Form in which water-soluble zinc protoporphyrin IX (ZPP) exists in Parma ham

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Abstract

The aim of this study was to elucidate the form in which zinc protoporphyrin IX (ZPP) exists in Parma ham. Despite the fact that ZPP is water-insoluble, ZPP in Parma ham can be extracted with water. We therefore hypothesized that water-soluble ZPP in Parma ham is bound to a water-soluble protein so that it dissolves in water. Parma ham was found to include water-soluble ZPP as well as water-insoluble ZPP. We tried to purify the water-soluble ZPP from the water extract of Parma ham by using protein separation procedures. The water-soluble ZPP focused at pH 6.5 by preparative isoelectric focusing electrophoresis. Most of the water-soluble ZPP was collected between 60-80% saturation fraction of ammonium sulfate. By using centrifugal ultrafiltration, the molecular weight of the water-soluble ZPP was estimated to be 30-50 kDa. These results suggested that the ZPP in Parma ham forms a complex with water-soluble protein.

Introduction

Parma ham is an Italian traditional dry-cured ham made only from the leg of a pig and sea salt. Despite a lack of nitrite or nitrate, the color of Parma ham is bright red and very stable (Morita et al., 1996; Adamsen et al., 2004). Wakamatsu et al. (2004, 2007) found that zinc protoporphyrin IX (ZPP) is a major red pigment of Parma ham and suggested that ZPP was derived not from heme but from protoporphyrin IX (PPIX) generated independently. On the other hand, Ishikawa et al. (2007) showed that ZPP and PPIX are formed from heme of oxymyoglobin. The mechanism by which ZPP is formed in Parma ham is still not clear. It is important to clarify the form in which ZPP exists in Parma ham in order to elucidate the mechanism of ZPP formation. ZPP can be extracted from Parma ham by the use of acetone as well as water (Morita et al., 1996). Since ZPP is insoluble in water, ZPP extracted with water might not be a simple substance. We therefore hypothesized that water-soluble ZPP in Parma ham was bound to a water-soluble protein so that it dissolved in water. The aim of this study was to elucidate the form in which water-soluble ZPP exists in Parma ham.

Materials and methods

Whole Parma ham (deboned) was purchased from Galloni F.lli Spa. Parma ham with connective and adipose tissue cut off was minced and then homogenized in 10 volumes of distilled water. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C to obtain a supernatant (water extract of Parma ham) and a pellet. The first pellet was homogenized in 10 volumes of distilled water and centrifuged again. The second pellet obtained was homogenized in 10 volumes of 75% acetone and centrifuged to obtain the acetone extract and the third pellet. This extraction with 75% acetone was carried out once more.

The water extract of Parma ham was ultracentrifuged (50,000 rpm, 30 min, 4°C) and the

supernatant was dialyzed overnight in 5 mM NaCl and 5 mM Tis-HCl (pH 6.8). After dialysis, urea and carrier ampholyte (pH 3-10) (final concentration of 5M and 1%, respectively) were added to the samples. Preparative isoelectric focusing electrophoresis was carried out at 12 W and 4°C for 4 hours by using Rotofor (Bio-Rad). The samples were separated into 20 fractions, and pH and fluorescence intensity of each fraction were measured.

Ammonium sulfate was added to the water extract of Parma ham and the water extract was left at room temperature for 30 min. After centrifugation at 12,000 rpm for 30 min at 25°C, the pellet was dissolved in distilled water and fluorescence intensity was determined. The solution of 60-80% saturation fraction of ammonium sulfate was poured into a centrifugal ultrafiltration tube (Vivaspin 500, Sartorius AG) and centrifuged at 5,000 rpm for 30 min at 4°C. Fluorescence intensity of the filtrate was determined, and centrifugal ultrafiltration was carried out with a filter tube with smaller fractionation molecular weight.

Fluorescent spectra of the extracts were measured from 500 to 700 nm for excitation at 420 nm using a fluorescence spectrophotometer (RF-5300PC, Shimadzu). Fluorescence intensity at 590 nm for excitation at 420 nm was regarded as the amount of ZPP.

Results and discussion

Most of the water-soluble ZPP was extracted with water once (Fig. 1-I, II). However, a large amount of ZPP was extracted with acetone after that (Fig. 1-III, IV). This result suggested that Parma ham contains both water-soluble ZPP and water-insoluble ZPP. In this study, we investigated the form in which water-soluble ZPP exists in Parma ham.

Since ZPP is water-insoluble, we hypothesized that water-soluble ZPP in Parma ham is bound to a water-soluble protein so that it dissolves in water. We tried to purify the water-soluble ZPP from the water extract of Parma ham by using protein separation procedures. By using a preparative isoelectric focusing electrophoresis, the water-soluble ZPP focused at pH 6.5 (Fig. 2), suggesting that the isoelectric point of the water-soluble protein bound to ZPP is approximately pH 6.5. However, a specific protein was not identified from the electrophoregrams of the sample focused at pH 6.5 (data not shown).

Next, the water-soluble ZPP was separated by ammonium sulfate fraction. Most of the ZPP was collected between 60-80% saturation fraction of ammonium sulfate (Fig. 3). The variety of proteins in the water extract-dissolved ZPP was considerably decreased, but a specific protein was not identified from electrophoregrams (data not shown). Centrifugal ultrafiltration of the 60-80% saturation fraction of ammonium sulfate was then carried out. The amount of ZPP gradually decreased with decrease in fractionation molecular weight, and ZPP was hardly observed at all in the filtrate of less than 30 kDa (Fig. 4). The results suggested that the molecular weight of the water-soluble ZPP was 30-50 kDa. We are now trying to observe a fluorescent spot characteristic of ZPP in two-dimensional gel electrophoresis (2DE) of water-soluble ZPP and identify the spot by MALDI-TOF/MS.

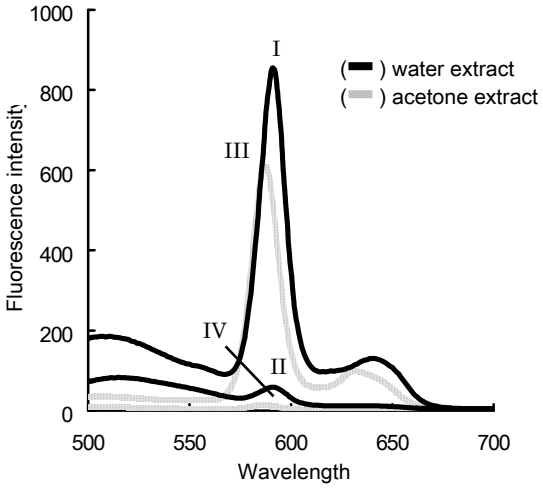

Figure 1. Fluorescent spectra (Ex: 420 nm) of water extract of Parma ham (I), water extract of the first pellet (II), acetone extract of the second pellet (III) and acetone extract of the third pellet (IV).

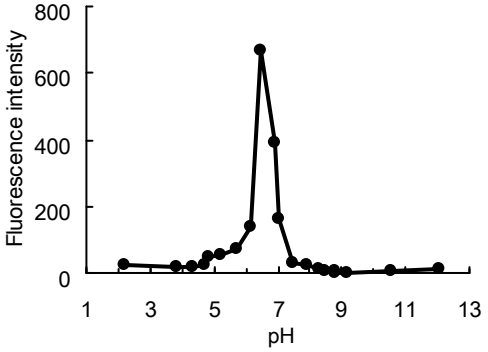

Figure 2. Amount of ZPP and pH of samples separated from water extract of Parma ham by isoelectric focusing electrophoresis (Ex/Em: 420/590 nm).

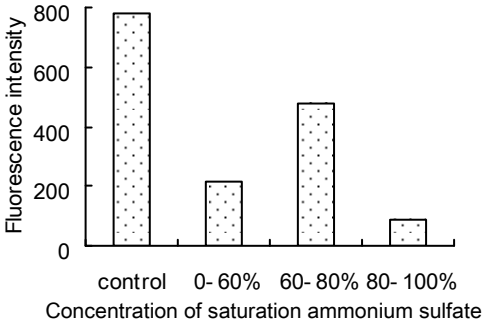

Figure 3. Amount of ZPP in samples separated from water extract of Parma ham by ammonium sulfate fraction (Ex/Em: 420/590 nm).

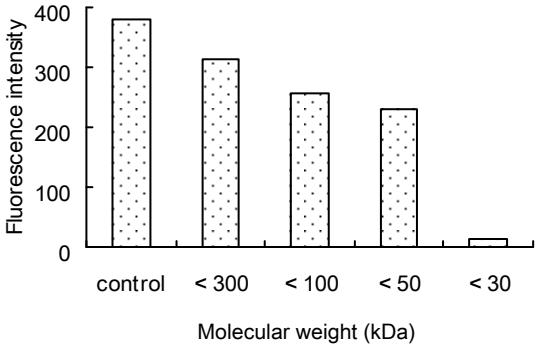

Figure 4. Amount of ZPP in the filtrate obtained by ultrafiltration of the 60-80% saturation fraction of ammonium sulfate (Ex/Em: 420/590 nm).

Conclusions

The molecular weight of the water-soluble ZPP in Parma ham was estimated to be 30-50 kDa and the isoelectric point of it was approximately pH 6.5. The water-soluble ZPP was collected between 60-80% saturation fraction of ammonium sulfate. The results suggest that the water-soluble ZPP in Parma ham forms a complex with a water-soluble protein.

Acknowledgements

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References

Adamsen, C. E., Møller, J. K. S., Hismani, R., and Skibsted, L. H. (2004). Thermal and photochemical degradation of myoglobin pigments in relation to colour stability of sliced dry-cured Parma ham and sliced dry-cured ham produced with nitrite salt. Eur. Food Res. Technol., 218, 403-409

Ishikawa. H., Kawabuchi. T., Sato. M., Numata. M., and Matsumoto. K., (2007). Formation of zinc protoporphyrin IX and protoporphyrin IX from oxymyoglobin in porcine heart mitochondria. Food Sci. Technol. Res., 13, 85-88

Morita, H., Niu, J., Sakata, R., and Nagata, Y. (1996). Red pigment of Parma ham and bacterial influence on its formation. J. Food Sci., 61, 1021-1023

Wakamatsu, J., Nishimura, T., and Hattori, A. (2004). A Zn-porphyrin complex contributes to bright red color in Parma ham. Meat Sci., 67, 95-100

Wakamatsu, J., Okui, J., Hayashi, N., Nishimura, T., and Hattori, A. (2007). Zn protoporphyrin IX is formed not from heme but from protoporphyrin IX, Meat Sci., 77, 580-586