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Unraveling proteome changes of Holstein beef M. *semitendinosus* and its relationship to meat discoloration during post-mortem storage analyzed by label-free mass spectrometry

Qianqian Yu, Wei Wu, Xiaojing Tian, Man Hou, Ruitong Dai *, Xingmin Li

College of Food Science and Nutritional Engineering, China Agricultural University, 17 Qinghua East Road, Haidian District, Beijing 100083, PR China
Beijing Higher Institution Engineering Research Center of Animal Product, China Agricultural University, No. 17 Qinghua East Road, Haidian District, Beijing 100083, PR China

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ABSTRACT

Label-free proteomics was applied to characterize the effect of post-mortem storage time (0, 4, and 9 days at 4 °C ± 1 °C) on the proteome changes of M. *semitendinosus* (SM) in Holstein cattle, and correlations between differentially abundant proteins and meat color traits were investigated. The redness (a*) value decreased significantly ($P < 0.05$) during post-mortem storage, meanwhile, the relative proportion of metmyoglobin increased significantly ($P < 0.05$) from 16.99% at day 0 to 40.26% at day 9. A total of 118 proteins with significant changes (fold change > 1.5, $P < 0.05$) was identified by comparisons of day 4 vs. day 0, day 9 vs. day 0, and day 9 vs. day 4. Principal component and hierarchical cluster analyses of these proteins were performed, and results exhibited clear distinctions among samples from different storage times. Eighteen differentially abundant proteins were correlated closely with the a* value of meat. Bioinformatics analyses revealed that most of these proteins were involved in glycolysis and energy metabolism, electron-transfer processes, and the antioxidation function, which implied an underlying connection between meat discoloration and these biological processes.

Significance: It is always a challenge for scientists to improve the stability of meat color during post-mortem storage and retail display. However, the mechanism involved in meat discoloration has not been unraveled completely, and the application of label-free proteomics in studying meat discoloration has not been reported. Our work discovers some key proteins in SM muscle of Holstein cattle that were correlated with a* value of meat via label-free proteomics. Bioinformatics analyses revealed that some of these differentially abundant proteins were involved in glycolysis and energy metabolism, electron-transfer processes, and the antioxidation function, which implied an underlying connection between meat discoloration and these biological processes. These results provide the theoretic basis on understanding of complicated biochemical changes and underlying molecular mechanisms responsible for meat discoloration.

1. Introduction

Meat color is one of the most critical characteristics that influence the purchase decisions of customers at the point of sale [1], and customers often relate discoloration to unwholesomeness and staleness. Therefore, it is always a challenge for scientists to improve the stability of meat color during post-mortem storage and retail display. Myoglobin is the principle protein responsible for meat color, and this has received extensive attention [2–4]. However, many intrinsic factors (breed, age of animal, muscle type, metabolism, the ultimate pH of meat, etc.) and extrinsic factors (temperature, O_2 availability, packaging, etc.) affect meat color [5]. Suman et al. summarized *pre*-harvest (i.e., diet, animal management) and *post*-harvest (i.e., packaging, aging, antioxidants) strategies to improve color stability in fresh and cooked beef, and suggested that these strategies are specific to the type of animal, feeding regimen, packaging system, and muscle source [6].

With rapid development of analytical techniques, proteomics, a powerful and promising post-genomic tool, has been used in meat science research to discover molecular biomarkers and to illustrate complicated biological mechanisms involved in the development of meat quality traits. Meat tenderness has been studied comprehensively using proteomics approaches [7–9], and some potential protein biomarkers have been discovered, which belong mainly to structural proteins, heat shock proteins, oxidative resistance proteins, and proteins involved in apoptosis, proteolysis, and energy metabolism. Canto et al. classified 10 color-stable and 10 color-labile steaks from 73 beef carcasses based on redness and color stability on day 11 [10]. They identified successfully nine differentially abundant proteins; among them,

* Corresponding author at: College of Food Science and Nutritional Engineering, China Agricultural University, 17 Qinghua East Road, Haidian District, Beijing 100083, PR China.
E-mail address: dairuitong@hotmail.com (R. Dai).

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three glycolytic enzymes (phosphoglucomutase-1, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase M2) were over-abundant in color-stable steaks and were correlated positively ($P < 0.05$) with redness and color stability. Similarly, Joseph et al. identified 16 differentially abundant proteins in color-stable (*Longissimus lumborum*) and color-labile (*Psoas major*) beef muscles, and they reported that overabundance of antioxidant proteins and chaperones could contribute to meat color stability [11]. Meat color stability and changes in sarcoplasmic proteomes of different bovine muscle types during post-mortem storage were also analyzed and investigated by Wu et al. [12, 13]

However, only gel-based approaches were used in these studies. Recently, label-free quantitative proteomics has gained much attention due to its notable superiority in discovering biomarkers [14,15]. The label-free approach does not need any isotopic or chemical labeling [16], and it has the largest dynamic range and the highest proteome coverage for identification [14]. Gallego et al. optimized a label-free quantitative proteomic methodology for the relative quantification of proteins that were extracted from raw pork meat, and this yielded high repeatability, linearity, and accuracy [17]. This result suggested that the label-free method could be applied to study the changes of proteomes in meat products. Piovesana et al. characterized the sarcoplasmic proteome of muscle tissue from farmed and wild gilthead sea bream by labeling and by label-free proteomics strategies, and they concluded that the label-free approach provided more comprehensive protein identification with good analytical reproducibility and sample throughput [18].

Although proteomics has been used to identify potential biomarkers and to investigate molecular mechanisms that affect meat quality, application of label-free proteomics in studying meat discoloration has not been reported, and the mechanism involved in meat discoloration has not been unraveled completely. Therefore, the objectives of the present study were i) to analyze the changes of proteomes in M. *semitendinosus* (SM) muscles during post-mortem storage by the label-free approach, and ii) to assess correlations between meat color parameters and differentially abundant proteins to discover the underlying mechanism for meat discoloration.

2. Materials and methods

2.1. Materials

This study was carried out using beef from Holstein cattle with same feeding strategy. Animals came from a farm in Dachang country, Hebei Province, China. The chemicals and reagents used in this study were applied by: Tris-HCl (Solarbio, CAS: 1185-53-1), dithiothreitol (Genview, CD116-25g), urea (sigma, U5378-500g), iodoacetamide (Vetec, V900335-5g), NH_4HCO_3 (Sigma, 09830-500G), trypsin (Promega, V5113), Protease Arrest (G-Biosciences, 786-437), formic acid (Sigma, 94318), acetonitrile (Sigma, 34851), ENO3 monoclonal antibody (Abnova, H00002027-M01), PGM1 monoclonal antibody (Abnova, H00005236-M01), HSP60/HSPD1 polyclonal antibody (abcam, ab46798), β-actin monoclonal antibody (Immunoway, YM3028), goat anti-mouse IgG (H + L) (Beijing TDY Biotech Co., Ltd., S001), and goat anti-rabbit IgG (H + L) (Beijing TDY Biotech Co., Ltd., S004).

2.2. Sample preparation

Muscle samples of SM were harvested from three Holstein cattles (n = 3), which were slaughtered at the age of approximately 36 months and with a live weight of 450 ± 5 kg. After 36 h post-mortem, the SM muscles from the three animals were obtained. Muscles from the same animal were considered as one biological replicates in our study. All the muscle samples were cut into 3 cm steaks, kept in styrofoam trays, and overwrapped with polyethylene (PE) film (13,000 ± 20% cm^3/m^2/24 h oxygen transmission rate). All trays were stored in a refrigerator at 4 °C ± 1 °C and samples were collected at 0, 4, and 9 days of storage for subsequent determination of quality. The proteome samples (2 g from each storage time) were frozen immediately in liquid nitrogen until analyses.

2.3. pH of meat

The pH of samples (3 biological replicates × 3 technical replicates) from post-mortem storage time (0, 4, and 9 days) was evaluated according to the method of Strange et al. [19]. Meat samples (5 g) were homogenized with 50 mL deionized water, and the pH was measured using a FE-20 pH-meter (Mettler Toledo, Zurich, Switzerland).

2.4. Instrumental evaluation of color

The surface CIE lightness (L*), redness (a*), and yellowness (b*) values of meat samples were determined utilizing a CR-400 Minolta colorimeter (Konica Minolta Sensing Americas Inc., Ramsey, NJ, USA) with illuminant of D_{65}. The instrument was calibrated by white plate prior to measurement. Three biological replicates (3 × 3 × 3 cm) were provided, and each of them was evaluated at six locations on the meat surface.

2.5. Determination of myoglobin redox forms

The proportion of myoglobin redox forms of samples (3 biological replicates × 3 technical replicates) for each storage time was determined according to Tang et al. [20] and Wu et al. [13]. Briefly, 5 g muscle samples were homogenized with 25 mL phosphate buffer (40 mM, pH 6.8) for 25 s, and then the homogenate was centrifuged at 4 °C, 4500*g*, for 30 min. The supernatant was filtered and compensated with phosphate buffer to 25 mL, and the absorbance of mixture at 503, 525, 557, and 582 nm was measured utilizing a spectrometer (Evolution 60S, Thermo Scientific, USA). The proportions of deoxymyoglobin (DeoMb), oxymyoglobin (OxyMb), and metmyoglobin (MetMb) were calculated according to the following equations:

$$[\mathrm{DeoMb}] = \frac{C_{\mathrm{DeoMb}}}{C_{\mathrm{Mb}}} = -0.543R_1 + 1.594R_2 + 0.552R_3 - 1.329$$

$$[\mathrm{OxyMb}] = \frac{C_{\mathrm{OxyMb}}}{C_{\mathrm{Mb}}} = 0.722R_1 - 1.432R_2 - 1.659R_3 + 2.599$$

$$[\mathrm{MetMb}] = \frac{C_{\mathrm{MetMb}}}{C_{\mathrm{Mb}}} = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$$

where $R_1 = \frac{A_{582}}{A_{525}}$, $R_2 = \frac{A_{557}}{A_{525}}$, $R_3 = \frac{A_{503}}{A_{525}}$.

2.6. Analysis of proteomics

2.6.1. Protein extraction and digestion

Samples of SM from 3 biological groups were collected at day 0, 4, and 9 after 36 h post-mortem and performed to proteomics analyses. Frozen muscle samples were ground in the cold extraction buffer (8.0 M urea, 100 mM Tris-HCl, 10 mM dithiothreitol (DTT), Protease Arrest (1×, acting as protease inhibitors, G-Biosciences, St. Louis, MO, USA), pH 8.0). After sonication (2 s each time, repeated 5 times), the homogenate was centrifuged at 4 °C, 10,000*g*, for 30 min, and the supernatants were collected and stored at −80 °C for further analysis. The protein concentration of muscle sample was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc. Waltham, MA, USA). Muscle proteins were digested as described by Shi et al. [21] with some modifications. Briefly, the extract solution of each sample (100 μg protein) was reduced by DTT with a final concentration of 40 mM at 60 °C for 1 h. Iodoacetamide (50 mM) was added to alkylate cysteines and then incubated for 1 h in a darkroom at 20 °C. Proteins were diluted with 200 μL of NH_4HCO_3 (50 mM) and digested with