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In situ FTIR assessment of dried *Lactobacillus bulgaricus*: KBr disk formation affects physical properties

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Abstract. The overall protein secondary structure, heat-induced protein denaturation, membrane phase behaviour, and glassy behaviour of *Lactobacillus bulgaricus* dried with sucrose were studied by Fourier transform infrared spectroscopy (FTIR) using two sample preparation methods. Samples for FTIR analysis were either prepared by mixing dried sample with KBr and compression into disks or by air-drying of cell/sucrose suspensions on CaF_2 windows. The results show that KBr compression causes protein unfolding and affects the thermo-physical properties of dried cells when compared with cells that were dried on CaF_2 windows and directly used for FTIR analysis without further manipulation. The protein denaturation temperature of samples prepared in KBr disks was decreased by more than 70°C compared to that of samples dried on CaF_2 windows. In addition, hydrogen bonding interactions of the glassy cell/sucrose matrix were drastically affected by KBr compression. In contrast with samples dried on CaF_2 windows, samples in KBr disks were not in a glassy state at room temperature. The membrane phase behaviour of the dried cells was also affected by preparation of the sample into KBr disks. We conclude that the KBr compression method for preparing samples for FTIR analysis affects conformation and physical properties of biomolecules in the dried state.

Keywords: Protein structure, FTIR, lyophilization, glass transition, membrane phase transition, air drying, lactic acid bacteria

1. Introduction

Lactic acid bacteria play a central role in dairy fermentation processes, which encompasses the production of cheeses and yoghurts. Lactic acid bacteria may be preserved and distributed in liquid, frozen, spray-dried or freeze-dried form [5]. Storage of the cells in the dried state is preferable, because this allows room temperature storage and makes transport easier. Research on the behaviour of several lactic acid bacteria has identified factors such as protective excipients, storage temperature and water activity of the dried powders to be critical parameters that affect survival [5,15]. Protective excipients often exist of combinations of disaccharides, polysaccharides, and proteins. The requirements for protectants of biological materials in the dried state are understood to some extent: good protectants are generally good glass formers and are able to form hydrogen bonds with polar residues of biomolecules in the dried state [7,8].

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The dried state limits the number of techniques that can be applied to study conformation and stability of intracellular biomolecules. One of the few suitable techniques for *in situ* analysis of biomolecules in the dried state is Fourier transform infrared spectroscopy (FTIR) [18]. The advantage of FTIR is that it can be used, irrespective of the hydration state of the sample. On account of characteristic molecular vibrations that absorb in the infrared region, information can be derived on molecular conformation and intermolecular interactions of biomolecules in their native environment.

Proteins in biological tissues can be detected on account of two characteristic absorption bands at around 1650 cm^{-1} (amide-I band) and 1550 cm^{-1} (amide-II band), arising from the peptide backbone. The amide-I band, which is most often used for protein analysis is composed of different IR transitions that reflect different types of protein secondary structure [2,10,14]. Dehydration-induced changes in protein amide bands have been assigned to structural changes of proteins in the dried state [1,4,12]. Other studies have implicated that the removal of water *per se* can also induce spectral changes of protein FTIR spectra that may not be directly associated with protein structural changes [16].

The temperature dependence of a molecular vibration can be used to show shifts of bands, associated with the melting of lipids in membranes (CH-stretching vibrations), with protein denaturation (C=O-stretching vibration) and with the melting of glasses (OH-stretching vibration), which can be measured simultaneously [17,18,20].

The most common method of sample preparation for FTIR analysis of freeze-dried proteins in the solid state is compression after mixing with potassium bromide (KBr). Freeze-dried samples often require compression into KBr pellets in order to increase translucency for FTIR analysis. Dispersing dry protein samples in material such as KBr after grinding and compressing this mixture, however, could result in structural alterations. Thus, questions have arisen if the KBr compression method is a suitable method to study proteins or protein/excipient formulations in the solid state [13]. Chan et al. [6] demonstrated a correlation between the loss of activity of rhDNase and the pressure used for the compression of KBr pellets. Protein unfolding can potentially occur due to the pressure effects and grinding of the sample and mixing with KBr [13]. By contrast, other studies have shown that compression of KBr/protein mixtures only results in minor structural alterations [11,12]. The effects of KBr compression on the thermo-physical properties of proteins or cells in the dried state have not been reported thus far.

In this study we have investigated the effects of KBr compression on the overall protein secondary structure, heat-induced protein denaturation, membrane phase behaviour and glassy behaviour of freeze-dried *Lactobacillus bulgaricus* using sucrose as excipient. FTIR studies on dried samples prepared in KBr disks were compared with those of thin air-dried layers of cell/sucrose suspension on CaF_2 windows. The advantage of air drying the sample directly on CaF_2 windows is that it results in an IR transparent layer that does not require further sample preparation. We show that the overall protein secondary structure and the thermo-physical properties of dried cell/sucrose suspension are strongly affected by preparation of the sample into KBr disks.

2. Materials and methods

2.1. Bacteria material and culture conditions

Lactobacillus delbrueckii subspecies *bulgaricus* CFL1 was used for all experiments. Cultures were grown in supplemented whey medium (60 $g\,l^{-1}$ whey, 20 $g\,l^{-1}$ lactose, 5 $g\,l^{-1}$ yeast extract) in a 2 l fermentor at 42°C, as previously described [9]. The pH was controlled at 5.5 by addition of 1.44 M

NaOH. Cells were harvested by centrifugation at the end of the exponential growth phase, which was when the NaOH consumption rate started to decrease.

2.2. Air drying and freeze drying of L. bulgaricus, and preparation of samples for FTIR analysis

Cells were washed twice in 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). Cell pellets were then diluted once in various protective formulations and in water, resulting in an approximate cell concentration of 1.4×10^7 colony forming units ml^{-1}. Sucrose was purchased from Merck (Darmstadt, Germany). Sucrose was added at a final concentration of 5% (w/v).

Air drying was done in a desiccator that was continuously flushed with dry air of less than 3% RH. Ten μl of the cell/sucrose suspension was evenly spread on a CaF_2 window, and dried overnight. This results in a residual water content of 0.020 ± 0.005 g H_2O per g dry weight. For studies on the effect of KBr compression on air-dried samples, 10 μl of the cell/sucrose suspension was dried in a mortar that was subsequently used for mixing with KBr.

Freeze drying was done using a SMH15 freeze-drier (Usifroid, Maurepas, France). Seven hundred μl cell/sucrose suspension was added into 3 ml vials, and frozen at −50°C on the shelves of the freeze-drier. Primary drying was performed at a shelf temperature of −30°C at 15 Pa for 45 h. Then the shelf temperature was increased with 0.2°C/min to 25°C, and kept at 25°C for 7 h at 10 Pa. At the end of the freeze-drying cycle the vacuum was broken with dry nitrogen and the vials were stoppered. The water content of the freeze-dried samples was 0.024 ± 0.002 g H_2O per g dry weight as determined by the Karl Fischer method.

A standard procedure was used for processing dried samples into KBr disks. An approximate amount of 1 mg of freeze-dried product was mixed with 300 mg dry KBr powder using a pestle and mortar. The resulting mixture was transferred to a stainless steel holder (13 mm inner diameter), and the holder was placed in a hydraulic press (RIIC-Beckmann, Glenrothes, Scotland) and evacuated with an attached vacuum pomp. The press was adjusted to 10,000, 8000, 6000, 4000 or 2000 kg, generating a pressure of approximately 7500, 6000, 4500, 3000 or 1500 $kg\,cm^{-2}$ on the pellet within the holder, respectively. Pressure was maintained for approximately 3 minutes.

2.3. Fourier transform infrared spectroscopy studies

Infrared absorption measurements were carried out with a Nicolet Magna 750 Fourier transform infrared spectrometer (Nicolet, Madison, WI, USA), equipped with a narrow band mercury/cadmium/telluride liquid-nitrogen-cooled IR-detector. KBr pellets of freeze-dried bacterial/sucrose samples or sandwiches of two CaF_2 windows with the air-dried samples were mounted into a Specac variable temperature cell (Specac Ltd, Orpington, Kent, UK). Liquid nitrogen was used as a coolant in the refrigerant chamber, and the temperature was regulated by the temperature controller of the cell. The temperature of the sample was recorded separately using an extra thermocouple that was located very close to the sample. The temperature dependence of the FTIR spectra was studied starting with the lowest temperature, with a scanning rate of $1.8°C\,min^{-1}$. The optical bench was continuously purged with dry air (Balston, Haverhill, MA, USA). The acquisition parameters were: 4 cm^{-1} resolution, 32 co-added interferograms, 4000–900 cm^{-1} wavenumber range. Spectral analysis and display were carried out using Omnic software (Nicolet, Madison, WI, USA) and Perkin-Elmer software (Perkin-Elmer, Norwalk, CT, USA).

For protein studies the spectral region between 1700 and 1600 cm^{-1} was selected, which contains the amide-I absorption band. Protein denaturation was monitored by observing the position of the amide-I

band around 1635 cm^{-1}. For this purpose, the inverted second derivative spectrum was calculated, and the band at 1635 cm^{-1} was selected and normalized. Band positions were calculated as the average of the spectral positions at 90% of the peak height, and νCO versus temperature plots were constructed. Protein denaturation temperatures were determined from the minima in the first derivatives of the νCO versus temperature plots.

Membrane fluidity was monitored by observing the position of the CH_2 symmetric stretching band at approximately 2850 cm^{-1}. The inverted second derivative spectrum was calculated, and the lipid band was selected and normalized. Band positions were calculated as the average of the spectral positions at 80% of the peak height, and νCH_2 versus temperature plots were constructed. Phase transition temperatures were determined from the maxima in the first derivatives of the νCH_2 versus temperature plots.

Possible glass transitions were studied by observing the band position of the OH stretching band around 3300 cm^{-1}. The spectral region between 3600 and 3000 cm^{-1} was selected and normalised. The band position was calculated as the average of the spectral positions at 80% of the peak height. Glass transition temperatures were determined from νOH versus temperature plots according to Wolkers et al. [20]. The melting of glasses is associated with an abrupt change in the wavenumber temperature coefficient (WTC), the slope in a νOH versus temperature plot. The WTC is indicative of the rotational freedom of the OH stretching mode, and thus is a measure for the average strength of hydrogen bonding.

2.4. Thermal analysis

Differential scanning calorimetry (DSC) was used to determine the glass transition temperature and protein denaturation temperature of the freeze-dried cell/sucrose suspension. DSC measurements were carried out using a Pyris 1 instrument (Perkin-Elmer, Norwalk, CT, USA). About 10 mg of sample was placed in DSC aluminium pans and the pans were sealed. An empty pan was used as a reference. Samples were cooled to $-80°C$ and heated up to 250°C with a heating rate of $10°C\,min^{-1}$. The glass transition is reported as the midpoint temperature of the change in heat capacity that is associated with the glass transition. The protein denaturation temperature was determined as the midpoint of the endothermic peak associated with protein denaturation. Results were obtained from at least two replicates.

3. Results

In Fig. 1 the *in situ* IR spectra of *L. bulgaricus* cells that were dried in the presence of sucrose are shown. Freeze-dried cells were compressed into KBr disks, and air drying was done directly on a CaF_2 window without further sample preparation. The band at 3330 cm^{-1} in the air-dried sample corresponds to OH-stretching vibrations mainly arising from the excipient sucrose. In the KBr disk the OH band is sharper and shifted to higher wavenumber position (3420 cm^{-1}) compared to the sample on the CaF_2 window. The two bands at 2929 and 2852 cm^{-1} in the CH-stretching region (3000–2800 cm^{-1}) of the spectra denote the asymmetric and symmetric lipid CH_2 stretching vibrations, respectively. The amide-I and amide-II bands arising from endogenous proteins can be seen at 1656 cm^{-1} and 1547 cm^{-1}, respectively. The amide bands appear to be broader in the KBr disk. In addition, the amide-II/amide-I line height ratio is decreased from 0.46 in the air-dried sample to 0.37 in the KBr disk. In the region below 1500 cm^{-1}, a variety of characteristic IR group frequencies can be observed, that are difficult to assign. The shape of the spectra below 1500 cm^{-1} is characteristic and can be used as a 'finger print' of the sample.

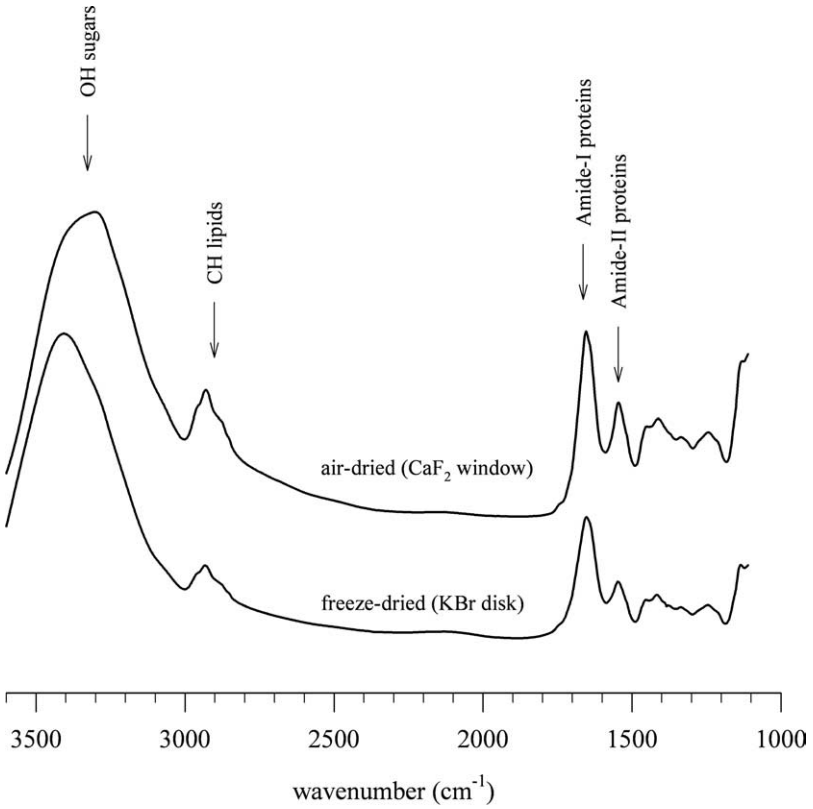

Fig. 1. *In situ* infrared absorption spectra of *L. bulgaricus*/sucrose suspension air-dried on CaF_2 windows and of freeze-dried sample prepared into KBr disks, at 20°C. The OH-stretching band arising from sugars (excipient), the lipid CH_2 stretching bands, and the amide-I and amide-II bands of proteins are indicated.

The differences in overall protein secondary structure between the freeze-dried sample in the KBr disk and the non-manipulated air-dried sample on the CaF_2 window are illustrated in Fig. 2. Differences in amide-I band profile are already visible directly after sample preparation (compare spectra at 20°C). Second derivative spectra were calculated to show these differences more clearly (Figs 2B and 4A). The line height ratio of the band at 1638 cm^{-1} (denoting turn/β-sheet structures) and the band at 1657 cm^{-1} (denoting α-helical structures) is increased from 0.80 on the CaF_2 window to 0.88 in the KBr disk. This indicates that the relative proportion of β-sheet structures is increased by KBr compression. Figure 2 also shows that the amide-I band profile of the sample in the KBr disk drastically changes above 60°C: bands at 1629 cm^{-1} and 1692 cm^{-1} start to appear, indicative for the formation of extended β-sheet structures of denatured/aggregated protein. The amide-I band profile of the air-dried sample only shows signs of protein denaturation above 160°C. In addition, the changes in overall protein secondary structure that are associated with protein denaturation are less pronounced than that of the sample in the KBr disk.

In order to verify that the differences in heat induced protein denaturation were due to KBr compression and were not due to differences in the method of drying, air-dried samples were also prepared into KBr disks. Heat-induced protein denaturation of air-dried sample in KBr disks was very similar to that of the freeze-dried sample in KBr disks. Figure 3A shows wavenumber versus temperature plots of the band at 1638 cm^{-1} for the three samples. In KBr disks protein denaturation already starts just above

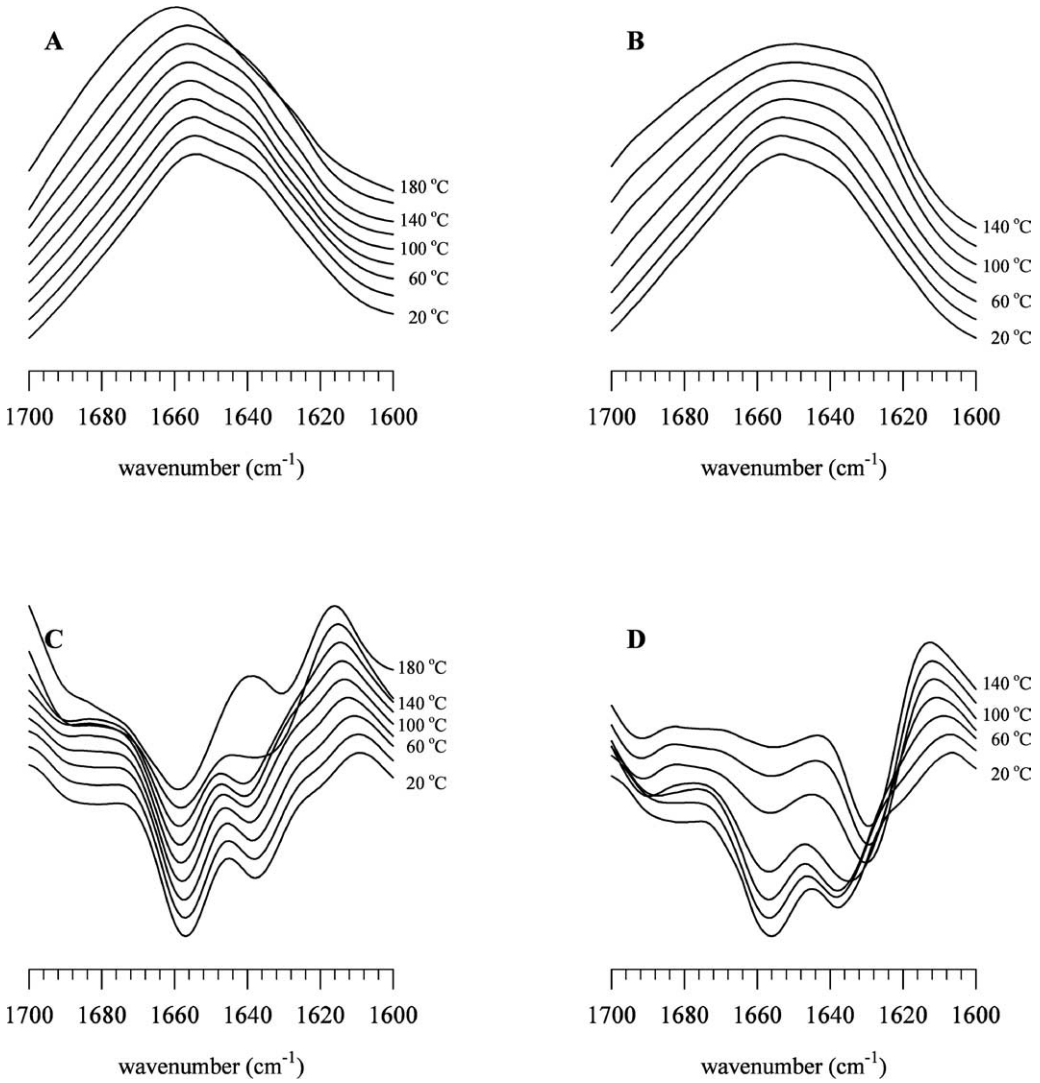

Fig. 2. *In situ* infrared absorption spectra of the amide-I region (1700–1600 cm^{-1}) at various temperatures of *L. bulgaricus*/sucrose suspension air-dried on CaF_2 windows (A) and of freeze-dried sample in KBr disks (B), illustrating the effects of KBr compression on the overall protein secondary structure during thermal denaturation. Second derivative spectra (C (sample on CaF_2 window), D (sample in KBr disk)) were calculated to resolve the different bands in the amide-I region, representing different types of protein secondary structure.

60°C, for both the freeze-dried and the air-dried sample, with a denaturation temperature (T_d) of 83°C, whereas the air-dried sample on CaF_2 windows exhibits a T_d at 168°C.

Also the membrane phase behaviour is affected by KBr compression (Fig. 3B). The membranes of freeze- and air-dried cells prepared into KBr disks exhibit phase transitions at 44°C and 40°C, respectively, whereas air-dried cells on CaF_2 windows show a phase transition at 53°C. In addition, the phase transition of the sample dried on CaF_2 windows is more cooperative as is evident from the height of the maximum of $d\nu CH_2/dT$ in the first derivative of the wavenumber versus temperature plot (Fig. 3B, inset-figure).

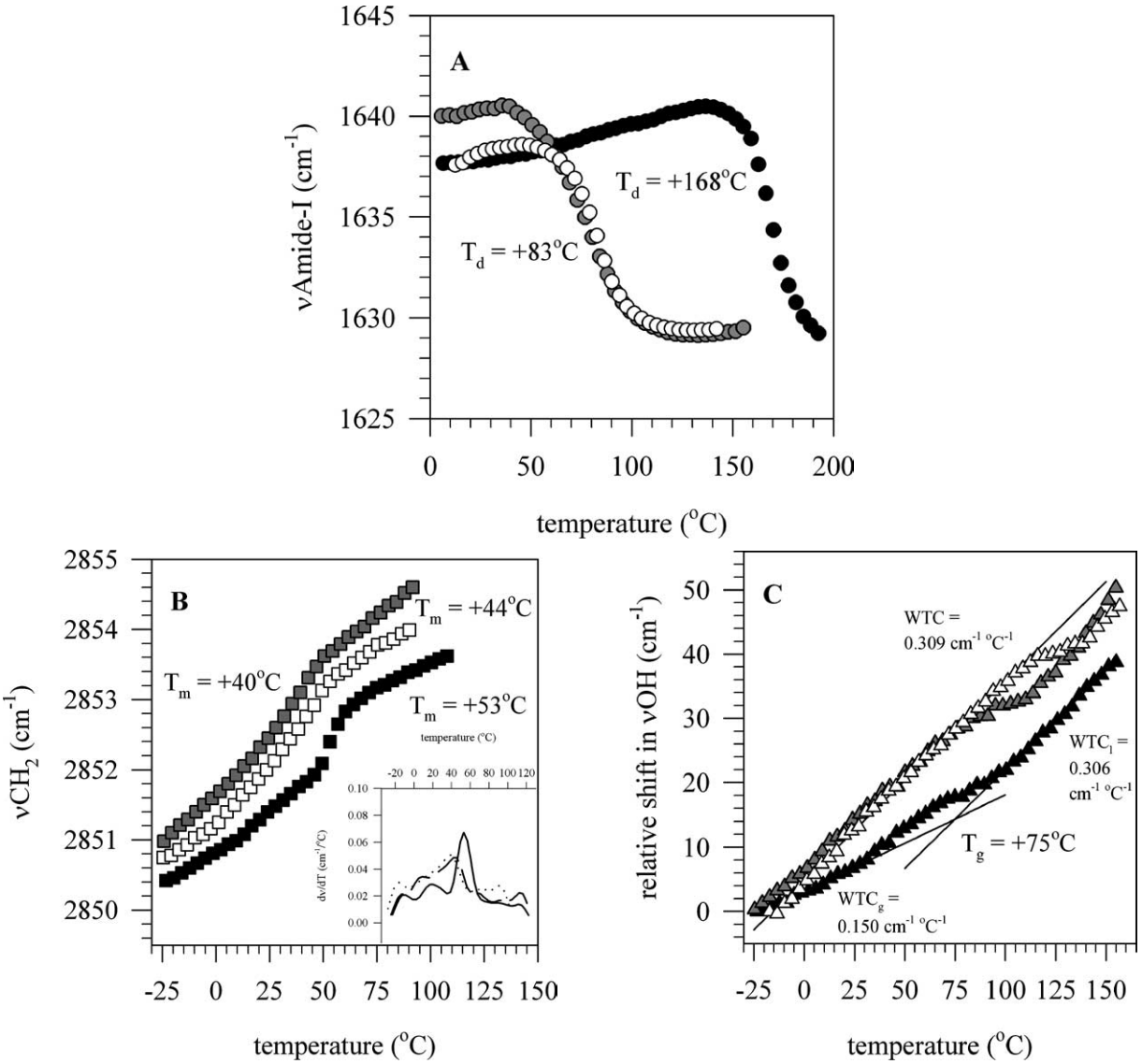

Fig. 3. Protein denaturation (A), membrane phase behaviour (B) and glassy behaviour (C) of *L. bulgaricus*/sucrose suspension air-dried on CaF_2 windows (black filled symbols, solid line) and of air- (grey filled symbols, dotted line) and freeze-dried (open symbols, dashed line) sample prepared in KBr disks. The data points reflect the band position of the protein amide-I band around 1635 cm^{-1} (circles), the lipid symmetric CH_2 stretching band around 2850 cm^{-1} (squares), and the sugar OH stretching band around 3350 cm^{-1} (triangles), respectively. The first derivatives of the νCH_2 versus temperature plots were calculated to determine the membrane phase transition temperatures and the cooperativity of the transitions (3B inset-figure).

Possible glass transitions of the dried cell/sucrose suspension were determined from νOH versus temperature plots. Cell suspensions that were directly dried on a CaF_2 window show a glass transition at approximately 75°C, as determined from the intersection of linear regression lines in the glassy and liquid state of the plot. By contrast, no glass transition was observed for freeze- or air-dried cells that were prepared in KBr disks. The slope of the samples in the KBr disks (indicated as WTC in Fig. 3C) is almost the same as the slope of the air-dried sample on the CaF_2 window at temperatures above the

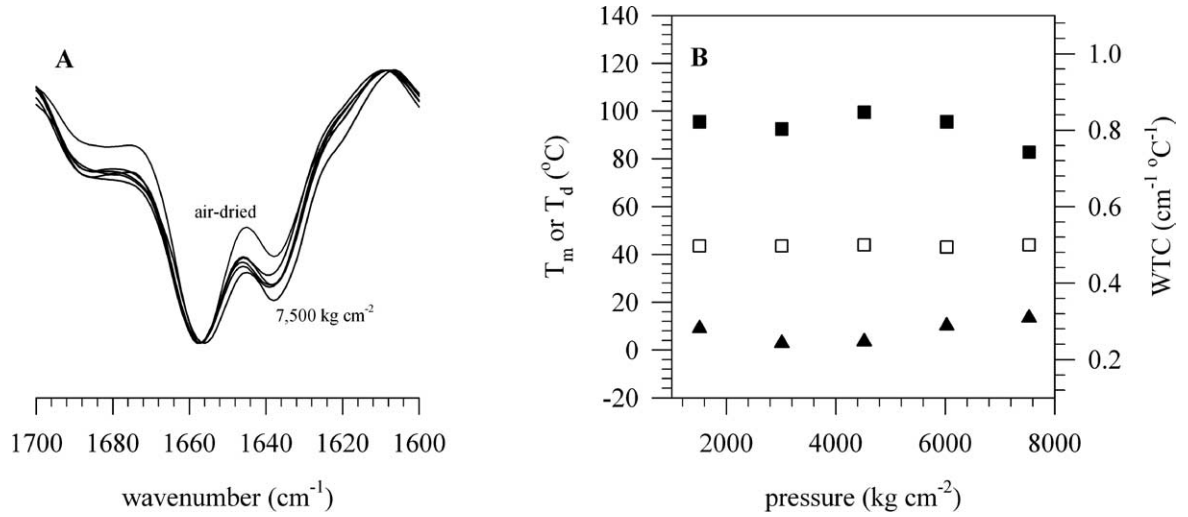

Fig. 4. (A) Second derivative infrared absorption spectra of the amide-I region (1700–1600 cm^{-1}) of freeze-dried *L. bulgaricus*/sucrose suspensions in KBr disks at 20°C. Freeze-dried samples were compressed into KBr disks using various pressures (7500, 6000, 4500, 3000 or 1500 $kg\,cm^{-2}$). The amide-I band of air-dried sample on CaF_2 windows is also shown for comparison. (B) The protein denaturation temperature (closed squares), membrane phase transition temperature (open squares) and the WTC of νOH (closed triangles) of freeze-dried sample in KBr disks compressed at different pressures.

glass transition temperature, T_g (indicated as WTC_l). This indicates that KBr compression decreases the strength of hydrogen bonding interactions of the dried matrix resulting in hydrogen bonding interactions that normally occur in the liquid state above T_g. The T_g of the samples in the KBr disks was decreased to temperatures below −25°C.

Thermal analysis using DSC was done to confirm that the low denaturation temperature and the low T_g of the freeze-dried sample in the KBr disks were due to the procedure to prepare KBr disks. Thermal analysis revealed a change in heat capacity with a midpoint at approximately 51°C that was assigned to the glass transition of the freeze-dried cell/sucrose suspension and a small endothermic peak with a midpoint at 160°C that was assigned to denaturation of cellular proteins. As expected, the T_g and T_d values of the freeze-dried sample are close to those of the air-dried sample as analyzed by FTIR on CaF_2 windows. This also indicates that the KBr compression method to prepare samples for FTIR analysis introduces artefacts in the thermo-physical properties of freeze-dried biological samples.

In order to investigate if the disturbed thermo-physical properties of dried samples in KBr disks were caused by the applied pressure in the hydraulic press that was used to make the KBr disks, the effect of pressure on the thermo-physical properties was studied. Figure 4A shows the effects of the applied pressure on the amide-I band profile. The pressure was varied between 7500 and 1500 $kg\,cm^{-2}$. The amide-I band profile remained virtually the same as a function of the pressure. The relative proportion of β-sheet structures in the KBr disks was increased compared to that of the air-dried sample on CaF_2 windows. Figure 4B shows the effects of pressure on the protein denaturation temperature, membrane phase transition temperature (T_m) and glassy behaviour. The T_m, T_d and WTC of νOH did not differ as a function of the applied pressure on the disks: T_d varied between 83°C and 99°C, T_m remained 44°C, and the WTC of νOH varied between 0.25 and 0.30 $cm^{-1}\,°C^{-1}$ (no glass transitions were observed). We were not able to perform FTIR analysis using pressures lower than 1500 $kg\,cm^{-2}$, because the translucency of the disks was not sufficient to obtain good quality spectra. Based on these results it

cannot be concluded if the abnormal physical properties in the KBr disks are due to the applied pressure on the disks in the hydraulic press (pressure effects may occur below 1500 kg cm^{-2}) or to the grinding and mixing of the sample with KBr.

4. Discussion

Thermo-physical properties of *L. bulgaricus* dried with sucrose were studied by FTIR using two sample preparation methods. The results show that the protein denaturation temperature of samples that were prepared in KBr disks was decreased by more than 70°C compared to that of non-manipulated samples dried on CaF_2 windows. The glassy behaviour of dried cell/sucrose suspensions and the membrane phase behaviour of the cells were also affected by preparation of the sample into KBr disks. Cells that were dried with sugars and transferred into KBr disks were not in a protective glassy state at ambient temperature.

The KBr compression method to prepare samples of dried proteins for FTIR analysis is widely used, particularly in studies of pharmaceutical proteins [4]. Using KBr disks fundamental insights have been obtained in dehydration induced conformational changes of certain proteins and how they can be prevented by sugars [12]. Most studies on dried proteins using KBr disks, however, are on protein secondary structure at one temperature. Although the effects of KBr compression on the protein amide-I band at first sight appear to be minor at room temperature (increase in relative proportion of β-sheet structures), temperature dependent FTIR analysis clearly shows that KBr disk formation introduces artefacts in the physical properties of dried *L. bulgaricus*/sucrose suspension. DSC analysis revealed that the freeze-dried sample was in glassy state at room temperature as expected when using sucrose as excipient, whereas FTIR analysis using KBr disks suggested a T_g below -25°C. The studies on the OH-stretching band showed that hydrogen bonding interactions of the glassy sugar matrix are drastically disturbed in KBr disks. The effects of KBr compression on the glassy matrix could be correlated with the low protein denaturation temperature that was measured for the dried cells in KBr disks. In addition, DSC analysis indicated that the denaturation temperature of the freeze-dried sample was considerably higher than the denaturation temperature that was determined by FTIR using KBr disks.

FTIR analysis on the glassy behaviour of the dried sample on CaF_2 windows showed a broad glass transition from 45°C to 90°C with a T_g of 75°C, which is higher than the T_g of 57°C of anhydrous sucrose [20]. The higher T_g of the air-dried cell/sucrose suspension compared to that of sucrose alone is likely the result of hydrogen bonding interactions between sucrose and cellular proteins [19]. The denaturation temperature that was determined for the air-dried sample falls within the range that can be expected for dried proteins [3]. It has been shown that the denaturation temperature of dehydrated proteins is correlated with the glass transition temperature of excipients [3].

The possible effects of KBr compression on protein secondary structure have led to the development of alternative methods to study dried materials with low translucency. Air drying directly on CaF_2 windows can be used as an alternative sample preparation method for FTIR analysis of biological samples in the dried state. Air drying has the advantage that it does not require manipulation of the sample. Souillac et al. [13] have evaluated diffuse reflectance FTIR spectroscopy to study freeze-dried proteins. No sample preparation is necessary in this case and the reflectance spectrum is determined by the scattering characteristics of the sample. Also an attached FTIR microscope with a more intense beam could be useful in studies of dried biological cells or tissues [17,20].

5. Conclusion

In conclusion, we show that temperature-dependent FTIR analysis can be used to reveal physical properties of lactic acid bacterial cells in the dried state, but that care must be taken with the sample preparation method. Preparation of dried samples in KBr disks for FTIR analysis introduces artefacts in conformation and physical properties of biomolecules in the dried state. FTIR allows for simultaneous *in situ* analysis of membrane phase transition temperatures, protein denaturation temperatures and glassy behaviour. The combination of structural information and physical properties of biomolecules in their native environment that can be derived from FTIR analysis make FTIR one of the most powerful techniques to study dried biological materials.

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