Summary of PhD thesis

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Study on the ZnPP formation mechanism in two different optimum pH at 4.75 and 5.5 in pork

(異なる2 つの至適pH 4.75 と5.5 における豚肉のZnPP 形成機構に関する研究)

1. Introduction

Zinc protoporphyrin IX (ZnPP) is a bright red pigment formed in meat products without nitrate or nitrite. Wakamatsu et al. (2004) identified ZnPP as the main pigment in Italian dry-cured Parma ham (*Prosciutto di Parma*). ZnPP formation was also confirmed in several other studies focusing on production of nitrite-free meat products, such as dry-cured Iberian hams and dry-fermented sausages (Adamsen et al., 2006). Therefore, there is significant interest in clarifying the mechanisms of ZnPP formation in meat products, since it can play an important role in coloration without nitrite or nitrate.

For several years researchers have studied the mechanism of ZnPP formation in meat products. Although ZnPP formation mechanisms in meat products are not completely elucidated yet; three possible mechanisms have been suggested for this red pigment formation in meat products (Wakamatsu et al., 2004): a) a non-enzymatic reaction in which ZnPP is formed under anaerobic conditions; b) enzymatic reactions where FECH is directly involved; and c) bacterial enzymatic reactions. Nevertheless, ZnPP formation mechanism in meat and meat products is still imprecise.

In Parma ham, ZnPP was first proposed to be formed from myoglobin by a transmetallation process in which iron (II) ion is substituted by zinc (II) (Wakamatsu et al., 2004). Myoglobin is a major heme protein in meat and has been considered as a heme donor in ZnPP formation. It was also reported that exogenous recombinant yeast FECH facilitates ZnPP formation from myoglobin-heme and heme in meat via the replacement of iron in the protoporphyrin ring by zinc ions (Chau et al., 2011). However, FECH (42 kDa) is considerably greater than that of myoglobin (17 kDa) since it is suspected that it would be difficult for FECH to assist the de-ironing of heme located in the globin pocket of myoglobin. Subsequently, Wakamatsu et al. (2019) reported that significant myoglobin degradation was observed at pH 4.75 but the degradation of myoglobin did not occur during ZnPP formation in model solution. Therefore, more detail examination about the role of myoglobin is required.

It was also reported that the amount of ZnPP peaked at pH 5.5 using *longissimus* muscle and decreased considerably at lower or higher pH (Wakamatsu et al., 2007). Recently, it has found a new optimum pH of ZnPP formation at 4.75 using *infraspinatus* muscle (Wakamatsu et al., 2019). The pH considerably influences enzymatic activity in biological processes. The pH of fresh meat is considered a critical factor for FECH activity in ZnPP formation.

PPIX is an important precursor to biologically essential prosthetic groups such as heme. When the amounts of bioavailable iron in the reticulocyte are low, FECH inserts zinc into PPIX instead of iron and raised ZnPP (Lamola & Yamane, 1974). Investigations of PPIX formation using the *longissimus* muscle as an experimental system have shown that ZnPP and PPIX formation pattern are similar (Wakamatsu et al., 2007). Therefore, a full understanding of PPIX formation will be worthwhile to elucidate ZnPP formation mechanism in meat products.

Although it is assumed that FECH, myoglobin as heme donor, and Zn^{2+} play important roles in ZnPP formation in meat, the precise mechanism is not well understood. FECH is located at the inner membrane of the mitochondria in mammalian cells (Taketani, 1993) and hence is relatively insoluble. On the other hand, myoglobin is distributed throughout the cytoplasm (Ordway, 2004) and is a water-soluble component. FECH and myoglobin are in different locations in muscle; hence, separation of muscle fractions that contains FECH and myoglobin is important to clarify their contribution to ZnPP formation in meat.

In this study, it was hypothesized that ZnPP formation depends on PPIX formation at pH 4.75, multiple components in pork homogenate are essential to ZnPP and PPIX formation at pH 4.75 and 5.5, and the contributors or precursors for ZnPP formation are different between both pH conditions. Therefore, to elucidate ZnPP formation mechanism, the objectives of this research were (a) to investigate the factors that affect ZnPP and PPIX formation at the newly discovered optimum pH 4.75, (b) to search for the contributors present in pork responsible for ZnPP and PPIX formation at pH 4.75 and 5.5, (c) to verify the involvement of myoglobin or other water-soluble protein(s) contributed to ZnPP and PPIX formation at pH 4.75 and 5.5.

2. Materials and methods

2.1. Materials

Porcine *longissimus dorsi* (LD) and *infraspinatus* (IS) muscle were used from three primal cuts of common pigs produced in Hokkaido.

2.2. ZnPP and PPIX formation model system using pork homogenate

Pork homogenates were prepared as previously described (Wakamatsu et al., 2007). PPIX formation in the homogenates was investigated after adding 0.5 mM EDTA. The solutions were incubated anaerobically at 25°C for 5 and 10 days in darkness. $NaNO_2$ (final concentrations of 0,

5, 10, 15, 20, 25, and 30 μM) and N-methyl mesoporphyrin (N-MMP; final concentrations of 0, 0.05, 0.1, 0.5, 1, and 5 μM) were added to the homogenates before incubation.

2.3. Fractionation of IS and LD muscle

Pork IS and LD (20%) were separately homogenized with ultrapure water and adjusted to pH 4.75 and 5.5 respectively with diluted hydrochloric acid (HCl). Then the homogenate was centrifuged and the supernatant was filtered through a filter paper to yield the filtrate as "the soluble fraction". The precipitate was diluted with ultrapure water up to initial volume and then homogenized and centrifuged in the same as before for two more times to completely remove the water-soluble compounds. The precipitate was homogenized with ultrapure water up to initial volume to yield "the insoluble fraction". Moreover, the soluble fraction was dispensed into an ultrafiltration spin column (VIVASPIN 20; 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd.) through a sterile syringe filter. It was then separated by centrifugation and the filtrate was "the <10 kDa soluble fraction" and the residue was diluted with ultrapure water up to initial volume and regarded "the >10 kDa soluble fraction". After separation, the >10 and <10 kDa soluble fractions were heat-treated separately for 30 minutes at 100°C.

2.4. Separation of water-soluble protein by gel filtration chromatography

The water-soluble fraction of IS and LD were concentrated separately using an ultrafiltration spin column (VIVASPIN 20; 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd.) by centrifugation. The concentrated >10 kDa soluble fraction was applied to a gel filtration column. The gel filtration column for IS at pH 4.75 was used Toyopearl HW 55F (3.3 φ × 60 cm, Tosoh Corporation, Tokyo, Japan) and for LD at pH 5.5 was used Toyopearl HW 50 (2.5 φ × 50 cm, Tosoh Corporation). The mobile phase consisted of 50 mM sodium chloride and 10 mM citrate buffer (pH 4.75 for IS and 5.5 for LD). The flow rate was 15 ml/h. The absorbance of the collected fractions was measured at 280 nm for protein and at 400 nm for myoglobin content.

2.5. Separation of water-soluble proteins by cation exchange chromatography

First, the LD water-soluble fraction was subjected to a CM-Toyopearl gel column equilibrated with 10 mM citrate buffer (pH 4.75-8.0) and unadsorbed fractions were collected.

Then, the LD water-soluble fraction was subjected to a CM-Toyopearl gel column equilibrated with 10 mM citrate buffer of pH 8.0 (0 M NaCl). The flow rate was 1.0 ml/min. The unadsorbed fraction was concentrated and adjusted to pH 7.5. Then the unadsorbed fraction was again applied to the gel column equilibrated with 10 mM citrate buffer of pH 7.5 (0 M NaCl) and subsequently eluted with 10 mM citrate buffer pH 7.5 (0.6 M NaCl). The obtained fractions were concentrated and the pH 7.5 buffer (0.6 M NaCl) eluted fraction was dialyzed against ultrapure water to remove the salt.

2.6. Model experiment for verification of ZnPP and PPIX formation by separated fractions

Regarding IS and LD muscle homogenate fractions, single fraction and mixed fractions were applied to ZnPP and PPIX formation model experiment systems. The amount of separated fractions to model experiments were 0.25 ml insoluble fraction, 0.5 ml soluble fraction, 0.5 ml >10 kDa soluble fraction and 0.5 ml <10 kDa soluble fraction in the single or mixed fractions. Other separated fractions were applied to the model experiment as separated fraction 0.5 ml, insoluble fraction 0.25 ml, <10 kDa soluble fraction 0.5 ml. Then, antibiotics and EDTA were added to the mixture of fractions and the final volume of each sample was diluted up to 1.5 ml with ultrapure water. The incubation condition for IS was at 37°C and LD was 25°C for 5 days.

2.7. Fluorescence analysis

Acetone extraction and fluorescence analysis were performed as previously described (Wakamatsu et al., 2004).

2.8. Statistical analysis

Statistical analyses were performed using Microsoft Excel 2007 with Ekuseru-Toukei 2006 (Social Survey Research Information Co., Ltd., Tokyo, Japan) for add-in software. Differences among individuals were evaluated by one-way analysis of variance with Tukey's multiple comparison tests. $P < 0.05$ was considered statistically significant.

Results

3.1. Mechanism of ZnPP formation in IS muscle at pH 4.75

3.1.1. Factors affecting ZnPP and PPIX formation

In order to clarify the factors that affect ZnPP and PPIX formation at the optimum pH 4.75, the effect of temperature, incubation time, oxygen, and FECH inhibitors ($NaNO_2$ and N-MMP) on ZnPP formation was observed by using an established model experiment system (Wakamatsu et al., 2004). After incubation at 4, 18, 25 and 37°C for 5 days, ZnPP and PPIX formation were increased to the greatest extent at 37°C. The ZnPP and PPIX formation were slightly increased with the increase of incubation time. Oxygen, $NaNO_2$ and FECH inhibitor (N-MMP) also significantly inhibited ZnPP and PPIX formation at pH 4.75. Temperature, incubation time, oxygen, $NaNO_2$ and N-MMP affected not only ZnPP formation but also PPIX formation at pH 4.75, the aspects of ZnPP and PPIX formation were almost similar. Therefore, it was suggested that PPIX is a precursor of ZnPP and PPIX formation at pH 4.75.

3.1.2. Effect of fractionation on ZnPP and PPIX formation

In order to find out the contributors to ZnPP and PPIX formation at pH 4.75, IS muscle homogenate was fractionated and the effect of separated fractions on ZnPP and PPIX formation

were investigated. When IS muscle homogenate was fractionated into soluble and insoluble fractions and incubated separately, ZnPP and PPIX formation were significantly suppressed. However, when they were mixed together, ZnPP and PPIX formation were rescued. When the soluble fraction was further fractionated into the >10 and <10 kDa soluble fractions and incubated with the insoluble fraction separately, ZnPP and PPIX formation were significantly suppressed. When the separated two soluble fractions were mixed and incubated together with the insoluble fraction, ZnPP and PPIX were rescued. Therefore, two water-soluble fractions (>10 and <10 kDa) and insoluble fractions of IS homogenate are essential in model solution to ZnPP and PPIX formation at pH 4.75.

3.1.3. Effect of heat treatment of fractionated water-soluble fractions on ZnPP and PPIX formation

Hamm and Deatherage (1960) reported that about 77% of the water-soluble globular proteins are denatured by heating at 60°C for 30 minutes. Since most of the proteins are present in the >10 kDa soluble fraction of pork homogenate, heating effect of the >10 kDa soluble fraction was examined to investigate the contribution of proteins on ZnPP and PPIX formation. When the heated >10 kDa soluble fraction was incubated with the insoluble and <10 kDa soluble fractions, ZnPP and PPIX were significantly inhibited. Therefore, water-soluble protein(s) present in the >10 kDa soluble fraction contribute to ZnPP and PPIX formation at pH 4.75.

Next, in order to clarify whether the heat-stable or heat-labile <10 kDa soluble component contributes to ZnPP and PPIX formation, heating effect of the <10 kDa soluble fraction on ZnPP and PPIX formation were also examined. When the heated <10 kDa soluble fraction was incubated with the insoluble and >10 kDa soluble fractions, ZnPP and PPIX formation were not suppressed. Thus, heat-stable components present in the <10 kDa soluble fraction might contribute to ZnPP and PPIX formation at pH 4.75.

3.1.4. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by gel filtration chromatography at pH 4.75

In order to investigate the contribution of myoglobin or other water-soluble protein(s) in the >10 kDa soluble fraction to ZnPP and PPIX formation, protein(s) from the water-soluble fraction was separated using gel filtration chromatography and applied to ZnPP and PPIX formation model experiment systems. When the separated fractions were incubated with the insoluble and <10 kDa soluble fractions separately, high ZnPP and PPIX were formed with myoglobin eluted fractions and optimum in myoglobin absorbance peak. The amount of ZnPP and PPIX formed were significantly correlated with the myoglobin contents. Therefore, myoglobin contributes to ZnPP and PPIX formation at pH 4.75.

3.2. Mechanism of ZnPP formation in IS muscle at pH 5.5

3.2.1. Effect of fractionation on ZnPP and PPIX formation

First, to check the contributors to ZnPP and PPIX formation at pH 5.5, LD muscle homogenate was fractionated into different fractions and the effect of separated fractions on ZnPP and PPIX formation were investigated. When LD homogenate was fractionated into the insoluble and soluble fractions and incubated individually, ZnPP and PPIX formation were significantly suppressed. When they were mixed together, ZnPP and PPIX were rescued. When the soluble fraction was further fractionated into the >10 and <10 kDa soluble fractions and incubated with the insoluble fraction separately, ZnPP and PPIX formation were significantly suppressed. When they were mixed together with the insoluble fraction, ZnPP and PPIX formation were rescued. Therefore, one or more components in each fraction from LD muscle homogenate are essential for ZnPP and PPIX formation at pH 5.5.

3.2.2. Effect of heat treatment of fractionated water-soluble fraction on ZnPP and PPIX formation

Next, heating effect of the >10 kDa and <10 kDa soluble fraction soluble fraction were examined on ZnPP and PPIX formation at pH 5.5. When the heated >10 kDa soluble fraction of LD muscle was incubated with the insoluble and <10 kDa soluble fractions, ZnPP formation and PPIX were significantly suppressed. When the heated <10 kDa soluble fraction was incubated with the insoluble and >10 kDa soluble fractions, ZnPP and PPIX were not so suppressed. Therefore, water-soluble protein presents in the >10 kDa soluble fraction and heat-stable component(s) in the <10 kDa water-soluble fraction contributed to ZnPP and PPIX formation.

3.2.3. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by gel filtration chromatography

Similarly to section 3.1.5., the LD water-soluble protein was separated by a gel filtration chromatography and applied to ZnPP and PPIX formation model experiment systems. When the separated fractions were incubated with the insoluble and <10 kDa soluble fractions individually, higher ZnPP and PPIX formation were observed in earlier eluted fractions than the myoglobin-eluted fractions. ZnPP and PPIX were formed in the absence of myoglobin. Therefore, higher molecular weight protein(s) compared with myoglobin in the water-soluble fraction contributed to ZnPP and PPIX formation at pH 5.5.

3.2.4. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by cation exchange chromatography (CIEX)

To explore the contributing protein to ZnPP and PPIX formation, LD water-soluble protein was separated by CIEX with higher pH (up to 8.0) buffers. When the collected sample was

incubated with the insoluble and <10 kDa soluble fractions, ZnPP formation was suppressed up to pH 7.5 but rescued at pH 8.0. Then, the separation of the contributing protein that was not adsorbed with pH 8.0 buffer was tried and SDS-PAGE was performed. The band pattern of proteins that was not adsorbed with pH 8.0 (0 M NaCl) buffer was almost same as that of washed with pH 7.5 (0 M NaCl) buffer, whereas the two 42 and 60 kDa bands were observed in the fraction eluted with pH 7.5 (0.6 M NaCl) buffer. In model experiment, the amount of ZnPP formed in pH 7.5 (0.6 M NaCl) buffer-eluted fraction group was significantly higher than that in the pH 7.5 (0 M NaCl) buffer-washed fraction. However, the amount of PPIX formed was not significant between the two fractions. Thus, water-soluble proteins 42 and/or 60 kDa contribute to ZnPP formation but do not contribute to PPIX formation at pH 5.5.

4. Discussion

4.1. Mechanism of ZnPP formation at pH 4.75

4.1.1. ZnPP-forming route

Recently, a new optimum pH for ZnPP formation was observed at pH 4.75 (Wakamatsu et al., 2019). But, from where ZnPP is derived at pH 4.75? However, the investigations of the effects of various factors on ZnPP and PPIX formation revealed that ZnPP formation was almost the same as that of PPIX formation at pH 4.75. This result is consistent with the result at pH 5.5 (Wakamatsu et al., 2007). Therefore, ZnPP was suggested to derive from PPIX at pH 4.75.

The next question is from where PPIX is derived? At pH 5.5, PPIX is not from heme in myoglobin (Wakamatsu et al., 2007). However, this study indicated that fractions eluted myoglobin by gel filtration increased PPIX formation. Ishikawa et al. (2007) suggested that mitochondria have the ability to form PPIX from oxymyoglobin and are directly related to the release of Fe^{2+} from porphyrin ring in myoglobin. Therefore, PPIX suggested to be derived from heme in myoglobin in ZnPP formation mechanism at pH 4.75.

4.1.2. Mechanism of PPIX formation from heme derived myoglobin

In order to clarify the mechanism of PPIX formation from heme derived from myoglobin, IS muscle homogenate was fractionized. When IS muscle homogenate was separated into three fractions i.e. insoluble fraction, >10 kDa and <10 kDa soluble fraction, PPIX formation was inhibited in the absence of one of the three fractions. Thus, one or more components in each fraction of these three fractions contribute to form PPIX.

FECH enzymes are located in the inner mitochondrial membrane. Because FECH that is considered to be involved in ZnPP formation (Becker et al., 2012). FECH would be exist in the insoluble fraction which is essential for PPIX formation at pH 4.75. Hence FECH from the insoluble fraction is supported to be one of the contributors to PPIX formation.

It was revealed that myoglobin from the >10 kDa soluble fraction contribute to PPIX formation at pH 4.75. Myoglobin is a sarcoplasmic heme protein responsible for the color of meat and according to its molecular weight (17 kDa), would be in the >10 kDa soluble fraction. Gel filtrated-fraction with myoglobin promoted to form PPIX. Wakamatsu et al. (2019) also suggested that ZnPP formation at pH 4.75 is derived from myoglobin. Thus, myoglobin in the >10 kDa soluble fraction plays a role to form PPIX.

The heat-stable component present in the <10 kDa soluble fraction was essential for PPIX formation. If Zn^{2+} in the <10 kDa soluble fraction is the contributor, mixing of the insoluble and >10 kDa soluble fractions with EDTA would increase PPIX formation. Therefore, heat-stable components in the <10 kDa fraction except Zn^{2+} contributes to ZnPP formation.

4.1.3. Mechanism of ZnPP formation from PPIX

It was reported that ZnPP in a model solution was the insertion of Zn into PPIX (Wakamatsu et al., 2007). In this study, addition of $NaNO_2$ and N-MMP inhibited ZnPP and PPIX formation indicating that FECH is deeply involved in ZnPP and PPIX formation at pH 4.75. Indeed, FECH catalyzes the insertion of divalent metal into PPIX. On the other hand, zinc is present in muscles both in insoluble and soluble component (Hazell, 1982). Therefore, Zn^{2+} either from the insoluble or soluble fraction contributes to ZnPP formation from PPIX at pH 4.75 with FECH.

4.1.4. Proposed ZnPP formation mechanism at pH 4.75

FECH from the insoluble fraction catalyzes the iron removal reaction from heme derived from myoglobin in association with the heat-stable <10 kDa soluble compounds (except Zn^{2+}) and PPIX is formed. Then, the Zn^{2+} is inserted into PPIX either from insoluble or >10 kDa soluble fraction and produced ZnPP by the catalysis of FECH. The heat-stable <10 kDa soluble component facilitates ZnPP formation from PPIX in accordance with FECH.

4.2. Mechanism of ZnPP formation at pH 5.5

4.2.1. ZnPP-forming route

It was reported that PPIX is a precursor of ZnPP formation at pH 5.5 (Wakamatsu et al., 2007). It was also suggested that PPIX is formed by the heme biosynthetic pathway (Shirashi, 2010). Therefore, PPIX in model solution might be accumulated via a precursor(s) or produced from the heme biosynthetic pathway but not from heme in myoglobin.

4.2.2. Mechanism of PPIX formation

In order to know the formation mechanism of PPIX, this study fractionized the LD muscle homogenate. When LD muscle homogenate was separated into three fractions i.e. the insoluble fraction, >10 kDa and <10 kDa soluble fraction, PPIX formation was suppressed in the lack of

one of those fractions. These results are similar to the results at pH 4.75. Thus, one or more components in each separated fraction are contributed to form PPIX at pH 5.5.

FECH would be present inner mitochondrial membrane of the insoluble fraction. It was reported that FECH from porcine muscle mitochondria catalyzes the insertion of zinc into PPIX to form ZnPP (Chau et al., 2011). Thus, FECH contributed to PPIX formation at pH 5.5.

Next, the contribution of water-soluble protein was observed for PPIX formation at pH 5.5. Gel filtrated fractions having higher molecular weight protein than that of myoglobin promoted PPIX formation. Thus, water-soluble protein except myoglobin contributes to PPIX formation.

Heat-stable components in the <10 kDa soluble fraction was essential to form PPIX at pH 5.5. In this experiment mixing of the insoluble and >10 kDa soluble fractions with EDTA did not increase PPIX formation similarly as at pH 4.75. Therefore, divalent metal Zn^{2+} in the <10 kDa soluble fraction might not contribute to PPIX formation at pH 5.5.

Therefore, it is suggested that FECH from the insoluble fraction, soluble protein other than myoglobin and heat-stable components except Zn^{2+} also contribute to PPIX formation.

4.2.3. Mechanism of ZnPP formation from PPIX

The final step in ZnPP formation is the insertion of Zn^{2+} to PPIX. ZnPP formation was suppressed in the absence of one of the three LD homogenate fractions. But it was not shown if all fractions of LD homogenate are essential to insert of Zn^{2+} into PPIX. This study also found that 42 and/or 60 kDa soluble components promoted to ZnPP formation at pH 5.5. Thus, 42 and/or 60 kDa protein might also contribute to the insertion of Zn^{2+} to PPIX.

4.2.4. Proposed ZnPP formation mechanism at pH 5.5

PPIX is a precursor of ZnPP formation at pH 5.5 which is formed by the heme biosynthetic pathway. FECH from the insoluble fraction and water-soluble protein except myoglobin and heat-stable components in the <10 kDa soluble fraction were contributed to PPIX formation from heme biosynthetic pathway. Then, Zn^{2+} either from the insoluble fraction or >10 kDa soluble fraction inserted into PPIX and produced ZnPP by FECH. Moreover, 42 and/or 60 kDa components in the soluble fraction might play a role to insertion of Zn^{2+} into PPIX. Heat-stable components in the <10 kDa soluble fraction also contributed to ZnPP formation from PPIX.

4.3. ZnPP formation mechanism in Parma ham

The formation mechanism of ZnPP is different between at pH 4.75 and 5.5 in pork in terms of incubation time, temperature, precursor level and the contribution of water-soluble proteins, However, which mechanism is dominant in Parma ham? The pH of Parma ham is about 5.5-6.0. Since the ability of ZnPP formation at pH 4.75 is much higher than that at 5.5, the ZnPP mechanism at optimum pH 4.75 cannot be ignored in Parma ham.

5. References

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