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Evaluation of activity of selected antioxidants on proteins in solution and in emulsions

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Abstract

Protection against protein oxidation by lipophilic and hydrophilic antioxidants in model systems using bovine serum albumin (BSA) in solution alone, or in an emulsion with linolenic acid methyl ester (LnMe) was found to be strongly dependent on the oxidation initiator. Tocopherol, Trolox, or the carotenoids astaxanthin and canthaxanthin were incubated with BSA or BSA/LnMe and oxidation was initiated either with the water-soluble azo-initiator 2,2′ azo-bis-(2-amidinopropane) hydrochloride (AAPH), or $FeCl_3$ and ascorbate, or the Fenton system using $FeCl_2$/EDTA/H_2O_2, or with the singlet oxygen generating species anthracene-9,10-dipropionic acid disodium 1,4 endoperoxide ($NDPO_2$).

The results show that all the antioxidants tested were inefficient in the system with $FeCl_3$/ascorbate. However, with the other initiating agents, the hydrophilic antioxidant, Trolox, was the most effective in preventing both protein and lipid oxidation. In contrast the lipophilic antioxidants were ineffective in preventing oxidation of BSA in aqueous solution, but did show some moderate antioxidative activity on protein and lipid in the BSA/LnMe system. Using the singlet oxygen generating system it was also demonstrated that Trolox always provided better protection of the protein than tocopherol and the carotenoids in both the BSA and the BSA/LnMe systems. In conclusion, prevention of protein oxidation using a water-soluble antioxidant has a protective effect on the lipid fraction and this approach deserves further attention in complex biological systems.

Keywords: *Protein oxidation, tocopherol, trolox, astaxanthin, canthaxanthin, carbonyl groups*

Introduction

Reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals have the ability to damage unsaturated lipids and other biomolecules directly or indirectly via free radical processes [1–3]. Under normal conditions various antioxidative enzymatic systems and pools of low molecular weight antioxidants, e.g. ascorbic acid, tocopherols, and carotenoids, are capable of scavenging and eliminating ROS to an extent where their damaging effect is prevented [4,5]. However, today there is a strong evidence that free radical damage is implicated in aging [6,7], and in the pathological conditions of a number of diseases, e.g. atherosclerosis, cancer, rheumatoid arthritis [8,9].

For decades, studies have concentrated exclusively on free radical induced lipid oxidation. Recently, however, the number of reports supporting that protein and lipid oxidation are coupled processes has increased [10,11]. Lipids have been believed to be the main target of free radical mediated oxidation. Furthermore, lipid oxidation products have been shown to induce tissues

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damage [12], and more specifically to alter DNA [13,14] and proteins [15–17]. It has been shown that polyunsaturated fatty acid oxidation products such as peroxides and aldehydes, i.e. malonaldehyde and 4-hydroxynonenal can modify protein amino acid side chains by Michael addition and formation of Shiff bases [18–20]. It is often assumed that protein oxidation is subsequent to lipid oxidation, however, the interaction between lipids and proteins during oxidation is not well understood.

Free radicals can modify macromolecule structure to an extent where cellular functions are impaired [21,22]. However, the existence of a direct radical mediated protein oxidation has been suggested in LDL at early stages of oxidation. Furthermore, other studies have suggested that protein oxidation occurs prior to lipid oxidation [11,22]. This is further supported by studies indicating that oxidized proteins can transfer radicals to proteins and oxidize a broad range of cellular molecules [23,24]. It has also been proposed that proteins are directly oxidized by metal-catalysed oxidation or radiation, via production of highly reactive hydroxyl radicals or singlet oxygen rather than by free radicals generated during lipid oxidation [25,26]. Oxidation of proteins often results in accumulation of carbonyl groups, formation of protein hydroperoxides, protein backbone fragmentation or protein crosslinking (for review see Davies and Dean [27] or Stadtman and Levine [28]). Accumulation of protein carbonyl groups has been associated with the pathology of many age-related diseases [2,29], and protein hydroperoxides have been recognized as important reactive intermediate species in the course of oxidative damage in biological systems [30]. Protein hydroperoxides have also been shown to be stable intermediates, which under specific conditions decompose and may further induce oxidative damage to other macromolecules [31].

The hindrance of protein oxidation has received little attention and the activity of antioxidants on proteins has rarely been assessed. While tocopherols and carotenoids have been shown to be effective antioxidant scavenging free radicals mediated during lipid peroxidation [32] and quenching singlet oxygen [33–35], the antioxidative activity of tocopherol and carotenoids on proteins has hardly been investigated. Therefore, our study was undertaken in order to evaluate the potential antioxidative activity of tocopherol, the water-soluble tocopherol analog Trolox, and the seafood carotenoids astaxanthin and canthaxanthin on protein. These studies were performed in an aqueous solution of a single protein and in a lipid–protein model system in order to get a better insight of the interaction between lipids, proteins, and antioxidants. Additionally, different types of free radical generating systems were used to investigate the potential of the antioxidants tested to prevent protein oxidation, either via direct free radical scavenging or via singlet oxygen quenching. The obtained results allow a better understanding of the free radical mechanisms involved in tissue damage and the potential of specific antioxidants in preventing such damage by protecting the proteins.

Materials and methods

Materials

Bovine serum albumin (BSA), hydrogen peroxide, linolenic acid methyl ester (LnMe), 2 [N-morpholino] ethane sulfonic acid (MES) and sodium dodecyl sulphate (SDS) were from Sigma (St Louis, MO, USA). Xylenol orange, 2,4-dinitrophenylhydrazine (DNPH), fluoresceinamine isomer II ($FINH_2$), 2,2′ Azo-bis-(2-amidinopropane) hydrochloride (AAPH), and sodium cyanoborohydride, were from Aldrich (Milwaukee, WI, USA). Vitamin E (D,L α-tocopherol) and Trolox were from Flucka GmbH (Buchs, Switzerland). Astaxanthin and canthaxanthin were from Dr Ehrenstorfer GmbH (Augsburg, Germany). Anthracene-9,10-dipropionic acid disodium salt (NDP) was from Molecular Probes (Leiden, The Netherlands). All chemicals were of analytical grade and double deionized water was used throughout.

$NDPO_2$-synthesis

Chemical generation of singlet oxygen was performed by thermal decomposition of anthracene-9,10-dipropionic acid disodium 1,4 endoperoxide ($NDPO_2$). The enodoperoxide $NDPO_2$ was synthesized from anthracene-9,10-dipropionic acid disodium (NDP) according to the protocol described by Aubry [36]. Briefly, NDP (5 mM) was incubated with hydrogen peroxide (1 M) and NaOH (0.1 M) in the presence of sodium molybdate (0.01 M) for 3 h at room temperature. A control was prepared by incubating an aliquot of the reaction mixture at 50°C for 1 h in order to regenerate NDP. Aliquots of $NDPO_2$ and NDP were stored at −80°C until use.

Incubations

A protein/lipid emulsion was prepared by mixing 9 ml BSA (10 mg/ml) with linolenic acid methyl ester (10 mM) for 90 s using an Ultra Turrax T25 (Janke & Kunkel GmbH, Staufen, Germany) at 13500 rpm. BSA (9 mg/ml) alone or BSA/LnMe emulsions were incubated in a shaking water bath at 37°C in 50 mM phosphate buffer pH 7.4. The antioxidant solutions were prepared in THF and added to the reaction mixture prior to initiation at final concentration of 20, 100, 200 or 400 μM for tocopherol and Trolox and 20 and 2 μM for astaxanthin and canthaxanthin. In all samples THF represents 1% of the total volume of

the reaction mixture. Blanks with no antioxidant but containing THF were always run in parallel. The reactions were initiated by adding AAPH (50 mM) or $FeCl_3$ and ascorbic acid (0.1 and 25 mM, respectively) or $FeCl_2$, EDTA and H_2O_2 (1, 1 and 2 mM, respectively). After, 30, 90, 180 and 360 min of incubation, a 2.5 ml sample was eluted on a PD 10 column (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with phosphate buffer (50 mM, pH 7.4) in order to remove initiating agents and lipids. The collected protein fractions (3.5 ml) were further analyzed for protein bound hydroperoxides, and carbonyls groups, and the protein concentration was determined. For singlet oxygen induced oxidation, $NDPO_2$ (0.5 mM) was added to the reaction mixtures described above and the reaction was allowed to proceed for 90 min in a shaking water bath at 37°C with a simultaneous control containing NDP (0.5 mM) instead of $NDPO_2$. Subsequently, carbonyl groups were directly measured on the protein fractions.

Carbonyl groups: DNPH derivatization

The protein carbonyl content was determined using DNPH, as previously described by Levine [37]. The protein fractions (200 μl) were precipitated with trichloroacetic acid (TCA) and incubated for 10 min with DNPH (10 mM) in 2 M HCl. A protein fraction incubated without DNPH was always investigated in parallel for each sample. After precipitation with TCA, the pellet was washed three times with ethanol/ethylacetate (1:1 vol/vol) containing 10 mM HCl. The carbonyl content was calculated as mol carbonyls per mol BSA using the absorbance at 370 nm for the dinitrohydrazone derivate.

$FINH_2$ derivatization

Protein fractions of 2 ml were freeze-dried and stored at −80°C until derivatization. The freeze-dried protein fractions were solubilized and boiled for 1 min in 700 μl of MES–SDS buffer (0.25 M, 1% SDS). Thereafter, the mixture was incubated for 1 h with 50 μl fluoresceinamine (0.25 M in 0.5 M NaOH) and 50 μl $NaCNBH_3$ (0.4 M in 0.25 M MES buffer) in a shaking water bath at 37°C. To remove excess $FINH_2$ and $NaCNBH_3$, the sample was diluted to 2.5 ml and eluted on a PD 10 column equilibrated with double deionized water. A 100 μl sample of the collected fraction was diluted in NaOH (0.1 M) and absorbance recorded on a Shimadzu UV-160 spectrophotometer (Kyoto, Japan). Carbonyl groups were calculated using maximum absorbance at 290 nm for derivatized protein and at 490 nm for fluoresceinamine using $\varepsilon_{490\,nm}$ for fluoresceinamine in 0.1 M NaOH of 86,800 $M^{-1}\,cm^{-1}$ [38].

Protein hydroperoxides (FOX1)

The protein fraction (50 μl) was incubated with 950 μl of ferrous ammonium sulfate/xylenol orange reagent (2.5 and 1 mM, respectively) and incubated at room temperature for 30 min. The absorbance was read at 560 nm on the Shimadzu UV-160 spectrophotometer using xylenol orange reagent as a blank. A standard curve was prepared using hydrogen peroxide.

Conjugated dienes

Conjugated dienes were determined by mixing 1 ml of the reaction mixture with 2.5 ml of ice cold phosphate buffer buffer (50 mM, pH 7.4) and with 3.5 ml heptane. After vigorous shaking the samples were centrifuged at 5°C (2000*g* for 3 min) and the organic phase was read at 210 and 234 nm using the Shimadzu UV-160 spectrophotometer. Conjugated dienes concentration was estimated as a ratio between the absorbance at 234 and 210 nm.

Volatile aldehydic fraction

Aldehydes were collected from the sample by dynamic headspace sampling. The sample was purged with nitrogen at 340 mg/min for 10 min at 37° C and the volatiles were trapped on Tenax-GR traps column (Varian Chrompack International, Bergen op Zoom, The Netherlands). Volatiles were released from the column by thermal desorption (ATD-400, Perkin-Elmer, MA, USA) and analyzed by GC-MS on a 30 m DB 1701 capillary column (J & W Scientific, Folsom CA, USA,). The temperature program used was 65°C for 1 min, ramping 4° C/min to 90° C and 20°C/ min to 240 °C and holding for 5 min at the final temperature. The ionization energy of the mass range of 30–250 atomic mass units with repetition rate at 3.4 scans per s. For quantification, aldehyde standards were directly injected to the Tenax-GR traps column and analyzed as described above for the samples. Detection limits were determined as 3 × noise. The noise was determined from six GC–MS analyses on blank tubes and was recorded over a period of 1 min. The response calculated as 3 × noise was converted to concentrations by use of the calibration curve prepared for each standard compound.

Protein determination

Protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) according to the manufacturers specifications.

Statistics

The experiments were carried out at least in triplicate (unless otherwise stated) on independent days. The results are given as mean values of triplicates

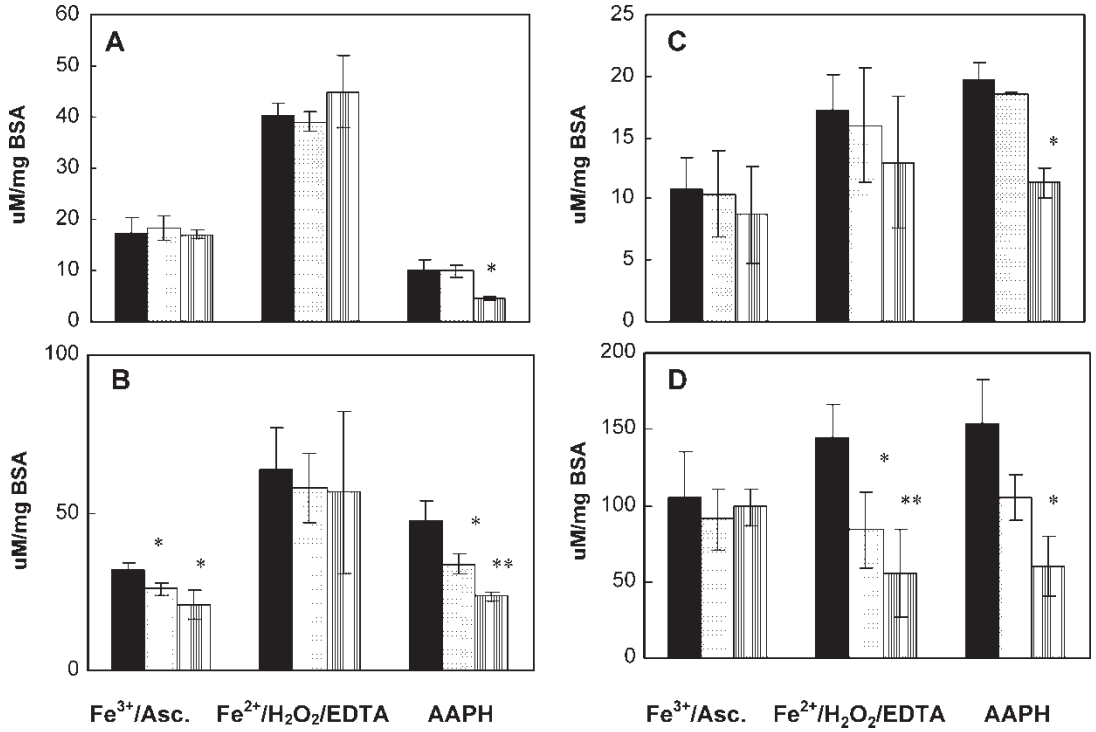

Figure 1. Protein hydroperoxides detected after 30 min (A, B) and 6 h (C, D) incubation of BSA in the absence (A, C) or in the presence (B, D) of linolenic acid methyl ester at 37°C when oxidation was initiated with either Fe^{3+}/asorbic acid, or Fe^{2+}/H_2O_2/EDTA or AAPH and with no antioxidants (solid bars), 400 μM tocopherol (dotted bars) and 400 μM Trolox (striped bars). Native BSA: 8.7 μM/mg (± 2.4). $^{*}p < 0.05$ and $^{**}p < 0.01$ comparing samples with antioxidant with samples with no antioxidants.

with indication of the standard deviation. Evaluation of statistical significance of differences was performed using the Student's *t* test.

Results

Protein in solution (BSA)

Protein oxidation was initiated with different initiators and oxidation of BSA, measured as carbonyl groups and protein bound peroxides, that was found to depend on the type of initiator used. For the initiating agents Fenton and Fe^{3+}/Asc the level of protein bound peroxides was highest after 30 min of incubation compared with AAPH as initiator (Figure 1). The Fenton reagent gave the highest initial level of protein bound peroxides but resulted in an overall lower level of protein carbonyls after 6 h of incubation when compared to AAPH and Fe^{3+}/Ascorbate (Table I). However, the level of carbonyl groups on BSA was the

Table I. Protein carbonyl groups detected using DNPH derivatisation of BSA after 6 h incubation with or without lipids and using three different initiating agents. Native BSA 0.14 mol/mol (± 0.06).

	Protein carbonyls (mol/mol BSA)		
	AAPH	Fe^{3+}/Asc	Fe^{2+}/EDTA/H_2O_2
No lipids			
No antioxidant	1.2 (± 0.1)	1.4 (± 0.1)	0.7 (± 0.3)
α-Tocopherol 100 μM	1.1 (± 0.2)	1.3 (± 0.1)	0.6 (± 0.2)
α-Tocopherol 400 μM	1.1 (± 0.2)	1.2 (± 0.1)	0.7 (± 0.3)
Trolox 100 μM	1.1 (± 0.1)	1.2 (± 0.2)	0.5 (± 0.1)
Trolox 400 μM	0.7 (± 0.2)	1.4 (± 0.2)	0.5 (± 0.2)
Lipids			
No antioxidant	0.8 (± 0.2)	1.3 (± 0.6)	0.7 (± 0.1)
α-Tocopherol 100 μM	0.8 (± 0.2)	1.3 (± 0.8)	0.7 (± 0.1)
α-Tocopherol 400 μM	0.8 (± 0.2)	1.1 (± 0.2)	0.7 (± 0.1)
Trolox 100 μM	0.8 (± 0.1)	1.5 (± 0.4)	0.6 (± 0.1)
Trolox 400 μM	0.4 (± 0.1)	1.6 (± 0.3)	0.6 (± 0.1)
Astaxanthin 20 μM	0.7 (± 0.2)	1.4 (± 0.2)	0.8 (± 0.1)

Table II. Protein carbonyl groups on BSA after 90 min incubation at 37°C in the absence or in the presence of linolenic acid methyl ester and using $NDPO_2$ as singlet oxygen generating species.

	Protein carbonyls (mol/mol BSA)	
	No lipids	Lipids
Controls		
Native BSA	0.17 (± 0.02)	nd
NDP	0.19 (± 0.02)	0.17 (± 0.01)
$NDPO_2$		
No antioxidant	0.28 (± 0.01)	0.22 (± 0.01)
α-Tocopherol 100 μM	0.26 (± 0.01)	0.18 (± 0.01)
α-Tocopherol 20 μM	0.27 (± 0.01)	0.19 (± 0.01)
Trolox 100 μM	0.24 (± 0.01)	0.16 (± 0.01)
Trolox 20 μM	0.27 (± 0.02)	0.16 (± 0.02)
Astaxanthin 20 μM	0.25 (± 0.03)	0.19 (± 0.02)
Astaxanthin 2 μM	0.25 (± 0.02)	0.22 (± 0.02)
Canthaxanthin 20 μM	0.27 (± 0.02)	0.18 (± 0.01)
Canthaxanthin 2 μM	0.26 (± 0.04)	0.19 (± 0.01)

nd: not determined.

lowest for the singlet oxygen generating system when compared with other free radical generating species (Table II). This indicates that oxidative damage of proteins is highly dependent on the type of free radical generating system and the fluxes of the production of free radicals. None of the antioxidants were effective at preventing oxidation of BSA when initiation was performed with Fe^{3+}/Asc probably due to the efficiency of this system at continuously generating free radicals in the homogenous solution as also reported by others [10,38].

Addition of α-tocopherol at 100 or 400 μM did not significantly affect the level of protein carbonyls nor the level of protein bound peroxides irrespective of the type of initiating agent used (Table I). In contrast, when initiation was performed with $NDPO_2$, tocopherol did show a tendency to protect BSA even at concentration as low as 20 μM. Additionally, increasing the concentration of tocopherol to 100 μM seemed to further protect BSA from oxidative damage generated by singlet oxygen (Table II). When the water-soluble AAPH was used as the initiating agent, addition of Trolox did result in a significantly lower level of carbonyl groups and protein bound peroxides (Table I and Figure 1), already after 30 min incubation. Trolox was also found to provide better protection of BSA against oxidation when initiation was dependent on $NDPO_2$ (Table II). For the carotenoids, astaxanthin and canthaxanthin a tendency to prevent the formation of carbonyl groups on the protein was observed at low concentration (2 μM). Increasing the concentration from 2 to 20 μM did not further reduce formation of carbonyl groups on BSA. In contrast, no antioxidative activity of the carotenoids was observed on BSA after 90 min incubation when initiation was performed with the other three initiators (results not shown).

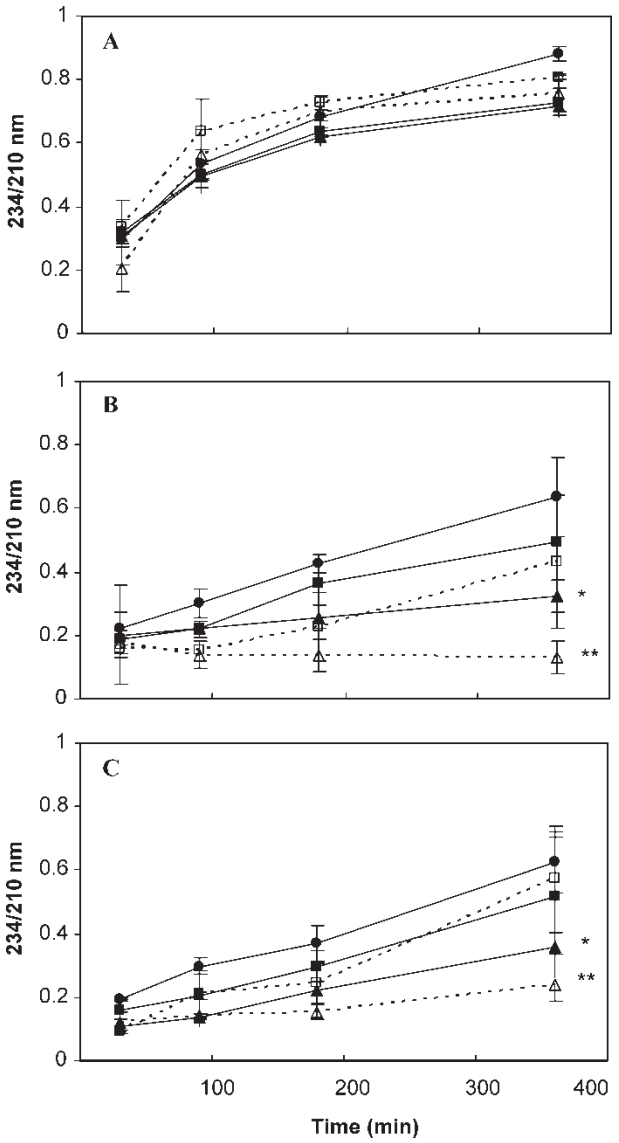

Figure 2. Time course of conjugated diene formation during incubation of BSA/linolenic acid at 37°C in the absence of antioxidant (●) or in the presence of (■) 100 μM, or (▲) 400 μM α-tocopherol (—) or of (□) 100 μM, or (△) 400 μM Trolox (...) when oxidation was initiated either with A: Fe^{3+}/ascorbic acid; B: Fe^{2+}/H_2O_2/EDTA; or C: AAPH. $*p < 0.05$ and $**p < 0.01$ comparing samples with antioxidant with samples with no antioxidants after 360 min incubation.

Protein in emulsions (BSA/LnMe)

In the presence of lipid, the level of protein bound peroxides increased with time irrespective of the type of initiator. The level of protein hydroperoxides was almost 10 times higher after 6 h incubation in the presence of lipid compared to the system without lipids (Figure 1). The level of protein carbonyls was not different in the presence and absence of lipid for Fenton type initiator, initiation by Fe^{3+}/Asc, and initiation by singlet oxygen but was reduced in the presence of lipid with AAPH as the initiator (Tables I and II). The antioxidants tested were not

Table III. Aldehydes formed during linolenic acid methyl ester oxidation in the presence or in the absence of antioxidants.

	t-butenal (ng/mg lipid)		t-2-pentenal (ng/mg lipid)		t,t-2,4-Hexadienal (ng/mg lipid)		t,t-2,4-Heptadienal (ng/mg lipid)	
	30 min	360 min	30 min	360 min	30 min	360 min	30 min	360 min
AAPH								
No Antioxidant	0.61 (± 0.08)	0.81 (± 0.05)	1.04 (± 0.08)	2.18 (± 0.21)	0.23 (± 0.02)	0.69 (± 0.09)	1.67 (± 0.40)	3.13 (± 0.54)
Tocopherol 400 μM	0.60 (±0.03)	0.66* (±0.07)	0.87 (±0.03)	1.73* (±0.15)	0.25 (±0.01)	0.51* (±0.04)	1.94 (±0.26)	2.91 (±0.50)
Trolox 400 μM	0.52 (±0.01)	0.46** (±0.05)	< 0.05	0.78** (±0.13)	0.21 (±0.00)	0.31** (±0.02)	1.3 (±0.10)	2.13* (±0.12)
Fe^{3+}/Asc								
No antioxidant	1.43 (±0.53)	4.42 (±1.07)	7.10 (±2.88)	26.99 (±6.08)	0.40 (±0.10)	5.21 (±1.76)	9.26 (±4.90)	41.56 (±12.76)
Tocopherol 400 μM	1.27 (±0.34)	3.47 (±0.88)	6.61 (±2.16)	22.82 (±5.71)	0.40 (±0.10)	3.92 (±1.25)	8.76 (±3.55)	38.52 (±9.07)
Trolox 400 μM	1.03 (±0.26)	3.89 (±0.98)	4.74 (±1.43)	23.21 (±4.15)	0.34 (±0.05)	4.07 (±0.85)	5.68 (±2.12)	37.87 (±9.04)
Fe^{2+}/H_2O_2/EDTA								
No antioxidant	0.62 (±0.03)	0.76 (±0.02)	1.60 (±0.20)	2.46 (±0.11)	0.26 (±0.01)	0.91 (±0.05)	1.70 (±0.21)	4.65 (±0.23)
Tocopherol 400 μM	0.56 (±0.04)	0.49** (±0.08)	1.42 (±0.06)	1.47** (±0.21)	0.25 (±0.00)	0.56** (±0.03)	1.35* (±0.20)	2.9** (±0.37)
Trolox 400 μM	0.52* (±0.05)	0.49** (±0.02)	1.08** (±0.11)	1.31** (±0.19)	0.22** (±0.01)	0.49** (±0.09)	0.85** (±0.09)	2.36** (±0.61)

*$p < 0.05$ and **$p < 0.01$ comparing no antioxidant with addition of antioxidant.

able to prevent oxidative damage to BSA, nor to the lipid when oxidation was performed using the iron ascorbate initiating system as also found for the pure protein system even if a significant reduction of protein peroxides was observed after 30 min incubation. In the presence of lipid, both tocopherol and Trolox were able to reduce the formation of conjugated dienes after 90 min, but only when the initiating agents were either AAPH or Fenton reagent (Figure 2). The effect was even more pronounced after 6 h of incubation. The effect of Trolox was more significant than tocopherol at a similar concentration.

Secondary oxidation products, formed during the course of the experiment are presented in Table III for 30 min and 6 h of incubation. Aldehyde development was reduced in presence of tocopherol or Trolox when the initiation was performed with the water-soluble AAPH initiator. Similarly, when initiation was performed with the Fenton reagent both antioxidants were able to prevent the development of aldehydes, with Trolox being more efficient than tocopherol. Prevention of lipid oxidation also resulted in lower protein peroxide levels when compared to the system with no antioxidant (Figure 1B and D), but surprisingly the level of carbonyl groups was not systematically reduced (Table I).

Protein carbonyls were only reduced after 6 h of incubation when the initiating agent was AAPH in the presence of Trolox, but not affected when the antioxidant present in the system was tocopherol. These results were further confirmed by measuring carbonyl groups using fluoresceinamine derivatization, as tocopherol at the highest concentration was found to protect the protein moderately, in contrast to Trolox, which was always found to be more effective with both initiating species (Fenton and AAPH) (Figure 3). When initiation was performed using $NDPO_2$ both tocopherol and Trolox seemed to prevent oxidative damage to BSA. However, Trolox appeared more effective than tocopherol at protecting

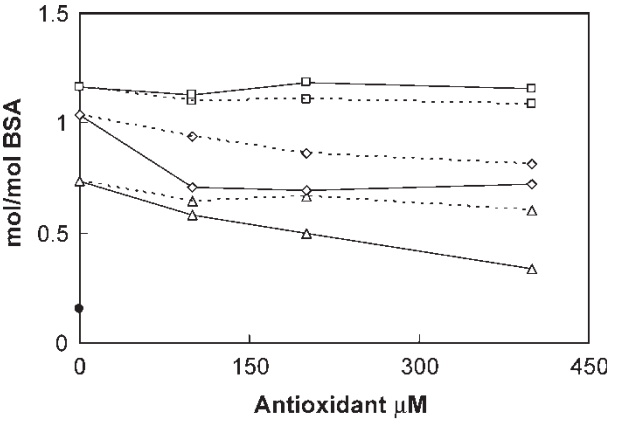

Figure 3. Effect of α-tocopherol (—) and Trolox (...) on the development of carbonyl groups on BSA in BSA/linolenic acid emulsions when oxidation was initiated with either AAPH (△), or Fe^{2+}/H_2O_2 /EDTA (◇) or Fe^{3+}/Ascorbic acid (□) and after 6 h incubation at 37°C. Control (•) Native BSA. Standard deviation <10%.

the protein and supports the results obtained with other initiating species. Interestingly, astaxanthin and canthaxanthin were not able to inhibit protein oxidation in the BSA/LnMe system when initiation was performed with either AAPH or the Fenton reagent, but were able to protect BSA even in the presence of lipid when initiation was performed with the singlet generating species $NDPO_2$ (Table II).

Discussion

Interaction between protein and lipid oxidation products has received increasing attention in recent decades and there is strong evidence that lipid oxidation products are able to induce protein damage [10,15]. However, the sequence of events that lead to oxidative changes on the protein is still ambiguous. It is clear that the extent of oxidative damage on protein is dependent on the type of radicals generated, their localization and the kinetics of their formation [16,39]. The system iron/ascorbate was found to be the most powerful initiator of oxidative damage and surprisingly, none of the tested antioxidants, even at high concentration, protected the protein efficiently, irrespective of the presence of lipid. Additionally, none of the lipophilic antioxidants tested showed any antioxidative activity on protein in a pure protein system when initiation was performed using free radical generating system such as AAPH or the Fenton reagent. However, it was expected that the lipophilic antioxidants would be buried in the hydrophobic core of the protein, thereby protecting the amino acid residues typically buried within protein (Tyr, Trp, Phe) from oxidative damage [27].

It has been recognized that carotenoid pigments associate with protein via hydrophobic interactions, for example in plasma, astaxanthin is associated with albumin [40] and in muscle tissue with the acto–myosin complex [41]. Carotenoids may consequently be expected to be involved in protecting the protein from oxidative damage, an aspect that has not been thoroughly investigated. Lipophilic antioxidants were not found to protect the protein from oxidative damage, and oxidation of proteins is most likely induced on the amino groups exposed to the aqueous environment [42]. This was confirmed with the findings that indicated that the only antioxidant able to provide some protein protection was the water-soluble antioxidant Trolox. Our result shows that Trolox was able to protect protein from oxidation in a dose dependent manner by preventing the formation of protein hydroperoxides and protein carbonyl groups. A similar study using human serum albumin also showed that water soluble antioxidant are far better than lipophilic antioxidants in protecting protein from oxidative damage [43].

In contrast, all antioxidants tested were able to prevent oxidative damage to the protein when the initiating species was singlet oxygen. Singlet oxygen quenching constants for the antioxidants tested were reported by Di Mascio et al. [33]. According to their ranking astaxanthin and canthaxanthin are the best singlet oxygen quenchers followed by Trolox and α-tocopherol. This is in agreement with our findings with the pure protein system for the same concentration of antioxidants (20 μM). Additionally, the amino acids, which are primary targets for oxidation with singlet oxygen, were recently reported to be buried in the core of the protein structure [26]. As such it is expected that the lipophilic antioxidant would prevent protein oxidation. Interestingly, increasing carotenoid concentration did not seem to prevent further protein from oxidative damage whilst the response seems to be concentration dependent with both Trolox and tocopherol. This might be explained by the mechanisms by which the tested antioxidants prevent oxidative damage. Tocopherol and Trolox can scavenge both the initiating radical species and the radical generated during the propagation phase of oxidative damage. However, it may be concluded that the carotenoids are not able to act as chain breaking antioxidants in the present system.

In the presence of lipids, and when singlet oxygen was the initiating species, Trolox was the best in protecting the protein, as it scavenges free radicals, which damage exposed amino groups, singlet oxygen, and free radicals generated during lipid peroxidation, which could further promote protein damage. This was also reflected in the results obtained from the free radical generating systems such as AAPH and the Fenton reagent, as Trolox was much more efficient than tocopherol in preventing protein oxidation even in the presence of lipids.

Proteins have also been found to act as antioxidants, as they form stable and long-lived protein hydroperoxides and prevent propagation reactions, but in the presence of heat, light or transition metals it can propagate oxidative damage to lipid, antioxidants or DNA [30,31,44]. However, as reported by Gieseg et al. [45] some protein hydroperoxides are formed during lipid oxidation, but tocopherol will simultaneously reduce the level of protein hydroperoxides by quenching lipid derived radicals as this antioxidant protects the lipid fraction. Our results support the hypothesis that proteins are involved in protection of lipid from oxidative damage. Accordingly, preventing the formation of protein hydroperoxides or deactivating protein hydroperoxides effectively protects the lipids. In contrast, protecting the lipid from oxidation, in an oil-in-water emulsion, did not provide any significant protein protection.

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