博 士 論 文 の 要 約

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学 位 論 文 題 名

Studies on the formation mechanism of zinc protoporphyrin IX from heme proteins *via* the heme dissociation in nitrite/nitrate-free meat products

亜硝酸塩/硝酸塩無添加食肉製品中におけるヘムの解離を介したヘムタンパク質からの亜鉛プロトポルフィリン IX の形成機構に関する研究

[Backgrounds and objectives]

Color is one of the most important factors of meat and meat products affecting consumer buying decisions. In the meat processing industry, synthetic nitrate and nitrite are widely used in meat products for their color-fixative properties and their inhibitory effects on bacteria growth, particularly *Clostridium botulinum*. Nitrate and nitrite are also known for their antioxidant and anti-rancidity properties, as well as their contribution to the characteristic cured meat flavor. However, the use of nitrate and nitrite has raised concerns due to its potential to form carcinogenic nitrosamines. Since the requirements of consumers for organic and clean-label meat products have increased due to the concerns about the health risk of synthetic nitrate and nitrite, the meat industry has currently concerned with the development of their alternatives. Zinc protoporphyrin IX (ZnPP) is a distinct red pigment in traditional Italian Parma ham (*Prosciutto di Parma*), that has matured over one year after curing with only sea salt. In addition, the stable bright red color of Parma ham is mainly due to the formation of ZnPP. Since the formation of ZnPP is also found in other nitrite-free dry-cured meat products, such as the dry-cured Iberian hams and the dry-cured fermented sausages, ZnPP is considered as a potential pigment to improve the color of dry-cured meat products without adding nitrite and nitrate or any coloring agents.

In recent years, it has been proposed that ZnPP in Parma ham is mainly formed through the conversion of heme, which comes from the endogenous heme proteins, such as hemoglobin (Hb) and myoglobin (Mb). After heme is dissociated from heme proteins, it is first converted into protoporphyrin IX (PPIX) through an iron-removal reaction. Subsequently, the formation of ZnPP is explained by the insertion of the zinc ion into PPIX. In addition, although ZnPP itself is water-insoluble, ZnPP mainly exists in Parma ham as water-soluble complexes by binding with Hb (ZnPP-Hb) and Mb (ZnPP-Mb). However, the specific formation pathway of these water-soluble ZnPP complexes in Parma ham remains unclear. Additionally, the formation of ZnPP is proposed to be strongly associated

with Mb owing to its highest content in meat than other heme proteins, such as Hb. Also, after slaughter, only a small amount of Hb remains in the meat. However, the amount of ZnPP-Hb is approximately threefold higher than that of ZnPP-Mb in Parma ham, indicating Hb may be a more crucial hemeprotein in the formation of the water-soluble ZnPP in Parma ham compared to Mb. Therefore, in the first part of this study, we attempt to elucidate the formation pathway of the water-soluble ZnPP complex in Parma ham and the mechanism by which ZnPP-Hb is dominated in Parma ham compared with ZnPP-Mb.

On the other hand, nitrite or/and nitrate, which are added to common cured meat products, have been found to inhibit the formation of ZnPP in meat. This phenomenon is proposed as the nitric oxide (NO), which is derived from nitrite, can degrade the [2Fe–2S] cluster of mammalian FECH, further suppressing its catalytic activity of iron-removal and zinc insertion to form ZnPP in meat. However, NO as a kind of heme ligand can also strongly bind to the heme iron of the meat-endogenous heme proteins to form the structurally stable nitrosyl heme proteins, which bring a long-lasting, bright red color for the nitrite-added cured meat. This phenomenon indicated that NO derived from nitrite may also inhibit ZnPP formation by limiting the heme dissociation from the heme proteins in meat. Therefore, the significance of heme dissociation from heme proteins on ZnPP formation may be investigated using NO to interact with endogenous heme proteins. Nevertheless, since NO can bind with either the heme (Fe^{2+}) or the hematin (Fe^{3+}) in heme proteins to make them structurally stable, it is hard to elucidate the specific pathway of heme or hematin dissociation from heme proteins to form ZnPP only using NO. However, the carbon monoxide (CO) can only bind with the heme of heme proteins to form the highly structurally stable carboxy heme proteins, while the azide ion can bind with the hematin of the oxidized heme proteins to form the structurally stable azide heme proteins. Besides, CO and azide ion does not cause disassembly of the FECH [2Fe-2S] cluster in meat, which is different from that of NO. Therefore, in the second part of this study, we aim to clarify the significance of heme dissociation from the heme proteins in the formation mechanism of ZnPP in meat by investigating the inhibitory pathway of NO on ZnPP formation from heme proteins compared with CO and sodium azide. Additionally, some reductants have been reported to be essential for the iron-removal reaction of hematin to form ZnPP under anaerobic conditions, but the over-reduction might inhibit ZnPP formation. Therefore, the effects of the free heme reduction and the over-reduction on ZnPP formation were also evaluated in the second part of this study.

1. Investigating the mechanism of the water-soluble ZnPP formation in Parma ham using a new experimental model

[Materials and Methods]

To investigate the formation mechanism of ZnPP in Parma ham more efficiently and conveniently, based on previous ZnPP-forming experimental models, a new experimental model producing plenty of water-soluble ZnPP like Parma ham need to establish first. Single-factor experiments were carried out to determine the optimum level of each parameter in the new experimental model (meat content, incubation temperature, pH, and

incubation time) with respect to ZnPP formation. The fluorescent intensity of the model supernatant was used as a metric for the quantity of water-soluble ZnPP formed, and the fluorescence intensity of the model precipitate was used as a metric for the quantity of water-insoluble ZnPP formed. The water-soluble ZnPP complexes formed in the new experimental model were then verified whether they were the same as those in Parma ham using SEC-HPLC, urea-PAGE, and western blotting analysis. To clarify how ZnPP-Hb is dominant over ZnPP-Mb in Parma ham, exogenous Hb or Mb standard was added to the experimental model at the same final concentration, and the changes in water-soluble ZnPP and total ZnPP (the sum of water-soluble and water-insoluble ZnPP) were monitored by measuring their fluorescent intensities during anaerobic incubation. The released non-heme iron content in the model supernatant was measured using a 1,10-phenanthroline colorimetric method. The protein stabilities of Hb and Mb standards were evaluated by measuring the changes in their heme absorbance spectrum and tryptophan (Trp) fluorescence spectrum after 3 days of anaerobic incubation. Finally, to investigate the binding reaction of ZnPP with heme proteins to form water-soluble ZnPP complexes, the apo-form of Hb (Apo-Hb), which was prepared by removing the heme from the Hb, was mixed with the ZnPP standard, and the mixture was then detected using urea-PAGE.

[Results and discussion]

The optimal condition for the new experimental model was found as incubating 50% porcine *longissimus thoracis et lumborum* muscle at pH 5.5 for 10 days at 35°C. Compared with the previous models, the water-soluble ZnPP in the new model increased seven-fold, and the water-insoluble ZnPP increased four-fold, indicating that plenty of water-soluble ZnPP complexes could form in the new experimental model. According to the results of urea-PAGE, the fluorescent bands of water-soluble ZnPP complexes in the new experimental model were almost the same as the main fluorescent bands of water-soluble ZnPP complex in Parma ham, and these bands were detected as ZnPP-Hb using western blotting. In HPLC results, two peak fractions that related to the water-soluble ZnPP complexes were observed in both the new experimental model and Parma ham. After calculating their molecular weights, one peak fraction was considered as Hb dimer and the other peak fraction was considered as the free ZnPP. These results indicated that ZnPP-Hb dimer was the main water-soluble ZnPP complex in the new experimental model which was consistent with that in Parma ham. Therefore, the new experimental model was proposed to be suitable for investigating the formation mechanism of water-soluble ZnPP in Parma ham. The addition of the exogenous Mb in the new experimental model had no effect on the total ZnPP, water-soluble ZnPP, and released non-heme iron increase compared with those in the control group, in which no heme substrates was added. In contrast, when exogenous Hb was added into the new experimental model, it significantly promoted the increase of the total ZnPP, water-soluble ZnPP, and released non-heme iron compared with both control and Mb-added groups. These results indicated that, compared with Mb, the heme in Hb was easier to dissociate and convert into ZnPP and water-soluble ZnPP complexes. The results of the protein structural stability changes of Hb and Mb

showed that after 3 days of anaerobic incubation, little increase of the Trp fluorescence intensity in Mb was observed, indicating its globin unfolding was very low. Also, almost no absorption change of Mb specific peaks was observed, indicating its heme-globin was stable. In contrast, the Trp fluorescence intensity of Hb was significantly increased after anaerobic incubation, indicating the protein unfolding of a part of Hb was increased. Meanwhile, the absorption of the specific peaks in Hb was notably decreased, indicating the heme-globin binding of Hb was destroyed. Therefore, due to the stable heme-globin binding of Mb, less heme could dissociate from it, resulting in less contribution of Mb on ZnPP formation. As for Hb, due to its high protein unfolding and unstable heme-globin binding, a large amount of heme could dissociate from it and then convert to ZnPP. Subsequently, with the heme dissociation from Hb, the apo-Hb with a vacant heme pocket could be produced simultaneously. After only mixing the apo-Hb solution with the ZnPP standard, the ZnPP-Hb fluorescent bands were detected in urea-PAGE and showed almost the same position as those in the new experimental model supernatant and Parma ham. Therefore, ZnPP can non-enzymatically bind with apo-Hb to form ZnPP-Hb complex. In summary, in Parma ham, due to the high protein unfolding and unstable heme-globin binding of Hb than Mb, heme was easily dissociated from the heme pocket of Hb, and the apo-Hb dimer was simultaneously produced. The heme mainly dissociated from Hb was converted to ZnPP and then non-enzymatically inserted into the apo-Hb to form a large amount of ZnPP-Hb as the predominate water-soluble ZnPP complex in Parma ham.

2. Investigating the inhibitory mechanism of nitrite on ZnPP formation in dry-cured meat products

[Materials and Methods]

To verify whether NO, CO, and azide as heme ligands could bind with endogenous heme proteins and whether their existences inhibit the formation of ZnPP in meat homogenate, the sodium nitrite, CO, and sodium azide were separately added into the ZnPP-forming experimental model that was established in the first part of this study. After 10 days of anaerobic incubation, the ZnPP fluorescent intensity of each treatment was evaluated and the heme absorbance spectrum of the model supernatant after CO and azide addition were measured. Meanwhile, the UV absorbance of nitrosyl heme in the experimental model, which was added with the sodium nitrite, was measured at 395 nm. To confirm whether the addition of NO, CO, and azide could stably bind with the free heme/hematin to inhibit ZnPP formation by suppressing their iron-removal reaction, the sodium nitrite, sodium azide, and CO were separately added into the hemin-1-methylimidazole (1MI) solution, which was prepared by dissolving the hemin standard into the aqueous buffer using 1MI. After the reaction, the UV spectrum of each treatment was measured. In addition, since Hb was found as the main substrate to dissociate heme to form ZnPP in the first part of this study and to eliminate the effect of each free ligand molecule on some endogenous enzymes, such as FECH, various Hb derivatives were used to investigate the significance of heme dissociation from the heme proteins in the formation mechanism of ZnPP. The OxyHb, nitrosyl Hb (NOHb), carboxy Hb (COHb), and azide metHb (N_3mHb), which were

prepared by reacting oxygen, sodium nitrite, CO, and sodium azide with Hb standard *in vitro*, were separately added into the experimental model as the exogenous heme substrate with equal final concertation. The supplemental effects of each Hb derivative on ZnPP formation, PPIX formation, and non-heme iron content in the model were evaluated during anaerobic incubation. Meanwhile, the protein stability of each Hb derivative was evaluated by measuring the changes in their heme absorbance spectrum and Trp fluorescence spectrum during anaerobic incubation. The heme dissociation amount of each Hb derivative during anaerobic incubation was also evaluated using RP-HPLC. To investigate the effect of free heme reduction on ZnPP formation, the hemin-1MI solution was separately incubated with the crude FECH extract and zinc ions under two conditions, with or without the addition of ascorbic acid as the reductant. After a 24 hours anaerobic incubation, the ZnPP fluorescence intensity of different treatments was evaluated. To confirm whether the over-reduction affected ZnPP formation in the experimental model, sodium hydrosulfite was added to the model as the strong reductant, and the ZnPP fluorescence intensity of different treatments was evaluated after 10 days of anaerobic incubation.

[Results and discussion]

With the addition of sodium nitrite and sodium azide into the experimental model, the formation of ZnPP was inhibited does-dependently. At the same time, the absorbance of nitrosyl heme was correspondingly increased with nitrite addition, whereas the specific peaks of N_3mHb were observed and increased with azide addition. After CO treatment, compared with the control group, the formation of ZnPP was significantly decreased. Meanwhile, the specific peaks of COHb were observed in the CO-treated group. These results indicated that the conversion of the endogenous Hb to its derivatives stably bound by NO, azide, and CO could inhibit ZnPP formation in meat. Moreover, after the addition of OxyHb, which is the main state of Hb in fresh meat, into the experimental model, during anaerobic incubation, the formation of ZnPP, PPIX and released non-heme iron increased significantly compared with the control group in which no exogenous Hb was added. However, the addition of exogenous NOHb, COHb, and N_3mHb into the experimental model did not affect ZnPP formation, PPIX formation, and released non-heme iron increase compared with those in the control group during anaerobic incubation. These results suggested that, compared with OxyHb, the heme/hematin in NOHb, COHb, and N_3mHb could not be deironized to form ZnPP *via* PPIX under anaerobic conditions. The results of the protein structural stability changes of Hb derivatives showed that, during anaerobic incubation, the absorption of the specific peaks in OxyHb was significantly decreased, while the Trp fluorescence intensity of OxyHb was significantly increased. This phenomenon indicated that the globin protein of OxyHb was easily unfolding and its heme-globin binding was unstable during anaerobic incubation. In contrast, almost no changes in the heme absorption and Trp fluorescence intensity were observed in NOHb, COHb, and N_3mHb, indicating their protein structures were stable during anaerobic incubation. Also, the heme dissociation amount of OxyHb was constantly increasing throughout the

anaerobic incubation, while the heme dissociation contents of NOHb, COHb, and N_3mHb increased slightly and were much lower than that of OxyHb during anaerobic incubation. These results indicated that heme/hematin was hard to dissociate from the NOHb, COHb, and N_3mHb under the anaerobic conditions due to their stable protein structures compared with the OxyHb. Furthermore, compared with the heme absorption spectrum of the hemin-1MI solution without any treatment, no changes in the heme absorption spectrum of the hemin-1MI solution added with NO, CO, and azide were observed, indicating the NO, azide, and CO ligands could not bind to the free heme/hematin. Therefore, NO, CO, and azide stably bound with the heme/hematin in endogenous Hb inhibited ZnPP formation by suppressing its heme/hematin dissociation and heme iron-removal reaction. These results indicated that the heme dissociation from the endogenous Hb, and the hematin dissociation from the oxidized mHb, which may be induced by the heme scavenging system, were significant for ZnPP formation in meat. On the other hand, the results of the effect of free heme reduction on ZnPP formation showed that, ZnPP was not formed without the reduction of hematin to heme induced by ascorbic acid, whereas ZnPP increased significantly when ascorbic acid was added to reduce hematin to heme. This phenomenon indicated that the hematin reduction to heme was essential for ZnPP formation. However, when sodium hydrosulfite was used as the strong reductant, the formation of ZnPP was inhibited with its addition. This phenomenon was speculated as the over-reduction may inhibit the oxidation of Hb to mHb, thus, less hematin can dissociate from mHb, resulting in the formation of ZnPP being inhibited. Therefore, after hematin was dissociated from the oxidized heme proteins, the hematin reduction to heme, which may be induced by some reductases in meat, such as myoglobin reductase, was proposed to be previously needed for ZnPP formation in meat. The over-reduction is speculated to suppress ZnPP formation by preventing the oxidation of endogenous Hb. In summary, the heme/hematin dissociation from the endogenous heme proteins were proposed to be significant for the formation of ZnPP *via* PPIX in meat. On the other hand, oxidation and reduction and the balance between them were suggested to be important for the formation of ZnPP in meat.

In conclusion, the suggested pathway for the ZnPP formation from heme proteins in nitrite/nitrate-free dry-cured meat products was proposed. Although Mb is the most abundant heme protein in meat, ZnPP was proposed mainly formed from Hb rather than Mb and existed as the ZnPP-Hb in the nitrite/nitrate-free dry-cured meat products. Compared with Mb, the Hb that entered the muscle tissue dissociated a large amount of heme due to its unstable heme-globin binding and derived apo-Hb simultaneously. Meanwhile, the rapid autoxidation of Hb in meat, which might be induced by the heme scavenging system, resulted in hematin being easily dissociated from the oxidized mHb. After the dissociated hematin from mHb was reduced to heme by the meat-inherent reductase, the free heme including the directly dissociated part was converted to ZnPP *via* PPIX. The over-reduction may inhibit ZnPP formation by preventing the oxidation of endogenous Hb. Subsequently, the ZnPP-Hb, which was formed by non-enzymatically inserting ZnPP into apo-Hb, could rapidly produce and as the dominating water-soluble ZnPP complexes in the nitrite/nitrate-free dry-cured meat products.