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Abstract

The Italian traditional dry-cured ham (Parma ham) shows a stable bright red color that is achieved without the use of nitrite and/or nitrate. In this study we examined the pigment spectroscopically, fluoroscopically and by using HPLC and ESI-HR-MASS analysis. Porphyrin derivative other than acid hematin were contained in the HCl-containing acetone extract from Parma ham. A strong fluorescence peak at 588 nm and a weak fluorescence peak at 641 nm were observed. By HPLC analysis the acetone extract of Parma ham was observed at the single peak, which eluted at the same time as Zn protoporphyrin IX and emitted fluorescence. The results of ESI-HR-MS analysis showed both agreement with the molecular weight of Zn protoporphyrin IX and the characteristic isotope pattern caused by Zn isotopes. These results suggest that the bright red color in Parma ham is caused by Zn-protoporphyrin IX.

Keywords: Zn-protoporphyrin IX, Parma ham, heme, zinc, substitution

1. Introduction

The color of meat and meat products are one of the important factors that determine the quality and is an important factor in the criteria by which consumers make selections. Myoglobin, which causes the red color of meat, is a heme protein with the iron complex of porphyrin, and the color of myoglobin depends on the kind of molecule coordinating to iron (Fox 1966; Ledward 1992; Sakata 2000). Deoxymyoglobin, without a ligand attached to iron, is the main constituent of meat that has just been cut, and deoxymyoglobin is later converted into oxymyoglobin, with oxygen. With the passage of time or with heating, oxymyoglobin is auto-oxidized and changes into metmyoglobin.

Myoglobin in meat products adding nitrate and/or nitrite is converted into stable red nitrosylmyoglobin coordinated to nitric oxide, and nitrosylmyoglobin changes into pink-reddish nitrosohemochromogen after the meat has been cooked. By the way the north Italian traditional dry-cured ham “Prosciutto di Parma (Parma ham)” is made from only the legs of fattened pigs and is salted with sea salt, dried, and matured over a period of one year. Despite the fact that nitrite and/or nitrate have not been added, the color is extremely stable bright red and is not changed by exposure of the ham to light or heat. Morita, Niu, Sakata & Nagata (1996) reported that the red heme pigment was easily extracted with 75% acetone and that it is a new myoglobin derivative that is unknown in meat and meat products. Recently it was reported that this lipophylic stable red pigment in Parma ham increased with aging. (Parolari, Gabba, & Saccani 2003) It has

also been reported that bacteria obtained from meat products convert myoglobin to the desirable red myoglobin derivatives (Faustman Johnson, Cassens & Doyle, 1990; Arihara, Kushida, Kondo, Itoh, Luchansky & Cassens, 1993; Arihara, Cassens & Luchansky, 1994; Morita, Sakata, Sonoki & Nagata, 1994), but it is not clear what the pigment is.

The objective of this study is to identify the stable red pigment in Parma ham in order to obtain information for producing bright red meat products without nitrite and/or nitrate.

2. Materials and methods

2.1. Materials

Zn protoporphyrin IX and hemin were purchased from Aldrich Chem. Co. (WI, U.S.A.). Protoporphyrin IX 2Na salt was purchased from ICN Pharmaceuticals Inc. (CA, U.S.A.) An entire piece of deboned Parma ham was purchased from UNIBON Salumi S.C.ar.l. (Emilia Romagna, Italy). All other chemicals and solvents used in this study were of analytical grade.

2.2. Extraction of porphyrins

The thickest portion, which mainly consists of *M. Semimembranosus*, *M.*

1 *Semitendinosus*, *M. Biceps femoris*, *M. Rectus femoris*, was transversely cut out
2 from an entire piece of deboned Parma ham and minced after removal of adipose
3 and connective tissue as much as possible. Minced sample (5 g) was
4 homogenized in 10 volumes of each of three kinds of ice-cooled solvents (distilled
5 water, 75% acetone (Okayama and Nagata 1978), and 75% acetone containing
6 0.7% HCl (Okayama and Nagata 1979)) for 5 min, and the homogenate was
7 filtered through a filter paper (No. 5C Toyo Roshi Co., Ltd., Tokyo, Japan). The
8 absorption and fluorescent spectrum of the filtrate were measured.

9 The porphyrins were also extracted using the acid-butanone method (Teale
10 1959). Minced sample (5 g) was homogenized in 10 volumes of ice-cooled
11 distilled water, and the homogenate was centrifuged (3,000 rpm, 5 min, 4 °C) and
12 then filtered through a filter paper (No. 5C Toyo Roshi Co., Ltd., Tokyo, Japan).
13 The pH of the filtrate was adjusted to 2 by using 0.1 N HCl, and then the
14 equivalent of 2-butanone was added. The 2-butanone layer was collected after
15 strong mixture agitation. Demetalation of porphyrins was carried out by adding
16 6 N HCl. All of the operations were carried out under shading as much as
17 possible.

18 19 *2.3. Isolation of the unknown red pigment from Parma ham*

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21 Minced Parma ham (5 g) was homogenized in 20 ml distilled water, and the
22 homogenate was centrifuged (3,000 rpm, 5 min, 4 °C) and then filtered through a
23 filter paper (No. 5C Toyo Roshi Co., Ltd., Tokyo, Japan). Three volumes of

ice-cooled acetone were added to the filtrate, and the mixture was placed in ice for 15 minutes. The mixture was centrifuged at 3,000 rpm for 5 min at 4 °C. An equal amount of distilled water was added to the supernatant, and the mixture was applied to a disposable C18 column, Sep-Pak® Vac C18 Cartridge (12 cc/ 2g; Waters Co., MA U.S.A.) prewashed with 15 ml of methanol and 15 ml of distilled water. The column was washed with 25 ml distilled water and then the red pigment was eluted with 10 ml of 75% acetone.

2.4. Absorption and fluorescent spectral analysis

The absorption spectra of the extracts were measured from 380 to 700 nm using a Model U-3210 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The fluorescent spectra of the extracts were measured from 500 to 700 nm at 420 nm for excitation using a Model 650-60 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan).

2.5. HPLC analysis

HPLC was used to separate the red pigments in Parma ham by the method of Oshima, Yamada, Saito & Hayakawa (1996). The HPLC system from Jasco Co. (Tokyo, Japan) was consisted of a Model 880-PU pump, a Model 870-UVi UV/VIS detector, a Model 820-FPi spectrofluorometer and a Model 802-SC system controller. The unknown red pigment isolated from Parma ham was dried up using a centrifugal evaporator (CVE-3100, Tokyo Rikakikai Co., Ltd., Tokyo,

Japan) and solubilized in methanol:0.01 M Na₂HPO₄ (76:24, v/v, pH 9). After solubilization, the sample was filtered through 0.45 µm filter (DISMIC-3, Toyo Roshi Co., Ltd., Tokyo, Japan). A Shodex Asahipak ODP-50 4D column (4.6 × 150 mm, Showa Denko K.K., Tokyo, Japan) was used for separation of the red pigment, and the separation was carried out by isocratic elution using the mobile phase (methanol:0.01 M Na₂HPO₄=76:24, v/v, pH 9) at a flow rate of 0.5 ml/min at an ambient temperature. Twenty microliters of each sample was injected. The eluent was monitored at 415 nm for excitation and at 590 nm for emission wavelengths.

2.6. Electrospray ionization high resolution mass spectrometry (ESI-HR-MS) analysis

ESI-HR-MS analysis of the unknown red pigment isolated from Parma ham was carried out using a JMS-SX120A (JEOL Ltd., Tokyo, Japan) equipped with an ESI ion source (JEOL MS-ESI 10, JEOL Ltd., Tokyo, Japan). The sample diluted in a chloroform/methanol/acetone (1:1:8 v/v) solvent mixture was infused into the ESI ion source at a flow rate of 1 µl/min. The needle voltage and capillary voltage were 2681 V and -1230 V, respectively. The chamber temperature was set to 105 °C. A mixture of PEGs was used as an internal standard.

3. Results and discussion

The absorption spectra of the red pigments extracted from Parma ham by various solvents are shown in Fig. 1. The spectra of heme pigment extracted with water or 75% acetone were the same as those reported by Morita, Niu, Sakata & Nagata (1996). On the other hand, when the red pigment was extracted with 75% acetone containing 0.7% HCl, the spectral pattern showed one absorption peak, at 409 nm, in the Soret band and four peaks, at 509, 553, 600 and 637 nm, in the visible range (Q band). All heme pigments in meat products are extracted as acid hematin by 65-80% acetone containing 0.7-2% HCl (Hornsey 1956; Okayama and Nagata 1979). Hornsey (1956) reported that the absorption peaks of acid hematin are observed only at 512 and 640 nm in the case of extraction with 80% of acetone containing 2% HCl. Our present result shows that a part of the red pigment in Parma ham is not transformed into acid hematin.

To determine the porphyrin structure of the red pigment in Parma ham, we attempted to remove the coordinated metal. The metal in the porphyrin metal complex is generally removed by treatment with a strong acid. Fig. 2 shows the absorption spectrum of the solution extracted with the acid-butanone. Although it was the same as that of the solution extracted with 75% acetone shown in Fig. 1, the addition of 6N HCl resulted in a remarkable change in the absorption spectrum (Fig. 2). Although porphyrins generally have four absorption peaks in the Q band, metalloporphyrin shows on two peaks in the Q band due to the

1 symmetry of the molecular structure. The spectral pattern after the addition of
2 6N HCl was the same as that of protoporphyrin IX (data not shown). These
3 facts suggest that the red pigment in Parma ham is a metal complex of
4 protoporphyrin IX.

5 Although porphyrins generally show strong red fluorescence, the fluorescence
6 of metal complexes of porphyrin such as an iron complex (e.g., protoheme)
7 disappears. However, Zn, Mg and Cd complexes emit fluorescence. Fig. 3
8 shows fluorescence spectra of red pigments extracted with distilled water or 75%
9 acetone. A strong and a weak fluorescence peak at 588 nm and 641 nm were
10 observed in both water- and acetone-extracts, respectively. Although data are
11 not shown, fluorescence was not detected in 75% acetone extract from raw pork.
12 It is suggested that the Parma ham red pigment is not iron complex like
13 protoheme, because it was no fluorescence. Mg protoporphyrin IX is an
14 intermediate in chlorophyll biosynthesis. Mg insertion into protoporphyrin IX is
15 catalyzed by Mg chelatase (Walker and Willows 1997). The fluorescence
16 emission peak of Mg protoporphyrin IX is 593-599 nm and that of Zn
17 protoporphyrin IX is 583-589 nm (Castelfranco, Weinstein, Schwatz, Pardo &
18 Wezelman, 1979; Fuesler, Wright & Castelfranco, 1981; Masuda et al., 1999).
19 Each peak wavelength is different and our result was similar with that of Zn
20 protoporphyrin IX.

21 Therefore, we examined the possibility of the red pigment being Zn
22 protoporphyrin IX using the HPLC method of Oshima, Yamada, Saito &
23 Hayakawa (1996). Hemin (Fe complex), Zn protoporphyrin IX (Zn complex) and

1 protoporphyrin IX (metal free) were able to be distinctly separated, and
2 fluorescence of Zn protoporphyrin IX was only detected at 415 nm for excitation
3 (Fig. 4, *traces 1 and 1'*). In HPLC analysis of the unknown red pigment isolated
4 from Parma ham, a single peak with fluorescence was observed at the same
5 retention time as that of Zn protoporphyrin IX (Fig. 4, *traces 2 and 2'*).
6 Additionally the reagent Zn protoporphyrin IX was diluted in 75% acetone, and
7 the spectral pattern was same as that of 75% acetone extract of Parma ham (data
8 not shown).

9 By ESI-HR-MS analysis of the unknown red pigment isolated from Parma
10 ham, the highest molecular ion peak was detected at m/z 624 (Fig. 5). Six main
11 peaks were found when the peak region was expanded (Fig. 5 Insert). This peak
12 pattern was agreed well with that of Zn protoporphyrin IX ($C_{34}H_{32}N_4O_4Zn$) (Fig. 5,
13 Insert), because Zn has five isotopes ($m/z = 64, 66, 67, 68$ and 70) and the isotopic
14 ratio is characteristic. Fe has four isotopes ($m/z = 54, 56, 57$ and 58) and its
15 isotopic ratio entirely differ from that of Zn. The exact mass of the principal
16 molecular ion computed from the internal standard was 624.1711 and differed by
17 only 0.4 milli- mass units from the monoisotopic mass (624.1715) of Zn
18 protoporphyrin IX. On the other hand, peaks originating in Fe protoporphyrin
19 IX (MW 616.49), Mg protoporphyrin IX (MW 584.95) and Cd protoporphyrin IX
20 (MW 673.05) were not observed. Our findings indicate that the red derivative in
21 Parma ham is not only a Fe-porphyrin complex but also a Zn-porphyrin complex,
22 namely, Zn protoporphyrin IX. Based on the results described above, it was
23 speculated that the stable red pigment in Parma ham was produced by

1 conversion of an endogenous Fe-porphyrin complex, heme pigment, to a
2 Zn-porphyrin complex during the maturing process.

3 Zn is the most abundant metal after iron in a living body, but the amount of
4 Zn in meat, i.e., in skeletal muscle, is greater than that of iron (Hazell 1982,
5 Kagawa 2001). Since Parma ham is manufactured using only pork and sea salt,
6 it is thought that something in these two raw materials or microorganism
7 changes the Fe-porphyrin complex into a Zn-porphyrin complex. Ferrochelatase
8 (EC 4.99.1.1) catalyzes the insertion of Fe^{2+} into protoporphyrin IX in the final
9 step of heme synthesis (Porra and Jones, 1963a; Porra and Jones, 1963b).
10 However, this enzyme is involved in the insertion of not only Fe^{2+} but various
11 divalent metal ions, *e.g.*, Zn^{2+} , Co^{2+} , Ni^{2+} or Mn^{2+} into porphyrins (Taketani and
12 Tokunaga, 1981; Camadro and Labbe 1982; Bloomer, Reuter, Morton & Wehner,
13 1983). The level of Zn-chelating activity of ferrochelatase isolated from bovine
14 liver was higher than that of Fe-chelating activity (Taketani and Tokunaga 1982).
15 On the other hand, Zn is the second easiest metal after copper for insertion into
16 porphyrins and is easily inserted into porphyrin without the requirement of a
17 catalyst. Possible substitution systems are a non-enzymatic reaction, bacterial
18 enzymatic reaction or endogenous enzymatic reaction. However, if it is an
19 enzyme reaction, questions remain as to whether the endogenous enzyme activity
20 is maintained during the long manufacturing process of Parma ham and whether
21 bacterial enzymes permeate to the interior of ham. Future studies will focus on
22 mechanism of Fe-Zn substitution that occurs in Parma ham during the
23 manufacturing.

4. Conclusions

The absorption and fluorescence spectra of the red pigment extracted from Parma ham are very similar to those of Zn protoporphyrin IX. The results of HPLC analysis showed that the red pigment isolated from Parma ham with acetone had the same retention time as that of Zn protoporphyrin IX. The results of ESI-HR-MS analysis showed that the red pigment not only had a molecular weight similar to that of Zn protoporphyrin IX but also showed the characteristic pattern resulting from Zn. It is suggested that the bright red color in Parma ham is caused by Zn protoporphyrin IX in which the iron in the heme of myoglobin has been replaced by Zn.

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Figures and tables

Fig. 1 Absorption spectra of heme pigments extracted from Parma ham with distilled water (a), 75% acetone (b) and 75% acetone containing 0.7% HCl (c). The maximum absorption wavelengths are shown for the extracts.

Fig. 2 Absorption spectra of heme pigments extracted by the acid-butanone method (a) and then added one drop of conc. HCl (b). The maximum absorption wavelengths are shown for the extracts.

Fig. 3. Fluorescent spectra of heme pigments extracted from Parma ham with distilled water (a) and 75% acetone (b). The maximum absorption wavelengths are shown for the extracts.

Fig. 4. HPCL elution profile of the red pigment from Parma ham. *Traces 1 and 1'*, Standard solutions. *Traces 2 and 2'*, Parma ham extract. Peaks are as follows; *H*: hemin, *Z*: zinc protoporphyrin IX, *P*: protoporphyrin IX.

Fig. 5. ESI-HR mass spectra of the red pigment from Parma ham and the magnification (insert).

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