoparticle yield.

In Vitro Release Studies

Known weights of legumin nanoparticles (2 mg) were placed in vials containing 3 ml of release medium (PBS) which were agitated at 37°C. Samples were taken at appropriate time intervals and centrifuged at 20,000 rpm for 15 min, and the amount of MB released was determined spectrophotometrically as described above.

RESULTS AND DISCUSSION

To prepare small particles by 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 (1).

Legumin is a natural protein whose aqueous solubility is pH dependent (14). Thus, pH values less than 2 or greater than 7 enable its solubilization. In this study, the appearance of the coacervate was assessed over the pH range 4.5 to 7 (all buffers were prepared at ionic strength 0.15 M with NaCl). At pH values ranging from 4.5 to 5.5 P [values close to the isoelectric point of legumin, pI = 4.8 (11)], the system separated rapidly into a viscous coacervate phase and a dilute equilibrium phase. At pH values close to the neutrality, an opalescent system was formed.

In all cases, these coacervates had to be hardened to avoid particle aggregation and consequent flocculation. In our work, glutaraldehyde was chosen for this pur-

pose, because it is very effective and widely used as a cross-linking agent. Thus, it has been reported that cross-linking by glutaraldehyde only involves lysine residues and that the number of modified lysine residues increases with glutaraldehyde concentration (15). This degree of cross-linking affects the stability of the particles and the drug release characteristics (16). For legumin, 1 mg of this protein contains 0.33 μmol lysine (12).

Legumin nanoparticles, produced at pH 6.8, were found to be around 250 nm in diameter, with a narrow size distribution (Table 1). SEM measurements of the same samples showed the particle size to be smaller (approximately 150–200 nm) than that yielded by photon correlation spectroscopy (PCS). This is probably explained by shrinkage of the nanoparticles on drying: the particle size measured by PCS is that of hydrated legumin nanoparticles. The SEM micrographs revealed that legumin nanoparticles obtained by this coacervation method were spherical in shape (Fig. 1).

Zeta potential determinations of legumin nanoparticles (Table 1), measured in NaCl 0.9%, show that these particles are negatively charged, and no significant differences ($p < 0.05$) were found between nanoparticles prepared with different concentrations of cross-linking agent. Unfortunately, this coacervation method only yielded 27.15 ± 0.92% of the initial concentration of legumin (from spectrophotometry) or 28.22 ± 1.51% (from gravimetry). It was apparent that no significant change in legumin nanoparticle yield occurred during the cross-linking step and, in all cases, the differences between the two methods were not significant ($p <$ 0.05), but large amounts of sample were necessary to obtain accurate values by gravimetry.

Table 1

Mean Diameters (Mean ± Standard Deviation, n = 6) and Zeta Potentials (in NaCl 0.9% w/v; Mean ± Standard Deviation, n = 6) of Legumin Nanoparticles Stabilized with Different Concentration of Glutaraldehyde, Expressed as Aldehyde/Lysine Ratio

Aldehyde/Lysine Ratio (μmol/μmol)	Particle Size (nm)	Zeta Potential (mV)
3.04	252 ± 29	−19.1 ± 1.4
4.55	243 ± 30	−18.7 ± 1.6
9.09	228 ± 27	−19.3 ± 1.1

Figure 2 illustrates the stability of legumin nanoparticles in PBS. They exhibited a good stability, and after incubation at 37°C for 4 days, less than 30% were degraded.

For comparative purposes, we have defined two parameters (see Table 2): k_D, which reflects the degradation rate of nanoparticles (calculated from the slopes of the curves presented in Fig. 2), and t_{50}, which reflects the time taken to reduce the nanoparticle concentration by 50% of its initial value.

In all cases, legumin nanoparticle degradation follows zero-order kinetics, and increasing the amount of cross-linking agent leads to a decrease in the degradation rate of these particles. Thus, the time for 50% disintegration (t_{50}) clearly indicates that the more intense the reaction (higher concentration of glutaraldehyde), the greater is

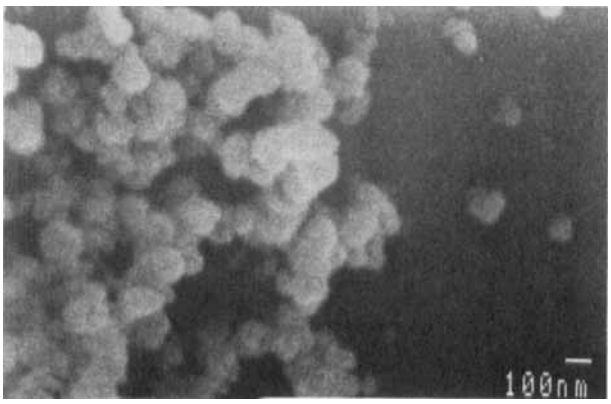

Figure 1. SEM micrographs of legumin nanoparticles with average diameters below 300 nm.

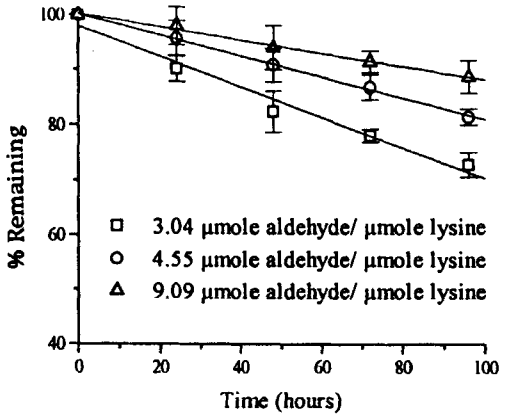

Figure 2. Degradation of legumin nanoparticles in PBS (pH 7.4; ionic strength 0.15 M).

Table 2

Stability Parameters for Legumin Nanoparticles: k_D (degradation ratio in % hr^{-1}) and t_{50} (days)

Aldehyde/Lysine Ratio (μmol/μmol)	k_D (% hr^{-1})	t_{50} (days)
3.04	0.277	7
4.55	0.191	11
9.09	0.120	17

the degree of cross-linking and, therefore, the slower the disintegration.

The amount of MB entrapped in legumin nanoparticles was relatively low; only about 6.25% of the initial dye was associated with the nanoparticles (Table 3). The percentage of loaded MB was almost the same, regardless of the glutaraldehyde concentration used.

The MB release profiles for legumin nanoparticles are reported in Fig. 3. Under the study conditions, a biphasic pattern of methylene blue release was observed from all legumin nanoparticles, independently of the glutaraldehyde concentration. A similar pattern of release has been observed for other natural colloidal carriers, for example, albumin. It has been suggested that the drug (MB, in this case) can be associated with the particle in two ways: on or at the surface (initial brief period of rapid release), and entrapped in the inner matrix (second period during which slower release by diffusion took place) (17,18). Increasing the cross-linking agent concentration from 3.04 to 9.09 μmol glutaraldehyde/μmol lysine decreased the amount released in the first 30 min from 64% to 52%. For the second, more prolonged period, the rate of release was reduced more than 1.5 times, from 0.155 % min^{-1} (ratio aldehyde/lysine = 3.04) to 0.105 % min^{-1} (ratio aldehyde/lysine = 9.09).

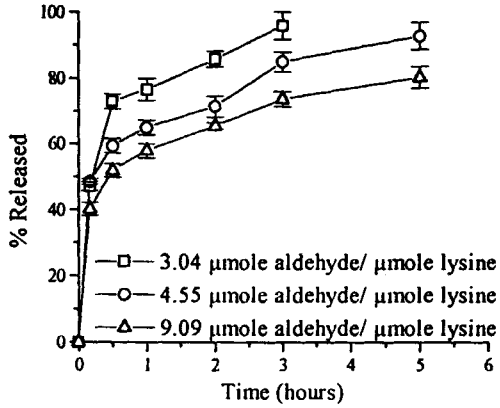

Figure 3. In vitro release profile of methylene blue from legumin nanoparticles in PBS (pH 7.4; ionic strength 0.15 M) at 37°C (expressed as the percentage of total MB content released plotted against time).

CONCLUSIONS

In conclusion, legumin nanoparticles with a size of about 250 nm may be produced by means of a pH-coacervation method and chemical cross-linking. This method avoids the use of organic solvents but only yields about 27% of protein added as nanoparticles. The amount of glutaraldehyde used as cross-linking agent appreciably modifies the stability of the legumin nanoparticles. The release rate of methylene blue from legumin nanoparticles is also dependent on the cross-linking agent concentration. Typically, the release increased when the glutaraldehyde/lysine ratio decreased.

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Table 3

Characteristics of MB-Loaded Legumin Nanoparticles (Mean ± Standard Deviation, n = 4)

Aldehyde/Lysine Ratio (μmol/μmol)	Particle Size (nm)	Particle Yield (%)	MB-Loaded (μg/mg nanoparticles)	Entrapment Efficiency (%)
3.04	305 ± 36	29.11 ± 0.14	38.74 ± 2.21	6.05
4.55	327 ± 41	28.57 ± 0.09	40.03 ± 1.57	6.25
9.09	311 ± 29	27.54 ± 0.12	40.81 ± 1.63	6.38

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