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Microstructural Changes in High-Protein Nutrition Bars Formulated with Extruded or Toasted Milk Protein Concentrate

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Abstract

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Keywords

confocal laser scanning microscopy, extrusion, free sulfhydryl, milk protein concentrate, protein bar

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Microstructural Changes in High-protein Nutrition Bars Formulated with Extruded or Toasted Milk Protein Concentrate

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Keywords: Milk protein concentrate, protein bar, extrusion, free sulfhydryl, confocal laser scanning microscopy

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Practical Application

High-protein nutrition bars formulated with extruded MPC80 underwent fewer microstructural changes during storage. Disulfide crosslink formation and free sulfhydryl content changes were not always indicative of texture changes in high-protein nutrition bars. Texture change in high-protein nutrition bars formulated with MPC80 was, thus, only partly due to these aggregations. Pre-extruded MPC80 may produce high-protein nutrition bars with an extended textural shelf life compared to those produced with unmodified MPC80.

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Introduction

Powder milk protein concentrates (MPCs), particularly those with more than 80 g protein per 100 g product (i.e., MPC80), possess poor rehydration and solubility characteristics that worsen during storage (Havea 2006; Anema and others 2006; Haque and others 2010). High-protein nutrition (HPN) bars, which contain 20-50% protein (w/w), are intermediate moisture systems that do not require complete protein solubility and are a potential application for MPCs (Cho 2010). However, when utilized in HPN bars, MPCs present challenges in balancing cohesiveness (e.g., too crumbly), firmness (e.g., too hard), and texture change over the product's shelf life (Baldwin and Pearce 2005; Imtiaz and others 2012; Li and others 2008; Loveday and others 2009). Texture change of HPN bars during storage is likely due to a combination of different phenomena, for example, moisture migration between constituents, macronutrient phase separations, and disulfide bond- and Maillard-induced protein aggregations (Zhou and others 2008a; Loveday and others 2009; McMahon and others 2009; Zhou and others 2013).

In addition to protein, HPN bars are comprised of 10-50 g carbohydrate and 10-15 g fat per 100 g (Zhu and Labuza 2010). Free water is minimized and water activity is kept less than 0.65 to ensure microbial shelf stability (Loveday and others 2009). While other ingredients (e.g., sugar alcohols) and other factors (e.g., storage conditions) can influence HPN bar texture, protein source (e.g., dairy, soy) and type (e.g., concentrate, hydrolysate, crisp) have direct impact (Childs and others 2007; McMahon and others 2009; Imtiaz and others 2012). The physicochemical properties of MPC can be tailored for HPN bars using physical, chemical, or enzymatic modifications (Imtiaz and others 2012). The texture of HPN bars formulated at 30% protein (w/w) with physically modified MPC80 was evaluated over 42 days storage at 22°C, 32°C, and 42°C (Banach and others 2014). HPN bars produced with extruded MPC80 hardened slower

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than those made with toasted or unmodified MPC80. MPC80 toasted at 75°C or 110°C for 4 h produced HPN bars that had minimal texture change or increased fracture force, respectively, when compared to those formulated with control MPC80. Extruded MPC80s had reduced protein solubility and, based on the rate of free amine reduction during HPN bar storage, were less chemically reactive (Banach and others 2013; Banach and others 2014).

Free amine reduction was one chemical change that occurred during storage of HPN bars, but it insufficiently explains texture change (Rao and others 2013; McMahon and others 2009; Baier and others 2007; Banach and others 2014). Protein aggregations, including those from disulfide crosslink formations and Maillard reactions, during storage have also been implicated in texture change (Zhou and others 2008a; Zhou and others 2008b; Zhou and others 2013). N-ethylmaleimide prevented disulfide bond formation and extended textural shelf life of a model intermediate moisture food (IMF) 6-times the control (Zhu and Labuza 2010). Free sulfhydryl interactions were texturally relevant in the same IMF, as molecular cysteine slowed or accelerated hardening when added at low or high levels, respectively (Zhu and Labuza 2010). The objective of the present study was to determine the effect extrusion and toasting had on the free sulfhydryl content of MPC80 and to verify the occurrence of disulfide crosslinking within HPN bars formulated with those modified protein ingredients. Additionally, confocal laser scanning microscopy (CLSM) was used to study macronutrient phase separations in these HPN bars. Instrumental texture properties were presented in detail elsewhere (Banach and others 2014); however, they are related to the microstructural changes presented in this study.

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Materials and Methods

Materials and Reagents

MPC80 (79.9% protein, 4.6% moisture) was purchased from Idaho Milk Products (Jerome, ID). Glycerol, boric acid, sodium chloride, ethylenediaminetetraacetic acid (EDTA), urea, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Pierce™ BCA protein assay, and Nile red (MP Biomedicals, LLC) were purchased from Fisher Scientific (Waltham, MA). L-cysteine hydrochloride monohydrate, sodium tetraborate decahydrate, and fluorescein isothiocyanate (FITC) isomer 1 were purchased from Sigma-Aldrich (St. Louis, MO). The reducing agent compatible bicinchoninic acid (BCA) protein assay was purchased from G-Biosciences® (St. Louis, MO). The 2x Laemmli Sample Buffer, precast 4-20% gradient Mini-Protean® TGX™ gels, Bio-Safe™ Coomassie Stain, and Precision Plus Protein™ Standards were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

MPC Modification and HPN Bar Preparation

MPC80 was modified with extrusion or dry-heat toasting. MPC80 moisture content was adjusted to 38% and extruded at die-temperature of 65°C or 120°C using a low-shear screw profile. The extrudate was dried, milled, and sieved through a 250 µm mesh, as detailed elsewhere (Banach and others 2014; Banach and others 2013). For dry-heat toasting, MPC80 was put in a laboratory oven at 75°C or 110°C for 4 h and passed through the same screen. These modified proteins are referred to as E65 (78.4% protein, 7.3% moisture), E120 (79.5% protein, 5.8% moisture), T75 (80.6% protein, 4.1% moisture), and T110 (81.7% protein, 3.0% moisture), respectively.

HPN bars, with protein and moisture content indicated, were prepared by Banach and others (2014) using control MPC80 (31.4% protein, 14.4% moisture), E65 (31.7% protein,

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14.2% moisture), E120 (31.6% protein, 13.6% moisture), T75 (31.6% protein, 13.4% moisture), and T110 (31.5% protein, 13.5% moisture). After 0, 6, 13, 22, or 42 days storage at 32°C, the HPN bars were frozen in liquid nitrogen, ground with a laboratory blender, and kept at -80°C until free sulfhydryl measurement and SDS-PAGE in the present study.

Free Sulfhydryl Measurement

The free sulfhydryl content of each protein ingredient and HPN bar was determined by Ellman's assay with modifications (Beveridge and others 1974). Free sulfhydryl extraction buffer (pH 8.5) contained 8 mol urea plus 4.1 mmol EDTA per L and was prepared in borate buffer (100 mmol boric acid, 75 mmol sodium chloride, and 25 mmol sodium tetraborate decahydrate per L). Protein ingredients (0.75 g) were mixed with degassed extraction buffer (11.25 g) for 2 h in 15-mL centrifuge tubes. HPN bars (2.04 g) and degassed extraction buffer (9.96 g) were mixed in 25-mL Erlenmeyer flasks for the same time. For the HPN bars prepared with T110, 2.55 g was mixed with 12.45 g extraction buffer. Protein ingredient and HPN bar dispersions were centrifuged for 15 min at 12,000 g and 15,000 g, respectively.

Sample supernatants (0.5 mL) or cysteine standards (0.5 mL) were vortexed with 50 µL of 10 mmol DTNB L^{-1} and 2.5 mL extraction buffer, which was held at room temperature for 15 min and absorbance read at 412 nm. Sample and standard blanks were prepared by substituting DTNB with extraction buffer. Standard net absorbance was plotted against seven free sulfhydryl concentrations (25 to 493 µmole L^{-1}) and was fitted with a linear ($R^2 \geq 0.995$) curve (not shown) used to determine sample free sulfhydryl concentration. These values were divided by the BCA assay determined soluble protein (g L^{-1}) and free sulfhydryl content was reported as µmole per g protein.

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Non-reduced and Reduced SDS-PAGE

Sample supernatants from the free sulfhydryl assay (above) were used for non-reduced SDS-PAGE. Reduced extraction followed the same procedures except the extraction buffer contained 50 mL β-mercaptoethanol L^{-1}. Soluble protein was diluted to 4 mg mL^{-1} and was verified using the appropriate BCA assay. Non-reduced dilutions contained 3.7-4.4 mg protein mL^{-1} whereas the reduced dilutions contained 3.8-5.6 mg protein mL^{-1}. The non-reduced samples were diluted 1:2 with both reducing and non-reducing 2x Laemmli Sample Buffer. The reduced samples were only diluted 1:2 with reducing 2x Laemmli Sample Buffer. The protein standard and samples were loaded onto the gel at equal volume (10 µL) and were electrophoresed at 150 V for 50 min using standard SDS-PAGE running buffer (250 mmol tris, 1.92 mol glycine, and 10 g SDS per L). The gels were fixed in methanol/acetic acid/Millipore water (40/10/50) for 30 min, stained for 1 h, and de-stained with Millipore water.

Confocal Laser Scanning Microscopy of the HPN Bars

CLSM methodologies were adapted from literature to detect possible macronutrient phase separations within the HPN bars during storage (McMahon and others 2009). A separate 50 g batch of each HPN bar was prepared with the same lot of ingredients. In addition to the protein ingredients described above, each model contained 21.5 g glycerol (99.8% glycerol, 0.1% water), 18.4 g palm kernel stearin, 12.0 g maltitol syrup (Lycasin®80/55, 51.7% D-maltitol, 3.0% D-sorbitol, 24.5% water, Roquette America, Keokuk, IA), 10.0 g high-fructose corn syrup (CornSweet®55, 55% fructose, 41% dextrose, 4% higher saccharides, 23% water, Archer Daniels Midland, Decatur, IL), and distilled water to standardize protein ingredient moisture content per 100 g. A mechanical stand mixer was used to combine the ingredients, according to Banach and others (2014), and a small portion was leveled into a press-to-seal

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silicone isolator (13 mm diam. × 2 mm depth, Grace™ Bio-Labs, Bend, OR) mounted on a glass microscope slide. One drop of FITC-acetone solution (0.2 g FITC kg^{-1}) and one drop of Nile red-acetone solution (0.2 g Nile red kg^{-1}) were applied to the HPN bar surface with a glass Pasteur pipette. A glass coverslip was placed over the sample and, along with the base of the push-to-seal isolator, was sealed into place with silicone. The freshly prepared slides were kept at room temperature (~22°C) overnight and day 0 images were acquired the following day.

CLSM micrographs were acquired with a SP5 X MPC confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) using the 10x objective lens with 2x digital zoom. Three representative images (775 µm × 775 µm, 1024 px × 1024 px) of each HPN bar were acquired using filters to capture FITC (i.e., protein) and Nile red (i.e., lipid) fluorescence. The fluorescence signals were auto-contrasted and overlaid in Leica LAS AF Lite software. The same slides were imaged after 6, 22, and 42 days at 32°C after equilibrating to room temperature.

Statistical Analyses

A mixed linear model was used to discern free sulfhydryl content differences between the protein ingredients. Independent variables were protein ingredient and ingredient preparation, and their interaction was the random term. HPN bar free sulfhydryl content was also modeled using the mixed linear method. The independent variables were protein ingredient, storage time, and their interaction. Protein ingredient and storage time slicing factors were applied separately to analyze changes within each HPN bar throughout storage and between HPN bars at fixed time, respectively. In each model, Satterthwaite's method was used to compute denominator degrees of freedom and means were compared using Tukey's adjusted p-value. All statistical analyses were performed with SAS® software (version 9.3, SAS Institute Inc., Cary, NC).

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Results and Discussion

Free Sulfhydryl Content of Modified MPC80 Ingredients

We have hypothesized that the textural performance of MPC80 protein ingredients in HPN bars is related to their initial free sulfhydryl content. Protein modifications that increase free sulfhydryl concentration or increase exposure by way of protein unfolding could accelerate disulfide bond formation during HPN bar storage. Free sulfhydryl content of the protein ingredients and their corresponding HPN bars after storage at 32°C is shown in Table 1. Control MPC80 in the present study had 4 µmole free sulfhydryl per g soluble protein. Mao and others (2012) reported that MPC80 had approximately 9.5 µmole free sulfhydryl per g soluble protein, while MPC with 62% protein (w/w) had 4.8 µmole free sulfhydryl per g soluble protein (Cao and others 2015). While on the same order of magnitude, free sulfhydryl differences can be attributed to production scale, storage time and conditions, and modifications made to Ellman's assay.

Extrusion reduced the free sulfhydryl content of MPC80 by imparting both heat and shear force (Table 1); E65 and E120 had 3.0 and 0.7 µmole per g soluble protein, respectively. Higher extrusion temperatures reportedly caused greater free sulfhydryl loss in texturized whey protein concentrate (WPC) and whey protein isolate (WPI) (Qi and Onwulata 2011a; Qi and Onwulata 2011b; Manoi and Rizvi 2009; Nor Afizah and Rizvi 2014). The die-end melt temperature of E120 was greater than that of E65 and it was this temperature difference that significantly reduced E120's free sulfhydryl content ($P < 0.05$).

T75 and T110 had 4.5 and 5.6 µmole free sulfhydryl per g soluble protein, respectively (Table 1). Dry heating beta-lactoglobulin (β-lg) and WPI caused partial protein unfolding and increased free sulfhydryl accessibility to DTNB in the absence of SDS (Gulzar and others 2011a;

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Gulzar and others 2011b). When the assay buffer included SDS, which increased DTNB access to the protein's buried free sulfhydryl groups via denaturation, the measured free sulfhydryl content of the same proteins decreased, which was the result of disulfide bond formation and free sulfhydryl oxidation (Gulzar and others 2011a; Gulzar and others 2011b). Although urea denatures proteins differently than SDS, it should have sufficiently solubilized and unmasked the buried free sulfhydryl groups found within the toasted MPC80. Increased free sulfhydryl content in the toasted MPC80 did not align with previous results (Gulzar and others 2011a; Gulzar and others 2011b). Sulfhydryl-disulfide and free sulfhydryl oxidations occurred minimally in toasted MPC80s since free sulfhydryl content increased in the presence of urea and greater exposure occurred at the higher toasting temperature. Reduced free sulfhydryl content, as was the case with extruded MPC80, produced softer and more texturally stable HPN bars than those formulated with T75 and T110, which had relatively unaltered and increased free sulfhydryl content, respectively (Banach and others 2014).

SDS-PAGE Protein Profiles of the Modified MPC80 Ingredients

SDS-PAGE protein profiles of toasted, extruded, and unmodified MPC80 were used to explain their measured free sulfhydryl content (Figure 1). The protein ingredients were solubilized in either non-reducing (Figure 1A, B) or reducing (Figure 1C) extraction buffer, without (1A) or with β-mercaptoethanol (1B and 1C) added to the SDS-PAGE sample buffer. The profiles of T75 matched those found in unmodified MPC80 under the same set of running conditions. Therefore, the fact that these two protein ingredients had statistically equivalent free sulfhydryl content (Table 1) and that they produced HPN bars with similar textural properties was not surprising (Banach and others 2014). More noticeable differences were visualized for T110, E65, and E120, and are discussed below.

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Measured free sulfhydryl interpretation was the primary purpose for SDS-PAGE comparison and hence discussion will focus on the free sulfhydryl-containing proteins in MPC, including bovine serum albumin (BSA) (Cys34) and β-lg (Cys121), which have the potential to form disulfide bonds during HPN bar storage. Protein disulfide bond formations can be visualized on SDS-PAGE gels by disappearance or reappearance of bands when a reducing agent is excluded or included (Onwulata and others 2010). BSA (66 kDa) remained soluble in each modified MPC80 and, with the exception of T110, its appearance remained the same with fixed SDS-PAGE conditions. BSA contains 17 disulfide bonds and so partial reduction, as indicated by fading band intensity, occurred on the gels that included β-mercaptoethanol (Figure 1B, C). Disulfide bond formation involving BSA as a participant in T110 was unlikely, as solubility was not regained with reduced extraction (Figure 1C).

Under non-reduced conditions, the soluble β-lg in E65 was limited and it was almost nonexistent in E120 when compared with MPC80 (Figure 1A). Extrusion of MPC80 at a die temperature of 120°C made β-lg insoluble, which corroborates its low, yet detectable, free sulfhydryl content (Table 1). Soluble disulfide linked protein aggregates (DLPA) too large to enter the gel were noted in E65, but were absent in E120 (Figure 1A). β-mercaptoethanol reduced the DLPA found in E65 and helped identify the participating proteins (Figure 1B). β-lg band intensity in E65 was regained, resembling that found in MPC80, and confirmed its involvement in the DLPA that resulted from extrusion at 65°C (Figure 1B). DLPA are also found in the region labeled simply as protein aggregates (PA) for E65 and E120 as protein band smearing occurred vertically in these lanes (Figure 1A) and clarity was regained with reducing agent addition (Figure 1B, C). Intensity in the region labeled PA was greater in E65 than in E120. However, the figure was labeled with PA versus DLPA, as some aggregates remain in

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this region for some of the proteins (i.e., T110) after reduction. The β-lg band was still absent in E120 after reducing agent addition to the SDS-PAGE sample buffer, thus, did not participate as heavily in the formation of soluble DLPA (Figure 1B).

The casein proteins, including the α_{S2}, α_{S1}, β, and κ units, found between 37 kDa and 25 kDa, were altered more by toasting at 110°C than the other treatments. Casein in T110 was less soluble, as indicated by reduced band intensity, than in MPC80 under the same conditions. The casein proteins do not contain any free sulfhydryl groups, but as solubility decreased under strictly non-reduced conditions, the β-lg in T110 became more concentrated when compared with the visual band intensity of β-lg in MPC80 (Figure 1A). PA in T110 remained after reduction (Figure 1B, C), which suggested resultant aggregation involved Maillard-type aggregations that involved the casein proteins more than the whey proteins. Although T110's free sulfhydryl content was not significantly greater than MPC80's (Table 1), its elevated magnitude likely resulted from increased β-lg and less casein in solution.

Dissolution of E65, E120, and T75 in reducing buffer produced protein profiles almost identical to unmodified MPC80 (Figure 1C). β-lg in E120 solubilized under these conditions, which indicated that insolubility under non-reduced conditions was from disulfide cross-linked aggregations that formed during extrusion. Unlike the soluble DLPA in E65, those found in E120 were mostly insoluble under non-reduced conditions, which was attributed to the higher extrusion temperature. The β-lg bands for E65, E120, and T110 on this gel are broader and shifted upwards, and their α-la bands lacked definition compared with MPC80 (Figure 1C). T110 still had a vertically smeared SDS-PAGE protein profile, which indicated that non-reducible Maillard induced PA formed during modification.

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Free Sulfhydryl Content of the HPN Bars during Storage

Changes in protein solubility during storage might influence HPN bar free sulfhydryl measurements. Soluble protein extractable from the HPN bars was significantly influenced by protein ingredient and storage time. Soluble protein ranged from 40-45, 32-37, 44-46, 29-39, and 42-50 mg mL^{-1} for the HPN bars formulated with E65, E120, T75, T110, and MPC80, respectively, during 42 d storage. Measured protein solubility was the lowest on day 42 for the HPN bars prepared with T75, T110, E65, and MPC80. However, protein solubility in the E120 formulated HPN bars tended to increase with storage time, a trend that made the interaction term significant ($P < 0.05$). When day 0 protein solubility was compared with day 42 protein solubility, only the T110 formulated HPN bar had significantly lower solubility on day 42. While the T110 formulated HPN bars produced less supernatant overall, the soluble protein concentration was only significantly lower than all other samples on day 42. Soluble protein extractable from an IMF reportedly decreased during storage and was related to matrix hardening (Zhou and others 2008a). In the present study, a significant reduction in protein solubility was not observed for all HPN bars during storage even though they all underwent significant texture change during the same time (Banach and others 2014).

Only the second preparation of the HPN bars made by Banach and others (2014) was used to evaluate free sulfhydryl change during storage (Table 1), which was satisfactory since protein ingredient preparation (n = 2) did not influence free sulfhydryl content ($P > 0.05$). No difference between the measured free sulfhydryl content of a protein ingredient and its respective HPN bar was expected on day 0. While differences were observed in the extruded MPC80s, larger deviations were found between the protein ingredient and the HPN bar free sulfhydryl content when prepared with toasted and unmodified MPC80. Initially, the HPN bar formulated

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with T110 had lower free sulfhydryl content than the HPN bars formulated with MPC80 and T75, a trend that was reversed within the protein ingredient category. While the HPN bar was more complex than the protein ingredient, any background noise from the extra constituents was subtracted from the sample prior to calculating free sulfhydryl content with the standard curve.

Free sulfhydryl content in HPN bar was significantly affected by the protein ingredient used and its interaction with storage time ($P < 0.05$), but storage time alone did not have a significant effect ($P > 0.05$). No initial differences were detected between the HPN bars formulated with MPC80, T75, T110, and E65 ($P > 0.05$), whereas the E120 formulated HPN bars had significantly lower free sulfhydryl content. Although the numbers trended towards reduction, significant free sulfhydryl change was not detected during HPN bar storage when formulated with MPC80, T75, or E120 (Table 1). Free sulfhydryl content in E65 formulated HPN bars decreased significantly ($P < 0.05$) after 13 days and did not differ from the one formulated with E120 for the remainder of the study. The free sulfhydryl concentration in T110 formulated HPN bars increased ($P < 0.05$) with storage and was significantly greater than the other HPN bars on day 42 (Table 1).

Decreasing free sulfhydryl concentration during storage would indicate free sulfhydryl oxidation or the formation of disulfide bonds and that the HPN bar texture changes observed by Banach and others (2014) followed the protein aggregation mechanism previously reported (Zhou and others 2008a; Zhou and others 2008b). While all the HPN bars analyzed by Banach and others (2014) hardened, the HPN bar formulated with E65 was the softest and hardened the slowest. Yet, the present study revealed a significant free sulfhydryl content decrease in this sample within the same storage period. On the other hand, the T110 formulated HPN bars performed poorly from a texture standpoint and had increased free sulfhydryl concentration

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during storage. The insignificant free sulfhydryl decrease observed in the HPN bars formulated with MPC80 and T75, which behaved similarly from a texture standpoint, may or may not be sufficient to induce textural change. However, the significant interaction between protein ingredient and storage time disproves disulfide bond formation as the main mechanism of HPN bar texture change when formulated with MPC80.

SDS-PAGE Protein Profiles of the HPN Bars during Storage

Reduced and non-reduced SDS-PAGE protein profiles for the HPN bars formulated with unmodified (Figure 2), toasted (Figure 3), and extruded (Figures 4 and 5) MPC80 were used to verify disulfide bond formation during storage. In Figures 2-5, images A and B show the proteins extractable under non-reduced conditions whereas C shows the proteins soluble in a reducing buffer. Gel A was run without β-mercaptoethanol, but it was included in the SDS-PAGE sample buffer for gels B and C. Under the same SDS-PAGE conditions, the protein profiles of the HPN bars prepared with T75 matched those prepared with the control MPC80 and thus are not shown.

DLPA accumulated just below the loading well for the HPN bars formulated with MPC80, T75, T110, and E65 (Figures 2A, 3A, and 4A). In the HPN bars formulated with MPC80 or T75, the formation of soluble DLPA increased throughout storage period, as indicated by band intensity (Figures 2A). However, the same protein aggregations decreased during storage in the T110 formulated HPN bars (Figure 3A). The DLPA in E65 were of higher molecular weight, as the band was highly concentrated at the top of the gel and DLPA migration into the gel was virtually nonexistent (Figure 4A). In this case, the DLPA remained nearly constant and thus these aggregations did not change during storage as they did in the HPN bars formulated with toasted and unmodified MPC80. These DLPA, especially those that did not

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enter the gels, were inferred due to disulfide crosslink formation, as a reducing agent in the sample buffer allowed the proteins involved to enter the gel (Figures 2B, 3B, and 4B). The HPN bars formulated with E120, in line with the protein ingredient, did not show any soluble DLPA initially nor were any formed during storage (Figure 5A).

Directly below the DLPA region, a strip labeled PA, which consists of both disulfide crosslinked aggregates as well as those due to Maillard-induced protein aggregations, was identified (Figures 2-5). Vertical band smearing on each storage day became less intense when a reducing agent was added to the SDS-PAGE sample buffer or both the SDS-PAGE sample and extraction buffers. Disruption of these PA was from reduction of disulfide bonds that were present initially (i.e., Day 0) in each HPN bar from protein ingredient modification or natively found in MPC80. Disulfide linked aggregates were less common in the PA region for the T110 formulated HPN bars, as reducing agent addition did not decrease vertical band smearing and thus was inferred to be from non-reducible, Maillard-induced PA formed during initial protein modification (Figure 1). However, on the gels with a reducing agent, vertical band smearing within the lanes increased with the storage time when formulated with extruded (Figures 4 and 5 B or C) or unmodified MPC80 (Figure 2 B or C) and remained constant when formulated with the heavily pre-aggregated T110 (Figure 3 B or C). The development of non-reducible, Maillard-induced PA with storage may have contributed to HPN bar texture change as previously reported (Banach and others 2014; Zhou and others 2013), even though this was suggested not to be a mechanism of texture change by McMahon and others (2009).

Individual protein bands (e.g., casein, β-lg) on the non-reduced gels were slightly smeared; however, their resolution improved with reducing agent addition to the SDS-PAGE sample buffer alone or to both extraction and SDS-PAGE sample buffers (Figures 2-5). The

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casein proteins, including α_{s1}-, α_{s2}-, β-, and κ-casein, separated at lower resolution on the non-reduced gels when compared to the reduced gels, especially as storage time increased. Decreased casein mobility after day 0 on the non-reduced SDS-PAGE gels for the HPN bars formulated with MPC80 (Figure 2A) and T75 (not shown) was due to increased molecular weight from protein glycation that occurred during storage (Loveday and others 2009; Zhou and others 2013). With longer storage, the caseins in the HPN bars formulated with MPC80 (Figure 2), T75 (not shown), and to a lesser extent, those with extruded MPC80 (Figures 4 and 5) had improved resolution on the reduced SDS-PAGE gels. The caseins, which account for 80% protein in any membrane concentrated MPC, do not contain any free sulfhydryl groups, but the α_{s2}-casein (Cys36–Cys40) and the κ-casein (Cys11–Cys88) each have a disulfide bond (Bouguyon and others 2006; Rasmussen and others 1992). Since improved casein separation occurred only when a reducing agent was added, it might involve sulfhydryl-disulfide interchange amongst cysteine-containing β-lg, κ-casein, α_{s2}-casein, and α-la. However, the small change in molecular weight that improved casein separation may have been from glycation of the protein.

The observed β-lg, which contains one free sulfhydryl group, on the non-reduced SDS-PAGE gels, was relatable to the free sulfhydryl content of the HPN bars on each respective storage day. β-lg band intensity from the HPN bars formulated with MPC80 (Figure 2A) or T75 (not shown) remained fairly constant throughout storage, as did the measured free sulfhydryl concentration (Table 1). β-lg solubility decreased with storage for the HPN bar formulated with E65 (Figure 4A) and was absent in the samples prepared with E120 (Figure 5A). The extractable β-lg content increased with storage for the HPN bars formulated with T110 (Figure 3A). The decreasing, missing, and increasing β-lg within the HPN bars formulated with E65, E120, and

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T110, respectively, corresponded with free sulfhydryl content (Table 1). While disulfide bond formation occurred during HPN bar storage, the differences in the SDS-PAGE protein profiles and free sulfhydryl contents show that it cannot be the only source of texture change. The non-reducible PA, represented by band smearing on the SDS-PAGE gels, and especially prevalent in the HPN bars formulated with T110, also played a role in both initial texture and change during storage.

Confocal Micrographs of the HPN Bars during Storage

Initial differences in HPN bar microstructure were more apparent when formulated with extruded MPC80 versus toasted MPC80 and compared with unmodified MPC80 (Figure 6). Similar to published CLSM images of HPN bars formulated with MPC80 (Loveday and others 2009), a green proteinaceous continuous phase was observed on day 0. The intense FITC background staining may have hindered the appearance of Nile red. Its intensity decreased with storage, which allowed for lipid depiction (Loveday and others 2010).

The larger black regions present on the micrographs of the HPN bars formulated with control MPC80, T75, or T110 are non-fluorescing components (McMahon and others 2009). The smaller unstained regions with circular or concave shape might be undissolved, unmodified or toasted MPC80 powder since there was not enough free water in this formulation for complete protein hydration (McMahon and others 2009; Loveday and others 2009). The slightly larger unstained regions with concave shape on the micrographs for the HPN bars formulated with extruded MPC80 are likely undissolved protein particles with limited FITC uptake. Although all protein ingredients were passed through a 250 µm mesh, the extruded MPC80 had a larger size distribution and average diameter when compared with control MPC80. The particles in the control MPC80 were no larger than 100 µm (Crowley and others 2014). Extruded MPC80,

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which was milled using centrifugal mill equipped with a 500 µm mesh, had approximate d_{80} of 250 µm (Vargo 2014). The larger protein particles served as inert structural elements, or structure breakers, that physically disrupted the HPN bar matrix and with limited solubility were less likely to participate in chemical reactions during storage (Purwanti and others 2010). Larger particle size and decreased surface area was one factor that slowed free amine reduction in the HPN bars formulated with extruded MPC80 (Banach and others 2014). The larger sized particles found in E65 did not slow free sulfhydryl content reduction between day 6 and day 13 in the HPN bar formulated with that protein ingredient (Table 1).

Limited microstructural changes were observed in the HPN bars formulated with extruded MPC80 through the 42 day storage period (Figure 6). The green, protein-based continuous phase remained prominent in the HPN bars formulated with E65 or E120. On day 22 and day 42, larger lipid droplets and what appeared to be lipid coated protein particles were seen for these HPN bars. McMahon and others (2009) saw more lipid coalescence in HPN bars that contained more WPI hydrolysate versus native WPI, and those samples remained softer during storage. Additionally, the HPN bars formulated with lower weight percentages of hydrolyzed WPI hardened quicker and the CLSM images showed the development of protein-rich and carbohydrate-rich regions (McMahon and others 2009). The HPN bars formulated with extruded MPC80 maintained an unvarying protein-rich phase throughout storage and HPN bar hardening was slowed by preventing macronutrient (i.e., protein, carbohydrate, fat) phase separation.

CLSM also revealed that microstructural changes were more conspicuous in HPN bars formulated with unmodified or toasted MPC80, which were less texturally stable (Banach and others 2014). During storage, the continuous protein-rich phase on day 0 was penetrated by Nile red stained lipids and blackened, particle-clustered regions. Loveday and others (2010; 2009)

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also reported decreased protein solubility and increased particle clustering during storage of HPN bars formulated with MPC80 or calcium caseinate as their pourable HPN bar formulation set into a firm matrix within a day of manufacture. Although particle clustering was not apparent in WPI formulated HPN bars, unstained regions did develop in those that hardened more rapidly, which were suggested to be carbohydrate-rich regions (McMahon and others 2009). The MPC80 particle surfaces were hydrated during protein bar production, but this surface layer hydration was lost as water molecules moved to associate with polyhydroxy compounds used in the model (Loveday and others 2009). Inadequate protein particle surface hydration in the present study potentially limited fluorescence in the HPN bars formulated with unmodified or toasted MPC80. If water molecules continued to disassociate from the particle surface, it partially explains why more unstained regions appeared during storage.

The water activity of the HPN bars formulated with unmodified or toasted MPC80 increased quickly during the first 4 days at 32°C and then remained fairly constant (Banach and others 2014). Increased water activity would support the notion of water molecule movement to the bulk phase and concurrently less association with the protein. The water activity of the HPN bars formulated with extruded MPC80 did not increase rapidly during the first 4 days of storage, rather it increased slowly and approached the plateau value obtained for the other HPN bars (Banach and others 2014). Water activity measurement employed lacked sensitivity and even though it plateaued early on for the HPN bars formulated with unmodified or toasted MPC80, a slow yet continual shift of water molecules to the bulk phase might be one reason for the disappearance of the continuous green background on the micrographs during storage (Figure 6). On the contrary, CLSM images for the HPN bars formulated extruded MPC80, especially those formulated with E120 and stored 22 and 42 days, had small regions with high levels of FITC

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fluorescence, which confirmed that these regions were not becoming moisture depleted. Therefore, extruded MPC80 was better able to utilize water molecules as a plasticizer in their intermediately bound state, which helped maintain the soluble protein network and improved textural stability during HPN bar storage (McMahon and others 2009; Li and others 2008).

Conclusions

Extrusion decreased and toasting increased the free sulfhydryl content of MPC80. The HPN bars produced with extruded or toasted MPC80 were less and more prone, respectively, to texture change when compared to each other and the control MPC80. The free sulfhydryl content during HPN bar storage increased when formulated with T110, decreased when formulated with E65, and did not change significantly when formulated with T75, E120, or unmodified MPC80. During HPN bar storage, soluble DLPA increased for MPC80 and T75, decreased for T110, remained constant for E65, and were absent in E120. The formation of soluble DLPA and free sulfhydryl change during storage were not consistently relatable to HPN bar texture change. Microstructurally and texturally, the HPN bars formulated with extruded MPC80 exhibited greater stability, and use of this modified protein in HPN bars may be useful in extending textural shelf life.

Acknowledgement

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Tables

Table 1. Free sulfhydryl (SH) content (µmole/g protein ± SD) of the protein ingredients and high-protein nutrition (HPN) bars after storage at 32°C.

Protein	Ingredient SH	HPN Bar SH after Storage				
		day 0	*day 6*	*day 13*	*day 22*	*day 42*
MPC80	4.0 ± 0.3^{bc}	$5.3\pm1.3^{a,z}$	$5.3\pm1.4^{ab,z}$	$5.4\pm1.2^{a,z}$	$4.9\pm0.9^{a,z}$	$5.0\pm1.8^{b,z}$
T75	4.5 ± 0.1^{bc}	$5.3\pm0.9^{a,z}$	$5.5\pm1.4^{a,z}$	$5.2\pm0.9^{a,z}$	$4.7\pm1.0^{a,z}$	$4.5\pm0.7^{b,z}$
T110	5.6 ± 0.7^{c}	$4.0\pm0.9^{a,y}$	$5.5\pm0.8^{a,yz}$	$5.6\pm0.9^{a,yz}$	$6.0\pm0.9^{a,yz}$	$7.1\pm1.2^{a,z}$
E65	3.0 ± 0.2^{b}	$3.7\pm0.8^{a,z}$	$3.4\pm0.9^{b,yz}$	$1.5\pm0.3^{b,y}$	$1.7\pm1.3^{b,y}$	$1.8\pm0.7^{c,yz}$
E120	0.7 ± 0.3^{a}	$0.6\pm0.7^{b,z}$	$0.7\pm0.4^{c,z}$	$0.6\pm0.5^{b,z}$	$0.5\pm0.7^{b,z}$	$0.2\pm0.5^{c,z}$

MPC80, unmodified MPC80. T75 and T110, MPC80 toasted for 4 h at 75°C and 110°C, respectively. E65 and E120, MPC80 extruded at die temperatures of 65°C and 120°C, respectively.

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

^{y,z} Means are significantly different ($P < 0.05$) if they do not share a common superscript within the same row.

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Figures

(Note: All figures color on web but grayscale in print)

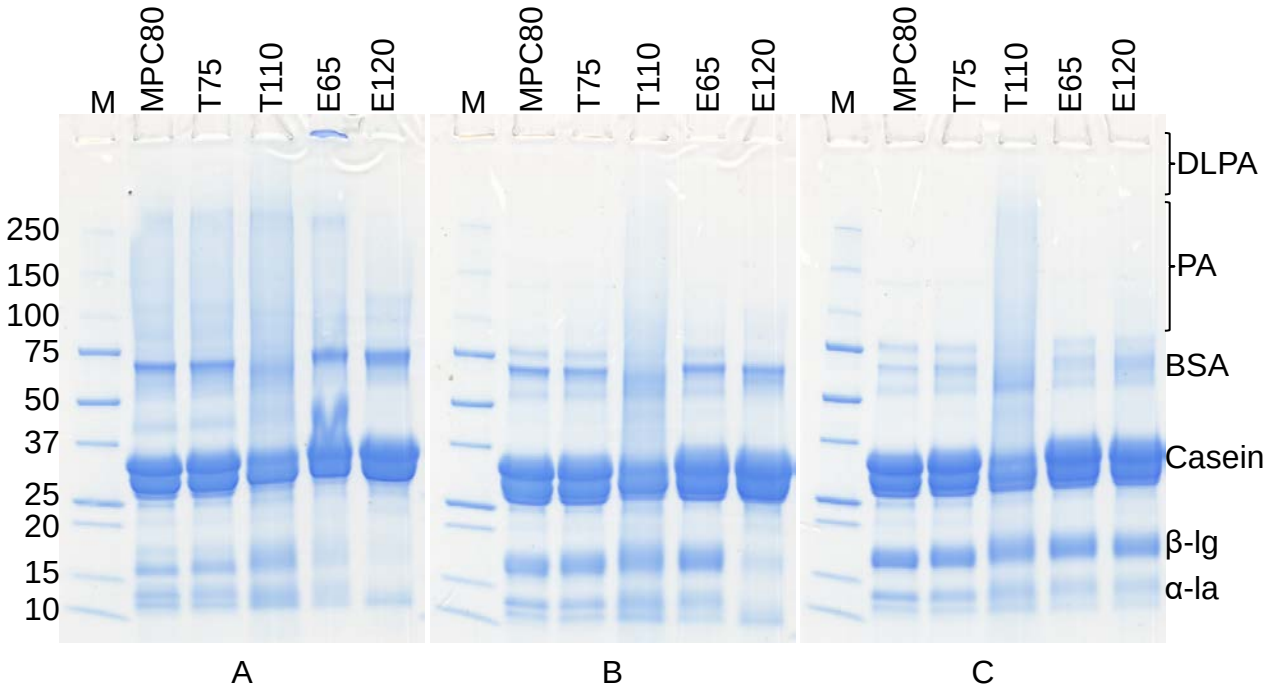

Figure 1–Non-reduced (A) and reduced (B, C) SDS-PAGE protein profiles for MPC80, T75, T110, E65, and E120 extracted with non-reducing (A, B) or reducing (C) buffer. MPC80, unmodified MPC80. T75 and T110, MPC80 toasted 4 h at 75°C and 110°C, respectively. E65 and E120, MPC80 extruded at die temperature of 65°C and 120°C, respectively. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include: α_{S2}, α_{S1}, β, and κ. β-lg, beta-lactoglobulin. α-la, alpha-lactalbumin.

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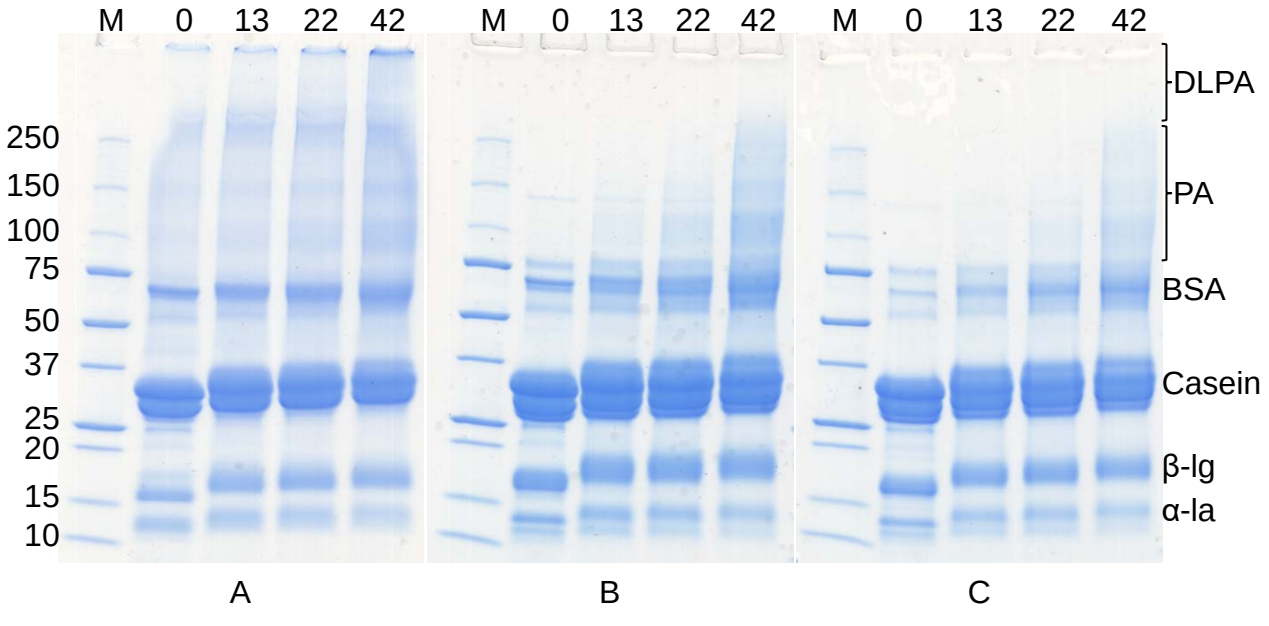

Figure 2–Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with unmodified MPC80 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. Caseins, from high to low molecular weight, include: α_{S2}, α_{S1}, β, and κ. β-lg, beta-lactoglobulin. α-la, alpha-lactalbumin

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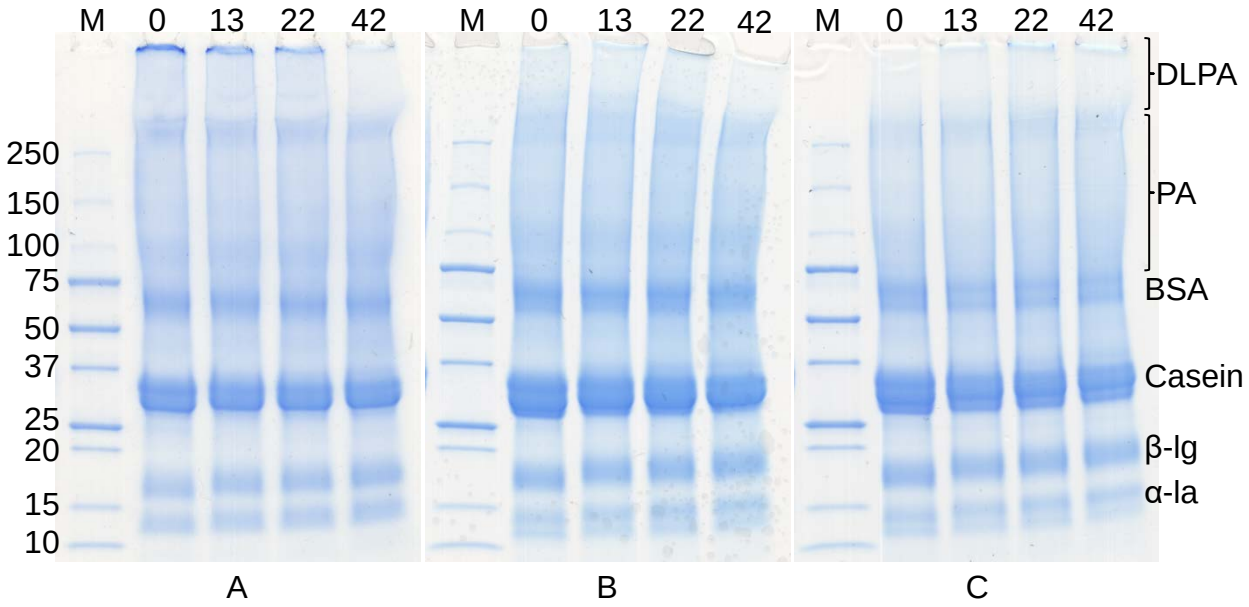

Figure 3–Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with T110 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel. T110, MPC80 toasted at 110°C for 4 h. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include: α_{S2}, α_{S1}, β, and κ. β-lg, beta-lactoglobulin. α-la, alpha-lactalbumin.

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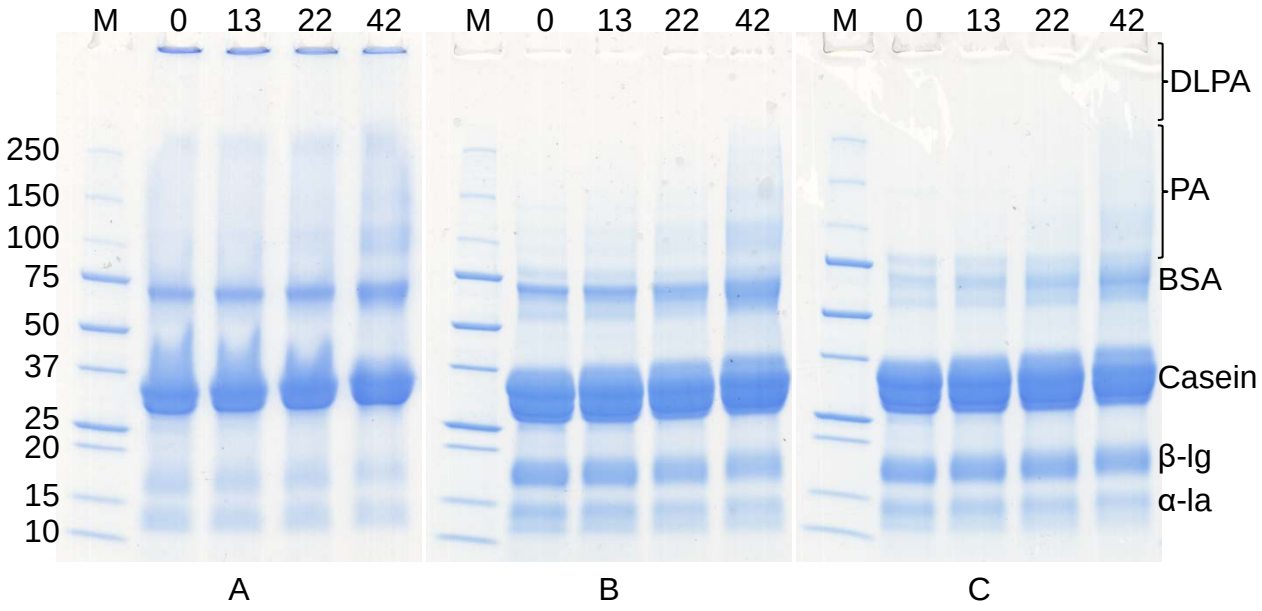

Figure 4–Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with E65 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel. E65, MPC80 extruded at a die temperature of 65°C. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include: α_{S2}, α_{S1}, β, and κ. β-lg, beta-lactoglobulin. α-la, alpha-lactalbumin.

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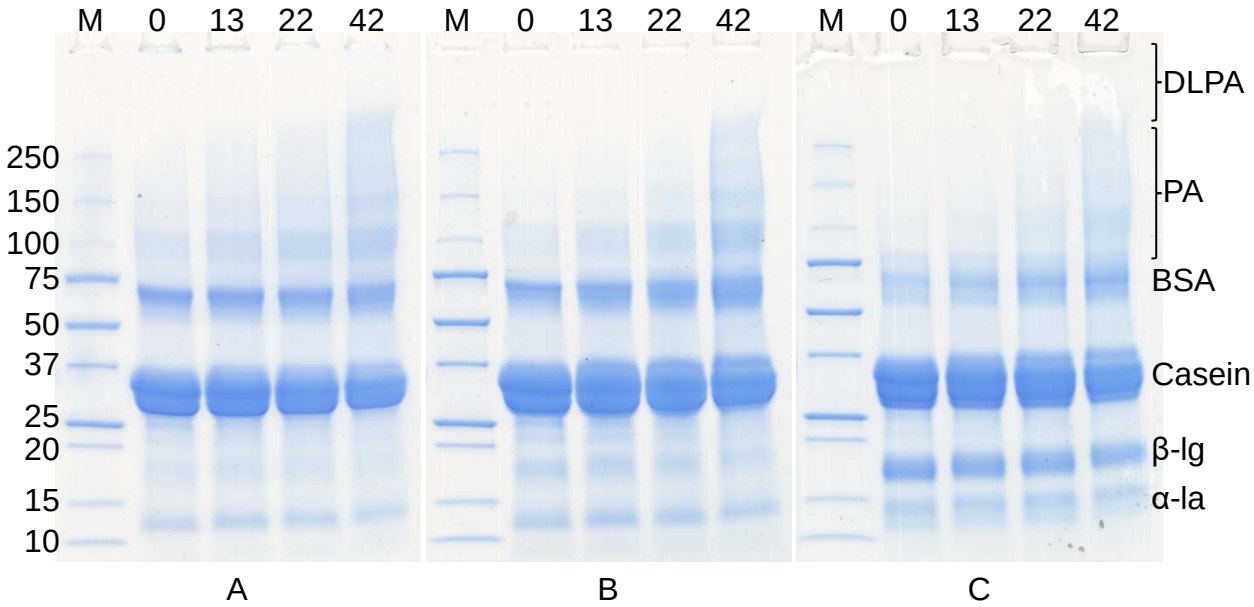

Figure 5–Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with E120 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel. E120, MPC80 extruded at a die temperature of 120°C. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include: α_{S2}, α_{S1}, β, and κ. β-lg, beta-lactoglobulin. α-la, alpha-lactalbumin.

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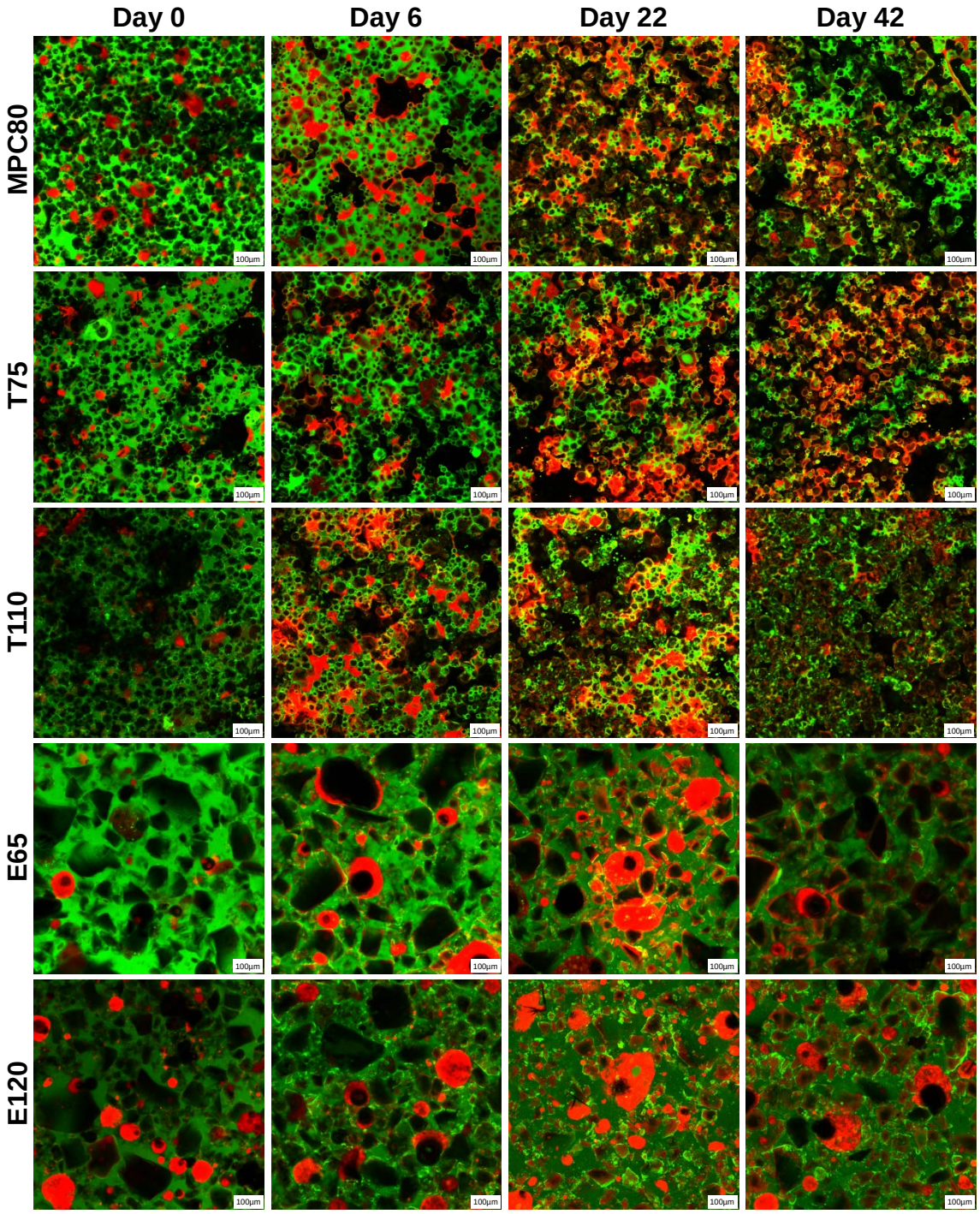

Figure 6–Confocal micrographs (775 µm x 775 µm) of high-protein nutrition (HPN) bars formulated with unmodified (MPC80), toasted (T75 and T110), or extruded (E65 and E120) MPC80. HPN bars (30% protein (w/w)) were stored for 0, 13, 22, or 42 days at 32°C**.** MPC80, unmodified MPC80. T75 and T110, MPC80 toasted 4 h at 75°C and 110°C,

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respectively. E65 and E120, MPC80 extruded at die temperature of 65°C and 120°C, respectively. Fluorescein isothiocyanate (FITC) stained the protein component green and Nile red stained the lipid component red. The length of the white bar on each micrograph represents 100 µm.

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