Influence of Fat-Replacing Ingredients on Process and Age Induced Soluble Nitrogen Content and Ultrastructure of Lowfat Cheddar Cheese

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Influence of fat replacers on process and age (up to 6 months at 5°C) induced water soluble nitrogen generation in lowfat (5%, w/w) Cheddar cheese was investigated. Treatments with cellulose plus guar gum based Novagel NC200 (NOV), starch based Stellar (STEL), and protein based Dairy Lo (DL) and Simplesse (SIMP) were compared with lowfat (LFC) and fullfat (32%) controls. Nitrogen contents in aqueous extract were determined by Kjeldahl method. Process induced proteolysis followed the order DL > NOV > SIMP > STEL > LFC > FFC. Age related proteolysis, which generated hydrophobic peptides, was rapid in the first 3 months, and resulted in an increase of non-protein nitrogen compared to post-process content by 113, 50, 17 and 14%, respectively for LFC, STEL, FFC, and NOV. Protein based DL showed a reduction in this value by 22% and SIMP showed no change. The ultrastructure of the DL treated cheese matrix appeared closer to FFC compared to all other treatments and the LFC in that smaller (<3μ) globular occlusions, representing butter-fat droplets, were seen compared to the dominance of larger (>3μ) non-globular occlusions in the NOV and SIMP treatments. While the LFC showed signs of globular coalescence and enlargement, DL, and to a lesser extent STEL treatments showed markedly less. The possible reason for the treatment effect on the cheese matrix ultrastructure is discussed.

Keywords: Proteolysis, peptide, fat-replacer, microstructure

Introduction

The US production of cheese is higher than ever before. In 2004, more than one-third of all cow’s milk was used to make more than 4000 metric tons of cheese (Anonymous, 2005). Between 1990 and 2000, the per capita consumption of cheese increased by more than 21% making it a favorite dairy food (Anonymous, 2002). This makes cheese manufacturing a potentially lucrative growth area for the dairy industry. Since high fat diets are associated with chronic illnesses (Anonymous, 1996) and the US food intake guidelines recommend consumption of lowfat dairy products, the demand for lowfat products is also ever increasing. Unfortunately, acceptability of low fat products is limited since fat plays an important role in texture and flavor development and release. Lowfat cheese in particular tends to be devoid of flavor and acceptable organoleptic quality is difficult to produce (Foda et al., 1974, Olson and Johnson, 1990). Full-fat Cheddar cheese contains around 32% fat which influences body and texture by filling interstitial spaces in the protein and mineral structural mesh (Aryana and Haque, 2001a). Fat also affects the water to protein ratio that regulates firmness and elasticity of cheese by increasing moisture retaining property of the curd (Olson and Johnson, 1990).

One approach to development of lowfat cheeses is by using different fat replacing ingredients. This may involve use of already available fat-replacers; creative use of functional ingredients such as proteins, emulsifiers, stabilizers and fibers; use of new or not normally used technologies in the preparation of full-fat product; or combinations of these approaches. The mechanism of how texture and volatiles are influenced by far-replacers is not clearly elucidated in the literature. Process and age induced proteolysis is also imperative since water soluble nitrogen contented of cheese profoundly impacts flavor perception (Molina et al., 1999). While proteins provide body and flavor fullness, peptides and free amino acids such as methionine and leucine contribute most to cheese flavor (Aston and Creamer, 1986). How age and process (i.e., ripening, cooking and renneting) related generation of soluble non-protein nitrogen is affected by ingredients is not known. It is conceivable that cheese matrix proteins may be more readily solubilized in the aqueous environment of the mouth when amphipathic or soap-like peptides are present as a result of casein proteolysis (Haque,
In addition to two intrinsic enzyme activity intensive steps in cheese manufacture, ripening and cooking, an extra process parameter that is unique to Cheddar cheese manufacture is Cheddaring; a multi-step process that gives Cheddar its unique flavor (Kosikowski, 1982). During Cheddaring, the drained curd is cut into loaves, stacked, turned every 10 min and restacked until organic acid content of whey is between 0.5 and 0.7%. Such high starter and non-starter activity implies high level of hydrolysis prior to the aging process.

Based on the above observations, we hypothesize that process and age related cheese proteolysis is impacted differently by fat-replacers resulting in differences in textural and organoleptic quality of the end product. Since proteolysis takes place in the aqueous phase, solubility of the substrate protein is a parameter of interest. The objective of this study was therefore to investigate the influence of some commercially available protein and carbohydrate based fat-replacers, used previously in our related studies, on changes in water soluble protein and water soluble non-protein nitrogen content of lowfat Cheddar cheese and their apparent influence on ultrastructure of the cheese matrix.

Materials and Methods

Materials  Protein based fat-replacers, Dairy Lo (DL) and Simplesse (SIMP) were obtained from Pfizer Inc., Groton, CT and Nutrasweet Company, Deerfield, IL, respectively. Stellar (STEL), which is a starch-based, was from A.E. Staley Manufacturing Company, Decatur, IL. Novagel (NOV), which is a mixture of 90% cellulose and 10% guar gum, was from FMC Corp., Philadelphia, PA. Mixed strain starter culture (#98) (Lactococcus lactis and Lactococcus cremoris) and rennet were from Chr. Hansens Lab., Milwaukee, WI. Annato color was from Miles Lab., Inc., Elkhart, IN. Nonfat dry milk powder (NFDM) was from the Mississippi State University Dairy Plant and skim milk was obtained after separating (centrifuging) fresh milk from a mixed cattle herd (16.7% Jersey and 83.3% Holstein) at the Mississippi State University South Dairy Farm. Trichloroacetic acid (TCA) (#T8657) was from Sigma-Aldrich, St. Louis, MO, USA. Chloroform was from Fisher Scientific, Fair Lawn, NJ. Ethanol (100%) was purchased from Electronic Microscopy Sciences, Fort Washington, PA. All other reagents were analytical grade.

Cheddar cheese manufacturing  Cheddar cheese manufacturing procedure of Kosikowski (1982) was used. Raw and skim milk was standardized to 0.5% milk fat for lowfat Cheddar control (LFC) and for the lowfat treatments, and to 3.2% milk fat for full-fat Cheddar control (FFC). The 0.5% milk fat containing standardized milk was divided into five portions. One portion was used to manufacture the LFC and remaining four were for the treatments. As per manufacturers’ recommendations, SIMP, DL, STEL, and NOV were respectively added (w/v) to the cheese milk at the rate of 1.5, 1.0, 1.2 and 0.2% (plus 0.5% NFDM). Solids content was adjusted to the same starting level with NFDM for all experiments. The cheese milks were pasteurized using a high temperature short time plate heat exchanger system (Yunior SS, AVP Crepaco, Tonawanda, NY) at 71°C for 15 sec, equilibrated to 32°C, mixed with mixed strain starter culture (0.35% v/v), set for one hour followed by addition of 20 mL/100 kg of Annato color and 19 g per 100 kg of single strength rennet. Cutting was after 30 min at 32°C with vertical and horizontal curd knives having 6.35 mm between the wires. The cut curd was allowed to settle for 10 min. Cooking was by increasing temperature from 32°C to 38°C within 30 min (1°C/5 min) and holding at that point for 75 min. Whey was drained while the curd was cut into approximately 15 cm wide loaves, stacked, and turned every 10 min. This was repeated until the titratable acidity of the whey reached 0.48% (v/v). The curd loaves were mechanically milled and salted at the rate of 2% (w/w) of the weight of the milled curd. The salted curd as allowed to sit for 15 min after which it was hooped and pressed overnight (14 h) at 1.75 kg/cm². The pressed cheese was cut into 0.32 kg blocks, vacuum packaged in clear poly vacuum bags, sealed and placed in a curing room (5°C) for aging up to 6 mos.

Compositional analysis  Fat content of cheese was analyzed using the Babcock method as described by Richardson (1985). All pH values were determined using a Fisher Accumet model 610 pH meter (Fisher Scientific, Inc., Norcross, GA). Total solids and moisture content were determined by weight difference following 24 h oven (Napco Model 630) drying. The cheese samples were analyzed for ash content using a muffle furnace (Blue M Electric Corp. Blue Island, IL) (Anonymous, 1990a).

Aqueous extraction (AE) of cheese  A cheese sample (4 g) was suspended in distilled water by grinding with a pestle and mortar. The suspension was quantitatively transferred into a 100 mL volumetric flask. The mortar was rinsed several times with additional water and each rinsing dilution was added into the flask until volume was adjusted to 100 mL. The flask was equilibrated to 50°C for 1 h in a thermostated water bath (±0.1°C), filtered through a Whatman No 40 filter paper (Whatman International Ltd., Maidstone, England), flash frozen in liquid nitrogen, lyophilized, and stored at -4°C over desiccants in an evacuated bell jar until needed. This powder is referred to as the aqueous extract (AE).

Nitrogen determinations  Total nitrogen in cheese  Samples were analyzed for protein using Labconco Semi-Micro Kjeldahl digestion and distillation units (Labconco Co., Kansas City, MO) using a modification of AOAC method (Anonymous, 1990b) as follows: Two grams of Kjeltab potassium sulfate/copper sulfate (Fisher Scientific Co., Ltd., Fair Lawn, NJ) were added along with 0.25 g of cheese sample to a 100-mL semi micro Kjeldahl flask. Digestion was accomplished with 5 mL of concentrated sulfuric acid followed by distillation using a Labconco (Model 6500) distillation unit. Sodium hydroxide (50% w/w) was used to neutralize the sample during distillation. Tashiros indicator was used to determine the distillation end point. Hydrochloric acid was used for titration. This is referred to as the “Total N”.
**Total soluble nitrogen in aqueous extract (TNAE)** A 100 mg sample of freeze dried AE was used to determine TNAE of the sample by Kjeldahl method as described above. The TNAE, which comprised primarily of water soluble proteins, small and large peptides and free amino acids, is expressed as a percentage of total nitrogen in cheese.

**Soluble large peptide and protein nitrogen in aqueous extract (Pro-NAE)** A 100 mg sample of freeze dried AE was dissolved in 10 mL distilled water, 10 mL of 30% (w/v) TCA was added and the solution was kept at 22°C for 4 h. The suspension was centrifuged at 10,000 rpm, 25°C for 10 min. The supernatant was discarded and precipitate, comprising of large peptides and proteins, was used to determine Pro-NAE by Kjeldahl method as described above. Results are expressed as a percentage of the total nitrogen.

**Soluble non-protein nitrogen in aqueous extract (Pep-NAE)** Soluble non-protein nitrogen content in aqueous extract, comprising mainly of small peptides that are not precipitated by 15% (w/v) TCA and free amino acids, was calculated by difference (Pep-NAE = TNAE-Pro-NAE) and expressed as a percentage of the total nitrogen in cheese.

**Scanning Electron Microscopy (SEM)** Sample preparation was according to Aryana and Haque (2001b). Cheese samples were cut at 22°C into 4 mm cubes, fixed in 1.4% glutaraldehyde overnight at 4°C, rinsed in six changes of distilled water during a 30 min period, defatted in six changes of chloroform over 1 h and dehydrated in an increasing gradient series of 35, 50, 70 and 95% (15 min per change) ethanol extraction steps. Final dehydration was in three 20 min interval changes of 100% ethanol followed by critical point drying (Polaron critical point dryer, Ted Pella, Inc., Redding, CA) and mounted with double sided carbon sticky tape on aluminum stubs (Electron Microscopy Sciences, Fort Washington, PA). Mounted samples were sputter coated (Polaron sputter coater, Watford, UK) with 20 nm gold palladium and stored until needed in a desiccator at 22°C. Five representative cheese blocks of each age group of each of the three replications were mounted as one replication per stub. From these five blocks per stub, one representative block was selected at random. On this block many fields were looked at : a, b, c, d. Rows without common superscript are significantly different (P<0.05).

**Results and Discussion**

There were no significant differences in the fat content and pH of lowfat treatments and control after processing (0 month) (Table 1). All treatments significantly increased the moisture content of the lowfat cheeses compared to controls. The DL and STEL treatments had significantly higher moisture content than all the rest.

Increasing moisture is one way to improve the hard, firm body that is typical of lowfat cheese (McGregor and White, 1990; Olson, 1984). These fat-replacers appeared to increase the water holding capacity of the cheese matrix. Cheddar cheese is a viscoelastic solid in which the caseins form protein matrix with entrapped fat and moisture. This core matrix of cheese consists of an extensive network of $\alpha_s$ casein molecules and the strength of this network is dependent on water, fat and mineral content of cheese (Creamer and Olson, 1982).

**Table 1. Composition of lowfat (5%) Cheddar cheese at the beginning of aging**

<table>
<thead>
<tr>
<th>Component</th>
<th>Controls 2</th>
<th>FFC</th>
<th>DL</th>
<th>Treatment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>46.0± 39.0*</td>
<td>50.0± 47.4± 49.0± 50.2±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (%)</td>
<td>5.0± 32.0±</td>
<td>5.2± 5.2± 5.0± 5.0±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>3.4± 3.2±</td>
<td>3.3± 4.0± 3.6± 3.7±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>5.0± 5.1±</td>
<td>5.1± 4.9± 5.0± 5.2±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (%)</td>
<td>32.8± 26.4±</td>
<td>32.9± 33.8± 32.9± 34.0±</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rows without common superscript are significantly different (P<0.05).

1 Mean of three replicates.
2 The LFC was the lowfat control (5%) containing the same amount of solids, fat and approximately the same amount of protein. The FFC, was the full-fat control (32% fat) cheese produced by exactly the same method.
3 Treatments are lowfat (5%) Cheddar cheese containing the fat-replacers; DL = Dairy Lo NOV = Novagel, SIMP = Simplesse, and STEL = Stellan.

Data from three replicates were used and analysis of variance (ANOVA) was done with the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) (Anonymous, 2001). Means were separated by use of least significant difference (LSD) test. Significant differences were determined at $a = 0.05$ (Steele and Torrie, 1980).
from hydrolysis, this could be contributed to by increased cheese matrix solubilization or soap-like amphipathicity of the ingredients. Formation of a looser cheese matrix that fell apart more easily would also potentially facilitate rapid solubilization. The highest TNAE was seen for DL followed by the other treatments in the order: NOV > SIMP > STEL > LFC = FFC. This order was not comparable to the initial protein or moisture content (Table 1).

At this post-process and pre-aging stage, all treatments had similar Pro-NAE (Fig. 1B), and significantly higher Pep-NAE content (Fig. 2) than the controls. On aging, the average Pro-NAE content for all the cheeses almost doubled after 6 months of maturation as did TNAE. After 6 month, Pro-NAE concentrations were closer though still significantly different, and varied in the order: SIMP > NOV > DL > STEL > LFC > FFC. It is interesting that the position of FFC in the order was reversed after 4 months of ripening.

The intensity of the difference of Pep-NAE, both inter-treatment and treatment vs. the controls was significant (Fig. 2). This reflected marked differences in the rate and degree of proteolysis. At the beginning of aging Pep-NAE was significantly high being 2.4, 5.4, 4.2, 4.0, 3.4, 1.9% (of total cheese nitrogen), respectively for LFC, DL, NOV, SIMP, STEL and FFC (Fig. 2).

Peptide content in FFC increased slowly at the start of aging process and became rapid after about 2 months (Fig. 2). In the LFC and all treatments except DL, increase in Pep-NAE was very rapid in the first two months and continuous until the end. The DL treatment, which had significantly higher initial peptide content, showed a gradual decrease in the peptide content with aging. This reflected residual peptidase activity that may be from the starter lactococci and the numerically dominant non-starter lactobacilli (Williams et al., 2002).

Much of the rapid change during aging, as reflected by the Pep-NAE (Fig. 2) formation, took place in the first 3 months of aging for the treatments and the LFC. Increase of non-protein nitrogen compared to post-process content was by 113, 50, 17 and 14%, respectively for LFC, STEL, FFC, and NOV. Protein based DL showed a reduction in this value by 22% and SIMP showed no change. There was little or non change in the proteolytic status of the treatments and LFC after the aging period. On the other hand, the FFC continued to age, and at 6 months, its peptide content reached 3.9% (compared to 1.8% at 0 month) and was still rising (Fig. 2). This indicates that unlike in the lowfat cheeses, residual bacterial activity persisted in the FFC even=6 months of aging. Is our study related to aging-induced changes in population of Lactococci, Lactobacilli and aerobic microorganisms in lowfat and fullfat Cheddar cheese, we noted that the bacteria preferred the cheese matrix/butterfat interface and persisted longer in full fat cheese than in lowfat cheese (Haque et al., 1997a, Haque et al., 1997b).

Over the full aging period, the average TNAE for all the cheeses almost doubled (Fig. 1). The sharpest increase was seen for the LFC that doubled its TNAE content.

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**Fig. 1.** Effect of treatments and aging on, (A), total nitrogen in aqueous extract (TNAE) and, (B), protein nitrogen in aqueous extract (Pro-NAE) of Cheddar cheese with and without fat-replacers. The x-axis represents the age in months and the y-axis represents soluble nitrogen as percentage of the total nitrogen in the cheese. The abbreviation LFC, DL, NOV, SIMP, STEL and FFC, represent, lowfat control, Dairy Lo, Novagel, Simplesse, Stellar and full-fat control cheeses, respectively. Dissimilar letters above bar in each age cluster denotes statistical difference at α = 0.05.

**Fig. 2.** Effect of treatments and aging on peptide and non-protein nitrogen content in aqueous extract (Pep-NAE). The x-axis represents the age in months and the y-axis represents nitrogen as a percentage of the total nitrogen in the cheese. The abbreviations are the same as in Fig. 1.
within 3 months and the order of intensity was: LFC > STEL > NOV > DL > SIMP > FFC. With further increase in age, TNAE of LFC gradually decreased to levels closer to the treatments and the FFC though the differences were still notable (Fig. 1).

Kuchroo and Fox (1982) noted that water-soluble fraction of Cheddar cheese was a very heterogeneous mixture of peptide as indicated by SDS-gel electrophoresis, high voltage paper electrophoresis, thin layer and paper chromatography. Grierson (Grierson, 1985) reported that water-soluble nitrogen (WSN) and 12.5% TCA soluble extracts increase during the storage of cheese at 4°C from 1.66% (0 day) to 1.84% in 7 days.

The apparent difference in the ultrastructure of FFC and LFC was in the occurrence of small (<5μm) globular void spaces left by the fat-globules during the solvent extraction steps of the micrograph sample preparation (Fig. 3). Much larger number of these in varying globular sizes are seen in FFC (Fig. 3, A) compared to the LFC (Fig. 3, B). This was obviously because the FFC had more than six fold higher content of emulsified butterfat. The extent of size distribution and occurrence of globular voids that were ≈3μm in diameter indicated that cheese milk emulsion of the FFC was unstable before the cheese matrix hardened. The arrow on the right points to a cluster of large coalesced globular voids with a combines length of >10μm (Fig. 3, A). The period in the manufacturing process during which much of the destabilization could have taken place was the “ripening” step where the pasteurized milk was inoculated with the lactic starter culture and held at 32°C for 60 min according to standard procedure (Kosikowski, 1982). Part of the coalescence could also have proceeded during the initial stages of the renneting process when gentle agitation was used at 32°C to add the rennet and Annatto color Emulsions become unstable under these conditions (Walstra et al., 1987). The LFC (Fig. 3, B), which only had 5% fat, as expected showed much fewer globular voids. Again, most of these were large (diameter ≈3μm) indicating early coalescence. The protein matrix of the LFC appeared “flaky” and layered. Interestingly, among the treatments, DL (Fig. 3, C), which was seen to have by far the highest process induced peptide content and largest distribution of hydrophilic and amphipathic peptides (Fig. 2), also showed the highest number of globular voids and many of them were ≈3 μm in diameter indicating greater stability of the DL containing cheese milk emulsion. Some levels of globular clustering is pointed to by an arrow but it is apparent that coalescence did not occur (Fig. 3, C) conceivably reflective of a stable interface. The cheese matrix was apparently less flaky or layered. The STEL treatment was next to DL in terms of the occurrence of globular voids. Globules that were smaller than 3μm were not that evident and evidence of resistance to coalescence are pointed to by an arrow (Fig. 3, D).

The higher occurrence of globular occlusions in the DL and STEL treatments may reflect better emulsifying activity and stability of the ingredients or the process induced formation of amphipathic peptides. This premise is supported by the above observation of resistance to coalescence. More and smaller fat globules meant increased butter-oil and protein matrix interface. Though ever so subtle, this would have an effect on the textural perception as notice by our rheological experiments using the same ingredients where we found DL to the best followed by STEL (Kucukoner, 1998). In addition, formation of flavor volatiles involves lipase activity. Lipases are soluble enzymes that act on insoluble substrates and therefore perform interfacial catalysis (Chahinian et al., 2006). Larger the interface, greater is the potential for catalysis. Our previous work with the same ingredients have shown that flavor volatiles development was affected by the treatments (Haque and Aryana, 2002).

The SIMP treatment had few voids, and these were mostly non-globular, even thought the fat contents of all the treatments was the same (Table 1). This could be attributed to a high level of emulsion instability during lactic culture incubation, stirred cooking and Cheddaring steps. This could have lead to phase separation and accumulation of the dispersed phase in non-globular strata-like layers that would not lend itself to visualization at such high magnification (Fig. 3, E). The undulated surface appearance of the cheese matrix may be due to the presence of these non-globular voids. The NOV treatment showed non-globular elongated voids (pointed by arrows) (Fig. 3, F). Emulsion droplets can assume such a shape when the surface tension is significantly reduced causing a reduction in the outward Laplace pressure from within.

![Figure 3](image-url)
the dispersed phase globule (8). This ingredient, NOV, was composed of 90 cellulose and 10% guar gum.

Dickinson et al., (1991) reported significant reduction in the surface tension while working with acacia gums. Based on a simple model, when two emulsion globules are apart at a distance, h, the free energy of coalescence may be approximated by the expression, $\Delta G = \alpha h^2$, where, $\alpha$ is the inter-globular distance and $\Delta G$ is the surface tension (Walstra and Dickinson, 1987). Therefore, amphiphiles that strongly reduce surface tension will destabilize emulsions.

The carbohydrate-based fat-replacers, STEL and NOV, did not contain peptides or protein hydrolyzates according to the manufacturers. Therefore, higher initial peptide content compared to LFC reflected increased proteolysis during the manufacturing steps. The protein-based fat-replacers, SIMP and DL also showed high initial Pep-NAE content with DL being the highest. SIMP reportedly does not have any added peptides and is composed of whey protein concentrate that is cooked and blended in a patented process to create particles distributed in a way similar to fat globules in stable oil-in-water emulsions.

Since DL is protein based, it is conceivable that it contained peptides that result in a better emulsification and softer curd due to reduced tenacity of protein-protein interactions (Haque, 1993). However, the >2 fold greater peptide content of the DL treatment at 0 month compared to LFC is too large to have originated solely from the ingredient even if it were to contain peptides and must have been partly process based in origin.

Conclusion

The processes steps in the manufacture of Cheddar cheese are intrinsically enzyme activity intensive leading to generation of soluble non-protein and protein nitrogen. There was significant inter-treatment and treatment vs. control difference in this process related formation of TNAE and Pep-NAE. Treatments showed significantly higher process induced Pep-NAE content compared to LFC and FFC. All treatments except STEL significantly reduced age related proteolysis compared to LFC. Age related proteolysis was the highest for LFC followed by STEL and FFC. Data showed that the fat-replacers selectively modulated process induced peptide generation and that high initial non-protein nitrogen content reduced subsequent age related proteolysis. Scanning Electron Microscopy indicated that DL gave microstructure that was closer to that of FFC compared to all other treatments.

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References


