Interactions among lactose, β -lactoglobulin and starch in co-lyophilized mixtures as determined by Fourier Transform Infrared Spectroscopy

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Abstract Processing and storage change food powders containing a large quantity of lactose due to lactose crystallization and interactions among components. Model food systems were prepared by co-lyophilization of lactose, β -lactoglobulin (BLG), and gelatinized starch. A mixture design was used to define the percentage of each mixture component to simulate a wide range of food powders. Interactions among lactose, BLG and starch were studied using Fourier Transform Infrared (FT-IR) at different relative humidities (RH), before and after 3 months storage. Results showed the presence of hydrogen bonds among these components. Moreover, interactions or formation of hydrogen bonds among lactose, starch and BLG preserved BLG against freezing and freeze-drying shocks. Lactose crystallization could be identified by comparing infrared spectra of amorphous and crystallized lactose at O–H and C–H stretching vibration bands.

Keywords Solid state interaction · Crystallization · Lactose · Spectroscopy · Storage

Introduction

Interactions among proteins and polysaccharides have been studied by numerous authors in different systems especially

in emulsions and colloids. Solid state interactions are very important to protect activity and protein structure in pharmaceutical products (for example; enzymes and hormones). Moreover, solid state interactions affect physicochemical properties of final products. Drying processes like spray drying, drum drying and freeze-drying affect protein structure and nutritional quality of foods. Saccharides protect proteins against some process such as freezing and freeze-drying. Protecting effect of saccharides was explained by numerous authors (Carpenter et al. 1998; Imamura et al. 2003). Some saccharides, for example glucose, protect quaternary structure of some enzymes (Anchordoquy et al. 2001). Several conditions are necessary to protect the protein structure by saccharides. First, creation of amorphous sugar network is essential. For example, mannitol crystallizes during freeze drying and it cannot protect the protein structure such as growth hormone rh-GH (Costantino et al. 1998; Izutsu and Kojima 2002). There are two hypotheses that explain protection effect of amorphous structure (or glassy matrix). Franks (1994) contends that sugars provide a ‘glassy’ matrix in which protein mobility and reactivity are reduced. Another hypothesis is that this matrix dilutes proteins in the solid state, reducing intermolecular contacts and thus inhibiting deleterious intermolecular pathways (i.e. aggregation) (Liu et al. 1991). Second, direct interactions among proteins and saccharides, particularly by hydrogen bonds, are necessary to stabilize proteins against drying or freezing shocks. It seems that saccharides as lactose interact with polar groups of proteins by hydrogen bonds, and replace water molecules eliminated during drying or freeze-drying (Allison et al. 1999; Arakawa et al. 2001).

Interactions between water and protein are important especially when second or tertiary structure of protein should be protected. To preserve proteins structure, nutrient bioavailability and physicochemical and functional properties of foods, it is necessary to optimize the quantity of each

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component and the process parameters. Moreover, in some foods such as infant formula, a large quantity of lactose is present, which is in both amorphous and crystallized state. Amorphous lactose is meta-stable and may be softened or plasticized by temperature and water. Lactose crystallization causes the most drastic changes during storage of powder containing lactose such as stickiness, caking, collapse and acceleration of non-enzymatic browning. Solid state interactions can delay or inhibit lactose crystallization thus unwanted changes will not happen.

In this study, the model food containing different quantities of lactose, BLG and starch was chosen to study the interactions among these components. Structure changes and interactions observed in this study can be developed for other foods containing proteins, polysaccharides and disaccharides. The aim of this research was to study the interactions among BLG, lactose and starch using Fourier Transform Infrared spectroscopy (FTIR), as well as the effect of relative humidity and freeze-drying shocks on structural changes of BLG.

Materials and methods

Model infant formulation preparation

Model formulations were prepared by mixing 3 major components of foods (as previously explained by Nasirpour et al. (2006)). The samples were stored for 2 weeks on phosphorous pentoxide to complete drying process.

In this work, the co-lyophilized formulations contained three components and they are noted as FLac/Starch/BLG (w/w %). For example, F80/10/10 means formulation containing 80 % lactose, 10 % gelatinized starch and 10 % BLG (w/w).

Infrared spectroscopy

Samples of lactose, BLG, gelatinized starch and the formulations stored at different RH were prepared as potassium bromide (KBr) pellets by grinding of 25 mg samples and 475 mg KBr. KBr is transparent to infrared waves. This ratio was chosen to achieve absorption between 1 and 2. This powder mixture was then crushed in a mechanical die press to form a translucent pellet through which the beam of the spectrometer can pass. The samples were dried for 1 week on the P_2O_5 (P_2O_5 was previously dried) before mixing with KBr for decreasing water effect on IR spectra. The absorbance spectra were recorded with a Tensor 27 (Bruker, Wissembourg, France) Fourier Transform Infrared (FTIR) in the region from $4,000\text{ cm}^{-1}$ to 400 cm^{-1} using 4 cm^{-1} resolution and an accumulation of 64 scans. The system was continuously purged with dry air. The spectra were treated with different methods using OPUS/IR (Bruker, Wissembourg, France).

Firstly, baseline of spectra was corrected and they were smoothed by 11 points using the Savitzky-Golay algorithm. For comparing, the spectra were normalized by two methods: min-max and vector normalization. In the normalization method used for each spectrum is explained under the figures. In some regions of spectra, the second derivative was carried out to show more details about the regions.

Results and discussion

IR spectroscopy of pure lactose

It is important to know structural changes of lactose at different RH, Fig. 1 shows the spectra of amorphous and crystallized lactose. Amorphous lactose was stored at several RH ranging from 0 % to 85 % for 3 months and spectra bands were studied. Crystallized lactose showed the presence of one sharp peak around $3,380\text{ cm}^{-1}$ and a few shoulders in O–H stretching vibrations ($\nu\text{O–H}$) region between $3,050\text{ cm}^{-1}$ and $3,600\text{ cm}^{-1}$ showing the presence of hydrogen bonds. The broad form of $\nu\text{O–H}$ band of amorphous lactose indicated a wide range of hydrogen bond lengths and orientations. The main reason for sharper peaks in the crystalline state is the higher degree of homogeneity of intramolecular interactions (Wolkers et al. 2004). This leads to less dispersion of vibrational levels and a higher conformational selectivity. Crystallization caused a decrease in $\nu\text{O–H}$ band intensity, suggesting an increase in hydrogen bond density and strength (Ottenhof et al. 2003). Numerous researchers (Ottenhof et al. 2003; Maalouly and Jaillais 2005) interpreted changes in wavenumber of the $\nu\text{O–H}$

Fig. 1 Infrared spectra of pure lactose in amorphous state and crystallized state at $20\text{ }^{\circ}\text{C}$. Baselines of spectra were corrected and then all spectra were smoothed by 9 points and were normalized by min-max method

Fig. 2 **a** Second derivative of spectral region of 3,460 to 3,560 (cm^{-1}) of lactose, **b** and **c** absorbance and second derivative of spectral region of 2,950 to 2,990 (cm^{-1}) of lactose respectively, relative humidity

varies between 0 and 85 % and all spectra were smoothed by 9 points and were normalized by vector method

band, in the region 3,000 to 3,600 cm^{-1} , as being mainly due to hydrogen bonds. Indeed, the $\nu\text{O}-\text{H}$ band involved in a hydrogen bonds is expected to have a lower wavenumber than its non-hydrogen bonded counterpart as it will have a

lower bond force constant due to electron delocalisation in hydrogen bonded structures.

By increasing the RH, a new peak was observed at 3,525 (cm^{-1}) indicating hydration of lactose at high RH.

Fig. 3 **a** Infrared spectra of gelatinized starch, β -lactoglobulin and lactose at about 0 and 75 % relative humidity and 20 °C after 3 months storage and **b** infrared spectra of F65/10/25 at different relative

humidity and 20 °C after 3 months storage. The baseline of spectra were corrected, then the spectra were smoothed by 9 points and the spectra region were cut and normalized by vector method

Fig. 4 Second derivative of F65/10/25 stored 3 months at different relative humidity. The baselines were corrected then the spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method

The second derivative of this region give more details (Fig. 2a). Figure 2a shows that by increasing the RH, the intensity of the peak at $3,527\text{ cm}^{-1}$ increased. Moreover, the peak at $3,509\text{ cm}^{-1}$ was observed in crystallized state samples (high RH). This region is interesting for lactose crystallization.

The second region of infrared spectra of lactose which was studied between $2,800\text{ cm}^{-1}$ and $3,000\text{ cm}^{-1}$, corresponded to C–H bonds. The analysis of crystallized lactose resulted in the presence of four peaks in this region, whereas that of amorphous lactose showed one broad and one weak peak in this region.

Moreover, between the two broad peaks ($2,900$ and $3,370\text{ cm}^{-1}$), a weak peak was observed at $2,978\text{ cm}^{-1}$. This peak was sensitive to RH and appeared as RH increased. Its intensity also increased as RH increased (Fig. 2b and c). For example, in the case of the sample stored at 0 % RH, there was no peak in this region but by increasing the RH to more than 43 % this peak was clearly observed. It was shown previously that lactose crystallized at 43 % (Nasirpour et al. 2006).

Fig. 5 Amide I band of freeze-dried and without treatment β -lactoglobulin, **a**; absorbance and **b**; second derivative. The baselines were corrected and the spectra were smoothed by 9 points and were cut and normalized by vector method

Non-covalent interactions among lactose/BLG/gelatinized starch

Non-covalent interactions among lactose, BLG and gelatinized starch were studied using mid infrared spectroscopy. Firstly, each component spectrum was obtained at any RH and secondly, model formulations were analyzed at different RH ranging from about 0 to 85 %.

Spectral region of $1,700$ to $1,600\text{ cm}^{-1}$

For proteins, infrared signal is principally due to the vibrations of the amide groups of the polypeptide backbone. An amide group gives rise to specific vibrational modes (Bandeekar 1992), that so-called amide modes, among which the amide I band is the most intense and the most studied. It originates from the C=O stretching vibration of the amide group weakly coupled to the N–H bending and the C–N stretching vibrations. The amide I mode is very sensitive to the geometry, local environment, and hydrogen bond strength of the amide groups. Each type of secondary structure is thought to absorb at a specific frequency (Jackson and Mantsch 1995). Moreover, this region corresponds to O–H scissoring mode of water that corresponds to lactose crystallization water. The presence of a number of amide I band frequencies and been correlated with the presence of α -helical, antiparallel and parallel β -sheets and random coil structures (Byler and Susi 1986). Figure 3a shows absorbance bands of lactose, BLG and gelatinized starch in this region.

Amorphous and crystallized lactose were different in this region and H₂O scissoring mode of residual water shifted from $1,653\text{ cm}^{-1}$ in the crystalline form to $1,639\text{ cm}^{-1}$ in the amorphous form, indicating that the water molecules in crystalline lactose are more strongly hydrogen bonded to the sugar molecules compared to residual water in amorphous lactose. Wolkers et al. (2004) found the same results for trehalose. Moreover, amorphous lactose had one sharp peak and one shoulder in this region compared to crystallized lactose that had only one sharp peak.

Infrared spectra of formulations

Figure 3b shows 3,600 to 3,100 cm^{-1} band of infrared spectra of F65/10/25 at four RHs and 20 °C after 3 months storage. The formulations stored at about 0 and 43 % are amorphous and it can be seen in $\nu\text{O-H}$ at around 3,200 to 3,600 cm^{-1} . These formulations had a sharp, strong peak without any shoulder at around 3,410 cm^{-1} . By increasing the RH to 54 %, a small shoulder appeared at around 3,300 cm^{-1} and it was clearer in formulation stored at higher RH for example, 59 and 75 % RH. As explained in the case of F65/10/25, water liberation was not observed at 54 % RH, thus by analyzing this region, it is possible to know the crystallized or amorphous state of lactose in formulation. For example, F65/10/25 was amorphous after 3 months storage at 43 % RH and in $\nu\text{O-H}$ (3,200–3,600 cm^{-1}), a sharp peak could be observed, and as it is clearly seen in Fig. 3b, by increasing the RH to 54 % a small shoulder could be seen. By increasing the RH to 59 and 75 %, these shoulders became clearer. It means that crystallization started in this formulation at 54 % RH after 3 months storage but it was not complete. These results agreed with result found for crystallization by moisture sorption isotherm (Nasirpour et al. 2006).

Figure 4 shows the second derivative of amide I of F65/10/25 stored at different RH. The sample stored at 0 % RH showed a peak at 1,632 cm^{-1} and by increasing the RH, the wavenumber increased. When crystallization occurred in samples, for example in the case of F65/10/25 stored at 59 % or higher RH, the increase in wavenumber was more important.

Influence of lactose and starch on BLG spectrum

Figure 5 shows the infrared spectra of freeze-dried BLG and BLG without treatment. The amide I of BLG and formulations were compared to show the influence of lactose and starch on BLG structure. The BLG spectra, before and after freeze-drying, were first compared. The sharp peak at 1,638 cm^{-1} denotes β -sheet structure of BLG, which is a predominant structure in this protein (Fig. 5a and b). This peak shifted to 1,635 cm^{-1} after freeze-drying and as it was shown by numerous authors that freeze-drying induced changes in secondary structure (Allison et al. 1999; Anchordoquy et al. 2001). The intensity of peak at 1,638 cm^{-1} decreased after freeze-drying of BLG, showing the decrease in β -sheet structure. Moreover, reduction in intensity of β -sheet band in freeze-dried BLG indicates perturbation of the protein secondary structure (Izutsu and Kojima 2002). In addition, the second peak around 1,693 cm^{-1} shows the antiparallel β -sheet structure or protein aggregation (Jackson and Mantsch 1995). The intensity

Fig. 6 Second derivative of formulations containing 65 % lactose and different β -lactoglobulin and starch quantities stored 3 months at 11 % relative humidity. The baselines were corrected then the spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method. Lactose and gelatinized starch spectra were subtracted from formulation spectra

of this peak was more important in the case of freeze-dried BLG. This is due to aggregation of BLG after freezing, indicated by a small shoulder in Fig. 5a.

Figure 6 shows the second derivative of amide I band of infrared spectra of formulations containing 65 % lactose and different quantity of BLG and gelatinized starch after 3 months storage at 11 % RH. All formulations had a peak at 1,631 cm^{-1} corresponding to β -sheet structure of BLG. Freeze-dried BLG had a peak at 1,635 cm^{-1} (Fig. 5). The decrease in wavenumber in formulation containing 65 % lactose shows the presence of hydrogen bonds among BLG, lactose and gelatinized starch. This phenomenon was observed by numerous authors (Kreilgaard et al. 1998;

Fig. 7 Second derivatives of F65/10/25 and freeze-dried β -lactoglobulin. The baselines were adjusted then all spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method. Lactose and gelatinized starch spectra were subtracted from formulation spectra

Jovanović et al. 2006) who indicated that carbohydrate especially lactose can replace water in solid state. In addition, lactose and gelatinized starch protect BLG against aggregation and in all formulations containing gelatinized starch and lactose, the small shoulder that was shown in freeze-dried BLG, was not observed. In all processes including freeze-drying, spray drying or other process, stresses associated with freezing and drying may cause irreversible damage to the protein, seen as structural denaturation and aggregation (Heller et al. 1999).

By comparing the results of the others formulations stored for 3 months at 75 % RH. It was observed that there was a little shift in amide I of all formulations compared to freeze-dried BLG, but the peak at $1,638\text{ cm}^{-1}$ in BLG without treatment remained more or less the same when lactose and gelatinized starch were added. Moreover, the intensity of this peak decreased after freeze-drying in all samples compared to BLG without treatment. As explained in the spectra of BLG, freeze-dried BLG had a more intense peak at $1,693\text{ cm}^{-1}$. This peak was less intense after 3 months storage at 75 % RH and even disappeared in the case of F65/10/25. In addition, this antiparallel β -sheet structure was not observed in the samples stored at 11 % RH.

Figure 7 shows BLG structure changes in formulation F65/10/25 before storage and after storage at 11 % and 75 % RH. The peak at $1,635\text{ cm}^{-1}$ for BLG after freeze-drying shifted to $1,631\text{ cm}^{-1}$ for sample after freeze-drying and after 3 months storage at 11 % RH. This is because of the formation of hydrogen bonds among BLG and gelatinized starch and lactose. Therefore, direct sugar-protein interactions are responsible for the observed hydrogen bonding to BLG in the dried preparation and storage at 11 % RH. However, by increasing storage RH to 75 %, the peak at $1,636\text{ cm}^{-1}$ was nearly the same as that for freeze-dried BLG. The intensity of this peak increased in the formulations after freeze-drying and stored at 11 % RH, but for freeze-dried BLG and sample stored at 75 % RH, the peak had the same intensity. By adding gelatinized starch and lactose to formulations, after freeze-drying and storage at 11 % RH, the intensity of the peak at around $1,635\text{ cm}^{-1}$ increased. Moreover, antiparallel β -sheet structure of BLG (peak at around $1,694\text{ cm}^{-1}$) nearly did not exist in these formulations.

Conclusion

Results obtained from infrared spectroscopy showed that lactose crystallization can be identified by changes in different infrared spectra such as stretching vibration of O–H bonds at around $3,600$ to $3,200\text{ cm}^{-1}$ and stretching vibration of C–H bonds at around $3,000$ to $2,800\text{ cm}^{-1}$. In addition, crystallized lactose showed some shoulders in stretching vibration of O–H bonds at around $3,600$ to

$3,200\text{ cm}^{-1}$ and stretching vibration of C–H bonds, showing rearrangement of lactose crystals. Freeze-drying caused aggregation of BLG and it can be seen in amide I region of proteins as a shoulder in infrared spectra of BLG. By increasing the RH from ~ 0 to 75 %, changes in wavenumber, were not observed. In formulation containing lactose, BLG and gelatinized starch several changes were observed before and after storage at different RHs. In all formulations, the amide I band of BLG shifted to smaller wavenumbers showing the presence of hydrogen bonds among these components. By increasing the RH from ~ 0 or 11 % to 75 or 85 % the amide I band of BLG shifted to wavenumber greater than BLG. In this condition, perhaps the hydrogen bonds that existed among BLG and lactose, gelatinized starch were replaced by water. In high RH conditions, lactose is in crystallized state and it is not hydrophilic. Thus, the number of hydrogen bonds present between BLG and lactose decreased. Another hypothesis that can be suggested for this phenomenon is that in high RH, nonenzymatic browning occurred between lactose and BLG and may influence the changes in wavenumber of amide I band of BLG. Relative humidity at which lactose crystallization occurs is changed by adding proteins or saccharides. This phenomenon was observed previously, and some researchers believe that changes in the Tg of lactose mixtures are responsible. However, in some systems, such as those with rhGH and trehalose, the lactose crystallization RH is changed without changing the system Tg. In this condition, other parameters such as molecular environment changes and interactions among the components affect lactose crystallization. During lyophilization, water is replaced by lactose and starch in the β -LG structure, and the long chain length may interfere with intermolecular hydrogen bonding between lactose and β -LG. Therefore, these bonds prevent or retard lactose crystallization.

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