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Importance of elasticity on calcium caseinate fiber formation

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ABSTRACT

This study aims to gain a better understanding of the structuring mechanisms in roller-dried calcium caseinate by using H₂O and D₂O as protein solvents. Calcium caseinate mixed with H₂O gives a layered material, while the use of D₂O results in a fibrous material. The D₂O hydrated protein network is more elastic and can hold more air, which could contribute to the fibrous appearance and a greater mechanical anisotropy of the sheared material. These results highlighted that the elasticity of the calcium caseinate dispersion is important for stabilising air bubbles upon shear deformation, which is expected to play a major role in the fibre formation of calcium caseinate.

1. Introduction

The creation of fibre structures within proteins is of great relevance, as these structures are promising candidates for the next generation of meat analogues. A novel technology based on well-defined shear flow was introduced to create fibrous structures from calcium caseinate (Manski, van der Goot, & Boom, 2007b,c) and plant proteins, such as soy protein (Dekkers, Nikiforidis, & van der Goot, 2016) and pea protein (Schreuders et al., 2019). With regards to the meat-like structures, the fibrousness of calcium caseinate material is much more pronounced than those of plant proteins. To better translate the results obtained from calcium caseinate to plant proteins, it is important to investigate the mechanism of fibre formation in calcium caseinate.

The first step towards understanding is a quantitative description of the structures formed. Tensile tests were used to characterise the mechanical properties, while confocal scanning laser microscopy and scanning electron microscopy (SEM) revealed structure at micro- and nanoscale (Manski, van der Zalm, van der Goot, & Boom, 2008; Wang, Dekkers, Boom, & van der Goot, 2019; Wang, Tian, Boom, & van der Goot, 2019a). X-ray tomography (XRT) was used to describe void fractions and air bubble shapes, while spin-echo small angle neutron scattering provides complementary information to XRT (Tian, Wang, van der Goot, & Bouwman, 2018; Wang, Dekkers, & van der Goot, 2020; Wang, Tian, Boom, & van der Goot, 2019b). More recent small angle neutron scattering turned out to be a promising method to quantify the

orientation and anisotropy of the fibres down to 100 nm (Tian et al., 2020). In these neutron scattering techniques, D₂O rather than H₂O is often used as a solvent to enhance the contrast between protein and solvent (Lopez-Rubio & Gilbert, 2009; Tromp & Bouwman, 2007). Generally, it is assumed that the replacement of H₂O by D₂O has limited effect on the protein structure (Bouchoux et al., 2015). However, we found that the roller-dried calcium caseinate fibre formation was strongly influenced by the solvent isotope effect (Tian, Garcia Sakai, Pappas, van der Goot, & Bouwman, 2019; Tian et al., 2018). Shearing 30 wt% roller-dried calcium caseinate gave more anisotropic fibres with more deformed air bubbles after replacing H₂O with D₂O.

A possible explanation for the solvent isotope effect is that proteins become more compact in D₂O than that in H₂O (Cioni & Strambini, 2002; Efimova, Haemers, Wierczinski, Norde, & van Well, 2007; Sasikanker, Oleinikova, Weingärtner, Ravindra, & Winter, 2004). Gelatin gels in D₂O are more rigid than those gels in H₂O (Oakenfull & Scott, 2003). Besides, D₂O can promote the self-association of several proteins, such as β -casein (Evans, Phillips, & Jones, 1979) and tubulin (Chakrabarti, Kim, Gupta, Barton, & Himes, 1999). The mechanism is attributed to the enhancement of hydrophobic interactions and stronger hydrogen bonds in D₂O than in H₂O (Efimova et al., 2007; Sheu, Schlag, Selzle, & Yang, 2008; Tian et al., 2019).

Here, we studied the mechanism of fibre formation in roller-dried calcium caseinate by taking advantage of the different properties of H₂O and D₂O as protein solvents. The sheared materials were prepared

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in a shear cell device with roller-dried calcium caseinate dispersion, in which H₂O was partially or fully replaced with D₂O. The resulting structures of protein and air phase were investigated with SEM and XRT, respectively. The mechanical properties of the materials were determined with a texture analyser. Further, the linear viscoelastic behaviour of the calcium caseinate dispersions was characterised using small-amplitude oscillatory shear measurement. It should be noted that D₂O is used as a route to investigate the importance of protein-solvent interactions, but we do not advocate it as a food solution.

2. Materials and methods

2.1. Materials

Roller-dried calcium caseinate powder was kindly provided by DMV International (Veghel, the Netherlands). The calcium caseinate powder (dry base) contained at least 88 wt% protein and 1.20 wt% calcium according to the manufacturer's specifications. The dry matter content of the calcium caseinate powder was 93.5 wt%. Heavy water (Deuterium Oxide, D₂O, $\rho = 1.107 \text{ g} \cdot \text{mL}^{-1}$) with 99.9% atom% D was purchased from Sigma-Aldrich. The conductivity of demineralized water (H₂O) and heavy water (D₂O) were 0.36 and 2.92 $\mu\text{S}/\text{cm}$, respectively. All other chemicals used for microscopy were of reagent grade (Sigma-Aldrich).

2.2. Sample preparation in the shear cell device

Protein dispersions were prepared by manually mixing 30 g calcium caseinate powder with 70 g demineralized water (100% H₂O), 35 g demineralized water + 38.9 g heavy water (50% v/v H₂O + 50% v/v D₂O) or 77.7 g heavy water (100% D₂O). Each dispersion was transferred to a pre-heated (50 °C) shear cell device with gap angle of 2.5° (Wageningen University, the Netherlands). More details of the shear cell device is described in an earlier publication (Van der Zalm, Berghout, van der Goot, & Boom, 2012). The shear process was carried out at a rotating speed of 150 rpm for 5 min. After shearing, the materials were cooled down to 4 °C within 10 min before the cell was opened. Tensile tests were performed on materials within 1 h after removal from the device. Other samples were stored at −20 °C until further analysis. Each material was prepared and analysed twice independently.

2.3. Tensile strength analysis

A texture analyser (Instron Testing System, table model 5564) was used with a load cell of 100 N. A dog-bone-shaped mould was used to cut samples for the tensile test. The samples were 15.2 mm long and 3.18 mm wide; the thickness varied between 4 and 6 mm. The samples were parallel or perpendicular to the shear flow (based on the shear–vorticity plane). The tensile tests were conducted at a constant deformation speed of 3 mm/s. Grips with abrasive paper were used to prevent slipping during testing. At least two specimens per direction were measured. The results obtained from these tensile tests were depicted as force–displacement curves. The true stress (σ , kPa) and Hencky strain (ϵ , %) were calculated. The point at which a dramatic decrease in stress in the stress–strain curve was observed, was taken as the point of fracture, at which we measured the fracture stress (σ , kPa) and fracture strain (ϵ , %). The ratio of the average data for mechanical properties measured in the parallel and perpendicular directions to the shear flow were calculated and used as an indication of the mechanical anisotropy of the sample.

2.4. X-ray tomography

The void fraction and geometry of air bubbles in the sheared materials were studied by X-ray tomography. Samples were scanned with a GE Phoenix v|tome|x m tomographer (General Electric, Wunstorf, Germany) set at 80 kV/90 μA . The system contains two X-ray sources; the 240 kV micro-focus tube with a tungsten target was used. The images

were recorded by a GE DXR detector array with 2024×2024 pixels (pixel size, 200 μm). The detector and object were located 815 mm and 28.55 mm from the X-ray source, respectively, resulting in a spatial resolution of 7.00 μm . A full scan consists of 750 projections over 360°. The first image was skipped; images 2–4 were averaged into one projection. GE reconstruction software (Wunstorf, Germany) was used to calculate the 3D structure via back projection. The 3D images were analysed using Avizo imaging software version 9.3.0. Three samples of each material were measured.

2.5. Scanning electron microscopy

The sheared materials were torn apart manually along the shear flow direction (~ 2 mm, based on the shear flow–vorticity plane), and rinsed with demineralized water. Subsequently, the samples were fixated in a solution of glutaraldehyde (1.25% v/v) and formaldehyde (1.25% v/v) in a vacuum chamber for overnight. Follow by, the fixed samples were dehydrated in a graded series of ethanol solutions (10%, 30%, 50%, 70%, 90% and 100% v/v, 20 min each). Critical point drying with carbon dioxide (CPD 300, Leica, Vienna, Austria) was performed. The dried samples were then glued to sample holders using conductive carbon cement (Leit-C, Neubauer Chemicalien, Germany), and sputter-coated with 15 nm of iridium (EM QSG100, Leica, Vienna, Austria). The surface was analysed with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands) at a magnification of 100,000 times at a working distance of 4–6 mm, with secondary electron detection at 2 kV and 6.3 Pa.

2.6. Rheology

To carefully measure the rheological properties of the calcium caseinate premixes, a slightly different sample preparation was performed. Main reason was that remaining air bubbles will strongly influence the rheological measurement. Previous experiments revealed that slight heating helps to remove most of the air, but it does not change the structuring properties (Wang et al., 2019a). Calcium caseinate premixes were prepared at 70 °C for 15 min to obtain homogeneous dispersions. Care was taken to remove any air. The mixtures were kept at 50 °C prior to the rheological measurements. Strain sweep measurements within a strain range of 0.1–100% were performed at 6.28 rad/s to determine the linear viscoelastic region at 50 °C using a Paar MCR 301 rheometer (Anton Paar, Graz, Austria) with serrated parallel plate geometry (diameter 25 mm, gap 1mm). Frequency sweeps were performed at a constant strain of 1% (based on strain amplitude sweeps) within an angular frequency (ω) range of 0.1–62.8 rad/s. The samples were covered with silicone oil to reduce water evaporation and rested for 1 min before testing.

3. Results and discussion

3.1. Structure formation

Fig. 1 shows the materials obtained after shearing 30 wt% dense calcium caseinate dispersion made of different solvents, namely 100% H₂O, 50% D₂O and 100% D₂O. With only H₂O in the calcium caseinate dispersion, a layered material was obtained after shearing at 150 rpm for 5 min (Fig. 1A). This material did not show any detached fibres upon tearing. Partially or full replacement of H₂O with D₂O in calcium caseinate dispersions resulted in fibrous materials, demonstrated by isolated thin fibres after tearing (Fig. 1B and C1). The use of D₂O led to expelled water during shearing. When prepared with 100% D₂O, some large holes were present in the material as displayed in Fig. 1C2 (based on the velocity gradient–vorticity plane).

The inclusion of air of the sheared material was analysed with X-ray tomography (XRT) (Figs. 2 and 3). It is obvious that the calcium caseinate material made with H₂O contained the lowest air inclusion

Fig. 1. Macrostructure of sheared 30 wt% calcium caseinate dispersions made of (A) 100% H₂O, (B) 50% D₂O and (C) 100% D₂O; (C1) material after tearing; (C2) cross section of the material in the velocity gradient–vorticity plane.

Fig. 2. Reproduction of XRT images of air bubble morphology in sheared calcium caseinate materials with 100% H₂O, 50% D₂O and 100% D₂O (Tian et al., 2018). Copyright 2018, Elsevier. All the materials were obtained after shearing at 150 rpm for 5 min. Blue represents air bubbles in three-dimensional images; black represents air bubbles in two-dimensional images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

levels ($2.6 \pm 0.5\%$, v/v). The air bubbles are small ($<0.01 \text{ mm}^3$) and spherical in shape. The use of 50% and 100% D₂O largely increased the air inclusion level to $15.1 \pm 1.3\%$ (v/v) and $13.8 \pm 2.3\%$ (v/v), respectively. These materials mainly contained large ($>0.01 \text{ mm}^3$) and elliptical air bubbles. Those results are in line with previous study using XRT data and neutron refraction model in which it was shown that large air bubbles in sheared materials were deformed and aligned in the shear flow direction when D₂O was present in the material (Tian et al., 2018). According to the neutron refraction data, the degree of deformation of air bubble in 50% and 100% D₂O samples (0.23 and 0.25) had no significant difference (Tian et al., 2018), suggesting that air bubbles in these two samples have a similar degree of anisotropy.

The SEM images in Fig. 4 show the microstructure of sheared material with 100% H₂O, 50% D₂O and 100% D₂O. No protein alignment at a submicron scale was achieved in these samples. This indicated that the presence of D₂O in roller-dried calcium caseinate had little effect on the alignment of the aggregates at the submicron length scale in the flow direction.

3.2. Tensile properties

As described in the previous section, the use of H₂O yielded a layered structure, while the use of D₂O promoted fibre formation at the

macroscale (Fig. 1). These materials were subjected to tensile analysis parallel and perpendicular to the shear flow direction. The ratio of the stress σ or strain ϵ in the parallel and perpendicular directions was used as an index for anisotropy. In the absence of D₂O, the sheared material showed slight anisotropy with an index of 2.0 and 1.3 for σ and ϵ , respectively (Fig. 5). This anisotropic index of σ and ϵ increased to 4.6 and 2.2 when 50% of D₂O was used. There was no significant difference on the anisotropic index between the material with 50% and 100% D₂O. The use of D₂O enhanced the strength of materials in the parallel direction but weakened in the perpendicular direction. In both directions, the high porosity indeed decreased the strength of the materials (Fig. 2), but expelled water during shearing resulted in higher dry matter content that also enhances it. Thus, the large difference between parallel and perpendicular direction could be attributed to the anisotropic morphology of air bubbles in these materials. The large and deformed air bubbles work as a weak phase in the perpendicular direction, which promotes crack propagation along the fibre direction.

3.3. Rheological properties

Fig. 6 depicts the frequency dependency of storage modulus G' , loss modulus G'' and tangent of phase shift $\tan \delta$ (G''/G') of calcium caseinate dispersions with 100% H₂O, 50% D₂O and 100% D₂O measured at 50 °C.

Fig. 3. Overall cumulative void fractions (as the void fraction below the bubble volume in mm^3). The error bars for the total void fraction below volume are $\pm$ standard deviation; where not shown they were smaller than the marker used.

Fig. 6A shows that the use of D_2O in calcium caseinate resulted in increased values of G' at low frequency range ($0.1 < \omega < 10 \text{ rad/s}$) and less frequency dependency of G'' . All dense calcium caseinate dispersions show crossovers of G' and G'' ($\tan \delta = 1$) at measured frequency range, except for the dispersion with 100% D_2O which had a crossover at a lower frequency of 0.25 rad/s (data not shown). The inverse of the frequency at the crossover is defined as the terminal relaxation time (τ).

The τ increased with the use of D_2O , indicating that calcium caseinate dispersion with D_2O has much stronger physical interactions. This is most likely caused by the enhancement of hydrophobic interactions and hydrogen bonding (Efimova et al., 2007; Sheu et al., 2008; Tian et al., 2019). Previously, differences in relaxation time were used to explain differences in structuring behaviour of spray dried sodium caseinate and calcium caseinate (Manski, van Riemsdijk, Boom, & van der Goot, 2007). Calcium caseinate had a higher relaxation time. This longer relaxation time implied that upon shearing, microstructural change could be induced in calcium caseinate, while not in sodium caseinate at moderate shear rates. Here, the use of D_2O led to increased relaxation time as well and led to similar outcomes with respect to fibre formation. The $\tan \delta$ decreased with increasing D_2O concentration through the measured frequency range, indicating that D_2O promoted the elasticity of the material (Fig. 6B). This may be related to more compact and rigid protein particles, which results in a more 'entangled' calcium caseinate dispersion.

A previous study found that the addition of maltodextrin in roller-dried calcium caseinate could stabilise air bubbles upon shear deformation. The explanation was that maltodextrin increased the viscosity and created a more elastic network. Here, we see a similar trend for the elasticity when using D_2O , but not for the viscosity. The viscosity of dispersions with 50% and 100% D_2O was lower (82 and $128 \text{ Pa}\cdot\text{s}$) than that with H_2O ($474 \text{ Pa}\cdot\text{s}$) at a shear rate of 10 s^{-1} . It is likely that the elasticity of the matrix plays a more important role in air holdup than the viscosity. A similar effect has been reported in the dough systems: the elasticity of dough could help to maintain the thickness of solid phase between bubbles upon deformation, and thus would suppress coalescence of bubbles (Chakrabarti-Bell, Wang, Patel, Weiss, & Austin, 2013; Chakrabarti-Bell, Wang, & Siddique, 2014; Ishwarya, Desai, Naladala, & Anandharamakrishnan, 2017).

Fig. 4. SEM images of the fractured surface parallel to the shear flow direction of the sheared calcium caseinate materials. The samples are made of (A) 100% H_2O ; (B) 50% D_2O and (C) 100% D_2O . Scale bars indicate 500 nm.

Fig. 5. Tensile properties: (A) fracture stress σ , (B) fracture strain ε of sheared calcium caseinate materials (100% H_2O , 50% D_2O and 100% D_2O) deformed parallel (filled bars) and perpendicular (open bars) to the shear direction. The error bars for the mechanical properties are $\pm$ standard deviation; where not visible, they were smaller than the marker used. The anisotropy index (AI, diamonds) is the ratio between the average values measured in the parallel and perpendicular directions.

Fig. 6. (A) Storage modulus G' (closed symbols) and loss modulus G'' (open symbols) as a function of angular frequency of calcium caseinate-maltodextrin dispersions. (B) Tangent of phase shift $\tan \delta$ as a function of angular frequency. The lines are guide to the eye.

4. Conclusions

The creation of fibre structures within proteins is important, as these structures are promising candidates for the next generation of meat analogues. The use of D₂O promotes the fibre formation of roller-dried calcium caseinate upon shearing. D₂O leads to increased calcium-protein interactions and more compact protein structure on molecular level. This results in a more elastic dispersion with longer relaxation time, which makes it more prone for shear alignment. The increased elasticity of the calcium caseinate dispersion also enhanced air inclusion in the protein dispersion. With D₂O, air was included as large and deformed air bubbles that contributed to the fibrous appearance and a greater mechanical anisotropy of the sheared material. These results indicated that the elasticity of the dispersion is important for fibre formation in roller-dried calcium caseinate. This study also draws attention to the solvent quality effects. This suggests on one hand that it is possible through adjusting solvent quality, it is possible to make fibrous materials that were not created when using water, but on the other hand it shows that results obtained with small-angle neutron scattering or nuclear magnetic resonance techniques that used D₂O as solvent to enhance the quality of the measured should be interpreted carefully.

Declaration of competing interest

None.

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