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Stability of α-Tocopherol in Freeze-Dried Sugar–Protein–Oil Emulsion Solids as Affected by Water Plasticization and Sugar Crystallization

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ABSTRACT: Water plasticization of sugar–protein encapsulants may cause structural changes and decrease the stability of encapsulated compounds during storage. The retention of α-tocopherol in freeze-dried lactose–milk protein–oil, lactose–soy protein–oil, trehalose–milk protein–oil, and trehalose–soy protein–oil systems at various water activities (a_w) and in the presence of sugar crystallization was studied. Water sorption was determined gravimetrically. Glass transition and sugar crystallization were studied using differential scanning calorimetry and the retention of α-tocopherol spectrophotometrically. The loss of α-tocopherol followed lipid oxidation, but the greatest stability was found at 0 a_w presumably because of α-tocopherol immobilization at interfaces and consequent reduction in antioxidant activity. A considerable loss of α-tocopherol coincided with sugar crystallization. The results showed that glassy matrices may protect encapsulated α-tocopherol; however, its role as an antioxidant at increasing a_w accelerated its loss. Sugar crystallization excluded the oil-containing α-tocopherol from the protecting matrices and exposed it to surroundings, which decreased the stability of α-tocopherol.

KEYWORDS: *Encapsulation, α-tocopherol, carbohydrate–protein, water activity, crystallization*

INTRODUCTION

Vitamin E contributes to many biological functions, including enzymatic activities, gene expression activity, neurological function, and antioxidant activity.[1] α-Tocopherol is the main vitamin E compound, with antioxidant activity that inhibits the formation and decomposition of hydroperoxides,[2] although it may become pro-oxidative at elevated concentrations.[3,4] Losses of α-tocopherol in food systems could result from (i) degradation, which was affected by exposures to oxygen, light, and high temperatures,[5,6] as well as from (ii) its consumption as an antioxidant reactant in the presence of lipids, i.e., accelerated degradation during lipid oxidation.[7−9] Freeze-drying entraps and encapsulates sensitive compounds into a dry wall material, which may also enhance stability of bioactive components.[10,11] Encapsulation of the hydrophobic α-tocopherol requires (i) breakdown of the lipid phase into small droplets by emulsification and dispersion of these droplets into the continuous aqueous phase, (ii) freezing to immobilize and encapsulate the lipid droplets in freeze-concentrated solutes, and (iii) stabilization of the system by sublimation of the ice.[10,12] Sugars are effective encapsulating agents because they form amorphous glassy solids that restrict the movement of entrapped and encapsulated compounds.[10,11,13−17] The glass formation occurs in freezing, and the stability of freeze-dried solid structures accounted for their glassy state. Plasticization resulting in glass transition of freeze-dried materials affects numerous changes in the storage of dried solids, e.g., crystallization of sugars.[10,11,18] Dehydrated sugar–protein mixtures showed delayed crystallization of component sugars[19] and showed reduced changes in solid structures. This suggested carbohydrate–protein systems as potential encapsulants. Proteins may also act as emulsifiers,[20] and the emulsification properties of proteins are important in the stabilization of hydrophobic nutrient delivery systems.

Widicus and others[6] reported first-order kinetics of degradation of α-tocopherol in freeze-dried model systems containing no fat. The degradation rate of α-tocopherol increased with increasing water activity (a_w), and it was diffusion-dependent. Widicus and Kirk[8] found that, in freeze-dried model systems containing fat, α-tocopherol degraded more rapidly than in systems containing no fat and followed the progression of lipid oxidation. In fat-containing systems, water affected the stability of α-tocopherol through protecting lipids against oxidation by (i) hydrogen bonding to the hydroperoxides and (ii) hydration of metal catalysts, which made them less effective as a result of changes in their coordination sphere[21] up to 0.5 a_w. At higher a_w, the rate of lipid oxidation increased with increasing a_w, because of the mobilization of catalysts and exposure of lipids to an increased number of catalysts (especially in swelling systems). However, the effects of physical changes, e.g., glass transition and crystallization of matrices, as a result of water sorption on the stability of α-tocopherol have not been studied intensively. Especially at 0 a_w, when lipid oxidation is rapid, the consumption of antioxidants may be assumed to be the most rapid. However, because of the polar paradox that hydrophobic antioxidants, e.g., α-tocopherol, are more active in emulsions than in oils,[22,23] we would expect α-tocopherol to show less antioxidant activity in anhydrous systems, i.e., systems containing no water (similar to bulk oil systems).

The objectives of the present study were to encapsulate and stabilize emulsified oil droplets containing α-tocopherol in amorphous carbohydrate–protein matrices by freezing and

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freeze-drying. The physicochemical properties (water sorption, glass transition, and crystallization) of the matrices and the stability of α-tocopherol in these matrices, as affected by water activity and physical changes, were determined.

MATERIALS AND METHODS

Materials. α-Lactose monohydrate (Sigma-Aldrich, St. Louis, MO), trehalose dihydrate (Cargill, Inc., Minneapolis, MN), milk protein isolate (MPI; Kerry Ingredients, Listowel, Co., Kerry, Ireland), and soy protein isolate (SPI; PRO-FAM891, Archer Daniels Midland, Decatur, IL) were used as encapsulating matrices. Olive oil (OO; Extra Virgin Olive Oil, Don Carlos, Hacienda Don Carlos, purchased from the local market) was used as a lipid carrier for the hydrophobic α-tocopherol (Sigma-Aldrich, St. Louis, MO). Reagents used for extraction of α-tocopherol, including ascorbic acid, potassium hydroxide (KOH), methanol, butylated hydroxytoluene (BHT), *n*-hexane, and anhydrous sodium sulfate (Na_2SO_4), were all purchased from Sigma-Aldrich (St. Louis, MO). Ethanol (Ethanol 100), which was also used for extraction of α-tocopherol, was purchased from Carbon Group (Carbon Group, Ringaskiddy, Co., Cork, Ireland).

Preparation of Emulsions and the Freeze-Dried Materials. Lactose−MPI (3:1), lactose−SPI (3:1), trehalose−MPI (3:1), and trehalose−SPI (3:1) suspensions were prepared [250 g, 20% (w/w) of solids] using distilled water. All materials were allowed to hydrate at 45 °C for at least 3 h, with an assumption of sufficient hydration of materials. The suspensions were reweighed, and the amount of water equivalent to the amount of evaporated water was added. The suspensions were left to cool to room temperature (24 ± 1 °C). α-Tocopherol (5%, w/w) was mixed with olive oil, which naturally contains a smaller amount of α-tocopherol than other oils,[24] under stirring at room temperature. The olive oil with α-tocopherol (25 g) was mixed into the carbohydrate−protein suspensions (250 g), pre-homogenized using Ultra-Turrax (T25 Digital, Staufen, Germany) at 10 000 rpm for 30 s, and immediately homogenized at room temperature (APV-1000 high-pressure homogenizer, Wilmington, MA) at 24 MPa (two stages: 20 and 4 MPa) for 3 cycles.

Freshly prepared emulsions [275 g, 9.1% (w/w) oil phase] were transferred using an adjustable-volume pipet (Pipetman P5000, Gilson, Inc., Middleton, WI) into glass vials (clear glass ND18, 10 mL, VWR, U.K.) (5 mL per vial), frozen at −35 °C ($T_g' < T < T_m'$[19]) for 24 h, and subsequently transferred to a −80 °C freezer for 5 h to avoid ice melting during transfer to a freeze-dryer (Lyovac GT2, STERIS, Hürth, Germany). Samples in glass vials with semi-closed rubber septa were freeze-dried for ≥72 h at <0.1 mbar (T < −40 °C). All vials at completion of freeze-drying were hermetically closed inside the freeze-dryer using the rubber septa prior to breaking the vacuum with ambient air. The freeze-dried lactose−MPI−OO (3:1:2), lactose−SPI−OO (3:1:2), trehalose−MPI−OO (3:1:2), and trehalose−SPI−OO (3:1:2) systems were theoretically composed of two parts of nonfat solids and one part of lipid, and α-tocopherol accounted for 1.67% (w/w) of the total solids.

Characterization of the Nonfat Solids: Water Sorption and Sorption Isotherms. Triplicate samples of each freeze-dried emulsion in open vials were equilibrated up to 5 days at room temperature (in general, 24 ± 1 °C) in evacuated desiccators over various saturated salt solutions, LiCl, CH_3COOK, $MgCl_2$, and K_2CO_3, giving respective water activities (a_w) of 0.11, 0.23, 0.33, and 0.44 at equilibrium.[25] The samples were weighed at 0, 3, 6, 9, 12, 24, 48, 72, 96, and 120 h. A gradual decrease of sorbed water indicated time-dependent lactose or trehalose crystallization. This was monitored during storage over saturated solutions of $Mg(NO_3)_2$, $NaNO_2$, and NaCl, giving respective a_w of 0.54, 0.65, and 0.76 at equilibrium.[25] The vials with samples were weighed every 1 h up to 6 h, at 8, 10, 12, 24, 48, 72, 96, and 120 h. The water activity of the saturated salt solutions was confirmed using an Aqua Lab 4TE instrument (Decagon Devices, Inc., Pullman, WA). The water content of each material was measured gravimetrically as a function of time, and the mean weight of triplicate samples was calculated.[26]

The Guggenheim−Anderson−de Boer (GAB) equation was used to model water sorption.[27] The GAB isotherm parameters, m_m (monolayer value) and K, were obtained by plotting a_w against a_w/m and from a second-order polynomial regression over the a_w range from 0.11 to 0.44.

Characterization of the Nonfat Solids: Glass Transition and Crystallization. The onset, T_g (onset), and endset, T_g (endset), temperatures of glass transition and the instant crystallization temperature, T_{ic}, were measured using differential scanning calorimetry (DSC; Mettler Toledo 821e with liquid N_2 cooling, Switzerland). The thermograms were analyzed using STAR thermal analysis software, version 6.0 (Mettler Toledo, Switzerland), as reported by Roos and Karel.[28] The freeze-dried materials were powdered using a spatula in glass vials. To determine the T_g (onset) and T_{ic}, 10−15 mg of the powdered freeze-dried materials were prepared in open DSC pans and equilibrated over P_2O_5 and saturated salt solutions to a_w of 0−0.44 at room temperature. After equilibration, the pans were hermitically sealed and the samples were analyzed (Table 2) according to the report by Zhou and Roos.[19] Triplicate samples were analyzed for each material.

Oil Droplet Size Distribution. The droplet size distribution of emulsions was analyzed by laser diffraction (Malvern Mastersizer S, Malvern Instruments, Ltd., Malvern, U.K.) according to O'Regan and Mulvihill.[29] Fresh emulsions were analyzed within 1 h after preparation. Frozen emulsions after 24 h of storage at −35 °C were thawed (room temperature for 1 h) and analyzed. Freeze-dried materials were reconstituted to the original weight, gently mixed, and analyzed. The droplet size (μm) was reported as the volume-weight mean diameter, $D[4, 3]$, and the volume median diameter, $D[v, 0.5]$.

Spectrophotometry of α-Tocopherol. The α-tocopherol content was measured spectrophotometrically. The freeze-dried materials before and after storage were reconstituted to the original weight and vortexed for 30 s at high speed for mixing. Aliquots of 1 mL of the reconstituted emulsions were transferred into test tubes (20 mL) and destabilized by ethanol (3 mL). Ascorbic acid solution [5% (w/v), freshly made, 2 mL] was added to the mixtures to prevent oxidation in the liquids. Separation of α-tocopherol (unsaponified fraction) from the oil (saponified fraction) was achieved by adding saturated KOH−methanol (1 mL) into the mixes and vortexing for 15 s, followed by holding at 45 °C in a water bath for 30 min. After cooling to room temperature, α-tocopherol was extracted 3 times with *n*-hexane [with 0.1% (w/v) BHT, 2 mL], which was added into the mixes followed by vortexing for 30 s to extract α-tocopherol. The mixes were left to stand for 10 min for the separation of the organic layer containing α-tocopherol on the top and the water layer on the bottom. The organic layer was removed carefully using a glass Pasteur pipet (9 in., Corning, Inc., Corning, NY) into a glass vial containing anhydrous Na_2SO_4 powder (about 0.5 g), which absorbed possible residual water (modified from the report by Cornacchia and Roos[30]). After 1:10 dilution with *n*-hexane (with BHT), the extracts were transferred into cuvettes (semi-micro, low-form, UV grade, Kartell, Italy), and the absorbance was measured at 297.7 nm using a spectrophotometer (Varian Cary 300, Bio UV−vis spectrophotometer, Agilent Technologies, Santa Clara, CA). This wavelength was found to correspond to the maximum absorbance of α-tocopherol in *n*-hexane (with BHT) over the spectrum of wavelengths from 200 to 400 nm.[31] A standard curve was obtained by measuring absorbance of α-tocopherol in *n*-hexane (with BHT) at various concentrations (0−125 μg/mL). The amount of α-tocopherol in the olive oil was measured to adjust the actual amount of added α-tocopherol, but this was, however, undetectable.

Storage of the Freeze-Dried Materials and Stability of α-Tocopherol. To study the stability of α-tocopherol in different encapsulants, the freeze-dried materials in closed vials were stored in an incubator at 60 °C for 40 days. The α-tocopherol content was measured at intervals during storage. To study the effect of water activity and structural changes on the stability of α-tocopherol, the freeze-dried materials in open vials were equilibrated for 5 days at room temperature (24 ± 1 °C) in evacuated desiccators over P_2O_5 and various saturated salt solutions [LiCl, CH_3COOK, $MgCl_2$, K_2CO_3,

$Mg(NO_3)_2$, $NaNO_2$, and NaCl], giving respective water activities (a_w) of 0, 0.11, 0.23, 0.33, 0.44, 0.54, 0.65, and 0.76 at equilibrium, according to the water sorption results. The sample vials were closed manually with septa, sealed with parafilm, and then transferred into the incubator at 60 °C for 10 days. Because of the high porosity of the freeze-dried materials, the total volume of the glass vials (10 mL) was considered as the headspace volume during storage. The α-tocopherol content was measured after (i) equilibration and (ii) incubation, and the ratio [content after incubation/content after equilibration] × 100 was calculated as percent retention.

Statistics. All measurements carried out in this study were performed in triplicate. The mean values of triplicate samples ± 1 standard deviation (SD) are reported as results for water sorption, glass transition and crystallization, and the stability study.

RESULTS AND DISCUSSION

Water Sorption and Sorption Isotherms. Water sorption of freeze-dried lactose−MPI−OO, lactose−SPI−OO, trehalose−MPI−OO, and trehalose−SPI−OO systems over various a_w conditions as a function of time was plotted, as shown in Figure 1. The final water contents are given in Table 1. At low water activities up to 0.44 a_w, sugar−protein systems showed the most rapid water sorption and the highest amounts of sorbed water, followed by sugar−protein−oil systems (panels A and C of Figure 1). Less water sorption in sugar−protein−oil systems compared to sugar−protein systems was possibly because of hydrophobic interactions between proteins and oils at the interface. This decreased the quantity of proteins in the sugar−protein-rich continuous phase.

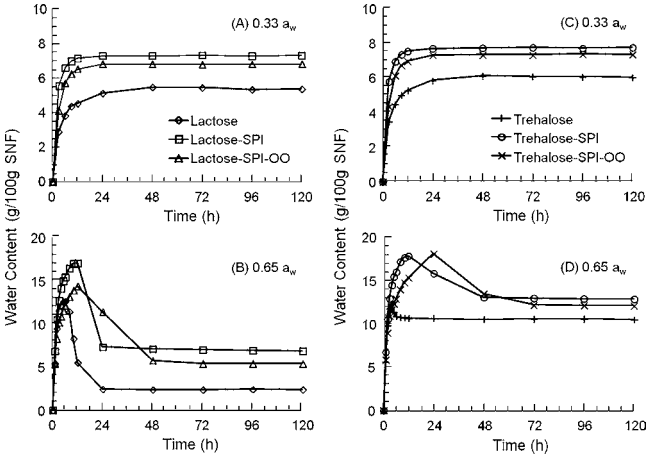

Figure 1. Water sorption [g/100 g of solid nonfat (SNF)] of freeze-dried lactose,[19] trehalose,[19] lactose−SPI (3:1),[19] trehalose−SPI (3:1),[19] lactose−SPI−OO (3:1:2), and trehalose−SPI−OO (3:1:2) systems at (A and C) 0.33 a_w and (B and D) 0.65 a_w at room temperature (24 ± 1 °C). The loss of water at 0.65 a_w indicated sugar crystallization.

Sugar crystallization was found at 0.54 a_w and above for lactose and trehalose.[19] Delayed sugar crystallization occurred in sugar−protein and sugar−protein−oil systems as a result of interference with the mobility of lactose and trehalose molecules and possible interactions between sugars and proteins (panels B and D of Figure 1), e.g., by hydrogen bonding between sugars and proteins. A limited amount of hydrogen bonding between sugars and proteins, above which sugars and proteins exist as free components without interaction, has been reported, although the components were not necessarily completely immiscible, i.e., phase-separated but forming a homogeneous bulk.[32] Our earlier studies showed that at 3:1 ratio of sugars and proteins existed as phase-separated, largely immiscible glass formers,[19,33] which excluded major molecular interaction effects on sugar crystallization. At 0.65 a_w, lactose crystallization was found after 6, 12, and 12 h for lactose, lactose−SPI, and lactose−SPI−OO systems, respectively. It appeared that lactose crystallization responsible for changes in sorbed water contents in the lactose−SPI−OO system (Figure 1B) proceeded more slowly at a lower water content than in the lactose−SPI system. Trehalose crystallization was found after 4, 12, and 24 h in trehalose, trehalose−protein, and trehalose−protein−oil systems, respectively (Figure 1D). Trehalose crystallization was less rapid in the sugar−protein−oil systems than in the sugar−protein systems. We assume that the dispersed oil phases with protein interfaces disturbed the continuous hydrophilic sugar−protein phase and hindered the movement of sugar molecules to form crystals. The molecular assemblies of the components occur in emulsification prior to freezing (protein−oil) and as a result of freeze concentration (protein−sugar). The protein molecules are likely to become covered by sugar molecules during freezing, and this structure can be retained to the freeze-dried systems. Diffusion of the sugar molecules at the molecular surfaces of proteins and the hydrophobic environment of oil droplets can reduce their nucleation and crystal growth rates.

The GAB model was fitted to the water sorption data. Sugar−protein systems at the low experimental a_w range showed the sigmoid sorption isotherms and highest water sorption, followed closely by sugar−protein−oil systems and then pure sugar systems (Figure 2). The lower amount of sorbed water in the nonfat solids of systems containing oil at each a_w condition could be due to the hydrophobic interactions between protein and oil, which decreased the amount of protein in the sugar−protein phases and their hydrogen bonding with water. The monolayer water contents (m_m, g/100 g of SNF) were slightly different, which were 4.96, 5.51, 5.30, and 5.57 g/100 g of SNF for lactose−MPI−OO, lactose−SPI−OO, trehalose−MPI−OO, and trehalose−SPI−OO systems, respectively, but similar corresponding a_w values (0.21−0.23) were obtained for all systems.

Table 1. Water Content (Mean Values ± SD) in Freeze-Dried Carbohydrate−Protein−Oil (3:1:2) Systems after Equilibration at Various Water Activities (a_w) for 5 Days at Room Temperature (24 ± 1 °C)

	water content (g/100 g of SNF)							
system	0.00 a_w	0.11 a_w	0.23 a_w	0.33 a_w	0.44 a_w	0.54 a_w	0.65 a_w	0.76 a_w
lactose−MPI−OO	0.00 ± 0.00	3.44 ± 0.03	5.45 ± 0.02	7.00 ± 0.01	9.61 ± 0.03	12.62 ± 0.04	5.58 ± 0.06	8.21 ± 0.04
lactose−SPI−OO	0.00 ± 0.00	3.29 ± 0.04	5.39 ± 0.01	6.85 ± 0.02	9.18 ± 0.02	11.90 ± 0.08	5.39 ± 0.09	7.78 ± 0.05
trehalose−MPI−OO	0.00 ± 0.00	3.66 ± 0.02	5.82 ± 0.07	7.48 ± 0.04	10.28 ± 0.04	14.27 ± 0.06	12.61 ± 0.02	14.23 ± 0.11
trehalose−SPI−OO	0.00 ± 0.00	3.57 ± 0.03	5.76 ± 0.02	7.34 ± 0.07	9.93 ± 0.01	13.78 ± 0.03	12.08 ± 0.03	13.07 ± 0.02

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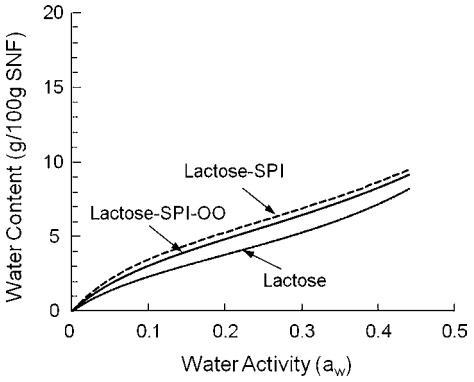

Figure 2. Water sorption isotherms of freeze-dried lactose,[19] lactose–SPI (3:1),[19] and lactose–SPI–OO (3:1:2) systems. The isotherms were modeled using the GAB relationship with experimental data.

Glass Transition and Instant Sugar Crystallization. The glass transition and sugar crystallization for anhydrous and humidified lactose–MPI–OO, lactose–SPI–OO, trehalose–MPI–OO, and trehalose–SPI–OO systems were measured, and the glass transition temperatures and instant sugar crystallization temperatures were read from the DSC thermographs. The second heating scans of DSC thermographs for lactose–SPI–OO and trehalose–SPI–OO systems at 0.33 a_w are given in Figure 3 as examples, in which oil melting occurred

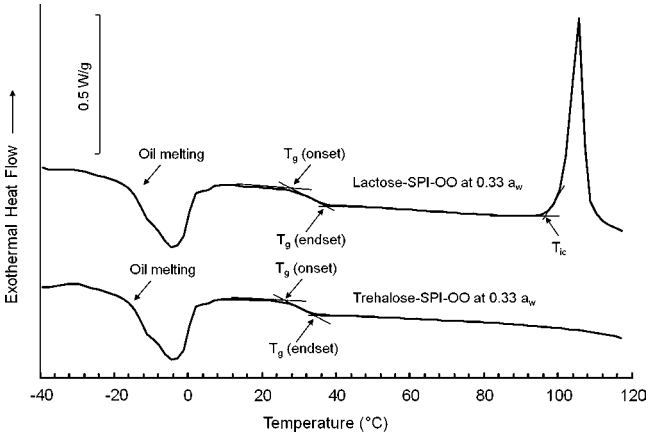

Figure 3. DSC thermographs of lactose–SPI–OO and trehalose–SPI–OO at 0.33 a_w, showing oil melting, glass transition, and sugar crystallization. The thermographs were taken from the second heating scans (heating rate at 5 °C/min).

and was followed by a step endothermal change for glass transition. An exothermal peak, representing sugar crystallization, was observed only for lactose-containing systems at a_w above 0. The T_g of all systems, as shown in Table 2, was depressed as a result of water plasticization.[11,28] The T_g values of anhydrous lactose–MPI–OO and lactose–SPI–OO were similar to that of pure lactose. The T_g values of anhydrous trehalose–MPI–OO and trehalose–SPI–OO were slightly higher than that of pure trehalose, suggesting a possible better compatibility of trehalose with proteins, which may also contribute to the excellent protecting effects of trehalose on protein structures.[34] Proteins may affect the T_g of anhydrous systems by hydrogen bonding to sugar molecules, depending upon the type of proteins and sugars and the amount of proteins in the sugar–protein phase after emulsification, and self-assembly of proteins at the oil droplet interfaces. The hydrogen bonding between sugars and proteins may be diminished in the presence of water. The plasticizing effect of water was dominant at increasing a_w, and in humidified systems, the T_g values were dependent upon the a_w and the component sugar (Figure 4B) rather than the water content (Figure 4A), regardless of the presence of protein and oil. As shown in Figure 4, it should be noted that the glass transition occurred only in the miscible nonfat solids components and the T_g values at varying a_w followed closely those of the component sugars.[19] This confirmed that the protein and carbohydrate components were largely phase-separated, and the T_g values corresponded to those of carbohydrates at most a_w; i.e., the presence of protein affected the quantity of sorbed water, but the T_g corresponded to that of the carbohydrate at any constant $a_w > 0$.

The instant crystallization at T_{ic}, showing rapid amorphous lactose crystallization, occurred in lactose–MPI–OO and lactose–SPI–OO systems at 0.11–0.44 a_w (Table 3), but no T_{ic} was found for anhydrous systems. Our earlier studies reported that lactose alone crystallized at 174 °C at 0 a_w, but no T_{ic} was observed for anhydrous systems in the presence of other components, e.g., protein[19] and hydrophilic vitamins.[35] These inhibiting effects of proteins and vitamins on sugar crystallization result from numerous factors, including restricted diffusion and retarded arrangements of sugar molecules to nucleate and form crystals. The T_{ic} decreased with increasing a_w as a result of water plasticization and confirmed phase separation of the sugar components. No instant crystallization of trehalose was found in trehalose–protein–oil systems (Figure 3), probably because an insufficient amount of water for trehalose to form dihydrate crystals was present and the interactions between trehalose and other components inhibited trehalose molecules to crystallize.[19]

Oil Droplet Size Distribution. The oil droplet size distributions for the emulsified systems are given in Figure 5. The volume weight mean diameter, $D[4, 3]$, and the median diameter, $D[v, 0.5]$, of emulsions are given in Table 4. The fresh lactose–MPI–OO and trehalose–MPI–OO emulsions showed monomodal distribution of droplets (curve a in panels A and C of Figure 5, respectively), while tail distribution was found in lactose–SPI–OO and trehalose–SPI–OO systems (curve a in panels B and D of Figure 5, respectively). During freezing, the oil droplets were immobilized and encapsulated in the maximally freeze-concentrated solutes. Freezing at −35 °C for 24 h and thawing at room temperature did not cause significant changes of the $D[4, 3]$ and the oil droplet distributions (data not shown) in all systems, except in the trehalose–SPI–OO system, which showed an increase of $D[4, 3]$ but similar $D[v, 0.5]$, indicating the increase of tail distribution (Table 4). The structure of freeze-dried materials was determined by (i) the oil droplet size distribution (size and number) during homogenization and (ii) the ice crystals (size and number) formed during freezing. After the sublimation of ice crystals, pores were left and a porous structure was obtained. Because of the low molecular mobility in the glassy matrices, the oil droplet size distribution was retained in the freeze-dried materials. Freeze-dried lactose–MPI–OO and trehalose–MPI–OO systems showed bimodal distribution (curve b in panels A and C of Figure 5, respectively), and freeze-dried lactose–SPI–OO and trehalose–SPI–OO showed increased tail distribution (curve b in panels B and D of Figure 5, respectively), of oil droplets, which could be a result of the

Table 2. Onset and Endset Temperatures of Glass Transition (T_g, °C) at Various Water Activities (a_w) for Freeze-Dried Lactose−MPI−OO (3:1:2), Lactose−SPI−OO (3:1:2), Trehalose−MPI−OO (3:1:2), and Trehalose−SPI−OO (3:1:2) Systems

system		T_g[a] (°C)				
		0.00 a_w[b]	0.11 a_w[c]	0.23 a_w[d]	0.33 a_w[e]	0.44 a_w[f]
lactose−MPI−OO	onset	101	52	35	27	15
	endset	111	60	44	34	24
lactose−SPI−OO	onset	106	50	37	29	16
	endset	118	59	45	37	25
trehalose−MPI−OO	onset	111	53	33	25	14
	endset	119	60	42	33	21
trehalose−SPI−OO	onset	116	49	38	28	15
	endset	124	58	47	35	23

[a] T_g, ±1°C. [b] DSC method: (i) from 25 to 150 °C at 5 °C/min, (ii) from 150 to −50 °C at 10 °C/min, and (iii) from −50 to 200 °C at 5 °C/min. [c] DSC method: (i) from 25 to 90 °C at 5 °C/min, (ii) from 90 to −50 °C at 10 °C/min, and (iii) from −50 to 150 °C at 5 °C/min. [d] DSC method: (i) from 10 to 70 °C at 5 °C/min, (ii) from 70 to −50 °C at 10 °C/min, and (iii) from −50 to 140 °C at 5 °C/min. [e] DSC method: (i) from 0 to 60 °C at 5 °C/min, (ii) from 60 to −50 °C at 10 °C/min, and (iii) from −50 to 130 °C at 5 °C/min. [f] DSC method: (i) from −10 to 50 °C at 5 °C/min, (ii) from 50 to −50 °C at 10 °C/min, and (iii) from −50 to 120 °C at 5 °C/min.

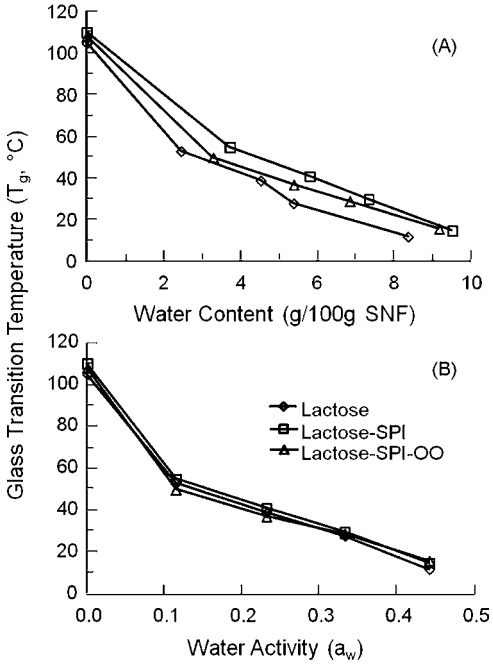

Figure 4. Effects of (A) water content (g/100 g of SNF) and (B) water activity (a_w) on the onset temperature of glass transition (T_g, °C) in freeze-dried lactose,[19] lactose−SPI (3:1),[19] and lactose−SPI−OO (3:1:2) systems.

Table 3. Onset Temperature of Crystallization (T_{ic}, °C) in Freeze-Dried Lactose−MPI−OO (3:1:2) and Lactose−SPI−OO (3:1:2) Systems

system	T_{ic}[a] (°C)				
	0.00 a_w	0.11 a_w	0.23 a_w	0.33 a_w	0.44 a_w
lactose−MPI−OO	N/O[b]	124	108	91	83
lactose−SPI−OO	N/O[b]	124	107	94	75

[a] T_{ic}, ±1°C. [b] N/O = not observed.

breakdown of the thin interface during reconstitution. After storage at 0.76 a_w for 5 days, a dramatic change of droplet size distribution was observed, corresponding to the crystallization of component sugar, which caused a complete rupture of the interfaces between the continuous and dispersed phases. Oil droplets were excluded from the encapsulants, and coalescence occurred.

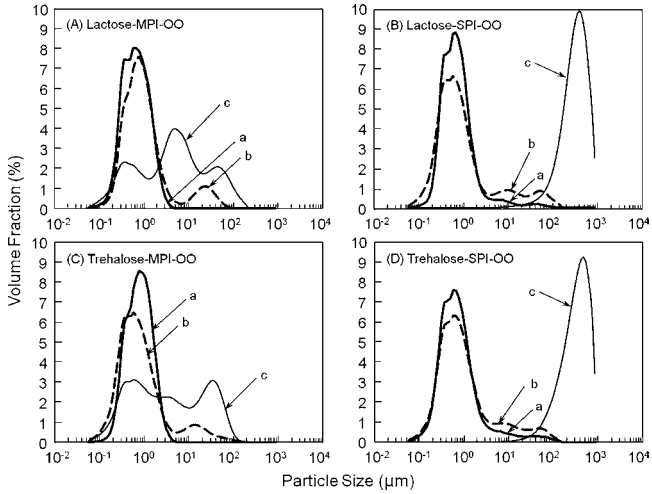

Figure 5. Emulsion droplet size distribution of (A) lactose−MPI−OO (3:1:2), (B) lactose−SPI−OO (3:1:2), (C) trehalose−MPI−OO (3:1:2), and (D) trehalose−SPI−OO (3:1:2) systems. The emulsion samples for each system were (a) fresh emulsions that were measured within 1 h after homogenization, (b) freeze-dried materials that were reconstituted and measured, and (c) freeze-dried materials that were stored at 0.76 a_w for 5 days, reconstituted and measured.

Stability of α-Tocopherol as Affected by Water Activity. The retention of α-tocopherol in the freeze-dried materials was measured right after freeze-drying, which were 94.8 ± 0.8%, 93.9 ± 1.5%, 95.1 ± 1.6%, and 92.4 ± 1.2% for lactose−MPI−OO, lactose−SPI−OO, trehalose−MPI−OO, and trehalose−SPI−OO systems, respectively. These amounts of retained α-tocopherol were taken as 100% for the storage study.

During 40 days of storage at 60 °C, rapid loss of α-tocopherol occurred in all anhydrous systems within 3 days of storage, followed by less rapid loss up to 25 days of storage. From 25 to 40 days, constant retention of α-tocopherol was found, except for the lactose−SPI−OO system, in which the α-tocopherol content decreased but at a lower rate (Figure 6). After 40 days of storage, lactose−MPI−OO and trehalose−MPI−OO systems retained 84.6 ± 1.5% and 82.8 ± 1.2% of α-

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Table 4. Volume Weight Mean Diameter, $D[4, 3]$ (Mean ± SD, μm) and 50% Volume Percentile of Droplet Size Distribution, $D[v, 0.5]$ (Median Mean Diameter ± SD, μm) of Lactose−MPI−OO (3:1:2), Lactose−SPI−OO (3:1:2), Trehalose−MPI−OO (3:1:2), and Trehalose−SPI−OO (3:1:2) Systems

	fresh emulsions[a]		thawed emulsions[b]		freeze-dried emulsions[c]	
system	$D[4, 3]$ (μm)	$D[v, 0.5]$ (μm)	$D[4, 3]$ (μm)	$D[v, 0.5]$ (μm)	$D[4, 3]$ (μm)	$D[v, 0.5]$ (μm)
lactose−MPI−OO	0.75 ± 0.00	0.60 ± 0.00	0.88 ± 0.10	0.72 ± 0.01	3.26 ± 0.63	0.77 ± 0.01
lactose−SPI−OO	1.82 ± 0.03	0.59 ± 0.02	1.95 ± 0.20	0.62 ± 0.01	5.35 ± 0.15	0.62 ± 0.01
trehalose−MPI−OO	0.85 ± 0.02	0.61 ± 0.02	1.03 ± 0.43	0.61 ± 0.01	3.03 ± 0.33	0.62 ± 0.01
trehalose−SPI−OO	2.53 ± 0.16	0.60 ± 0.02	3.92 ± 0.70	0.63 ± 0.01	5.47 ± 1.27	0.67 ± 0.02

[a]Measurements were carried out within 1 h after homogenization (fresh emulsions). [b]Measurements were carried out after thawing of frozen emulsions. Fresh emulsions were frozen at −35 °C for 24 h and then thawed at room temperature for 1 h. [c]Measurements were carried out after reconstitution of freeze-dried materials.

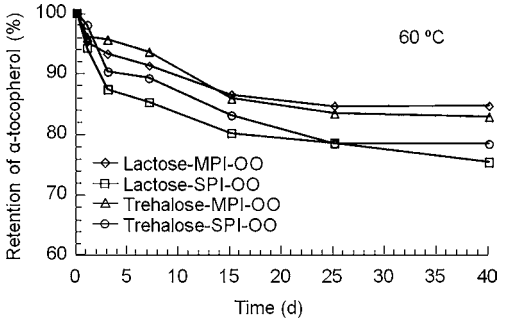

Figure 6. Retention (%) of α-tocopherol in anhydrous freeze-dried lactose−MPI−OO (3:1:2), lactose−SPI−OO (3:1:2), trehalose−MPI−OO (3:1:2), and trehalose−SPI−OO (3:1:2) systems during 40 days of storage at 60 °C.

tocopherol, respectively, while lactose−SPI−OO and trehalose−SPI−OO systems retained smaller amounts of α-tocopherol, which were 75.3 ± 2.1% and 78.3 ± 1.7%, respectively.

The retention of α-tocopherol after 5 days of storage at various a_w conditions and the water content of systems at corresponding a_w were plotted as a function of a_w (Figure 7). α-Tocopherol was the most stable at 0 a_w. MPI-stabilized systems showed higher retention. This high retention of α-tocopherol confirmed our hypothesis that α-tocopherol did not show good

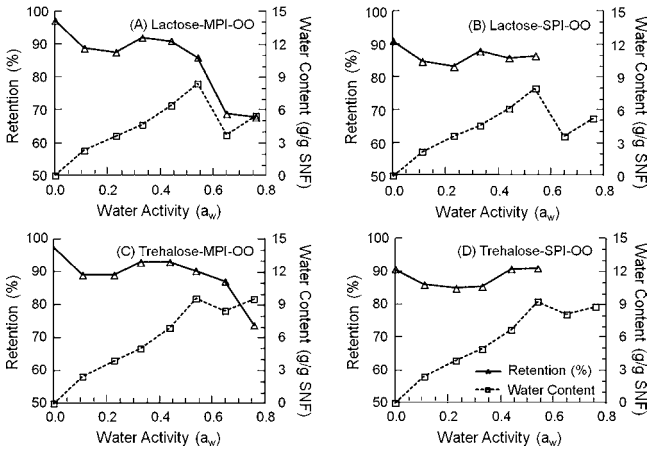

Figure 7. Retention (%) of α-tocopherol and water content (g/100 g of SNF) for freeze-dried (A) lactose−MPI−OO (3:1:2), (B) lactose−SPI−OO (3:1:2), (C) trehalose−MPI−OO (3:1:2), and (D) trehalose−SPI−OO (3:1:2) systems after storage at various a_w (0.0−0.76) for 5 days.

antioxidant activity in anhydrous systems, which could be similar to bulk oil systems, and the stability of α-tocopherol was assured by its immobilization in the glassy solid matrices. α-Tocopherol is a hydrophobic compound but contains a hydrophilic OH group, which is surface-active to be oriented in the oil−water interface to protect oil against oxidation.[23] During freezing, water molecules formed ice crystals and the glassy solids with α-tocopherol at interfaces were formed. Freeze-drying removed ice crystals by sublimation and retained the structures formed during freezing, explaining a relatively high stability of α-tocopherol at low a_w.

α-Tocopherol showed highest stability at 0 a_w in all of the systems. A minimum retention of α-tocopherol was found at 0.11−0.23 a_w (at 0.11−0.33 a_w for the trehalose−SPI−OO system), which agreed with rapid lipid oxidation at low a_w.[7] At increasing a_w (0.33−0.44 a_w for the trehalose−SPI−OO system and from 0.23 to 0.33 a_w for all other systems), the retention of α-tocopherol increased, showing that the loss of hydrophobic α-tocopherol coincided with the oxidation of the oil.[6−8] At higher than 0.54 a_w, the loss of α-tocopherol was concomitant to component sugar crystallization. The reasons for the loss of α-tocopherol could be (i) exclusion of oil droplets from the matrices causing a loss of protection, (ii) increased availability of oxygen from the environment, and (iii) increased mobilization of α-tocopherol at the interfaces and its consumption as an antioxidant. Some differences in α-tocopherol stability at crystallizing conditions may also relate to higher solubility of trehalose and retention of a protective trehalose "syrup" on entrapped oil particles. SPI-containing systems at a_w > 0.54 showed a difficulty in reconstitution of freeze-dried materials and, therefore, a difficulty in the extraction of α-tocopherol, possibly because of the gelation of SPI with a sufficient amount of water, which was released from sugar crystallization. Extraction of oil from such systems, however, was technically difficult, and data at higher a_w values were not included.

Materials equilibrated at various a_w (referring to a_w at room temperature) were transferred to 60 °C and kept for 10 days. At 60 °C, systems with T_g above (0 a_w) and around (0.11 a_w) the storage temperature retained α-tocopherol in the glassy structures, indicating that the glassy state protected α-tocopherol. The loss of α-tocopherol occurred at 0.23 a_w and above, at which the heat-induced plasticization depressed the T_g of the systems to below the storage temperature, followed by component sugar crystallization (Figure 8). Lactose crystallization had a more severe effect on the loss of α-tocopherol compared to trehalose crystallization. Trehalose crystallized to a lesser extent as a dihydrate and retained a higher amount of water in the crystals. The higher solubility of trehalose

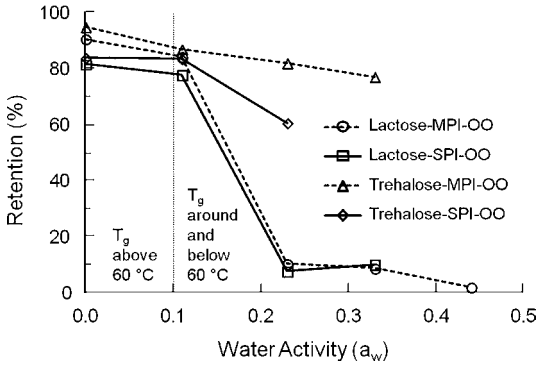

Figure 8. Retention (%) of α-tocopherol for freeze-dried lactose–MPI–OO (3:1:2), lactose–SPI–OO (3:1:2), trehalose–MPI–OO (3:1:2), and trehalose–SPI–OO (3:1:2) systems after storage at 60 °C for 10 days. The materials were equilibrated to various a_w (0.0–0.44) for 5 days before transportation to 60 °C.

dihydrate also allowed trehalose to dissolve in water and form trehalose syrup, which possibly protected the sensitive compounds.[19] The rapid loss of α-tocopherol at sugar crystallization further suggested that a relatively large quantity of α-tocopherol could be located at the oil–water interface.

Significant color changes (browning) were visually observed at $a_w \geq 0.23$ after storage at 60 °C for lactose-containing systems. Slight color changes were found in trehalose-containing systems at $a_w \geq 0.65$ after storage at 60 °C, which could be due to the presence of traces of reducing sugars in proteins. SPI-containing systems at $a_w > 0.33$ in storage at 60 °C showed a difficulty in reconstitution of freeze-dried materials, which was due to the possible gelation of SPI at these conditions.

Matrix Structure and Stability of α-Tocopherol. Schematic diagrams of the structure formation during processing and structural changes during storage for model systems are shown in Figure 9. Homogenization broke down the oil phase into small droplets and stabilized the dispersed phase in the water phase by the amphiphilic proteins[36] (panels A and B of Figure 9). During freezing, ice crystals were formed and the solutes (sugar–protein) were freeze-concentrated. The oil droplets were immobilized and encapsulated in the continuous unfrozen solute phase (Figure 9C). Pores were left after the sublimation of ice crystals, and the size of ice crystals formed during freezing determined the porosity of the freeze-dried materials. The oil droplets were encapsulated in the matrices, although partial exposure to the pores was possible (Figure 9D). During storage at various a_w conditions, water was sorbed by the amorphous solutes because of the high hygroscopy and porosity of the freeze-dried materials (Figure 9E). Labuza[7] has found that at 0 and low a_w, lipid oxidation occurred rapidly. At increasing a_w, water exerted a protective effect, which attributed to the hydration of metal catalysts decreasing their effectiveness and hydrogen bonding to peroxides. This explained the low retention of hydrophobic α-tocopherol at low a_w. α-Tocopherol could be consumed as an antioxidant and showed lower retention, accounting for the decreased oxidation of the solvent lipid. In our study, the stability of α-tocopherol at 0 a_w was possibly enhanced by partial immobilization at interfaces of the oil and the glassy matrices that could reduce the antioxidant activity of α-tocopherol, i.e., corresponding to lower consumption as an antioxidant, at 0 a_w. At water activities that depressed the T_g to below the storage temperatures (room temperature or 60 °C) and allowed the component sugar to crystallize time-dependently, the stability of α-tocopherol was no longer retained because of the increased exposure of lipids to the surrounding atmosphere. Structural changes, e.g., collapse (Figure 9F) and sugar crystallization (Figure 9G), caused the exclusion of the oil droplets from the encapsulants to the environment and coalescence of the oil droplets, leading to losses of α-tocopherol.[35]

In conclusion, this study showed that stability of α-tocopherol in freeze-dried carbohydrate–protein–oil emulsions was affected by water plasticization and physical changes. This information contributes to the use of carbohydrate–protein systems as structure-forming matrices for stabilization of

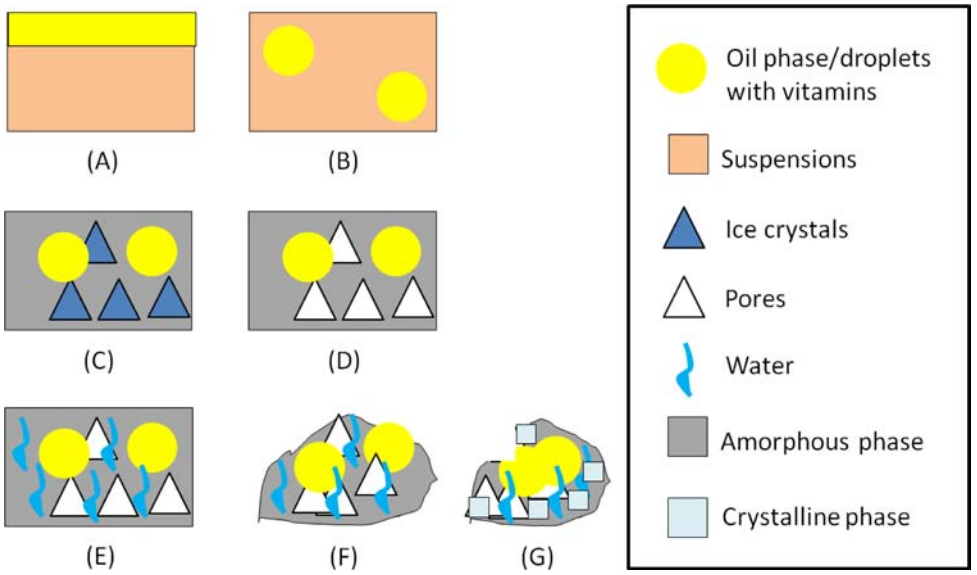

Figure 9. Diagrams of the model systems (A) before and (B) after homogenization, (C) frozen, and (D) freeze-dried. During storage, (E) water-plasticization and (F) collapse occurred because of water sorption. (G) Crystallization of sugars may lead to exclusion and coalescence of the oil droplets. The symbols do not represent the real size of components.

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hydrophobic nutrients. Freezing and freeze-drying of emulsions encapsulated oil droplets in structure-forming sugar−protein matrices. Milk proteins and soy proteins showed good emulsifying properties as well as contribution to the matrix properties. The structure of the systems was well-retained during processing, although changes of oil droplet size distribution occurred because of the ruptures of the interfaces on reconstitution. The presence of oil slightly altered the water sorption behavior of the matrices but reduced the crystallization rate of the component sugars at high water activity conditions. α-Tocopherol was probably immobilized at anhydrous conditions at interfaces and did not show antioxidant activity, and the stability of α-tocopherol was retained in anhydrous freeze-dried sugar−protein matrices. Water activity affected the stability of α-tocopherol by affecting the rate of lipid oxidation and the antioxidant activity of α-tocopherol. Water-sorption- and thermal-induced crystallization of the component sugars caused dramatic structural changes of the matrices, which were believed to be the main cause of the loss of the stability of α-tocopherol. Dependent upon the properties (type of crystals and their solubility) of the component sugars, the effects of sugar crystallization differed. Because sugar crystallization is a time-dependent phenomenon above the T_g of the matrices, more studies are required to understand the changes above T_g of the systems and the effects of structural changes on the stability of encapsulated particles.

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Notes

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ABBREVIATIONS USED

a_w, water activity; MPI, milk protein isolate; SPI, soy protein isolate; OO, olive oil; w/w, weight per weight; T_g', glass transition temperature of the maximally freeze-concentrated solutes; T_m', ice-melting temperature in the maximally freeze-concentrated systems; SNF, solid nonfat; GAB, Guggenheim−Anderson−de Boer relationship; m_m, monolayer water content; T_g, glass transition temperature; T_{ic}, instant crystallization temperature; w/v, weight per volume

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