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Author(s)	Wakamatsu, J.; Nishimura, T.; Hattori, A.
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A Zn-porphyrin complex contributes to bright red color in Parma ham

J. Wakamatsu*, T. Nishimura and A. Hattori

Meat Science Laboratory, Division of Bioresource & Product Science
Graduate School of Agriculture, Hokkaido University
N-9, W-9, Kita-ku, Sapporo, Hokkaido 060-8589, JAPAN

* Corresponding Author

Jun-ichi Wakamatsu

Meat Science Laboratory, Division of Bioresource & Product Science
Graduate School of Agriculture, Hokkaido University
N-9, W-9, Kita-ku, Sapporo, Hokkaido 060-8589, JAPAN
(tel) +81-11-706-2547 (fax) +81-11-716-0879
(e-mail) jwaka@anim.agr.hokudai.ac.jp

1 # Abstract

2

3 The Italian traditional dry-cured ham (Parma ham) shows a stable bright red
4 color that is achieved without the use of nitrite and/or ni0trate. In this study we
5 examined the pigment spectroscopically, fluoroscopically and by using HPLC and
6 ESI-HR-MASS analysis. Porphyrin derivative other than acid hematin were
7 contained in the HCl-containing acetone extract from Parma ham. A strong
8 fluorescence peak at 588 nm and a weak fluorescence peak at 641 nm were
9 observed. By HPLC analysis the acetone extract of Parma ham was observed at
10 the single peak, which eluted at the same time as Zn protoporphyrin IX and
11 emitted fluorescence. The results of ESI-HR-MS analysis showed both
12 agreement with the molecular weight of Zn protoporphyrin IX and the
13 characteristic isotope pattern caused by Zn isotopes. These results suggest that
14 the bright red color in Parma ham is caused by Zn-protoporphyrin IX.

15

16

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

1 ## 1. Introduction

2

3 The color of meat and meat products are one of the important factors that
4 determine the quality and is an important factor in the criteria by which
5 consumers make selections. Myoglobin, which causes the red color of meat, is a
6 heme protein with the iron complex of porphyrin, and the color of myoglobin
7 depends on the kind of molecule coordinating to iron (Fox 1966; Ledward 1992;
8 Sakata 2000). Deoxymyoglobin, without a ligand attached to iron, is the main
9 constituent of meat that has just been cut, and deoxymyoglobin is later converted
10 into oxymyoglobin, with oxygen. With the passage of time or with heating,
11 oxymyoglobin is auto-oxidized and changes into metmyoglobin.
12 Myoglobin in meat products adding nitrate and/or nitrite is converted into
13 stable red nitrosylmyoglobin coordinated to nitric oxide, and nitrosylmyoglobin
14 changes into pink-reddish nitrosohemochromogen after the meat has been cooked.
15 By the way the north Italian traditional dry-cured ham "Prosciutto di Parma
16 (Parma ham)" is made from only the legs of fattened pigs and is salted with sea
17 salt, dried, and matured over a period of one year. Despite the fact that nitrite
18 and/or nitrate have not been added, the color is extremely stable bright red and is
19 not changed by exposure of the ham to light or heat. Morita, Niu, Sakata &
20 Nagata (1996) reported that the red heme pigment was easily extracted with 75%
21 acetone and that it is a new myoglobin derivative that is unknown in meat and
22 meat products. Recently it was reported that this lipophylic stable red pigment
23 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*
20
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,

1 Japan) and solubilized in methanol:0.01 M Na_2HPO_4 (76:24, v/v, pH 9). After
2 solubilization, the sample was filtered through 0.45 μm filter (DISMIC-3, Toyo
3 Roshi Co., Ltd., Tokyo, Japan). A Shodex Asahipak ODP-50 4D column (4.6 ×
4 150 mm, Showa Denko K.K., Tokyo, Japan) was used for separation of the red
5 pigment, and the separation was carried out by isocratic elution using the mobile
6 phase (methanol:0.01 M Na_2HPO_4=76:24, v/v, pH 9) at a flow rate of 0.5 ml/min
7 at an ambient temperature. Twenty microliters of each sample was injected.
8 The eluent was monitored at 415 nm for excitation and at 590 nm for emission
9 wavelengths.
10
11 *2.6. Electrospray ionization high resolution mass spectrometry (ESI-HR-MS) analysis*
12
13 ESI-HR-MS analysis of the unknown red pigment isolated from Parma ham
14 was carried out using a JMS-SX120A (JEOL Ltd., Tokyo, Japan) equipped with
15 an ESI ion source (JEOL MS-ESI 10, JEOL Ltd., Tokyo, Japan). The sample
16 diluted in a chloroform/methanol/acetone (1:1:8 v/v) solvent mixture was infused
17 into the ESI ion source at a flow rate of 1 μl/min. The needle voltage and
18 capillary voltage were 2681 V and -1230 V, respectively. The chamber
19 temperature was set to 105 ˚C. A mixture of PEGs was used as an internal
20 standard.
21
22

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, Schwatcz, 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

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

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

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

Zn is the most abundant metal after iron in a living body, but the amount of Zn in meat, i.e., in skeletal muscle, is greater than that of iron (Hazell 1982, Kagawa 2001). Since Parma ham is manufactured using only pork and sea salt, it is thought that something in these two raw materials or microorganism changes the Fe-porphyrin complex into a Zn-porphyrin complex. Ferrochelatase (EC 4.99.1.1) catalyzes the insertion of Fe^{2+} into protoporphyrin IX in the final step of heme synthesis (Porra and Jones, 1963a; Porra and Jones, 1963b). However, this enzyme is involved in the insertion of not only Fe^{2+} but various divalent metal ions, *e.g.,* Zn^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+} into porphyrins (Taketani and Tokunaga, 1981; Camadro and Labbe 1982; Bloomer, Reuter, Morton & Wehner, 1983). The level of Zn-chelating activity of ferrochelatase isolated from bovine liver was higher than that of Fe-chelating activity (Taketani and Tokunaga 1982). On the other hand, Zn is the second easiest metal after copper for insertion into porphyrins and is easily inserted into porphyrin without the requirement of a catalyst. Possible substitution systems are a non-enzymatic reaction, bacterial enzymatic reaction or endogenous enzymatic reaction. However, if it is an enzyme reaction, questions remain as to whether the endogenous enzyme activity is maintained during the long manufacturing process of Parma ham and whether bacterial enzymes permeate to the interior of ham. Future studies will focus on mechanism of Fe-Zn substitution that occurs in Parma ham during the 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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1 Figures and tables
2
3 Fig. 1 Absorption spectra of heme pigments extracted from Parma ham with
4 distilled water (a), 75% acetone (b) and 75% acetone containing 0.7% HCl
5 (c). The maximum absorption wavelengths are shown for the extracts.
6
7 Fig. 2 Absorption spectra of heme pigments extracted by the acid-butanone
8 method (a) and then added one drop of conc. HCl (b). The maximum
9 absorption wavelengths are shown for the extracts.
10
11 Fig. 3. Fluorescent spectra of heme pigments extracted from Parma ham with
12 distilled water (a) and 75% acetone (b). The maximum absorption
13 wavelengths are shown for the extracts.
14
15 Fig. 4. HPCL elution profile of the red pigment from Parma ham.
16 *Traces 1 and 1'*, Standard solutions. *Traces 2 and 2'*, Parma ham extract.
17 Peaks are as follows; *H*: hemin, *Z*: zinc protoporphyrin IX, *P*:
18 protoporphyrin IX.
19
20 Fig. 5. ESI-HR mass spectra of the red pigment from Parma ham and the
21 magnification (insert).

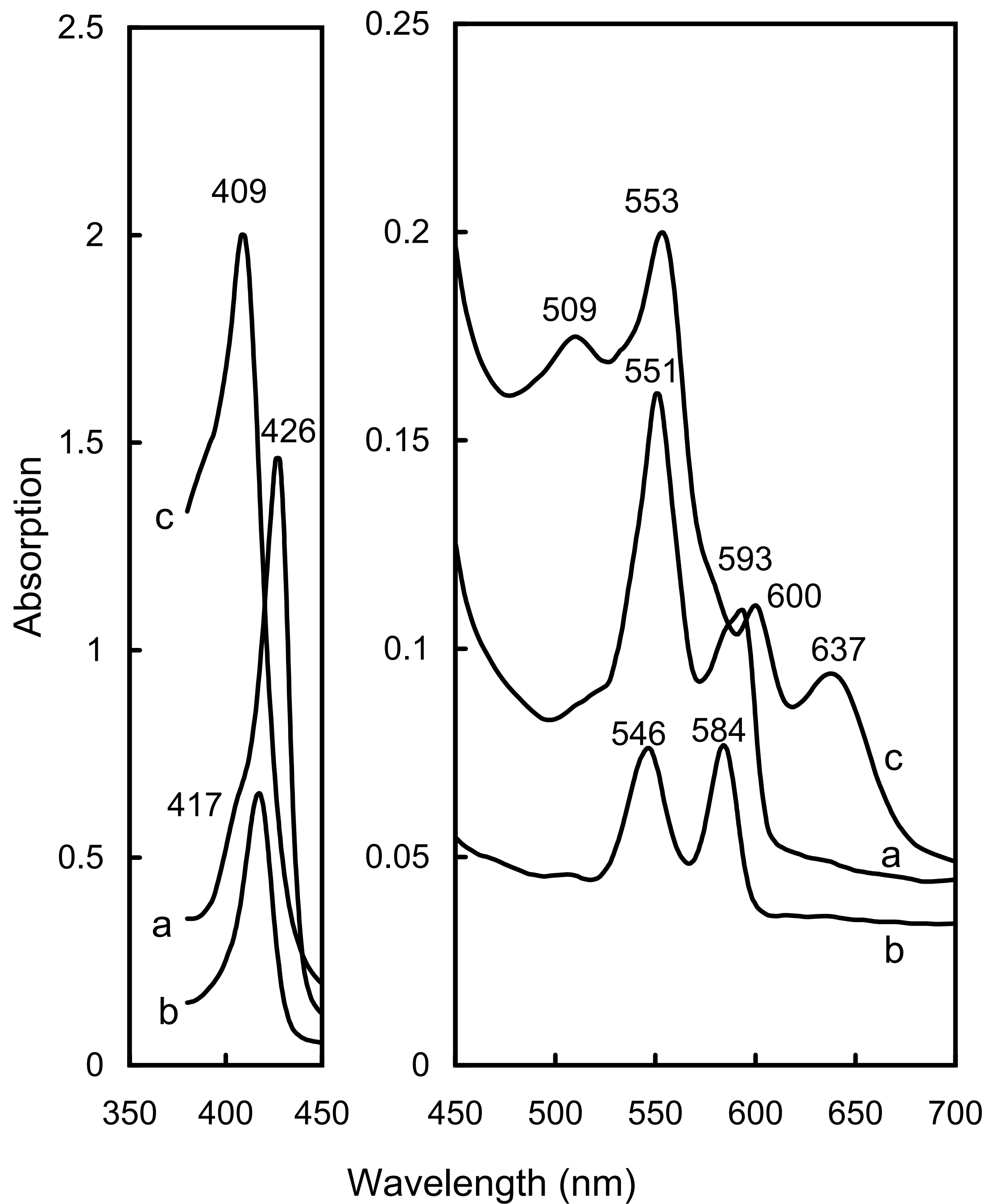

Fig. 1 Absorption spectra of heme pigments extracted from Parma ham with distilled water (a), 75% acetone (b) and 75% acetone containing 0.7% HCl (c). The maximum absorption wavelengths are shown for the extracts.

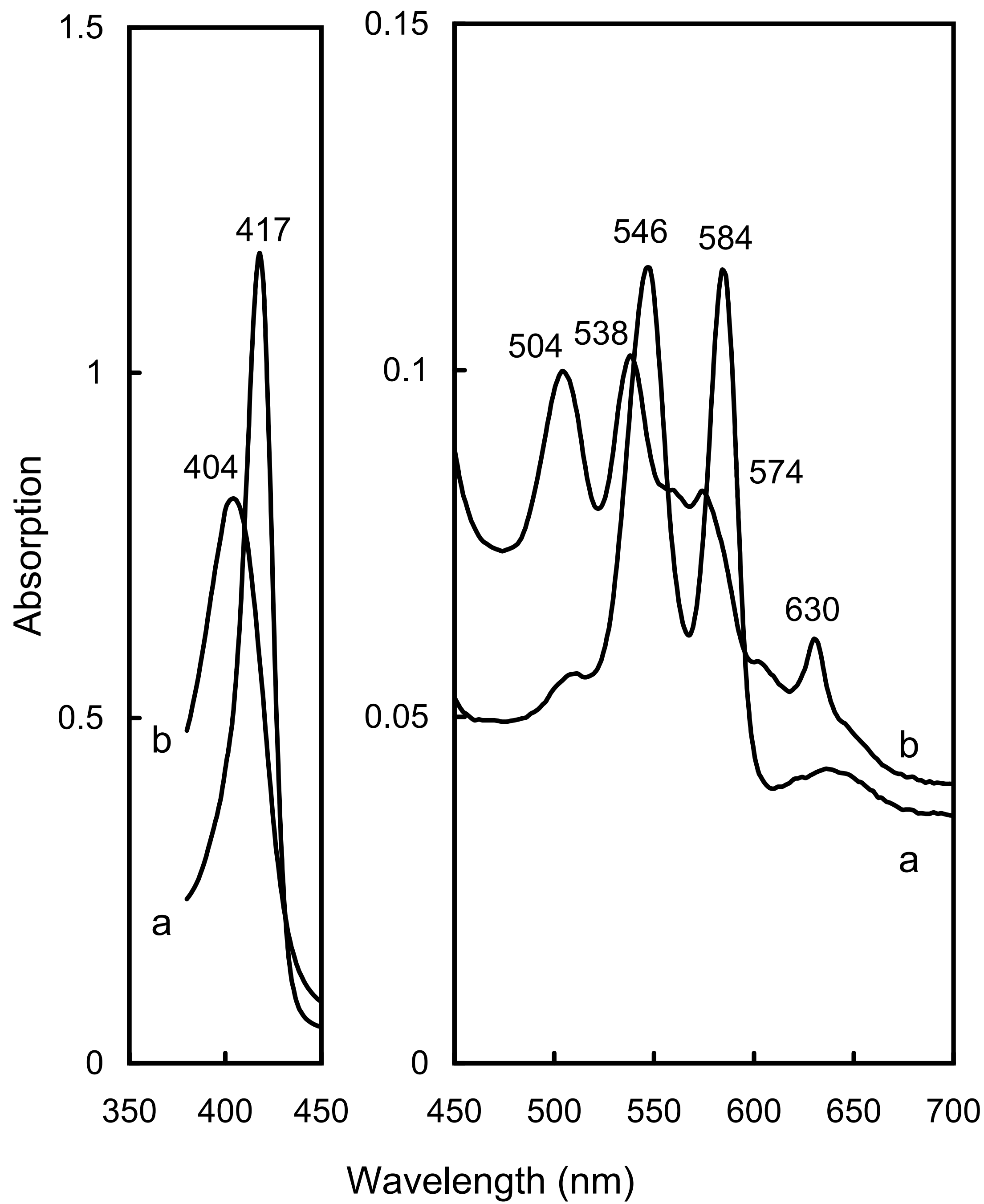

Fig. 2 Absorption spectra of heme pigments extracted by the acid-butanone method (a) and then with one drop of conc. HCl added (b). The maximum absorption wavelengths are shown for the extracts.

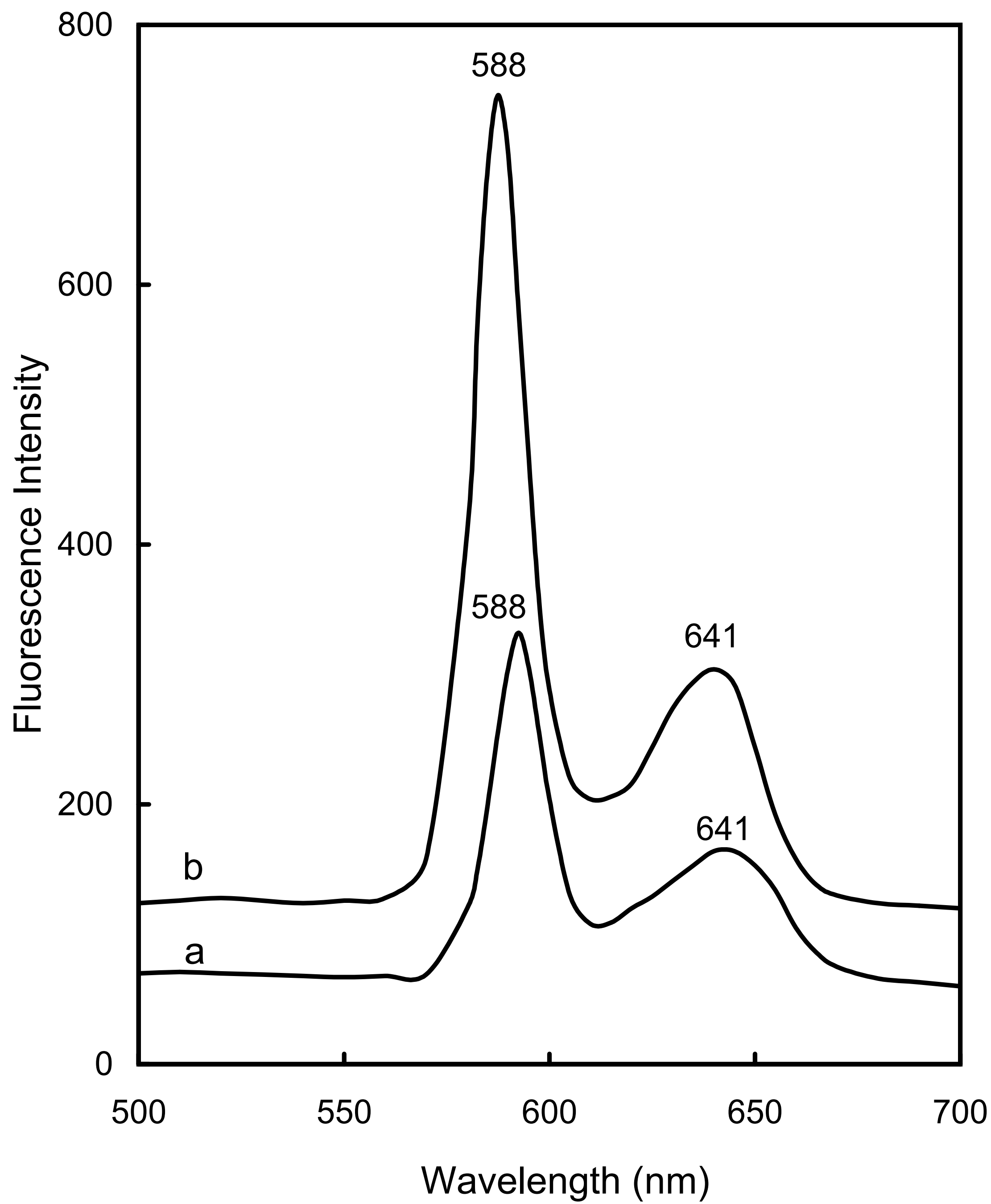

Fig. 3 Fluorescent spectra of heme pigments extracted from Parma ham with distilled water (a) and 75% acetone (b). The maximum absorption wavelengths are shown for the extracts.

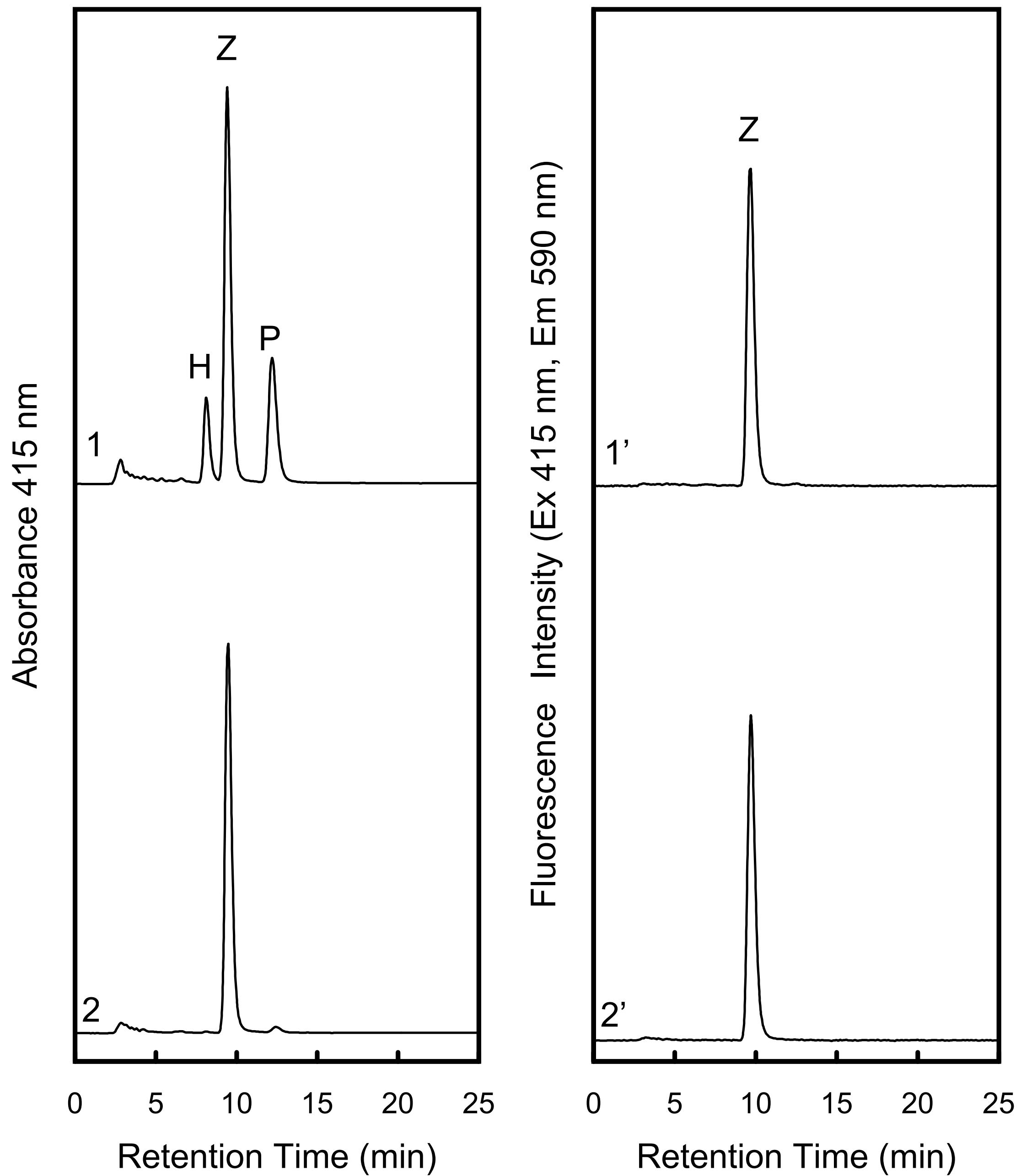

Fig. 4 HPCL elution profile of the red pigment from Parma ham. Traces 1 and 1’, Standard solutions. Traces 2 and 2’, Parma ham extract. Peaks are as follows; H: hemin, Z: zinc protoporphyrin IX, P: protoporphyrin IX.

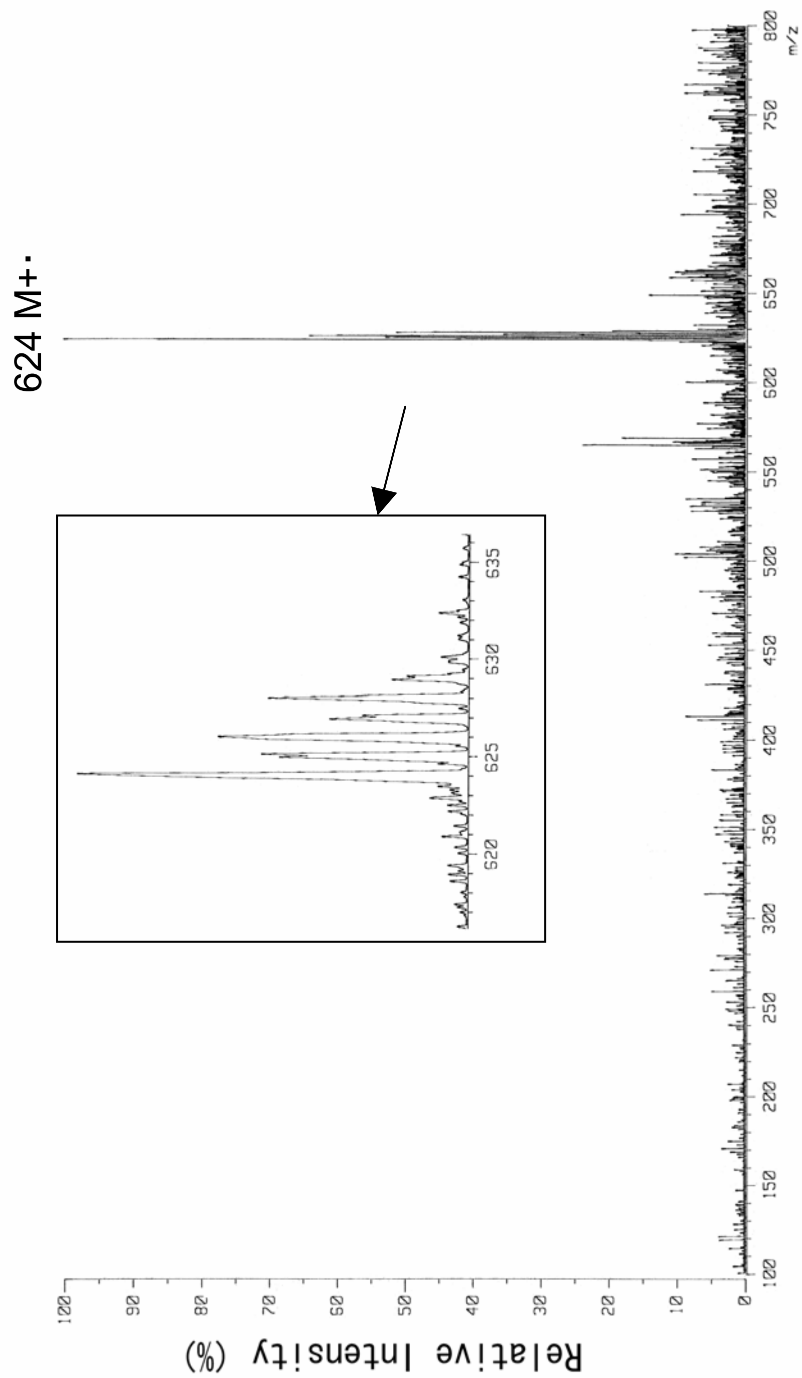

Fig. 5 ESI-HR mass spectra of the red pigment from Parma ham and the magnification (insert).