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The antioxidant activity of carnosine and its consequences on the volatile profiles of liposomes during iron/ascorbate induced phospholipid oxidation

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The natural dipeptide L-carnosine (β-Ala-His) exhibits antioxidative properties and can be used as an antioxidant in food products. Its antioxidant activity and its effect on the volatile compounds produced during lipid oxidation was studied in a meat-related model system (liposomes of muscle phospholipids). Oxygen uptake, conjugated dienes, trienes and ketodienes, thiobarbituric acid reactive substances (TBARS) and volatile compounds were measured after induction of oxidation by equimolar Fe(III)/ascorbate (45 μM).

Inclusion of carnosine (2–10 mM) lead to a decrease in all indices of lipid oxidation, except for the initial rate of oxygen uptake, which increased, and the rates of oxygen uptake 5 or more min after catalyst injection, which remained constant. The decrease varied as a function of carnosine concentration, method of measurement and incubation time. When carnosine was added to previously oxidised liposomes, TBARS, t-2-undecenal, total 2-alkenals and hexanol amounts decreased significantly.

Carnosine antioxidant activity is multifunctional: it has a buffering effect; it interferes in the initiation step of oxidation; it decreases the amount of preformed peroxides; it reacts with some secondary products. © 1997 Elsevier Science Ltd

INTRODUCTION

Lipid oxidation in meat and meat products is one of the major causes of spoilage and deterioration of organoleptic properties, leading to off-flavour development, colour deterioration and loss of nutritive value (Kanner *et al.*, 1988). Lipid oxidation can be inhibited by the addition of antioxidants during food processing. However, efficient synthetic antioxidants such as butylhydroxytoluene and butylhydroxyanisole exhibit toxic properties and they cannot be recommended for general usage. Other antioxidants are naturally present in raw food materials. For example, L-carnosine (β-Ala-His) is a dipeptide found at high levels (1–20 mM) in skeletal muscle of several vertebrate species (Crush, 1970; Kohen *et al.*, 1988). It has been shown to be a useful antioxidant in processed foods (Decker & Faraji, 1990; Decker & Crum, 1991). Carnosine (1.5%) is more efficient than α-tocopherol and butylhydroxytoluene (0.02%) in preventing colour changes and rancidity of salted ground pork in frozen storage for up to 6 months, and in the inhibition of lipid oxidation in cooked salted ground pork stored for 7 days at 4°C (Decker & Crum, 1991, 1993).

The antioxidant activity of carnosine has been studied by several authors. The results reveal that the antioxidant behaviour of carnosine in model systems depends on factors such as the catalyst of oxidation and the method used to assess oxidation. For instance, on the basis of malonaldehyde release in a liposome system, carnosine exhibits good antioxidant activity during methylene blue photosensitised oxidation, weak antioxidant activity during riboflavine 5′-phosphate sensitised oxidation and even a pro-oxidant effect during Cu(II)-catalysed oxidation (Yamashoji & Kajimoto, 1980). The antioxidant effect of carnosine in liposomes decreases according to the catalyst in the following order: copper/ascorbate, iron/ascorbate, hydrogen peroxide-activated haemoglobin, photoactivated riboflavin and lipoxygerase (Decker & Faraji, 1990; Decker *et al.*, 1992). Carnosine inhibits the formation of thiobarbituric reactive substances (TBARS)

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in various model systems such as fatty acid alcohol/aqueous solution (Yamashoji & Kajimoto, 1980; Kohen *et al.*, 1988), liposomes (Decker & Faraji, 1990), sarcoplasmic reticulum membranes (Boldyrev *et al.*, 1988, 1989) and microsomes (Decker *et al.*, 1992). The antioxidant effect of carnosine is also related to the method used to assess lipid oxidation. For example, TBARS formation is inhibited less than lipid peroxide release during copper/ascorbate and iron/ascorbate induced peroxidation of liposomes (Decker *et al.*, 1992).

Several mechanisms have been proposed to explain the inhibition of lipid oxidation by carnosine. One hypothesis is related to the buffering capacity of the peptide. Carnosine helps to keep the pH of the system at physiological values (Kohen *et al.*, 1988), at which lipid peroxidation is reduced (Misik *et al.*, 1991). It has also been suggested that carnosine acts as a chelator of pro-oxidant metals. However, the effectiveness of carnosine as a chelator is limited because it chelates copper (Brown, 1981; Decker *et al.*, 1992) but not iron (Decker *et al.*, 1992; Aruoma *et al.*, 1989), and the copper-chelating property does not prevent Cu(II) reactivity using the phenanthroline assay (Aruoma *et al.*, 1989). Another possible mechanism relates to the ability of carnosine to scavenge the hydroxyl radical (Aruoma *et al.*, 1989; Chan *et al.*, 1994), singlet oxygen (Dahl *et al.*, 1988) and the peroxy radical (Kohen *et al.*, 1988). Carnosine can also act as hydrogen donor through the side-chain of its His residue (Kohen *et al.*, 1988; Dahl *et al.*, 1988). Another theory is that carnosine interacts with primary products of peroxidation (Boldyrev *et al.*, 1989; Boldyrev & Severin, 1990), with some volatile compounds involved in organoleptic deterioration (Aruoma *et al.*, 1989; Decker & Crum, 1991; Kansci *et al.*, 1994), or with other compounds to generate good flavour compounds (Decker & Crum, 1991). This effect could explain the large improvement of organoleptic properties of meat processed with carnosine (Decker & Crum, 1991). Thus, the relative contribution of the various mechanisms to the antioxidant activity of carnosine is not clearly defined. In particular, the effect of the peptide on formation and/or trapping of volatile compounds has not been investigated. This is an aspect particularly worthy of consideration since volatiles make a major contribution to food flavour.

The aim of this paper is to evaluate the antioxidant activity of carnosine, the different proposed mechanisms of action, and the effects of carnosine on volatile compound production. This work was performed on a meat-related model system consisting of muscle phospholipid liposomes and iron/ascorbate to induce oxidation. Lipid oxidation was assessed by several methods to obtain information on the different stages of the lipid oxidation reaction. In addition, the consequences of carnosine addition on previously oxidised lipids, especially on the profile of volatiles, yielded information about the ability of the peptide to trap volatile compounds.

MATERIALS AND METHODS

Materials

Lipids were extracted from fresh pork muscles (*longissimus dorsi*) according to Folch *et al.* (1957). Phospholipids were purified from total lipid extract on a silicic acid column (Kates, 1982). The purity of the phospholipids was checked by normal-phase high-performance liquid chromatography paired with a light scattering detector (Leseigneur-Meynier & Gandemer, 1991). No cholesterol or triglycerides were present in this fraction which contained 1.8–4.9% cardiolipin, 20–28% phosphatidylethanolamine, 7.1–10.8% phosphatidylinositol, 52–58% phosphatidylcholine and 1.2–1.8% sphingomyelin, depending on the phospholipid preparation. The fatty acid composition of phospholipid was determined by gas–liquid chromatography (GLC) of fatty acid methyl esters (FAME) prepared as described in Berry *et al.* (1965), using the GLC procedure proposed by Leseigneur-Meynier & Gandemer (1991). The phospholipids contained 34.6–38.7% saturated fatty acids, 22.5–31.2% monounsaturated fatty acids and 32.3–38.1% polyunsaturated fatty acids. The polyunsaturated fatty acids were composed mainly of linoleic and arachidonic acid, accounting for 26–32% and 7–11% of the total FAME, respectively. The purified phospholipids were kept in chloroform at −20°C prior to liposome preparation.

L-Carnosine (purity 99%) was obtained from Sigma (ref. C-9625). Its absorbance spectra in PIPES solution was close to that described by Boldyrev *et al.* (1993). All other chemical reagents were of analytical grade. Piperazine-N-N′-bis(2-ethanesulfonate) (PIPES) solutions (10 mM, 0.15 M NaCl) were prepared with ultra-pure Millipore water (18 Mohm). The pH of the PIPES solution, generally 6.0, was adjusted with NaOH. Each day, sodium ascorbate and $FeCl_3$ solutions were prepared separately in degassed ultra-pure water. The solutions were mixed in equimolar concentrations just before use.

Liposome preparation and general procedure

Large unilamellar vesicles (LUV) were prepared according to the procedure of Hope *et al.* (1985) to obtain liposomes with well-defined size and stable structure and to minimise oxidation during the preparation (Genot *et al.*, 1992). LUV (1 mg phospholipid ml^{-1}) were prepared in degassed PIPES solution by extrusion through two polycarbonate membranes (Poretics Corp.) of 0.4–0.45 μm porosity. The liposomes were air-saturated by bubbling filtered air at room temperature for 10 min.

Carnosine was dissolved in the LUV suspension to give a final concentration of 2–10 mM. After 10 min, phospholipid oxidation was induced by injection of equimolar Fe(III)/ascorbate (final concentration 45 μM). The liposomes were incubated at 25°C in the dark. Lipid oxidation was assessed by one to four methods.

Measurement of lipid oxidation

Oxygen consumption

The oxygen uptake was measured with an oxygen monitor (Strathkelvin, Glasgow) equipped with a Clark-type electrode and connected to a computer (Genot *et al.*, 1994). The electrode was calibrated with sodium dithionite (0% saturation) and 25°C air-saturated water (100% saturation: 0.253 mmol O_2 litre^{-1}). One millilitre of the solution was dispersed in a 25°C thermostatted cell which was airtight. The data were recorded for 25–30 min after injection of the Fe(III)/ascorbate mixture. The acquisition parameters were adjusted according to Genot *et al.* (1994). Oxygen uptake was calculated after 1, 5, 10, 15 and 20 min of liposome incubation with Fe(III)/ascorbate. The rates of oxygen uptake before catalyst injection (V_0) and 1, 5, 10, 15, 20 min after Fe(III)/ascorbate injection and the maximum oxygen uptake rate (V_{max}) were determined from the slopes of the curve. Apparent pseudo first-order rate constants (K_{app1} and K_{app2}) were calculated in the fast and slow stages of the reaction from the slopes of the linear parts of the plot of ln [O_2] as a function of time. Thus, the first pseudo first-order constant (K_{app1}) was calculated within the first 2.5 s of the reaction (13–20 degrees of freedom; $r^2 > 0.98$) and K_{app2} between 10 and 20 min after catalyst injection (49 degrees of freedom, $r^2 > 0.99$). The measurements were performed on liposomes with and without carnosine (2–10 mM) and also on blanks, composed of the buffer, alone or with carnosine, to which the Fe(III)/ascorbate mixture was added.

Thiobarbituric acid reactive substances (TBARS)

TBARS were evaluated using the method of Buege & Aust (1978). Aliquots (0.25 ml) of the incubation mixture were sampled from a closed flask stored at 25°C at various times after catalyst injection (0.1, 1, 2, 4, 6 and 21 h). Samples were dispersed into test tubes, and 0.75 ml of water followed by 2 ml of thiobarbituric acid (TBA) reagent (0.375% (w/v) TBA, 15% (w/v) trichloroacetic acid in HCl 0.25 N) were added. The tightly closed tubes were heated in a boiling bath for 15 min, cooled and centrifuged (2500*g*, 10 min). The absorbance of the supernatant was read at 532 nm against a blank. The results were expressed as TBARS (nmol malonaldehyde (MDA) mg^{-1} phospholipid) using the molar extinction coefficient of the TBA–MDA complex at 532 nm (1.56×10^5 M^{-1} cm^{-1}). The measurements were performed in triplicate on at least two different liposome preparations.

Ultraviolet spectra of lipids

The formation of conjugated dienes and of ketodienes and conjugated trienes was evaluated after extraction of the lipids contained in 500 μl of incubation medium following the method of Folch *et al.* (1957). The solvent was evaporated under a stream of nitrogen and the phospholipids were dissolved in 2 ml of ethanol. The lipid absorbance spectrum was recorded against ethanol from 200 to 350 nm using a double beam UV–Vis spectrophotometer (Varian, Model Cary 13). Conjugated diene formation is characterised by an increase of the absorbance at 233 nm (A_{233}). Ketodienes and conjugated trienes, as well as ethylenic diketones and α-diketones, absorb around 270–280 nm (Recknagel & Glende, 1984). Thus, the ratios A_{233}/A_{214} and A_{275}/A_{214} nm (A_{214} is isobestic point) were taken as indices of lipid oxidation (Klein, 1970). Second derivative spectra were calculated in the 210–250 nm region to evaluate the changes in the ratio of *t,t* and *c,t* isomers of hydroperoxides. *t,t* and *c,t* isomers exhibit minima in the second derivative spectra set at 233 and 242 nm, respectively (Corongiu *et al.*, 1986). The ratio of *t,t*/*c,t* isomers was evaluated as described by Sergent *et al.* (1993). Each measurement was performed in duplicate.

For a given parameter, the percentage of inhibition was calculated from the values measured in liposomes in the absence and the presence of carnosine at the same incubation time.

Analysis of volatile compounds

The analyses were performed on three samples. Liposomes alone (control) and liposomes with 10 mM carnosine were incubated with 45 μM Fe(III)/ascorbate (1:1, mol:mol) at 25°C for 24 h in closed flasks. The third sample contained liposomes oxidised with 45 μM Fe(III)/ascorbate to which 10 mM carnosine was added after 24 h. Volatile compounds were identified and quantified using a purge and trap system paired with a gas–liquid chromatograph and a mass spectrometer (GC–MS). This experiment was performed separately three times.

A Tekmar dynamic headspace concentrator (Model 2000) was used with a 25 ml sparger. One millilitre of the liposomes was introduced into the sampler and the headspace swept with nitrogen at 40°C. The purge flow rate was set at 50 ml min^{-1} for 30 min. The volatile compounds were trapped on a Tenax column and swept after cryofocalisation at −100°C into the injection port, set at 200°C, of a capillary gas chromatograph paired with a mass detector (Hewlett-Packard 5971A). The operational parameters used for the concentration of the volatile compounds were: prepurge with nitrogen, 5 min at 50 ml min^{-1} at room temperature; purge preheat, 5 min, 40°C; purge with nitrogen, 30 min at 50 ml min^{-1}, 40°C; desorb preheat, 195°C; Tenax trap heated at 200°C.

A fused silica capillary column (60 m×0.32 mm i.d.; film thickness 1 µm) coated with 5% phenylsiloxane–95% methylsiloxane phase (Durabond DB-5) was used to separate the volatile compounds by GLC. The oven temperature was programmed as follows: the initial temperature was set at 40°C for 5 min and then increased to 210°C at 3°C min^{-1}. The carrier gas was helium (5 p.s.i.). The electron impact ionisation was set to 70 eV and the interface temperature at 280°C (source temperature 167°C). Mass spectra scanned between mass/charge 33 and 300 were recorded on a computer (Vectra QS/20) and identified with available libraries (NBS, NIST, TNO).

Table 1. Effect of carnosine on rates of oxygen consumption and apparent pseudo first-order constants (K_{app1}, K_{app2}) during oxidation of phospholipid liposomes [1 mg ml^{-1} in PIPES solution (10 mM, pH 6.0, 0.15 M NaCl)] induced by Fe(III)/ascorbate (1:1, mol:mol, 45 μM) at 25°C

Carnosine (mM)	Rate of oxygen consumption (nmol O_2 min^{-1})							K_{app1} (min^{-1})	K_{app2} (min^{-1})
	V_0	V_{max}	Time (min)						
			1	5	10	15	20		
0	0.65	146.3^c	3.83^a	1.27	0.90	0.80	0.74	0.605^c	0.0038
2	0.47	183.3^a	3.24^b	1.01	0.78	0.74	0.60	0.787^b	0.0036
6	0.56	192.6^a	2.79^b	1.05	0.83	0.79	0.73	0.874^a	0.0038
10	0.53	169.7^b	2.97^b	1.03	0.87	0.81	0.85	0.757^b	0.0039

The parameters (V_0, V_{max}, K_{app}) are calculated as indicated in the text. Time 0 corresponds to Fe(III)/ascorbate injection.
In a same column, numbers with different superscript letters are significantly different ($P < 0.05$; $n = 4$).

The amounts of volatile compounds were expressed in arbitrary units corresponding to the integrated areas of the peaks. The results were expressed in two ways. Firstly, the proportion of each compound was calculated as a percentage of the area of all identified peaks (repartition, % total area). Secondly, for each replicate the area due to a particular compound (A) was related to the area of that compound in the control and expressed in relative amount (% control) according to the following expression:

$$\text{Relative amount (A)} =$$

$$[\text{area (A)}]_{\text{model}} \times 100/[\text{area (A)}]_{\text{control}}$$

As each sample was opened prior to headspace collection, the odour was assessed in the laboratory by five members of a trained panel and their comments were noted.

Statistical analysis

One-way or two-way analysis of variance was performed according to the method of Snedecor & Cochran (1980).

RESULTS

Oxygen consumption

Before Fe(III)/ascorbate (45 μM) injection, oxygen uptake was very low in all systems tested as shown by the initial rate of oxygen uptake ($V_0 = 0.5 - 0.6$ nmol O_2 min^{-1}; Table 1). After Fe(III)/ascorbate injection, oxygen consumption in solutions containing only PIPES or PIPES + carnosine remained very low (data not shown). This indicates that oxygen consumption due to Fe(III)/ascorbate and to carnosine alone can be neglected. When liposomes were present, the oxygen uptake increased with no lag time, regardless of the carnosine concentration (0–10 mM) (Fig. 1). After the initial surge, oxygen uptake decreased with increasing carnosine concentration. Oxygen uptake was inhibited by about 10–22% of the total uptake when carnosine concentration increased from 2 to 10 mM. The inhibitory effect of carnosine on oxygen uptake was independent of incubation time. Surprisingly, the maximal rate of oxygen uptake (V_{max}) and K_{app1} were significantly higher in the presence of carnosine than in the control (Table 1). On the contrary, the rate of oxygen uptake after 1 min was significantly decreased, whatever the peptide concentration. The rates of oxygen consumption measured 5, 10, 15 and 20 min after catalyst injection decreased slowly with time but were similar at each carnosine concentration. Similarly, K_{app2} was unaffected by addition of carnosine.

Conjugated dienes, and trienes and ketonic dienes

In lipids re-extracted from fresh liposomal suspension, conjugated dienes were not detected. The injection of Fe(III)/ascorbate mixture in the liposomes led to an early increase of the A_{233}/A_{214} ratio due to the appear-

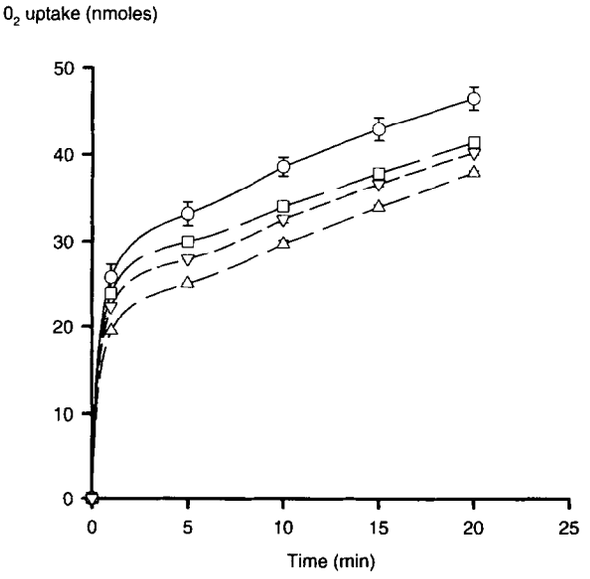

Fig. 1. Inhibition of oxygen uptake by carnosine, during Fe(III)/ascorbate (1:1, mol:mol, 45 μM) induced oxidation of pork muscle phospholipid liposomes (1 mg ml^{-1}) in PIPES solution (10 mM, pH 6.0, 0.15 M NaCl) at 25°C. ○, 0 mM carnosine (control); □, 2 mM carnosine; ▽, 6 mM carnosine; △, 10 mM carnosine. $n = 4$.

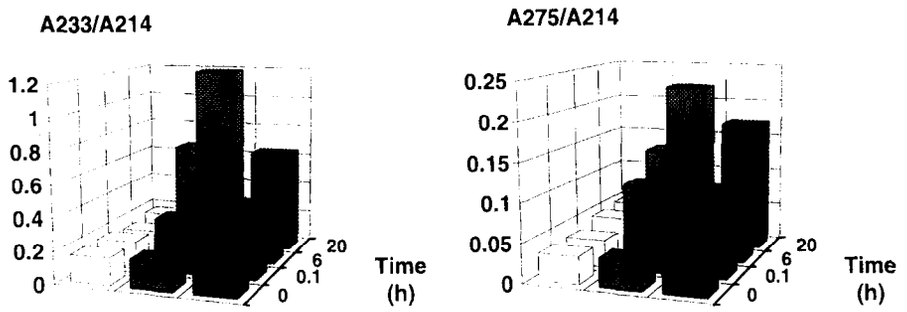

Fig. 2. Effect of carnosine (10 mM) on formation of conjugated dienes (left) and trienes and ketodienes (right) during Fe(III)/ascorbate induced oxidation of phospholipid liposomes (1 mg ml^{-1}) in PIPES solution (10 mM, pH 6.0, 0.15 M NaCl) at 25°C. No shading, large unilamellar vesicles (LUV); light shading, LUV + iron/ascorbate; dark shading, LUV + 10 mM carnosine + iron/ascorbate. Mean of two series of experiments.

ance of conjugated dienes. When 10 mM carnosine was added to the liposomes, A_{233}/A_{214} was lower than that of the control (Fig. 2, left). The inhibition of conjugated diene formation varied from 24% just after Fe(III)/ascorbate injection to 50% after 21 h. Similarly, the absorbance of conjugated trienes and ketonic dienes (A_{275}/A_{214}) was lower in the presence of carnosine than in the control (Fig. 2, right). The UV spectra of all samples exhibited the same absorption maxima and no significant difference was found on the second derivative spectra, particularly on the relative heights of the minima corresponding to *t,t* and *c,t* isomers of hydroperoxides (data not shown).

TBA test

Just after addition of the Fe(III)/ascorbate mixture (45 μM) to liposomes, TBARS dramatically increased. They then continued to increase more gradually and tended to level off progressively (Fig. 3). In the presence of 2–10 mM carnosine, TBARS production was greatly reduced. This decrease was a function of peptide concentration and, to a lesser extent, of time (Table 2). The highest carnosine concentration (2.26 mg ml^{-1}, 10 mM) almost completely inhibited TBARS formation during 20 h at 25°C. For the lower concentrations, the inhibition was around 50% for 2 mM, 70% for 4 mM and 85% for 8 mM carnosine. Percentage of inhibition was relatively stable over the first 6 h at 25°C, but was lower after 20 h for 2 and 4 mM carnosine. This suggests that the inhibition of TBARS formation by carnosine is partially overcome with time for the lowest carnosine concentrations.

To test the possible trapping of TBARS by carnosine, the peptide was added to liposomes previously oxidised for about 15 h using iron/ascorbate. Then TBARS were measured, over a 6 h storage period at 25°C, on oxidised liposomes (control) and oxidised liposomes to which carnosine was added (Fig. 4). In the control, TBARS continued to increase slightly whereas TBARS decreased with time in the presence of carnosine. When

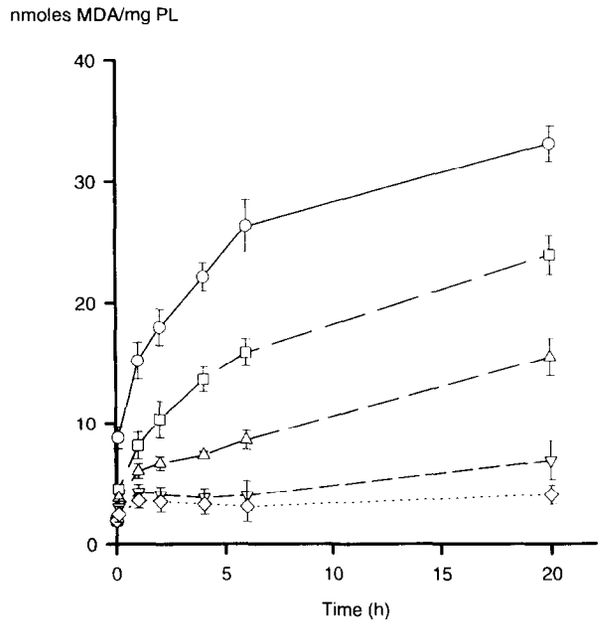

Fig. 3. Effect of carnosine on release of thiobarbituric acid reactive substances during Fe(III)/ascorbate (45 μM) induced oxidation of phospholipid liposomes (1 mg ml^{-1}) in PIPES solution (10 mM, pH 6.0, 0.15 M NaCl) at 25°C. ○, 0 mM carnosine; □, 2 mM carnosine; △, 4 mM carnosine; ▽, 8 mM carnosine; ◇, 10 mM carnosine. $n = 3$.

Table 2. Inhibition of thiobarbituric acid reactive substances (TBARS) release by carnosine during iron/ascorbate induced phospholipid oxidation

Carnosine (mM)	Inhibition of TBARS (%)						
	Time (h)						
	0.1	1	2	4	8	20	Mean
2	62	53	48	42	43	29	46[a]
4	72	70	71	73	73	56	69[b]
8	83	82	87	91	91	94	86[c]
10	92	88	90	93	95	93	91[d]
Mean	77[a]	75[a]	75[a]	74[a]	73[a]	66[b]	73

Experimental conditions as in Table 1.
TBARS values are the mean of three separate experiments in which measurements were performed in triplicate. Percentage of inhibition was calculated using the formula: % inhibition = $100[(1 - (X/X_0)]$, where X and X_0 are the TBARS (initial value of non-oxidized lipids subtracted) measured in the presence and absence of carnosine, respectively, at a given incubation time. Means in the same column or row with different superscripts are significantly different at the 5% threshold level. Some interactions between time and carnosine concentration were found.

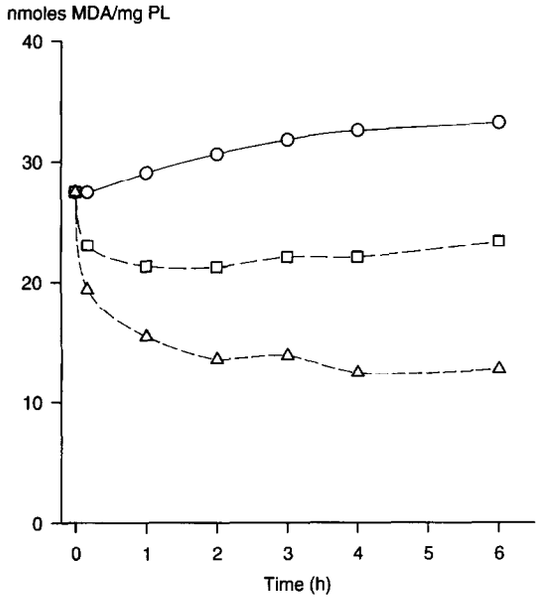

Fig. 4. Effect of carnosine added to previously oxidised phospholipid liposomes (1 mg ml^{-1}) on thiobarbituric acid reactive substances. ○, 0 mM carnosine; □, 4 mM carnosine; △, 10 mM carnosine. Results of an experiment performed in duplicate. All analyses were performed in triplicate.

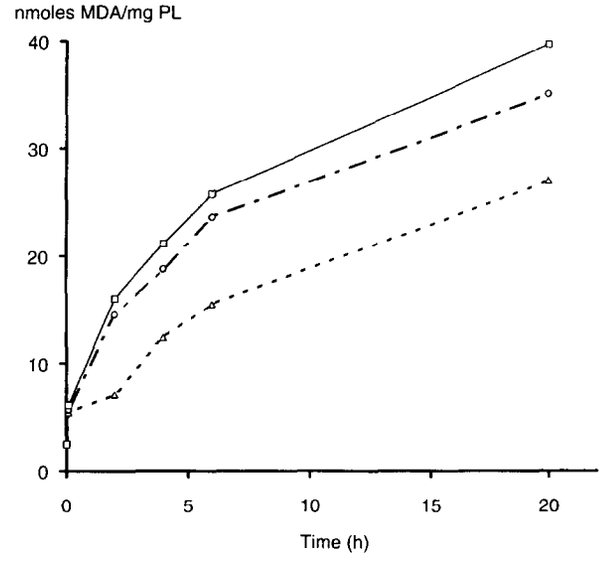

Fig. 5. Effect of pH on Fe(III)/ascorbate induced oxidation of phospholipid liposomes (1 mg ml^{-1}). □,: pH 6.0; ○, pH 6.5; △, pH 7.0. Results of an experiment performed in duplicate. All analyses were performed in triplicate.

the peptide concentration was 4 mM, the decrease was about 15% of the initial TBA value of oxidised lipids and remained stable with time. With 10 mM carnosine, the reduction of TBARS was initially around 25% but reached 50% after 4 h. These results suggest that some preformed TBARS react with carnosine. However, the reduction in TBARS observed in this experiment was less than that measured when carnosine was added to the liposomes before induction of lipid oxidation.

Since carnosine has been proposed to act as antioxidant by a buffering effect (Kohen *et al.*, 1988), the pH of the liposome + carnosine solutions was controlled. The pH of the solutions increased from 6.0 in the control to 6.4–7.0 in the presence of 2–10 mM carnosine and remained steady over the oxidation experiments. TBARS formation during phospholipid oxidation was studied between pH 6.0 and 7.0 (Fig. 5). At pH 7.0, TBARS formation showed a lag time which was not observed at lower pHs (Fig. 5) or with carnosine (Fig. 3). When compared to TBARS measured at pH 6.0, TBARS formation was inhibited by 10–12% at pH 6.5 and by 30–45% at pH 7.0. These values are lower compared to the 50% and 95% inhibition observed with 2 mM (pH 6.4) and 10 mM (pH 7.0) carnosine, respectively (Table 2). In addition, TBARS measured when the pHs of the different reaction mixtures were adjusted to 6.0 confirmed the inhibitory effect of carnosine towards TBARS (results not shown).

Volatile compounds

The main class of volatile compounds found after 24 h of Fe(III)/ascorbate induced oxidation at 25°C of phospholipid liposomes was saturated aldehydes (alkanals), which represented about 65% of the total volatile compounds (Table 3). The major compound in this class was hexanal, accounting for more than 45% of the total area of the chromatogram. It was followed by pentanal (8%).

The second class was 2-alkenals which represented around 15% of total volatiles. Their most representative compounds were 2-octenal and 2-heptenal (about 4% for both compounds).

1-Octen-3-ol, whose perception threshold is very low (around 30 p.p.b. in water), accounted for nearly 7% of total volatiles. 2,4-Alkadienals and alcohols represented respectively 4.6% and 5.7% of the total volatile compounds. The proportions of volatiles were stable from one experiment to another (average deviation less than 5%), whereas the total volatile amounts varied as much as three fold. The initial oxidation state of the lipids probably accounted for a large part of this variation.

When carnosine was added to liposomes before Fe(III)/ascorbate addition, the total amount of volatile compounds produced 24 h later was reduced by 62% as compared to the amount extracted from liposomes oxidised without the peptide (Fig. 6 and Table 3). Reduction due to the inclusion of 10 mM carnosine was recorded in alkanals (−44% to −66% of control, depending on the alkanal), 2-alkenals (−86% to −100% of control, except for *t*-2-decenal which was unaffected), 2,4-nonadienal (disappearance of total *t,c* isomer and loss of 78% of *t,t* isomer), hexanol (−89%), heptanol (−55%), 2-pentylfuran + 2,4-heptadienal (−73%).

When carnosine was added to oxidised liposomes, total volatiles remained stable (non-significant decrease), but there were some changes in amounts and therefore in the distribution of the volatile compounds (Table 3 and Fig. 6). On one hand relative amounts of *t*-2-undecenal and hexanol were 25% and 61% of the control, respectively. When expressed in percent of total volatiles, *t*-2-heptenal and *t*-2-octenal were halved.

Table 3. Effect of 10 mM carnosine on volatile compounds produced during Fe(III)/ascorbate induced oxidation of phospholipids at 25°C

Compound	Repartition (% total area)			Relative amounts (% control)		
	Oxidised liposomes (control)	Liposomes + 10 mM carnosine added at $t = 24\ h$	Oxidised liposomes + 10 mM carnosine added at $t = 24\ h$	Control	Liposomes + 10 mM carnosine added at $t = 0\ h$	Oxidised liposomes + 10 mM carnosine added at $t = 24\ h$
Pentanal	8.0	7.1 NS	8.0 NS	100	33.6 ***	96.8 NS
Hexanal	47.5	49.8 NS	53.2 NS	100	39.7 ***	107.0 NS
Heptanal	2.5	1.9 NS	1.8 NS	100	36.3 **	74.9 NS
Octanal	2.5	2.2 NS	1.8 NS	100	36.7 ***	73.5 NS
Nonanal	4.3	6.2 NS	3.6 NS	100	56.0 ***	77.2 NS
Alkanals	64.8	67.2 NS	68.4 NS	100	39.0 ***	99.8 NS
t-2-Pentenal	1.0	0.0 *	0.9 NS	100	0.0 ***	87.2 NS
t-2-Hexenal	2.5	0.0 NS	0.4 NS	100	0.0	44.6 NS
t-2-Heptenal	4.0	1.9 **	2.0 **	100	19.1 ***	47.1 NS
t-2-Octenal	4.3	2.2 **	2.0 **	100	18.7 ***	43.2 NS
t-2-Nonenal	0.7	0.3 NS	0.4 NS	100	14.4 ***	51.2 NS
t-2-Decenal	1.1	1.7 NS	0.9 NS	100	59.0 NS	76.1 NS
t-2-Undecenal	1.1	0.0 ***	0.3 ***	100	0.0 ***	24.5 ***
2-Alkenals	14.7	6.1 ***	6.8 ***	100	16.2 ***	45.0 **
t,t-2,4-Heptadienal	0.4	0.3 NS	0.5 NS	100	33.6 NS	132.1 NS
t,c-2,4-Nonadienal	0.8	0.0 **	0.3 **	100	0.0 ***	35.2 NS
t,t-2,4-Nonadienal	1.1	0.8 NS	0.7 NS	100	28.1 ***	53.8 NS
t,c-2,4-Decadienal	0.9	2.0 **	1.2 NS	100	93.5 NS	131.7 NS
t,t-2,4-Decadienal	1.3	4.4 **	2.2 NS	100	179.8 NS	215.1 NS
2,4-Alkadienals	4.6	7.6 *	4.9 NS	100	68.9 NS	107.0 NS
Pentanol	2.6	2.6 NS	5.0 NS	100	47.2 NS	170.3 NS
Hexanol	1.1	0.4 NS	0.8 NS	100	11.0 *	61.3 **
Heptanol	0.7	0.5 NS	0.7 NS	100	44.7 ***	113.0 NS
Octanol + 3,5-octadien-2-one	1.3	2.0 *	1.4 NS	100	65.3 NS	112.0 NS
Alcohols	5.7	5.6 NS	7.9 NS	100	40.7 NS	121.1 NS
2-Heptanone	0.4	0.0 NS	0.2 NS	100	0.0 NS	32.8 NS
1-Octen-3-ol	6.7	10.4 **	8.9 **	100	63.2 NS	133.0 NS
2-Pentylfuran + 2,4-heptadienal	2.5	1.8 NS	1.9 NS	100	27.1 ***	72.7 NS
3-Octen-2-one	0.6	1.4 **	0.9 NS	100	88.0 NS	162.2 NS
Miscellaneous	3.1	3.2 NS	2.8 NS	100	38.0 ***	86.7 NS
Total	100.0	100.0	100.0	100	38.0 ***	93.9 NS

Liposomes (control) and liposomes + 10 mM carnosine were stored for 24 h at 25°C in the presence of 45 μM Fe(III)/ascorbate (1:1, mol:mol), $n = 3$.
NS, not significantly different from control. Different from control at: *, 10% threshold; **, 5% threshold; ***, 1% threshold.

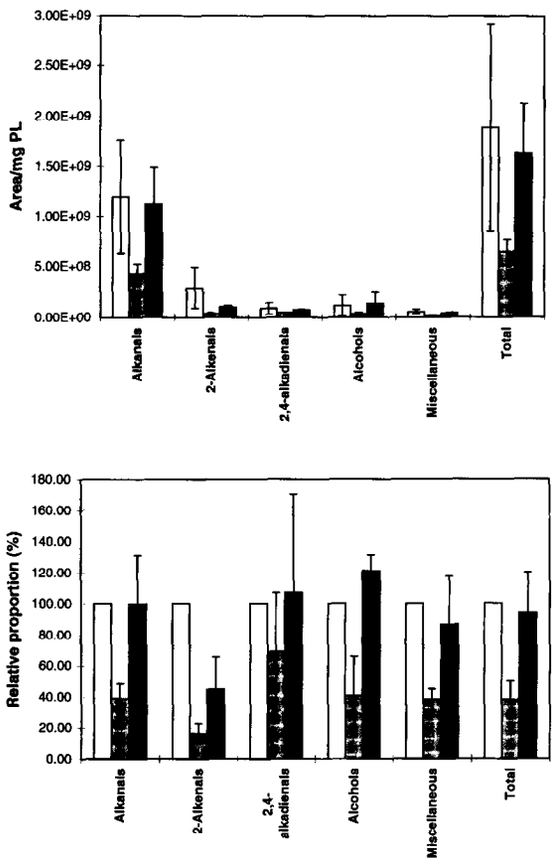

Fig. 6. Effect of carnosine (10 mM) on volatile compounds generated during Fe(III)/ascorbate induced oxidation of phospholipid liposomes. (A: top) Absolute quantities as detected by CG–MS (area mg^{-1} phospholipid); (B: bottom) relative proportions. no shading, liposomes (1 mg phospholipid ml^{-1}) oxidised for 24 h at 25°C with 45 μM iron/ascorbate (1:1, mol:mol) (control); light shading, liposomes + carnosine (10 mM) incubated as control; dark shading, control + carnosine (10 mM).

Total 2-alkenals were 45% of the control, due mainly to the decrease in *t*-2-undecenal. On the other hand, the percentage of 1-octen-3-ol increased from 6.7% to 8.9% of total volatiles when carnosine was added after 24 h of oxidation.

DISCUSSION

The multistage process of the free radical peroxidation of unsaturated lipids can be controlled at different levels from the generation of active oxygen species to the inactivation of lipid peroxidation products. Therefore, only complete kinetic curves provide reliable information about antioxidant effects. The methods we have chosen to test the activity of carnosine take into account the time course of lipid peroxidation. Since in meat and meat products free iron is one of the main pro-oxidants (Kanner *et al.*, 1988) and phospholipids are the main subtrates of lipid oxidation (Gandemer, 1990), the oxidation of muscle phospholipid liposomes was induced by the Fe(III)/ascorbate redox system. The initial pH of the medium (6.0) and its ionic strength (0.15 M NaCl) were chosen to be as near as possible to the conditions found in meat, taking into account the characteristics of PIPES, a buffer which does not inhibit lipid peroxidation (Boldyrev & Severin, 1990; Genot *et al.*, 1994).

Iron/ascorbate induced oxidation of muscle phospholipid liposomes

Fe(III)/ascorbate induced oxidation of muscle phospholipid liposomes was characterised by a rapid initial rate with no lag phase, as shown by oxygen uptake measurements (Fig. 1 and Table 1), and a rapid increase in conjugated dienes, conjugated trienes and ketodienes (Fig. 2) and TBARS (Fig. 3) after catalyst injection. The lag phase of lipid oxidation is absent when the ratio of Fe(II) to Fe(III) is close to one (Samokyszyn *et al.*, 1990), which is approached with the equimolar Fe(III)/ascorbate mixture, or when an iron-dependent breakdown of pre-existent hydroperoxides induces a very fast initial rate of lipid oxidation (Fukuzawa *et al.*, 1993; Wang *et al.*, 1994). The latter assumption is supported by results showing that the extent of oxygen consumption during Fe(III)/ascorbate oxidation of liposomes depends on the initial oxidation state of the lipids (Genot *et al.*, 1994; Wang *et al.*, 1994). Lower amounts of TBARS were produced when the pH was raised from 6.0 to 7.0, with the emergence of a lag period at pH 7.0 (Fig. 5). According to Grosch (1982), the iron-catalysed decomposition of hydroperoxides is markedly pH-dependent with a maximum activity in the range 5.0–5.5. Kawakatsu *et al.* (1984) attributed the effect of pH on iron-induced phospholipid oxidation to a stabilisation of ferrous ion in more acidic medium.

After this very rapid induction, phospholipid oxidation rapidly reached a steady-state phase where oxidation indices increased slowly (Figs 1–5 and Table 1).

The composition in volatile compounds arising from muscle phospholipid oxidation (Table 3) is roughly in accordance with previous works on the type of secondary products of peroxidation of PUFA (Grosch, 1982, 1987). Because pork phospholipids contain a large majority of $n-6$ PUFA, the formation of hexanal is favoured with respect to propanal. Pentane, which has often been taken as an index of oxidation, was not found. This finding is not related to the extraction method as we have identified pentane in other studies on meat and meat products using the same procedure (Meynier *et al.*, unpublished results). Compounds such as 1-octen-3-ol and 2-pentylfuran arise from the decomposition of 10-hydroperoxide octadecadienoic acid (10-HPOD) after a homolytic cleavage between C10 and C11 or from autoxidation of methyl arachidonate (Taylor & Mottram, 1990).

Effect of carnosine

When carnosine (2–10 mM) was added to the liposomes, oxygen uptake, conjugated dienes, conjugated trienes and ketodienes, TBARS and total volatile compounds were decreased as compared to the same

index measured without peptide. The decrease varied as a function of carnosine concentration, method of measurement and, to some extent, incubation time.

In contrast to oxygen uptake, V_{max} and K_{appl}, observed within the first seconds after Fe(III)/ascorbate addition, were significantly higher in the presence of carnosine, with no effect of peptide concentration (Table 1). Working on microsomes in pH 7.4 phosphate buffer, Aruoma *et al.* (1989) also observed an increase of the initial rate of oxygen consumption during Fe(III)/ascorbate induced peroxidation in the presence of 10 mM carnosine. They deduced from their data that carnosine stimulates lipid peroxidation. In our opinion, the decreases in oxygen uptake as soon as 1 min after catalyst injection (Fig. 1) demonstrates that carnosine exerts an antioxidant effect. This apparent discrepancy results, firstly, because of a lack of data concerning the oxygen uptake at longer incubation time in the study of Aruoma *et al.* (1989), and, secondly, because of the use of a microsomal system in which both autoxidation and enzymatic oxidation take place. In liposomes only non-enzymatic oxidation is involved.

A possible mechanism to explain the early effect of carnosine on oxygen consumption and on lipid oxidation is that the dipeptide interferes with the production of free radicals by the Fe(III)/ascorbate redox system. The solubility of the peptide allows it to partially inactivate the Fe(III)/ascorbate system dissolved in the aqueous medium. In support of this contention, the inhibitory effect of carnosine was reduced when Fe(III)/ascorbate induced oxidation was performed on muscle phosphatidylethanolamine (Kansci *et al.*, 1994). In that system, the iron was chelated by phosphatidylethanolamine and therefore the initiating free radicals were generated in the lipid hydrophobic core, which was not accessible to the dipeptide. The interaction between carnosine and the Fe(III)/ascorbate system can also modify the oxidation rate of ascorbate, and thus increase K_{appl} and V_{max}. In this way, Decker *et al.* (1992) found that low concentrations of carnosine (0.05–0.1 mM) slightly accelerated ascorbate oxidation by copper and iron, but no effect was found for higher concentrations.

In addition to the above mechanism, the buffering effect of the peptide, which leads to an increase of the pH of the medium, diminishes the efficiency of iron in producing free radicals and consequently lipid oxidation (Fig. 5).

Carnosine decreased the rate of oxygen consumption 1 min after Fe(III)/ascorbate addition and had no effect on later rates. This shows that the peptide weakly interferes in the propagation steps of oxidation involving oxygen uptake.

Carnosine (10 mM) led to a 24–50% reduction in the formation of conjugated dienes as function of time (Fig. 2). The 24% value, obtained a few minutes after Fe(III)/ascorbate addition, agrees with the 22% inhibition of oxygen uptake obtained with the same carnosine concentration (Fig. 1). Since the rates of oxygen uptake 5 min and later after catalyst injection was similar whatever the carnosine concentration (Table 1), one can assume that the rate of hydroperoxide appearance was constant. Thus, the inhibition of conjugated dienes should have been constant with time. As this was not the case, the enhanced inhibition of conjugated dienes could be due to reaction of preformed hydroperoxides with the dipeptide. Carnosine has been assumed to react with primary products of oxidation (dienes, ketodienes) (Boldyrev *et al.*, 1989; Boldyrev & Severin, 1990). These authors found changes in the UV spectra we did not observe. This difference can be explained by the procedure used to record the spectra in both studies. Boldyrev and co-workers performed difference spectra on the lipid + carnosine mixtures whereas we analysed the spectra of the re-extracted phospholipids. Therefore, in our study, interaction products of carnosine and oxidising lipids, if any, were possibly removed in the aqueous layer of Folch mixture.

Kohen *et al.* (1988), on the basis of the comparison of antioxidant activities of histidine- and alanine-containing peptides, concluded that the His residue of carnosine is able to donate a proton which inactivates peroxy radicals. As the ratio between *t,t* and *c,t* isomers of hydroperoxides produced during lipid peroxidation depends on the hydrogen donor concentration (Porter *et al.*, 1980), we tested the ability of carnosine to be a hydrogen donor using second derivative UV spectra of lipids. No significant difference was found in the relative heights of the minima corresponding to *t,t* and *c,t* isomers of hydroperoxides. Therefore, no evidence for carnosine to act as a hydrogen donor was obtained in this study.

The TBA assay measures some secondary products of oxidation, such as malonaldehyde, as well as 2,4-decadienals and, to a lesser extent, saturated aldehydes (Hoyland & Taylor, 1991). The results obtained (Fig. 3 and Table 2) show an inhibitory effect of carnosine on TBARS release. The inhibition of TBARS formation was very high compared to the values obtained for oxygen consumption and conjugated dienes measurements. For instance, the presence of 10 mM carnosine led to an almost complete inhibition (95%) of TBARS. This marked inhibition could be the result of different phenomena.

Firstly, the addition of the peptide leads to a shift of the pH towards less acidic values, reducing lipid oxidation as previously described. The buffering effect, however, only explains a part of the inhibitory effect of carnosine on TBARS formation.

Secondly, as carnosine has been assumed to interfere with free radical generation in the first step of oxidation and later to react with hydroperoxides, one would expect less TBARS production from peroxide breakdown.

Thirdly, according to Aruoma *et al.* (1989), carnosine could interfere with the TBA test. Trichloroacetic acid was used in this assay to minimise interference as suggested by these authors. However, when carnosine was added to oxidised liposomes, the TBA value measured a few minutes after peptide addition was lowered by 25% for 10 mM carnosine compared with that obtained for

the control (Fig. 4). Moreover, it continued to decrease some hours later. This result confirms previous observations showing that the addition of carnosine to oxidised liposomes decreases indices of lipid oxidation (Boldyrev *et al.*, 1988; Boldyrev & Severin, 1990; Aruoma *et al.*, 1989). Thus, more than an interference with the TBA test, one can conclude that carnosine prevents the reaction between some compounds and TBA. To explain this phenomenon, some TBARS may be trapped or react with carnosine to form TBA unreactive adducts. Various oxidation products may be involved. Firstly, as previously stated, carnosine can react with preformed hydroperoxides which would otherwise decompose into TBARS. Secondly, the amino acid residues of the dipeptide (βAla-His) are able to form addition derivatives with malonaldehyde (Nair *et al.*, 1981) or with other reactive secondary products of lipid oxidation (Grosch, 1982). In agreement with this last assumption, a significant decrease of 2-alkenals, *t*-2-undecenal particularly, was observed when carnosine was added to oxidised lipids (Table 3 and Fig. 6A).

All these quantitative and qualitative changes in volatile compounds must lead to changes in the odour of the model systems. Consequently, all the members of the panel described the 24 h oxidised liposomes as very rancid. The oxidised liposomes with 10 mM carnosine added after oxidation were found to be weakly rancid and the perceived odour was even qualified as 'not bad'. The 'burned rubber' term was employed once for the latter sample. Finally, no rancid flavour was found in the liposomes oxidised with 10 mM carnosine, 'plastic' or 'burned-rubber' odours being mentioned twice for this sample. These results are in good agreement with the analysis of volatile compounds performed on the same samples and showed the value of carnosine in the improvement of the flavour of food products as proposed by Decker & Crum (1991, 1993).

In conclusion, inhibition of oxygen consumption, conjugated dienes, TBARS and total volatile compound formation by carnosine demonstrates that the peptide actually inhibits iron-induced peroxidation of muscle phospholipids. Besides its buffering effect, carnosine has a multifunctional effect on lipid oxidation: it interferes in the initiation step of oxidation; it decreases the amount of preformed peroxides; it reacts with some reactive secondary products, among them 2-alkenals. The selective effect of carnosine on volatile compound production leads us to predict a considerable improvement in the flavour of meat products through the use of this dipeptide.

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