Advances in Dairy Research
ISSN: 2329-888X

Sharma et al., J Adv Dairy Res 2017, 5:4
DOI: 10.4172/2329-888X.1000191

Research Article Open Access

Identification of Bioactive Peptides in Goat Milk and Their Health Application

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Received date: 18 August, 2017; **Accepted date:** 03 October, 2017; **Published date:** 10 October, 2017

Abstract

We have identified the bioactive peptides in goat milk using two dimensional gel electrophoresis and mass spectrometry. The milk samples were collected from three healthy breeds of goats at CIRG farms. The milk proteins were isolated using simple centrifugation and were evaluated on the basis of presence of other proteins variants using SDS PAGE and two dimensional gel electrophoresis. The protein profiles of all the milk proteins were same showing the presence of all the major caseins proteins and whey proteins whereas the two dimensional gel electrophoresis resulted gels has shown the presence of various protein spots. These protein spots were analysed under mass spectrometry and found biologically active peptides dipeptidyl peptidase, transcription factor A, Interleukin 12 subunit, oligodendrocyte transcription factor 2. In our study we have reported the biological significance of these peptides identified and their role in human health such as mitochondrial disease, brain malignancies, signaling processes of cytokines and help in generating cytotoxic lymphocyte helps enhance immunity.

Keywords: Bioactive peptides; Milk proteins; Two dimensional gel electrophoresis; Mass spectrometry; MALDI-TOF

Abbreviations MALDI: Mass Assisted Lasor Desorption/ Ionization; TOF: Time of Flight; 2-DE: Two dimensional gel electrophoresis; SDS: Sodium do decyl sulphate; PAGE: Polyacrylamide gel electrophoresis; DTT: Dithiothreotol; IAA: iododacetamide; MS: Mass spectrometry; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information

Introduction

Goat milk has been as rich supplier of proteins which possesses high immunogenicity, important nutritive value, great biological value, incredible amino acid profile and various bioactive peptides which show great properties. The main protein sections in goat milk include caseins, whey proteins, immunoglobulins, lactoferrin and many peptide fractions. Milk proteins and their peptides exert a wide range of biological functions [1-3].

Milk derived bioactive peptides are the specific peptides which are produced by the degradation of milk proteins by different proteases and possesses different positive properties which benefits the different health functions of the living organisms [4,5]. These peptides bears the antihypertensive, antithrombotic, immunomodulatory, antibacterial, antifungal, antiviral, antioxidant, binding and transporting metals, preventing amnesia and causing smooth muscle contractions properties [6-8].

Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is the potential tool to identify and quantify the various proteins present in mixture [9-15]. The protein samples are absorbed in the matrix composed of non-volatile material which is then irradiated using lasor beam which thus results in the formation of vaporized ion. The main advantage of using this technique is only small quantity of sample is required for the experimentation and used for the analysis of heterogenous samples such as milk [16]. In addition, it possesses a very high sensitivity of mass range of up to 300,000 Da for proteins [17].Thus, this study is entitled to study the presence of bioactive peptides in goat milk proteins using two dimensional gel electrophoresis (2-DE) and Mass spectrometry (MS) and their influence on human health.

Material and Methods

Sample collection

Milk samples were collected from lactating healthy goat Jamnapari (n=35), Barbari (n=35), Jakhrana (n=20) and maintained at -20°C for further analysis at CIRG laboratories, Makhdoom, Mathura. These milk samples were centrifuged at 12,000 g for 10 minutes at -4°C. The fat layer was then carefully removed. The milk serum was extracted from below the creamy layer and was used for further protein characterization processes. The concentrations of protein of milk samples were estimated by Lowry et al. [18].

SDS PAGE

The protein milk samples were analyzed on SDS-PAGE. The variant gene products at casein loci and whey proteins were identified and selected for two dimensional gel electrophoresis for further quantification of milk proteins.

Two dimensional gel electrophoresis

The two dimensional gel electrophoresis (2-DE) experimentation separates the milk proteins on the basis of two dimensions i.e. Iso electric point and Molecular weight. The first dimension, Iso Electric Focusing was performed on Ettan IPGphor system (IPG strip, serva, pH range 3-11). The milk samples were centrifuged at 12,000 g for 10 minutes at -4°C and the serum layer was carefully taken out and quantified [18]. The 100 µg of protein samples were subjected to the analytical gels after the dilution with rehydration buffer (8 M Urea, 0.002% bromophenol blue, 2% w/v CHAPS, 3 mg dithiothretol, 0.5-2% ampholyte pH 3-10) (GE healthcare handbook). After a short vortex, 130 µl of the sample buffer were subjected to in gel-rehydration of each IPG strips (pH 3-11, 7 cm long IPG strips) and kept overnight for 16 hours at room temperature. After full incorporation of the sample buffer, IPG strips were transferred to IPGphor II. Then, iso electro focusing was then performed at 20°C by a series of constant watt voltage steps as follows: 0.1 W, 1:00 hrs; 0.5 W, 8000 Vhrs; 1000 V, 1:00 hours. After the first dimension gel run, strips were prepared for second dimension run. In the Second dimension, SDS-PAGE was performed on mini gel SE 260. Strips were initially immersed into a SDS Equilibration Buffer (Urea 6 M, Tris HCl 2%, Bromophenol Blue 0.002%) for 15-20 minutes and then loaded into a SDS Gel [19].

Protein visualization and image analysis

After the 2-DE process, gels were silver stained [20], so as the low amount of protein spots can be visible. Spot detection and quantification were performed with Image Master-II software (Amersham). The volume of each spot (integrated optical density) was calculated as the product of spot area and spot intensity. After intensity calibration, spot detection, background subtraction, normalization and one dimension calibration, the pI and molecular weight of each spot were calculated, followed by the identification based on MALDI-TOF-MS.

Mass spectrometry

Trypsin digestion: The selected spots were excised from the 2-DE gels followed by digestion of the proteins by trypsin enzyme. The gel pieces were de-stained using de-staining solution for 10 minutes and this process was repeated 3-4 times until the gel pieces become translucent white. The gels were dehydrated using acetonitrile and speedvac till complete dryness. The gel pieces were rehydrated with dithiothretol (DTT) and incubated for one hour. After incubation the DTT solution was removed. The gel pieces were now incubated with iodoacetamide (IAA) for 45 minutes. The supernatant was removed and the gel was incubated with ammonium bicarbonate solution for 10 minutes. The supernatant was removed and the gel was dehydrated with acetonitrile for 10 minutes and speedvac till complete dryness. The trypsin solution was added and incubated overnight at 37°C.The digest solution was transferred to fresh eppendorf tubes. The gel pieces were extracted thrice with extraction buffer and the supernatant was collected each time into the eppendorf above and then dried completely (speedvac). The dried peptide mixture was suspended in tris acetate (pH 8.3) buffer.

MALDI TOF: The peptide solution obtained after trypsinization was mixed with a matrix solution of α-cyano-4-hydroxycynnamic acid (HCCA) solution in a 1:1 ratio and the resulting 2 µl was spotted onto the MALDI plate. The lasor pulse duration was 1-5 nanoseconds (ns) and laser frequency was 100 Hz. After air drying the sample, it was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument and further analysis was done with ULTRAFLEX ANALYSIS SOFTWARE for obtaining the PEPTIDE MASS FINGERPRINT. The masses obtained in the peptide mass fingerprint were submitted for Mascot search in concerned database for identification of the protein. For the Mascot searches of the peptide other mammalian parameter was used. Chemical modifications such as carbamidomethylation of cysteine and oxidation of methionine were taken into account. For Protein Identification, MASCOT was found to be most suitable database search engine. The peptides which were identified using MASCOT were then underwent BLAST to determine the peptides are conservative or non-conservative.

Results

Two dimensional gel electrophoresis analysis

The 2-DE resulted gels were analyzed under Gel Documentation system Alpha Innotech Corporation (USA) corresponds to the protein which has been identified by MALDI TOF. Two-dimensional electrophoresis of milk samples from goat breeds shown in Figure 1 illustrated the presence of protein spots in individual milk protein sample. Each protein milk sample bears many minute proteins and the major protein spots which have been identified on 2-DE gels were α-S2 casein, β-casein, κ-casein, β-lactoglobulin and α-lactalalbumin. Casein proteins showed wavy bands on gels where as whey proteins were more distinct. Each protein variant comprises of two parts-the concentrated form and the diffused part, which were unidentified. Two large spots which dominated the gel analyzed as β-casein whereas κ-casein protein showed a complex picture. It is clear that the vast majority of protein spots on the 2-DE gels of milk samples identified. The analysis of gels using Image Master II software has identified the presence of total 49 protein spots in gel 1, 40 protein spots in gel 2 and 43 protein spots in gel 3. Different protein patterns in 2-DE gels were studied from different species shows that animal may possesses differences may be due to heterogeneity in isoforms of milk proteins which may be subjected to post translational modifications.

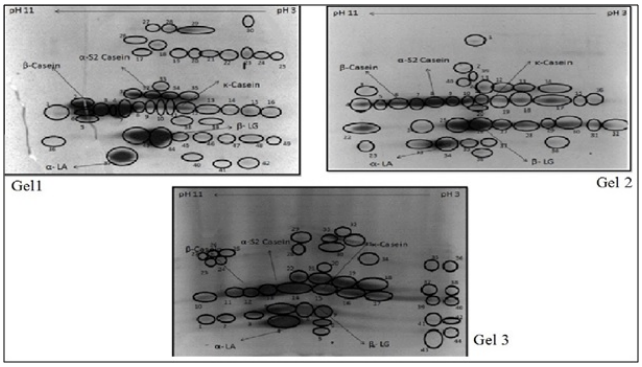

Figure 1: Electrophoretic pattern of Milk Proteins (Gel 1, Gel 2, Gel 3): This image shows the silver stained electrophoretic pattern of goat milk protein on two dimensional gel electrophoresis. The major protein sections of the milk proteins have been labelled and the circled spots show the protein spots present in the protein.

Protein Identification by mass spectrometry

The trypsin digested peptides have been analyzed under MALDI TOF to generate the peptide sequence and to identify the peptides using NCBI BLAST under the mammalian protein database. Here, we have identified four new peptides in Jamnapari goat breed and are listed in Table 1; and their respective mass spectra have been shown in Figures 2-10. The identified peptides are Transcription factor A showing the peptide sequence DTEEC, Oligodendrocyte transcription factor 2 (IATLLLAR), Interleukin-12 subunit alpha precursor (IL-12A) (TSETK), Dipeptidyl-peptidase 1 precursor (IATLLLAR).

Figure No.	Spot No	Mass	Protein Name	Peptide Sequence	Status
Gel 1	4	24104	Oligodendrocyte transcription factor 2	IATLLLAR	Unique peptide
Gel 1	44	28879	Transcription factor A	DTEEC	Unique peptide
Gel 2	22	25272	Interleukin-12 subunit alpha precursor	TSETK	Unique peptide
Gel 2	32	50121	Dipeptidyl - peptidase 1 precursor	IATLLLAR	Unique peptide
Gel 2	36	31685	Caspase-3 precursors	TSETK	100% similarity with Caspase-3 precursor
Gel3	5	24347	α-S1 Casein precursors	YLGYLEQLLR	100% similarity with α S1 Casein precursor
Gel 3	22	26486	α-S2 Casein	TNAIPYVR	100% similarity with α S2 Casein
Gel 3	21	26528	α-S2 Casein	NMAIHPR	100% similarity with α S2 Casein
Gel 3	19	26486	α-S2 Casein type c	NMAIHPR	100% similarity with α S2 Casein type A, α S2 Casein

Table 1: Bioactive Peptides: List of bioactive peptides found in Goat Milk.

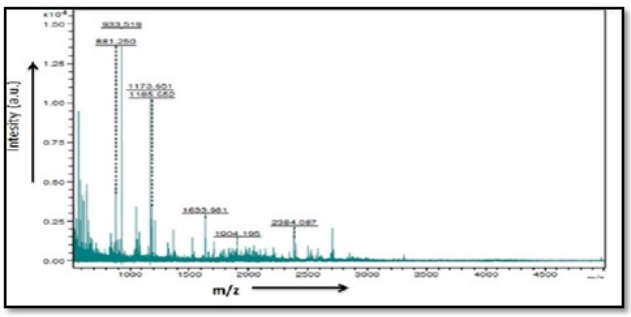

Figure 2: Mass Spectrum of protein α-S2 casein c peptide.

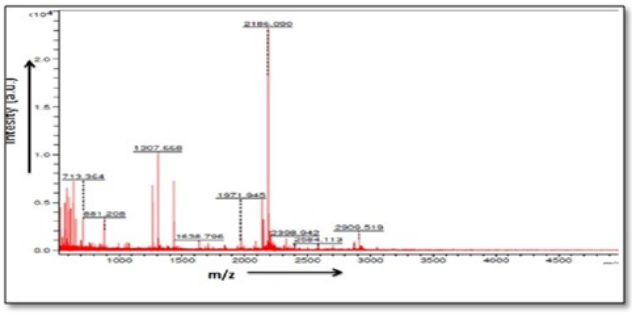

Figure 3: Mass Spectrum of protein α-S1 Casein precursor.

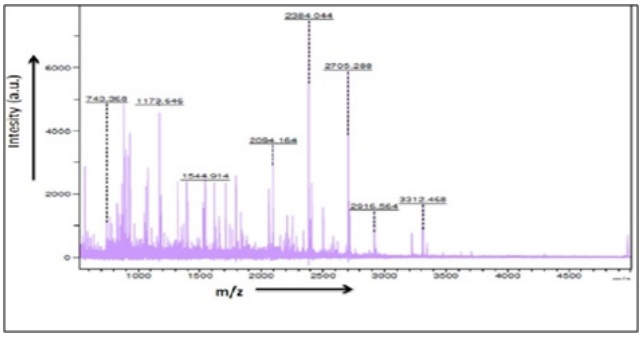

Figure 4: Mass Spectrum of protein Alpha S2 Casein.

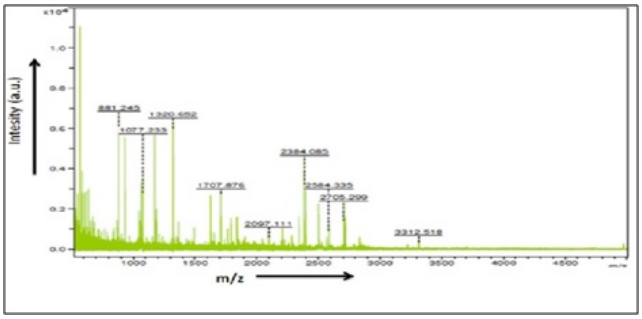

Figure 5: Mass Spectrum of protein Alpha S2 Casein.

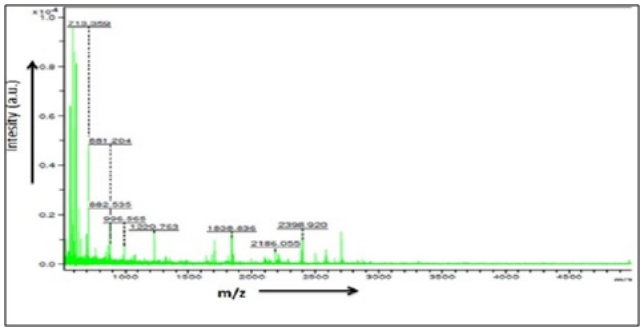

Figure 6: Mass Spectrum of protein Caspase 3 precursor.

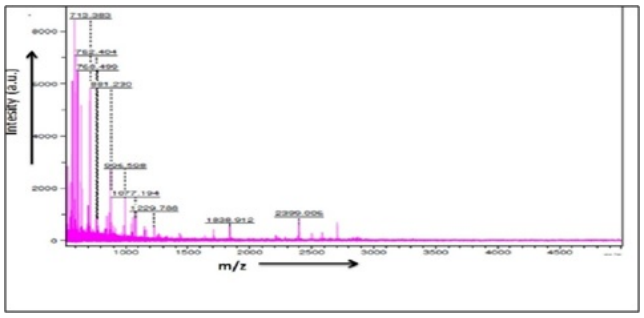

Figure 7: Mass Spectrum of protein Dipeptidyl peptidase 1 precursor.

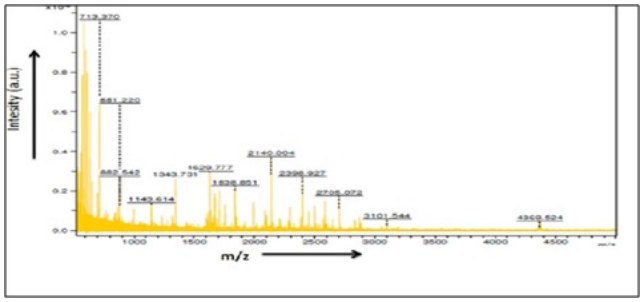

Figure 8: Mass Spectrum of protein Interleukin 12 subunit alpha precursor.

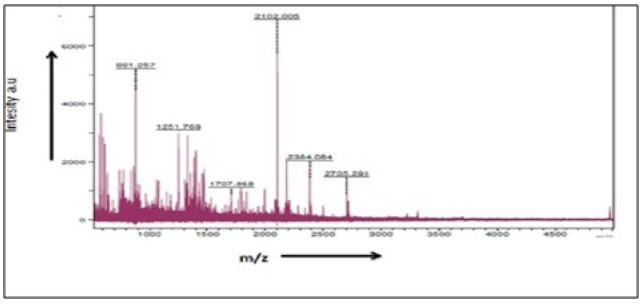

Figure 9: Mass Spectrum of protein Oligodendrocyte transcription factor 2.

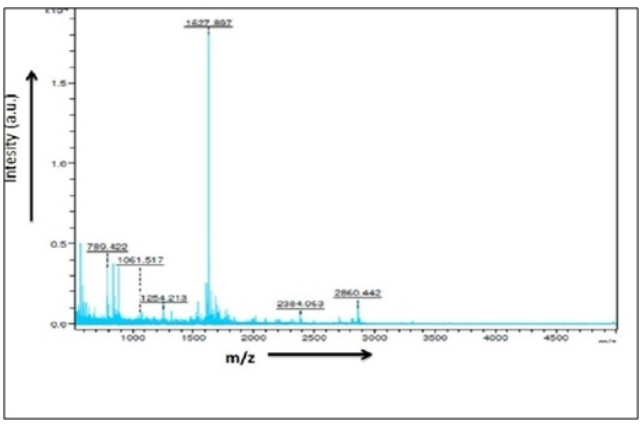

Figure 10: Mass Spectrum of protein Transcription factor 2.

These identified peptides have not been reported yet in *Capra hircus* and thus we have reported them as unique peptides and explained their biological significances in other species. The other identified peptides have been identified as known peptides. The Alpha S2-casein type c of Jamunapari breed with peptide sequence NMAIHPR have 100% similarity with alpha S2-casein type A, alpha S2-casein *Ovis aries* (sheep) and alpha S2-casein of *Capra hircus* (goat). No variations among these peptides were observed indicating that the peptide can highly conserved. The Alpha S2-casein of Jamunapari breed with peptide sequence NMAIHPR has 100% similarity with Alpha-S2-casein *Ovis aries*. The Alpha-S2-casein of Jamunapari breed with peptide sequence TNAIPYVR have 100% similarity with Alpha-S2-casein *Ovis aries*. The Alpha S1-casein precursors of Jamunapari breed with peptide sequence YLGYLEQLLR had 100% similarity with Alpha-S1-casein precursor *Ovis aries*. The Caspase-3 precursors of Jamunapari breed with peptide sequence TSETK have 100% similarity with Caspase-3 precursor *Bos taurus* (cattle).

Discussion

The most familiar and general approach to predict protein function is to trace the functions of the proteins with similar peptide sequences carrying out similar functions [21]. Majority of proteins has shown approximate similar function on the basis of sequence similarity to proteins with known functions [22-24]. Here, in our current study, we

have found peptides which may show a similar function as with similar sequence.

Transcription factor A (DTEEC) gene encodes a mitochondrial transcription factor protein which is a prime activator of mitochondrial transcription process as well as it participates in mitochondrial genome replication and repair. It bends mitochondrial promoter DNA to aid transcription of the mitochondrial genome. Many studies has supported that the gene product attains a role in regulating the mitochondrial genome copy number and is essential for embryonic development [25]. It may play a role in organizing and compacting mitochondrial DNA. Decrease in the activity of Transcription Factor A is linked with Kearns-Sayre syndrome which is a mitochondrial disease characterized by pigmentary retinitis and progressive external ophthalmoplegia (PEO). Oligodendrocyte transcription factor (OLIG2) is encoded by the Olig2 gene and comprises of 329 amino acids in length, 32kDa in size and contains 1 basic helix-loop-helix DNA-binding domain. The OLIG2 acts as an anti-neurigenic and a neurigenic factor at different stages of development in central nervous system. It is known for determining motor neuron and oligodendrocyte differentiation [26] and has a potent role in sustaining replication in early development. It is mainly involved in diseases such as brain tumor and Down syndrome. Interleukin 12 subunit alpha Precursor (Cytotoxic Lymphocyte) gene encodes a subunit of a cytokine that acts on T and natural killer cells, and has a multiple biological activities. The cytokine is a disulfide-linked heterodimer composed of the 35-kD subunit encoded by IL-12 gene, and the other 40-kD subunit is a member of the cytokine receptor family. This cytokine is required for the T-cell-independent induction of interferon (IFN)-gamma, and is important for the differentiation of both Th1 and Th2 cells. Dipeptidyl peptidase (DPPI) is a lysosomal cysteine protease plays an important role in the processing of granzymes which are neutral serine proteases exclusively expressed in the granules of activated cytotoxic lymphocytes. Cytotoxic assays with DPPI effector cells reveal severe defects in the induction of target cell apoptosis at both early and late time points. DPPI therefore plays an essential role in the in vivo processing and activation of granzymes A and B, which are required for cytotoxic lymphocyte granule-mediated apoptosis [27]. Caspase 3 is a member of the cysteine-aspartic acid protease which is encoded by the casp3 orthologs. Caspases are sequencially activated and plays an important role in the cell apoptiosis. They exist as inactive proenzymes which undergo proteolytic processing at aspartic residues to produce two subunits large and small which dimerizes to form the active enzyme. It is the predominant caspase responsible for the cleavage of amyloid-beta 4A precursor protein which is linked with neuronal death in Alzheimer disease. Milk derived [28] peptides play vital roles in human health and nutrition. Numerous studies are dedicated to the casein fractions of the milk protein which accounts for 80% of milk total protein and is a rich source of bioactive peptides that stimulate and aid the immune system [29-32].

Conclusion

This study shows the advancement of proteomic studies in milk proteins and explains the major application to search the biological efficiency of the milk proteins. We have concluded the presence of novel bioactive peptides in goat milk showing the biological importance of goat milk. The peptides found in our study have shown a parallel connectivity with the same peptides present in other mammals and lays a foundation of expected effective biological significance for human health. Oligodendrocyte Transcription factor 2 peptide shows an effective role in repairing of the central demyelinating after injury in other organisms. Various other studies has supported that Olig2 has the potential to enhance the recovery period. Thus this study ensures the presence of cure of central demyelinating diseases from Olig2 peptides. IL-12 alpha precursors peptide shows an association with primary biliary cirrhosis and many other autoimmune diseases. DPPI plays a potential role in activating granzymes A and B which tends to activate the apoptosis in mitochondria. Thus, this study opens the gateways for further scope of this research. Further clinical trials should be carried out for the novel milk peptides to understand the clear biological role of these peptides in goat milk for the human health benefits.

Acknowledgements

We thank Dr. Rout (CIRG, Makhdoom, Uttar Pradesh) for their excellent technical assistance; Dr. Gajendra Singh for showering his guidance to achieve the best work out of all and Mr. Rakesh and all the Laboratories staff who worked collectively to make this study progressive.

Ethics Approval and Consent to Participate

This study is entitled to study the goat milk proteins. These goats are available in the CIRG farms, Makhdoom and are in healthy condition. To conduct our study we just withdraw milk from goats which doesn't made any harm to them and thus the ethics approval is applicable here.

Funding

The work is solely funded by own for the study design, collection and analysis of data, data interpretation or in writing the report.

Competing Interests

The authors declare that they have no competing interests.

Consent for Publication

This manuscript does not contain any details, images, or videos relating to individual participants.

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