To my family

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Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente

Doctoral Thesis in
Management of Innovation in the Agricultural and Food Systems of the Mediterranean Region

– XXVIII cycle –

Study of chemical-physical and biochemical changes in food of animal origin by proteomic approach

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Part One – General

1. Introduction

Food industry is undergoing a reorganization of its structure due to changing of market needs. Consumers ask for an increase in product typicalness, in fact the “Italian Delicatessen”, with its traditional and safer products, shows a constant growth despite a delicate macroeconomic situation.

The meat sector is supported from cooked ham and uncooked ham. Both the data and the volumes of the industry reflect this aspect. The hams, followed by salami, mortadella, bacon and other meats, occupy the 50% of the meat sector.

Meat has a very complex composition that varies with animal species, its age and, especially, the fat content. In the past, meat consumption was associated with the demand for energy and constructive activity for the body; today, however, meat consumption is linked to its high nutritional impact. The amount of meat energy or calorie depends, above all, by its fat content. Its plastic or constructive value is due, mainly, to the percentage of proteins, vitamins and minerals and it is linked to their digestibility and their similarity with the of the human organism components. In fact, the animal proteins, ranging from 20 to 30% of the edible part, are highly digestible. The essential amino acids that are in muscle, which are substances necessary for life and give to the sausage its characteristic taste, are also of utmost importance. The meat has a high nutritional value, not only for the high content of protein, but also because it is the only food that provides the iron in a form that can be assimilated from the body. The pork meat contains many nutrients recommended by the guidelines for a healthy diet and the RDA (Recommended daily intake levels of energy and nutrients for the Italian population). In particular, in addition to high quality proteins and iron, it is an important source of zinc, selenium, vitamins A, B12, folic acid and arachidonic acid. These features make the pork meat very suitable to be part of a balanced diet with cereals, fruit,

vegetables, milk and dairy products, and oils, that provide all in the necessary elements for the body welfare.

“Made in Italy” label is famous in the world of food also for dairy products, which represent a major portion of the export.

In recent years, the buffalo herd, in the production of PDO Buffalo Mozzarella cheese, has greatly increased, while the number of cattle has been reduced. This process has favored the development of the entire production chain, creating an induced, which involves more than 20 thousand employees in the PDO Buffalo Mozzarella cheese production.

About 80% of the buffalo herd is distributed as part of Campania, the remaining 20% is spread almost exclusively in southern Lazio, mainly near Frosinone and Latina, and Puglia mainly province of Foggia. Today, according to the data released by the Consortium in the southern Lazio, they would count eleven manufacturers of PDO Buffalo Mozzarella cheese. The markets expansion, including foreign markets, is a good opportunity for buffalo’s industry and for dairies producing milk, although it has to be solved the problem of synchronization between the period of greatest demand of mozzarella, the summer, and the period in which the largest quantities of milk are produced, the winter. In Campania the livestock of buffalo is concentrated near Caserta and Salerno. The presence of traditional and recognized products is a point of strength in a competitive market that turns to globalization. It helps to affecting the indices of unemployment in areas related to dairies.

The importance of milk in our diet is also demonstrated by the fact that the market is full of products (skimmed milk, milk easily digestible) that are designed to keep some nutrients of milk, removing some negative factors. The knowledge of this products in compositional terms can valorize products and establish their exact role in human nutrition.

This research project aimed to carry out:

- A systematic study of Italian meat products, to a better understanding of the different organization that proteins have after technological processes; this organization differs depending on the type of products. The study was faced by proteomic approach using electrophoretic techniques mono and two-dimensional coupled to mass spectrometry.

- A systematic study of proteolysis of buffalo frozen milk, curds and mozzarella, using electrophoretic techniques mono and two-dimensional coupled to mass spectrometry and the determination of an index able to define the freshness of the product.

- To establish quantitative parameters to identify possible use of frozen milk in the production of buffalo mozzarella cheese, trying to reveal the extent of hydrolysis effected by plasmin on β-CN by ELISA TEST.

2. Meat products and technological processes

2.1 Cooked Ham

Once considered food for poor people, today cooked ham is the most consumed among cooked meat products, especially after significant improvements in production technology, which managed to standardize the product at higher levels of quality.

According to the DM 21/09/05 the name "Ham" is reserved for the meat product obtained from pork: leg boned, defatted, without tendons and rind, with the use of water, salt, sodium nitrite, potassium nitrite (possibly in combination with each other or with sodium nitrate and potassium nitrate). Pork leg means the hind limb eventually dissected "transversely from the remaining part of the carcass before the end of hipbone". In the production of cooked ham other ingredients may be employed, such as wine (including aromatized wines and liqueur), sugar (including dextrose, fructose, lactose, glucose syrup), milk protein, soy protein, starch, spices, jellies food, flavorings, additives.

The main phases of production are (Fig. 2.1): reception of fresh raw material; fresh legs are selected according to rigorous criteria and sanitary and quality controls. Among the required standards for the raw material, it is important the presence of fat, which must be of high consistency (hard) and pink (the fresh leg must not be too lean). This is synonymous of a high quality raw material, of a mature pig and then of a high nutritional quality meat. If the thighs don't meet these standards, they are discarded and returned to the supplier.

Boning. Boning can be done manually – this way is best suited for quality products - or by machine. This operation is done by hand or by specialized operators, according to a particular technique that preserves the integrity of the thigh during processing. In this phase, fat and rind can be eliminated in different percentages and ham so take its own characteristics, mainly commercial, "degreased" or "semi-defatted".

Syringing. This is the phase in which the meat is flavored. The "brine" (a composition of water, salt, natural flavors etc.) is infused within the meat. If some substances called "thickening agents" (chemical or natural origin) are not added during cooking, owing to a phenomenon of natural osmosis, much of the injected brine exits, otherwise it remains in the product, giving to the cooked ham the characteristics of a lesser value product than those of the product without thickeners.

Churning. After the syringing, thighs are put into containers of stainless steel and thanks to an alternation of phases of rest and massage, made by a machine called "churn, the proteins are extracted from the meat to give to the finished product the right firmness. The period of this phase, greatly depends on the required quality of the finished product. The product passes through long periods of rest to short cycles of massage. The period of the churning phase can be about of 12/24 hours. This massage is very slow and entails that the thigh remains anatomically intact during processing.

Pressing and cooking: This is the stage of the production where the meat is put into the mold to give shape to the leg. A sheet of microforated paper is placed between ham and mold to provide maximum hygiene of product, an interface that will be removed at the time of packaging. Well accommodated in the mold still open, ham enters in a tunnel where vacuum is created, the remaining air is removed from the muscles. It is, therefore, the moment to put the lid on the mold and to start the pressing step: a mechanically strong pressure which helps the further compaction of the meat. In the ovens there is saturated steam, with internal ventilation that allows even cooking; They are controlled by probes that follow the curve of the temperature rise within the product: the temperature must reach 67/68 °C at the heart of the product.

The ham "rests" for 24 hours in a cold room, at 0 °C, to cool and compact, before it can leaving the mold.

Packaging and sterilization: at the end of cooking, molds are cooled, then the rind is marked with the name of the product and it is packed in a multi-layer aluminum vacuum bag To increase the storage of the product the cooked ham is subjected to a heat treatment which may be of pasteurizing or sterilization. In the first case, the deadline is shorter (three / four months), in the second case, which is the one most used, the deadline is six months. At the end of sterilization, the product undergoes cooling in a cell.

Storage. After the product has been cooled, it is packaged in cardboard boxes and stored within the cell and after a few days is ready to be shipped.

The quality of cooked hams is directly related to the meat quality of the, because meat affects the color, the seal of the slice, the final performance.

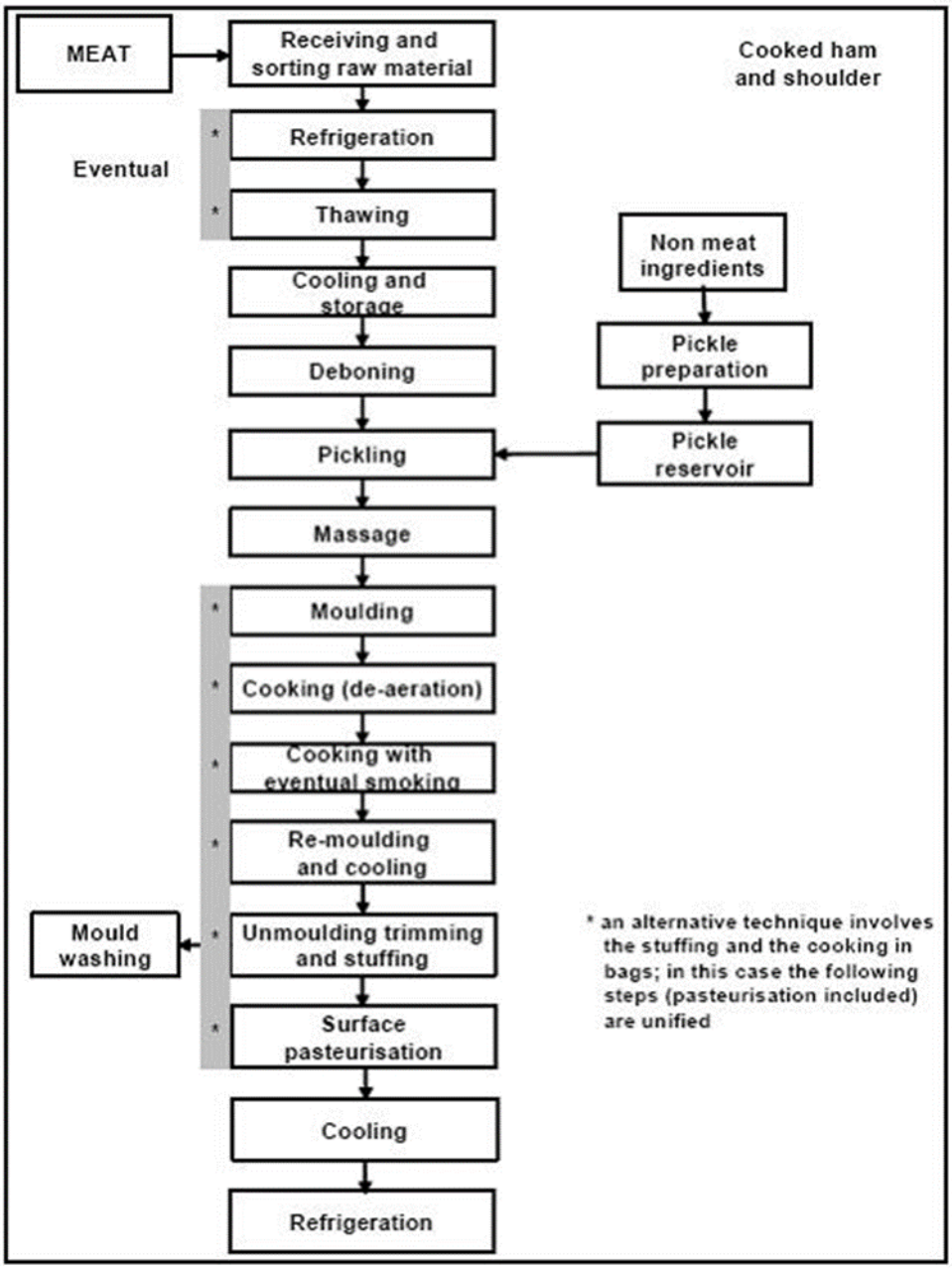

Fig 2.1: Production of cooked hams

Presentation. The ham is sold in vacuum packs or in a protective atmosphere, as whole cut, in pieces, sliced or otherwise portioned. The addition of ingredients and foods, different from those referred to Legislative Decree of 25 January 1992, n.107, for the purposes of flavouring are signaled by specific integration of the sales description. Analogous integration is necessary when the cooked ham is subjected to smoking.

Sale. The cooked ham is kept at a temperature not exceeding + 4°C. Ham packaged in slices, which has not undergone the pasteurization process after packaging, reports the date of minimum storage that it has to be no more than 60 days from the date of packaging.

Other types of ham. It is allowed to include the name "ham" with the word "choice" if, in the middle part of the product, at least three of the four main muscles (semi-tendinous, semi-membraneous, quadriceps and hamstring) of the whole leg of pork can be clearly identified and the moisture on degreased and without additives product (UPSD), is less than or equal to 78,5%. In the production of chosen cooked ham it is permitted to use the ingredients used for the production of cooked ham.

It is allowed to include the name "ham" with the words "high quality" if, in the middle part of the finished product, at least three of the four main muscles of the whole leg of pork can be clearly identified and the moisture of the degreased and without additives product (UPSD) is less than or equal to 75.5%. In high quality ham it is allowed to use only the following ingredients: wine, including flavored and fortified wine; sugar: dextrose, fructose, lactose at the maximum dose of 1.5% of the finished product; aromas: except flavoring substances obtained by chemical synthesis; species and herbs; ascorbic acid and erythorbic acid and sodium salts - monosodium glutamate - sodium lactate. The high quality ham has the following characteristics: the cooking treatment must be strong enough to ensure the achievement of a temperature at least of 69 °C at the core of the product; the cooled and packaged product undergone pasteurization.

2.2 Prague ham

The traditional cooked ham can be prepared with numerous variants. One of the most important is Prague Ham. The production of cooked ham has an old tradition in different countries of Europe, where different qualities and technologies can be found. Many times the adjective Prague ham is used, although it is less known that the true original Prague ham is a small smoked ham from the bone leg (4 – 5 kg) produced from small pigs. It is the traditional product of the province of Trieste. It is lightly smoked and it is also produced in Lombardy and Emilia with the following phases (6-10 kg or 10-12 kg):

- selection of the thighs by size;
- Cooling at 4 ° C for 18-24 hours;
- boning (not always);
- skinning;
- degreasing;
- Weighing the hams;
- liquoring;
- syringing with multi needles;
- tenderizing;
- churning;
- smoking;
- baking in ovens for 7 hours until reaching 72 ° C at the core (that is, at the center).

A different method of preparation is well executable: the pig leg carefully selected, is deboned and then trimmed, eliminating the so-called "fleshings" (pieces of meat attached to the bone). Then, the salting is performed by immersion in a brine solution for several hours. The thigh is massaged to distribute the brine within the meat in a uniform way, and then "formed". It is inserted into a metal container and subjected to pressing, to remove the air retained inside the

muscle mass. After washing and drying, the ham is smoked, stewed (steamed) and then left to rest for at least a couple of weeks.

2.3 Mortadella

Mortadella is an emulsified and stuffed meat product which was traditionally produced from horse meat, but it is now produced from pork or beef or a mixture of these. The amount of added fats depends on the quality required. Meat quality is related to the amount of muscle protein and to the proportion of fat and collagen; as this ratio increase, the amount of protein decreases and so the meat quality is reduced. The addition of a fat emulsion improves the meat properties, including taste.

The production of Italian Mortadella follows the principles of raw-cooked meat products, with meat, animal fats and water as basic raw materials. The meat component does usually not only include lean meat, but also offals such as spleen, oesophagus and sometimes even udder.

Small dices of fat are also often embedded in the batter in combination with green peas or black peppers. The fillers used are usually starches and flours. The cohesiveness of the mortadella is achieved partly by the network of muscle proteins, but to a certain extent also through the stickiness of the fillers. Mortadella is stuffed in large calibre casings (up to 200 mm).

In order to not expose the outer zone of the sausage for too long time to excessive temperatures, a heat treatment is required. The heat treatment usually starts with a temperature of water around of +60°C. This temperature is maintained until the core temperature in the sausage has reached +35°C. Then, the water temperature is raised continuously, always maintaining a difference with the core temperature (usually 25°C) until the final water temperature (“cooking”) is reached.

Production of Mortadella.

In order to produce Italian mortadella, several cuts are used, such as shoulder, lean grounded meat, stomach and lard. In the process, the first step is the finely grinding of the lean pork to a paste, then its addition to the mixer along with the fat squares and the rest of the ingredients (salt, pepper, sodium ascorbate, sodium nitrite, and in some cases pistachios). The uncooked mixture is then stuffed into a natural or synthetic cellulose casings. The mortadella is then cooked in hot dry air ovens: the final temperature at the centre of the product is at least 70°C for large mortadella and 74°C for smaller ones. As a general guide, about one hour of cooking time is needed for each centimetre of mortadella diameter. After cooking, the mortadella is then cooled and either vacuum or protective-atmosphere packed; it takes 3 days to complete the production cycle. Packing the mortadella in this way, at the correct temperature, stabilizes and locks the organoleptic properties for the entire lifetime of the product.

2.4 Wurstel

The quality of the finished products depends greatly on the percentage of the different cuts of meat: this percentage can change from 20% of only beef in poor frankfurters (i.e wurstel), to a 45-50% of pork for finest sausages. The fat can range from 20 to 40% and water from 20 to 30%. In the finest sausages, the proportion of pork, synonymous of higher quality, reaches the 50%, but it is the production process to determine the final quality. Since in the traditional production cheap cuts are used, it is required a careful preparation to get high quality products. The production technology, in fact, greatly influences the characteristics of the final product.

Meat. The raw material can be pork, beef, horse, or poultry-chicken and turkey. Pork, frozen or not, is represented by comminuted meat, cuts of bacon, Italian hams and shoulders, trimmings from foreign hams or shoulders, cuts from "muscular" or from the end of the

shoulder muscles. This last cut, with a high content of proteins, gives stability to the emulsion. Comminuted meat is produced by trimming the main anatomical cuts, whose commercial value is linked to the origin and composition. Their fat content can range from 16 to 24%, while the connective tissue content between 1 and 7%. The composition can be very different; in particular the lean of the head, for its blood residual content, is richer in pigments, such as the hemoglobin, and it is able to confer a deep red color to the finished product. Using comminuted meats can lead to a problem: with the same parameters of formulation, obtained by using mixtures of different cuts, comminuted meat may give rise, with the same operations, to finished products not equal to each other. The different anatomical origins may cause variations in aroma. The composition of the frankfurters (i.e wurstel) also includes the hard fat pork, chilled or frozen, cuts of bacon, gorges and lards, trimmings of fat ham. The emulsions of fat and rind in water, already frozen in tablets, contribute to the presence of soluble collagen and increase the texture of the product, in proportion to their percentage of use. The so-called "reworked" mortadella, sausages and other meat products can be used. Water can be also added as cold water or better as ice flakes. This addition has the double purpose to maintain low the temperature of meat during processing in the cutter (which must not be higher than 12 °C) and then to keep the dough, making the finished product more pasty and reducing the drops by smoking and baking.

The additives used and their doses, according to the regulations, are: salt 2%, Sodium nitrite 0.015%, sugar 0.15-0.5%, ascorbic acid or sodium L-ascorbate 0.05-0.75% , skim milk powder 1-2-4%, sodium caseinate 1.5%, polyphosphates 0.15 – 0.2 – 0.3%, pepper powder 0.1%, sweet paprika (liquid) 0.1% sodium glutamate 0.15 – 0.2%, spices 0.3% (nutmeg, mace, ginger, cinnamon, red sweet onion, white pepper, oregano, sage, clove, pigment, paprika, cardamom, etc.). In recent years, it is to point out, the general tendency to lower the fat content, which is reduced from 35% to less than 25% and that also

the quality of frankfurters, like the other cooked products, provides a poor use of polyphosphates.

Stages of processing

The production process consists of four main steps: grinding, stuffing, cooking and packaging. Basically, the raw material is frozen.

Grinding. Preventively, the packages of frozen meat are reduced to shavings, through a machine called “roughing”, then they are weighed, in order to guarantee the perfect matching to the product recipes. The meat is then loaded on the cutter, which grinds the meat very finely, thanks to its eight knives that work with a speed of 3600 rpm, obtaining a soft dough.

Bagging. The phase of grinding ends with the refinement of the dough and then, it is loaded into the hopper for the bagging. The filler is connected to a special machine that, thanks to a pump, predetermines the weight of the frankfurters sausage.

Cooking. The operator verifies the right weight and then, after having slipped the neck of the sausages on a metal rod, he takes them into the oven. The cooking step lasts about three hours and reaches a temperature of 80 °C. After the cooking step, the products are brought to the cooling cell.

Packaging. The elimination of the cellulosic casings of stuffing and cooking, is called "peeling" and it is the first step towards the packaging. Wurstel are brought to the autoloader, through conveyor, which groups them and lay them in the thermoforming machine. An operator separates and eliminates products with abnormal shape, while the other operators check the number and the right location of the wurstel in the slots of the packaging machine. The packages of wurstel are finally boxed and placed on pallets.

3 Meat proteins and proteolysis

3.1 Proteins

A protein is built up from a long polymer chain of amino acids, a polypeptide chain. The variable side chains give each protein chain its distinctive character. There are three general categories of side chains: nonpolar uncharged and charged polar (Dickerson & Geis, 1969). The build-up of the polypeptide chain of the proteins is called the primary structure (Fig. 3.1). This polypeptide chain forms specific conformations in solutions, the so called localised secondary structures, i.e., α-helix, β-pleated sheet or random coil (Fig. 3.1). Tertiary and quaternary structures of proteins are the denomination of the three-dimensional structure and the association of protein entities in solution, respectively (Fig. 3.1). The stabilisation of these structures of a given protein system is dependent mostly on non-covalent forces, such as hydrogen bonding, van der Waal's forces, electrostatic and hydrophobic interactions. The formation of globular proteins is a typical example of how hydrophobic interactions stabilises this type of tertiary structure. There is a driving force for the non-polar, hydrophobic side chains of a protein to be removed from an aqueous to a non-polar environment. Among the meat proteins myoglobin, giving meat its colour, is a typical example of a globular protein (Fig. 3.2(A)). Another structural form of proteins, which is highly prevalent among meat proteins, is the fibrous form of proteins. Actin, myosin and collagen in meat are typical fibrous proteins. These proteins are built up from three main structures, namely the a-helix, the antiparallel βpleated sheet and the triple helix (the last one only collagen). Myosin is a helical, whereas collagen uses the triple helix (Fig. 3.2(B)). To stabilise these structures hydrogen bonding is frequent (Dickerson & Geis, 1969). At increased temperatures the hydrophobic side chains, for entropy reasons, can more favourably stay in the aqueous environment and in the case of the compact globular proteins that lead to an expansion and partial unfolding. However, a free energy gain can be achieved by the association of two

partially unfolded proteins, thereby shielding the hydrophobic side chains from the aqueous environment (Dickerson & Geis, 1969). If the degree of association of protein entities is too large, leading to less colloidal stability of the system, the solubility of the proteins is lowered, and a precipitate is formed. If, however, the three-dimensional association of the proteins occurs in such a way that the attractive and the repulsive forces are so well balanced that a three-dimensional network is formed, a gel will set. This gel binds the water in the former solution, mainly by capillary forces (Hermansson, 1986). It is also solid-like in its mechanical behaviour. The gels can in turn vary from being transparent, containing a network of strands of small cross-sections to opaque gels containing much coarser aggregated structures (Hermansson, 1986). For one of the latest review on thermally-induced gelling of globular proteins, see Clark (1998). For the fibrous proteins the large amount of hydrogen bonds and electrostatic interactions that keep the stretched molecules in register in the large building blocks, where the fibrous proteins take part, are broken on heating. This results in the molecules having a greater freedom to form any random configuration, as driven by entropy. Since the proteins are relatively stretched in the fibrous form, fibrous proteins contract on cooking in contrast to globular proteins, which expand.

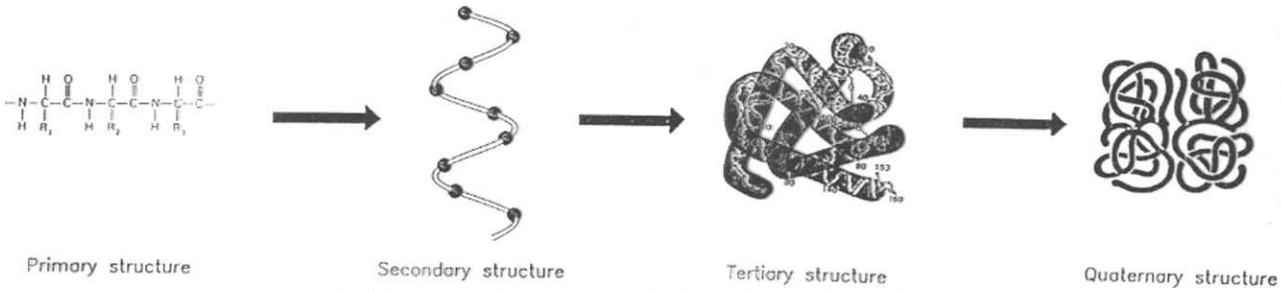

Fig. 3.1. The different type of structure of proteins in solution (Tornberg et al., 1990).

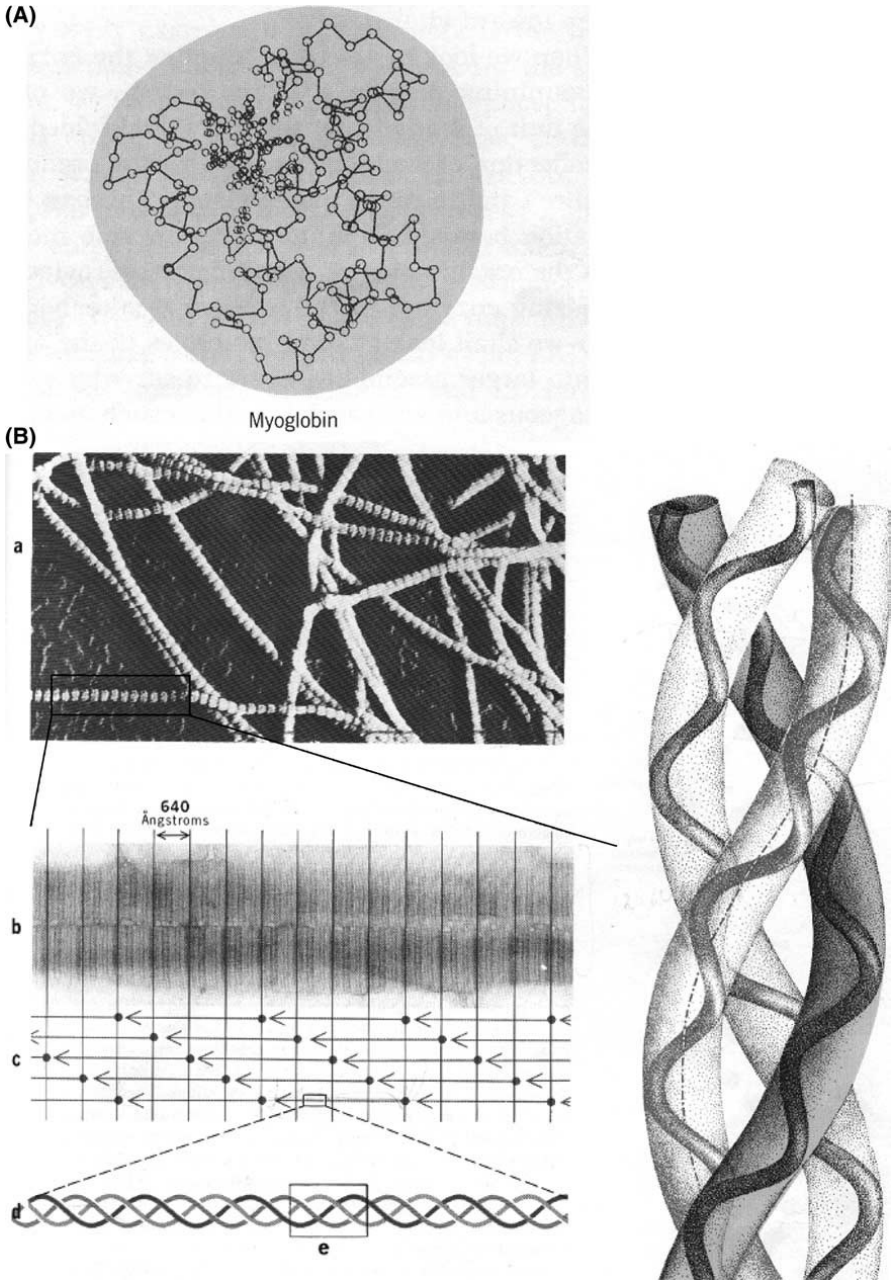

Fig. 3.2. Example of meat proteins being: (A) globular: myoglobin; (B) fibrous: the build-up of a collagen fibril (a) from the tropocollagen molecule (e) (Dickerson and Geis, 1969).

3.2 Meat proteins: composition and structure

The muscle consists of 75% water, 20% protein, 3% fat and 2% soluble non-protein substances. Out of the latter 2%, metals and vitamins constitute 3%, non-protein nitrogen-containing substances 45%, carbohydrates 34% and inorganic compounds 18%. The proteins can be divided into three groups; myofibrillar, sarcoplasmic and connective tissue proteins.

The myofibrillar proteins constitute between 50 and 55% of the total protein content, while the sarcoplasmic proteins account for approximately 30–34%. The remaining 10–15% of the proteins are the connective tissue proteins. The myofibrillar proteins are further divided into three subclasses: the myofilamentous fibrous proteins myosin and actin building up the myofibrillar structure, the regulatory proteins including the tropomyosin- troponin complex, α- and β-actinin, M-protein and C-protein and ultimately the scaffold proteins, such as titin, nebulin, desmin, vimentin and synemin, supporting the whole myofibrillar structure. Titin is a massive protein with a molecular weight of around 1 million Dalton. The sarcoplasmic proteins are the soluble proteins of the sarcoplasma, to which belong most of the enzymes of the glycolytic pathway, creatine kinase and myoglobin. About 100 different proteins are known to be present in the sarcoplasmic fraction and they are globular proteins of relatively low molecular weight ranging from 17.000 (Myoglobin) to 92.500 (Phophorylase b). The structures built up by the connective tissue proteins starts with an external covering sheet of connective tissue, the epimysium, around the whole muscle. This layer of connective tissue binds the individual bundles of muscle fibres into place and also binds groups of muscles together. The muscle fibre, which is the muscle cell, can vary in diameter from 10 to 100 μm and have a length of up to 30 cm. The cell has a membrane, called the sarcolemma, and is also surrounded by another type of connective tissue called the endomysium. The fibres are collected into fibre bundles, where the third type of connective tissue (perimysium) envelopes the fibre bundle (Ashgar & Pearson, 1980). The connective tissue proteins collagen, reticulin and elastin are all fibrous proteins. Collagen, a glycoprotein, is the main structural component of the connective tissues (55–95% of the dry matter content) and is composed of tropocollagen monomers about 2800 Å long and 14–15 Å in diameter with a molecular weight of 300.000. The tropocollagen molecules aggregate to form either extended fibres in the epimysium and perimysium or mainly as a structural matrix in the endomysium. Collagen

exists in several different genetic forms (I–V), which are present in muscle. Bailey, Restall, Sims, and Duance (1979) used immmunoflourescence to show that type I is present in the epimysium, types I and III are present in the perimysium and types III, IV and V collagen are present in the endomysium. The muscle fibres constitute 75–92% of the total muscle volume, and they hold long, thread-like structures, the myofibrils, wherein the sarcomere, the smallest contractile unit, is lined up. The structural build-up of the sarcomere can be overviewed in Fig. 3.3. The diameter of the myofibrils is about 1 μm and the length of a sarcomere is about 2.2 μm in a resting muscle. According to Fig. 3.3 the sarcomere is built from two ‘‘building blocks’’, that consist of a thick filament, extending over the A-band and a thin filament, extending from the Z-line towards the A-band in the H-zone. The thick filaments are composed of myosin. There are 200–400 molecules of myosin in each thick filament, with each being 1.5 μm long and 130 Å in diameter (Knight & Trinick, 1987). By tryptic digestion, the myosin filament can be split into a heavy head, called heavy meromyosin (HMM), and a tail called light meromyosin (LMM). The water-insoluble LMM fraction has a molecular weight of 150.000 and is composed of either a double or triple-stranded α-helical structure. The head region consists of two globular units, each about 70 Å in diameter with 45% α-helical content (Knight & Trinick, 1987). The second major myofibrillar protein is actin. The fibrous actin (F-actin) is formed from longitudinal polymerization of globular actin (G-form, Mw 47.000 Da). In solution at low ionic strength actin exists in the monomeric globular form. When the ionic strength is raised, the monomers are polymerized into the fibrous structure, consisting of a double, twisted helix with a diameter of 70 Å (Fig. 3.3; Ashgar & Pearson, 1980).

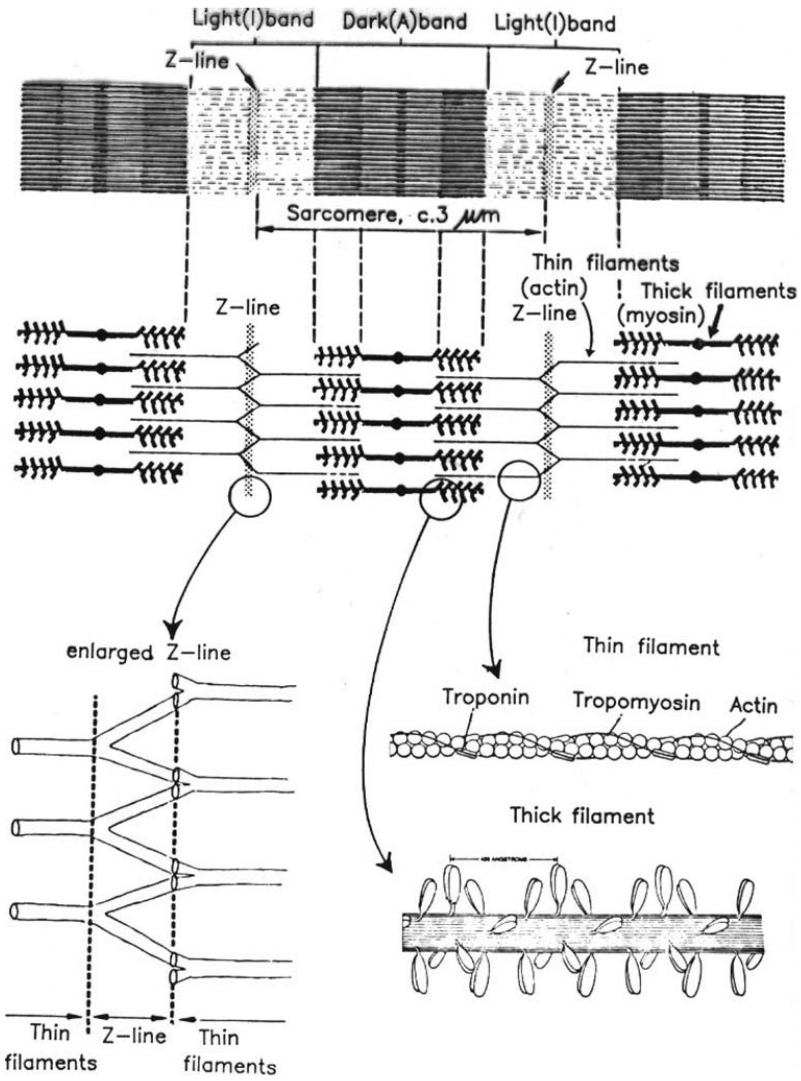

Fig. 3.3. The structural build-up of the sarcomere, the thin and thick filaments (Tornberg et al., 1990).

3.3 Behaviour of meat proteins on heating

Conformational changes of the proteins occurring on heating are usually called denaturation. The cooking temperature, where conformation changes occur is commonly called denaturation temperature and has been mostly investigated using differential scanning calorimetry (DSC). The unfolding of the proteins (the loss of helical structure) can also be followed by optical rotary dispersion (ORD) and circular dichroism (CD). Another way to follow the unfolding of the proteins is to measure the surface hydrophobicity of the proteins, using a fluorescent probe 8-anilino-1-naphtalene sulfonate (ANS). The next step in the

structural changes to occur on heating are the protein protein interactions, resulting in the aggregation of proteins. These processes are mainly studied by turbidity measurements and loss in protein solubility. The gel forming ability and the type of gels formed by the proteins are usually studied, using some sort of mechanical and micro-structural measurements.

3.4 Sarcoplasmic proteins

According to the review by Hamm (1977) many of the researchers have found that most sarcoplasmic proteins (i.e., those muscle proteins soluble in water or at low ionic strength) aggregate between 40 and 60 °C. Davey and Gilbert (1974) found for the bull neck muscle that the heat aggregation of the sarcoplasmic proteins could extend up to 90 °C. They were also the first to suggest that the sarcoplasmic proteins might have a role in the consistency of cooked meat in such a way that the heat-induced aggregated sarcoplasmic proteins can form a gel in between the structural meat elements and thereby link them together. In our investigations on tenderness of meat cooked to different temperatures (Tornberg, Andersson, & Josell, 1997), our measurements suggest that might well be the case. The mechanism by which this will operate will further be elaborated on later in this review. Another interesting aspect of the sarcoplasmic proteins is the tenderizing effect some of these enzymes can have, using low temperature long time heating (heating rate of 0.1 °C/min) on beef muscles. Laakkonen, Sherbon, and Wellington (1970) have shown that collagenase could remain active in the meat at cooking temperatures < 60 °C, whereas at faster heating and reaching a higher end temperature of 70–80 °C they were inactivated. They also showed that a heating time of at least 6 h was needed to achieve a substantial lowering of the shear force, i.e., a tenderizing effect. Over the same time most of the water losses between 25 and 30% (w/w) had occurred.

3.5 Myofibrillar proteins

With regard to changes in secondary structure on heating of myosin Morita and Yasui (1991) have for example measured the change in helix content (circular dichroism) and surface hydrophobicity (ANS) of LMM, light meromyosin, at pH 6 and 0.6 M KCl. The helix content of LMM began to decrease at about 30 °C and attained a minimum at 70 °C. Simultaneously, heating to 65 °C progressively increased the surface hydrophobicity, whereas at higher temperatures it decreased again. The decrease in hydrophobicity observed at the higher temperatures suggests that part of the hydrophobic residues take part in protein–protein interactions leading to a network formation of aggregates, a gel. The advantage of the DSC method is that it can be used in complex mixtures and at high concentrations of proteins, which is the situation occurring in meat. A typical curve (Fig. 3.4) from thermal transitions found in a muscle is composed of three major transition zones A, B and C. The first transition displays its maximum between 54 and 58 °C and has been attributed to myosin (Martens & Vold, 1976; Wright, Leach, & Wilding, 1977). The second transition, which occurs between 65 and 67 °C, was assigned to collagen (Martens & Vold, 1976; Stabursvik & Martens, 1980) and to sarcoplasmic proteins (Wright et al., 1977). The third transition has been assigned to actin and is found between 80 and 83 °C (Wright et al., 1977). For the second transition it has also been shown that both isolated actomyosin and myosin and its sub-units undergo transitions in the same temperature range (Wright & Wilding, 1984). Recently, the thermal denaturation of titin from pork and beef has been investigated, using DSC. Denaturation was characterised by a single DSC peak at 78.4 and 75.6 °C for beef and pork titin, respectively (Pospiech, Greaser, Mikolajczak, Chiang, & Krzywdzinska, 2002).

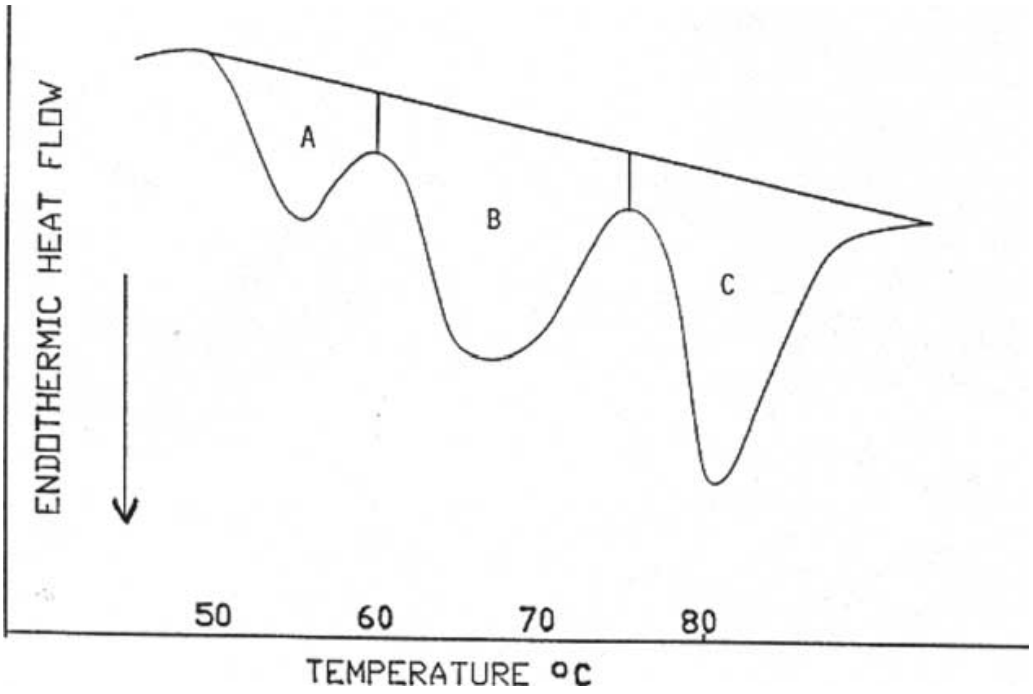

Fig. 3.4. A typical thermal curve of muscle is composed of three major zones: A, Myosin subunits; B, Sarcoplasmic proteins and collagen, and C, Actin (Findlay et al., 1989).

3.6 Connective tissue proteins

At temperatures between 53 and 63 °C the collagen denaturation occurs according to DSC measurements (Martens, Stabursvik, & Martens, 1982), which probably involves first the breakage of hydrogen bonds loosing up the fibrillar structure and then the contraction of the collagen molecule. If unrestrained, collagen fibres shrink to one-quarter of its resting length on heating to temperatures between 60 and 70 °C. If the collagen fibres then are not stabilised by heat-resistant intermolecular bonds, it dissolves and forms gelatine on further heating. The presence of heat-stable bonds means that intermolecular linkages are retained at these temperatures and Fig. 3.5. Different type of gel formation on heating of globular proteins with varying degree of aggregation. Freely after Hermansson (1982). E. Tornberg / Meat Science 70 (2005) 493–508 499 a proportion of the fibre matrix does not dissolve (Light, Champion, Voyle, & Bailey, 1985). In young animals the epimysium contains primarily of thermally labile crosslinks, the perimysium a mixture of thermally labile and stable cross-links and the

endomysium of thermally stable cross-links (Sims & Bailey, 1981). As the animal age increases the thermally labile increasingly converts into thermally stable cross-links (Shimokomaki, Eisden, & Bailey, 1972). Higher levels of heat-stable cross-links lead to the development of greater tension in the connective tissue during cooking (Sims & Bailey, 1981). Wu, Dutson, and Smith (1985) have followed the structural alterations, using scanning electron microscopy, of the epimysium, perimysium and endomysium from bovine sternomandibularis caused by heating to 60 and 80 °C for 1 h. The epimysium did not show large alterations after cooking, whereas the perimysial collagen and endomysial collagen became granular at 60 °C and started to gelatinise at 80 °C. There are also differences in solubilization for the different type of collagens, where type I is more easily solubilized on heating than type III (Burson & Hunt, 1986).

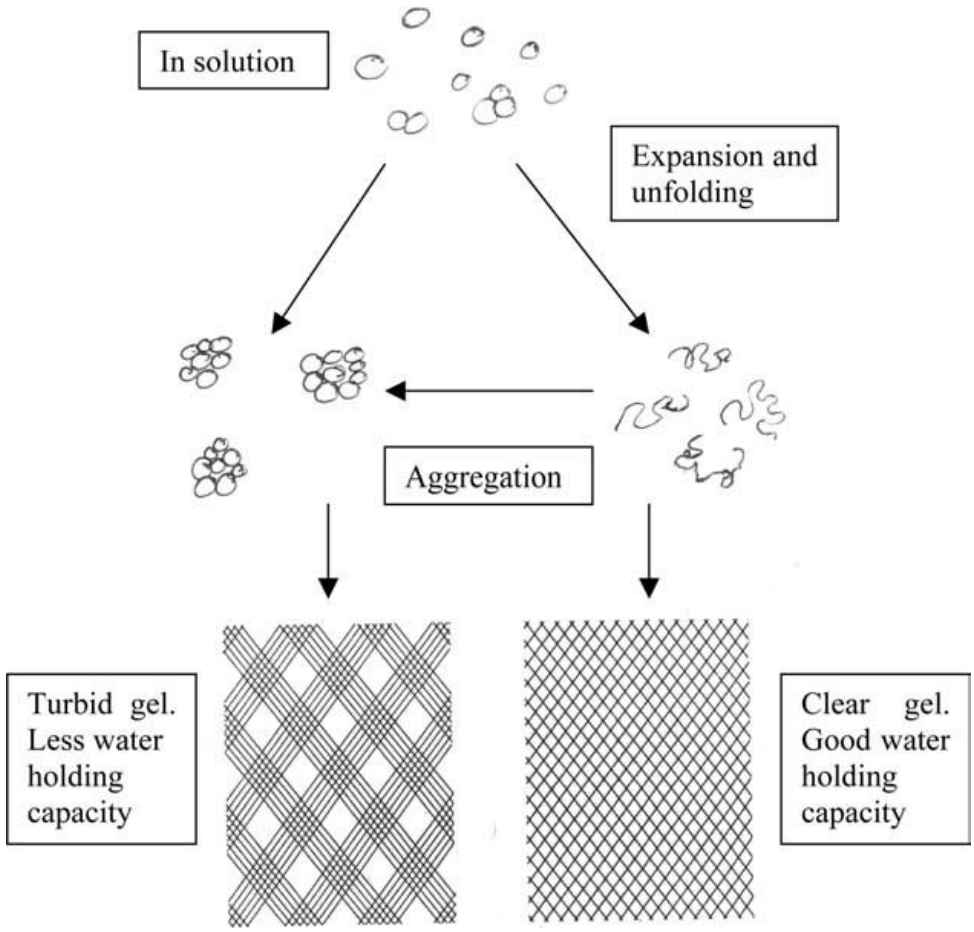

Fig. 3.5. Different type of gel formation on heating of globular proteins with varying degree of aggregation. Freely after Hermansson (1982).

4. Dairy products and technological processes

4.1 Mozzarella cheese

Mozzarella cheese is a soft, unripened cheese variety of the Pasta-filata family which had its origin in the Battipaglia region of Italy (Citro, 1981). The cheese is white, soft with a glossy surface and is valued for its stretch property. In the cheesemaking of 'Pasta filata' the smooth texture and grain of the cheese are achieved through a skillful stretching of curd in hot water. The desirable characteristics of Mozzarella cheese are obtained by the action of lactic acid on dicalcium-para-caseinate. At a pH of 5.2-5.4, much of this compound gets converted to mono-calcium paracaseinate which provides the strings and sheen to the cheese (Kosikowski, 1958). The term Mozzarella derives from the Italian word "mozza", which refers to the final phase of the dairy process when the curd is cut into shapes of established size ("mozzatura"). Buffalo milk is the raw material used to prepare buffalo Mozzarella cheese. The first technological phase is the straining of the milk through filters or sheets. This phase eliminates any big particles that could be present in the milk.

At this point it is possible:

1) to start the dairy process as soon as the milking is finished: this is what usually happens in smaller dairies that do not have structures and systems for milk preservation; in this way many problems due to microbial contamination can be avoided;

2) to refrigerate the milk at 4°C and start the dairy process later; semi-industrial dairies use this procedure.

Another very important parameter for the production of buffalo Mozzarella cheese is the milk's acidity: the cheese quality depends on its evolution. The acidity must be kept under control during all phases, from the milking to the stretching. If the milk has the right degree of acidity it coagulates well: the acidity and the temperature of the milk are the main factors affecting the renneting in its coagulation effect. If the curd does not reach the right degree of

acidity during the maturation phase, it may not be possible to stretch; the proper acidity allows the chemical-structural alterations that are necessary for the entire stretching process.

4.2. Composition of mozzarella cheese

The composition of Mozzarella cheese depends on the type of milk and procedure used for cheese. The buffalo milk Mozzarella cheese contains 23.0-25.0% fat and 50.0% moisture on fat free cheese basis. The composition of Mozzarella cheese reported by some workers is depicted in Table 4.1.

Constituents	El-Owni and Oswan (2009)	Jana (1992a)	Pizaia *et al.* (2003)
Moisture (%)	48.59	50.51	49.30
Fat (%)	27.25	26.34	25.85
Protein (%)	20.06	20.48	20.83
Lactose (%)	1.59	-	-
Ash (%)	2.25	2.00	3.62
Salt (NaCl) (%)		0.90	2.17
Acidity (% LA)	0.66	0.61	0.76

Table 4.1: Composition of Mozzarella cheese

4.3. Mozzarella cheese manufacture

Buffalo milk is reported to be more suitable for Mozzarella cheese than cow milk due to high yield, its characteristic aroma and physical attributes. The cheese yield and fat content were significantly higher for buffalo milk product but the difference in pH, Titratable Acidity (TA), moisture, protein, soluble nitrogen and ash content were not significant. Singh and Ladkani (1984) standardized the method for Mozzarella cheese from cow and buffalo milk, with and without cheddaring. The stretchability was relatively inferior in buffalo cheese. Patel et al. (1986) standardized the Starter Culture (SC) and Direct Acidification (DA) method for the Mozzarella cheese manufacture from buffalo milk. The type of milk (viz., cow, buffalo and their admixture) used for Mozzarella had a great influence on the cheese composition and

sensory properties. The cheese obtained from a blend of cow and buffalo milk had superior organoleptic quality as well as meltability compared to those made from individual milks; buffalo cheese had superior nutritional value (Sameen et al., 2008). Traditionally, Mozzarella cheese was manufactured from raw milk. However, milk pasteurization is recommended for Mozzarella cheese that is to be consumed fresh, because the plasticizing process does not destroy always pathogens (Anonymous, 1977a; Caserio et al., 1977). Heat treatment of milk (72°C, no hold) meant, for Mozzarella, improved protein and Total Solids (TS) recovery but decreased the fat recovery, gave soft-bodied cheese, improved flavor score, kept quality and ensured public health safety (Patel et al., 1986). Schafer (1975) and Olson (1980) made satisfactory quality Mozzarella cheese from pasteurized and/or UHT treated milks with increased yield compared to the one made from raw milk. Partridge et al. (1982) did not find any significant difference in the quality of Mozzarella cheese made from fresh or stored (0-10 days) pasteurized milk. UHT heat treatment of milk resulted in a greater recovery of the milk proteins in directly acidified Mozzarella cheese leading to the 3.4% increase in cheese yield (Schafer and Olson, 1975). Homogenization of milk in Mozzarella cheese manufacture led to whiter appearance and improved flavor (Kosikowski, 1958, 1960; Ernstrom, 1965; Jana and Upadhyay, 1992a, b), reduced fat losses in whey and moulding water (Maxcy et al., 1955; Breene et al., 1964; Quarne et al., 1968a; Jana and Upadhyay, 1992a), increased cheese yield (Schafer and Olson, 1975; Jana and Upadhyay, 1992a,b).

Starter cultures. Traditionally used, whey starters have been gradually replaced by more carefully maintained and cultivated starters. The starter used for fresh Italian Mozzarella is only *Streptococcus thermophilus*. To achieve consistent quality in the end product, the parameters to be controlled include avoiding batch-to-batch variation in the starter strain ratio and constant acidification (Sigsgaard, 1994). Manufacture of high-moisture Mozzarella entails the use of starters like *S. lactis*, *S. durans* or *S. faecalis*, whereas for low-moisture

Mozzarella the starters recommended are *S. thermophilus*, *L. bulgaricus* and/*or L. helveticus* (Christensen, 1966; Anonymous, 1977a). Mozzarella cheese when made with the combination of *S. thermophilus* Non-Galactose (NG) and Galactose Fermenting (GF) strains and *L. helveticus* (GF) and *L. bulgaricus* (NG) reduced the incidence of non-enzymatic browning of Mozzarella cheese (Johnson and Olson, 1985; Hutkins et al., 1986).

4.4. Methods of manufacture of mozzarella cheese

The methods of manufacture of Mozzarella cheese change considerably according to the market of the cheese (Fig. 4.1).

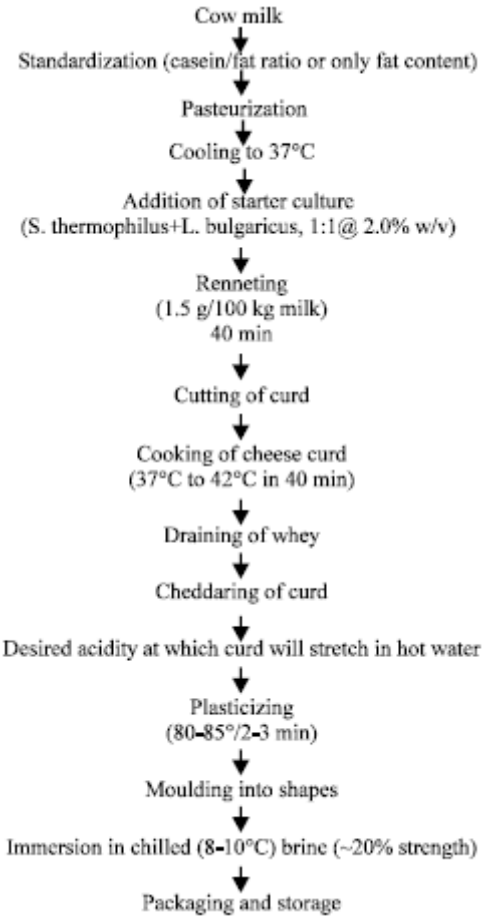

Fig. 4.1: Flow chart for preparation of Mozzarella cheese

Coagulation. The milk is heated up to 36-38°C (this temperature supports the coagulating effect of the rennet enzymes) and the rennet is added. The rennet is a mixture of various

enzymes, each of which has a specific function on the milk components (especially on fats and proteins). The rennet can be of animal or plant origin. It is extracted from the abomasum (stomach) of ruminants, specifically from calves, kids and lambs (the rennet is never extracted from adult animals because theirs has a different composition which make it less active for dairy processing). Many plants produce enzymes that can cause milk coagulation. Two plants have traditionally been used for this purpose: the thistle (*Cynara Cardunculus*) and the fig (*Ficus carica*).

4.5. The cutting of the curd

The cutting expels the whey from the curd. Depending on the kind of cheese that we want to produce, the cutting can be of two different types. If we want to make soft cheese (like Mozzarella cheese) the cutting process must be divided in two phases separated by a more or less long lapse of time: in the first phase the curd is cut into big cubes using a knife; during the following pause the separation between whey and curd can be noticed. During the second cutting (with a curd knife) the cubes are reduced into nut-size granules, soft and very moist because of the whey, that will led to a soft cheese. Soft cheeses are very rich in whey and must be eaten almost immediately, otherwise the fermentation process spoils the cheese.

4.6. The maturation of the curd under whey

In the specific case of buffalo Mozzarella cheese, maturation takes place under whey. Once the cutting is finished, 60% of the whey is withdrawn (the whey is extracted until the curd is almost visible); part of this whey is heated up to 46°C and then added again to the mass, this operation allows the curd to be kept at a temperature of about 36°C. Lactic acid starts being produced during this rest (the pH value goes down and the curd becomes more acid); the acid captures the calcium ions (the mineral that allows casein to aggregate and thus form curd) and

causes curd demineralisation, which acquires flexibility. Practically, a reduction of the calcium in the curd and its increase in the whey take place during the maturation.

4.7. Fusion

On average, fusion takes 3 hours (at a room temperature of 20°C). The optimal pH for the curd, at the end of the maturation stage, is of 4.9. The maturation can also be natural; in detail, the curd does not become acid under whey, but it is put on a table where it is left to ripen for a length of time that mainly depends on the room temperature. Usually, when we have a room temperature of 21°C the mass reaches its right acidity after 12-18 hours.

4.8. Stretching trial

The matured curd is cast in boiling water; the melted paste is then transferred on a stick and pulled by hand, if it stretches in unbroken threads that are more than one metre long, it can be considered ready for the stretching process. The curd, ready for the stretching, is put on a curd-draining table on which it is left for 10 minutes, then the curd is cut into small pieces by a machine with rotary blades. Boiling water (95°C) is added manual and the stretching is done with the help of mechanic equipment of wooden sticks and a ladle. The stretching is usually considered finished when the cheese becomes shiny and homogeneous. The manual stretching is a very difficult stage and it is necessary to pay attention to many small, but extremely important, details. When the paste is in the vat it is necessary to add water in an amount that is double the quantity of paste and continuously stir the mixture with a stick. When the cheese starts to be stretched, it needs to keep on stirring and with a ladle to pour enough water out until the cheese is nearly. At this point, the cheese is gathered around the stick and stretched with the ladle; if some more whey should come out during these movements, it is eliminated.

The cheese is now ready for the shaping or “mozzatura” (the cutting of the curd into pieces of established size).

4.9. Shaping or “mozzatura”

In this phase, the handcraft-technology differs from the semi-industrial one. At dairy farms, the stretched cheese is handled with great care, operators performing characteristic movements that finish with the “mozzatura” to obtain buffalo Mozzarella cheese. In semi-industrial dairies instead, the whole operation is totally mechanized and when coming out of the dairy, the buffalo Mozzarella cheese has a pre-established size.

4.10. The salting

Nowadays there is a great trend towards the elimination of this phase. There are various ways in which one can operate:

- salting in brine: to prepare the brine it is necessary to boil the water, add salt (usually about 200 g of salt per litre of water) and then cool the solution taking it down to 20°C. The produced brine can be used until a contamination phenomenon can be noticed (the brine becomes cloudy). After the cooling the buffalo Mozzarella cheese is soaked in the brine for a time that varies according to its size (e.g., for a cheese that weighs 400-500 g, the time is of about 2 hours);
- stretching the curd with salted water (1% of salt);
- using a “sauce”: the stretching water is used to prepare a solution (1 part) + water (1 part) + acid whey collected at the beginning of the maturation (just enough to take the acidity of the solution to pH 4; 8°SH) + salt (2%); the buffalo Mozzarella cheese is soaked in this solution up to when it is sold. The sauce will have a whitish colour because of the stretching water. Basically the sauce acts as a conservation liquid;

- using a solution made of water + citric acid (an amount sufficient to take the solution to a pH 4; 8°SH) +salt (2%); in this case the solution will be clear.

4.11. The packaging and sale

It is very usual to keep buffalo Mozzarella cheese in solutions that contain salt, amongst other ingredients. This allows to enclose in just one phase both the salting and the packaging; in particular, until it is consumed, the salt acts on the product. Buffalo Mozzarella cheese is then kept at 15°C. The product is usually eaten within 1 or 2 days.

5. Proteolysis in cheese

5.1 Advances in the study of proteolysis

An important phenomenon that occurs during cheese manufacture is the proteolysis, which is due to the action of milk endogenous enzymes as well as rennet and microbial enzymes. Therefore, enzymatic protein fragmentation can be an effective way to control the preservation state of dairy products, because product history can be reconstructed from proteolysis levels and the formation of specific fragments (Gaiaschi et al., 2001; Mc Sweeney et al., 1993, Fox et al., 1989). Proteolysis in cheese (Fox and Mc Sweeney, 1996) is produced by endo- and exopeptidases having different origins: plasmin, which is derived from milk, pepsin and chymosin, which are derived from rennet, as well as indigenous and starter microflora enzymes. Proteolysis in cheese during ripening plays a vital role in the development of texture as well as flavour and has been the subject of several reviews (e.g., Fox, Singh, & McSweeney, 1995b; Fox & McSweeney, 1996). Proteolysis contributes to textural changes of the cheese matrix, due to breakdown of the protein network, to a decrease in a_w through water binding by liberated carboxyl and amino groups and on increase in pH (in particular in surface mould-ripened varieties), which facilitates the release of sapid

compounds during mastication. It contributes directly to flavour and to off-flavour (e.g., bitterness) of cheese through the formation of peptides and free amino acids as well as liberation of substrates (amino acids) for secondary catabolic changes, i.e., transamination, deamination, decarboxylation, desulphuration, catabolism of aromatic amino acids and reactions of amino acids with other compounds. During ripening, proteolysis in cheese is catalysed by enzymes from coagulant (e.g., chymosin, pepsin, microbial or plant acid proteinases), milk (plasmin and perhaps cathepsin D and other somatic cell proteinases), enzymes from the starter, nonstarter, or secondary cultures (e.g., *P. camemberti*, *P. roqueforti*, *Propionibacterium sp.*, *B. linens* and other coryneforms) and exogenous proteinases or peptidases, or both, used to accelerate ripening (Fig 5.1).

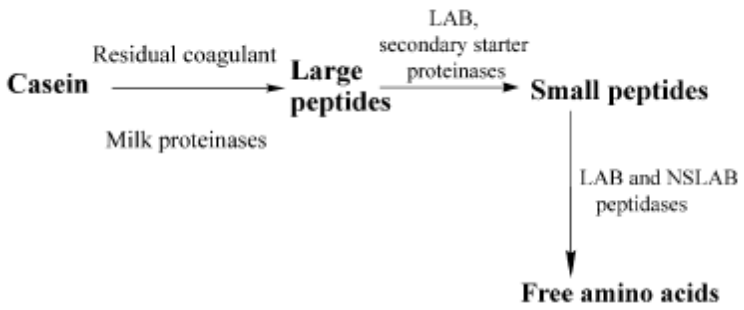

Fig. 5.1. Proteolytic agents in cheese during ripening.

In many cheese varieties, the initial hydrolysis of caseins is caused by the coagulant and to a lesser extent by plasmin, which results in the formation of large (water-insoluble) and intermediate-sized (water-soluble) peptides which are degraded subsequently by the coagulant and enzymes from the starter and non-starter microflora of the cheese. The extracellular, cell envelope-associated proteinase of *Lactococcus* (lactocepin, PrtP) contributes to the formation of small peptides in cheese probably by hydrolysing the larger peptides produced from αs1-casein by chymosin or from β-casein by plasmin, whereas the peptidases (which are intracellular) are released after the cells have lysed and are responsible for the degradation of

short peptides and the production of free amino acids. The final products of proteolysis are free amino acids and their concentration in cheese at any stage of ripening is the net result of the liberation of amino acids from casein, their degradation to catabolic products and perhaps some synthesis by the cheese microflora.

5.2. Coagulant

Majority of cheeses produced around the world were manufactured traditionally, and in many cases still are manufactured, using an enzymatic coagulant extracted from the abomasa of milk-fed calves. This extract, known as calf rennet, consists of two proteolytic enzymes: chymosin (EC 3.4.23.4), the major component (88–94% milk clotting activity, MCA) and bovine pepsin (EC 3.4.23.1; 6–12% MCA). The relative proportion of these enzymes varies with individuality and age of calves, the method of rennet separation and the conditions and pH values at which the milk clotting activity is measured (Guinee & Wilkinson, 1992). The principal role of chymosin in cheesemaking is to coagulate milk by specifically hydrolysing the Phe_{105}- Met_{106} bond of the micelle-stabilising protein, κ-casein, which is many times more susceptible to chymosin than any other bond in milk proteins and leads to the coagulation of the milk (see Fox, Guinee, Cogan, & McSweeney, 2000). Most of the coagulant activity added to the milk is lost in the whey; only 0–15% of the rennet activity added to the milk remains in the curd after manufacture, depending on factors including type of coagulant, ratio of different enzymes in blends, cooking temperature, the cheese variety and the moisture level of the final cheese (Guinee & Wilkinson, 1992). Pepsins are more sensitive to denaturation by pH than chymosin and hence the amount of activity of these coagulants retained in the curd is very strongly dependent on the pH of the milk at setting and shortly thereafter (Fox & McSweeney, 1996); in fact, increasing the pH of the curds-whey mixture to around 7 after

milk coagulation using porcine pepsin is one of the methods used to produce rennet-free cheese curd (e.g., Lane, Fox, Johnston, & McSweeney, 1997).

In the latter part of the last century, cheese consumption increased while the availability of calf rennet decreased, which led to rennet shortages and subsequent price increases. In addition, more restrictive ethical concerns associated with production of such animal rennets led to a search for suitable rennet substitutes for cheese making. Several proteases from animal, microbial and plant sources were investigated as likely substitutes and have been reviewed by Guinee and Wilkinson (1992), Broome and Limsowtin (1998) and Fox et al. (2000).

5.3. Plasmin

The dominant indigenous milk proteinase, plasmin (fibrinolysin, EC 3.4.21.7), has been the subject of much study and throughly described in recent reviews (see Bastian & Brown, 1996; Kelly & McSweeney, 2001 for reviews). The elevated plasmin activity in cheese varieties has been attributed to thermal inactivation of inhibitors of plasminogen activators, resulting in the increased conversion of plasminogen, the inactive precursor of plasmin, to the active enzyme. The primary cleavage sites of plasmin on β-casein are Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108} the cleavage of which yields β-CN(f29–209) (γ_1-CN), β-CN(f106–209) (γ_2-CN) and β-CN(f108–209) (γ_3-CN) (Eigel et al., 1984). The proteose-peptone (PP) components 5 (β-CN(f1–105) and β-CN(f1–107)), PP-8 fast β-CN(f1–28) and PP-8 slow (β-CN(f29–105) and β-CN(f29–107)) are the corresponding N-terminal fragments which accumulate in milk on storage. Plasmin cleaves α_{s2}-casein in solution at eight sites. Although κ-casein contains several Lys and Arg residues, it appears to be quite resistant to plasmin action.

5.4. Cathepsin D

Milk contains an indigenous acid proteinase, first recognised by Kaminogawa and Yamauchi (1972) as cathepsin D, and later identified as procathepsin D (procathepsin is the proenzyme form of the lysosomal proteinase, cathepsin D; Larsen, Benfeldt, Rasmussen, & Petersen, 1996). The literature on cathepsin D has been reviewed recently by Hurley, Larsen, Kelly, and McSweeney (2000a) and Kelly and McSweeney (2001). Cathepsin D (EC 3.4.23.5) is an aspartic proteinase with an optimum pH of 4.0 on haemoglobin and optimum temperature of 37°C (Kaminogawa & Yamauchi, 1972; Barrett, 1972). Cathepsin D produces the glycomacropeptide, κ-CN(f106–169), that also is produced by chymosin by the enzymatic cleavage of the Phe_{105}-Met_{106} bond (McSweeney, Fox, & Olson, 1995), and two more cleavage sites of cathepsin D on κ-casein have been identified, i.e., Leu_{32}-Ser_{33} and Leu_{79}-Ser_{80} (Larsen et al.,1996). As the specificity of cathepsin D is similar to that of chymosin, one might expect that it possesses the ability to coagulate milk. The milk clotting potential of cathepsin D, however, has been reported to be very poor (McSweeney et al., 1995; Larsen et al., 1996). Larsen et al. (1996) reported that the enzyme was capable of coagulating milk over the pH values examined (pH 5.0–6.5), with coagulation time decreasing as expected with decreasing pH. The level of cathepsin D present (around 0.4 mg/mL) in milk though, is far too low to be of significance with respect to milk coagulation. Cathepsin D and chymosin had similar cleavage sites on α_{s1}-casein, i.e., Phe_{23}-Phe_{24}, Phe_{24}-Val_{25}, Leu_{98}-Leu_{99} and Leu_{149}-Phe_{150} (Larsen et al., 1996), cathepsin D hydrolysates of α_{s2}-casein differ markedly from those produced by chymosin (McSweeney et al., 1995). The enzyme cleaves α_{s2}-casein at Leu_{99}-Tyr_{100}, Leu_{123}-Asn_{124}, Leu_{180}-Lys_{181} and Thr_{182}-Val_{183} (Larsen et al.,1996). Proteolysis of β-casein by cathepsin D is similar to that by chymosin, with β-CN(f1–192) being the primary product and β-CN(f1–163/165/167) also being formed. In total, 13 sites have been identified in β-casein that are cleaved by cathepsin D, viz., Phe_{52}-Ala_{53}, Leu_{58}-Val_{59}, Pro_{81}-Val_{82}, Ser_{96}-

Lys_{97}, Leu_{125}-Thr_{126}, Leu_{127}-Thr_{128}, Trp_{143}-Met_{144}, Phe_{157}-Pro_{158}, Ser_{161}-Val_{162}, Leu_{165}-Ser_{166}, Leu_{191}-Leu_{192}, Leu_{192}-Tyr_{193} and Phe_{205}-Pro_{206} (Larsen et al., 1996). Hurley, Larsen, Kelly, and McSweeney (2000b) reported the presence of cathepsin D or procathepsin D in Quarg cheese samples produced from raw, pasteurised or raw ultrafiltered skim (except those to which pepstain had been added) and peptides thought to be produced as result of cathepsin D were observed in cheese made from raw and pasteurised milk. In addition to cathepsin D, other proteolytic enzymes are present in lysosomes of somatic cells and may contribute to proteolysis in cheese. One of the principal enzymes found in polymorphonuclear granulocytes (PMN cells or neutrophils) is the serine proteinase, elastase (Verdi & Barbano, 1991). Elastase has a broad specificity on β- and αs1-caseins, cleaving 19 and 25 sites, respectively (Considine, Healy, Kelly, & McSweeney, 1999, 2000). Elastase hydrolyses b-casein and some of the cleavage sites are identical to or near those cleaved by plasmin, chymosin or cell envelope-associated proteinases of several strains of Lactococcus. Most of the cleavage sites were found to be located near the N- or C-termini of the molecule, viz., Ile_{26}-Asn_{27}, Gln_{40}-Thr_{41}, Ile_{49}-His_{50}, Phe_{52}-Ala_{53}, Gln_{56}-Ser_{57}, Leu_{58}-Val_{59}, Asn_{68}-Ser_{69}, Val_{82}-Val_{83}, Val_{95}-Ser_{96}, Ser_{96}-Lys_{97}, Lys_{97}- Val_{98}, Ala_{101}-Met_{102}, Glu_{108}-Met_{109}, Phe_{119}-Thr_{120}, Glu_{131}-Asn_{132}, Leu_{163}-Ser_{164}, Ala_{189}-Phe_{190}, Phe_{190}- Leu_{191} and Pro_{204}-Phe_{205} (Considine et al., 1999). Therefore, it is possible that indigenous elastase in milk may be of significance to the proteolysis of milk proteins.

5.5. Starter proteinases and peptidases from Lactococcus and Lactobacillus

The starter cultures commonly used in cheese manufacture include mesophilic Lactococcus and Leuconostoc species, thermophilic Lactobacillus species and Streptococcus thermophilus. The principal role of the starter culture is in the production of lactic acid, causing a decrease in pH. Although lactic acid bacteria (LAB) are weakly proteolytic, they possess a very

comprehensive proteinase/peptidase system (Fig. 5.2) capable of hydrolysingolig opeptides to small peptides and amino acids (Fox & McSweeney, 1996; Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Law & Haandrikman, 1997; Christensen, Dudley, Pederson, & Steele, 1999).

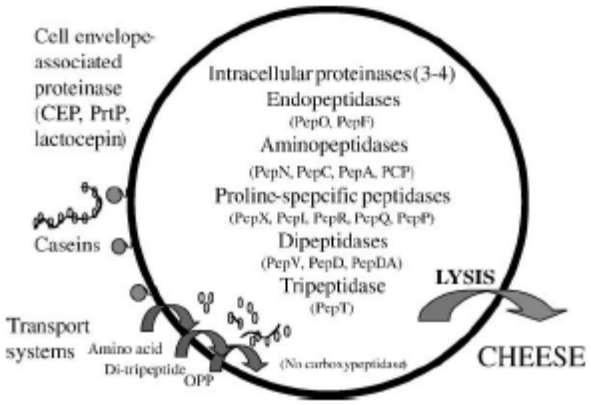

Fig. 5.2. Schematic representation of the proteolytic system of lactococcus.

5.6. Comparison of proteolysis within and between cheese varieties

Proteolysis has been considered by some researchers as a basis for the classification of cheese. Several indices of proteolysis could be useful for classification, however an obvious difficulty is the fact that cheese is a dynamic system and therefore the results obtained depend to a large extent on the age of the cheese when analysed (Marcos, Esteban, Leòn, & Fernàndez-Salguero, 1979; Smith & Nakai, 1990; Fox, 1993; McGoldrick & Fox, 1999; Ardö, 1999a; Pripp, Stepaniak, & SØrhaug, 2000c). Several studies have shown differences in proteolysis between cheese varieties. Marcos et al. (1979) compared proteolysis in several cheese varieties by analysingg el electrophoregrams and reported that, in general, α_{s1}-casein was degraded more extensively than β-casein. These authors reported that in cheeses in which β-casein was degraded less extensively (e.g., Parmesan, Emmental, Gruyere and Tilsit), the concentrations of γ_1- and γ_2-caseins were high, while in cheeses were almost all β-casein had been degraded (i.e., Roquefort), less γ_1-casein and more γ_2- and in particular, γ_3-casein were

present indicatingmore extensive plasmin action. The ratio of β- to γ-caseins has been suggested as a basis for cheese classification (Fox, 1993). Analysis of proteolytic profiles can be used generally as a powerful method to better understand proteolysis in cheese and how this process is influenced by factors including type of starter, physico-chemical variables, microbial counts, cheese making parameters, age, quality and sensory characteristics.

5.7. Identification of peptides and patterns of proteolysis in cheese

The extent and type of proteolysis in a number of the principal cheese varieties has been characterised. However, complete characterisation of proteolysis in cheese requires isolation and identification of individual peptides. Using various extraction techniques and methods to isolate individual peptides (i.e., urea-PAGE, HPLC and CE), many of the water-insoluble and water-soluble peptides have been isolated from cheese and identified, using amino acid sequencing and mass spectrometry.

Proteolysis in Cheddar cheese is well characterised as reviewed by Fox, Singh, and McSweeney (1994), McSweeney, Pochet, Fox, and Healy (1994), Singh et al. (1994) Singh, Fox, and Healy (1995, 1997), Fernàndez, Singh, and Fox (1998) and Mooney, Fox, Healy, and Leaver (1998) and peptides isolated are summarised in Fig. 5.3 and 5.4. The major water-insoluble peptides are produced either from α_{s1}-casein (fig.5.3.A) by chymosin or from β-casein (Fig. 5.3.B) by plasmin and some are degraded further by the lactococcal CEP (Fig. 5.3).

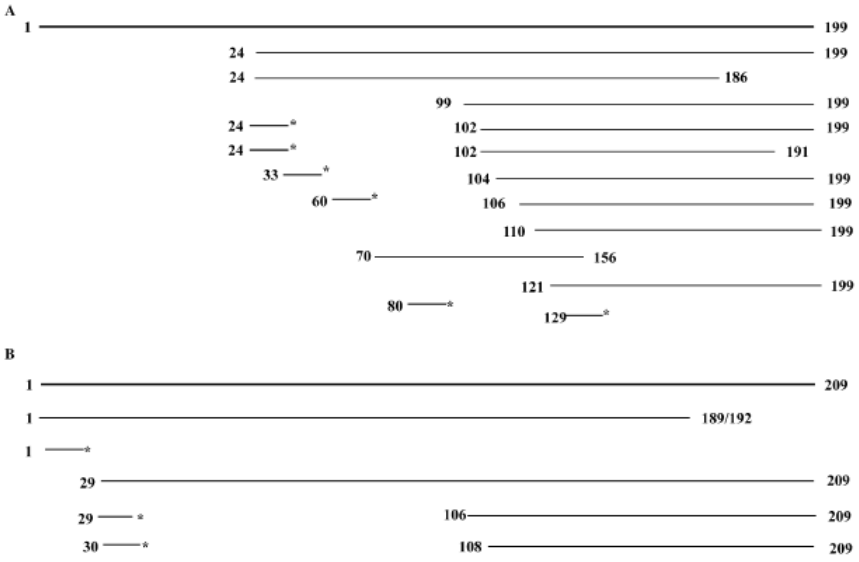

Fig. 5.3. Principal water-insoluble peptides derived from αs1-casein (A) and β-casein (B) isolated from Cheddar cheese (McSweeney et al., 1994; Mooney et al., 1998).

α_{s1}-Casein in Cheddar cheese is rapidly and completely hydrolysed by chymosin at the Phe_{23}-Phe_{24} bond. The larger peptide, α_{s1}-CN(f24–199) produced by the cleavage of Phe_{23}-Phe_{24}, is further hydrolysed by chymosin at the bond Leu_{101}-Lys_{102} and, to a lesser extent, at Phe_{32}-Gly_{33} and Leu_{109}-Glu_{110}, and perhaps by plasmin at Lys_{103}-Tyr_{104} and Lys_{105}-Val_{106}. The large C-terminal peptides, α_{s1}-CN(f24–199), α_{s1}-CN(f33–199), α_{s1}-CN(f102–199) and α_{s1}-CN(f110–199) and α_{s1}-CN(f99–199), α_{s1}-CN(f104–199) and α_{s1}-CN(f106–199) have been identified in the water-insoluble fraction of Cheddar cheese (McSweeney et al., 1994; Mooney et al., 1998). The complementary N-terminal peptides (e.g., α_{s1}-CN(f24–98), α_{s1}-CN(f24–101) and α_{s1}-CN(f24–109)) could not be identified in the water-insoluble fraction, but since these are highly phosphorylated, they may be in the water-soluble extract. Surprisingly, the bond Trp_{164}-Tyr_{165}, which is hydrolysed rapidly by chymosin in α_{s1}-casein in solution (McSweeney, Olson, Fox, Healy, & HØjrup, 1993b), does not appear to be hydrolysed in cheese; at least a peptide with Tyr_{165} as its N-terminal has not yet been identified neither in water-soluble fraction nor in model cheese systems (Singh et al., 1994,

1995, 1997; Exterkate, Alting, & Slangen, 1995; Exterkate, Lagerwerf, Haverkamp, & Schalkwijk, 1997; Fernàndez et al., 1998). Perhaps, the bond Trp_{164}-Tyr_{165} is difficult for chymosin to access in cheese, perhaps due to intermolecular interactions. The peptide α_{s1}-CN(f1–23) is hydrolysed rapidly by the lactococcal CEP at the bonds Gln_9-Gly_{10}, Gln_{13}-Glu_{14}, Glu_{14}-Val_{15} and Leu_{16}-Asn_{17}, and probably at other sites, depending on the specificity of the enzyme (Exterkate & Alting, 1993; Exterkate et al., 1995). β-Casein is hydrolysed mainly by plasmin at Lys_{28} Lys_{29}, Lys_{105}-Gln_{106} and Lys_{107}-Glu_{108}, producing the fragments β-CN(f29–209), β-CN(f106–209) and β-CN(f108–209) (γ_1-, γ_2-, and γ_3-, respectively). The γ-caseins are present in the water-insoluble fraction of Cheddar (McSweeney et al., 1994; McGoldrick & Fox, 1999). Most of the peptides that have been identified are produced from β-casein by the action of lactococcal CEP, probably on proteose peptones rather than on intact β-casein, since none of the peptides identified contained an intact plasmin cleavage site (Singh et al., 1997; Mooney et al., 1998; Fox & Wallace, 1997). The concentration of α_{s2}-casein appears to decrease During ripening but no large peptides derived from α_{s2}-casein have yet been reported (Mooney et al., 1998), and only a few small peptides derived from α_{s2}-casein have been identified in the WSE (Singh et al., 1995, 1997, 1999). Most of the peptides of Cheddar are derived from the N-terminal half of β-casein (especially from residues 53 to 91), with a smaller number from the N-terminal half of α_{s1}-casein (Singh et al., 1997) (see Fig. 5.4c).

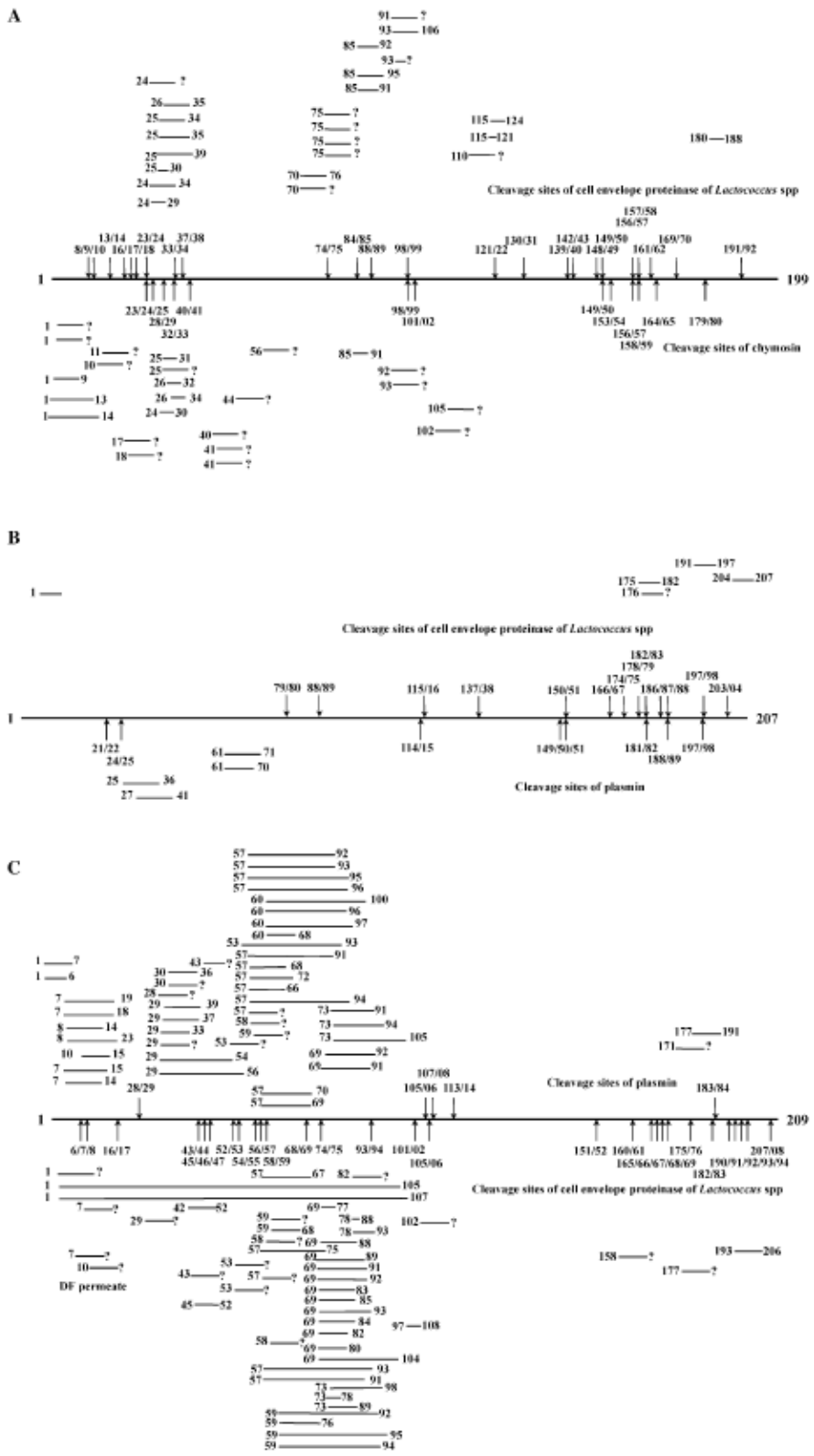

Fig. 5.4. Water-soluble peptides derived from α_{s1}-casein (A), α_{s2}-casein (B), and β-casein (C) isolated from Cheddar cheese. The principal chymosin, plasmin and lactococcal cell-envelope proteinase cleavage sites are indicated (Singh et al., 1994, 1995, 1997; Breen et al., 1995; Fernòndez et al., 1998).

The N-terminal of most of these peptides corresponds to a cleavage site for chymosin (α_{s1}-casein), plasmin (β-casein), or lactococcal CEP. The large water-insoluble peptides in Cheddar are from the C-terminal segments of α_{s1}-casein produced mainly by chymosin or from β-casein (γ-caseins) produced by plasmin. Para-κ-casein (κ-CN(f1–105)) is not hydrolysed during ripening. The small water-soluble peptides appear to be peptides produced by the action of the lactococcal CEP, or perhaps endo-peptidases, from products of chymosin or plasmin action.

5.8. Contribution of proteolysis to the development of taste and flavour compounds

Proteolysis contributes to the taste of cheese by the production of peptides and free amino acids and the sapid flavour compounds generally partition into the soluble fraction on extraction of cheese with water. Large peptides do not contribute directly to cheese flavour, but are important for the development of the correct texture; however, large peptides can be hydrolysed by proteinases to shorter peptides that may be sapid. The composition of the amino acid fraction and the relative proportions of individual amino acids are thought to be important for the development of the characteristic flavour (e.g., Broome, Krause, & Hickey, 1990; Engels & Visser, 1994; Molina et al., 1999b). However, the relative proportion of individual amino acids appears to be similar in many varieties and increasing the concentration of free amino acids in cheese does not necessarily accelerate ripening or flavour intensity (McGarry et al., 1994; Christensen, et al., 1995). Fox and Wallace (1997) suggested that cheese flavour and the concentration of free amino acids not be correlated, since different cheeses (e.g., Cheddar, Gouda and Edam) have very different flavours, although the concentration and relative proportions of free amino acids were generally similar. Bitterness in cheese is most often due to hydrophobic peptides and is generally regarded as a defect, although bitter notes may contribute to the desirable flavour of mature cheese. Certain

sequences in the caseins are particularly hydrophobic and, when excised by proteinases, can lead to bitterness. Bitter peptides from αs1-casein are predominantly from the region of residues 14–34, 91–101 and 143–151, while bitter peptides from β-casein are mostly from the region of residues 46–90, and particularly from the hydrophobic C-terminus. Chymosin (or rennet substitutes) is important in the production of bitter peptides, since residual coagulant is the principal proteinase in many cheese varieties and its primary action on β-casein releases extremely hydrophobic peptides. Thus factors that affect the retention and activity of coagulant in the curd (e.g., pH or salt) may influence the development of bitterness. In the future, work may be expected to develop further methodology for studying proteolysis. Although there have been notable advances in their application and in data interpretation (chemometrics), the common analytical techniques for proteolysis (e.g., urea-PAGE, RP-HPLC and quantification of soluble N and free amino acids) have remained relatively unchanged over the last number of years. A notable trend in recent years, which we feel will continue in the future, has been the study of proteolysis in different cheese varieties. This includes many cheese varieties produced in smaller quantities or in restricted geographical areas that now have been characterised with respect to proteolysis, including the isolation and identification of some significant peptides. The identification of peptides from cheese using mass spectrometry and amino acid sequencing has only begun. Future work on the identification of peptides has the potential to increase our understanding of the ongoing processes in cheese and, as a consequence, also how to control them. The kinetics of peptide production and degradation is not well understood and thus it is likely that mathematical modelling techniques will be applied to study proteolysis in cheese during ripening.

Part two - Experimental

1. Protein modifications in cooked pork products investigated by a proteomic approach

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1.1 Abstract

To evaluate process-induced protein modifications in cooked ham and emulsion sausages, the proteomes of whole-cut (Parma and ‘‘Praga’’ cooked hams) and comminuted pork (mortadella and würstel) products were compared to raw pork using two-dimensional gel electrophoresis (2-DE) coupled to image analysis and mass spectrometry (MS). Other than heat-induced breakdown of part of the myosin heavy chains, the 2-DE pattern of cooked ham was substantially similar to that of raw pork. However, the MS-based analysis showed minor modifications, including the extensive oxidation of methionines. In contrast, likely due to emulsification, comminuted sausages were characterized by an abundant insoluble protein fraction (IPF). Interestingly, tropomyosin and myosin light chains in comminuted sausages were exclusively found in the IPF. Our results indicate that the protein aggregation systems of cooked hams and emulsion sausages reflect the processing conditions and are definitely different, the former being characterized mainly by disulphide bridges and the latter by additional covalent inter-protein links.

1.2. Introduction

Cooking meat and meat products is commonly practiced to obtain safer products and to develop sensory attributes (Mottram, 1998). In cooking procedures, the heat treatment induces modifications to the structural setting of the main meat constituents (Barbera & Tassone, 2006; Tornberg, 2005), especially proteins, as a consequence of degradation, denaturation, oxidation and polymerization events (Barbieri & Rivaldi, 2008; Gatellier, Santé-Lhoutellier, Portanguen, & Kondjoyan, 2009). Proteins (~20% of the muscle) represent the primary constituents of meat. Skeletal muscle proteins include the salt-soluble structural components, (myofibrillar proteins; ~45–55%), the water-soluble metabolic enzymes which constitute the sarcoplasmic fraction (~30–35%), and the connective tissue (or stroma) proteins (~10– 15%), which envelope fibres (holding them together and are soluble in dilute acidic solutions). Upon cooking (37–80 °C), the muscle protein fractions undergo conformational, chemical, and physical modifications to a variable extent. Structural proteins of thick (myosin heavy chain) and thin filaments (actin, tropomyosins, and troponins) and Z-disk proteins (desmin and α-actinin) respond differently to cooking temperatures in the 40–80 °C range; α-actinin is the most heat sensitive and becomes insoluble at 50 °C, myosin at 55 °C, actin between 70 and 80 °C, and tropomyosins (TPMs) and troponin at higher temperatures, greater than 80 °C (Cheng & Parrish, 1979). Denaturation of other sarcomeric proteins, such as titin, occurs at 73 °C (Fritz, Dietrich, & Greaser, 1992), while nebulin withstands cooking at 80 °C (Locker, 1984). Most sarcoplasmic proteins undergo aggregation between 40 and 60 °C, even though, for some of them, coagulation can occur up to 90 °C (Laakkonen, 1973; Tornberg, 2005). Collagen, the predominant connective protein, denatures at about 65 °C (Laakkonen, 1973) and gelatinizes at 80 °C. These events trigger transversal and longitudinal shrinkage as well as the flaking of muscle fibres and connective tissue, as a consequence of the heat treatment (Palka & Daun, 1999). Thus, each fraction affects the organoleptic traits of cooked meat

products in a way that is strictly related to its structure, and that in turn is determined by the cooking process. In the processing of cooked pork products, which includes the production of a large variety of traditional meat preparations worldwide, whole anatomical cuts, generally deriving from pork legs, or assembled muscle pieces, are salted, spiced, rubbed with specific aromas, and injected with brine. However, to produce emulsioned sausages, meat pieces from different cuts are finely comminuted, salted, flavoured, and cased. Muscle fibres of cooked ham undergo biochemical and shape modifications similar to those in cooked meat (transversal and longitudinal shrinkage, aggregation and gel formation of the sarcoplasmic proteins; Tornberg, 2005). For this reason, a ham press mould is used to prevent shape loss and to adhere muscle fibres during the cooking phase; this step is simply called ‘‘forming’’ or ‘‘stuffing’’. The salting step and subsequent heating of the comminuted emulsion sausages, (such as mortadella and ‘‘frankfurter’’, also called würstel), promote the effective solubilization of myofibrillar proteins (Di Luccia et al., 2005; Trani et al., 2010), which form a dense gel protein network that retains water by capillary force (Tornberg, 2005). The type of gel matrix is related to the balance between disperse or aggregated muscle proteins prior to this. The cross-linking of proteins in the gel network is triggered by free radicals (Promeyrat et al., 2010) and by the conformational transitions (Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008) that affect several properties of the proteins, including their solubility, hydrophobicity, and emulsion stabilizing power (Sun, Zhou, Zhao, Yang, & Cui, 2011). The physico-chemical properties of proteins can also be altered due to covalent coupling of reactive amino acid side chains (e.g. lysine, cysteine) to the secondary products of lipid peroxidation, especially carbonyl derivatives such as malondialdehyde and 4-hydroxynonenal (Gardner, 1979). Myosin denaturation involves a two-step gel formation that occurs in different temperature ranges, between 30 and 50 °C and above 50 °C, respectively, resulting in further aggregation. In all of these events, the use of varying recipes, salt

concentration, and pH determine the properties of the protein networks, which are what primarily govern the overall sensory traits of the cooked meat products (Tornberg, 2005). Cooked hams are produced by heating in an oven to a core temperature of 70 °C, following brine injection and tumbling. Generally, a cooking time of 1 h per kg of meat product is required, which means an overall time of 10–12 h. The origin of cooked ham dates back to the mid-fifteenth century, as quoted noted in Libro de coquinaria art by Martino of Como (about 1465), where it was obtained from the hind limb of a pig, from which the fat was removed, cut, boned, massaged, processed and, finally, steamed. ‘‘Praga’’ ham is a related specialty from the Trieste area of central Europe, which formerly belonged to the Austro-Hungarian Empire. For this style of ham, the cooking stage is carried out in special hot-air ovens rather than by exposure to wet steam. Following this, the smoking stage is entirely natural and is based exclusively on beech wood. The comminuted meat products analysed in this study are mortadella di Bologna, a typical Italian cooked sausage, and würstel, a sausage manufactured worldwide. The origin of the word ‘‘mortadella’’ is somewhat controversial, but one of the most reliable hypotheses is that it derives from the late Latin ‘‘mortarium’’, which described the pestle and mortar, in which the friars in Bologna (Italy) prepared the mixture of pounded meat mixed with fat and spices. Nowadays, comminution is accomplished by grinders that reduce the granulometry of the meat to less than 0.9 mm, and the cooking is performed in stages (drying, pre-cooking, firing, and second firing) with temperatures as high as 80 °C for 19–20 h overall (Barbieri, Bergamaschi, Barbieri, & Franceschini, 2013). The würstel is linked to the butcher Johann Georg Lahner, who in 1807, invented the frankfurter sausage (today’s frankfurters) that gradually spread to the entire Austro-Hungarian Empire (Lahner, 1969). Würstels are cooked at variable times and temperatures, typically for about two hours or until the entire product reaches a temperature of 70 °C. Following this, the sausages are traditionally smoked with beech wood, to give them a characteristic flavour. In this paper, we

compared the proteomes of cooked salami obtained from whole cuts or comminuted meat as determined by different processing technologies, and in particular, investigated heat-induced protein modifications with respect to raw pork.

1.3. Materials and methods

1.3.1. Sample preparation

Traditional meat products, manufactured according to welldefined process guidelines, were selected in this study. Three samples each of Parma cooked ham, ‘‘Praga’’ cooked ham, mortadella di Bologna, würstel, and raw pork were purchased at a local supermarket. The samples (50 g) were mixed with a 0.03 M phosphate buffer (pH 7.2) to obtain a homogeneous slurry; they were then centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant, containing the sarcoplasmic fraction, was recovered for electrophoresisanalysis. Each resulting pellet, about 1 g, was suspended in 10 ml of extraction buffer (8 M urea, 2 M thiourea, 2% NP-40, 10% dithiothreitol, DTT), stirred for 4 h, and centrifuged at 10,000 rpm for 10 min. The supernatant, which contained the myofibrillar fraction, was recovered and used for successive electrophoresis analyses. Total meat proteins (myofibrillar and sarcoplasmic proteins) were extracted by homogenizing the cooked and raw products with a denaturing and reducing buffer (DRB; 8 M urea, 2 M thiourea, 2% NP-40, 10% DTT) or with a nonreducing buffer (DB; 8 M Urea, 2 M thiourea, 2% NP-40). The homogenates were magnetically stirred for 12 h prior to clarification (by centrifugation, 12,000g for 20 min). The resulting supernatant (S1) was recovered, and the resulting pellet was dried in an oven until it reached a stable weight. It was then weighed and resuspended in 2.5 ml of both DRB and DB, sonicated 3 times for 10 min each, centrifuged at 13,000 rpm at 4 °C; and the resulting supernatant (S2) was analysed by 2-DE. The pellet was resuspended three times with distilled water, centrifuged after each washing, and dried in an oven until it reached a stable weight, which was then recorded.

1.3.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The protein sample (1:1 vol:vol) was diluted with 10 mM Tris– HCl, 1 mM EDTA, 2.5% SDS, 1% DTT, and 0.01% bromophenol blue (BPB), pH 8.0, and loaded onto a 8–18% polyacrylamide gel gradient (10 µl after 3 min heating in a boiling water bath). Separation was performed using a Multiphor II (GE-Healthcare Bio-Sciences, Little Chalfont, U.K.), at 200 V, and 20 mA for the stacking gel, and 600 V, 50 mA for the resolving gel. The gels were stained with G-250 Coomassie (blue silver).

1.3.3. Two-dimensional gel electrophoresis (2-DE)

The two-dimensional gel electrophoresis of protein extracts were carried out in triplicate. Briefly, a sample volume equivalent to 100 µg of total protein extract, quantified with the Bradford assay, was loaded onto immobilized pH gradient (IPG) strips, pH 3–10, 13 cm long (GE-Healthcare Bio-Sciences, Little Chalfont, U.K). Following rehydration (7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% carrier ampholyte, 0.001% BPB), isoelectrofocusing (IEF) was carried out at 20 °C using a unit Ettan IPGphor 3 (GEHealthcare Bio-Sciences, Little Chalfont, U.K.); the voltage was increased stepwise to 8000 V, reaching a total of 40,000 Vhrs. Afterwards, the IPG strips were reduced and alkylated using buffers containing 6 M urea, 50 mM pH 8.8 Tris–HCl, 2% SDS, 30% glycerol, 5 mM tributyl phosphine (TBP), and 0.002% BPB, successively supplemented with 1% w/v DTT and 2.5% w/v iodoacetamide for 25 min each. The SDS–PAGE separation was performed using hand-cast 8–18% polyacrylamide gels and placed in a Hoefer SE 600 (GE-Healthcare Bio-Sciences, Little Chalfont, U.K.) electrophoresis chamber. The electrophoresis was run at 30 mA per gel and 10 °C. The gels were visualized with Coomassie G-250 (Sigma Aldrich, Steinheim, Germany) and digitalized using an ImageMaster Scanner (GEHealthcare Bio-Sciences, Little Chalfont,

U.K.). The detection and quantification of spots were performed using the ImageMaster 2D Platinum 7.0 software (GE-Healthcare Bio-Sciences, Little Chalfont, U.K.).

1.3.4. In-gel tryptic digestion of protein spots

Protein spots were in-gel digested according to the method of Di Luccia et al. (2005). Briefly, gel spots were manually excised and destained by repeated washing with 50% acetonitrile (v/v) in 25 mM NH_4HCO_3. The proteins were then reduced by incubation with 10 mM DTT (1 h, 56 °C), and then alkylated with 55 mM iodoacetamide (45 min at room temperature in the dark). The gel plugs were thoroughly washed with NH_4HCO_3, dehydrated with 100 µl of 100% acetonitrile, and then dried in a speed-vac. The proteins were digested overnight at 37 °C after the gel pieces were rehydrated with 10–12 µl of a trypsin solution (Promega, Madison, WI, USA, 12.5 ng/ml in 25 mM NH_4HCO_3). The resulting peptides were extracted three times with 40 µl of 50% acetonitrile/5% formic acid (v/v). Extracts were combined, dried in a vacuum centrifuge, and re-dissolved in 15 µl of 50% acetonitrile (v/v)/0.1% trifluoroacetic acid (TFA) prior to MS analysis.

1.3.6 MALDI-TOF MS-based peptide mass fingerprinting

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) experiments were carried out using a Voyager DE Pro mass spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with an N_2 laser (k = 337 nm). The instrument operated at an accelerating voltage of 20 kV in the positive reflector ion mode. The matrix was α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile/0.1% TFA). Typically, 250 laser shots were accumulated for each spectrum. External mass calibration was performed with commercial standard peptide mixtures (Sigma, Milan, Italy). The mass spectra were elaborated using the Data Explorer 4.0 software (Applied BioSystems, Carlsbad,

CA), which was furnished with the instrument. Peptide mass fingerprinting (PMF)-based identifications were carried out by interrogating the nonredundant National Center for Biotechnology Information (nrNCBI) and Swiss-Prot/TrEMBL databases, using the Mascot (Matrix Science, London, UK) and Protein Prospector MS-FIT (http://prospector.ucsf.edu/) search engines. Mass tolerance of 0.4 Da, fixed carbamidomethylation of cysteines, variable formation of pyroglutamic acid at the N-terminal Gln, and possible methionine oxidation were set as the protein modifications of interest. A single missed tryptic cleavage was accepted. Searches were taxonomically restricted to Sus scrofa or extended to mammalian, in the case of missed identification. Protein identifications were considered successful when the top-score entries were significantly higher than the threshold ($p < 0.05$), and were validated by manual inspection, checking for the presence of additional minor signals not previously included into the peak list, and compared to the expected pI/MW coordinates on the 2-DE maps.

1.4. Results

1.4.1. Extraction and SDS–PAGE of muscle proteins from cooked pork products

Muscle protein fractions were sequentially extracted from cooked pork products according to their different solubility and analysed by SDS–PAGE, as shown in Fig. 1 (Di Luccia et al., 2005; Picariello et al., 2006; and Trani et al., 2010). Unlike raw pork, the sarcoplasmic proteins of cooked hams and emulsion sausages exhibited a very low extractability by a weak phosphate buffer (Fig. 1, lanes 5–8). This was most likely due to deep heat-induced conformational transitions, possible coagulation, aggregation, and, especially in comminuted products, lipid-protein interactions. Myofibrillar proteins of cooked pork products, extracted by DRB after depletion of the sarcoplasmic counterparts, showed an unexpected complex electrophoretic protein pattern (Fig. 1). Indeed, the cooked pork products contained

sarcoplasmic protein bands that co-migrated with several myofibrillar components in the 25–50 kDa MW range. In other words, a part of the sarcoplasmic proteins of cooked products remained unextracted when using saline buffers at a low concentration, but they were solubilized by DRB (Supplementary Information Fig. S1).

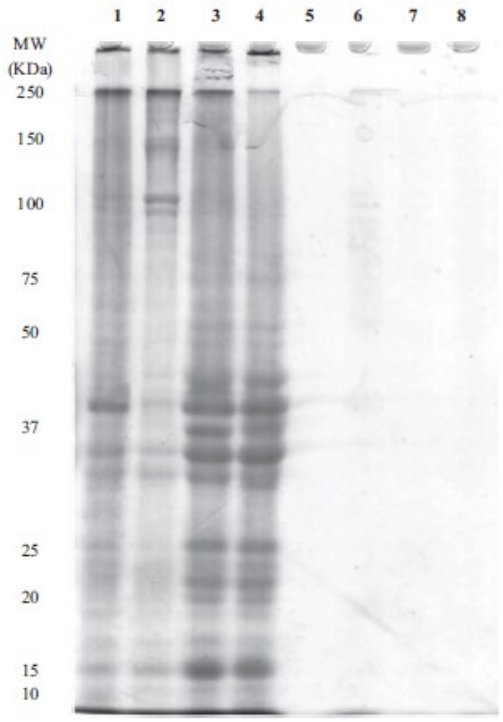

Fig. 1. SDS–PAGE patterns of myofibrillar (lanes 1–4) and sarcoplasmic (lanes 5–8) fractions: lane 1, mortadella; lane 2, würstel; lane 3, ‘‘Praga’’ cooked ham; lane 4, Parma cooked ham; lane 5, mortadella; lane 6, würstel; lane 7, ‘‘Praga’’ cooked ham; lane 8, Parma cooked ham.

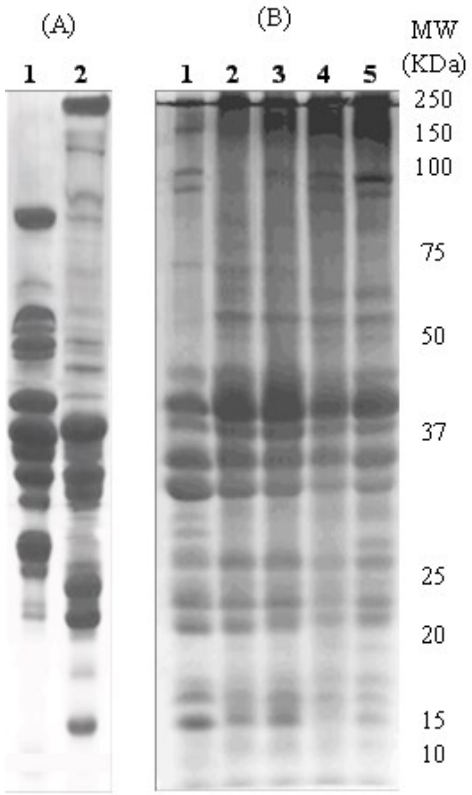

Figure S1. SDS-PAGE patterns of raw pork (panel A): lane 1, sarcoplasmatic fraction and lane 2, myofibrillar fraction. SDS-PAGE patterns of total proteins (panel B): lane 1, raw pork; lane 2, Parma cooked ham; lane 3, "Praga" cooked ham; lane 4, mortadella, and lane 5,würstel.

The electrophoretic patterns of DRB-extracted cooked products showed a protein smear for a high molecular mass (>100 kDa); this was probably due to large aggregate networks of neo-formed protein (Fig. S1). To investigate the nature of the polymeric aggregates, we compared the myofibrillar protein profiles from cooked products by using a gradient SDS–PAGE performed either in the presence or absence of the disulphide reducing agent (Supplementary Information, Fig. S2).

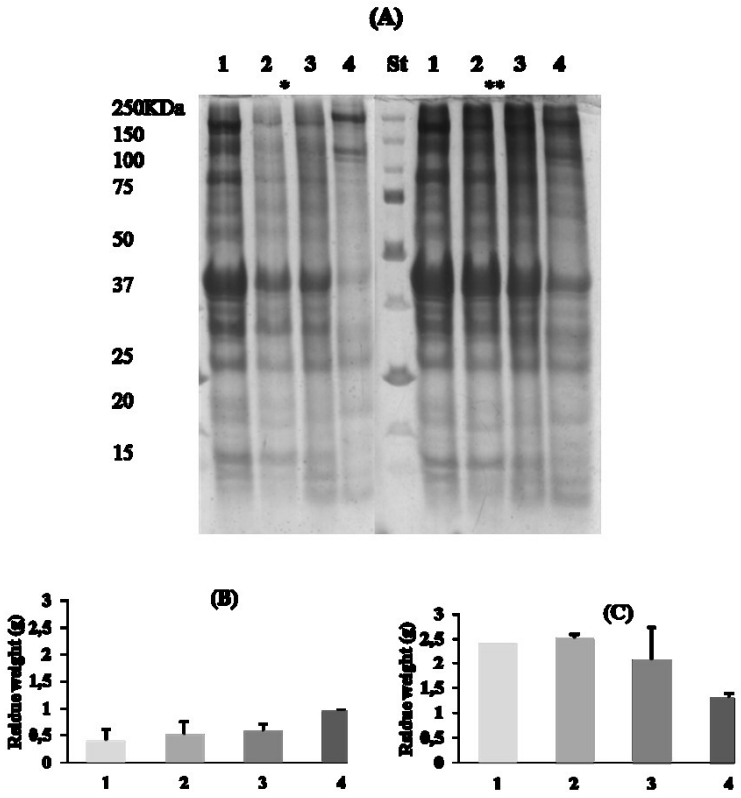

Figure S2. Protein extraction from cooked hams and emulsion sausages in presence (*) or absence (**) of DTT. Panel A, SDS-PAGE patterns of Parma cooked ham (lane 1), "Praga" cooked ham (lane 2), mortadella (lane 3), and würstel (lane 4). Changes of dry residue after the extraction of total proteins in presence of DTT (panel B) and in absence of DTT (panel C) of Parma cooked ham (1), "Praga" cooked ham (2), mortadella (3), and würstel (4).

In the case of würstel (cfr. lane n. 4 of Fig. S2 panel A), the relative increase of the high-MW bands (estimated 250 and 140 kDa) was particularly striking. Upon reduction they released lower-MW protein chains, thus supporting the formation of polymeric aggregates via covalent disulphide bonds. Aside from the expected band of myosin heavy chain (210 kDa), which is also detected in cooked hams, mortadella contained a diffused band at a higher MW, most likely due to protein aggregates. In any case, the permanence of the protein aggregates even under reducing conditions suggested that nonreducible covalent bonds are involved in the formation of polymeric protein networks in cooked products (Sharp & Offer, 1992). The weight variation of the dry residues after extraction by denaturing buffer with or without DTT

is schematized in Fig. S2. It is noteworthy that there were no significant discrepancies between the DTT extracts of cooked hams and mortadella, while the yields from würstel were significantly different (Fig. S2, panel C). When the extraction in DRB was performed following extraction in a nonreducing buffer, there was a greater increase in the weight of the residues for cooked hams and mortadella than there was for würstel. More precisely, the increase was 83.3% and 66.7% for Parma and ‘‘Praga’’ cooked hams, respectively, 54.9% for mortadella, and only 15.4% for würstel. From these results, it appears that different compositions of raw matter were used to produce the emulsioned sausages, since würstel was richer in insoluble proteins (probably collagen). Furthermore, the data confirmed that proteins establish covalent inter-protein links that assist the disulphide bridges in the formation of insoluble gel networks. This was further confirmed by 2-DE analysis, image analysis, and MS-based protein identification.

1.4.2. Proteomes of raw pork and cooked pork products

The 2-DE map of the muscle proteins from raw pork and cooked samples is shown in Fig. 2. As expected, myofibrillar proteins migrated within the acidic side of the 2-DE map, whereas sarcoplasmic proteins were tendentially spread towards the alkaline side, above pH 7.0. In this case, 156 spots were counted in the 2-DE raw pork meat fraction and most of them were tentatively assigned on the basis of their relative coordinates (pI and MW), based on results from previous studies (Di Luccia et al., 1992; Lametsch, Roepstorff, & Bendixen, 2002). Despite the tumbling and heat-treatments that cooked hams undergo, the main myofibrillar and sarcoplasmic proteins were detected as unmodified in the 2-DE maps. However, Parma and ‘‘Praga’’ cooked hams showed a higher total number of detectable protein spots (211 and 242, respectively) than raw pork (156). On the other hand, protein extracts from emulsion sausages showed only a very limited number of detectable spots (70 and 68, respectively),

compared to raw pork and cooked hams. In these cases, surprisingly, myofibrillar proteins such as TPMs, myosin light chains (MLCs), and other minor proteins involved in muscle contraction were almost completely missing. Table 1 shows the total number of spots (ns) for the various cooked products, the percentage of variation compared to raw pork, and the total volume of the spots. It is interesting to note that the values of ns for Parma and ‘‘Praga’’ cooked hams were 135% and 156%, respectively, of the ns for raw pork; by comparison, the values of ns of mortadella and würstel were 45% and 44%, respectively, that of raw pork. The total volumes increased as the number of spots increased. In order to evaluate the correlation between the qualitative and quantitative parameters and to better describe the phenomenon, we defined the variation of spot density as the ratio ($\Delta ns/|\Delta V|$), where Δns is the variation in spots and $|\Delta V|$ is the absolute value of the variation in the volume. These were calculated by image analysis software. The variation in spot density determines the degree to which the protein changes in the cooked products with respect to raw pork. The variation of spot density was negative for emulsified sausages and positive for cooked hams. Spot matching, as assessed by image analysis, showed the similarities and differences between the cooked products and raw pork. Parma and ‘‘Praga’’ cooked hams matched raw pork for 58% and 53% of the spots, respectively, while mortadella and würstel matched for 22% and 21%, respectively.

Gel-map matching	**Nb Matches**	**Matches (%)**
Pork – Parma cooked ham	107	58.31
Pork – Praga cooked ham	106	53.27
Pork – Mortadella	25	22.12
Pork – Würstel	24	21.43

Table S1. Spots matching among cooked pork product and raw pork

Table 1

Number and volume of spots detected by 2-DE gel electrophoresis and image analysis of total protein obtained from first extraction.

Samples	Number of total spot (n_s)	%	Total volume (V_t)	$\Delta n/\lvert\Delta V\rvert^{*} \cdot 10^3$
Raw pork	156	100	87383.47	0.00
Parma cooked ham	211	135	116648.36	1.88
Praga cooked ham	242	155	116752.10	2.93
Mortadella	70	45	35920.16	−1.67
Würstel	68	44	34399.42	−1.66

* Δn is the difference between total spot number of samples and total spot number of raw pork and |ΔV| is absolute value of the difference between total volume of samples and total volume of raw pork.

The lower matching of mortadella and würstel was due to a smaller number of detectable spots, and, in particular, due to the lack of several expected myofibrillar chains, which were represented primarily by actin (Supplementary Information, Table S2). The sarcoplasmic proteins of emulsion sausages also occurred at lower spot numbers and intensities than in cooked hams (Fig. 2).

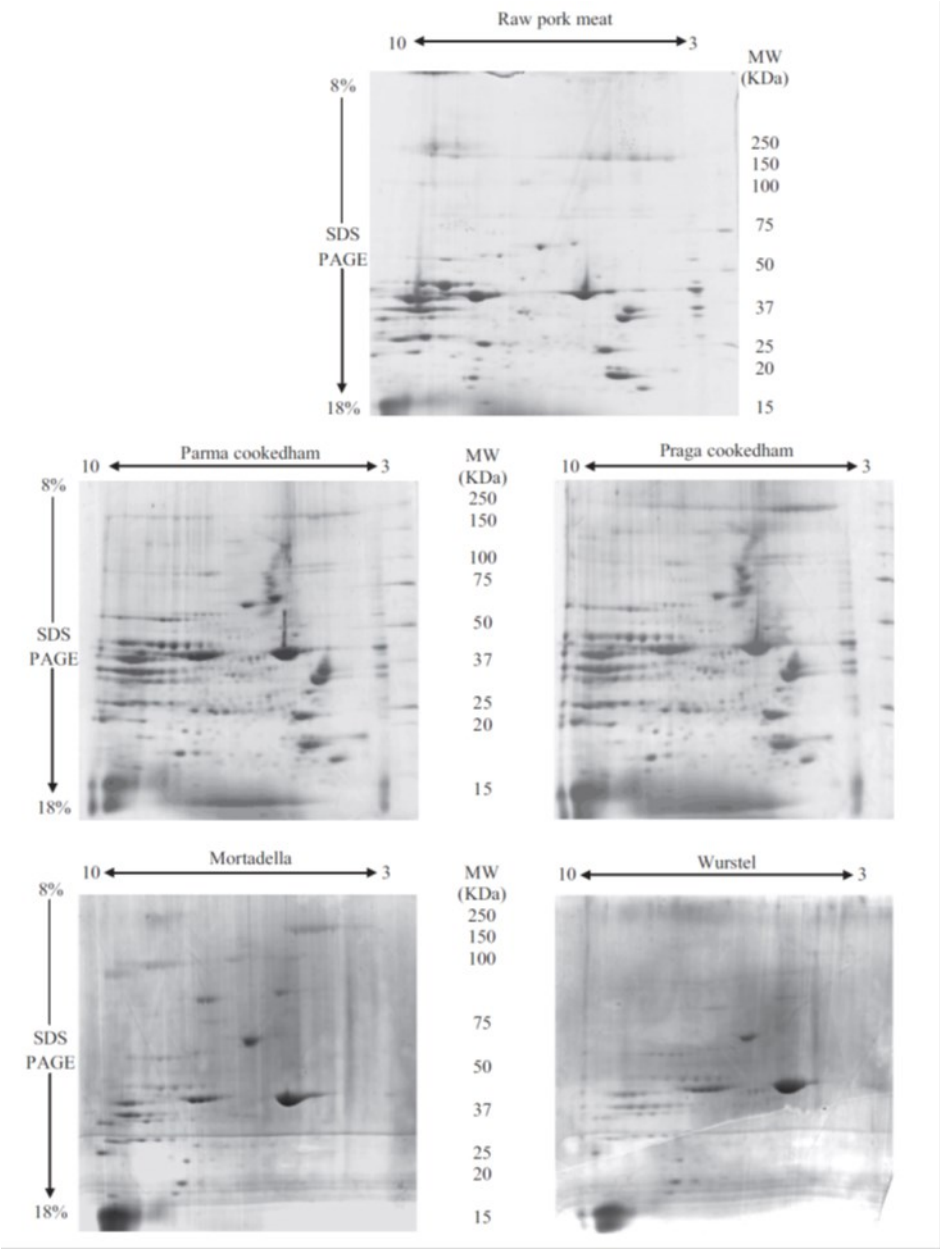

Fig. 2. 2-DE analysis of meat proteins extracted with denaturing and reducing buffer (DRB) from raw meat, cooked hams and emulsion sausages (I extraction).

These qualitative and quantitative differences in the 2-DE protein patterns depended on a series of factors, including:

(i) Decrement of the stain intensity of sarcoplasmic spots that made it easier to single out individual proteoforms within a contiguous train of spots.

(ii) Appearance of neo-formed spots around pI 7.0 in cooked hams, which probably arose due to the heat-induced breakdown of large protein chains (e.g., myosin).

(iii) Detection of additional spots in the 50–100 kDa MW range, probably due to partial protein aggregation.

1.4.3. Spot identification by MALDI-TOF MS

Muscle proteins from Parma cooked ham separated by 2-DE and identified by MALDI-TOF MS-based PMF are shown in Fig. 3 and listed in Table 2. Protein identification has been also referred to by previous 2-DE analysis (pI range 3–10) for muscle proteins from different mammals (Bendixen, 2005). We note the occurrence in cooked pork products of a neo-formed series of spots in the 110– 65 kDa range; this is particularly evident in the 2-DE maps of cooked hams. They were identified as follows: spot No. 1: α-actinin; spots Nos. 2–5 and No. 7: myosin heavy chains (MHC); and spot No. 6: BSA. The MHC spots migrated at similar pIs, but they had lower MW than the parent protein, which indicated an extensive cooking-induced breakdown of the chain during the cooking process. Interestingly, almost all of the tryptic peptides of the MHC fragments mapped within the central and C-terminal protein region, indicating that the 2-DE spot arose from the fibrous (C-terminal) region of myosin. On the other hand, the N-terminal heads (up to the actin-binding domains) of MHC are for the most part engaged in the formation of the coalesced heat-induced gel networks, and this could be why they are less extractable (Sharp & Offer, 1992; Tornberg, 2005). The majority of the other identified spots corresponded to entire protein chains. This was also established by comparing the expected and experimental 2-DE coordinates (MW/pI), which demonstrated that heat-induced proteolysis is a rather uncommon event and affects MHC almost exclusively. Many of the methionine residues were partially oxidized, as demonstrated by MALDI-TOF MS mapping of the tryptic peptides of actin (mass increment by 16 Da; Supplementary Information Fig. S3).

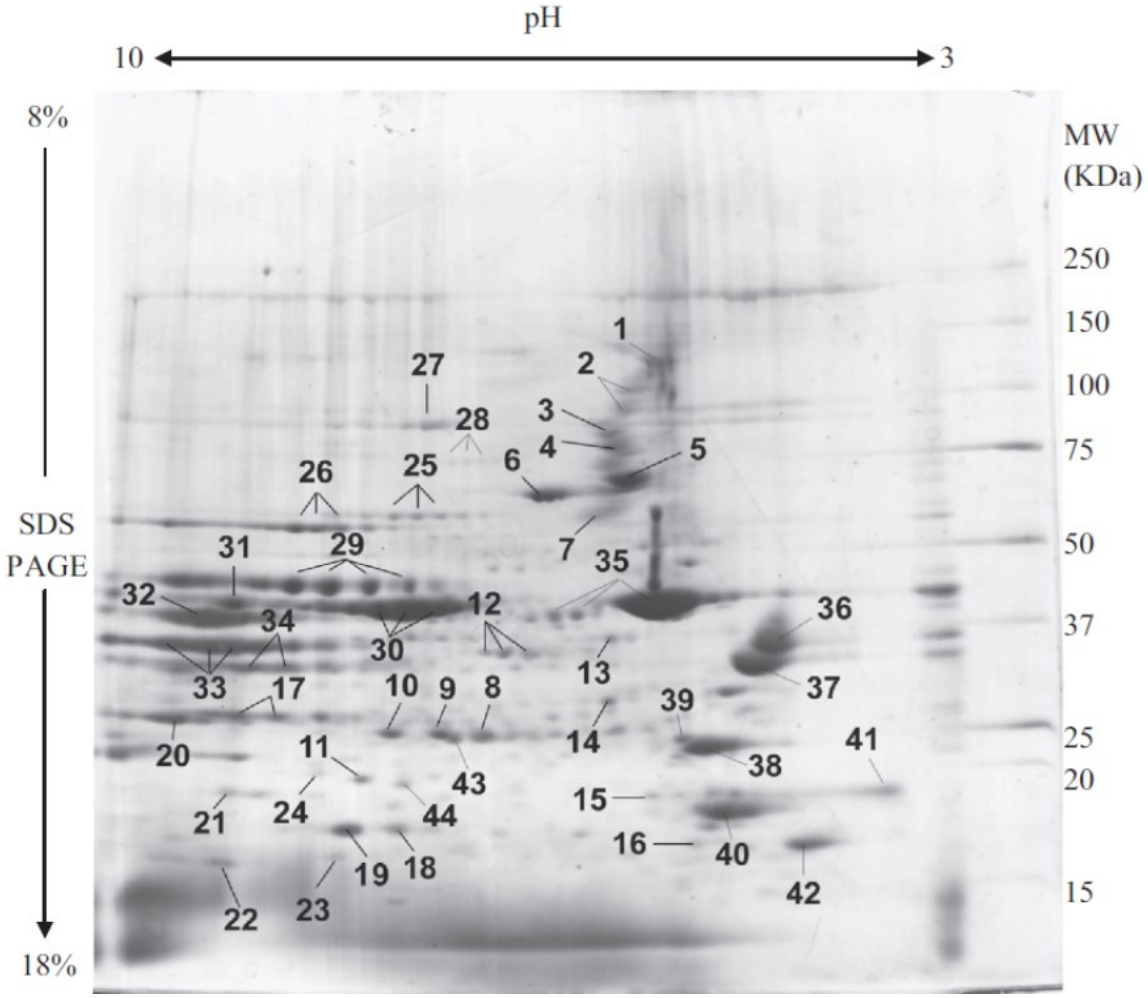

Fig. 3. Identification by MALDI-TOF tryptic peptide mass fingerprinting of spots excised from 2-DE gels of Parma cooked ham. The identified spots are assigned in Table 2.

Spot	Identification	PAN	Notes	MW (kDa)/pI
1	α-Actinin-3 skeletal muscle isoform (F actin cross-linking protein)	Q0III9	By homology with Bos taurus	
2	Myosin heavy chain 1 fragment	Q9TV61		
3	Myosin heavy chain 1 fragment	Q9TV61		
4	Myosin heavy chain 1 fragment	Q9TV61		
5	Myosin heavy chain 1 fragment	Q9TV61		
6	Serum albumin	P08835		66.8/5.84
7	Myosin heavy chain 1 fragment	Q9TV61		
8	Triosephosphate isomerase	Q29371		26.7/7.00
9	Triosephosphate isomerase	Q29371		26.7/7.00
10	Triosephosphate isomerase	Q29371		26.7/7.00
11	α-Crystallin β-subunit	Q7M2W6	HSP-27 kDa related protein	20.1/6.80
12	Troponin T, slow skeletal muscle	Q75ZZ6		31.2/5.9
13	Actin, skeletal muscle	P68137	C-term fragment	42.1/5.23
14	Actin, skeletal muscle	P68137	C-term fragment	42.1/5.23
15	Myosin light chain 1f (fragment)	A1XQT6		20.9/4.90
16	Carnitine O-palmitoyltransferase 1, muscle isoform (fragment)	Q8HY46		
17	Carbonic anhydrase 3	Q5S1S4		29.4/7.70
18	Myoglobin	P02189		16.9/6.83
19	Myoglobin	P02189		16.9/6.83
20	Phosphoglycerate mutase 2	B5KJG2		28.7/8.86
21	Adenylate kinase isoenzyme 1 (myokinase)	P00571		21.6/8.38
22	Haemoglobin α -chain	P01965		15.0/8.76
23	Haemoglobin β -chain	P02067		16.0/7.25
24	Phosphatidylethanolamine-binding protein 1	F1RKG8	By homology with Bos taurus coded P13696	20.9/7.00
25	Phosphoglucomutase-1	G0Z3A1		61.7/6.07
26	Pyruvate kinase, M2 isozyme	F1SHL9		56.1/8.09
27	Serotransferrin (transferrin)	P09571		80.7/7.62
28	Succinate dehydrogenase	Q0QF01		72.8/7.20
29	B-enolase muscle isoform	Q1KYT0		47.1/8.11
30	Creatine kinase M-chain	Q5XLD3		43.0/6.61
31	Phosphoglycerate kinase 1	Q7SIB7		44.4/8.01
32	Fructose-bisphosphate aldolase A		By homology with rabbit	39.3/8.31
33	Glyceraldheyde-3-phosphate dehydrogenase	P00355		35.8/8.51
34	Lactate dehydrogenase	P00339		36.6/8.18
35	Actin, alpha skeletal muscle	P68137		41.8/5.23
36	Tropomyosin β -chain	A1X899		33.3/4.62
37	Tropomyosin α -chain	P42639		32.7/4.71
38	Myosin light chain 1f	A1XQT6		20.9/4.90
39	Myosin light chain 3 (MYL3)	F1SNW4		21.8/5.00
40	Myosin light chain 2	Q5XLD2		19.0/4.87
41	Troponin C, skeletal muscle	P02587		18.0/4.06
42	Myosin light chain 3, skeletal muscle isoform (MLC3F)	Q29069		16.7/4.93
43	Heat shock protein 27 (HSP-27)	Q5S1U1		22.9/6.20
44	Phosphatidylethanolamine-binding protein 1 (basic cytosolic 21 kDa protein)	P13696	By homology with Bos taurus (see spot 24)	20.9/7.00

Table 2. Proteins identified from the 2-DE gel spots of Fig. 3 by MALDI-TOF-MS analysis in positive ion mode of the in-gel tryptic digest. PAN: Primary Accession Number in the Uniprot Protein database P00883.

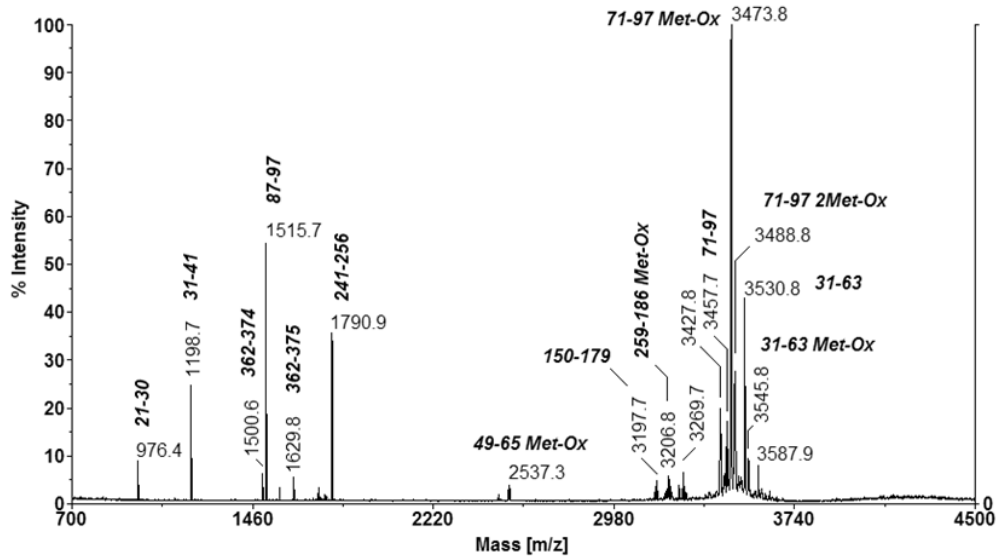

Figure S3. MALDI-TOF tryptic peptide mass mapping of the 2-DE spot of skeletal muscle actin (P68137) from cooked ham. The peptide identifications are indicated in italics through the position within the protein chain.

Sulphur-containing methionine is particularly susceptible to oxidation by reactive oxygen species (ROS) to form methionine sulphoxide and sulphone (Vogt, 1995). The ROS formation is catalyzed by the presence of free transition metal ions and oxygen, which creates hydroxyl free radicals, especially at high temperatures. In this case, heat treatment in the presence of pro-oxidative Fe^{2+} or Fe^{3+} from heme and heme-free groups, as well as H_2O_2 as a cell metabolism by-product, determines the oxidation of methionine to a significant extent, regardless of the sarcoplasmic or myofibrillar nature of the proteins (Lund, Heinonen, Baron, & Estevez, 2011; Lund, Luxford, Skibsted, & Davies, 2008).

1.4.4. Proteome study of a second extraction of cooked hams and comminuted sausages

The missed detection of several expected major myofibrillar and sarcoplasmic proteins in the 2-DE of emulsion sausages (Fig. 2) suggested that they probably constituted an insoluble protein fraction (IPF). Indeed, the 2-DE analysis, aided by ultrasonication, of cooked pork products that were extracted by DRB, revealed several additional proteins for mortadella and würstel (Fig. 4) and, to a lesser extent, for cooked hams (not shown). In particular, the analysis of the DRB-extracted proteins of emulsion sausages showed the presence of intense spots of both sarcoplasmic and myofibrillar proteins, primarily actin. The qualitative and quantitative results of two sequential protein extractions from emulsion sausages are summarized in the Supplementary Information, Table S2: DRB (extraction I) and ultrasonication- aided DRB (extraction II). Ultrasonication increased the ratio of myofibrillar-to-sarcoplasmic ns values (M/S) from 0.23 to 0.93 and from 0.17 to 0.88 for mortadella and würstel, respectively. However, the total spot volumes were stunningly different between the two products, the figures for mortadella being much higher (Fig. 4). The percentage contribution of actin to the total volumes also increased more than twofold with ultrasonication (extraction II), indicating that it has an important role in the formation of

soluble and insoluble protein aggregates in emulsion sausages. The different ratios and compositions of the two protein fractions reported in Table S2 suggested that IPF aggregates were formed by non-reducing covalent cross-linking and differed both qualitatively and quantitatively between the two emulsion sausages. These findings indicated that the meat preparation and heat treatment of the production processes induced the formation of peculiar gel networks due to protein aggregation, and that this was mainly through the mechanisms of coagulation, denaturation, oxidation, and inter-chain cross-linking of polymers.

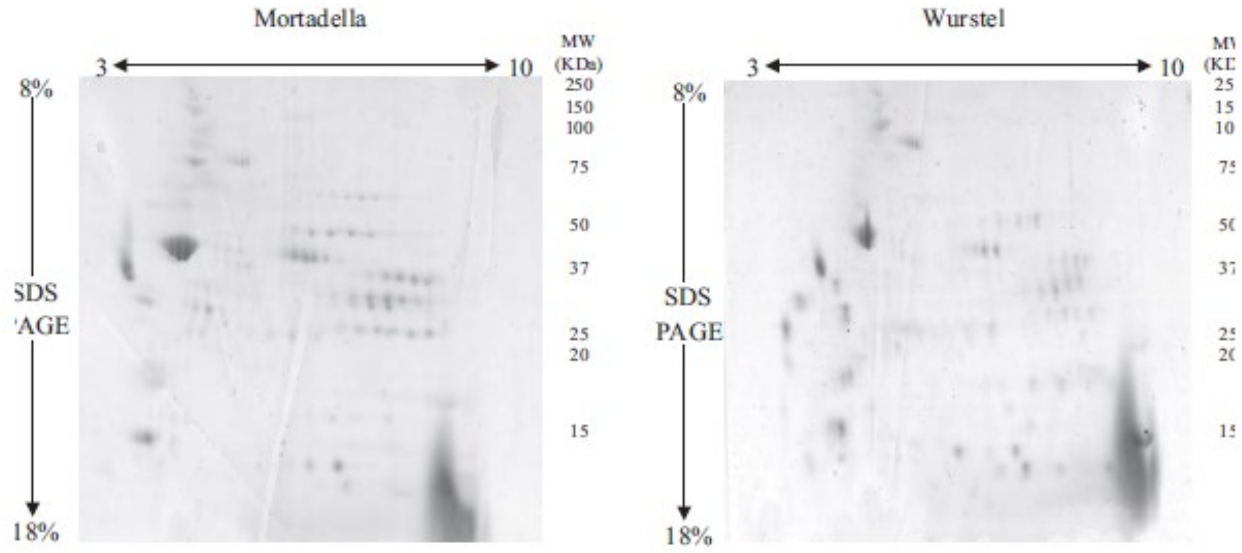

Fig. 4. 2-DE analysis of proteins obtained from sonication-aided denaturing and reducing buffer (DRB) extraction (II extraction) of mortadella and würstel

Samples	Extraction	Number of total spot (n_s)		Spot ratio	Total volume (V_t)		% Actin
		M	S	M/S	M	S	
mortadella	I	13	57	0.23	18731.59	15823.19	22.33
	II	42	45	0.93	1999945.42	1739384.1	53.45
würstel	I	9	59	0.17	15576.15	20066.77	29.18
	II	30	34	0.88	1.07	0.55	66.48

Table S2. Image analyses of 2-DE gel electrophoresis maps of total protein obtained from extraction with only DRB (I extraction) and with DRB and sonication (II extraction).

1.5. Discussion

Cooked hams and emulsion sausages represent two different models of cooked meat products, the former being generally manufactured from a whole anatomical cut (leg) that preserves the structural arrangement of the muscle, and the latter produced with comminuted pork pieces from different anatomical cuts that lose the muscular filamentous network due to extensive grinding (with particles less than 0.9 mm). Therefore, the heat-induced effects on the structural and conformational arrangement of the muscle proteins are expected to be different. Results obtained in this study confirmed this expectation, and revealed different patterns of protein modifications as a result of heat treatments and processing. One of the main results is the almost complete insolubility of sarcoplasmic proteins in cooked ham and comminuted products, which we attributed to the increased hydrophobicity, as a consequence of heat denaturation and coagulation (Promeyrat et al., 2010). This is caused by the breaking up of hydrogen and/or electrostatic bonds at temperatures that are sufficiently high that the orientation of the dipole of water is disturbed, which destabilizes the protein–water solvation system of the ‘‘native’’ state. The conformational transitions and the exposure of hydrophobic

amino acids, which, in normal conditions, are buried within the inner core of the globular proteins (Chelh, Gatellier, & Santé-Lhoutellier, 2007; Santé-Lhoutellier et al., 2008), promote the aggregation of proteins due to hydrophobic interactions. Davey and Gilbert (1974) found that heat aggregation of sarcoplasmic proteins could extend up to 90 °C, and they suggested that during cooking, sarcoplasmic proteins might contribute to the consistency of the meat by forming a gel that links together several structural elements. Even so, cooked ham showed a complete profile of muscle proteins in 2-DE maps, whereas emulsion sausages unexpectedly exhibited both a reduced number of sarcoplasmic proteins and the absence of several structural (myofibrillar) protein chains, in particular, the tropomyosins TPMs and MLCs. A further unexpected finding was the drastic relative increase of the actin spot in emulsion sausages, which suggested an engagement of other myofibrillar proteins in polymeric networks through the formation of covalent links. Lund et al. (2008), which used the oxidation of MHC as a model, established that the type of cross-linking could be highly dependent on the triggering events of oxidation. It has been reported that oxidation with myoglobin/H_2O_2 results in the formation of non-disulphide bonds, whereas oxidation with a metal-catalysed (Fenton-like) system results primarily in the formation of disulphide bonds (Hanan & Shaklai, 1995). Oxidative protein cross-linking can involve different amino acids: basic and aromatic amino acids and cysteine are particularly prone to reacting with free radicals during the cooking process (Petruk et al., 2012; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008). Basic amino acids are oxidized to generate carbonyl products (Stadtman & Levine, 2003; Uchida, 2003). The thiol groups of cysteine can be oxidized with the formation of disulphide bridges (Lund et al., 2011). Tyrosine in myofibrillar proteins can generate dityrosine bridges (Gerrard, 2002; Lund et al., 2011), and the combination of thiol radicals can lead to thiol-tyrosine bonds (Lund et al., 2008; Martinie et al., 2012; Petruk et al., 2012). The presence of these nonreducible inter-chain bonds has already been described for

several processed foods, such as bread, pasta, and meat (Gerrard, 2002; Lund et al., 2008, 2011). Finally, the interaction of reactive amino acids, such as lysine and cysteine, with (bifunctional) aldehyde products arising from lipid oxidation can also induce protein networking through the formation of Schiff bases (Gardner, 1979; Refsgaard, Tsai, & Stadtman, 2000). All of these links likely concur to the formation of larger aggregates or/ and insoluble polymeric protein networks. In the light of our results, it is possible to hypothesize that the cooking of a whole anatomical cut or of comminuted pork pieces generates different aggregates and/or insoluble polymeric protein networks. In recent proteomic studies, performed to define the quality and optimize the process parameters through the identification and monitoring of molecular markers, Barbieri and Rivaldi (2008) and Pioselli, Paredi, and Mozzarelli (2011) analysed the exudate during the manufacturing of cooked ham. The exudate is formed due to the injection of brine, the extensive tumbling and the increased temperature. Basically, three steps are crucial to this process (Barbieri & Rivaldi, 2008): (i) formation of an exudate rich in myofibrillar proteins; (ii) the formation of a network at a temperature near 62 °C; (iii) the loss of exudate in cooking subsequent to the formation of a myofibrillar matrix. These authors identified desmin in the exudate as a marker of the heat-induced modifications. Desmin steadily increased in any sample of the exudate of the cooked ham sample, but it decreased or even disappeared once the meat structure reached a steady state, and the cooking process could be stopped. On the other hand, the lack of desmin in the exudate indicates that the meat is not sufficiently cooked, or that the meat structure is too strong for a good network to be formed. In the cooking phases of production, raw ham and raw pork undergo changes. Muscle meat proteins denature which causes deep structural changes, such as the destruction of cell membranes (Rowe, 1989), the transversal and longitudinal shrinkage of meat fibres, the aggregation and gel formation of sarcoplasmic proteins, and the gelation and solubilization of connective tissue (Tornberg, 2005). To avoid

shrinkage and fibre separation, and to obtain homogenous products, the application of pressure on the pork leg is necessary during cooking in order to make the muscle fibres cohere by gel formation, due to the denaturation of proteins and the solubilization of connective tissue. In a system in which the fibres are forced to remain in a firm structure, an increase in temperature first causes MHC denaturation, followed by an increase in emulsification, sarcoplasmic coagulation and successive incorporation within the myofibrillar network. Finally, the fibres adhere to each other due to a collagen gelation that acts like glue. The result is a compact and homogenous product in which the fibres are no longer distinguishable. In this case, protein networks are generated mainly through disulphide links and hydrophobic interactions, as demonstrated by a very small insoluble residue obtained from total protein extraction in the DRB (Table S2). This also explains the progressive decrease of myofibrillar proteins in the exudate from ham during the cooking process. Emulsion sausages, (mortadella and würstel), lack the fibre structure that increases the solubilizing effect on the muscle proteins. Moreover, the comminution causes the incorporation of air into a system that is composed primarily by fat, proteins, and water. In such a system the conditions occur for generating both the metal-catalysed and the myoglobin/H_2O_2 oxidative phenomena that underlies the formation of reducible and non-reducible link formations (Bhoite-Solomon, Kessler-Icekson, & Shaklai, 1992). The dissolving of myofibrillar protein into the water-phase during comminution and blending represents the most important factor for judging the quality of the protein network and the structure of the emulsion sausage. Solubilization and heat denaturation of myofibrillar and soluble sarcoplasmic proteins form a protein solution with an emulsifier property that can conglomerate the exposed hydrophobic residues with fats, and is also prone to oxidation. These events underlie the formation of aggregates, and, likely, the polymeric protein networks. With extraction by DBR, we obtained larger amounts of protein residue from the

emulsion sausages than from the cooked hams. Moreover, the complete aggregate solubilization by breaking the disulphide bonds (Singh, Donovan, Batey, & MacRitchie, 1990) and, probably, other covalent links, was achieved by a combination of SDS and sonication. This explains the different 2-DE patterns of proteins obtained from the total protein extraction and from the IPF (data not shown). This supports the existence of two types of aggregation in the gel matrix residue, one containing only actin and the other containing actin and the remaining myofibrillar proteins and some of the associated sarcoplasmic proteins. This hypothesis is also supported by the results of Findlay, Parkin, and Stanley (1989), who demonstrated the occurrence of three major transition areas (A, B, and C) as determined by differential scanning calorimetry. The first transition had a maximum between 54 and 58 °C and was attributed to myosin; the second transition was between 65 and 67 °C and was ascribed to collagen, sarcoplasmic proteins, actomyosin, myosin, and its sub-units; the third transition, found between 80 and 83 °C, was attributed to titin and actin (reviewed in Tornberg, 2005).

1.6. Conclusion

A proteomic study of cooked pork products allowed us to determine qualitative and quantitative differences that are related to the processing parameters, including cooking, if it is whole cut or comminuted, salt solubilization of myofibrillar protein, protein aggregation, and collagen gelation.

In particular, 2-DE maps reflect the temperature effects on raw matter and the processing conditions, depicting the overall protein changes occurring in the cooked products, as compared to raw pork. Results obtained in this work also proved that:

- Emulsion sausages contained larger amounts of insoluble residue, such as gelled collagen, than cooked hams.

- An IPF with a high myofibrillar-to-sarcoplasmic protein ratio can be recovered from the insoluble residue of emulsion sausages.
- Actin is the main protein in emulsion sausages, and it forms aggregates with TPMs and MLCs that occur almost exclusively in the IPF.
- Within cooked products, there are relevant oxidation phenomena, and this is also evidenced by the MS identification of oxidized methionine in the muscle proteins.

The differences observed between the protein composition of cooked ham and emulsion sausages were ascribed to the degreeof solubilization of the myofibrillar proteins, their distribution in the network gel of cooked products, the temperature cycle utilized, the presence of fats, and the status of the pork cut (entire or comminuted). The gel properties of cooked pork products determined the nature of the myofibrillar network, and their inclusion within the coagulated sarcoplasmic protein matrices, gave rise to a finally homogenous gel matter bonded by covalent and noncovalent links. Denatured MHCs, and probably solubilized actin, contributed to the homogeneity of these products by emulsifying the fat matter with the myofibrillar and sarcoplasmic gel.

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Publication

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1.8 Study of proteolysis in river buffalo mozzarella cheese by a proteomic approach

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1.8.1. Abstract

The guarantee of the origin and quality of raw material is essential for the protection and valorization of Campana buffalo mozzarella cheese. The risk of utilization of semifinished products and stored milk in substitution for fresh milk is increasing, due to the continuous desire to reduce production costs. A proteomics approach and electrophoresis survey of retail mozzarella cheeses indicated different rates of proteolysis in the production of dairy industries. The use of fresh milk and correct cheesemaking protocol yielded only γ-caseins, which are derived from β-casein by plasmin, and para-κ-casein, which is derived from κ-casein by chymosin. The detection of abnormal hydrolysis resulting in β- and α_{s1}-casein fragments, identified by mass spectrometry, indicates the use of stored milk or stored and pressed curd, or the reuse of unsold mozzarella cheese, to produce mozzarella. The formation of γ-caseins and other fragments during a long storage of raw materials at room or refrigeration temperature was ascribed to plasmin (endogenous milk enzyme), whereas formation of αs1-casein fragments, mainly α_{s1}-I^{6P}- and α_{s1}-I^{7P}-casein during the storage of curd was ascribed to the action of chymosin (exogenous enzyme) from rennet. Sodium dodecyl sulfate-PAGE and alkaline urea-PAGE permitted us to evaluate the freshness of the

raw materials used in the manufacturing of buffalo mozzarella cheese and to reveal possible inappropriate preservation.

1.8.2. Introduction

Campana buffalo mozzarella cheese is a Protected Designation of Origin (PDO) product registered under Commission Regulation (EC) no. 1107/1996. According to the regulations, PDO buffalo mozzarella must be produced with whole, fresh river buffalo milk, coming from river buffalo reared in geographical areas given by the PDO specification; its unique characteristics are attributable to both the raw material and to the production technology. In recent years, price-based competition has led many dairies to lower production costs bychoosing technological solutions that are not compatible with the typical product characteristics, such as the use of semi-manufactured materials in substitution for fresh milk. On the other hand, consumers cannot choose between a mozzarella produced from fresh milk and one produced from semi-manufactured materials, as the EU regulation no. 1107/1996 does not allow the use of semi-finished products. This undoubtedly creates unfair competition for conventional producers; therefore, dairy operators are increasingly interested in finding solutions to safeguard product typicality. An important phenomenon that occurs during cheese manufacture is proteolysis, which is due to the action of endogenous milk enzymes as well as that of rennet and microbial enzymes. In fresh cheese, such as mozzarella, mainly primary proteolysis is expected. Therefore, enzymatic protein fragmentation can be an effective way to control the preservation state of dairy products, because product history can be reconstructed from proteolysis levels and the formation of specific fragments (Fox, 1989; Mc Sweeney et al., 1993; Gaiaschi et al., 2000). Proteolysis in cheese (Fox and McSweeney, 1996) is produced by endo- and exopeptidases of different origins: plasmin, which is derived from milk; pepsin and chymosin, which are derived from rennet; as well as indigenous and

starter microflora enzymes. Plasmin is active on all caseins, but especially on β-CN (Eigel et al., 1984; Fox et al., 1994) and α_{s2}-CN (Le Bars and Gripon, 1989); its primary cleavage sites in β-CN are Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, and Lys_{107}-Glu_{108}, with the formation of the fragments β-CN f29–209 (γ_1-CN), f106–209 (γ_2-CN), and f108–209 (γ_3-CN). γ-Casein fragments have been proposed as markers of Grana Padano cheese ripening (Gaiaschi et al., 2000).

Chymosin is the main enzyme responsible for the primary proteolysis in most cheese varieties. This aspartyl protease is contained in the rennet together with pepsin; the main target of chymosin is peptide bond Phe_{105}-Met_{106} of the κ-CN fraction, cleavage of which determines milk coagulation and curd formation. Chymosin is active also on both α_{s1}-CN and β-CN in solution (Carles and Ribadeau-Dumas, 1984) but in cheese it appears to hydrolyze mainly α_{s1}-CN (Fox, 1989; McSweeney et al., 1993; Mulvihill and McCarthy, 1993; Scherze et al., 1994; Sienkiewicz et al., 1994). The primary site of chymosin action on α_{s1}-CN is Phe_{23}-Phe_{24}, yielding the fragment α_{s1}-I-CN f(24–199) (Hill et al., 1974; Carles and Ribadeau-Dumas, 1985; McSweeney et al., 1993); cleavage of the Phe_{23}-Phe_{24} bond is believed to be responsible for softening of cheese texture (De Jong, 1976; Creamer and Olson, 1982). Faccia et al. (2014) reported that quantification of the fragment α_{s1}-I-CN can be effective for revealing the use of stored curd in high-moisture cow mozzarella because chymosin is denatured by the high temperature (90°C) used during the stretching phases, it results in a low level of α_{s1}-CN hydrolysis (Hayes et al., 2002; Sheehan et al., 2007; Faccia et al., 2014). Chymosin also cleaves α_{s1}-casein at Phe_{28}-Phe_{29}, Leu_{40}-Ser_{41}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160}, Try_{164}-Tyr_{165}, Leu_{11}-Pro_{12}, Phe_{32}-Gly_{33}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143}, and Phe_{179}-Ser_{180}, with the cleavage rate depending on the ionic strength and pH (Mulvihill and Fox, 1979; McSweeney et al., 1993). Finally, starter bacteria enzymes are known to affect secondary proteolysis (Yun et al., 1995; Gagnaire et al., 2004). Primary proteolysis in mozzarella cheese is due to the incomplete thermal inactivation of enzymes

during the stretching process (Richardson, 1983; McGoldrick and Fox, 1999); the production of α_{s1}-I-CN takes place in the earliest stages of production and proceeds at a rate that depends on curd cooking temperature and water activity (Tunick et al. 1993; Yun et al., 1993), on the amount of the residual rennet (Dave et al. 2003), as well as on pH and NaCl concentration (Feeney et al., 2002). Even though the primary proteolysis rate can be affected by several factors, it progresses quite slowly in both low- and high-moisture mozzarella (Kindstedt et al., 1988; Tunick et al., 1993; Faccia et al., 2014). River buffalo milk proteins are analogous to bovine αs- and β-CN. However, caseins from the 2 species are not identical because of small differences in primary structure (Addeo and Mercier 1977; Chikuni et al., 1995; Ferranti et al., 1998; Sukla et al., 2006), which may differently affect the proteolysis of casein fractions (Ganguli et al., 1965; Di Luccia et al., 2009), milk clotting (El-Shibiny and Abd El-Salam, 1977), and proteolytic enzymes from the 2 species. Trieu-Cuot and Addeo (1981) reported the formation of a fragment, named compound B, produced in river buffalo milk by the action of plasmin on β-CN, which was identified by Di Luccia et al. (2009) as a fourth fragment originating from the presence of a plasminsensitive Lys bond at position 68 that is not present in bovine milk. The presence of β-CN f(68–209), or γ_4-CN, in river buffalo milk was also reported by Somma et al. (2008). Furthermore, Di Luccia et al. (2009) established that this fragment was produced during the first fragmentation of β-CN, unlike what occurs in bovine milk where γ_1 is the first fragment produced, and that γ_4 increases in refrigerated and frozen river buffalo milk and in curd and mozzarella cheese. The long action of rennet involves the production of α_{sl} fragments during storage of mozzarella cheese (Farkye et al., 1991; Di Matteo et al. (1982) estimated that about 55.4% of α_{sl} casein was hydrolyzed to α_{sl}-I and α_{sl}-II peptides. In the same way, semi-finished products contain increased levels of α_{sl}-I and α_{sl}-II peptides in mozzarella cheese (Faccia et al., 2014); such products include pressed curds, which are produced by pressing curd into molds to remove most of the whey (Everard et al.,

2011) to make their transport and storage easier. As far as "Mozzarella di Bufala Campana" is concerned, no recent and sound scientific study to date has specifically assessed proteolysis. In this paper, we provide an overview on casein proteolysis of buffalo mozzarella to study the relationship between proteolysis and quality and to identify molecular markers of product freshness. The analytical techniques used for this work were 1- and 2-dimensional PAGE compined with detection by mass spectrometry and multivariate data analysis.

1.8.3. Materials and methods

1.8.3.1. Reagents and samples

Acetonitrile, formic acid, tris (hydroxymethyl) aminomethane (Tris), HCl, trichloroacetic acid, urea, dithiothreitol (DTT), bromophenol blue sodium salt, SDS, acrylamide/bis-acrylamide 30% solution (37.5% T; 1% C), ammonium bicarbonate phosphate, glycerol, iodoacetamide, acetone, Coomassie Brilliant Blue G (CBB), silver blue, and trypsin singles (Proteomics grade kit) were purchased from Sigma (St. Louis, MO) and used as received. DeStreak Rehydration Solution, immobiline DryStrip Cover Fluid, and bromophenol blue were from GE Healthcare Biosciences (Uppsala, Sweden); rennet with a strength of 1/10,000 was bought at a local market. Sixty samples of fresh PDO buffalo mozzarella, produced in Campania, were kindly furnished by Istituto Zooprofilattico (Portici, Napoli, Italy). Ten samples obtained from Campanian factories producing buffalo mozzarella under specific PDO requirements were used as control.

1.8.3.2 SDS–PAGE analysis

Each sample (0.10 g) was solubilized in 1.0 mL of 9 M urea and an aliquot was added to a sample buffer containing Tris-HCl (0.6 M, pH 6.8), 2% SDS, 10% (vol/vol) glycerol, and 1.5% (wt/vol) DTT to obtain a final protein concentration of 4 mg/mL. Then, 10 μL of this

solution was loaded on a 15% polyacrylamide pore gel (1.5 × 18 × 16 mm), and electrophoresis wascarried out in a Hoefer SE 600 Series Ruby Standard Vertical Electrophoresis Unit (GE Healthcare BioSciences, Little Chalfont, UK), at 25 mA for 3 h at room temperature. The gels were stained with 0.25% (wt/ vol) CBB overnight (Candiano et al., 2004) and destained with water. Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used as protein molecular mass markers.

1.8.3.3. Alkaline PAGE in the presence of urea

Each sample (0.10 g) was dissolved in 1.0 mL of 9 M urea. An accurate volume of solution (100 μL) was mixed with 100 μL of urea sample buffer (Tris-HCl, urea, traces of bromophenol blue sodium salt) containing 2% DTT. Duplicate samples (7 μL) were loaded onto an 8–4% polyacrylamide gel (1.5 × 18 × 16mm) in the presence of urea, using a Hoefer SE 600 Series Ruby Standard Vertical Electrophoresis Unit (GE Healthcare BioSciences). Urea-PAGE analysis was conducted as reported by Andrews (1983) and the gels were stained with CBB and destained in water. Image analysis was carried out using Quantity One software (Bio-Rad); the optical densities obtained were expressed as relativequantity compared with the total area of the identified bands in the electrophoretic pattern of each sample.

1.8.3.4. Two dimensional gel electrophoresis

Each sample (0.10 g) was mixed with 1.0 mL of 9 M urea, and a 100 μL aliquot was dissolved in 150 μL of DeStreak Rehydration Solution and 100 mM DTT. The sample was hydrated with a 13-cm pH 3–10 immobilized pH gradient (IPG) strip (Amersham Biosciences, Melbourne, VIC, Australia) for a minimum of 8 h at room temperature. The first dimension was provided using an Ettan IPGphor 3 (GE Healthcare BioSciences)

electrophoresis unit (Amersham Biosciences) in 4 steps: 500 V/h followed by 1.000 V/h, a gradient to 8.000 V over 90 min, and 8.000 V for 3 h. After the IPG electrophoresis, the strips were equilibrated, first in a solution containing 1.5 M Tris-HCl, pH 6.8, 6 M urea, 30% glycerol (wt/vol), 2% SDS, 64 mM DTT, and traces of bromophenol blue, for 15 min, and then, in a solution containing 1.5 M Tris-HCl, pH 6.8, 6 M urea, 30% glycerol (wt/vol), 2% SDS, 135 mM iodoacetamide, and traces of bromophenol blue, for a further 15 min. The equilibrated IPG dry strips were loaded on top of the 8–18% SDS-PAGE gradient polyacrylamide gel (1.5 × 18 × 16mm), embedded with 1% agarose. The electrophoresis was carried out in an Hoefer SE 600 Series Ruby Standard Vertical Electrophoresis Unit (GE Healthcare BioSciences) at a constant voltage of 100 V, at 30 mA/gel, for 16 h at 15°C. Gels were stained with 0.25% (wt/vol) CBB overnight and destained in water. Images were captured on an ImageMaster Scanner (GE Healthcare BioSciences) in transmission mode, at a resolution of 300 dpi.

1.8.3.5. Proteolysis of buffalo milk α_{s1}-casein by rennet

α_{s1}-Casein, purified according to Di Luccia et al. (2009), was hydrolyzed by using rennet (strength of 1/10,000) and purified α_{s1}-casein at an enzyme:protein ratio of 1/500 (wt/wt) in 50 mM phosphate buffer (pH 6) at 32°C. The final concentration of α_{s1}-casein was 15 mg/mL. Aliquots were taken for analysis at 15, 30, 60, and 120 min, and the reaction was stopped by dilution with a 24% trichloroacetic acid solution. Samples were then centrifuged at 12,000 × g for 10 min at 4°C; the pellet washed 3 times with cold acetone, dissolved in 1.0 mL of 9 M urea, and analyzed by alkaline urea PAGE as described above.

1.8.3.6. In-gel digests

Protein spots from 2-dimensional gel electrophoresis (2D-PAGE) were excised and an in situ digestion was applied. Spots were destained with a 25 mM ammonium bicarbonate: 50% acetonitrile solution, reduced in a solution of 10 mM DTT: 25 mM ammonium bicarbonate for 1 h at 56°C, and alkylated by carboxymethylation in a 55 mM iodoacetammide and 25 mM ammonium bicarbonate solution for 45 min, in the dark. Then, protein spots were washed with a 25 mM ammonium bicarbonate: 50% acetonitrile solution and digested in situ by trypsin at 37°C overnight. The peptides resulting from the digestion step were extracted 3 times with 40 μL of water:acetonitrile: formic acid solution (50:45:5, vol/vol/vol) and then dried by vacuum centrifugation. Finally, the peptide mixtures were dissolved in water: acetonitrile (50:50, vol/vol).

1.8.3.7. Liquid Chromatography/Electrospray Ionization – Tandem Mass Spectrometry

The HPLC-MS system adopted in this work consisted of an HPLC 1200 Series system (Agilent Technologies, Palo Alto, CA), equipped with a vacuum degasser (G1322A, Agilent), an autosampler (G1377A, Agilent), a quaternary pump, and a thermostatically controlled column compartment. The chromatographic separation was obtained with a Zorbax SB-C18 column (2.1 × 100 mm i.d., 1.8 m packing; Agilent) protected by a guard cartridge of the same packing, and maintained at 25°C. The HPLC device was connected online to a MicrOTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source (ESI). The injection volume of the samples was 15 μL. The mobile phase, consisting of water with formic acid (0.1%; A) and acetonitrile with formic acid (0.1%; B), was pumped at 0.2 mL/ min into the HPLC system with the following gradient elution program: 0–3 min, isocratic 5% B; 3–33 min, linear from 5 to 50% B; 33–38 min, isocratic 50% B; 20–21 min, linear from 95 to 5% B; 21–25 min, isocratic 5% B,

followed by column washing and re-equilibration The time-of-flight (TOF) detector, used for accurate mass measurements, operated in positive mode (nebulizer gas, nitrogen, 1 bar; dry gas, nitrogen, 6.5 L/min, 200°C; endplate offset 500 V; capillary voltage 4.5 kV). External calibrations were made using a 100 L KD Scientific (Holliston, MA) syringe pump with a reference solution made up of 10 μL of formic acid (98%), 10 μL of aqueous sodium hydroxide (1.0 M), 490 μL of isopropanol and 490 μL of deionized water. The rawfile data were collected as continuum mass spectrum at a regular time interval. Typically, 2 runs were performed for the HPLC-ESI-MS analysis of each sample. First, an MS full-scan acquisition (spectral rate of 1 spectrum/s with rolling averages of 3, m/z range of 100–2,000) was performed to obtain preliminary information on the predominant m/z ratios observed during the elution. Then, the mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition selecting the 3 most abundant precursor ions. The tandem MS data were deconvoluted and deisotoped and exported in a generic Mascot format before database searching. Tandem MS search parameters had a mass accuracy of 0.025 Da and carboxymethyl as constant modification.

Proteins were tentatively identified by peptide mass fingerprinting by mass searches in the database Swiss Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland) or by fragmentation of their parent ions in MS/MS mode followed by mass searches in the Mascot database (Matrix Science, Boston, MA).

1.8.3.8. Statistical analysis

The relative intensities of urea-PAGE bands were subjected to principal component analysis (PCA), excluding after a first screening all variables with scores <0.5. The data were analyzed with Statistica software package (version 7, StatSoft, Tulsa, OK).

1.9. Results and discussion

Proteolysis was investigated in 60 samples of PDO buffalo mozzarella cheese to highlight possible situations of fraud for consumers. As control, we considered 10 samples of PDO buffalo mozzarella cheese manufactured in controlled conditions. Information from SDSPAGE, 2D-PAGE, urea-PAGE, and MS were combined to obtain insight into proteolysis phenomena.

1.9.1. Screening of protein profile of PDO mozzarella samples by SDS-PAGE

Results of SDS-PAGE patterns that are representative of caseins from unknown PDO samples (lanes S1 to S5) and control (lane C1) mozzarella samples are shown in Figure 1. The estimated molecular weight (MW) of bands was calculated by using a Precision Plus Protein Dual Xtra Standard (Bio-Rad). Although the separation of β-CN and αs-CN fractions of river buffalo casein was very poor by SDS-PAGE, the bands from proteolysis fragmentation of parent caseins were well distinguished. Profiles of samples S1 and S3 were similar to those of the control, showing the expected presence of intense bands with estimated MW of 30–33 kDa ascribable to β-CN and αs-CN fractions, a low-intensity band at 15 kDa, ascribable to β-CN f(69–209), named γ_4 according to Di Luccia et al. (2009), and a band at 13 kDa corresponding to para-κ-CN. Unlike S3, samples S2, S4, and S5 showed numerous intense protein bands at low MW (<25 kDa), which suggests secondary proteolytic activity. In particular, S2 showed a strong reduction of β-CN as well as the presence of low MW bands of high intensity and an additional intense band at an estimated MW of 17.5 kDa. This band could be due to the fragment α_{s1}-I-CN f(35–199), which appears to be a proteolytic product obtained by plasmin digestion of α_{s1}-CN, identified by Gaiaschi et al. (2000) as αb. However, the co-migration of a β-CN fragment cannot be ruled out. The S4 lane also showed a strong reduction of β-CN but with lessintense bands at MW <25 kDa, suggesting a different

proteolytic activity. It is interesting to note the disappearance of γ_1 and γ_4 and concomitant increase of γ_2 and γ_3 bands in S2 and S4, implying the occurrence of further proteolysis on γ_1 and γ_4 fragments. Finally, the protein pattern of S5 showed more β-CN than those of S2 and S4, even though it showed the presence of γ_4 together with the presence of low MW bands, as also seen in S2, suggesting a different level of enzymatic activity. Because it is possible to study only primary proteolysis by conventional monodimensional electrophoresis, our results on samples S2, S4, and S5 showed differences in migration and intensity of bands, suggesting the occurrence of a primary proteolysis activity at a different level. Thus, we submitted the most hydrolyzed sample, S2, and the least hydrolyzed sample, S1, to further 2DPAGE and MS investigations.

1.9.2. 2D-PAGE and MS investigation

Separations by 2D-PAGE of S1 and S2 caseins are shown in Figures 2A and 2B, respectively. For S2, 29 protein spots could be detected compared with the 8 spots revealed for S1. For identification purposes, all gel spots were cut and digested with trypsin; tentative identifications, reported in Table 1, were achieved by combining information of ESI-quadrupole (Q)-TOF peptide mass fingerprints (MS full-scan data), ESIQ-TOF MS/MS analysis of some peptides, and spot position in terms of MW and isoelectric point (pI). The pI and MW were estimated by 2D-PAGE and by Protein Prospector database (http://prospector.ucsf.edu/prospector/mshome.htm) output when simulating peptide digestion. The latter approach is to be considered reliable if the investigated peptide is thought to have a few phosphorylations or other modifications. We assumed MW estimations herein to be theoretical MW (Table 1). The positions of the major caseins in the 2D-PAGE gels are indicated in Figure 2. Although S1 showed only a few spots easily ascribable to α_{s1}-, α_{s2}-, β-, and para-κ-CN based on the literature and MS information, 2D-PAGE of S2 exhibited several

spots at lower MW. Moreover, it is noteworthy that the α_{s2}-CN fractions in the S2 2D-PAGE map exhibited a higher content compared with α_{s1}-CN and, in agreement with the monodimensional SDS-PAGE, a marked diminution of β-CN fraction.

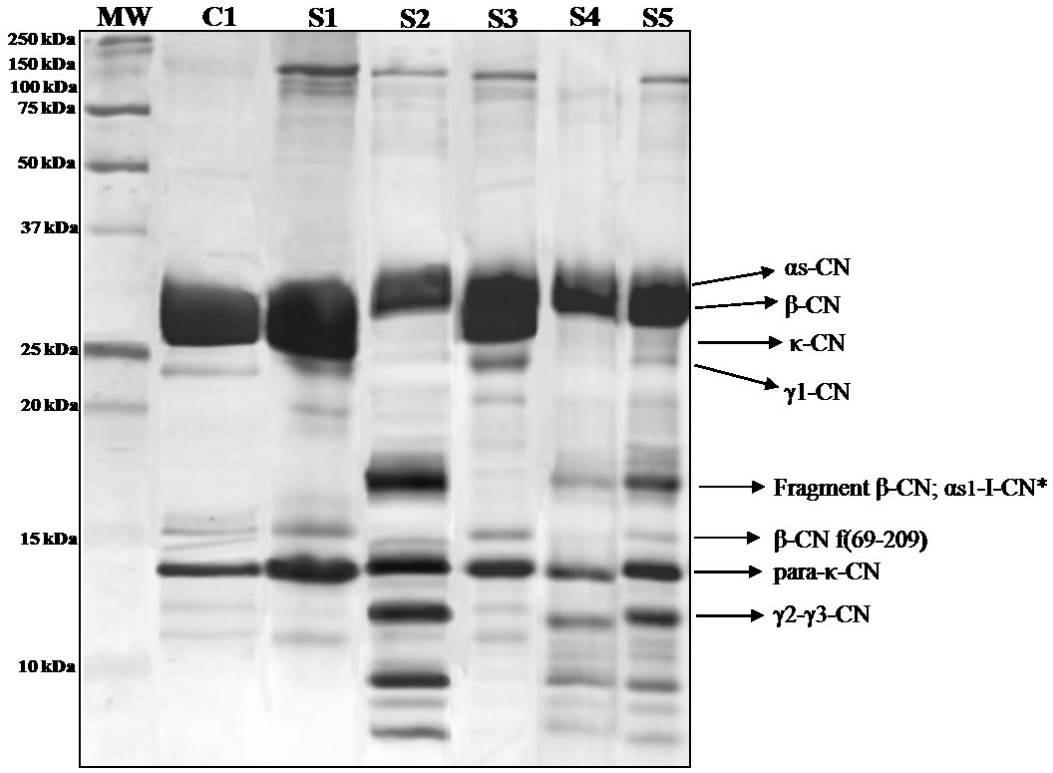

Figure 1. Sodium dodecyl sulfate-PAGE pattern of Protected Designation of Origin (PDO) buffalo mozzarella caseins. Lane MW = molecular mass standard; lane C1 = fresh PDO buffalo mozzarella caseins; lanes S1 to S5 = caseins of samples with different levels of proteolysis. Asterisk (*) indicates tentative attribution based on the literature.

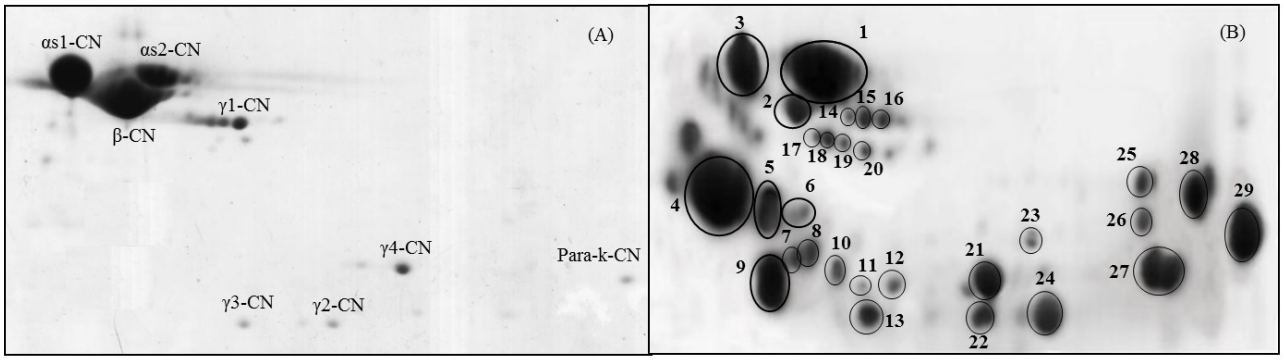

Figure 2. Two-dimensional PAGE of caseins from fresh Protected Designation of Origin buffalo mozzarella (A) and from the highly proteolysed sample 5 (B). Identification of the bands is reported in Table 1.

This could indicate intense proteolysis due to plasmin during inappropriate storage of mozzarella cheese or due to the use of poor quality, semi-finished products. Based on MS results, spots 4 to 13 were ascribed to α_{s1}-CN fragments. In particular, spot 4 was assigned to α_{s1}-I-CN f(24–199), as that was the most intense band and, as expected, its estimated pI and MW were, respectively, more acidic and lower than that of α_{s1}-CN. In analogy to bovine caseins, the α_{s1}-I-CN fragment appears to be the main proteolytic product obtained by rennet digestion of the parent casein fraction (Mulvihill and Fox, 1979; McSweeney et al., 1993). In cheesemaking, production of this large peptide has been closely associated with chymosin: in bovine mozzarella cheese, its formation is reported to occur at the earliest stage of production and to proceed at a rate that depends on cooking temperature and water activity (Tunick et al. 1993; Yun et al., 1993), amount of residual rennet (Dave et al. 2003), pH and NaCl concentration (Feeney et al., 2002). Faccia et al. (2014) found that α_{s1}-I-CN appeared at a low level in fresh milk and increased with inappropriate conservation of milk. The formation of this fragment in the milk could be due to enzymes that recognize the same cleavage sites as chymosin, such as enzymes of psychrotrophic bacteria (Barry, 1979; Law et al., 1979) and cathepsin D. Enzymes from psychrotrophic bacteria represent a major spoilage factor ofstored milk before processing (Sorhaug and Stepaniak 1997), and cathepsin D, an endogenous aspartic protease in milk, appears correlated with somatic cell count (Hurley et al. 2000). Other α_{s1}-CN fragments were found, suggesting an intense proteolytic activity likely related to the different ages of the curds used for production of the analyzed samples: the greater the age of the curd, the greater the presence of products of proteolysis, as has also been reported for bovine curds (Faccia et al., 2014). Spot 5 matched well with α_{s1}-CN f(35–199), identified by Gaiaschi et al. (2000) as αb, and generated by plasmin. Moreover, the co-migration in the second dimension (SDS-PAGE) of spot 5 with spot 26, ascribed to β-CN fragments, supports our previous suggestion of co-migration of α_{s1}-CN and β-CN fragments. Spot 9 matched well

with α_{S1}-CN f(80–199), reported by Gaiaschi et al. (2000) as αc, and generated by plasmin; spot 21 was ascribed to β-CN f(78–209) with a theoretical pI and MW of 7.03 and 14.677; and spot 27, based on pI and MW, might be β-CN f(67–209) with a theoretical pI and MW of 8.57 and 14.392, and originating from the primary action of milk and bacterial acid proteinase (Kaminogawa et al., 1980; Juillard et al., 1995). Spots 14 to 20 were attributed to fragments of α_{s2}- CN with a lower level of phosphorylation, being the fraction most subjected to phosphorilation during milk synthesis (Swaisgood, 1992; Mamone et al., 2003). Spot 29 was attributed to para-κ-CN, κ-CN f(1–105), (pI 9.3, MW 12,366), based on a combination of MS and 2D PAGE data (pI 9.24, MW 14,500). By 2D-PAGE and monodimensional SDS-PAGE (see Figure 1), the MW of para-κ-CN was somewhat different from expected, probably because of an interaction of the protein with SDS. Moreover, the recognition of only 2 peptides belonging to κ-CN suggests that spot 29 could represent more than one protein. Finally, spots 21 to 28 were attributed to fragments of β-CN, originating from plasmin activity, which continues during cold storage and survives the high temperature treatment during the processing of dairy products (Richardson, 1983b). The action of plasmin on lysine residues is well documented (Sherry et al., 1966), and it has been shown to produce γ_1 f(29–209), γ_2 f(106–209), γ_3 f(108–209), and γ_4 f(69–209) (Eigel,1977; Trieu-Cuot and Addeo, 1981).

Table 1. Summary of spots and MS information obtained by HPLC-MS and HPLC-MS/MS.

Spot number	2D pI/MW estimated[1]	PProspector pI/MWth[2]	Number of peptides matchig protein[3]	peptides matching protein[3]	Coverage of the matching protein[4] (%)	Number of matched peptides MS/MS[5]	Accession number and name[6]
1	5.09/23500	6.4/24179	13	71-76;81-91;115-125;126-136; 126-137; 137-149; 138-149; 138-150;153-	42.2	5 (1)	4584554 BUBALUS BUBALIS aS2-casein

				158;161-165; 174-181;182-188;198-205			
2	4.84/22500	5.1/23583	5	29-32;100-107;170-176;177-183;203-209	15.7	4 (4)	Q9TSI0 O62824 CASB BUBBU
3	4.29/23000	4.8/22773	9	37-58;80-83;84-90;91-100;91-102;104-124;125-132;133-151;194-199	49.5	5 (1)	O62823 CASA1_B UBBU
4	**4/20000**	**4.5/20108**	**8**	**80-83; 84-90;91-100;103-124;104-124;125-132;133-151;194-199**	**43.2**	**3 (1)**	**α_{s1}-I-CN f(24-199)/O62823 CASA1_B UBBU**
5	4.99/18900	4.8/24369	3	91-100;104-124;133-151	24.5	3 (3)	O62823 CASA1_B UBBU
6	4.89/18500	4.8/24369	2	84-90;91-100	8.5	-	O62823 CASA1_B UBBU
7	4.79/14800	4.8/24369	7	59-79;84-90;91-100;104-124;125-132;133-151;194-199	46.0	2 (2)	O62823 CASA1_B UBBU
8	4.98/14900	4.8/24369	8	59-79;80-83;84-90;91-100;104-124;125-132;133-151;194-199	48.0	3 (3)	O62823 CASA1_B UBBU
9	4.52/13500	4.8/24369	4	91-100; 125-132;133-151;194-199	21.5	2(2)	O62823 CASA1_B UBBU
10	5.24/13800	4.8/24369	2	91-100;104-124	15.5	-	O62823 CASA1_B UBBU
11	5.44/13500	4.8/24369	2	84-90;91-100	8.5	-	O62823 CASA1_B UBBU
12	5.79/13700	4.8/24369	1	91-100	5.0	-	O62823 CASA1_B

							UBBU
13	5.50/11700	4.8/24369	1	91-100	5.0	-	O62823 CASA1_B UBBU
14	5.34/22300	6.4/24800	8	71-76; 71-80; 81-91;92-113;115-125; 174-181; 182-188; 198-205	37.9	2(2)	4584554 BUBALUS BUBALIS aS2-casein
15	5.44/22300	7.2/24800	9	71-76; 81-91; 92-113;115-125; 153-158; 167-173; 174-181; 182-188; 198-205	41.7	2(2)	4584554 BUBALUS BUBALIS aS2-casein
16	5.69/22200	7.2/24800	11	71-76; 81-91; 92-113; 115-125; 126-136; 153-158; 161-165;167-173; 174-181; 182-188; 198-205	50.5	2(2)	4584554 BUBALUS BUBALIS aS2-casein
17	5.00/21500	7.2/24800	7	71-76; 81-91; 92-113;115-125; 153-158; 174-181; 182-188	34.5	2(2)	4584554 BUBALUS BUBALIS aS2-casein
18	5.14/21500	7.2/24800	11	71-76; 71-80; 81-91; 92-113; 92-114; 115-125; 153-158; 167-173; 174-181; 182-188; 198-205	44.2	3(1)	4584554 BUBALUS BUBALIS aS2-casein
19	5.29/21500	7.2/24800	14	71-76; 71-80; 81-91; 92-113; 114-125; 115-125; 126-136; 138-149; 153-158; 153-160; 167-173; 174-	56.3	2(2)	4584554 BUBALUS BUBALIS aS2-casein

				181; 182-188; 198-205			
20	5.45/21300	7.2/24800	8	71-76; 81-91; 92-113; 115-125; 153-158; 174-181; 182-188; 198-205	38.8	2(2)	4584554 BUBALUS BUBALIS aS2-casein
21	6.74/13500	5.3/25107	5	98-105;170-176;177-183;184-202;203-209	22.8	2(2)	CASB BUBBU
22	**6.73/11700**	**5.5/11519**	**3**	**170-176;177-183;203-209**	**20.6**	**2(2)**	**f(108-209)CASB BUBBU**
23	**7.14/14000**	**6.3/12412**	**3**	**170-176;177-183;203-209**	**18.9**	**2(2)**	**f(98-209)/CASB BUBBU**
24	**7.29/12000**	**6.3/11784**	**4**	**170-176;177-183;184-202;203-209**	**38.5**	**2(2)**	**f(106-209)/CASB BUBBU**
25	8.24/20500	5.3/25107	3	170-183;184-202;203-209	19.0	1(1)	CASB BUBBU
26	**8.24/14500**	**6.8/15749**	**3**	**170-176;177-183; 203-209**	**14.9**	**2(2)**	**f(69-209)/CASB BUBBU**
27	**8.44/14000**	**8.57/14392**	**4**	**98-105;170-176;177-183; 203-209**	**25.7**	**3(2)**	**f(67-209)/CASB BUBBU**
28	**8.79/19000**	**8.2/17968**	**2**	**184-202; 203-209**	**16.1**	**1(1)**	**f(49-209)/CASB BUBBU**
29	**9.24/14500**	**9.3/12366**	**2**	**11-16;69-86**	**22.6**	**-**	**para-k-CN f(1-105)/P11840 CASK_BUBBU**

[1] Apparent isolelectric point (pI) and molecular weight (MW) values estimated from 2D-PAGE. The pI values correspond to the middle of the spots.
[2] pI and MW as estimated by Mascot/Protein Prospector databases; in bold tentative identifications, pI and MW as estimated by Protein Prospector database output when simulating the peptide digestion.
[3] number and peptides matching protein as resulted by Protein Prospector search with MS full scan data as entry
[4] calculated as number of matched aminoacids/total aminoacids
[5] number and peptides matching protein as resulted by Mascot search with MS/MS data as entry, in the brackets unique peptides
[6] SwissProt database; for α_{S2}-CN, NCBInr.2013.6.17 database

Thus, the majority of β-CN spots were tentatively attributed by simulating the cleavage of each Lys residue in β-CN and matching information of theoretical pI and MW with 2D-PAGE

data. Spot 28 was assigned to f(49–209) (pI 8.2, MW 17,968), spot 27 to β-CN f(67–209) (pI 8.57, MW 16,184), spot 26 to γ_4 f(69–209) (pI 6.8, MW 15,749), spot 23 to f(98–209) (pI 6.3, MW 12,412), spot 24 to γ_2 f(106–209) (pI 6.3, MW 11,784), and spot 22 to γ_3 f(108–209) (pI 5.5, MW 11,519). The fragments relevant to spots 21 and 25 could not be identified; due to chymosin action, other cleavage sites could be hypothesized. Based on the above considerations, it was evident that sample S2 was severely proteolyzed; therefore, bands present in SDS-PAGE (Figure 1) were likely composed of more fragments coeluting together.

1.9.3. Alkaline Urea-PAGE analysis

All samples were also analyzed by alkaline urea- PAGE, the most widely used electrophoretic technique to investigate primary proteolysis in cheese and which is able to reveal several large casein fragments (Ledford et al., 1966; Fox, 1989); in Figure 3, results from unknown PDO (lanes S1 to S5) and control (lane C1) mozzarella samples are shown. Sample C1 exhibited β-CN, α_{s2}- CN, and α_{s1}-CN bands, faint bands of similar intensity in the area of γ-CN, and no further band corresponding to peptides with higher negative net charge. α_{s1}-Casein showed 2 major bands due to its heterogeneity for different degrees of phosphorylation and we named these α_{s1}-CN^{6P} and α_{s1}-CN^{7P} (Figure 3) according to the findings of Chianese et al. (2009). Samples S2 and S5 showed more intense and numerous bands at electrophoretic mobility of α_{s1} fragments and γ-CN with the exception of γ_4 and γ_1, which were reduced or disappeared, in agreement with MS data. The 2 major bands with the highest electrophoretic mobility, together with a reduction of β-CN and α_{s1}-CN intensities, suggest the presence of intense primary proteolysis. Furthermore, it is worth noting the presence of an intense band that migrated between β-CN and α_{s1}-CN fractions. This band could correspond to co-migrating fragments from β- and α_{s1}-CN as deduced from pI of the 2D-PAGE map and identified by MS as spots 11, 12, 13, and 21 (Table 1).

The bands with the highest mobility, in analogy to cow mozzarella, could be similar to ALMI that was identified as α_{s1}-I-CN (Faccia et al., 2014). However, the river buffalo α_{s1}-I-CN presented 2 bands that we named α_{s1}-I^{6P} and α_{s1}-I^{7P} based on their respective electrophoretic mobility deriving from parent protein (Figure 3). These could be derived by fragmentation of parent casein with different phosphorylation degree as shown by their heterogeneity (Figure 3). Bovine α_{s1}- I-CN presented one band (Faccia et al., 2014), which increased very slowly in low-moisture mozzarella, and even though it could be detected in significant amounts after several week of ripening, it is present only in small amounts in fresh cheese (Sheehan et al., 2004; Costabel et al., 2007). Bovine α_{s1}-I-CN is even less abundant in high-moisture cheese, and is barely detectable in cheeses aged for only a few days (Di Matteo et al., 1982; Baruzzi et al., 2012; Faccia et al., 2014). Unlike in fresh cow mozzarella, no α_{s1}-CN fragment was detected in fresh buffalo mozzarella samples, suggesting that chymosin action is slower (El-Shibiny and El-Salam, 1977; Gaiaschi et al., 2000; Bonfatti et al., 2013). Therefore, the α_{s1}-I-CN fragments were detectable only in mozzarella cheese when the proteolysis occurred at much higher level, indicating poor milk preservation or the use of semi-manufactured curd; the intensity among the samples was different, probably due to the age andmanufacturing or storage conditions. Principal component analysis (Figure 4) was carried out, with the relative intensities of urea-PAGE bands relevant to all samples as variables (scores >0.5). Principal components (PC) 1 and PC2 accounted for 53.61 and 17.72% of total variance, respectively. On PC1, PCA discriminated samples according to proteolysis phenomena: samples with a low abundance of proteolysis products (group 1) showed negative high scores in the first component, whereas proteolyzed samples (group 2) had positive PC1 scores (Figure 4A). It is interesting to note that group 2 was not as homogeneous as group 1, although it was well separated from group 1, likely reflecting the numerous causes of proteolysis. The most discriminant variables were the main caseins αS1-CN and β-CN, which showed high scores

for group 1, and α_{s1}-I-CN, γ-CN, and other β-CN fragments (fβ-CN) for group 2 (Figure 4B). Principal component 2 seems to discriminate samples according to different proteolysis products, especially within group 2. Results suggests that the presence of fragments α_{s1}-I-CN, γ-CN, and fβ-CN could be markers of "poor production practice" for PDO buffalo mozzarella, as all control samples had a reduced amount or complete lack of these fragments.

1.9.4. Proteolysis of buffalo milk α_{s1}-casein by rennet

To assess whether the α_{s1}-I-CN fragments originated from α_{s1}-casein, as a consequence of rennet activity, we carried out an in vitro hydrolysis of purified α_{s1}-casein. The kinetics reaction showed that α_{s1}-casein decreased with time and disappeared after 30 min (Figure 5).

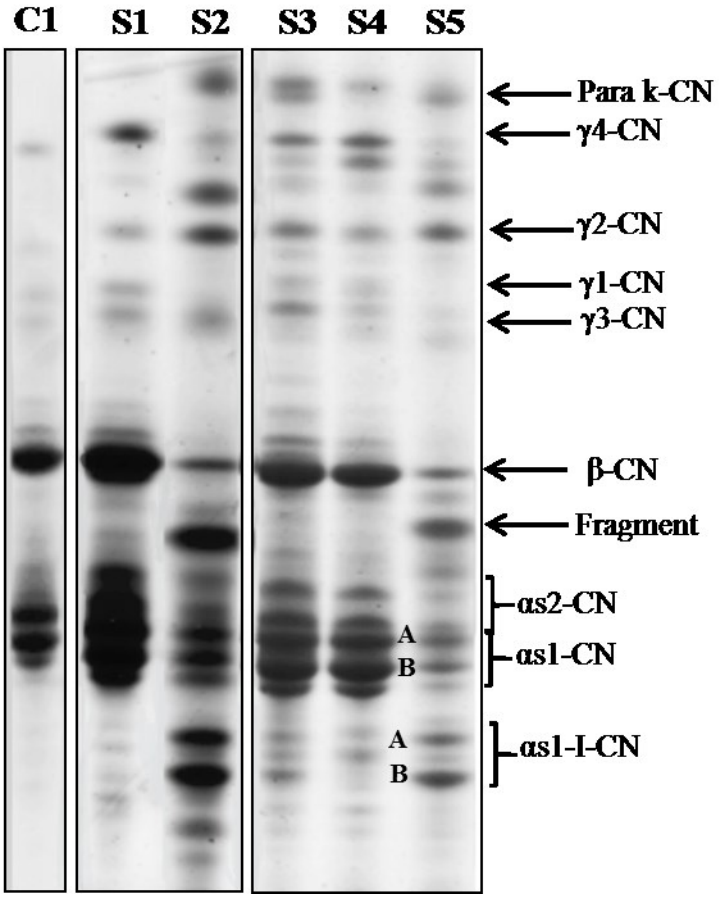

Figure 3. Urea-PAGE pattern of Protected Designation of Origin (PDO) buffalo mozzarella caseins. Lane C1 = fresh PDO buffalo mozzarella caseins; lanes S1 to S5 = caseins of samples S3 to S6 with different levels of proteolysis.

with time and disappeared after 30 min (Figure 5). The α_{s1}-I^{6P} and α_{s1}-I^{7P} fragments were already formed by 15 min (lane 2 in Figure 5) and decreased with increasing time of rennet action, up to 2 h, when they almost completely disappeared, whereas fragments of lower MW continued to increase. Results confirmed that the α_{s1}-I^{6P} and α_{s1}-I^{7P} fragments were derived from chymosin activity on α_{S1}-casein and they were attributable to α_{s1}-I (α_{s1} fragment 24–199). In analogy to bovine casein proteolysis, the other faster bands were sequentially formed by rennet action at pH 6.0; they were designated α_{s1}-II^{6P}, α_{s1}-II^{7P}, α_{s1}-III^{6P}, and α_{s1}-III^{7P} and attributed to the fragments α_{s1}-CN f(24/25–169) and α_{s1}-CN f(24/25–149/150), respectively (Mulvihill and Fox, 1979). However, our observations in mozzarella cheese proteolysis were limited mainly to α_{s1}-I^{6P} and α_{s1}-I^{7P} due to denaturation of chymosin during the stretching phase at about 90°C (Hayes et al., 2002; Sheehan et al., 2007; Faccia et al., 2014).

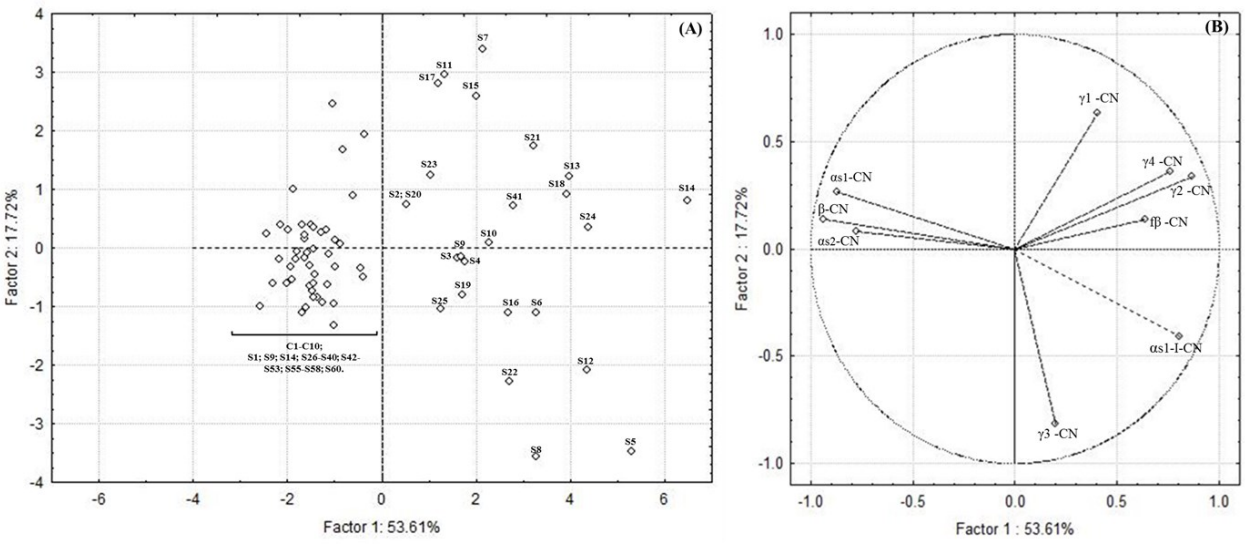

Figure 4. Principal component analysis biplots of urea-PAGE bands of 60 Protected Designation of Origin buffalo mozzarella samples (S) and 10 controls (C).

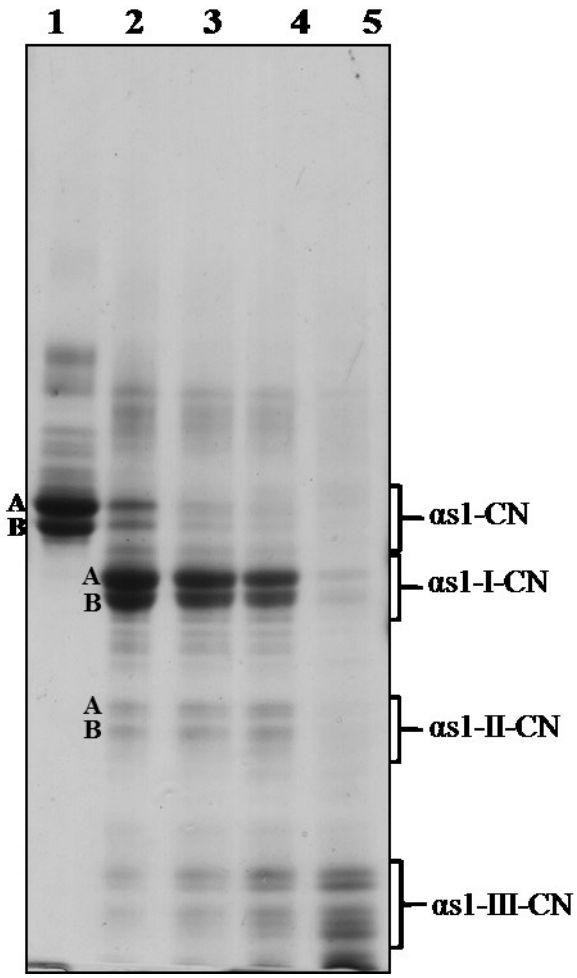

Figure 5. Urea-PAGE of buffalo α_{s1}-casein hydrolyzed in vitro by the use of rennet. Lane 1 = pure α_{s1}-CN of buffalo milk; lane 2 = α_{s1}- CN of buffalo milk hydrolyzed by the use of rennet for 15 min; lane 3 = α_{s1}-CN of buffalo milk hydrolyzed by the use of rennet for 30 min; lane 4 = α_{s1}-CN of buffalo milk hydrolyzed by the use of rennet for 60 min; lane 5 = α_{s1}-CN of buffalo milk hydrolyzed for 120 min.

1.10. Conclusion

This survey of river buffalo mozzarella cheeses by SDS-PAGE and urea-PAGE showed unusual hydrolysis. The proteolysis in this cheese generated a numbers of fragments identified by MS as derived from β- and α_{s1}-CN. The presence of many of the fragments indicated that proteolysis in river buffalo mozzarella cheese occurred at different levels due to endogenous and exogenous enzymes during cheesemaking and storage. The proteolysis occurred at 2 different stages: (1) plasmin action progressively increased during long storage at room or low temperatures with increasing amounts of γ-CN and concomitant reduction of β-CN; and (2) chymosin action increases α_{s1}-I-CN fragment in the curd until stretching,

whereas it is slow or absent in cheese from stretching until consumption. Under normal conditions, the formation of the α_{s1}-I-CN fragment is not detectable. Indeed, the intensity of the β- and α_{s1}-CN fragments in the finished product mainly depends on the storage and kind of raw matter. The proteomics approach we used allowed us to identify casein fragments to use as potential molecular markers. Finally, we can assert that SDS-PAGE revealed fragments from plasmin activity and its proteolysis rate, and alkaline urea- PAGE is better able to reveal fragments from chymosin action to use in the case of stored or pressed curd, a material usually imported as semi-finished product from other countries. The proposed methods can be useful to verify qualitatively the freshness of raw materials and conformity to PDO buffalo mozzarella, as is often required by the official protocol of production.

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Publication

Study of proteolysis in river buffalo mozzarella cheese by a proteomic approach

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1.12. Determination of a freshness index for Buffalo Mozzarella cheese by electrophoretic study: preliminary results

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1.12.1. Abstract

To find a possible freshness index for buffalo mozzarella cheese were analysed 75 samples from 5 different dairies, 3 located in the province of Caserta and 2 in the province of Salerno. Three fresh mozzarella cheeses were also prepared as a electrophoretic reference to establish a range of normal quantitative values. The results obtained showed that the fragments mostly involved in the state of freshness of mozzarella cheese derived from the action of plasmin, that generates the γ-caseins from β-casein. Comparing the measure of the intensity of the bands of the γ-caseins to the intensity of the parent protein, the β-casein, it was possible to calculate an index of freshness called Φ. The higher value of Φ was, the greater was the freshness of the mozzarella. It needs to increase the number of observations such that they are representative of production in the various areas included in the DOP displinary, and to establish, a range of values that indicate with certainty the freshness of the product.

1.12.2. Introduction

One of the major sensory attributes requested by the consumer for cheeses with a short shelf-life is the freshness. For this reason it is necessary to use in the production of these cheeses, and in particular for the mozzarella cheese, fresh milk and the appropriate storage conditions .

Mozzarella di Bufala Campana, valuing the typical production of buffalo milk for its chemical and physical properties, in June 12, 1996 has gained recognition of the DOP due to the reg. Ce 1107/96 on the basis of a product specification. Article 3 of the specification states that the "Mozzarella di Bufala Campana" must be produced exclusively with whole buffalo milk fresh. The process involves the use of raw milk, thermised or pasteurized if necessary, from buffalo reared in the production areas listed in art. 2. However, today the competition and the economic crisis push some manufacturers to use together milk, does not always fresh, semi-finished products to reduce costs of production and selling mozzarella at competitive prices. Added to this the increasing demand for the product and the necessity to cover distant markets from the production area stimulate producers to find solutions that are not always in line with the specification. These market requirements have become a source of debate in the Consortium of Mozzarella di Bufala Campana to make discipline more elastic especially in the use of raw materials, such as the use of frozen milk or curd. In the absence of analytic methodologies to protect the freshness of the product DOP it becomes difficult to ensure to the consumer a correspondence between those stated on the label and real raw matter used to manufacture mozzarella cheese.

Several studies have shown that the formation of the casein fragments and their content can indicate whether fresh cheeses such as mozzarella, have been produced and / or stored in accordance with good processing technology (Di Matteo et al. 1982; Farkye et al. 1991; Di Luccia et al. 2009; Face et al. 2014). In fact, the presence of endogenous enzymes in milk, mainly plasmin and cathepsin, together with those present in the rennet, chymosin and pepsin, fragment the caseins as increases storage time (Fox et al., 1989; Mc Sweeney et al., 1993; McSweeney & Fox, 1995; Kelly et al. 2006.

In order to fill the gap in the analytical evaluation of the freshness of the mozzarella di Bufala with respect for tradition, the product specification and to ensure the freshness of the cheese

to the consumer it has started a study on the buffalo mozzarella.

1.13. Materials and methods

Sample preparation: The samples of mozzarella, 50 g, were homogenized in a solution of sodium acetate and 1N acetic acid to 10%, centrifuged at 9,500 rpm for 10 min, at 4 ° C and solid surfaced fat milk was removed with a spatula. The precipitate was washed twice with distilled water and centrifuged after each wash. From the obtained residue were taken 100 mg and dissolved in 1 ml of urea 9 M. The solution was centrifuged at 6,000 rpm for 10 min at 10 ° C and subjected to protein assay, using Direct Detect Spectrometer Quick-Start (Millipor, Corporation, Billerica, Ma, USA).

1.13.1. SDS-PAGE

The discontinuous electrophoresis at alkaline pH in the presence of sodium dodecyl sulfate was carried out according to the indications of Laemmli (1970). The caseins have been fractionated in a gel of 15% polyacrylamide T of dimensions 16 × 18 × 0.15 cm. Electrophoresis was performed at 600 V, 25 mA, 10 W and 10 ° C.

Casein fractions were revealed by staining gels with Blue Silver, colloidal Coomassie G-250 (Candiano et al. 2004). The gels were acquired and processed by image analysis with the software Quantity One 1-D (Bio-Rad Laboratories, Richmond, CA, USA).

1.14. Results and discussion

Figure 1 shows some of representative electrophoretic patterns of the casein fraction extracted from 75 samples of bufala Campana mozzarella cheese DOP. The profiles are characterized by a high molecular weight band of approximately 130 kDa that represents the membrane proteins of the fat globule (Conway et al., 2010). The most intense bands represent the

fraction α_s ($\alpha_{s1}+\alpha_{s2}$) and β-CN with estimated molecular weights of 35 and 30 kDa, respectively.

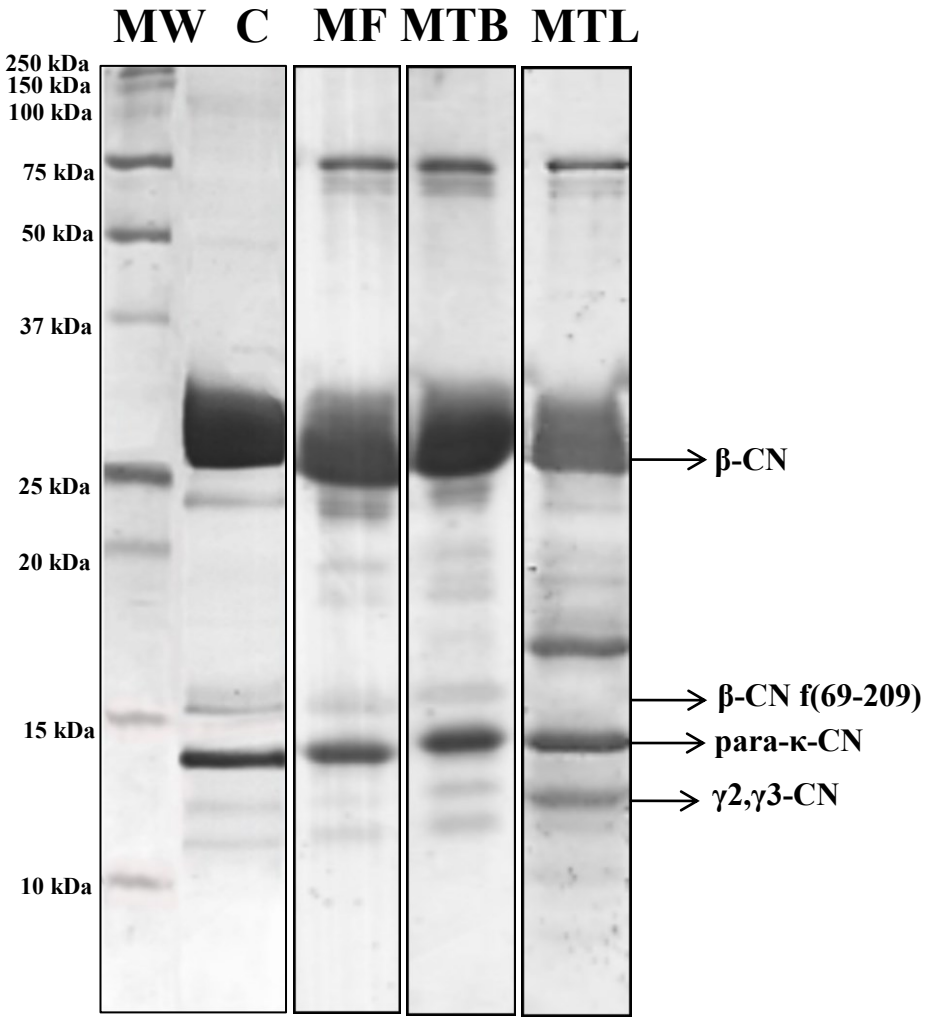

Figure 1: polyacrylamide gel electrophoresis (SDS-PAGE 15%) of buffalo mozzarella caseins. C: control; MF = fresh mozzarella; MTB = mozzarella with short retention; MTL = mozzarella with long times of preservation.

The band at 25 kDa was identified as κ-CN while the band at 20 kDa represents the γ_1-CN, the 16-kDa band is the γ_4-CN, the band at 14 kDa is the para-κ-CN and the band 11 kDa is the mixture of fragments β-CN, γ_2 and γ3. It is noteworthy as the intensity of staining of γ-CN was variable. This has focused our attention on the action of the endogenous enzyme milk, plasmin, and on the measure of the band intensities related to the β-CN and its fragments γ_2, γ_3 and γ_4. The γ_1 was not considered because in the fresh samples is not observed, or is present in negligible amounts, because the first γ to form is the γ_4 (Di Luccia et al. 2009) and not the

γ_1 as occurs in bovine milk where the γ_4 is absent. Table 1 shows the relative amounts of the bands, obtained by image analysis of individual samples loaded on the gels in duplicate. Based on the values of relative intensity of the bands we calculated an freshness index of Bufala Campana mozzarella cheese DOP expressed in % and indicated as (Φ) in the following formula:

$$\frac{\beta\text{-CN}}{\beta\text{-CN}+\gamma 2+\gamma 3+\gamma 4} \times 100 = \text{indice di freschezza} = \Phi$$

our control is represented by the value of the freshness index Φ obtained from measurements of buffalo mozzarella cheese prepared from fresh raw milk, the recorded value was 84.49%. Considering that our survey shows the preliminary results and considering that the minimum value observed is of 48.54% we felt it appropriate to consider, in the field of our observations, a 30% variability. Therefore they were considered fresh samples that fall in the range from 85.24 to 78.05%; samples of mozzarella whose values fall in an a range from 76.49 to 69.08% were deemed little fresh for not properly storage; values less than 36.9% were considered not fresh deriving from doubtful raw material or from mozzarella cheese poorly preserved. On the basis of these considerations were stated fresh 37% of the examined samples of mozzarella, and just fresh 36.9% and not fresh 26.1%. It is to be measured between the latter the presence of samples with values between 68.03 and 48.54% which leaves suppose the a poor preservation of the raw material or of the finished product, mozzarella cheese.

Classification of Buffalo Mozzarella cheese related to **index of freshness**	Minimum value	Maximum value	Φ **(Index of freshness)**	%
Mozzarella Fresh	78,05	85,24	$82,65 \pm 3,24^{A}$	37,0
Mozzarella with short retention	69,08	76,49	$74,05 \pm 2.25^{B}$	36,9
Mozzarella with long times of preservation	48,54	68,03	$60,37 \pm 6.28^{C}$	26,1

A, B, C = $P<0.001$

Table 1: Values of the index fresh mozzarella from the measurements determined by the ratio of the fractions of β-cn and γ-cn.

1.15. Conclusion

The obtained results showed that the fragments mostly involved in the state of freshness of a mozzarella cheese derived from the action of plasmin, namely the γ-caseins. The ratio between the measure of the staining intensity of the γ-caseins bands and the intensity of the parent protein, the β-casein, allowed us to calculate an index of freshness, called Φ. The higher the value of Φ is, the greater is the freshness of mozzarella cheese. On the basis of these considerations were stated fresh 37% of 75 the examined samples of mozzarella, and just fresh 36.9% and not fresh 26.1%,with a 30% variability. To establish unequivocally range of values to ensuring the freshness of the product, it needs to increase the number of samples representative of the production in the various PDO areas.

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1.17. Elisa test to detect beta casein in buffalo milk subjected to different freezing times

1.17.1. Introduction

Traditional southern mozzarella cheese, like Fior di Latte cheese and buffalo Mozzarella cheese have unique characteristics, mainly due to raw materials and production technologies. In the last years, the pressure exerted by the dynamics of the market has resulted in a deep "evolution" of these products. In fact, many small and medium dairies met a competition on product price, orienting them towards the reduction of production costs to regain competitiveness. This led to search technological solutions, which are not always compatible with the typicality of products. In particular, as regards the buffalo mozzarella cheese, the increased demand of these product, the limited availability of the raw material in the summer period, togheter with the increase of economic profits, have made mozzarella cheese a potential target for adulteration (Di Luccia et al., 2009). The use of semi-finished instead of fresh milk for mozzarella cheeses production is one of the technological solutions adopted. This is currently widespread and it has led to a real "parallel industry", namely that of "preserved curds". Curds pressed and chilled (whose shelf life is generally attested up to three months) or frozen are now widely used in the production of mozzarella cheeses. Moreover, the spread of semi-finished product is related to the satisfaction of two aspects:

1) from an economic standpoint, there is the opportunity to purchase these semi-finished abroad at extremely competitive costs;

2) from a technological point of view, all stages inherent the gathering discharge and storage of milk are eliminated, including its coagulation for the curd production.

This can bring to a reduction of production costs over 50%, compared to the traditional process, resulting in unfair competition against traditional producers (Faccia et al., 2011, 2014). There is also the potential risk of importation of frozen curds from geographical areas outside from those defined for the production of DOP mozzarella cheeses (Di Luccia et al.,

2009). This also damages the livestock sector (Faccia et al., 2011, 2014) and it is clear a lack of clarity in regard to the consumer, who is not able to discern and to make an informed choice, between mozzarella cheese produced by fresh milk and mozzarella cheese produced by semi-finished (Di Luccia et al., 2009; Faccia et al., 2011 2014). In fact, the cheeses obtained by semi-finished, if properly stored, mixed with fresh milk or even alone, can achieve quality levels apparently noticeable, so it can easily mislead the consumer (Faccia et al., 2011, 2014). Therefore, it is necessary to protect consumers from misleading actions and preserve the link of DOP products to specific geographical areas (Di Luccia et al., 2009). Currently, for national dairy operators is opened a double challenge:

1) regain the quality and characteristics of traditional products, as a factor of competitiveness "no price";

2) identify solutions to protect the typicality of the products.

However, to protect typical Italian cheeses, it is necessary also to find a way to guarantee the origin, quality and freshness of the raw material, which have to come from farms located in the geographical areas provided by the traditional production disciplinary and/or protocols (Faccia et al., 2011). In light of this, the emerging analytics challenge is to trace the "curd history" in dairy products (Di luccia et al., 2009).

Proteolysis in milk occurs mainly by plasmin action, a blood enzyme (Politis et al., 1992). It is an alkaline serine protease (Eigel, 1977), transported from plasma across mammary epithelial cells. Its activity is determined by interactions between the inactive precursors (plasminogen) and plasminogen activators associated with casein micelles or somatic cells (Politis et al., 1992). Plasmin is concentrated in cheese curd obtained by rennet, while inhibitors of plasmin and the plasminogen activators, remain in the whey (Kelly & McSweeney, 2003). Plasmin activity continues during cold storage, causing changes in the coagulation properties of milk, because plasmin survives to the heat treatment during the

processing of dairy products (Richardson, 1983b). It preferentially hydrolyzes β-CN, α_{s2}-CN, and to a lesser extent α_{s1}-CN (Senoq et al., 2002); instead, k-CN, β-LG, and α-LA are resistant to its action (Kelly and McSweeney, 2003). Plasmin is highly specific for lysine-X type peptide bonds and, to a lesser extent, arginine-X. Indeed, plasmin cleaves all 15 potential sites in the sequence of the β-CN in a recycle reactor. However, there are three bonds that are mainly cleaved in milk and cheese:

- Lys_{28}-Lys_{29},
- Lys_{105}-His_{106},
- Lys_{107}-Glu_{108}.

From these cleavages are released polypeptides, f(106-209) and f(108-209), known as γ_1-CN, γ_2-CN and γ_3-CN, respectively, and their complementary peptides that contribute to the proteose peptone fraction of milk (Eigel, 1977). Ovine and goat milks are different from those vaccines and buffaloes, because in these two types of milks, β-CN is composed of 207 amino acids, with deletion of two residues (namely Pro_{179}-Tyr_{180} or Tyr_{180}-Pro_{181}); consequently, the derived γ-CN are shorter for 2 missing amino acids than their bovine and buffalo counterparts (Roberts et al., 1992). In river buffalo it was observed the formation of fourth γ-CN, fragment of β-CN (29-209), named γ_4-CN (Trieu-Cuot and Addeo, 1981; Di Luccia et al., 2009. Fresh cheese, such as mozzarella cheese, undergoes a primary proteolysis due to action chymosin and plasmin on α_{s1}-CN and β-CN, respectively, it occurs mainly during the storage where the levels of α_{s1}I-CN and γ-CN fragments increase progressively. In this regard, some authors have proposed the use of the γ-CN formation as markers of quality and ripening cheese (Gaiaschi et al. 2000 and 2001; Di Luccia et al., 2009). Although it has been dedicated a considerable efforts to evaluate the progress of plasmin activity in cheese, the analytical techniques able to characterize the proteolytic complex model reached in cured products are limited to monitoring the first stages carried out by plasmin (Di luccia et al., 2009; Senoq et

al, 2002). In recent years, immunoassays offered good prospects for achieving this goal, because they are characterized by high sensitivity and specificity compared to classical biochemical techniques. (Senoq et al., 2002). In fact, to evaluate these phenomena in Swiss-type cheese, Senoq et al. (2002) and Dupont et al. (2003) have produced antibodies against the principal peptide cleavage sites of plasmin in the sequence of β-CN. This approach can also be applied for the quantitative assessment of proteolysis during storage of raw materials (essential for the achievement of dairy products DOP, where it is essential the control of origin of raw material). In particular, the anti-β-CN f (20-39) CEES1 has been specifically designed to recognize the binding of β-CN Lys^{28}-Lys^{29} (Gagliardi et al., 2009; Di Luccia et al., 2009). On the basis of these considerations, we proceeded to prepare an indirect ELISA, using this antibody. Through this analysis, we tried to detect the hydrolysis effected by plasmin in β-CN, on buffalo milk stored for different times at -20 °C. From this investigation, we aims to achieve a protocol able to discriminate between dairy preparations obtained by fresh milk (presumably with high levels of β-CN) and dairy preparations obtained by semi-finished (where we expect levels of β-CN hydrolysis higher than those obtained by fresh milk). This analysis tool would arise as a screening technique for the detection of fraud about fresh cheeses, especially those which have certification of origin or traditional speciality, thus ensuring more effective protection for the consumer and traditional dairy producers Italian.

1.18. Materials and methods

1.18.1. Purification of β-CN

Buffalo raw milk (whole) was collected from a dairy farm located in Campania (Southern Italy). Immediately after collection, it was added the phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO) at a final concentration of 1 mM. Milk was transported to the laboratory at refrigeration temperature. All this was done to limit the proteolysis of β-CN. Once in the

laboratory, milk was skimmed by centrifugation at 4500 x g at 4 °C for 30 min and it was divided into aliquots of 10 ml. Then the total caseins were separated by adding acetic acid to pH 4,6. The pellet thus obtained was stored at -80 °C until its subsequent treatment. Of the total caseins were used 3 g, which have been solubilized in 40 ml of buffer containing 0,2% β-mercaptoethanol. This solution was loaded onto the column of ion-exchange chromatography (IEC) using a SP Sepharose Fast Flow strong cation exchange resin (Amersham Biosciences) packed in a column 26 × 230 mm, as described by Andrews et al. (1985) and Di Luccia et al. (2009). The elution was performed with a gradient from 0,24 to 0,50 M NaCl with a flow rate of 3,0 ml/min. The effluent was monitored by UV detection (280 nm), and the peaks were collected manually, dialyzed, and freeze-dried.

1.19. Treatment of samples

1.19.1. Preparation of PBS, PBST and blocking solution

To prepare PBS were used:

- 0.1370 M of sodium chloride (NaCl), min. conc. 99.0% (Titolchimica, Pontecchio Polesine, Italy);
- 8.0 mM of potassium phosphate dibasic anhydrous (Na_2HPO_4), min. conc. 99.0% (Carlo Erba Reagents SpA, Milan, Italy);
- 1.5 mM of potassium dihydrogen phosphate (KH_2PO_4), min. conc. 99.0% (Carlo Erba Reagents SpA, Milan, Italy);
- 2.7 mM of potassium chloride (KCl), min. conc. 99.5% (Sigma-Aldrich Chemie GmbH, Steinheim, Germania; Sigma-Aldrich, St. Louis, MO 63178, USA).

The buffer was set at pH 7.2 immediately after its preparation and before each experiment.

For the washing buffer was applied 0.55% (v/v) Tween-20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germania; Sigma-Aldrich, St. Louis, MO 63178, USA) in PBS (PBST). Also in

this case the pH was set at 7.2 as above.

The blocking solution was made by dissolving (in a thermostatically-controlled bath at 40 °C) 2.5% (w/v) gelatin (Bio-Rad Laboratories, USA) in PBST. Once occurred its complete solubilization, the solution was allowed to cool at room temperature.

The preservation of all these solutions was carried out at refrigeration temperature and before each experiment temperature was brought to ambient levels. In specific, the blocking solution in the gel state, resulting from storage at refrigeration temperature, was first brought to the liquid state (always in a thermostatically-controlled bath at 40 °C), then it was allowed to cool at room temperature before its use.

1.19.2. Preparation of the primary antibody

In this trial, it was used as primary antibody, purified anti-peptide polyclonal antibody 0.75 mg/ml from rabbit (Prim r.s.l., Milan, Italy), specifically designed to recognize the binding of β-CN Lys_{28}-Lys_{29}, such as one that Senoq et al. (2002) have used in their research. This was stored at a temperature of -80 °C and thawed before every analysis, leaving it at room temperature.

For its preparation, it was provided for a first dilution by adding 12 µl of primary antibody in 3.475 ml of PBST (dilution level 1:290.583; approximated 1:300000). Then, it was made a second dilution by transferring 3 ml of the first into 9 ml of PBST (dilution level of 1:1162.33; approximated to 1:1200).

1.19.3. Preparation of the secondary antibody

In this trial, it was used as secondary antibody Anti-Rabbit IgG (whole molecule) -Alkaline Phosphatase (Sigma-Aldrich, St. Louis, MO 63103, USA) produced in goat, affinity isolated antibody, adsorbed with human IgG; whose storage occurred at refrigeration temperature. For

its preparation, it was provided for a first dilution by adding 2.5 µl of secondary antibody in 10 ml of PBST (dilution level 1:4001,000; approximated 1:4000.000). Then, it was made a second dilution by transferring first 1 ml of first into 9 ml of PBST (dilution level 1:40010.00; approximated 1:40000.00).

1.19.4. Preparation of solutions of antigen

We weighed 0.0018 g from purified buffalo β-CN, in a eppendorf tube. Subsequently, in the same tube was added 1 ml of PBS (pH 7.2). The heterogenous solution thus obtained was stirred until complete dissolution of the solute. Then, we have detected the concentration of antigen by Spectrometer (Direct Detect, Germany), which resulted:

- Average Concentration (mg/ml) – 1.947
- Standard Deviation – 0.09
- Coefficient of Variation% - 4.9

We have considered a concentration of the purified buffalo β-CN solution, equal to 1.8 mg/ml, by convention.

From buffalo casein from fresh milk (03 2015 Feb.), we have weighed 0.006 g in a eppendorf tube; we added 1 ml of PBS (pH 7.2), and then we dissolved the solute as described above.Then, the peptides concentration was detected:

- Average Concentration (mg/ml) – 4.393
- Standard Deviation – 0.03
- Coefficient of Variation% - 0.6

We have considered a concentration of casein solution buffalo fresh milk, equal to 4.0 mg/ml, by convention.

From buffalo Casein from frozen milk at -20 °C for about 4 years (2011), we have weighed 0.0060 g in a eppendorf tube, proceeding as described above. From detection the

concentration of peptides, was resulted:

- Average Concentration (mg / ml) – 3.790
- Standard Deviation – 0.03
- Coefficient of Variation% - 0.8

We used as reference for the concentration of caseins in buffalo milk a value of 3.6 mg/ml. These 3 stock solutions were stored at -80 °C, and thawed before proceeding with the experiment, leaving them at room temperature. For the execution of the analysis, 2 dilutions were made in eppendorf, for each of them:

1) dilution 1: 100 - 10 µl of stock solution were added to 990 µl of PBS (pH 7.2);

2) dilution 1: 1000-100 µl of the first dilution were added to 900 µl of PBS.

From the latter dilution was obtained, for each sample, the concentration level necessary for the performance of the analysis:

- purified buffalo β-CN - approximate level of concentration 1.8 µg / ml (1800.00 ng/ml);
- buffalo casein from fresh milk (03 2015 Feb.) - approximate level of concentration 4.0 µg/ml (4000,00 ng/ml);
- buffalo casein from milk frozen at -20 ° C for about 4 years (2011) - approximate level of concentration 4.0 µg/ml (4000,00 ng/ml).

1.19.5. Indirect ELISA

A flat-bottomed ELISA plate of 96 wells (Nunc, Nunc-Immuno Plate, MaxiSorp Surface, Denmark) was prepared before starting the experiment, filling the bottom of the wells with 50 µl of PBS (pH 7.2). Then it was discharged by slamming on paper. Once conditioned, from eppendorf tubes were taken aliquots of 200 µl of antigen solutions, which they were:

- purified buffalo β-CN, used as a reference, at a concentration of about 1.8 µg/ml of PBS;

- buffalo casein from fresh milk (03.2015.Feb.), to the approximate concentration of 4.0 µg/ml of PBS;
- buffalo casein from frozen milk at -20 °C for about 4 years (2011), to the approximate concentration of 4.0 µg/ml of PBS.

With these solutions (samples), the bottom of the wells were coated for the dilutions. Each sample was applied simultaneously into two adjacent wells, so that each detection was in duplicate. The bottom of the wells of the series, however, was coated with 50 µl of PBS (pH 7.2). From the wells filled with antigen solutions, 150 µl were taken and added to 50 µl of PBS in the wells in the following series. Then, from the latter were taken another 150 µl of solution and transferred into subsequent wells, repeating the operation until completion of the series, so that each well there were 50 µl of antigen solution at different levels of dilution. In this way, on the ELISA plate were made 8 different concentrations for each sample and zero was represented by white and negative. These last two were separately prepared on the plate, by reserving 2 wells each, with 50 µl of PBS. White and negative have not received the antigen, but only the solvent (PBS), while the former differs from the latter, since it not receives the antibody solutions, but only PBST.

Thus prepared, the plate was covered with parafilm and incubated for 2 h at 37 °C. After this time, we proceeded to remove the parafilm and wash the plate 4 times with washing buffer at pH 7.2 (PBST 200 µl/well). For each washing, the plate was agitated manually, emptied and slammed on paper as indicated above. After this, the bottom of the wells was coated with 200 µl of blocking solution. The plate was incubated for 1 h at 37 °C and it was covered with parafilm. After incubation, the plate was again washed as described above. At this point, we proceeded in the coating the bottom of the wells with 100 µl of antibody solution (anti-peptide CEES1 1:12.000 v/v in PBST). The plate was incubated overnight at 37 ° C (always wrapped with parafilm). After incubation, the parafilm was removed from the plate and this

was washed as described above. Then, the bottom of the wells was coated with 100 µl of alkaline phosphatase conjugated antibody solution. At this point, the plate was covered with parafilm and incubated for 1 hour at 37 °C. After having washed again, we applied the reagent/substrate, dinitro-phenylphosphate (200 µl/well) to the plate. This latter was left at room temperature, so it can take place the enzyme reaction. After 50 min from application the reagent/substrate, it was performed a first detection of absorbance at 405 nm by iMarkTM Microplate Absorbance Reader (Bio-Rad). Immediately after the reading, we added 50 µl/well of stop solution (3 M sodium hydroxide) to stop the enzymatic reaction and performed a second absorbance detection, at 405 nm.

1.20 Results and discussion

The research of frozen milk is enough hard because freezing tends to block or to slow significantly the enzymatic actions. However, using an high sensitivity immunochemistry methodology, such as the ELISA test, it was be possible to obtain good and promising results, albeit preliminary. Analyses were conducted using the indirect ELISA and they have shown that the concentration of β-CN tends to decrease increasing the time of freezing. Results obtained are listed in the table 1 below:

Table1: the obtained data by the quantification of the β-CN by means of Elisa test.

	Mean concentration of β-CN in ng/ml	**DEV. ST.**	**Weight fraction of β-CN**	**DEV. ST. the Weight fraction of β-CN**	**Mean Weight fractions of β-CN (%)**
β-CN buffalo purified (standard)	959.471	23.2928	0.948	0.0233	**95.6±2,01**
	766.529	2.9119	1.009	0.0042	
	577.118	29.9481	1.013	0.0530	
	374.176	2.9112	0.876	0.0071	
	298.882	4.1599	0.933	0.0127	
β-CN buffalo milk fresh	1147.193	53.784	0.433	0.0345	**32.8±2,11[a]**
	826.039	13.847	0.349	0.0061	

	687.114	8.642	0.316	0.0411	
	532.868	12.385	0.305	0.0059	
	442.033	7.598	0.275	0.0117	
	410.983	14.295	0.287	0.0274	
β-CN buffalo milk frozen at -20 ° C for 3 months	897.118	15.8067	0.183	0.0028	**25.1±0,38[b]**
	838.294	5.882	0.228	0.0021	
	716.529	12.4783	0.260	0.0042	
	589.706	0.8323	0.285	0	
	438.294	16.6382	0.283	0.0106	
	307.118	3.7441	0.264	0.0028	
β-CN buffalo milk frozen at -20°C for 2 years.	862.412	31.1954	0.163	0.0057	**19,1±0,37[c]**
	701.412	4.5757	0.177	0.0007	
	601.824	5.4072	0.202	0.0021	
	473.000	18.7171	0.212	0.0085	
	301.825	2.9112	0.2	0.0014	
β-CN buffalo milk frozen at -20°C for 4 years	794.176	20.7967	0.156	0.0042	**18.4±0,39[c]**
	666.529	4.5757	0.174	0.0014	
	551.824	6.6546	0.192	0.0021	
	459.471	17.4698	0.214	0.0078	

a, b, c=P<0,05

Mean Weight fractions of β-CN (%) was obtained multiplying by 100 the mean of weight fraction of β-CN. The results of this first investigation were significantly different up to 2years (P<0,05). They showed that it is possible to distinguish the fresh milk from frozen milk after 3 months, although, in a previous work Gagliardi et al. (2009) had shown that this methodology could reveal variation in the concentration of the β-CN after one month. Moreover the results also indicated that isn't possible to reveal frozen milk over 2 years. This finding demonstrated that the endogenous enzyme of the milk, plasmin, acts on the samples during freezing.

It is necessary to implement this technique increasing the number of obserbvation, however, at the moment these results were promising to continue the ELISA study and to propose this methodology as a valuable quantitative tool for the detection of the frozen milk.

1.21. Conclusions

The electrophoretic techniques have been excellent qualitative analytical instruments, which allowed to obtain informations on the quality of the mozzarella that we found in commerce and, sometimes, which is difficult to obtain with the laboratory preparations. The immunochemical technique has been a promising tool for analysis for detecting the frozen milk.

With this project we have achieved interesting results, which will surely represent the foundation for the development of tools to protect the " Mozzarella di Bufala Campana " PDO and the whole Italian production of fresh cheeses, especially in this moment, where a policy of globalization tends to flatten the differences that are, however, the strength of our cultural and economic heritage .

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