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https://doi.org/10.5109/4716

出版情報：九州大学大学院農学研究院紀要. 51 (1), pp.93-97, 2006-02-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **51** (1), 93–97 (2006)

Formation of Zinc Protoporphyrin IX from Myoglobin with Pork Loin Extract

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(Received October 27, 2005 and accepted November 16, 2005)

The formation of zinc protoporphyrin IX (ZPP) with pork loin extract was determined by visible absorption and fluorescent spectral analyses. After the anaerobic incubation (in the dark at pH 5.5 and 30°C) of the extract with metmyoglobin and $ZnCl_2$, characteristic peaks in absorption and spectra were observed at 417, 546, and 584 nm. In fluorescent spectra, a peak was observed at 589 nm. Formed amounts of ZPP estimated from the fluorescent intensity at 589 nm was 15.5 nmol/mL–pork extract. The difference in the formations of ZPP under aerobic and anaerobic incubations was not significant. The ZPP formation was significantly facilitated by the use of ATP. Zinc chelatase activity of loin extract was assayed with protoporphyrin IX and it was estimated as 42 mU/mL–extract. The Fe–Zn substituting activity of the extract was assayed with myoglobin as a substrate. Under the assay conditions at pH 5.5 and 40°C, ZPP was markedly increased with increasing time by using oxymyoglobin reduced with ascorbate, while little increase was observed with metmyoglobin. The activity was estimated as 4.1 mU/mL–extract with oxymyoglobin. At pH 5.5–7.0, higher activities were observed at lower pH.

INTRODUCTION

Myoglobin is a main contributing factor to the color of meat and meat products, and therefore their sensory quality. Myoglobin is liable to oxidize during storage or heating (Sakata, 2000). Undesirable discoloration of meat is due to the oxidation of myoglobin (accumulation of brown metmyoglobin) (Renerre, 1990). Nitrite is usually added to cured–meat products to prevent the oxidation of myoglobin. However, nitrate has a possibility of risk for human health (Cassens *et al.*, 1979, and Shahidi, 1991), and hence, there has been a growing demand for nitrite–free meat products and an intensified effort to develop a substitute for nitrite.

Recently, highly stable red pigment to light and heating has been found in Parma ham, a traditional dry cured ham of Parma (Italy) (Sakata, 2000). Parma ham is produced using only sea salt (from the Mediterranean Sea) without nitrate. The red pigment of Parma ham is easily extracted with 75% acetone, and the visible absorption spectral pattern is distinct from those of other known myoglobin derivatives (Sakata, 2000). The red pigment is gradually transformed from a myoglobin derivative into a thermostable non–protein heme complex (Moller *et al.*, 2003). Wakamatsu *et al.* (2004a) has been identified that the red pigment as Zn–protoporphyrin IX (ZPP), in which the heme iron is substituted by zinc. In addition, they reported the formation behavior of ZPP with pork loin homogenates (Wakamatsu *et al.*, 2004b).

The purpose of this paper is to clarify the ability of pork loin extract to form ZPP. The formation of ZPP with the loin extract was invetigate in detail, and then determined the several factors affecting on the ZPP formation. In addition, we determined zinc chelatase activity of the loin extract with protoporphyrin IX as a substrate to clarify the mechanism of the ZPP formation from myoglobin. Wakamatsu *et al.* (2004b) also investigated the ZPP formation with pork loin extract, but zinc chelatase activity have not been estimated. Thus, this is the first report on zinc chelatase activity of the loin extract. In addition, the Fe–Zn substituting activity, which is the combined activity of Fe–releasing from heme and Zn–chelating into heme, was assayed. Then, we investigated some factors affecting on zinc chelatase and the Fe–Zn substituting activities to characterize the ability of the extract on the ZPP formation.

MATERIALS AND METHODS

Materials

Fresh pork loin (Landrace, 6 months old, 110–115 kg of body weight) sample was obtained from Itoham Foods Inc. (Ibaraki, Japan), and stored at −40°C until use. Myoglobin (metmyoglobin) from horse skeletal muscle was purchased from Wako Pure Chemical Industries, Itd. (Osaka, Japan). Zn–protoporphyrin IX disodium salt was purchased from Aldrich Chem.Co. (Wisconsin, USA) and used as a standard of ZPP. Disposable O_2 absorbing and CO_2 generating agent and oxygen indicator tablet were purchased from Mitsubishi Gas Chemical Co., Inc. (Tokyo, Japan). All other chemicals used in this study were of analytical reagent grade.

Preparation of pork loin extract

Pork loin was homogenized with an equal volume of 50 mM of sodium phosphate buffer (pH 5.5) containing 150 mM NaCl. Each homogenization was performed at 10,000 rpm for 5 min with polytron homogenizer (Kinematica, Inc. USA). Each homogenate was soni-

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cated (Branson Sonifier 250, Branson Ultrasonics Co., USA) at 200 W for 1 min, and then centrifuged at 10,000×g for 20 min. Each supernatant (pork extract) was filtrated with the membrane filter (0.45 μm) and used in the following study. The protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, USA) using bovine serum albumin as a standard.

Anaerobic incubation of sample solution

Each pork extract (2 mL) was mixed with 1 mL of metmyoglobin solution (final conc. of 2 mg/mL) in a 10 mL-container. In the case with reduced-myoglobin (oxymyoglobin) as a substrate, reduced-myoglobin was prepared by the addition of sodium ascorbate (4 mg/mg-myoglobin) before the mixing with pork extract. Then, 100 μL of $ZnCl_2$ (final conc. of 250 μg/mL) and 1.9 mL of 50 mM sodium phosphate buffer were added. Each solution was put into the gas-impermeable container and kept in dark under anaerobic condition at 30 °C. The anaerobic condition (oxygen concentration in the container; <0.1%) was achieved by using the disposable O_2 absorbing and CO_2 generating agent and checked with an oxygen indicator tablet. After incubation, each solution was centrifuged at 3,000×g for 20 min. The supernatant containing myoglobin was applied to the visible absorption spectral analysis. On the other hand, water-insoluble red pigment (ZPP) was extracted from the precipitate with 75% acetone and applied to visible absorption and fluorescent spectral analyses.

Visible absorption and fluorescent spectral analyses

Visible absorption spectra of myoglobin and ZPP were measured at 400-700 nm by using a spectrophotometer with the photodiode array (Multispec 1500, Shimadzu Co., Ltd., Kyoto, Japan). Fluorescent spectra of ZPP were measured at 550-650 nm at 410 nm for excitation by using a fluorescence spectrophotometer (Model 5300, Shimadzu Co., Ltd., Kyoto, Japan). The formed amount of ZPP (nmol/mL-pork extract) after the storage was determined by using the standard curve for ZPP preparation (0.08-1.6 μmol/mL-75% acetone, r =0.999).

Assays of zinc chelatase and Fe-Zn substituting activities

Assay of zinc chelatase activity was performed by modifying the fluorimetric method described by Goldin and Little (1969). Each pork extract (0.4 mL) was mixed with equal volume of 50 mM sodium phosphate buffer containing 2.5 mg of ATP, and then 100 μL of 1 mM $ZnCl_2$ and 100 μL of 90 μM protoporphyrin IX solution were added. The solutions were kept at 40 °C in water bath under aerobic condition. On the other hand, the anaerobic incubation was performed by using the solution degassed with a vacuum pump, and the container of the solution was sealed and kept at 40 °C in a water bath. After incubation, 3 mL of acetone was added to the solution and each solution was centrifuged at 3,000×g for 20 min. Formed red pigment (ZPP) in 75% acetone was measured by the fluorescent spectral analysis. In addition, Fe-Zn substituting activity was assayed by using oxymyoglobin reduced by sodium ascorbate as a substrate in a similar manner as above.

Formed amount of ZPP (nmol/mL-pork extract) after the incubation was determined by fluorescent spectral analysis as described above. One unit of the activity (zinc chelatase activity and Fe-Zn substituting activity) is defined as the amount of enzyme that catalyzes the formation of one nmol of ZPP in one minute at 40 °C under assay condition.

Statistical analysis. Results are presented as means± SD and analyzed using paired Student's t-test. All statistical analyses were performed using Stat View software version 5.0J (SAS institute Inc. USA).

RESULTS AND DISCUSSION

ZPP formation with pork loin extract after the anaerobic incubation

The ZPP formation with pork loin extract was determined by visible absorption and fluorescent spectral analyses. Each incubation was performed at pH 5.5 because the ZPP formation occurrs in Parma ham, whose pH was reported as 5.7 (dry-cured ham; pH 5.5) by Adamsen *et al.* (2004). Fig. 1 shows the visible absorption and fluorescent spectra of red pigment (in 75% acetone extracts) formed after the incubation the loin extract. The incubation time was set at 168 h, because very slow formation of ZPP was observed with loin extract. Characteristic peaks in absorption spectra were observed at 417, 546, and 584 nm in both samples. In fluorescent spectra (Ex; 410 nm), a peak at 589 nm was observed. We confirmed that these spectral patterns coincide with that of ZPP preparation and the red pigment extracted from Parma ham (Data not shown). These results were in agreement with those of Wakamatsu *et al.* (2004a, b). Formed amounts of ZPP

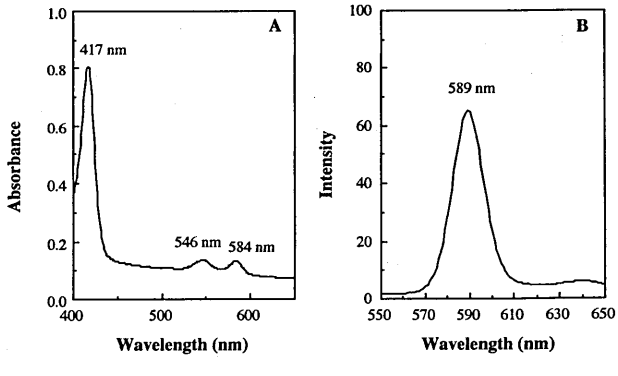

Fig. 1. The visible absorption (A) and fluorescent spectra (B) of red pigment (in 75% acetone) formed after the anaerobic incubation with pork loin extract. Each incubation was done in the dark at pH 5.5 and 30 °C with metmyoglobin and $ZnCl_2$ (final conc.; 2 mg/mL and 250 μg/mL, respectively).

estimated from the fluorescent intensity at 589 nm was 15.5 nmol/mL–pork extract.

ZPP formations with pork loin extract under the aerobic and anaerobic incubations

The ZPP formation behaviors under anaerobic and aerobic conditions were compared. In both incubations, metmyoglobin was incubated with the loin extract and $ZnCl_2$. The aerobic incubation was done without O_2 absorbing and CO_2 generating agent. Fig. 2 shows the formed amounts of ZPP after the anaerobic and aerobic incubations for 168 h. In both incubations, similar intensity was observed, and aerobic incubation resulted in the formation of 95% of anaerobic incubation. These results suggested that anaerobic condition is not essential for the formation of ZPP. Wakamatsu *et al.* (2004b) studied the effects of oxygen on the formation of ZPP with pork loin homogenates. They concluded that oxygen inhibits the ZPP formation because this formation appeared to be minimal after aerobic incubation. However, the formation of ZPP was not appreciably inhibited by oxygen in our present study. Moreover, the reduction of metmyoglobin was also observed under aerobic incubation. After aerobic condition, the sample solution changed to bright red color and the reduction of metmyoglobin was confirmed by visible absorption spectra (Data not shown). Dean and Ball (1960) have been also reported that metmyoglobin is reduced during the aerobic storage by a natural metmyoglobin–reducing system in meat. Hence, oxygen would little affects the reduction of metmyoglobin. As described above, we assumed that zinc chelatase is responsible for the formation of ZPP. Zinc chelatase acts even under the aerobic condition (Nunn *et al.*, 1988), and hence, the ZPP formation would be little affected by oxygen.

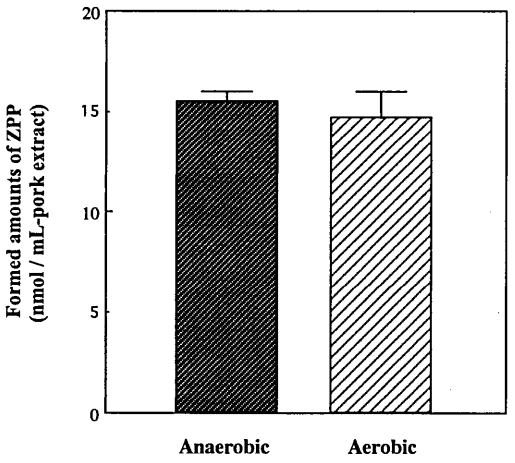

Fig. 2. Formed amounts of ZPP after the anaerobic and aerobic incubations. Both incubations were carried out as same in Fig. 1. Data represent the mean±SD (n=5).

The effect of ATP on the formation of ZPP with the loin extract

The effect of ATP on the formation of ZPP was examined because zinc chelatase activity was increased by the addition of ATP (Goldin and Little, 1969). They demonstrated that ATP consistently stimulates the activity 1.5–2.5 fold at the concentration of 2.5 mg/mL–reaction mixture. In the present study, metmyoglobin was incubated with the extract and $ZnCl_2$ in the dark at 30 °C under anaerobic condition for 168 h, and ATP was added to the incubating–sample at the concentration of 2.5 mg/mL. Fig. 3 shows the formation of ZPP with and without ATP. As a result, the formation was significantly increased by the addition of ATP. This result suggested that zinc chelatase in heart extract could be activated by the addition of ATP. Goldin and Little (1969) suggested that Zn–ATP complex may serve as a more effective metal donor to form ZPP than free–Zn. However, Zn–ATP complex would not be formed under our assay condition at pH 5.5, because ATP would be little ionized at the acidic pH. Thus, the direct binding of ATP to zinc chelatase may stimulate the formation of ZPP.

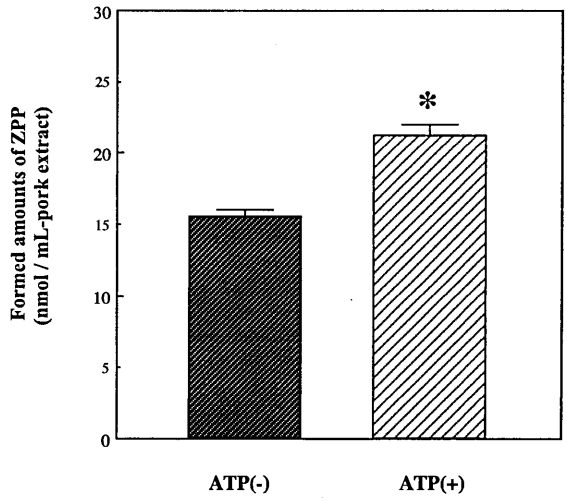

Fig. 3. The effect of ATP on the formation of ZPP by the anaerobic incubation. Data represent the mean±SD (n=5). * $P<0.01$ vs. ATP (–).

Zinc chelatase activity of the pork extract

We determined zinc chelatase activity of pork loin extract with protoporphyrin IX as a substrate. Fig. 4 shows the formation behavior of ZPP during the aerobic incubation. ZPP was increased with increasing time, and linear ZPP formation was observed until 120 min. Zinc chelatase activity was calculated from the slope of the linear curve ($r=1.000$). The activity of the extract was estimated as 42 mU/mL–extract (Fig. 5). In addition, the specific activity was estimated as 0.81 mU/mg–protein. On the other hand, little formation of ZPP was observed with the heart extract treated by heating at 80 °C and 15 min (Data not shown). This result suggested that nonenzymatically insertion of Zn into protoporphyrin IX would not occur under our

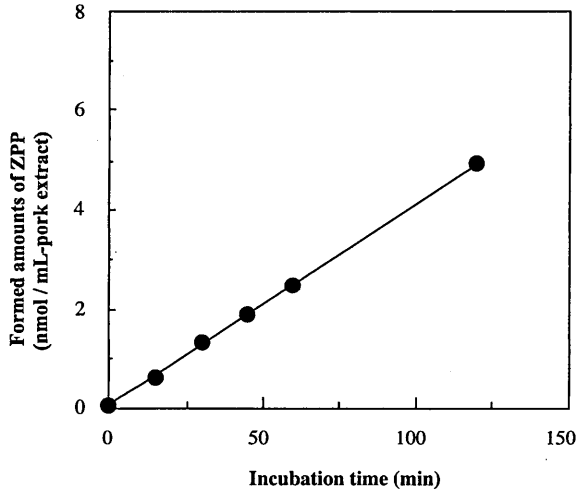

Fig. 4. The formation behavior of ZPP from protoporphyrin IX with the loin extract under the aerobic incubation.

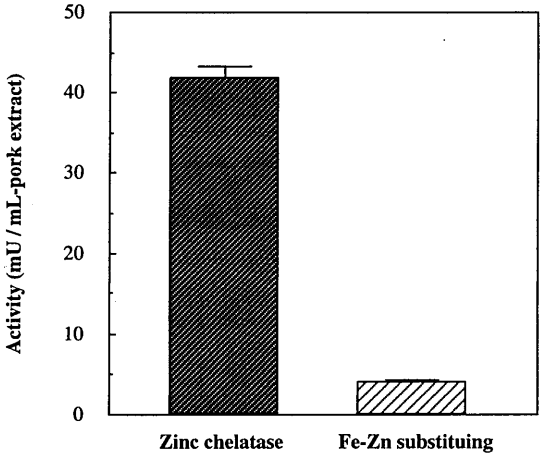

Fig. 5. Zinc chelatase and Fe–Zn substituting activities of the loin extract under aerobic condition. Data represent the mean±SD (n=5).

experimental conditions. Wakamatsu *et al.* (2004b) was investigated the effects of pre–heating of pork loin homogenate on the ZPP formation from myoglobin. It has been shown that the ZPP formation was decreased with increasing of the pre–heating temperature, and no formation was observed at higher than 60 °C. They also suggested that the ZPP formation would be caused by the action of pork meat endogenous enzymes.

We examined the effect of oxygen on zinc chelatase activity. The activities under both conditions were nearly equal and there was no significant difference (Data not shown). Zinc chelatase from other source has been also assayed under aerobic condition (Nunn *et al.*, 1988; Camadro and Labbe, 1982, and Goldin and Little, 1969). Their results showed that oxygen inhibits ferrochelatase activity, but does not inhibit zinc chelatase activity, and suggest that anaerobic condition is not an essential condition for the ZPP formation with zinc chelatase.

The Fe–Zn substituting activity of the pork extract

The Fe–Zn substituting activitiy of the pork extract was assayed with oxymyoglobin as a substrate. Oxymyoglobin was prepared by reducing metmyoglobin with sodium ascorbate (4 mg/mg–myoglobin), and confirmed by the visible absorption analysis. Under the assay conditions at pH 5.5 and 40 °C, the linear formation of ZPP was observed with oxymyoglobin and the activity was calculated from the slope of the linear formation curve, while little formation of ZPP was observed with metmyoglobin (data not shown). In the present assay system with oxymyoglobin, it was possible to estimated the activity of the loin extract within 6 hours, while 5 days–incubation was needed for the assay system by Wakamatsu *et al.* (2004b). The Fe–Zn substituting activity of the loin extract was shown in Fig. 5. The activity was estimated as 4.1 mU/mL–extract with oxymyoglobin. The specific activity was 0.08 mU/mg–protein. The Fe–Zn substituting activity of the extract was ~10 times lower than their zinc chelatase activity. The release of Fe from heme would be rate–determining step on the ZPP formation from myoglobin.

Effect of pH on zinc chelatase and Fe–Zn substituting activities of the loin extract

The effect of pH on zinc chelatase and Fe–Zn substituting activities of pork extract were investigated. Fig. 6 shows these activities of the loin extract over the range of pH 5.5–7.0. As a result, increase in zinc chelatase activity of the extract were observed at lower pH. At pH 5.5, ~1.4 times higher activity was observed compared with that of at pH 7.0. The optimum pH of zinc chelatase varies according to their origin. Zinc chelatase from barley (Goldin and Little, 1969) and yeast (Camadro and Labbe, 1982) have the optimum pH at 8.0 and 7.5, respectively. On the other hand, zinc chelatase of human lymphocytes has the optimum pH at 5.5 (Nunn *et al.*, 1988). The activity profiles of the porcine extracts resemble their activity profile. Dailey *et al.* (1986) suggested that mammalian ferrochelatase

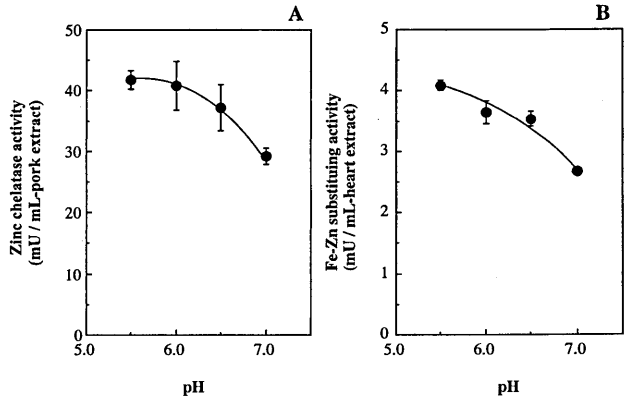

Fig. 6. The effect of pH on zinc chelatase activity (A) and Fe–Zn substituting activity (B) of the loin extract. Each incubation was carried out at 40 °C. Data represent the mean±SD (n=3).

shares many common characteristics. Similarly, mammalian zinc chelatase may exhibit similar activity profile on pH. The Fe–Zn substituting activity of the extract also increased with the decrease in pH, and the profiles were similar to those of zinc chelatase activity. Lower pH may also have effect on the release of iron from porphyrin ring of myoglobin.

Conclusions

The anaerobic incubation with pork loin extract resulted in the formation of ZPP. The formation was little affected by oxygen. The ZPP formation was stimulated in the presence of ATP. Zinc chelatase and Fe–Zn substituting activities were determined in the present study. The lower pH resulted in the higher activities. These activity profiles suggested that zinc chelatase would be closely related to ZPP formation.

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