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Bioactive peptides in beef: Endogenous generation through postmortem aging

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ABSTRACT

The present research was performed to investigate endogenous release of bioactive peptides in beef during postmortem aging times (1, 10 and 20 days). Gradually decreased Warner-Bratzler shear force (WBSF) values of *longissimus thoracis* (LT) and *semitendinosus* (ST) muscles were observed and the degradation of structural proteins and collagen led to release of low-molecular weight (<3 kDa) peptides. These peptides exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, ACE- and renin-inhibitory activities. The peptide sequences were identified by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). *In silico* analysis (PeptideRanker and BIOPEP) of their bioactivity potentials demonstrated peptides with the predicted bioactivity scores (>0.8) as well as collagen peptides with bioactivity scores (0.6–0.8). The present findings provide insights on development of healthy beef through postmortem aging at 4 °C.

1. Introduction

Tenderness, juiciness, color, flavor and aroma are all key attributes of meat palatability with regards to consumers' satisfaction, with tenderness being the most significant factor (Huffman et al., 1996; Miller, Carr, Ramsey, Crockett, & Hoover, 2001; Wu, Fu, Therkildsen, Li, & Dai, 2015). As important as this attribute is, it seems to be the most variable of all meat palatability traits and has been attributed to several factors, including degradation of myofibrillar proteins and to a large extent the connective tissue. Postmortem aging of beef is a very effective method to improve tenderness (Huff-Lonergan, Zhang, & Lonergan, 2010). This process allows the natural enzymatic and biochemical processes to take place resulting in increased tenderness (Nishimura, Hattori, & Takahashi, 1995) due to the weakening of the myofibrils and the intramuscular connective tissue (Dransfield, 1994). During this process, postmortem aging not only contributes to meat tenderness but it may also generate peptide fractions with physiological significance, such as antioxidant and blood pressure lowering effects.

Hypertension is a global leading risk factor for cardiovascular diseases (Ahhmed & Muguruma, 2010). The renin-angiotensin system (RAS) is a main pathway responsible for regulating blood pressure and ensuring fluid homeostasis. Moderate hypertension can be controlled through dietary approaches and a number of investigations have reported the antihypertensive and ACE-inhibitory peptides of different food sources (Kim & Wijesekara, 2010). Oxidative stress, an imbalance between oxidants and antioxidants, might further induce and exacerbate hypertension (Kizhakekuttu & Widlansky, 2010). However, administration of food-derived peptidic antioxidants contributes to a lower occurrence of oxidative stress (Samaranayaka & Li-Chan, 2011). In recent years, proteolysis of meat proteins (myofibrillar, sarcoplasmic or collagen) has been documented to release several potential bioactive sequences to exhibit *in vitro* antihypertensive and antioxidant activities (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011; Mora et al., 2014; Escudero et al., 2013; Xing et al., 2016).

Although some health-promoting compounds from meat with positive physiological effects have been reported (Decker & Park, 2010; Young et al., 2013), few studies focused on the peptides generated during postmortem aging and their potential as a natural source of antihypertensive and antioxidant peptides to maintain blood pressure and health. Therefore, the aim of this study is to investigate inherent bioactivity developed through aging/tenderization in beef. In this study, beef was aged for 1, 10 or 20 days postmortem and the bioactivity of the released peptides was determined. In addition, their peptide sequences were characterized and bioactivity potentials from the individual peptides were predicted based on *in silico* analysis in order to estimate their relative contribution to the sample bioactivity.

2. Materials and methods

2.1. Materials and sample preparation

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ACE from rabbit lung were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human recombinant renin inhibitor screening assay kit was purchased from

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Cayman Chemicals (Ann Arbor, MI, USA). Amicon Ultra-15 centrifugal filters (3 kDa molecular weight cut-off) were purchased from Merck Millipore (Cork, Ireland). The Oasis HLB cartridges (1 mL) were obtained from Waters (Dublin, Ireland).

Six bulls (3 Danish Holstein + 3 Danish Holstein cross breeds; age: 16 ± 2 months; live weight: 245 ± 35 kg) were slaughtered at a Danish Crown slaughter house (Aalborg, Denmark). The animals were stunned by captive bolt pistol, hung and bled. After 15 min postmortem, the carcasses were electrically stimulated with low voltage (78 V, 200 mA) for 35 s. Carcasses were hung in the chiller at 4 °C. After one day postmortem, *longissimus thoracis* (LT) (from the 1st thoracic vertebrae to the 5th thoracic vertebrae) and *semitendinosus* (ST) muscles were removed from each carcass and transported for 1 h in insulated shipping boxes filled with ice bags to the lab. The temperatures of the LT and ST muscles after arrival in the lab were determined to be 5.1 ± 0.3 °C and 5.3 ± 0.4 °C with Testo 110 thermometer (Testo GmbH & Co., Lenzkirch, Germany). The pH value was measured in each muscle with a PHM201 pH meter (Radiometer, Denmark) equipped with Metrohm probe type glass electrode (Metrohm, Switzerland). The electrode was calibrated in pH 4.01 and 7.00 IUPAC buffers. Subsequently, each muscle was divided into three (8 cm × 5 cm × 5 cm) blocks, labelled and randomly distributed in three groups of aging (day 1, day 10 and day 20). The samples were packed in the vacuum bags (Lava Vakuumverpackung, Bad Saulgau, Germany), which consists of polyethylene and polyamide (O_2 permeability: 50 $cm^3/m^2 * d * bar$). The vacuum bags were evacuated and heat-sealed using a vacuum packaging machine (Komet, Stuttgart, Germany) under 1.0 bar of vacuum. Day 1 samples were analyzed for texture at the same day and sub-samples from both raw and cooked samples (described in Section 2.2) at day 1 were stored at −20 °C for further bioactivity analyses, whereas day 10 and 20 samples were stored at 4 °C for additional 9 or 19 days before texture analysis and subsampling for bioactivity measurement as described below.

2.2. Warner-Bratzler shear force (WBSF)

The WBSF of aged beef samples was determined as per the method of Honikel (1998). Briefly, the beef samples were heated in a thermostat bath (GD100, Grant Instruments, Shepreth, UK) after reaching core temperature (63 °C). The heat treatment was terminated by immersing the samples in ice water for 15 min. Thereafter, the samples were stored in a water bath at 4 °C until next day. The next day rectangular blocks (1 * 1 cm thick) were cut parallel to the longitudinal orientation of the muscle fibres and the shear force was measured using a Texture Analyzer TX-T2 (Stable Micro Systems, Godalming, U.K.) with a Warner-Bratzler shear blade with a rectangular hole. The blade speed was set to 100 mm/min and the average maximum force (N/cm^2) of 6 replicates cut from each sample was used.

2.3. Peptide extraction

The peptides were extracted from beef samples according to Bauchart et al. (2006) with slight modifications. Frozen beef samples of both raw and cooked origin (2.5 g) were homogenized in 12.5 mL of 3% perchloric acid in centrifuge tubes on ice for 2 min using a Polytron PT 2100 homogenizer from Kinematic AG (Luzern, Switzerland). Subsequently, the homogenate was centrifuged at 10,000*g* for 20 min at 4 °C and the supernatant was collected and filtered using a cellulose acetate filter of 0.2 μm pore size (Frisenette, Denmark). The extracts were neutralized to pH 7 using sodium hydroxide. The salt precipitate was eliminated using the cellulose acetate filter twice. Subsequently, the supernatant was subjected to ultra-filtration using 3 kDa cut-off centrifugal filters at 10,000*g* for 30 min. The resulting filtrates were lyophilized and stored at −20 °C for further analysis.

2.4. Peptide identification by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)

Prior to MS analysis, the extracted peptide samples were subjected to purification using Oasis HLB (C18 solid phase) cartridges in order to remove the salts and impurity. Afterwards, the samples were lyophilized and re-diluted using 0.1% formic acid before injection into LC-ESI-MS. The LC system (Agilent Technologies, Waldbronn, Germany) was equipped with Jupiter Proteo C18 column of dimensions 150 mm × 0.5 mm (Phenomenex, Denmark). The percentage of solvent B (90% acetonitrile, 0.1% formic acid) in solvent A (0.1% formic acid) was based on a linear gradient. The flow rate was fixed at 100 μL/min for 110 min. Tandem MS spectra were further analyzed by PEAKS Studio 7.5 (Waterloo, ON, Canada) and searched against the customized bovine family (*Bos taurus*) from UniProt database. The search was implemented using no specific enzyme cleavage sites and an MS/MS mass tolerance of 0.5 Da. The peptides with average local confidence (ALC) over 75% were used for further analysis. The analysis was performed using two individual samples and only peptides positively identified in both samples were acceptable. Peptide sequences, their position in the parent proteins and the observed masses and retention times were collected from the PEAKS. Basic Local Alignment Search Tool (BLAST) was employed to search for regions of local similarities between the identified peptides and the parent protein sequences within the *Bos taurus* database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences of the peptides identified in this work were searched and revealed 100% homology with proteins of *Bos taurus*.

2.5. Peptide concentration

The concentration of the extracted peptides was determined by calculating the amount of N-terminal amines using fluorescamine according to Petrat-Melin et al. (2015). The extracted peptide concentration was fixed at 30 mM for further determination of bioactivity.

2.6. Bioactivity determination of the extracted peptides

2.6.1. DPPH radical scavenging capacity

DPPH radical scavenging activity of isolated peptides was determined according to Li, Chen, Wang, Ji, and Wu (2007) with slight modifications. 500 μL test sample (peptide fraction of 30 μM) was mixed with 500 μL of 99.5% ethanol and 125 μL of 99.5% ethanol containing 0.01% DPPH. This mixture was kept in the dark at room temperature for 60 min before determination of absorbance at 517 nm. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavening capacity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the control.

2.6.2. ACE inhibitory-activity

ACE-inhibitory activity was determined according to the approach of Petrat-Melin et al. (2015). Briefly, ACE working solution (7.1 U/mL) of 50 μL in borate buffer (50 mmol/L, pH 8.3) was added to wells of a 96-well microplate, followed by addition of 50 μL of either sample or control (Milli-Q water). The enzyme reaction was initiated by addition (200 μL) of 0.45 mmol/L Abz-Gly-Phe(NO_2)-Pro dissolved in Tris-base buffer (150 mmol/L, pH 8.3). The resultant reagents were immediately mixed and incubated at 37 °C. The generated fluorescence was measured using a microplate reader (BioTek Instruments, Winooski, USA) with excitation and emission wavelengths of 355 and 405 nm, respectively.