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Evaluation of oxidative damage in mozzarella cheese produced from bovine or water buffalo milk

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Abstract

Technological processes are the main sources of protein and lipid oxidation in food. The oxidative status was determined in a soft Italian cheese, namely mozzarella, produced from water buffalo or bovine milk. The amount of protein-bound carbonyls, dityrosine and α-lactalbumin aggregates were measured to evaluate the extent of protein oxidation. The α-tocopherylquinone/α-tocopherol ratio and the trolox-equivalent antioxidant capacity were used as redox markers in the fat fraction. The levels of protein-bound carbonyls and α-lactalbumin aggregates were found significantly higher in bovine mozzarella than in buffalo mozzarella. On the other hand, higher amounts of redox markers were found in buffalo mozzarella. The levels of dityrosine aggregates were similar in the two types of cheese. The data suggest that protein and fat are more protected against oxidative structure alterations in buffalo mozzarella than in bovine mozzarella.

Keywords: Protein oxidation; Lipid oxidation; Mozzarella cheese

1. Introduction

Biochemical reactions, caused by processing and storage of dairy products, improve food safety, organoleptic properties and shelf life. However, heat treatment, light exposure and oxygen may cause oxidative damage to food lipids and proteins.

Lipid oxidation is a major factor affecting the quality of processed dairy products, especially during long periods of storage (Kristensen & Skibsted, 1999). Lipid oxidation starts with the production of free radicals, inducing the synthesis of hydroperoxides which, in turn, produce volatile carbonyl compounds responsible for off-flavours (Finlay, 1993; Kristensen & Skibsted, 1999). In particular, oxidants in dairy products are scavenged by β-carotene and reducing vitamins, which become oxidised (O'Connor & O'Brian, 1995). Food antioxidants might play a beneficial role in protecting lipids from oxidative degradation. In particular α-tocopherol (TH), the most abundant hydrophobic antioxidant in milk (Erickson, Dunkley, & Smith, 1964), is mainly involved in scavenging peroxyl radicals (Burton & Ingold, 1986). Thus, early oxidative stress in lipids is associated with TH oxidation and leads to the formation of a number of by-products (Ha & Csallany, 1988), including α-tocopherylquinone (TQ) as a major form (Bieri & Tolliver, 1981). Therefore, the titration of TQ or, better, the determination of the TQ/TH ratio can be used to evaluate the extent of lipid oxidation in dairy products (Faustman, Liebler, & Burr, 1999).

Another target of oxidative damage in dairy products is the protein matrix. The Maillard reaction (MR), occurring in food processing and storage, as well as in vivo, is involved in mechanisms of protein oxidation. In this reaction, the proteins undergo structure modifications which are responsible for lowered digestibility (Umetsu & Chuyen, 1998) and, consequently, reduced amino acid availability for absorption (Moreaux & Birlouez-Aragon, 1997; Naranjo, Malec, & Vigo, 1998). Toxic end-products also result from oxidative damage of proteins

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(Koschinsky et al., 1997). Dicarbonyl intermediates, produced by sugar reaction in the early steps of MR (Liggins & Furth, 1997), modify protein structure, causing protein–protein cross-linkage. Therefore, the content of protein-bound carbonyls (PC) is routinely used as biomarker of protein oxidation (Stadtman & Oliver, 1991). Protein structure alteration also occurs by metal-catalysed oxidation, which induces dityrosine (DT) production (Huggins, Wells-Knecht, Detorie, Baynes, & Thorpe, 1993) and, consequently, formation of inter- and intra-molecular cross-links. The level of DT and PC in dairy products can be considered as a further marker of protein oxidative damage (Huggins et al., 1993; Stadtman, 1995).

In this study, we determined the levels of TQ, DT, PC and protein aggregates to evaluate the oxidative stress in mozzarella, a typical Italian cheese from milk of water buffalo (*Bubalus bubalis*), manufactured by unchanged manual cheesemaking steps (Mucchetti, Carminati, & Addeo, 1997). The aim of the present study, was to evaluate the effect of oxidative reactions in mozzarella from different sources or cheesemaking procedures. Buffalo milk (alone) is actually used in small dairies producing mozzarella by manual operations (Addeo, Emaldi, & Masi, 1995). Industries which have introduced slight technological modifications, consisting mainly in the mechanisation of stretching and moulding operations (Addeo et al., 1995), use bovine milk or mixtures of bovine milk with minor amounts of buffalo milk. Chemical composition differences between water buffalo milk and bovine milk consist essentially in higher contents of fat, protein, calcium and phosphate in the former (Addeo et al., 1995). In addition, water buffalo milk fat has a higher melting point and has been found to be more resistant to oxidative changes than bovine milk fat (Ganguli, 1974).

2. Materials and methods

2.1. Materials

Mozzarella, produced with buffalo milk, was purchased from seven different artisanal cheesemakers in the province of Salerno (southern Italy). Mozzarella from bovine milk, produced by six Italian industries, using the same manufacturing process (Addeo et al., 1995), was bought at local supermarkets linked to Europe-wide networks of distribution. Guanidine-HCl and 2,4-dinitrophenylhydrazine (DNPH) were obtained from ICN (Costa Mesa, CA, USA). The Sephacryl S-200 was purchased from Amersham-Pharmacia Biotech Italia (Cologno Monzese, Italy). Chemicals of highest purity, Bovine Serum Albumin fraction V (BSA), TH standard, anti α-lactalbumin antibodies (developed in rabbit), and goat anti-rabbit antibodies, conjugated with horseradish peroxidase (GAR–HRP), were purchased from Sigma (St. Louis, MO, USA). The protein assay reagent was supplied by Bio-Rad (Richmond, CA). D-α-tocopheryl-quinone from Acros-Organics (Carlo Erba, Milan, Italy) was used. HPLC solvents were obtained from Romil (Cambridge, UK). Milli-Q purified water (Millipore, Bedford, MA, USA) was used.

2.2. Homogenate preparation

Cheeses were obtained on the same day of production, 2–3 h after they were put on the market. Cubic aliquots (2 g) were excised from the cheese at a distance of 2 cm from the surface, transferred into glass centrifuge tubes containing 10 ml of 0.2 M sodium citrate buffer (pH 8) and then immediately homogenised. Particulate material was removed by centrifugation (10,000 ***g***, 10 min, 4 °C) and the clear supernatant was analysed without delay.

2.3. Carbonyl assay

PC, in cheese homogenates, was determined spectrophotometrically, essentially according to Reznick and Packer (1994). PC content was calculated by using the molar extinction coefficient (22,000 M^{-1} cm^{-1}; Levine et al., 1990) on blank subtracted data. The protein concentration was determined (Bradford, 1976) and mixtures containing known amounts of BSA, in the presence of 22 mM guanidine HCl, were used for the calibration. The amount of PC in cheese samples was expressed as nmols/mg of protein.

2.4. Titration of fat content

Fat content was determined in the samples of water buffalo and bovine cheese, according to a published procedure (Brown-Thomas, Moustafà, Wise, & May, 1988).

2.5. Analysis of fat-soluble antioxidants

Fat-soluble vitamins were extracted essentially according to a published procedure (Panfili, Manzi, & Pizzoferrato, 1994) but the saponification mixture was supplemented with 0.07% Butylated Hydroxy Toluene (BHT) and 11.5% ascorbic acid (Vatassery, Smith, & Quach, 1993). Aliquots (20 μl) were loaded onto a Nova-Pak C18 steel column (3.9×150 mm; Waters, Milford, MA), equilibrated and eluted with methanol/water (95/5) at 1.0 ml/min flow rate and 30 °C (Stump, Roth, & Gilbert, 1984). TQ elution was monitored spectrophotometrically at 265 nm; retinol and TH were detected by fluorescence at λ_{EX} 325–λ_{EM} 465 and λ_{EX} 295–λ_{EM} 335 nm, respectively. TQ, TH and retinol were calculated on the basis of calibration curves prepared with pure standards and expressed as μg/100 g of product. Since TQ is produced by TH oxidation, the TQ/TH ratio (TQ/TH×100) was used as index of oxidative stress.

2.6. TEAC assay

Total antioxidant capacity in mozzarella homogenates was measured, as Trolox-equivalents, according to a published procedure (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993; Rice-Evans & Miller, 1994) and is called TEAC here.

2.7. Dityrosine assay

The concentration of dityrosine in mozzarella homogenates was evaluated in accordance with a published procedure (Huggins et al., 1993). Dityrosine was expressed as μg/mg of protein.

2.8. Determination of α-lactalbumin aggregates

Mozzarella proteins were analysed by gel filtration chromatography under denaturing and reducing conditions. Samples of solubilised proteins (100 μl) were supplemented with 0.1% β-mercaptoethanol and loaded onto a Sephacryl S-200 column (1.5×12 cm), equilibrated and eluted with 15 mM Tris–HCl buffer, containing 50 mM NaCl and 0.02% Sodium Dodecyl Sulfate (SDS) (pH7.3) at 0.9 ml/min flow rate. The presence of α-lactalbumin and α-lactalbumin-like antigens in the eluted fractions was detected by Western blotting (Towbin, Staehelin, & Gordon, 1979). The amounts of these proteins were measured by ELISA. In detail, fractions were mixed with 1/9 volumes of 70 mM Na_2CO_3, 170 mM $NaHCO_3$, 15 mM NaN_3. Then triplicate aliquots (50 μl) were loaded into microtitre plate wells. The plates were incubated overnight at 4 °C. Remaining plastic reactive sites were blocked by incubation (1 h, 37 °C) with Tris Buffered Saline (TBS) buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.3) supplemented with 0.5% BSA. After blocking, the wells were loaded with 50 μl of the anti α-lactalbumin antibody, diluted 1:3000 with T-TBS buffer (TBS containing 0.05% Tween-20) supplemented with 0.25% BSA. After 1 h of incubation at 37 °C, aliquots (50 μl/well) of GAR–HRP antibody, diluted 1:1000 with T-TBS, were treated for 1 h at 37 °C. The plates were washed twice with T-TBS and twice with TBS, following each of the earlier described steps. Finally, each well was loaded with 100 μl of a colour development solution consisting of 20 mg of *o*-phenylendiamine in 50 ml of 0.1 M sodium phosphate-citrate buffer (pH 5), supplemented with 120 μl of 3% H_2O_2. After 60 min of incubation at 37 °C for colour developing, the reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4. The absorbance was measured at 492 nm by a microplate spectrophotometer.

2.9. Statistics

Cheese samples were analysed in duplicate and the averages were calculated. From these values the mean (M) and the standard deviation (S.D.) were obtained. The results were statistically analysed using the Student's *t* test. Trend curves, regression analysis, and correlation test were done by commercially available software. Significance was determined according to the test of Sokal and Rohlf (1994).

3. Results

3.1. Protein oxidation markers

The extent of protein oxidation in mozzarella samples was analysed by measuring the PC and DT amount (Table 1). The PC level in cheese samples from bovine milk fluctuated in a wide range (1.71–15.00 nMol/mg protein). However, the mean value was significantly higher ($P<0.05$) than that from water buffalo milk. No significant difference was found in DT levels between the two groups of samples. Within the group of samples from water buffalo, a positive correlation between PC and DT content was found ($P<0.05$), whereas no correlation was found in the other group of samples.

The cheese homogenates were first analysed by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting, and the presence of α-lactalbumin antigens on high molecular weight aggregates was seen (data not shown). The level of α-lactalbumin was determined after gel filtration chromatography. The proteins from each homogenate were separated into two peaks, whose elution was monitored by measuring the absorbance at 280 nm in the fractions collected. Each fraction was analysed by ELISA for its content of α-lactalbumin or α-lactalbumin-containing antigens. The chromatograms from bovine samples were similar to those from water buffalo. The immunochemical detection of α-lactalbumin epitopes in the eluted material showed a positive reaction

Table 1
Protein oxidation markers[a]

Sample	Buffalo cheese		Bovine cheese	
	PC	DT	PC	DT
1	1.60	0.11	1.87	0.10
2	1.56	0.08	1.71	0.12
3	1.72	0.13	8.75	0.11
4	2.48	0.25	1.73	0.12
5	2.14	0.12	4.35	0.15
6	2.07	0.11	15.0	0.13
7	2.11	0.09		
Mean	1.95	0.12	5.57	0.12
S.D.	0.34	0.06	5.36	0.02

[a] PC, protein-bound carbonyls (nmol per mg of protein); DT, dityrosine (μg per mg of protein).

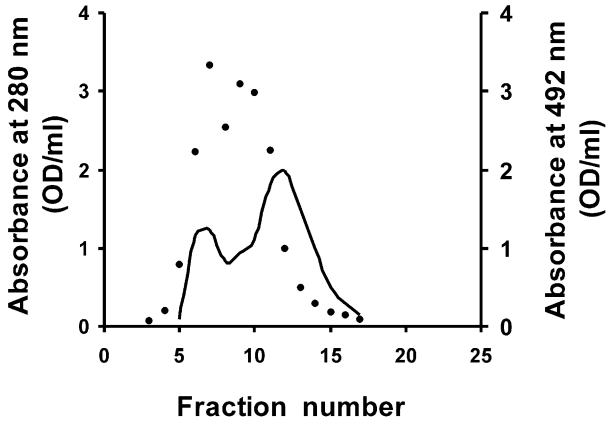

Fig. 1. Chromatography of the proteins solubilised from mozzarella. The proteins were fractionated by gel filtration, on a column of Sephacryl S-200, under reducing and denaturing conditions. The absorbance at 280 nm was measured in the eluted fractions (dotted line). Fractions containing α-lactalbumin epitopes were detected by ELISA at 492 nm (full line). The figure shows a typical chromatogram with fast-migrating material (fractions 5–8) and slow-migrating material (fractions 10–15).

for proteins eluted with both the fast-migrating peak (namely "F") and the slow-migrating peak (namely "S") (Fig. 1). The peak S contained, as assessed by Western blotting, antigens with the same electrophoretic mobility of α-lactalbumin (data not shown). Protein material eluted with the peak F, containing highly aggregated material, was more abundant in samples from bovine cheese than in those from water buffalo cheese. In fact when the protein amount in this peak was calculated as a percentage of the total eluted material, the samples from bovine cheese displayed a significantly higher F level ($P<0.02$). A correlation was found between the F amount and the DT amount ($P<0.05$), in both the cheese from water buffalo ($r=-0.701$; $P<0.05$) (Fig. 2A) and that from bovine milk ($r=0.903$; $P<0.05$) (Fig. 2B). A correlation was found also between the F and the PC amounts in the group of cheese from water buffalo milk ($r=0.818$; $P<0.05$) (Fig. 3).

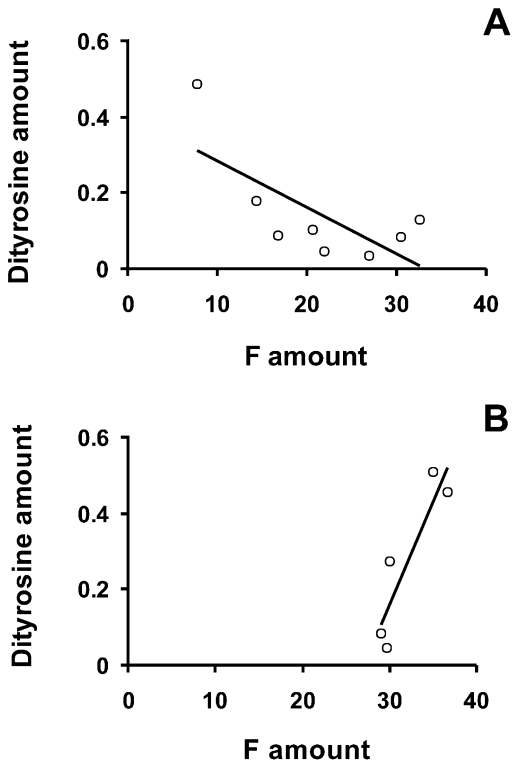

Fig. 2. Correlation of the dityrosine (DT) level (expressed as g/mg of protein) with the fast-migrating material collected by chromatography (peak S in the text; data expressed as per cent of the total eluted material). Panel A: correlation of parameters from water buffalo cheese ($r=-0.701$, $P<0.05$). Panel B: correlation of parameters from bovine cheese ($r=0.903$, $P<0.05$).

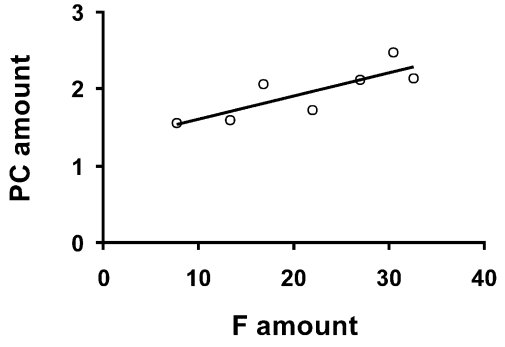

Fig. 3. Correlation of the protein-bound carbonyls (PC) level (expressed as nmols/mg of protein) with the fast-migrating material collected by chromatography (peak S in the text; data expressed as per cent of the total eluted material). Parameters from water buffalo are shown ($r=0.818$, $P<0.05$).

3.2. Fat-soluble antioxidants

The protein and fat contents were determined in mozzarella samples from the two types of milk. The fat/protein ratio (weight/weight) in the group of samples from water buffalo (1.66 ± 0.33; range: 1.32–2.02) was significantly different from that in the other group (1.12 ± 0.16; range: 0.94–1.32; $P=0.008$).

TEAC, and levels of retinol, TH and TQ in mozzarella were determined (Table 2). TEAC in samples from water buffalo cheese was higher than in those from bovine cheese (7.86 ± 1.35 nmol/mg of protein versus 5.88 ± 1.88 nmol/mg of protein; $P<0.04$). Significant differences in the titres of retinol, TH and TQ, between the two cheese groups, were not found. On the other hand, the TQ/TH ratio, which can be assumed as an index of lipid oxidation (and therefore used to evaluate the TH consumption in cheese), was significantly higher in samples from water buffalo than in those from bovine (6.32 ± 2.53 versus 1.34 ± 0.77; $P<0.001$).

Table 2
Antioxidants in mozzarella cheese[a]

Sample	Buffalo cheese				Bovine cheese			
	TEAC	R	TH	TQ/TH[b]	TEAC	R	TH	TQ/TH[b]
1	0.60	229	268	8.91	0.40	324	412	0.68
2	0.70	222	130	6.79	0.30	141	132	2.37
3	0.70	213	105	7.12	0.70	254	244	2.24
4	0.80	137	94.5	9.16	0.70	242	174	0.75
5	0.80	200	111	6.34	0.70	267	245	1.24
6	1.00	231	188	3.34	0.73	286	288	0.77
7	0.90	236	240	2.58				
Mean	0.79	210	162	6.32	0.59	252	249	1.34
S.D.	0.14	34.4	70.0	2.53	0.19	61.8	97.6	0.77

[a] TEAC, Trolox-equivalent antioxidant capacity (nmol/mg of protein); R, retinol (μg/100 g of sample); TH, α-Tocopherol (μg/100 g of sample); TQ, α-Tocopherylquinone (μg/100 g of sample).
[b] The ratio is expressed as (μg/mg)×100.

4. Discussion

Milk and dairy products are good sources of excellent quality proteins (Rosenthal, 1991) and fat-soluble antioxidants, such as TH, β-carotene and retinol (Panfili et al., 1994). Tocopherols inhibit the chain reaction of lipid peroxidation by scavenging several free-radicals that catalyse the initiation and propagation steps (Burton & Ingold, 1986). Similarly β-carotene and retinol, which is produced from carotene, scavenge both singlet oxygen and lipoperoxides, thus preventing or limiting the oxidation of the fatty acids (Donnelly & Robinson, 1995). The production of TQ reflects the consumption of TH, resulting from antioxidant activity (Faustman, Liebler, & Burr, 1999). Thus a higher TQ/TH ratio should be expected when higher fat levels and lower liposoluble antioxidant levels are present in milk or cheese. Fat content in milk of water buffalo is about twice as high as in bovine milk (Addeo et al., 1995) and it is lacking in β-carotene. Therefore, owing to the oxidant conditions during cheese manufacturing, namely heat-treatment and exposure to oxygen, higher amounts of lipoperoxides should be produced and higher TH consumption should occur in the material processed from milk of water buffalo. This might explain why we found the TQ/TH ratio higher in cheese from water buffalo than in that from bovine cows.

The levels of PC and DT are usually used to evaluate the extent of protein oxidation (Huggins et al., 1993; Stadtman, 1995). PC and DT are markers of two different oxidative events. PC is produced by heat-induced oxidation of carbohydrates (Maillard chemistry). DT is formed by metal-catalysed reactions, which are based on the formation of hydroxyl radicals from hydrogen peroxide or lipoperoxides (Fenton chemistry). The finding of comparable levels of DT in both groups of mozzarella strongly suggests that such a hydroxyl-driven oxidation is not the major cause of the higher level of α-lactalbumin-containing protein aggregates in cheese from bovine milk. Conversely, the higher PC content in proteins of this group of cheese might be responsible for the sugar-dependent formation of molecular cross-links. Also, hydrophobic interactions and disulphide cross-linking can produce whey protein aggregates with high molecular mass, particularly during heat-treatment (Dalgleish, Senaratne, & Francois, 1997; Havea, Singh, Creamer, & Campanella, 1998). However, in the denaturing and reducing conditions used to separate α-lactalbumin from α-lactalbumin-containing aggregates, PC-based cross-links are resistant while hydrophobic interactions or disulphide bridges are broken. This result, together with the finding of a positive correlation of PC level with the amount of α-lactalbumin-containing aggregates in mozzarella from water buffalo milk, supports the hypothesis that PC might play a role in the formation of stable protein aggregates. In this context, the variability of the PC values found in the group of mozzarella samples from bovine milk should be noted. Such variability might reflect heterogeneity in heat-treatment by the different industries. However, differently from the result obtained from the water buffalo samples, there was no correlation of PC level with the amount of α-lactalbumin-containing aggregates. It is possible that other chemical or physical factor(s) acting during the cheese production, but to a lesser extent when water buffalo milk is used, participate to the formation of the protein aggregates. Protein aggregates should not be formed in cheese, because they alter the product consistency and, therefore, its organoleptic properties by enhancing the phenomenon of the so-called gelification (Baer, Oroz, & Blanc, 1976; Parris, Anema, Singh, & Creamer, 1993; Parris, Purcell, & Ptashkin, 1991). In addition, the presence of intermolecular bonds, such as DT- and PC-based cross-links, in food proteins does not allow digestion and absorption in the gastrointestinal tract (Umetsu & Chuyen, 1998). As a matter of fact, proteases breaking the polypeptide structure at sites containing oxidised amino acid do not exist in digestive juices or blood. Furthermore, transport systems for dityrosines or dicarbonyl-bound amino acids through the cell plasma membrane have never been reported. Therefore it is conceivable that these amino acid derivatives, even though transported across the intercellular junctions in the intestine, would be addressed to the liver and targeted for elimination. In this event, the possibility exists that the amino acid derivatives might overwork the liver and even be toxic. In conclusion, we suggest that oxidative factors trigger radical-based mechanisms, leading to protein structure alteration. This results in aggregate formation, lowering the cheese quality in terms of consistency and loss of amino acids as nutrient factors (Moreaux & Birlouez-Aragon, 1997; Naranjo, Malec, & Vigo, 1998). In this

context, the quality of mozzarella produced from water buffalo milk should be considered higher than that of mozzarella from bovine milk. Whether differences between the two types of products analysed have to be ascribed to handling differences in cheesemaking technology, or just to biochemical differences of the two types of milk used, remains to be ascertained.

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