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The influence of microsomal and cytosolic components on the oxidation of myoglobin and lipid *in vitro*

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Muscle microsomes were isolated from beef, pork and tuna and combined with oxymyoglobin to study oxymyoglobin and lipid oxidation interactions. Tuna muscle microsomes contained higher concentrations of long chain fatty acids and polyunsaturated fatty acids, and a lower α-tocopherol content than microsomes from the other two species ($P < 0.05$). Oxymyoglobin and lipid oxidation were greater in tuna followed by pork and beef ($P < 0.05$). The influence of high (HMW) and low molecular weight (LMW) cytosolic fractions from muscles of these species on oxymyoglobin and lipid oxidation was studied in an oxymyoglobin–phosphatidylcholine-liposome system. In each species, the LMW fraction resulted in greater OxyMb and lipid oxidation than the HMW fraction ($P < 0.05$). Within a given fraction type, there was no difference between species ($P < 0.05$). In lamb liver oxymyoglobin–microsomes, oxymyoglobin and lipid oxidation were delayed with increased microsomal α-tocopherol content ($P < 0.05$). These results suggest that differences in oxymyoglobin and lipid oxidation *in vitro* were more strongly influenced by oxidative stability of membrane components rather than cytosolic components.

INTRODUCTION

The oxidation of both oxymyoglobin (OxyMb) and lipid affects meat product quality (Faustman & Cassens, 1990) and the two processes appear to be related (Yin & Faustman, 1993). Both membrane and cytosol components appear to influence lipid oxidation in muscle foods (Borhan *et al.*, 1990; Anton *et al.*, 1991). However, the relative effects of membrane and cytosol components on OxyMb oxidation in the presence of lipid has received less attention.

Cellular and subcellular membranes are particularly susceptible to lipid oxidation due to their relatively high concentration of polyunsaturated fatty acids (PUFAs) and close proximity to oxygen, transition metals and peroxidase enzymes (Vladimirov *et al.*, 1980). The high concentration of muscle membrane PUFAs in fish muscle appears responsible for the faster lipid oxidation rate observed in this species (Khayat & Schwall, 1983). In muscle, α-tocopherol is located close to membrane-bound enzymes (e.g. NADPH oxidase) that generate free radicals; it acts to protect membrane lipids by scavenging free radicals (Machlin, 1984). Meat with a higher concentration of α-tocopherol is more resistant to OxyMb and lipid oxidation (Faustman *et al.*, 1989; Asghar *et al.*, 1990; Arnold *et al.*, 1993).

Microsomes are useful for studying membrane-related processes as they represent small authentic versions of cell membranes still capable of biochemical function (Albert *et al.*, 1989). Although Anton *et al.* (1991) obtained microsomes from beef muscles of differing color stability and incubated these with myoglobin, no differences in myoglobin oxidation were observed. The influence of membrane lipid components such as fatty acids and α-tocopherol on OxyMb and lipid oxidation has been studied *in vitro* using a liposome model (Yin & Faustman, 1993; Yin *et al.*, 1993). While useful as a model approach, the liposome system may not truly represent membrane-related activity *in vivo* as it is highly controlled. That is, relative concentrations of fatty acids and α-tocopherol are not necessarily representative of those *in vivo*; in addition, membrane-bound proteins are absent. A reasonable approach for studying the effect of these lipid components on OxyMb and lipid oxidation would be to use a microsome–myoglobin system.

The cytosolic fraction of muscle tissues contains antioxidant and prooxidant enzymes, H_2O_2, metal ions, reducing compounds and chelating agents which may affect the overall catalysis of muscle lipid oxidation (Borhan *et al.*, 1990). The cytosolic fraction can be

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divided into a low (LMW) and a high molecular weight (HMW) fraction. Kanner *et al.* (1991) reported that H_2O_2-activated metmyoglobin (MetMb) dependent lipid oxidation was inhibited by both LMW and HMW compounds. However, Seman *et al.* (1991) reported that while iron-stimulated lipid oxidation was inhibited partially by the HMW fraction, it was accelerated by the LMW fraction. The LMW fraction contains most of the reducing compounds such as ascorbic acid and free iron ions (Kanner *et al.*, 1991) which are believed to accelerate lipid oxidation (Decker *et al.*, 1993). The HMW fraction contains chelators and antioxidant enzymes, especially catalase, which can inhibit lipid oxidation (Han & Liston, 1989). Since cytosolic components influence lipid oxidation, they may also affect OxyMb oxidation since lipid oxidation and OxyMb oxidation appear to be coupled (Yin *et al.*, 1993). The objective of this research was to study and compare the influence of lipid and cytosolic components on OxyMb and lipid oxidation. Lipid components were studied using an OxyMb–microsome system, while cytosolic components were studied in an OxyMb–liposome system.

MATERIALS AND METHODS

Muscle oxymyoglobin–microsome preparation

Microsomes were prepared according to the method of Schenkman and Cinti (1978). Beef and pork gluteus medius muscles and bluefin tuna steak (n = 3) were purchased from a local grocery store. Three replicate microsomal fractions were prepared from each muscle. Muscle (100 g) was minced with scissors and homogenized in three volumes of sucrose buffer (250 mM sucrose–10 mM Tris–HCl, pH 7·4) using a Teflon and glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 600 *g* for 5 min. The supernatant was removed and centrifuged at 12000 *g* for 10 min. Calcium chloride ($CaCl_2$) was added to the resulting supernatant to a final concentration of 8 mM and centrifuged at 25000 *g* for 15 min. The resulting microsomal pellet was resuspended in 150 mM KCl–10 mM Tris–HCl (pH 7·4) and followed by a final centrifugation at 25000 *g* for 15 min. The washed microsomal pellet was resuspended in sodium citrate buffer, pH 5·6. Microsomal protein concentration was determined by the modified Lowry procedure (Markwell *et al.*, 1978). In the reaction solution, muscle microsomal protein concentration was adjusted to 0·7 mg/ml. Oxymyoglobin was prepared as described by Yin and Faustman (1993) and adjusted to a final concentration of 2·5 mg/ml in the microsome-containing solution.

Liver oxymyoglobin–microsome preparation

Livers were obtained from lambs supplemented with (2000 IU/day, n = 6) or without (20 IU/day, n = 6) α-tocopheryl acetate in the diet. Liver (50 g) was used to prepare microsomes according to the above procedure. Three replicate microsomal fractions were prepared from each liver. Liver microsomal protein concentration was adjusted to 1 mg/ml (Kanner *et al.*, 1986) for the OxyMb–microsome preparation.

α-Tocopherol and fatty acid analysis

The α-tocopherol content of microsomes was analyzed by the procedure of Lang *et al.* (1992). Microsomes (1 ml) were combined with 0·5 ml hexane, mixed vigorously, and the clear hexane layer collected; the procedure was repeated a total of three times. The collected hexane layer was dried by nitrogen, and followed by dissolving in 1·5 ml hexane. The resulting α-tocopherol extraction (25 μl) was analyzed by reverse phase high performance liquid chromatography with methanol/methylene chloride (70:30, v/v), as mobile phase. Fatty acid composition of liver and muscle microsomes was determined by gas chromatography (LePage & Roy, 1986).

Cytosolic fraction preparation

Cytosolic fractions were prepared according to Kanner *et al.* (1991). Muscle tissue (10 g; beef, pork or tuna) was minced in a blender for 30 s. The minced tissues were further homogenized in 40 ml 0·05 M sodium citrate buffer (pH 5·6) with a Teflon and glass Potter–Elvehjem homogenizer. The homogenate was centrifuged for 30 min at 100000*g* at 4°C. The resulting supernatant was used as the soluble cytosolic extract. Separation of the cytosolic fraction into low and high molecular weight fractions was accomplished with an Amicon Model 12 instrument, using a molecular weight cutoff membrane of 10000 with nitrogen flush at 30 psi.

Oxymyoglobin–liposome preparation

Both LMW and HMW fractions were used to prepare OxyMb–liposomes as described by Yin and Faustman (1993). Native myoglobin (Mb), a component of the HMW fraction, was highest in beef followed by pork and tuna. The Mb concentrations of all fractions (HMW and LMW fractions) were adjusted to be equivalent to that in the pork HMW fraction by adding 55 μl of 38 mg/ml concentrated OxyMb solution to 10 ml of the tuna HMW fraction; and adding 165 μl of 110 mg/ml OxyMb solution to all LMW fractions. Sodium citrate buffer (0·05 M, 165 μl) was added into 10 ml beef HMW fraction to obtain the desired Mb concentration. The maximum dilution for these manipulations was 1·6%.

OxyMb and lipid oxidation measurements

OxyMb and lipid oxidation of OxyMb–microsomes and OxyMb–liposomes were measured as described by Yin and Faustman (1993). Oxymyoglobin oxidation

was measured spectrophotometrically from 650 to 500 nm by use of a diffuse-integrating sphere; per cent metmyoglobin was determined by the method of Krzywicki (1982). Lipid oxidation was measured by the thiobarbituric acid assay (TBA assay) of Schmedes and Holmer (1989).

Statistical analysis

Data were treated by analysis of variance (ANOVA) and computed by using the SAS General Linear Model (GLM) procedure (SAS, 1985). Differences among means at the 5% level were determined by the least significant difference test (Steel & Torrie, 1980).

RESULTS AND DISCUSSION

The influence of muscle microsomal components from different species on OxyMb and lipid oxidation are presented in Fig. 1(A) and (B), respectively. Both OxyMb and lipid oxidation of muscle microsomes followed the order tuna > pork > beef ($P < 0.05$). The α-tocopherol content and fatty acid composition of these muscle microsomes are presented in Table 1. The α-tocopherol content of muscle microsomes followed the order beef > pork > tuna ($P < 0.05$), which is the reverse of that observed for oxidation. The PUFA content of muscles microsomes followed the order tuna > pork > beef ($P < 0.05$), which is in the same order as OxyMb and lipid oxidation of muscle microsomes.

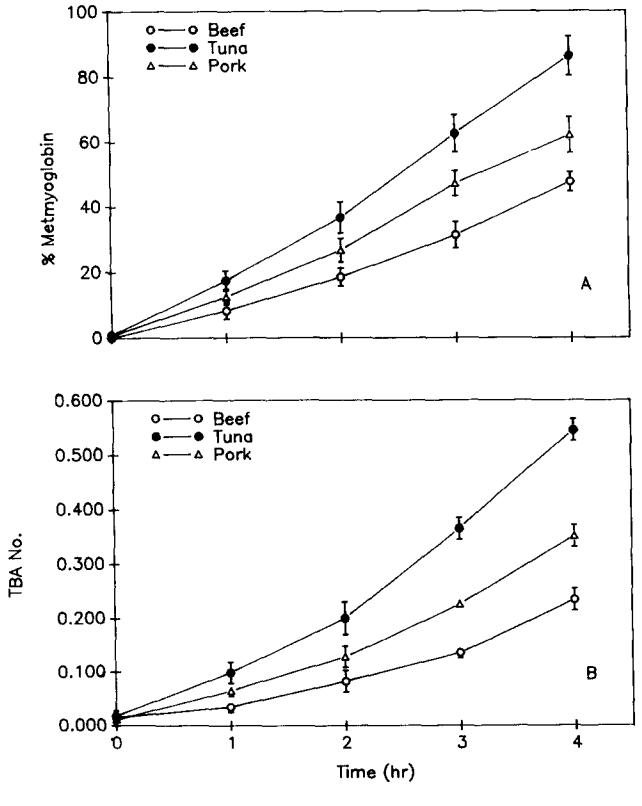

Fig. 1. Metmyoglobin formation (%) (A) and lipid oxidation (TBA) (B) in muscle OxyMb–microsomes during incubation at pH 5·6, 25°C. Standard deviation bars are indicated ($n = 3$).

Table 1. α-Tocopherol content and fatty acid composition (%) of muscle microsomes[a]

	Muscle microsomes		
	Beef	Pork	Tuna
α-Tocopherol[b]	11·6c ± 0·15	6·2b ± 0·21	4·4a ± 0·32
Fatty acid			
14:0	1·5	1·1	1·0
14:1	4·6	2·5	4·9
16:0	23·0	20·6	19·3
16:1	5·2	3·1	3·2
18:0	9·8	12·3	9·4
18:1	35·7	34·5	20·5
18:2	14·1	16·6	2·1
18:3	0·5	1·0	1·4
20:1	—	—	1·3
20:4	3·6	5·8	5·0
20:5	—	—	3·4
22:4	—	—	2·8
22:5	—	—	2·0
22:6	—	—	21·2
SAT	34·3b	34·0b	29·7a
MONO	45·5c	40·1b	29·9a
POLY	18·2a	23·4b	37·9c

SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; POLY, total polyunsaturated fatty acids.
abc, values in rows with different superscripts are different ($P < 0.05$).
[a] $n = 3$ muscles from each of beef, pork and tuna.
[b] μg/g microsomal proteins.

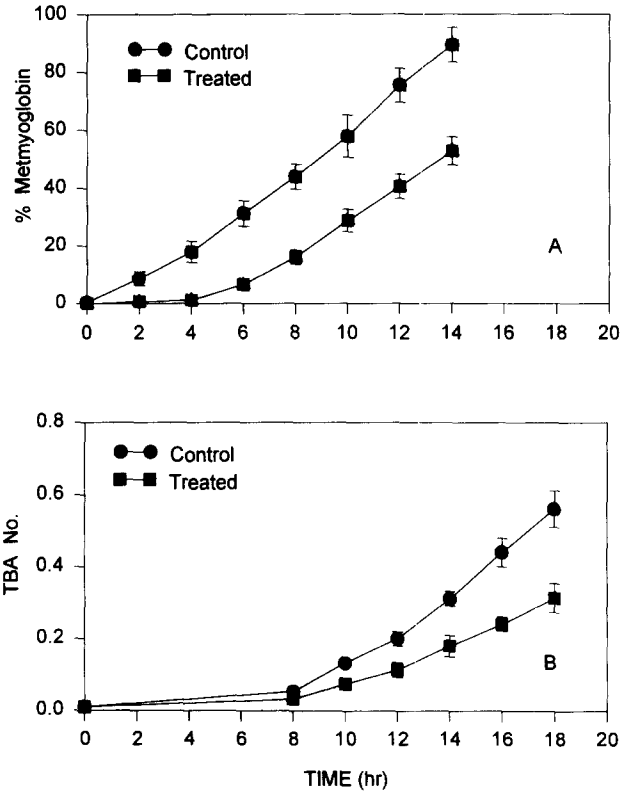

Fig. 2. Metmyoglobin formation (%) (A) and lipid oxidation (TBA) (B) in lamb liver OxyMb–microsomes during incubation at pH 5·6, 25°C. Microsomes were isolated from lambs on vitamin E-supplemented (treated) or non-supplemented (control) diets. Standard deviation bars are indicated ($n = 6$).

The influence of liver microsomal α-tocopherol on OxyMb and lipid oxidation are presented in Figs 2(A) and 2(B), respectively. The relationship between liver microsomal α-tocopherol concentration, OxyMb and lipid oxidation is presented in Figs 3(A) and 3(B), respectively. OxyMb and lipid oxidation were delayed by increased microsomal α-tocopherol content ($P<0.05$). The α-tocopherol content and fatty acid composition of liver microsomes are presented in Table 2. Liver microsomes from α-tocopherol-supplemented lambs contained higher concentrations of α-tocopherol than those from control animals. There was no difference in fatty acid composition of liver microsomes between treated and control animals ($P<0.05$). Thus, it appeared that the increased oxidative stability of both OxyMb and lipid in treated microsomes was due to greater content of α-tocopherol.

Dietary supplementation of α-tocopherol in animal diets has been demonstrated to delay lipid oxidation in meat-producing livestock (Faustman, 1993). In addition, dietary supplementation of α-tocopherol has also resulted in elevated α-tocopherol concentration in microsomal and mitochondrial membranes (Asghar *et al.*, 1991; Arnold *et al.*, 1993). Yin *et al.* (1993) reported that incorporation of α-tocopherol into phosphatidylcholine–myoglobin liposomes significantly delayed both OxyMb and phospholipid (PL) oxidation. The protective effect of the lipid-soluble antioxidant, α-tocopherol, toward water-soluble OxyMb in both the liposome and microsome model systems may be due to direct scavenging of free radicals produced in the membrane lipid, which resulted in the subsequent protection of OxyMb against free radical-initiated oxidation in the aqueous phase.

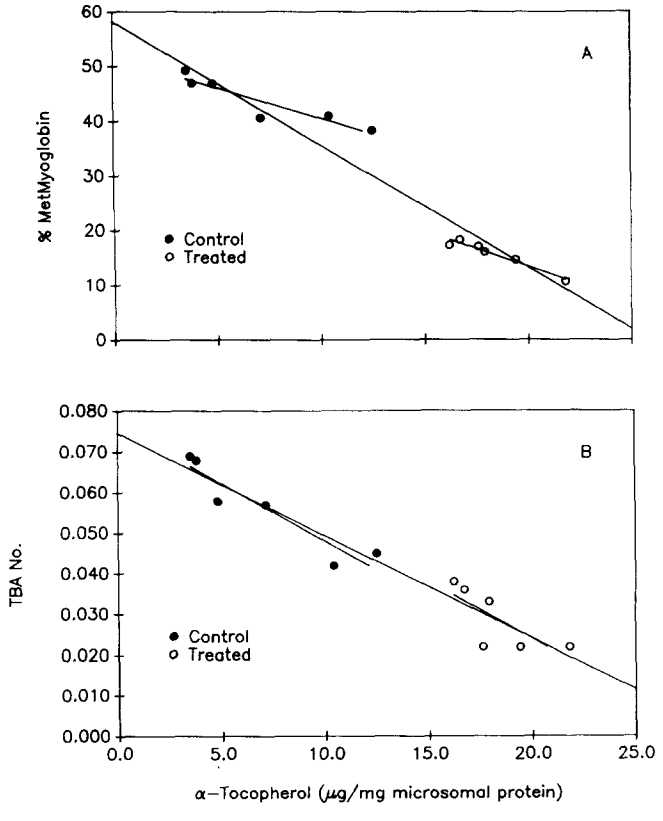

Fig. 3. Correlation between microsomal α-tocopherol content and metmyoglobin formation (A) and lipid oxidation (TBA) (B) in lamb liver OxyMb–microsomes at 8 h incubation (25°C). Microsomes were isolated from lambs on vitamin E-supplemented (treated) or non-supplemented (control) diets. First-order regression lines are indicated within each treatment and for both treatments combined (full graph).

Table 2. α-Tocopherol content and fatty acid composition (%) of liver microsomes[a]

	Liver microsomes	
	Control	Treated
α-Tocopherol[b]	7·1a ± 2·2	18·3b ± 2·6
Fatty acid		
14:0	0·5	0·7
14:1	0·4	0·4
16:0	13·6	14·6
16:1	1·6	2·1
18:0	23·8	22·9
18:1	21·7	23·1
18:2	14·6	12·6
18:3	1·6	1·5
20:1	1·7	1·2
20:4	11·2	10·8
20:5	0·6	0·7
22:4	1·4	1·7
22:5	2·3	2·4
22:6	2·8	2·8
SAT	37·9a	38·2a
MONO	25·4a	26·8a
POLY	34·5a	32·5a

SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; POLY, total polyunsaturated fatty acids.
abc, values in rows with different superscripts are different ($P<0.05$).
[a] $n = 6$ livers from each of control and treated lambs.
[b] μg/mg microsomal proteins.

The oxidation of microsomal and mitochondrial PLs has been reported to be responsible for the development of flavor deterioration in meat (Pearson *et al.*, 1977; Asghar *et al.*, 1990). PUFAs have been indicated to be the major substrates in oxidative deterioration of muscle PLs (Kanner *et al.*, 1987). Yin and Faustman (1993) reported that higher concentrations of PUFAs and long chain fatty acids (>18°C) resulted in faster OxyMb and PL oxidation rates in OxyMb–liposomes. In the present study, tuna muscle microsomes demonstrated the greatest extent of OxyMb and lipid oxidation. This was apparently due to lower α-tocopherol content and higher concentrations of PUFAs and long chain fatty acids in tuna than in beef and pork (Table 1). The content of long chain fatty acids was not different between beef and pork ($P>0.05$). However, beef muscle microsomes had higher α-tocopherol content and lower PUFA content than pork muscle microsomes which favored the greater stability of OxyMb and membrane lipid of beef muscle microsomes ($P<0.05$). One potential explanation for the observed differences in oxidation would be that pre-existing lipid peroxides in the different muscle foods were

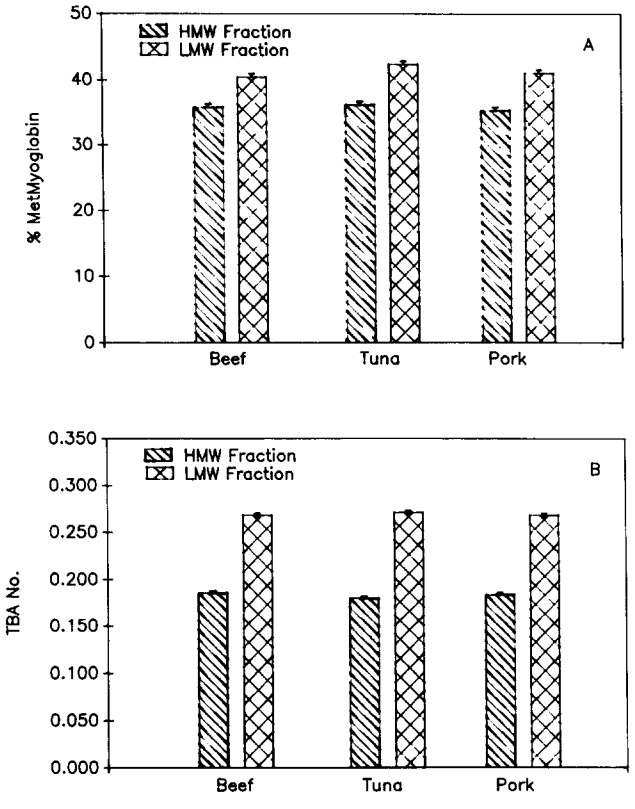

Fig. 4. Effect of high (HMW) and low molecular weight (LMW) cytosolic fractions from muscle on metmyoglobin formation (%) (A) and lipid oxidation (TBA) (B) in OxyMb–phosphatidylcholine-liposomes during incubation at pH 5·6, 25°C. Bars present LSMEANS; standard error bars are indicated (n = 3).

responsible. The history of handling and storage of these prior to acquisition is unknown, nor were peroxide values determined. However, the proposed hypothesis is still consistent with the observed differences in fatty acid profiles; fatty acid peroxides would not be detected by this methodology.

The influence of muscle cytosolic fractions on OxyMb and lipid oxidation is presented in Figs 4(A) and 4(B), respectively. For each species, the LMW fraction resulted in greater OxyMb and lipid oxidation than the HMW fraction ($P<0·05$). However, within a fraction type ($P>0·05$), there was no difference between species for OxyMb and lipid oxidation.

Lipid oxidation in muscle foods can be initiated by free iron ions, especially ferrous iron, and hydrogen peroxide (Kanner & Harel, 1985). Ferrous ions propagate lipid oxidation by breakdown of hydroperoxides to free radicals. The inhibitory effect of the HMW fraction toward lipid oxidation stimulated by the iron–redox cycle is derived from the capability of this fraction to chelate iron ions and prevent their penetration into the membrane (Harel & Kanner, 1985). Iron ions in the HMW fraction are associated primarily with proteins such as hemoglobin (Hb), Mb, ferritin and hemosiderin (Hazell, 1982) and as such are less catalytically active than when free. The HMW fraction contains enzymes such as superoxide dismutase (SOD), catalase and glutathione-*S*-transferase which are believed to provide a protective effect toward membrane lipids (Williamson & Ball, 1988; Seman *et al.*, 1991). The addition of SOD to myoglobin liposomes resulted in reduced oxidation of both pigment and lipid (Yin & Faustman, 1994). The LMW contains most of the reducing compounds capable of releasing iron from iron storage proteins such as ferritin and Mb, which would accelerate the oxidative process (Halliwell & Gutteridge, 1986). In the present study, the greater OxyMb and microsomal lipid oxidation observed with muscle LMW fractions likely resulted from the lower content of antioxidant enzymes, and higher content of reducing compounds and catalytic iron than in HMW fractions. The extent to which differences observed between the HMW and LMW are applicable to other oxidative interactions is not clear. It may be that the HMW fraction was more effective than the LMW because of the specificity of HMW antioxidants towards pro-oxidant species generated in the Mb–liposome system.

The extent of oxidation that occurs in postmortem muscle may be determined by the balance of prooxidants and antioxidants (endogenous and exogenous) in the tissue. In this study, a myoglobin–microsome model was used to characterize the role of membrane components on OxyMb and lipid oxidation, while a liposome model was used to characterize the influence of cytosolic fractions on OxyMb and lipid oxidation. Results of this study demonstrated that microsomal components (i.e. fatty acids, α-tocopherol) play a determinant role in OxyMb and lipid oxidation rate *in vitro*.

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