MICROWAVE-ASSISTED TRYPTIC DIGESTION OF THERMALLY TREATED SKELETAL MUSCLE PROTEINS

Magdalena Montowska, Edward Pospiech

Institute of Meat Technology, Poznan University of Life Sciences, Poznan, Poland

Abstract – The aim of the study was to examine the influence of microwave irradiation on the acceleration of enzymatic cleavage and enzymatic digestion of thermally treated myofibrillar proteins. The effect of the thermal treatment on protein denaturation as well as microwave-assisted digestion was analysed by SDS-PAGE. The heating had a substantial impact on the formation of protein aggregates as well as degradation of high molecular weight proteins. The preliminary results indicate that the application of microwaves reduced the digestion process of severely denatured samples to 1 h. The method shows great potential as a tool for the rapid and high-throughput identification and quantification of denatured skeletal muscle proteins in processed samples.

Key Words – beef, horse meat, microwave irradiation, proteomics, speed up enzymatic cleavage, thermal denaturation.

I. INTRODUCTION

Mass spectrometry-based proteomics has become an increasingly popular area of research in food science for protein interaction studies, protein identification and their quantification. Recently developed ambient mass spectrometry technique, such as liquid extraction surface analysis and direct infusion, to direct examination of proteins from complex samples, e.g. processed meat products, can results in a novel, fast method of quantification and authentication based on particular peptides [1,2]. However, high-throughput protein quantification of processed food is severely hampered by thermal treatment during food processing as well as long duration of the digestion step in any traditional workflow for protein identification.

High temperature influences the conformational changes of secondary, tertiary and quaternary protein structures. Although the thermal denaturation does not affect the primary structure of proteins, it causes a loss of their solubility, i.e. increase in the surface hydrophobicity of proteins, and therefore their aggregation. Formation and insolubility of protein aggregates may have a negative effect on enzymatic digestion, and consequently on the sequence coverage of protein [2]. Complete digestion of the analyzed material is essential to develop an efficient method for quantification of proteins in processed food. For processed meat products, such as sausages, pâtés and ready-to-eat meat dishes, it is difficult to conduct large scale profiling of proteins due to the sample complexity, heterogeneity and considerable degradation of muscle proteins.

On the other hand, new and robust methods for studies of protein interactions or food safety require fast and effective protocols. The ability for a rapid analysis is nowadays a crucial issue in the case of perishable food products. In traditional procedure, enzymatic digestion of proteins is carried out overnight or for several hours at 37 °C, to produce a high amounts of peptides with a high coverage of peptide sequence. Recently, microwave technology has been introduced as an alternative approach to speed up the enzymatic cleavage of protein and considerably reduce time of digestion in many biological applications [3].

During the last decade, several studies have been carried out to adapt the microwave-assisted digestion for mass spectrometry-based proteomics. Microwaves were applied to digest standard protein solutions, such as cytochrome c, myoglobin, lysozyme, ubiquitin [4,5], BSA, human urinary proteins, yeast lysate [6], and proteins captured on the affinity surfaces of protein chips [7]. However, no one has used the microwave irradiation to analyze denatured skeletal muscle proteins.

The aim of the study is to develop a rapid, sensitive, and high-throughput method of protein digestion after thermally induced denaturation in response to the need for quantitative analysis of highly processed proteins in complex food products. We want to speed workflow for mass spectrometry-based quantitative approaches in food science. In this preliminary study, the effect

of high temperature on degradation and aggregation of beef proteins was examined as well as the application of microwaves to accelerate the digestion of protein aggregates is discussed.

II. MATERIALS AND METHODS

Sample Preparation

Longissimus muscles of cattle and horse were purchased locally. Meat slices of about 25 mm in thickness were wrapped in aluminum foil and heated in a Rational Combi convection oven at three cooking variants presented in Table 1. Samples of about 5 g were cut from the raw and cooked meat and kept at -80 °C until further SDS-PAGE analysis.

Table 1. Cooking conditions

Variant	Temperature [°C]	Time [min]
I	190	10
II	160	30
III	190	until reaching core temp. of 99 °C

In-solution digestion

Washing of meat samples and protein digestion were performed according to our procedure described previously [2] with minor changes.

Dried samples (2 mg) were rehydrated in 100 µL of 100 mM ammonium bicarbonate or lysis buffer (7 M urea, 2 M thiourea, 50 mM Tris-HCl, pH 8.8), 200 mM DTT was added as a reducing agent and incubated for 1 h at 56 °C and further alkylated by addition of 200 mM iodoacetamide (IAA) and incubated in the dark for 30 min at room temperature. The excesses of DTT and IAA were removed by filtration using 3 kDa Amicon Ultra-0.5 centrifugal filters (MilliporeMerck, Darmstadt, Germany), followed by washing twice with water. The concentrated samples were digested in a solution containing 0.083 µg/µL of trypsin in ammonium bicarbonate in the domestic microwave oven at 138 W for 40 s and 303 W for 20 s. The standard/control sample was digested at 37 °C over a period of 20 h. Digested solution was then centrifuged for 10 min at 13400 rpm, and the supernatant was stored at -20 °C for subsequent electrophoretic analysis.

SDS-PAGE

Electrophoresis was performed to establish the extent of protein denaturation as well as to assess the use of microwave technology in acceleration of protein digestion. Dried meat sample of 10 mg was solubilized with lysis buffer (8 mol/L urea, 2 mol/L thiourea, 0.05 mmol/L Tris, 75 mmol/L DTT, 3% SDS, 0.05% bromophenol blue, pH 6.8). Samples were heated at 98 °C for 4 min. Protein concentration was determined using a 2-D Quant Kit (GE Healthcare Bio-Sciences). Meat sample extracts (12 µg protein) or meat digests (15 µL) were loaded onto 15% polyacrylamide gels prepared in Hoefer SE250 systems (GE Healthcare). Gels were run at 20 mA/gel constant current until the dye front reached the end of the gel. The gels were stained using Coomassie Brillant Blue (Sigma-Aldrich, Germany) and scanned on an ImageMaster Scanner (GE Healthcare Bio-Sciences).

III. RESULTS AND DISCUSSION

The effect of high temperature on protein aggregation

Thermal treatment had a substantial effect on myofibrillar proteins which is in agreement with previously reported results [8]. In our previous study, we have observed a lower sequence coverage for cooked meat in comparison with the raw meat [2].

The present study shows a high degree of protein degradation and aggregation for meat heated over 10 min at a temperature above 160 °C (Fig. 1). The high molecular weight proteins were gradually degraded as the exposure to heat increased. Figure 1 shows that the amount of myosin heavy chain (MHC) cooked at harsh conditions of Variant III considerably decreased compared to raw meat. Above MHC, a bond of nebulin and/or titin degradation products was severely altered even at Variant I and completely disappeared at Variants II and III. Less visible changes occurred in proteins of lower mass weight; actin, one of the most thermostable proteins, turned out to be more resistant even under the Variant III.

The high temperature had a large influence on the formation of protein aggregates (Fig. 1). The aggregates occurred at Variant I, and further, as

the time of thermal treatment increased at Variants II and III, become more condensed.

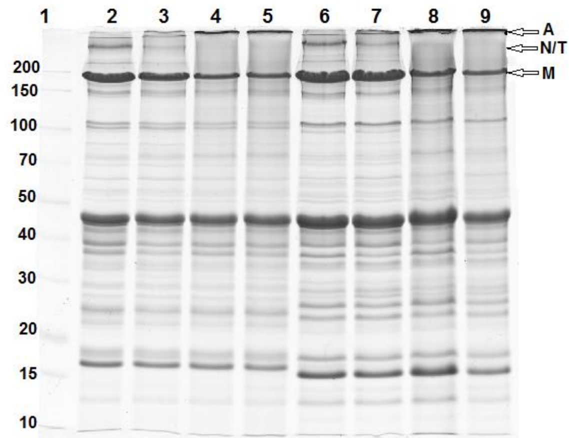

Figure 1. Representative SDS-PAGE at different thermal conditions. Line 1, Thermo Scientific Unstained Protein Ladder (kDa); line 2, raw beef; line 3, beef Variant I; line 4, beef V.II; line 5, beef V.III; line 6, raw horse meat; line 7, horse meat V.I; line 8, horse meat V.II; line 9, horse meat V.III. Protein bands show substantial protein aggregation (A), degradation of nebulin/titin degradation products (N/T) and degradation of MHC (M).

Microwave-assisted tryptic digestion of cooked beef

In this preliminary study we evaluated the application of microwave technique for the rapid digestion of highly processed and denatured meat proteins. It is a potentially desirable approach for high-throughput analysis because sample preparation would be reduced dramatically, which would radically speed up time of the entire analysis.

The results presented in the above section showed that thermal denaturation causes protein aggregation. The lower sequence coverage observed in our previous study [2] in complex cooked meat digests seems to be caused by the insolubility of protein aggregates due to the conformational changes of proteins during thermal treatment. Chemical denaturants such as urea, thiourea or guanidine hydrochloride can increase the efficiency of digestion. That is why in this study, we examined the extraction with the addition of urea as well as chemical reduction and alkylation, which cause the breakdown of protein aggregates and protect against re-formation of disulfide bridges during the digestion process. As a result lysine and arginine residues are more exposed to trypsin. However, our intention is to keep the procedure as simple as possible so as not to compromise analytical throughput of the method.

Figure 2 presents protein profiles from the tryptic digests of beef cooked under Variant III from conventional (2a) and microwave-assisted digestion (2b). Microwave irradiation generated similar results than conventional procedure. In the conventional methods, time needed to completely digest the sample ranges on average from 6 to 18 h. Our preliminary work shows that even short exposure of sample to microwave irradiation may reduce the required time to digest the sample of cooked beef to approximately 1 h. The sample was incubated at 37 °C for 30 min in a heating block, microwaved for 20 or 40 s and then incubated for another 30 min.

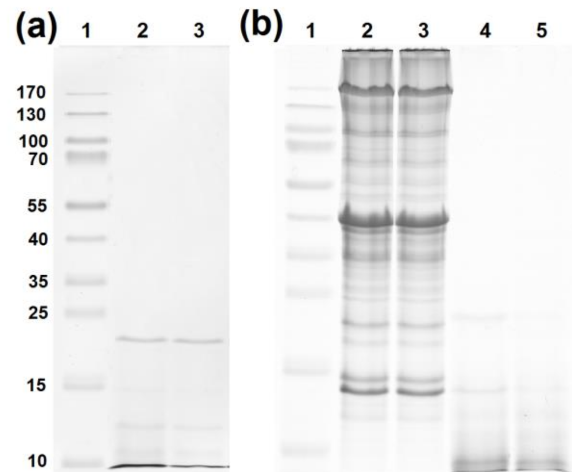

Figure 2. SDS-PAGE gel of cooked beef (Variant III) before and after tryptic digestion using the conventional and microwave-assisted protocols; (a) line 1, Thermo Scientific Prestained Protein Ladder (kDa); line 2, conventional digestion protocol without protein reduction and alkylation; line 3, conventional digestion with protein reduction and alkylation; (b) line 1, Thermo MW Ladder; lines 2 and 3, undigested beef; line 4, microwave-assisted digestion at 138 W for 40 s; line 5, microwave-assisted digestion at 303 W for 20 s.

When the sample was microwaved at 303 W, the reaction temperature turned out to be 85 °C. At high temperature the reaction proceeds rapidly [5] but the trypsin activity in our sample decreased

(Fig. 2b, Line 5). The rapid loss of enzymatic activity may increase the number of trypsin miscleavage sites, and therefore the percentage of incomplete digestion. Further mass spectrometry analysis is needed to examine the digestion completeness and the sequence coverage of key myofibrillar proteins.

IV. CONCLUSION

The preliminary results demonstrate the suitability of microwave technology to speed up enzymatic cleavage of protein aggregates. The microwave irradiation shortened time of protein digestion to approximately 1 h. The method shows great potential as a tool for the rapid identification and quantification of peptides from processed meat products. Our future direction is to perform mass spectrometry analysis to compare the number of miscleavaged peptides to make sure the completeness of sample digestion.

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