Handbook of Muscle Foods Analysis
Leo M.L. Nollet, Fidel Toldrá

Peptides

Publication details
María-Concepción Aristoy, Miguel A. Sentandreu, Fidel Toldrá
Published online on: 10 Nov 2008

How to cite :- María-Concepción Aristoy, Miguel A. Sentandreu, Fidel Toldrá. 10 Nov 2008, Peptides from: Handbook of Muscle Foods Analysis CRC Press
Accessed on: 16 Oct 2021

PLEASE SCROLL DOWN FOR DOCUMENT

Full terms and conditions of use: https://www.routledgehandbooks.com/legal-notices/terms

This Document PDF may be used for research, teaching and private study purposes. Any substantial or systematic reproductions, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The publisher shall not be liable for an loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.
Chapter 3

Peptides

María-Concepción Aristoy, Miguel A. Sentandreu, and Fidel Toldrà

Contents

3.1 Introduction .............................................................................................................. 42
3.2 Sample Preparation and Extraction ................................................................................ 42
3.3 Fractionation ............................................................................................................. 43
  3.3.1 Ultrafiltration ....................................................................................................... 43
  3.3.2 Gel Filtration Chromatography ............................................................................ 43
3.4 Analysis ............................................................................................................................ 44
  3.4.1 Analysis of Dipeptides .......................................................................................... 44
  3.4.2 Analysis of Glutathione .........................................................................................45
  3.4.3 Analysis of Other Peptides .................................................................................... 46
    3.4.3.1 Reversed-Phase HPLC ........................................................................... 46
    3.4.3.2 Ion-Exchange Chromatography ..............................................................47
    3.4.3.3 Capillary Electrophoresis ........................................................................47
    3.4.3.4 Gel Electrophoresis ................................................................................ 48
3.5 Peptide Sequencing ........................................................................................................ .. 48
  3.5.1 Peptides from Proteins.......................................................................................... 49
    3.5.1.1 Polypeptide Digestion ............................................................................ 49
    3.5.1.2 Sequencing of Tryptic Peptides ..............................................................50
  3.5.2 Free Peptides .........................................................................................................51
References ..................................................................................................................................52
3.1 Introduction

There are several peptides naturally present in muscle. Carnosine (β-alanyl-l-histidine), anserine (β-alanyl-l-1-methylhistidine), and balenine (β-alanyl-l-3-methylhistidine) are dipeptides that express some physiological properties in muscle. The content of these dipeptides is especially high in muscles with glycolytic metabolism, though this also depends on the animal species, age, and diet. Beef and pork contain more carnosine and less anserine, lamb has similar amounts of carnosine and anserine, while poultry is very rich in anserine. Balenine is present in minor amounts in pork muscle. Glutathione (GSH) is a cysteine-containing tripeptide (glutamine, glycine, and cysteine) naturally present in fresh meats. This tripeptide plays an essential role in the antioxidant system, as well as in the intercellular redox state, by the blockage of reactive oxygen species and free radicals. This thiol compound exists in two forms, the reduced (GSH) and the oxidized form (GSSG), and the ratio of the two forms is crucial for the characterization of the oxidative stress in cells. The interrelation among tissue GSH, nutrition, and oxidative stress is widely discussed elsewhere. Most meats have been found to contain approximately twice the GSH found in poultry and 2–10 times more GSH than fish products.

Other peptides have been reported to be generated during the aging of beef, pork, chicken, and rabbit. Some of these peptides have been characterized, and some of them are related to meat tenderness or flavor: for instance, the 30-kDa peptide originated from troponin T through the action of calpain, which is related to meat tenderization, and more recently a 32-kDa peptide, a 110-kDa polypeptide from the degradation of C protein, and some peptides of 1282.8 Da resulting from the sarcoplasmic protein glyceraldehyde 3-phosphate dehydrogenase, 1734.8 Da from troponin T, and 5712.9 Da from creatine kinase.

The study of peptides in meat has focused on several goals: (i) The detection of natural muscle dipeptides such as carnosine, anserine, and balenine, or the tripeptide GSH, which have some functional properties in postmortem muscle; (ii) the detection of muscle dipeptides as biochemical markers for the detection of meat in feeds; (iii) the prediction of tenderness based on the appearance of a 30-kDa fragment from the degradation of troponin T, or the generation of other small peptides from the degradation of sarcoplasmic proteins; (iv) the contribution of small peptides to flavor; (v) the assessment of muscle metabolism pattern based on peptide levels; and (vi) changes in the peptide fractions during the aging of meat as potential markers of meat quality. Thus, the isolation and analysis of muscle peptides may have a broad range of relevant applications. The most important techniques for the extraction and analysis of muscle peptides are described in this chapter.

3.2 Sample Preparation and Extraction

The procedure for sample preparation is quite detailed in the literature and is quite similar to those followed for other meat-soluble compounds such as free amino acids or nucleotides. Muscle tissue must be excised from fat and other visible connective tissues. The muscle has to be finely ground and a representative sample, at least 5–10 g, taken for the analysis. The weighed tissue is homogenized with sufficient (typically in the range of 1/2, 1/5, or 1/10 p/v) of redistilled water, dilute saline solutions, acidic solutions (0.1N hydrochloric acid), or neutral phosphate buffer by vortex-mixing, or by using several disposals, including Polytron™, ultra-turrax, Stomacher™, and so on. The homogenate is then centrifuged (typically at 10,000 × g for 20 min) in cold and the supernatant is filtered through glass-wool or cheesecloth. The supernatant is collected and usually
deproteinized by means of protein precipitation in 2.5–3-fold volume of acetonitrile, methanol, or ethanol or by lowering the pH by adding perchloric acid (PCA) up to 0.5 M or trichloroacetic acid (TCA) up to 4–5%. Nevertheless, different acid concentrations have been used to fractionate the peptidic extract from cheese, making use of the different solubilities of the peptides as a function of their size or amino acid composition.

A comparison of six methods for the extraction of meat peptides in the molecular weight range of 3–17 kDa has been reported. In many cases, extraction and deproteinization stages are achieved in only one step by extracting the sample with a deproteinizing solvent such as 0.6N PCA or 5% TCA. Precipitated proteins are separated by centrifugation; the supernatant can be dried in a speed-vacuum or by lyophilization or directly stored in the freezer until the analysis.

3.3 Fractionation

While dipeptides and tripeptides can be readily analyzed in the obtained extract (methods for the analysis of these compounds are described in the next section), larger peptides may usually need further fractionation previous to the analysis.

Several methods of fractionation have been described based upon their differential properties, including size, charge, or polarity. In this section, methods based on size are described; methods based on charge and polarity are described in Sections 3.4.3.1 and 3.4.3.2.

3.3.1 Ultrafiltration

Ultrafiltration is a preparative technique with several applications in peptide analysis. With this technique it is possible to isolate the peptide fraction of interest based on size, but it has also been used to fractionate or to concentrate peptide extracts. A wide variety of membranes of several different materials, filtration surfaces, and especially different cut-off sizes are available.

3.3.2 Gel Filtration Chromatography

This technique is usually performed for preparative purposes. The sample containing the extracted peptides can be applied on a gel filtration column for further fractionation. Peptides are separated by size, eluting first the biggest peptides. In this chromatography, neither mobile nor stationary phases must interact with the sample peptides. The range of size for fractionation depends on the type of gel. For example, Sephadex G-25 gel (Pharmacia) is adequate to separate peptides within the range 500–5,000 Da, while G-10 (Pharmacia) is adequate for much smaller peptides (below 700 Da), as in meat extracts or in cheese extracts. Cheese peptides smaller than 1000 Da were also fractionated using a Toyopearl HW-40S (Tosoh Corp., Tokyo, Japan). This type of chromatography requires the gel be packaged into long and narrow columns (typically 2.6 × 60 cm) for maximal separation efficiency.

The elution is made with water, 0.01N HCl, or diluted phosphate buffers at low flow rates (i.e., 30–120 mL/h) under refrigeration conditions. The eluent is monitored by ultraviolet (UV) absorption at 214, 254, or 280 nm to track the elution of the compounds of interest. A typical chromatogram of a pork meat sample is shown in Figure 3.1. The column can be calibrated with standards of known molecular mass, and within the desired molecular weight range. Typical
standards for the G-25 gel column are bovine serum albumin (68 kDa), egg albumin (45 kDa), chymotrypsinogen A (25 kDa), myoglobin (18 kDa), cytochrome c (12.5 kDa), aprotinin (6.5 kDa), ristocetin A sulfate (2.5 kDa), pepstatin (686 Da), and glycine (75 Da).

Gel filtration can also be performed by HPLC, in which the injection volumes used to be substantially lower. In this process, columns such as TSK-2000 SW (Tosoh Corp.) or Superdex peptide (Amersham Biosciences, Uppsala, Sweden) among others can be used. The eluent should be neutral to acid diluted phosphate or acetate buffers. Gel filtration can also be performed by HPLC, in which the injection volumes used to be substantially lower. In this process, columns such as TSK-2000 SW (Tosoh Corp.) or Superdex peptide (Amersham Biosciences, Uppsala, Sweden) among others can be used. The eluent should be neutral to acid diluted phosphate or acetate buffers. The column can also be calibrated with standards of known molecular mass as described above. Trademark catalogs are usually a good guide for these applications.

Fractions are usually collected for further analysis (sensory, further peptides separation, etc.). The size of the fractions depends on the specific purpose.

### 3.4 Analysis

Small peptides such as di- or tripeptides can be analyzed directly in the deproteinized extract (Section 3.2), while bigger peptides may be analyzed after fractionation of such deproteinized extract (Section 3.3).

#### 3.4.1 Analysis of Dipeptides

The cationic nature of meat dipeptides (carnosine, anserine, and balenine) makes cation exchange HPLC the method of choice for its analysis. Postcolumn UV detection at 214 nm is enough for most of the applications in meat, but when higher sensitivity is required, the limit of detection in complex samples can be substantially enhanced with o-phthalaldehyde (OPA) postcolumn...
derivatization.\(^5,6\) In this case, fluorescence detection is possible (\(\lambda_{\text{ex}} = 340, \lambda_{\text{em}} = 450\) nm). A cation exchange chromatographic separation of muscle dipeptides is shown in Figure 3.2. The silica-based SCX column (spherisorb) employed in this analysis renders a very robust analysis. For the postcolumn derivatization with OPA, a T-zero death volume piece connects the outlet from the column with the OPA-derivatization-reagent inlet and to a 200 cm long and 0.01 in. i.d. stainless steel reaction coil, kept at room temperature, which takes the derivatizing column effluent to the fluorescence detector set at 340 and 445 nm for excitation and emission wavelengths, respectively. The preparation of the OPA reactive is reported elsewhere.\(^{40}\) Precolumn OPA\(^{25,41}\) or PITC\(^3\) derivatization and reversed-phase (RP) HPLC separation may also be used.

The quantitation of balenine has an added difficulty due to the absence of a commercially available balenine standard. To overcome this problem, Aristoy et al.\(^5\) obtained a solution of balenine from pork loin which was further valorized by hydrolysis and analysis of the constituent amino acids (\(\beta\)-alanine and 3-methylhistidine) as described in that manuscript. Successful separation of carnosine, anserine, and balenine through hydrophilic interaction chromatography (HILIC) has been recently reported.\(^{42}\)

### 3.4.2 Analysis of Glutathione

Some methods have been published on the analysis of GSH, including direct spectral,\(^{43}\) separation (HPLC or capillary electrophoresis [CE]), and enzymatic methods. Separation HPLC methods are linked to different types of detection such as electrochemical,\(^{39}\) UV/Vis,\(^{44}\) or fluorimetric detection. When spectrophotometric detection (direct or combined with chromatographic separation) is used, the derivatization of the GSH molecule may be required, where some reagents are proposed, including iodoacetic acid and 1-fluoro-2,4-dinitrobenzene for UV/Vis.
3.4.3 Analysis of Other Peptides

The whole or fractionated meat peptidic extract needs further analysis through high-performance techniques such as HPLC (reversed-phase and cation exchange), CE, or polyacrylamide gel electrophoresis.

3.4.3.1 Reversed-Phase HPLC

Owing to its high resolutive power, this is the most common HPLC methodology for analyzing peptidic extracts. Indeed, RP-HPLC is widely used to generate peptide maps from digested proteins or peptidic extracts. With this technique, peptides are separated as a function of their polarity, which is directly related to their amino acid composition. There are many types of available reverse-phase columns, including those based on silica support with octadecylsilane (C-18)
covalently bonded and packaged in 250 × 4.6 mm columns, the most often used. The typical silica pore size for small-peptide analysis ranges from 60 to 100 Å, reserving 300 Å for polypeptide analysis.\textsuperscript{29,50} A mobile phase gradient using acetonitrile as organic modifier is typically used, while 0.1% trifluoroacetic acid is the preferable volatile buffer, although 0.1% formic acid can also be used.\textsuperscript{29} The eluent can be monitored at different wavelengths (214, 254, and 280 nm) or spectra can be obtained if using a diode array detector. Hydrophilic peptides elute first while hydrophobic peptides are retained in the column and take longer to elute. A chromatogram of pork muscle peptides is shown in Figure 3.4.

### 3.4.3.2 Ion-Exchange Chromatography

This type of chromatography also offers good separation of peptides and may be complementary to the RP-HPLC technique (Section 3.4.3.1) for peptide analysis. An example is shown in Figure 3.2. Acid peptides are separated better in anion exchange columns,\textsuperscript{34} while neutral or basic peptides are separated better in cation exchange columns. In general, silica-based columns offer better performance than polymeric ones. The best results are obtained using a nonvolatile salt such as NaCl to achieve the elution of the retained peptides. This is a drawback when peptide identification by mass spectrometry is required, because in this case volatile additives must be used. To overcome this problem, the separation used to be followed by an RP-HPLC or normal-phase HPLC\textsuperscript{34} in which salt is separated from the retained peptides.

### 3.4.3.3 Capillary Electrophoresis

CE has been applied to obtain peptide mapping based on its high efficiency and speed. Its application to the characterization of proteins by mean of their peptide mapping hydrolyzates has been
demonstrated in the literature.\textsuperscript{51} Its application to the analysis of peptides from crude extracts may be useful in the case of dairy foods such as milk or cheese\textsuperscript{52,53} because of the simplicity of the milk protein composition. However, in the case of meat or meat products,\textsuperscript{16,36,54} it is very complicated due to the more complex protein and peptide composition, the presence of interference (mainly amino acids and nucleotides), the scant information giving by this technique, and the difficulty of collecting fractions that permit further purification and characterization of the separated peptides. The widespread use of the combination of CE and mass spectrometry may enhance its applicability in the meat peptides area. However, this technique has shown very good utility for peptidase kinetic studies, where peptide hydrolysis can be controlled in very short time spans.\textsuperscript{55–58}

3.4.3.4 Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is useful for the separation of peptides in the range of 1–30 kDa present in meat. However, some cautions are necessary, including the use of low acrylamide concentration (below 10%) and tricine-SDS-PAGE. The separation is related to the pK values of the functional groups of tricine that define the electrophoretic mobility of peptides.\textsuperscript{59} This system is also useful for the isolation of extremely hydrophobic peptides that will be identified by mass spectrometry. Applications to meat peptides ranging from 3 to 17 kDa have recently been reported.\textsuperscript{24} 2-D electrophoresis is being used for proteomics as described in Chapter 5.

3.5 Peptide Sequencing

The main step for the structural characterization of proteins and peptides is the determination of their amino acid sequences. This is of crucial importance when the main functional properties are investigated since it can help to deduce some peptide/protein characteristics. Currently, there is an enormous and increasing interest in the characterization of the primary sequence of many food proteins as a basis for better knowledge of food properties. The interest in proteomics (analysis of the protein sequence) is common to other scientific fields, such as cell biology. This phenomenon is in close relation to the important development of modern mass spectrometry detectors applied to proteomics, experienced during the last decades. In fact, this discipline constitutes one of the key areas for biological research, together with genomics (gene analysis), transcriptomics (analysis of gene expression), and metabolomics (analysis of metabolite profiles).\textsuperscript{60} The determination of the amino acid sequence of peptides and proteins is today much more feasible than just 15 years ago. In addition, the remarkable developments in cDNA sequencing allow the deduction of the corresponding protein chain, making it unnecessary to determine the entire amino acid sequence of a given protein for its identification, especially in the case of large structures.\textsuperscript{61,62} In the case of free peptides, however, sequencing of the amino acid chain continues to be the method of choice to get the primary structure. This is particularly true in the food science field since, for example, many foods contain a large number of peptides resulting from hydrolysis of proteins during processing. This is the case for products obtained from milk or fish hydrolyzates,\textsuperscript{63} or for the case of dry-cured meat products such as dry-fermented sausages or dry-cured ham.\textsuperscript{64} The different alternatives that have been used for peptide/protein sequencing are described, with special emphasis on current trends.

Before the advent of the modern proteomic technology, Edman degradation has been extensively used to determine the amino acid sequence of peptides and proteins. This technique consists
in the sequential cleavage of the N-terminal amino acid through the formation of a phenylthiohydantoin (PTH)-amino acid derivative that is further identified by its specific retention time in HPLC. The automation of this process, which constitutes the basis of protein sequencers, made it more affordable to perform many cycles of this reaction, identifying at each step the corresponding N-terminal amino acid of the peptide/protein of interest.\(^6\) Despite its utility, now decades old, this technique has some important limitations such as the time required for each cycle, the absolute requirement for a free amino terminus to perform the cleavage, and the necessity of working with highly purified samples. Researchers having much expertise in this technique can deduce the sequencing of a mixture of two peptides, but only if these peptides are in such concentrations in the mixture sufficiently different as to allow for clear different HPLC detection responses at each cycle. All these features make this process quite tedious and time consuming.

Another approach to peptide sequencing was developed just 3 years after Edman degradation, involving the derivatization of peptides into more volatile compounds suitable to analysis by GC-MS in order to obtain a mass spectrum, from which the peptide sequence could be deduced.\(^6\) The technique was somewhat complementary to Edman sequencing in those cases where the latter showed important limitations, such as N-terminal blocked, highly hydrophobic peptides or peptides containing certain posttranslational modifications.\(^6\)

These strategies were later displaced by the development of the so-called soft ionization techniques for peptide identification, which made possible the ionization of intact peptides without the need for derivatization. The first step was the invention of “fast atom bombardment” (FAB) ionization,\(^6\) followed more recently by matrix-assisted laser desorption ionization (MALDI)\(^6\) and electrospray ionization (ESI).\(^6\) These two techniques are the principal ionization methods used for the actual mass spectrometers applied to proteomics. Peptides are ionized and their molecular masses are determined with high accuracy by following their trajectories in a vacuum system. Peptide ions can subsequently be fragmented in order to obtain the necessary structural information to determine their amino acid sequence. The main advantages compared to the previous methods are the high sensitivity, short time to collect data, and relatively easy sample preparation. Other important advantages are the possibility of analyzing complex peptide mixtures together with N-terminal blocked or other modified peptides.

Sequencing using mass spectrometry mainly uses peptides instead of proteins as a starting material for getting the sequence. As a general rule, mass spectrometers appear to be more efficient in obtaining sequence information from peptides no longer than 20 amino acids.\(^7\) Furthermore, peptides are easier to keep in solution. For that reason, the first step after protein purification is to digest the target protein into peptides by using a specific endopeptidase such as trypsin. This enzyme specifically cleaves proteins at the C-terminus of arginine and lysine, allowing a predictable generation of peptides and an easier interpretation of mass spectra.

In view of the complexity of actual proteomic technology using mass spectrometry, only the most common situations and strategies that can be followed for obtaining the sequence of the peptides/polypeptides of interest are discussed in this chapter.

### 3.5.1 Peptides from Proteins

#### 3.5.1.1 Polypeptide Digestion

The polypeptides of interest could have been purified previously by different chromatographic procedures, but the last purification step usually consists in SDS-PAGE. The portion of gel
corresponding to the polypeptide is excised to perform an in-gel protein digestion using trypsin or any other suitable enzyme. If SDS-PAGE is not the last purification step, then trypsin will be added to the purified sample (in-solution digestion).

3.5.1.2 Sequencing of Tryptic Peptides

From this point, the obtained peptides can be identified in two main ways:

1. **Peptide mass fingerprinting using MALDI-TOF MS**: The peptide mixture is spotted onto a special metal plate and then combined with a matrix, normally a low-molecular-weight aromatic acid such as α-cyano-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid, in order to promote the ionization of peptides when receiving the impact of a laser beam. Single-charged intact peptides are generated in this way, and then a time-of-flight (TOF) mass spectrometer will generate a mass spectrum showing the molecular masses (m/z values) of the peptides present in the mixture. The set of the whole tryptic peptide masses constitutes the “mass fingerprint” that will be specific to the analyzed protein. Thus, matching the obtained peptide masses to the theoretical tryptic peptide masses calculated for each protein in the database will allow the identification of the protein.

2. **Capillary HPLC coupled to the mass spectrometer**: The peptide fragments are previously separated in a capillary HPLC column that is directly coupled to the electrospray (ESI) ion source of a mass spectrometer. The column outlet is directly connected to the needle of the ESI ion source, so that the peptide flows through the needle and is ionized by the application of high voltage near the entrance of the mass spectrometer. The ions enter the vacuum of the mass spectrometer for analysis. There are different types of mass spectrometers that can be coupled to this system, including the quadrupole-ion trap, quadrupole-TOF, or triple quadrupole. The main difference with respect to the mass fingerprinting approach is that these systems allow tandem mass spectrometry, also known as MS/MS. In this mode, a first mass spectrum is carried out to obtain the m/z values of the intact peptides. Contrary to MALDI, ESI tends to generate double or triple-charged intact peptide ions, and this must be taken into consideration at the time to obtain the molecular mass of the peptides from the m/z values obtained in this first spectrum. In a second step, one particular peptide ion (called “precursor ion”) is isolated and fragmented in order to obtain a second mass spectrum of the resulting fragments (called “product ions”), from which the sequence of the original peptide can be deduced. This is possible because the fragmentation of peptides in the mass spectrometer is adjusted to occur mainly through the cleavage of the amide bonds, which generates a b-type ion series when the charge is retained by the N-terminal fragment or a y-type ion series when it is retained by the C-terminal fragment. Apart from these, other kind of fragments can occur if other type of bonds are cleaved into the peptide, as illustrated in Figure 3.5. The mass difference between neighboring peaks in either the y- or b-ion series is directly related to the amino acid composition of the precursor peptide. Interpretation of the spectrum can be made by using some specific computer algorithms, such as Sequest or Mascot, but one must keep in mind that this approach can fail under some circumstances. For such situations, an expert in the interpretation of tandem-MS spectra would probably solve the problem if data are of sufficient quality and with enough information.
3.5.2 Free Peptides

In many cases the research is focused on the identification of peptides present in complex matrices, which have been generated by the action of proteolytical processes that are not known in detail. This is the case of many processed foods such as fermented milk products or dry-cured meat products. In these cases there is no need to generate peptides by trypsin digestion or other methods because peptides are already present in the sample. However, since they have not been generated by digestion with a known enzyme, the identification of the peptide sequence will be more complicated. In such cases, the "peptide mass fingerprinting" approach will not be useful, and the only alternative is the development of different purification procedures able to fractionate the peptides as much as possible but without the need to purify to homogeneity. The first step normally implies a clean-up procedure to obtain a peptide extract devoid of the contaminants (proteins, lipids, sugars) that can be present in the initial crude extract. The obtained peptide extract is normally fractionated by different types of chromatography, including size exclusion, ion exchange, or reverse-phase. The way the sample elutes from the last purification step, which can be either online or off-line coupled to the mass spectrometer, must be compatible with MS analysis. This can be done performing an HPLC reverse-phase chromatography, for example, in which samples are eluted by increasing the organic content of the mobile phase and so avoiding the use of salt, which can suppress the further ionization of peptides. As very hydrophilic peptides are not retained in reverse-phase columns, a good alternative in those cases would be the use of hydrophilic interaction chromatography, which is also compatible with MS analysis. The sequence of the eluted peptides can be determined by tandem mass spectrometry, as explained in Section 3.5.1.2. For the obtained peptide sequences, the protein of origin can be identified by searching for amino acid sequence similarity against online protein databases. The BLAST search engine (http://www.bork.embl-heidelberg.de) can be used for this purpose.

© 2009 by Taylor & Francis Group, LLC
References


