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DETERMINATION OF APHRODISIN GENE OF SYRIAN HAMSTER BY MOLECULAR TECHNIQUE

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ABSTRACT

Aphrodisin is a secretory protein expressed in the reproductive organs and parotid salivary gland of the female hamster and acts as an aphrodisiac pheromone. RT-PCR and cloning was applied for the determination of aphrodisin gene from the vaginal tissues of the hamster. In the present study, total RNA was extracted from hamster uterus by Trizol method and apparently undegraded 28S, 18S and 5S species of ribosomal RNA was clearly visible. After performing RT-PCR a distinct band of ~475 bp was obtained and showed in electrophoresis gel analysis which was the partial cDNA of aphrodisin protein. After purification of PCR product and its sequencing using OE-F and OE-R primers confirmed the PCR product was the full-length cDNA of mature aphrodisin of 151 amino acids. The pET 21 plasmid purified from DH5-α cell and ligated with Aph-insert of ~475 bp. Subsequent sequencing confirmed error free ligation and presence of aphrodisin insert. No sequence discrepancy was noted with the published cDNA sequence of mature aphrodisin except for a single base mismatch (GGg for GGA), which did not result in any change in the coded amino acid (glycine). SDS-PAGE analysis showed a major protein greater than 17-kDa was observed in the protein profiles of post-lysis pellet of IPTG induced BL-21 cells but not in their lysates. This 17 kDa protein band should represent recombinant aphrodisin protein which is present in the post-lysis pellet since it is not solubilized by simple sonication. Peptide mass fingerprinting and matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectra analysis of the trypsin digested ~17 kDa recombinant protein, and mass spectrophotometrical (MS) analysis indicated that the overexpressed ~17 kDa protein was closely similar to aphrodisin of Syrian hamster.

Key words: Syrian hamster, aphrodisin protein, overexpression

INTRODUCTION

Aphrodisin is a 17-kDa soluble glycosylated secretory protein expressed in vagina, uterus, Bartholin's gland of the female hamster and also in the female's parotid salivary glands (Magert *et al*., 1999), and in the bank vole (Stopkova et al., 2010). Aphrodisin acts as an aphrodisiac pheromone that is deposited upon the snout and tongue of the male by contact during normal chemosensory investigation of the female's vaginal discharge by the male hamster, and it is probably transported in the nasal mucus to chemoreceptors in the male's vomeronasal organ (Clancy *et al*., 1984).

Aphrodisin consists of 151 amino acids with two disulfide bonds and a blocked *N*-terminus (pyroglutamic acid). Each N-glycosylation site (N41 and N69) is linked with only one *N*-acetylglucosamine residue (Singer et al., 1986). Aphrodisin belongs to lipocalin family, members of which are known to be carrier proteins and they transport low molecular weight hydrophobic molecules. Lipocalins display a remarkable range of different molecular recognition properties and are variable in sequence, but share a typical 3D structure (Flower *et al*., 1993; Flower, 1995 and 1996). Among lipocalins, aphrodisin is highly homologous (40% sequence identity) to rat odorant binding proteins OBP-1 and IF, expressed and secreted by nasal glands of rat in both sexes, which are thought to carry volatile hydrophobic odorants towards olfactory receptors across the aqueous nasal mucus (Pevsner *et al*., 1988; Briand *et al*., 2000). Aphrodisin has also 39% identity with a male-specific lipocalin (MSP) expressed in submandibular glands of male hamster, which is secreted in saliva (Thavathiru *et al*., 1999; Srikantan *et al*., 2005).

Although physical contact with aphrodisin is believed to have an aphrodisiac like effect on the male hamster, it is unclear whether this effect is due to the protein itself or whether it is due to a pheromonal ligand bound to this lipocalin. To investigate in this direction, studies need to be done using the recombinant aphrodisin protein, which will not have any bound pheromonal ligand of hamster origin. Thus, there was a need to obtain recombinant aphrodisin protein.

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The present study was carried out for the isolation of total RNA from the vaginal tissues of the hamster, RT-PCR and cloning of the *cDNA* of aphrodisin, preparation of appropriate plasmid, constructs for overexpression of aphrodisin in *E. coli* and purification of the recombinanat aphrodisin.

MATERIALS AND METHODS

Animals

The study was carried out in the Centre for Cellular and Molecular Biology, Hyderabad, India. In the present study the Syrian (golden) female hamsters *(Mesocricetus auratus)* were bred and maintained in the institutional animal facility. They were reared in a case with food and water *ad libitum.*

Sample collection, RNA extraction, and conduction of RT-PCR

Upper vagina and utero-vaginal junction were taken and total RNA was extracted by Trizol$^{(R)}$ reagent (GIBCO BRL, Grand Island, NY, USA) based on the procedure of Srikantan *et al*. (2005). In brief 100 mg of vaginal tissue close to the external os was dissected out with sharp surgical instrument and immediately homogenized (**Kinematica GmbH homogenizer, Switzerland**) in a Sorvall tube containing 1 ml of TRIzol Reagent (a monophasic solution of phenol and guanidine isothiocyanate). Following homogenization insoluble materials was removed from the homogenate by centrifugation (Eppendorf centrifuge 5810 R) at 12,000 x *g* for 10 minutes at 4^0 C. Transferred the clear homogenates to a fresh centrifuge tube (1.5 ml tube) and incubated it for 5 minutes at 15–30^0 C to permit complete dissociation of nucleoprotein complexes. Added 0.2 ml of chloroform per 1 ml of Trizol reagent for phase separation. Cap microfuge tube to secure. Shake the tube vigorously by hand for 15 seconds and incubate at room temperature for 2–3 minutes. Centrifuged the sample at 12,000 x *g* for 15 minutes at 2-8^0 C. Following centrifugation, the mixture separated into a lower (red) phenol-chloroform phase, an interphase, and a clear upper aqueous phase. RNA remains in the upper aqueous phase, which was 60 % of the volume of TRIzol Reagent used for homogenization. Aqueous phase solution was transferred to a fresh centrifuge tube. Added 0.5 ml of isopropyl alcohol (per 1 ml. of TRIzol Reagent) for precipitation of RNA. Incubated the samples at 15–30^0 C for 10 minute, centrifuged at 12,000 x *g* for 10 minutes at 2–8^0 C. The supernatant fluid was discarded. Wash RNA pellet with 75% ethanol. At least 1ml of 75% ethanol per 1 ml of TRIzol Reagent used for initial homogenization. Mixed the sample by vortexing and centrifuged at no more than 7500 x *g* for 5 minutes at 2–8^0 C. RNA pellet was dried briefly in air, 20–30 µl of RNAse free autoclaved water was added to dissolve and then stored at –70^0 C. Checking of quality of total RNA by agarose gel electrophoresis. A reverse transcription was done using oligo-dT and then PCR was done using the two primers consisted of APH-OE-F: 5'-ATCAATCATATGCAGGATTTTGCAGAGCTT-3' (Tm=58) (30mer) APH-OE-R: 5'-ACTCTCGAGTTCAGGACAAGTATCTGTAGC-3'(Tm=62) (30mer). Forward primer is complimentary to the sequence of aphrodisin (APH) gene encoding the N-terminal end of the mature aphrodisin protein. Reverse primer is complimentary to the gene sequence encoding the C-terminal end of aphrodisin. Both forward and reverse primers have extra bases at their 5'-ends, which include restriction enzyme sites for Nde1 and Xho1 respectively (underlined) to facilitate cloning into vector plasmid. The PCR procedure was conducted at 94^0C for 15 sec, 55^0 C for 30 sec, and 72^0 C for 1 min for 40 cycles.

Purification, quantification, and sequencing of DNA (PCR product)

The DNA fragment (band of PCR product) was excised from the agarose gel under brief UV illumination with a clean, sharp scalpel and the DNA was purified using QIAquick gel extraction kit (Qiagen, Germany). The DNA was quantified by Nano Drop Spectrophotometer (ND 1000, USA) and nucleotide sequence of the DNA was performed directly from PCR products by the didoxy nucleotide chain termination method using ABI Prism 3730 DNA Analyzer (Applied Biosystems, USA).

Digestion of the PCR product (Aphrodisin) by restriction enzyme and circular pET-21a vector

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double stranded DNA following its specific recognition of short nucleotide sequences, known as restriction sites, in the DNA. The PCR product was digested to remove its overhang on both sides. Xho1 and Nde1 was used in the present study. The DNA template (20 µl), Xho1 (1 µl), Nde1 (1 µl), Bovine serum albumin (10x, 2.5 µl), and NEB4 buffer (2.5 µl) were incubated in water bath (Julabo F 12) for overnight at 37^0 C. The digested product was run through 1.3% agarose gel.

The double digested DNA band (Aph-insert) was collected from agarose gel (using QIAquick gel extraction kit) (Qiagen, Germany). Circular pET-21a vector also digested by restriction enzyme to make it linearization.

Ligation of Aph-insert with the linearized pET-21a vector:

Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. The enzyme used in the present study to ligate DNA fragments is T4 DNA ligase, which originates from the T4 bacteriophage. This enzyme ligated DNA fragments having overhanging, cohesive ends that are annealed together. The ligation reaction requires double digested linearlize pET-21a vector (10.5 µl), APH-insert (3.2 µl), T4 DNA ligase enzyme (0.1µl), T4 DNA ligase buffer (2.0 µl), and DDW (4.2 µl). The digestion mixture was incubated in water bath (Julabo F 12) for overnight at 18^0 C.

Transformation of ligated mixture (APH-insert + pET-21 vector) in DH5α for cloning purpose

Transfer 2 µl of ligated mixture to 200 µl of DH5α cells in a vial and immediately place into ice for 30 min. followed by heat shock at 42^0 C for exactly 90 sec in water bath and immediately transferred again the vial into ice for few minutes. Add 800 µl LB broth and mix gently (no ampicillin). Incubate the mixture at 37^0 C in Julabo water bath for 1 h and spin at 8000 rpm for 1 min. at room temperature. The supernatant discarded and the pellet was suspended in 100-200 µl of LB broth. The suspension of LB broth was plated in LB agar (containing ampicillin) at different concentrations with appropriate controls. The plate was incubated at 37^0 C overnight. The colonies of transformants were observed in next morning.

Patching of primary culture and colony direct PCR to confirm presence of insert:

Colonies of potential transformants were further streaked and patched in the same media and were incubated at 37^0C overnight to get secondary isolated colonies. Isolated single colonies were checked by PCR for presence of aphrodisin insert. In brief a small amount of cells was picked from each colony using a sterile toothpick and suspended in 50 µl of sterile DDW. This was heated for 10 min. at 95^0 C followed by centrifugation in room temperature for 10 min. at 10000 RPM. 1 µl of the supernatant (obtained from separate colonies) was used as a template for PCR using OE-F and OE-R primers. If the correct size of PCR product was obtained then the original colony was considered to be 'positive' (i.e. containing the appropriate plasmid with aphrodisin insert). A single 'positive' colony was then inoculated in LB broth with ampicillin and isolation and purification of plasmid was done by QIA prep spin miniprep kit (Qiagen, Germany). The OD of DNA per µl was quantified by Nano Drop Spectrophotometer (ND 1000) and stored at –30 ^{0}C. Sequencing of the plasmid was done using OE-F, OE-R and vector-specific T7 promoter and T7 terminator primers to confirm sequence and reading frame of Aph insert.

Induction with IPTG, sonication, SDS-PAGE gel electrophoresis

The cloned plasmid then transformed into BL-21 cells and inducted with IPTG (isopropyl β-D thiogalacto pyranoside) for over-expression of aphrodisin which then sonicated (Srikantan *et al.* (2005) and run through SDS-PAGE gels at 7mA constant current. The gel was removed and stained in 0.25% coomassie blue dye (made in 45% Methanol, 45% water and 10% acetic acid) for 3-4 hrs. Destaining was done in 45 methanol, 45% water and 10% acetic acid mixture.

Mass-spectrometric analysis

Matrix-assisted laser-desorption ionization-time-of-flight **(MALDI-TOF)** mass spectra were recorded in a Voyager-DE STR mass spectrometer (Applied Biosystem) to check the homogeneity of the purified protein and to determine their masses.

RESULTS AND DISCUSSION

Extraction of Total RNA:

Extracted RNA from hamster uterus by Trizol method was run in agarose gel and the total RNA preparation was apparently undegraded with 28S, 18S and 5S species of ribosomal RNA clearly visible (Figure 1).

RT-PCR:

After performing RT on hamster uterus total RNA using oligo-dT as primer, PCR was performed on the RT-product (cDNA) using Aph-OE-F and Aph-OE-R primers. PCR product was run in agarose gel. As shown in **Figure-2** a distinct band of ~475 bp was obtained, which was as expected and should represent the partial cDNA of aphrodisin (encoding the complete mature aphrodisin protein).

Purification of PCR product and its sequencing using OE-F and OE-R primers:

The 475 bp PCR products was gel eluted and its DNA concentration estimated using nanodrop spectrophotometer and was submitted for sequencing using the PCR primers.

Sequencing results confirmed that the PCR product indeed encoded the full-length cDNA of mature aphrodisin (151 amino acids) including restriction enzyme sites of Nde1 and Xho1 at each end.

Purification of pET21a plasmid:

From a glycerol stock of DH5-alpha cells containing pET21a plasmid, streaks were made on LB-amp plates to isolate single colonies and inoculation done in LB-amp broth to isolate pET21a plasmid. The final DNA concentration of the plasmid preparation was determined.

Double digestion with Nde1 and Xho1 restriction enzymes of the RT-PCR product and pET21a plasmid:

The pET21a plasmid was double digested with Nde1 and Xho1 resulting in its linearization. The double digested and linearized vector showed up as a single band of ~5.4 kb in agarose gel as shown in **Figure 3**. The linearized vector band was gel purified and the DNA concentration was quantitiated using nano drop spectrophotometer. Similarly, double digestion of the purified RT-PCR product (Aph-insert) was done after which it was run on agarose gel and purified. The final insert should have Nde1 and Xho1 sticky ends on either side with a length slightly shorter than 475 bp.

Ligation of Aph-insert with the linearized pET-21a Vector and transformation into DH5-alpha cells:

Several potential transformant colonies were obtained, which needed to be checked for presence of pET21a-Aph plasmid (i.e. plasmid containing aphrodisin insert).

Colonies direct PCR to screen for presence of pET21a-Aph plasmids:

Colony PCR using Aph-OE-F and Aph-OE-R primers was used to check several potential transformant colonies. As shown in **Figure-4**, out of 10 colonies of transformants tested, colony 1 and 6 turned out to be negative while the remaining eight colonies were positive and they showed a distinct PCR product of ~475 bp. Moreover, when colony PCR was done using vector specific primers (T7 promoter and T7 terminator) PCR products were obtained, which were as expected of slightly higher size (not shown).

Plasmid isolation and sequencing:

After isolation of plasmid containing the aphrodisin insert from DH5-alpha cells it was submitted for sequencing using OE-F/OE-R as well as vector-specific primers T7 promoter and terminator primers (**Figure-5**). Results of sequencing confirmed error free ligation and presence of aphrodisin insert. No sequence discrepancy was noted with the published cDNA sequence of mature aphrodisin except for a single base mismatch (GGG for GGA), which did not result in any change in the coded amino acid (glycine). The recombinant pET21a-Aph plasmid encoded the full-length sequence of mature aphrodisin protein (2^{nd} paragraph in the sequence below) preceded at its N-terminal end by a methionine (translation start). At the C-terminal end of aphrodisin a vector encoded extension of eight amino acids was present, which had the sequence: leucine, glutamine and 6 consecutive histidines were present before the translation stop codon (tga).

Over-expression of aphrodisin:

The bacterial expression constructs in pET-21 were transformed into competent *E. coli* strain BL-21 (DE3) cells (expression host) prepared by calcium chloride method. Their transformation procedure has described in Materials and Methods section. After induction by IPTG the bacterial pellets and lysates was run through SDS-PAGE. A major protein band with mobility somewhat greater than 17-kDa was observed in the protein profiles of post-lysis pellet of IPTG induced BL-21 cells but not in their lysates (**Figure 6**). However, such a protein band was absent in lysates and post-lysis pellets of uninduced BL-21 cells (**Figure 6**). Needless to say, that no major 17-kDa protein was detected in IPTG-induced cultures of untransformed BL-21 cells (not shown). This 17 kDa protein band should represent recombinant aphrodisin protein which is present in the post-lysis pellet since it is not solubilized by simple sonication.

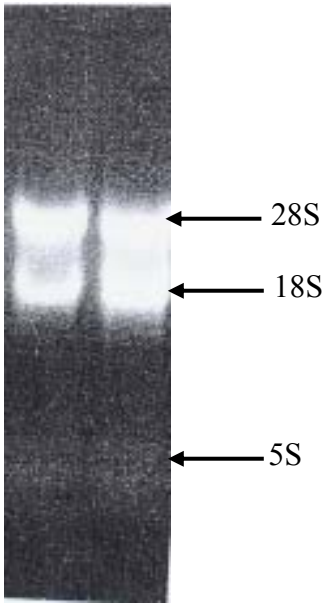

Fig. 1. Both lanes show total RNA isolate from hamster uterus by Trizol method, which were run in 1.3% TAE-agarose gel. The total RNA preparation was apparently undegraded with 28S, 18S and 5S species of ribosomal RNA clearly visible.

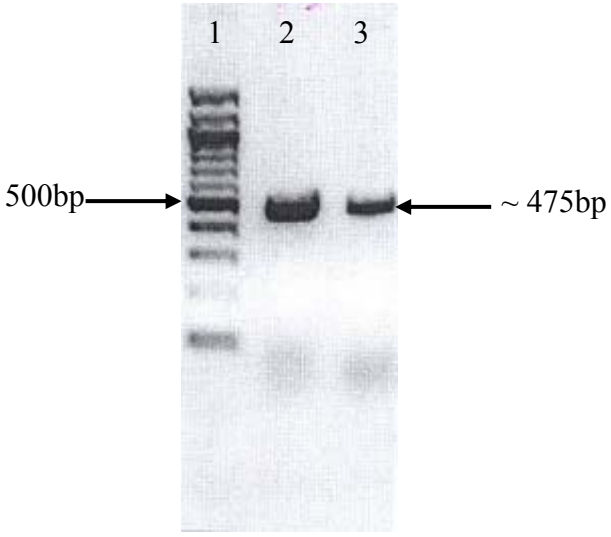

Fig. 2. Lane 2 and 3 show a distinct band of ~ 457 bp (1.3% agarose gel) of the particle cDNA of aphrodisin obtained after PCR using RT-product (cDNA) of hamster uterus as a template and Aph-OE-F and Aph-OE-R as primers. Lane 1 shows DNA markets.

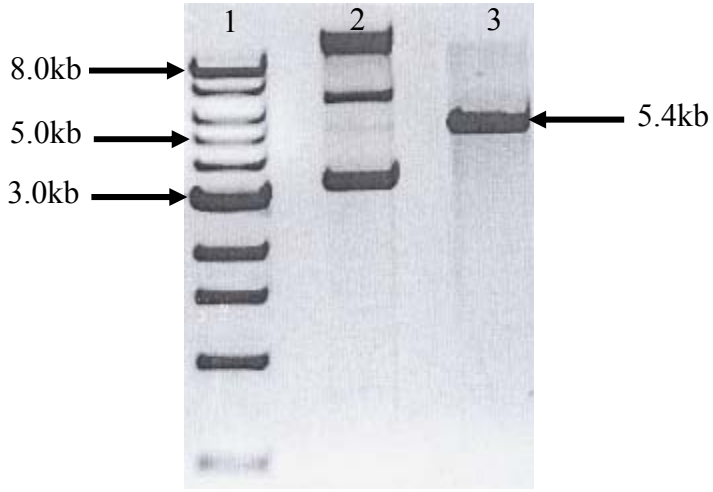

Fig. 3. Lane 3 shows the single band of ~ 5.4 kb of linearized pET21a plasmid obtained after double digestion of the circular plasmid with Nde1 and Xho1. The lane 2, is shows intact (undigested) pET21a plasmid vector. Electrophoresis was in 0.8% agarose gel. Lane 1 shows resolved DNA molecular weight markers.

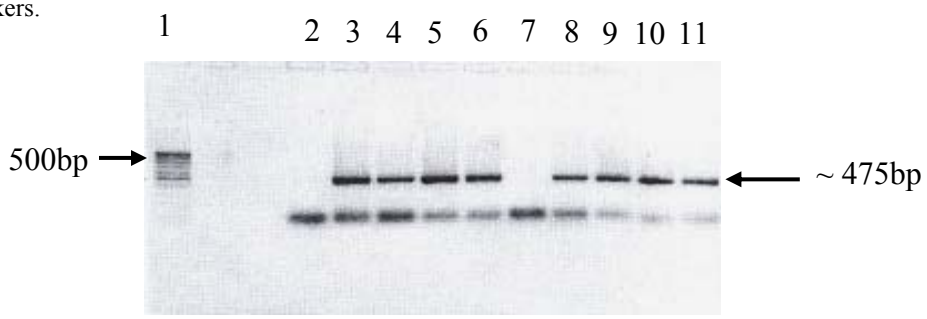

Fig. 4. Colony PCR using Aph-OE-F and Aph-OE-R primers. Colony PCR was performed to check for potential transformant colonies. The lanes 3 to 6, and 8 to 11 show a distinct PCR product of ~ 475 bp (indicating a positive transformant colony), while colonies tested in lanes 2 and 7 show no such PCR product (negative reaction). Lane 1 is DNA markers.

AATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTT
TGTTTAACTTTAACTTTAAGAAGGAGATATACAT***ATG***(Nde1 site)

CAGGATTTTGCAGAGCTTCAAGGAAAATGGTATACCATTGTCATTGCTGCTGACAATCT
TGAAAAGATAGAAGAAGGAGG**g**CCACTGAGATTCTATTTTCGTCATATTGATTGTTATA
AAAACTGCAGTGAAATGGAAATCACATTTTATGTCATTACAAACAACCAGTGCTCCAAG
ACCACAGTCATTGGGTACTTGAAAGGAAATGGAACCTACCAAACCCAGTTTGAAGGTAA
CAATATATTTCAACCTTTGTATATAACATCAGACAAGATTTTCTTTACCAACAAGAACA
TGGATAGAGCTGGCCAGGAAACGAACATGATTGTTGTTGCTGGAAAAGGTAATGCTTTG
ACACCTGAAGAAAATGAAATACTTGTGCAATTTGCTCATGAAAAGAAAATTCCAGTGGA
AAACATTCTCAATATTCTTGCTACAGATACTTGTCCTGAA

(Xho1
site)CTCGAG**CACCACCACCACCACCAC**tga**GATCCGGCTGCTAACAAAGCCCGAAAG
CTGGGAAAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGC

Fig. 5. Mature aphrodisin cDNA sequence inserted in pET21a plasmid between Nde1 and Xho1 sites

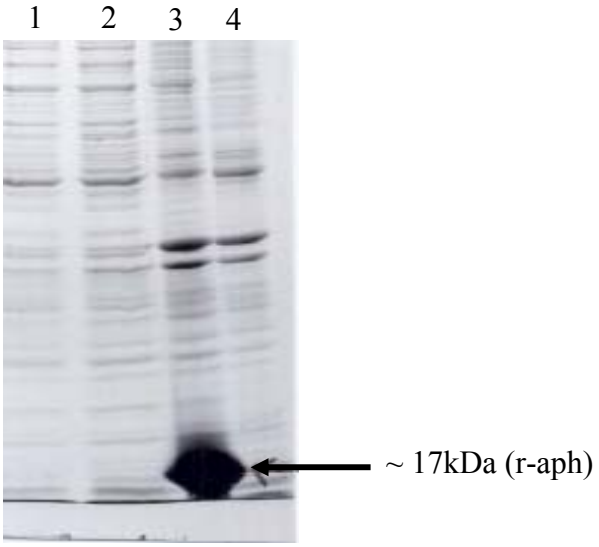

Fig. 6. 10.5% SDS-PAGE profiles are shown. Lysates of uninduced and uninduced cultures are shown in lanes 1 and 2 and post-lysis pellets of induced and uninduced cultures are shown in lanes 3 and 4. Induction was with IPTG. The bacterial cells were lysed by sonication and then centrifuged. Supernatants (lysates) and residual post-lysis pellets were run in gel. A major protein band, with mobility slightly greater than 17kDa, is present in protein profiles of post-lysis pellet of induced BL21 cells (lane 3). Such a protein band was absent in lysates (of induced or uninduced BL21cells) and also in post-lysis pellets of uninduced BL-21 cells.

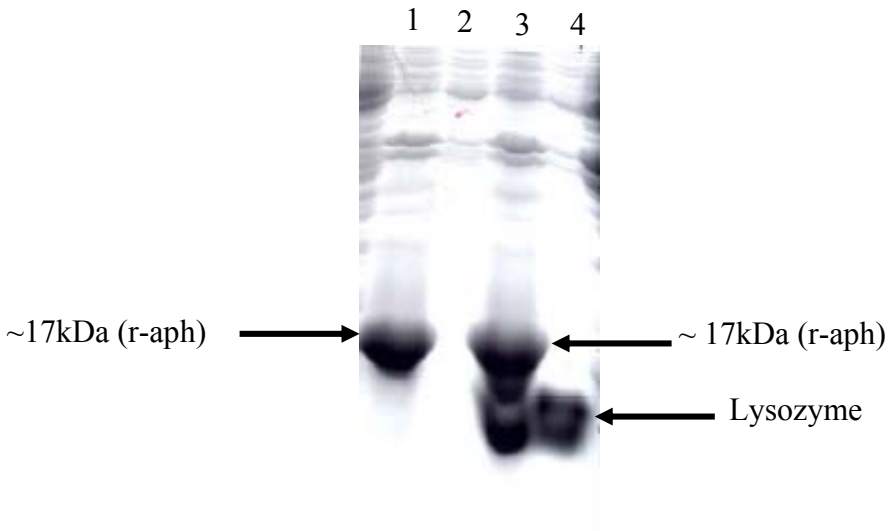

Fig. 7. Incubation of IPTG induced bacterial cells with lysozyme before lysis by sonication. The lane 1, post-sonication pellet of induced cells with no prior lysozyme treatment; lane 2, post-sonication supernatant (lysate) of induced cells with no prior lysozyme treatment; lane 3 and 4 are same as lanes 1 and 2 except that lysozyme incubation was done prior to sonication. The 17kDa band (r-aphrodisin) in the 12.5 % SDS-PAGE profiles is present only in pellets in both lysozyme unincubated as well as incubted cells storngly suggesting that r-aphrodisin is present in inclusion bodies.

Lysozyme treatment of induced BL-21 cells does not release r-aphrodisin into supernatant.

Figure 7 show that lysozyme treatment prior to sonication does not result in extraction of r-aphrodisin in the post-sonication lysate which strongly suggesting that r-aphrodisin is present in inclusion bodies. Extraction of r-aphrodisin from the residual post-sonication pellet would require treatments with denaturants like urea/guanidium hydrochloride or treatments with other detergents.

Tryptic digestion and mass-spectrometric analysis of the ~17 kDa recombinant protein:

The molecular weight of the overexpressed Aphrodisin in the present study was ~17kDa, which is similar to earlier reports of molecular weight of natural aphrodisin of Syrian hamster (Singer *et al.,* 1986; Magert *et al.,* 1999; Briand *et al.,* 2000). Peptide mass fingerprinting and matrix-assisted laser-desorption ionization-time-of-flight **(MALDI-TOF)** mass spectra analysis of the trypsin digested ~17 kDa recombinant protein, and MS/MS analysis indicated that the overexpressed ~17 kDa protein was closely similar to aphrodisin of Syrian hamster **(Figure-8 a-b)**.

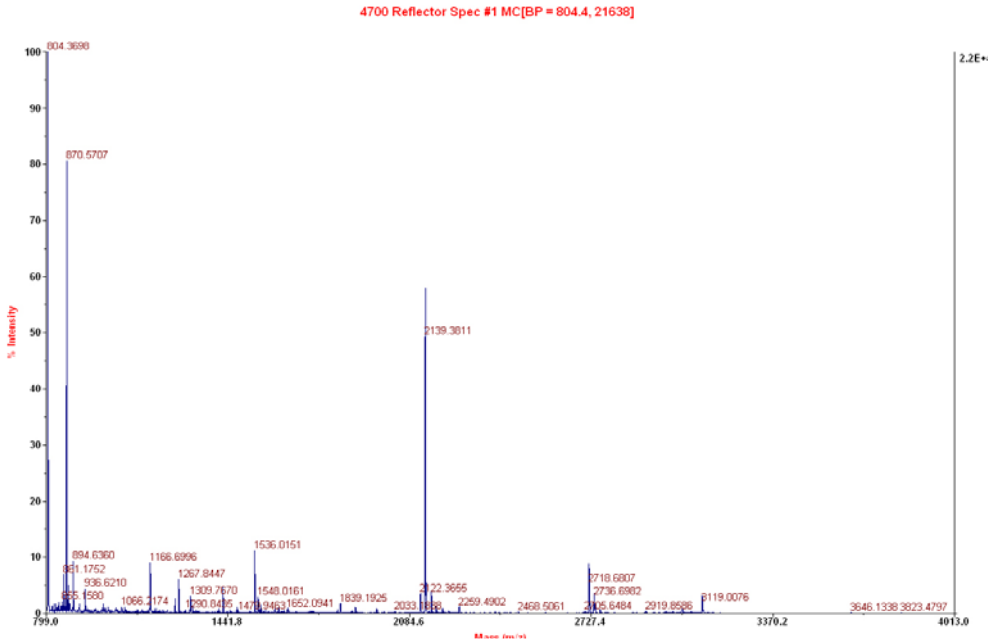

Fig. 8a. The mass spectrum showing peptide mass fingerprint (PMF) of ~17 kDa, recombinant aphrodisin protein band present in post-sonication pellets of IPTG induced cultures of BL21 cells. The peptide mixture was mass analyzed using AB4800 MALDI-TOF/TOF and the peptide masses were recorded as shown in the figure.

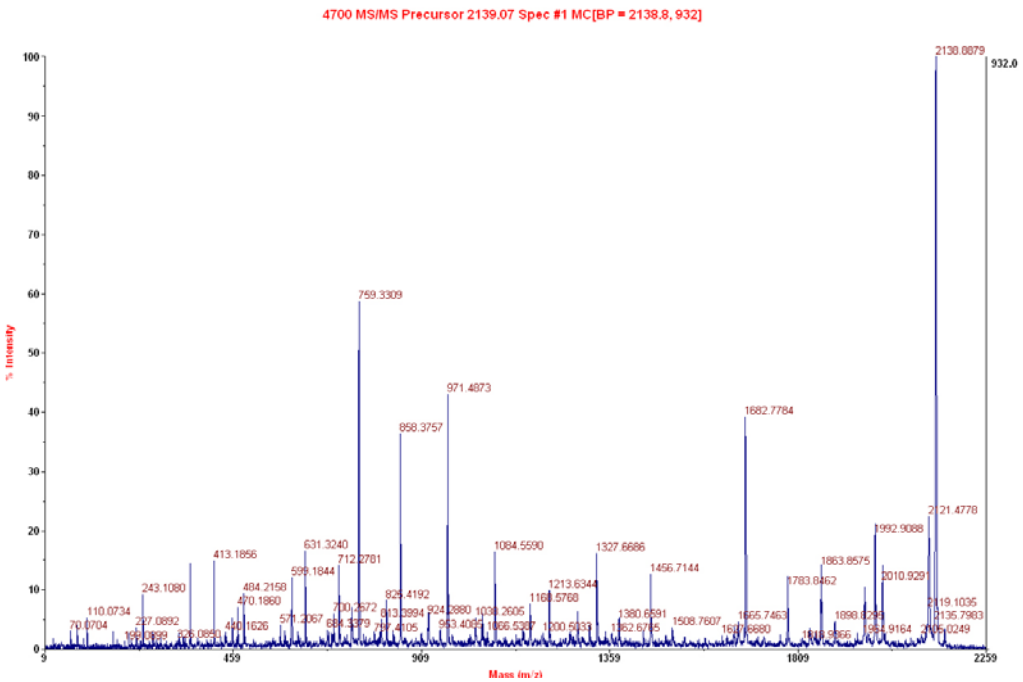

Fig. 8b. The MS/MS spectrum showing the fragmentation pattern of the peptide 2139.0688 m/z. This spectrum when searched in the database was found to match the peptide sequence, `GNALTPEENEIL VQFAHEK' Derived from the protein aphrodisin of syrian hamster

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