Effect of bovine casein and its hydrolysates on hardening in protein dough model systems during storage

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19 Abstract

20 In recent years, high protein food bars (HPFB) are a rapidly growing market. One major problem
21 for commercial HPFB is that they become harder over time without moisture loss, making the
22 product unacceptable to consumers. In this study, the effect of bovine casein and its hydrolysates
23 on bar hardening during storage was studied. Five formulations of protein dough model systems
24 were prepared from five commercial spray-dried dairy protein powders. According to our results,
25 moisture migration should be the decisive factor of bar hardening in the moisture nonequilibrium
26 systems during storage. Furthermore, disulfide-induced protein/peptide aggregation could also
27 cause bar hardening. The substitution of a portion of whey protein isolates with either intact
28 casein or hydrolyzed casein proteins could effectively reduce bar hardening during storage. In
29 addition, the effect of the degree of hydrolysis on selected physicochemical properties (moisture
30 sorption and glass transition) and bar hardening was also investigated.

1. Introduction

In recent years, high protein food bars (HPFB) are a rapidly growing sector of the sports nutrition, muscle building, health supplement, and weight reduction markets (Mintel, 2012; Wright, 2011). The global market size of protein bars for sports nutrition is projected to grow at an annual average growth rate about 9.8% between 1997 and 2016 (Euromonitor International, 2012). The size of the HPFB market in the USA has grown by 71% between 2006 and 2011, with annual growth of 14.7% and 13.9% in 2010 and 2011, respectively. This market in the USA was estimated to grow to about \$3 billion in 2016 (Mintel, 2012).

HPFB are typically formulated to have an a_w of about 0.6 at room temperature to ensure microbial stability (Davis, 2005; Hazen, 2010). One major problem for commercial HPFB is that they generally become harder over time without moisture loss, making the product unacceptable to consumers (Ahmed, 2004; Berry, 2011; Hazen, 2010; Hutchinson, 2009; Wade, 2005). Several possible mechanisms for bar hardening have been proposed, which can be classified into two categories: physical and chemical reactions. As to physical reactions, they include 1) moisture migration (Hogan, Chaurin, O'Kennedy, & Kelly, 2012; Li, Szlachetka, Chen, Lin, & Ruan, 2008; Loveday, Hindmarsh, Creamer, & Singh, 2009, 2010), and 2) phase separation (Loveday, Hindmarsh, Creamer, & Singh, 2010; McMahon, Adams, & McManus, 2009). As to chemical reactions, they include 1) disulfide-induced protein aggregation (Zhou, Liu, & Labuza, 2008a; Zhu & Labuza, 2010), and 2) Maillard-induced protein polymerization (Rao, Fisher, Guo, & Labuza, 2013; Rao, Rocca-Smith, & Labuza, 2013). Actually, as discussed in the study of Hogan, Chaurin, O'Kennedy, and Kelly (2012), no single causative mechanism should be solely responsible for hardening in HPFB. It should occur due to many different chemical, physical, thermodynamic and process related factors.

In order to control bar hardening in HPFB, several solutions are being used by the bar industry or proposed by the researchers: 1) substituting protein hydrolysates for some intact proteins, which serves as a plasticizer to decrease the glass transition temperature (T_g) of the bar matrix (Rao, Rocca-Smith, & Labuza, 2013; Tran, 2009); 2) addition of cysteine, a food grade additive, into the bar matrix to prevent disulfide-induced aggregation (Zhu & Labuza, 2010); 3) using non-reducing sugar alcohols such as maltitol as a humectant to minimize Maillard-induced protein polymerization (Tran, 2009); and 4) substituting some protein ingredients with protein sources containing high fat content such as hen egg yolk (Rao, Fisher, Guo, & Labuza, 2013).

In this study, we further studied the effect of bovine casein and its hydrolysates on bar hardening during storage. Bovine caseins are those phosphoproteins that precipitate from raw skim milk by acidification to pH 4.6 at 20 °C (Jenness, Larson, Mcmeekin, Swanson, Whitnah, & Whitney, 1956). Casein accounts for 78.3% of the protein in bovine milk (Rao, Klaassen Kamdar, & Labuza, 2013). As expected, similar to other protein hydrolysates from whey protein and hen egg white, hydrolyzed casein can also be used to substitute some intact proteins to decrease the bar hardening during storage. In addition, casein protein molecules have two major inherent characteristics. One is that they have relatively little ordered secondary structure, which leads to having no characteristic denaturation temperature (Dickinson, 2006). The other is that bovine casein has no free sulfhydryl group and has only two disulfide groups (Owusu-Apenten, 2005). Therefore, compared with other proteins in whey, soy, and hen egg white containing many sulfhydryl-disulfide groups (Rao, Klaassen Kamdar, & Labuza, 2013), the effect of disulfide-induced protein aggregation in casein during storage should be smaller. Hence, in this study, we expect both intact casein and its hydrolysates can reduce bar hardening during storage.

2. Materials and methods

2.1. Materials

Five spray-dried dairy protein powders were kindly provided by two food companies. WPI (BiPRO) was obtained from Davisco Foods International, Inc. (Eden Prairie, MN, USA). The intact casein powder, Excellion EM 9 Calcium Caseinate (CN), and three hydrolyzed sodium caseinate powders, Hyvital Casein Easy-to-Digest 100 (HCN-1), Hyvital Casein Phosphopeptides (HCN-2), and Hyvital Casein HA 300 (HCN-3), were obtained from FrieslandCampina Domo (Paramus, NJ, USA). After receiving these products, several sample characteristics were analyzed. The initial moisture content was determined using the Aquatest cma Karl Fischer Coulometric Titrator (Photovolt Co., Minneapolis, MN, USA). The a_w at room temperature (25 °C) was determined using the AquaLab Vapor Sorption Analyzer (Decagon Devices, Inc., Pullman, WA, USA). Protein content was determined according to the Dumas combustion method (AOAC, 2007) using a nitrogen determinator, TruSpec N (LECO Corp., St. Joseph, MI, USA). The specific factor for the conversion of nitrogen content to dairy protein content is 6.38 (FAO, 2003). Several other characteristics (DH, contents of fat, lactose, free amino acids, calcium and sodium, average molecular weight, and pH) were given by the manufacturer. The major characteristics of these powders are summarized in Table 1. All protein powders were kept at −20 °C until use. Sodium azide was purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA).

2.2. Moisture sorption isotherm

To generate the moisture sorption isotherms of five powders at 25 °C, the method built in the AquaLab Vapor Sorption Analyzer (Decagon Devices), namely the dynamic vapor sorption (DVS) method for static isotherm, was used. The Guggenheim-Anderson-de Boer (GAB)

isotherm model was used to describe the dry basis (db) moisture content (m) as a function of a_w (Labuza & Altunakar, 2007).

2.3. Glass transition

The T_g as a function of solid component was analyzed using differential scanning calorimetry (DSC). Briefly, a quantitative amount (~7 mg) of each powder sample was transferred into an aluminum DSC sample pan (Mettler-Toledo Inc., Columbus, OH, USA). These samples were then equilibrated in eight sealed desiccators containing either Drierite desiccant or saturated salt slurries (LiCl, MgCl₂, K₂CO₃, Mg(NO₃)₂, CoCl₂, NaCl and KCl) at a_{ws} from 0.05 to 0.85 (Greenspan, 1977) at 23 °C for 28 days, respectively. Before and after hermetically sealing the sample pan, each sample was weighed. The DSC measurement was done using the DSC1 STAR^e System (Mettler-Toledo) which was calibrated using indium for its temperature and energy axis. Each sample was scanned twice at the same scan rate (5 °C/min). The range of scanning temperature was from 0 to 150 °C. After scanning, all samples were weighed to check whether mass was lost or not. If so, the test was repeated.

The data was analyzed using the thermal analysis software, STAR^e Software (version 11.00a, Mettler-Toledo). The T_g was reported as the inflection point of the slope [dT/dt] from the second scan, which is the point between the limits at which the slope of the curve changes from increasing to decreasing or vice versa. The Gordon-Taylor equation was used to model the changes in T_g with the increasing of moisture content (Rao & Labuza, 2012).

2.4. Preparation of protein dough model systems

To study the effect of bovine casein and its hydrolysates on dough hardening, five formulations (WPI, WPI/CN, WPI/HCN-1, WPI/HCN-2, and WPI/HCN-3) of protein dough model systems were prepared. Briefly, WPI was mixed well with either CN or different HCNs listed in Table 1

in a ratio of 9:1 (g/g) using a powder mixer (IKA Works, Inc., Wilmington, USA), respectively. Each protein powder blend was mixed with distilled and deionized water containing 0.05% sodium azide (g/g) to control microbial growth in a ratio of 3:2 (g/g) at room temperature. After mixed until a uniform dough texture was achieved, the dough was then sealed in a plastic bag (Thermo Fisher Scientific Inc., Rockford, IL, USA) and kept at 4 °C for 2 days for moisture equilibration. Before packaging, the dough was kept at room temperature for 2 h. The resultant dough (about 10 g) was weighed and pressed into a plastic disposable sample cup (Decagon Devices), and then immediately covered with the lid (Decagon Devices). All finished samples were placed in moisture barrier pouches (IMPAK Corporation, Los Angeles, CA, USA), then heat-sealed for storage. The water vapor transmission rate of the pouch is 0.009 g/m²/24 h. The packaged samples were stored at 45 °C for up to 35 days. The samples were removed at designated time intervals and cooled at room temperature for at least 2 h before being analyzed immediately or frozen at −45 °C for later analysis.

2.5. Physicochemical changes of protein dough model systems

During storage, three physicochemical properties of the dough systems were analyzed at designated time intervals. First, the a_w of the samples was determined using the AquaLab Vapor Sorption Analyzer (Decagon Devices). The initial a_w at room temperature of five dough model systems was about 0.99. Second, the color of the samples was analyzed using the Minolta Chroma Meter CR-200 (Minolta Camera Co., Osaka, Japan). All relevant methods are described in detail in our previous studies (Rao & Labuza, 2012). Third, the degree of hardness of the samples was analyzed using the TA.XT*Plus* Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA) fitted with a flat-ended cylinder stainless steel probe (3 mm diameter).

2.6. Analysis of buffer-soluble proteins in different protein dough model systems

145 The buffer-soluble proteins in different protein dough model systems stored at 45 °C for 0, 14
146 and 35 days were analyzed according to the method described in our previous study (Rao,
147 Rocca-Smith, & Labuza, 2012) with modifications. Briefly, the protein solubility in PBS-SDS
148 (phosphate buffered saline [PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, and 137
149 mM sodium chloride, pH 7.4 (Fisher Scientific, Fair Lawn, NJ)] containing 1% sodium dodecyl
150 sulfate [SDS, g/ml]) was determined by adding a certain amount of dough sample (5 mg, db) into
151 1 ml PBS-SDS. The mixture was mixed thoroughly using a vortex mixer (Henry Troemner LLC,
152 Thorofare, NJ, USA) for 30 s at 3000 rpm. The suspension was then mixed on a rotator (Fisher
153 Scientific) at 50 rpm overnight. After centrifuging at 15000g for 15 min, the supernatant was
154 collected. The concentration of soluble proteins in the supernatant was determined using the
155 Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific). Bovine serum
156 albumin (BSA) was used as the protein standard (working range: 20–2000 µg/ml). The protein
157 solubility as a function of storage time was calculated.

158 Simultaneously, the precipitate was washed three times with 1 ml PBS-SDS. For each wash,
159 after the mixture was mixed thoroughly on a rotator (Fisher Scientific) for 5 min at 50 rpm, the
160 precipitate of each sample was collected using an Amicon Ultra-0.5 Centrifugal Filter device
161 with 100 kDa NMWL (Merck Millipore Ltd., Billerica, MA, USA) according to the
162 manufacturer's instruction. After three washes, the precipitate was then mixed with 250 µl PBS-
163 SDS containing 5% 2-mercaptoethanol (PBS-SDS-2-ME, ml/ml). The mixture was then shaken
164 on a shaker (Thermo Fisher Scientific) at 200 rpm until no solid was visible by the naked eye.
165 After centrifuging at 14000g for 15 min, the supernatant was collected. All steps were carried out
166 at room temperature unless otherwise specified.

167 For two models, WPI and WPI/CN, the buffer-soluble proteins from both the supernatant and the
168 precipitate were further analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
169 according to the method of Laemmli (1970). Before heating at 60°C for 1 h, one part of sample
170 was mixed with one part of Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA,
171 USA) with or without 5% 2-ME (ml/ml). The heated sample (10 µl/lane) was then added into the
172 4-20% Mini-PROTEAN TGX Precast Gel (Bio-Rad). The SDS-PAGE Molecular Weight
173 Standards (broad range, Bio-Rad) were used for accurate molecular weight estimation on the gel.
174 The running buffer was 25 mM Tris (pH 8.3) containing 192 mM glycine and 0.1% SDS (g/ml).
175 After running at 200 V for a certain time at 4 °C, the gel was stained with the EZBlue Gel
176 Staining Reagent (Sigma-Aldrich).

177 For three HCN models (WPI/HCN-1, WPI/HCN-2, and WPI/HCN-3), the buffer-soluble
178 proteins/peptides from both the supernatant and the precipitate were further analyzed using
179 tricine-SDS-PAGE according to the method of Schagger (2006). Before heating at 60 °C for 1 h,
180 one part of sample was mixed with one part of tricine sample buffer (Bio-Rad) with or without 2%
181 2-ME (ml/ml). The heated sample (10 µl/lane) was then added into the 10-20% Mini-PROTEAN
182 Tris-Tricine Precast Gel (Bio-Rad). The Spectra Multicolor Low Range Protein Ladder (Thermo
183 Fisher Scientific) was used for accurate molecular weight estimation on the gel. The running
184 buffer was 100 mM Tris (pH 8.3) containing 100 mM tricine and 0.1% SDS (g/ml). After
185 running at 100 V for a certain time at 4 °C, the gel was fixed using a fixative solution containing
186 40% methanol and 10% acetic acid (ml/ml) for 30 min. Before staining, the gel was washed with
187 an excess of water to remove the fixing solution for 15 min, and then stained with the EZBlue
188 Gel Staining Reagent (Sigma-Aldrich).

After destained with deionized water, the gels of five dough model systems were scanned by the Gel Doc XR system (Bio-Rad) and analyzed with the Image Lab software (version 5.1, Bio-Rad). The trace quantity of a band was measured by the area under its intensity profile curve. The relative quantity (RQ) of defined bands in lanes was calculated using the equation: $RQ = \text{trace quantity at day 14 or day 35} / \text{trace quantity at day 0}$.

2.7. Statistical analysis

Each sample condition was tested at least in duplicate at each time. To compare the change in the buffer-soluble proteins during storage, two-way ANOVA with Tukey's multiple comparisons test was performed. $P < 0.05$ was considered to be statistically significant. The software, GraphPad Prism for Windows (version 6.05, GraphPad Software, La Jolla, CA, USA), was used to analyze the data.

3. Results and discussion

3.1. Moisture sorption isotherm and glass transition

Moisture sorption isotherms of five commercial powders at 25 °C were generated using the GAB model (Figure 1). All of them showed a type II isotherm (Van den Berg & Bruin, 1981). The GAB monolayer moisture values (m_0) of different HCNs were similar to that of CN at 25 °C (~5.3 g H₂O/100 g solid, Figure 1), indicating that HCNs expose few, if any, new adsorption sites (Rao, Klaassen Kamdar, & Labuza, 2013). Their m_0 is generally around an a_w of 0.2–0.3 at 25°C (Figure 1). As expected, when the degree of hydrolysis (DH) increases, the average molecular weight decreases while the percentage of free amino acids increases (Table 1). DH is defined as the proportion of cleaved peptide bonds in a protein hydrolysate (Rutherford, 2010). It must be noted that when the a_w is higher than 0.7, the higher the DH, the greater is the moisture holding capacity in the HCN powder (Figure 1). The similar situation has been observed in our

previous study using other commercial HCNs (Netto, Desobry, & Labuza, 1998) and whey protein hydrolysates (WPH) (Zhou, Liu, Chen, Chen, & Labuza, 2014). It seems that at the higher DH, more hydrophilic groups in the hydrolyzed protein are generated during enzymatic hydrolysis. This improved hygroscopicity could further increase the plasticizing effect at higher DH thereby lowering the T_g (Figure 2). The T_g in an amorphous system is a temperature-, time-, and composition-dependent, material-specific change in physical state, from a "glassy" state to a "rubbery" state (Slade & Levine, 1988). At the same weight fraction of the solid component, when the DH increases, the average molecular weight decreases, the T_g decreases (Figure 2). The effect of molecular weight on T_g can be predicted using the Fox and Flory equation (Fox & Flory, 1950). A similar result has also been observed in other powders such as WPH (Zhou, Liu, Chen, Chen, & Labuza, 2014) and poly(vinylpyrrolidone) (Buera, Levi, & Karel, 1992).

3.2. Physicochemical changes of protein dough model systems

During storage, two physicochemical properties, a_w and color, were not observed any significant changes in any model systems ($P > 0.05$, data not shown). It must be noted that the moisture content of each model was at least 40% (g/g), resulting that the a_w of the system analyzed at any designated time intervals was very close to one at 25 °C (about 0.99). It actually was out of the effective measurement range (0.03 to 0.95) of the instrument.

On the other hand, the hardness increased as a function of time in all models (Figure 3). It must also be noted that the extent of the increase in the hardness was different between these models. At the last day of storage (day 35), the hardness of the models containing either CN or HCN was lower than 12 N (Figure 3). It took about 28 days when the hardness of the WPI model was beyond 12 N (Figure 3). According to our previous study (Tran, 2009), at a hardness value of 12 N, consumers would indicate the hardened bar was unacceptable. As mentioned previously, it is

well known that the substitution of a portion of the intact protein with protein hydrolysates in HPFB, which serve as plasticizers by effectively reducing bar hardening due to the decrease in the T_g (Rao, Klaassen Kamdar, & Labuza, 2013). For example, the hardness of the WPI/HCN-3 model was about four times smaller than that of the WPI model at day 35 (Figure 3).

In addition, when substituting 10% (g/g) of WPI with CN, i.e. calcium caseinate, the hardness of the WPI/CN model decreased about 54% compared with that of the WPI model at the last day of storage (Figure 3). It has been reported that calcium caseinate had a synergistic softening effect on bars when combined with soy proteins (Li, Szlachetka, Chen, Lin, & Ruan, 2008). Therefore, the substitution of a portion of protein with intact casein could be another effective approach to reduce bar hardening during storage. It is worth mentioning that unlike protein hydrolysates which have a bitter flavor, intact casein does not have this undesirable taste when using as an ingredient. However, for the HPFB containing either calcium caseinate or sodium caseinate, one must be pay attention to the effect of glycerol on the hardening during storage. Glycerol has been being used as a popular plasticizer to soften the texture in the bar industry. However, it has been found that it could extensively increase the hardness during storage in the bar model containing either calcium caseinate (Loveday, Hindmarsh, Creamer, & Singh, 2010) or sodium caseinate (Hogan, Chaurin, O'Kennedy, & Kelly, 2012). Interestingly, this negative effect was not significant in the model containing micellar casein (Hogan, Chaurin, O'Kennedy, & Kelly, 2012). The relevant mechanism is worthy of further study in the future.

3.3. Analysis of buffer-soluble proteins

In order to study the effect of protein-protein interactions through disulfide bond formation/exchange, buffer-soluble proteins in five dough models were extracted with PBS-SDS. From SDS-PAGE, three major whey proteins, bovine serum albumin (BSA), β -lactoglobulin

(BLG) and α -lactalbumin (ALA), were clearly observed in all reduced supernatant samples at day 0 (Figure 4, lane 4). As expected, the relative protein solubility (RPS, %) in PBS-SDS decreased as a function of storage time (Figure 4, lines 1-3), which reduced about 26% in the WPI/CN model and about 62% in the WPI/HCN-2 model compared with that at day 0 after 35 days of storage, respectively. Similar results were also observed in a WPI/buffer model system (Zhou, Liu, & Labuza, 2008d; Zhu & Labuza, 2010). This RPS reduction is mainly due to the disulfide-induced protein aggregation.

Because of disulfide interaction, the solubility of the two major whey proteins (BLG and ALA) in PBS-SDS changed as a function of storage time, i.e. decreased in the supernatant while increased in the precipitate (Table 2 and Figure 4). However, the effect of CN or HCN on the solubility of ALA was much greater than that of BLG during storage. Compared with the WPI model, the addition of CN in the WPI/CN model increased 10% of the ALA solubility in PBS-SDS after 35 days of storage (Table 2 and Figure 4A-B, line 3). Between three HCN models, the relative amount of PBS-SDS buffer-soluble ALA decreased about 50%, 90%, and 60% in the WPI/HCN-1, WPI/HCN-2, and WPI/HCN-3 models, respectively, compared with that at day 0 after 35 days of storage (Table 2 and Figure 4C-E, line 3). One of the major reasons for this change should lie in the different number of disulfide groups in both proteins. Compared with BLG which has two disulfide groups, ALA has four (Owusu-Apenten, 2005), indicating that ALA should have greater chance to be involved in disulfide interaction during storage.

As expected, the supernatant, i.e. PBS-SDS buffer-soluble proteins, contained a portion of protein aggregates (> 200 kDa). They were on the top of the stacking gel before treated with 2-ME (Figure 4, lanes 1-3), which were not visible i.e. they were dissolved after treated with 2-ME (Figure 4, lanes 4-6). Additionally, compared with the supernatant in the absence of 2-ME, the

relative quantity of the major protein monomers (BSA, BLG and ALA) increased, respectively, in the supernatant in the presence of 2-ME (data not shown). These results indicate that the PBS-SDS buffer-soluble aggregates could be not only intermolecular disulfide-linked but also a mixture of both homopolymers which consisted of identical protein monomer units and heteropolymers which were derived from two or more different protein/peptide monomer units. Interestingly, the trends of the relative amount of the PBS-SDS buffer-soluble aggregates in five models were different during storage (Table 2 and Figure 4, lines 1-3). Compared with the WPI model, the addition of CN in the WPI/CN model decreased the formation of the PBS-SDS buffer-soluble aggregates after 35 days of storage (Table 2 and Figure 4A-B, line 3). Between three HCN models, after 35 days of storage, the relative amount of the PBS-SDS buffer-soluble aggregates increased about 160% in the WPI/HCN-1 model, while it decreased about 80% in both the WPI/HCN-2 and the WPI/HCN-3 models as compared with that at day 0 (Table 2 and Figure 4C-E, line 3). However, the relative amount of both BLG and ALA in the precipitates, i.e. PBS-SDS buffer-insoluble aggregates after 35 days of storage, increased at least 9 fold more in the three HCN models as compared with those at day 0, respectively (Table 2 and Figure 4C-E, line 9). Moreover, it seems like that at the higher DH, it was easier to form the precipitates during storage. This indicates that depending on the enzymes used during protein hydrolysis and/or the DH, different peptides in different HCNs could interact with different whey proteins through disulfide bond formation/exchange, especially with ALA, and formed the precipitates (Figure 4C-E, lines 7-9). The precipitate from each model could easily dissolve in PBS-SDS-2-ME (Figure 4, lanes 7-9). The major components in the precipitate were three protein monomers, i.e. BSA, BLG and ALA. These results showed that the proteins in the precipitate also contained intermolecular disulfide bonds. In addition, because a trace amount of lactose (reducing sugar)

was in these dairy powders (Table 1), the Maillard-induced protein aggregation could also occur during storage, which needs to further experimentally confirm.

It has been reported that mainly due to disulfide-induced protein aggregation, the protein aggregates were responsible for the bar hardening in a WPI/buffer model (Zhou, Liu, & Labuza, 2008a; Zhu & Labuza, 2010). In this study, there is a controversy between bar hardening and disulfide-induced protein aggregation when substituting a portion of WPI with HCN. Our results clearly showed that the addition of HCN could effectively reduce the dough hardness (Figure 3). Simultaneously, it could form various disulfide-induced protein aggregates (buffer-soluble or buffer-insoluble) during storage (Figure 4). Not only that, the higher the DH, the softer the dough (Figure 3) and the more disulfide-linked PBS-SDS buffer-insoluble protein aggregates were present after 35 days of storage (Table 2 and Figure 4C-E, lines 7-9). Therefore, the relationship between disulfide-induced protein/peptide aggregation and bar hardening needs to be further elucidated when protein hydrolysates serve as a plasticizer in HPFB. According to the upward trends of the hardness shown in Figure 3 and other relevant studies (Zhou, Liu, & Labuza, 2008a; Zhu & Labuza, 2010), the addition of protein hydrolysates can effectively reduce the rate of hardening during storage, but this approach can not completely stop bar hardening. One of the reasons could be due to the effect of disulfide-induced protein/peptide aggregation on bar hardening during storage.

It has been reported that when the same dairy protein powder was binarily mixed with different plasticizers such as water, high fructose corn syrup, glycerol, or sunflower oil, respectively, in different bar models, the hardness values were totally different under the same storage condition (Hogan, Chaurin, O'Kennedy, & Kelly, 2012). Their findings indicates that bar hardness is dependent on many factors such as protein type, protein content, and the property of the

plasticizer. In addition, it is been found that pre-equilibration of WPI powder at a_w 0.77 prior to bar manufacturing could effectively reduce the rate of hardening during storage, that is, the bar hardness decreased about 75% after 14 days of storage at 37 °C (Hogan, Chaurin, O'Kennedy, & Kelly, 2012). These results provides a strong evidence that bar hardening in HPFB is mainly caused by nonequilibrium changes due to the hydration behavior of individual ingredients and the competition for available moisture. The mechanism related to moisture migration and control in multi-domain foods has been comprehensively elaborated in our previous study (Labuza & Hyman, 1998).

From Figure 1, when the a_w is higher than 0.7, the hygroscopicity of three HCNs is better than that of WPI. Moreover, the higher the DH, the greater is the moisture holding capacity of HCN. Although WPI used in this study did not pre-equilibrate at high a_w , the substitution of CN with HCN could effectively accelerate the rate of moisture migration in these nonequilibrium systems and then decrease the T_g of the bar matrix, resulting in the reduction of bar hardening during storage (Figure 3). On the other hand, the substitution of CN with HCN could also extensively increase the rate of disulfide-induced protein/peptide aggregation (Table 2 and Figure 4). However, in these nonequilibrium systems, the rate of moisture migration should prevail over the rate of disulfide-induced protein/peptide aggregation. Therefore, minimization of the a_w difference between different ingredients in HPFB could provide a useful means of reducing bar hardening during storage, although doing so does not normalize the effects of protein type.

4. Conclusions

Overall, it was found that a wide range of interactive physical and chemical factors can contribute to the hardening in HPFB during storage. In those moisture nonequilibrium systems, the physical reaction, moisture migration from domains of high to low a_w , should be the decisive

factor of bar hardening. Besides that, other chemical reactions such as disulfide-induced protein/peptide aggregation can also cause bar hardening. To effectively reduce bar hardening during storage, besides substitution of some of the intact protein with protein hydrolysates, two other approaches, substitution of a portion of protein with intact casein and minimization of the a_w difference between different ingredients before making HPFB, should be useful. However, the bar manufacturer should avoid adding glycerol when calcium caseinate or sodium caseinate is used in their products. In summary, based on the better understanding of the interactions between different ingredients at the molecular level, followed by the optimization of the formulation, the manufacture should be able to minimize the deteriorative textural change in HPFB and ensure that the final product has a good quality acceptance over its shelf-life.

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467 **Table 1.** Characteristics of five commercial spray-dried dairy protein powders.

Sample*	Commercial product name	a_w	Moisture content (% dry basis)	Degree of hydrolysis (%)	Protein (% dry basis)	Fat (%)	Lactose (%)	Average molecular weight (Dalton)	Free amino acids (%)	Calcium (mg/ 100 g)	Sodium (mg/ 100 g)	pH (6% solution)
WPI	BiPRO Whey Protein Isolate	0.22	4.6		96.9	0.3	<1.0					
CN	Excellion EM 9 Calcium Caseinate	0.43	6.2		97.2	0.8	0.25			1,200	22	
HCN-1	Hyvital Casein Easy-to-Digest 100	0.38	5.9	7	99.1			11,912	2.0	53	1,073	7.2
HCN-2	Hyvital Casein Phosphopeptides	0.33	4.7	14	96.1	0.1		655	2.1	55	3,560	7.3
HCN-3	Hyvital Casein HA 300	0.40	4.6	21	96.9	0.1	<0.5	450	11.0	210	1,050	6.9

468 * WPI: whey protein isolate; CN: calcium caseinate; HCN: hydrolyzed casein

469 **Table 2.** Change in the relative quantity (RQ)^{*} of the two major whey proteins and the aggregates in the buffer-soluble proteins
470 extracted from different protein dough model systems stored at 45 °C.

Models	Storage time (days)	Supernatant (RQ±SEM [*])			Precipitate (RQ±SEM)	
		BLG [*]	ALA [*]	Aggregates	BLG	ALA
WPI	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	14	0.95±0.01	0.94±0.02	2.29±0.06	9.88±0.17	7.11±0.02
	35	0.80±0.02	0.65±0.01	2.96±0.29	11.88±0.13	8.70±0.02
WPI/CN	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	14	0.92±0.02	0.90±0.01	1.24±0.02	3.39±0.11	6.74±0.06
	35	0.82±0.03	0.75±0.01	1.87±0.04	3.78±0.18	6.66±0.16
WPI/HCN-1	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	14	1.01±0.01	0.96±0.02	2.43±1.43	8.63±0.48	10.94±0.33
	35	0.91±0.04	0.53±0.08	2.63±0.15	9.72±0.51	12.82±0.34
WPI/HCN-2	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	14	0.83±0.01	0.22±0.02	0.33±0.12	11.11±0.39	15.69±0.46
	35	0.70±0.02	0.11±0.01	0.22±0.03	11.49±0.31	16.35±0.46
WPI/HCN-3	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	14	0.94±0.00	0.49±0.01	0.31±0.05	11.74±0.36	15.18±0.32
	35	0.90±0.01	0.42±0.02	0.25±0.08	12.10±0.56	15.34±0.40

471 ^{*} The RQ of defined bands in lanes (Figure 4) was calculated using the equation: RQ = trace quantity at day 14 or day 35/trace
472 quantity at day 0. SEM: standard error of the mean; BLG: β-lactoglobulin; ALA: α-lactalbumin.

Figure 1. Moisture sorption isotherms of four commercial spray-dried casein protein powders. The calculated GAB model parameters of the powders are shown in the inserted table. m_0 is the monolayer moisture value, k is a multilayer factor, C is the surface heat constant, MAPE is the mean absolute percentage error, and r^2 is the goodness of fit.

Figure 2. The glass transition diagrams of four commercial spray-dried casein protein powders. The calculated Gordon–Taylor equation parameters are shown in the inserted table. T_{g1} is the T_g of the solid component in its dry form, K is a constant, and r^2 is the goodness of fit. The horizontal dotted line indicates 0°C.

482 **Figure 3.** Effect of degree of hydrolysis on the hardness of different protein dough model systems during storage at 45 °C. The
 483 horizontal dotted line indicates 12 N.
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Figure 4. SDS-PAGE of the buffer-soluble proteins in different protein dough model systems stored at 45 °C in the absence (nonreducing) and the presence (reducing) of 2-mercaptoethanol. (A): WPI; (B): WPI/CN; (C): WPI/HCN-1; (D): WPI/HCN-2; and (E): WPI/HCN-3. The relative protein solubility (RPS, %) of the buffer-soluble proteins compared with that of the relevant models system at day 0 is labeled. ^{a, b, c} Values with different letters are significantly different ($P < 0.05$). Agg: buffer-soluble protein

490 aggregates (> 200 kDa); BSA: bovine serum albumin; CN: α_{S1} -, α_{S2} -, and/or β -casein; BLG: β -lactoglobulin; ALA: α -lactalbumin;
491 peptides: buffer-soluble aggregates (< 10 kDa); D0: at day 0; D14: at day 14; D35: at day 35.