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Enzyme Dehydration Using MicroglassificationTM Preserves the Protein’s Structure and Function

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ABSTRACT: Controlled enzyme dehydration using a new processing technique of Microglassification1,4 has been investigated. Aqueous solution microdroplets of lysozyme, α-chymotrypsin, catalase, and horseradish peroxidase were dehydrated in n-pentanol, n-octanol, n-decanol, triacetin, or butyl lactate, and changes in their structure and function were analyzed upon rehydration. Water solubility and microdroplet dissolution rate in each solvent decreased in the order: butyl lactate > n-pentanol > triacetin > n-octanol > n-decanol. Enzymes MicroglassifiedTM in n-pentanol retained higher activity (93%–98%) than n-octanol (78%–85%) or n-decanol (75%–89%), whereas those MicroglassifiedTM in triacetin (36%–75%) and butyl lactate (48%–79%) retained markedly lower activity. FTIR spectroscopy analyses showed α-helix to β-sheet transformation for all enzymes upon Microglassification1,6, reflecting a loss of bound water in the dried state; however, the enzymes reverted to native-like conformation upon rehydration. Accelerated stressed-storage tests using MicroglassifiedTM lysozyme showed a significant (p < 0.01) decrease in enzymatic activity from 46,560 ± 2736 to 31,060 ± 4327 units/mg after 3 months of incubation; however, it was comparable to the activity of the lyophilized formulation throughout the test period. These results establish MicroglassificationTM as a viable technique for enzyme preservation without affecting its structure or function. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: dehydration; enzymes; FTIR; microparticles; protein formulation; protein structure; drying

INTRODUCTION

The development of enzyme formulations that are stable during shipping and long-term storage is one of the most important considerations in developing enzymes as commercial products.1 The chemical complexity and marginal stability of higher order or multi domain protein structure often presents significant stability problems.3 For ease of preparation and handling, liquid preparations are generally preferred. However, the presence of water in liquid preparations has the potential to encourage structural changes or even serve as a reactant for chemical degradation.2 Stress conditions in liquid media, such as heating and freezing, shaking, pH variations, and changes in amino acid side-chain structures may result in undesirable by-products and compromised functionality.4,5 Shear forces associated with mechanical shaking can partition proteins to the air–water interface and thus expose them to degrading interfacial tensions. Gidalevitz et al.4 studied the behavior of glucose oxidase, alcohol dehydrogenase, and urease, and found that the exposure of enzymes to such tensions and the more hydrophobic air phase encouraged partial unfolding. Variations in liquid media pH can change the oxidation potential of oxidants, altering the binding affinity between the catalytic metal ions and ionizable amino acids, and modify the stability of the oxidation intermediates.8 Therefore, when the inherent stability and function of the protein, or the economics and logistics of product shipping (e.g., maintaining a cold chain) and use (e.g., loss of soluble protein because of surface or interfacial adsorption) preclude storage in the liquid form, the protein product is generally dried and stored in a solid form.6 Traditional drying methods are often accompanied by a modification in the product’s thermal history, which may impact thermo-mechanical properties, such as diffusivity and stability, as well as product quality or activity.7 Enzymes such as lysozyme and catalase undergo an irreversible loss in activity when freeze-dried in the absence of excipients, with activity losses of up to ~7% for lysozyme and 35%–50% for catalase.8 In the food processing industry, spray drying of whey proteins has been known to result in partial denaturation and increased insolubility of the dried product, even at modest outlet temperatures of 60 °C.9,10 Freeze drying lignin peroxidase, an enzyme used in degradation of lignin in wood, has been shown to result in ~30% loss of activity, although the inclusion of sucrose improved activity retention.11 Lyophilization and spray drying methods have been very well developed and customized within the pharmaceutical industry; the use of excipients and stabilizers in therapeutic protein formulations have generally been very successful in minimizing loss in functional activity.1,12 Nevertheless, challenges remain in addressing the dehydration of complex biologics, such as recombinant human interferon-γ, which is prone to aggregation induced, in part, because of adsorption at air–liquid and solid–air interfaces during the drying procedure.13,14 Therefore, a successful drying technique should be able to minimize process dependent stresses such as thermal, mechanical, interfacial adsorption, and pressure induced instability without irreversible changes to enzyme function.15

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Environmental changes involving temperature, salt concentration, or addition of hydrophobic interfaces are needed to relieve stresses imposed by dynamic structural and environmental changes involving temperature, salt concentration, or addition of hydrophobic interfaces. The removal of the surrounding water, in particular, the partial removal of water of hydration, can improve protein storage and stability by restricting intra-domain mobility and alleviating water-dependent physicochemical degradation pathways. The loss of enzyme functionality is a two-step phenomenon that involves reversible unfolding followed by kinetically irreversible steps that may lead to aggregation or covalent structural changes. We have recently reported that a new dehydration technology, called Microglassification, can controllably remove water from proteins leading to rapid solidification at ambient temperature. Studies on single lysozyme microdroplet dehydration have shown that by adjusting the water activity of an organic medium, such as n-decanol, the microdroplet can be dehydrated to sub-monolayer hydration levels in the form of a solid microsphere having a concentration of 700–1150 mg/mL. Globular proteins, such as bovine serum albumin (BSA) can be similarly dehydrated to sub-monolayer water coverage with concomitant and reversible structural changes upon rehydration and only minor levels of irreversible aggregation. In this work, we report the continued evaluation and optimization of Microglassification on a series of enzymes: lysozyme, α-chymotrypsin, catalase, and horseradish peroxidase (HRP), using a range of dehydrating solvents. The enzymatic activity of each of the Microglassified enzymes has been analyzed upon rehydration and compared with the activity of a commercially available lyophilized formulation. Moreover, comprehensive analyses of the effect of Microglassification on enzyme secondary structure have been carried out using Fourier transform infrared spectroscopy (FTIR). In a preliminary study, we have also performed accelerated storage tests to evaluate the effect of stressed storage conditions on the structure and function of Microglassified lysozyme powder.

MATERIALS AND METHODS

Materials

All reagents and enzymes including chicken egg white lysozyme, bovine pancreas α-chymotrypsin, bovine liver catalase, and HRP were purchased in lyophilized form from Sigma–Aldrich (St. Louis, Missouri). The solvents n-pentanol, n-octanol, n-decanol, triacetin, and butyl lactate were used as received without further purification. Enzyme solutions were obtained by dissolving the lyophilized samples in phosphate buffer saline.

Water Solubility in Organic Phase

Prior to dehydrating the protein microdroplets, it is necessary to measure the solubility of water in the dehydrating solvents in order to determine water-saturation levels and fractional water saturation (f). The f value represents the ratio of the experimental water concentration to saturated water concentration in a particular solvent and thus establishes the correlation between the water removal and its chemical potential. Water solubility was measured and verified using two independent techniques: Refractive index method and Karl Fischer titration.

Refractive Index Method

A series of standard solutions (water-in-solvent) of the drying solvents were prepared by mixing the drying solvent with DI water (0–100 mg/mL). The solution was allowed to equilibrate at room temperature (23°C) for 24 h and its refractive index was measured in triplicate using a digital DR-A1 Abbe refractometer (Atago, Bellevue, Washington). The refractive indices were adjusted to the corresponding value at 22°C using the equation:

\[ n_w = n_x - 0.00045 (T_x - 22) \]

where nx is the refractive index of the solution at temperature Tx. The refractive index values were plotted against water concentration in that solvent, and the refractive indices of the solvent, which were partially saturated (one phase), were fit to a second-order polynomial trend line. The intersection of this trend line with the average refractive index of the fully saturated solvent (two phases) was taken to be the solubility limit of water in that drying solvent.

Karl Fisher Titration

The water content of the saturated solvent/water mixtures was measured by Karl Fisher titration using Aquastar AQ C34 coulometer (Mettler Toledo, Columbus, Ohio). A 0.05–0.10 g sample of the solvent-rich phase was titrated against Aquastar CombiCoulomat fritless reagent (n = 5), and the water content was reported in units of (g water/100 g solution).

To compare the enzyme hydration in different solvents, it is important to correlate water activity (aw) to fractional water saturation (f) value. For the solvents used in this study, such correlations have already been reported for n-pentanol and n-decanol by our group, and for n-octanol by Segatin et al. The aw of water–triacetin and water–butyl lactate solutions were set between 0.11 and 0.90 by saturated salt solutions using previously described isopiestic protocols and equilibrated for 1 month at 22°C. The water content of the partially saturated triacetin or butyl lactate phase corresponding to each aw was determined by Karl Fischer titration (n = 5).

Single Microdroplet Dissolution

A micropipette consisting of a single tapered glass capillary with a tip diameter of 4–10 μm was employed to study the dissolution of a single water droplet in each drying solvent. Following the methods established in our laboratory, the micropipette was mounted upon a micromanipulation system attached to a microscope equipped with a digital camera. The advantage of this setup is that it permits the manipulation of single pure-aqueous or aqueous protein solution microdroplets, or gas micro-bubbles of the fluid, which are typically tens of microns in diameter. Because each of the solvents in this study was mutually soluble with, but not miscible in, water, it was possible to clearly define an observable water–solvent interface. In separate experiments, single ~80-μm diameter water microdroplets were expelled in each of the organic phases and the time-dependent dissolution of the droplets was recorded. The time required for complete droplet dissolution was calculated by extrapolating the droplet diameter to zero. The diffusion coefficient of water in the drying solvent was calculated as described by Su et al.

Enzyme Microglassification

Microglassification of each enzyme was carried out by creating a water-in-oil emulsion of each enzyme solution (20 mg/mL) with 1.5 mL of the dehydrating medium in a microcentrifuge.
the premise that one unit $\alpha$-chymotrypsin hydrolyzes 1.0 $\mu$mol BTEE per min at pH 7.8 in a 3.0 mL reaction mixture at 25 $^\circ$C.  

Catalase

Microglassified™ and lyophilized catalase samples ($n = 5$) were rehydrated in 50 $\mu$L phosphate buffer (pH 7.0) at a concentration of 1.0 mg/mL and then diluted to 25 $\mu$L/mL. 100 $\mu$L test solution was added to 2.9 $\mu$L of buffer followed by 5 $\mu$L of 30% H$_2$O$_2$ solution. The contents were mixed by inversion and the rate of change of absorbance was monitored at 240 nm. The activity for catalase was determined on the premise that one unit catalase decomposes 1.0 $\mu$mol of H$_2$O$_2$ per min at pH 7.0 in a 3.0 mL reaction mixture at 25 $^\circ$C.  

Horseradish Peroxidase

Microglassified™ and lyophilized HRP samples ($n = 5$) were dissolved in 0.1 M phosphate buffer (pH 6.0) at a concentration of 100 $\mu$g/mL. A 100 $\mu$L test solution was added to 160 $\mu$L of 50% H$_2$O$_2$, 2.10 mL of DI water, and 320 $\mu$L each of phosphate buffer and pyrogallol (substrate). The contents were mixed by inversion at 20 $^\circ$C and the enzymatic conversion of pyrogallol to purpurogallin was monitored at 420 nm. The determination of the enzyme activity was based on the premise that one unit HRP forms 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 in a 3.0 mL reaction mixture at 25 $^\circ$C.  

Protein Concentration

To normalize the enzymatic activity, the total protein content of each sample was determined using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Thermo Scientific, Rockford, Illinois). Known concentrations of BSA served as standards.  

Analyses of Enzyme Secondary Structure

The secondary structure of both dried and rehydrated Microglassified™ enzymes ($n = 5$) was analyzed by FTIR using FTLA2000 spectrometer (ABB, Wickliffe, Ohio) equipped with a diamond crystal attenuated total reflectance (ATR) accessory. Samples were rehydrated at a concentration of 50 mg/mL. Lyophilized enzyme formulations, both dried and rehydrated, served as controls. For each sample, a 200-scan interferogram was collected at a resolution of 4 cm$^{-1}$ using an empty cell as the background. The spectra of the sample buffer were subtracted from the spectra of the rehydrated samples using PROTA software package implemented within Bomem-GRAMS/32 AI. The baseline of the spectra was adjusted from 1460 to 1720 cm$^{-1}$ to accommodate the amide I and amide II regions. A combination of Fourier self-deconvolution (FSD), second derivatization, and curve fitting algorithms were employed to elucidate enzyme secondary structure, as described by Dong et al.  

Briefly, resolution enhancement of the original spectra was carried out in the amide I region (1600–1700 cm$^{-1}$) by FSD at an enhancement factor of 2.6. The secondary derivatives of the deconvoluted spectra were obtained, baseline corrected, inverted, and smoothed using seven-point Savitsky–Golay function. The resolved spectra were fitted to a Gaussian curve profile and the peaks were assigned as per the convention described in the literature. The relative area of each of the resolved peaks was used for quantitative analyses.
Accelerated Storage Test

The effect of stressed storage conditions on enzyme activity and structure was evaluated for lysozyme as a representative model. We realize that real-time stability studies are obviously the most reliable demonstration of a product’s shelf life, and accelerated storage testing, based on the Arrhenius equation, is a practical means of quality assurance for biological standards. However, we chose to use a simpler test for preliminary analysis, which has been previously described in the literature. Briefly, ~30 mg of dried Microglassified™ lysozyme prepared in n-pentanol was aliquoted in glass vials and secured with rubber-lined plastic caps. The lyophilized lysozyme samples served as control. The samples were stored at 40°C for 3 months in a hot air oven (n = 3/time-point), and the enzyme activity and secondary structure of the powders were analyzed at the end of each month using the methods described earlier. Although the exact level of hydration of these samples was not determined, both the Microglassified™ and lyophilized samples were stored under vacuum prior to sealing the vials. We have previously shown that Microglassified™ lysozyme and lyophilized lysozyme powders absorb the same amount of water as a function of water activity, so it is assumed that both samples had the same low level of residual moisture.

Statistical Analyses

Where appropriate, the data were expressed as the means ± SD and analyzed statistically by one-way ANOVA using Tukey’s post-hoc test. A p value <0.05 was considered statistically significant.

RESULTS

Water Solubility Limit and Dehydration Rate

Figure 1 shows the variation in refractive index of the water–n-pentanol system with increasing water concentration. A second-order polynomial trend-line was fit to the data representing the single-phase system, and its intersection with the average of the two-phase system represents the solubility limit of water in n-pentanol. Note that this slightly nonlinear trend for refractive index versus water concentration is in agreement with other measures of the same property for isomeric pentanol in water. The solubility limit of water in n-pentanol was found to be 11.43 g water/100 g n-pentanol, which corresponds to 10.31 g water/100 g solution. The solubility limit values calculated using refractive index and Karl Fischer titration methods, and the diffusion coefficient of water in each solvent are shown in Table 1. The diffusion coefficient of water in n-pentanol and n-octanol has been previously reported by Su et al. Overall, our results show that in the alcohol homologous series, an increase in the chain length of the primary alkyl-alcohols decreases their water solubility limit. We have included literature values for the solubility limit, solvent’s refractive index, and dielectric constant—which will be used in discussing the dehydration mechanism. Figure 2 shows the microdroplet dissolution curves for 80-µm diameter water droplets for each of the drying solvents tested. It was found that the solubility of water as well as the initial water dissolution rate in the drying media decreased in the order: butyl lactate > n-pentanol > triacetin > n-octanol > n-decanol; time required for complete dissolution of such an 80-µm diameter water microdroplet were

![Figure 1](https://example.com/figure1.png)

**Figure 1.** The variation of refractive index of water–n-pentanol system with increasing concentration of water in n-pentanol. The refractive index of the partially saturated n-pentanol (one phase) was fit to a second-order polynomial trend line, and the intersection of this trend line with the average refractive index of the fully saturated n-pentanol (two phases) was taken to be the solubility limit of water in n-pentanol. Similar curves were constructed for all the solvents in this study.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** The particle size distribution of the Microglassified™ enzyme beads in n-pentanol. It was found that just less than half (47%, 43%, 45%, and 46%) of the Microglassified™ lysozyme, α-chymotrypsin, catalase, and HRP particles were in the size range of 5–10 μm, respectively. About one third (28%, 31%, 26%, and 27%) were in the size range of 0–5 μm, whereas the remainder of the Microglassified™ particles of each enzyme (25%, 26%, 29%, and 27%) was >10 μm in size.

Particle Size Distribution

Enzymatic activity assays of Microglassified™ lysozyme, α-chymotrypsin, catalase, and HRP upon reconstitution in buffer are shown in Figure 4. In general, enzymes Microglassified™ in primary alkyl-alcohols, particularly in n-pentanol, demonstrated higher activity upon reconstitution than those Microglassified™ in triacetin or butyl lactate. The activities have been normalized to total protein content and also expressed as percentage of lyophilized control.

Lysozyme

The average enzyme activity of rehydrated lysozyme decreased in the series: control > n-pentanol > n-octanol > n-decanol > triacetin > butyl lactate (Fig. 4a). Samples Microglassified™ in n-pentanol (46,560 ± 2736 units/mg; 94%), n-octanol (42,061 ± 4207 units/mg; 85%), n-decanol (41,323 ± 4811 units/mg; 84%), or triacetin (40,585 ± 2609 units/mg; 82%) demonstrated...
Table 1. Table Showing the Measured Values of the Solubility Limit and Diffusion Coefficient of Water in Various Organic Solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>RI Method</th>
<th>Karl Fischer</th>
<th>Published Water Solubility</th>
<th>Refractive Index (20°C)</th>
<th>Dielectric Constant (20°C)</th>
<th>Diffusion Coefficient (cm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Pentanol</td>
<td>10.31 ± 0.02</td>
<td>10.79 ± 0.02</td>
<td>10.21 (Ref. 35)</td>
<td>1.410</td>
<td>15.1</td>
<td>0.52 × 10⁻⁵ (Ref. 23)</td>
</tr>
<tr>
<td>n-Octanol</td>
<td>4.83 ± 0.04</td>
<td>4.82 ± 0.08</td>
<td>4.35 (Ref. 35)</td>
<td>1.429</td>
<td>10.3</td>
<td>0.20 × 10⁻⁵ (Ref. 23)</td>
</tr>
<tr>
<td>n-Decanol</td>
<td>3.63 ± 0.07</td>
<td>3.78 ± 0.03</td>
<td>3.68 (Ref. 36)</td>
<td>1.437</td>
<td>8.1</td>
<td>0.97 × 10⁻⁶</td>
</tr>
<tr>
<td>Triacetin</td>
<td>4.15 ± 0.05</td>
<td>4.56 ± 0.14</td>
<td>–</td>
<td>1.430</td>
<td>7.1</td>
<td>0.36 × 10⁻⁵</td>
</tr>
<tr>
<td>Butyl lactate</td>
<td>15.88 ± 0.04</td>
<td>15.23 ± 0.24</td>
<td>–</td>
<td>1.421</td>
<td>5.1</td>
<td>0.30 × 10⁻⁵</td>
</tr>
</tbody>
</table>

The refractive index and dielectric constant values have been adopted from Landolt–Bornstein tables.³⁷

Figure 2. Measurement of the dissolution rate of a water droplet in the organic solvent. The time required for the complete dissolution of an 80 µm diameter water microdroplet held on the end of a micropipette in an organic solvent increased in the order: butyl lactate < n-pentanol < triacetin < n-octanol < n-decanol.

The activity data for α-chymotrypsin displayed a similar trend to that of lysozyme. As shown in Figure 4b, chymotrypsin activity measured for the enzyme solutions that had been Microglassified™ in n-pentanol (44 ± 3 units/mg; 96%), n-octanol (42 ± 2 units/mg; 91%), or n-decanol (39 ± 5 units/mg; 85%) was comparable to the control (46 ± 7 units/mg; 100%). On the other hand, samples Microglassified™ in triacetin (34 ± 4 units/mg; 75%) or butyl lactate (31 ± 3 units/mg; 67%) were significantly lower (p < 0.05) activity than the control.

α-Chymotrypsin

Figure 3. Particle size distribution of Microglassified™ enzyme beads. The size fraction was similar across all the enzymes.

Horseradish Peroxidase

The enzymatic activity of rehydrated HRP samples showed mixed results. As shown in Figure 4d, samples that had been Microglassified™ in n-pentanol (241 ± 36 units/mg; 98%), n-octanol (210 ± 47 units/mg; 85%), or n-decanol (219 ± 33 units/mg; 89%) were comparable to the control (246 ± 25 units/mg; 100%). In contrast, samples Microglassified™ in triacetin (89 ± 27 units/mg; 36%) or butyl lactate (118 ± 24 units/mg; 48%) exhibited a very significant reduction (p < 0.01) in activity as compared with the samples Microglassified™ in other solvents or the control.

Catalase

The overall trend for enzymatic activity of catalase samples was similar to lysozyme and α-chymotrypsin; however, only samples Microglassified™ in n-pentanol (14,451 ± 973 units/mg; 93%) were comparable to the control (15,551 ± 1235 units/mg; 100%) as shown in Figure 4c. Samples Microglassified™ in n-octanol (12,165 ± 1314 units/mg; 78%), n-decanol (11,581 ± 1200 units/mg; 75%), or triacetin (10,563 ± 470 units/mg; 68%) showed significantly lower (p < 0.05) activity than the control. Samples Microglassified™ in butyl lactate (7854 ± 750 units/mg; 51%) exhibited significantly lower activity than the control (p < 0.01) or any of the other solvents (p < 0.05).

Structural Analyses of Microglassified™ Enzymes

A combination of FSD, secondary derivatives, and curve-fitting algorithms were used on the FTIR spectra to determine the secondary structure of the Microglassified™ enzymes. The secondary structures of dehydrated enzyme powders (lyophilized control and Microglassified™ samples) are shown in Table 2 and upon rehydration in buffer in Table 3.
Figure 4. Enzymatic assays of microglassified (a) lysozyme, (b) α-chymotrypsin, (c) catalase, and (d) HRP, showing the recovery of enzymatic activity upon rehydration. Commercially available lyophilized enzymes were used as control. (*p < 0.05), (**p < 0.01).

Lysozyme

**Microglassified™ Powder.** The amide I peak center of all Microglassified™ and lyophilized lysozyme samples occurred at 1648 cm⁻¹. Overall, it was found that the secondary structure of Microglassified™ enzyme prepared in each solvent was comparable to the secondary structure of the lyophilized sample (Table 2), and were consistent with previously published literature values for the lyophilized formulation (α-helix: 22%–27%; β-sheet: 21%–23%; others 33%–51%). As a representative example, lysozyme Microglassified™ in n-pentanol was composed of 25 ± 2%, 22 ± 3%, 30 ± 3%, and 22 ± 3% α-helix, β-sheet, β-turn, and random structures, respectively. These data are comparable to the lyophilized sample’s content of 28 ± 2%, 20 ± 5%, 34 ± 4%, and 18 ± 4%, respectively, for similar secondary structural elements.

**Enzyme Solution.** The amide I peak shifted slightly from 1648 cm⁻¹ for the dried Microglassified™ powder to 1651–1653 cm⁻¹ for the rehydrated samples. The secondary structure of rehydrated lysozyme that had been Microglassified™ in n-pentanol, n-octanol, or n-decanol was comparable to each other, to the control (Table 3), as well as to previously published values for lysozyme solution (α-helix: 40%–45%; β-sheet: 19%–41%; β-turn: 19%–32%; random: 0%–14%). Rehydrated lysozyme that had been Microglassified™ in triacetin or butyl lactate demonstrated significantly higher (p < 0.05) β-sheet content of 36 ± 7% and 30 ± 5%, respectively, as compared with samples Microglassified™ in pentanol (22 ± 5%) or the control (23 ± 5%). It was also found that the increase in β-sheet content in these samples was accompanied by a decrease in β-turns and random structures, as compared with other samples or the control.

α-Chymotrypsin

**Microglassified™ Powder.** The peak center of the amide I region of the dried Microglassified™ and control (lyophilized) α-chymotrypsin was located at 1635 cm⁻¹ indicating a major presence of β-sheet. It was found that the secondary structure of the Microglassified™ α-chymotrypsin, with the exception of those Microglassified™ in triacetin, was comparable to that of the control (Table 2), and previously published values for the lyophilized formulation (α-helix: 13%–19%; β-sheet: 30%–59%; β-turn: 19%–41%; random: 9%–14%). As a representative example, α-chymotrypsin Microglassified™ in n-pentanol contained 54 ± 4% β-sheet and 18 ± 3% α-helix, which was comparable to the 58 ± 4% β-sheet and 14 ± 2% α-helix of the control. Samples Microglassified™ in triacetin demonstrated a significant increase (p < 0.05) in α-helix (24 ± 2%) largely at the expense of lower content of β-sheet (49 ± 2%) and β-turn (17 ± 3%), as compared with samples Microglassified™ in n-pentanol or the control.

**Enzyme Solution.** The amide I peak center continued to occur at 1634–1637 cm⁻¹ indicating that the molecule was still predominantly composed of β-sheet. The overall secondary structure of the rehydrated samples that had been Microglassified™ in n-pentanol, n-octanol, and n-decanol were comparable to each other, to the control samples (Table 3), as well as to the previously published literature values for α-chymotrypsin in solution (α-helix: 9%–15%; β-sheet: 47%–59%; β-turn: 17%–30%; random: 0%–15%). In contrast, rehydrated
samples obtained after Microglassification\textsuperscript{TM} in triacetin or butyl lactate demonstrated a significantly higher (\(p < 0.05\)) content of \(\alpha\)-helix (17 ± 4\%) and 27 ± 3\%, respectively, as compared to the rehydrated \(\alpha\)-chymotrypsin samples that had been Microglassified\textsuperscript{TM} in \(n\)-pentanol (\(\alpha\)-helix: 10 ± 3\%) or the control (\(\alpha\)-helix: 11 ± 3\%). The content of \(\beta\)-turn and random structures among all rehydrated samples was comparable.

**Catalase**

**Microglassified\textsuperscript{TM} Powder**

The amide I peak center of all Microglassified\textsuperscript{TM} and control (lyophilized) catalase samples occurred at 1638 cm\(^{-1}\) indicating a major presence of random structures in the molecule. Overall, the secondary structure of catalase Microglassified\textsuperscript{TM} in \(n\)-pentanol, \(n\)-decanol, and \(n\)-octanol was similar to the control (Table 2), and to a previously published study on lyophilized catalase\textsuperscript{40} (\(\alpha\)-helix: 25\%; \(\beta\)-sheet: 19\%; others: 56\%). As a representative example, catalase Microglassified\textsuperscript{TM} in \(n\)-pentanol was composed of 26 ± 3\%, 22 ± 4\%, 11 ± 3\%, and 41 ± 4\% \(\alpha\)-helix, \(\beta\)-sheet, \(\beta\)-turn, and random type structures, which was comparable to control's content of 26 ± 2\%, 17 ± 2\%, 10 ± 3\%, and 46 ± 4\% of these secondary structural elements, respectively. However, the random structure content of catalase Microglassified\textsuperscript{TM} in triacetin (37 ± 3\%) or butyl lactate (34 ± 3\%) was significantly less (\(p < 0.05\)) than that of the control samples.

**Enzyme Solution.** The amide I peak center for all rehydrated samples occurred at 1642 cm\(^{-1}\) indicating that random structures were still the major constituents in the molecule. The overall secondary structure of the rehydrated samples previously Microglassified\textsuperscript{TM} in \(n\)-pentanol, \(n\)-octanol, \(n\)-decanol, and butyl lactate (Table 3) were comparable to each other, to the control, and to previously published values for catalase in solution\textsuperscript{40,43} (\(\alpha\)-helix: 25\%–26\%; \(\beta\)-sheet: 12\%–19\%; others 55\%–63\%). On the other hand, samples that were Microglassified\textsuperscript{TM} in triacetin demonstrated a significantly higher (\(p < 0.05\)) content of random structures (47 ± 3\%) at the expense of \(\beta\)-turn (14 ± 2\%) as compared with the samples that had been Microglassified\textsuperscript{TM} in \(n\)-pentanol (\(\beta\)-turn: 19 ± 5\%; random: 35 ± 4\%) or the control (\(\beta\)-turn: 18 ± 3\%; random: 40 ± 5\%).

**Horseradish Peroxidase**

**Microglassified\textsuperscript{TM} Powder.** The peak center of the amide I region of the dried Microglassified\textsuperscript{TM} and control (lyophilized) HRP samples occurred at 1646 cm\(^{-1}\) indicating a major presence of \(\alpha\)-helix in these samples. The overall secondary structure of the Microglassified\textsuperscript{TM} HRP, with the exception of HRP Microglassified\textsuperscript{TM} in butyl lactate, was similar to that of the

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**Table 2. FTIR Analyses of Microglassified\textsuperscript{TM} Enzyme Powders**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Solvent</th>
<th>(\alpha)-Helix (%)</th>
<th>(\beta)-Sheet (%)</th>
<th>(\beta)-Turn (%)</th>
<th>Random (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>(n)-Pentanol</td>
<td>25 ± 2</td>
<td>22 ± 3</td>
<td>30 ± 3</td>
<td>22 ± 3</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>28 ± 4</td>
<td>20 ± 3</td>
<td>32 ± 2</td>
<td>19 ± 5</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>33 ± 4</td>
<td>22 ± 1</td>
<td>29 ± 1</td>
<td>16 ± 4</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>30 ± 3</td>
<td>23 ± 3</td>
<td>29 ± 3</td>
<td>18 ± 5</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>32 ± 3</td>
<td>20 ± 2</td>
<td>28 ± 2</td>
<td>20 ± 5</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>28 ± 2</td>
<td>20 ± 5</td>
<td>34 ± 4</td>
<td>18 ± 4</td>
</tr>
<tr>
<td></td>
<td>Literature\textsuperscript{a}</td>
<td>22–27</td>
<td>21–23</td>
<td></td>
<td>33–51\textsuperscript{b}</td>
</tr>
<tr>
<td>(\alpha)-Chymotrypsin</td>
<td>(n)-Pentanol</td>
<td>18 ± 3</td>
<td>54 ± 4</td>
<td>18 ± 2</td>
<td>10 ± 4</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>16 ± 4</td>
<td>49 ± 4</td>
<td>26 ± 5</td>
<td>9 ± 3</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>17 ± 2</td>
<td>52 ± 3</td>
<td>21 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>24 ± 2</td>
<td>49 ± 2</td>
<td>17 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>16 ± 1</td>
<td>53 ± 2</td>
<td>24 ± 3</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>14 ± 2</td>
<td>58 ± 4</td>
<td>21 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>Literature\textsuperscript{a}</td>
<td>13–19</td>
<td>30–59</td>
<td>19–41\textsuperscript{c}</td>
<td>9–14</td>
</tr>
<tr>
<td>Catalase</td>
<td>(n)-Pentanol</td>
<td>26 ± 3</td>
<td>22 ± 4</td>
<td>11 ± 3</td>
<td>41 ± 4</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>26 ± 2</td>
<td>16 ± 3</td>
<td>10 ± 4</td>
<td>48 ± 5</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>25 ± 4</td>
<td>20 ± 2</td>
<td>14 ± 2</td>
<td>41 ± 4</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>20 ± 3</td>
<td>23 ± 4</td>
<td>16 ± 4</td>
<td>37 ± 3</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>23 ± 4</td>
<td>26 ± 5</td>
<td>16 ± 2</td>
<td>34 ± 3</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>26 ± 2</td>
<td>17 ± 3</td>
<td>10 ± 3</td>
<td>46 ± 4</td>
</tr>
<tr>
<td></td>
<td>Literature\textsuperscript{a}</td>
<td>25</td>
<td>19</td>
<td>56\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>(n)-Pentanol</td>
<td>40 ± 4</td>
<td>29 ± 2</td>
<td>20 ± 3</td>
<td>11 ± 4</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>42 ± 3</td>
<td>36 ± 5</td>
<td>16 ± 3</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>43 ± 5</td>
<td>36 ± 2</td>
<td>14 ± 2</td>
<td>7 ± 3</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>38 ± 2</td>
<td>39 ± 4</td>
<td>21 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>27 ± 5</td>
<td>19 ± 6</td>
<td>15 ± 1</td>
<td>36 ± 5</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>43 ± 4</td>
<td>30 ± 3</td>
<td>9 ± 2</td>
<td>18 ± 5</td>
</tr>
<tr>
<td></td>
<td>Literature\textsuperscript{a}</td>
<td>32–40</td>
<td>22–24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\)The literature values have been taken from Refs. 38–41.

\(b\)Sum of random coils, \(\beta\)-turns, and other undefined contents.

\(c\)Sum of \(\beta\)-turns, intermolecular \(\beta\)-sheets, and undefined contents.
control (Table 2), and previously published literature values for the lyophilized formulation\(^9,41\) (\(\alpha\)-helix: 32\%–40\%; \(\beta\)-sheet: 22\%–24\%; others 37\%–46\%). Thus, HRP Microglassified\(^\text{TM}\) in \(n\)-pentanol was composed of 40 \%\+ 4\%, 29 \%\+ 2\%, 20 \%\+ 3\%, and 11 \%\+ 4\% \(\alpha\)-helix, \(\beta\)-sheet, \(\beta\)-turn, and random structures, which was comparable to control’s content of 43 \%\+ 4\%, 30 \%\+ 3\%, 9 \%\+ 2\%, and 18 \%\+ 5\% of these structural elements, respectively. However, HRP Microglassified\(^\text{TM}\) in butyl lactate demonstrated a significant \((p < 0.01)\) twofold to threefold increase in random structures reduced \((p < 0.05)\) as compared with HRP Microglassified\(^\text{TM}\) in \(n\)-pentanol or the control.

Table 3. FTIR Analyses of Rehydrated Microglassified\(^\text{TM}\) Enzyme Solutions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Solvent</th>
<th>(\alpha)-Helix</th>
<th>(\beta)-Sheet</th>
<th>(\beta)-Turn</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>(n)-Pentanol</td>
<td>39 %+ 4</td>
<td>22 %+ 5</td>
<td>26 %+ 4</td>
<td>15 %+ 2</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>42 %+ 7</td>
<td>25 %+ 7</td>
<td>20 %+ 2</td>
<td>12 %+ 4</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>42 %+ 3</td>
<td>22 %+ 4</td>
<td>15 %+ 4</td>
<td>16 %+ 3</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>40 %+ 5</td>
<td>36 %+ 7</td>
<td>13 %+ 4</td>
<td>10 %+ 5</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>41 %+ 5</td>
<td>34 %+ 5</td>
<td>16 %+ 2</td>
<td>9 %+ 3</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>41 %+ 4</td>
<td>23 %+ 5</td>
<td>23 %+ 3</td>
<td>12 %+ 2</td>
</tr>
<tr>
<td>Literature(^a)</td>
<td></td>
<td>40–45</td>
<td>19–41</td>
<td>19–32</td>
<td>0–14</td>
</tr>
<tr>
<td>(\alpha)-Chymotrypsin</td>
<td>(n)-Pentanol</td>
<td>10 %+ 3</td>
<td>48 %+ 6</td>
<td>27 %+ 2</td>
<td>15 %+ 3</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>12 %+ 4</td>
<td>43 %+ 6</td>
<td>25 %+ 6</td>
<td>12 %+ 2</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>13 %+ 3</td>
<td>50 %+ 5</td>
<td>27 %+ 4</td>
<td>10 %+ 4</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>17 %+ 4</td>
<td>44 %+ 3</td>
<td>25 %+ 5</td>
<td>14 %+ 5</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>27 %+ 3</td>
<td>40 %+ 7</td>
<td>18 %+ 2</td>
<td>12 %+ 4</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>11 %+ 3</td>
<td>50 %+ 6</td>
<td>25 %+ 3</td>
<td>14 %+ 2</td>
</tr>
<tr>
<td>Literature(^a)</td>
<td></td>
<td>9–15</td>
<td>47–59</td>
<td>17–30</td>
<td>0–15</td>
</tr>
<tr>
<td>Catalase</td>
<td>(n)-Pentanol</td>
<td>26 %+ 3</td>
<td>20 %+ 4</td>
<td>19 %+ 5</td>
<td>35 %+ 4</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>23 %+ 5</td>
<td>22 %+ 5</td>
<td>20 %+ 6</td>
<td>36 %+ 5</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>21 %+ 3</td>
<td>18 %+ 6</td>
<td>20 %+ 4</td>
<td>41 %+ 3</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>23 %+ 5</td>
<td>15 %+ 5</td>
<td>14 %+ 2</td>
<td>47 %+ 3</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>23 %+ 6</td>
<td>22 %+ 7</td>
<td>24 %+ 4</td>
<td>31 %+ 5</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>26 %+ 2</td>
<td>20 %+ 3</td>
<td>18 %+ 3</td>
<td>40 %+ 5</td>
</tr>
<tr>
<td>Literature(^a)</td>
<td></td>
<td>25–33</td>
<td>12–19</td>
<td>55–63(^b)</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>(n)-Pentanol</td>
<td>49 %+ 3</td>
<td>8 %+ 3</td>
<td>24 %+ 3</td>
<td>19 %+ 3</td>
</tr>
<tr>
<td></td>
<td>(n)-Octanol</td>
<td>43 %+ 5</td>
<td>12 %+ 4</td>
<td>19 %+ 4</td>
<td>26 %+ 5</td>
</tr>
<tr>
<td></td>
<td>(n)-Decanol</td>
<td>45 %+ 4</td>
<td>12 %+ 3</td>
<td>22 %+ 3</td>
<td>20 %+ 4</td>
</tr>
<tr>
<td></td>
<td>Triacetin</td>
<td>57 %+ 7</td>
<td>9 %+ 4</td>
<td>12 %+ 5</td>
<td>21 %+ 4</td>
</tr>
<tr>
<td></td>
<td>Butyl lactate</td>
<td>41 %+ 6</td>
<td>10 %+ 5</td>
<td>26 %+ 2</td>
<td>22 %+ 6</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>48 %+ 4</td>
<td>7 %+ 2</td>
<td>24 %+ 1</td>
<td>20 %+ 3</td>
</tr>
<tr>
<td>Literature(^a)</td>
<td></td>
<td>45–51</td>
<td>2–11</td>
<td>38–47(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)The literature values have been taken from Refs. 28 and 39–43

\(^b\)Sum of \(\beta\)-turns, intermolecular \(\beta\)-sheets, and random contents.

**Enzyme Solution.** For the rehydrated HRP samples, the amide I peak center showed a major shift from 1645 cm\(^{-1}\) upon Microglassification\(^\text{TM}\) to 1650–1652 cm\(^{-1}\) after rehydration. The overall secondary structure of the rehydrated HRP samples Microglassified\(^\text{TM}\) in any of the solvents (Table 3), with the exception of triacetin, was comparable to each other, to the control, and to previously published values for HRP in solution\(^9,41\) (\(\alpha\)-helix: 45\%–51\%; \(\beta\)-sheet: 2\%–11\%; others 38\%–47\%). Rehydrated HRP that had been Microglassified\(^\text{TM}\) in triacetin demonstrated a significantly higher \((p < 0.05)\) content of \(\alpha\)-helix \((57 \%\+ 7\%)\) and a lower content of \(\beta\)-turn \((12 \%\+ 5\%)\) as compared with rehydrated samples that were Microglassified\(^\text{TM}\) in \(n\)-pentanol \((\alpha\)-helix: 49 \%\+ 3\%; \(\beta\)-turn: 24 \%\+ 3\%) or the control \((\alpha\)-helix: 48 \%\+ 4\%; \(\beta\)-turn: 24 \%\+ 1\%). The content of \(\beta\)-sheet and random structures among all rehydrated samples were comparable.

**Accelerated Storage Test Results**

In order to gain an early evaluation of the stability of the Microglassified\(^\text{TM}\) enzyme powders in these initial studies, we measured if and to what extent there were any changes in enzymatic activity and secondary structure of Microglassified\(^\text{TM}\) and lyophilized lysozyme over a 3-month period at just one elevated temperature of 40 °C.

**Lysozyme Activity upon Stress Storage**

The enzymatic activity of the Microglassified\(^\text{TM}\) and lyophilized lysozyme (control) samples decreased significantly \((p < 0.01)\) (by ~33\%) from 46,560 \pm 2736 and 49,440 \pm 6702 units/mg at month 0, to 30,000 \pm 1385 and 30,600 \pm 3600 units/mg, respectively, after incubation at 40 °C for 1 month (Fig. 5). However, upon further incubation, the Microglassified\(^\text{TM}\) and lyophilized samples showed minimal changes in their enzymatic activity. The activities of the Microglassified\(^\text{TM}\) and lyophilized samples were 30,215 \pm 5438 and 31,060 \pm 4327 units/mg after month 2, and 26,780 \pm 3018 and 27,225 \pm 2910 units/mg after month 3, respectively. It is noteworthy that the enzymatic activities of the Microglassified\(^\text{TM}\) and lyophilized samples were comparable at all time points.
Changes in Secondary Structure

As shown in Table 4, FTIR analyses of the Microglassified™ and lyophilized lysozyme powders incubated at 40°C for 3 months showed major changes in the structure of the enzyme. After 1 month of incubation, the content of β-sheet in the samples decreased significantly (p < 0.05) from 22 ± 3% and 20 ± 5% to 14 ± 4% and 13 ± 2% in the Microglassified™ and lyophilized samples, respectively. The decrease in the β-sheet content was accompanied by a significant (p < 0.05) increase in the content of random structures, which increased from 22 ± 3% and 18 ± 4% to 35 ± 5% and 27 ± 4% in the Microglassified™ and lyophilized samples, respectively, during the same time period. Thereafter, minimal structural changes in the powders, both Microglassified™ and lyophilized, were observed. It was also observed that throughout the 3-month storage period, the content of α-helix and β-turn structures, in both the Microglassified™ and lyophilized samples, remained nearly identical.

DISCUSSION

The main objectives of this work were to test the feasibility of Microglassification™ to controllably dehydrate enzymes in a series of dehydrating solvents, and evaluate the changes in enzyme structure and function upon rehydration. Using our signature micropipet manipulation technique, it was found that butyl lactate and n-pentanol had higher dehydration rates than n-octanol, n-decanol, or triacetin. Enzymatic activity assays showed that catalase, lysozyme, α-chymotrypsin, and HRP that had been Microglassified™ in n-pentanol, retained an average of 93%, 94%, 96%, and 98% of their activity upon rehydration, respectively. FTIR analyses indicated that all the enzymes underwent significant structural rearrangement upon Microglassification™. Upon reconstitution in buffer, the enzymes Microglassified™ in n-pentanol, n-decanol, or n-octanol generally reverted to a native-like conformation, whereas the enzymes Microglassified™ in triacetin or butyl lactate demonstrated structural deviations from the native conformation. Accelerated storage tests for both Microglassified™ and lyophilized lysozyme showed that although the enzymatic activity reduced significantly after incubation at 40°C for 3 months, the activities of both formulations were comparable to each other throughout the test period. Taken together, these results demonstrate that Microglassification™ is an attractive method to successfully and conveniently dehydrate enzymes for long-term preservation.

The removal of water from an aqueous protein microdroplet immersed in an organic drying medium depends on the droplet’s dissolution rate, and is proportional to the solubility and diffusion of water in that drying solvent. Water dissolution is primarily driven by enthalpic and entropic effects arising from the difference in water activity (a_w) between the two phases. In our earlier work, we have established that water continues to dissolve into the organic phase until a_w of both phases are in equilibrium, resulting in protein solidification in the form of a Microglassified™ bead. As shown in Table 1 and Figure 2, water dissolution rates generally increased with increasing solubility, and can be successfully modeled by the Epstein–Plesset equation:

\[
\frac{dR}{dt} = -\frac{DC_s(1-f)}{\rho} \left( \frac{1}{R} + \frac{1}{\sqrt{\pi Dt}} \right)
\]

where \( R \) is the droplet radius, \( D \) is the diffusion coefficient of water in the surrounding medium, \( C_s \) is water solubility, \( \rho \) is droplet density, and \( t \) is time.

The Epstein–Plesset equation and our micropipet assay on single microdroplets of pure water provide fundamental guiding principles for dehydration procedures. The mass transfer rate of water (dR/dt) is dependent on the product of water solubility (C_s) and its diffusion coefficient (D). Water can form a hydrogen bond with the (–OH) group of n-octanol, and this hydrogen bonding between a solute and a solvent decreases D by an order of magnitude and slows the dissolution process. Therefore, for similar solubilities, a faster diffusion rate leads to a greater overall mass transfer rate of water out of the microdroplet. Similarly, a

Table 4. Accelerated Stability Test—Lysozyme Secondary Structure (%)

<table>
<thead>
<tr>
<th>Month</th>
<th>Microglassified™ Lysozyme</th>
<th>Lyophilized Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>α-Helix</td>
<td>25 ± 2</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>β-Sheet</td>
<td>22 ± 3</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>β-Turn</td>
<td>30 ± 3</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Random</td>
<td>22 ± 3</td>
<td>35 ± 5</td>
</tr>
</tbody>
</table>

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Aniket et al., JOURNAL OF PHARMACEUTICAL SCIENCES
drying solvent that has greater solubility for water increases the dissolution rate, thereby reducing the time required for equilibration. Our results showed that solvents with higher dielectric constant (ε) also demonstrated a higher water solubility limit (Table 1). A high dielectric solvent, because of its greater charge carrying capacity, and therefore a greater ability to offer a transition-state stabilization, weakens the electrostatic forces between polar residues and water. Therefore, because of the concomitant contributions from several factors including hydrogen bonding, diffusivity, and dielectric properties, water solubility and dissolution rate increased in the order: n-decanol < n-octanol < triacetin < n-pentanol < butyl lactate (Fig. 2).

Following the vortex procedure to produce water-in-oil emulsions from which the Microglassified™ beads formed, size analyses showed that the particle size distributions of all enzyme beads were quite similar to each other (Fig. 3). The large differences in molecular weights of the enzymes—lysozyme (14 kDa) is approximately twofold and threefold smaller than α-chymotrypsin (25 kDa) and HRP (44 kDa), and ~18-fold smaller than catalase (250 kDa)—was inconsequential, and this was accompanied by a decrease in the content of α-helix structures, although the magnitude of this transformation varied among different enzymes. For instance, β-sheet content of catalase tripled upon dehydration, from 22 ± 3% to 66 ± 4%, whereas its α-helix content decreased from 26 ± 2% to 16 ± 3%. Water molecules can form hydrogen bonds more easily with the (C=O) groups of the peptide backbone that constitutes the α-helix but not with the β-sheets, hence, water loss upon dehydration disrupts α-helix structure more than β-sheet. These results are consistent with other reports on structural changes during lyophilization of insulin and poly(t-lysine), where a similar increase in β-sheet was observed mostly at the expense of α-helix. Nevertheless, it should be noted that the changes in enzyme conformation were largely reversible, as the secondary structure of the enzymes before and after Microglassification™, and subsequent rehydration, were comparable.

Accelerated storage test results showed that enzymatic activity of Microglassified™ and lyophilized lysozyme samples reduced by almost one-third after 1 month of incubation at 40 °C (Fig. 5). The reduction in the enzymatic activity can be attributed to the structural changes in these molecules during storage, in particular, ~50% loss of β-sheet observed in both samples (Table 4). The active-site of lysozyme is located within a cleft formed by α- and β-domain, wherein the former domain consists primarily of α-helices and the latter domain consists of three β-sheets. Therefore, the significant loss of β-sheet under the stressed storage conditions likely induced irreversible modifications in the enzyme’s active site that resulted in reduced activity.

**CONCLUSIONS**

The results of this study demonstrate the feasibility of Microglassification™ to rapidly and controllably dehydrate a series of common enzymes while largely preserving their structure and enzymatic activity. It was found that the primary alkyl-alcohols, in particular n-pentanol, were the most optimal solvents for enzyme dehydration. FTIR analyses of the enzyme secondary structure showed that, as expected, all the enzymes underwent structural rearrangements upon dehydration by Microglassification™ and subsequent drying. Most prominently, this resulted in α-helix to β-sheet type transformation as the enzymes solidified from solution into a Microglassified™ state. The changes in enzyme secondary structure were essentially reversible as indicated by reorganization to a near native-like conformation upon rehydration. Accelerated storage test results showed that the enzymatic activity of Microglassified™...
and lyophilized lysozyme reduced significantly upon storage at 40 °C; however, their activities were comparable to each other. Thus, Microglassification™ can have direct application for the preservation of enzymes, proteins, and other biomacromolecules. We envision that this method can also be used as a formulation technique to develop high-concentration injectable biotherapeutics in the form of micro- and nano-sized Microglassified™ beads, including encapsulation in slow release polymer formulations.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Ryan Denkewicz for assistance with FTIR spectra analyses. This work was supported by grant no. IIP-0848968 from the National Science Foundation.

REFERENCES


