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The role of myoglobin degradation in the formation of zinc protoporphyrin IX in the longissimus lumborum of pork

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ABSTRACT

Investigations on the post mortal formation of fluorescent zinc protoporphyrin (ZnPP) IX in pork meat are currently in focus of meat science research. The role of myoglobin degradation in this context appears to be one of the most diversely discussed issues. To address this question meat-extracts of *longissimus lumborum* (LL) muscle (0.8 mg/mL) were incubated at 30 °C for up to 72 h and investigated by HPSEC-UV-fluorescence, SDS-PAGE and MALDI-TOF-MS. Between 0 and 72 h of incubation the fluorescence intensity ($\lambda_{ex./em.}$ = 420/590 nm) of the meat-extracts rose significantly ($p < 0.001$) from 10.9 ± 0.8 to 34.8 ± 0.3 (rel. units) while the staining intensity of the SDS-PAGE of myoglobin non-significantly ($p > 0.4$) changed from 6.2 ± 0.5 × 10^5 to 5.0 ± 0.3 × 10^5 (rel. units). The results indicate that ZnPP is formed by a Fe(II)-Zn(II)-substitution in myoglobin heme where an accompanying myoglobin degradation is not necessarily obligatory.

1. Introduction

The luminescent metalloporphyrin ZnPP is a by-product of the heme biosynthesis, which is present in almost all forms of life (Heinemann, Jahn, & Jahn, 2008). Due to its color and its specific fluorescence at the excitation and emission wavelengths $\lambda_{ex.}$ = 420 nm and $\lambda_{em.}$ = 590 nm (Camadro, Ibraham, & Levere, 1984; Wakamatsu, Okui, Ikeda, Nishimura, & Hattori, 2004) ZnPP has technologically become a subject of interest, as its physical features have been discussed in terms of their potential benefits during meat processing: ZnPP has been proven as the main chromophore in Parma ham giving the product its characteristic red color (Adamsen, Moller, Laursen, Olsen, & Skibsted, 2006; Moller, Adamsen, Catharino, Skibsted, & Eberlin, 2007; Wakamatsu, Nishimura, & Hattori, 2004). Parma ham traditionally is cured without the addition of nitrite salts, which are typically used to achieve a stable red color in meat, but also represent a source of concern due their role in the formation of carcinogenic N-nitrosamines (Pegg & Shahidi, 2008, p. 259). In this context ZnPP might represent a nitrite free alternative for the reddening of meat and meat products (Tuan Thanh, Ishigaki, Kataoka, & Taketani, 2011). On the other hand the accumulation of ZnPP during the storage of the *longissimus lumborum* (LL) of pork has been attested by Schneider et al. (2008), so that ZnPP has been discussed as a potential indicator in the evaluation of LL quality.

Nevertheless, the biochemical reaction pathway leading to the formation of ZnPP in the aforementioned kinds of pork meat (Parma ham and LL) still has to be clarified. There are three assumptions on the post mortem formation of ZnPP, which have been discussed using the example of Parma ham:

- i) a non-enzymatic chelation of Zn(II) by protoporphyrin IX (PP) (Becker, Westermann, Hansson, & Skibsted, 2012)

Abbreviations: CA, cellulose acetate; HPSEC, high performance size exclusion chromatography; ZnPP, zinc protoporphyrin IX; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MWCO, molecular weight cut off; DHAP, 2,5-dihydroxyacetophenone; FePP, iron protoporphyrin IX; LL, longissimus lumborum; PP, protoporphyrin IX; FECH, ferrochelatase; RT, room temperature.

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- ii) an enzyme-induced substitution of Fe(II) from heme with Zn(II) by endogenous ferrochelatase (FECH) (Becker et al., 2012; Benedini, Raja, & Parolari, 2008; Parolari, Benedini, & Toscani, 2009; Wakamatsu, Okui, Hayashi, Nishimura, & Hattori, 2007),
- iii) a bacteria-induced enzymatic reaction (Morita, Niu, Sakata, & Nagata, 1996; Wakamatsu, Nishimura, et al., 2004; Wakamatsu, Okui, et al., 2004),

whereby the possibility of an enzymatic and the non-enzymatic pathway, respectively, is assumed to be affected by the storage temperature (Parolari, Aguzzoni, & Toscani, 2016). Myoglobin is supposed to be the heme-donor for the ZnPP formation (Wakamatsu, Nishimura, et al., 2004; Wakamatsu, Okui, et al., 2004). Using the example of Parma ham Grossi, do Nascimento, Cardoso, and Skibsted (2014) have reported, that the degradation of myoglobin is essential for the formation of ZnPP, as the degraded myoglobin would allow a Fe(II)-Zn(II)-transmetallation in myoglobin heme. However, the increase of ZnPP concentrations in LL within the storage at room temperature (RT) for 72 h has been attested in our recent research works taking the view that the accumulation of ZnPP has not been associated with a degradation of myoglobin in LL. Thus the aim of this study was to find out, whether myoglobin degradation, as it had been described in the case of Parma ham, could also be observed in LL samples in spite of the very short storage time as compared to Parma ham (>9 month).

To pursue this question LL extracts were incubated at RT for up to 72 h and analysed by various analytical techniques: a HPSEC-UV-fluorescence setup was used in order to separate the myoglobin from most of other meat-inherent proteins and to determine the occurrence of ZnPP fluorescence in the myoglobin during the incubation of the extracts. For the determination of a probable myoglobin degradation during the storage of the LL extracts the myoglobin-containing protein bands of the LL extracts were collected after HPSEC by a fraction collector and investigated qualitatively and semi-quantitatively by MALDI-TOF-MS and SDS-PAGE, respectively: MALDI-TOF-MS spectra were supposed to enable the detection of lower molecular myoglobin fragments, which might indicate a myoglobin degradation during the storage of the LL extracts. SDS-PAGE analyses were supposed to enable the detection of a probable decrease of the myoglobin concentration during the storage of the LL extracts.

The present research study delivers new insights into the chemical nature of the ZnPP formation during the storage of LL, in which the fluorophore is accumulated at RT within a few days of storage.

2. Materials and methods

2.1. Preparation and incubation of the water-soluble LL proteins

LL of pork was purchased from a local butcher (Potsdam, Germany) one day after slaughter and minced with a hand mixer. Thirty two g of that meat homogenate were weighed into a beaker and diluted with 40 mL of a pH 7.4, 4 °C phosphate buffer (10 mmol/L Na_2HPO_4, 1.8 mmol/L NaH_2PO_4, 9 g/L NaCl, 0.2 g/L NaN_3, all from Carl Roth, Karlsruhe, Germany). Protein extraction was performed on ice by treating the meat homogenate with an Ultra Turax (IKA GmbH, Staufen, Germany) at 12.000 rpm for 1 min. Afterwards the obtained meat-suspension was evenly transferred into two 50 mL Falcon tubes and centrifuged at 5200×*g*, RT for 5 min. After centrifugation the supernatants of both Falcon tubes were collected to obtain the clear stock solution of the extracted LL proteins. Aliquots of 1 mL each were transferred from the stock solution into 2 mL Eppendorf tubes (n = 4), which were then incubated at 30 °C for up 72 h.

2.2. Spectroscopic analyses of protein bands by HPSEC-UV-fluorescence

After 0, 24, 48 and 72 h of incubation the Eppendorf tubes were centrifuged at 5200×*g*, RT for 5 min. The clear extracts were syringe-filtered through 0.2 μm cellulose acetate (CA) -filters (Carl Roth, Karlsruhe, Germany) into HPLC glass-vials. Fifty μL of each extract were injected into a Shimadzu Nexera HPLC device (Berlin, Germany), which was equipped with both an UV (SPD M20A) and a fluorescence (RF 20A) detector, respectively, and a fraction collector (FRC 10A). Protein separation was performed by HPSEC using a Tosohaas tsk gel column (G4000WXL, 6 μm, Griesheim, Germany) at a flow rate of 0.4 mL/min. The eluent was a pH 7.4 phosphate buffer solution (10 mmol/L Na_2HPO_4, 1.8 mmol/L NaH_2PO_4, 9 g/L NaCl, 0.2 g/L NaN_3, all from Carl Roth, Karlsruhe, Germany). The sample's absorbance was detected at A = 254 nm (UV) and the fluorescence was measured at the excitation/emission wavelengths $\lambda_{ex.}$ = 420 nm and λ_{em} = 590 nm (specific ZnPP fluorescence), respectively. A 5 g/L myoglobin (equine heart, Sigma-Aldrich, Steinheim, Germany) standard solution was used to identify the myoglobin-containing band in the separated LL proteins.

2.3. Collection of the myoglobin for qualitative and semi-quantitative analyses after HPSEC

The myoglobin-containing protein band was collected by the integrated fraction collector (FRC 10 A) after HPSEC separation at 0, 24, 48, 72 h of extract's incubation (section 2.2). An aliquot of 300 μL was taken from each collected solution and concentrated by a Vivaspin® 500 centrifugation filter (3 kDa MWCO, GE healthcare, Solingen, Germany) at 10,600×*g*, RT for 60 min. Afterwards the retentate was washed twice with 100 μL ultra-pure water at 10,600×*g*, RT for 30 min. Each concentrated and washed sample was immediately refrigerated at −20 °C until the day of analyse.

2.4. Qualitative analyses of the myoglobin-containing protein bands (MALDI-TOF-MS)

For MALDI-TOF-MS analysis 2 μL of each concentrated and washed sample (section 2.3) was mixed with 2 μL of a 2,5-dihydroxyactetophenone (DHAP, Bruker Daltonik GmbH, Bremen, Germany) solution as matrix (7.6 mg of 2,5-DHAP was dissolved in 375 μL of ethanol, Sigma-Aldrich, Steinheim, Germany, and 125 μL of a solution containing 18 mg/mL diammonium hydrogen citrate, Sigma-Aldrich, Steinheim, Germany, dissolved in water) and 2 μL of 0.26 mol/L trifluoroacetic acid (Sigma-Aldrich, Steinheim, Germany). One μL of the mixture was droped onto an Anchor Chip 800 Target (Bruker Daltonik GmbH, Bremen, Germany). After crystallization of the sample by air-drying, measurements were carried out on AUTOFLEX-III LRF200-CID, equipped with Smartbeam-Laser 200 (Bruker Daltonik GmbH, Bremen, Germany) in the mass range of 5000–30,000 m/z. The instrument was internally calibrated using the signals of the positive $[M+H]^+$ monoisotopic ions of a protein 1 calibration standard (Bruker Daltonik GmbH, Bremen, Germany).

2.5. Semi-quantitative analysis of the myoglobin-containing protein band (SDS-PAGE)

SDS-PAGE, according to the method of Laemmli (1970), was applied for determining the molecular weight distribution using Quantity One 1-D Analysis Software, version 4.5.2 (Bio-Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy). Aliquots of the concentrated and washed samples (section 2.3) were mixed in different ratios with sample buffer (50 mmol/L Tris-HCl buffer at pH 6.8 containing 4 g of sodium dodecyl sulfate, 12 g of glycerol, 5 g