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A Review of Methods for Genotyping Milk Proteins in Cattle

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Abstract

It is well known that first study of animal biodiversity is based on morphological, chromosomal and biochemical markers. Most of them are limited by gender, age and environment. In the last years with a significant progress in molecular biological techniques are discovered the molecular markers. They play a key role on population and species diversity. Molecular markers are specific pieces of DNA that can be identified within the genome and are inherited according to Mendel's laws. They can be successfully utilized to detect mechanisms of polymorphic traits that contribute to genetic diversity. Moreover, molecular markers enable the detection of genetic variants at DNA sequence level are devoid of these limitations typical for morphological, chromosomal, protein markers and not influenced by environment.

Keywords: Milk proteins; Molecular markers; Genotyping

Why Milk Protein Polymorphism is Important?

It is well known that milk proteins are divided into two main groups depending on their behaviour at pH 4.6. The soluble fraction, called "whey protein", is constituted of several different proteins, the most important ones being β-lactoglobulin and α-lactalbumin. The remaining fraction is insoluble and precipitate at this condition, named "whole casein", and consists of four different caseins αS1-CN, αS2-CN, β-CN, and κ-CN. Among them a noticeable genetic variation has been identified and characterized. Cow milk is an important alimentary source for offspring but also in human diet. Compared with other agricultural species, dairy cattle possesses numerous distinctive features like the long generation interval, the limited female fertility and the fact that nearly all economic traits are expressed only in females. Due to this reasons the search of genetic markers for genetic elaboration of milk herds plays an important role for the prosperity of every dairy farm.

During the last decades a significant attention was drawn on the possibility of milk proteins to influence milk qualitative and quantitative traits as well as on milk products like cheese, yellow cheese etc. [1-4] These effects are determined to some functional modification mainly point mutations and deletions in coded genes, which lead to alteration of biological properties of the encoded protein. Another point of view is the utilization of milk protein variant for breed characterization [4-7], biodiversity investigations [8,9], as well as biogeography and domestication studies [7,10,11]. Moreover, milk protein polymorphisms influence human nutrition in various aspects: i) the hypoallergenic properties of particular milk, i.e. most milk proteins are potential allergens, mainly αs1-CN and αs2-CN, and β-LG, which are absent, in human milk [12]. Searching of variant which determines hypoallergenic properties of milk is a significant issue to human diet. ii) the release of peptides with biological functions from milk proteins. In the last years it has been recognized that proteins provide a rich source of biologically active peptides which act positively on body conditions and even human health [13]. Among all milk proteins the caseins seem to be a source of various bioactive peptides or with hormone-like activity [14].

In this review, we concentrate our attention on main approaches for distinguishing of milk protein variants in cattle.

PCR based molecular approaches for genotyping milk proteins

Several PCR based methods are developed to separate allele variants in milk proteins. These include Simple Sequence Repeat polymorphism (SSR or microsatellite markers), Single Strand Conformation Polymorphism (PCR-SSCP), Restriction Fragment Length Polymorphism (PCR–RFLP analysis),

PCR–SSR (simple sequence repeat polymorphism or microsatellite markers)

The microsatellites are short segments of DNA, which are tandemly repeated. DNA motifs range in length from two to five nucleotides, and are typically repeated 5-50 times. For example, the sequence TATATATATA is a dinucleotide microsatellite, and GTCGTCGTCGTCGTC is a trinucleotide microsatellite. Microsatellites are distributed throughout the genome. They are usually located in non-coding parts of the genome and are therefore biologically silent. This allows them to accumulate mutations unhindered over the generations and gives rise to variability, which can be used for DNA fingerprinting and identification purposes. Other microsatellites are located in regulatory flanking or intronic regions of genes or directly in codons of genes - microsatellite mutations in such cases can lead to phenotypic changes and diseases.

Often, microsatellite DNA is established depending on the difference in repeat number visualized at different bands on acrylamide gel electrophoresis.

Microsatellites are widely used for mapping locations within the genome, specifically in genetic linkage analysis/marker assisted selection to locate a gene or a mutation responsible for a given trait.

During the last decades it has become possible to determine genetic variability of complex traits into quantitative trait (QTL). QTL could enhance selection efficiency when the trait is difficult or expensive to measure. Most researches on QTL mapping of milk production traits of dairy cattle had been reported. The results of Reinecke et al. [15] and Olsen et al. [16] showed that the effect of QTL was significant in total milk fat on chromosome 20. Also Ashwell et al. [17] and Viitalla et al. [18] showed that some QTL had significant effect in milk fat percent on the same chromosome. Heyen et al. [19] found out that QTL on chromosome 7 influences milk fat. Freyer et al. [20], Szyda and Komisarek [21], showed most significant QTL effect on chromosome 6 in.

PCR-SSCP (Single Strand Conformation Polymorphism)

Single-strand conformation polymorphism (SSCP), or single-strand chain polymorphism, is a technique which uses the differences of single-stranded nucleotide sequences. The technique relies on the fact that denatured DNA molecules migrate across a nondenaturing polyacrylamide gel according to their size and their sequence. Hence, two PCR fragments which differ in their sequence may display mobility shifts on the gel. The PCR-SSCP approach does not require expensive restriction enzymes, and a purification step is not necessary, the cost of genotyping is reduced, compared with PCR-RFLP [22]. With this technique Barroso et al. [22] and Dhanammal et al. [23] successfully detected bovine κ-casein and β-casein variants, respectively.

Restriction fragment length polymorphism (PCR–RFLP analysis)

This technique also known as cleaved amplified polymorphic sequence (CAPS), is a mostly used for genotyping of milk proteins in dairy cattle. The technique exploits the fact that single nucleotide polymorphism (SNP) is often associated with the creation or abolishment of a restriction enzyme recognition site. The main steps of PCR-RFLP analysis include: i) amplification of a target sequence containing variable position; ii) PCR amplification of polymorphic region; iii) use of specific endonuclease that recognizes specific sequence and as a result the restriction fragments are with different size; iiii) electrophoretic separation and visualizing of restriction fragments. Important advantages of the PCR-RFLP technique include inexpensiveness and lack of requirement for advanced instruments. Disadvantages include the requirement for specific endonucleases and difficulties in identifying the exact variation in the event that several SNPs affect the same restriction enzyme recognition site. Moreover, since PCR-RFLP consists of several steps including an electrophoretic separation step, it is relatively time-consuming. The main applications of PCR-RFLP analysis are in the identification of individuals in paternity and maternity cases, population genetics structure, and in the diagnosis of a variety of diseases. Concerning milk protein polymorphism this approaches are used to determine influence of particular variant of milk protein gene on milk qualitative and qualitative traits. For example, it is well known that milk with the CSN3*B variant possesses short coagulation time significantly shorter than milk with CSN3*A (1). Moreover, variants CSN3*A and CSN3*B are strictly connected to the micelle size [24]. Although, κ-casein is considered a crucial factor in renneting, ther milk proteins have to be taken into account too. In general, CSN2*B and LGB*B were found to be more favorable for rennet coagulation and the cheese making quality of milk [1]. On the other hand, the PCR-RFLP analysis is useful technique for determination of population structure, origin and domestication of Bos taurus [7,10].

Single nucleotide polymorphism (SNP) assay followed by direct sequencing

A Single Nucleotide Polymorphism, also known as Simple Nucleotide Polymorphism, (SNP) is a DNA sequence variation occurring commonly within a population (e.g. 1%) in which a single nucleotide — A, T, C or G — in the genome differs between members of a biological species or paired chromosomes. For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. In this case two alleles exist. Almost all common SNPs have only two alleles. Single-nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions. SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. SNPs in the coding region are of two types, synonymous and nonsynonymous SNPs. Synonymous SNPs do not affect the protein sequence while nonsynonymous SNPs change the amino acid sequence of proteins.

The most precise method for search of unknown SNP is PCR analysis followed by direct sequencing. This technique is based on the amplification of specific parts of the genome in the PCR reaction and sequencing of the obtained product. A comparison between electrophoregram images of sequencing products is performed, which allows determining whether a mutation in a given region has occurred. The great advantage of this polymorphism is its university in the genome between particular species and highly efficient identification of polymorphism within the tested sequence. On the other hand, the high cost of the analysis represents a disadvantage. The high density of SNP markers in the genome leads to their extensive utilization in the genetic and population analysis.

Based on this technique Chessa et al. [25] and Zepeda-Batista et al. [26] have estimated the allelic frequency of αS1-CN, αS2-CN, β-CN and κ-CN genes. SNPs analysis was used to reveal its influence on milk production traits [27]. The authors have investigated 423 SNPs across 411 Holstein bulls, and their association with 5 milk production traits - protein, fat, and milk yields, and protein and fat composition. The analysis showed positive effect of SNPs variation of chromosome 20 on protein composition and protein and milk yields.

In addition to DNA based techniques for genotyping of milk proteins, genetic variations can be detected at the phenotypic level by various protein identification techniques as well. These techniques include acrylamide electrophoresis in denaturing (SDS-PAGE) or native conditions, isoelectric focusing (IEF), chromatography etc. (Table 1).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

This technique is a variation of gel electrophoresis where sodium dodecyl sulfate (SDS) is applied. SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. In addition to SDS, proteins may optionally be briefly heated in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol/BME), which further denaturate the proteins by reducing disulfide linkages. An electric field

is applied across the gel, causing the negatively charged proteins to migrate across the gel from the negative electrode (the cathode) towards the positive electrode (the anode). Depending on their size, each protein moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. The proteins may therefore be separated roughly according to their size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions.

Methods	Advantages	Disadvantages
IEF	High resolution, resulting in greater separation of solutes Easier, because the placement of sample application is not important The sample and ampholytes can be mixed before application	Carrier ampholyte batch-to-batch reproducibility of the ampholytes Phenomenon of cathodic drift owing to electroendosmosis in the gel causes an eventual breakdown of the basic end of the pH gradient with time
SDS-PAGE	Highly reproducible Inexpensive, fast, sensitive, expressive Economic (no sophisticated equipment required) Easy (clear, one-dimensional separation principle)•	Limited pI range (4-8) Proteins >150 kD not seen in 2D gels Difficult to see membrane proteins (>30% of all proteins) Only detects high abundance proteins (top 30% typically) Time consuming
RT-HPLC	Speed (minutes) High resolution Sensitivity (ng to fg) Reproducibility of +/- 1% Accuracy Automation	Cost Complexity Low sensitivity for some compounds Irreversibly adsorbed compounds not detected Coelution difficult to detect
PCR-SSR	Require very little and not necessarily high quality DNA Highly polymorphic evenly distributed throughout the genome Simple interpretation of results Easily automated, allowing multiplexing Good analytical resolution and high reproduci	Complex discovery procedure Expensive

Table 1: Advantages and disadvantages of methods for genotyping of milk proteins.

Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue R-250), allowing visualization of the separated proteins. After staining, different proteins appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane of the gel to calibrate the gel and determine the approximate molecular mass of a particular protein.

Regarding milk proteins in cattle there were numerous studies referring to this method not only for raw milk but also for milk products (beverage, yoghurt, cottage cheese, hard cheese, etc.). For example, Zagorchev et al. [28] successfully identified casein fraction and β-lactoglobulin from raw milk. The authors determined even protein isoforms (A, B and AB) on 14%-SDS PAGE. Tricine-SDS-PAGE was used for separation, identification and quantification of bovine milk casein fractions [29]. SDS-PAGE was applied to clarify whether milk with a modified casein:whey protein ratio was less allergenic than native cow's milk [30].

Isoelectric focusing (IEF)

Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separation of molecules based on differences in their isoelectric point (pI). It is usually performed on proteins in a gel that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH. IEF involves the addition of an ampholyte solution into immobilized pH gradient (IPG) gels. An electric current is passed through the gel, creating a "positive" anode and "negative" cathode end. Negatively charged molecules migrate through the pH gradient in the medium toward the "positive" end, while positively charged molecules move toward the "negative" end. As a particle moves towards the pole opposite of its charge, it moves through the changing pH gradient until it reaches a point in which the pH of the respective molecule isoelectric point is reached. At this point, the molecule no longer has a net electric charge and as such will not proceed any further within the gel.

Preparative isoelectric focusing followed by polyacrylamide gel electrophoresis are most often methods used for determination of milk proteins and their variants.

Erhardt and Giambra [31] analyzed milk proteins in about 1800 milk samples from 11 different farms by using IEF. The obtained results clearly distinguished A1 and A2 variants of β-CN. Balteanu et al. [32] determined together with common B and C variant a new one named αS1-casein IRV. The isoelectric point of this new protein was situated between the B and C alleles closer to the C. Also, the authors successfully determined A1 and A2 alleles of β casein; A allele of αS2-casein and α-lactoalbumin genes and A and B variants of β-lactoglobulin locus.

Reversed phase high performance liquid chromatography (RT-HPLC)

High-performance liquid chromatography (HPLC; formerly known as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate the components in a mixture, to identify and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column. In recent years, with the advent of high performance media and instrumentation, reversed phase chromatography has been applied to the purification of biomolecules such as peptides, proteins and oligonucleotides. Reversed phase chromatography has proven so successful for biomolecule purification in the research laboratory that it is now routinely applied for process scale purification of synthetic peptides, and recombinant peptides and proteins.

Reversed phase is usually combined with other chromatography techniques such as gel filtration and ion exchange chromatography. RT-HPLC chromatography focused on following biomolecules:

- Naturally occurring peptides and proteins
- Recombinant peptides and proteins
- Chemically synthesised peptides
- Protein fragments from enzyme digests
- Chemically synthesised oligonucleotides

All these groups of biomolecules differ in several ways which prove important in their purification.

This chromatographic technique is a successful method for separation and quantification of bovine milk proteins, even milk protein variants [33,34]. By using HPLC techniques, the authors have separated and quantified the six major bovine milk proteins and several genetic variants. They share that the precision of the method depends on the chosen commercial protein standards.

Concluding Remarks

It can be concluded that the main methods for genotyping of milk proteins in cattle are based on phenotypic and genotyping level. As concern phenotypic level the main techniques which used are acrylamide electrophoresis in denaturing (SDS PAGE) or native conditions, IEF, chromatography etc. The basic methods for genotyping of DNA level include PCR-SSCP, PCR_RFLP analysis, SNPs determination etc.

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