

Research Article

Identification of Milk Protein Polymorphism in Indian Goats by 2D Gel Electrophoresis

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Abstract

Analysis of casein and whey protein was carried out in six Indian goats by both SDS-PAGE and 2-DE analysis. The variation was observed mainly in α_{s_1} , α_{s_2} , β , κ -casein and β -lacto globulin locus in these breeds. Proteome analysis showed the presence of high number of spots in α_{s_1} -casein, and κ -casein showed highest number of spots in all the goat breeds. The gels were showing remarkably both variability and similarity suggesting that the heterogeneity in protein forms in individual milk samples exist, and milk protein represents a common pattern of post-translational modification.

Keywords: Goat; Milk protein polymorphism; Quantitative variation; 2D gel electrophoresis; Milk proteome

Abbreviation: 2-DE: 2-Dimensional Gel Electrophoresis; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Introduction

Casein, the main protein fraction of ruminant milk, is one of the valuable components of milk due to its nutritional value and processing properties. Genetic variants of αs_1 , αs_2 , β , and κ -casein have been characterized both at protein and DNA level in different goat breeds [1,2]. The caprine casein analysis is quite complex due to large number of mutations in the coding region due to interallelic recombination [3]. The as, casein exhibited extensive polymorphism and is also associated with differential level of protein synthesis [4,5]. Similarly as2-casein locus also showed seven alleles and they are associated with differential synthesis level [6]. ĸ-casein locus also showed polymorphism and associated with milk quality and cheese making properties [7,8]. At CSN3 locus, 15 different polymorphic alleles have been identified by analysing isoelectro focussing, PCR-SSCP and PCR-RFLP [9,10]. The whey protein, β -lactoglobulin and α -lactalbumin, also showed polymorphism and the distribution of different variants have been investigated in different breeds and population [9,10].

It is also not possible to analyze the variation of casein cluster by any single approach, therefore various approaches have been applied to determine variability at casein loci. The protein variability has been characterized by SDS-PAGE analysis, however the effect of deletion/ substitution have no effect on net charge of proteins. Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a powerful technique that separates the proteins on the basis of their isoelectric point (pI) in the first dimension and on the basis of their molecular weight in the second dimension [11]. It allows the separation of individual proteins that are defined by their size and chemical structure [12]. Milk casein presents some interesting findings with multiple forms present at both high and low amount. In the present study, we analyzed the major variants of goat milk casein complex by SDS-PAGE and the resolution of different goat milk casein forms using 2-DE.

Materials and Methods

Genetic stock and experimental design

The six goat breeds namely Jamunapari, Barbari, Jakhrana, Ganjam, Beetal and Sirohi have been included in this study. The total

number of samples 160, 337, 72, 42, 30 and 16 were collected from Jamunapari, Barbari, Marwari, Jakhrana, Sirohi, Beetal and Ganjam goats, respectively. The details of the goat breeds and their utility have described in table 1.

Sample collection and processing of samples

Milk samples were collected from six Indian goat breeds from their natural habitat and transported to the laboratory and stored at -20°C for further analysis.

Milk samples

The milk samples stored at -20°C were allowed to thaw and then centrifuged at 12000 rpm for 10 min at 4°C. The milk serum was

Breed	Native tract	Utility
Barbari	Mathura, Agra, Etah (U.P.)	Medium size, known for both milk and meat
Jamunapari	Chakarnagar, Etawah (U.P.)	Large size known for milk production
Beetal	Ambala, Gurdaspur, Punjab	Large size breed known for milk yield
Ganjam	Rambha Chhatrapur (in Ganjam district of Orissa)	Medium size known for meat production but milk fat is valued high
Jakhrana	Jhakarana, Behror, Alwar (Rajasthan)	Large size known for milk production
Marwari	Desh-Nokh, Bikaneri Barmer, Nagaur (Rajasthan)	Large size breed known for meat, milk and coarse fibre
Sirohi	Tonk, Ajmer, Bhilwara, Udaipur (Rajasthan)	Large size known for meat and milk

Table 1: Breeds of goats and their utility in India.

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aspirated out from below the surface creamy layer and was used for all further analysis

Protein determination

Protein concentration of the samples was determined by Folinphenol method [13], using BSA as the standard.

SDS-PAGE and image analysis

SDS-PAGE was carried out in a Vertical Gel Electrophoresis apparatus (plate size: 160 mm×160 mm) (ATTO Corp., Japan) using 5% (w/v) stacking gel and 12% (w/v) separating gel [14]. After electrophoresis, the gels were stained with Coomassie blue R-250 for visualization of the proteins. Molecular weight of the protein bands was determined with reference to standards (SIGMAMARKER, M-4038). Densitometric scanning of the gels was carried out using a Gel Analysis Software, Gene Tool (Mascon Global Ltd.)

2-DE

The samples which showed more variability in casein loci in SDS-PAGE were examined by 2-DE. For 2-DE [15], isoelectric focusing (first dimension) was performed in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. The gel solution was prepared as follows: 9.2 M urea, 4% acrylamide, 2.0% (w/v) CHAPS, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte. Polymerization was initiated by 0.01% ammonium persulfate and 0.1% (v/v) TEMED. The upper chamber was filled with cathode electrode buffer (100 mM NaOH). The anode electrode buffer was 10 mM ortho-phosphoric acid. First-dimension gels were prefocussed at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min. The electrode buffers were exchanged against fresh and degassed buffers. Samples were prepared by adding an equal volume of sample buffer (final conc.: 9.5 M urea, 2.0% CHAPS, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte) and incubated at room temperature for 10-15 min after which sample proteins (80-100 μ g) were loaded into the sample reservoirs. The samples were overlaid with 20-40 µl of sample overlay buffer (final conc.: 9M urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte, and 0.05% bromophenol blue). 2D SDS-PAGE Standards (Bio-Rad) were co-run parallel to samples. The proteins were electrophoresed at 500 V for 10 min followed by 750 V for 3.5 hr. After the first dimension run, the gels were pressed out of the tubes with the help of a tube gel ejector filled with second dimension sample buffer and were equilibrated for 10-15 min at room temperature. They were loaded on to the slab gel for second-dimension run. The IEF gels not used immediately were stored at -70°C in sample equilibration buffer. The second dimension run (SDS-PAGE) was carried out in a Mini-Protean 3 electrophoresis cell (Bio-Rad Laboratories) using 5% (w/v) stacking gel and 12% (w/v) separating gel, as described above. The gels were silver stained.

2-D Gel image analysis

Analysis of gel samples and matching between gels were carried out by Gel Fox 2D V.3.01 (Alpha Innotech, USA). The 2-DE of bovine milk proteins was used as reference gel [16].

Results and Discussion

Analysis of milk protein variants by SDS-PAGE

SDS-PAGE was carried out to analyze the milk sample for genetic polymorphism of six Indian goat breeds. The SDS-PAGE profile of

milk protein variants of $\alpha s_{1,} \alpha s_{2,} \beta$ -Cn, β -LG and α -LA are presented in figure 1. In as,-casein, the A variant was most frequent in all the breeds and majority of goat were found as homozygous AA. The B variant was observed in heterozygous from (AB). The F variant was found as heterozygous AF in some of the samples. In as,-casein, the most prevalent variant was A in all the samples and mostly observed as homozygous AA. The B variant was found as homozygous (BB). The monomorphic pattern was observed in the k-casein locus and found as homozygous AA in all the samples. The SDS-PAGE pattern of β -LG was almost similar in all the samples analyzed in this study. It exhibited homozygous AA, however, B variant was also observed in individuals in all the breeds as heterozygous AB. The electrophoretic pattern revealed that the variant 'A' was most frequent than variant 'B' and was observed as homozygous AA in all the samples at α-LA locus. The milk protein polymorphism indicates that each milk protein has two or more forms, which is genetically determined by autosomal gene and co-dominant alleles. The samples which showed more variability in SDS-PAGE were examined by 2-DE.

Separation of goat milk proteins by two dimensional gel electrophoresis (2-DE)

The complement of proteins from individual milk sample of different goat breeds with pI values between 4 and 6 are presented in figure 2. Analysis of gel samples and matching between gels were carried out by Gel Fox 2D V.3.01. The 2-DE gel presents the complex heterogeneity of milk proteins in goat. The analysis revealed various spots in individual samples of different goat breeds and spots varied from 25 to 102 in different breeds.

There are number of protein spots resolved in milk protein area and large spots identified as α_{s_1} -casein and β -casein dominated the gel. The two-dimensional gel electrophoresis profile of milk protein variants showed sharp, distinct bands for whey protein and their more diffuse, wavy bands could recognize the casein. The 2-DE patterns of individual milk sample are presented in figure 3 in different Indian breeds. The high degree of heterogeneity with goat milk protein explains the different band intensities. The α_{s_1} -casein focused anodic end of the gel. Each variant consists of two fractions i.e. the more concentrated α_{s_1} -casein and diffuse fraction, which was not identified. The variant A and B could be clearly distinguished. α_{s_2} -casein was found in two variants





A, B and each variant revealed one major band situated cathodically, and two minor fractions, which focused more anodally. Two large spots identified as β -casein, dominated the gel. κ -casein presents an even more complex picture with at least 6 forms present with pI values ranging from 5.81 to 4.47 (Figure 3). A similar gel obtained with milk from a single goat electrophoretically typed as having both the A and B variants of κ -casein. The major whey proteins, β -lactoglobulin and α -lactalbumin, were also observed. Ganjam goat showed the absence or very low intensity spot of α s₁-casein locus, where as β -casein and κ -casein is showing higher number of spots in the sample. κ -casein locus

showed less number of spots in Jamunapari goats. It is clear that the vast majority of protein spots on the 2-D gels of milk samples occurred due to proteolysis, phosphorylation or glycosylation. The gels showed some similarity suggesting that milk protein represents a common pattern of post-translational modification among individuals (Figure 3). With its high resolution, 2-DE allows separation of protein isoforms containing the slightest differences, a key in the comprehensive analysis of individual milk samples.

Discussion

The important contribution of milk to human nutrition and its availability in large amounts have made it the subject of biochemical studies for many decades. The protein in milk is just the product of six genes, which constitutes about 95% of milk protein. The proteome of milk is showing complicated spots with the presence of numerous low-abundance gene products and a high level of post-translational modifications including phosphorylation and glycosylation. There has been considerable interest in goat milk protein due to availability of different bioactive peptide derived from milk proteins, which have pharmaceutical and nutraceutical applications and have future commercial importance [17,18]. Secondly, goat milk is used for treatment of different disease, allergy and heat stress and can have future health application. Therefore, the milk proteome analysis appears to be timely and required for future industrial application with respect to human health and nutrition [19].

Milk proteome is complex and this complexity is the consequence of post-translational modifications and due to the presence of numerous genetic variants of protein and bioactive peptides in milk. The 2-DE gel analysis presented the complex heterogeneity in goat milk



proteins in different breeds. The variation was observed between SDS-PAGE and 2-DE analysis mainly at κ -casein, α_{s1} -casein and β -LG and α -LA loci. Despite the presence of hundreds of proteins, no study was able to identify any proteins other than caseins, β -LG, α -LA. It is also clear that numerous protein spots in 2-DE gels of milk samples are due to proteolysis, phosphorylation and glycosylation. Attempts have been made to analyze different low abundance protein through different approaches [20-22].

Conclusion

2-DE is one of the powerful techniques used for characterizing milk proteome in goats. A large variation in protein spots was observed in gel analysis indicating differential expression due to the presence of hundreds of protein and bioactive peptide in milk.

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