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Peptides as antioxidants and carbonyl quenchers in biological model systems

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Abstract
Subjecting selected peptides to *in vitro* analyses covering their ability to interfere with the lipid oxidation chain reaction as well as to protect proteins from direct and indirect oxidation has provided the basis for a more detailed understanding of peptide-mediated protection in biological systems. The efficiency of peptides as radical scavengers and chain-breaking antioxidants in oxidizing lipid membranes was found to be low. Previous studies on antioxidative activity of peptides tend not to include comparisons with efficiencies of more well-documented antioxidants and/or use irrelevantly high dosages of peptides. The present study demonstrates that the effect of the investigated peptides towards oxidation in biological membrane systems is mainly a protection of vital proteins from being oxidatively modified. This protection is obtained through a prevention of lipid oxidation derived carbonylation (indirect protein oxidation) and through interference with aqueous radical species (direct protein oxidation), and it is only achieved if the peptides are present in high concentrations as sacrificial antioxidants.

Keywords: *Antioxidative mechanisms, peptides, lipid oxidation, protein oxidation, protein carbonylation, carnosine, DNPH*

Introduction

Antioxidative activity of peptides from dietary protein sources is in focus due to the increasing demand for natural bioactive ingredients. Peptides from a variety of high-protein sources have been reported to show antioxidative effects, for example peptides recovered from hydrolysed soy protein and soy-fermented foods [1–3], hydrolysed zein [4], hydrolysed fish skin gelatin [5], hydrolysed giant squid muscle [6], protease digest of prawn muscle [7], enzyme hydrolysed tuna backbone protein [8], muscle protein from bullfrog [9], whey protein [10], casein [11], pork cracklings and feathers from chickens [12].

Different techniques have been used in the search for antioxidative peptides and protein hydrolysates. Numerous studies have documented the radical-scavenging effect of peptides by subjecting isolated peptides or hydrolysed protein to assays based on free radical generation, such as the ORAC (oxygen radical absorbance capacity) [13,14], TEAC (Trolox equivalent antioxidant capacity) [15], DPPH (1,1-diphenyl-2-picrylhydrazyl) [3,8,9,16–18] and hydroxyl- and superoxide radical quenching assay [9,17]. A radical-scavenger is a compound which inactivates radicals, whereas an antioxidant is commonly accepted as being a compound, which when added in a relatively small concentration compared to the substrates, delays or inhibits oxidation [19]. Thus, antioxidative activity of peptides has also been evaluated by lipid peroxidation inhibition assays [8,9,18], including the ferric thiocyanate method [6,7,20], and the inhibiting effect on formation of secondary lipid oxidation products has been evaluated by measuring TBARS (thiobarbituric acid reactive substances) in lipid emulsions [1,14], liposome systems [10,18] and meat systems [11,17]. Finally, *ex vivo* experiments have been conducted to verify the protective effect of

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peptides, e.g. on DNA [21] and human lung fibroblast cells [6,22].

The mechanism behind the antioxidative activity of peptides is yet to be elucidated. It is discussed in the literature whether the antioxidative effect evolves from single amino acids in the peptide sequence or if the effect is a result of interactions between multiple amino acids side chains, although most researchers agree upon the latter [7,15,23]. Antioxidative activity of peptides is frequently explained by a radical-scavenging effect [4–6], whereas other studies point towards several antioxidant mechanisms, including a physical shielding effect [1,4] and metal-chelating effects [4,21]. It seems accordingly safe to assume that antioxidative activity of peptides originates from more than one single reaction type or mechanism.

Oxidative stress resulting from elevated levels of reactive oxygen species (ROS) does not only affect lipids. Unsaturated membrane lipids are easily oxidized and when lipid oxidation is initiated in model systems it propagates in a radical chain reaction. Lipid membrane oxidation as a deteriorating phenomenon in biological systems has therefore received much attention, while oxidative modification of other vital cell components like proteins and enzymes has been less investigated. Protein damage due to oxidative stress can be divided into two categories based on the reaction pathways, (i) direct protein oxidation and (ii) indirect protein oxidation, which both can lead to protein carbonylation, fragmentation, dimerization, peroxidation and cross-linking [24–27]. Direct protein oxidation occurs through a variety of reactions initiated by radicals and reactive oxygen species and includes side chain- and backbone oxidation, while indirect protein oxidation involves oxidative modification of protein side chains by reactive carbonyl species (RCS) [24,27]. RCS, such as 4-hydroxy-2-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE), and acrolein originate from lipid oxidation and are capable of forming adducts with the protein side chains of cysteine, histidine and lysine [25,27,28]. Such lipid oxidation derived protein modifications are, together with protein glycations, involved in age associated diseases, such as atherosclerosis, diabetes, Alzheimer's and Parkinson's disease [24,26] and new studies indicate that protein carbonyls formed in reactions with lipid oxidation-derived aldehydes are prevalent compared to carbonyls formed by direct oxidation of the protein [29].

The aim of the present study was to investigate the antioxidative mechanisms of peptides in samples with relevance for food and biological systems. Peptides were selected based on their amino acid composition and length and were, along with a whey protein and free amino acids, subjected to *in vitro* analyses with the purpose of measuring antioxidative activity. The methods were chosen based on their ability to express antioxidative activity of peptides in systems of increasing structural organization and at different stages of the oxidation process. Hence, a more complete understanding of the antioxidative mechanisms of peptides can be achieved, both in relation to lipid oxidation chain reactions and protection of proteins from direct and indirect oxidation.

Materials and methods

Chemicals

β-Casomorphin fragment 1–5 hydrochloride, $\geq 97\%$ peptide content (Peptide 1); Bradykinin acetate, $\geq 98\%$ peptide content (Peptide 2); Tyr-Bradykinin acetate hydrate, $\geq 97\%$ peptide content (Peptide 3); Renin substrate tetradecapeptide porcine, $\geq 97\%$ peptide content (Peptide 4); and Valosin, $\geq 97\%$ peptide content (Peptide 5) were all purchased from Sigma-Aldrich Denmark A/S (Brøndby, Denmark). The peptides 1–5 (Table I) were dissolved in 10.0 mM phosphate buffer (pH 7.4) and stored at -18°C until use. L-α-phosphatidylcholine (PC); bovine serum albumin (BSA), $\geq 96\%$; guanidine hydrochloride; L-methionine and $(\pm)$-α-tocopherol, $\geq 98.0\%$ were purchased from Fluka Chemie AG (Neu Ulm, Switzerland). L-Carnosine; 2,4-Dinitrophenylhydrazine (DNPH); Tween-20 (polyoxyethylenesorbitan monolaurate) and metmyoglobin from horse heart, $\geq 90\%$, were purchased from Sigma Chemie Gmbh (Steinheim, Germany). Peptigen® IF-3090, $\geq 87\%$, were obtained from Arla Foods Ingredients amba (Viby J., Denmark). L-Glutathione, reduced, $\geq 98.0\%$, was obtained from Bulk Medicines & Pharmaceuticals (Norderstedt GmbH, Germany). β-Lactoglobulin from bovine milk was isolated and purified according to Kristiansen et al. [30]. L-Tyrosine and L-cysteine, reduced $\geq 99\%$, were purchased from Merck (Darmstadt, Germany). (R)-(+)-Trolox® (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), $\geq 98\%$, was purchased from Aldrich Chem. Co. (Milwaukee, WI, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Wako Chemicals (Richmond, WA). All other chemicals were of analytical grade and water was purified

Table I. Amino acid composition of peptides 1–5.[a]

Peptide	Amino acid composition[b]
1	Tyr-Pro-Phe-Pro-Gly
2	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
3	Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
4	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser
5	Val-Gln-Tyr-Pro-Val-Glu-His-Pro-Asp-Lys-Phe-Leu-Lys-Phe-Gly-Met-Thr-Pro-Ser-Lys-Gly-Val-Leu-Phe-Tyr

[a]The peptides are listed from N- to C-terminal.
[b]Peptide sequences are obtained from the manufacturer.

through a MilliQ Plus water system from Millipore (Bedford, MA).

Radical-scavenging assay

A radical-scavenging assay, based on reduction of Fremy's salt radicals was set up according to Rødtjer et al. [31]. The diluted peptides 1–5, Peptigen (a hydrolysed whey protein), β-lactoglobulin, glutathione, tyrosine, methionine, cysteine, carnosine and Trolox were diluted with 25% (v/v) ethanol to a final sample concentration of 25.0 μM. For additional samples, Peptigen, carnosine, glutathione and Trolox were diluted to 2.5 mM; 3.00 ml diluted sample was mixed with 200 μl of 139.0 μM Fremy's salt dissolved in a 25% (v/v) solution of saturated sodium carbonate, resulting in 8.7 μM Fremy's salt. The concentration of Fremy's salt was adjusted based on spectrophotometric measurements, using the extinction coefficient, $\varepsilon_{270} = 933\ M^{-1}\ cm^{-1}$ for the anion of Fremy's salt [31]. Exactly 5 min after mixing, the samples were analysed at room temperature using a JEOL JES-FR30X ESR spectrophotometer (JEOL Ltd., Tokyo, Japan) with the microwave power set at 4 mW, sweep width of 7.5 mT, sweep time of 30 s, modulation width of 0.20 mT and time constant of 0.03 s. The signal intensity of the obtained ESR signal was measured as the height of the central line relative to the height of a Mn(II)-marker (JEOL Ltd., Tokyo, Japan) attached to the cavity of the spectrophotometer. Radical-scavenging in the presence of antioxidants was expressed relative to the ESR signal intensity from Fremy's salt radicals in control samples without antioxidant ($[\text{signal intensity}_{\text{antiox}}/\text{signal intensity}_{\text{control}}]\cdot 100\%$).

Oxygen consumption assay

Oxygen consumption analysis was carried out according to Hu and Skibsted [32] with minor modifications; 230 μl 28.20 mM linoleic acid dissolved in methanol was mixed with 62.5 μl 0.04 g/ml Tween-20 in methanol. The solvent was removed with a stream of nitrogen and replaced with 2.50 ml buffer with or without peptide/antioxidant. Peptide 1–5, Peptigen, β-lactoglobulin, glutathione, tyrosine, methionine, cysteine, carnosine and Trolox were pre-diluted in 10.0 mM phosphate buffer and suitable volumes (10–500 μl) were mixed with 5.0 mM thermostatted (25°C) air-saturated phosphate buffer (pH 6.8) to achieve 12.0 μM peptide or antioxidant in the final sample volume of 2.50 ml. An additional sample with 0.12 mM carnosine was prepared. In order to initiate oxidation, 25 μl 0.20 mM metmyoglobin in 5.0 mM phosphate buffer was added and immediately after initiation the oxygen consumption was measured by injecting the sample into a thermostatted (25.0 ± 0.1°C) measuring cell (IKA-Labortechnik, Staufen, Germany) with no headspace. The oxygen consumption was measured with a Clark electrode connected to a multichannel analyser (Unisense Picoammeter PA2000, Unisense Aps, Århus, Denmark). The electrode was calibrated by a two-point calibration with anoxic (0% O_2) and air-saturated (100% O_2) buffers thermostatted at 25°C. The oxygen percentage in the cell was measured as a function of time and the slope of the curve (between 80% and 40% O_2) was used to calculate the oxygen consumption rate ($v(\text{oxygen}) = -\text{slope}[O_2]_{\text{initial}}/100$), where $[O_2]_{\text{initial}}$ is the initial 100% oxygen concentration corresponding to water saturated with air, $[O_2]_{\text{initial}} = 2.6\cdot 10^{-4}$ M at 25°C [32]. The oxygen consumption in the presence of antioxidant was expressed as a percentage of the oxygen consumption rate of a control sample without antioxidant, $([v(\text{oxygen})_{\text{antiox}}/v(\text{oxygen})_{\text{control}}]\cdot 100\%)$.

Conjugated dienes formation in a peroxidizing liposome model system

A liposome model system was prepared according to the method of Roberts and Gordon [33] with minor modifications; 2.0 ml solution containing 1.50 μmole phosphatidylcholine (PC) dissolved in chloroform was mixed with 1.0 ml hexane in a 25.0 ml round bottomed flask. Solvent was removed under vacuum on a rotary evaporator (Büchi Rotavapor R-144, Bie & Berntsen A/S, Rødovre, Denmark) with the water bath set at 30°C. Nitrogen was introduced to re-establish atmospheric pressure and to avoid oxidation. Furthermore, the flask was covered with aluminum foil to prevent light induced oxidation. Subsequently, the lipid residue was rehydrated in 10.0 ml 10.0 mM phosphate buffer solution (pH 7.4). When required, this buffer solution was replaced with 1.0 mole% (antioxidant:lipid) of peptide 1–5, Peptigen, β-lactoglobulin, glutathione, tyrosine, methionine, cysteine, carnosine or Trolox diluted in 10.0 ml phosphate buffer. A sample containing 100.0 mole% (antioxidant:lipid) carnosine was additionally prepared. The final liposome solution contained 150 μM PC, as the relative molecular mass of the soy bean PC is ~ 900 Da. The flask, still under nitrogen, was vortexed for 10 min, producing an homogenous white suspension of multilamellar liposomes, and the flask was placed for 30 s in an ultrasonic bath to ensure complete recovery of the lipids from the flask wall. Large unilamellar liposomes were obtained by passing the liposome suspension 20 times through an Avestin Liposofast Basic small volume extrusion device (Avestin Europe GmbH, Mannheim, Germany) using a double layer of polycarbonate membranes (100 nm pore size).

A 2.50 ml unilamellar liposome suspension was incubated for 10 min at 37°C in a quartz cuvette within the water-jacket temperature regulated cell holder of a HP 8453 UV-Visible spectrophotometer

(Hewlett-Packard GmbH, Waldbronn, Germany). Lipid oxidation was initiated by addition of the hydrophilic initiator AAPH to a final concentration of 0.75 mM and the absorbance at 234 nm was recorded every 10 min. An induction time was determined as the on-set of conjugated dienes formation (the point of interception between the tangent to the propagation phase and the primary axis). Induction times were compared after adjustment with induction time for control samples without antioxidant ($\triangle_{\text{induction time}}$ [min] = Induction $\text{time}_{\text{antiox.}}$ – Induction $\text{time}_{\text{control}}$).

Protein carbonyl formation in a peroxidizing liposome/BSA model system

A liposome/BSA model system, showing similarities with the one used by Dean et al. [34] and the oil-in-water emulsion systems used by Baron et al. [35], was constructed. Liposome suspensions for the liposome/BSA model system were prepared as described above with minor modifications; 10.0 ml 3.33 mM PC dissolved in chloroform was added to 100 µl 73.3 mM of the hydrophobic initiator AMVN diluted in absolute ethanol. The solvent was evaporated on a rotary evaporator (Büchi Rotavapor R-144, Bie & Berntsen A/S, Rødovre, Denmark) and the lipid residue was rehydrated in 10.0 ml 50.0 mM phosphate buffer pre-heated to 37°C. In the samples pure buffer solution was replaced with appropriate dilutions of glutathione, carnosine, Peptigen or Trolox in 50.0 mM phosphate buffer. The liposome suspensions were vortexed for 5 min and subsequently kept in a water bath set at 37°C for 20 min. Extrusion to obtain unilameller liposomes was performed by passing the suspensions 12 times through a double layer of membranes (200 nm pore size).

A 333 µl PC liposome suspension (333 µl with or without peptide or trolox) containing AMVN, 100 µl 10.0 mg/ml BSA and 67 µl 50.0 mM phosphate buffer was mixed, vortexed and oxidized in a water bath at 37°C for 24 h. This model system contained 1 mg BSA, 1 mg lipid residue (2.2 mM) and 2.2 mM Peptigen, carnosine, glutathione or Trolox.

The carbonyl concentration in the liposome/BSA model system was determined by DNPH analysis. Derivatization was conducted according to Levine et al. [36]; 500 µl sample (liposome/BSA model system) was mixed with 500 µl 10.0 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.0 M HCl. Blank samples were prepared by mixing a 500 µl sample and 500 µl 2.0 M HCl without DNPH. After vortexing, the samples were placed in a water bath at 37°C for 1 h to derivatize. All samples were vortexed every 10 min. Subsequently, the samples were added to 325 µl 50% (w/v) trichloroacetic acid (TCA), vortexed, placed on ice for 10 min and spun in a Microcentrifuge 154 (Ole Dich Instrumentmakers ApS, Hvidovre, Denmark) for 15 min at 11 000 g, after which the supernatant was decanted. The protein pellet was washed according to Fagan et al. [37] by adding 5.0 ml ethanol:ethylacetate (1:1), vortexing, letting it react for 10 min and then spun for 10 min at 11 000 g. This wash procedure was repeated three times (3 × 5.0 ml ethanol:ethylacetate). After the final wash, the pellet was dissolved in 1.0 ml 6.0 M guanidine hydrochloride dissolved in 20.0 mM potassium dihydrogen phosphate (pH 2.3), placed in water bath at 37°C for 30 min and finally centrifuged to remove insoluble material [36]. The carbonyl content in the samples was determined spectrophotometrically by measuring the absorbance at 375 nm on a Cary 3 UV-Visible Spectrophotometer (Varian, Herlev, Denmark). The concentrations of protein carbonyl (nmole carbonyl per mg protein) were calculated using $\varepsilon = 22\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ as extinction coefficient [37,38]. Various test samples were prepared to verify that the carbonyl content determined by the method was exclusively a result of carbonyl formation on BSA.

Protein carbonyl formation in a peroxidizing BSA model system

One hundred microlitres of 10.0 mg/ml BSA dissolved in 50.0 mM phosphate buffer, 100 µl 27.5 mM AAPH dissolved in 50.0 mM phosphate buffer, 200 µl 50.0 mM phosphate buffer and 100 µl 11.0 mM Peptigen, carnosine, glutathione or Trolox dissolved in 50.0 mM phosphate buffer were mixed, vortexed and oxidized in a water bath at 37°C for 0, 5, 7, 22.5 and 30 h. The carbonyl concentration on BSA was determined by DNPH analysis as described above for the liposome/BSA model system.

Results

Radical-scavenging effects of peptides

Figure 1 (upper panel) shows the quantity of spins measured in Fremy's salt assay following addition of peptides, proteins and amino acids. The results are given as relative measurements as the ESR signal intensity is compared to a control sample without antioxidant (100%). Glutathione reduced the quantity of spins to 72%, indicating a significant radical-scavenging effect ($p \leq 0.05$), and cysteine scavenged the radicals to a level comparable to Trolox by reducing the quantity of spins to 27% ($p \leq 0.05$). None of the other samples showed radical scavenging capacity.

Lipid peroxidation inhibition

Figure 1 (lower panel) shows the effect of 12.0 µM peptide, protein or amino acid on oxygen consumption in a 2.60 mM linoleic acid emulsion. None of the samples effectively decreased the rate of oxygen consumption in the peroxidizing lipid emulsion

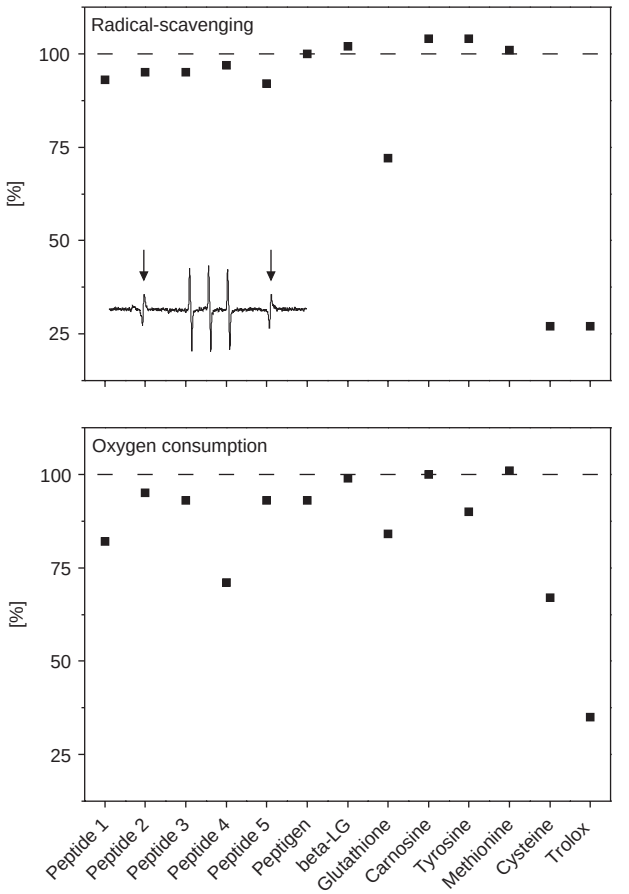


Figure 1. Upper panel: Radical-scavenging activity expressed as the ESR signal intensity of Fremy's salt radicals in the presence of 25 μM antioxidant relative to a control sample without antioxidant (100%). Peptide 1–5 ($n=1$), Peptigen, β-lactoglobulin, glutathione, tyrosine, methionine, cysteine, carnosine and Trolox ($n=3$). CV <5.0% for all samples, except for those containing glutathione and cysteine, which had CV <20.0%. Inset: ESR-signal as obtained in the analysis. Lines from the manganese marker are marked with an arrow. Lower panel: Rate of oxygen consumption in the presence of 12.0 μM peptide or antioxidant in a 2.6 mM linoleic acid emulsion (pH 6.8, 25°C). Data is expressed in percentages relative to the rate in the absence of antioxidant (100%). Peptide 1–5, carnosine and Trolox ($n=3$); Peptigen, β-lactoglobulin, glutathione, tyrosine, methionine and cysteine ($n=2$). CV <5.0% for all samples, except for peptide 5, carnosine, tyrosine and Trolox, which were ≤15.0%.

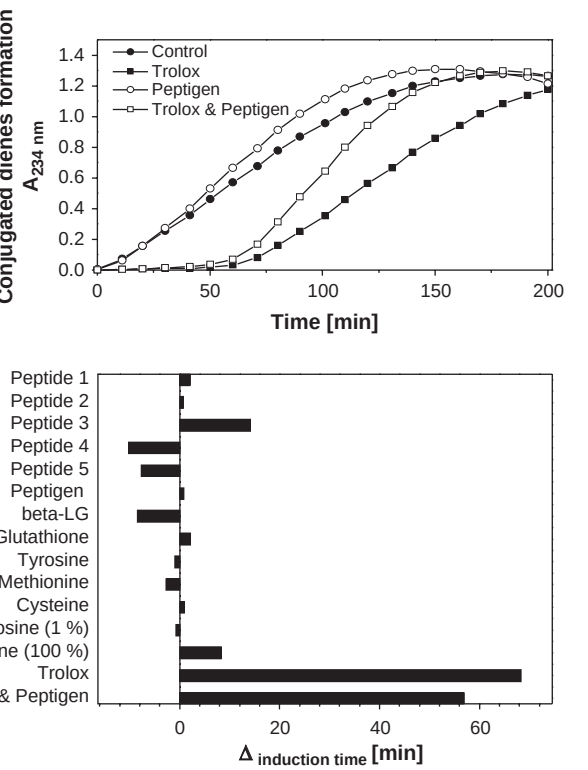


Figure 2. Upper panel: Conjugated dienes formation in a peroxidizing liposome model system containing 150.0 μM lipid, 0.75 mM AAPH and 1.5 μM of either Trolox or Peptigen or 1.5 μM Trolox and 1.5 μM Peptigen in combination. Lower panel: Induction time for samples containing similar concentrations as above adjusted by subtracting the induction time of a control sample without antioxidant. Carnosine was additionally tested in a 150.0 μM concentration (lower panel). All samples were measured in duplicates. CV <10.0% for all samples, except for peptide 4 and 5, which were <25.0% and <15.0%, respectively.

compared to Trolox, which was able to reduce the oxygen consumption rate to 35% (relative to a control sample without antioxidant). However, cysteine, peptide 1 and peptide 4 showed significant effects ($p \leq 0.05$) with reductions to 67%, 82% and 71%, respectively. Further, glutathione was able to reduce the oxygen consumption slightly (86%), but not to a significant level ($p = 0.064$).

As seen from the lag-phases in Figure 2 (upper panel), the formation of conjugated dienes was postponed when Trolox is present in a liposome model system. None of the peptides were able to prolong the lag-phase as effectively as Trolox, for which an induction time of 68.3 min in the liposomes samples was obtained (lower panel). Minor pro-oxidative effects resulting in negative induction times were even observed for some of the peptide/amino acids samples. It should be noted that for both the liposome model system and the oxygen consumption assay all peptides and antioxidants were added in 1.0 mole% in relation to lipid in order to investigate their effects under conditions where traditional lipid antioxidants are effective. However, it was additionally observed that not even a 100-fold increase of the carnosine concentration was able to delay the on-set of lipid oxidation in the liposomes (Figure 2, lower panel) or to decrease the oxygen consumption rate (data not shown) to levels comparable to samples with 1.0 mole% Trolox.

A possible interaction between the hydrolysed whey protein Peptigen and Trolox in the liposome samples was examined, as a regeneration effect of peptides on traditional phenolic antioxidants previously has been suggested for α-tocopherol combined with tri- and tetrapeptides [39,40]. As seen in Figure 2 (lower panel), a small decrease in the induction time was observed for liposome samples with both Trolox and Peptigen compared to samples containing only Trolox. This antagonistic effect was

not only expressed as a decrease in the induction time but rather as an increased rate of conjugated dienes formation (see upper panel). A similar phenomenon was observed when Peptigen alone was added to the liposome samples, resulting in a higher rate of conjugated dienes formation when compared to control samples without antioxidant (upper panel).

Inhibition of protein carbonyl formation

Figure 3 shows that carnosine ($p \leq 0.05$) and Peptigen ($p = 0.07$) decreased the formation of protein carbonyls at 37°C in a peroxidizing liposome/BSA model system compared to a control sample without peptide or antioxidant. A mole ratio of 1:1 (peptide lipid) was chosen for this model system, as one peptide molecule is expected to prevent one protein carbonylation reaction and not the production of a cascade of radicals as formed in a chain reaction. Oxidation in the liposome/BSA model system was initiated by the lipophilic initiator AMVN in order to generate the initial radicals within the liposomal membrane and a concomitant analysis of the formation of conjugated dienes (Figure 3, inset) showed lipid oxidation propagating in the system. Carbonyl formation in test samples containing liposomes, BSA, AMVN and Peptigen alone or in combination (only data for samples containing BSA alone ($t = 0$ h) or BSA + AMVN ($t = 24$ h) are included in Figure 3) verified that protein carbonyls were not formed on BSA by direct reaction with AMVN-generated radicals. Furthermore, analyses of the test samples showed that carbonyls derived either from lipid oxidation in the liposome membranes (lipid aldehydes) or formed upon reaction with the added peptides were removed in the washing procedure. Therefore only carbonyls on BSA contributed to the measured carbonyl concentration. The formation of BSA carbonyls during the incubation time was thus due to reactions of lipid oxidation-derived reactive species with BSA and hence the peroxidizing liposome/BSA model system reflects the influence of the peptides on indirect oxidation of proteins.

Finally, the inhibition of direct protein oxidation by the peptides was investigated in a peroxidizing BSA model system without liposomes. The concentrations of BSA and peptide or antioxidant were similar to those in the liposome/BSA model system, whereas oxidation in this system was initiated by the hydrophilic initiator AAPH. Accordingly, a comparison of the carbonyl formation on BSA in the two systems with and without liposomes is possible, although only qualitative comparisons can be done due to the different peroxyl radicals generated from AAPH and AMVN. As seen in Figure 4 (left panel), Peptigen reduced the formation of protein carbonyls and Trolox was able to prevent protein carbonyl formation on BSA for up to 30 h of incubation. Carnosine and glutathione only reduced protein oxidation moderately, being much less efficient than Trolox

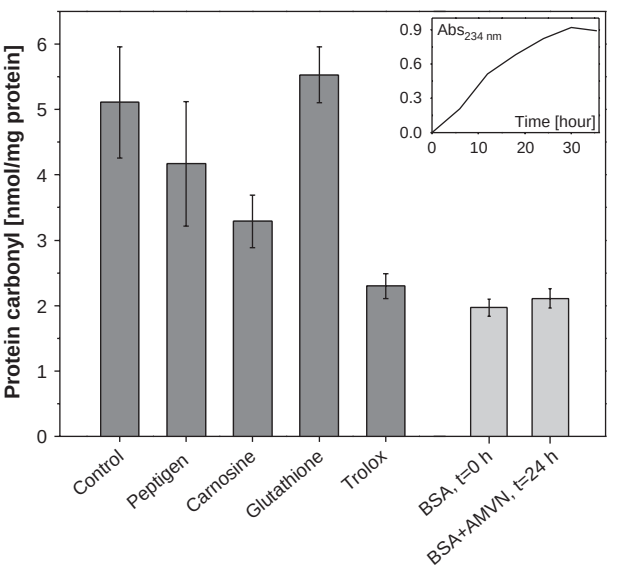


Figure 3. Protein carbonyl groups formed on BSA in a peroxidizing liposome/BSA model system after 24 h incubation at 37°C. Dark columns represent the model system containing 2.2 mM liposome suspension, 2.0 mg/ml BSA and 14.7 mM AMVN (control). This model system was added 2.2 mM peptide or antioxidant. Light columns represent test samples without liposomes containing 2.0 mg/ml BSA ($t = 0$ h) or 2.0 mg/ml BSA and 14.7 mM AMVN ($t = 24$ h). The protein carbonyls were determined by DNPH-analysis. Control (no antioxidant) ($n = 17$), Peptigen and carnosine ($n = 5$), glutathione, Trolox, BSA and BSA + AMVN ($n = 3$). Inset: Formation of conjugated dienes in the liposome/BSA model system during 36 h incubation at 37°C.

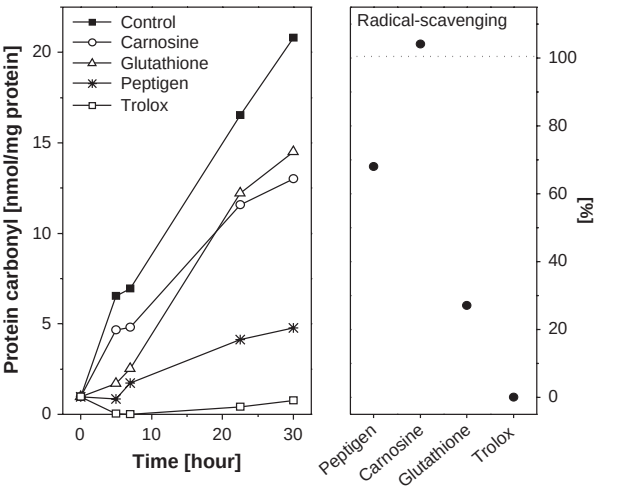


Figure 4. Left panel: Protein carbonyl groups formed on BSA in a peroxidizing BSA model system without liposomes when incubated at 37°C. The model system contained 2.0 mg/ml BSA, 2.2 mM peptide or antioxidant and 5.5 mM AAPH. Samples were collected at $t = 0$, 5, 7, 22.5 and 30 h and protein carbonyls were determined by DNPH-analysis. Each data point represents one sample. Right panel: Radical-scavenging activity expressed as the ESR signal intensity of Fremy's salt radicals in the presence of 2.5 mM antioxidant relative to a control sample without antioxidant (100%). All samples are measured in triplicate. CV < 5.0% for all samples, except for glutathione, which was < 25%.

and Peptigen. Further, Figure 4 shows the scavenging of Fremy's salt radicals by peptides when added in the high concentrations as used in the BSA-model system. It was seen that, even at this high concentration level, carnosine was unable to scavenge radicals in aqueous solution, whereas the effect of Peptigen was somewhat improved (cf. Figure 1). Glutathione and Trolox, which showed significant radical scavenging effects already at low concentrations, were as expected even more efficient radical scavengers at high concentrations.

Discussion

In order to optimize the selection and use of peptides for controlling oxidative degradation of food and biological systems, a full understanding of their mechanisms of action is necessary. Previous studies have proposed antioxidative capacities of peptides. However, these studies did not identify exact mechanisms behind the antioxidative activity, but found dose-dependent capacities [14,17] or applied very high peptide concentrations relative to the lipid fraction in order to detect an antioxidative effect [1,3,4,41]. Further, new studies have stressed the importance of protecting proteins in biological systems from oxidative modifications, such as protein carbonyl formation [25,26,29], and this leads to new prospectives regarding the protective effects of peptides in biological systems.

In the present study we have investigated the effect of selected peptides at different stages of the oxidation process. Figure 5 illustrates how elevated levels of ROS in a biological system may lead to free radical ($R^{\bullet}$) formation both in the hydrophobic and hydrophilic phase of a membranal system. Radical generation may initiate the chain-reaction of lipid peroxidation (I) and the accessibility of oxygen leads to formation of lipid peroxyl radicals ($LOO^{\bullet}$), which may propagate a chain-reaction by reacting with other lipid residues (II). The formed lipid hydroperoxides (LOOH) decompose into secondary oxidation products, including reactive carbonyl species (RCS), such as α,β-unsaturated aldehydes and di-aldehydes [42], and these will, due to their change in hydrophobicity, orientate towards the aqueous phase. The RCS may indirectly oxidize proteins by forming adducts with especially cysteine-, histidine- and lysine side chains (V). Proteins may additionally be oxidatively modified by direct peroxidation by radical species in the aqueous phase (III), which in the presence of oxygen leads to oxidized protein and eventually protein carbonyl formation [43]. The lipid oxidation products, $LOO^{\bullet}$, $LO^{\bullet}$ and $L^{\bullet}$, have also been proposed to be able to modify proteins (IV), even though reactive lipid radicals preferentially react in the lipid bilayer due to their high reactivity. Peptides may protect membranal systems from oxidation by interfering with one or more of the different reactions I–V in Figure 5, and a combination of the results obtained from each of the analyses included in this study reveals an interesting picture of the antioxidative mechanisms of peptides.

The first important observation was that the radical scavenging activity of the peptides was almost negligible. This is in contrast to what has been reported elsewhere [8,9,13,15–17] and especially the comparison to the activity of the well-known antioxidant Trolox emphasizes the very weak radical scavenging effect. Only cysteine and the cysteine-containing peptide glutathione showed some interaction with the Fremy's salt radicals, whereas tyrosine residues as present in peptides 1, 3, 4 and 5 had no effect.

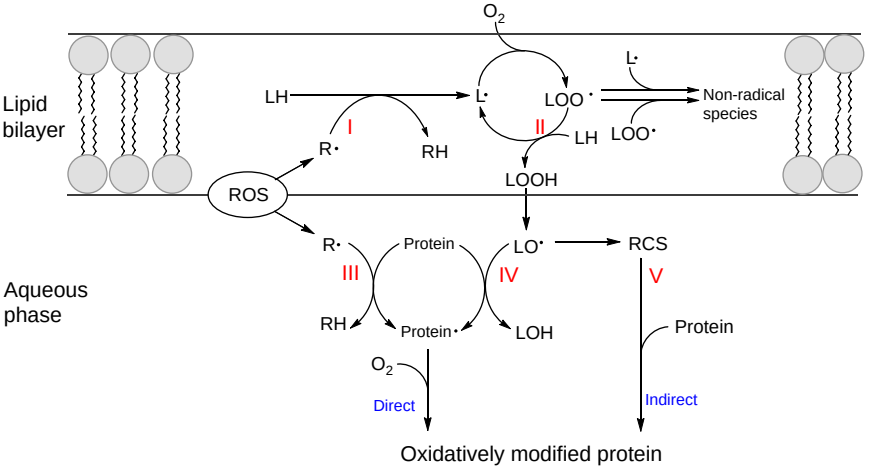


Figure 5. Potential antioxidative mechanisms of peptides in biological membrane systems. The interactions between lipids and proteins during peroxidation of a liposome/BSA model system are illustrated together with the possible antioxidative reaction pathways of peptides: (I) radical-scavenging activity in the lipid phase, (II) inhibition of lipid peroxidation chain-reaction, (III) inhibition of protein peroxidation by radical attack, (IV) lipid radical-scavenging activity and (V) inhibition of protein carbonyl formation by quenching reactive carbonyl species derived from lipid peroxidation.

Additionally, the investigated peptides showed very little efficiency in interfering with the lipid peroxidation chain reaction, as seen from the results in Figure 1 (lower panel) and Figure 2. For both the oxygen consumption assay and the liposome model system the antioxidative effect of Trolox was superior to the tested peptides and amino acids and only small effects were observed depending on the system. The amino acid cysteine and glutathione showed some activity in the oxygen consumption assay, an observation which indicates the reducing ability of the thiol-group. However, it is interesting that the antioxidative effect of cysteine and glutathione was less pronounced in the oxygen consumption assay, which has a higher structural organization than the homogenous radical-scavenging Fremy's salt assay, and that a different effect was observed in the liposome model system (with yet another structural organization) where none of the two showed significant effect. As documented in various studies [44–46] the distribution of compounds in heterogeneous systems is significant for the obtained antioxidative effects.

Antioxidative activity of tyrosine side-chains has been reported, also in more structured systems like dispersed food lipids [40,47]. The inclusion of peptides 1–5 in the present study was expected to provide information in this respect. However, the weak and indistinct effect of peptides 1–5 in the two peroxidizing lipid systems did not give any important information (cf. Figures 1 and 2) and the overall conclusion was that the peptides 1–5 are inefficient as antioxidants against lipid membrane oxidation.

The observed antagonism between Peptigen and Trolox in the liposome model system (Figure 2, upper panel) contradicts the results from another study [40] and deserves further attention. The antagonism may be due to an embedment of peptides in the liposome membrane which interferes with the liposome structure and increases the accessibility of radicals generated from AAPH in the water phase. The same theory may explain the small pro-oxidant effects observed for the longer peptides, peptide 4 and peptide 5, in the liposome model system. A location of amino acid residues at both the outside and the inside of the liposome membrane may create a 'pathway' from the water phase to the lipid phase, enabling the transfer of radicals between the two phases.

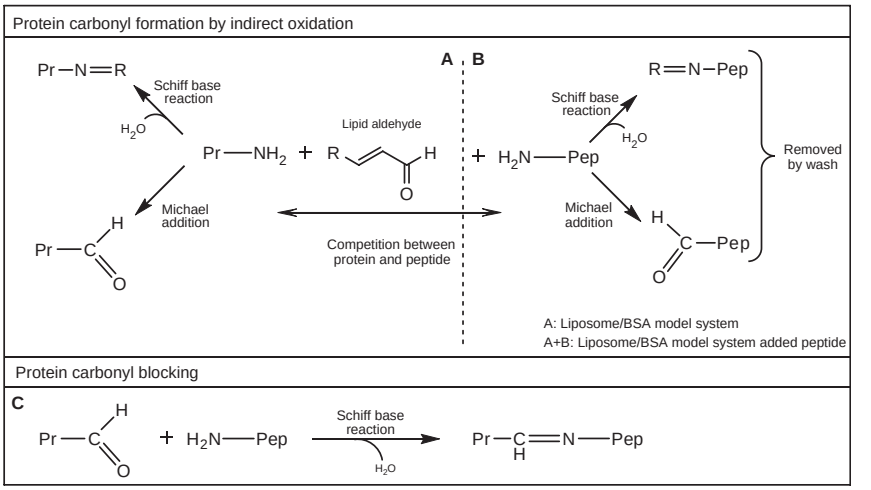
This suggestion is somewhat in disagreement with a theory based on a shielding effect of peptides [4,48], which was thought to result from the creation of a protective shield of peptides around the water–lipid interface. This shield is thought to scavenge any approaching radical species. However, such a shielding effect most likely requires a concentration of peptides which is higher than 1 mole% (peptide:lipid) as used in the present study and this consideration is also supported by the fact that no radical scavenging activity was observed for the peptides in the Fremy's salt assay (Figure 1).

The peptide concentration of 1 mole% (peptide: lipid) used in this study (corresponding to 1% (w/w) in a PC liposome system with M_w(peptide) $\sim$ 1 kDa) resembles the effective concentrations of phenolic antioxidants, such as α-tocopherol [49,50]. Notably, it is reflective that studies demonstrating the antioxidative effect of peptides in complex matrices such as foods or biological tissues generally use very high concentrations of peptides relative to the lipid fraction. Concentrations of up to 5-, 10-, 20- and 30-fold peptide compared to lipid (w/w) have actually been applied by Kitts [41], Park et al. [3], Peña-Ramos and Xiong [1], and Kong and Xiong [4], respectively. Less elevated (but still high) peptide concentrations relative to the lipid fraction have also been used, such as addition of 10–160% (w/w) in ground beef homogenates [14,17]. Inhibition of oxidative deterioration upon addition of such high amounts of peptide might well be due to the above-mentioned shielding effect, even though matrix effects introduced by very high peptide concentrations cannot be excluded to influence the tendency of oxidation in these investigations.

4-HNE, 4-ONE and acrolein originating from lipid oxidation have been found to form adducts with proteins [26]. Small peptides, such as carnosine and glutathione, have been suggested as protectors of proteins in biological membrane systems by being carbonyl quenching agents able to react with RCS by Michael additions or in Schiff base reactions [26]. The expansion of the liposome model system to contain a protein-source (BSA) was done in order to examine the significance of peptides as inhibitors of such an indirect protein oxidation (reaction V in Figure 5) and the results in Figure 3 point at this reaction mechanism as being important for the positive effect of using peptides as antioxidants. Both Peptigen and carnosine showed the ability to decrease the formation of protein carbonyls in the model system and hence they seem to interfere with indirect protein oxidation.

It should be noted that only the Michael addition type reaction (and not Schiff base formation) between protein side chains and lipid carbonyls leads to the formation of protein carbonyls (see Scheme 1A). The DNPH-analysis only determines carbonyls and Schiff base formation between the lipid carbonyls and BSA side chains in the liposome/BSA model system is therefore not detected. Reduced protein carbonyl formation following addition of peptides may thus be attributed to quenching of lipid carbonyls (Scheme 1B) or blocking of protein carbonyl groups (Scheme 1C).

Glutathione did not reduce protein carbonyl formation in the liposome/BSA model system. This may be due to the fact that the thiol-group on the cysteine



Scheme 1. Reaction mechanisms in the liposome/BSA model system, showing the protein carbonyl formation by indirect oxidation (A), as well as the protective effect of a lysine side chain of a peptide by either reacting preferentially with the lipid carbonyls (B) and/or blocking protein carbonyls formed by indirect oxidation of BSA (C).

residue of glutathione reacted in Michael additions and not in Schiff base reactions [27]. This may have limited the number of lipid carbonyls quenched by glutathione, as well as it is expected that the cysteine side chain will be less efficient in blocking already formed carbonyls on BSA (Scheme 1).

The effect of peptides on indirect protein oxidation was found for carnosine and Peptigen present in 100 mole% relative to lipid (mole ratio 1:1) and in this respect such a high peptide concentration seems reasonable because one peptide molecule is intended to prevent one protein carbonylation reaction and not the production of a cascade of radicals, as is the case for a lipid peroxidation chain-breaking antioxidant.

To finalize the investigation of the effects of the peptides, their interference with a direct oxidation of proteins (reaction III in Figure 5) was taken into further consideration. The use of higher concentrations as was found necessary for interference with indirect protein oxidation may also introduce a prevention of direct protein oxidation, probably due to a shielding effect where radical scavenging becomes significant due the abundant number of peptide molecules in the system. As can be evaluated from the results in Figure 4 (right panel), some radical scavenging effect was obtained for some of the peptides at higher concentrations and, even though glutathione and carnosine reduced the carbonyl formation on BSA equally (left panel), they showed very different radical-scavenging capacities (right panel). Hence, a radical-scavenging effect may explain the protective effect of glutathione against direct protein oxidation, but not the effect of carnosine. The effect of carnosine in the BSA model system must therefore be ascribed to adduct formation through Schiff base reactions with already formed protein carbonyls on BSA (Scheme 1C) and not to scavenging of protein peroxyl- or AAPH-derived radicals. Peptigen seemed to be able to react both in Schiff base reactions and as a scavenger of aqueous radicals when applied in high concentrations.

A final curiosum to be mentioned is the probability of the reaction between malondialdehyde and peptides in Michael type additions; reactions which are favoured at lower pH as found in many food products [26]. Hence, there is a probability of interference of peptides with the traditionally used TBARS-analysis for measurement of secondary lipid oxidation products. TBARS-analysis has been used in many studies for evaluation of the antioxidative activity of peptides and protein hydrolysates [4,10,11,14,51] and interference from reactions between malondialdehyde and peptide side chains may have led to misinterpretations of the effect of peptides as lipid antioxidants.

Conclusion

As illustrated in Figure 5, the preventive effect of peptides against oxidation of biological membranes may be attributed to several reaction pathways. The present study shows that the investigated peptides are not effective as electron or hydrogen donating species in membrane systems. However, if applied in higher concentrations they can act as protectors of specific indirect protein oxidation reactions or by creating a shield, which is able to scavenge some of the reactive oxygen species formed in the aqueous phase.

The set-up in the present study is relevant also for investigation of other possible antioxidants than peptides, as it will provide important information on their most important mechanisms of action and form a basis for choosing appropriate dosage levels and analysis methods.

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