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# Protein oxidative changes in whole and skim milk after ultraviolet or fluorescent light exposure

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## ABSTRACT

We investigated how protein changes occur, at the primary or higher structural levels, when proteins are exposed to UV or fluorescent (FL) light while in the complex matrix, milk. Whole milk (WM) or skim milk (SM) samples were exposed to FL or UV light from 0 to 24 h at 4°C. Protein oxidation was evaluated by the formation of protein carbonyls (PC), dityrosine bond (DiTyr), and changes in molecular weight (protein fragmentation and polymerization). Oxidative changes in AA residues were measured by PC. Dityrosine and N′-formylkynurenine (NFK), a carbonylation derivative of Trp, were measured by fluorometry. Protein carbonyls increased as a function of irradiation time for both WM and SM. The initial rate for PC formation by exposure to FL light (0.25 or 0.27 nmol/h for WM and SM, respectively) was slower than that following exposure to UV light (1.95 or 1.20 nmol/h, respectively). The time course of NFK formation resembled that of PC. After 24 h of UV exposure, SM had significantly higher levels of NFK than did WM. In contrast, WM samples irradiated with UV had higher levels of DiTyr than did SM samples, indicating different molecular pathways. The formation of intra- or intermolecular DiTyr bonds could be indicative of changes in the tertiary structure or oligomerization of proteins. The existence of NFK suggests the occurrence of protein fragmentation. Thus, proteolysis and oligomerization were analyzed by sodium dodecyl sulfate-PAGE. After 24 h of exposing WM to UV or FL light, all the proteins were affected by both types of light, as evidenced by loss of material in most of the bands. Aggregates were produced only by UV irradiation. Hydrolysis by pepsin and enzyme-induced coagulation by rennet were performed to evaluate altered biological properties of the oxidized proteins. No effect on pepsin digestion or rennet coagulation was found in irradiated SM or WM. The oxidative status of proteins in milk and dairy products is of interest to the dairy industry and consumers. These findings provide knowledge that could be useful in determining the optimal lighting conditions in the dairy industry in general and in cheese making in particular.
**Key words:** milk protein, protein oxidation, light exposure

## INTRODUCTION

The oxidative status of milk and dairy products is of interest to the dairy industry and consumers. Oxidation of milk can result in off-flavors, making the oxidized milk unacceptable to consumers and causing a decrease in the nutritional quality (Dunckley et al., 1962; Dimick and Kilara, 1983; Marsili, 1999). Both UV and visible light wavelengths contribute to the development of aroma compounds in milk (Rosenthal, 1992; Bosset et al., 1993). Singlet oxygen, created during the cascade of photochemical reactions, reacts with lipids, proteins, and vitamin compounds to initiate formation of oxidation products with unpleasant off-flavors (Hansen and Skibsted, 2000; Davies, 2003). Free radicals, generated by autooxidation reactions, are unstable and contribute further to the accumulation of secondary metabolites with effects on flavor and aroma.

Exposure of milk to light can take place at several stages from the time of milking until it is consumed. Previous works (Dimick and Kilara, 1983) report that light-induced protein oxidation in dairy products proceeds quickly and is detected after only a short exposure to light. Sandmeier (1996) noted that the reaction had no induction time.

Photooxidation affects the primary structure of proteins, producing changes in individual amino acids as carbonyl formation, and loss of aromatic amino acids has been described for several complex food matrices (Viljanen et al., 2005). Formation of protein carbonyls (**PC**) has been shown to be one of the salient changes in oxidized proteins and the concentration of PC is highly indicative of protein oxidation (Fenaille et al., 2006). Kynurenine and N'-formylkynurenine (**NFK**), a

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carbonylation product of Trp, have been used as reporters of hen lysozyme denaturation by thermal treatment (Fukunaga et al., 1982).

The aromatic amino acids Trp, Tyr, and Phe have absorption maxima at wavelengths around 280 to 290 nm (Wetlaufer, 1962). Direct photochemical changes to protein are restricted to high-energy UV light because they have very limited absorption at wavelengths longer than 310 nm (Mortensen et al., 2004). Some earlier investigations on the effects of light on milk dealt with sensory aspects and the ability of milk to clot when renneted (Wishner, 1964; White and Bulthaus, 1982). Photooxidation of His residues causes κ-CN and whole CN to lose their ability to clot when renneted (Hill and Wake, 1969). In those works, the causes of the deleterious aromas were assigned mainly to the oxidation of lipid and riboflavin. Catalytic photoaggregation and photodegradation of purified α-LA, β-LG, and acid whey proteins isolated from homogenized milk have been described (Gilmore and Dimick, 1979). More recently, the effect of irradiation on individual isolated protein oxidation has been studied. Oxidative changes involving Trp residues and formation of dityrosines (**DiTyr**) have been noted (Dalsgaard et al., 2008), along with decreased accessibility of chymosin to oxidized caseins (Dalsgaard and Larsen, 2009).

The formation of protein polymers between whey proteins and casein components has been described for thermal-treated milk (Singh et al., 1996). Denaturation of β-LG seems to be the key phenomenon that initiates polymer formation (Anema, 2000) and has been proposed as a marker of heat processed milk (Chen et al., 2005). Heat induces a conformational change that results in the exposure of a reactive thiol group, which forms disulfide bonds with other cysteine-containing proteins such as BSA, or with proteins having disulfide bridges, such as α-LA, κ-CN, and $\alpha_{S2}$-CN (Roefs and de Kruif, 1994; Vasbinder et al., 2001). Heat treatment of milk results in a complex mixture of native whey proteins and denatured whey proteins present as whey protein aggregates, CN–whey protein aggregates, and whey protein-coated CN micelles (Vasbinder et al., 2003).

Therefore, this study aimed to investigate the changes introduced into milk proteins at the primary or higher structural levels by UV or fluorescent (**FL**) light oxidation while in their natural complex matrix—whole milk (**WM**) or skim milk (**SM**). Oxidation was determined by the formation of PC and DiTyr, and by alteration in the molecular weights of proteins to detect polymerization and proteolysis. In addition, we assayed hydrolysis by pepsin and clotting by rennet because it has been demonstrated that photooxidation influences hydrolysis by proteases. The relationship between our observations and milk quality preservation is discussed. These findings provide knowledge that could aid in determining optimal lighting conditions in the dairy industry in general and in cheese making in particular.

## MATERIALS AND METHODS

### Materials

2,4-Dinitrophenylhydrazin (**DNPH**) and pepsin (P6887, activity: 3,260 units/mg protein) were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany). N,N′-Methylene-bis-acrylamide and acrylamide were from Sigma (Sigma Chemical Co., St. Louis, MO). Guanidine hydrochloride (ultrapure) was from Genbiotech (Capital Federal, Argentina), TCA, glacial acetic acid, ethyl acetate, ethyl alcohol, and hydrochloride acid were from Cicarelli (San Lorenzo, Argentina). All of these products are of analytical grade.

### Milk Samples

Commercially processed spray-dried WM was used (SanCor, Sunchales, Argentina).Whole milk powder was reconstituted to 13% total solids as indicated on the label. To obtain SM, reconstituted WM was defatted by centrifugation at 7,500 × *g* for 15 min at 4°C (centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) and then filtered through glass wool.

### Experimental Design

Samples of 5 mL (WM or SM) were poured into 6-cm-diameter Petri dishes and exposed to FL or UV light inside a chamber (30 cm high, 50 cm long, and 20 cm wide) from 0 to 24 h at 4°C. Simultaneously, control samples were exposed to the same conditions but were wrapped in aluminum foil to avoid exposure to light.

The FL lamp was an Alic Daylight (15 W/F15T8, 230 V; Alic, Hefei, China), with an intensity of 2,090 to 2,100 lx, and the UV lamp was an Hg UV-C TUV Philips tube (15 W/G15T8; Philips, Eindhoven, Holland), with an intensity of $2.34 \times 10^{19}$ quanta/s as assessed by ferrioxalate actinometry.

### Protein Quantification

Protein concentration was determined by UV spectroscopy at 280 nm or by following the Bradford method (Bradford, 1976) using BSA as standard.

### Measurement of Dityrosine and NFK

Protein solutions (1 mg/mL) were analyzed for the presence of dityrosine (DiTyr) by fluorometry, and