EPR STUDIES OF ALTERNATIVE COOKED CURED MEAT PIGMENT (CCMP)

Marjeta Stevanović[1], Marjeta Šentjurc[2]

[1]*University of Ljubljana, Biotechnical faculty, Jamnikarjeva 101, 1000 Ljubljana, Slovenia*
[2]*J. Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*

Received 5.8.1999

Abstract

Sodium nitrite has a multifunctional role in the meat curing process: it imparts characteristic pink colour to cooked cured meat products, it gives the typical flavour to cured meats, it acts as an antioxidant and most importantly, it is a very effective antimicrobial agent. Unfortunately, nitrite can also react with amines, amides and amino acids in meats forming carcinogenic N-nitroso compounds. The residual nitrite present in cured meats may also lead to the formation of carcinogenic N-nitroso compounds in the gastrointestinal tract. Therefore, there is a need to develop an alternative to the use of nitrite. The cooked cured meat pigment (CCMP) was synthesized from bovine hemin chloride using nitrosating (NO) and reducing agent (Na-ascorbate). CCMP is a low spin (S=1/2) ferrous protoporphyrin complex with odd number of electrons. It was qualitatively analysed with electron paramagnetic resonance. The results showed that CCMP is a five-coordinate mononitrosylprotohaem complex. EPR spectra of CCMP obtained in our synthesis closely resembled those reported in the literature. Our results also indicated that freshly prepared CCMP is unstable: with longer time of storage the intensity of EPR spectra decreases. Accordingly, we could not detect the EPR spectrum of CCMP after 14 days of storage, suggesting that the iron ion in CCMP complex oxidized back to the ferric (3+) state.

Introduction

Nitrite was and still is, one of the most widely used of all food additives. Nitrite as a traditional agent for curing meat products has multifunctional properties: it imparts the characteristic pink colour to cooked cured meat products, it contributes to the typical flavour of cured meat products, it acts as an antioxidant and most importantly, it has a strong antimicrobial effect in retarding germination of spores and the formation of *Clostridium botulinum* toxin [1], [2], [3], [4].

Unfortunately, during last 20 years nitrite has become the source of serious concerns. Nitrite may react with amines, amides and amino acids present in meats, leading to the formation of carcinogenic N-nitroso compounds, such as N-nitrosamines (for example N-nitrosopyrolidine and N-nitrosodimethylamine). Moreover, the residual nitrite present in cured meat may also lead to the formation of carcinogenic N-nitroso compounds in the gastrointestinal tract [4], [5]. Therefore, it is necessary to develop alternatives to nitrite. Researchers proposed different methods to inhibit the possibility of N-nitrosamine formation in cured meat products. These include a decrease in the level of added nitrite or the use of N-nitrosamine blocking agent such as ascorbate and α-tocopherol. However, as far as N-nitrosamines are concerned, the most attractive and reliable method is total elimination of nitrite from the curing process [4], [6].

The alternative cooked cured meat pigment (CCMP) synthesised directly from bovine red blood cells or through a hemin intermediate was found to be a viable colorant for application to comminuted meat as a substitute for nitrite [7], [8].

The structure of the cooked cured meat pigment extracted from thermally processed meat has long been a subject of controversy. The resultant pigment either will be a five-coordinate mononitrosylprotohaem or may acquire a second molecule of nitric oxide forming a six-coordinate dinitrosylprotohaem (Fig. 1) [9], [10].

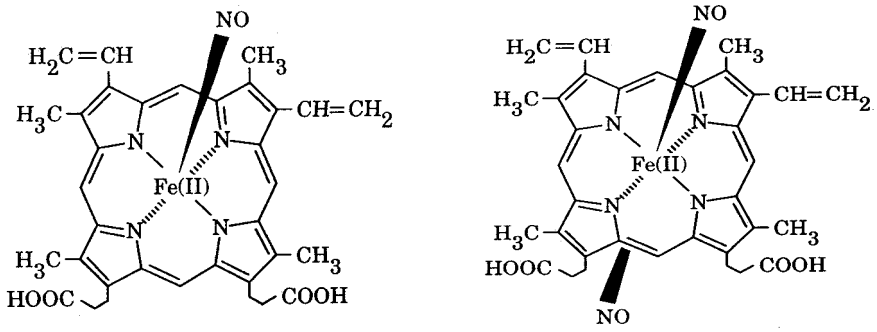

Fig. 1: The chemical structure of five-coordinate mononitrosylprotohaem and six-coordinate dinitrosylprotohaem [9], [10]

Recently, very extensive and numerous studies based on different analytical technique (infrared; visible spectroscopies; mass spectrometry and electron paramagnetic resonance - EPR) have confirmed that the chemical structure of CCMP is mononitrosylprotohaem [9], [10], [11], [12], [13], [14]. Disadvantage of this pigment is

high reactivity with oxygen which diminishes its applicability in practice. Therefore, further investigations of this pigment are necessary and a procedure for stabilization of CCMP has to be developed [2]. In this respect we have synthesised CCMP and investigated its stability as well as its influence on the colour of meat emulsion coagulates. The genotoxic activity of meat emulsion coagulates prepared with CCMP was investigated, too.

The aim of this study was to prove the successfulness of our synthesis of CCMP, to determine its structure, and to study its stability on air.

Experimental

Synthesis of CCMP is based on the reduction of hemin by means of a reducing agent - Na-ascorbate - and introduction of gaseous nitric oxide (NO) into the mixture as shown on the Fig. 2. CCMP was synthesised as described in [2], with slight modification. Briefly, 120 mg of bovine hemin chloride (Sigma Chemical Co., USA) were dissolved in 20 ml of 0,04 M Na_2CO_3 solution. N_2 was introduced in this solution for 5 minutes and the solution afterwards kept in dark for 30 minutes. 2 g of Na-ascorbate and 180 ml of 0,2 M acetate buffer (pH=6,5) were added to the mixture.

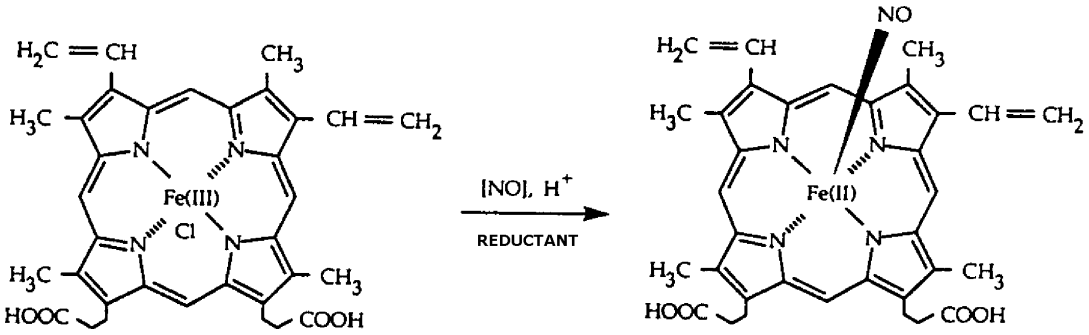

Fig. 2: Synthesis of CCMP from hemin chloride [4]

Nitric oxide was then slowly bubbled into the mixture for 5 minutes and then centrifuged at 4221 x g in centrifuge (Sorvall Instruments model RC5C in rotor GS3) at 4 °C. Precipitate from the mixture was washed three times with 2% (w/v) ascorbic acid to ensure elimination of any traces of nitrite from the mixture.

Hemin chloride and alternative pigment of cured meat (CCMP) were qualitatively analysed with electron paramagnetic resonance (EPR) method on an X-band spectrometer BRUKER ESP 300. All spectra were measured at 180 K.

Preparation of samples:

- 20 mg of hemin were dissolved in 2 ml dimethyl sulfoxide (DMSO)
- freshly prepared CCMP from 20 mg of hemin was dissolved in 2 ml DMSO

Samples were poured into quartz capillary (inner diameter 3,0 mm) and frozen in liquid nitrogen before measurements. Spectra were measured at different time intervals after synthesis. During storage the samples were kept at room temperature in open quartz capillaries.

Results and discussion

EPR spectra of hemin and CCMP are presented in Fig. 3.

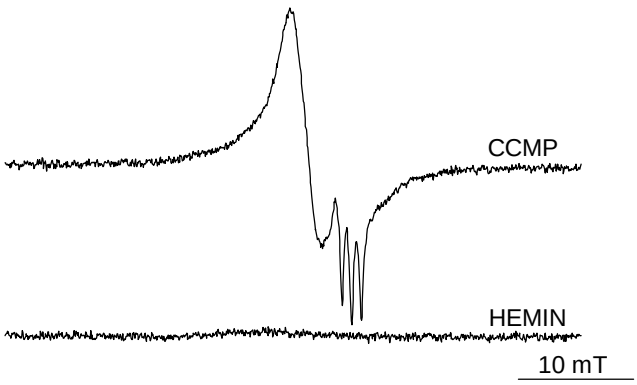

Fig. 3: EPR spectra of hemin chloride and freshly prepared CCMP
Conditions of measurements: T = 180K; microwave frequency = 9,62 GHz, microwave power = 10 mW, modulation frequency = 100 kHz; modulation amplitude = 0,1 mT: sweep width = 315 - 365 mT.

From Fig. 3 it is evident that for hemin no EPR signal was observed in the magnetic field region. However, an EPR signal with $g_{\perp} = 6$ typical for ferric haem complexes was detected in the magnetic field range from 0 to 400 mT (results not shown).

The EPR spectrum of freshly prepared CCMP in DMSO (dimethyl sulfoxide) shows a triplet signal due to interaction of unpaired electron of ferrous (2+) protoporphyrin complex with the nitrogen nucleus from the NO group. The spectrum allowed determination of the g tensor components and of the hyperfine splitting tensor A. We obtained g_3 = 2,013, while the components g_1 and g_2 could not be determined accurately from the spectra and were determined only approximately ($g_1 \approx 2{,}080$; $g_2 \approx 2{,}067$). The component A_3 of freshly prepared CCMP was 1,710 mT. These parameters as well as the line-shape of the spectra closely resembled those published in the literature [10], [13], [15], [16], [17], and are typical for the five-coordinate mononitrosylprotohaem complex. This proves successfulness of our synthesis of CCMP.

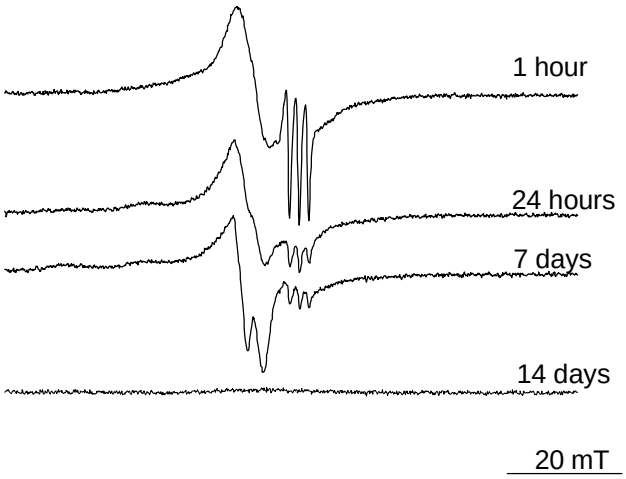

Fig. 4: The influence of storage on EPR spectra of CCMP
The experimental conditions were the same as described for Fig. 3.

Fig. 4 shows the EPR spectra of CCMP 1 hour, 24 hours, 7 days and 14 days after the synthesis. It is evident that with longer storage time the intensity of EPR spectra decreases. After 14 days an EPR spectrum could no longer be detected. This shows that the complex is very unstable; it already changes within 24 hours, and within 14 days the iron ion of the CCMP complex is oxidised back to a ferric (3+) state.

Another step in our study was to test the use of CCMP in model meat emulsion coagulates. Briefly, analyses of meat emulsion coagulates made with CCMP showed that:

- CCMP is a possible alternative to nitrite;
- the colour of meat emulsion coagulates depends on the original myoglobin content of meat as well as on the addition level of CCMP;
- coagulates made with CCMP contain minimal quantities of residual nitrite and do not show genotoxic activity.

Conclusions

The EPR studies of CCMP have confirmed previous studies of its chemical nature: CCMP is a five-coordinate mononitrosylprotohaem.

Although CCMP was found as a possible alternative to nitrite for commercial production, stability of this pigment is required. The study of its stabilization is in progress. The EPR technique is found to be a suitable method to follow this process.

Acknowledgement: This work was supported by the Ministry of Science and Technology of the Republic of Slovenia and by JATA MESO, d.d., Slovenia.

References

[1] J.H. Hotchkiss, *Cancer surv.*, **1989,** *8*, 295 - 321.
[2] United States Patent US 5 230 915 1993; F. Shahidi, R.B. Pegg, **1993**, 46 p.
[3] R.G. Cassens, *Food technol,*. **1997**, *51*, 53 - 55.
[4] F. Shahidi, R.B. Pegg, *Meat focus int.*, **1993**, *2*, 407 - 414.
[5] F. Shahidi, R.B. Pegg, *J. food sci.*, **1991**, *56*, 1205 - 1208.
[6] F. Shahidi, R.B. Pegg, *Food chem.*, **1992**, *43*, 185 - 191.
[7] F. Shahidi, R.B. Pegg, *Food chem.*, **1990**, *38*, 61 - 68.
[8] F. Shahidi, R.B. Pegg, *J. muscle foods*, **1991**, *2*, 297 - 304.
[9] R.B. Pegg, F. Shahidi, *Food chem.*, **1996**, *56*, 105 - 110.
[10] R.B. Pegg, F. Shahidi, N.J. Gogan, S.I. DeSilva, *J. agr. food chem.*, **1996**, *44*, 416 - 421.
[11] K.B. Killday, M.S. Tempesta, M.E. Bailey, C.J. Metral, *J. agr. food chem.*, **1988**, *36*, 909 - 914.
[12] A. Jankiewicz, M. Kwasny, K. Wasylik, A. Graczyk, *J. food sci.*, **1994**, *59*, 57 - 59.
[13] F. Shahidi, R.B. Pegg, N.J. Gogan, S.I. DeSilva, *40th international congress of meat science and technology*, Den Haag, Netherlands, **1994**, 1 - 5.
[14] F. Shahidi, R.B. Pegg, *41st international congress of meat science and technology*. Proceedings. Volume II. San Antonio, USA, **1995**, 406 - 407.
[15] B.B. Wayland, L.W. Olson, *J. Chem. Soc. Chem. Communi.*, **1973**, 897 - 898.
[16] B.B. Wayland, L.W. Olson, *J. Am. Chem. Soc.*, **1974**, *96*, 6037 - 6041.
[17] R. Bonnett, S. Chandra, A.A. Charalambides, K.D.Sales, P.A. Scourides, *J. Chem. Soc., Perk. 1*, **1980**, 1706 - 1710.

Povzetek

Natrijev nitrit ima v procesu razsoljevanja mesa multifunkcionalno vlogo: tvori značilno rožnato barvo kuhanega razsoljenega mesa, oblikuje tipično aromo razsoljenih izdelkov, ima antioksidativen učinek in najbolj pomembno - je zelo učinkovito antimikrobno sredstvo. Na žalost nitrit lahko reagira z amini, amidi in amino kislinami prisotnimi v mesu in tvori kancerogene N-nitrozo spojine. Rezidualni nitrit, prisoten v razsoljenih izdelkih prav tako lahko vodi do tvorbe kancerogenih N-nitrozo spojin tudi v gastrointestinalnem traktu. Zato je nujno potrebno razviti alternativo nitritu. Pigment kuhanega razsoljenega mesa (CCMP) smo sintetizirali s pomočjo nitrozirnega sredstva (NO) in reducenta (Na-askorbata). CCMP je nizko spinski (S=1/2) fero protoporfirinski kompleks z lihim številom elektronov. Kvalitativno smo ga analizirali z elektronsko paramagnetno resonanco. Rezultati so pokazali, da je CCMP mononitrozo fero protoporfirinski kompleks s koordinacijskim številom 5. Dobljeni EPR spektri tega pigmenta se niso bistveno razlikovali od podatkov iz literature, kar potrjuje uspešnost naše sinteze CCMP. Sveže pripravljen CCMP je nestabilen: z daljšim časom skladiščenja se zmanjšuje intenziteta EPR spektra. Po 14 dneh EPR spekter ni več zaznaven, kar pomeni, da se je v tem času Fe v CCMP oksidiralo nazaj v feri (3+) stanje.