ANALYTICAL METHODS USED FOR MILK PROTEINS
SEPARATION AND IDENTIFICATION (minireview)

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Abstract: Milk proteins have been studies in the last decade, but many questions concerning milk protein expression, structure and post-translational modification remain unexplained. In recent years, the methods used to analyze the proteins from milk are electrophoresis techniques using polyacrylamide gels and isoelectric focusing (IEF); liquid chromatography in different systems (ion-exange, hydrophobic interaction and reversed phase); immunological methods; identifications of proteins on the basis of UV spectra and identification of proteins by mass spectrometry. In this minireview it is provide a description of the most important methods used for milk proteins separation and identification.

1. INTRODUCTION

Physiologically speaking, milk is a complex biological fluid, constituted mainly of water and very valuable nutrients: proteins, fat, lactose and minerals.

Milk proteins are today largely known for their high nutritional value. They include remarkable techno-functional properties largely exploited by food industries. Since the end of the 1980s, both research scientists and the industry have become increasingly interested in both the biological and physiological properties of these proteins. Indeed, over the last several years, it has been shown that these proteins (caseins, lactoserum proteins) have different biological activities in vitro and in vivo [1, 2].

From the solubility point of view, at pH 4.6 and 20°C, the protein fraction can be divided into two different fractions. The soluble fraction, named "whey protein", is constituted by several different proteins, the most important ones are α-lactalbumin (a-La) and β-lactoglobulin (b-Lg). The fraction insoluble, named "whole casein", is constituted of four different caseins (Cn): αs1-Cn, αs2-Cn, β-Cn and k-Cn. These proteins are associated with a variable number of phosphate groups and, in the case of k-casein, with a carbohydrate moiety. The caseins αs1-, αs2- and β-caseins, richer of phosphate groups, is distinguishing from k-casein for their more or less marked tendency to "precipitate" in presence of calcium ions.

Caseins are phosphoproteins synthesized by mammary epithelial cells under multi-hormonal control. There are presented in milk as large spherical particles, casein micelles, as the results of aggregation of smaller subunits (submicelles) in the presence of calcium phosphate salt [3]. Caseins are present in the milk of all mammals and their total concentration and relative proportions are dependent by species. For example the women breast milk has one of the lowest levels of proteins and the main casein is represented by β-casein. Contrary, rabbit milk has one of the highest protein content and the predominant
casein is αs1-casein. In the case of dairying species the highest protein content is present in sheep milk, more de 50g/kg [4].

2. MILK PROTEOMICS

PROTEOME analysis is the analysis of the entire PROTEin complement expressed by a genome. The term proteomics was first coined in the 1990s and it is defined as the systematic separation, identification and characterization of proteins from different sources as cell type, secretion or subcellular compartment [5, 6].

Milk proteins have been studied for well over 50 years but many questions concerning milk protein expression, structure and post-translational modification remain unanswered. In recent years, the methods performed to analyze the proteins from milk are electrophoresis techniques using ployacrylamide gels (UREA-PAGE) with urea or sodium dodecyl sulphate (SDS-PAGE) and isoelectric focusing (IEF); high performance liquid chromatography (HPLC) in different systems like ion-exange, hydrophobic interaction and reversed phase; immunological methods; identifications of proteins on the basis of UV spectra AND identification of proteins by mass spectrometry [7 – 10].

3. TWO-DIMENSIONAL POLYCRYLAMIDE GEL ELECTROPHORESIS (2D-PAGE)

Two-dimensional electrophoresis (2D-electrophoresis) is a widely used methods for the analysis of complex protein mixtures extracted biological samples and was first introduces by P.H.O’Farrell in the beginning of 1970s [11].

This technique sorts protein mixtures according with two different properties. The first dimension step separates proteins according to their isolectric points (isolectric focusing, IEF) and the second, SDS-polycrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights [12].

3.1. Sample preparation

There are used different treatments and conditions to solubilized different types of protein sample depends on the choice of cell disruption method (gentle or vigorous lysis), protection against proteolysis, protein concentration and dissolution method, choice of detergents and composition of the sample solution.

The sample solution must contain certain components, like urea and detergents, to ensure complete solubilization and denaturation prior to the first dimension IEF.

Usually, urea, a neutral chaotrope, solubilisez and denatures proteins unfolding them to expose internal ionizable aminoacids. Commonly 8M urea is used but the concentration can be increased and also thiourea, in addition to urea, can be used to further improve protein solubilization [13].

Non-ionic detergents (NP-40, Triton X100 or zwitterionic CHAPS), in the range of 0.5 to 4%, are used to ensure complete sample solubilisation and to prevent aggregation through hydrophobic interactions, to minimizes protein aggregation.

Reducing agents like dithiothreitol (DTT) and dithioerythreitol (DTE) are included in the sample solution (at concentrations ranging from 20 to 100 mM) to brake the disulfide bonds present and to maintained all proteins in their fully reduced state [14].
3.2. First-dimension Isolecetric focusing (IEF)

The isoelectric point (pI) is the specific pH at which the net charge of protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero.

IEF is an electrophoretic method that separates proteins according to their isoelectric points (pI). The resolution of this separation is determined by the slope of the pH gradient and the electric field strength (typically high voltages, in excess of 1000V).

3.3. Second dimension SDS-PAGE

The second dimension separation, SDS-polyacrylamide gel electrophoresis, separates polypeptides according to their molecular weights.

SDS is an anionic detergent, that, when in solution in water, forms globular micelles composed of 70-80 molecules with the dodecyl hydrocarbon moiety in the core and the sulfate head groups in the hydrophilic shell. SDS and polypeptides form complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments. The results of this structure are that large amounts of SDS are incorporated in the SDS-proteins complex in ratio of approximately 1.4 gSDS/g protein [15]. Depending by the polycrylamide percentage, there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide complex.

For the second dimension, SDS-PAGE, the most used buffer system is the tris-glycine system described by Laemmli [16].

Electrophoresis is performed usually at constant current (or constant power) in two steps. During the initial migration and stacking period, the current is approximately half of the value requires for the separation (15-30mA/gel for 15-90 minutes).

3.4. Visualization of separated proteins

There is different detection methods used for SDS gels. For all the methods the following feature are desired: a high sensitivity, a wide linear range for quantification and compatibility with mass spectrometry.

The most sensitive detection methods are autoradiography and fluorography. The most sensitive non-radioactive methods (below 1ng) are silver staining. Utilizing two-dimensional separation with a sensitive detection method such as silver stain, complex mixtures of whole cell extract can produce as many as 1000-2000 protein spots [17].

Coomassie staining is 50 less sensitive than silver staining (bellow 100 ng/protein spot). Coomassie blue is preferable when the relative amounts of protein are to be determined by densitometry [18].

4. LIQUID PHASE SEPARATION

Liquid phase separation of proteins presents different advantages over 2D-PAGE like a higher sensitivity, superior dynamic range and is more easily automated and faster.

Liquid phase separation can utilize different separation mechanism like size exclusion, ion exchange or reversed phase.

Reversed-phase high performance liquid chromatography (RP-HPLC) is usually used for routine separation and quantification of κ-, α- and β-caseins in raw and processed milk. These methods were successfully applied for authenticity evaluation and quantitative
determination of ovine and caprine milk percentages of commercial protected denomination of origin cheeses [19, 20].

5. PROTEINS IDENTIFICATION BY MASS SPECTROMETRY

Currently, the uses of MS in proteomics are in three major areas. MS is the preferred technique for characterization and quality control of recombinant proteins and other macromolecules, an important task in the field of biotechnology. It is also commonly used for protein identification, either in classical biochemical projects or in large-scale proteomic ones. Finally, because MS measures the molecular weight of a protein, it is the method of choice for the detection and characterization of posttranslational modifications and potentially can identify any covalent modification that alters the mass of a protein. Identification of proteins is usually achieved via a process consisting of protein separation, protein digestion, and mass spectrometry analysis of resulting peptides and comparison of observed peptides to those in a database [21].

Electrospray (ES) and matrix-assisted laser desorption ionization (MALDI) are the ionization techniques used in the most mass spectrometers designed for biological applications [9].

In MALDI method, in order to generate gas phase, protonated molecules, a large excess of matrix material is coprecipitated with analyte molecules The resulting solid is then irradiated by nanosecond laser pulses, usually from small nitrogen lasers with a wavelength of 337 nm. The matrix is typically a small organic molecule with absorbance at the wavelength of the laser employed like cyano-4-hydroxycinnamic acid or dihydrobenzoic acid (DHB). The cyano matrix, which generally leads to the highest sensitivity in MALDI, is “hotter” than DHB, so the latter is preferred when the ions need to be stable for milliseconds in trapping experiments rather than microseconds in time-of-flight experiments. The mass range below 500 daltons (Da) is often obscured by matrix-related ions in MALDI. Proteins generally undergo fragmentation to some extent during MALDI, resulting in broad peaks and loss in sensitivity; therefore MALDI is mostly applied to the analysis of peptides.

Electrospray mass spectrometry (ES) has been developed for use in biological mass spectrometry by Fenn [22].

Liquid containing the analyte is pumped at low microliter-per-minute flow rates through a hypodermic needle a high voltage to electrostatically disperse, or electrospray, small, micrometer-sized droplets, which rapidly evaporate and which impart their charge into the analyte molecules. This ionization process takes place in atmosphere and is therefore very gentle.

A wide range of compounds, proteins, oligonucleotides, sugars (with less sensitivity, as sodium rather than hydrogen is the charging agent), and polar lipids, can be analyzed by ES. The only requirement is that the molecule be sufficiently polar to allow attachment of a charge. For a given compound, the signal strength (peak height in the spectrum) increases linearly with the analyte concentration over a wide range until saturation occurs. However, the signal is to a first approximation independent of liquid flow rate, which makes it desirable to operate at the lowest flow rate possible. There does not seem to be an upper mass limit to analysis by ES. Large ions are typically multiply charged (proteins and peptides by added protons in the positive mode and abstracted protons in the negative mode), which brings them into the range of mass-to-charge (m/z) ratios of typical mass spectrometers. Very complex mixtures can be analyzed by ES, but the spectra become increasingly difficult to interpret as the molecular weight of the components and their number increases.
BIBLIOGRAPHY