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Analytical Methods

Optimisation of a simple and reliable label-free methodology for the relative quantitation of raw pork meat proteins

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ABSTRACT

Recent advances in proteomics have become an indispensable tool for a fast, precise and sensitive analysis of proteins in complex biological samples at both, qualitative and quantitative level. In this study, a label-free quantitative proteomic methodology has been optimised for the relative quantitation of proteins extracted from raw pork meat. So, after the separation of proteins by one-dimensional gel electrophoresis and trypsin digestion, their identification and quantitation have been done using nanoliquid chromatography coupled to a quadrupole/time-of-flight (Q/ToF) mass spectrometer. Relative quantitation has been based on the measurement of mass spectral peak intensities, which have been described that are correlated with protein abundances. The results obtained regarding linearity, robustness, repeatability and accuracy show that this procedure could be used as a fast, simple, and reliable method to quantify changes in protein abundance in meat samples.

1. Introduction

Mass spectrometry has become a fundamental tool among proteomic techniques to identify and precisely quantify proteins of complex biological samples such as meat and meat products (Aebersold & Mann, 2003; Cravatt, Simon, & Yates, 2007).

Classical methodologies using one-dimensional (1D) or two-dimensional (2D) gel electrophoresis with different detection methods such as dyes, fluorophores or radioactivity have allowed the separation and quantitation of proteins through the measurement of stained spot intensities, providing good sensibility and linearity. However, the applicability of these methods is limited to abundant and soluble proteins when the aim is to achieve high-resolution protein separation, as well as they do not reveal the identity of the underlying proteins, and neither provide accurate results on changes of protein expression levels, especially in the case of overlapping proteins (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007; Szabo, Szomor, Foeldi, & Janaky, 2012). These difficulties are overcome by modern mass-spectrometry-based quantitation techniques, which can be separated into two categories: (i) the use of labelling methodologies that involve stable isotopes, and (ii) the use of label-free techniques. Labelling techniques are considered to be the most accurate in quantitating protein abundances, but they present some limitations as well as require expensive isotope labels, a large amount of starting material, and an increased complexity of experimental protocols. Moreover, some of the labelling techniques cannot be used in all types of samples due to the restricted number of available labels, which is deficient for the simultaneous study of multiple samples (Aebersold & Mann, 2003; Bantscheff et al., 2007; Neilson et al., 2011; Schulze & Usadel, 2010). On the other hand, label-free methods are considered to be less accurate, but they are a simple, reliable, versatile, and cost-effective alternative to labelled quantitation. There are currently two strategies extensively implemented as label-free approaches: (1) quantitation based on the signal intensity measurement based on precursor ion spectra; and (2) spectral counting (Neilson et al., 2011; Zhu, Smith, & Huang, 2010). Focusing quantitation on the basis of peak intensity, it has been demonstrated that ion amount and signal are linearly correlated within the dynamic range of a mass spectrometer. In fact, despite spectral counting such as Exponentially Modified Protein Abundance Index (emPAI) or Absolute Protein Expression (APEX) techniques are very useful in the estimation of the relative amounts of proteins in a single sample, MS1 quantitation results more precise and accurate when aim is to estimate changes in protein from sample to sample (Levin, Hradetzky, & Bahn, 2011; Wang et al., 2003).

Numerous recent studies describe quantitative proteomic analysis in plants (Mora, Bramley, & Fraser, 2013; Schaff, Mbeunkui,

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Blackburn, Bird, & Goshe, 2008; Stevenson, Chu, Ozias-Akins, & Thelen, 2009), but to the best of our knowledge, there are not many studies in meat or meat products. Thus, the purpose of the present study is the optimisation of a label-free procedure, using ion peak intensity-based comparative nLC–MS/MS, for the relative quantitation of proteins extracted from raw pork meat.

2. Materials and methods

2.1. Preparation of a mixture of protein standards for the optimisation of the methodology

The viability and practicability of the methodology were proved using a mixture of six standard proteins typically found in muscle and meat with a wide range of molecular weights, containing myoglobin (MYG, 17 kDa), tropomyosin (TPM, 33 kDa), actin (ACT, 43 kDa), troponin (TNN, 52 kDa), and alpha-actinin (ACTN, 103 kDa). Beta-lactoglobulin protein (LACB, 19 kDa) was also included in the mixture as normaliser of data as is not naturally present in meat. All protein standards were purchased from Sigma–Aldrich, Co. (St. Louis, MO, USA). Working solutions of 5 nmol for each protein were prepared with 50 mM ammonium bicarbonate (ABC) at pH 8, and subsequently an in-solution digestion was carried out using trypsin enzyme (Sequencing grade modified trypsin; Promega Corp., Madison, WI, USA). Samples were reduced with dithiothreitol (DTT) and cysteins were alkylated by using iodoacetamide (IAA). Finally, the digestion was started by adding 0.125 μg/μL trypsin to obtain a final enzyme:substrate ratio of 1:50 (w/w), and the sample was incubated overnight at 37 °C. After incubation, 10% formic acid (FA; v/v) was added to stop the digestion. The digested proteins were used to prepare standard proteins mixtures at different proportions as indicate the ratios shown in Table 1. The concentration of beta-lactoglobulin was kept constant for the normalisation of quantitative data. Moreover, working solutions at concentrations of 100, 50, 20, 10, 5, 2, and 1 fmol/μL of the digested LACB were prepared to test the linearity under the experimental conditions.

2.2. Preparation of raw meat samples and extraction of proteins

Optimised methodology for protein quantitation was carried out using raw meat from 6 months old pig (Landrace × Large White) at 24 h post-mortem. Extraction of sarcoplasmic and myofibrillar proteins was done in triplicate according to Sentandreu, Fraser, Halket, Patel, and Bramley (2010), and protein concentrations were determined by using the Bradford protein assay (Bradford, 1976).

2.3. Separation of raw meat myofibrillar and sarcoplasmic proteins by 1D-SDS–PAGE

Solutions with sarcoplasmic and myofibrillar proteins were diluted at concentrations of *N*, *N*/2, and *N*/4 (*N* = 2 mg/mL) with regard to the concentration values obtained by the Bradford assay. A total of 100 μL of each dilution was mixed with 100 μL of sample buffer (containing 0.5 M Tris–HCl pH 6.8, 10% w/v SDS, 50% v/v glycerol, 0.2 M DTT and 0.05% v/v bromophenol blue) and the homogenate was heat denatured at 95 °C for 4 min. Then, 10 μL of each sample was loaded onto the gel, and the electrophoresis was carried out at 120 V and 50 W, using a separation gel (12% acrylamide) and a stacking gel (4% acrylamide) (Laemmli, 1970). The ProteoSilver plus silver stain kit (Sigma, St. Louis, MO, USA) was employed to develop the gel, and SDS–PAGE molecular weight standards, broad range (161-0317; Bio-Rad Laboratories, Inc., CA, USA) were used to assess them molecular weights of the proteins.

Table 1
Composition of each protein mixture containing six standard proteins.

Protein name	Control	Set 1	Set 2	Set 3
LACB	1	1	1	1
ACT	1	0.5	0.5	1.5
TPM	1	1	0.5	1
MYG	1	1.5	1	0.5
TNN	1	0.5	1.5	1.5
ACTN	1	1	1.5	0.5

LACB, beta-lactoglobulin; ACT, actin; TPM, tropomyosin; MYG, myoglobin; TNN, troponin C; ACTN, alpha-actinin.

2.4. In-gel digestion of raw meat myofibrillar and sarcoplasmic proteins

After the separation by SDS–PAGE, one section from the gel of sarcoplasmic proteins and another section from the gel of myofibrillar proteins at the three concentrations assayed (*N*, *N*/2, and *N*/4) were selected for in-gel digestion and the posterior quantitation, as can be seen in Fig. 1.

The stained bands were excised into small pieces, and then reduced and alkylated by using DTT and IAA, respectively. Gel pieces were dried three times for 10 min with 100 μL of ACN. Once the gel fragments became dry and opaque, they were placed in ice for 10 min, and 1 μL of freshly prepared LACB protein solution of 500 fmol/μL was added. The digestion was started by adding 12.5 ng/μL of trypsin enzyme dissolved in 50 mM ABC pH 8, in order to obtain an enzyme:substrate ratio of 1:50 (w/w), and maintaining the samples in ice for 30 min to allow the enzyme to come into the gel. Samples were incubated at 37 °C overnight, and then 10% (v/v) FA was added to stop the enzyme activity. Peptides were extracted from the gel pieces after sonication for 10 min with 50 μL of 0.1% v/v TFA in ACN:H_2O (50:50, v/v), and

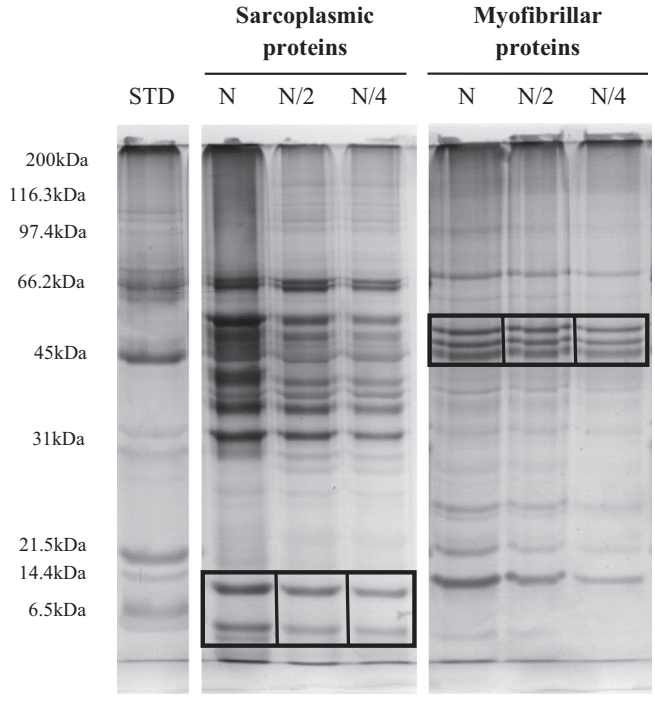

Fig. 1. Separation of sarcoplasmic and myofibrillar proteins from raw pork meat using SDS–PAGE at three different concentrations (*N*, *N*/2, *N*/4). Sections indicated in rectangles contain those bands selected for the label-free quantitation of each group of proteins. Molecular weights of the standards (STD) are indicated.