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International Journal of Pharmaceutics 242 (2002) 405–409

international journal of pharmaceutics

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Note

Influence of some formulation parameters on lysozyme adsorption and on its stability in solution

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Received 24 December 2001; received in revised form 18 January 2002; accepted 29 January 2002

Abstract

According to our results concerning the behavior of lysozyme at interfaces, its secondary structure and its enzymatic activity, successful protein encapsulation would need to maintain a pH value far from the enzyme isoelectric point value during the formulation to reduce, in particular, the adsorption of lysozyme molecules at the created interfaces. Moreover, buffers or salt solution must be used in order to keep intact the native secondary conformation of lysozyme, and preserve its enzymatic activity.

Keywords: Lysozyme; Dichloromethane–water interface; Enzymatic activity; FTIR

Drug encapsulation into biocompatible and bioresorbable polymers, such as poly(α-hydroxy acid)s (PAHA), represents an interesting way to preserve drug activity, to control its release and to achieve targeting to specific tissues (Bazile et al., 1992; Stolnik et al., 1995). Among manufacturing processes, the double emulsion, water-in-oil-in-water method, followed by a solvent extraction and freeze-drying steps, is one of the most common techniques to encapsulate a wide range of drugs (Pistel et al., 1999). In the case of therapeutic proteins, the microencapsulation process, and particularly the primary emulsion step, involves exposition of proteins to mechanical stresses and to organic solvents leading to aggregation and denaturation processes (Morlock et al., 1998; Putney and Burke, 1998). The amphipatic protein segments adsorb at liquid interfaces and penetrate the interfacial region, causing the entanglement of the protein within the segments of PAHA and its irreversible denaturation (Boury et al., 1995). During freeze-drying, some additives, such as polyols are known to be potentially efficient in preventing dehydratation-induced protein structural changes (Burke et al., 1992). Moreover, during encapsulation of biologically active proteins in sustained delivery systems by emulsion processes, physico-chemical parameters such as ionic strength, type of salts, presence of additives may alter the stability of proteins and modify their conformation (Putney and Burke, 1998). The influence of these critical parameters must be under-

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00226-0

stood and controlled since proteins must keep unchanged their biological function to preserve their pharmacological activity. In this paper, we present some results concerning a model protein, the hen egg white lysozyme. By coupling interfacial tension measurements, enzymatic activity and characterization of the secondary structure of the protein in the bulk, we obtained some preliminary information on the effects of some formulation parameters.

Hen egg white lysozyme (prod. no. L-6876) and *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and used without any further purification. Ultrapure water was obtained from a Millipore® system (Milli-Q Plus 185, Molsheim, France). Dichloromethane (DCM) and NaCl were supplied from Prolabo (Paris, France). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Acros Organics (Noisy-le-Grand, France). pH values were adjusted by addition of NaOH 1M or HCl 0.1M and measured using a pH-meter Mettler Delta 320.

The adsorption and interfacial properties of lysozyme were analyzed at the DCM water interface (1 mg ml^{-1}) by means of a pendant drop method (Tracker, ITConcept, Longessaigne, France) (Fig. 1). From numeric analysis of the pendant drop (DCM) profile, the drop being formed at the extremity of a syringe immersed in a bowl containing water, characteristic parameters (interfacial tension, area, volume) were determined in real time. By controlled movements of the syringe piston, driven by a step by step motor, surface area can be maintained constant during the whole experiment. All glass containers were washed with a solution of sulfochromic acid saturated in potassium dichromate and then, were rinsed with ultrapure water before to be dried in an oven at 50 °C.

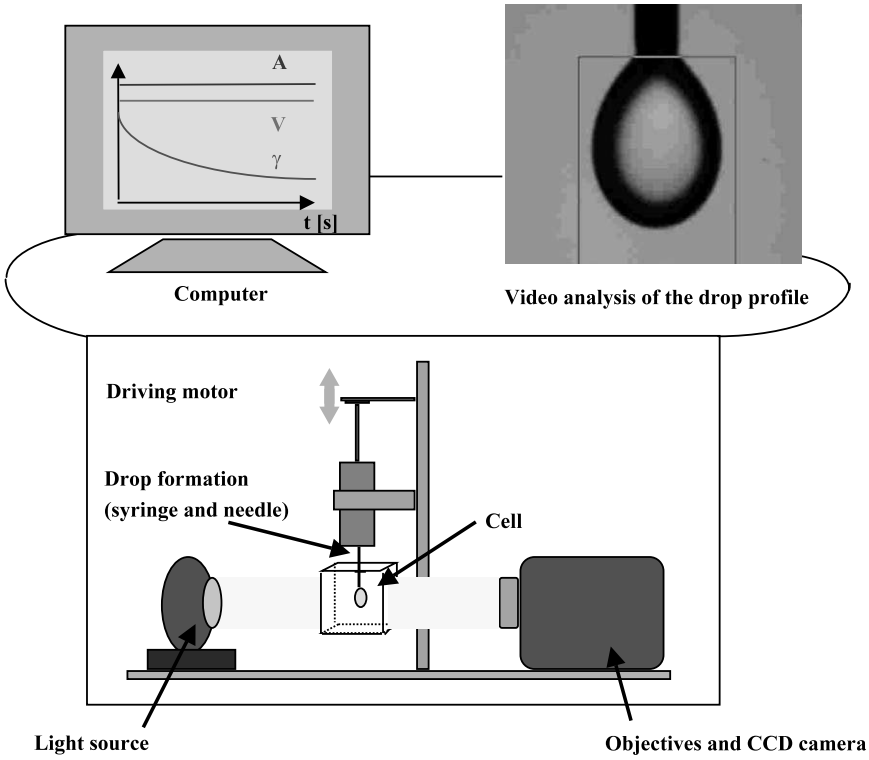

Fig. 1. Schematical representation of the pendant drop method. The numerical analysis of the drop profile allowed us to control, in real time, the interfacial area of the surface film.

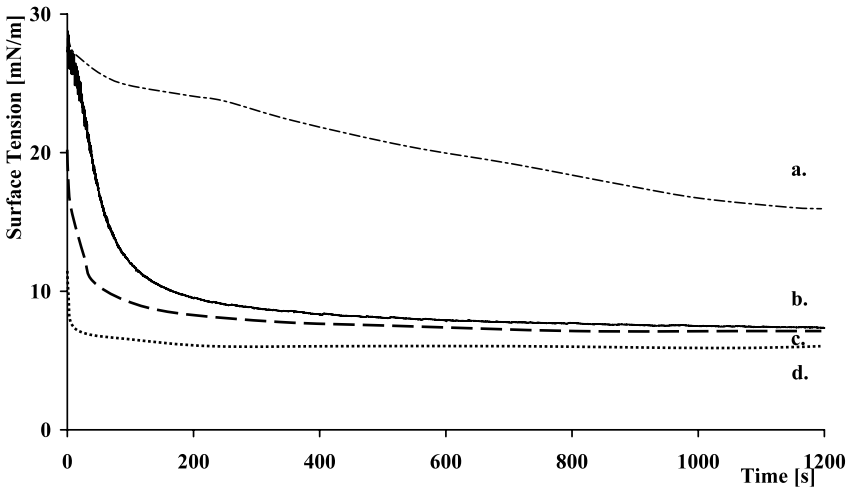

Fig. 2. Adsorption kinetics of lysozyme (1 mg ml^{-1}) at the DCM–water interface. (a) pH 2(–·–·–·–); (b) pH 6 (——); (c) pH 6 + NaCl 0.1 M(– – –); (d) pH 11 (……).

The enzymatic activity of lysozyme, using *Micrococcus lysodeikticus* as a substrate, was determined in different solvents: ultrapure water (pH 6.0); 0.1M NaCl (pH 6.0); and 0.1M Tris buffer (pH 7.4). The absorbance change, reliable to the rate of lysis of the substrate, was monitored at 450 nm using a spectrophotometer Bio-tek 922 (Kontron Instruments).

Spectra of lysozyme were recorded in solution at 10 mg ml^{-1} between 400 and 4000 cm^{-1} at 4 cm^{-1} intervals by Fourier Transform Infrared spectrometry (FTIR) (Brücker Vector 22 spectrometer) using an attenuated total reflection cell. All spectra were corrected for the solvent (water or Tris buffer) and water vapor contributions. All spectra were analyzed by correcting spectrum baseline, by normalizing and calculating the second derivate spectra for their component composition in the amide I (1720–1580 cm^{-1}) band. Second derivate spectra were smoothed with a 9-point smoothing function.

Fig. 2 shows adsorption kinetics of lysozyme at different pH values (2, 6, 11), in water or 0.1M NaCl. The adsorption rate increased as the pH became close to the isoelectric point of lysozyme (IEP = 11.2). The increase of pH, corresponding to a decrease of the protein net charge, decreased electrostatic repulsions that slowed down the adsorption. When salt was added to the aqueous phase, the rate of adsorption of lysozyme at the interface increased (curves c and d). Higher ionic strength, corresponding to lower electrostatic repulsions within lysozyme molecules, led to a further adsorption of the protein molecules toward the air–water interface (Damodaran et al., 1998). Lu et al. (1999) determined by neutron reflectivity the thickness of the adsorbed interfacial film of lysozyme at the air–water interface. They found that the film was thicker at pH 11, pH value close to the IEP of lysozyme. Charge screening effect induced by adding salt led to the same observation. This corroborates our results: the adsorption of lysozyme molecules at the air–water interface increased when electrostatic interactions decreased.

Fig. 3 shows that the enzymatic activity of lysozyme was maximal in Tris and 0.1 M NaCl but was cancelled in pure water. These results showed that ionic strength has a preponderant effect upon enzymatic activity. As it was already shown by Chang and Carr (1971), at similar pHs, no marked difference was observed between the calibration curve of the enzymatic activity of lysozyme in Tris and the one obtained in 0.1 M NaCl (curves a and b). The results confirmed that lysozyme was inactive in distilled water but was active towards its natural substrate with a suitable ionic strength (Davies et al., 1969; Chang and Carr, 1971).

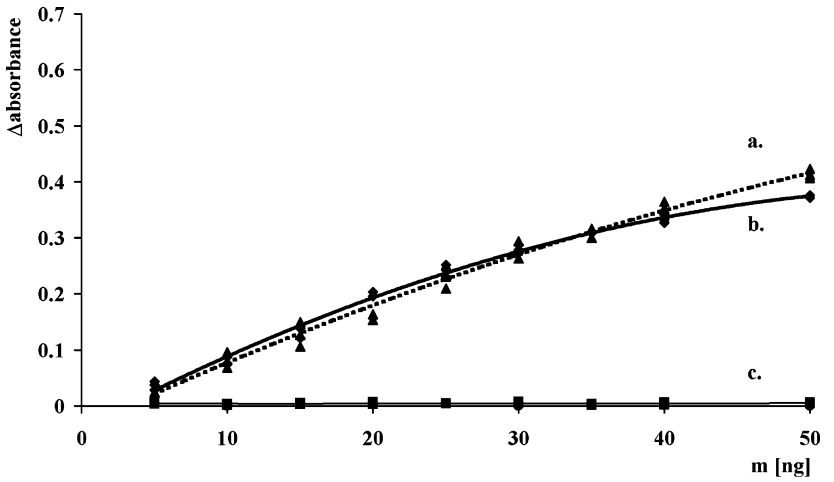

Fig. 3. Calibration curves of the enzymatic activity of lysozyme in different media. (a) Tris pH 7.4 (– –▲– –); (b) NaCl 0.1M pH 6 (—◆—); (c) water pH 6 (—■—).

The secondary structure of lysozyme was analysed by means of FTIR spectroscopy (Fig. 4). Experimental results showed a maximum at about 1650 cm^{-1} of lysozyme in dry state, or dissolved in pure water, or in Tris buffer. This absorption band was assigned to α-helix and was in accordance with the literature (Dufour and Robert, 2000). An absorption band at about 1620 cm^{-1} that was assigned to aggregation (intermolecular β-sheets) was only observed in the case of lysozyme in water. According to our other experimental results, this may correspond to an insolubilization of the protein in pure water.

The present study points out the importance of some formulation parameters on the relationship existing between the behavior of lysozyme at interfaces, its secondary structure and its enzymatic activity. According to our results, successful protein encapsulation would need to maintain a pH value far from the enzyme IEP value during the formulation to reduce, in particular, the adsorption of lysozyme molecules at the created interfaces. Moreover, buffers or salt solution must be used in order to keep intact the native secondary conformation of lysozyme, and preserve its enzymatic activity. The role of additives on the stability of lysozyme will be considered in a further work.

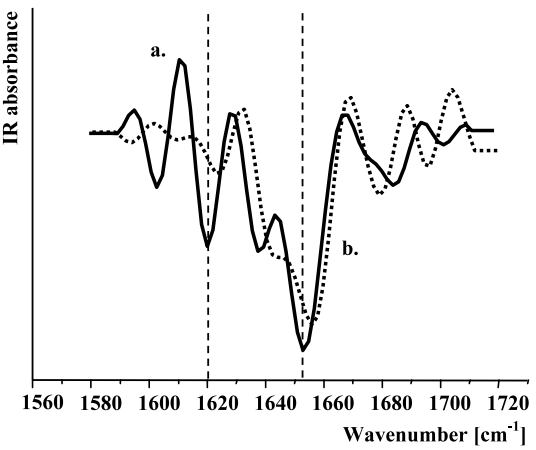

Fig. 4. Second derivate spectra of lysozyme (a) in water (——); (b) in Tris buffer (– – – – –).

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