JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Encapsulation of Essential Oils in Zein Nanospherical Particles

NICHOLAS PARRIS,* PETER H. COOKE, AND KEVIN B. HICKS

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038

Three essential oils, oregano, red thyme, and cassia (100% pure oil), were encapsulated by phase separation into zein nanospheres. Topographical images indicated that the powders were made up of irregularly shaped particles (~50 μm) containing close-packed nanospheres. Approximately 31% of the oregano encapsulated particles had mean diameters greater than 100 nm compared to 19% for the zein alone particles. In vitro digestion of zein particles with pepsin at a concentration ratio of 10:1 was complete after 52 h in phosphate−citrate buffer, pH 3.5, at 37 °C by spectroscopic analysis. Nonenzymatic, aqueous in vitro release of essential oils from encapsulated zein particles was carried out in phosphate buffered saline at pH 7.4 and 37 °C. Release occurred at varying rates over 20 h probably from different locations within the closely packed nanospheres of different sizes. Gel electrophoresis SDS−PAGE of zein incubated with freeze-dried swine manure solids at 37 °C indicated that preformed microbial enzymes capable of digesting zein within minutes were present in the manure. Except for differences in size of nanospheres, no structural differences were resolved by several microscopic methods, suggesting that the oil and proteins phases were blended during phase separation.

KEYWORDS: Oregano; red thyme; cassia oil; SEM images; in vitro digestion; pepsin; in vitro release

INTRODUCTION

Spice essential oils are important products that are valued for their flavor and aroma. Grinding of spices breaks down the secretory cells, glands, and other tissues in which the oil is located (*1*). Essential oils are slightly soluble in water and impart to the water their odor and taste. They contain terpenes, alcohols, esters, aldehydes, ketones, phenols, ethers, and other minor compounds. Essential oils have a broad spectrum of biological activities, including growth inhibition observed against bacteria, yeasts, and fungi. Bose et al. (*2*, *3*) measured the bactericidal efficiency of several essential oils and found their effects to be high against Gram-negative bacteria but very low against Gram-positive organisms. In addition, they found that essential oils containing aldehydes were more effective than those containing alcohols. A significant number of essential oils have been shown to be inhibitory to both bacteria and fungi (*4*). Although the mechanism of action is not completely understood, essential oils containing terpenoids and phenolics are thought to act against microorganisms through membrane disruption (*5*).

Han (*6*) reviewed the use of antimicrobial packaging in the food industry. Unlike traditional packaging materials that primarily provide barrier and protective properties, antimicrobial food packaging adds a new dimension or function by allowing the antimicrobials to interact directly with the food contents. Padgett et al. (*7*) incorporated lysozyme or nisin into biodegradable protein films. They demonstrated that films made from soy protein or corn zein were effective carriers for these biocides and that packaging used as a postprocessing measure could effectively reduce the rate of bacterial growth. Carlin et al. (*8*) demonstrated the surface antimicrobial effect of sorbic acid in zein when applied as a coating to cooked sweet corn for reducing the growth of *Listeria monocytogenes*. Corn zein microspheres have been proposed as a novel delivery system for ovalbumin as a parenteral antigen (*9*), ivermectin, a highly effective parasiticide for use in farm animals (*10*), antitumor drugs for delivery into the tumor-feeding arteries (*11*), abamectin, a light-sensitive lactone used to control pests (*12*), drugs using a solvent-evaporation process for the preparation of injectable controlled-release microcapsules (*13*), and zein microspheres containing an entrapped protein polysaccharide, PS-K, used for cancer immunotherapy (*14*). To the best of our knowledge, zein has not been used as a microencapsulating agent for essential oils.

In this study we investigated the encapsulation of essential oils into zein nanospheres as a potential controlled release vehicle with site-specific delivery to maximize the antimicrobial properties of the oils.

MATERIALS AND METHODS

Materials. Decolorized corn zein was obtained from Showa Sangyo Co. Ltd., Tokyo, Japan, distributed by Chugai Boyeki (America) Corp., New York, and zein F-4000 was obtained from Freeman Industries, Tuckahoe, NY. Oregano and cassia 100% pure oils were obtained from Footeandjenks, Camden, NJ; red thyme 100% pure oil was from Frontier Aromatherapy, Norway, IA. Thymol and pepsin A (min

* Author to whom correspondence should be addressed [telephone (215) 233-6453; fax (215) 233-6559; e-mail nparris@errc.ars.usda.gov].

10.1021/jf040492p
Published on Web 05/12/2005

99.5%), EC 3.4.23.1 (1:10000), were from Sigma, St. Louis MO, and silicone fluid SF96/50 was from Thomas Scientific, Swedesboro, NJ. An Ultra Turrax T 25 high-speed dispersing apparatus was manufactured by Jamke & Kunkel GMBH & Co. KG, Staufen, Germany, and a reciprocal shaking bath (model 25) was from Precision Scientific, Winchester, VA. A spectrophotometer UV-160 was from Shimadzu Corp., Kyoto, Japan. Freeze-dried swine manure solids were generously donated by Dr. Patrick Hunt, ARS, USDA, Florence, SC.

Encapsulation of Essential Oils in Zein Nanospheres. Particles containing approximately 10−20% essential oil were prepared by dissolving 250 mg of oil and 1.0 g of zein in 15 mL of 85% ethanol. The solution was rapidly dispersed with high-speed mixing into 40 mL of water containing 0.01% silicone fluid until a single phase was formed (approximately 1 min). The opaque solution containing the encapsulated oil particles was lyophilized overnight. The dry powder, which was loosely attached to the lyophilizing bottle, was collected and stored in desiccators held at 0% relative humidity. Typical yields were between 65% and 75% of product. Zein nanospheres containing no oil were also prepared.

Electron Microscopy and Particle Size Distribution. Thin layers of powder particles were glued to carbon adhesive tabs (Electron Microscopy Sciences, Ft. Washington, PA) mounted on specimen stubs, and the samples were coated with a thin layer of gold by sputter-coating. Digital images of particle topographies were collected using a Quanta 200 FEG scanning electron microscope (FEI, Hillsboro, OR) operated in the secondary electron imaging mode.

Dispersions of powder particles in 24% aqueous ethanol solutions were applied as 10 μL aliquots to Formvar coated specimen screens and negatively stained with a solution of 2% uranyl acetate. Excess stain was adsorbed with filter paper, and the samples were air-dried before being viewed with a CM12 transmission electron microscope (FEI, Hillsboro, OR) operated in the bright-field mode. Photographic images were recorded and digitized using a SprintScan Ultra 45 film scanner (Polaroid Corp., Waltham, MA).

Particle size distributions from topographical images were processed and analyzed using the Fovea Pro v. 3 plug-ins (Reindeer Graphics, Asheville, NC) and PhotoShop v. 7 (Adobe Systems Inc., San Jose, CA). Images were flattened, and contrast and brightness were adjusted to facilitate segmentation of the nanostructural features accurately. One cycle of dilation and a watershed segmentation were performed to improve the match between the edges of the nanospheres in the original images and the apparent edges in the segmented, binary images. Finally, equivalent diameters were computed and plotted by grouping 25 measurements.

Determination of Encapsulated Oil. The amount of oil entrapped in the zein particles was quantified after all the oil was removed by extracting the oil from 10 mg of the lyophilized particles 3 times with 1 mL of ethyl acetate and comparing its absorbance to standard curves constructed at 275 nm for red thyme and oregano, at 276 nm for thymol, and at 281 nm for cassia oil. Zein particles were not soluble in ethyl acetate and did not interfere with the spectrophotometric determination.

Enzymatic Digestion of Zein Nanospheres. In vitro digestion of zein particles was accomplished using a modification of the methods described by Johnston (*15*) and Liu et al. (*10*). Zein particles (100 mg) and 10 mg of pepsin were suspended in a flask containing 100 mL of 0.01 M KH_2PO_4−citrate buffer + 0.5% Tween-20, pH 3.5. The flask was agitated at 50 rpm and 37 °C. Three milliliter samples were removed periodically. The reaction was quenched with 0.3 mL of 0.1 N NaOH, and the absorbance was measured at 280 nm. Three milliliters of sample buffer was replaced.

Nonenzymatic in Vitro Release of Essential Oils from Zein Nanospheres. Particles containing encapsulated oil, 100 mg, were placed into a flask containing 100 mL of 80% phosphate buffer saline (pH 7.4) + 24% ethanol and agitated at 50 rpm and 37 °C. Samples (3 mL) were withdrawn periodically. Their absorbance was measured at the appropriate wavelength, and 3 mL of the sample buffer was replaced.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of corn zein incubated in the presence of swine manure was carried out on a Phast System Pharmacia (Piscataway, NJ) with a phast gel of 20% acrylamide. Fifty milligrams of swine solids and 40 mg of zein was placed in five Eppendorf tubes. Similarly, swine solids were either heated at 60 °C for 30 min or autoclaved (121 °C, 15.5 psi) before mixing with zein solids. To each tube were added 0.5 mL of 10% SDS and 0.5 mL of 0.44M Tris buffer, pH 8, and then they were mixed. A total of 50 μL of 2-mercaptoethanol (2-ME) was added to the tubes after incubation at 37 °C for 0, 15, 30, 45, and 60 min and then heated at 100 °C for 10 min to stop digestion of zein. After cooling, the tubes were centrifuged at 14000*g* for 4 min, the supernatant was passed through a 50 000 nominal molecular weight limit (NMWL) filter unit, Millipore Corporation (Bedford, MA), and the filtrate was analyzed. Gels were stained with 0.2% (w/v) Coomassie R350 dye. Molecular weight standards, Bio-Rad (Richmond, CA), and their corresponding molecular weights were as follows: phosphorylase *b* 97 000; bovine serum albumin (BSA), 66 200; ovalbumin, 42 699; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme 14 400.

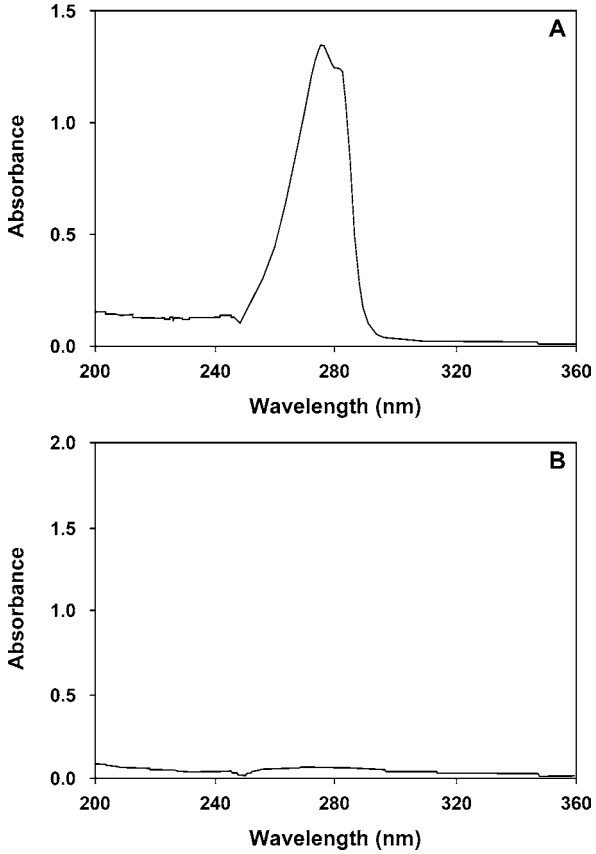

Figure 1. UV–visible spectra of ethyl acetate extract from (A) oregano encapsulated in zein nanospheres and (B) zein nanospheres alone.

RESULTS AND DISCUSSION

The preparation of essential oil encapsulated zein nanospheres by phase separation was found to be more rapid and less tedious, as an encapsulating agent for biodegradable microspheres, than solid-in-oil-in-water (S/O/W) emulsion (*16*) or chemical conjugation (*11*) methods previously reported. The oil and corn zein were dissolved in aqueous alcohol, as described in Materials and Methods, and rapidly mixed in water containing a small amount of dispersant. The entire process takes a few minutes and the suspension of particles is lyophilized overnight. The principal component in red thyme and oregano is thymol (λ_{max} = 275 nm) and cinnamaldehyde (λ_{max} = 280 nm) in cassia oil. The oils were quantitatively extracted from the zein particles using ethyl acetate without interference from zein protein (**Figure 1A**) and without interference from zein proteins, which were not soluble in ethyl acetate at the same concentration

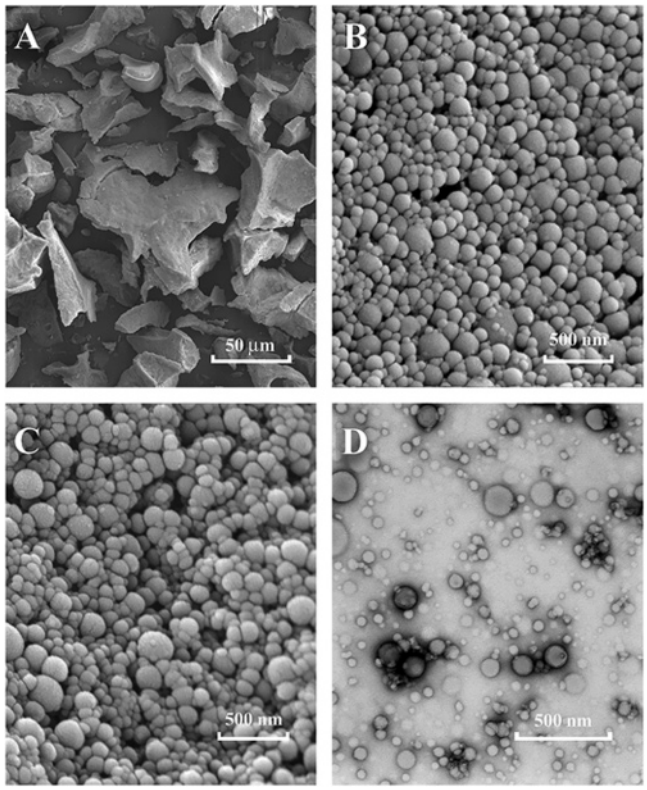

Figure 2. SEM images of zein nanospheres: (A) 500×; (B) oregano oil encapsulated in zein nanospheres at 50000×; (C) zein nanospheres at 50000×; (D) zein nanospheres dispersed in 24% ethanol at 50000×.

(**Figure 1B**). It was, however, necessary to use decolorized zein for this study because compounds present in zein from yellow corn were soluble in ethyl acetate and interfered with the determination. Typically, the yields of zein particles alone and zein particles containing oil ranged between 65% and 75%. In general the zein oil particles contained approximately 20% each of oregano, thyme, and thymol and 13% of cassia oil.

Topographical SEM images of the lyophilized suspension at low magnification (500×) indicated that the powder consists of irregularly shaped particles (**Figure 2A**). At a greater magnification (5000×) the particles appear to be composed of aggregated spheres and the outline of their surfaces forms the shape of the particle (not shown). At 50000× the individual nanospheres are visible and appear to be closely packed for both oregano encapsulated particles (**Figure 2B**) and zein particles (**Figure 2C**). Suspended nanospheres are evident (**Figure 2D**) when the lyophilized powder is dispersed in 24% ethanol, the final alcohol concentration of the preparation before lyophilizing. Although SEM images show little difference in particle size between oregano encapsulated and zein nanospheres (parts **B** and **C** of **Figure 2**), analysis of the particle size distribution in several images indicated that the oregano oil encapsulated particle population had larger nanospheres. The equivalent diameter for the oregano encapsulated zein was shifted to nanospheres with greater mean diameter than the zein particles alone (compare **Figure 3A** and **Figure 3B**). In other words, 31% of the oregano encapsulated nanospheres had mean diameters greater than 100 nm compared to 19% for the zein alone particles.

To determine the stability of the zein particles under physiological conditions, the particles were subjected to digestion in the presence of pepsin, pH 3.5. Although the pH of the stomach may be as low as 1.0, food that enters the

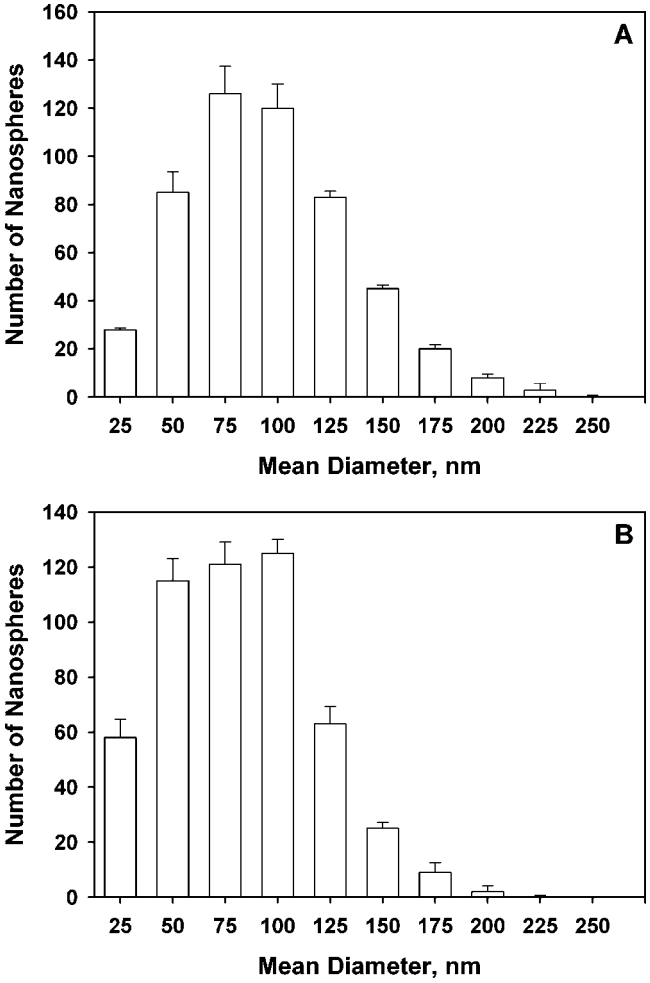

Figure 3. Particle size distribution from three preparations of (A) oregano oil encapsulated in zein nanospheres and (B) zein nanospheres alone. Particles with mean diameter less than 15 nm were not counted.

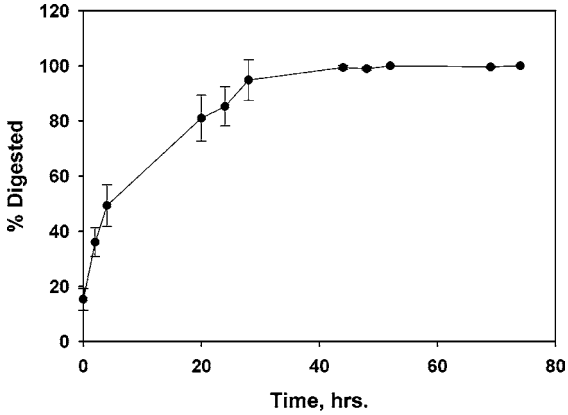

Figure 4. Enzymatic in vitro digestion of zein nanospheres with pepsin (10:1) in 0.01 M KH_2PO_4–citrate buffer + 0.5% Tween-20, pH 3.5, at 37 °C monitored at 280 nm. Data points are the mean of 3 replicates ± 1 standard deviation.

stomach may cause the pH to rise to 3.0−4.0 because of the buffering capacity of proteins (*15*). When zein samples were first introduced into the buffered solution, the particles formed aggregates that gradually dispersed and completely dissolved after 52 h (**Figure 4**). Typically, gastric emptying time for humans and some monogastric animals higher up the food chain can range between 1 and 4 h. Since, after 4 h, less than half the particles were dissolved, it is possible that zein particles

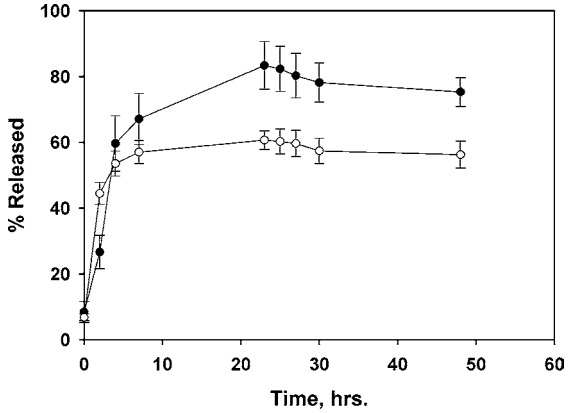

Figure 5. Nonenzymatic in vitro release of oregano oil from zein nanospheres in phosphate buffered saline, pH 7.4, at 37 °C (○) and in phosphate buffered saline, pH 7.4, at 37 °C plus 24% ethanol (●). Data points are the mean of 3 replicates ± 1 standard deviation.

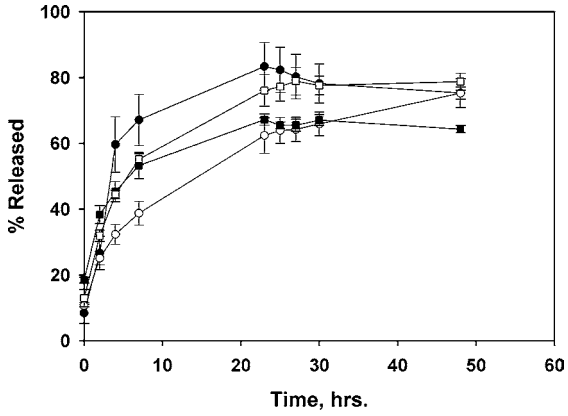

Figure 6. Nonenzymatic in vitro release of oregano (●), red thyme (○), cassia oil (■), and thymol (□) from zein nanospheres using conditions described for ● in Figure 5. Data points are the mean of 3 replicates ± 1 standard deviation.

will protect most of the essential oil from being released in the stomach; it will be released later in the small and large intestines.

After food enters the duodenum, bicarbonate ions (*15*) are secreted to neutralize the food mixture; therefore, it would be necessary to determine the effectiveness of the essential oils as an antimicrobial under neutral conditions. **Figure 5** compares the nonenzymatic in vitro release of oregano from zein nanospherical particles in phosphate-buffered saline, pH 7.2, with and without ethanol. The ethanol content of the buffer was adjusted to 24% to minimize aggregation of the particles (**Figure 2D**) and to release the oregano oil more uniformly. As can be seen in **Figure 5**, 83% of the oil was released after 24 h in the presence of ethanol compared to 61% without alcohol during the same period of time. Apparently, little if any oregano coated the particle surface because there was no initial burst of oregano released. In the presence of alcohol, approximately 60% of the oil was released at a relatively constant rate for 4 h and at a slower rate for the next 20 h, after which no more oregano oil was released. Similar nonenzymatic in vitro release patterns were observed for red thyme, cassia oil, and thymol (**Figure 6**). In the large intestine of all animals, microbes may digest the zein and increase the rate of release of essential oils. Gel electrophoresis of zein incubated in the presence of swine manure solids at 37 °C indicated that digestion of zein occurred within minutes (compare lanes 2 and 3, **Figure 7**). No zein was

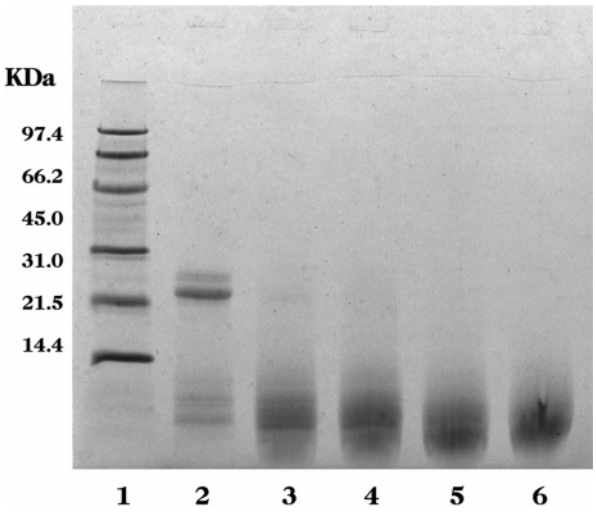

Figure 7. SDS–polyacrylamide gel electrophoresis of zein incubated with swine manure solids at 37 °C: (lane 1) molecular weight standards; (lanes 2–6) zein–manure samples after incubation for 0, 15, 30, 45, and 60 min, respectively.

detected after 30 min, and more lower molecular weight peptides were present (lanes 5 and 6). The fact that hydrolysis of zein to peptides occurred rapidly indicated that zein digestion was due to the presence of enzymes in the freeze-dried swine manure solids and not microbes. Further experimental verification was carried out by heating the solids at 60 °C for 30 min (to denature the enzymes) or autoclaving the solids (to inactivate the bacteria) before mixing with zein. In both cases the zein was not hydrolyzed, indicating that preformed microbial enzymes, in the freeze-dried manure, were responsible for hydrolysis of zein initially observed.

Attempts to stain the oil differentially with osmium tetroxide solutions on dispersed nanoparticles resulted in uniform electron density except for variations expected from particles with different diameters. Also, efforts to localize oil in powder particles by fluorescence from Nile Red stain using confocal laser scanning microscopy were equivocal because the (hydrophobic) zein particles fluoresced at equal intensities as the zein−oil powder particles over the range of emission expected for oil or lipid (*16*). Atomic force microscopy in the intermittent contact mode of operation also revealed similar patterns of organization of the nanoparticles; no differences in particle contents were observed in phase deflection images of zein−oil samples, and images of zein were similar to those of zein−oil samples when compared over micrometer-sized areas.

In conclusion, encapsulation of essential oils in zein nanospheres by phase separation is a rapid and simple method. These particles appear to have limited digestibility in the stomach, slow release in the small intestine, and more rapid release in the large intestine. They could be useful for oral or injectable administration of biological materials. In their encapsulated form, there should be little interaction of the essential oil with other components in the feed. The effects of these organisms or enzymes on release of essential oils, microbial growth inhibition, and probiotic effects will be studied before final formulations for feed applications are optimized.

ACKNOWLEDGMENT

We thank John Minutolo, Paul Pierlott. and Dr. Guy Miller (BioMatrix Internation) for their technical assistance.

LITERATURE CITED

(1) Parry, J. W. The Chemical Composition of the Spices. In *Spices Their Morphology, Histology and Chemistry*; Parry, J. W., Eds.; Chemical Publishing Co., Inc.: New York, 1962; pp 187−189.

(2) Bose, S. M.; BhimaRao, C. N.; Subramanyan, V. Influence of organic matter on bactericidal efficiency of Indian-essential oils. *J. Sci. Ind. Res.* **1949**, *8*, 157.

(3) Bose, S. M.; BhimaRao, C. N.; Subramanyan, V. Some factors affecting the bactericidal property of lemongrass oil emulsions. *J. Sci. Ind. Res.* **1950**, *9B*, 12.

(4) Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564−582.

(5) Lambert, R. J. W.; Skandamis, P. N.; Coote, P. J.; Nychas, G. J. E. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* **2001**, *91*, 453−562.

(6) Han, J. H. Antimicrobial food packaging. *Food Technol.* **2000**, *54*, 56−65.

(7) Pagett, T.; Han, I. Y.; Dawson, P. L. Incorporation of food-grade antimicrobial compounds into biodegradable packaging films. *J. Food Prot.* **1998**, *61*, 1330−1335.

(8) Carlin, F.; Gontard, M.; Reich, M.; Nguten-The, C. Utilization of zein coating and sorbic acid to reduce *Listeria monocytogenes* growth on cooked sweet corn. *J. Food Sci.* **2001**, *66*, 1385−1386.

(9) Hurtado-Lopez, P.; Somavarapu, S.; Murdan, S. Immunognicity of zein microspheres. *J. Pharm. Pharmacol.* **2003**, *55* (Suppl.), S-14.

(10) Liu, X.; Sun, W.; Wang, H.; Zhang, L.; Wang, J. Microspheres of corn, zein, for an ivermectin drug delivery system. *Biomaterials* **2005**, *26*, 109−115.

(11) Suzuki, T.; Sato, E.; Matsuda, Y.; Tada, H.; Unno, K.; Kato, T. Preparation of zein microspheres conjugated with antitumor drugs, available for selective cancer chemotherapy and development of a simple colorimetric determination of drugs in microspheres. *Chem. Pharm. Bull.* **1989**, *37*, 1051−1054.

(12) Demchak, R. J.; Dybas, R. A. Photostability of Abamectin/zein microspheres. *J. Agric. Food Chem.* **1997**, *45*, 260−262.

(13) Tice, T. R.; Gilley, R. M. Preparation of injectable controlled-release microcapsules by a solvent-evaporation process. *J. Controlled Release* **1985**, *2*, 343−352.

(14) Matsuda, Y.; Suzuki, T.; Sato, E.; Sato, M.; Kotzumi, S.; Unno, K.; Kato, T.; Nakai, K. Novel preparation of zein microsphere conjugated with PS-K available for cancer immunotherapy. *Chem. Pharm. Bull.* **1989**, *37* (3), 757−759.

(15) Johnson, L. R. Digestion and Absorption. In *Gastrointestinal Physiology*, 6th ed.; Johnson, L. R., Ed.; Mosby, Inc.: St Louis, MO, 2001.

(16) Morita, T.; Sakamura, Y.; Horikiri, Y.; Suzuki, T.; Yoshino, H. Protein encapsulation into biodegradable microspheres by a novel S/O/W emulsion method using poly(ethylene glycol) as a protein micronization adjuvant. *J. Controlled Release* **2000**, *69*, 435−444.

(17) Greenspan, P.; Fowler, S. Spectrofluorometric studies of the lipid probe, nile red. *J. Lipid Res.* **1985**, *26*, 781−789.

Received for review December 22, 2004. Revised manuscript received April 11, 2005. Accepted April 13, 2005.

JF040492P