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Proteomic approach for the detection of chicken mechanically recovered meat

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ABSTRACT

Mechanically recovered meat is cheaper than raw meat and thus has been incorporated into many meat-derived products. EU regulations exclude mechanically recovered meat from the definition of meat; as a consequence analytical procedures are needed to differentiate it from hand-deboned meat. The present pilot study has utilized a proteomic approach to find potential markers for the detection of chicken mechanically recovered meat. Intact proteins were extracted from raw meat and then analyzed with OFF-GEL electrophoresis followed by SDS-PAGE and identification of potential markers by nano-LC-MS/MS. It was shown that it is possible to extract, separate and identify key proteins from processed meat material. Potential chicken mechanically recovered meat markers — hemoglobin subunits and those similar to myosin-binding protein C were also identified.

1. Introduction

Mechanically recovered meat (MRM) is obtained by recovering residual raw meat from animal bones or poultry carcasses from which the bulk of the meat has been removed. This is typically achieved using a machine that applies high pressure or shear forces to the animal bones or poultry carcasses (BMMA, 1991). Such machines allow most of the residual meat, which would otherwise be difficult or uneconomical to obtain, to be recovered. MRM has the appearance of finely comminuted meat and is used in a wide range of meat products, as an inexpensive source of meat. Although MRM has a similar chemical composition to authentic or hand de-boned meat (HDM), consumers see MRM as a cheap, inferior material and treat it with suspicion. This has led to the exclusion of MRM from the EU definition of meat (Directive No 101/2001). In doing so, clear and separate labeling of MRM in products is required. Therefore there is a need for reliable analytical methods that can differentiate MRM from HDM.

Numerous approaches have been employed to differentiate MRM from HDM. Histological approaches have been developed that exploit changes in meat properties arising during the mechanical production process which after the appropriate staining can be visualized under the microscope (Pickering, Evans, Hargin, & Stewart, 1995a; Tremlova, Sarha, Pospiech, Buchtova, & Randulova, 2006). Although this method is so far the most commonly used in MRM detection, it does not give reliable results and is non quantitative. In addition, it is time consuming and requires considerable expertise. These properties preclude the methodology as a robust, routine mode of analysis for the food industry.

Another approach is based on the assumption that during MRM production fluids from bone structures are released into the meat derived material. These fluids can be immunologically different from meat itself, and even from the residual blood that is always found in hand-deboned meat. Potential MRM specific polyclonal antibodies were obtained by raising them against a low molecular weight fraction of chicken bone marrow, and then used to screen MRM, HDM and MRM-HDM mixtures using ELISA based assays (Pickering, Griffin, Smethurst, Hargin, & Stewart, 1995b). Results were, however, equivocal, mainly due to the low selectivity of the procedure which was highly influenced by residual blood, skin and other tissues. This approach indicated that immunological tests cannot be used for MRM detection, unless further optimization of the procedure is achieved, for example by production of more specific antibodies.

Another approach for MRM identification is based on the application of protein analysis methods. It is based on the assumption that some bone proteins, not found in raw meat, can be released into the final product during MRM production or relative quantities of some proteins can differ between both kinds of material. Electrophoretic techniques have been used to separate meat proteins including SDS-PAGE (Field, Sanchez, Ji, Chang, & Smith, 1978; Savage, Richardson, Jolley, Hargin, & Stewart, 1995), capillary gel electrophoresis (Day & Brown, 2001) or isoelectric focusing followed by multivariate data analysis (Skarpeid, Moe, & Indahl, 2001). Differences in the relative concentrations of several proteins were observed, with hemoglobin content higher in marrow than in meat, and hence also higher in MRM than HDM. On the other hand, HDM was characterized by higher amounts of actin, myosin and myoglobin. Some other distinct protein bands were also noticed, but

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they were not identified. Since very limited numbers of samples were included in these studies and no information about repeatability of the results was given, the approach needs further validation.

There are few aspects to consider when applying methods of protein analysis for meat samples. When the whole proteome analysis is concerned, 1-D gel electrophoresis cannot provide sufficient resolution and less abundant, but potentially significant, proteins can be missed when SDS-PAGE alone is used. Therefore proteomics relies heavily on 2-D gel electrophoresis, which is laborious. An alternative approach to 2-D gel electrophoresis is OFF-GEL electrophoresis, where proteins after separation according to their pI values, are recovered from solution and can be directly used for SDS-PAGE separation, enzyme digestion, crystallization or mass spectrometry (Michel et al., 2003). The OFF-GEL approach is a good alternative to classical 2-D gel electrophoresis and has successfully been used in protein purification in *E. coli* extracts (Ros et al., 2002) and proteome analysis of body fluids (Burgess et al., 2006; Heller et al., 2005).

In the current pilot study, OFF-GEL electrophoresis, followed by SDS-PAGE and nano-LC-MS/MS identification of proteins, was applied to identify biomarkers that could be potentially used for the detection of chicken MRM in meat products.

2. Material and methods

2.1. Reagents and standards

Hand-deboned and mechanically recovered chicken meat samples were obtained from Leatherhead Food International (Leatherhead, UK) and prepared according to the Regulation (EC) No 853/2004. Samples were produced on a commercial scale using batches of 10 to 20 kg of chickens. Hand deboned meat material was removed and the remainder used to prepare MRM. The apparatus used was a Lima (RM500) set at 18 bars and 3 mm. The material was placed onto large stainless steel trays at a depth of 1 in. The pooled sample was prepared from at least three trays. After manufacturing, 28 kg of each meat product were frozen at once and delivered to Royal Holloway, University of London, where they were stored at −20 °C until further treatment. Small pieces of samples were taken from different parts of the large blocks, homogenized with Waring Commercial Laboratory Blender (Waring Products, Torrington, UK) and stored at −80 °C until use. Three samples of each kind of meat were used.

The following chemicals were used: sodium dodecyl sulfate (app. 99%), ammonium bicarbonate (minimum 99.0%), urea (98+%), thiourea (ACS reagent), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (≥98% TLC), dithiothreitol (DTT) (for electrophoresis, 99%), glycerol for molecular biology (minimum 99%), bromophenol blue sodium salt (for molecular biology, for electrophoresis) and ampholyte 3–10 for isoelectric focusing from Aldrich (Steinheim, Germany), water (for HPLC) and TRIS (molecular biology grade, minimum 99%) from BDH (Poole, England), trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland), formic acid (Super Purity Solvent) from Romile Pure Chemistry (Cambridge, UK), acetonitrile (HPLC gradient grade) from J. T. Baker (Deventer, Holland), trypsin, modified (sequencing grade) from Promega (Madison, WI, USA) and hydrochloric acid (analytical grade) from Fischer Scientific (Loughborough, UK).

2.2. Preparation of protein extracts from meat samples

Immediately prior to extraction, samples were allowed to thaw at room temperature. To raw meat material (1 g), 3 mL of extraction buffer (7 M urea, 2 M thiourea, 2% CHAPS and 10 mM DTT) were added and samples were shaken gently for 30 min at RT. Then, samples were centrifuged at 3000 ×g for 10 min, two 1 mL aliquots of the supernatant were taken and centrifuged at 16,000 ×g for 10 min. Supernatants were combined, and proteins precipitated with 100% ice cold acetone (10 mL). Samples were then incubated overnight at −20 °C. Next day, the protein pellet was washed three times with ice cold acetone and centrifuged at 16,000 ×g for 10 min, the acetone decanted and sample dried in vacuum centrifugal evaporator (Genevac EZ-2 Evaporator from Genevac Inc (New York, USA)) for 15 min. The dry pellet was dissolved in 1 mL of OFFGEL buffer containing 7 M urea, 2 M thiourea, 65 mM DTT, 5% glycerol and 0.5% (v/v) of ampholytes (pH 3.0–10.0). Protein concentration was measured using the Bio-Rad Protein Assay Dye reagent. A total of 500 μg protein was loaded into each IPG strip (Immobiline™ DryStrip) from GE Healthcare (Uppsala, Sweden).

2.3. OFFGEL electrophoresis (OGE)

The OGE fractionation was performed as previously described (Heller et al., 2005) using a 3100 OFFGEL Fractionator from Agilent Technologies (Morges, Switzerland). Each OGE fraction contained proteins spanning a pI range of approximately 0.3 units, as can be assumed from the configuration of the OGE device: 24 equal wells corresponding to 24 fractions obtained for each sample distributed over the pH 3–10 range. IPG strips (24 cm, pH 3.0–10.0) were rehydrated in a solution containing 7 M urea, 2 M thiourea, 65 mM DTT, 0.5% ampholytes and 5% (v/v) glycerol. A 24-well device was then placed on the rehydrated IPG and 50 μL of sample was loaded in each well across the whole strip. The separation was carried on at constant current (50 μA) for 64 kVh with maximum voltage equal to 8 kV. Fractions were recovered from each of the wells and run on SDS-PAGE gels. Each type of meat (HDM and MRM) was analyzed three times to check the repeatability of the results.

2.4. SDS-PAGE and in-gel digestion

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was run in the Mini-PROTEAN® 3 from BioRad Laboratories (Hercules, CA, USA) according to manufacturer's instructions. 10 μL of solution made from 8 μL off OFFGEL fraction and 2 μL of sample loading buffer (0.25 M Tris–HCl, pH 6, 8, 10% SDS, 50% glycerol, 0.05% bromophenol blue) was loaded onto a 12% gel. Gels were stained with a silver stain kit ProteoSilver™ from Sigma-Aldrich (Steinheim, Germany) according to the manufacturer's protocol. In-gel digestion was performed according to Koistinen et al. (2002). Peptides were reconstituted in 65 μL of 0.1% TFA water solution and 60 μL were injected into the LC column.

2.5. Protein identification with nano-LC-MS/MS analysis

Peptides were separated using Ultimate/Famos nano LC system from LC Packings (Amsterdam, The Netherlands). The sample was loaded onto a 200 μm i.d. × 5 mm PS-DVB monolithic trap column from Dionex (Sunnyvale, CA, USA) with a flow rate of 10 μL/min of 0.1% TFA for 30 min. After preconcentration, the trap column was automatically switched in-line with the 100 μm i.d. × 50 mm PS-DVB monolithic analytical column from Dionex and the peptides were eluted with a linear gradient starting from 95% eluent A (0.1% formic acid in water) to 40% of eluent B (0.1% formic acid in ACN) in 40 or 120 min, the flow rate being 200 nL/min. The LC was connected to mass spectrometer with a nanoES ion source from Protana (Odense, Denmark) using 10 μm PicoTip from New Objective (Woburn, MA, USA). The positive TOF mass spectra were recorded on a QSTAR Pulsar *i* hybrid quadrupole TOF instrument from Applied Biosystems (Foster City, CA, USA) using information-dependent acquisition (IDA). TOF MS survey scan was recorded for mass range m/z 400 to 1600 followed by MS/MS scans of the two most intense peaks. Typical ion spray voltage was in the range of 2.0 to 2.4 kV and N_2 was used as collision gas. Other source parameters and spray position were optimized with the tryptic digest of bovine serum albumin.