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Original article

Effects of cysteine addition and heat treatment during non-fat dry milk processing on the plasmin enzyme system

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Abstract – The objective of this study was to measure the effects of cysteine addition and heat treatment on plasmin (PL) activity and plasminogen (PG) activation in reconstituted non-fat dry milk (NFDM). Cysteine was added to a final concentration of 0.16 mg·mL^{-1} to raw, defatted milk and pasteurized at 72 °C for 15 s (low heat, LH) or 100 °C for 30 s (medium heat, MH) prior to spray drying. Both MH fluid milk and NFDM had no PL activity or PG activation, regardless of cysteine addition. For both LH fluid milk and NFDM, there was a significant decrease in PL activity upon cysteine addition ($P < 0.0001$ for both), as well as in PG activation ($P = 0.0003$ for fluid milk, $P < 0.0001$ for NFDM), likely due to the polymerization of PG or binding of PL and PG with other milk proteins. Upon storage of reconstituted powders at 21 °C and 4 °C, PL activity increased with a complimentary decrease in PG-derived PL activity in LH samples, though these changes slowed in samples with added cysteine. Urea-PAGE showed proteolysis of β-casein consistent with PL activities in LH samples. No PL or PG-derived PL activities or proteolysis were observed in stored MH samples. These results suggest that the addition of cysteine prior to the pasteurization and manufacture of LH-NFDM significantly decreases the potential for proteolysis during storage of reconstituted powder caused by the PL enzyme system.

plasmin / plasminogen / cysteine / heat treatment / non-fat dry milk

摘要 – 脱脂乳粉生产过程中半胱氨酸和热处理对血浆酶系的影响。本研究目的是测定添加半胱氨酸和热处理对还原脱脂乳中血浆酶 (PL) 活性的影响和对血浆酶原 (PG) 的激活。在脱脂原料乳中添加 0.16 mg·mL^{-1} 的半胱氨酸, 先经过 72 °C、15 s (低热处理) 和 100 °C、30 s (中度热处理) 的巴氏杀菌,然后进行喷雾干燥。如果不考虑半胱氨酸的添加, 在中度热处理的液态奶及其干燥脱脂粉中都没有检测到血浆酶活性和血浆酶原的激活。而在低热处理的液态奶及其干燥的脱脂粉中, 两个样品中血浆酶活性均随着半胱氨酸的添加呈显著性的降低 ($P < 0.0001$) , 同样两个样品中血浆酶原的激活趋势也相同 (液态奶的 $P = 0.0003$, 干燥脱脂粉的 $P < 0.0001$), 产生着种现象的原因可能是由于血浆酶原的聚合或者血浆酶和血浆酶原与乳中其他蛋白质结合在一起。将还原的脱脂奶分别在 21 °C 和 4 °C 下贮藏, 在低热处理的样品中, 血浆酶的活性和源于血浆酶原的血浆酶活性显著地增加, 但是在添加了半胱氨酸的样品中, 则显著地抑制了血浆酶的活性。 Urea-PAGE 证明了 β-酪蛋白的水解程度与低热处理样品中血浆酶的活性一致。在贮藏的中度热处理样品中, 没有检测到血浆酶或源于血浆酶原的血浆酶活性, 也没有检测到蛋白质的水解。上述试验结果证明预先在脱脂原料乳中添加半胱氨酸, 然后采用低热巴氏杀菌处理和喷雾干燥生产脱脂粉, 这一加工过程可以显著地降低还原脱脂奶在贮藏过程中由于血浆酶系造成的蛋白水解。

血浆酶 / 血浆酶原 / 半胱氨酸 / 热处理 / 脱脂乳粉

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Résumé – Effets de l'addition de cystéine et du traitement thermique au cours de la fabrication de poudre de lait écrémé sur le système enzymatique plasmine. L'objectif de cette étude était de mesurer les effets de l'addition de cystéine et du traitement thermique sur l'activité de la plasmine et sur l'activation du plasminogène dans des laits écrémés en poudre reconstitués. La cystéine était ajoutée à une concentration finale de 0,16 mg·mL^{-1} à du lait cru écrémé et pasteurisé à 72 °C-15 s ("low heat", LH) ou 100 °C-30 s ("medium heat", MH) avant séchage par atomisation. Le lait MH n'avait pas d'activité plasmine ou d'activation du plasminogène, indépendamment de l'addition de cystéine. Pour le lait LH, il y avait une diminution significative de l'activité plasmine après addition de cystéine ($P < 0,0001$), ainsi que de l'activation du plasminogène ($P = 0,0003$ pour le lait avant séchage, $P < 0,0001$ pour le lait reconstitué), probablement due à la polymérisation du plasminogène ou à la liaison de la plasmine et du plasminogène à d'autres protéines laitières. Au cours du stockage à 21 °C et 4 °C, l'activité plasmine augmentait tandis que celle dérivée du plasminogène diminuait dans les échantillons LH, ces changements étant cependant radicalement ralentis dans les échantillons avec ajout de cystéine. L'électrophorèse urée-PAGE montrait une protéolyse de la caséine β en accord avec les activités plasmine dans les échantillons LH. Aucune activité plasmine ou plasmine dérivée du plasminogène ou protéolyse n'était observée dans les échantillons MH au cours du stockage. Ces résultats suggèrent que l'addition de cystéine avant la pasteurisation et la fabrication de poudre LH diminue significativement le risque de protéolyse due au système enzymatique plasmine du lait reconstitué, au cours du stockage de la poudre.

plasmine / plasminogène / cystéine / traitement thermique / poudre de lait écrémé

1. INTRODUCTION

Non-fat dry milk (NFDM) is a widely used dairy ingredient in the food industry, with applications varying from baked goods and confections to soups and sauces [21]. Non-fat dry milk is manufactured by preheating, concentrating, and spray drying pasteurized skim milk [14], which often is reconstituted to produce ultra-high temperature (UHT) milk [19] or utilized for its functional properties in other food formulations. Depending upon the preheat treatment that fluid milk receives during processing, the reconstituted powder exhibits varying degrees of water absorption, emulsion stability, and gelation [22]. Heat treatment of NFDM is classified according to the whey protein nitrogen index [14], as more whey proteins, specifically β-lactoglobulin, bind to caseins with increasing temperatures [18, 21, 26].

Proteolysis of milk proteins, specifically α- and β-casein, has both positive and negative effects on the quality of milk and dairy products. While proteolysis often is caused by bacterial proteases, milk of high microbiological quality still can undergo proteolysis due to the indigenous plasmin (PL) enzyme system [5, 15, 24]. This system consists of plasminogen (PG), the inactive zymogen, which is converted to active PL (EC 3.4.21.7), a serine proteinase, by the action of plasminogen activators (PA), both tissue-type (tPA) and urokinase-type (uPA). Plasmin mainly hydrolyzes β-casein, causing various effects on the quality of dairy products, such as cheese and yogurt, which have been previously reported in the literature [6, 10, 15, 20]. Plasmin clearly has been shown to survive high temperature, short time (72 °C, 15 s) pasteurization conditions [24], though decreases in PL activity and PG activation have been found in milk processed at higher temperatures because of disulfide interactions between PL system components and β-lactoglobulin [26]. However, PG concentrations in fluid milk are 2–20 times that of PL [24]; thus, controlling the activation of PG is of utmost importance for maintaining the quality of stored dairy products.

Prior research has shown that reconstituted milk powders are capable of undergoing PL-like proteolysis [19]. However, research on PG activation in reconstituted

powders has not been reported. This is of particular importance, since activation of PG in powders would increase PL levels, potentially resulting in reduced quality. Prior research in our laboratory has shown that the addition of cysteine before thermal treatment essentially eliminates PL activity and PG activation in model systems and fluid milk due to induced thiol-disulfide interchange reactions, resulting in the polymerization of PG [30]. Thus, cysteine addition may provide a means of controlling PL-induced proteolysis in dairy products. However, the effect of cysteine addition on PL system components in NFDM has not been studied previously. Therefore, the objective of our study was to measure the effects of cysteine addition and heat treatment on PL activity and PG activation in reconstituted NFDM.

2. MATERIALS AND METHODS

2.1. Materials and sources

Bovine PL (5 U·mL^{-1}, #602370) was obtained from Roche Applied Science (Indianapolis, IN, USA). Bovine PG (#416), human urokinase (uPA, #124), and Spectrozyme® PL (SpecPL, #251B) were obtained from American Diagnostica (Stamford, CT, USA). Solutions of PL, PG, uPA, and SpecPL were prepared in modified tris buffer (MTB, 0.1 mol·L^{-1} sodium chloride, 0.05 mol·L^{-1} tris, 0.01% Tween 80, pH 7.6). A micro bicinchoninic acid (BCA) protein assay kit (#23235) was obtained from Pierce Biotechnology (Rockford, IL, USA). Acrylamide (40% with bis, #161-0148), Criterion empty gel cassettes (18-well, #345-9902; and 26-well, #345-9903), Criterion tris-HCl 10.5–14% gradient precast gels (#345-9949), 10 X tris/glycine running buffer (#161-0771), 10 X tris/glycine/SDS running buffer (#161-0732), Laemmli buffer (#161-0737), pre-stained broad range molecular weight standards (#161-0318), ammonium persulfate (APS, #161-0700), and N,N,N',N'-tetramethyl-1,2-ethanediamine (TEMED, #161-0801) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). β-casein (#C6905), β-lactoglobulin (#L3908), κ-casein (#C0406), β-mercaptoethanol (#M7154), and cysteine (#C8755) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Aerobic plate count petrifilms (#6400) were obtained from 3M Microbiology (St. Paul, MN, USA). Fresh raw milk was obtained from the Purdue University Dairy Research and Education Center (West Lafayette, IN, USA).

2.2. Experimental designs and milk preparation

2.2.1. Study 1: Comparison of liquid and non-fat dry milk

Fresh raw milk was collected in triplicate, defatted, and divided into two aliquots. Cysteine was added to one aliquot to a final concentration of 0.16 mg·mL^{-1}. Each aliquot was further divided into two aliquots and pasteurized at 72 °C for 15 s (low heat treatment, LH) or 100 °C for 30 s (medium heat treatment, MH). Total plate counts were completed for raw and pasteurized milk samples according to AOAC Official Method 986.33 [4]. Milk samples then were spray dried using a Niro mobile minor spray dryer (Copenhagen, Denmark) with inlet and outlet temperatures of 120 °C and 80 °C, respectively. Heat classification of the milk powders was determined using the whey protein nitrogen index assay [2]. Both liquid milk and NFDM samples were retained for enzymatic activity analyses. NFDM samples were reconstituted to 10% w/v in deionized-distilled water (DDW) prior to analysis.

2.2.2. Study 2: Storage study of reconstituted non-fat dry milk

All NFDM samples prepared according to Section 2.2.1 (LH, LH with cysteine, MH, and MH with cysteine) were reconstituted, in duplicate, to 10% w/v in 0.02% w/v sodium azide (to prevent microbial growth) and dosed with uPA to a final concentration of 0 or 1 IU·mL^{-1}. Plasmin activity, PG-derived PL activity, and β-casein hydrolysis were measured at 0, 5 and 10 days after storage at 21 °C, and at 0, 2 and 4 weeks after storage at 4 °C. Total plate counts were completed for all samples at the beginning and end of storage at both temperatures.

2.3. Enzymatic activity assays

Liquid milk and reconstituted NFDM samples were prepared in triplicate (Study 1) or duplicate (Study 2) for analysis using the method of Richardson and Pearce [25] with minor modifications. The milk samples (0.75 mL) were vortexed with 0.4 mol·L^{-1} trisodium citrate (0.25 mL) in an Eppendorf tube (1.5 mL capacity) and incubated for 15 min at 37 °C. The samples were centrifuged in the Eppendorf tubes at 15 600× *g* for 15 min, and the supernatant was retained for activity analyses.

To measure PL and PG-derived PL activities, chromogenic activity assays were carried out according to the method of Fajardo-Lira and Nielsen [8] with modifications. For the PL activity assay, supernatant samples (100 μL) were mixed with 1.6 mmol·L^{-1} SpecPL (300 μL) in Eppendorf tubes. For the PG-derived PL activity assay, supernatant samples (100 μL) were mixed with 3.2 mmol·L^{-1} SpecPL (100 μL), 280 IU·mL^{-1} uPA (100 μL), and MTB (100 μL). Both PL and PG-derived PL assay samples were incubated for 1 h at 37 °C and centrifuged at 15 600× *g* for 5 min. Supernatant from the PL and PG-derived PL assay samples (100 μL) were pipetted in triplicate into a 96-well microtiter plate and the absorbance was read at 405 nm and 490 nm with a V_{max} kinetic plate reader (Molecular Devices Co., Menlo Park, CA, USA). The absorbance at 490 nm was subtracted from the absorbance at 405 nm to correct for turbidity. The absorbance values then were converted to PL and PG concentrations (in μg·mL^{-1}) using standard curves [8]. A micro BCA assay was performed on PL used in the standard curve according to the manufacturer's directions to convert U PL·mL^{-1} to μg PL·mL^{-1}. For PG determination, the absorbance value was corrected by subtracting the absorbance obtained from the PL assay to account for pre-existing PL activity in the tested sample.

2.4. Two-dimensional alkaline/SDS-PAGE

Two-dimensional electrophoresis was employed, using alkaline-PAGE in the first dimension and SDS-PAGE under reducing conditions in the second dimension, following similar methods published previously by Havea et al. [12] and Manderson et al. [17] to analyze milk protein complexes formed during heating and processing of NFDM. Reconstituted NFDM samples (10 μL) were added to 240 μL of alkaline-PAGE sample buffer (20% v/v 0.5 mol·L^{-1} tris, pH 6.8; 8% v/v glycerol; 0.009% w/v bromophenol blue). Samples (35 μL) were loaded onto 10.5–14% gradient tris-HCl gels and electrophoresed in 10 X tris/glycine running buffer (diluted 1:10 v/v with DDW) at 200 V for 60 min. Individual lanes from the gels were excised, boiled for 5 min in SDS-PAGE reducing sample buffer (62.5 mmol·L^{-1} tris HCl, 2% v/v sodium dodecyl sulfate, 25% v/v

Table I. Urea-PAGE gel formulation.

Ingredients	Resolving gel (10%)	Stacking gel (4.2%)
Deionized distilled water, mL	1.2	-
Resolving buffer[1], mL	14	-
Stacking buffer[2], mL	-	9
Acrylamide[3] (40% w/v), mL	4.8	1
TEMED[4], μL	10	10
APS[5], μL	80	50
Total volume, mL	20.09	10.06

[1] Resolving buffer = 6.43 mol·L^{-1} urea, 0.557 mol·L^{-1} tris, 0.056 mol·L^{-1} HCl, pH 8.9.
[2] Stacking buffer = 5 mol·L^{-1} urea, 0.067 mol·L^{-1} tris, 0.044 mol·L^{-1} HCl, pH 7.6.
[3] 40% w/v Acrylamide/bis solution (2.6% C).
[4] TEMED = N,N,N',N'-tetramethyl-1,2-ethanediamine.
[5] APS = 10% w/v ammonium persulfate, prepared fresh.

glycerol, 0.5% v/v β-mercaptoethanol, trace bromophenol blue, pH 6.8), and set into a SDS-PAGE gel according to Larson et al. [16]. Protein standards of β-lactoglobulin, κ-casein, PL, and PG were prepared by making 0.25 mg·mL^{-1} solutions in DDW of each protein. An aliquot of each protein standard solution (100 μL) was diluted with Laemmli buffer (190 μL) and β-mercaptoethanol (10 μL) and boiled for 5 min. Molecular weight standards (5 μL) and individual protein standards (10 μL) were loaded onto the gels in addition to the alkaline PAGE strip previously set in the stacking gel. Gels then were electrophoresed at 200 V for 60 min, stained with Coomassie blue (50% v/v methanol, 10% v/v glacial acetic acid, 0.25% w/v Coomassie Brilliant Blue G-250) for 1 h and destained (10% v/v glacial acetic acid, 5% v/v methanol) overnight [9].

2.5. Urea-PAGE and β-casein hydrolysis

Urea-PAGE analysis of the milk samples was used to quantify β-casein according to the method of Andrews [3] with modifications. Standard solutions of 0.4, 0.6, 0.8 and 1.0% w/v β-casein were prepared, in triplicate, using DDW, diluted 1:25 with sample buffer (8 mol·L^{-1} urea, 0.06 mol·L^{-1} tris, 0.04 mol·L^{-1} HCl, 0.1 mol·L^{-1}β-mercaptoethanol, trace bromophenol blue, pH 8.9), and boiled for 5 min. Reconstituted NFDM samples (5 μL) were prepared in triplicate by dilution with DDW (5 μL) and sample buffer (240 μL) and also were boiled for 5 min. Standards and samples (10 μL) were loaded onto hand-cast urea gels (Tab. I). The gels were electrophoresed in 10 X tris/glycine running buffer (diluted 1:10 v/v with DDW) at 200 V for 60–90 min. Gels then were stained and destained as previously described in Section 2.4. Destained gels were scanned using a Bio-Rad Laboratories GS-800 densitometer. Peak optical density of β-casein bands were analyzed, after subtracting the background density, using Quantity-One 1-D software (version 4.5.1, Bio-Rad Laboratories). The densities of the β-casein bands for the reconstituted NFDM samples were converted to concentrations using the standard curve.

2.6. Statistical analysis

Data were analyzed using the general linear model and mixed procedures of SAS

version 9.1 [27]. For data from Study 1, a 2-way analysis of variance (ANOVA) was used to test the main effects (heat treatment and cysteine addition) and interaction for significance. For data from Study 2, a 3-way repeated measures ANOVA was used to test the main effects (heat treatment, cysteine addition, uPA addition, and time) and all possible interactions for significance. The Tukey-Kramer multiple means comparison test was used to compare means at 95% confidence when the model was shown to be significant ($P \leqslant 0.05$). Results for all analyses were considered significant if $P \leqslant 0.05$.

3. RESULTS AND DISCUSSION

3.1. Study 1: Comparison of liquid and non-fat dry milk

Total plate counts for raw and pasteurized fluid milk were below 10^4 and 10^3 CFU·mL^{-1}, respectively (data not shown), demonstrating the milk was of good microbiological quality. Whey protein nitrogen index results confirmed that the milk samples heated at 72 °C for 15 s or 100 °C for 30 s yielded LH- and MH-NFDM, respectively (data not shown).

For both liquid and powder samples, the interaction between heat treatment and cysteine addition was significant for PL activity ($P < 0.0001$ for both), as well as for PG-derived PL activity ($P = 0.0003$ and $P < 0.0001$, respectively). The addition of cysteine significantly decreased PL and PG-derived PL activities in LH fluid milk and reconstituted NFDM (Fig. 1), likely due to the polymerization of PG as previously shown by Stevenson [30]. However, MH fluid milk and reconstituted NFDM showed no PL or PG-derived PL activity, regardless of cysteine addition (Fig. 1). This observation is likely due to the denaturation of whey proteins at high temperatures, specifically β-lactoglobulin [21]. The unfolding of β-lactoglobulin exposes a free sulfhydryl group that binds to PL and PG via sulfhydryl/disulfide interchanges, thus rendering it inactive, which has been demonstrated by several authors [1, 18, 26]. The addition of cysteine decreased PL and PG-derived activities without higher heat treatments, as in the LH fluid milk and NFDM, because cysteine initiates thiol-disulfide interchange reactions, resulting in polymerization of PG or binding of PL and PG with other free sulfhydryls in milk proteins. However, heat is necessary to induce this reaction, as the addition of cysteine without heat did not inactivate PL or PG in model systems [30]. This could be because PG must be heated in excess of 62 °C to unfold and expose its disulfide bonds [7].

Two-dimensional gel electrophoresis of reconstituted NFDM samples revealed several protein agglomerates, especially within the second band from the 1-dimensional gel strip (Fig. 2). In both LH samples, a band parallel with the PG standard was present near the middle of the 1-dimensional strip, corresponding with band 2 in the 1-dimensional gel. Additionally, there was another band with similar molecular weight as the PG standard for the LH control sample (circled), corresponding to band 1 in the 1-dimensional gel, while this band was not present in the LH sample with cysteine. The difference in band patterns between the LH samples with and without cysteine could indicate that PG binds with other milk proteins when heated with cysteine, resulting in the observed decrease in PG-derived PL activity. Also present in band 1 from the 1-dimensional gel was κ-casein, which might have been associated with PG. This is consistent with findings that PL and PG are known to associate with casein micelles, of which κ-casein constitutes the exterior [5]. Cysteine addition might have disrupted any association between

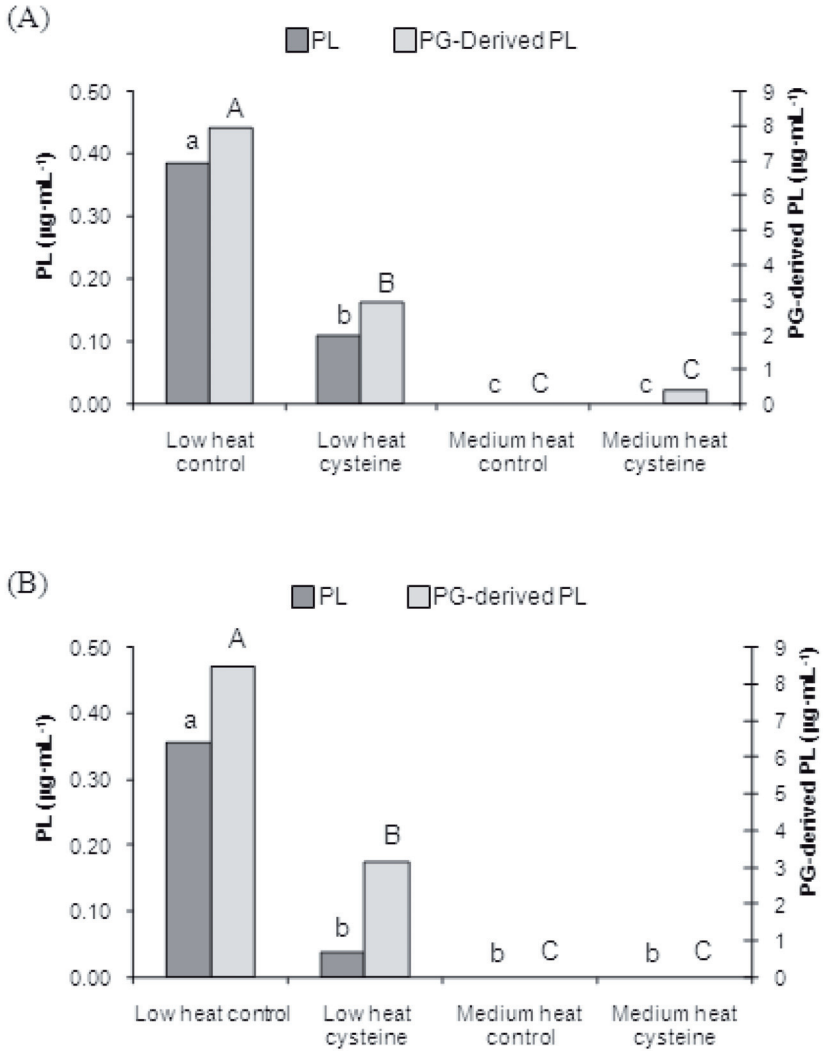

Figure 1. Plasmin (PL) and plasminogen (PG)-derived PL in low and medium fluid milk (A) and reconstituted NFDM (B). Bars represent the mean of triplicate results. Within each graph, lower case letters indicate statistical significance for PL, while upper case letters indicate statistical for PG-derived PL ($P \leqslant 0.05$).

κ-casein and PG, allowing PG to participate in disulfide linkages with itself or other proteins. However, it cannot be ascertained from the 2-dimensional gels whether PG polymerized or bound with other milk proteins. While PG polymerizes in a model system [30], it is more likely that PG bound with other milk proteins, as each PG molecule would have a greater chance of coming into contact with casein

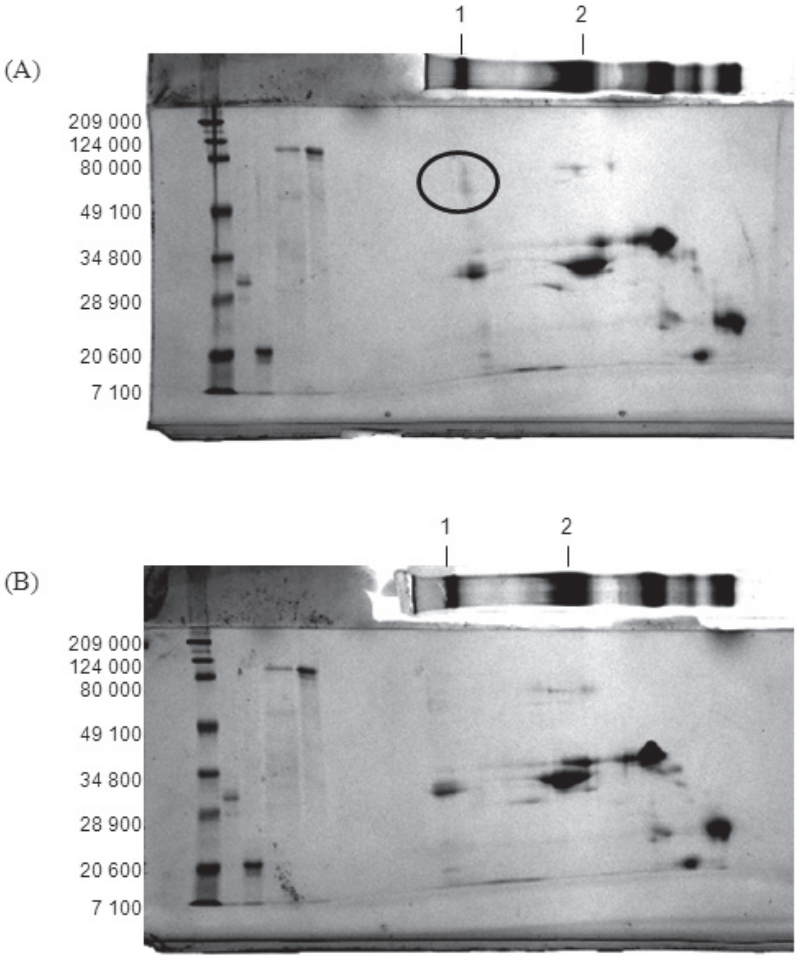

Figure 2. Two-dimensional alkaline/SDS-PAGE of reconstituted low heat NFDM without cysteine (A) and with cysteine (B). For all gels, lanes: 1 = molecular mass standards (in $g \cdot mol^{-1}$), 2 = κ-casein, 3 = β-lactoglobulin, 4 = plasmin, 5 = plasminogen.

or whey proteins than with another PG molecule. Whey and casein agglomerates have been reported previously in NFDM [22, 31]; however, this is the first report that suggests PG could be present in such agglomerates. No differences in band patterns were apparent between the MH samples with and without cysteine (data not shown), which was consistent with absence of PG-derived PL activity in the two samples.

3.2. Study 2: Storage study of reconstituted non-fat dry milk

TPC on reconstituted NFDM samples were < 1 $CFU \cdot mL^{-1}$ at both the beginning and end of storage studies at 4 °C and 21 °C (data not shown). Thus, any proteolysis observed in the samples was likely not due to bacterial proteases, but rather the result of indigenous enzymatic activity. Though PL exhibits proteolytic

activity against β-casein, other native milk enzymes such as cathepsin D and cysteine protease have been shown to degrade milk proteins [29].

Plasmin and PG-derived PL activities for reconstituted NFDM samples stored at 21 °C and 4 °C are shown in Figure 3. Cysteine reduced PG activation over time in LH-NFDM with and without uPA at both storage temperatures. Little PL or PG-derived PL activities were detected in MH-NFDM, likely due to disulfide interactions between milk proteins, as previously discussed. It is relevant that cysteine decreases PG activation, especially in the presence of exogenous uPA, as several studies have confirmed that uPA are heat stable and associated with somatic cells [11, 13]. This implies that milk with higher somatic cell counts has a greater propensity for PL-induced proteolysis, even after pasteurization. Even though milk does contain PL inhibitors, which have recently been shown to be more heat stable than previously thought, PG activation into greater quantities of PL can overcome the competitive inhibition afforded by PL inhibitors [23].

For PL activity in samples stored at 21 °C, the main effects of heat treatment, uPA addition, and time and all interactions were significant ($P \leqslant 0.05$), but cysteine addition was not significant. However, all main effects and interactions were significant for samples stored at 4 °C ($P \leqslant 0.05$). Plasminogen activation occurs at a much slower rate under refrigerated conditions [28], yielding less active PL and increasing the relative effect observed due to cysteine addition. For PG-derived PL activity in samples stored at 21 °C, the model was not significant ($P > 0.05$), although decreases in activity were shown for all LH samples, regardless of cysteine and uPA addition, complimenting the increases observed in PL activity (Fig. 3B). All main effects and interactions were significant for samples stored at 4 °C ($P \leqslant 0.05$).

Urea-PAGE of samples stored at 21 °C clearly showed increased proteolysis over time in LH control samples (Fig. 4A) because of the increase in PG activation and subsequent PL activity. Cysteine greatly decreased proteolysis in LH samples, leaving more β-casein than present in control samples, even in the presence of exogenous uPA (Fig. 4A). Similar trends were observed in samples stored at 4 °C (Fig. 4B), although proteolysis was less severe. Little or no proteolysis was apparent in any MH-NFDM samples, regardless of storage temperature, cysteine addition, and uPA addition. Residual β-casein remaining in samples stored at 21 °C and 4 °C is shown in Figure 5. While the model was not significant for either temperature ($P > 0.05$), the decreases in β-casein content over time clearly compliment the increases observed in PL activity (Fig. 3).

Several studies have been conducted on the effect of heat treatment on PL enzyme system in fluid milk, including the effect of various UHT preheat treatments on PL-like proteolysis in reconstituted LH-NFDM [19]. However, this is the first report of directly measuring PL activity and PG activation in NFDM of different heat classifications. It is clear from this study that PG activation occurs in reconstituted NFDM, which increases PL activity to result in greater proteolysis. This also is the first report that cysteine decreases indigenous enzymatic activity in NFDM, thus reducing proteolysis. This finding is especially important considering that NFDM is added to several food formulations in which enzymatic activity could have potentially adverse effects on product quality.

4. CONCLUSIONS

This study measured PL and PG-derived PL activities in both LH and MH-NFDM and the fluid milk from which

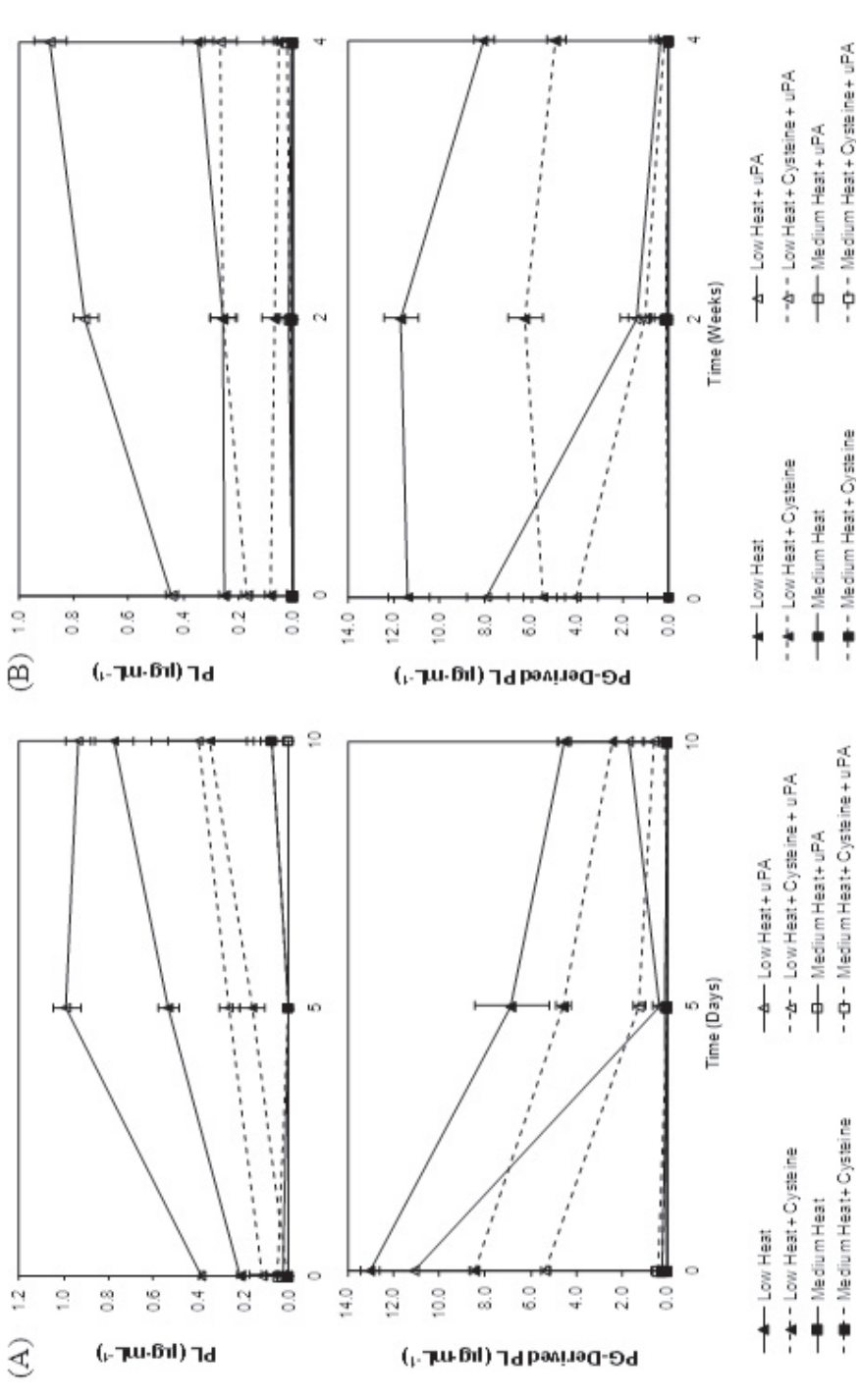

Figure 3. Plasmin (PL) and plasminogen (PG)-derived PL in reconstituted low and medium heat NFDM with and without cysteine and urokinase-type PG activator (uPA) stored at 21 °C (A) and 4 °C (B). Data points represent the mean of duplicate results. Error bars represent the standard error of the mean.

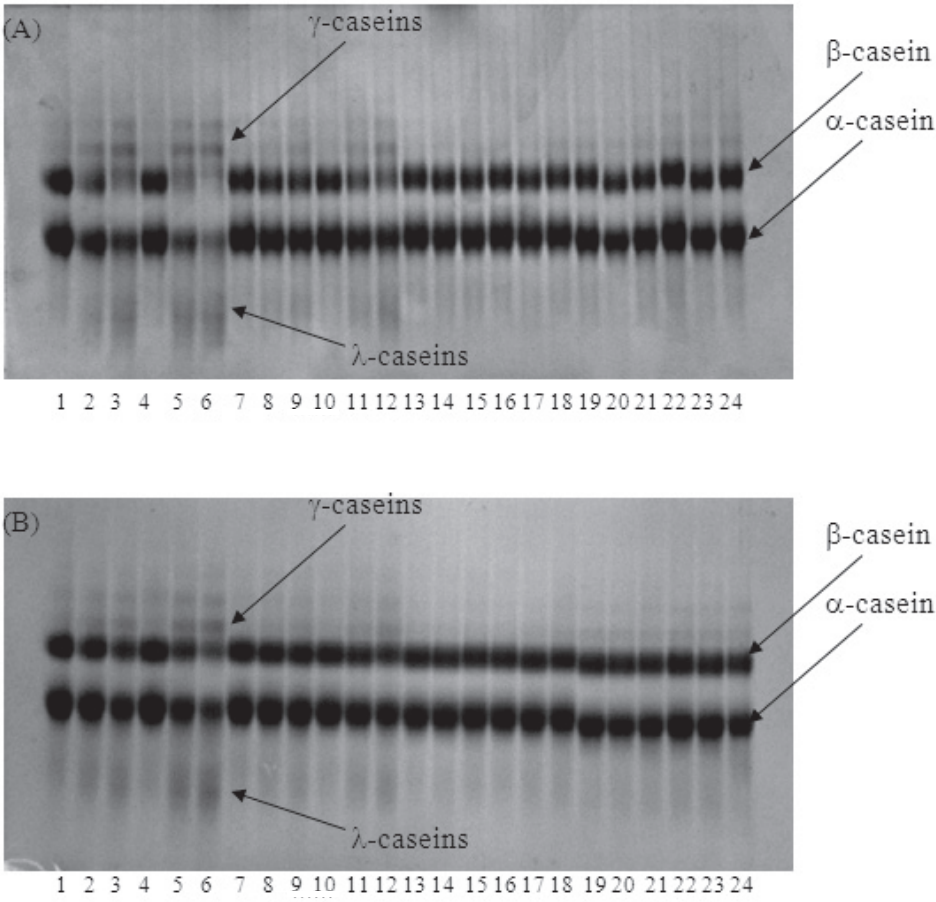

Figure 4. Urea-PAGE of reconstituted low heat (LH) and medium heat (MH) NFDM samples with and without cysteine and urokinase-type plasminogen activator (uPA) stored at 21 °C (A) and 4 °C (B). Every three lanes represents samples stored for 0, 5, and 10 days at 21 °C or 0, 2, and 4 weeks at 4 °C. Lanes: 1–3 = LH, 4–6 = LH + uPA, 7–9 = LH + cysteine, 10–12 = LH + cysteine + uPA, 13–15 = MH, 16–18 = MH + uPA, 19–21 = MH + cysteine, 22–24 = MH + cysteine + uPA.

it was made. While LH samples exhibited enzymatic activity, none was detected in MH samples, due to induced disulfide bonding between PL, PG, and milk proteins. It is possible that the addition of cysteine induced thiol-disulfide interchange reactions at lower heating temperatures, thus decreasing observed PL and PG-derived PL activities in LH samples, while not affecting the already undetectable activity in MH samples. Storage studies showed that PG conversion to active PL occurred over time in LH samples, which increased with the addition of exogenous uPA, while cysteine reduced PL activity over time. Proteolysis results

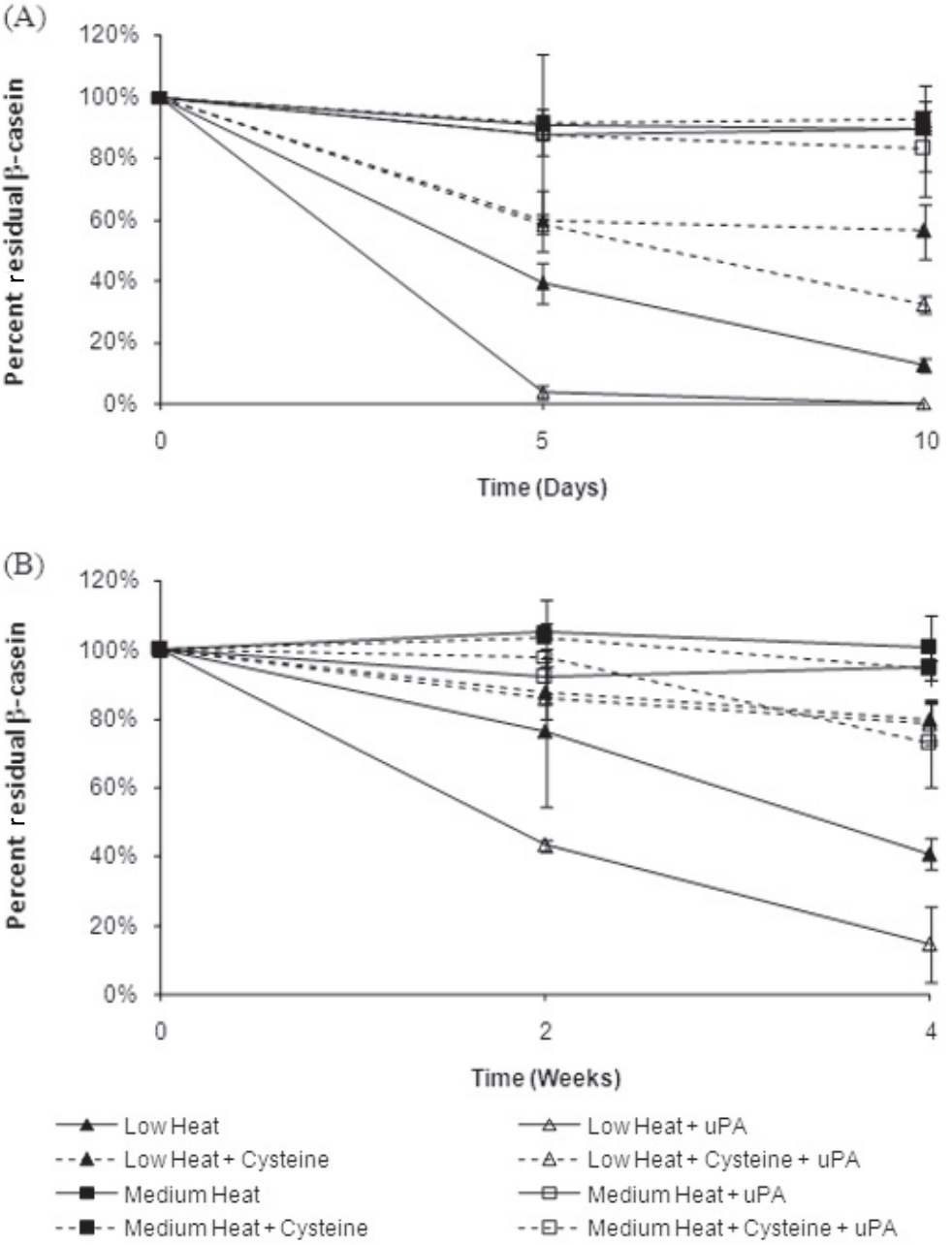

Figure 5. Percent residual β-casein in reconstituted low and medium heat NFDM with and without cysteine and urokinase-type PG activator (uPA) stored at 21 °C (A) and 4 °C (B). Data points represent the mean of duplicate results. Error bars represent the standard error of the mean.

complemented the observed increases in PG activation and PL activity, demonstrating that cysteine decreases proteolysis due to PL and PG present in reconstituted NFDM. These results imply that the addition of cysteine prior to the pasteurization and manufacture of LH NFDM is an effective means of decreasing the potential for PL-induced proteolysis in any product to which LH-NFDM is added.

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