8-2016

Textural performance of crosslinked or reduced-calcium milk protein ingredients in model high-protein nutrition bars.

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Abstract
Transglutaminase (Tgase) crosslinking and calcium reduction were investigated as ways to improve the texture and storage stability of high-protein nutrition (HPN) bars formulated with milk protein concentrate (MPC) and micellar casein concentrate (MCC). The MPC and MCC crosslinked at none, low, and high levels, and a reduced-calcium MPC (RCMPC) were each formulated into model HPN bars. Hardness, crumbliness, moisture content, pH, color, and water activity of the HPN bars were measured during accelerated storage. The HPN bars prepared with MPC were harder and more cohesive than those prepared with MCC. Higher levels of Tgase crosslinking improved HPN bar cohesiveness and decreased hardening during storage. The RCMPC produced softer, yet crumblier HPN bars. Small textural differences were observed for the HPN bars formulated with the transglutaminase crosslinked proteins or RCMPC when compared with their respective controls. However, modification only slightly improved protein ingredient ability to slow hardening while balancing cohesion and likely requires further improvement for increased applicability in soft-texture HPN bars.

Keywords
micellar casein concentrate, milk protein concentrate, transglutaminase, protein bar

Comments
This accepted article is published as Banach, J.C., Clark, S., and Lamsal, B.P.* 2016. Textural performance of crosslinked or reduced-calcium milk protein ingredients in model high-protein nutrition bars, *Journal of Dairy Science*, 99(8);6061–6070. Doi: 10.3168/jds.2016-10995. Posted with permission.

Interpretive summary: Textural performance of crosslinked or reduced-calcium milk protein ingredients in model high-protein nutrition bars. By Banach et al., . High-protein nutrition (HPN) bars are susceptible to increased hardness and decreased cohesiveness during storage which make the product unpalatable and decrease its shelf life. Milk protein concentrates (MPC) accelerate HPN bar texture change and less is known about the performance of micellar casein concentrate (MCC) in these applications. We evaluated the textural stability of HPN bars formulated with transglutaminase crosslinked MPC and MCC, and reduced-calcium MPC (RCMPC). HPN bars made with MCC were also crumbly. Crosslinking produced slightly softer, yet brittle HPN bars. RCMPC produced soft, crumbly, and powdery HPN bars.

Textural performance of crosslinked or reduced-calcium milk protein ingredients in model high-protein nutrition bars

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Section: Dairy Foods; Subsection: Chemistry and Materials Science

ABSTRACT

Transglutaminase (Tgase) crosslinking, and calcium-reduction were investigated as ways to improve the texture and storage stability of high-protein nutrition (HPN) bars formulated with milk protein concentrate (MPC) and micellar casein concentrate (MCC). MPC and MCC crosslinked at 'none,' 'low,' and 'high' levels, and a reduced-calcium MPC (RCMPC) were each formulated into model HPN bars. HPN bar hardness, crumbliness, moisture content, pH, color, and water activity were measured during accelerated storage. HPN bars prepared with MPC were harder and more cohesive than those prepared with MCC. Higher levels of Tgase crosslinking decreased HPN bar hardening and led to improved cohesiveness during storage. RCMPC produced softer, yet crumblier HPN bars. Small textural differences were observed for the HPN bars formulated with the transglutaminase crosslinked proteins or RCMPC when compared with their respective controls. However, modification only slightly improved protein ingredient ability to slow hardening while balancing cohesion and likely require further improvement for increased applicability in soft-texture HPN bars.

Keywords: micellar casein concentrate, milk protein concentrate, transglutaminase, protein bar

INTRODUCTION

High-protein foods are popular amongst consumers seeking satiety, increased muscle mass, or decreased risk of sarcopenia (Sloan, 2012). Consumers are turning to high-protein nutrition (HPN) bars to conveniently add more protein to their diet. HPN bars have utilized new, trendy protein sources (e.g., insect), but have traditionally relied on dairy and soy ingredients such as concentrates, isolates, and hydrolysates. Protein content typically ranges from 20-50% (w/w) whereas carbohydrates (e.g., high-fructose corn syrup), polyols (e.g., glycerol), sugar alcohols (e.g., sorbitol), and lipids (e.g., palm oil) comprise the rest of the formulation (McMahon et al., 2009, Imtiaz et al., 2012).

It is well known that HPN bars, especially those prepared with high-protein milk protein concentrates (MPCs; ≥ 80% protein w/w), are texturally unstable during storage (Loveday et al., 2009, Imtiaz et al., 2012). Specifically, HPN bars formulated at 30% protein (w/w) using MPC that contained 80% protein (MPC80) rapidly hardened and lost cohesiveness during storage (Banach et al., 2014, Banach et al., 2016a). Nutritionally, MPCs maintain the casein-to-whey protein ratio (80:20) of typical bovine skim milk and are a complete protein with higher digestible indispensable amino acid score (1.18) than whey protein isolate (WPI; 1.09), whey protein concentrate (WPC; 0.97), soy protein isolate (SPI; 0.90), and pea protein concentrate (PPC; 0.82) (Rutherfurd et al., 2015). MPCs' nutritional aspects and their ability to be ultra-filtered directly from skim milk independent of other processes make HPN bars a primary target application.

Micellar casein concentrates (MCCs) are produced by micro-filtering skim milk such that the final spray dried powder has an elevated casein-to-whey protein ratio (92:8) (Dairy Management Inc., 2015). MCCs, which are undefined by the global trade atlas and the United

States Food and Drug Administration (FDA), are less studied than MPCs (Lagrange et al., 2015). Model HPN bars (45% protein w/w) prepared with MCC remained softer than those formulated whey protein hydrolysate, β-lactoglobulin, α-lactalbumin, WPI, or sodium caseinate after 10 d at 37°C (Hogan et al., 2012). Agglomerated MCC produced HPN bars (40-50% MCC powder w/w) that were less dough-like and less prone to hardening than those prepared with non-agglomerated MCC over 7 d storage at 37°C (Hogan et al., 2012). Further validation of MCC in HPN bars is needed since based on protein composition similar textural performance as MPCs would be expected in these applications.

HPN bar texture changes during storage cannot be attributed to one mechanistic cause, and although multicomponent (e.g., protein, carbohydrate, fats, minerals, vitamins), most work has focused on the protein source and ingredient type while the system hardens. Suggested HPN bar hardening mechanisms include moisture migration between constituents, limited free water for complete protein plasticization, entropy-driven macronutrient phase separations, internal disulfide bond formations, and Maillard-induced protein aggregations (Zhou et al., 2008, Loveday et al., 2009, McMahon et al., 2009, Zhou et al., 2013). Mineral (e.g., Na^{+}, K^{+}, Mg^{2+}) addition or removal, including those natively associated with the protein (e.g., Ca^{2+}), may alter the protein's structure, increase internal moisture migration, and subsequently accelerate HPN bar texture change (Book, 2008). Protein hydrolysis has been the main modification technique to impart textural stability during HPN bar storage (McMahon et al., 2009, Rao et al., 2016). Proprietarily modified (Imtiaz et al., 2012) and extruded MPCs (Banach et al., 2014) also improved textural stability when incorporated into model HPN bars. MPC and MCC must be modified to not only slow hardening, but also to maintain cohesion during HPN bar storage in order to be a preferred protein source for these applications.

Most protein powders, especially MPCs, are modified to improve solubility (Mao et al., 2012, Sikand et al., 2013) as well as dependent functional properties (e.g., emulsification, foaming). However, there is no clear relation between these properties and performance in intermediate-moisture foods (IMFs) such as HPN bars. Transglutaminase (Tgase), an enzyme produced by *Streptoverticillium mobaraense*, has been used to improve the texture of solid foods such as restructured meats, fish pastes, yogurts, breads, and confectionaries (Kieliszek and Misiewicz, 2014, Gaspar and de Góes-Favoni, 2015). Tgase builds texture by crosslinking glutamine residues with intra- or inter-protein lysine residues, which occurs faster and with greater specificity than its acyl transfer and deamidation processes (DeJong and Koppelman, 2002, Gaspar and de Góes-Favoni, 2015). Tgase treatment has historically been applied to processed foods seeking textural improvement, but is not commonly used to functionalize protein ingredients for multiple applications (DeJong and Koppelman, 2002). Previously, MPC and MCC were crosslinked by Tgase and functionality was evaluated in processed cheese and yogurt (Salunke, 2013, Salunke et al., 2013a, b), but they were not evaluated in HPN bars.

Tgase crosslinked proteins typically have increased water holding capacity (WHC) (Gaspar and de Góes-Favoni, 2015). The effect of increased WHC on HPN bar texture is unknown as water may move towards the protein as driven by water activity (a_w) gradient (Gautam et al., 2006, Book, 2008, Li et al., 2008, Hazen, 2010) or towards the low molecular weight, poly-hydroxyl compounds by osmotic pull (Loveday et al., 2009). Reduced-calcium MPC (RCMPC) was manufactured by carbon dioxide acidification of milk protein retentate during ultra-filtration which solubilized micellar calcium and phosphate (Marella et al., 2015). RCMPC had improved solubility which may allow for more rapid hydration during HPN bar

production that along with its lower calcium, ash, and net negative charge may limit moisture migration and slow moisture-induced hardening during HPN bar storage.

This study was designed to compare relative textural performance of Tgase crosslinked MPC and MCC, and RCMPC, in a previously used model HPN bar formulation (Banach et al., 2014). Crosslinked protein ingredients will have fewer amine groups available for participation in the Maillard browning reaction (Gerrard, 2002), which may limit formation of protein aggregates that have been associated with HPN bar texture change (Zhou et al., 2013, Banach et al., 2016b). Model HPN bars (30% protein w/w) were prepared with MPC and MCC previously Tgase crosslinked at 'none,' 'low,' and 'high' levels and RCMPC, and hardness, crumbliness, moisture content, pH, color, and a_w were measured during storage.

MATERIALS AND METHODS

Materials

The MPC and MCC powders with 'none' (N), 'low' (L), and 'high' (H) Tgase crosslink levels, including MPC-N (74.4% protein, 3.7% moisture, 8.9% lactose), MPC-L (74.4% protein, 3.9% moisture, 8.7% lactose), MPC-H (74.3% protein, 2.7% moisture, 8.6% lactose), MCC-N (77.6% protein, 3.2% moisture, 4.4% lactose), MCC-L (77.6% protein, 3.6% moisture, 4.5% lactose), and MCC-H (76.9% protein, 3.2% moisture, 4.5% lactose), and the RCMPC (71.9% protein, 3.4% moisture, 14.4% lactose) were previously produced (Salunke, 2013, Marella et al., 2015). Urea, SDS, β-mercaptoethanol, bromophenol blue, and glycerol (99.8% glycerol, 0.1% water) were obtained from Fisher Scientific (Waltham, MA). Supplies for SDS-PAGE, including tris, Precision Plus Protein™ Standard, Any kD™ TGX™ precast gels, Bio-Safe™ Coomassie Stain, and 10x tris/glycine/SDS running buffer, were obtained from Bio-Rad, Inc. (Hercules, CA). Lactose (200-mesh, 99.8% lactose, Glanbia Nutritionals, Twin Falls, ID),

maltitol syrup (Lycasin®80/55, 51.7% D-maltitol, 3.0% D-sorbitol, 24.5% water, Roquette America, Keokuk, IA), non-hydrogenated palm oil (SansTrans®39, IOI Loders Croklaan, Channahon, IL), and high-fructose corn syrup (CornSweet®55, 55% fructose, 41% dextrose, 4% higher saccharides, 23% water, Archer Daniels Midland, Decatur, IL) were donated for use in this study.

Brief Description of Milk Protein Ingredient Modification

A full description of protein powder production and modification is available elsewhere (Salunke et al., 2012, Salunke, 2013, Marella et al., 2015). Low (i.e., MPC-L, MCC-L) and high (i.e., MPC-H, MCC-H) crosslinking was accomplished by treating retentates with 0.3 and 3.0 Tgase units per g protein, respectively, for 25 min at 50°C, which was followed by enzyme inactivation at 72°C for 10 min. The controls (i.e., MPC-N, MCC-N) were not treated with Tgase. Separately, RCMPC was produced by injecting skim milk with carbon dioxide gas (2,200 ppm), which was then ultrafiltered and diafiltered (pH 5.7), and, like all the protein powders used in this study, was spray dried.

Tgase Crosslink Verification by SDS-PAGE

Modified Proteins were dissolved at 6.7 mg protein per mL in tris buffer (50 m*M*; pH 8.0) with denaturants (8 *M* urea, 2% SDS, 5% β-mercaptoethanol). After being mixed for 4.5 h, protein was diluted to ~4 mg per mL. The solutions were centrifuged at 15,000×g for 15 min and the supernatant was diluted two-fold with 2x reduced sample buffer (125 m*M* tris, 8 *M* urea, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue). Samples (4 µL) and a molecular weight standard (10µL) were loaded onto precast gels and were electrophoresed at 100 V for 70 min. The proteins were fixed, stained, and de-stained as described elsewhere (Banach et al., 2016b).

Model High-protein Nutrition Bar Preparation

HPN bars (30% protein w/w) were prepared (n = 3) with each control, Tgase crosslinked, and RCMPC ingredient serving as the sole protein source in each 250 g batch. Each HPN bar formulation was first standardized to 6% lactose (w/w) by combining the protein powder (251-271 g) with lactose (0-28 g). 50.6 g Glycerol, 26.9 g maltitol syrup, and 1.0-2.2 g distilled water were stirred into the dry ingredients. Forty-three g non-hydrogenated palm oil and 21.8 g high-fructose corn syrup were heated together until all the fat melted, which was then mixed into the other constituents. HPN bar dough was pressed into cylindrical molds (ID = 21 mm; H = 13 mm) and a_w sample cups, and were transferred to 32°C storage the following day. More details about HPN bar production are available elsewhere (Banach et al., 2014).

High-protein Nutrition Bar Texture (Hardness and Crumbliness) Measurement

Measurements were made on day 0, 7, 16, 28, and 42 after equilibrating the HPN bars to room temperature (22°C). Each cylindrical HPN bar sample was compressed two times (i.e., texture profile analysis; TPA) to 60% strain at crosshead speed of 2 mm s^{-1} with a flat plate while force versus time data were recorded (TA-XT2, Texture Technologies, Scarsdale, NY). Hardness was reported as the maximum force (N) during the first compression. After compression, the sample was transferred to a stack of 3-inch sieves and was mechanically shaken for 30 s (speed 3, Shaker #18480, CSC Scientific Sieve, Fairfax, VA). HPN bar crumbliness was reported as the mass percent finer than the top sieve (No. 3.5) with 5.6 mm aperture (Banach et al., 2016a). HPN bar samples that were too hard for the analyzer to compress to 60% strain were not analyzed for crumbliness. When texture analyzer's load cell maxed out, hardness was specified as the force just prior to stopping. Additional sample measurements ($n \geq 3$) were attempted as availability allowed.

High-protein Nutrition Bar Color, Water Activity, pH, and Moisture Content Measurement

HPN bar color and a_w were measured on day 0, 2, 7, 16, and 42 as previously described (Banach et al., 2014). a_w was also measured immediately after manufacture (day -1). HPN bar dispersions were prepared in Millipore water (20% w/w) and pH was measured after mixing for 16 h. 2 g of each HPN bar (n = 2) was dried at 102°C for 24 h on day 0, 7, 16, and 42 and moisture content was calculated by difference.

Statistical Analyses

All statistical analyses were conducted using SAS® software (version 9.4, SAS Institute Inc., Cary, NC). Log-transformed hardness measurements were analyzed using the Lifereg procedure. Protein (i.e., MPC, MCC), crosslink level (i.e., none, low, high), storage day (i.e., 0, 7, 16, 28, 42), all two-way interactions, and preparation were the independent variables. In instances when the load cell maxed out (~240 N), the measurement was designated as the right-censoring value. Differences between least squares means (ls-means) were determined, unless otherwise stated, using Tukey's adjusted *P*-value ($P < 0.05$). For HPN bar crumbliness analysis, protein, crosslink, and day were categorized into one variable since some protein × crosslink × storage day combinations were inestimable. That is every HPN bar sample tested on that day from each preparation failed to fracture. Ls-mean estimate statements were written to determine if differences between relevant ls-means were significant ($P < 0.05$). Moisture content, a_w, pH, and L* measurements of all the HPN bars were modeled using the mixed procedure. Protein ingredient (i.e., MPC-N, MPC-L, MPC-H, MCC-N, MCC-L, MCC-H, RCMPC) and time were the independent variables, and HPN bar preparation was set as the random effect.

RESULTS AND DISCUSSION

Verification of Tgase Mediated Crosslink Formation with SDS-PAGE

As expected, the SDS-PAGE profiles of the controls and RCMPC did not contain any polymerized or aggregated proteins (Figure 1). MPC and MCC were both crosslinked by Tgase and the portion of crosslinked protein increased with applied enzyme concentration. Highly crosslinked protein polymers, with molecular weight greater than 250 kDa, were unable to enter the gel and were only found in MPC-H and MCC-H. Vertical protein band smearing, an indicator of protein polymerization (Hsieh and Pan, 2012), occurred between the 50 kDa marker through just above or just below the 250 kDa maker for the high-level or low-level Tgase crosslinked protein ingredients, respectively. A ten-fold increase in Tgase application increased protein polymer formation between 50-250 kDa, as visualized by increased stain intensity, and produced high molecular weight polymers incapable of permeating into the gel. However, when MPC-L and MCC-L are compared to their controls, that is MPC-N and MCC-N, respectively, they each contained a higher concentration of crosslinked protein with molecular weight between 50-250 kDa.

β-, κ-, α_{s1}-, and α_{s2}-casein in MPC and MCC served as the primary substrates for Tgase to crosslink since the globular whey proteins, including β-lactoglobulin (β-lg), α-lactalbumin (α-la), and bovine serum albumin (BSA), are less crosslinkable due to structural constraints (Hsieh and Pan, 2012). Since MCC is richer in casein compared to MPC, it should be more susceptible to Tgase crosslinking, but this was not readily apparent by SDS-PAGE. Corresponding with the newly formed protein polymer concentration, Tgase only slightly polymerized the caseins when applied at a low concentration and hence the SDS-PAGE protein profiles of MPC-L and MCC-L closely matched their controls. Tgase treatment polymerized essentially all the κ-casein in MPC-

H and MCC-H, whereas the β-casein and the α_s-caseins were only partially crosslinked. Fresh raw skim milk casein susceptibility to Tgase crosslinking was previously determined as β > κ > α_{s1} > α_{s2} (Hsieh and Pan, 2012). Another study revealed κ-casein was polymerized prior to all the β-casein in Tgase-treated reconstituted milk (Smiddy et al., 2006). MPC and MCC κ-casein was polymerized more easily than the other caseins since it preferentially exists on the outside of the micelle and was more accessible to Tgase than the interiorly located caseins (Smiddy et al., 2006). A truncated Tgase polymerization time of 30 min, which is more conducive for mass production, was insufficient to crosslink all the β-casein in either the MPC or MCC retentate, even though a portion of it is located on the micelle's exterior (Smiddy et al., 2006). β-lg and α-la were also polymerized by Tgase, as was previously observed (Hsieh and Pan, 2012), but not nearly to the same extent as the caseins as their bands persisted on SDS-PAGE gel. Whey protein polymerization might contribute to the increased concentration of crosslinked protein polymers in MPC-H when compared to MCC-H.

SDS-PAGE analysis confirmed that MPC and MCC were both crosslinked at 'high' and 'low' levels. It is not possible to predict protein ingredient performance in HPN bars based solely upon their SDS-PAGE profiles. Protein hydrolysates soften initial HPN bar texture (Rao et al., 2013), but with lower molecular weight and no protein aggregates, the system exists in the rubbery state which is prone to disulfide and Maillard browning induced protein aggregations that have been related to textural hardening during storage (Zhou et al., 2008, Zhou et al., 2013). Tgase modified MPC and MCC possess altered functionality (Salunke, 2013) which will alter HPN bar texture. HPN bar stability might be conferred by limiting chemical reactivity by way of increased molecular weight and by preventing the internal production of Maillard- induced protein aggregates.

High-protein Nutrition Bar Moisture Content, pH, and L* Color Values during Storage

HPN bar moisture content, averaged across days 0 and 42, was 16.7% and was not significantly influenced by protein ingredient or storage time (Table 1), which ruled out moisture loss as a contributor to texture change. HPN bar pH did not change during storage ($P > 0.05$) (Table 1). On days 0 and 42, the HPN bar made with RCMPC, which was acidified during protein ingredient production, had lower pH than the other HPN bars ($P < 0.05$). L* lightness values decreased ($P < 0.05$) as the samples browned by the Maillard reaction during storage (Table 1). On days 0 and 42, the HPN bar prepared with RCMPC had the lowest L* value since slightly acidified dairy powders brown faster during storage (Dattatreya and Rankin, 2006). Similar to L*, the a* and b* color values (data not shown) of each HPN bar did not differ from their control after equivalent storage. Lower pH of RCMPC and fewer free amines present in the crosslinked protein ingredients did not slow the visual aspect of Maillard browning. Color compounds do not show through until the late stages of the reaction; regardless, it was unlikely that the development of Maillard-induced protein aggregates (Zhou et al., 2013) was slowed by using these modified protein ingredients. After equivalent storage, each HPN bar likely contained a similar concentration of Maillard-induced protein aggregates and any apparent textural differences would be attributable to another aspect of the modified protein ingredient. The aesthetic aspect of color change is of minor importance as it and any potential off-flavors generated are masked by colorings and flavorings added to commercial products (Rao et al., 2013).

Average HPN bar a_w on day manufacture (i.e., day -1) was 0.40, which increased to 0.43 in less than 24 h (i.e., day 0) and to 0.45 after 2 days (Table 2). These low magnitude increases in a_w were similar to those observed for other HPN bars, but such a small increase is difficult to

relate to overall texture change (McMahon et al., 2009, Banach et al., 2014). a_w of each HPN bar was lower than expected, which may have factored into the low level of sample browning.

Texture (Hardness and Crumbliness) Changes in HPN Bar during Storage

Transglutaminase Crosslinked MPC and MCC. The HPN bars hardened during storage (Figure 2) and in addition to time, hardness was significantly influenced by protein, crosslink level, and their two-way interactions ($P < 0.05$). The HPN bars hardened quicker than expected based on a previous report (Banach et al., 2014). Incompressibility occurred earlier in storage, around day 16, for the HPN bars formulated with MPC-N, for which additional sample measurements did not initiate sample fracture. HPN bars from different preparations became too hard for the texture analyzer on different testing days which was due to the effect of preparation ($P < 0.05$). When additional samples were measured, some tended to fracture while others remained incompressible. Inconsistency made it statistically unjustified to include the three-way interaction term (i.e., protein × crosslink × day) in the Lifereg model and limited hardness contrasts to main effects and two-way interactions.

HPN bar storage for 42 d at 32°C has been used to approximate 1 year at 22°C (Li et al., 2008, McMahon et al., 2009) and at that rate 1 week at 32°C is ~8.7 weeks or ~2 months at 22°C. Any substantial hardening within 2 months of manufacture would be unacceptable for a product whose target shelf life is 1 year. On day 0, MPC formulated HPN bars had mean hardness of 113 N and were not significantly ($P > 0.05$) softer than the MCC formulated HPN bars that had mean hardness of 121 N. On all other days tested, the HPN bars prepared with MCC were softer than those prepared with MPC ($P < 0.05$). MCC produced softer HPN bars than several other dairy proteins (Hogan et al., 2102), but those particular MCC-formulated samples hardened substantially less over 10 d at 37°C than the present samples did over 6 d at 32°C. MPC-H

hardened more gradually than MPC-N and MPC-L (Figure 2A) and more similar to the MCC formulated HPN bars (Figure 2B). On average, the HPN bars formulated with MPC-N were harder ($P < 0.05$) than those formulated with MPC-H and MPC-L. Although significant, the small magnitude difference between MPC-N and MPC-L has no practical ability to reduce HPN bar hardness on each storage day (Figure 2A). Even the practicality of MPC-H to reduce HPN bar hardness on each day could be questioned, but it does produce a softer ($P < 0.05$) HPN bar than MPC-N and MPC-L when averaged over the storage period. There was no difference in HPN bar hardness between MCC-L and MCC-H, but they were both softer ($P < 0.05$) than the MCC-N. After equivalent storage, hardness of the MCC-formulated HPN bars all but matched one another (Figure 2B) and such small differences imparted by Tgase crosslinking did not impart practical softening.

Average HPN bar hardness was inversely related with level of crosslink, increasing from 175 N for MPC-H/MCC-H to 193 N for MPC-L/MCC-L to 218 N for MPC-N/MCC-N, and all the contrasts between levels were significant ($P < 0.05$). The day × crosslink interactions were compared using Bonferroni's adjustment. The different levels of Tgase crosslinking did not have an effect ($P > 0.05$) on day 0 HPN bar hardness and if use of Tgase crosslinked proteins did not affect textural stability, this would be seen on each testing day. However, after 7 d the 'high' Tgase crosslinked proteins produced softer HPN bars than the non-crosslinked proteins ($P < 0.05$), but maintained similar hardness to those prepared with the 'low' crosslinked proteins ($P > 0.05$). On day 42 the HPN bars formulated with 'low' and 'high' Tgase crosslinked proteins were both softer ($P < 0.05$) than those made with non-crosslinked proteins, but there was no difference ($P > 0.05$) between the Tgase levels. Tgase crosslinked proteins induced HPN bar brittleness and since max force during compression frequently occurred at the point of fracture,

the modification imparted a softening effect. MPC-H/MCC-H each contained high molecular weight protein polymers (Figure 1) that imparted structural heterogeneity which created internal weak spots and allowed the system to fracture under lower compressive force (Purwanti et al., 2010). HPN bars formulated with low molecular weight hydrolysates are soft and pliable, but they are susceptible to chemical changes, such as disulfide bond formations (Zhou et al., 2008) and Maillard-induced protein aggregations (Zhou et al., 2013), that occur with hardening. These changes, as well as free amine reduction, were not related to the texture change of MPC-formulated HPN bars, but they did occur during storage (Loveday et al., 2009, Banach et al., 2016b). Tgase crosslinking of the protein ingredients increases their average molecular weight, but decreases their molecular mobility and internal chemical reactivity. If these reactions do in fact play a role in HPN bar texture change, this would be mean that disulfide bond formations and Maillard-induced protein aggregations would be slowed. Maillard browning-induced protein aggregations would also be slowed since the Tgase crosslinked proteins have lower initial free amine content when made into HPN bars.

Overall, the model HPN bars prepared with either MPC or MCC were crumbly and lacked cohesion. Crumbliness and cohesiveness are sparsely reported in the HPN bar based literature. Results from a sieve analysis of twice-compressed HPN bars were previously correlated with trained panel measured in-hand crumbliness and in-mouth cohesiveness (Banach et al., 2016a). HPN bar crumbliness increased substantially after 1 week and then increased at a much slower rate (Figure 3). MCC produced HPN bars that were, on average, more crumbly than those made with MPC ($P < 0.05$). A drawback of using MPCs in HPN bars is that they decrease cohesiveness (Imtiaz et al., 2012, Banach et al., 2016a) and the MCC under current study only worsened this texture attribute. Proprietarily functionalized WPC added to MPC

decreased crumbliness and increased cohesiveness of a HPN bar (Imtiaz et al., 2012). Whey proteins are removed during MCC production; since they possess an ability to impart cohesiveness, it was not surprising that MCC produced crumblier HPN bars.

Tgase crosslinking of protein was expected to improve HPN bar cohesiveness/crumbliness by adding structure. Tgase crosslinked proteins produced HPN bars that were less crumbly than the control ($P < 0.05$). The higher level of crosslinking imparted greater cohesion than the lower level of crosslinking ($P < 0.05$). Data required careful analysis since HPN bars became incompressible at different storage times. Some crumbliness estimates were based on a single preparation while others were inestimable, for example, the HPN bar formulated with MPC-N after day 16. Mechanical force generated during sieving/shaking was insufficient to break an incompressible sample and it was completely retained on the top sieve. While not crumbly in terms of the assay, these samples would be deemed unacceptable by hardness alone, and being texturally irrelevant, crumbliness was not reported for samples that did not break during compression. Crumbliness of the HPN bars prepared with MCC-N and MCC-L did not differ ($P > 0.05$) on each day tested (Figure 3B). HPN bar crumbliness values of MPC-H were compared with MPC-L and those for MCC-H were compared with MCC-L. HPN bars formulated with MPC-H or MCC-H regularly fractured during TPA and while fines persisted, they were more cohesive than MPC-L or MCC-L, respectively, yet contrast significance varied with testing day. MPC-H or MCC-H HPN crumbliness was not different ($P > 0.05$) than MPC-L or MCC-L on day 0, respectively, but on day 7 and day 16 those differences were significant ($P < 0.05$). The HPN bar formulated with MCC-H was also less crumbly than MCC-L on day 28 ($P < 0.05$). HPN bar crumbliness leveled off as day 42 approached and on that day, no difference ($P > 0.05$) were found between MPC-H or MCC-H and MPC-L or MCC-L, respectively. Using

Tgase crosslinked protein ingredients in HPN bars reduced the rate in which crumbliness developed and improved overall cohesiveness. Tgase was inactivated after MPC and MCC were crosslinked and so internal Tgase crosslinking does not occur within the HPN bar. Tgase improved the cohesiveness of an emulsified meat system when added in its active form (Herrero et al., 2008). Since the HPN bars had low moisture (Table 1), low a_w (Table 2), and stable pH (Table 1), protein gelation cannot occur during storage. Caseinate gels produced by glucono delta-lactone (GDL) acidification were more cohesive when produced with Tgase-crosslinked caseinate (Song and Zhao, 2013). Other than inhibition or slowing of the texture change mechanisms discussed for hardening, it was not possible to pinpoint why MPC-H and MCC-H produced a more cohesive HPN bar.

Reduced-Calcium MPC. RCMPC produced a HPN bar that was more powdery, drier to the touch, and less adhesive (data not shown) on each testing day when compared with all the other model HPN bars. It was important to balance constituents for shelf stability (i.e., $a_w < 0.65$) while maintaining a formula suitable for all the protein ingredients being evaluated in the current study, yet similar to those previously used for MPC-formulated HPN bars (Imtiaz et al., 2012, Banach et al., 2014). MPC-N was not produced from the same lot of skim milk as RCMPC, but it sufficed as its control in this study. RCMPC slowed HPN bar hardening (Figure 2A), especially when compared with MPC-N, but values still approached the maximum measurable by the texture analyzer utilized as storage time neared 42 d. Standard deviation between preparations was high and thus it was unlikely that the hardness of the RCMPC formulated HPN bar differed with the MPC-N on day 0, 16, 28, and 42. Apparently its hardness was only lower than MPC-N on day 7 (Figure 2) or ~2 months at 22°C. While RCMPC produced a softer HPN bar for the short term, it was the crumbliest one evaluated in this study

(Figure 3A). While softness was imparted initially, RCMPC did not improve HPN bar cohesiveness and thus reducing the calcium content of MPC will not improve its ability to serve as a predominant protein in these applications. However, RCMPC might be blended with other protein ingredients to potentially impart softening or, in an instance desired, a crumbling effect.

CONCLUSIONS

In this study, MPC and MCC, previously crosslinked at 'low' and 'high' levels, plus one RCMPC were texturally evaluated in a model HPN bar. MPC and MCC produced HPN bars that progressively hardened and lost cohesion during storage. Overall, those formulated with MPC were harder and more cohesive than those made with MCC. Tgase crosslinked proteins decreased HPN bar hardness and decreased the development of crumbliness during storage. More protein crosslinking lowered peak force during compression, after which the sample was characterized as being less crumbly. However, as storage time progressed, the HPN bars formulated with the modified protein ingredients behaved with greater textural similarity as their respective controls. The RCMPC produced a softer and crumblier HPN bar when compared with control MPC. We conclude that the small magnitude changes in HPN bar texture that resulted from utilizing Tgase crosslinked MPC or MCC, or RCMPC, did not improve stability during storage and that these modified protein ingredients have no practical advantage over their unmodified controls in HPN bars.

ACKNOWLEDGEMENT

The protein ingredients used in this study were produced at South Dakota State University by Prafulla Salunke and Chenchaiah Marella. This project was partially supported by Dairy Research Institute award #H003889501 through the University of Minnesota and partially by the Iowa State University Agricultural Experiment Station.

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Banach, Table 1

	Moisture		pH		L*	
Protein[1]	Day 0	Day 42	Day 0	Day 42	Day 0	Day 42
MPC-N	16.1^{a,z}	16.7^{a,z}	6.6^{a,z}	6.5^{a,z}	87.5^{a,z}	79.5^{ab,y}
MPC-L	16.4^{a,z}	17.0^{a,z}	6.5^{a,z}	6.5^{a,z}	87.7^{a,z}	78.5^{bc,y}
MPC-H	17.5^{a,z}	16.3^{a,z}	6.5^{a,z}	6.4^{a,z}	88.5^{a,z}	79.0^{abc,y}
MCC-N	17.0^{a,z}	16.8^{a,z}	6.3^{ab,z}	6.5^{a,z}	88.3^{a,z}	79.9^{ab,y}
MCC-L	16.5^{a,z}	17.0^{a,z}	6.6^{a,z}	6.6^{a,z}	87.7^{a,z}	81.3^{a,y}
MCC-H	16.9^{a,z}	17.4^{a,z}	6.4^{a,z}	6.5^{a,z}	88.9^{a,z}	81.2^{a,y}
RCMPC	16.4^{a,z}	16.0^{a,z}	6.0^{b,z}	5.9^{b,z}	84.4^{b,z}	76.8^{c,y}

$^{a\text{-}c}$ Least squares means are significantly different ($P < 0.05$) if they do not share a common superscript within the same column.

$^{y\text{-}z}$ Least squares means are significantly different ($P < 0.05$) if they do not share a common superscript within the same row for each attribute.

[1] The HPN bars (30% protein w/w) were formulated with milk protein concentrate (MPC), micellar casein concentrate (MCC), or reduced-calcium MPC (RCMPC). N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively.

Banach, Table 2

	Day					
Protein[1]	-1[2]	0	2	7	16	42
MPC-N	0.39^{b,x}	0.43^{bc,y}	0.44^{b,yz}	0.44^{bcd,yz}	0.46^{ab,z}	0.45^{a,z}
MPC-L	0.39^{ab,x}	0.42^{cd,y}	0.44^{b,z}	0.44^{cd,z}	0.45^{b,z}	0.45^{a,z}
MPC-H	0.41^{a,x}	0.44^{b,y}	0.45^{ab,yz}	0.45^{abc,yz}	0.47^{ab,z}	0.46^{a,yz}
MCC-N	0.41^{a,y}	0.46^{a,z}	0.47^{a,z}	0.46^{a,z}	0.47^{a,z}	0.47^{a,z}
MCC-L	0.40^{ab,x}	0.44^{b,b}	0.46^{ab,z}	0.45^{ab,z}	0.46^{ab,z}	0.46^{a,z}
MCC-H	0.40^{ab,x}	0.43^{b,y}	0.46^{ab,z}	0.46^{ab,z}	0.46^{ab,z}	0.46^{a,z}
RCMPC	0.36^{c,w}	0.40^{d,x}	0.42^{c,yz}	0.43^{d,z}	0.43^{c,yz}	0.41^{b,xy}

a-d Least squares means are significantly different ($P < 0.05$) if they do not share a common superscript within the same column.

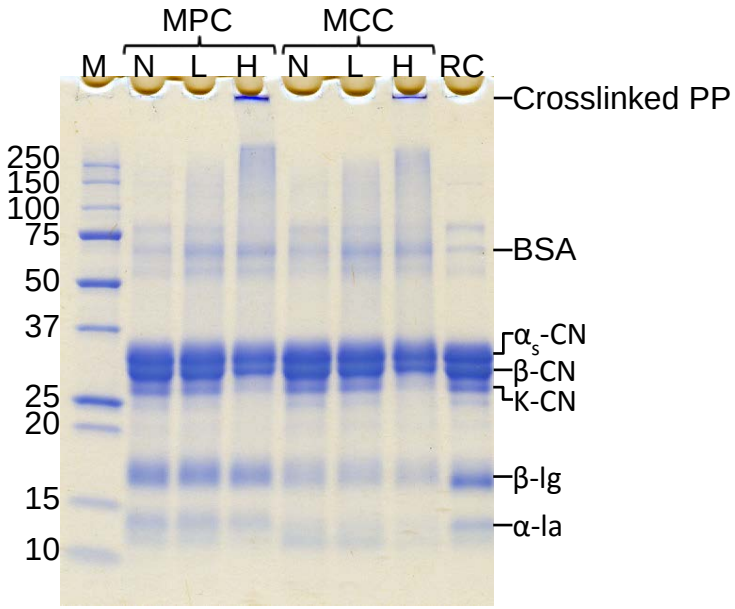
y-z Least squares means are significantly different ($P < 0.05$) if they do not share a common superscript within the same row.

[1] The HPN bars were formulated with milk protein concentrate (MPC), micellar casein concentrate (MCC), or reduced-calcium MPC (RCMPC). N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively.

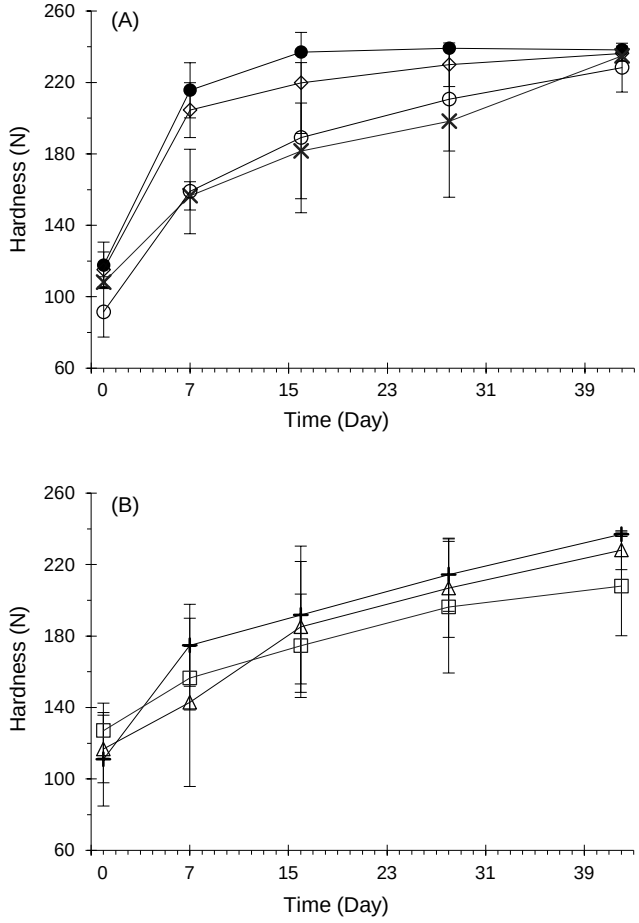
[2] Day -1 indicates the day of HPN bar manufacture whereas day 0 was when samples were moved into 32°C storage.

FIGURES

Banach, Figure 1

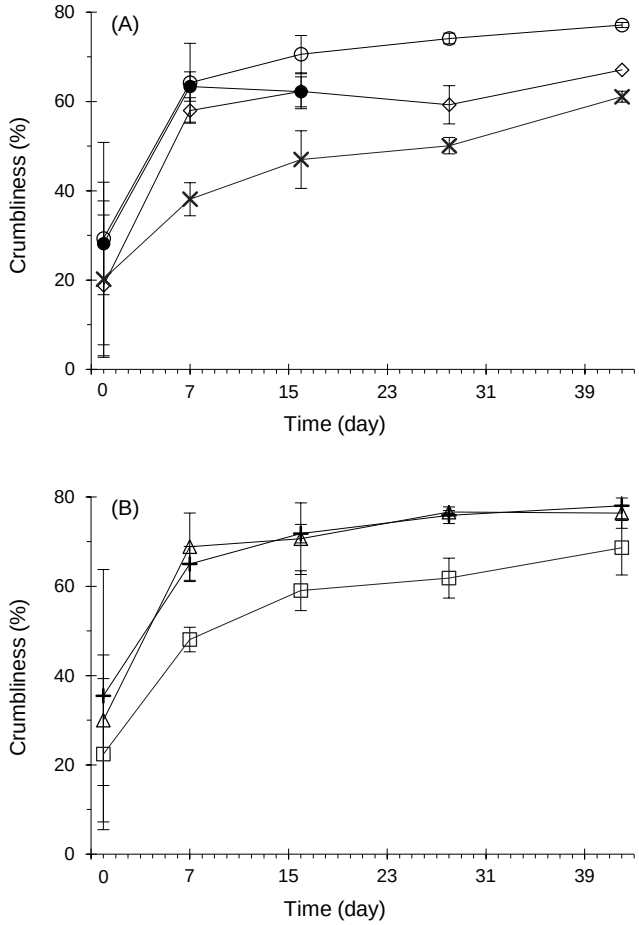


6 Banach, Figure 2



7

8 Banach, Figure 3



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