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

Ajay Kumar1, P.K. Rout** and B.P. Mohanty2

1Genetics and Breeding Division, Central Institute for Research on Goats, Makhdoom, Farah, Mathura-281122, India
2Biochemistry Laboratory, Central Inland Fisheries Research Institute, Barrackpore, Kolkata-700120, India

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 αs₁, αs₂, β, κ-casein and β-lactoglobulin locus in these breeds. Proteome analysis showed the presence of high number of spots in αs₁-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 αs₁, αs₂, β, κ-casein and β-lactoglobulin 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 αs₁ casein exhibited extensive polymorphism and is also associated with differential level of protein synthesis [4,5]. Similarly αs₂-casein locus also showed seven alleles and they are associated with differential synthesis level [6]. κ-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 allows the separation of individual proteins that are 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 allows the separation of individual proteins that are characterized by SDS-PAGE analysis, however the effect of deletion/substitution have no effect on net charge of proteins.

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.

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

| 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 milk production but milk fat is valued high |
| Jakhana       | Jhakarana, Behror, Alwar (Rajasthan) | Large size known for milk production |
| Marwari       | Desh-Nokh, Bikaner, Nagaur (Rajasthan) | Large size breed known for milk and meat, milk and coarse fibre |
| Sirohi        | Tonk, Ajmer, Bihwara, Udaipur (Rajasthan) | Large size known for meat and milk |

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...
Identification of Milk Protein Polymorphism in Indian Goats by 2D Gel Electrophoresis. J Proteomics Bioinform 6: 001-004. doi:10.4172/jpb.1000252

Protein determination

Protein concentration of the samples was determined by Folin-phenol 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 amphotolyte, 0.4% Bio-Lyte 3/10 amphotolyte. 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 orth-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 amphotolyte, 0.4% Bio-Lyte 3/10 amphotolyte) and incubated at room temperature for 10-15 min after which sample proteins (80-100 µg) 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 amphotolyte, 0.2% Bio-Lyte 3/10 amphotolyte, 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 αs1, αs2, β-Cn, β-LG and α-LA are presented in figure 1. In αs1-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 αs2-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 κ-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 αs1-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 αs1-casein focused anodic end of the gel. Each variant consists of two fractions i.e. the more concentrated αs1-casein and diffuse fraction, which was not identified. The variant A and B could be clearly distinguished. αs2-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 αs1-casein locus, whereas 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.

Acknowledgement

The authors are thankful to the Director, CIRG for providing necessary facilities to carry out the work.

References

1. Rout PK, Kumar A, Mandal A, Laloe D, Singh SK, et al. (2010) Characterization of casein gene complex and genetic diversity analysis in Indian goats. Anim Biotechnol 21: 122-134
2. Kumar A, Rout PK, Mandal A, Roy R (2007) Identification of the CSN1S1 allele in Indian goats by the PCR-RFLP method. Animal 1: 1099-1104.
3. Martin P (1993) Genetic polymorphism of goat milk proteins. Le Lait 73: 511-532.
4. Feligini M, Silvia F, Cubric-Curik V, Brambilla A, Parma P, et al. (2005) Caprine αs1-Casein Polymorphism: Characterisation of A, B, E and F Variants by Means of Various Biochemical and Molecular Techniques. Food Technol Biotechnol 43: 123-132.
5. Caroli A, Chiatti F, Chessa S, Rignanese D, Rignanese D, et al. (2006) Focusing on the goat casein complex. J Dairy Sci 89: 3178-3187.
6. Boulanger A, Grosclaude, F, Mahe MF (1984) α-S1 and α-S2 casein polymorphism in goats. Genetics Selection Evaluation 16: 157-175.
7. Eigle WN, Butler JE, Emstrom CA, Farrell Jr HM, Harwalkar VR, et al. (1984) Nomenclature of proteins of cow’s milk: fifth revision. J Dairy Sci 67: 1599-1631.
8. Martin P, Leroux C (1994) Characterization of a further α_{s1} casein variant generated by exon skipping. In: XXIV International Conference of Animal Genetics, Prague.
9. Prinzenberg EM, Gutscher K, Chessa S, Caroli A, Erhardt G (2005) Caprine kappa-casein (CSN3) polymorphism: new developments in molecular knowledge. J Dairy Sci 88: 1490-1498.
10. Yahyaoui MH, Pena RN, Sánchez A, Folich JM (2000) Rapid communication: polymorphism in the goat beta-lactoglobulin proximal promoter region. J Anim Sci 78: 1100-1101.
11. Grosclaude F, Ricordeau G, Martin P, Remue F, Vassal L, et al. (1994) Du gene au fromage: le polymorphisme de la caseine α-S1 caprine, ses effets, son evolution. INRA Productions Animales 7: 3-19.
12. Grosclaude F, Mahe MF, Brignon G, Di-Stasio L, Jeunet R (1987) A Mendelian polymorphism underlying quantitative variations of goat αS1-casein. Genetics Selection Evaluation 19: 399-412.
13. Lowry OH, Rosenbrough NJ, Farr AL, Randall RL (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
15. O’Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007-4021.
16. Holland JW, Deeth HC, Alewood PF (2004) Proteomic analysis of kappa-casein micro-heterogeneity. Proteomics 4: 743-752.
17. Clare DA, Swaisgood HE (2000) Bioactive milk peptides: a prospectus. J Dairy Sci 83: 1187-1195.
18. Brody EP (2000) Biological activities of bovine glycomacropeptide. Br J Nutr 84: S39-S46.
19. Fox PF, McSweeney PLH (1998) Dairy chemistry and biochemistry. Kluwer Academic Publishers, New York.
20. Baeker R, Haebel S, Schlatterer K, Schlatterer B (2002) Lipocalin-type prostaglandin D synthase in milk: a new biomarker for bovine mastitis. Prostaglandins Other Lipid Mediat 67: 75-88.
21. Yamada M, Murakami K, Wallingford JC, Yuki Y (2002) Identification of low-abundance proteins of bovine colostral and mature milk using two-dimensional electrophoresis followed by microsequencing and mass spectrometry. Electrophoresis 23: 1153-1160.
22. Morakami K, Lagarde M, Yuki Y (1998) Identification of minor proteins of human colostrum and mature milk by two-dimensional electrophoresis. Electrophoresis 19: 2521-2527.