THE COOKED CURED-MEAT PIGMENT - ESR STUDIES

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SUMMARY

Preparation of the cooked cured-meat pigment (CCMP) from red blood cells or from haemin has previously been reported from our laboratories, and its stabilization by microencapsulation in food-grade wall materials has been achieved. However, the exact chemical nature of CCMP has remained elusive. Electron spin resonance (ESR) spectra of CCMP in acetone or pyridine were recorded. The CCMP in acetone showed a well resolved triplet due to hyperfine splitting of one NO group. This may imply the existence of a pentacoordinated mononitrosyl haem complex. However, a hexacoordinated mononitrosyl haem compound with participation of a pyridine molecule existed when pyridine was used as a solvent and loss of resolution of the hyperfine structure occurred. The ESR spectra of the cooked nitrite-cured and CCMP-treated meats, *in situ*, were similar to one another. Therefore, the pigment formed from nitrite-curing of meat and that introduced to meat by the preformed CCMP, as part of a nitrite-free multi-component curing system, were considered identical.

INTRODUCTION

The structure of the nitrosylprotohaem pigment of cooked cured meat has long been a subject of dispute (Tarladgis, 1962; Lee and Cassens, 1976; Renerre and Rougie, 1978; Bonnett *et al.*, 1980). In 1901, Haldane was the first to attribute the pink colour of cured meat to a nitric oxide-haemoprotein complex. This hypothesis was based on the fact that the visible absorption bands of extracts of cured meat resembled those of the reaction product of nitric oxide and haemoglobin. Based on numerous spectroscopic investigations of nitrosylhaem complexes, at present, it is believed that during thermal processing of nitrite-cured meat, the globin portion of nitrosylmyoglobin denatures and subsequently detaches itself from the haem moiety. Yet, this is where the controversy remains. The resultant pigment will be either a five-coordinate mononitrosylprotohaem complex (Fig. 1) or it may acquire a second molecule of nitric oxide forming a six-coordinate dinitrosylprotohaem compound.

Wayland and Olson (1973, 1974) showed that $Fe^{II}TPP(NO)$ had a strong ν_{NO} at 1700 cm^{-1} while its ESR spectrum in a toluene glass showed 3 g values with NO ^{14}N hyperfine splitting characteristic of a pentacoordinate haem complex as reported by Bonnett *et al.* (1980). They also reported that in the presence of excess NO, $Fe^{II}TPP(NO)_2$ may form. Two IR N-O stretching frequencies were found at 1870 cm^{-1} and 1690 cm^{-1} (*i.e.* $Fe^{II}TPP(NO^-)(NO^+)$). The band at 1870 cm^{-1} is in the range expected for a linear $Fe^{II}NO^+$ moiety and the 1690 cm^{-1} band is consistent with a bent $Fe^{II}NO^-$ fragment.

Bonnett *et al.* (1980) attempted to characterize the pigment of cooked cured meat, nitrosylprotohaem, from the reaction of NO with protohaem dimethyl ester and with methoxyiron (III)-protoporphyrin dimethyl ester. A strong IR band at about 1660 cm^{-1} was diagnostic of the stretching mode of a bent Fe-NO moiety and a pentacoordinate complex. The ESR spectrum of the nitrosylhaem in an acetone glass showed a triplet signal due to hyperfine splitting by a single axial nitrogenous ligand of NO indicating a pentacoordinate nitrosylhaem system (*i.e.* $g_1 = 2.102$, $g_2 = 2.064$, $g_3 = 2.010$). However, when the ESR spectrum of the nitrosylhaem was monitored in a solvent providing a second nitrogenous base such as pyridine, the g_1, g_2 and the hyperfine structure at g_3 were no longer resolved ($g_1 = 2.08$, $g_2 = 2.04$, $g_3 = 2.003$). These authors also examined various cured meat samples directly by ESR spectroscopy as opposed to their extracts and observed a signal with the hyperfine splitting characteristic of a pentacoordinate nitrosylhaem. More recently, Killday *et al.* (1988) isolated and characterized an extract of the cooked cured-meat pigment (CCMP) from thermally processed

corned beef by IR and VIS spectroscopies and thin-layer chromatography. They further identified the pigment by fast atom bombardment mass spectrometry as being a mononitrosyl ferrous protoporphyrin.

We have reported the preparation of the CCMP directly from bovine red blood cells (Shahidi and Pegg, 1991) or through a haemin intermediate (Shahidi *et al.*, 1984; 1985) in the presence of reductants and a nitrosating agent. This pigment, preformed outside of the meat matrix, is used as part of a composite package for nitrite-free curing systems, but its chemical nature has not been adequately elucidated. The purpose of this study was to investigate the chemical nature of this pigment through ESR spectroscopy.

MATERIALS & METHODS

The CCMP was prepared from haemin and nitric oxide essentially as described by Shahidi *et al.* (1985). In some experiments, pigment samples were centrifuged using an IEC clinical centrifuge at 3000 rpm (905xg) for 5 min. The CCMP was recovered as a precipitate from the mixture after centrifugation. After washing with a 2% (w/v) sodium ascorbate solution, the pigment was frozen (77 K) using liquid nitrogen and then lyophilized with a Labconco freeze-dryer 5 (Labconco Corp., Kansas City, MO) for 12 h.

Preparation of Meat Systems and Application of CCMP: Ground meat was mixed with 20% by weight of distilled water and 550 ppm sodium ascorbate. Sodium nitrite and preformed CCMP were added directly to meat samples at levels of 200 and 30 ppm, respectively. Beef slurries were thoroughly homogenized and then cooked at 85±2°C in a thermostated water bath for 40 min, while stirring occasionally with a glass rod. After cooling to room temperature, cooked meat samples were covered with Parafilm and stored in a refrigerator at 4 °C until used. Nitrosylprotohaem pigments were extracted from treated cooked beef systems according to the method of Hornsey (1956), but at a much smaller scale.

ESR Studies: Approximately 10 mL of acetone (or pyridine) were added to lyophilized pigment, or freshly prepared CCMP after the supernatant wash was discarded. A deep red-coloured solution was formed. Using a Pasteur pipette, a 1.5 mL aliquot of the extract was transferred to a reservoir connected to an ESR tube (i.d. 2.16 mm). The pigment extract was frozen and the glassware was attached to a vacuum line. Eventually the contents in the reservoir were thawed, and then transferred to the evacuated ESR tube by opening one of the stopcocks. The pigment extract in the ESR tube was frozen under liquid nitrogen forming an acetone glass. All spectra were recorded at 77 K by immersing the tubes in an insertion Dewar flask filled with liquid nitrogen. The flask was placed in the centre of the resonant cavity of the ESR spectrometer. ESR spectra were recorded on a Bruker ESP-300 X-band spectrometer at *ca.* 9.46 GHz using a ER 4102ST cavity. Field calibrations were made with DPPH dissolved in benzene (g = 2.0036). A modulation frequency of 3.13 kHz was used. In other experiments, moist nitrite-cured and CCMP-treated cooked meat samples were transferred to ESR tubes and examined as plugs (ca. 20 mm x 4.2 mm). Finally, acetone extracts of nitrite-cured or CCMP-treated cooked beef systems were transferred to the resevoir and attached to the vacuum line as described above without further treatment.

RESULTS AND DISCUSSION

The ESR spectral parameters of an acetone extract of a lyophilized sample of CCMP were compared with those of nitrosylprotohaem dimethyl ester investigated by Bonnett *et al.* (1980) and to $Fe^{II}TPP(NO)$ and $Fe^{II}TPP(NO)_2$ systems reported by Wayland and Olson (1974) (Fig. 1). In all cases examined, the ESR parameters of these systems were similar and possessed characteristics recognized as those of a pentacoordinate nitrosylprotohaem system. ESR spectra of the nitrosylprotohaem dimethyl ester, $Fe^{II}TPP(NO)$ and the freeze-dried CCMP in a solvent glass showed g_1, g_2 and g_3 values characteristic of a rhombic g tensor due to the anisotropic nature of the system, with hyperfine splitting in the g_3 region from the nitric oxide ligand. The hyperfine coupling by a single nitrogenous ligand (I=1) would produce a signal of a triplet nature with equal line intensities (Fig. 1C). According to Wayland and Olson (1974), this ^{14}N hyperfine coupling provides evidence for placing the odd electron in a molecular orbital with substantial iron dz^2 character. The odd electron of nitric oxide thus becomes highly delocalized on the ferrous ion.

The ESR spectrum of the preformed pigment was compared to those of nitrite-cured and CCMP-treated cooked beef systems *in situ* as well as their acetone extracts. Similar ESR parameters for these nitrosylprotohaems were observed in all cases. The position of the g signals and the associated hyperfine splitting a_3 value are provided in Table 1. The observed triplet in nitrite-cured meat systems, due to the hyperfine splitting of NO in the g_3 region suggests that the iron-imidazole bond is effectively cleaved from

nitrosylmyoglobin during thermal processing (Bonnett *et al.*, 1980). If this was not so, the hyperfine structure at g_3 would no longer be resolved. A second nitrogenous ligand (*i.e.* from imidazole) bound to the iron would provide ESR characteristics of a hexacoordinate system with a shift in the g_3 value. However, spectra obtained clearly indicate that the pigment of cooked cured meat is a mononitrosylprotohaem compound.

The ESR spectrum of CCMP in an acetone glass showed ^{14}N hyperfine splitting in the g_3 region with a_3 of 17.1 G, but a small shoulder with two other splittings of low intensity was also observed. The existence of dinitrosylprotohaem complexes has been reported in literature (Wayland and Olson, 1974; Olson *et al.*, 1982; Lançon and Kadish, 1983), but such complexes do not correspond with the observed ESR spectrum of preformed CCMP. Dissolution of the CCMP precipitate in pyridine, a solvent which provides a second nitrogenous ligand, caused a dramatic change in the ESR spectrum of CCMP. The resulting spectrum was identical to that observed for the nitrosylprotohaem dimethyl ester in piperidine reported by Bonnett *et al.* (1980). The hyperfine structure at g_3 was no longer resolved. In the pyridine glass, if two NO moieties were coordinated to the protohaem system, the second one is easily labile and replaced with pyridine. Further research on ESR characteristics of various nitrosylprotohaem complexes is in progress.

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