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Trine Kastrup Dalsgaard, John Sørensen, Mette Bakman, Caroline Nebel, Rita Albrechtsen, et al.. Light-induced protein and lipid oxidation in low-fat cheeses: whey proteins as antioxidants. Dairy Science & Technology, 2011, 91 (2), pp.171-183. 10.1007/s13594-011-0001-1 . hal-00874108

**HAL Id: hal-00874108**

**<https://hal.science/hal-00874108>**

Submitted on 17 Oct 2013

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## Light-induced protein and lipid oxidation in low-fat cheeses: whey proteins as antioxidants

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Received: 18 February 2010 / Accepted: 3 August 2010 /  
Published online: 22 March 2011  
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**Abstract** Photo-oxidation of cheese products has become an issue due to the fact that packaging of cheeses in transparent materials is very frequently used. The present study aimed to give new aspects of the possible antioxidative activity of whey proteins in photo-oxidation of cheese and the whey proteins were expected to act as scavenger and thereby reduce lipid oxidation. Oxidation was investigated in low-fat model cheese with whey protein isolate (WPI) added and compared to a low-fat control cheese. The cheeses were packed in air or in vacuum, respectively, and stored under light. Accumulations of lipid hydroperoxides, pentanal, hexanal, heptanal, 1-hexanol, dimethyl disulfide and dihydroxyacetone were all found to be dependent on the availability of oxygen. The presence of WPI reduced the accumulation of both lipid and protein oxidation products. The consumption of the naturally occurring antioxidant  $\alpha$ -tocopherol was not affected by the added WPI. After the seventh day of light-exposed storage during which all riboflavin was decomposed and the concentration of  $\alpha$ -tocopherol had found a stable level, the presence of WPI resulted in an extension of the observed lag phase in the accumulation of secondary lipid oxidation products. As WPI reduced the accumulation of protein oxidation products as well, it is suggested that WPI is not a typical radical scavenger.

低脂干酪中光诱导的蛋白和脂肪氧化：乳清蛋白的抗氧化作用

**摘要** 由于在流通领域中包装干酪的普遍使用，干酪产品的光氧化问题已经成为重要的议题。本文探讨了在低脂干酪中乳清蛋白作为抗氧化剂对光氧化的抑制作用。乳清蛋白可能

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具有自由基清除的作用, 因此, 有可能具有降低脂肪氧化的作用。将乳清分离蛋白添加到低脂模拟干酪中, 以纯低脂模拟干酪作为对照组。分别将干酪进行有空气和真空包装后在有光条件下贮藏。试验结果表明氧化产物, 如氢过氧化物、戊醛、己醛、庚醛、正己醇、二甲基二硫化物 (DMDS)、二酪氨酸均被检测出来, 其形成量大小取决于包装内可利用氧气的量。乳清分离蛋白可以使脂肪和蛋白氧化产物的量降低。而且乳清分离蛋白对干酪中自然存在的抗氧化剂  $\alpha$ -生育酚没有影响。在光照下贮藏 7d 后, 样品中核黄素完全分解了, 但是  $\alpha$ -生育酚的浓度仍保持恒定。乳清分离蛋白的使用可以延迟脂肪氧化, 也可以降低蛋白的氧化程度。本研究说明乳清蛋白并不是一种典型的自由基清除剂。

**Keywords** Antioxidant · Low-fat cheese · Photo-oxidation · Whey proteins

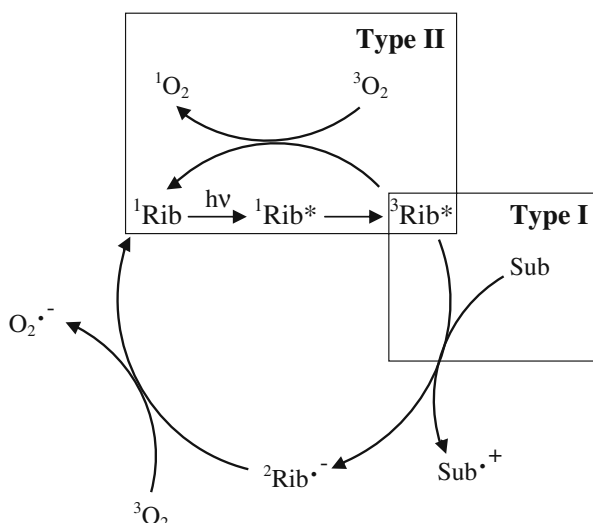
**关键词** 抗氧化剂 · 低脂干酪 · 光氧化 · 乳清蛋白

## 1 Introduction

Light-induced oxidation has become an issue in cheese manufacturing, as transparent materials are very frequently used. At the same time, low-fat content products have become more popular, and the flavour originating from protein oxidation may be more dominating in products with a low-fat content than in the products with high fat content in which the lipid oxidation is mainly responsible for the off-flavour formation.

The mechanisms by which the lipids become oxidised are well characterised, and for photo-oxidation the type I and type II mechanisms (Fig. 1) have been suggested to compete with each other both depending on the concentration of oxygen (Bradley and Min 1992; Foote 1968b) and the type of photo-sensitizer (Foote 1968a; Tsai et al. 1985). Proteins are oxidised through the same mechanisms as lipids. Singlet oxygen (type II) is, e.g., known to result in generation of protein carbonyls (Davies 2004), while generation of dityrosine has been suggested to be formed by the type I mechanism (Silva and Godoy 1994). Oxidation of the amino acid methionine to methional has been suggested to give off-flavour formation in milk (Allen et al. 1975; Patton 1954) but dimethyl disulfide (DMDS) was the major oxidation product generated from methionine in light-exposed milk (Kim and Morr 1996).

Proteins form cross-links between cysteines (Shen et al. 2000b), tyrosines (Adams et al. 1972; Mcarthur and Davies 1993; Shen et al. 2000b) or between histidine residues (Shen et al. 2000a) during oxidation processes, and these cross-links are suggested to be antioxidative mechanisms terminating radicals (Adams et al. 1972; Mcarthur and Davies 1993; Shen et al. 2000b). Oxidation of methionine in biological systems has been suggested to protect proteins from critical oxidative damage (Levine et al. 1996, 1999). Another amino acid susceptible to singlet-mediated oxidation, tryptophan has been demonstrated to reduce the accumulation of lipid hydroperoxides in emulsified linoleic acid (Toyosaki 2002).  $\beta$ -casein becomes oxidised before unsaturated fatty acids by triplet-excited riboflavin in light-induced oxidation, thereby indicating a scavenging effect of the proteins (Huvaere et al. 2010), and a former study showed similar reactivity between triplet-excited riboflavin and whey proteins (Cardoso et al. 2004). Whey proteins exerted antioxidative activity in model systems (Tong et al. 2000a), and chelation of metal ions has been suggested as a mechanism partly responsible for the antioxidative activity in metal-induced oxidation (Tong et al. 2000b).



**Fig. 1** Photo-oxidation initiated by the excitation of riboflavin (Rib). The excited triplet riboflavin ( $^3\text{Rib}^*$ ) can react with a substrate (Sub), and energy or electron transfer results in generation of free radical ( $\text{Sub}^{\bullet+}$ ) (type I). The excited triplet riboflavin ( $^3\text{Rib}^*$ ) transfer energy to oxygen (in its triplet state ( $^3\text{O}_2$ )) generating the reactive singlet oxygen ( $^1\text{O}_2$ ) through the type II mechanism

The effect of whey protein isolate (WPI) as antioxidants on light-induced oxidation has not been investigated in complex matrices. It was hypothesised that the presence of whey protein in low-fat cheese would reduce the generation of lipid oxidation products, while the generation of protein oxidation products would increase as a consequence of light exposure. The generation of the protein oxidation products dityrosine and DMDS as well as the lipid oxidation products lipid hydroperoxides, pentanal, hexanal, heptanal and 1-hexanol were measured in order to investigate which effect the addition of WPI had on the light-induced oxidation in low-fat cheese.

## 2 Materials and methods

### 2.1 Materials

A low-fat cheese, Cheasy 6% (6% fat), from ARLA Foods Amba (Hjørring, Denmark) purchasable on the Danish market was used as model for preparing low-fat content model cheeses. Neutrase was purchased from Novozymes A/S (Kalundborg, Denmark), and Natamycin (Natamax) from Danisco A/S, (Grindsted, Denmark). Ascorbic acid (99.7%),  $\alpha$ -,  $\delta$ , and  $\gamma$  tocopherol (all >96%), riboflavin (>98%), ethylenediaminetetraacetic acid (99%), citric acid monohydrate, iron-(III)-chloride hexahydrate (97%), hexanal (98%), 1-hexanol (99.5%), heptanal (>92%) were obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany).  $\text{H}_2\text{SO}_4$  (95–97%), HCl (37%), NaCl (99%), trichloroacetic acid (TCA; 99%),  $\text{BaCl}_2$  (99%), iron-(II)-sulphate (99.5%),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (99.5%),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

(99%) and  $\text{H}_2\text{O}_2$  (30%), NaOH (>99%) were obtained from Merck (Darmstadt, Germany). Sodium acetate, pentanal (97%) and 4-methyl-2-pentanone (99%) came from Fluka Chemie GmbH (Buchs, Germany). Acetonitrile, chloroform, hexane, 2-propanol and methanol (all HPLC-grade) and heptane (99%) were from Rathburn Chemicals (Walkerburn, Scotland).  $\text{NH}_4\text{SCN}$  (99%) was from VWR BHD Prolabo (Leuven, Belgium).

## 2.2 Production of model cheeses

Model cheeses were prepared from the semi-hard, rindless, low-fat cheese Cheasy 6% (Arla Foods, Denmark) ripened for approximately 6 weeks. A low-fat model control cheese, called control cheese hereafter, was made from Cheasy 6% cheese (28.6 kg), which was cut into pieces ( $10 \times 10 \times 4$  cm) and mixed with 14.5-L pasteurised tap water in a Scanima Mixer SRB-50 (Scanima A/S, Aalborg, Denmark) for 2 min at  $272 \times g$  through punching holes of 4 mm until a homogenous cheese paste was obtained. A low-fat model cheese with whey protein added, called cheese-WPI hereafter, was made from 16.8-kg cheese to which whey protein isolate, Lacprodan DI-9224 (Arla Foods, Videbæk, Denmark) 7% (w/w), was applied before mixing with 5.6 L of pasteurised tap water. To avoid microbial growth,  $12.5 \text{ mg} \cdot \text{kg}^{-1}$  Chricin C (Chr. Hansen, Hørsholm Denmark) was added and the paste was heated to  $80^\circ\text{C}$  for 5 min. The temperature of the cheese paste was reduced to  $45^\circ\text{C}$  before 0.08 AU of the proteolytic enzyme Neutrase (Novozymes, Kalundborg, Denmark) was added, and the paste was mixed for approximately 5 min.

The cheese paste was transferred to 1-kg sterile DUMA containers (Gerresheimer Værloese a/s, Værløse, Denmark) and cooled to  $4^\circ\text{C}$  before frozen at  $-20^\circ\text{C}$ . The final cheese paste produced from the control cheese contained 31.1% dry matter (IDF 1982), 5.4% fat (IDF 1997) and 21.0% protein (IDF 1986), respectively, while cheese paste produced from the cheese-WPI contained 37.5% dry matter, 5.2% fat and 27.3% protein. pH was 5.6 and 5.8, respectively, in cheese-WPI and control cheese, while the salt content (IDF 1988) was 1.3% and 1.2% in cheese-WPI and control cheese, respectively. The water activity was 0.989 in the control cheese, and 0.985 in the cheese-WPI when measured on an Aqualab model Series3TE (Decagon Devices, Inc. 950 NE Nelson Court Pullman WA 99163, USA). The cheese pastes were transferred into the lids (d. 9 mm) of sterile Petri dishes (Frisenette, Knebel, Denmark), treated with  $5 \text{ g} \cdot \text{L}^{-1}$  Natamycin (Natamax) and packed in vacuum or with air, respectively, in vacuum packs consisting of a layer of  $70\text{-}\mu\text{m}$  polyamid and  $20\text{-}\mu\text{m}$  polyethylene impermeable to air (PM Pack Service, Horsens, Denmark).

## 2.3 Experimental design

The cheeses were incubated at  $15^\circ\text{C}$  under fluorescent light (400–600 nm) from a light source (TL-D 90 de Luxe Pro 18 W/965 SLV from Philips, Frankfurt, Germany) similar to the light sources used in retail, with an intensity of 1,800 lux measured at the surface of the cheese samples. Samples wrapped in aluminium foil to avoid light exposure were incubated in parallel and used as blind controls. The samples were incubated for 0, 1, 2, 3, 7, 14, 21 and 28 days, homogenised and analysed for lipid hydroperoxides, volatile compounds, dityrosine, riboflavin and vitamin E.

## 2.4 Lipid hydroperoxides

Lipid hydroperoxides were measured according to Dalsgaard et al. (2010). Cheese (1 g) was dispersed in 5 mL of demineralised water and mixed by Ultraturax for 45 s. The lipid hydroperoxides were extracted into 10 mL (1:1) methanol/chloroform mixture. The samples were mixed for 30 s on a whirlmixer and finally centrifuged for 10 min at 1000×g. One millilitre of the chloroform phase was transferred into chloroform-cleaned glasses and mixed with 1 mL of ironII/thiocyanate mixture according to the IDF standard (74A:1991) modified by Østdal et al. (2000a). Absorbance was measured at 500 nm with 700 nm used as background subtraction on an HP-8453 diode array spectrophotometer from Agilent Technologies (Palo Alto, CA, USA), and quantification was performed according to external standards using a calibration curve made in the concentrations 0.5, 1.0, 2.5, 5.0, 7.5, 10.0 and 20.0  $\mu\text{g mL}^{-1}$  of iron (III). The samples were diluted properly to be within the range of the standard curve. Samples were analysed in triplicates.

## 2.5 Volatile compounds

The volatile compounds (pentanal, hexanal, heptanal and DMDS) were identified and quantified by gas chromatography coupled with mass spectrometry (GC–MS) head space analysis according to Dalsgaard et al. (2010). One millilitre of deionized (18.2 M $\Omega$ ) filtered water (0.22  $\mu\text{m}$ ) plus 1 mL of internal standard (100 ng.mL $^{-1}$  4-methyl-2-pentanone) were transferred to a 12.5-mL vial containing 2 g of light-treated cheese and sealed with Teflon-coated lids before incubation at 37 °C for 30 min. The headspace was analysed for volatile compounds using a Carboxen/polydimethylsiloxane solid phase micro extraction (SPME) fibre with a film thickness of 30  $\mu\text{m}$  from Supelco (Bellefonte PA, USA). The fibre was incubated at 60 °C for 30 min in the headspace of each sample. Desorption of the sample from the fibre was performed into the inlet of a GC 154ON from Agilent Technologies (Waldbronn, Germany) equipped with a Zebron, ZB-5HT column from Phenomenex (Torrance, CA, USA) and coated with a non-metal 5%-phenyl 95%-dimethylpolysiloxane phase with the dimensions: 0.25 mm i.d.; film thickness, 0.25  $\mu\text{m}$ ; length, 30 m. Helium was used as carrier gas with a constant flow of 1.2 mL.m $^{-1}$  in 100 N (m $^2$ ) $^{-1}$ . The splitless injector was kept at 250 °C. An SPME injection sleeve liner from Supelco (Bellefonte, USA) with an inner diameter of 0.75 mm was applied. The column temperature was programmed to stay at 40 °C for 4 min followed by an increase from 40 to 120 °C with a rate of 5 °C.min $^{-1}$ , a hold time of 5 min, and a subsequent temperature gradient from 120 to 300 °C with a rate of 20 °C.min $^{-1}$ . Mass spectral analysis was performed in selected ion monitoring according to the ions determined by the use of a standard for each compound on a quadrupole MSD 5975 (Agilent Technologies, Germany) with a quadrupole temperature of 150 °C and a fragmentation voltage of 70 eV according to Dalsgaard et al. (2010). The ion source temperature was 230 °C, and the interface was 280 °C. Quantification was performed relatively using external standard curves with concentrations of 1–500 ng.mL $^{-1}$  H $_2$ O of each compound to avoid day-to-day variation on individual compounds. Samples were measured in duplicate.

## 2.6 Dityrosine

Dityrosine was measured in 1 g of cheese according to Dalsgaard et al. (2010). The cheese was dispersed in 5 mL of demineralised (18.2 M $\Omega$ ), filtered water (0.22  $\mu$ m), mixed on a whirlmixer for 45 s. Before the cheeses were frozen to  $-20^{\circ}\text{C}$  for later analysis, 1 mL of 1 mol.L $^{-1}$  NaCl in 0.1 mol.L $^{-1}$  H $_2$ SO $_4$  was added to the samples. Twelve millilitres of 2-propanol was added to the thawed samples, and they were subsequently mixed for 3 min. Fat extraction was performed after addition of 9 mL of pentane. After another 3 min of mixing, the samples were centrifuged for 5 min at 1000 $\times g$ . The fat extraction was performed three times before the samples were dissolved in 4 mL of 50 mmol.L $^{-1}$  phosphate buffer and precipitated in 10% TCA and finally centrifuged for 10 min at 1000 $\times g$ . The pellet was acid hydrolysed in 1 mL of 6 mol.L $^{-1}$  HCl over night and finally neutralised by addition of 600  $\mu$ L of 6 mol.L $^{-1}$  NaOH. Measurements were performed according to Dalsgaard et al. (2007), using reverse phase HPLC combined with fluorescence detection with excitation and emission measured at 284 and 415 nm, respectively. External standard curve (0–2,000 nmol.mL $^{-1}$ ) of dityrosine was used for quantification and samples were measured in triplicates.

## 2.7 Riboflavin

Riboflavin was measured according to Dalsgaard et al. (2010). The cheese (1 g) was dispersed in 5 mL of demineralised (18.2 M $\Omega$ ), filtered water (0.22  $\mu$ m) and mixed for 30 s on a Whirl mixer. Protein was precipitated in 10% TCA on ice for 10 min and subsequently centrifuged at 1000 $\times g$  for 10 min. Approximately 1 mL of the supernatant was filtered through a cellulose filter (0.45  $\mu$ m) and transferred directly into brown-coloured vials to avoid further light exposure. The samples were injected onto a reverse phase column (Sorbax SB-C8 with the dimension 4.6 $\times$ 150 mm, 5  $\mu$ m from Agilent Technologies, USA). The riboflavin was quantified using fluorescence detection according to Silva et al. (2005). Quantification was performed using external riboflavin standards: 0.75, 0.5, 0.1, 0.05, 0.025, 0.01 and 0.005  $\mu$ g mL $^{-1}$  prepared from a 100  $\mu$ g mL $^{-1}$  riboflavin stock solution in 0.02 mol.L $^{-1}$  NaOH. Samples were measured in triplicates.

## 2.8 $\alpha$ -Tocopherol

The cheese (1 g) was dissolved in 1 mL of demineralised water mixed for 30 s and subsequently mixed for 10 min with 2 mL of ethanolic ascorbic acid (1%) according to Dalsgaard et al. (2010). Subsequent sample handling and measurements were performed according to Havemose et al. (2004). A saturated potassium hydroxide solution (0.3 mL) was added and mixed for 10 s before incubation for 30 min at 70  $^{\circ}\text{C}$ . While incubated, the samples were shaken every 5 min. Subsequently, the samples were cooled on ice, and 1 mL of demineralised water and 3 mL of heptane were added and mixed for 1 min. The samples were centrifuged for 3 min at 1,700 $\times g$ , and the upper phase was transferred into brown-coloured vials and injected onto a Hypersil Silica column (4.6 $\times$ 200 mm, 5  $\mu$ m from Thermo Fischer Scientific Inc. (Waltham, USA)), and eluted from the column using mobile phases

of 98% hexane and 2% 2-propanol. Fluorescence detection was performed using excitation and emission wavelength of 295 and 330 nm, respectively. Quantification was performed using an external standard curve. Samples were measured in duplicate.

## 2.9 Statistical analysis

Statistical analysis was performed using the Generalised Linear Models procedure of SAS version 9.2 (SAS Institute, INC., Cary, NC, USA). In order to obtain normality, the data were transformed by the  $\log(10)$  function. The LS-Means were calculated and differences regarded as significant at a minimum level of 95% ( $P < 0.05$ ). Differences were classified by the Ryan-Einot-Gabriel-Welsch (REGW) multiple range test (SAS Institute, INC., Cary, NC, USA).

## 3 Results

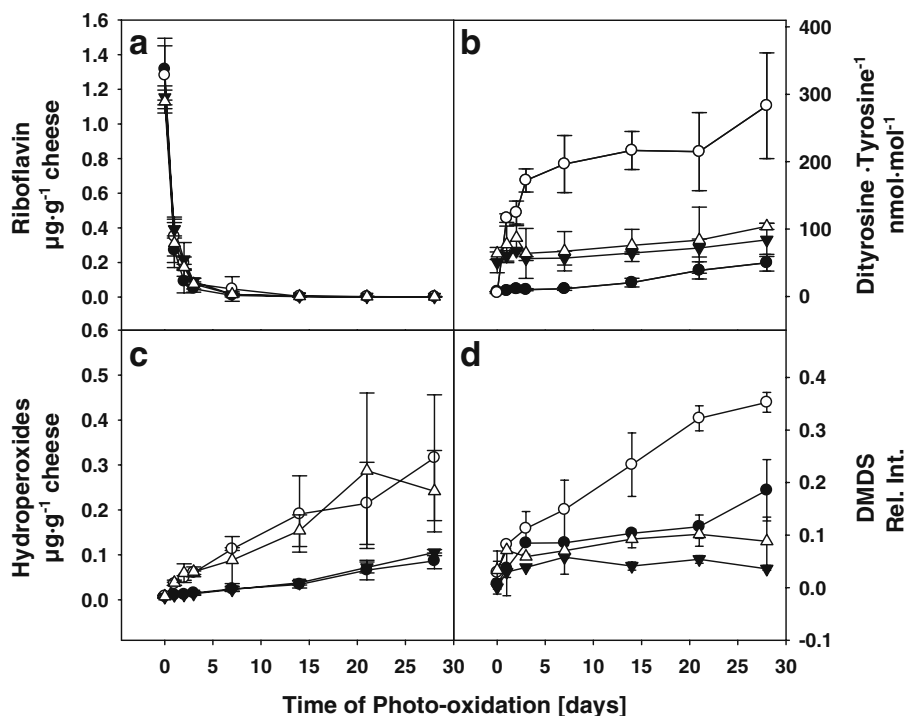
The aim of the present study was to investigate the effect of whey protein and package condition on the light-induced oxidation when low-fat cheese was stored and exposed to fluorescent light as used in retail. Oxidation in cheese-WPI was compared to oxidation in a similar control cheese.

The concentration of riboflavin was  $1.3 \mu\text{g.g}^{-1}$  cheese in control cheese and  $1.2 \mu\text{g.g}^{-1}$  cheese in the cheese-WPI at day 0 before exposure to fluorescent light (Fig. 2a). It was significantly reduced ( $P < 0.0001$ ) already within the first day of storage in fluorescent light in both types of cheese and completely absent within the first 2 to 3 days of storage in light, independent on the presence of oxygen in the packaging. No significance difference ( $\alpha = 0.05$ ) was measured in the degradation of riboflavin between the control and the cheese-WPI.

The accumulation of dityrosine mirrored the loss of riboflavin in the control cheese packed in air. It increased from  $8 \text{ pmol mg}^{-1}$  protein at day 0 to  $283 \text{ nmol.mol}^{-1}$  tyrosine at day 28 (Fig. 2b). After the first day of storage, it increased significantly ( $P = 0.0001$ ) in the control cheese packed in air, and in this control cheese a plateau was reached within the first 3 days of storage. In the cheese-WPI it was high ( $57 \text{ nmol.mol}^{-1}$  tyrosine) at day 0, where no photo-oxidation had occurred, and the concentration of dityrosine did not significantly change within the 28 days of storage in the cheese-WPI packed either in air or in vacuum. The dityrosine concentration in the cheese-WPI was  $104 \text{ nmol.mol}^{-1}$  tyrosine at day 28, which was significantly lower ( $P < 0.0001$ ) than the level of dityrosine seen for the control cheese packed in air. Over time of light exposure, insignificant changes ( $\alpha = 0.05$ ) were seen in the accumulation of dityrosine in the vacuum-packed control cheese.

The accumulation of lipid hydroperoxides in the cheeses was found to depend on storage time as well as package conditions (Fig. 2c). It followed the same pattern for both types of cheese. When stored in air and light, it increased steadily from  $0 \mu\text{g.g}^{-1}$  cheese at day 0 to  $\sim 0.3 \mu\text{g.g}^{-1}$  cheese at the 28th day of storage, both in the control cheese and the cheese-WPI. The levels of lipid hydroperoxides had increased significantly ( $P < 0.0001$ ) at the seventh day of storage in light as well as in air in both types of cheese. The cheese packed in air showed significantly higher



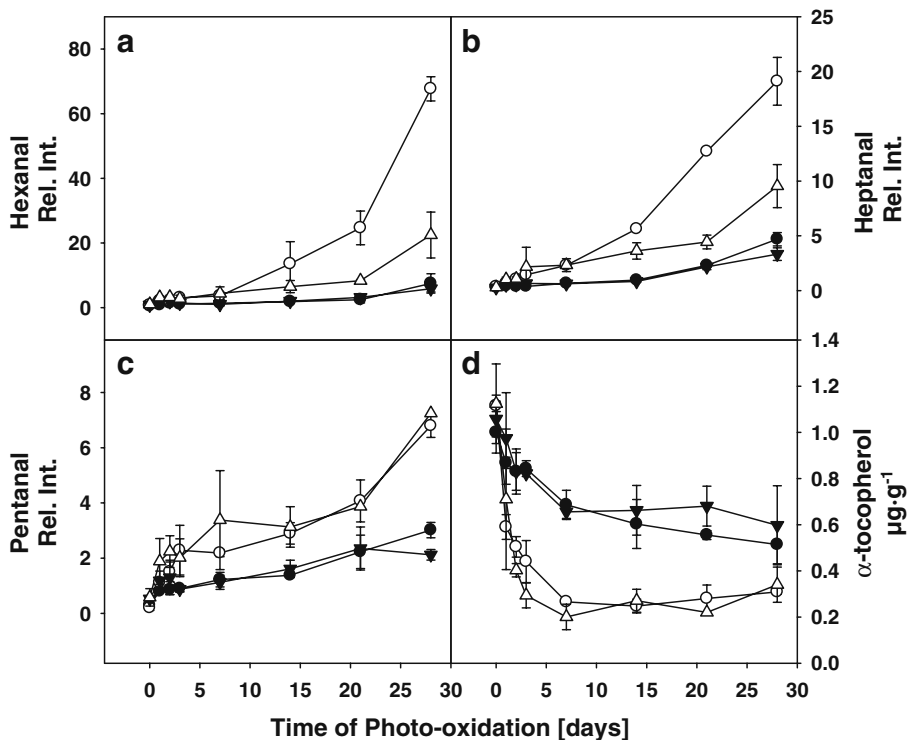


**Fig. 2** Decrease of the naturally occurring photo sensitizer riboflavin (**a**) and accumulation of dityrosine (**b**), lipid hydroperoxides (**c**) and dimethyl disulfide (**d**) in low-fat model cheese packed in air (white circle) or in vacuum (black circle) and cheese-WPI added also packed in air (white up-pointing triangle) or in vacuum (black down-pointing triangle). The cheeses were kept at 15 °C while exposed to fluorescent light (1,800 lux) for a period of 28 days. Bars indicate standard deviations of triplicates for riboflavin, dityrosine and lipid hydroperoxides and duplicates for dimethyl disulfide (DMDS)

accumulations of lipid hydroperoxides than the vacuum-packed cheeses. No significant difference ( $\alpha=0.05$ ) was seen between the two different types of model cheeses, neither when they were packed in vacuum, nor in air.

The accumulation of DMDS (Fig. 2D) was dependent on the addition of WPI. At day 28, the level of DMDS was approximately four times higher in the control cheese than in the cheese-WPI. The accumulation of DMDS in the vacuum-packed cheese-WPI was not recognised to be significantly different ( $\alpha=0.05$ ) from the vacuum-packed control cheese.

The accumulation of the secondary lipid oxidation products hexanal and heptanal was dependent on time of light exposure, package condition and the addition of WPI (Figs. 3a–b). Independently on the addition of WPI, the three lipid oxidation products (hexanal, heptanal and 1-heptanol) all showed a lag phase for the first 7 days of light exposure in the cheese packed in air. After the seventh day of storage, the accumulation of hexanal, heptanal and 1-hexanol increased significantly ( $P<0.0001$ ) in the control cheese. Conversely, the accumulation of the same three lipid oxidation products was time independent in the cheeses-WPI for the first 21 days of light exposure ( $\alpha=0.05$ ), indicating a continuous lag phase of hexanal, heptanal and 1-hexanol when WPI was added. At the 28th day of light exposure, the accumulation



**Fig. 3** Generation of hexanal (a), heptanal (b) and pentanal (c) and decrease of  $\alpha$ -tocopherol (d) in low-fat model cheeses with and without WPI added. The low-fat model cheeses were packed in air or in vacuum, respectively. Control cheese packed in air (white circle) or in vacuum (black circle) and low-fat cheese-WPI in air (white up-pointing triangle) or in vacuum (black down-pointing triangle) were kept at 15 °C while exposed to fluorescent light for a period of 28 days. Rel. Int.: calculated relatively to external standard curves (nanogrammes per litre) for hexanal, heptanal and pentanal. Rel. Int. for  $\alpha$ -tocopherol: calculated relatively to the content of  $\alpha$ -tocopherol at the beginning of the experiment. Bars indicate standard deviations of duplicates for all measurements

of hexanal, 1-hexanol and heptanal became significantly higher in the cheese-WPI when compared to the levels observed earlier in the experiment. Hence, addition of WPI to the cheese resulted in an extension of the lag phase that lasted for 14 days before the generation of the secondary lipid oxidation products hexanal, heptanal and 1-hexanol began to increase. Another secondary lipid oxidation product, pentanal, did not show the same lag phase as the other lipid oxidation products just mentioned (Fig 3c). Likewise, there was no significant difference ( $\alpha=0.05$ ) in the accumulation of pentanal between the control cheese and the cheese-WPI, whereas there was a significant dependence on time and package condition ( $P<0.0001$ ).

It was established that the accumulation of lipid hydroperoxides continued long after the riboflavin had completely vanished, and that the increase in the secondary lipid oxidation products hexanal, 1-hexanol and heptanal did not follow the same pattern as the accumulation of lipid hydroperoxides, but was lower for the first 7 days of light exposure than for the last 3 weeks. This indicates that naturally occurring antioxidants may be important. Vitamin E ( $\alpha$ -tocopherol) was therefore

measured in all the cheese samples as well (Fig. 3d). The concentration of  $\alpha$ -tocopherol was  $1.1 \mu\text{g}\cdot\text{g}^{-1}$  cheese in both types of cheeses, and the  $\alpha$ -tocopherol content decreased rapidly within the first 7 days of storage in the cheeses packed in air, independently on the presence of WPI, whereupon a stable level was reached. The content of  $\alpha$ -tocopherol decreased more slowly within the first 7 days of light exposure in the vacuum-packed cheese when compared with the cheese packed in air, and for the vacuum-packed cheeses, the decrease in  $\alpha$ -tocopherol was also independent on the type of cheese.

## 4 Discussion

In the present study, the vacuum-packed cheeses all showed very low accumulation of the measured oxidation products over time of storage under light, even though the riboflavin was lost at the same rate as in the cheese packed in air. Triplet-excited riboflavin is therefore not able to oxidise the substrates (lipids or proteins) to the same extent without the presence of oxygen, as it is when oxygen is present. Even dityrosine, which has been suggested to be formed through the type I mechanism (Silva and Godoy 1994), was generated to a much lower extent in the vacuum-packed cheese. Consumption of  $\alpha$ -tocopherol was also dependent on the concentration of oxygen. This result may reflect a higher consumption of  $\alpha$ -tocopherol in the cheese with air due to a higher generation of radicals. In contrast, consumption of  $\alpha$ -tocopherol was independent on the presence of WPI.

The accumulation of hexanal, heptanal and 1-hexanol was clearly reduced in the presence of WPI, while WPI had no effect on the accumulation of lipid hydroperoxides indicating that WPI did not quench singlet oxygen (Michaeli and Feitelson 1995; 1997) but rather the degradation of lipid hydroperoxides into aldehydes. Chelating or scavenging (Faraji et al. 2004) could potentially be responsible for the lower accumulation of secondary lipid oxidation products observed in the cheese-WPI when compared to the control cheese. As addition of WPI also decreased the accumulation of protein oxidation products, scavenging seems not to be the mechanism behind the lower accumulation of secondary lipid oxidation products. Chelating of transition metal ions by the whey proteins may be a mechanism by which a lower accumulation of secondary lipid oxidation products could occur. WPI, however, binds metal ions approximately ten times less efficiently than caseins (Faraji et al. 2004), and caseins, which are the dominating proteins in cheese, are therefore expected to bind more metal ions than WPI.

The lower accumulation of aldehydes could potentially be caused by a reaction between the aldehydes and the whey proteins through a Schiff base formation (Dalsgaard et al. 2006; Kikugawa et al. 1988) but this does not seem reasonable as the accumulation of pentanal was not affected by the addition of WPI. In contrast to the other lipid aldehydes no lag phase was observed in the accumulation of pentanal, and the accumulation seemed to follow the same pattern as seen for accumulation of lipid hydroperoxides. These results are hard to explain because pentanal and hexanal originate from the 14- and 13-hydroperoxides from linoleic acid (Belitz and Grosch 2004), and the results presented here indicate differences in the way they were generated. It has been shown that hexanal can be formed by both the type I and the

type II mechanisms in an oil–water emulsion with linoleic acid as substrate (Yang et al. 2007), while pentanal has been suggested to be formed by a type I mechanism (Lee H.H et al. 2002; Lee and Min 2009). The data presented in the present study can neither confirm nor reject the type I mechanism as being responsible for the generation of pentanal, but can only indicate that pentanal is formed through a different pathway than the other three secondary lipid oxidation products measured here.

Østdal et al. (2000b) has demonstrated the existence of long-lived radicals in whey proteins. These radicals could potentially explain the lower accumulation of dityrosine in the cheese-WPI, but the dityrosine concentration did not increase at all in the cheese-WPI during the 28 days of storage under light. Therefore tyrosine scavenging of triplet-excited riboflavin (Cardoso et al. 2004) reducing the type I mechanism seem unlikely. Radical transport between lipid and proteins has, however, also been suggested to be important for the generation of dityrosine (Saeed et al. 2006), and this mechanism seem to be of major importance in cheese (Dalsgaard et al. 2010), but no dityrosine generation was found to occur in the cheese-WPI. These results indicate that whey proteins prevented interaction between triplet-excited riboflavin and the highly labile caseins (Huvaere et al. 2010) in the cheese-WPI and also prevented interaction between caseins and lipids thus preventing radical transfer from the lipid phase to the casein fraction. The addition of WPI resulted in a lower accumulation of both dityrosine and DMDS indicating that scavenging was not the mechanism responsible for the antioxidative effect of WPI. The whey proteins did therefore not function as true antioxidants, but rather as retarders. The mechanism by which whey proteins act as antioxidants is still unclear, and more mechanistic studies are needed.

In conclusion, the present study showed that addition of WPI to low-fat cheeses effectively reduced accumulation of both lipid and oxidation products. Scavenging can therefore not be expected to be responsible for the antioxidative effect of whey proteins observed in low-fat cheeses.

**Acknowledgement** Heidi Taulborg Jørgensen is thanked for her skilful assistance in the pilot plant producing the model cheeses. The Danish Dairy Research Foundation and the Innovation Law under the auspices of the Ministry of Food, Agriculture and Fisheries, Denmark are thanked for financing the study.

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