Chapter 4

Research Methodology

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In order to accomplish the objectives mentioned in chapter 2, the procedures followed are detailed below.

4.1 Collection of milk sample

Camel milk samples were collected from Sarika Milk Bhandar, an outlet of NRCC, India from healthy *Camelus dromedarius.* The milk was collected aseptically into sterile containers directly by experts and transported to the laboratory in an ice box within few hours. It was then aliquoted and stored at -80ºC for further use.

4.2 Sample processing

4.2.1 Separation of whey and casein fractions from camel milk

Camel milk sample was defatted by centrifugation at 8000 rpm for 30 minutes at 4ºC to get skimmed milk. Casein was obtained from the skimmed milk by precipitation with 1 M hydrochloric acid till pH 4.6 at 22ºC. The samples were then centrifuged (Themoscientific) at 12000 rpm, 4ºC for 30 minutes. The precipitated casein was washed twice with distilled water and solubilized at pH 7.0 by addition of 1 M NaOH. The casein was then stored at -80ºC until analysis. The remaining supernatant, containing whey proteins was precipitated using ammonium sulphate. Whey supernatant was saturated with ammonium sulphate (273 g/L of whey supernatant) by constant stirring at 800 rpm to avoid foaming. The sample was kept at 4ºC, overnight. It was subsequently spun at 10,000 rpm, 15ºC for 15 minutes to obtain the whey pellet. Whey was further dialyzed using dialysis membrane-60 (HiMedia) with PBS buffer. This was followed by centrifugation at 10,000 rpm for 15 minutes. The pellet formed was diluted in 1X PBS Buffer.

4.2.2 Quantitation of milk proteins

Aliquots of skimmed milk, whey protein and casein protein were diluted 1:20 in PBS and protein content was estimated by Lowry's method using bovine serum albumin (Sigma, USA) as standard. The Lowry method of protein estimation is a colorimetric assay based on a blue purple color complex produced by the phenolic group of tyrosine and tryptophan residues present in the protein, when they react with Copper[II] ions and gets oxidized under alkaline conditions. This is followed by a subsequent reduction of phosphomolybdic phosphotungstic acid of Folin's reagent that gives rise to a blue purple color complex. The intensity of the color thus obtained is directly proportional to the concentration of aromatic amino acids present in the sample. This color complex shows a maximum absorption in the region of 740 nm. This method is sensitive down to even 10 µg/ml of protein concentration.

After preparation of 100 µl standard solution (BSA) at various concentrations and test solution of appropriate dilution in 1.5 ml Eppendorf tubes, 500 µl of Reagent C (*freshly prepared Reagent A and Reagent B mixed in the ratio of 50:1 respectively; Reagent A was prepared by adding 200 mg* Na_2CO_3 *and 40 mg NaOH to 10 ml distilled water; Reagent B was prepared by dissolving 50 mg* $CuSO_4.5H_2O$ *and 100mg Sodium potassium tartrate (*$C_4H_4KNaO_6$*) in 8 ml distilled water and then making up the volume to 10 ml*) was added to each sample followed by an incubation of 10 minutes. After which 50 µl of Reagent D was added to each sample. The samples were then mixed thoroughly and incubated for twenty minutes. This was followed by measurement of optical density (OD) of samples at 740 nm**.**

4.2.3 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of camel milk proteins

4.2.3.1. Protein separation

Proteins (skimmed camel milk, whey and casein) were separated by SDS-PAGE (Sodium dodecyl Sulphate - Polyacrylamide Gel Electrophoresis) on 12% gel. Prior to the electrophoresis, proteins were diluted to 2 µg/µl with the 5x sample buffer (reducing) and denaturation was done for 10 min. at 100°C. Twenty microliters of protein was added to each well, electrophoresis (Mini-protean 3 cell, Bio rad laboratories) was performed at 80 V in a vertical gel electrophoresis apparatus (Bio Rad). Separated proteins were identified using the wide range (6,500 – 200,000Da) molecular weight marker (Sigma #S8445).

Protein samples were separated with the aid of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 1.1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Electrophoresis was performed at 4.9% staking and 15.4% resolving polyacrylamide gels, running in 0.125M Tris–HCl pH 6.8 and 0.38M Tris–HCl pH 8.8 buffers, respectively.

The molecular weight standards (Sigma) that was used is given in Table 6

Table 6: Molecular weight standards

Marker	**Molecular weight (kDa)**
Myosin	200.0
Galactosidase	116.2
Phosphorylase b	97.4
Bovine serum albumin	66.2
Ovalbumin	45.0

Carbonic anhydrase	31.0
Trypsin inhibitor	21.5
Lysozyme	14.4
Aprotinin	6.5

4.2.3.2 Staining of polyacrylamide gel

Staining of gel was either done with Commassie blue or silver stain.

4.2.3.2.1 Commassie blue dye staining

The Coomassie staining works by binding of Coomassie dye to proteins via ionic interactions between the positively charged amine groups of proteins and sulfonic acid groups through Van der Waals attractions. In this procedure, first a 0.2% stock solution of Commassie stain was prepared by mixing 400mg of Commassie brilliant blue dye in 80ml sterile distilled water. It was then filtered and to the filtrate 120ml methanol was added, making up the volume to 200ml. A 0.1% working solution was prepared from this stock by adding 100ml of 20% acetic acid to 100ml of stock. After the SDS-PAGE run, the gels were transferred to 0.1% Coomassie solution which was then kept on a shaker for one hour at room temperature.

The stained gels were then transferred to Destaining solution 1 (for one hour) that was prepared by adding 40% methanol to 7% Glacial acetic acid in sterile distill water. The gels were then transferred to Destaining solution 2 (with gentle shaking for overnight) that had a composition of 5% methanol and 7% Glacial acetic acid.

Upon getting proper contrast between the gel and protein bands, the gels were photographed in Gel doc (BioRad)

4.2.3.2.2 Silver staining

After SDS-PAGE completion, the polyacrylamide gel was transferred to a tray containing 50ml fixing solution and was then incubated for ten minutes with continuous moderate shaking. Now the fixation solution was removed and the gel was given a wash with MilliQ water for 10 minutes in the same tray. The MilliQ water was discarded and 50ml of Fixing/Sensitizing solution was added to the tray containing the gel. It was then kept on a shaker for 5 minutes with gentle shaking. The fixing/Sensitizing solution was subsequently discarded followed by another wash with MilliQ water for ten minutes. Once more the gel was kept in 50ml of Fixing/Sensitizing solution for five minutes with continuous and gentle shaking. The fixing/Sensitizing solution was again discarded and the gel was given a wash with 40% ethanol and shaken for twenty minutes. This was followed by a wash of the gel with MilliQ water for twenty minutes. After the wash, the gel was treated with sensitizing solution and kept on a shaker for one minute. The sensitizing solution was then discarded and the gel was again given a wash twice with MilliQ water for one minute. The gel was then incubated in 50ml of silver stain for twenty minutes, ensuring the gel was completely submerged in the solution and was under continuous and gentle shaking. The gel was then removed from the staining solution and was washed with MilliQ water for one minute.

The gel was then transferred in a freshly prepared developing solution and kept on shaker with gentle shaking for one minute. This solution was then removed and fresh developing solution was added. The gel was kept in the solution until the color of separated protein bands developed and the bands were distinctly visible. The gel was then removed from the developing solution and was immediately transferred to the stopping solution with gentle shaking for 5 minutes to get sharp and distinct bands. The stopping solution was then discarded and the gel was washed with MilliQ water twice, each time for five minutes.

4.3 Column chromatography

4.3.1 Preparation of chromatography column

The chromatography column with diameter 1.5 cm and length 75 cm was properly washed with soap solution and rinsed thoroughly with autoclaved MilliQ water. It was then filled with 20% ethanol till use to avoid any microbial growth.

4.3.2 Preparation of gel beads (Sephadex G 100)

5g of Sephadex G 100 gel beads were submerged in 200ml sodium phosphate buffer. 0.02% sodium azide was added to the preparation. The whole preparation was then heated at 90°C for 5 hours. It was then allowed to settle and washed several times. After wash, the beads were degassed for 10 minutes and then gently poured into the column. The beads were then allowed to settle. After this the column containing beads was washed with three times the volume of the column.

4.4 FPLC

Camel whey sample and the buffer solutions were prepared by using standard protocols. Both the samples and buffers to be used with FPLC were filtered using 0.22µ filters and were the degassed before use.

The set protocols for pump and column wash of FPLC were then performed before loading the camel whey sample. The sample was then loaded onto the column of desired pore size. The flow rate was set to 0.5ml/minute. The column was then run and whey fractions were collected in sterile pre-labelled Eppendorf tubes at regular intervals.

Immediately after the procedure was over, the tubes of FPLC were washed with autoclaved milliQ water to avoid any protein precitation. After use, the column was washed with 5 water volume with autoclaved milliQ water. The column was then filtered with five column volume of 20% ethanol solution and was then stored in this 20% ethanol to avoid any bacterial growth.

4.5 Culture of HeLa cell line

4.5.1 Maintenance of cell line

HeLa, the human cervical cancer cell line was procured from NCCS, Pune, India. Cells were cultured at 37 ^{o}C, 5% CO_2 in RPMI (Invitrogen, ThermoFisher Scientific) supplemented with 10% fetal bovine serum or FBS (Invitrogen, ThermoFisher Scientific) and 1% penicillin-streptomycin mixture (Invitrogen, ThermoFisher Scientific). Cells were grown to 70-80% confluence prior to treatments. Trypsin- EDTA solution (sigma) was used for detachment of cells. A figure of cultured HeLa cells has been shown in Figure 5.

4.5.2 Cryopreservation of cells

Before cryopreservation, the cell sample was checked in advance for any sort of bacterial or fungal contamination by first growing the cells for two weeks in antibiotic free medium and then inspecting them under phase contrast microscope for any visual signs of contamination. Subsequently, the adherent cells were thoroughly washed with PBS to remove all dead cells and any traces of serum. 1 ml of 1X Trypsin EDTA solution (containing 0.05% Trypsin) was added and then evenly spread over the adhered cells in a 25 ml culture flask. The flask was then incubated in a CO_2 incubator for 1-2 minutes. As the cells detached from the surface of flask, 5ml of complete media was added to the incubated flask with gentle mixing to dilute trypsin EDTA and thus subside its activity. The contents of the flask were then transferred to sterile centrifugation tubes and were

centrifuged at 1500 rpm and 4°C for ten minutes. The supernatant was then discarded and the cells were resuspended in freezing media with gentle mixing. 1ml of freezing media containing cells was then transferred to each cryovial. The cryovials were properly labeled and were kept at -20°C for one hour. Then the vials were transferred to -80°C till further use.

4.5.3 Thawing of cells

The cryovials stored at -80°C were snap thawed by directly transferring the cryovials from -80°C to a 37°C waterbath. The contents of the thawed cryovials were transferred dropwise to a sterile centrifugation tube containing media to dilute the DMSO concentration. This was followed by centrifugation at 1500 rpm for 10 minutes and 4°C. The pellet of cells obtained was resuspended in 5 ml complete media and transferred to a 25 ml cell culture flask and the flask was then kept in CO_2 incubator. The media of flask was changed after 24 hours to remove any unadhered and dead cells as well as any traces of DMSO.

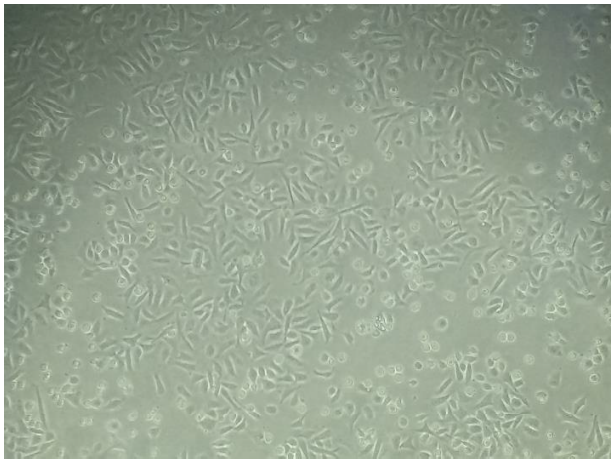

Figure 5: Cultured HeLa cells

4.6 MTT assay for cytotoxicity analysis

The cytotoxicity of samples against HeLa, the cervical cancer cell line was assessed by the MTT assay. The efficacy the samples to alter HeLa cells proliferation were determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) salt to its formazan crystals. HeLa cells were incubated at a concentration of 5.0 x 10^4 cells per well with increasing concentrations of camel milk, its whey and casein in a 96-well cell culture plate at 37ºC under a 5% CO_2 in a humidified incubator. After the required time of either 24 hours or 48 hours the media was aspirated. 20µl of MTT solution (5mg/ml in PBS) and 100µL of media was added to each well. The plate was then incubated in a 5% CO_2 incubator at 37ºC for 4 hours. To stop the reaction and dissolve the formed crystals, the media were then discarded and 150µL of DMSO was added to each well. The color intensity in each well was measured at 630 nm wavelength, using microplate reader (Multiskan#Thermo scientific). Each determination was carried out in triplicate and at least two independent experiments were carried out. Cell viability was calculated as percentage of the control wells using the following formula.

% Cell Viability = {O.D of treated cells/O.D of Control (without treatment)}*100

4.7 Cell migration assay

The scratch test was performed on HeLa cells to check the potential of milk or its derivative samples to control cell migration with respect to cisplatin and untreated control. For it, 3-4 ×10^6 cells were seeded in 6 well plate and treated for 24 hours. A scratch was made in the middle of plate and the cells were further incubated for 24 hours. After this the migration of cells across the boundary was observed microscopically to assess the inhibition of cell migration. Treatment of cells with cisplatin at its IC_{50} was used as a positive control and untreated cells were used as the negative control.

4.8 DAPI Staining

HeLa cells were treated with the IC_{50} of whey for 24 hours and the nuclear morphology of the cells was measured through fluorescence microscopy following DAPI staining. For this treated cells were washed with PBS, fixed with ice cold methanol for 5 minutes and washed 2 times with PBS. Next, cells were stained with 300nM DAPI stain, and incubated for 5 minutes at room temperature in dark. The DAPI stain was then removed and the cells were finally washed with PBS two times and visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The treated cells were compared with untreated control and cisplatin (IC_{50}) treated cells.

4.9 Caspase assay

Caspase-3 colorimetric protease assay kit (Invitrogen, ThermoFisher Scientific) was used to measure the caspase-3 enzyme activity that recognize the amino acid sequence (for caspase-3). The substrates provided for measuring the activity of these caspases are synthetic peptides which are labeled at their C-termini with para-nitroaniline (pNA). Upon cleavage of the substrates by caspases, absorption of light by free pNA can be quantified. For the measurement of caspase activity (effector of apoptosis) induced in treated cells, these cells were seeded in 60 mm dish at a concentration of 4×10^5 and treated with IC_{50} of whey and cisplatin respectively for 24 hours at 70% in eppendorf tubes. Isolated protein concentration was estimated by Bradford method. For obtaining the protein concentration of around 100µg/ml, 50µl of 2x reaction buffer with 10mM DTT was added and 5 µl of 4mM DEVD-PNA substrate was added and incubated for 2 hours at 37^{o}C in dark. Reading was taken at 405nm in microplate reader and fold change was calculated with reference to untreated control.

4.10 Fractionation of camel milk whey

Ten ml milk was centrifuged at 5000×g for 30 minutes at 10ºC, to remove the fat components. Whey was obtained after acid precipitation of caseins at a pH 4.6. This was done by adding 1ml of 10% (v/v) acetic acid and 1ml of 1M sodium acetate followed by centrifugation at 5000×*g* for 30 min at 10ºC. The pellet obtained was of casein and the whey proteins were present in the supernatant. The supernatant was extensively dialyzed against double-distilled water for 96 hours at 4ºC and applied to an ultra-filtration (UF) membrane of 30 kDa molecular mass cut-off (Amicon Ultra-15, Millipore, Carrigtwahill, Co. Cork, Ireland) and subsequently freeze dried using a lyophilizer.

4.11 LC-MS/MS analysis

This was used for identifying and determining the main characteristic features of proteins in camel milk whey samples. The whey sample for its analysis was outsourced to Vproteomics, New Delhi. For it, trypsin digestion of sample was followed by nano-ESI LC-MS/MS analysis.

4.11.1 Sample Preparation

25 microliter samples were taken and reduced with 5 mM TCEP and further alkylated with 50 mM iodoacetamide. It was then digested with Trypsin (1:50, Trypsin/lysate ratio) for 16 hours at 37°C. Digests were cleaned using a C18 silica cartridge to remove the salt and dried using a speed vac. The dried pellet was resuspended in buffer A (5% acetonitrile, 0.1% formic acid).

4.11.2 Mass Spectrometric Analysis of Peptide Mixtures

All the experiment was performed using EASY-nLC 1000 system (Thermo Fisher Scientific) coupled to Thermo Fisher-*QExactive* equipped with nanoelectrospray ion source. 1µg of the peptide mixture was resolved using 25cm PicoFrit column (360µm outer diameter, 75µm inner diameter, 10µm tip) filled with 1.8µm of C18-resin (Dr Maeisch, Germany). The peptides were

loaded with buffer A and eluted with a 0–40% gradient of buffer B (95% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min for 100 minutes. MS data was acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan.

4.11.3 Protein hydrolysate analysis by Proteome Discoverer

The whey sample were processed and RAW files generated were analyzed with Proteome Discoverer (v2.2) against the Uniprot *Camelus* reference proteome database. For Sequest search, the precursor and fragment mass tolerances were set at 10 ppm and 0.5 Da respectively. The protease used to generate peptides, *i.e.* enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") along with maximum missed cleavages value of two. Carbamidomethyl on cysteine as fixed modification and oxidation of methionine and N-terminal acetylation were considered as variable modifications for database search. Both peptide spectrum match and protein false discovery rate were set to 0.01 FDR.

4.12 In-silico comparative structural analysis of α-lactalbumin

4.12.1 Sequence Retrieval

The α-lactalbumin protein sequences are retrieved from NCBI for the four species, viz, *Camelus ferus* (camel), *Homo sapiens* (human), *Capra hircus* (goat), *Bos taurus* (cow). The sequence as well as structural similarity is computed between the four sequences, as explained subsequently.

4.12.2 Primary sequence comparison

Pairwise alignment of α-lactalbumin protein of all the selected species was constructed and assessed through the PRALINE multiple sequence alignment server (Bawono and Heringa, 2014). PRALINE is a fully customizable multiple and pairwise alignment application. Apart from several other currently available alignment strategies, it can integrate information from database homology

searches to generate a homology extended multiple alignment. The scoring scheme works within the range of 0 and 10, which respectively mark the least and the most conserved alignment position. The scoring is color-coded and it adds all the individual scores to obtain the final alignment score. The higher the final score, the more is the sequence identity.

4.12.3 Secondary structure comparison

The HHpred online server was used to screen the most closely related structural templates for all the selected protein sequences from the PDB database and the structures were retrieved. As the camel sequence showed a 70% sequence identity against the other three structures, it was modelled on the basis each of these templates on basis of their HHPred alignment through to further do a detailed assessment and select the best model. The constructed models were topologically compared through the TM-align server via the optimal structural superposition of their secondary structures, viz. calcium binding domains, α-helix and β-pleated sheets. TM-align deploys the TM-score rotation matrix. The alignment score and sequence similarity of the evaluated sequence pair, along with their conserved domain availability were retrieved from the same source. It quantitatively estimates the mutual structural similarity of the proteins against the camel and human sequences. TM-score is usually deployed for measuring the topological similarity of two protein structures, and it is less sensitive to the local conformational variations. The magnitude of TM-score for random structure pairs is independent of their sequence lengths and its score varies from 0 to 1, where the later defines the maximum similarity score attained by the two identical structures. Following the strict statistics of structures in the protein data bank (PDB), a score below 0.17 corresponded to the randomly chosen unrelated proteins whereas with a score more than 0.5 belonged to the same structural fold.

4.12.4 Comparative Conformational Analysis

The HHpred online server was used to screen the closest set of functionally related protein templates. The protein structures of the human, cow and goat α-lactalbumin were thus obtained from the PDB. To estimate the topological similarity of camel sequence with all these structures, its model was constructed by deploying each of these structures as a template. By using the HHPred alignment, the camel protein was modeled with each of the three templates through MODELLER. The constructed models were topologically compared through TM-Align via the optimal structural superposition of their secondary structures.