P-07-21

Investigation of zinc protoporphyrin IX-binding protein in water extract of Parma ham (#402)

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Introduction

Parma ham is an Italian traditional dry-cured ham processed without the addition of nitrate/nitrite, and its cherry-red color was attributed to zinc protoporphyrin IX (ZnPP) (Wakamatsu *et al.*, 2004). However, the mechanism by which ZnPP is formed in Parma ham has not been elucidated yet. Despite ZnPP is a water-insoluble compound with a molecular weight of merely 0.6 kDa, it was reported that approximately 60% of ZnPP existing in Parma ham was water-soluble, whose molecular weight was estimated to be 30-50 kDa (Hayashi *et al.,* 2008). Therefore, ZnPP might bind with water-soluble proteins in Parma ham. If the ZnPP-binding proteins are elucidated, it might be clarified where and how ZnPP is formed in Parma ham. Hence, the aim of this study was to investigate the ZnPP-binding proteins in the water extract of Parma ham.

Methods

Preparation of Parma ham water extract

Minced Parma ham was homogenized with 4 volumes of ultra-pure water at 10,000 rpm for 3 min. After the homogenate was centrifuged at 18,800 G for 60 min, the supernatant was filtered with a syringe filter and termed Parma ham water extract.

Size-exclusion high-performance liquid chromatography (SEC-HPLC)

SEC-HPLC was performed with eluent of citrate-phosphate buffer and the absorbance (280 nm) and fluorescence (Ex/Em: 420/590 nm) were monitored.

Purification of ZnPP complex with chromatography

The Parma ham water extract was previously mixed with citrate-phosphate buffer containing $(NH_4)_2SO_4$ and then loaded to hydrophobic interaction chromatography (HIC). HIC column was subsequently eluted using a step-gradient of $(NH_4)_2SO_4$ and then each fraction was collected. TheZnPP-abundant fraction collected from HIC was dialyzed overnight against ultra-pure water and then mixed with citrate-phosphate buffer for cation exchange chromatography (CIEX). After the sample was loaded to the CIEX column, ZnPP-binding proteins were eluted using a linear-gradient of NaCl and then collected. ZnPP fluorescence (Ex/Em: 420/590 nm) of each collected fraction was measured by a spectrofluorometer.

SDS-PAGE and amino acid sequencing

The fractions collected from each chromatography were loaded to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was stained by Coomassie brilliant blue R-250. After transferring from unstained gel to PVDF membrane, amino acid sequence of protein band was determined by Edman degradation and BLAST analysis.

Results

In preliminary experiment, the Parma ham water extract was applied to SEC directly for estimating the molecular weight of ZnPP-binding protein. Two main peaks were observed at 14.3 and 23.0 min by fluorescence detection (Fig. 1) and the fluorescence spectrum of these peaks were checked by the spectrofluorometer. However, since only the peak eluted at 14.3 min showed ZnPP specific fluorescence, the peak at 23 min might derive from impurity (data not shown). Molecular weight of ZnPP-binding protein candidates estimated by SEC was higher than that of myoglobin (17 kDa). In contrast, it coincided with the result which showed molecular weight of ZnPP-binding protein was around 30-50 kDa (Hayashi, 2008).

To further investigate the ZnPP-binding protein, the Parma ham water extract was purified with the order of HIC and CIEX. In HIC, 97% of ZnPP-binding protein was eluted in one fraction (Fig. 2). And then, approximately half amount of ZnPP-binding protein collected from HIC was adsorbed on CIEX column, whereas the remaining was not adsorbed (Fig. 3). When the adsorbed fraction collected from CIEX chromatography was applied to SDS-PAGE, one major band and two minor bands were separated (data not shown). The major band and one of the minor bands were identified by N-terminal amino acid sequencing successfully and the result showed that the minor band was the degraded product of the major band (data not shown).

Conclusion

In this research, the molecular weight of ZnPP-binding proteins in Parma ham were larger than that of myoglobin. The protein purified by HIC, CIEX and SDS-PAGE was suggested to be one of the ZnPP-binding proteins in Parma ham.

Notes

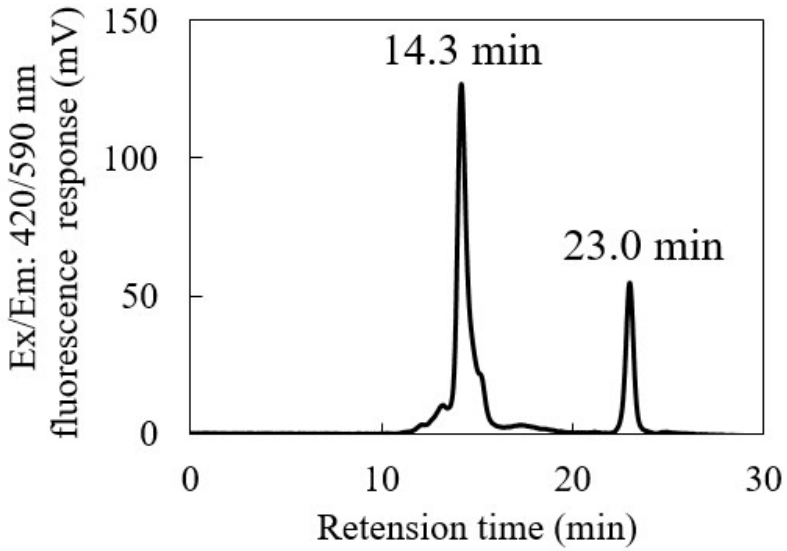

Fig. 1. SEC Chromatogram of the Parma ham water extract
SEC-HPLC was performed with eluent of citrate-phosphate buffer and the fluorescence (Ex/Em: 420/590 nm) were monitored.

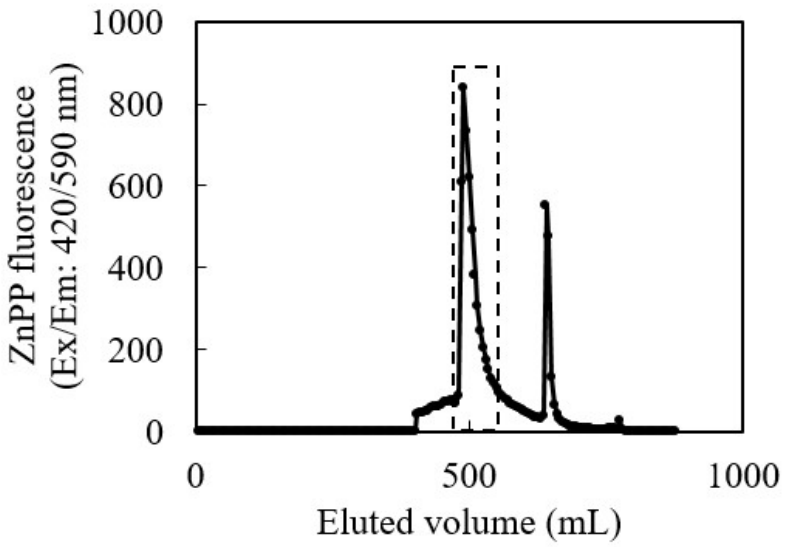

Fig. 2. HIC chromatogram of the Parma ham water extract
ZnPP fluorescence (Ex/Em: 420/590 nm) of each fractions were measured by the spectrofluorometer; the fractions within the dashed box were collected for CIEX process.

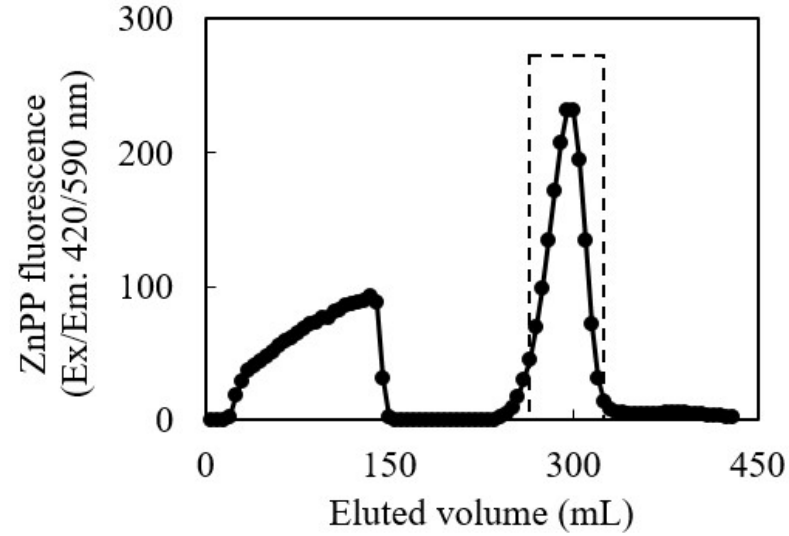

Fig. 3. CIEX Chromatogram of ZnPP-containing fraction collected from HIC chromatography ZnPP fluorescence (Ex/Em: 420/590 nm) of each fractions were measured by the spectrofluorometer; the fractions within the dashed box were collected for SDS-PAGE analysis.

Notes