Amino Acids (2012) 43:951–962
DOI 10.1007/s00726-011-1157-y

ORIGINAL ARTICLE

The impact of photo-induced molecular changes of dairy proteins on their ACE-inhibitory peptides and activity

Barbara Kerkaert · Frédéric Mestdagh · Tatiana Cucu · Kshitij Shrestha · John Van Camp · Bruno De Meulenaer

Received: 14 July 2011 / Accepted: 9 November 2011 / Published online: 25 November 2011

Abstract Among all dietary proteins, dairy proteins are the most important source of bio-active peptides which can, however, be affected by modifications upon processing and storage. Since it is still unknown to which extent the biological activity of dairy proteins is altered by chemical reactions, this study focuses on the effect of photo-induced molecular changes on the angiotensin I converting enzyme (ACE) inhibitory activity. Milk proteins were dissolved in phosphate buffer containing riboflavin and stored under light at 4°C for one month during which the molecular changes and the ACE-inhibitory activity were analysed. An increase in the total protein carbonyls and the *N*-formylkynurenine content was observed, besides a decrease in the free thiol, tryptophan, tyrosine and histidine content. These changes were more severe in caseins compared with whey proteins and resulted moreover in the aggregation of caseins. Due to these photo-induced molecular changes, a significant loss of the ACE-inhibitory activity was observed for casein peptides. A peptide analysis moreover illustrated that the decreased activity was not attributed to a reduced digestibility but to losses of specific ACE-inhibitory peptides. The observed molecular changes, more specifically the degradation of specific amino acids and the casein aggregation, could be assigned as the cause of the altered peptide pattern and as such of the loss in ACE-inhibitory activity.

Keywords Milk proteins · Photo-oxidation · ACE-inhibitory activity · ACE-inhibitory peptides · LC–TOF–MS

Abbreviations

ACE	Angiotensin I converting enzyme
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5-Dithiobis(2-nitro-benzoic acid)
OPA	Ortho-phthaldialdehyde
FMOC	9-Fluorenylmethylchloroformate
NFK	*N*-formylkynurenine
ALA	α-Lactalbumin
BLG	β-Lactoglobulin
BSA	Bovine serum albumin

Introduction

The importance of dairy proteins in the human diet has been recognized for many decades in view of their nutritional quality, versatile functionalities and more recently also for their physiological activities (Korhonen et al. 1998). The two major classes of milk proteins, whey (20%) and caseins (80%) differ greatly in their physicochemical properties, but are, however, both complex proteins, containing high levels of essential amino acids and having a high protein efficiency ratio (Hoffman and Falvo 2004). Besides, both types of milk proteins are known as major sources of bioactive peptides with a wide range of physiological activities (Hartmann and Meisel 2007; Korhonen

Electronic supplementary material The online version of this article (doi:10.1007/s00726-011-1157-y) contains supplementary material, which is available to authorized users.

B. Kerkaert · F. Mestdagh · T. Cucu · K. Shrestha · J. Van Camp · B. De Meulenaer (✉)
NutriFOODchem unit (partner in Food2Know), Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium
e-mail: Bruno.DeMeulenaer@UGent.be

et al. 1998; Shah 2000; Srinivas and Prakash 2010). Shah (2000) gave an overview of the bioactive peptides in milk and their respective physiological function.

One of the most important biological functions of these peptides is the inhibition of the angiotensin-I-converting enzyme (ACE) (Lopez-Fandino et al. 2006). ACE is associated with the angiotensin system, in which it is responsible for the release of the vasopressor angiotensin-II. In addition it is responsible for the inhibition of the vasodilator bradykinin in the kinin-kallikrein system (Lopez-Fandino et al. 2006). The combination of both ACE activities results in hypertension, which affects one-third of the Western population (Lopez-Fandino et al. 2006). Takano (1998) and FitzGerald et al. (2004) gave an overview of the milk-derived peptides with ACE-inhibitory activity. It was shown that these peptides are relatively small (<3 kDa) and have a hydrophobic C-terminal containing tryptophan, tyrosine, phenylalanine or proline (Miguel et al. 2009; Otte et al. 2007). Different studies have been performed on the production of dairy hydrolysates with an antihypertensive effect either by fermentation or by in vitro hydrolysis and enrichment (Miguel et al. 2009; Otte et al. 2007; Pihlanto et al. 2010; Srinivas and Prakash 2010; Vermeirssen et al. 2005). Since all ACE-inhibitory peptides have a relatively restricted size and a hydrophobic C-terminal, enrichment is achieved by ultrafiltration and size exclusion chromatography (Lopez-Fandino et al., 2006). Although it is well known how to isolate and produce milk hydrolysates with ACE-inhibitory activity, less is known about the stability of the inhibitory peptides after processing and storage (Korhonen et al. 1998).

Since dairy products are rich in the photo-sensitizer riboflavin, they are particularly prone to photo-oxidation (Dalsgaard et al. 2007; Mestdagh et al. 2005). Excited triplet riboflavin, produced under illumination, will be reduced either by direct reaction with food components (type I), or by triplet oxygen resulting in singlet oxygen which will readily attack the major food components (type II) (Choe et al. 2005). Proteins are known as major targets of singlet oxygen with a reactivity which depends strongly on the amino acid side chain composition, since only tryptophan, histidine, tyrosine, methionine and cysteine react at significant rates ($>0.7 \times 10^7$ dm^3 mol^{-1} s^{-1}) with singlet oxygen (Choe and Min 2006; Davies 2003). While the reaction products of singlet oxygen with specific amino acids have been clarified, questions arise about the singlet oxygen-induced molecular changes of the amino acid in a peptide or protein structure (Gracanin et al. 2009). Dalsgaard et al. (2007) recently studied the changes in the structure of milk proteins upon photo-oxidation and elucidated that the vulnerable amino acids mentioned before were oxidized to protein carbonyls and dityrosine resulting in the aggregation of α- and β- caseins. Light-induced changes in the structure of milk proteins were also reported by Jung et al. (Jung et al. 2000) who illuminated β-lactoglobulin. It has already been suggested that these changes will have their consequences on the biological activity: however, to which extent is still unknown (Chatterton et al. 2006; Korhonen et al. 1998). It was recently reported that light-induced changes affected both chymosin- and plasmin-mediated hydrolysis of milk proteins (Dalsgaard et al. 2008; Dalsgaard and Larsen 2009). It is, however, likely that photo-oxidation will also affect the ACE-inhibitory activity. Indeed, the aromatic amino acids tryptophan and tyrosine, which are vulnerable to photo-oxidation, are crucial amino acids at the C-terminal position of ACE-inhibitory peptides (Otte et al. 2007). Dalsgaard and Larsen (2009) already noticed a decrease in ACE-inhibitory casein peptides upon photo-oxidation of caseins and subsequent chymosin hydrolysis; however, they did not investigate if this had an effect on the ACE-inhibitory activity itself. Therefore, this study focuses on the molecular changes in whey and casein isolates upon photo-oxidation and their effect on the ACE-inhibitory activity of peptides released after gastro-intestinal digestion.

Materials and methods

Chemicals

Whey protein isolate (Lacprodan® DI-9224) and caseinate (Miproprodan 30) were delivered by Acatris Food Belgium (Londerzeel, Belgium). 2,4-dinitrophenylhydrazine (DNPH), 5,5-dithiobis(2-nitro-benzoic acid) (DTNB), ortho-phtaldialdehyde (OPA), 9-fluorenylmethylchloroformate (FMOC), sodium azide, phenol, angiotensin converting enzyme from rabbit lung (ACE), *N*-hippuryl-L-histidyl-L-leucine hydrate (Hip-His-Leu), amino acid standards, succinic anhydride and enzymes were purchased from Sigma Aldrich (Bornem, Belgium). Sodium sulphite, potassium phosphate, sodium dihydrogenphosphate, sodium tetraborate, hydrochloric acid, sodium chloride and urea were all of analytical grade and purchased from Chemlab (Zedelgem, Belgium). Acetic acid, mercaptoethanol, HPLC grade acetonitrile and HPLC grade methanol were obtained from VWR (Leuven, Belgium). Trichloroacetic acid (TCA), tris (hydroxymethyl) aminomethane (TRIS) and sodium dodecyl sulphate (SDS) were obtained from Acros Organics (Geel, Belgium). The precision plus protein standard and Biosafe Coomassie for SDS-PAGE were from Bio-Rad (Nazareth, Belgium).

Storage experiment

Protein solutions were prepared by dissolving 6 mg ml^{-1} whey isolate or caseinate, together with 0, 1.5 and

3.0 μg ml^{-1} riboflavin in 10 mM phosphate buffer at pH 6.8. The solutions were sterilised by filtration (CA, 0.45 μm, Novolab, Lokeren, Belgium) and incubated at 4°C under homogenous fluorescent tube illumination (1500 lux, 400–700 nm) on an orbital shaker (Edmund Bühler, Hechingen, Germany). Reference samples, not subjected to illumination, were stored in the dark. The light intensity was measured at the level of the bottlenecks using a Lux meter (PAR-cell 532, 400–700 nm, Skye Instruments, Llandrindod Wells, UK). The protein content was determined by Kjeldahl analysis, using a nitrogen-to-protein conversion factor of 6.38, to express the results per mg protein (AOAC International.Official Method 981.10 1981). All experiments were performed in triplicate.

Riboflavin analysis

The protein solutions were analysed for their riboflavin content by an HPLC method as previously described (Mestdagh et al. 2005). The solutions were injected as such on a LiChrosorb RP-C18 HPLC column (250 mm × 4.6 mm × 10 μm, Varian, Sint-Katelijne-Waver, Belgium) and detected fluorometrically at an excitation and emission wavelength of 450 and 530 nm, respectively. Quantification was performed using an external calibration curve.

Carbonyl assay

Protein carbonyls were determined following a protocol adapted from Fenaille et al. (2005). Briefly, 0.4 ml of DNPH (10 mM in 2 M HCl) was added to 0.3 ml oxidized protein. After 60 min of incubation in the dark, 0.7 ml of 20% TCA was added and samples were left on ice for 10 min followed by a centrifugation at 9,000*g* for 3 min. Protein pellets were washed three times with 1 ml ethanol:ethylacetate (1:1, v/v) to remove the excess DNPH. The final pellet was dissolved in 0.5 ml 6 M urea in 20 mM phosphate buffer (pH 2.3) and the absorbance was measured at 370 nm. Results were expressed in nmol carbonyls per mg protein using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} on blank subtracted data.

Determination of thiol groups

The free and total thiol content was determined by the Ellman method as previously described (Beveridge et al. 1974). For free thiols, 0.5 ml of oxidized protein was dissolved in 2.5 ml of 50 mM Tris–HCl buffer (pH8) containing 8 M urea. 20 μl of 10 mM of DTNB (in Tris–HCl) was added. After 5-min incubation, the absorbance of the samples was measured at 412 nm before and after adding DTNB against a blank without protein. The thiol content was calculated using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} on blank subtracted data. To determine the total thiol content, the disulfide bridges were first broken by adding 1 ml of 10 M urea (in Tris–HCl) and 20 μl of mercaptoethanol to 0.2 ml of oxidized protein. This mixture was incubated for 1 h after which the proteins were precipitated with 10 ml TCA (20%) and the excess mercaptoethanol removed. The final pellet was dissolved in 3 ml 8 M urea in 50 mM Tris–HCl buffer (pH 8) and the reaction with DTNB was performed as described above for the free thiol content. Results were expressed in μmol thiol groups per g protein.

Amino acid analysis

Proteins were hydrolysed to their constituent amino acids which were then derivatised with OPA and FMOC and separated on HPLC (Schuster 1988). For the acid hydrolysis, 2 ml of the protein solution was added to 2 ml 12 M HCl containing 0.1% phenol and 0.1% Na_2SO_3 in a glass tube with Teflon screw cap. For basic hydrolysis, 2 ml of protein solution was added to 2 ml 8 M NaOH. Both mixtures were vortexed, incubated for 24 h at 105°C, neutralized and further diluted to 20 ml. The final hydrolysate was filtered over a 0.45 μM PTFE syringe filter (Grace, Lokeren, Belgium). All the amino acids were automatically derivatised in the injector of an Agilent 1100 system (Agilent Technologies, Switzerland). Primary amino acids (0.5 μl sample) were derivatised with 0.5 μl of 75 mM OPA while the secondary amino acid proline was derivatised with 0.5 μl 10 mM FMOC. The derivatised amino acids were separated on a Zorbax Eclipse AAA Rapid Resolution column (4.6 × 150 mm, 3.5 micron, Agilent Technologies) which operated at 40°C. A flow rate of 2 ml min^{-1} was applied with a gradient of solvent A (45% methanol, 45% acetonitrile and 10% water) and solvent B (45 mM $NaH_2PO_4 \cdot H_2O$, 0.02% NaN_3, pH 7.8). The OPA and FMOC-derivatised amino acid were detected fluorometrically, at excitation and emission wavelengths of, respectively, 340/450 nm and 266/305 nm. Internal standards norvaline and sarcosine were used for the quantification.

Tryptophan and *N*-formylkynurenine (NFK)

The tryptophan and NFK content were measured fluorometrically on the intact protein (with a Spectramax Gemini XPS fluorimeter, Molecular Devices, Brussels, Belgium). Therefore, 50 μl of the protein solutions was diluted in 250 μl 6 M urea to completely unfold the protein structure. Tryptophan was measured at excitation and emission wavelengths of 280 and 330 nm, respectively. For NFK, 330 and 440 nm were used, respectively (Dalsgaard et al. 2007).

SDS-PAGE

Proteins, hydrolysed and non-hydrolysed, were subjected to SDS-PAGE under reducing conditions. Therefore, 10 µl of diluted sample (1 mg protein ml^{-1}) was mixed with 10 µl Laemmli buffer (Bio-Rad, Nazareth, Belgium) containing 0.05% mercaptoethanol. For the digested samples, the Laemmli buffer was replaced by Tricine buffer (Bio-Rad, Nazareth, Belgium). Subsequently, samples were heated for 5 min at 90°C and centrifuged for 3 min at 9,000*g*. The non-hydrolysed samples were loaded onto a 12% polyacrylamide Tris–HCl gel, while the digested samples were separated on a 16.5% Tris–Tricine gel (Bio-Rad, Nazareth, Belgium). Electrophoresis was performed at a constant voltage of 150 V. The gels were stained with Biosafe Coomassie (Bio-Rad) and the precision plus protein standard (Bio-Rad, Nazareth, Belgium) was used as a molecular weight marker.

Succinylation

Solid succinic anhydride (23 mg) was added to 2 ml of the photo-oxidized or non-oxidzed casein solutions to give a 50-fold excess of anhydride over protein amine groups (Aitken and Learmonth 2002). The anhydride was added slowly and the pH was monitored. To maintain the pH at 8, 200 µl of 2 M NaOH was added. After addition of anhydride, the solution was incubated for 30 min and subsequently diluted for SDS-PAGE analysis.

In vitro gastrointestinal digestion

To simulate the human gastrointestinal digestion process, a consecutive hydrolysis using pepsin, trypsin and α-chymotrypsin was performed (Vermeirssen et al. 2005). 50 ml of protein solution was transferred to an Erlenmeyer flask and brought to pH 2 (100 µl of 8 M HCl) to simulate the stomach digestion. Pepsin was added to the mixture in an enzyme/substrate ratio of 1/250, followed by an incubation of 2 h at 37°C. Afterwards, the pH was adjusted to 6.5 (100 µl of 10 M NaOH) which is required to simulate the small intestine digestion. Trypsin and chymotrypsin were added at an enzyme/substrate ratio of 1/250 and the solution was incubated for 2.5 h at 37°C. The pH was finally adjusted to 5, the hydrolysates were filtered, frozen at −20°C and lyophilized.

ACE-inhibitory activity

The determination of the in vitro ACE-inhibitory activity was performed by a spectrophotometric method described by Chang et al. (2001) with slight modifications. The method is based on a selective chromogenic reaction for histidyl-leucine using OPA. The lyophilized hydrolysate was dissolved in water at 100 mg ml^{-1} from which a dilution series was made. For each assay, 100 µl of the protein hydrolysate, 100 µl of Hip-His-Leu solution (15 mM in 20 mM sodium tetraborate and 0.3 M NaCl, pH 8.3) and 100 µl of ACE (40 mU ml^{-1} in 15 mM in 20 mM sodium tetraborate and 0.3 M NaCl, pH 8.3) were incubated for 2 h at 37°C. The enzymatic reaction was terminated and the chromogenic reaction started by adding 2 ml of alkaline OPA reagent. The OPA reagent consisted of 1.65 ml OPA (10 mg ml^{-1} in ethanol), 1.65 ml of 2-mercaptoethanol (5 ml ml^{-1} in ethanol) and 73 ml of sodium borate buffer (0.1 M sodium tetraborate and 0.2 M NaOH). After an incubation period (20 min, 25°C), the absorbance was measured at 390 nm. Appropriate blanks were analyzed. The IC_{50} value, defined as the concentration of sample that inhibits 50% of ACE activity, was determined by fitting the dose–response curve via a nonlinear regression analysis to a 4-parameter logistic model with the following equation:

$$Y = \left(A - B/\left(1 + (X/C)^{D}\right)\right) + B \quad (1)$$

with Y the percentage of ACE inhibition, X the concentration of the ACE-inhibitory peptides, A and B, respectively, the maximal and minimal percentage of ACE inhibition, C the IC_{50} value and D the slope of the dose–response curve.

LC–TOF–MS analysis of the ACE-inhibitory peptides

The lyophilized hydrolysates were dissolved in water and half-diluted in a solution of 3% acetic acid in 90% water and 10% acetonitrile. The diluted samples (10 µl) were injected on a Ultimate 3000 RSLC (Dionex, Germany) equipped with a Zorbax 300 SB-C8 column (3.5 micron, 2.1 × 150 mm, Agilent Technologies) applying a flow rate of 0.2 ml min^{-1}. The column was operated at a constant temperature of 40°C. A gradient between 0.1% acetic acid in 90% water/10% acetonitrile (solvent A) and 0.1% acetic acid in 10% water/90% acetonitrile (solvent B) was applied with from 0–1 min: 10% B; 1–11 min: 10–100% B; 11–16 min: 100% B; 16–16.5 min: 100–10% B and from 16.5–21 min: 10%B. The eluted peptides were analysed using a UV detector (Dionex, Germany) at 214, 280 nm and the microTOF II time-of-flight mass spectrometer (Bruker Daltonics, Germany). The electrospray ionization source was operated in the positive mode; the nebulizer (N_2) pressure was set at 2 bar, the nebulizer (N_2) gas flow at 4 L/min and the dry temperature at 200°C. The capillary voltage was maintained at 5,800 V, the capillary exit voltage at 150 V and the skimmer potential was 50 V. Conventional ESI–MS data were recorded using a scan range of *m/z* 100–3,000 and

screened for the masses of different ACE-inhibitory peptides given in Table 3. An internal mass calibration was performed with the ESI TOF tuning mix (Agilent Technologies). The mass resolution was about 8,000 (FWHM) while the mass accuracy varied between 0.1 and 40 ppm with an average of 20 ppm. A semi-quantitative analysis was performed by considering the hydrolysates of the casein solution without riboflavin as a reference and by assuming that the caseins were totally digested. The theoretical concentration of the ACE-inhibitory peptides identified in the non-oxidised casein hydrolysate was calculated and different dilutions of this reference were analysed in order to set-up a calibration curve for each peptide. From this curve, the amount of the peptides in the oxidized samples could be calculated and was expressed in μg peptide mg^{-1} protein.

Statistical analysis

The nonlinear regression was performed by the SPSS 16 statistics package. Statistical comparison between the IC_{50} values and the amino acid results was done by a one-way ANOVA test, applying a significance level of $p < 0.05$.

Results

Riboflavin

Riboflavin was stable upon storage in the dark as shown in Fig. 1. When stored in the light, however, an exponential decrease was observed in both the whey and casein solutions. Within 2 weeks, all the riboflavin was degraded even in the solutions with the highest riboflavin concentration. The reaction constants for the degradation in the whey solution with initially 1.5 and 3.0 μg ml^{-1} riboflavin were, respectively, 0.48 and 0.37 d^{-1}. In the casein solution, these reaction constants were significantly lower with 0.34 and 0.30 d^{-1} for respectively the 1.5 and 3.0 μg ml^{-1} riboflavin solutions.

Protein carbonyls

The protein carbonyl content, presented in Fig. 2, increased during the first 2 weeks of illuminated storage in the riboflavin-containing protein samples, followed by a stabilisation. In the sample without riboflavin stored under light and the samples with riboflavin stored in the dark no carbonyl formation was observed (data not shown). For the whey proteins, an increase up to 8 and 12 nmol carbonyls mg^{-1} protein was observed in, respectively, the 1.5 and 3.0 μg ml^{-1} riboflavin solutions. For the caseins, however, a stronger increase up to 20 nmol and 25 nmol carbonyls mg^{-1} was observed for, respectively, the 1.5 and 3.0 μg ml^{-1} riboflavin solutions.

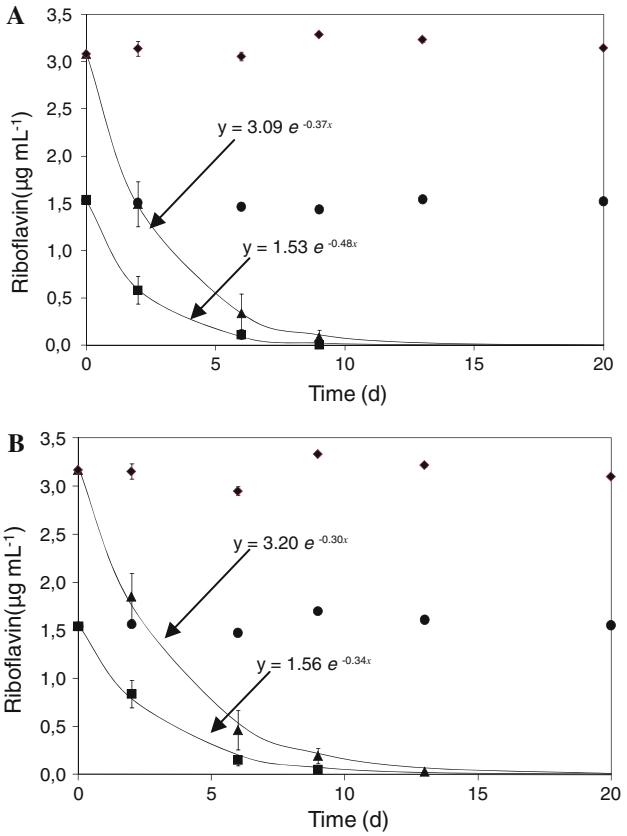

Fig. 1 Riboflavin concentration in function of storage time in whey solutions (**a**) and in casein solutions (**b**) with an initial concentration of 1.5 μg riboflavin ml^{-1} stored under continuous illumination (*filled square*) and in the dark (*filled circle*); with an initial concentration of 3.0 μg ml^{-1} stored under continuous illumination (*filled triangle*) and in the dark (*filled diamond*). *The data points* represent mean values ±SD of three independent experiments. *The lines* represent the exponential curve fitted to the data points

Protein thiols

While the total thiol content of 30 and 200 μmol g^{-1} protein for, respectively, caseins and whey proteins did not change upon photo-oxidation (data not shown), the free thiol content, only present in the whey proteins, decreased during the first 20 days of storage (Fig. 3). The initial amount of free thiols in whey of 25 μmol g^{-1} protein decreased to 10 μmol g^{-1} in the solutions with 3.0 μg ml^{-1} riboflavin and to 15 μmol g^{-1} in the solutions with 1.5 μg ml^{-1} riboflavin. After 20 days of storage no further degradation of the free thiol content was observed. In the reference samples, the free thiols only slightly decreased.

Tryptophan and *N*-formylkynurenine determined by direct fluorescent spectroscopy

The tryptophan content as function of the storage time is shown in Fig. 4a, b. Tryptophan decreased exponentially in

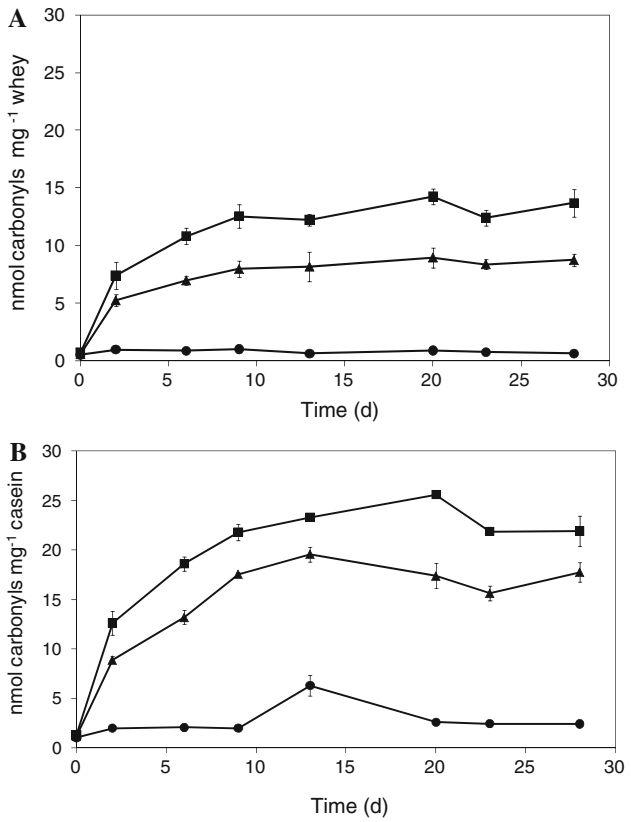

Fig. 2 Carbonyl content in function of storage time in whey solutions (**a**), in casein solutions (**b**) with an initial riboflavin concentration of 0 μg ml^{-1} (*filled circle*); 1.5 μg ml^{-1} (*filled triangle*) and 3.0 μg ml^{-1} (*filled square*). *The data points* represent mean values ±SD of three independent experiments

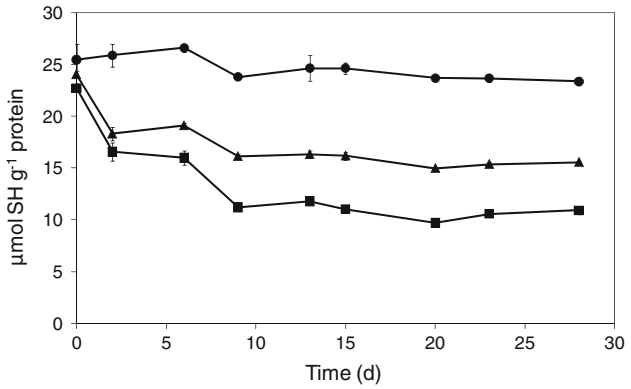

Fig. 3 Free thiol content in function of storage time in whey solutions with an initial riboflavin concentration of 0 μg ml^{-1} (*filled circle*); 1.5 μg ml^{-1} (*filled triangle*) and 3.0 μg ml^{-1} (*filled square*). *The data points* represent mean values ±SD of three independent experiments

the different model systems. The decrease was more pronounced in the solutions with a higher riboflavin content as can be seen from the first-order reaction rate constants. The reaction rate constants were, respectively, 0.011 and 0.020 d^{-1} in the whey solutions with 1.5 and 3.0 μg ml^{-1} riboflavin. In the casein solutions, the same trend was observed; however, the reaction rate constants were significantly higher with, respectively, 0.040 and 0.091 d^{-1} in the casein solutions with 1.5 and 3 μg ml^{-1} riboflavin. In parallel to the tryptophan degradation, an increase in the signal for NFK was observed as shown in Fig. 4c, d. In the first 2 weeks of storage, NFK was formed in both the whey and casein solutions; in the caseins yet to a faster extent than in the whey proteins as seen from the first-order reactions constants. After 15–20 days of storage, the NFK content stabilised in the whey solutions, while it significantly decreased in the casein solutions.

Amino acid composition

Apart from histidine, tyrosine and tryptophan, no significant changes in the amino acid composition were observed even not in the sulphur-containing amino acid, methionine. In both whey and casein proteins a significant decrease in tryptophan, histidine and tyrosine was observed after 15 days of storage, which was especially for tryptophan more pronounced in the caseins for which a decrease of 50% was observed after 15 days of storage, while only 27% of the tryptophan was lost in the whey proteins (Table 1). Between 15 and 30 days of storage, the histidine and tyrosine content further decreased particularly in the whey solutions resulting in a total degradation of, respectively, 39 and 19% for the highest riboflavin concentration. These losses were comparable with those measured in casein proteins in which losses of, respectively, 40 and 12% were observed after 30 days of illumination. A higher riboflavin content of 3.0 μg/ml instead of 1.5 μg/ml resulted in a slightly stronger degradation of histidine and tyrosine (data not shown). However, no significant impact of the riboflavin concentration was seen on the degradation of tryptophan measured after basic hydrolysis.

SDS-PAGE

The electrophoretic pattern of the whey and casein protein solutions with 3.0 μg/ml riboflavin is presented in Fig. 5 as function of the storage time under illumination conditions. For the whey proteins, a clear band for α-lactalbumin (ALA), β-lactoglobulin (BLG) and bovine serum albumin (BSA) was noticeable at, respectively, 14, 18 and 65 kDa. While the band for ALA was stable upon light oxidation, even after 28 days of light exposure, a slight decrease in the intensity of the BLG and BSA band was observed. The intensity of the bands for α_{s1}-casein (23 kDa), α_{s2}-casein (25 kDa) and β-casein (24 kDa) also decreased upon light oxidation and almost disappeared after 28 days of light exposure. After 6 days of illumination, a band of higher protein aggregates (>250 kDa) appeared in the case of caseins whose the intensity tended to increase upon further

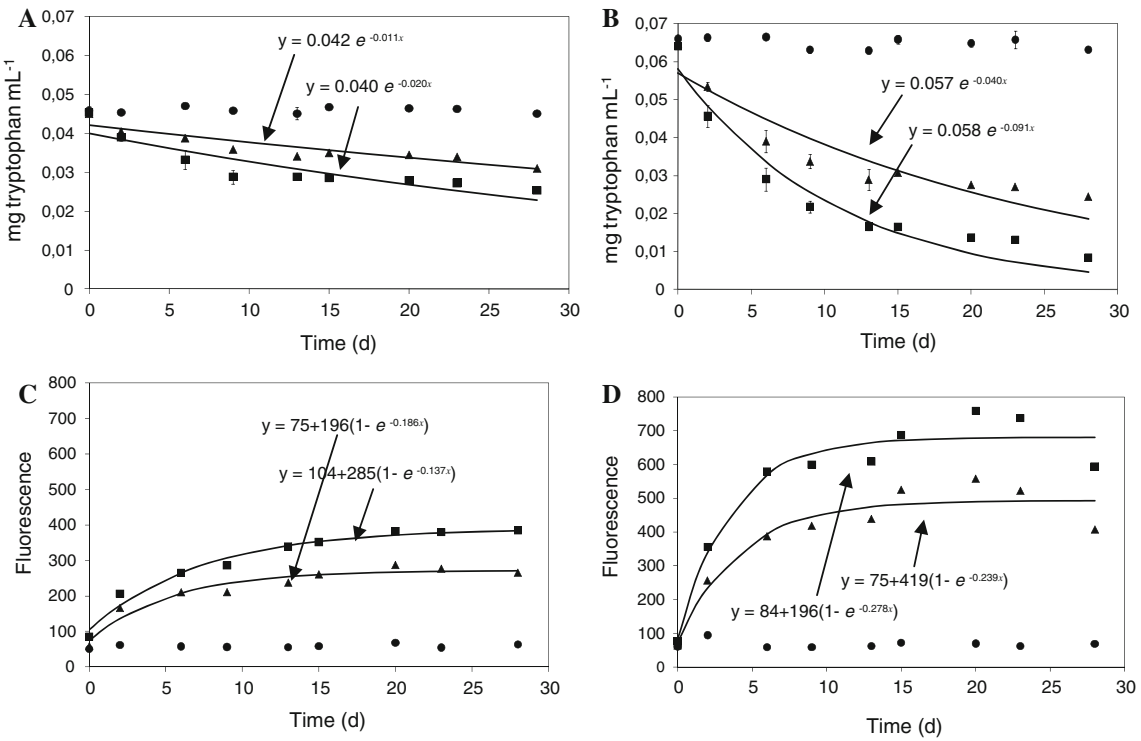

Fig. 4 Concentration of tryptophan in whey (**a**) and casein solutions (**b**) and the fluorescence signal for *N*-formylkynurenine in whey (**c**) and casein (**d**) solutions as function of the storage time for solutions with different riboflavin concentrations: 0 μg ml^{-1} (*filled circle*); 1.5 μg ml^{-1} (*filled triangle*) and 3.0 μg ml^{-1} (*filled square*). *The data points* represent mean values ±SD of three independent experiments

Table 1 Concentration of histidine; tyrosine; methionine and tryptophan (g 100 g^{-1} protein) in casein and whey protein solutions containing 3.0 μg ml^{-1} riboflavin after 0, 15 and 30 days of storage

	Day 0	Day 15	Day 28
Whey proteins			
Histidine	1.76 ± 0.02[a]	1.24 ± 0.00[b]	1.07 ± 0.01[c]
Tyrosine	3.27 ± 0.02[a]	2.94 ± 0.02[b]	2.64 ± 0.03[c]
Methionine	2.60 ± 0.03[a]	2.50 ± 0.01[a]	2.58 ± 0.10[a]
Tryptophan	1.64 ± 0.03[a]	1.19 ± 0.02[b]	1.15 ± 0.08[b]
Caseins			
Histidine	2.52 ± 0.07[a]	1.65 ± 0.12[b]	1.50 ± 0.06[b]
Tyrosine	5.39 ± 0.16[a]	4.77 ± 0.33[ab]	4.74 ± 0.12[b]
Methionine	3.02 ± 0.20[a]	2.80 ± 0.17[a]	2.92 ± 0.18[a]
Tryptophan	1.10 ± 0.03[a]	0.30 ± 0.20[b]	0.50 ± 0.11[b]

The data points represent mean values ± SD of three independent determinations

[a,b,c] Values in the same row with a different letter are significantly different (One way ANOVA, $p < 0.05$)

light exposure, while such aggregation was not observed for whey proteins. For both whey and casein proteins no precipitation was observed upon oxidation and the protein content remained as such constant.

Succinylation

Succinylation was performed in order to distinguish if the casein aggregates were a result of covalent or non-covalent linkage since it converts positively charged amines into negatively charged carboxylates, leading to increased electrostatic repulsion and expansion of the protein (Chapman et al. 2003). In the absence of succinylation, a typical pattern of aggregation was seen for the photo-oxidized casein samples. A similar pattern was observed after succinylation of these samples, indicating that the higher polymers were a result of covalent cross-linkage (Fig. 6). The bands for the non-aggregated

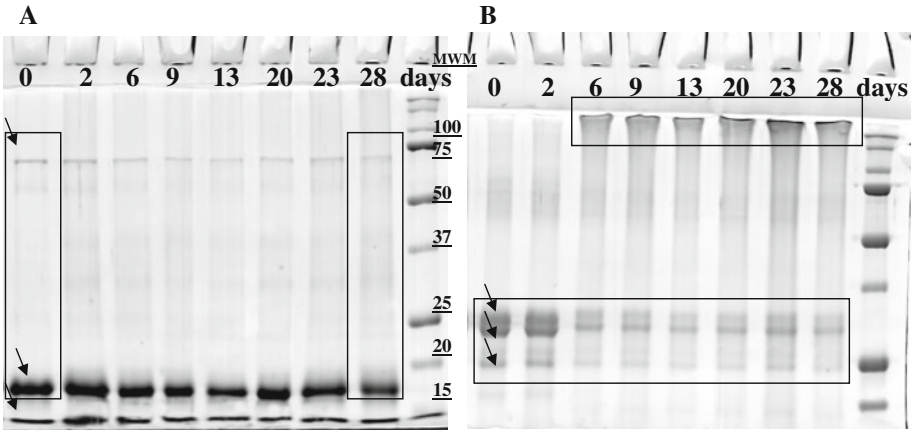

Fig. 5 SDS profile of whey (**a**) and casein (**b**) solutions with 3.0 μg ml^{-1} riboflavin in function of the storage time

proteins slightly increased due to the reaction with succinic anhydride.

ACE-inhibitory activity

At the end of the illumination experiment, the whey and casein solutions containing initially 0, 1.5 and 3.0 μg ml^{-1} riboflavin were subjected to a digestion procedure in order to assess the in vitro ACE-inhibitory assay. The IC_{50} values of the different hydrolysates are shown in Table 2A. The ACE-inhibitory activity of casein hydrolysates was not only determined for the solutions which were digested after 28 days of illumination but also for a casein solution containing 3 μg ml^{-1} riboflavin which was digested prior to an illumination period of 28 days (Table 2B). The IC_{50} value of the non-oxidized proteins was significantly lower for the casein hydrolysates (56 μg ml^{-1}) in comparison with the whey hydrolysates (95 μg ml^{-1}) demonstrating a higher ACE inhibition efficiency of the casein peptides compared with the whey peptides in the gastro-intestinal tract. Upon light exposure, the IC_{50} value significantly increased for the casein hydrolysates. Photo-oxidation decreased the ACE-inhibitory activity of the casein proteins up to 50% in a riboflavin concentration-dependent manner. This was observed both for the riboflavin-containing casein solutions which were digested after illumination, as for the solution which was digested prior to illumination. For the whey solutions containing riboflavin illuminated for 28 days and hydrolysed, the IC_{50} value significantly increased in the sample with 1.5 μg ml^{-1}, while no significant difference could be observed between the 0 and 3.0 μg ml^{-1} sample. From these results, no clear trend could be observed for whey proteins.

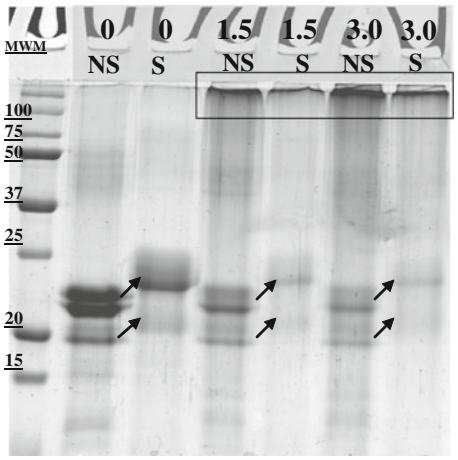

Fig. 6 SDS profile of casein solutions with 0, 1.5 and 3.0 μg ml^{-1} riboflavin illuminated for 28 days non-succinylated (NS) and succinylated (S)

Semi-quantitative peptide analysis

The hydrolysates were analysed by LC–TOF–MS in order to detect changes in the peptide profile upon photo-oxidation. The TOF–MS data were screened for all the masses mentioned in Table 3. This list was obtained by comparing the peptides obtained from an in silico gastro-intestinal digestion of caseins with the ACE-inhibitory peptides known in literature (FitzGerald et al. 2004; Hernandez-Ledesma et al. 2007; Pihlanto-Leppala et al. 1998). Besides a screening, the identified peptides were quantified by the use of the non-oxidized hydrolysate as a calibrant. The results of the semi-quantitative analysis are shown in Table 4. As can be observed not only ACE-inhibitory peptides but also peptides without activity (TDAPSF, TDVENL) were included in order to control the digestibility upon photo-oxidation. The ACE-inhibitory peptides all decreased significantly in the casein hydrolysate containing 1.5 μg ml^{-1} riboflavin and illuminated for 28 days prior to hydrolysis except HLPLP which only decreased when the riboflavin concentration was increased to 3.0 μg ml^{-1}. When illuminating in the presence of a higher riboflavin concentration, all ACE-inhibitory peptides further decreased except YQEPVL which was already

Table 2 In vitro angiotensin converting enzyme (ACE)-inhibitory activity of the hydrolysates of whey and casein solutions containing 0; 1.5 and 3.0 μg ml^{-1} riboflavin, (A) stored for 28 days under continuous illumination followed by gastro intestinal digestion, (B) gastro-intestinal digested followed by storage for 28 days under continuous illumination

	Riboflavin (μg ml^{-1})	IC_{50} (μg protein ml^{-1})*	
		Whey hydrolysates	Casein hydrolysates
A	0	95.0 ± 6.1[a]	55.8 ± 9.0[a]
	1.5	123.4 ± 4.3[b]	80.8 ± 1.0[b]
	3.0	106.9 ± 18.9[ab]	113.8 ± 13.9[c]
B	3.0	ND	97.9 ± 4.2[c]

The data points represent mean values ± SD of three independent determinations

ND not determined

* Concentration needed to inhibit ACE to 50% of its original activity

[a,b,c] Values in the same column with a different letter are significantly different (One way ANOVA, $p < 0.05$)

severely degraded in the presence of 1.5 μg ml^{-1} riboflavin. The degradation of the ACE-inhibitory peptides in the sample which was illuminated in the presence 3.0 μg ml^{-1} prior to digestion compared with the reference sample varied between 35% (HLPLP) and 100% (YQEPVL). The concentration of the peptide without ACE-inhibitory activity remained stable upon photo-oxidation, indicating that the digestibility was not affected.

Discussion

The degradation of riboflavin in the model systems followed an exponential decay as was observed previously in milk (Mestdagh et al. 2005). However, the degradation in these systems was significantly faster than in milk, since the first-order reaction rate constants in this study were around 0.30–0.48 d^{-1} while previously in milk, reaction constants of 0.05–0.08 d^{-1} were obtained (Mestdagh et al. 2005). This discrepancy can be explained by the fact that in milk more light scattering occurred compared with that in the model solutions. In addition riboflavin in this study was present in an unbound form in contrast to in milk. The slightly slower degradation in the casein solutions (0.34 d^{-1}) compared with that in the whey solutions (0.48 d^{-1}) could be due to the higher turbidity of the casein solutions caused their micelle structure. The degradation of riboflavin resulted in the formation of the highly reactive singlet oxygen which induced different molecular changes in the proteins. The generic marker for protein oxidation, the protein carbonyls, increased with the illumination time and this to a higher extent in the caseins in comparison with the carbonyl formation in whey proteins (Fig. 2). Dalsgaard et al. (2007) reported earlier that the formation of protein carbonyls upon photo-oxidation was remarkably more enhanced for the random coil α and β-caseins compared with BLG and ALA. The accumulation of protein carbonyls, which was moreover enhanced by a higher riboflavin concentration, stabilised after 2 weeks of light exposure. At the same moment, the riboflavin was depleted which indicates that the reactive oxygen species formed from the riboflavin degradation reacted readily with the proteins and were depleted as soon as riboflavin was degraded. When, however, unsaturated oils were added to the protein solutions, as done in a study of Mestdagh et al. (2011), protein carbonyl generation even continued after riboflavin depletion. The addition of unsaturated oils resulted in an extra source of reactive oxygen species and as such in a prolonged formation of protein carbonyls. Riboflavin depletion resulted not only in a stabilisation of the carbonyl content; it did moreover inhibit a further free thiol decrease which was observed during the first week of storage (Fig. 3). After 28 days of illumination, still 10–15 μmol thiols g^{-1} protein was detected which was unexpected since it was previously described that free cysteine reacts readily with singlet oxygen at a rate constant of 1.6×10^7 dm^3 mol^{-1} s^{-1} (Davies 2003). This can, however, be explained by the fact that amino acids are more protected once incorporated in a protein structure. Apart from reacting with cysteine, singlet oxygen will also react with tryptophan. A rate constant of 3×10^7 dm^3 mol^{-1} s^{-1} was earlier observed for the reaction between singlet oxygen and free tryptophan (Davies 2003). Tryptophan was determined on the intact protein by direct fluorescence spectroscopy (Fig. 4) and by amino acid analysis after basic hydrolysis (Table 1). From both methods, it was observed that tryptophan degraded to a higher extent in the caseins compared with the whey proteins as was also seen previously (Dalsgaard et al. 2007). However, the total degradation after 28 days of illumination measured by fluorescence spectroscopy was significantly stronger than if it was determined by amino acid analysis after hydrolysis and this especially for caseins. At the end of storage time, a total tryptophan decrease of 87% was determined with the

Table 3 The ACE-inhibitory peptides with their theoretical mono-isotopic masses obtained by in silico digestion of caseins using SWISS-PROT (http://www.expasy.org/cgi-bin/peptide-mass.pl) are given

	Sequence[a]	$[M + H]^+$	$[M + 2H]^{2+}/2$	IC_{50}(μg/ml)[b]	References[c]
α_{s1}-Casein					
f(117–123)	KKYKVPQL	1003.6300	502.3149	15	Hernandez-Ledesma et al. (2007)
f(119–124)	YKVPQL	747.4399	374.2200	16	FitzGerald et al. (2004)
f(172–179)	DAYPSGAW	866.3679	433.6840	84	Pihlanto-Leppala et al. (1998)
f(209–214)	TTMPLW	748.3698	374.6849	38	FitzGerald et al. (2004)
α_{s2}-Casein					
f(204–208)	AMKPW	632.3225	316.6613	367	FitzGerald et al. (2004)
β-Casein					
f(123–128)	EMPFPK	748.3698	374.6849	423	Pihlanto-Leppala et al. (1998)
f(149–153)	HLPLP	689.4344	345.2172	16	Hernandez-Ledesma et al. (2007)
f(155–158)	LQSW	533.2718	267.1359	266	FitzGerald et al. (2004)
f(192–198)	AVPYPQR	830.4519	415.7260	227	Pihlanto-Leppala et al. (1998)
f(208–213)	YQEPVL	748.3876	374.6938	209	Pihlanto-Leppala et al. (1998)

[a] The sequence of the ACE-inhibitory peptides

[b] The IC_{50} value is the concentration of peptide which inhibits 50% of the ACE activity

[c] The reference in which the ACE-inhibitory activity of the peptide is mentioned

Table 4 The concentration of the identified ACE-inhibitory peptides and two non ACE-inhibitory peptide (underlined) in the casein hydrolysates with different riboflavin concentrations are given

Sequence	$[M + H]^+$	0 μg ml^{-1} riboflavin μg peptide mg^{-1} protein	1.5 μg ml^{-1} riboflavin	3.0 μg ml^{-1} riboflavin
YQEPVL	748.3876	10.42[a]	0.31[b]	0.00[b]
AVP**Y**PQR	830.4519	11.56[a]	4.01[b]	2.64[c]
HLPLP	689.4344	9.60[a]	9.85[a]	6.20[b]
DA**Y**PSGA**W**	866.3679	15.52[a]	7.39[b]	3.98[c]
YKVPQL	747.4399	13.39[a]	8.10[b]	8.18[b]
KK**Y**KVPQ	502.3149	8.99[a]	7.00[b]	5.05[c]
TDAPSF	637.2828	11.41[a]	9.67[a]	9.31[a]
TDVENL	690.3304	9.61[a]	9.12[a]	8.76[a]

The casein solutions were stored for 28 days under continuous illumination prior to hydrolysis. The data points represent mean values of at least two independent determinations

[a,b,c] Values in the same row with a different letter are significantly different (One way ANOVA, $p < 0.05$)

fluorescence method in the casein solution containing 3.0 μg ml^{-1} riboflavin. A decrease of only 51% was found in the same solutions after amino acid analysis. This discrepancy was possibly due to an underestimation of the tryptophan content by fluorescence spectroscopy upon photo-oxidation. Since oxidation of caseins induced protein aggregation as was observed from the SDS profile (Fig. 5), some of the tryptophan residues in the caseins were shielded from the solvent. As a result of the shielding, a part of the tryptophan residues were not determined in the direct fluorescence spectroscopy assay in contrast to the amino acid analysis after basic hydrolysis which determined the total tryptophan content. Besides monitoring tryptophan losses, fluorescence spectroscopy thus revealed more information about the conformational changes and could as such be linked to the changes in the SDS profile. It was shown as well that the casein polymers formed were due to covalent linkages. For the whey proteins, however, no polymerisation could be observed upon photo-oxidation.

The two major degradation products of photo-oxidation of tryptophan, kynurenine and *N*-formylkynurenine (NFK), are both not stable upon basic hydrolysis in contrast to tryptophan (Kanner and Fennema 1987; Simat and Steinhart 1998). Therefore, the formation of NFK was measured in the protein solutions by direct fluorescence spectroscopy (Fig. 4). The signal for NFK increased significantly more in caseins compared with whey proteins upon photo-oxidation in parallel with the tryptophan decrease and the

other oxidative changes. Dalsgaard et al. (2007) also reported that the increase in NFK upon photo-oxidation was more pronounced in the random coil caseins compared with that in the globular whey proteins. From our results it was moreover seen that the formation of NFK was enhanced by a higher initial riboflavin concentration.

Besides tryptophan, tyrosine and histidine were also degraded, while in contrast methionine, also known to be prone to photo-oxidation (Davies 2003), remained stable (Table 1). Methionine, however, did degrade just as lysine when unsaturated oils were added to the model systems (Mestdagh et al. 2011). Interestingly, tyrosine and histidine degraded to the same extent in whey and casein proteins upon light oxidation in contrast to tryptophan. This discrepancy suggests that these two amino acids were not responsible for the casein aggregation which contrasts with previous studies in which protein aggregation was attributed to dityrosine formation (Dalsgaard et al. 2007). As the degradation of tryptophan and the formation of NFK were more pronounced in caseins compared with whey proteins as was protein aggregation, our observations seem to suggest that tryptophan and its degradation products, however, play a key role in the aggregate formation.

As it was the purpose of this study to investigate if the photo-induced molecular changes affected the bio-activity, the digestibility and the ACE-inhibitory activity of the photo-oxidized proteins were determined after gastro-intestinal digestion. Besides, a semi-quantitative analysis of specific ACE-inhibitory peptides was performed. The ACE-inhibitory activity of the casein peptides was significantly affected upon photo-oxidation (Table 2A), which contrasted with the whey peptides The ACE-inhibitory activity of the non photo-oxidized whey and casein proteins, expressed as the IC_{50} value (95 and 56 μg ml^{-1}, respectively), were comparable with earlier reported IC_{50} values of milk protein hydrolysates (Miguel et al. 2009; Otte et al. 2007; Pihlanto-Leppala et al. 1998; Vermeirssen et al. 2005). Upon photo-oxidation, the IC_{50} value of the casein hydrolysates significantly increased up to 114 μg ml^{-1} and this to a higher extent in the solutions with a higher riboflavin concentration. For the whey hydrolysates, however, no clear trend was observed. Given the fact that the reduction in ACE-inhibitory activity of caseins illuminated prior to digestion was comparable with the reduction observed for caseins digested prior to illumination (Table 2B), it could be concluded that the digestibility as such was probably not affected and was as such not the cause of the reduced ACE-inhibitory activity of caseins. This was moreover confirmed by the fact that the concentration of stable peptides as TDAPSF and TDVENL did not significantly decrease upon photo-oxidation (Table 4) and as well by the electrophoretic pattern which was comparable for the hydrolysates of the photo-oxidized and non-oxidized casein solutions (data not shown). These results indicate that the digestibility as such was not highly affected upon photo-oxidation but they do not exclude that there were minor changes in the proteolysis. This can only be evaluated by considering the total peptide profile which was, however, not possible due to the fact that not all peptides were well ionized. While the present results indicate that the gastro-intestinal enzymes were still able to hydrolyse photo-oxidized caseins, previous research indicated that plasmin hydrolysis was affected by photo-oxidation due to a changed accessibility of plasmin to specific peptide bonds (Dalsgaard et al. 2008).

Notwithstanding the fact that the digestibility was not severely affected upon photo-oxidation, an altered peptide profile was observed due to the decrease of specific peptides containing unstable amino acids. This could be concluded from a significant decrease in the concentration of several ACE-inhibitory peptides, which depended upon the riboflavin concentration (Table 4). A log-linear correlation was found between the sum of the molar concentration of the ACE-inhibitory peptides and the ACE-inhibitory activity ($R^2 = 0.943$) which is in line with the sigmoid curve between the % of ACE inhibition and the concentration of peptides. The loss of specific ACE-inhibitory peptides can be explained by the fact that these peptides contain light-sensitive amino acids as tryptophan, histidine and tyrosine which degraded after 28 days of photo-oxidation (Table 1). A peptide as DAYPSGAW (74%) degraded significantly more compared with HLPLP (35%) due to the fact that tryptophan was more sensitive to light compared with histidine (Table 1).

Photo-oxidation affected especially casein proteins resulting in the loss of specific amino acids, in particular tryptophan. Furthermore, it induced casein aggregation, most likely because of increased hydrophobic interaction and covalent cross-linkage between the oxidized proteins. Moreover, photo-oxidation resulted in a changed ACE-inhibitory peptide pattern, resulting in a seriously reduced ACE-inhibitory activity of the photo-oxidized casein proteins. While it was known that upon photo-oxidation, dairy proteins were severely modified (Dalsgaard et al. 2007), it was still unclear to which extent these modifications affected the bio-activity. From this research it was, however, observed that the photo-induced modifications resulted in a significant decrease of the ACE-inhibitory activity of caseins peptides. The dairy industry should be aware of the fact that storing in the light could affect not only the nutritional quality, but also the physiological activity. More research should be performed in order to evaluate if photo-oxidation of dairy proteins also affects other biological activities, as for example the antioxidant activity.

Acknowledgments Frédéric Mestdagh is post-doctoral researcher funded by the Research Foundation-Flanders (FWO). Tatiana Cucu is funded by the Belgian Science Policy (SD/AF/03A). Kshitij Shretsha is funded by the Flemish Interuniversity Council (VLIR). We thank Acatris Food Belgium for delivering the whey protein isolate and caseinate.

References

Aitken A, Learmonth M (2002) Succinylation of proteins. The protein protocols handbook. In: Walker JM (ed) Humana Press Inc., Totowa, pp 459–460

AOAC International.Official Method 981.10 (1981) Crude protein in meat: block digestion method. Official Methods of Analysis of AOAC, 16th edn, vol 2, pp 7–8

Beveridge T, Toma SJ, Nakai S (1974) Determination of Sh-groups and Ss-groups in some food proteins using Ellmans reagent. J Food Sci 39:49–51

Chang BW, Chen RLC, Huang IJ, Chang HC (2001) Assays for angiotensin converting enzyme inhibitory activity. Anal Biochem 291:84–88

Chapman ALP, Winterbourn CC, Brennan SO, Jordan TW, Kettle AJ (2003) Characterization of non-covalent oligomers of proteins treated with hypochlorous acid. Biochem J 375:33–40

Chatterton DEW, Smithers G, Roupas P, Brodkorb A (2006) Bioactivity of beta-lactoglobulin and alpha-lactalbumin—technological implications for processing. Int Dairy J 16:1229–1240

Choe E, Min DB (2006) Chemistry and reactions of reactive oxygen species in foods. Crit Rev Food Sci Nutr 46:1–22

Choe E, Huang RM, Min DB (2005) Chemical reactions and stability of riboflavin in foods. J Food Sci 70:R28–R36

Dalsgaard TK, Larsen LB (2009) Effect of photo-oxidation of major milk proteins on protein structure and hydrolysis by chymosin. Int Dairy J 19:362–371

Dalsgaard TK, Otzen D, Nielsen JH, Larsen LB (2007) Changes in structures of milk proteins upon photo-oxidation. J Agric Food Chem 55:10968–10976

Dalsgaard TK, Heegaard CW, Larsen LB (2008) Plasmin digestion of photooxidized milk proteins. J Dairy Sci 91:2175–2183

Davies MJ (2003) Singlet oxygen-mediated damage to proteins and its consequences. Biochem Biophys Res Commun 305:761–770

Fenaille F, Parisod V, Tabet JC, Guy PA (2005) Carbonylation of milk powder proteins as a consequence of processing conditions. Proteomics 5:3097–3104

FitzGerald RJ, Murray BA, Walsh DJ (2004) Hypotensive peptides from milk proteins. J Nutr 134:980S–988S

Gracanin M, Hawkins CL, Pattison DI, Davies MJ (2009) Singlet-oxygen-mediated amino acid and protein oxidation: formation of tryptophan peroxides and decomposition products. Free Radic Biol Med 47:92–102

Hartmann R, Meisel H (2007) Food-derived peptides with biological activity: from research to food applications. Curr Opin Biotechnol 18:163–169

Hernandez-Ledesma B, Quiros A, Amigo L, Recio I (2007) Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. Int Dairy J 17:42–49

Hoffman JR, Falvo MJ (2004) Protein - Which is best? J Sports Sci Med 3:118–130

Jung MY, Lee KH, Kim SY (2000) Riboflavin-sensitized photochemical changes in beta-lactoglobulin in an aqueous buffer solution as affected by ascorbic acid. J Agric Food Chem 48:3847–3850

Kanner JD, Fennema O (1987) Photooxidation of tryptophan in the presence of riboflavin. J Agric Food Chem 35:71–76

Korhonen H, Pihlanto-Leppala A, Rantamaki P, Tupasela T (1998) Impact of processing on bioactive proteins and peptides. Trends Food Sci Technol 9:307–319

Lopez-Fandino R, Otte J, Van Camp J (2006) Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. Int Dairy J 16:1277–1293

Mestdagh F, De Meulenaer B, De Clippeleer J, Devlieghere F, Huyghebaert A (2005) Protective influence of several packaging materials on light oxidation of milk. J Dairy Sci 88:499–510

Mestdagh F, Kerkaert B, Cucu T, De Meulenaer B (2011) Interaction between whey proteins and lipids during light-induced oxidation. Food Chem 126:1190–1197

Miguel M, Contreras MM, Recio I, Aleixandre A (2009) ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. Food Chem 112:211–214

Otte J, Shalaby SM, Zakora M, Pripp AH, EI-Shabrawy SA (2007) Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: effect of substrate, enzyme and time of hydrolysis. Int Dairy J 17:488–503

Pihlanto A, Virtanen T, Korhonen H (2010) Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. Int Dairy J 20:3–10

Pihlanto-Leppala A, Rokka T, Korhonen H (1998) Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. Int Dairy J 8(4):325–331

Schuster R (1988) Determination of amino-acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and high-performance liquid-chromatography. J Chromatogr Biomed Appl 431:271–284

Shah NP (2000) Effects of milk-derived bioactives: an overview. British J Nutr 84:S3–S10

Simat TJ, Steinhart H (1998) Oxidation of free tryptophan and tryptophan residues in peptides and proteins. J Agric Food Chem 46:490–498

Srinivas S, Prakash V (2010) Bioactive peptides from bovine milk alpha-casein: isolation, characterization and multifunctional properties. Int J Peptide Res Therapeutics 16:7–15

Takano T (1998) Milk derived peptides and hypertension reduction. Int Dairy J 8:375–381

Vermeirssen V, Van Camp J, Verstraete W (2005) Fractionation of angiotensin I converting enzyme inhibitory activity from pea and whey protein in vitro gastrointestinal digests. J Sci Food Agric 85:399–405