Determination of Milk Proteins in Dairy Products by Analytical Methods

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Abstract. Globally, milk is a commonly consumed food due to its high nutrient composition. Milk naturally contains a number of key nutrients, including protein, which is beneficial to humans regardless of their age. Proteins are macromolecules that play a crucial role in nutrition, growth and development. The percent of protein ranges from 3.0% to 3.6% in cow's milk. There are several methods used for the determination of milk proteins in dairy products, such as qualitative methods, determination of total organic nitrogen by Kjeldahl technique, colorimetric principles, enzymelinked immunosorbent assay (ELISA), electrophoresis, X-ray crystallography, nuclear magnetic resonance (NMR) and chromatographic methods. Milk proteins can be detected more easily by analytical instruments compatible with liquid chromatography due to polar ligands. Reversed-phase HPLC technique has become an essential technique in determination of milk proteins and peptides in dairy products. Reversed-phase HPLC combined with mass spectrometry (MS) provides a powerful technique for milk protein analysis. It is possible to determine also the animal origin of milk by detecting milk proteins. Chromatography combined mass verification technique is the leading technique for determination of milk proteins in dairy products.

INTRODUCTION

Milk proteins are the most important structures for the development, growth and self-renewal of the organism. Milk proteins are organic compounds that are essential for life in terms of their chemical composition. Milk proteins contain for the life all essential amino acids that cannot be synthesized by the human body and should be provided from everyday diet. Milk proteins mainly consist of 2 different groups; caseins and serum proteins (whey proteins). Casein is found only in milk in nature and is the main protein of milk. Whey protein is called noncoagulant and non-casein part of milk protein.

PROTEIN ANALYSIS

Qualitative methods

Proteins are determined by colour reactions.

Millon Test; when proteins are heated with concentrated nitric acid + mercury II Milan separator, the blood forms a red colour. This reaction results from the tyrosine amino acid. Ninhydrin Reaction; blue-violet colour occurs when proteins are heated with ninhydrin solution.

Xanthoprotein Reaction; proteins give a severe yellow colour with concentrated nitric acid (HNO3). If ammonia (NH3) is added to the medium, the colour turns orange. These colour transformations are due to the tyrosine and tryptophan amino acids. When the nitric acid gets into the hands, the reaction of the hands is yellow.

Diacetyl Reaction; arginine is a characteristic reaction for the amino acid. A dilute protein solution is mixed with 10% KOH solution and a dark pink colour with green fluorescence is formed if 1% diacetyl solution is added dropwise.

Lead Sulphur Reaction; if the alkaline solution of the protein is boiled with lead acetate solution, the sulfuric amino acids give a black lead sulphide precipitate or a brunette colour.

Methods for determination of total organic nitrogen

Proteins are composed of C, H, O, N, S and P. The amount of nitrogen in protein molecules is approximately 16%, this ratio is different in different foodstuffs. In the determination of the total organic nitrogen, there are two methods for the foods. Methods based on the conversion of nitrogen in natural form into elemental nitrogen in food. Methods based on converting nitrogen in natural form into ammonium salts in food.

There are three major methods developed on the basis of determination of gasified nitrogen or ammonium salts. Dumas method was developed in France in 1831. After that, Kjeldahl method was developed in Danish in 1883. The Ter Meulen method was developed in the Netherlands in 1924. Then, these methods are modified for several times.

Methods based on colorimetric principles

Colorimetric analyses can be applied to both macro and micro levels. By colorimetric methods, not only proteins in the dairy products, but also peptides and amino acids can be detected.

Protein determination by colorimetric method; it is the reaction of peptide bonds or amino acid residues with a suitable chemical chromophore group. The coloured proteins are measured by the spectrophotometer principle of light absorption.

Bi-urea method; in the strongly alkaline environment, the proteins in the food react with the copper compounds to form a red-violet or red-purple (purple) compounds. Since the intensity of the colour formed depends on the amount of protein in the environment, protein determination methods based on bi-urea reaction were developed.

FCL (Folin-Ciocalteau-Lowry) method; the Folin solution reacts with the proteins in the food and creates a blue colour in this method.

Formol titration method

This method is one of the fastest methods to determine the amount of milk proteins in dairy products. The amine group (-NH2) is converted to the methylene amino group (-N = CH2) by addition of formaldehyde to the amino acids in the proteins. The released carboxyl (COOH) group is titrated by the adjusted base and the result is calculated. In this method, the amount of base spent on titration is directly proportional to the amount of protein.

ELISA "Enzyme-linked Immuno-sorbent Assay" method

It is a biochemical-immunological method, allowing the antibody to bind with the antigen. It is very sensitive to antibody and antigen determination in the samples. Unbound or non-specific proteins are removed by washing. Enzyme-linked secondary antibodies are added and bound to the antigen-antibody construct (sandwich structure). The amount of protein is measured spectrophotometrically by adding the substrate to interact with the enzyme.

Electrophoresis method

Electrophoresis is the migration of solutes or particles loaded in a liquid medium under the influence of an electrical field. Since electrophoresis provides migration of all particulate species, the term "iontophoresis" refers to the migration of small ions in particular. The most common electrophoresis applications include whey proteins, hemoglobin and isoenzymes.

X-ray crystallography method

The method works on the basis of X-ray transmittance in the sample. α-strand and β-layer motifs contained in proteins can be determined by this method. Information about chemical bonds in the protein can be obtained. By collecting all data, the three-dimensional structure of the protein can be understood. The fact that some proteins do not crystallize restricts the use of this method.

Nuclear Magnetic Resonance (NMR)

NMR is used to investigate the three-dimensional structure of proteins. For NMR, it is necessary to use high purity protein sample. This method is applicable for natural or recombinant proteins and suitable for structure analysis of small proteins (35 kDa).

Chromatographic methods

The proteins can be detected by analytical devices compatible with liquid chromatography due to their lack of thermal stability and their polar ligands. Peptides and proteins are separated based on differences in surface hydrophobicities or surface charges. These methods are thin layer chromatography, ion exchange chromatography (IEXC), affinity chromatography, hydrophobic interaction chromatography (HIC), gel filtration chromatography, reverse phase chromatography (RPC).

Thin layer chromatography is decomposition of proteins according to their dissolution ability. The sample impregnated on a solid surface (cellulose) is placed in the solvent surface. By dissolving the solvent solution on the surface, the proteins in the sample are separated according to their dissolution ability. Ion exchange chromatography is separation of proteins according to ionic

loads. Hydrophobic interaction chromatography is chromatographic separation technique commonly used for purification of macromolecules such as proteins and polynucleotides. Purification schemes are mostly developed by combining HIC with ion exchange, size exclusion and affinity chromatography. Affinity chromatography ensures separation of proteins to chemical groups. Gel filtration chromatography allows the separation of proteins according to their size.

Reverse phase high performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the dissolved molecule to the immobilized hydrophobic ligands, which are bound to the stationary phase from the mobile phase to the sorbent (Mant & Hodges, 1996). In reverse phase method, analyses are performed
using C4, C8 and C18 filled non-polar columns. The C18 hydrophobic phase is suitable for separating peptides smaller than ~ 2000-3000 Da. The C4 hydrophobic phase is suitable for the separation of peptides and proteins larger than ~ 3000 Da (Aguilar & Hearn, 1996). Milk proteins can be detected by photo- diode array (PDA) and diode array (DAD) detectors with expanded UV and visible region properties in RP-HPLC technique. However, precise and accurate results are difficult to achieve with the combination of analytical devices that work only with light absorption. Due to interference elements and chromatographic separation difficulties, it is not possible to reach reliable analysis results. The exact solution of a correct analysis is possible by using mass selective detectors. The use of MS in chromatography has several advantages. MS is a very sensitive detection technique. MS provides the separation of peptides/proteins by molecular weights. MS can detect proteins or peptides as specific mass (Premstaller, Oberacher & Walcher, 2001). Finger print of proteins can help identify of peptides/proteins milk origin (ZACHAR, 2011).

CONCLUSION

Chromatographic techniques have developed into powerful separation techniques, capable of separating large numbers of proteins and peptides. As a result, combining chromatography techniques has become a widespread method for protein analyses and separations in dairy products. Reversed-phase HPLC technique has become an essential technique in the separation and analysis of milk proteins and peptides in dairy products. It is widely used in the life science to characterize proteins and to analyse them for product identity and impurities. Reversedphase HPLC combination of mass spectrometry provides a powerful technique for milk protein analysis. Mass spectrometry interfaces with reversed-phase HPLC by means of the electro spray ion (ESI) source. The polar and ionized groups scattered on the surface of the protein particles determine the electrical charge and electrical properties of the protein molecule. Amino groups take protons and form cations ($NH + 4$). These groups are soluble in aqueous media and form ions. Carboxyl and phosphate groups gain anionic property by giving proton (H+) to the environment. It is very difficult to ionize large molecules with ESI soft ionization technique. However, thanks to these ions, it is possible to analyze milk

proteins. Chromatography combined mass verification technique is the leading technique for determination of milk proteins in dairy products. It is possible to determine also the animal origin of milk by detecting milk proteins.

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