Physicochemical Changes in a Model Protein Bar during Storage

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Abstract

High-protein snack bars (protein bars) contain high-quality protein, sugars and other low molecular weight polyhydroxy compounds (PHCs), high-energy confectionary fats, and a minimum of water (water activity $\leq$ 0.65). The consequence of the intimate mixing of these components in protein bars is that they can react together, creating sensory characteristics that are unacceptable to consumers.. This study examined the changes occurring in a model protein bar during storage for 50 days at 20 °C.

Over this time, fracture stress increased from 20.1 $\pm$ 1.8 Pa to 201 $\pm$ 75 Pa at a rate that decreased slightly over time. ^{1}H nuclear magnetic resonance (NMR) showed that the molecular mobility of PHCs decreased dramatically over the first 5 days as the batter set into a solid bar. Over the first 17 hours after manufacturing, protein particles became more clustered, and soluble protein appeared to precipitate, as shown by confocal microscopy. Reactive lysine fell 38% in the first 10 days of storage and was approximately constant thereafter. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed little change in protein molecular weights. Following the initial 'setting' phase of 5-10 days, fracture stress continued to increase and the molecular mobility of PHCs decreased. Changes in PHC molecular mobility were consistent with glucose crystallisation. Chemical changes were minimal during this phase, which suggests that chemical reactions play little part in the hardening of protein bars and that changes in molecular mobility and changes in microstructure driven by moisture migration may be more important.

INTRODUCTION

Many processed foods are multicomponent heterogeneous systems that are far from thermodynamic equilibrium (Mezzenga, 2007). There is often a considerable time lag between manufacture and consumption, during which a product is transported and stored. During this storage time, multiple chemical, physical, and biological reactions occur serially and simultaneously. Some of these reactions lead to the development of desirable attributes, such as good flavors in aged wines and cheeses, but others create flavors, colors, or textures that impact negatively on quality, as perceived by consumers. The shelf life can be defined as the length of time for which a product can be stored before the appearance of the first characteristic that consumers find unappealing, e.g., texture that is too tough.

Intermediate-moisture foods generally have water activity (a_w) in the range 0.9–0.6 (Roos, 2001, p. 5). This is low enough to inhibit the growth of most micro-organisms, though some organisms will exhibit limited growth at the upper end of this range (Jay, 1996, p. 45). The factor that limits shelf life is more often chemical or physical reactions. The most important class of chemical reactions for intermediate-moisture foods is Maillard reactions (also known as nonenzymatic browning), in which exposed amino groups on proteins react with reducing sugars. Maillard reactions occur fastest at a_w in the range 0.65–0.75 and at high temperature (Lea & Hannan, 1949). Physical reactions include changes of state, such as melting or crystallisation, and transitions related to molecular mobility such as the glass transition.

High-protein snack bars (hereafter called 'protein bars') are a convenient and nutritious food format that was originally developed for athletes but is now formulated to appeal to a wide range of health-conscious consumers. These bars contain 15–35% protein, which consists almost exclusively of dairy or soy proteins because of their health benefits and cost effectiveness. Chocolate, sugars, and flavorings create an appealing taste and flavor. Nuts, wafers, nuggets, etc.

may be added for novel texture. Vitamins, minerals, and/or fiber are often added for enhanced nutritional value.

Products of this type are generally formulated to have a_w less than 0.65, and some have a_w as low as 0.3 (Doherty & Ward, 1997). The a_w can be depressed by removing water from the formulation and/or by adding low-molecular-weight solutes. The moisture content of protein bars is typically 10–15% w/w, and the humectants most commonly used are sorbitol, glucose, fructose, maltodextrin, glycerol, and high-fructose corn syrup.

The shelf life of protein bars is often limited by the development of a 'hard' or 'tough' texture that consumers find unpalatable. The mechanism leading to the hardening of protein bars has not been conclusively identified, although a number of hypotheses have been proposed. Zhou and Labuza (2008) suggested that hardening resulted from thiol–disulfide interchange reactions during storage. This was thought to lead to protein cross-linking, aggregation, and network formation, which resulted in a hard texture. Others have reported evidence that bar hardening during storage is favored by more ordered protein secondary structure and lower surface hydrophobicity of protein particles (Baier et al., 2007). This group cited moisture migration as a driving force for the hardening of protein bars, but did not specify the source and destination of migration.

It is also possible that Maillard reactions between reducing sugars and reactive lysine residues play a part in the hardening of protein bars. Cross-linking of proteins via Maillard reactions during food processing is widely acknowledged, but the specific reaction pathways are not well-known because of the multiplicity of possible reactions (Gerrard, 2002). Maillard reactions are favored by high temperature and alkaline pH (Lea & Hannan, 1949), and when an excess of reducing sugar is present (van Boekel, 2001). Rich and Foegeding (2000) reported that Maillard reactions in whey protein isolate gels were associated with strengthening of the gel. However, in

the work of Baier et al. (2007), when browning was inhibited with sulfites, the rate at which protein bars hardened was unaffected.

Li, Szlachetka, Chen, Lin, & Ruan (2008) suggested that the NMR relaxation behavior of protein powders as a function of temperature was an indication of their performance in protein bars. Higher values of T_2 were thought to indicate higher susceptibility to physicochemical changes over time, i.e. protein bar hardening.

In this study, we examined the contributions of various chemical and physical reactions to the hardening of a model protein bar stored for up to 50 days at 20 °C.

MATERIALS AND METHODS

Chemicals. Disodium tetraborate, tris, and sodium dodecyl sulfate (SDS) were obtained from BDH Chemical Ltd. (Poole, England) (AnalaR or molecular biology grade). Dithiothreitol (DTT), glycerol, glycine, and tetramethylethylenediamine (electrophoresis or spectrophotometric grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Ammonium persulfate and acrylamide/bis (37.5:1) were obtained from BioRad (Hercules, CA) and were electrophoresis grade.

Milk protein concentrate (MPC, ALAPRO™ 4850) (typical analysis 81.3% protein, 5.7% moisture, 3–5% lactose, 1.6% fat) was supplied by Fonterra Co-operative Limited, Auckland, New Zealand. Cocoa butter (ADM DeZaan pure prime pressed) and glucose were supplied by Hawkins Watts Ltd, Auckland, New Zealand. The water used to make protein bar material was supplied from an underground bore. All analyses used water filtered by reverse osmosis.

Product Manufacture. Bar material was made in 1 kg batches according to the formulation shown in **Table 1**. The ingredients were mixed with a Kenwood mixer (Kenwood Corporation, Long Beach, CA) fitted with a flat K-bar type blade.

Water and glucose at room temperature (~20 °C) were mixed and heated in a stainless steel container to 105 °C, then cocoa butter was added and the container was placed in a water bath at room temperature. While the sugar–water–cocoa butter syrup was cooling, MPC was weighed into the mixer bowl, followed by glycerol. The glycerol was mixed into the MPC with a spatula for approximately 30 s. When the syrup had cooled to 40 °C, at which temperature it was a slightly viscous liquid, it was added to the MPC and glycerol in the mixer bowl and mixed at 80 rpm.

These ingredients were mixed for 1 min and then rested for 1 min, during which the bowl was scraped with a spatula to remove powder caked against the surface. After resting for 1 min, the protein bar material was mixed for another 1 min.

Immediately after the second period of mixing, the viscous protein bar material was poured into 100 mL plastic containers (Lily brand, Huhtamaki NZ Ltd., Auckland New Zealand) or vertically oriented cylindrical plastic tubes with internal diameter 16 mm and length 80 mm, sealed at one end with tape. Care was taken not to entrap large air bubbles inside the tubes.

The lids were placed on the 100 mL containers and the tubes were sealed at the other end with tape. The samples were transferred to an incubator at 20 °C shortly after manufacture. The plastic tubes were vertically oriented throughout storage. The day following manufacture was designated 'day 1' of the storage trial.

Texture Analysis. A lubricated uniaxial compression test was adapted from methods described previously (Watkinson et al., 1997; Watkinson et al., 2001; Watkinson & Jackson, 1999). Each solidified cylindrical core of protein bar material was pushed out of its plastic tube with a

wooden rod, forming a plug approximately 70 mm long. The plug was sliced perpendicular to the long axis using a lubricated wire, giving three or four 20 mm long cylindrical sections.

Each section was placed upright (cylinder axis vertically oriented) on a smooth Teflon plate lubricated with mineral oil and then compressed vertically with a parallel lubricated Teflon plate. The samples were compressed with a TA.HD texture analyzer, equipped with a 500 N load cell (Stable Micro Systems, Godalming, UK) and driven by Texture Expert Exceed software (version 2.64, Stable Micro Systems), to 80% Cauchy strain at a crosshead speed of 0.83 mm/s. Each of the three or four cylindrical sections cut from a single sample plug was compressed in the same way.

Force–distance–time data were analyzed using proprietary software (Masterworks Software, Tawa, New Zealand) written in a functional programming language called J (version 4.06d, Jsoftware Inc., Shorewood, MN) (Iverson, 1991). The software algorithm converted the raw data into stress and Hencky strain according to the method of Watkinson et al. (1997), and the fracture stress was approximated by the local maximum in stress (Van Vliet, Lucisano, & Casiraghi, 1991). The modulus of deformability was measured from the maximum slope found at low strain (Watkinson & Jackson, 1999), and the fracture area was the area under the stress–strain curve (i.e., work) up to the point of maximum stress (Watkinson et al., 2001).

Confocal Laser Scanning Microscopy. During manufacture of the protein bars, a subsample was withdrawn after the final mixing and a few drops of dye were added. The dye was a mixture of Nile Blue and Fast Green FCF dissolved at 0.2% w/v in a commercial antifading mountant medium, Citifluor (Citifluor Ltd, Leicester, UK). The dye was mixed into the protein bar material with a knife until even coloring was achieved, as judged by eye, and then a drop of the mixture was placed on a glass cavity slide and a coverslip was applied. The slides were stored at 20 °C.

The slides were examined with a Leica model TCS SP5 DM6000B confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), initially approximately 30 min after the completion of mixing and then again the next day, approximately 17 h later.

The images were processed with Adobe Photoshop CS version 8.0 (Adobe Systems Incorporated, San Jose, CA) and ImageJ 1.38x (National Institutes of Health, USA; http://rsb.info.nih.gov/ij/).

Nuclear Magnetic Resonance (NMR). All NMR experiments were undertaken in a Bruker (Rheinstetten, Germany) AMX 200 MHz horizontal wide-bore magnet. The proton transverse (or spin–spin) relaxation was measured using the Carr Purcell Meiboom Gill (CPMG) spin-echo pulse sequence (Callaghan, 1991). The major components of the bar material – water, lipid, and polyhydroxy compounds (PHCs) – could be analyzed separately via their representative peaks in the proton NMR spectrum, as shown in **Figure 1**. The major PHCs, glucose and glycerol, could not be separated, as both appear at the –OH position in the proton spectrum.

The transverse relaxation signal (S) versus the CPMG time of each component was well-fitted in all cases by a bi-exponential model (Schuck et al., 2002):

$$S = A \exp\left(\frac{t}{T_{2,FAST}}\right) + B \exp\left(\frac{t}{T_{2,SLOW}}\right)$$

(1)

$T_{2,FAST}$ and $T_{2,SLOW}$ are the transverse relaxation rate constants of fast- and slow-relaxing protons of each compound, respectively. The values A and B are the proportions of each component. The T_2 of a compound is related to its molecular mobility and molecular interactions (Lin et al., 2006). As the molecular mobility decreases and/or the level of molecular interactions increases, T_2 will decrease. The presence of two relaxation rates corresponds to two coexisting states of a compound that differ in molecular mobility and/or levels of interaction. In semisolids, this

usually indicates compounds having both liquid-like amorphous and crystalline or glassy domains.

The standard Bruker phase-sensitive nuclear Overhauser effect (NOE) pulse sequence (NOESYPH) was used to acquire a two-dimensional nuclear Overhauser effect spectroscopy (2D NOESY) data set with a mixing time (τ_m) of 200 ms. In 2D NOESY, the NOEs appear as cross-peaks, indicating transfer of spin polarization from one spin population to another (cross-relaxation). NOEs can occur between adjacent nuclei within a molecule and/or between nuclei in different molecules. An NOE occurs when two nuclei are close in space (within 5 Å) (Otting & Liepinsh, 1995). The intensity of the NOE peak is proportional to the distance between the nuclei, and is inversely proportional to the spin exchange rate.

Protein Extraction and SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE). After storage for 1–50 days at 20 °C, it was necessary to prevent further chemical reactions so that the state of the sample was preserved. A procedure described earlier (Loveday & Winger, 2007) was adapted. A 100 mL plastic container was removed from storage and approximately 30 g of protein bar material was gradually added to liquid nitrogen in an aluminum dish. The remaining protein bar material was discarded. When boiling had subsided, the frozen protein bar material and the remaining liquid nitrogen were transferred to a pre-cooled laboratory Waring blender and blended at high speed until the sample had been shattered into a fine powder (30 s to 1 min). The powdered samples were stored in plastic bags at –80 °C until they were lyophilized several days later. The lyophilized samples were stored in plastic bags at –20 °C.

The protein extraction method was adapted from Poms et al. (2004). The extraction buffer was 50 mM borate at pH 9.0, and 0.1% w/v DTT was added for samples that were extracted for reduced SDS-PAGE or the reactive lysine assay.

Frozen lyophilized protein bar was added to extraction buffer at room temperature (~20 °C) at a ratio of 0.4 g of sample to 8 mL of buffer. The slurry was homogenized at high speed for 30 s (Diax 600, Heidolph, Schwabach, Germany), transferred to a 70 mL polystyrene pot (LabServ, Biolab, Auckland, New Zealand), and stirred at low speed overnight in a temperature-controlled room at 4 °C. After overnight stirring, a 5 mL aliquot of slurry was removed with a syringe (Terumo Corporation, Tokyo, Japan) and passed through a 0.8 μm syringe-driven filter (Minisart 26 mm diameter, Sartorius AG, Goettingen, Germany). The samples were filtered at room temperature (~20 °C) and the filtrate was stored at –20 °C.

Prior to SDS-PAGE, the filtrates were thawed and mixed with SDS sample buffer (1 volume of sample to 2 volumes of reducing buffer or 4 volumes of nonreducing buffer), which consisted of 62.5 mM tris-HCl pH 6.8, 10% glycerol, 2% SDS, and 0.01% bromophenol blue. For reduced SDS-PAGE, 0.1% DTT was included in the sample buffer.

The preparation and running of the SDS-PAGE gels followed the method of Anema and Klostermeyer (1997), except that the percentage of acrylamide in the gels was increased to 16%. Destaining and scanning of the gels were carried out as described by Considine et al. (2007).

Reactive Lysine Assay. The procedure used was adapted from Goodno et al. (1981). Each 100 mL of reagent was made up by dissolving 80 mg of orthophthaldialdehyde (OPA) in 2 mL of ethanol, adding this to 50 mL of 0.1 M borate buffer (pH 9.0), and then adding 10 mL of 10% w/v SDS solution, 100 mg of DTT, and sufficient water to bring the volume to 100 mL.

The protein bar extracts prepared for SDS-PAGE were also assayed for reactive lysine content. In a 1.5 mL acrylic cuvette (path length 10 mm), 50 μL of extract was added to 950 μL of reactive lysine reagent. The cuvette was covered with Parafilm (Alcan, Montreal, Quebec, Canada) and inverted several times. After incubation at 20 °C for 5 min, the absorbance at 335

nm was measured with a UV/visible spectrophotometer (PU8600, Philips, Amsterdam, The Netherlands).

The OPA reagent was used as a blank, and its absorbance was set to zero prior to assaying the protein bar extracts. In some cases, it was necessary to dilute the protein bar extracts with water before taking 50 μL for the assay so that the absorbances would be within the range that could be measured accurately. The absorbances were normalized against the average absorbance on day 1 and were expressed as percentages. The OPA assay was carried out three times on each protein bar extract.

RESULTS AND DISCUSSION

Texture Change. The texture of the protein bars changed substantially during storage; the fracture stress increased from 20.5 ± 1.8 Pa ($n = 4$) on day 1 to 201 ± 7.5 Pa ($n = 3$) on day 50 (**Figure 2**). Because fracture area and modulus of deformability were strongly and significantly correlated with fracture stress, only the fracture stress results are shown.

Baier et al. (2007) found a very similar pattern of hardening in protein bars stored at 37 °C and a_w of 0.11 or 0.42 for 28 days. Protein bars stored at a_w of 0.80 hardened for approximately 4 days and softened thereafter (Baier et al., 2007).

Microstructure Changes. Confocal micrographs of protein bar material taken approximately 30 min after mixing (**Figure 3**) showed rounded lipid bodies up to several hundred microns wide (labeled 'a' in **Figure 3**) and opaque proteinaceous particles 5–60 μm in diameter ('b'), surrounded by a continuous phase containing soluble protein ('c'). Some lipid bodies contained spherical vacuole-like unstained regions 50–130 μm in diameter ('d' in **Figure 4**) with some protein at the interface ('e'). These regions probably contain a mixture of water, glucose, and glycerol. The protein particles ranged in size from 5 to 60 μm (**Figure 5**). Some particles had convoluted concave surfaces, whereas others had smooth spherical surfaces. Other dried dairy

powders show very similar particle morphologies (McKenna, Lloyd, Munro, & Singh, 1999; Nijdam & Langrish, 2006), so it is likely that the opaque proteinaceous particles were undissolved powder particles.

Three segregated phases were evident after approximately 17 h at 20 °C

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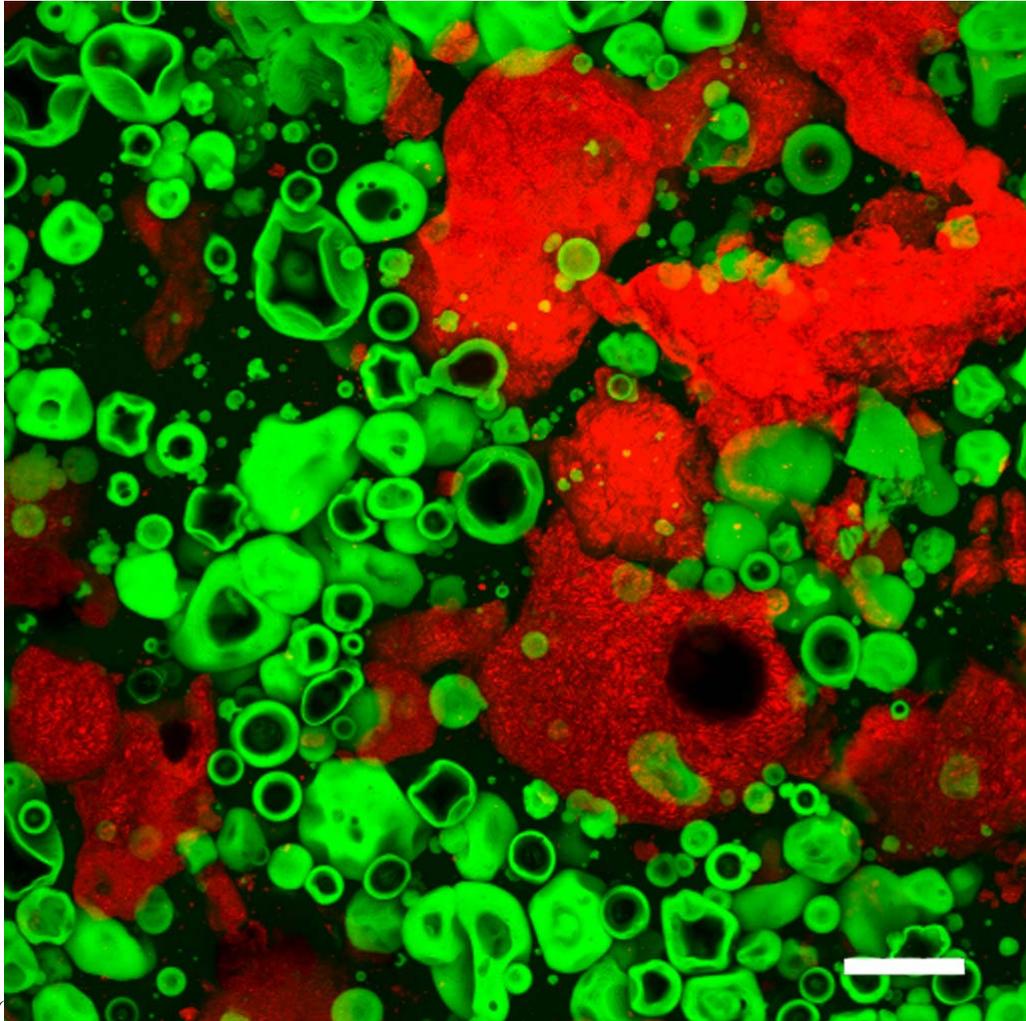
Figure 6). Lipid formed large elongated or bulbous structures with wrinkled surfaces, in some cases containing spherical unstained 'vacuoles'. The protein particles were unchanged in size and appearance, but were arranged in a more compact network-like phase. The continuous background of Fast Green staining (soluble protein) that was seen immediately after manufacture was replaced with unstained areas, which probably consisted of water and PHCs.

MPC contains a mixture of whey proteins and casein micelles in a weight ratio of approximately 20:80. It is likely that the dissolved protein seen shortly after manufacture consisted mainly of

whey proteins, which are generally much more soluble than caseins. During the first day after manufacture, the protein bar material hardened from a batter-like pourable material into a soft but cohesive solid. Over this time, two changes were observed in confocal micrographs: the 'clustering' of protein particles and the disappearance of soluble protein.

The bar mixture contained 15% w/w water, an amount insufficient to fully hydrate glucose (40% w/w), glycerol (15% w/w) and protein (20% w/w). In earlier work, bars made to the same formulation had water activity of 0.65. Under such conditions of limited water availability, there would be a competition between proteins and PHCs for hydration water.

Immediately after manufacture, the majority of the protein would exist as powder particles, but the surfaces of these particles are likely to be hydrated. During storage, the strong driving force for water to associate with the hydroxyl groups of low molecular weight PHCs (as they produce higher osmotic pressures) is likely to cause moisture migration away from particle surfaces, resulting in further association between the particles or some kind of phase separation. This is consistent with indications of phase separation observed by Chen, Zhang, and Cao (2005) in mixtures of soy protein isolate, glycerol and water. Those workers postulated the existence of glycerol-rich, protein-rich and protein-water microphases (Chen et al., 2005).

Some of the protein appears to dissolve during mixing, then precipitates during the following hours as it loses the competition for water of hydration, depositing at the surface of undissolved protein particles. PHCs may not be fully dissolved at first, but after some time they draw water away from dissolved protein.

Molecular Mobility and Spin Interactions. The NMR results indicate that lipids did not contribute to the hardening of the protein bars during storage. The NOESY measurements showed only weak protein–lipid, carbohydrate–lipid, and water–lipid interactions. The relaxation rate (T_2) measurements showed no significant changes in the molecular mobility of the lipids

during the aging of the protein bars (data not shown). The weak interactions and the unchanging

molecular mobility of the lipids were consistent with the phase separation seen in the confocal
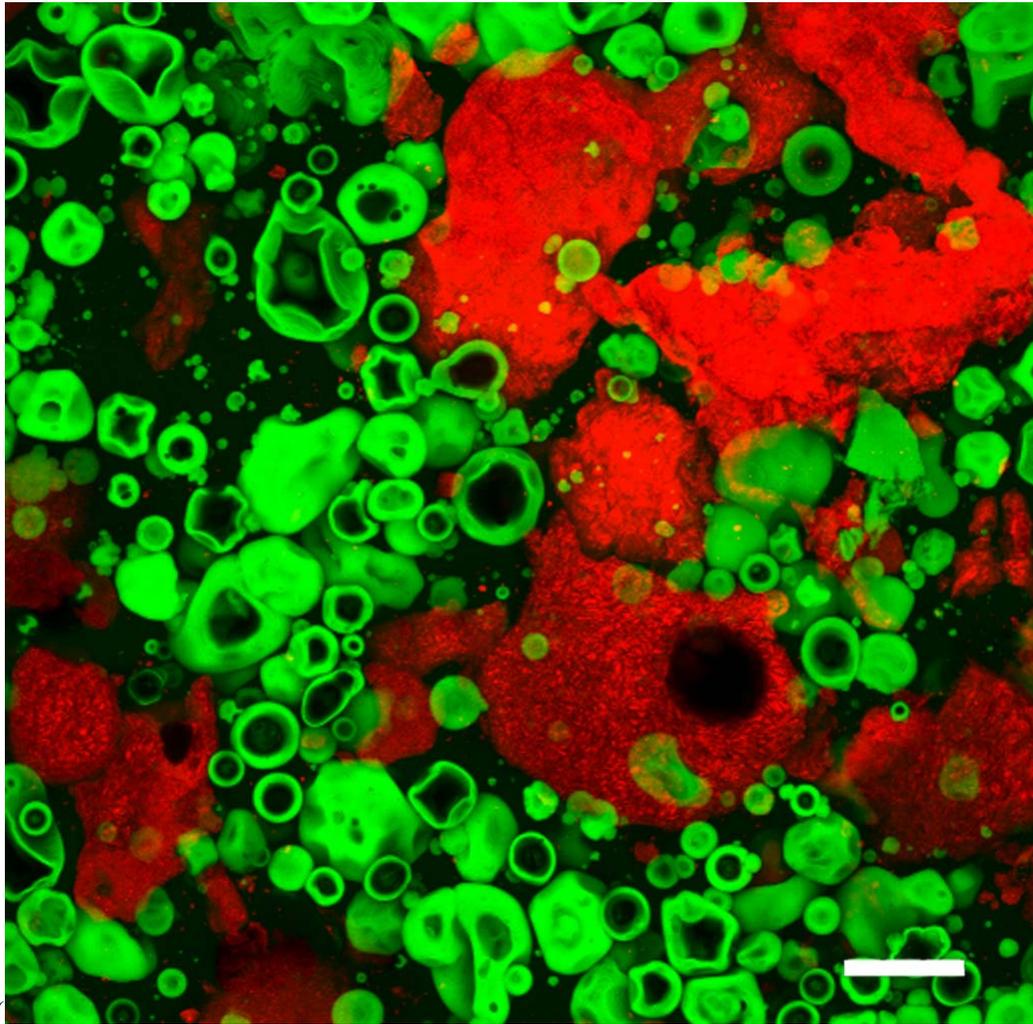
micrographs (

Figure 6), in which lipids appeared as independent bulbous structures within the protein bar

material.

The water and carbohydrates showed high NOE intensity levels, indicating strong molecular

interactions. However, there were no discernible trends in the NOE intensity levels during

storage of the protein bars (data not shown).

The relaxation rates of both fast- and slow-relaxing protons in the water and PHCs declined

sharply from day 1 to day 5. This was probably a reflection of the normal setting process for this

type of product, but may also reflect dissolution and hydration of PHCs at the expense of

dissolved protein, as noted above. In addition to the change over the first five days, there was a continued reduction in the mobility from day 5 to day 48 (**Figures 7, 8**), concomitant with the large increases in fracture stress (**Figure 2**). This shows that, after the initial setting, there was continued change over the longer term.

It was noted that, between days 5 and 48, the relaxation rate $T_{2,FAST}$ decreased by the largest proportion for both water and PHCs (37% and 76%, respectively), whereas the corresponding decreases in $T_{2,SLOW}$ were small for both water and PHCs (15% and 13%) (**Table 2**). The decreases in $T_{2,FAST}$ indicated that PHC and water domains of lower mobility became even less mobile, i.e., the solid-like regions became more solid-like. A possible cause for this hardening was a progressive crystallization of glucose in viscous, supersaturated PHC/water domains. Migration of either water or PHCs between the two fractions of different mobility did not seem to occur, judging by the unchanging proportions of each (*A* and *B*) (data not shown).

Protein Modifications. During the first 12 days of storage at 20 °C, reactive lysine fell 38% and plateaued thereafter (**Figure 9**). Confocal micrographs showed large undissolved protein particles, but proteins at the surface of particles would still be capable of undergoing chemical reactions with the surrounding medium.

Warmbier et al. (1976b) found a similar pattern of reactive lysine loss in a model intermediate-moisture food stored at 45 °C. In that study, the loss of reactive lysine was rapid during the first 7 days of storage and then slow or negligible during the subsequent 8 days (Warmbier et al., 1976b).

The active ingredient in the reactive lysine assay reagent, OPA, will react with both α-amino groups and ε-amino groups, so part of the assay response is due to N-terminal α-amino groups (Goodno et al., 1981). The contribution from α-amino groups is not negligible in mixtures of small peptides or where the lysine content is very low (Goodno et al., 1981), but neither of these

conditions applies to the mixture of milk proteins found in MPC (Farrell Jr. et al., 2004; Swaisgood, 1996).

It is likely that lysine residues reacted with added glucose or lactose in the MPC during storage, but this was not confirmed with other methods such as mass spectrometry. MPC contains 4.6% w/w lactose, (0.92% w/w in bar material), so lactose reactions are relatively minor. Other protein–protein and protein–sugar reactions were also possible (Gerrard, 2002), and may have occurred to some extent.

Residual unreacted lysine residues may have been located within the interior of proteins or in sterically inhibited positions, and were therefore unavailable to react with glucose or lactose molecules. Such a scenario would be consistent with the findings of Scaloni et al. (2002). In that work, the lactosylation sites on α_{s1}-casein from heated milks were identified by mass spectrometric analysis of enzyme-hydrolyzed casein. Lactosylation was site-specific, with lactosyl-lysine produced at only seven of the 14 lysine residues (Scaloni et al., 2002). Analogous results were reported for β-casein (Scaloni et al., 2002), α-lactalbumin, and β-lactoglobulin (Meltretter, Seeber, Humeny, Becker, & Pischetsrieder, 2007).

The samples had not changed color perceptibly after 50 days at 20 °C, in spite of the large decrease in reactive lysine (**Figure 9**). Warmbier et al. (1976a) reported an initial induction period without color formation before a model intermediate-moisture food began to brown. The duration of the induction period depended on temperature and a_w, and very little pigment formed when the product was stored for 53 days at 25 °C, similar to our findings after 50 days at 20 °C. Warmbier et al. (1976a) suggested that colorless reaction intermediates accumulated at first, and that pigment formation began only when the concentration of colorless intermediates was sufficient. The early stages of Maillard reactions, in which little or no color change is observed, were termed 'initial stage' and 'intermediate stage' by Hodge (1953).

Standard SDS-PAGE is a very powerful technique for separating a large number and type of proteins, primarily by molecular weight. Comparison of the patterns of reduced and nonreduced proteins can show the effect of reducing disulfide bonds. There is a possibility of Maillard-type reactions in protein bars, e.g., reactions in which glucose units form bonds with protein amino groups. However, such reactions will not greatly affect the molecular weight of the proteins because sugars are much smaller than proteins.

In the nonreduced SDS-PAGE gel (**Figure 10**), there was some high-molecular-weight material that either remained near the top of the resolving gel or did not enter the stacking gel at all but remained in the sample well. These features were absent from the reduced gel (**Figure 11**), indicating that they were disulfide-bonded aggregates.

In the control lanes, the κ-casein and β-lactoglobulin bands were darker in the reduced gel (**Figure 11**) than in the nonreduced gel (**Figure 10**), suggesting that these proteins were partially disulfide-bonded in MPC. In the reduced SDS-PAGE gels, the β-lactoglobulin band appeared to be slightly less mobile in the protein bar samples than in the control (MPC powder in water). The control lane was taken from a different gel, and the differences in β-lactoglobulin band position may have simply reflected slight differences in gel preparation.

Overall, the effect of storage time at 20 °C on protein molecular weights was minor. In the reduced SDS-PAGE gel (**Figure 11**), there was a suggestion of increased blurring above the major casein band with increasing storage time, but no conclusive changes. In the nonreduced gel (**Figure 10**), the κ-casein band became fainter with increasing storage time, and the other casein bands became blurred upwards, especially after 26 and 48 days of storage. The blurring of the α_s- and β-casein bands and the disappearance of the κ-casein band suggest an increase in molecular weight, which may have been due to the addition of one or more glucose or lactose molecules to lysine residues. The α-lactalbumin band on the nonreduced gel became

progressively fainter and more diffuse with storage time, and α-lactalbumin may have also undergone Maillard reactions.

The progressive upward blurring of bands with increasing storage time at 20 °C was similar to the effect of increasing heating time/temperature on milk proteins heated with reducing sugars (Ajandouz, Desseaux, Tazi, & Puigserver, 2008; Morris, Sims, Robertson, & Furneaux, 2004). However, molecular weight changes in the proteins in the protein bars were relatively small.

The model protein bars made in this investigation hardened substantially during storage for 50 days at 20 °C. Hardening occurred more rapidly than in commercial bars, but the model contained the main components of commercial protein bars in realistic ratios, and similar physicochemical reactions are likely to have occurred.

Protein bar manufacture can include different mixing methods and a different order of ingredient addition from that used here. Different rates and times of mixing or adding ingredients in a different order may affect the spatial distribution and hydration state of proteins and PHCs. If so, the driving forces for physicochemical reactions would be different. However, information on the effect of manufacturing variables on the rate of physicochemical reactions in protein bars is unavailable in the literature, therefore the magnitude of differences cannot be assessed.

The microstructure of the protein bars underwent a phase-separation within the first day, concomitant with a transformation from a liquid batter to a soft but cohesive solid. The NMR results showed a strong interaction between water and PHCs, and pointed to ongoing change in their molecular mobilities throughout 50 days of storage. Particularly notable was the large decrease in mobility of solid-like domains of water and PHCs, which may indicate that glucose was crystallizing.

A large proportion of reactive lysine reacted within the first 12 days, but there was little change in reactive lysine content thereafter. SDS-PAGE showed relatively minor changes in the molecular weights of proteins over 50 days at 20 °C.

The two-phase nature of the available lysine reactions and the negligible changes to protein molecular weights in SDS-PAGE were not consistent with the continuous increases in the fracture stress, fracture area, and modulus of deformability values of the protein bars. It appeared that the hardening of the protein bars was not associated with chemical modification of the proteins. However, the physicochemical changes to low-mobility PHC/water domains were concomitant with hardening, and may have been causally involved in the hardening of the protein bars.

This study of a model food product showed that several chemical and physical reactions occurred serially and simultaneously. It exemplified the diversity and complexity of aging reactions in multicomponent food systems, and illustrated the range of length- and time-scales at which they occur.

ABBREVIATIONS USED

a_w, water activity; 2D NOESY, two-dimensional nuclear Overhauser effect spectroscopy; CPMG; Carr Purcell Meiboom Gill; DTT, dithiothreitol; MPC, milk protein concentrate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; OPA, orthophthaldialdehyde; PHC, polyhydroxy compound; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Table 1. Model Protein Bar Recipe.

ingredient	% w/w
glucose	40
MPC	20
glycerol	15
water	15
cocoa butter	10
total	100

Table 2. Relaxation Rate Constants of the Fast-relaxing ($T_{2,FAST}$) and Slow-relaxing ($T_{2,SLOW}$) PHC and Water Protons in Protein Bar Material after 5 and 48 Days of Storage at 20 °C. Also Shown is the Percentage Change in the Relaxation Rates from Day 5 to Day 48.

	$T_{2,FAST}$ (ms)		$T_{2,SLOW}$ (ms)	
day	water	PHCs	water	PHCs
5	3.8	3.3	10	15
48	2.4	0.8	8.5	13
change	37%	76%	15%	13%
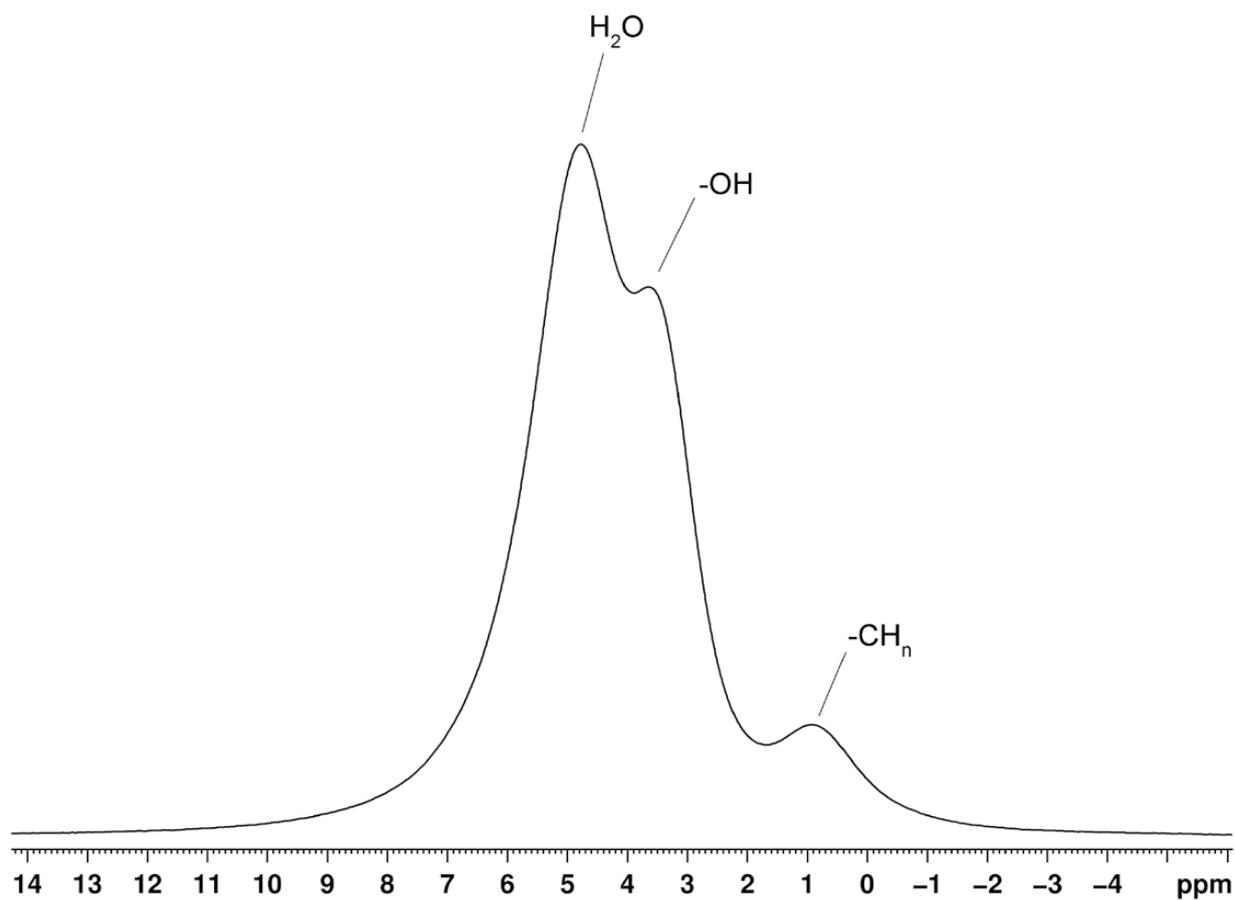
Figure 1. Typical ^{1}H NMR spectrum of protein bar material. The peaks are the proton signals from: H_2O (water), –OH (PHCs: glucose and glycerol), and –CH_n (lipids).
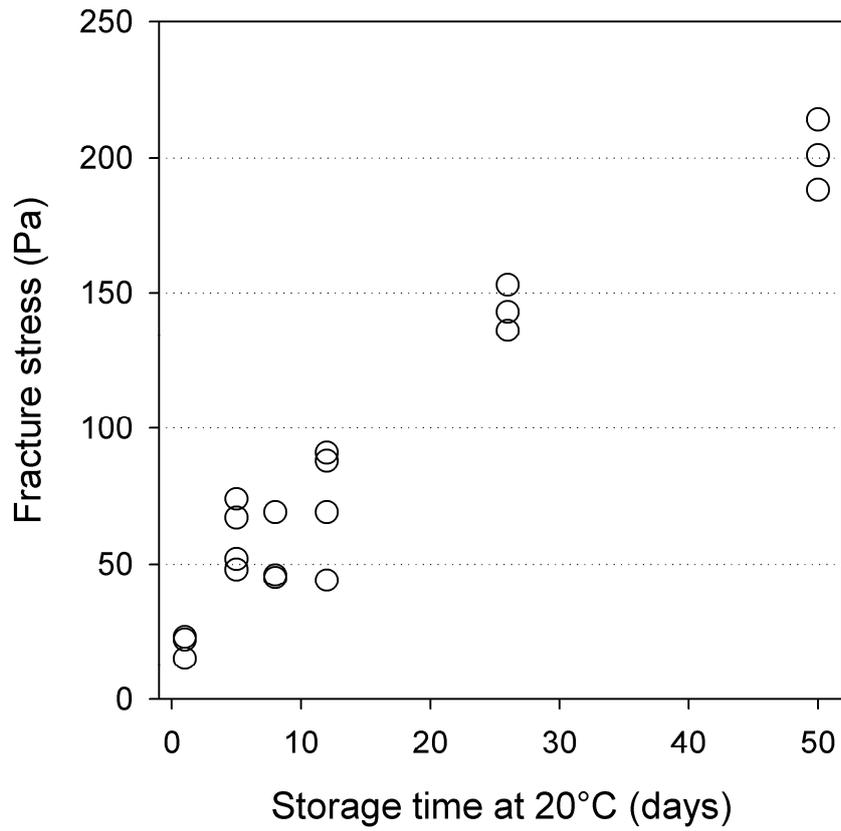
Figure 2. Fracture stress of protein bar samples in a lubricated uniaxial compression test. Data points are individual compression test results.
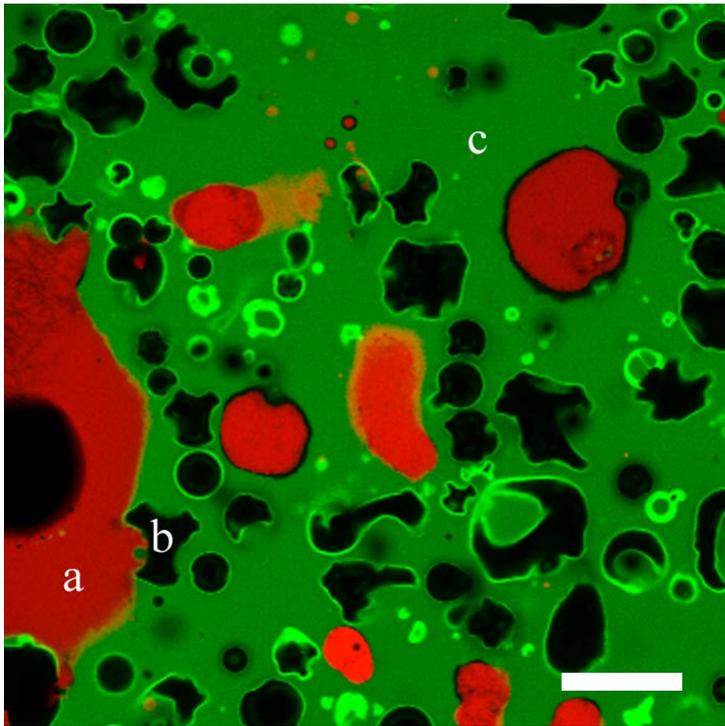
Figure 3. Confocal micrograph of protein bar material approximately 30 min after the completion of mixing. Red indicates Nile Blue staining (lipid) and green indicates Fast Green FCF staining (protein). Letters indicate: a, large lipid body; b, opaque protein particle with concave surface; c, continuous phase containing soluble protein. The scale bar is 45 μm.
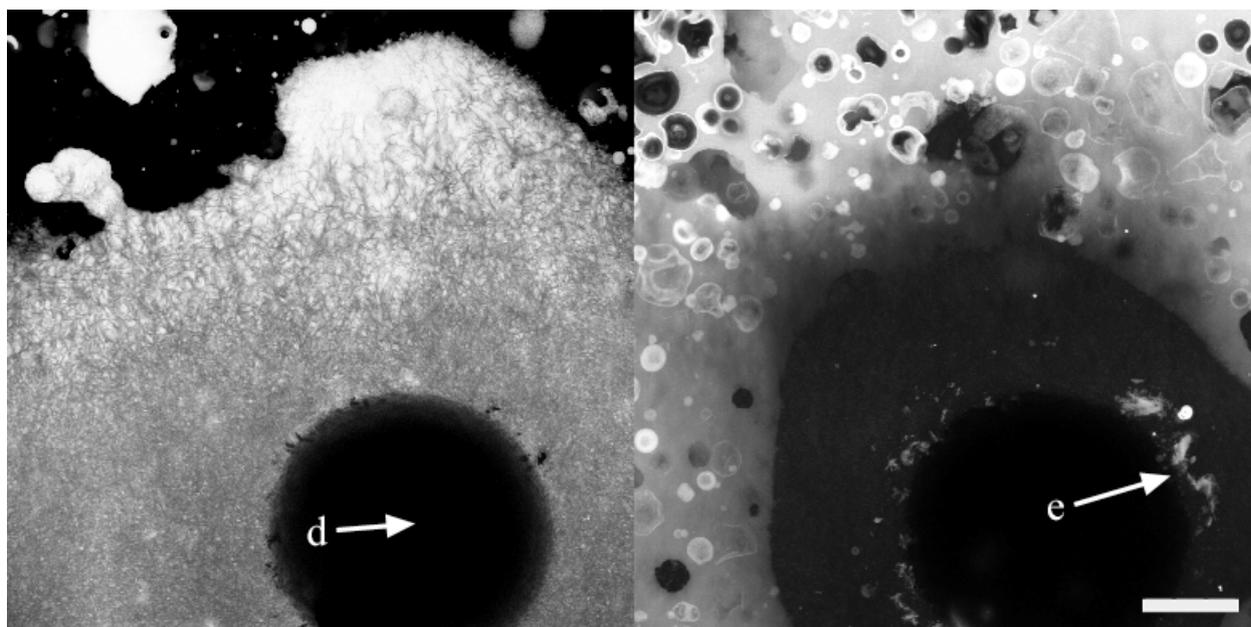
Figure 4. Confocal micrograph of protein bar material approximately 30 min after the completion of mixing: left, Nile Blue signal (lipid); right, Fast Green FCF signal (protein) from the same field of view. Letter 'd' indicates a spherical unstained area within a lipid body with protein (letter 'e') at the interface. The scale bars are 45 μm.
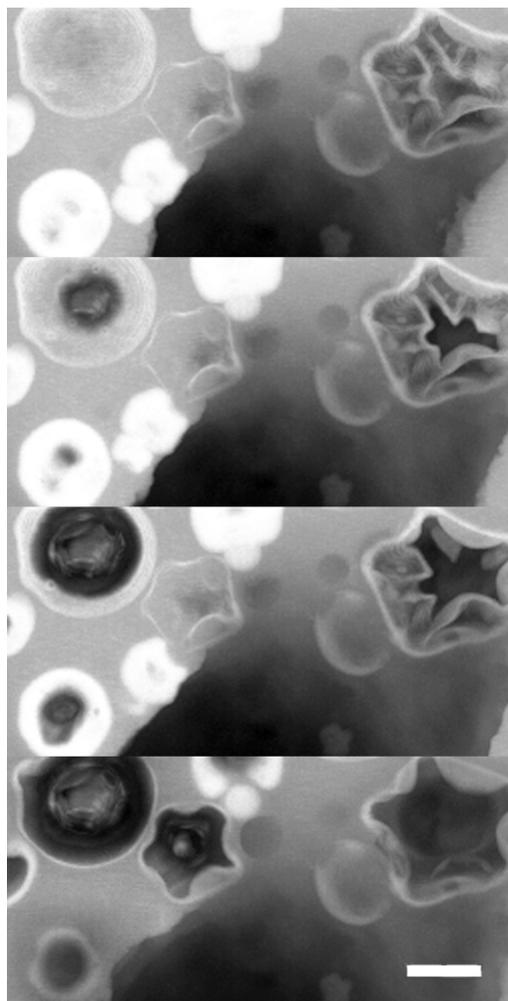
Figure 5. Confocal micrographs of protein particles in protein bar material approximately 30 min after the completion of mixing. The Fast Green FCF (protein) signal was converted to greyscale for clarity and the Nile Blue signal is not shown. A series of images were taken at different focal depths with spacing 0.82 μm and each of the four frames shows different subsets projected at maximum intensity. The scale bar is 10 μm.
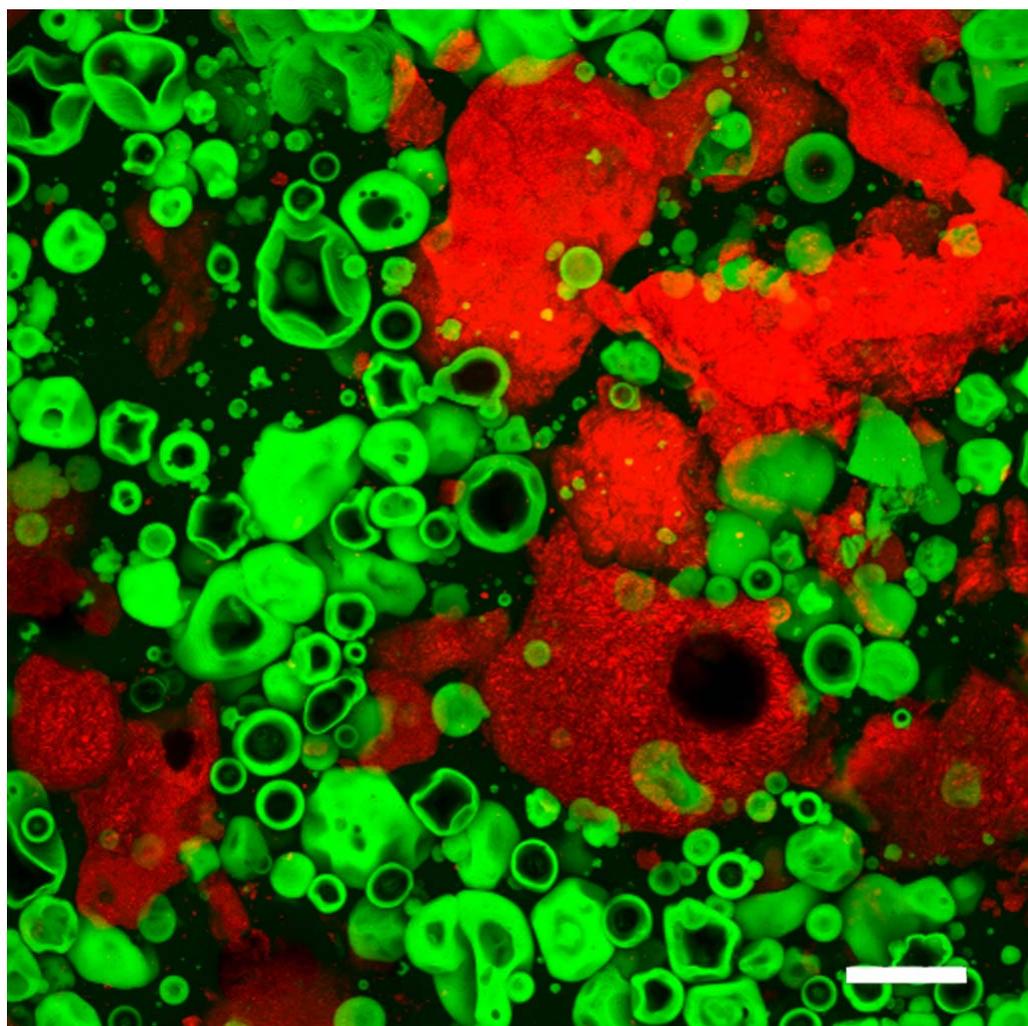
Figure 6. Confocal micrograph of protein bar material on the day after manufacture. Red indicates the signal from Nile Blue (lipid) and green indicates the signal from Fast Green FCF (protein). A series of images were taken at different focal depths with spacing 1.33 μm and were projected at maximum intensity. The scale bar is 45 μm.
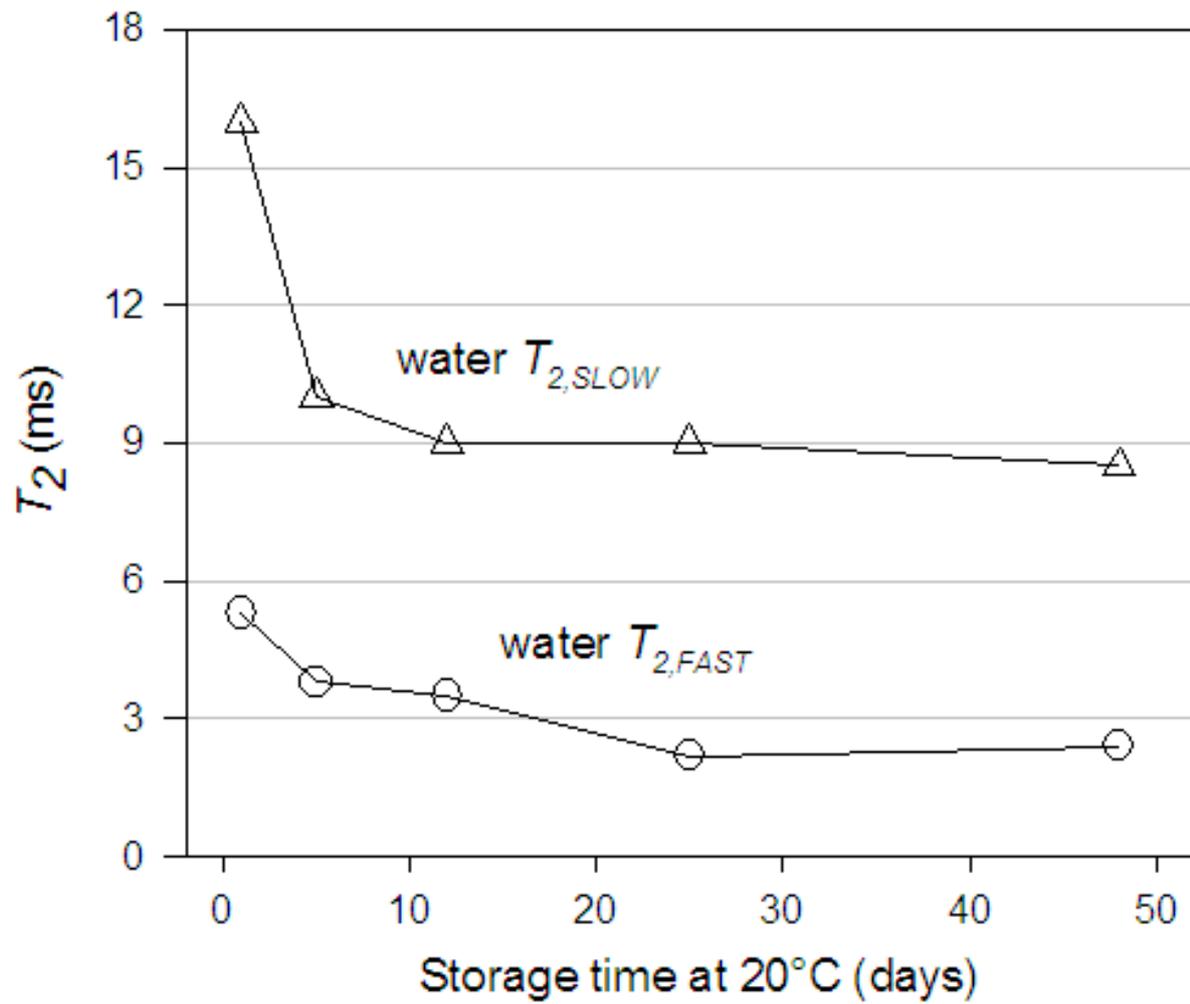
Figure 7. Relaxation rate constants of fast-relaxing ($T_{2,FAST}$) and slow-relaxing ($T_{2,SLOW}$) water protons in protein bar material during storage at 20 °C.
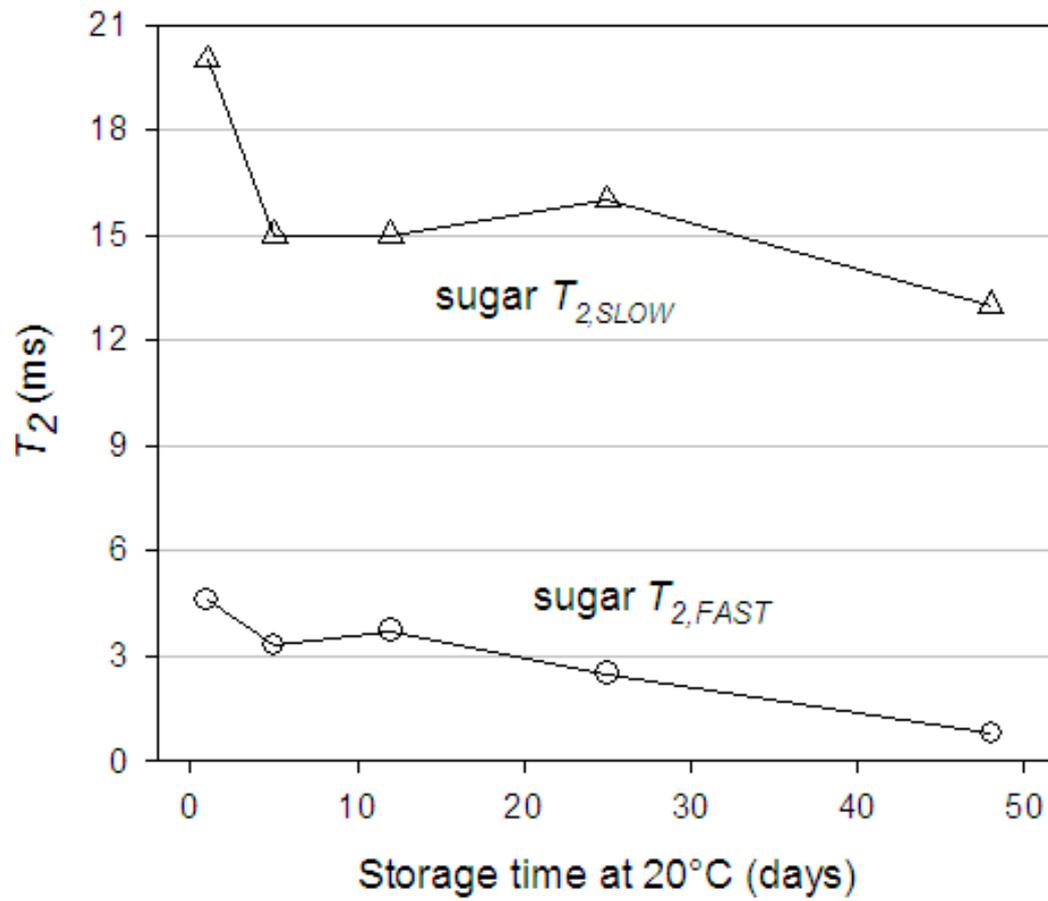
Figure 8. Relaxation rate constants of fast-relaxing ($T_{2,FAST}$) and slow-relaxing ($T_{2,SLOW}$) sugar (glucose + glycerol) protons in protein bar material during storage at 20 °C.
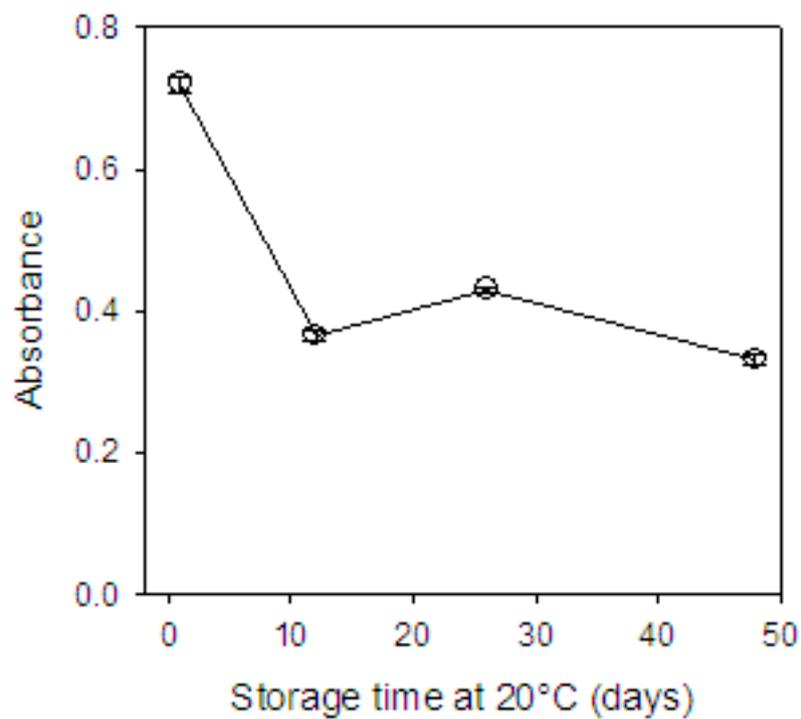
Figure 9. Reactive lysine content of protein bar material as absorbance in the orthophthalaldehyde (OPA) assay. The vertical bars are standard errors of triplicates.
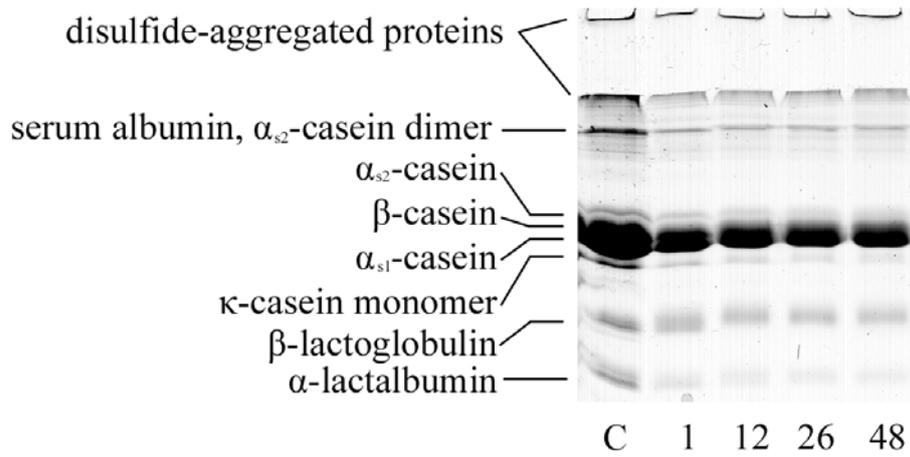
Figure 10. Nonreduced SDS-PAGE of MPC (control lane 1) and extracts from protein bars stored at 20 °C for 1, 12, 26, and 48 days.
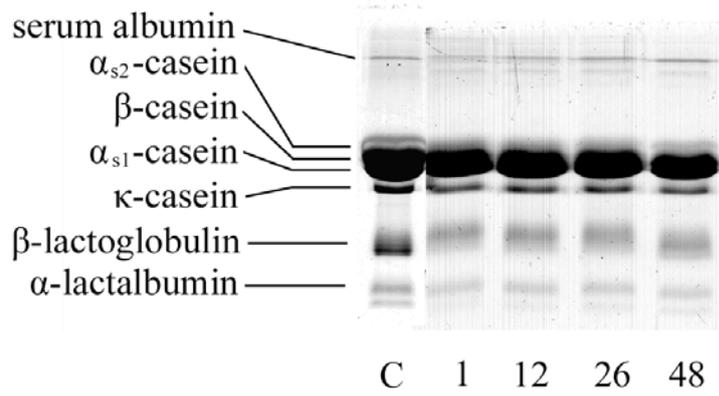
Figure 11. Reduced SDS-PAGE of MPC (control lane 1) and extracts from protein bars stored at 20 °C for 1, 12, 26, and 48 days.