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Effect of Water-soluble Haem and Non-haem Iron Complexes on Lipid Oxidation of Heated Muscle Systems

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ABSTRACT

The distribution of soluble iron between three main components—ferritin, haemoglobin plus myoglobin and a low molecular weight fraction—and the pro-oxidant activities of each fraction in heated water-washed muscle systems from beef, pork and mackerel was determined. Over 75% of soluble iron was associated with the haem fraction. Even so, in all meat systems, the low molecular weight fraction was the major catalyst for lipid oxidation. Ferritin contributed significantly to lipid oxidation in all systems. The addition of nitrite significantly inhibited oxidation in all systems.

INTRODUCTION

It has been generally accepted that haem compounds, especially met-myoglobin and methaemoglobin, are major catalysts of lipid oxidation in meat (Tappel, 1962; Greene, 1971). A number of workers (Wills, 1966; Liu & Watts, 1970) have attempted to assess the relative importance of haem and non-haem iron as catalysts of lipid oxidation in various animal tissues and concluded that both haem and non-haem iron have catalytic activity in raw and cooked systems. On the other hand, Sato & Hegarty (1971) and Love & Pearson (1974) presented evidence that non-haem iron, rather than haem iron, was involved in the oxidation of meat. Igene *et al.* (1979) also concluded that myoglobin *per se* is not the principal pro-oxidant in cooked meats. According to these authors, cooking destroys the haem pigments and releases a significant amount of non-haem iron from bound haem pigments

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and provides a source of free iron which accelerates lipid oxidation in cooked meats. Studies by Tichivangana & Morrissey (1985) showed that both metal ions and haem pigments catalysed lipid oxidation in various muscle systems. However, non-haem iron is the major pro-oxidant. In a recent study (Apte & Morrissey, 1987) the role of haem (haemoglobin) and non-haem (ferritin) iron complexes, as catalysts of lipid oxidation when added exogenously to model systems, was examined. It is evident from the results that haemoglobin was catalytic in both raw and heated systems, while ferritin exhibited pro-oxidant properties only in heated systems or in systems where iron was released by the action of ascorbic acid.

While the studies of Tichivangana & Morrissey (1985) and Apte & Morrissey (1987) helped to elucidate the role of haem and non-haem iron in lipid oxidation in various muscle systems, they failed to establish the relative importance of the various forms of iron, as present in muscle, to lipid oxidation.

The major part of iron in the human body is present as haem complexes in haemoglobin, myoglobin and many haem-containing enzymes and several iron-containing non-haem enzymes; also storage complexes such as ferritin and haemosiderin are present in tissue (Hallberg, 1982). However, in some species, e.g. chicken, the major part of body iron is present as haemosiderin (Hazell, 1982). Hazell (1982) used gel filtration to separate the soluble iron from beef, lamb, pork and chicken into four main components—ferritin, haemoglobin, myoglobin and low molecular weight compounds ($<12\,000$)—and observed that there are species differences in the way in which iron is distributed between the various fractions. Using the fractionation procedures of Hazell (1982), it may be possible to establish the relative contributions of the various iron fractions to lipid oxidation at the levels in which they exist in muscles. Thus, the present study was designed to fractionate and estimate the amount of iron present in the haem and non-haem fractions of beef, pork and fish, and to assess the relative contributions of each fraction to lipid oxidation in cooked muscle systems. In addition, the influence of nitrite and ascorbic acid on the catalytic activity of these iron fractions was investigated.

MATERIALS AND METHODS

Muscle samples

Beef (rump steak), pork (loin chops) and mackerel were purchased fresh from local meat and fish markets and were processed immediately on arrival at the laboratory.

Preparation of water-washed muscle fibres (WF)

Muscle samples were trimmed of extramuscular fat, minced and extracted with deionized water, as described by Tichivangana & Morrissey (1984).

Preparation and treatment of model systems

Various pro-oxidants were added to the WF system. A sample (~50 g) of extracted tissue was mixed thoroughly with the appropriate pro-oxidant or other reagents.

Reagents

All chemicals used were of 'AnalaR' grade obtained from British Drug Houses, Poole, Dorset, Great Britain, and Sigma Chemical Co. Ltd, Poole, Dorset, Great Britain.

Extraction of water-soluble iron-containing fractions

Samples (200 g wet weight) of lean muscle were cut into small pieces with a stainless steel scalpel blade, mixed with 200 ml of distilled water and homogenized in a Waring blender at medium speed for 2 min. The homogenates were centrifuged at 4000 *g* for 20 min. Supernatants were recovered, the residues were rehomogenized with another 200 ml of distilled water, recentrifuged and the supernatants again recovered. A total of four extractions were carried out. The pooled supernatant fractions were concentrated in a Busch Rotavapor-R evaporator before subsequent analysis.

Fractionation of iron-containing water-soluble components of muscle

A modification of the procedure outlined by Hazell (1982) was used for separation of soluble iron compounds in meat. Sephadex G-75 (column height, 125 cm; width, 2·5 cm; flow rate, ~30 ml h^{-1}; Pharmacia Fine Chemicals, Uppsala, Sweden) was used. Sephadex G-75 was considered appropriate for this particular study as it gave a rapid throughput and it resolved ferritin from the lower molecular weight fractions. The columns were eluted with distilled water. Fractions (~3 ml) were collected and analysed for iron by atomic absorption spectroscopy using a Pye Unicam SP-9 spectrophotometer. The fractions were pooled and fraction two, which contained haemoglobin, myoglobin and low molecular weight fractions, was dialysed against distilled water in the ratio 1:5 at 4°C for 24 h using Visking

tubing. Retentates and diffusates and the ferritin fractions were concentrated by rotary evaporation to a final volume of 25 ml at 25°C. The iron content of each fraction was determined by atomic absorption spectroscopy.

Method of cooking

Samples to be cooked were placed in 30 × 18 cm retortable bags (Seaward Laboratories, London) and placed in a hot-water bath and cooked to an internal temperature at 70°C for 30 min. Following cooking, the samples were cooled and immediately assessed for lipid oxidation. Portions were stored at 4°C for 48 h, after which the level of oxidation was again determined.

Measurement of lipid oxidation

Lipid oxidation was assessed by the 2-thiobarbituric acid (TBA) method of Ke *et al.* (1977). Sulfanilimide was added to all samples containing nitrite, to eliminate residual nitrite, by formation of a diazonium compound, which interferes with colour development in the TBA text (Zipser & Watts, 1962). The TBA values were expressed as milligrams of malonaldehyde per kilogram of tissue.

RESULTS AND DISCUSSION

Iron compounds in muscle

Table 1 shows the data for total soluble iron and the concentrations of ferritin, haem iron and dialysable iron for beef, pork and fish (mackerel). The

TABLE 1
The Concentration ($\mu g\,g^{-1}$) and Percentage Distribution of Iron in the Various Water-soluble Fractions of Beef, Pork and Fish[a]

Species	*Total soluble Fe*	*Ferritin Fe*	*%*	*Haem protein Fe*	*%*	*Diffusate Fe*	*%*
Beef	19·6 (18·6–20·8)	0·6 (0·48–0·68)	3·1	18·4 (17·5–19·4)	95·4	0·3 (0·2–0·32)	1·5
Pork	3·9 (3·2–4·2)	0·4 (0·36–0·42)	11·1	3·1 (2·4–3·5)	85·6	0·12 (0·11–0·14)	3·3
Mackerel	2·1 (1·9–2·2)	0·3 (0·25–0·39)	15·4	1·5 (1·4–1·6)	77·8	0·14 (0·11–0·16)	7·2

[a] Each value is the mean of three samples analysed in duplicate, with the range in parentheses.

concentrations of iron in the various soluble fractions of beef and pork are similar to those reported by Hazell (1982). The distribution of iron in the various fractions of fish has not been previously reported. The present results show, in line with those of Hazell (1982), that there are gross differences from one meat type to another in the total soluble iron content. In all three meats, the haem proteins, probably myoglobin, account for most of the soluble iron, with levels for low molecular weight iron and of ferritin being very low.

Effect of haem and non-haem fraction on lipid oxidation

Total soluble iron, ferritin, haem (haemoglobin plus myoglobin) or dialysable iron, isolated and fractionated from the three muscle systems, were added back to their respective WF system. The concentration of each fraction added corresponded to the mean values obtained for each component in the different muscle systems. Samples were then heated at 70°C for 30 min and stored at 4°C for 48 h and the extent of oxidation quantified. The objective here was to elucidate the role of the various iron fractions present in muscle in lipid oxidation. The results (Table 2) show that the WF system containing added total soluble iron gave TBA values similar to those obtained for the intact muscle systems. The low TBA for the WF system confirmed our earlier findings (Tichivangana & Morrissey, 1982) and the present results verified the validity of using WF systems in studying lipid oxidation. The results further suggest that the insoluble haemosiderin does not play any significant role in the lipid oxidation of muscle.

The results in Table 2 indicate that the dialysable fraction is the major pro-oxidant in the three muscle systems, accounting for ~45% of the overall TBA value in each muscle system. The haem fraction contributed ~33% to the oxidative process and the ferritin fraction accounted for between 18%

TABLE 2
Effect of Water-soluble Haem and Non-haem Iron Complexes on Lipid Oxidation in Heated Water-extracted Muscle Systems (WF) Stored at 4°C for 48 h

System	*Mean TBA values*[a]					
	Whole muscle	*WF*	*WF+ferritin fraction*	*WF+haem fraction*	*WF+dialyzable fraction*	*WF+total aqueous extract*
Beef	6·1	0·6	1·2	2·2	3·1	5·8
Pork	9·8	1·2	2·2	3·0	4·0	9·2
Fish	24·2	3·3	6·2	7·9	10·9	23·6

[a] Mean TBA values of four replicate analyses performed in duplicate. TBA values are expressed as milligrams of malonaldehyde per kilogram of tissue.

and 25% (depending on the particular muscle) of the overall TBA value. While it would be difficult to separate completely the effects due to haem and non-haem complexes in intact muscle systems, the above experiment does aid in assessing the relative contributions of each fraction as catalysts in heated muscle systems.

The present results do not rule out the possibility that a number of catalysts may be present in the low molecular weight fraction. However, on the basis of other studies (Sato & Hegarty, 1971; Igene *et al.*, 1979; Tichivangana & Morrissey, 1985) it is reasonable to assume that ferrous and ferric iron (Fe^{2+} and Fe^{3+}) are the primary catalysts in the low molecular weight fraction. In the earlier studies just quoted, iron was added exogenously as simple salts. However, in muscle systems it is likely that iron is chelated with amino acids and small peptides (Albert, 1950) which may have a specific influence on the catalytic activity of the element.

The present results also show that the haem fraction does exert considerable catalytic activity in heated muscle systems. This result is significant in view of the earlier findings by Sato & Hegarty (1971), Love & Pearson (1974) and Igene *et al.* (1979), who observed no acceleration of lipid oxidation when metmyoglobin was added to water-extracted muscle residues at levels of 1–10 mg/g muscle. On the other hand, Tichivangana & Morrissey (1984, 1985) showed that, while myoglobin-catalysed oxidation was slow in all raw muscle systems, some heated systems were very susceptible to oxidation. It was proposed (Tichivangana & Morrissey, 1985) that the discrepancy in results was related to the content of polyunsaturated fatty acids present in the muscle systems and the storage time after heating.

The results of both Igene *et al.* (1979) and Morrissey & Tichivangana (1985) showed that the proportion of non-haem to haem iron increases as a result of heating, and thus indicate that myoglobin is at least partially broken down during cooking. This increase in free iron in cooked meat is probably responsible for the rapid development of lipid oxidation compared with raw meat. Our earlier results (Apte & Morrissey, 1987) clearly indicate that intact ferritin is not a catalyst of lipid oxidation, i.e. ferritin does not contribute to lipid oxidation in raw muscle. However, earlier results (Apte & Morrissey, 1987) and those in Table 2 of this section clearly show that ferritin is highly catalytic in cooked systems. Heating probably denatures the ferritin molecule, probably releasing free iron which then catalyses lipid oxidation and the development of off-flavours.

The present results indicate that all the various forms of iron present in muscle contribute to the overall development of lipid oxidation in heated muscle. The order may be represented as follows: low molecular iron complexes $>$ total haem proteins $>$ ferritin $\gg$ haemosiderin. The present findings also clearly demonstrate that the susceptibility of muscle systems to

lipid oxidation is much more closely related to the polyunsaturated fatty acid content than to the concentration of catalyst present.

Effect of nitrite on lipid oxidation

This phase of the work was designed to investigate the effect of nitrite on lipid oxidation catalysed by the ferritin, haem and low molecular weight iron fractions of muscle. In addition, the effect of adding nitrite to muscle stored for various intervals after heating was examined. Table 3 shows data for nitrite-heated samples where the nitrite was added prior to heating or immediately after heating. In both cases, nitrite inhibited lipid oxidation. These results are highly significant for two reasons. First, nitrite is shown to inhibit oxidation catalysed by the ferritin, haem protein and low molecular weight iron fractions in the systems employed. Secondly, nitrite is also highly effective in preventing oxidation when it is added post-heating.

In a further series of experiments the activity of nitrite when added at various time intervals after heating was determined (Table 4). In all cases the added nitrite inhibited the auto-catalytic process; i.e. it appeared to either terminate the propagation stage of the reaction or neutralize substrate-free radicals. This anti-oxidative activity was maintained over the 48-h study period.

TABLE 3
Effect of Nitrite (100 mg/kg) Added Prior to and Immediately After Heating on Lipid Oxidation in Water-extracted Pork Muscle Systems Catalysed by Water-soluble Haem and Non-haem Fractions

Treatment	*Storage time (h)*	*TBA values*[a]		
		Control	*Nitrite added before heating*	*Nitrite added after heating*
Pork muscle	0	2·8	1·3	1·6
	48	9·8	3·2	3·9
WF only	0	0·9	0·6	0·7
	48	1·3	0·8	1·1
WF + ferritin fraction	0	1·2	0·7	0·9
	48	2·3	0·9	1·3
WF + haem fraction	0	1·4	0·8	0·9
	48	2·9	1·5	1·4
WF + dialysable fraction	0	1·5	0·9	1·0
	48	4·2	1·9	2·1
WF + total aqueous extract	0	2·9	1·3	1·5
	48	9·0	2·7	3·1

[a] Mean TBA values of four replicate analyses performed in duplicate.

TABLE 4
Effect of Nitrite (100 mg/kg) Added Prior to Heating, Immediately After Heating and After Storage at 4°C for 24 or 48 h on Lipid Oxidation of Pork (A) and Fish (B)

	Storage time (h)	*Mean TBA values*[a]				
		Control	*Nitrite added prior to heating*	*Nitrite added immediately after heating*	*Nitrite added 24 h after heating*	*Nitrite added 48 h after heating*
A	0	2·9	1·2	1·6	—	—
	24	4·5	1·6	2·2	4·7	—
	48	9·8	3·2	4·0	5·7	8·9
B	0	3·4	1·8	2·1	—	—
	24	8·2	3·8	4·5	7·6	—
	48	18·5	6·5	7·2	8·0	16·7

[a] Mean TBA values of four replicate analyses performed in duplicate. TBA values expressed as milligrams of malonaldehyde per kilogram of tissue.

Several theories have been presented for the probable function of nitrite as an anti-oxidant. Zipser *et al.* (1964) proposed that nitrite formed a complex with iron porphyrin in heat-treated meat, thereby inhibiting haem-catalysed lipid oxidation. Nitrosylmyoglobin has anti-oxidant properties *per se* (Kanner *et al.*, 1980; Morrissey & Tichivangana, 1985). This anti-oxidant is maintained even in the presence of pro-oxidants such as metmyoglobin and free metal ions. Lin *et al.* (1974) and Lin & Olcott (1975) showed that proline nitroxide and ethoxyquin nitroxide were quite effective anti-oxidants. Kanner (1979) and Kanner *et al.* (1984) showed a similar effect for *S*-nitrosocysteine, hemin nitroxide and cysteine-iron nitroxide. Kanner *et al.* (1984) proposed that the anti-oxidant effect of nitrosylmyoglobin and other nitroxides was due to quenching of substrate-free radicals. Morrissey & Tichivangana (1985) suggest that the specific anti-oxidative properties of nitrosylmyoglobin may be relatively insignificant in most cured meats. Nitrite also prevents the release of haem iron as non-haem, which is a highly effective catalyst (Morrissey & Tichivangana, 1985).

The stabilization and anti-oxidant properties of nitrosylmyoglobin cannot account for the reduced TBA values obtained where nitrite was added after heating (Table 3). In this case denaturation of myoglobin and ferritin and the release of free iron would have occurred prior to adding nitrite. MacDonald *et al.* (1980) found that nitrite substantially reduced oxidation in model systems containing such catalysts as Fe^{2+} or Fe^{2+}-EDTA. Similar effects were observed by Morrissey & Tichivangana (1985) when Fe^{2+}, Cu^{2+} or Co^{2+} were used as catalysts. The same effect is shown in

Table 3, where nitrite was also inhibitory towards the activity of the low molecular weight iron fraction.

Morrissey & Tichivangana (1985) postulated that nitrite probably formed inactive 'chelates' or complexes with non-haem iron, copper and cobalt, and in this manner inhibits catalytic activity and prevents oxidation. A similar explanation was also given by MacDonald *et al.* (1980) to explain the inhibitory effect of nitrite toward the activity of iron ions.

We believe, on the basis of earlier studies and the present work, that the complexing of either free or low molecular weight iron fractions by nitric oxide may be the critical reaction controlling lipid oxidation in cured meats. These complexes may indeed function as anti-oxidants by quenching alkyl and alkoxy radicals (Woolum *et al.*, 1968; Yonetani *et al.*, 1972; Kanner *et al.*, 1984).

The high reactivity of nitrite with iron complexes is further demonstrated by the recent work of Reddy *et al.* (1983), who reported that iron–sulphur complexes (especially feredoxin) react with added nitrite to form iron–nitrosyl complexes which inhibit the growth of *Clostridium botulinum*. These complexes gave electron spin resonance spectra similar to those of organic nitroxide-free radicals (Reddy *et al.*, 1983). This is further evidence that nitrite forms a complex with trace levels of iron in tissue and also perhaps forms an anti-oxidant in the process.

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