Zinc protoporphyrin IX predominantly exists as a complex non-enzymatically bound to apo-hemoglobin in Parma ham

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Background and Objectives: Zinc protoporphyrin IX (ZnPP) is a distinct red pigment in long-term matured meat products, such as Parma ham, which is manufactured without nitrate or nitrite. Because the use of nitrite in meat products is associated with a risk of cancer owing to its ability to form nitrosamines, nitrite-free meat products are preferred by some consumers. However, it is difficult to develop a favorable color for meat products without the addition of nitrite. Accordingly, ZnPP has become a subject of interest because of its safe and stable properties and applicability in the reddening of nitrite-free meat products. A previous study showed that ZnPP mainly exists in Parma ham as water-soluble complexes by binding to hemoglobin and myoglobin (ZnPP-Hb and ZnPP- Mb). However, their formation pathway remains unclear. Moreover, the iron removal reaction of heme, which mainly exists in hemeproteins, is considered a crucial step in the formation of ZnPP in meat products. However, direct evidence has been limitedly provided for this reaction. It has also been proposed that the formation of ZnPP in meat products is strongly associated with Mb be- cause of its much higher content in meat than Hb. Nevertheless, in Parma ham, the amount of ZnPP-Hb is approximately 3-fold higher than that of ZnPP-Mb. Therefore, in this study, we attempted to elucidate the formation pathway of the water-soluble ZnPP complex and the mechanism by which ZnPP-Hb is dominated in Parma ham compared with ZnPP-Mb. The findings in the present study provided a feasible water-soluble ZnPP complexes formation pathway, which could be utilized to improve the ZnPP forma- tion in nitrite-free meat products in future work.

Materials and Methods: An experimental model, which produces a large amount of water-soluble ZnPP by incubating 50% porcine *longissimus thoracis et lumborum* muscle anaerobically at pH 5.5 for 20 days at 35°C, was used to investigate the formation of wa- ter-soluble ZnPP in Parma ham. 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 a final concentration of 0.005% (w/w), and the changes in water-soluble ZnPP and total ZnPP (the sum of watersoluble and water-insoluble ZnPP) were monitored by measuring their fluorescent intensities during incuba- tion. The total non-heme iron content in the model supernatant was measured using a 1,10- phenanthroline colorimetric method. Apo-hemoglobin (apo-Hb) was prepared by removing the heme from the Hb protein using a cold (−20°C) acid acetone solution that contained 0.035% (v/v) HCl. The apo-Hb solution was mixed with the ZnPP standard at 4°C for 1 h to investigate the forma- tion of the ZnPP-Hb complexes, then detected using urea-PAGE.

Results and Discussion: The addition of Hb into the experimental model promoted ZnPP formation compared with that of Mb, which could be explained by the unstable heme-globin structure of Hb than Mb. In the crystal structures of Hb, the Leu(F7) and Leu(FG3) in both the Hb α and β chains could not form stable contacts with heme-7-propionate. This unstable structure resulted in low heme stability maintained within the Hb globin, further causing a high dissociation rate of heme in Hb. Therefore, Hb released heme more easily than Mb to be the substrate that converted into ZnPP. Subsequently, the increase in non-heme iron content with the accumulation of ZnPP during incubation indicated that the iron-release reaction of heme in hemeproteins made a substantial contribution to the formation of ZnPP. The high heme dissociation rate of Hb, owing to the low stability of heme, resulted in a higher increase in the non-heme iron content in the Hb-added group than in the Mb-added group. Finally, after only mixing the apo-Hb solution with the ZnPP standard, the ZnPP-Hb complex was formed, and its fluorescent bands in the urea-PAGE were similar to those in the Parma ham water extract. These results indicated that the formation of the ZnPP-Hb complex in Parma ham could occur by the non-enzymatically binding of ZnPP to apo-Hb. The formation pathway of the ZnPPMb complex was speculated to be similar to that of ZnPP-Hb, but the stable heme-protein structure of Mb resulted in less heme dissociation from the heme pocket, resulting in the reduced formation of ZnPP-Mb in Parma ham. These results revealed that ZnPP-Hb is dominant over ZnPP-Mb because Hb more easily releases heme as the substrate to be converted into ZnPP through the iron-removal reaction, fol- lowed by the non-enzymatic binding of ZnPP to apo-Hb to form ZnPP-Hb in Parma ham.

Key words: Zinc protoporphyrin IX, Myoglobin, Parma ham, Hemoglobin, Heme