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Insulin like protein from camel milk and similarity with human insulin

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ABSTRACT

From experimental studies, clinical studies and epidemiological studies it is proved beyond doubt that camel milk has potential role in prevention and treatment of diabetes. The main purpose of the study to isolate camel milk protein and compare its similarity with human insulin so that camel milk can be used as an adjunct therapy for diabetes. Raw camel milk (30 ml) was used for isolation of protein and peptides. The complete process included trypsin digestion, peptide fractionation and LC-MS technique. Digested and fractionized peptide sample was processed further for liquid chromatography and mass spectra/ tandem mass spectra were recorded in positive-ion and high sensitivity mode. MS/MS spectra were automatically calibrated during dynamic LC-MS. Raw data files were converted to Mascot Generic Format (MGF) and these MGF files were searched against UniPort, NCBI and common MS contaminant database. In our study 13 proteins and 22 peptide sequences were found similar to insulin/ insulin like growth factor and isoform. In our study some very large peptide sequence were identified which were seen similar to NUAK family SNF1-like kinase and this peptide sequence gives evidence of role of camel milk in cancer treatment. Observing so many similar peptides in camel milk sample with human insulin, isoform of insulin, receptors and others give strong evidence that camel milk have proteins/ peptides of such proteins similar to human insulin and give support to finding that camel milk contains insulin like molecule that mimics insulin interaction with its receptors.

Keywords: Camel milk, Diabetes, ILP, LCMS, Peptides

For centuries, camel (*Camelus dromadarium*) milk is used medicinally by the nomadic people as it is quite closer to human mother's milk and better than cow's milk. It is opaque with a sweet sharp taste but sometimes it can be salty. Its density ranges from 1.026 to 1.035 and the *p*H from 6.2 to 6.5, both are lower than those of the cow's milk. It sours slowly and can be kept longer without refrigeration as compared to cow's milk. It contains lower fat, cholesterol, and lactose. It also contains higher minerals (calcium, iron, magnesium, copper, zinc, and potassium) and vitamins A, B_2, C and E as compared to cow's milk and it contains no beta lactoglobulin and beta casein as are present in cow's milk which are the main causatives of allergy in humans.

Camel milk contains a number of protective proteins which keep the body healthy. Casein and whey proteins in camel's milk are between 72–76% and 22–28% respectively. They consist of strong antibacterial, antiviral and antifungal substances and immunoglobulins. There are also some tissue repair substances among the protein protectors in camel milk. Camel milk contains a protein which is similar to insulin, which has been found to be beneficial.

As camel milk contains tissue repairing proteins, insulin is not destroyed in the stomach and is absorbed rapidly from the intestine into the blood where it reduces blood sugar. It affects blood sugar and lipid profile of patients with type 2 diabetes.

Prior to protein identification of camel milk and observing its similarity with human insulin many clinical studies were done which gave a strong base to work in the same direction. One of the clinical studies was on camel milk effect on glycemic control of T1DM subjects and the result showed that after 3 months there was improvement in fasting blood glucose, HbA1c and reduction in mean dose of insulin. The study was further carried for 6 months, 1 year and 2 years as a randomized controlled trial. Some other studies were also done on effect of camel milk on residual beta cell function. It was found that Camel milk also have beneficial role in type 2 diabetes as well. After observing beneficial effect of camel milk, identification of peptides of camel milk protein which may be similar to human protein peptide was done.

MATERIALS AND METHODS

To isolate peptides and protein sequence of came milk 30 ml raw camel milk was used. For isolation and identification of camel milk protein various steps were performed which included: Trypsin digestion, Strong Cation Exchange (SCX) peptide fractionation, LC-MS/MS analysis

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by AB Sciex 6600 and Standard mascot search.

Trypsin digestion: Proteins from raw milk were resuspended in 8 M urea and reduced with 20 mM DTT in 60°C for 1 h. Proteins were then alkylated with 40 mM IAA at room temperature for 30 mins protected from light. Alkylation reaction was quenched by 10 mM DTT. Samples were diluted to 2 M urea with HPLC grade water. Protein concentration of the sample was determined by modified Lowry's assay (DC Assay Kit, Cat. 500-0111, BioRad) with the use of bovine serum albumin (BSA) to construct calibration curve. Appropriate amount of trypsin was then added to the sample in an enzyme-to-substrate ratio of 1 : 100. Digestion was performed in 100 mM triethylammonium bicarbonate (*p*H 8, T7408, Sigma) at 37°C for 18 h.

Strong Cation Exchange (SCX) Peptide Fractionation: After de-salting, the peptide samples were re-suspended in SCX buffer A (10 mM KH_2PO_3, 20% ACN, *p*H 2.7). SCX was performed with Polysulfoethyl A™ (200×4.6 mm, 200A) column using a step gradient (0–10 mins: 0% B, 29 mins: 15% B, 44 mins: 45% B, 46–53 min: 100% B) of buffer A and buffer B (10 mM KH_2PO_3, 20% ACN, 0.6 M KCl, *p*H 2.7) at a flow rate of 1 min/ml. In total, 53×1 ml fractions were collected. These fractions were further combined into 5 fractions and de-salted with ZipTip (Cat. ZTC18S960, Millipore) based on the amount of proteins in each fraction.

LC-MS/MS Analysis by AB Sciex 6600: Each dried peptide sample was dissolved in 12 µl of 0.1% FA. Samples were further analyzed by nanoLC-MS/MS using an Eksigent ekspert™ nanoLC 425 system coupled to AB Sciex TripleTOF® 6600 System. After sample was loaded, peptide is trapped (ChromXP nanoLC Trap column 350 µm id × 0.5 mm, ChromXP C18 3 µm 120Å) and elutes at a flow rate of 300 nL/min into a reverse phase C18 column (ChromXP nanoLC column 75 µm id × 15 cm, ChromXP C18 3um 120Å) using a linear gradient of acetonitrile (3–36%) in 0.1% formic acid with a total runtime of 120 mins including mobile phase equilibration. Mass spectra and tandem mass spectra were recorded in positive-ion and "high-sensitivity" mode with a resolution of ~35,000 full-width half-maximum. The nanospray needle voltage was typically 2,300 V in HPLC-MS mode. After acquisition of ~ 5 to 6 samples, TOF MS spectra and TOF MS/MS spectra were automatically calibrated during dynamic LC-MS and MS/MS auto-calibration acquisitions injecting 25 fmol alcohol dehydrogenase. For collision induced dissociation tandem mass spectrometry (CID-MS/MS), the mass window for precursor ion selection of the quadrupole mass analyzer was set to ± 2 m/z. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. Advanced information dependent acquisition (IDA) was used for MS/MS collection on the TripleTOF 6600 to obtain MS/MS spectra for the 20 most abundant and multiple charged (z = 2, 3 or 4) following each survey MS1 scan allowing typically for 250 msec acquisition time per each MS/MS. Dynamic exclusion was set for 30 secs after 2 repetitive occurrences.

Standard mascot search: Raw data files were converted to Mascot Generic Format (MGF) and mzXML format using msconvert. The MGF files were searched against the UniProt, NCBI and common MS contaminant database using Mascot 2.5 (Matrix Science) Software. The tolerance for MS1 and MS2 error was 50 ppm and 0.05 Da respectively. Carbamidomethylation (+57 Da) was added as fixed modification while oxidation (Specificity: M, Delta: +16 Da). A maximum of 2 trypsin miss cleavages are allowed in the process. Peptides were assumed to have a charge of 2+, 3+ or 4+. The instrument type was chosen as ESI-FTICR. The mass input was assumed to be monoisotopic mass. Decoy database was used to control the false discovery rate (FDR) under 1%.

RESULTS AND DISCUSSION

In our study 13 proteins and 22 peptide sequences were found similar to insulin/ insulin like growth factor and isoforms whereas, in another study by J Wangoh *et al.* identified 6 human peptides sequences in camel milk (3 types of camel) which were similar to human insulin.

In Table 1 peptide sequence SGMKELAVFR was found similar to human insulin like growth factor binding protein 2 (GN=IGFBP2). This insulin like growth factor works as binder to protein for further metabolism. On the other hand MNMLGGGGSAGRKPLK was also found in our camel milk protein sample and was seen similar to the GF binding protein 2 i.e. GN=IGFBP2. Peptide sequence KPCTRSLTCK was seen similar to isoform 3 of Ataxin 7, only Ataxin 7 and also with isoform B of Ataxin 7. This peptide sequence was seen more frequent in camel milk protein sample.

Whereas EMKDLVK similarity was seen in two different proteins of human that is isoform B of 1 phosphatidylinositol 4,5-bisphosphate phosphodiesterase and in 1 phosphatidylinositol 4,5-bisphosphate phosphodiesterase, i.e. it is similar to protein and its isoform and this gives more confirmation that the peptide sequences which were obtained from camel milk sample are seen similar to human insulin.

As we know, isoforms are functional similar proteins but they have different amino acid sequences so if any protein sequence is found similar to original protein and there isoform it gives conformational evidence of similarity between the same.

In our study we observed that some peptide sequences of camel milk sample were found similar to human insulin receptor substrate 1 OS whereas some peptide sequence like ENDFAQSGQDAVPESPSKLSSKRPK was longest among all the identified sequences and was seen similar to NUAK family SNF1-like kinase. This protein, i.e. NUAK family SNF1-like kinase helps in regulation of cell adhesion, proliferation, metal ion binding and p53 binding in humans. So this peptide sequence, which is the longest one, has similarity with NUAK family and gives evidence of role of camel milk effect in cancer treatment.

Table 1. Peptide sequence of camel milk and similarity with human insulin / receptors

Peptide Sequence	Human Insulin/ Receptors
Ser-Gly-Met-Lys-Glu-Leu-Ala-Val-Phe-Arg (SGMKELAVFR)	IGFBP2 PE=1
Met-Asn-Met-Len-Gly-Gly-Gly-Gly-Ser-Ala-Gly-Arg-Lys-Pro-Len-Lys (MNMLGGGGSAGRKPLK)	IGFBP2 PE=1
Lys-Pro-Cys-Thr-Arg-Ser-Leu-Thr-Cys-Lys (KPCTRSLTCK)	ATXN7 ISOFORM 3ATXN7 PE=1ATXN7 ISOFORM b
Glu-Met-Lys-Asp-Leu-Val-Lys (EMKDLVK)	PLCB1 ISOFORM bPLCB1
Glu-Asn-Asp-Phe-Ala-Gln-Ser-Gly-Gen-Asp-Ala-Val-Pro-Glu-Ser-Pro-Ser-Lys-Leu-Ser-Ser-Lys-Arg-Pro-Lys (ENDFAQSGQDAVPESPSKLSSKRPK)	NUAK1 PE=1
Gly-Val-Cys-Leu-Asn-Glu-Lys-Ser-Tyr-Arg (GVCLNEKSYR)	IGFBP5 PE=1
Glu-Gen-Val-Lys-Ile-Gly-Cys-Aea-Pro-Gly-Gly-Gen-Arg (EQVKIGCAPGGQR)	IGFBP5 PE=1
Ile-Ile-Ser-Ala-Pro-Glu-Met-Arg-Gen-Gen-Ser-Glu (IISAPEMRQESE)	IGFBP5 PE=1
Gen-Phe-Arg-Gly-Aea-Val-Gly-Glu-Gen-Leu-Gly-Iys (QFRGAVGEQLGK)	TRIM72 PE=1ISOFORM 2 OF TRIM72
Arg-Val-Glu-Cys-Sen-Glu-Gen-Lys-Ael-Pro-Pro-Ala-Gly-Geu-Asp-Pro-Arg (RVECSEQKAPPAGEDPR)	TRIM72 PE=1
Gen-Phe-Arg-Gly-Aea-Val-Gly-Glu-Gen-Ieu-Gly-Lys (QFRGAVGEQLGK)	TRIM72 PE=1ISOFORM 2 OF TRIM72
Leu-Pro-Ile-Leu-Cys-Thr-Lys (LPILCTK)	PAPPA2 PE=2
Met-Leu-Gen-Asn-Val-Gen-Met-Pro-Ser-Lys (MLQNVQMPSK)	DPP4 PE=1

Similarly, GVCLNEKSYR, EQVKIGCAPGGQR and IISAPEMRQESE all three were similar to insulin like growth factor binding protein 5. On the other hand, peptide sequences QFRGAVGEQLGK and RVECSEQKAPP-AGEDPR were similar to Tripartite motif-containing protein 72 of human. This tripartite motif is specific protein and helps in membrane repair by nucleating assembly of repair machines of injury site. Now similarity of our identified peptide sequence with motif protein 72 gives support in medicinal value of camel milk not only in diabetes but also in diabetic cardiomyopathy. Whereas QFRGAVGEQLGK is seen similar to Isoform 2 of Tripartite motif-containing protein 72.

Beg O U *et al.* also worked on camel milk whey protein and in this study they did complete structural comparison of camel protein with rat protein whereas in our study we compared raw camel milk with human insulin.

Lastly, LPILCTK was similar to Pappalysin-2 of humans. This pappalysin-2 is protein coding gene and helps in regulation of insulin like growth factors and their transportation. Peptide sequence MLQNVQMPSK was seen similar to Dipeptidyl peptidase 4 of humans.

A similar yet different study was done by Mallik *et al.* on antidiabetic agent of camel milk in which he had done sequence alignment of B and A chain of different types of insulin (which include camel milk and human) and found that camel milk contains insulin like molecule that mimics insulin interaction with its receptors. The following insulin similar peptides were identified having similarity with the samples (Table 1).

In this study we have observed similar peptides in camel milk with human insulin, isoform of insulin, receptors and others giving strong evidence that camel milk have proteins/ peptides of such proteins similar to human insulin and gives support to finding that camel milk contains insulin like molecule that mimics insulin interaction with its receptors.

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