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CHARACTERIZATION OF LYOPHILIZED LIPOSOMES PRODUCED WITH NON-PURIFIED SOY LECITHIN: A CASE STUDY OF CASEIN HYDROLYSATE MICROENCAPSULATION

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Abstract - A commercial casein hydrolysate was microencapsulated in liposomes produced with non-purified soy lecithin, cryoprotected with two different disaccharides and lyophilized. The encapsulation efficiency of casein hydrolysate ranged from 30 to 40%. The powders were analyzed by differential scanning calorimetry (DSC), scanning electron micrography (SEM), infrared spectroscopy (FTIR) and wide angle X-ray diffraction (WAXD). DSC data revealed the presence of an exothermal transition in empty lyophilized liposomes, which was ascribed to the presence of a quasicrystalline lamellar phase (intermediary characteristics between the L_β and L_c phases). The addition of peptides to the liposomal system caused the disappearance of this exothermic phenomenon, as they were located in the polar headgroup portion of the bilayer, causing disorder and preventing the formation of the quasicrystalline phase. Infrared data indicated the presence of the peptides in the lyophilized formulations and showed that the cryoprotectants interacted effectively with the polar heads of phospholipids in the bilayer.
Keywords: Casein hydrolysate; Liposomes; Lyophilized liposomes; Microencapsulation.

INTRODUCTION

Several protein hydrolysates are classified as bioactive and can be added to food formulations to provide functionality in the form of physiological benefits or to reduce the risk of certain diseases. Another important use for bioactive peptides is their addition to special formulations to support specific clinical needs of patients who are diagnosed with diseases, including phenylketonuria, acute and chronic liver disease, Crohn´s disease, pancreatitis and ulcerative colitis (Clemente, 2000). A wide range of beneficial effects have been described, including antimicrobial properties, blood-pressure-lowering effects, cholesterol-lowering ability, anti-thrombotic and antioxidant activities, enhancement of mineral absorption, cyto- or immunomodulatory effects, and opioid activities (Hartmann and Meisel, 2007). However, there are some issues with these protein derivatives, including unpleasant sensory properties (accentuated bitterness and strong odor), difficult dispersal in aqueous formulations due to their hydrophobicity, and allergenicity. These drawbacks often limit the incorporation of bioactive peptides in food products. In addition, protein hydrolysates may be hygroscopic and reactive (Fávaro-Trindade *et al.,* 2010). The hydrophobicity and the sensory characteristics are related to the exposed hydrophobic groups in the peptides that appear during the protein hydrolysation process

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(Yeom *et al*., 1994). Several studies have demonstrated that the bioavailability of peptides and proteins after oral administration can be low, due to their instability in the gastrointestinal tract and low permeability through the intestinal membrane (Lee and Yamamoto, 1990).

Microencapsulation is an alternative to overcome some of these drawbacks and to improve the resistance of the peptides to gastrointestinal conditions, as well as to increase their absorption in the intestine. One of the approaches frequently employed in the pharmaceutical field to attain the cited aims is LBDD (lipid-based drug delivery), i.e., the encapsulation of bioactive compounds in lipid-based structures (for instance, emulsions, lipid particles, or liposomes) (Jeong *et al*., 2007; Jannin *et al*., 2008; Trevaskis *et al*., 2008). This approach can be used to microencapsulate bioactives for food applications. One example of a suitable lipid-based system of microencapsulation for food applications is the liposome.

Liposomes are vesicular structures composed of a phospholipid membrane surrounding an aqueous core. Due to their peculiar structural characteristics, they can encapsulate or incorporate hydrophilic, hydrophobic or amphiphilic substances. A wide range of applications is possible in food using these versatile colloidal systems, and an increasing number of studies are available in the literature. These studies cover a large variety of substances, including enzymes (Benech *et al*., 2003; Kheadr *et al.,* 2003; Rodriguez-Nogales and López, 2006), protein hydrolysates (Morais *et al*., 2003), antioxidants (Kostecka-Gugala *et al*., 2003; McNulty *et al*., 2007), antimicrobials (Were *et al.,* 2004; Taylor *et al*., 2007), minerals (Ucich *et al.,* 1999; Kosajaru *et al*., 2006) and vegetable extracts and essential oils (Gortzi *et al*., 2007; Gortzi *et al*., 2008; Takahashi *et al*., 2008; Detoni *et al.,* 2009; Yoshida *et al.,* 2010).

The two main disadvantages of liposomal encapsulation for food applications are the scaling up of production processes and the cost of phospholipids (Taylor *et al*., 2005; Mozafari *et al.,* 2008). Most of the traditional methods used to produce liposomes require the use of organic solvents, and there are often reports of the high loss of material and the difficulty of scaling up high-pressure devices. The cost of raw materials (mainly phospholipids) is limiting even for high aggregated value ingredients or bioactive compounds. However, most of the studies of liposomes in food applications employ the same raw materials as in the pharmaceutical field, such as high-cost synthetic phospholipids or purified natural phospholipids (which are cheaper but still costly for large-scale production; for purified natural phospholipids, an average price is 980 euro/kg; non-purified soy lecithin costs approximately 20% of this value). In addition, despite being a promising microencapsulation system, liposomes have the disadvantage of instability (mainly multilamellar or multivesicular structures) and require the utilization of stabilization strategies, such as lyophilization or the addition of thickening agents in the aqueous phase. Lyophilization is the main approach used to extend the shelf life of liposomes, especially for those containing thermosensitive bioactive compounds (Chen *et al.,* 2010).

The main objective of this study was to verify the feasibility of producing liposomes with a lower cost phospholipid (non-purified soy lecithin) on a laboratory scale. Differently from most studies found in the literature regarding the application of liposomes in food (Benech *et al.,* 2003; Morais *et al.,* 2003; Were *et al*., 2004; Rodriguez-Nogales *et al*., 2006; Gortzi *et al*., 2007; Gortzi *et al*., 2008), which employ pharmaceutical-grade phospholipids (purified or synthetic), this study was carried out using a food-grade, non-purified and non-hydrogenated phospholipid (Lipoid S40, Lipoid GmbH, Germany). In addition, the liposomes were lyophilized and the effect of two disaccharides (trehalose and sucrose) as cryoprotectors was evaluated in terms of their influence on the structural characteristics of the liposomes. The phospholipid vesicles encapsulated casein hydrolysate (Hyprol 8052, Kerry Bioscience, Brazil) and were subjected to a lyophilization step. Hyprol is a hydrolyzed enzymatic digest of casein that provides high quality peptides, including casein phosphopeptides. The product is recommended as a soluble peptide source for enteral nutrition, infant food formulas, and for protein enrichment of food and beverages. However, it presents a strong bitter taste and a very unpleasant odor, limiting its direct use as a food additive (Kerry Bioscience, 2005; Fávaro-Trindade *et al*., 2010). After encapsulation and lyophilization, the microstructures of the systems were characterized by X-ray Diffractometry, Scanning Electron Microscopy, Differential Scanning Calorimetry and Fourier Transformed Infrared Spectroscopy.

MATERIALS AND METHODS

Chemicals

Soy lecithin (Lipoid S40, fat-free soybean lecithin with not less than 40% phosphatidylcholine, CAS number 8030-76-0) was used in the following experiments. According to the supplier (Lipoid

GmbH, Germany), the typical composition of this lecithin is no less than 97% total phospholipids (including at the minimum 40% phosphatidylcholine and 12-15% phosphatidylethanolamine, and a maximum of 3% of phosphatidylinositol and 4% of lysophosphatidylcholine). In terms of fatty acids, Lipoid S40 is composed of 17-20% palmitic acid, 2-5% stearic acid, 8-12% oleic acid, 58-65% linoleic acid and 4-6% linolenic acid. Hyprol 8052 (casein hydrolysate, in this text denominated as CH) was donated by Kerry Bioscience (Brazil). Its amino acid composition is shown in Figure 1. Hyprol 8052 also contains 9% CPP (casein phosphopeptides) and 21% free amino acids, and 92% of the peptides have molecular weights below 5 kDa. Other reagents and organic solvents were of analytical grade. Deionized water was used throughout.

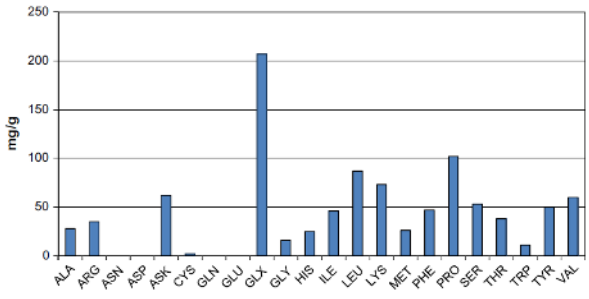


Figure 1: Typical amino acid profile of Hyprol 8052 (Data from Kerry Bioscience, 2005). Glx (glutamate + glutamic acid): 21%; proline: 10.2%; leucine: 8.7%; lysine: 7.3% (% in mass).

Liposome Preparation and Evaluation of Encapsulation Efficiency

The multilamellar liposomes composed of Lipoid S40 were prepared on a laboratory scale by the film hydration method, using a final phospholipid concentration of 7.5 mg.mL^{-1}, typical in liposome studies (Cabral *et al*., 2004; Detoni *et al*., 2009; Yoshida *et al*., 2010). Phospholipids were solubilized in a mixture of methanol:chloroform (1:9, v/v) and the organic solvent was completely evaporated under reduced pressure at 65°C in a rotary evaporator, resulting in a dry lipid film. The dry lipid film was then hydrated with a solution composed of casein hydrolysate (1 g.L^{-1}) solubilized in phosphate buffer (2 g.L^{-1}, pH 7.4) under agitation (150 rpm). Such concentrations of casein hydrolysate and phosphate buffer were based on the data presented by Morais *et al.* (2004), who also worked with liposomes encapsulating casein hydrolysates. When present in the formulation, the cryoprotectant – sucrose (Synth, Brazil) or trehalose (Vetec, Brazil) – was added to the hydration fluid at a concentration of 30 mg.mL^{-1} (cryoprotectant: phospholipid 4:1, mass ratio, value based on Cabral *et al*., 2004 and Yoshida *et al*., 2010). After the hydration step, the dispersion was submitted to bath sonication for 10 minutes and left for 24 at room temperature before storage under refrigeration.

Non-encapsulated casein hydrolysate was separated by ultrafiltration through a cellulose membrane (molecular weight cut-off of 100 kDa, YM100, Millipore) and quantified for total protein by the Lowry method as modified by Peterson (1977). The encapsulation efficiency was determined with the following equation:

$$EE(\%) = 100 * \left(\frac{\text{original mass of CH in the hydration solution} - \text{mass of non-encapsulated CH}}{\text{original mass of CH in the hydration solution}} \right)$$

Determination of Average Particle Size

The average particle size of liposomes in aqueous dispersions was obtained by photon correlation spectroscopy, using a Zetasizer Nano ZS (Malvern, UK), at 25°C, using a He-Ne laser at 633 nm. The samples were diluted with ultra-purified water to weaken the opalescence before measuring the particle mean diameter.

Differential Scanning Calorimetry (DSC)

Lyophilized liposome samples were analyzed in a microcalorimeter (TA2010, controlled by the system TA5000, both from TA Instruments, USA), with a temperature ramp of 10º C.min^{-1}, in an inert atmosphere (45 mL.min^{-1} of N_2). The reference was an empty aluminum pan. The glass transition (mid-point) temperature (T_g) and the melting (peak) temperature were calculated using the Universal Analysis V.7 software (TA Instruments).

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy of lyophilized liposome samples was performed using a LEO 440i (Leica Instruments, Germany) with an accelerating voltage of 15 kV. The samples were sputtered with gold, in a Pelaron SC7620 sputter coater (Ringmer, UK), at a covering rate of 0.51 angstrons.s^{-1}, for 180 s,

using a current of 3-5 mA, 1V and at a pressure of $2x10^{-2}$ Pa.

Fourier Transformed Infrared Spectroscopy (FTIR)

Infrared spectra were obtained in a Spectrum One (FTIR with ATR, Perkin Elmer, USA), with scans in the frequency range of 4000 to 600 cm^{-1} at room temperature, with a 4 cm^{-1} spectral resolution. For each spectrum, 16 scans were co-added. Data was analyzed using the software FTIR Spectrum (Perkin Elmer).

Wide Angle X-Ray Diffraction (WAXD)

Lyophilized liposomes were analyzed using an X-ray diffractometer (Phillips X´PERT, copper anode, λ=1.5418 angstroms), and the data were obtained from scans in the range of 2θ from 5 to 40º (steps of 0.02º), at room temperature.

RESULTS AND DISCUSSION

The encapsulation efficiency and the average particle size in an aqueous dispersion are shown in Table 1. The data indicate that non-cryoprotected liposomes produced with non-purified soy lecithin were smaller on average compared to non-cryoprotected liposomes produced with purified and hydrogenated soy lecithin (Lipoid S100-H, Lipoid GmbH, Germany). This effect can be explained by the presence of phosphatidylethanolamine (PE) in the Lipoid S40 (12-15% by mass). This PE concentration probably led to a tighter packing of the phospholipids in the bilayer, as the presence of PE avoids the kink of the acyl chains (Somerharju *et al*., 1999). The polar region of the liposome bilayers may have been less crowded, allowing the acyl chains to pack closer together in a more optimized configuration (Somerharju *et al.*, 1999), decreasing the average particle size significantly.

However, the average particle size of CH-loaded liposomes produced with non-purified soy lecithin increased when disaccharides were added to the formulations. This phenomenon may have occurred due to the presence of cryoprotectant molecules in the polar region of the liposome, separating the headgroups of the phospholipids as well as the Hyprol 8052 peptides. The decrease in the encapsulation efficiency may also be a consequence of this fact.

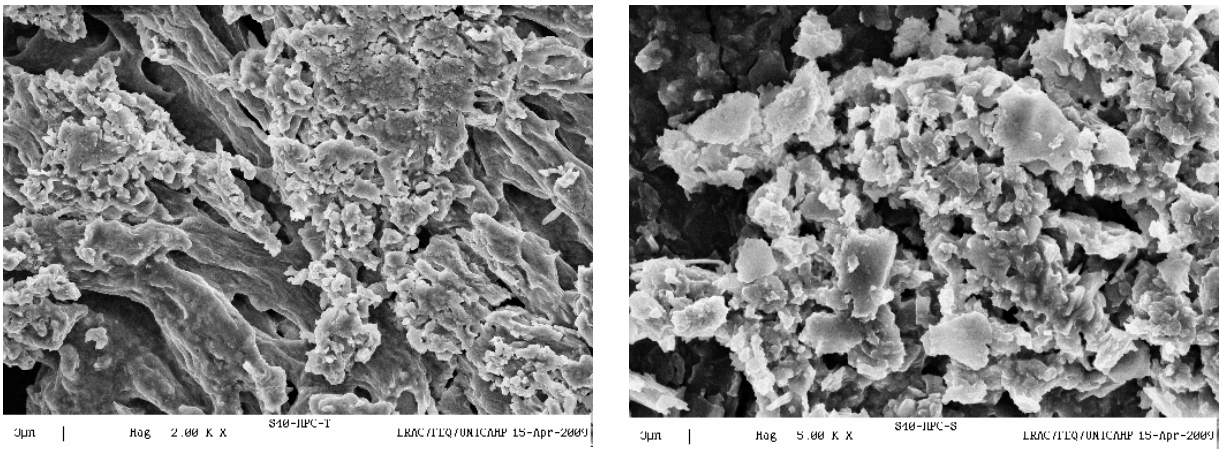
Scanning electron micrographs (Figure 2) of the powders obtained by lyophilization of the liposomes containing cryoprotectants indicated that extensive fusion of the phospholipid vesicles did not occur.

The lyophilized liposomes are visible in the micrographs as spherical structures and many of them are clearly incorporated into the amorphous mass of excess cryoprotectant. The addition of cryoprotectants such as sucrose and trehalose prevents leakage or fusion and plays an essential role in cake formation by creating an amorphous matrix in and around the phospholipid vesicles (Alexopoulou *et al*., 2006). Excess carbohydrate normally occurs with disaccharide/phospholipid ratios higher than 1 (Cacela and Hincha, 2006) and has a visible amorphous characteristic (*i.e*, a high viscosity and a low molecular mobility), protecting the liposomes during the drying process and decreasing the probability of fusion, as it makes the approximation between the vesicles almost impossible (Koster *et al.,* 2000).

Thermograms obtained by differential scanning calorimetry of lyophilized liposome samples showed some peculiar characteristics in the liposomal system produced with non-purified soy lecithin. Figure 3 shows the thermal behavior for lyophilized liposomes in the absence of casein hydrolysate.

Table 1: Encapsulation efficiencies and average particle size of the liposome formulations tested.

Liposome	Encapsulation efficiency (%)	Average particle size (μm)
CH-loaded, non-cryoprotected S100-H liposomes	43.4 ± 1.75	5.00 ± 1.21
CH-loaded, non-cryoprotected S40 liposomes	46.0 ± 1.0	0.53 ± 0.02
CH-loaded, cryoprotected with trehalose S40 liposomes	37.1 ± 0.80	2.15 ± 0.23
CH-loaded, cryoprotected with sucrose S40 liposomes	30.8 ± 1.22	2.27 ± 0. 31



(a) (b)

Figure 2: Scanning electron micrographs of cryoprotected CH-liposomes: (a) with trehalose; (b) with sucrose.

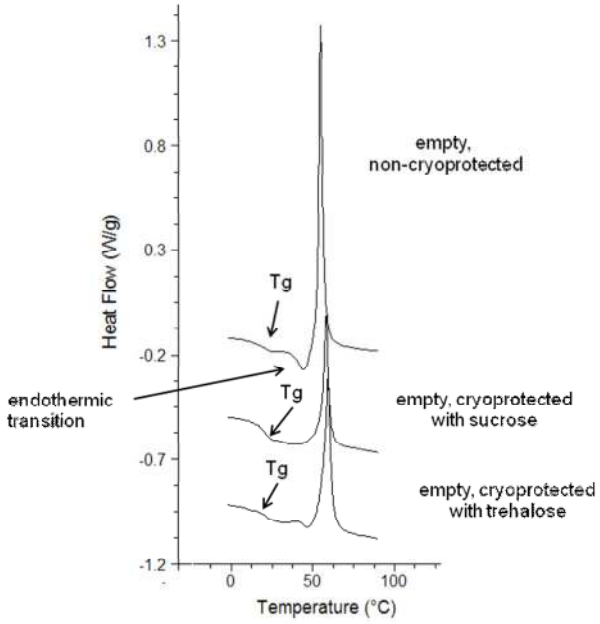


Figure 3: Thermograms obtained by DSC for empty lyophilized liposomes.

Some important information can be seen in the thermograms of empty liposomes. Non-cryoprotected lyophilized vesicles presented an endothermic peak at 44.2°C (pointed out in the thermogram), followed by an exothermic transition at 54.7°C. The endothermic peak may be due to a gel-liquid crystalline main transition, as dehydrated membranes were brought into close proximity with each other, and the hydration repulsion induced a compressive stress in the membrane, making the gel phase energetically favorable at higher temperatures (Koster *et al.,* 2000), thus causing the transition observed at 44.2ºC.

This endotherm disappeared in the thermograms obtained for cryoprotected empty liposomes. The addition of small solutes, such as disaccharides, to replace the original water molecules can increase the distance between membranes due to osmotic and volumetric effects, and the inter- and intra-membrane stress will decrease (Koster *et al*., 2000). These effects are expressed by a correspondent decrease (or even disappearance) in the T_m when

disaccharides, for example, are added to liposomal formulations. In the case of the systems analyzed here, the addition of both disaccharides promoted the total disappearance of the gel-liquid crystalline transition in lyophilized liposomes.

The exothermic peak present in the three samples of lyophilized liposomes in the absence of casein hydrolysate can be ascribed to the presence of phosphatidylethanolamine (PE) in the Lipoid S40. An exothermic transition indicates that the structure (or some domains in it) passed from a disordered to a more ordered phase, a phenomenon previously observed by Feng *et al*. (2004) in heating scans of the co-dispersions of phosphatidylethanolamine and glucocerebroside. Those authors detected the presence of a quasicrystalline phase (denominated as L_q) coexisting with a lamellar phase L_{β}. They hypothesized that the L_q phase is an intermediate lamellar phase between L_β and L_c that consists of a structure with highly ordered headgroups, as in the L_c phase, but with more disordered hydrocarbon chains in the apolar region of the liposome bilayer, as in the L_β phase (Koynova and Caffrey, 1998; Feng *et al*., 2004). It is quite reasonable to suppose that a similar arrangement was present in the samples analyzed here. Lipoid S40 is rich in unsaturated fatty acids (as listed in the Materials and Methods section), containing 12-15% of PE (by mass). Both characteristics are cited by Feng *et al*. (2004) as requisites for L_q phase existence. Phosphatidylethanolamine is composed of fewer hydrated polar groups than the choline phospholipids and has a greater tendency to form L_C phases. However, the need for accommodating the mismatch in fatty acid chain lengths between the two lipids of the complex in the apolar portion of the bilayer results in a decrease in the order of chain packing and an increase in the mobility in the bilayer core, exactly the configuration of the L_q lamellar phase.

The thermal behavior of cryoprotected, lyophilized CH-loaded liposomes obtained by DSC analysis is shown in Figure 4. The exothermic peaks previously described are not present, suggesting that the presence of the peptides probably induced disorder in the polar region of the liposome membrane. Some studies in the literature (Ringstad *et al*., 2008; Orädd *et al*., 2011) suggest that peptides tend to be localized near the polar group region of the phospholipid membrane, oriented parallel to the membrane surface. Depending on their hydrophobicity, they can penetrate the aliphatic region; however, this is not the case for Hyprol 8052, which is composed primarily of hydrophilic peptides.

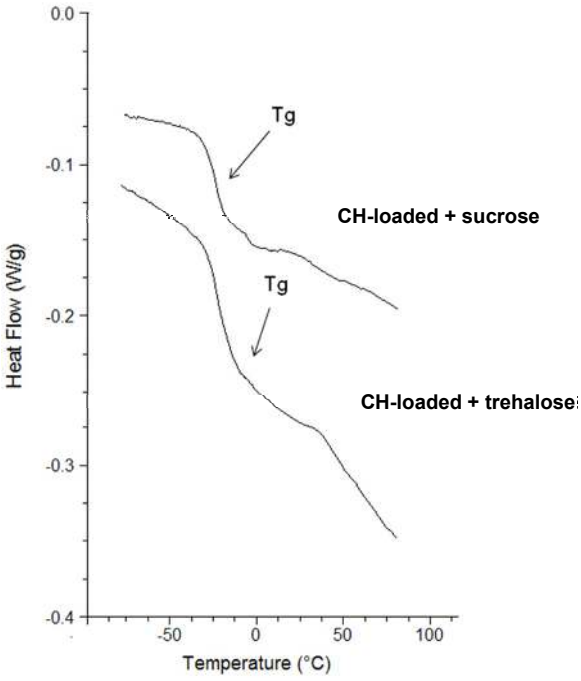


Figure 4: Thermograms obtained by DSC for lyophilized liposomes microencapsulating casein hydrolysate.

Another indication of the increased disorder due to the presence of peptides in lyophilized samples is the significant reduction in the value of T_g (glass transition temperature), suggesting that the solid matrix became more susceptible to molecular mobility at much lower temperatures, probably due to lower rigidity caused by the presence of the peptide in the bilayer polar region. The value of T_g decreased from 20.2°C in empty lyophilized trehalose-cryoprotected liposomes to -23.0°C in trehalose-cryoprotected CH-loaded liposomes and from 20.5°C in empty lyophilized sucrose-cryoprotected liposomes to -24.3°C in sucrose-cryoprotected CH-loaded liposomes.

Infrared spectroscopy was used to characterize the lyophilized liposomes microencapsulating casein hydrolysate. The spectra are shown in Figure 5.

The participation of the P=O in the phosphate group and the C=O in the ester groups of the polar head of the phospholipids in interactions with the other components of the liposomal formulations can be related to the peaks seen at 1232 cm^{-1} and 1737 cm^{-1}, respectively. Both peaks were lower in the spectra of cryoprotected CH-loaded liposomes, due to a restriction in the motion of the P=O and C=O groups that interact with the cryoprotectant (trehalose or sucrose) molecules.

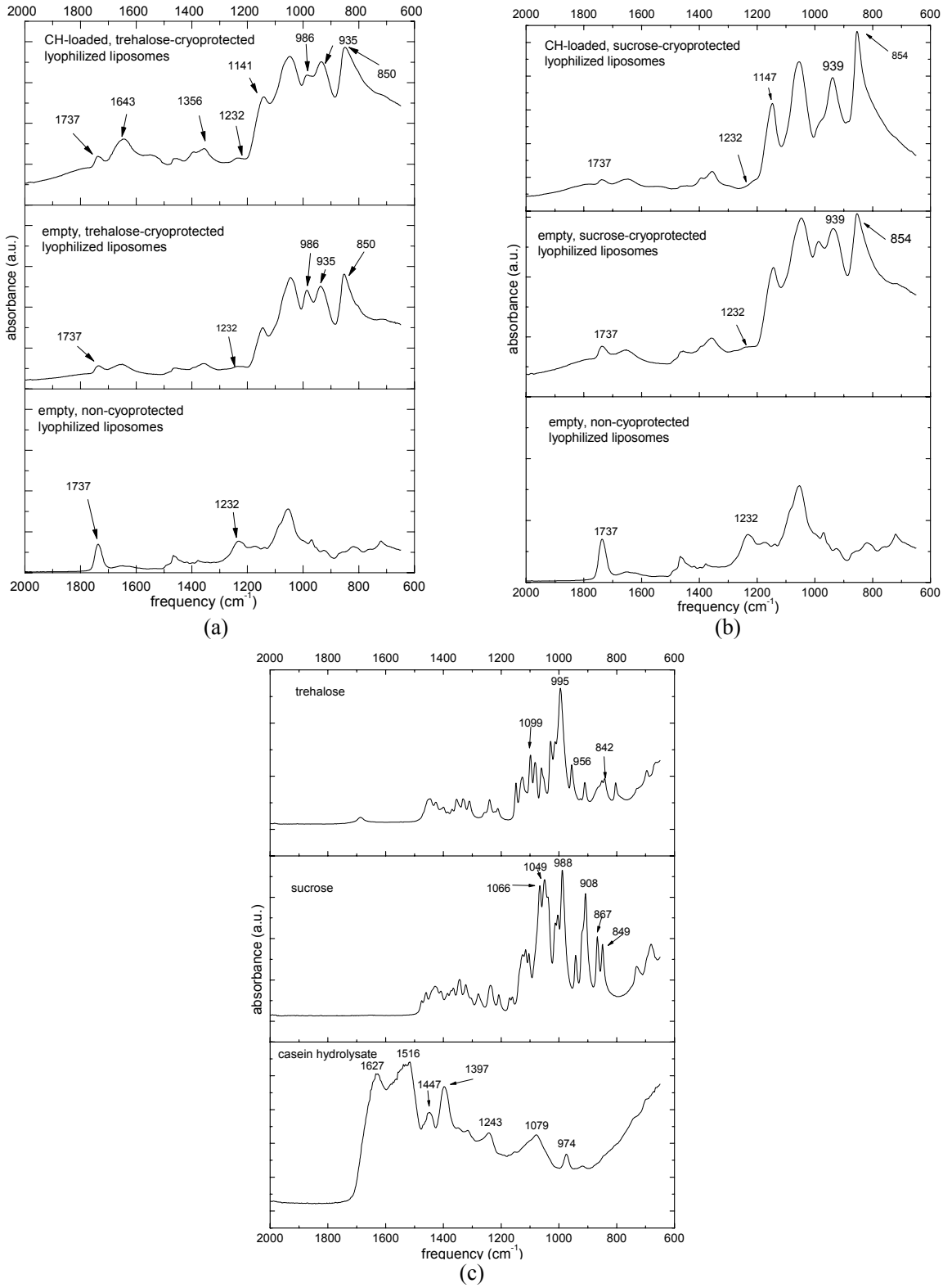


Figure 5: Fourier-transform spectra: (a) lyophilized liposomes; (b) pure casein hydrolysate (Hyprol 8052); peaks at 1627, 1516 and 1447 cm^{-1} are attributed to glx (glutamate+glutamic acid); at 1397 cm^{-1} to leucine and at 1243 and 1079 cm^{-1} to proline (suppositions based on Parker and Kirschenbaum, 1960, and Krimm and Bandekar, 1986). The peak at 1627 cm^{-1} could also be attributed to lysine (Krimm and Bandekar, 1986).

In the frequency range between 1650 and 1100 cm^{-1}, some peaks that were absent in the spectrum of the non-cryoprotected empty liposomes appeared in the spectra of CH-loaded liposomes (Figure 5). They are probably related to the amino acids present in the microencapsulated peptides, such as glx (glutamate and glutamic acid or/and lysine, peak at 1643 cm^{-1}), leucine (peaks at 1356 cm^{-1}) and proline (peaks at 1141 and 1147 cm^{-1}) (Parker and Kirschenbaum, 1960; Krimm and Banekar, 1986). Coincidently, these amino acids are among the most abundant in Hyprol 8052, according to Figure 1. It is useful to notice that these are thought to be the frequencies of these amino acid residues based on the literature, remembering that they were probably altered due to the fact the peptides/free amino acids are not in solution but interacting with the liposome bilayer.

Peaks between 990 and 840 cm^{-1} were also detected in the IR spectra of cryoprotected CH-loaded liposomes, which were all absent in the spectra of non-cryoprotected empty liposomes produced with non-purified lecithin. The cited peaks (indicated in the spectra in Figure 5) are probably due to the presence of amorphous sugar in excess, which is responsible for the formation of the glassy matrix seen in the micrographs (Figure 2).

Finally, the X-ray diffraction data confirmed that the lyophilization products of both cryoprotected liposomes (with trehalose and sucrose) were completely amorphous structures, as shown by the diffractograms in Figure 6. This is a clear indication that they are much more suitable for a successful rehydration step, as amorphous structures are known to be easier to rehydrate than crystalline arrangements (Kikuchi *et al.,* 1991; Alves and Santana, 2004).

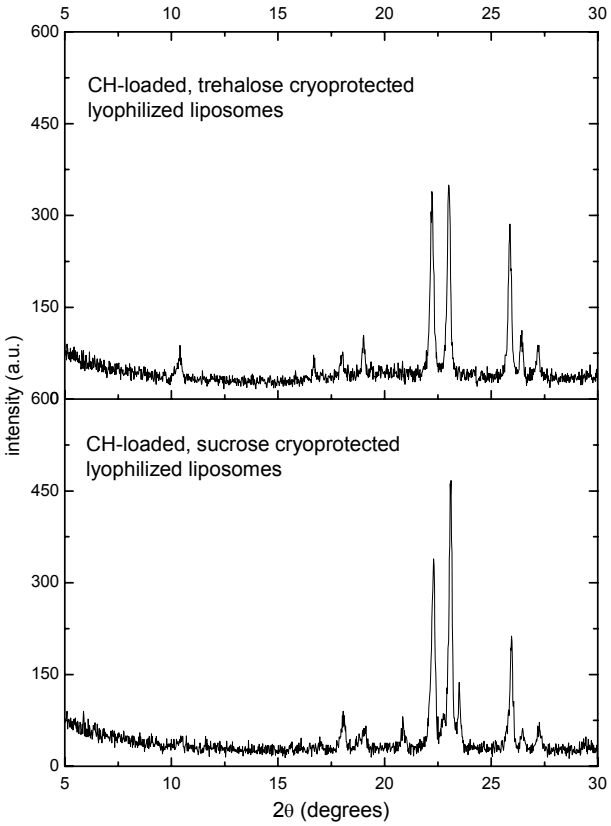


Figure 6: X-ray diffractograms of lyophilized liposomes produced with non-purified lecithin.

CONCLUSIONS

The results of this study confirm that liposomes produced with non-purified soy lecithin can be lyophilized using sucrose and trehalose as cryoprotectants and that both disaccharides are incorporated in a similar way in the liposome structure, according to infrared data. The resulting powders were completely amorphous, and morphological analyses by SEM did not indicate a fusion of the structures, showing the expected aspect of a glassy matrix around the liposomes.

Thermal analyses by DSC of lyophilized liposomes revealed an unexpected exothermic transition in the samples without casein hydrolysate. Such behavior was attributed to the formation of a quasicrystalline (L_q) lamellar phase due to the presence of a high percentage of phosphatidylethanolamine and unsaturated fatty acid chains in the non-purified soy lecithin chosen for this study (Lipoid S40). The microencapsulation of CH in the liposomes abolished the exothermic phenomenon and substantially diminished the glass transition temperature of the lyophilized systems, indicating that the amorphous structures were susceptible to higher molecular mobility due to the lower rigidity of the liposome bilayers.

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