Metalloproteomics Study of Bovine Longissimus dorsi Muscle Tissue in selected animals of the Nellore Breed (Bos indicus)

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ABSTRACT: The work describes a metalloproteomics study of bovine muscle tissue with different grades of meat tenderness from animals of the Nellore breed based on protein separation by two-dimensional gel electrophoresis, the identification of calcium ions in protein spots by X-ray fluorescence (SR-XRF) and the characterization of proteins by electrospray ionization mass spectrometry. Forty (40) specimens were selected and divided into two experimental groups: animals with tough meat and animals with tender meat. A third group of Piedmontese breed animals was included to serve as a comparative model for the level of meat tenderness. The procedures were efficient and preserved the metal-protein structure, enabling calcium detection in protein spots by SR-XRF at a given molecular weight range of 14 to 97 kDa. The proteins pyruvate kinase and albumin were inferred to be related to the phenotypical differences found in animals from the different groups.

Keywords: beef cattle, meat, proteomic

Introduction

The application of knowledge of the muscle proteome is a new tool in meat science. The greatest challenge of improving the quality of meat products using proteomics involves understanding which variations affect the tenderness of meat-producing species. In this context, changes in energy metabolism in the bovine Longissimus thoracis muscle proteome were evaluated by Jia et al. (2006) during the pre- and post-slaughter (ante mortem and post mortem) periods. The samples of this study were collected four days before slaughter and 60 minutes after slaughter. The results suggest that 24 proteins involved in the glycolytic pathway and/or heat-shock proteins (HSP) showed significant changes in expression between the periods assessed. Kim et al. (2008) conducted their study with the goal of testing the use of these proteins as biomarkers for meat tenderness and marbling and to determine if the quantitative expression of HSP27 and inositol 1,4,5-triphosphate receptor type 1 (IP3R1) was correlated with marbling, tenderness and free calcium levels; the authors found that these proteins could indeed be used as biomarkers of Hanwoo beef quality. However, few studies have been performed on animals of the Bos indicus genotype. The present study aimed to perform the separation of Longissimus dorsi (L. dorsi) muscle proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), to identify calcium ions in protein spots by Synchrotron Radiation X-ray Fluorescence (SR-XRF) and to characterize proteins by electrospray ionization mass spectrometry (ESI-MS) from Nellore breed (Bos indicus) cattle with different grades of meat tenderness.

Materials and Methods

Animals and sample collection. All procedures with animals were conducted from the ethical research standards, established by the São Paulo State University. Forty (40) specimens of the 1,084 slaughtered animals (bulls of the Nellore breed under the age of 24 months) were selected and divided into two experimental groups with different grades of meat tenderness: animals with tough meat (TO) and animals with tender meat (TE). A third group (P) of Piedmontese breed animals (Bos taurus) was included to serve as a comparative model for the level of meat tenderness and of accelerated and hypertrophic muscle tissue growth. Briefly, the carcasses were washed and chilled for 24h in a cold room at 1ºC for the onset of rigor mortis. After cooling, four samples of L. dorsi muscle with bone, measuring approximately 2.5 cm, were collected between the 12th and 13th ribs in the cranial direction from the left half-carcass of each animal. The method proposed by Wheeler, Kooymaarae & Shackelford, (1995) was used for shear force (SF) measurement. Rodas-Gonzalez et al. (2009), reported, based on customer panelists, that steaks with a Warner-Bratzler shear values less than 4.09 kg would produce customer satisfaction as high as 81%. Moreover, that 4.9 kg can be considered as the tenderness threshold (segregate tough to tender) for Latin American beef consumers. Another physicochemical analyses, including rib eye area and subcutaneous fat thickness (USDA - Quality and Yield Grade, 2000) were performed.

Protein extraction, precipitation and resolubilization. Approximately 3 g of muscle tissue from the pool of each experimental group (weighed in triplicate) were ground in 30 mL deionized water using an Ultra-Turrax high shear mixer (Marconi - MA102/E) at 20,000 rpm twice for 30 seconds. The protein extracts were separated from the solid parts by centrifugation at 13,000 rpm at 4º C in a refrigerated ultracentrifuge. The protein content of these protein extracts was precipitated using an ice-cold 80% (v/v) acetone solution at a 1:4 sample:acetone ratio. A portion of these precipitated proteins was resolubilized in 0.50 mol.L⁻¹ NaOH for total protein quantification. Another part was resolubilized in a specific buffer at 7 mol L⁻¹ with 2 mol L⁻¹ of thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 0.5% (w/v) ampholytes pH 3 and 0.02% bromophenol blue. Subsequently, 2.8 mg of dithiothreitol (DTT) were
added to this buffer, and this mixture was used in electrophoretic separations (Lima et al., 2010; Silva et al., 2013).

2D-PAGE. Approximately 250 µg of protein from the bovine muscle tissue protein extracts were loaded into 13-cm isoelectric focusing strips which contained the precast gel with an ampholyte-immobilized pH 3-10 gradient; 13-cm strips with an ampholyte-immobilized pH 4-7 gradient were also used. The first dimension runs were performed adapting the procedures reported by Lima et al. (2010) and Santos et al. (2011). The strips were placed on a polyacrylamide gel (12.5% w/v) in the second dimension of the electrophoretic process (SDS-PAGE), adapting the procedures recently reported by Lima et al. (2010) and Santos et al. (2011).

SR-XRF. The protein spots found in the bovine muscle tissue gels from the different experimental groups were taken to the Brazilian National Synchrotron Light Laboratory in Campinas-SP for the analysis of calcium ions by synchrotron radiation X-ray fluorescence (SR-XRF). The most intense protein spots, properly separated by pl and MW, were randomly selected for the evaluation and detection of calcium ions, as it would have been difficult to analyze all spots from all gels using the SR-XRF method.

ESI-MS. The protein characterization using the ESI-MS methods was made after cutting spots from the polyacrylamide gel. Then each spot was placed in a well of a Zip Plate (Millipore) for tryptic digestion. The tryptic digestion was performed using a specific kit (In-Gel Digest Kit) that digests proteins and purifies the obtained peptides. The ESI-MS runs were processed using the ProteinLynx Global Server v.2.2. software (Waters) and analyzed by database search using the Mascot v.2.2. system (Matrix Science Ltd). Trypsin digestions with up to one missed cleavage site, oxidations of methionine residues as variable modifications and peptide and fragment ion mass tolerance, all at a level of ±0.5 Da, were selected as search parameters. The searches were performed in the data bank of National Center for Biotechnology Information (NCBI database).

Statistical Analysis. The meat quality characteristics were evaluated using the GLM procedure (SAS, 2011). The FREQ procedure is used (SAS, 2011) for testing the difference between two frequencies of the incidence of calcium in protein spots based on the Chi square test. Each group (TE, TO and P) represents a random sample. If there are no differences among frequencies in the groups, the expected frequencies will be the same in all groups. The expected frequencies can be estimated by using the frequencies of successes (with calcium) in all groups together. The protein spot volume data were imported into ImageMaster Platinum (v. 7.0) - GE Healthcare (2007) - software and mean and standard deviation were calculated for selected spots.

Results and Discussion

Beef quality. The model detected differences in the grade of the animal meat tenderness among the experimental groups. The animals selected to form the TE group (tender meat) had SF values ranging from 2.40 to 4.70 kg, while the animals selected for the TO group (tough meat) had SF values ranging from 5.30 to 10.11 kg. The samples of P group had average SF of 2.76 kg.

Total protein and Electrophoretic separations. The value of the total protein concentration found in the bovine muscle tissue samples enabled the calculation of the protein extract volume (assessed from the solubilization of the acetone precipitate) to be loaded onto the gel strips prior to isoelectric focusing. The protein concentrations in the extracts of the TE, TO and P groups were 28.403 ± 0.970, 36.046 ± 0.841 and 23.803 ± 1.521 g.L⁻¹, respectively. The gels of electrophoretic runs showed good resolution (matching ≥ 50%), showing that the protein separation was efficient (Lima et al., 2010). There was a great diversity of protein spots, and it is noteworthy that the gels run in the pH gradient 4-7 also yielded good protein separation in that isoelectric point (pl) range. Most protein spots were found in the 20-to-66 kDa MW range, with the most frequent pIs in the approximate range of 5 to 7. The image processing of the electrophoretic runs of groups TE, TO and P showed a correlation between gels (n = 3) of 73%, 56% and 58%, respectively. Accordingly, this means that the protein spots were found in the three replicates of these gels. The mean number of protein spots found in the gel replicates of groups TE, TO and P were 186 ± 20, 146.5 ± 16.5 and 175 ± 15, respectively. The standard deviation below 12% can be considered a good indication of protein separation by 2D-PAGE (Brandão et al., 2010).

Qualitative analysis of calcium by SR-XRF. The results of the detection of calcium ions in protein spots from the different experimental groups are outlined in Table 1. The higher qualitative calcium detection (56%) by SR-XRF in protein spots from tender meat animals may be indicative of the occurrence of proteolytic activity in muscle tissue following the slaughter of these animals (post mortem period). In Table 1, deviations are significant, so there is influence of the samples of the three experimental groups on the outcome of the SR-XRF analysis. Thus, it is evident that the result of the detection of calcium ions depends on the experimental groups.

Proteins identified. The study of the differences in protein expression (based on normalized volume – %V and/or normalized intensity – %I) between gels from the TO, TE and P groups helps to choose the “different” protein spots with (or without) calcium ions from the gels of the experimental groups. Only pyruvate kinase (identified in spot 7, group P) and albumin (identified in spot 1, group P) were found to differ in expression, in %V and in %I between experimental groups. Pyruvate kinase (PK) is classified as a key skeletal muscle sarcoplasmic protein. This glycolytic enzyme catalyzes the transfer of a
phosphate group from phosphoenolpyruvate (PEP) to a molecule of ADP (adenosine diphosphate), yielding one molecule of pyruvic acid and one molecule of ATP (adenosine triphosphate). Higher PK concentrations and activities are hallmarks of a greater glycolytic metabolism in the muscle tissue, a characteristic of tissues with a higher proportion of fast twitch muscle fibers (FG (fast glycolytic) or Type IIx fibers) and elevated glycolytic metabolism. Animals with accelerated hypertrophic muscle growth have a higher concentration of type IIx fibers and, consequently, a greater post mortem meat tenderness (Picard & Cassar-Malek, 2009). Additionally, although no difference in expression, another important protein like heat shock protein (HSP) was identified. In a study by Kim et al. (2008), animals with tough meat and lower marbling expressed high heat shock protein 27 (HSP27), therefore, the authors found that protein could indeed be used as biomarker of Hanwoo beef quality. Similarly, decrease in the amount of HSP27 after 14 days of aging was observed in young bulls, which was correlated with meat tenderness (Morzel et al., 2008).

Table 1. Qualitative assessment of calcium in protein spots by Synchrotron Radiation X-ray Fluorescence (SR-XRF).

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Number of spots analyzed</th>
<th>Number of spots with calcium</th>
<th>Number of spots without calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE</td>
<td>107</td>
<td>60</td>
<td>47</td>
</tr>
<tr>
<td>TO</td>
<td>109</td>
<td>26</td>
<td>83</td>
</tr>
<tr>
<td>P</td>
<td>47</td>
<td>22</td>
<td>25</td>
</tr>
</tbody>
</table>

Statistics

\[ \begin{align*}
\text{df} & = 2 \\
\chi^2 & = 23,9437 \\
\text{P} & < 0.001
\end{align*} \]

The techniques 2D-PAGE used efficiently separated the proteins found in L. dorsi muscle tissue samples from groups of animals with different grades of meat tenderness in this metalloproteomics study of Nellore cattle. The correlations found in the gel replicates indicated that the total protein extraction procedures were efficient and preserved the metal-protein structure, enabling calcium detection in protein spots by SR-XRF at a given molecular weight range of 14 to 97 kDa. The protein pyruvate kinase was related to the phenotypic differences found at meat of animals and likely explains part of the variation in the tenderness.

Conclusion

The techniques 2D-PAGE used efficiently separated the proteins found in L. dorsi muscle tissue samples from groups of animals with different grades of meat tenderness in this metalloproteomics study of Nellore cattle. The correlations found in the gel replicates indicated that the total protein extraction procedures were efficient and preserved the metal-protein structure, enabling calcium detection in protein spots by SR-XRF at a given molecular weight range of 14 to 97 kDa. The protein pyruvate kinase was related to the phenotypic differences found at meat of animals and likely explains part of the variation in the tenderness.

Literature Cited


