Food Chemistry 115 (2009) 697–700

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Sustained release of lysozyme from zein microcapsules produced by a supercritical anti-solvent process

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ARTICLE INFO

Article history:
Received 5 September 2008
Received in revised form 5 December 2008
Accepted 17 December 2008

Keywords:
Delivery system
Lysozyme
Supercritical anti-solvent
Zein

ABSTRACT

Delivery systems with sustained release of antimicrobials are possible solutions to minimize the binding between antimicrobials and food matrices, thereby enhancing the efficacy of antimicrobials during shelf-lives of foods. In this work, GRAS (generally recognized as safe) corn zein was used as a carrier material and 90% aqueous ethanol was used as a solvent to microencapsulate GRAS hen egg white lysozyme using a supercritical anti-solvent (SAS) process. The microcapsules showed a continuous matrix with internal voids. The release of lysozyme was observed over 36 days at room temperature, with slower release at a higher pH between 2 and 8. At pH 4, release kinetics was further slowed by the addition of sodium chloride. Stronger molecular attraction between lysozyme and carrier zein was attributed to slower release at a higher pH and a higher ionic strength at the same pH. Our work demonstrated that SAS may be a feasible process to manufacture GRAS delivery systems to enhance antimicrobial efficacy.

1. Introduction

Many antimicrobials are hydrophobic or amphiphilic in nature (Davidson, 2001) and may bind food components such as lipids and proteins, which limits antimicrobial availability. As a result, traditional and naturally occurring food antimicrobials are used at high concentrations to achieve even moderate reductions in growth rates of pathogenic microorganisms in food products (Davidson, 2001). The incorporation of large amounts of antimicrobials may compromise sensory properties of products, not to mention the cost. Formulating delivery systems and developing microencapsulation techniques may be the key to solving the above challenges. Microemulsions and liposomes have been researched as antimicrobial delivery systems (Gaysinsky, Davidson, Bruce, & Weiss, 2005; Were, Bruce, Davidson, & Weiss, 2004). However, much work is needed for practical applications.

One promising category of scalable techniques applicable to food systems is the production of micro- and nanoparticles with supercritical carbon dioxide ($scCO_2$) as a solvent or an anti-solvent (non-solvent) (Jung & Perrut, 2001). Supercritical anti-solvent (SAS) is one technique analogous to spray drying in that a feed is continuously sprayed into $scCO_2$ (that acts an anti-solvent to most polymers including zein and lysozyme). The technique, and its variations, requires polymers dissolved in a solvent or solvent mixture (called co-solvent) miscible with CO_2. The feed stream is then sprayed into $scCO_2$. After the co-solvent in the atomized droplets is extracted out by CO_2, polymers precipitate into micro- and nanoparticles because of the insolubility in CO_2.

The potential of applying SAS in food applications has recently been shown for nisin microencapsulated in poly(L-lactide) (PLA) nanoparticles (Salmaso, Elvassore, Bertucco, Lante, & Caliceti, 2004). The hydrophobic nature of L-PLA enabled intermediate hydrophobic interactions with nisin, enabling the release in a controlled manner over 45 days. The gradually-released nisin was effective in inhibiting the growth of microorganism *Lactobacillus delbrueckii* subsp. *bulgaricus* for more than 40 days. In comparison, free nisin was only efficacious for four days. The sustained release of antimicrobials is particularly attractive for products that have a long shelf-life. However, PLA is not a food ingredient, and the organic solvents used, dichloromethane and dimethylsulfoxide, are of toxicity concerns although their concentrations in capsules produced from the SAS can be reduced to be below standards for pharmaceutical products (Elvassore, Bertucco, & Caliceti, 2001).

The objective of this work was to explore the possibility of using the SAS to manufacture food grade antimicrobial delivery systems. Corn zein (prolamines) was used as a carrier polymer for formation of the capsules matrix, and lysozyme was used as a model antimicrobial. A GRAS solvent, 90% aqueous ethanol was used as a co-solvent.

2. Materials and methods

2.1. Materials

Purified zein and ethanol (200 proof) were purchased from Acros Organics (Morris Plains, NJ). Hen egg white lysozyme, in

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doi:10.1016/j.foodchem.2008.12.063

lyophilized form with a measured specific activity of 76,133 U/mg, and the microorganism (*Micrococcus lysodeikticus*) used to measure lysozyme activity were purchased from Sigma–Aldrich Co. (St. Louis, MO). Chemicals were used directly without purification.

2.2. Apparatus and particle production protocol

The description of the apparatus and particle production procedures was detailed previously (Zhong, Jin, Xiao, Tian, & Zhang, 2008). Briefly, the system has a polymer feed stream and a CO_2 stream connected to a high pressure vessel. A sample collection basket with a 5 μm frit at the bottom is placed within the pressure vessel. During an operation, the pressure cell was filled with CO_2 and equilibrated to and maintained at a set temperature (40 °C) and pressure (10 MPa). The zein solution (5% w/v in 90% aqueous ethanol, with 0.1% w/v lysozyme) was injected via the polymer feed stream by a high pressure pump through a silica nozzle (inner diameter of 100 μm) into the pressure cell at 1 mL/min. The total solution volume injected was 15 mL. The CO_2 stream had a continuous flow of CO_2 (grade CD-50S, 99.9% pure, Airgas, Inc., Chicago, IL) at 50 g/min during equilibrium and spraying. After spraying the feed, CO_2 continued to flow through the pressure cell for 30 min to further extract residual solvent in the particles. The pressure cell was then gradually depressurized to the atmospheric pressure, followed by opening the pressure cell to harvest particles.

2.3. Measurement of encapsulation efficiency

To estimate the microencapsulation efficiency, 9 mg of zein capsules was dissolved in 5 mL 80% ethanol where zein and lysozyme were completely solubilized. The solution was then diluted to an appropriate ratio for the enzymatic assay. The encapsulation efficiency (η) was calculated by:

$$\eta(\%) = \frac{\text{Lysyzome units per mg of capsule} \times \text{Total mass of capsules}}{\text{Total lysozyme units injected}} \times 100\% \quad (1)$$

2.4. Evaluation of in vitro release kinetics

To characterize release kinetics, 27 mg capsules were dispersed in a microcentrifuge tube filled with 1.5 mL of a 66 mM potassium phosphate buffer (adjusted to pH 2–8 with 1 M HCl or 1 M KOH). The microcentrifuge tubes were continuously agitated on an end-to-end shaker (Lab Industries Inc., Berkeley, CA) at room temperature. At a predetermined time point, the dispersion was centrifuged at 5000*g* for 5 min (MiniSpin Personal, Eppendorf, Westbury, NY), and 1 mL of supernatant was sampled for assays. The remaining dispersion was supplemented with 1 mL of the corresponding fresh buffer and particles were re-dispersed for continued tests. The total amount of lysozyme in powders, i.e., 100% release, was measured by dissolving 27 mg of the powders in 15 mL 80% ethanol.

The accumulative lysozyme release at the *i*th sampling was calculated by:

$$R_{t_i}(\%) = \frac{\sum_{n=1}^{i-1} a_n + 1.5a_i}{U_o} \times 100\% \quad (2)$$

where U_o is the total lysozyme units included in the dispersion before release tests, and the prefix 1.5 before a_i is the total volume of dispersion.

2.5. Measurement of lysozyme activity

The activity of lysozyme was measured according to the protocol provided by Sigma for hen egg white lysozyme (product number L-6876). The test microorganism was *Micrococcus lysodeikticus*,

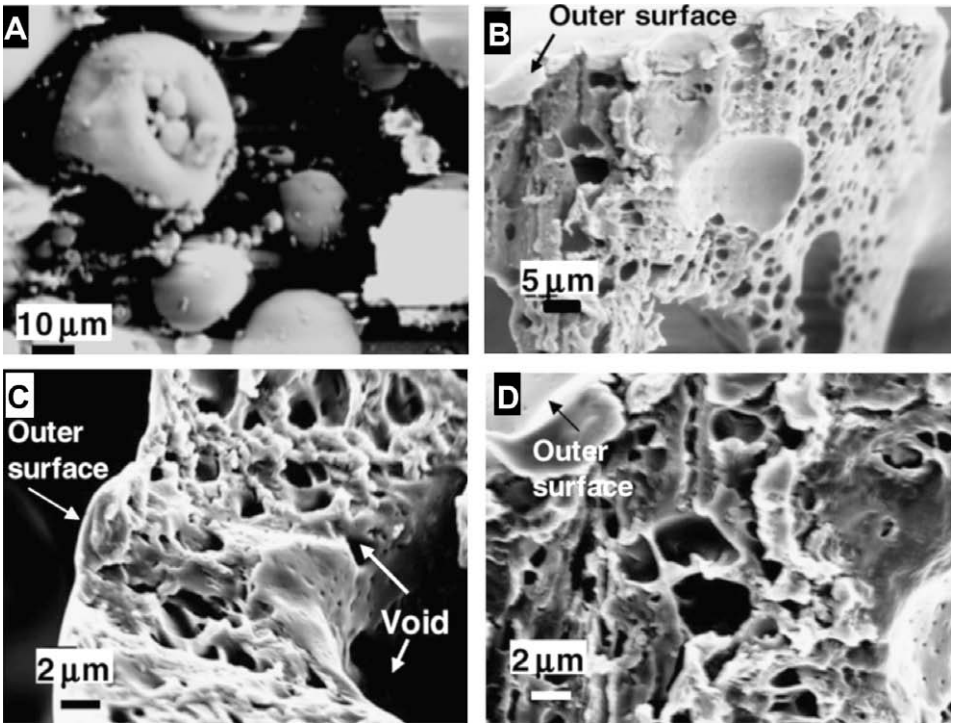

Fig. 1. SEM images of representative lysozyme-loaded zein microcapsules: surface morphology (A) and internal structure (B, C, D).

and measurements were based on the reduction rate of absorbance at 450 nm due to the lysis of cells by lysozyme at pH 6.24 and 25 °C. The lysis kinetics was measured using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA).

2.6. Scanning electron microscopy (SEM)

The SEM tests were performed with a LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany). The sample was sputter-coated with a ~5 nm-thick gold layer. Besides surface morphology, the inner structures of microcapsules were observed after fracturing capsules by a razor blade, following a literature method (Lee & Rosenberg, 2000).

3. Results and discussion

SEM images of microcapsules are shown in Fig. 1. Capsules are heterogeneous in size, ranging from as small as submicrometers to as big as ~50 micrometers in diameter (Fig. 1A). Big particles are red-blood-cell shaped but have smooth surfaces. The internal structure showed a continuous network with many heterogeneous voids (Figure B–D), but the outer particle surface is generally continuous and smooth.

The non-uniform size and irregular shape are similar to the observations from our previous study based on zein alone (Zhong et al., 2008). This may have been caused by the co-solvent used. Because water is a very polar solvent that has low miscibility with CO_2, extraction of the co-solvent (90% ethanol) by $scCO_2$ may be relatively slow, resulting in size variation and shape irregularity. When 95–100% aqueous methanol was used as co-solvents in our previous report (Zhong et al., 2008), zein particles were spherical and much more uniform, and a decreased average diameter (80–500 nm) and narrower distribution were observed when the zein solution had a higher methanol content and/or a lower zein concentration. Because lysozyme is insoluble in 95–100% methanol, production of zein nanocapsules with encapsulated lysozyme may not be feasible.

Approximately 0.5 g of powders was collected, which accounted for 65% of the non-solvent mass injected. One mg of powder contained 1063 U lysozyme, and the encapsulation efficiency was estimated to be 46.5%. The low encapsulation efficiency may arise from two factors. One is the difficulty to collect all particles (some stick to the wall of sample basket and frit), which gives a significant error for such a small amount of materials processed. The other factor may be the limitation of the frit used. The frit has a nominal opening of 5 μm, but the SEM image shows many particles smaller than 5 μm (Fig. 1A). A finer frit may be used in the future to improve the yield.

In vitro release kinetics in phosphate buffers at pH 2–8 are presented in Fig. 2A. Sustained release of lysozyme was observed at all pH conditions, with slower release kinetics (lower percentages of accumulative release at a same incubation time) at higher pH. For pH 2–5, it took a longer time to reach 100% release at a higher pH. At pH 6–8, continued release was still detected after incubation for 36 days (864 h). A slower release kinetics at a higher pH was also observed for nisin encapsulated in PLA nanoparticles, which was caused by stronger hydrophobic interactions between nisin and PLA at a higher pH (Salmaso et al., 2004). In our case, hydrophobic interactions may also have contributed to slower release at higher pH conditions because zein is water-insoluble (hydrophobic) and a higher pH closer to the isoelectric point of lysozyme, 10.5–11.0 (Cunningham, Proctor, & Goetsch, 1991), increases the hydrophobicity of lysozyme. In addition, because zein is a protein and has an isoelectric point of 6.8 (Cabra et al., 2005), zein is net

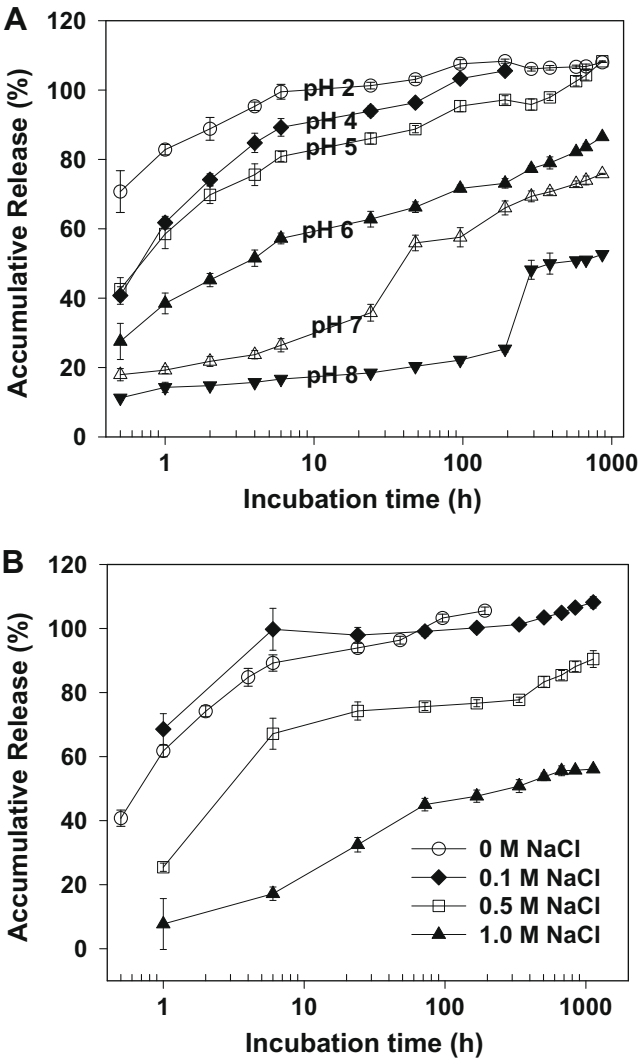

Fig. 2. *In vitro* release kinetics of lysozyme at room temperature from zein microcapsules suspended in buffers at (A) pH 2–8 and (B) pH 4 with different concentrations of NaCl. Error bars are 95% confidence intervals.

positively charged at pH 2–6 and negatively charged at pH 7 and 8. On the other hand, lysozyme is net positively charged at pH 2–8. Therefore, the overall electrostatic interactions between zein and lysozyme are repulsive at pH 2–6. However, gradual release of lysozyme was observed at pH 2–6 (Fig. 2), indicating the significance of attractive hydrophobic interactions. At pH 7 and 8, the overall electrostatic interactions between lysozyme and zein become attractive, which, together with stronger hydrophobic interactions, resulted in slower lysozyme release than at lower pH conditions.

The effect of ionic strength on lysozyme release kinetics was studied for buffers at pH 4, after adding 0.1, 0.5 and 1.0 M NaCl (Fig. 2B). Without NaCl, the accumulative release reached 100% in approximately 192 h (eight days). Addition of 0.1 M NaCl did not change too much release characteristics. When the NaCl concentration was increased to 0.5 M, continued release of lysozyme was measured even after 48 days (1152 h); even slower release was observed when the NaCl concentration was increased to 1.0 M. Increasing the ionic strength in buffers has two effects on molecular interactions between lysozyme and zein, which are both proteins and positively charged at pH 4. First, an increase in ionic strength suppresses the Debye length and thus the effective

distance of electrostatic repulsion (Israelachvili, 1992). Second, a higher ionic strength increases hydrophobicity of proteins. Combination of weaker electrostatic repulsion and stronger hydrophobic attraction contributed to slower release kinetics at higher ionic strengths in Fig. 2B.

In summary, microcapsules of zein with encapsulated lysozyme were produced using SAS. The microcapsules had a large variation in particle sizes based on SEM, possibly due to the co-solvent (90% ethanol) used. The capsules had smooth surfaces and a continuous matrix. The encapsulated lysozyme gradually released from microcapsules when suspended in aqueous buffers. A faster and more complete release was observed at a lower pH between 2 and 8. Addition of salt into buffers at pH 4 further slowed release kinetics. The correlation between molecular interactions and release profiles revealed that interactions between lysozyme and carrier zein were more attractive at a higher pH or at a higher ionic strength at the same pH. The long-time sustained release of antimicrobials, especially nearby neutral pH conditions and at the presence of salt, is particularly promising to extend the shelf-life of food products.

Acknowledgements

This research was funded by the University of Tennessee Agricultural Experiment Station through Hatch Project.

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