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Production of corn zein microparticles with loaded lysozyme directly extracted from hen egg white using spray drying: Extraction studies

Minfeng Jin, P. Michael Davidson, Svetlana Zivanovic, Qixin Zhong *

Department of Food Science and Technology, The University of Tennessee, 2605 River Drive, 23 FSPB, Knoxville, TN 37996-4591, United States

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ABSTRACT

Feasibility of achieving sustained release of lysozyme by encapsulation in corn zein using spray drying was examined. To reduce the materials cost, this part of work focused on partially purifying lysozyme from hen egg white using 30–90% (v/v) aqueous ethanol, adjusted to pH 3.0–9.24. After extraction for up to 24 h and centrifugation, the purification performance was evaluated for the supernatant. Extraction was insignificantly affected by kinetics ($P = 0.6186$) but was inefficient at pH above 5.0 and above 60% ethanol. The optimal extraction was achieved at 50% ethanol and pH 3.5. Further, most of the lysozyme precipitated from the 50% ethanol (at pH 3.5) extract after increasing ethanol concentration to 90% but was completely recovered after diluting the precipitate back to 50% ethanol. Findings from this work may lead to low-cost encapsulation technologies using partially-purified lysozyme, such as spray drying.

1. Introduction

Development of delivery systems for drugs and food antimicrobials has been active in recent years due to the potential benefits offered by controlled release of bioactive compounds (Al-Nabulsi & Holley, 2007; Bezemer et al., 2000; Del Nobile, Conte, Incoronato, & Panza, 2008; Mecitoglu et al., 2006). For example, sustained release of drugs or antimicrobials may improve the bioactivity or bioavailability by reduced binding with other components that are present in the matrices where bioactive compounds are expected to function. For food antimicrobials, the enhanced bioavailability may be needed to ensure the efficacy during the shelf-life of food products.

Several studies have used microemulsions and liposomes as antimicrobial delivery systems (Gaysinsky, Davidson, Bruce, & Weiss, 2005a, 2005b; Laridi et al., 2003; Were, Bruce, Davidson, & Weiss, 2003, 2004). Microemulsions have limitations in certain applications; liposomes are expensive as food carrier materials. Therefore, solutions are still needed for technologies that utilise naturally existing carrier materials to produce delivery systems using scalable processes, so that the eventual products are affordable for food applications. In addition, low-cost, scalable processes are required for extracting antimicrobial compounds from their natural resources before being incorporated in delivery systems.

This paper presents an exemplary approach for low-cost production of particulate delivery systems of lysozyme by integration of three major considerations. The first consideration was to select a potential carrier polymer that has right chemistry to control the diffusion kinetics of encapsulated lysozyme. We chose corn zein based on our separate study that showed sustained release of the encapsulated lysozyme from zein microcapsules produced by a supercritical anti-solvent process (Zhong, Jin, Davidson, & Zivanoric, 2009). Zein is insoluble in water and is thus suitable as a carrier material for antimicrobials to be delivered in food products such as beverages. Zein is soluble in aqueous ethanol that has been used to partially purify lysozyme from hen egg white (HEW) (Gemili et al., 2007; Mecitoglu et al., 2006), one of the major natural resources of lysozyme for applications of food antimicrobials (Johnson & Larson, 2004). Therefore, our second consideration was to extract lysozyme from HEW using aqueous ethanol and then dissolve zein in the extract for direct microencapsulation. Our third consideration was to use spray drying, a commercially feasible process to produce microcapsules. The overall strategy of this work may enable low-cost production of food grade delivery systems of antimicrobials.

Lysozyme is one of the extensively used model antimicrobials due to its general stability under a variety of conditions and a broad spectrum of antimicrobial activities against bacteria such as *Bacillus stearothermophilus*, *Micrococcus* spp., *Clostridium tyrobutyricum*, and *Listeria monocytogenes* and fungi (Johnson & Larson, 2004). Lysozyme is a single polypeptide chain of 129 amino acids cross-linked by four disulphide bridges, with a molecular weight of ~14.4 kDa and isoelectric point of ~10.5–11.0 (Johnson & Larson, 2004). The antibacterial property of lysozyme originates from the ability of the polypeptide to cleave the β-1, 4-glycosidic bonds between the C-1 of N-acetylmuramic acid and the C-4 on N-acetyl-glucosamine of bacterial peptidoglycan in the cell membrane (Johnson & Larson, 2004). Among several types of naturally-occurring lysozyme (type

* Corresponding author. Tel.: +1 (865) 974 6196; fax: +1 (865) 974 7332.
E-mail address: qzhong@utk.edu (Q. Zhong).

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G, C, etc.), only the type C enzyme from HEW is currently used in food preservation (Johnson & Larson, 2004) because of the relative ease of purification, low toxicity, low effective usage levels and low interference on sensory qualities of foods.

Different procedures or methods have been developed to purify lysozyme from HEW, including ion exchange chromatography (Banka, Petrovic, & Becarevic, 1993; Jiang, Wang, Chang, & Chang, 2001), gel-filtration chromatography (Islam, Kite, Baker, Ching, & Islam, 2006), dye-binding chromatography (Tejeda-Mansir, Montesinos, Magana-Plaza, & Guzman, 2003), membrane separation (Chiu, Lin, & Suen, 2007; Lee, Woo, & Park, 2003), reverse micelles (Noh & Imm, 2005), magnetic cation exchange (Safarik, Sabatkova, Tokar, & Safarikova, 2007), and ethanol precipitation (Gemili et al., 2007; Mecitoglu et al., 2006). Among these techniques, partial purification by the ethanol precipitation method has the advantages of low-cost, easy availability and convenience for processing. Partially-purified HEW lysozyme, compared to the more expensive purified, lyophilised product, can be used to reduce the materials cost for manufacturing delivery systems of lysozyme.

The ethanol precipitation method was used to partially purify lysozyme from HEW in a few studies for preparation of antimicrobial-loaded films (Jiang et al., 2001; Mecitoglu et al., 2006). In these studies, HEW proteins were precipitated at pH 4, further facilitated by 30% ethanol that acted as a non-solvent for some HEW proteins. However, 30% ethanol is not a solvent for zein. This paper was thus focused on extraction of lysozyme from HEW at various conditions, i.e. kinetics, pH, and ethanol concentrations to optimise extraction of lysozyme and removal of HEW proteins. The information from this part of work was used to prepare samples by directly dissolving zein in HEW extracts for spray drying, to be reported elsewhere.

2. Materials and methods

2.1. Materials

Fresh hen eggs were obtained from a grocery store. Ethanol (200 proof) was the product from Acros Organics (Morris Plains, NJ). Purified lyophilised HEW lysozyme (catalog number L6876) and *Micrococcus lysodeikticus*, the indicator microorganism for measuring lysozyme activities, were purchased from Sigma–Aldrich (St. Louis, MO). Bovine serum albumin and Coomassie® Plus Reagent (product 23236) were purchased from Pierce Biotechnology (Rockford, IL). Polyacrylamide gels, 15% Tris–HCl Ready Gel® Precast gels, were ordered from Bio-Rad Laboratories (Hercules, CA). Salts, bases, acids and other chemicals were from Fisher Scientific (Pittsburgh, PA).

2.2. Extraction protocol

The extraction protocol was a slight modification from a literature method (Jiang et al., 2001; Mecitoglu et al., 2006). Egg white was carefully separated from hen eggs and mixed with a 0.05 M NaCl solution at a volume ratio of 1:2 to threefold dilution. The pH of suspension was adjusted from 9.24 initially to 3.0–7.0 with 1 M acetic acid. An appropriate amount of ethanol was added to obtain a final ethanol concentration of 30-90% (v/v). While being continuously agitated, 1 ml of the suspension was sampled at a predetermined time point (i.e. shortly after adding ethanol and after mixing for 1–24 h) and centrifuged at 14,500*g* for 5 min (model MiniSpin Personal, Eppendorf, Westbury, NY). The supernatant was transferred for the following analyses.

2.3. Determination of lysozyme activity

Lysozyme activities in extracts were determined according to a method of Sigma–Aldrich (St. Louis, MO) for product HEW lysozyme (catalog number L6876), with slight modification in the measurement period and sample and reagent volumes. *Micrococcus lysodeikticus* was used as the test microorganism, suspended in a 66 mM potassium phosphate buffer at pH 6.24. The lysis of cells by lysozyme resulted in the reduction of absorbance, and the kinetics of absorbance reduction was used to estimate lysozyme activity. Experimentally, the absorbance of the suspension of the test microorganism after addition of lysozyme samples was monitored at 450 nm for 3 min by using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA) with a thermal jacket set at 25 °C. One unit of lysozyme is defined as the absorbance reduction rate of 0.001 per min at the above test conditions. Triplicate tests were performed for each sample.

2.4. Determination of total protein content

The total protein content of samples was determined by the Bradford method with a Coomassie® Plus Protein Assay kit (product 23236, Pierce Biotechnology, Rockford, IL), and bovine serum albumin was used as a reference. The absorbance was measured at 595 nm, and triple tests were performed for each sample.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Proteins in extracts were separated at denatured conditions on a 15% Tris–HCl gel (Ready Gel® Precast Gel from Bio-Rad Laboratories, Hercules, CA). Electrophoresis was performed with a Protean® II xi 2-D Cell (Bio-Rad) at a constant voltage of 200 V until the protein marker standards reached the gel bottom. The staining and destaining procedures followed the instruction manual of the Tris–HCl gel. The steps included staining in a mixture of methanol, acetic acid and Coomassie® Blue, destaining in a mixture of methanol and acetic acid until satisfactory visibility of protein bands, and rinsing with distilled water. The destained gels were dried and photographed.

2.6. Data analyses

Data analyses were carried out by using SAS software (version 9.1, SAS Institute, Cary, NC). Significant differences were analysed with a least-significant-difference ($P < 0.05$) mean separation method (LSD) from three replicates. Response surface regression was used to analyse the importance of independent variables (i.e. extraction time, pH, or ethanol concentration) for dependent variables (the extracted lysozyme units, total protein content, etc.) and to predict the optimum extraction conditions.

3. Results and discussion

3.1. Preliminary screening of extraction parameters: kinetics and pH

3.1.1. Extraction kinetics

Extract kinetics at various pH (3.5–9.24) and ethanol (30–50% v/v) proportions are plotted in Fig. 1. During extraction up to 24 h, there were slight fluctuations in detected lysozyme activities at different extraction time points for each pH and each ethanol concentration. To statistically understand the effect of kinetics on lysozyme extraction, a response surface analysis was performed. A slight increase in lysozyme extraction up to 24 h was predicted based on the actual experimental data (plot not shown). However, the response surface regression analysis showed the insignificance of extraction time ($P = 0.6186$, >0.05).

An extraction time of 6 h was sufficient to extract most lysozyme at all studied pH and ethanol concentrations, and was chosen

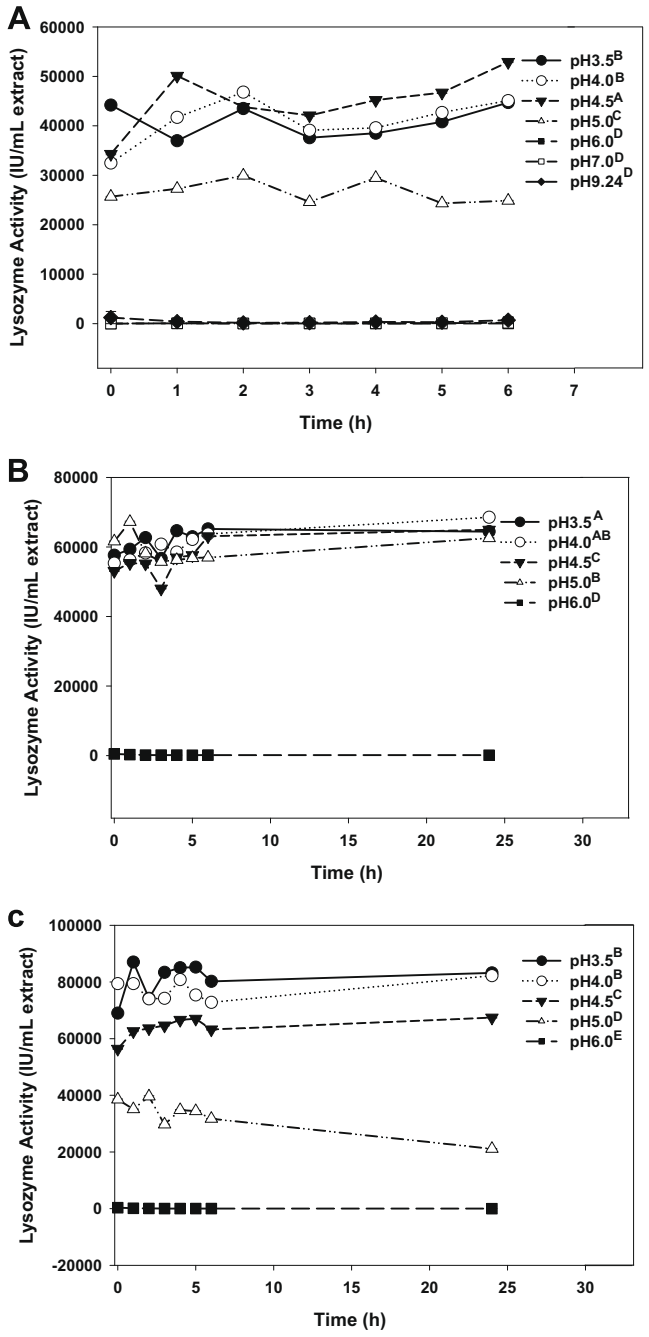

Fig. 1. Kinetics of lysozyme extraction from hen egg white adjusted to different pH conditions and ethanol concentration of: (A) 30%, (B) 40%, and (C) 50%. Superscripts with different letters in the legend indicate that data groups from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% confidence intervals from three independent measurements.

to screen extraction conditions in the next step. The selection from the tested pH range (3.5–9.24) and ethanol concentrations (30–50%) was similar to the literature recommendation (Gemili et al., 2007): 4–6 h for 30% ethanol and 6–8 h for 40% ethanol when the extraction pH was fixed at 4.0.

3.1.2. Extraction pH

At the three studied ethanol concentrations, i.e., 30%, 40% and 50%, the detected lysozyme activity was all below 500 IU/mL when the extraction pH was 6.0 and above (Fig. 1A). The inefficient extraction at pH 6 or above may have been caused by the co-precipitation of lysozyme with other HEW proteins, due to electrostatic attraction. Ovalbumins, the most abundant protein in HEW, have an isoelectric point (pI) of 4.7 (Moritz & Simpson, 2005), while lysozyme has a pI of ~10.5–11.0 (Johnson & Larson, 2004). Therefore, ovalbumins are overall positively charged below pH 4.7 and negatively charged above pH 4.7, while lysozyme always has a net positive charge at pH 3.5–9.24. The electrostatic attraction between lysozyme and ovalbumins may have caused the co-precipitation of lysozyme with ovalbumins: a reduced efficiency at pH 5 (ovalbumins are weakly negatively-charged) and inefficiency at pH 6 and above (when ovalbumins become more extensively charged). In addition, some precipitated HEW proteins are hydrophobic and their attraction with lysozyme may also be strengthened due to increased hydrophobicity of lysozyme at a pH condition closer to the pI of lysozyme.

The statistical analysis showed that pH is a significant independent variable for lysozyme extraction, as indicated by the significance of both linear and quadratic effects ($P < 0.0001$). The response surface regression predicted higher yields at lower extraction pH conditions (plot not shown). The analysis also showed that ethanol concentration (30–50%) and the interaction between pH and ethanol concentration were significant ($P < 0.0001$). However, the effect of ethanol concentrations in Fig. 1 was not compared because different batches of HEW were used for each ethanol concentration. We addressed this issue by reinvestigating effects of ethanol concentration and pH using HEW from a same batch of HEW at a fixed extraction time of 6 h.

3.2. Optimisation of extraction conditions

The same one batch of HEW was used to optimise extraction solvent conditions. In addition to 30–50% ethanol, ethanol concentrations greater than 60% (v/v) were studied for the possibility of using the centrifuged supernatant to directly dissolve zein for spray drying. A pH value higher than 5.0 was not studied further because of the poor extraction (Fig. 1). The extraction variables were compared using three parameters: number of lysozyme units (indicative of extractable lysozyme that is bioactive), total protein concentration in extracts (relative indication of the amount of impurities, i.e. non-lysozyme proteins), and specific activity (units of lysozyme per unit mass of protein, indication of purity).

3.2.1. Lysozyme activity in extracts

The amount of lysozyme in extracts, compared on the basis of extracted lysozyme units per mL HEW, showed that an ethanol concentration of 30–50% was generally effective for extraction (Fig. 2A). The highest lysozyme activity was detected in extracts prepared at pH 3.5 and 4.0 with 50% ethanol. When the pH was decreased to 3.0, the extraction became less efficient, especially for the 50% ethanol treatment. The SDS–PAGE experiment showed a band corresponding to lysozyme extracted at pH 3.0 and 50% ethanol (Fig. 3A), indicating the presence of lysozyme in the supernatant; the much lower activity tested may have been caused by the denaturation of lysozyme that resulted in the loss of activity.

When the ethanol concentration was increased to 60% and 70%, lysozyme activities in extracts dramatically decreased at all studied pH values (i.e. pH 3.0–4.5). When the ethanol concentration was increased to 90%, lysozyme activity in the supernatant fell below the detection limit of the assay method.

3.2.2. Total protein concentration in extracts

The total protein concentration in the supernatant decreased significantly ($P = 0.0007$) with an increase in ethanol concentration (Fig. 2B), while the effect of extraction pH was not significant ($P = 0.2268$). SD–PAGE showed similar band patterns and intensities for 30–50% ethanol treatments (Fig. 3B, Lanes 2–4). The band

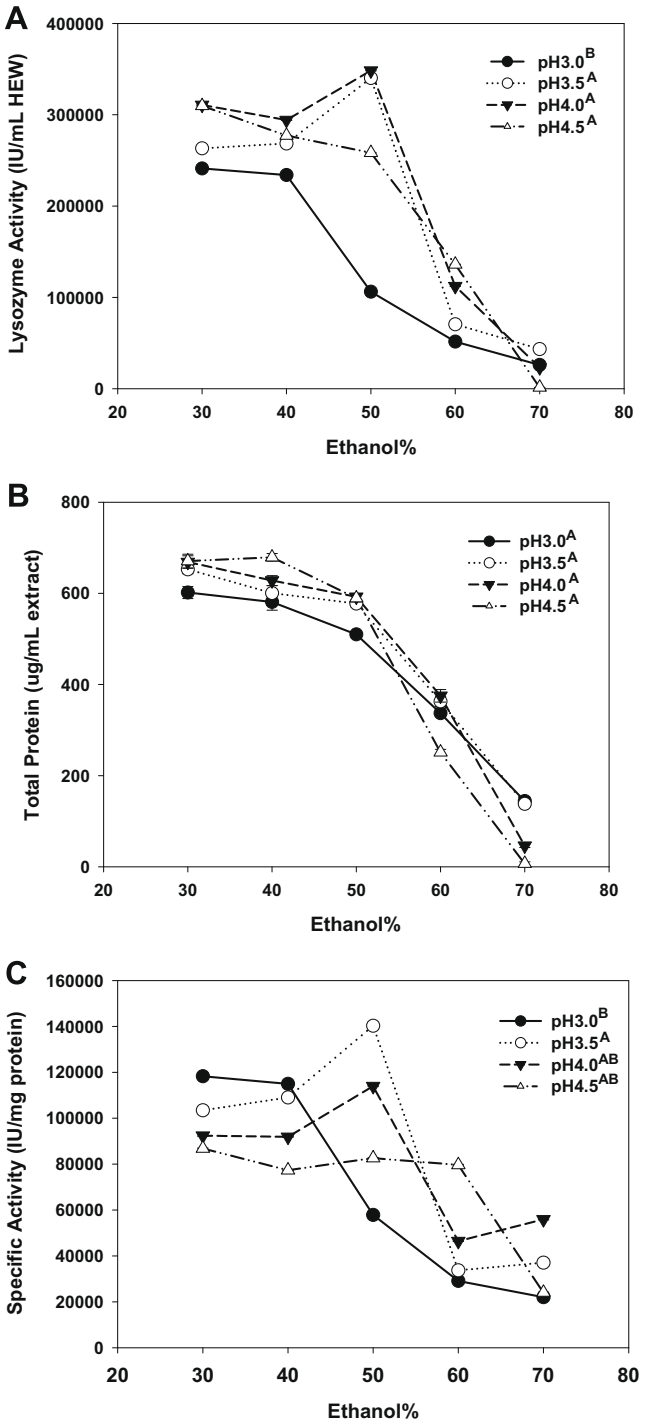

Fig. 2. Purification performance of 6-h extraction at various pH and ethanol conditions: (A) extractable lysozyme units (converted to per mL hen egg white, HEW), (B) total protein content in extracts, and (C) specific activity of extracts. Superscripts with different letters in the legend indicate that data groups from the corresponding pH conditions are statistically different ($P < 0.05$). Error bars are 95% confidence intervals from three independent measurements.

patterns were similar but the intensity was decreased when the ethanol concentration was increased to 60% (Fig. 3B, Lane 5). For the 70% ethanol treatment, there was only a visible band corresponding to lysozyme (Fig. 3B, Lane 6). However, it should be noted that volumetric protein concentrations (mg protein/mL) are expected to be lower when the same sample is diluted to higher ethanol concentrations with a larger amount of ethanol. We further examined the effect of ethanol concentration on precipitating HEW proteins by converting the results to total proteins extracted from each millilitre of HEW (Fig. 4). The data indicate that more HEW proteins precipitated at a higher ethanol concentration.

3.2.3. Specific activity of extracts

At pH 3.0, the effect of ethanol concentration on specific activity (Fig. 2C) was similar to that on the extracted lysozyme activity (Fig. 2A): a higher specific activity at a lower ethanol%. Similar changing trends of lysozyme activity and specific activity were observed at pH 3.5 and 4.0. The specific activity of extracts at pH 4.5 was unchanged when ethanol concentration was increased from 30% to 60% (Fig. 2C), different from a monotonic decrease in the extracted lysozyme activity (Fig. 2A). This observation indicates that, at pH 4.5, simultaneous precipitation of lysozyme (numerator in the definition of specific activity) and HEW proteins (denominator in the definition) occurred proportionally at 30–60% ethanol. Comparing all the studied conditions, the highest specific activity (best purity) and most lysozyme activity (best yield) was observed to be ~1,40,000 U/mg at a combination of pH 3.5 and 50% ethanol, which was concluded as the recommended extraction conditions for future work. A specific activity of 77,000 U/mg was reported when lysozyme was partially purified from HEW using 30% ethanol and pH 4.0 (Jiang et al., 2001), which is comparable with a specific activity of ~92,000 U/mg in our work (Fig. 2).

3.3. SDS–PAGE

SDS–PAGE was performed to examine the effects of pH and ethanol concentration on protein types in extracts based on molecular weight (MW). HEW has five major proteins – ovalbumins (64%, MW 45 kDa), conalbumin (12%, MW 76 kDa), lysozyme (3.5%, MW 14.4 kDa), ovomucin (11%) and avidin (0.05%, MW 68.3 kDa) (Li-Chan, Powrie, & Nakai, 1995). Crude ovomucin has two subunits, α- and β-. The α-ovomucin has two types: α1- and α2- with a MW of 150 and 220 kDa, respectively, based on SDS–PAGE; β-ovomucin has a MW of 400–720 kDa (Hiidenhovi, 2007).

Our SDS–PAGE result showed three major protein bands (Fig. 3), similar to a literature study (Raikos, Hansen, Campbell, & Euston, 2006) that suggested these proteins to be conalbumin (76 kDa), ovalbumin (45 kDa), and lysozyme (14.4 kDa). The largest band on the polyacrylamide gels may be residual ovalbumin after ethanol precipitation, because it is the most abundant among the five major HEW proteins and has a MW of ~40–50 kDa. However, the exact identification of these bands needs additional techniques such as Western blotting, 2-D gel electrophoresis and MALDI-TOF mass spectrometry (Raikos et al., 2006) and is beyond the scope of this work. Nevertheless, SDS–PAGE helped understand lysozyme extraction, as discussed in the above relevant sections.

3.4. Effect of ethanol concentration on the measured lysozyme activity

The possibility of enhanced lysozyme activity due to the presence of ethanol (Mecitoglu et al., 2006) may need to be examined for our extraction studies. We addressed this concern by measuring the activity of purified Sigma HEW lysozyme dissolved in 0–90% ethanol. The measured specific activities are listed in Table 1. The highest lysozyme activity was detected for the 60% ethanol treatment, followed by the 0% and 70% ethanol treatments, while there was no statistical difference for other ethanol concentrations (30%, 40%, 50% and 90%). The exact mechanism of enhanced lysozyme activity in 60% ethanol is beyond the scope of this work. Nevertheless, the effect of ethanol on the measured lysozyme activities should not affect the conclusions above, because 60% and 70% ethanol was observed to be less efficient than other lower ethanol concentrations (Fig. 2A).

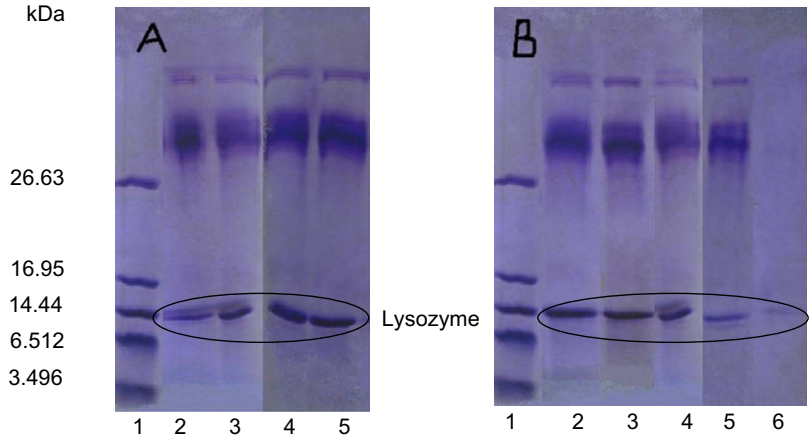

Fig. 3. Effects of solvent conditions on lysozyme extraction analysed by SDS–PAGE. (A) Effect of pH (Lane 1: molecular weight standards; Lanes 2–5: extracts with 50% ethanol at pH 3.0, 3.5, 4.0, and 4.5, loaded with 1.53, 1.73, 1.78, and 1.77 μg protein, respectively), and (B) effect of ethanol concentration (Lane 1: molecular weight standards; Lanes 2–6: extraction with 30%, 40%, 50%, 60%, and 70% ethanol at pH3.5, loaded with 1.96, 1.80, 1.73, 1.09, and 0.41 μg protein, respectively).

3.5. Precipitation of lysozyme at high ethanol concentrations

As shown in Fig. 2A, the efficiency of lysozyme extraction decreased dramatically when the ethanol concentration was increased to 60 and 70%, and no activity was detected in extracts when the ethanol concentration was increased to 90%. However, 60–90% ethanol is of interest to our work because zein is soluble in this ethanol concentration range. Because little or no lysozyme activity was detected in the supernatant after centrifugation of extracts prepared with 60–90% ethanol, the direct use of supernatant for microencapsulation is inappropriate. The situation may be resolved by first extraction with 50% ethanol (at pH 3.5) to maximise extraction of lysozyme and removal of HEW proteins, followed by increasing ethanol concentration to dissolve zein and subsequently spray drying the slurry. The two-step strategy should not affect our eventual goal of microencapsulation, if sustained release of lysozyme can be achieved. The question however remains regarding whether or not the precipitated lysozyme maintains activity or can be recovered after increasing the ethanol concentration above 60%.

To address the question of lysozyme recovery after adjustment of ethanol concentration, an extract was prepared by extraction for 6 h at 50% ethanol and pH 3.5 to remove a portion of HEW proteins.

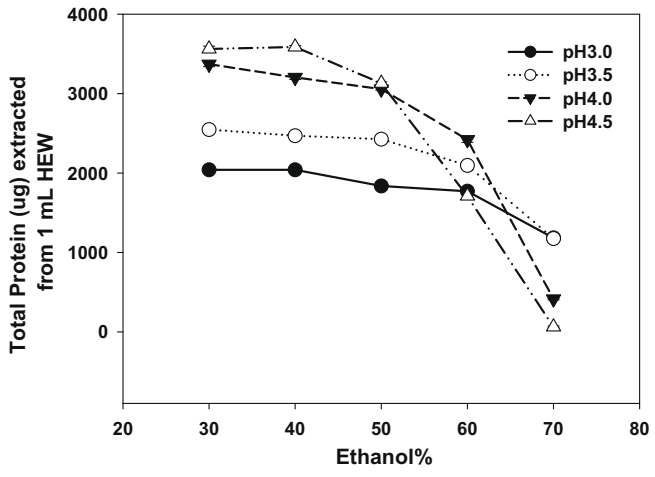

Fig. 4. Comparison of amount of proteins extracted from 1 mL hen egg white (HEW) at various pH and ethanol conditions. Error bars are 95% Confidence intervals from three independent measurements.

The supernatant after centrifugation (Treatment T0) was transferred and added with ethanol to 90%. The slurry was then centrifuged and a portion of supernatant (Treatment T1) was transferred for measurement of lysozyme activity. The remainder suspension was readjusted to 50% ethanol using deionized water (Treatment T2). A control was prepared using purified Sigma lysozyme, processed identically to the HEW extract.

The sample Treatment T1 showed significant precipitation, while samples Treatment T0 and Treatment T2 were clear, indicating that the precipitate at 90% ethanol was re-dissolved after the ethanol concentration was reduced to 50%. The purified Sigma lysozyme samples did not show any precipitation. The measured lysozyme activities of these treatments, after consideration of dilution factors and normalisation by the lysozyme activity before adjustment of ethanol concentration, are plotted in Fig. 5. The purified lysozyme did not show variations after decreasing or increasing the ethanol concentration, consistent with the results in Table 1. For the HEW extract, lysozyme activity in the supernatant after adjusting to 90% ethanol (Treatment T1) was below the detection limit of the assay method. However, after adjusting the ethanol concentration back to 50% (Treatment T2), an activity 229% that of the Treatment T0 was measured. Because the ethanol content had no effect on lysozyme activity, based on purified Sigma lysozyme, it may be possible that some lysozyme loosely bound with HEW proteins and was not available initially for lysing the test microorganism during the enzymatic assay; upon precipitation and dilution, this portion of lysozyme was released after re-dissolution and showed the cell-lysing activity. Nevertheless, Fig. 5 indicates the maintained lysozyme activity after changes of ethanol concentration in the HEW extract. In addition, Fig. 5 indicates an easy approach to concentrate lysozyme: the ethanol concentration

Table 1
Specific activity of purified Sigma lysozyme dissolved in different ethanol concentrations.

Ethanol concentration (%)	Specific activity (IU/mg)[a]
0	76133 ± 1473^{B}
30	63733 ± 3106^{C}
40	67333 ± 2893^{BC}
50	64696 ± 2503^{C}
60	113103 ± 10027^{A}
70	75454 ± 1388^{B}
90	63333 ± 5326^{C}

[a] Numbers are averages ± standard deviations from three measurements. Numbers with different superscripts are statistically different.

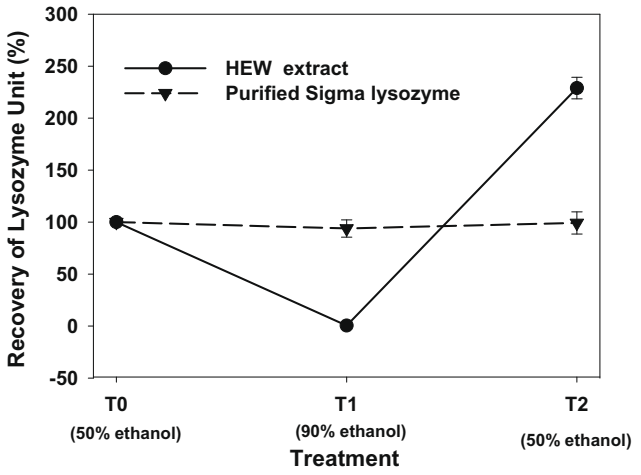

Fig. 5. Comparison of lysozyme activity in a 6-h extract prepared with 50% ethanol, pH 3.5 or a purified lysozyme dissolved in 50% ethanol (Treatment T0), in the supernatant after centrifuging a sample prepared by adjusting the Treatment T0 to 90% ethanol (Treatment T1), and in a sample after readjusting the Treatment T1 back to 50% ethanol (Treatment T2). The data points are normalised by the lysozyme activity of the Treatment T0, after consideration of dilution factors. Error bars are 95% confidence intervals from three independent measurements.

of HEW extracts can be increased to 90%, and the precipitates after centrifugation (and decanting the supernatant) can then be re-dissolved to different concentrations of lysozyme and ethanol.

The findings suggest that 50% ethanol and pH 3.5 can be used to extract lysozyme from HEW to remove a significant portion of HEW proteins. The extract can be adjusted to 60–90% ethanol to dissolve zein. The slurry can then be used for spray drying, to be reported elsewhere.

4. Conclusions

The pH and ethanol concentration were critical for extraction of lysozyme from HEW, while kinetics was not a significant parameter. At an ethanol concentration of 30–50%, poor extraction was observed at pH 5.0 and above. Poor extraction was also the case for extraction with an ethanol concentration higher than 60%. The recommended extraction condition was 50% ethanol at pH 3.5 for 6 h because of a good extraction and relatively high purity. Lysozyme in the extract prepared with 50% ethanol at pH 3.5 precipitated after the ethanol concentration was increased to 90%, but the lysozyme activity was completely recovered after dilution of the precipitates using deionized water to 50% ethanol. Findings from this part of the work may lead to low-cost encapsulation technologies using partially-purified lysozyme.

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