THE EXTRACTABILITY OF ZINC PROTOPORPHYRIN IX-MYOGLOBIN COMPLEX FROM PARMA HAM IS DEPENDENT ON THE pH

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I. INTRODUCTION

Parma ham, a traditional Italian dry-cured ham, is bright red without adding nitrite/nitrate. The redness is derived from zinc protoporphyrin IX (ZnPP), endogenously formed during long maturation periods [1]. Accelerating the formation of ZnPP in other nitrite/nitrate-free meat products can be a novel technique for colour improvement. Recently, myoglobin (Mb) and haemoglobin (Hb) were found to be the binding proteins of ZnPP extracted from Parma ham with water, and haem from these proteins was suggested to be the precursor of ZnPP [2]. In addition, the ZnPP-Hb complex was predominant over the ZnPP-Mb complex in the extract. A possible reason for this is that Hb was suggested to be a superior haem donor than Mb in ZnPP formation owing to its higher ability of haem dissociation [3]. However, these studies focused only on water-extractable ZnPP in Parma ham. Our preliminary investigation indicates that water-unextractable ZnPP also exists in Parma ham. The ratio of water-unextractable to -extractable ZnPP in Parma ham has yet to be investigated. In addition, it is unclear whether water-unextractable ZnPP is formed by the same mechanism as water-extractable ZnPP. In meat homogenates, the water-extractability of ZnPP differs depending on the pH or incubation periods [3]. These factors might affect the formation of ZnPP, which showed different water-extractability in Parma ham. In addition, the binding substance of water-unextractable ZnPP may provide new insights into its formation mechanism. Therefore, this study aimed to elucidate the factors that affect the water-extractability of ZnPP and the binding substances of water-unextractable ZnPP in Parma ham.

II. MATERIALS AND METHODS

Five Parma ham samples from three manufacturers with two different maturation periods (14 and 18 months) were used. From these hams, four major muscles (QF, *quadriceps femoris*; BF, *biceps femoris*; ST, *semitendinosus*; and SM, *semimembranosus*) were excised and analysed. Each muscle sample was homogenised with ultra-pure water and then fractionated into water-extractable and -unextractable fractions by centrifugation. ZnPP was extracted from each fraction using acetone, and the autofluorescence intensity (Ex/Em: 420/590 nm) was evaluated to determine the ZnPP amount. The water-extraction ratio of ZnPP was calculated as the ratio of ZnPP in the water-extractable fraction to the sum of ZnPP in the water-extractable and -unextractable fractions. The ZnPP-binding proteins that were extracted at pH 7.5 were separated by size exclusion-high-performance liquid chromatography (SEC-HPLC). The water-extractability of Mb from Parma ham was evaluated using western blotting method. Apo-Mb was prepared by the acid-acetone method and mixed with ZnPP in a buffer. After ultracentrifugation, the supernatant was collected, and the protein concentration was estimated using the Bradford method. Statistical analysis was done using testing for no correlation or paired T-test. $P < 0.05$ was considered as statistically significant.

III. RESULTS AND DISCUSSION

In four muscles from five Parma ham samples, 30-80% of ZnPP was present in the water-extractable state. The maturation period did not affect the water extractability of ZnPP from Parma ham. On the other hand, it differed between the muscles; QF showed higher water extractability than ST and SM. In addition, a positive correlation was observed between the muscle pH and the water extractability of

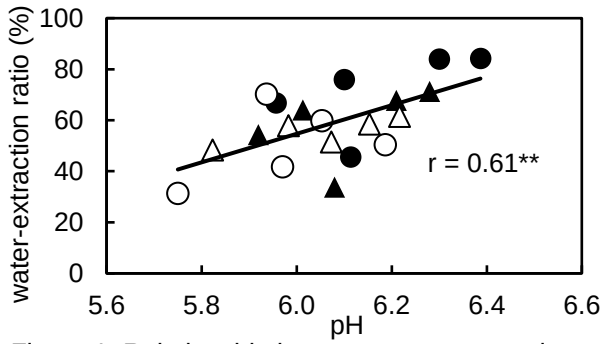

Figure 1. Relationship between water-extraction ratio of ZnPP and pH in Parma ham
●: QF, ▲: BF, ○: ST, △: SM. Approximate straight lines are obtained, and r represent the correlation coefficient. **: significant correlation between two variables (P<0.01).

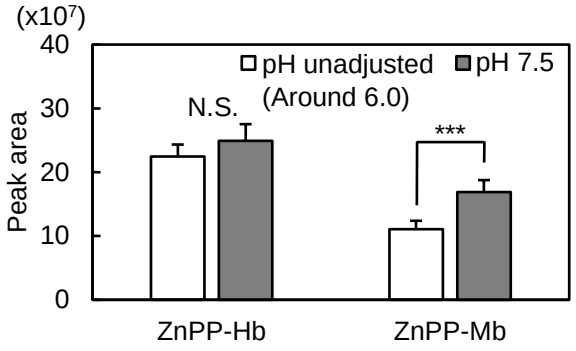

Figure 2. Peak areas of ZnPP autofluorescence which corresponds to ZnPP-Hb and -Mb complex separated with SEC-HPLC
Bars: SEM (n=20, 5 individuals, 4 muscles each),
***: significant difference (P<0.001),
N.S.: no significant difference between the groups.

ZnPP (Fig. 1). Considering the formation of ZnPP in the meat homogenate decreased remarkably at pH over 5.5 [4], this result suggested that, higher pH enhanced the extractability of ZnPP rather than the formation of water-extractable ZnPP. To confirm this, the pH at the extraction was changed, and the extractability of ZnPP was evaluated. As a result, the water extractability was maximum at pH 8.0-9.0 and reached 60-80%, significantly higher than that at unadjusted pH (around 6.0). Overall, the water-extractability of ZnPP depended on the pH during extraction, and much of the ZnPP, that could not be extracted with water at the pH of Parma ham could be extracted with weak alkalinity.

To investigate the ZnPP binding substance, the water extract at higher pH was subjected to SEC-HPLC. The retention time of ZnPP autofluorescence corresponded to those of Mb and Hb dimer indicating that, ZnPP existed as a complex with Mb or Hb, regardless of the pH during the extraction. Notably, the amount of the ZnPP-Mb complex was approximately 1.5 times higher at pH 7.5 than at the unadjusted pH. In contrast, the water-extractability of the ZnPP-Hb complex did not change between the pH values tested (Fig. 2). Therefore, Mb was suggested to be the primary binding protein for water-unextractable ZnPP in Parma ham. While Mb generally shows high water solubility, western blotting results indicated that most Mb in Parma ham existed in the water-unextractable state. Furthermore, when apo-Mb was mixed with ZnPP, its solubility decreased at pH 5.5-6.0, equivalent to the pH of meat or Parma ham. These results suggest that Mb was insolubilized by binding with ZnPP, and that the ZnPP-Mb complex existed in the water-unextractable state in Parma ham.

IV. CONCLUSION

The water extractability of ZnPP varied by 30-80%, depending on the pH of the Parma ham. Most of water-unextractable ZnPP were extracted with weak alkalinity, suggesting they mainly formed complexes with Mb. These results indicated that not only Hb but also Mb contribute to ZnPP formation in Parma ham.

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