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Research Article

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Identification and Characterisation of Moonlight Proteins from Insect Brain Tissue Lysate

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Abstract: Moonlight proteins are multifunctional proteins i.e, capable to perform multiple physiologically relevant biochemical or biophysical functions other than the one assigned in cell. Studies indicated that some of these play an important role in disease, identifying novel biochemical pathways, protein mechanisms in system biology and help in improving the prediction of protein functions. Discovery of antibiotics leads to the control of bacterial and fungal infections. Consistent over dose of antibiotics led to development of antibiotic resistance among pathogenic bacteria. Plants and animals based proteins proved to work against drug-resistant bacteria. For e.g., insects like cockroaches release antibacterial peptides in their hemolymph when induced with pathogenic bacteria like *E.coli*. In our previous study, the proteins isolated from brain tissue lysate of cockroaches also showed significant control on the growth of drug-resistant and pathogenic bacteria. Current study deals with identification and characterisation of two such proteins isolated from the brain tissue of cockroach. Their identification will help in bringing better understanding and assess to their future course of action. Mass spectrometer (MS) technique combined with high-resolution Q-Exactive orbitrap and liquid chromatography (LC) were considered for identification of protein in crude tissue lysate samples that were separated in the polyacrylamide gel. Of the 79 proteins identified from crude brain tissue lysate, the two proteins Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Transferrin isolated using gel based technique were identified by Q-TOF HRLC-MS/MS and their structure homology was modelled using SWISS MODEL . These two proteins have already been proved to behave as antibacterial, also they play a very important role in cellular glucose metabolism and iron transport in others, defining their moonlight property. Due to this property, they can as well be used to address the increasing problem of multidrug antibiotic resistance. These can be assessed and studied further for their possible action on bacteria which can help in the development of new peptide drugs.

Keywords: Drug resistant bacteria; antibacterial protein; mass spectrometer; structure homology modelling.

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1. INTRODUCTION

Multiple drug-resistant bacteria is one of the main threats in the current world scenario. These are maximally associated with the nosocomial infection. The prevalence of drug-resistant bacteria is mostly community-associated.[1] India carries one of the largest burdens of drug-resistant pathogens worldwide, including the highest burden of multidrug-resistant tuberculosis[2], alarmingly high resistance among gram-negative and gram-positive bacteria [3] even to newer antimicrobials such as carbapenems and faropenem since its introduction in 2010.[4,5] Regional studies report high AMR among pathogens such as *Salmonella typhi, Shigella, Pseudomonas,* and *Acinetobacter*.[6] Annually, more than 50,000 newborns are estimated to die from sepsis due to pathogens resistant to first-line antibiotics.[7] While exact population burden estimates are not available, neonates and the elderly are thought to be worse affected. Two million deaths are projected to occur in India due to antimicrobial resistance by the year 2050.[8] Available data indicates rising rates of antimicrobial resistance, across multiple pathogens of clinical importance, in the country. In 2008, about 29% of isolates of *Staphylococcus aureus* were methicillin-resistant, and by 2014, this had risen to 47%. Since conventional drugs are failing to control the infections by drug-resistant bacteria, alternative sources are approached for their control. Several antimicrobial peptides (AMPs) have been isolated from plants and animals. AMPs exist in all multicellular organisms and have evolved in living organisms over 2.6 billion years.[9] It is known from the beginning of the 20th century that body secretions, as well as blood and polymorphonuclear leukocytes, contain antimicrobial compounds[10]. The most well-known AMPs comprise lysozyme (isolated from the nasal mucous)[11], cecropins (from moths)[12], magainins (from frogs)[13], beta-defensins[14] and cathelicidins[15], of which the last two types are key components of the antimicrobial response in polymorphonuclear leukocytes in humans. AMPs typically possess a net positive charge and amphipathic properties. The positive charge of AMPs is believed to be key to their effect, interacting with and perturbing the negatively charged bacterial cell envelope.[16] In insects with complete metamorphosis, AMPs are rapidly and transiently synthesized by the fat body (tissue corresponding to the mammalian liver), and by various epithelia.[17] When produced by the fat body, the AMPs are secreted into the hemolymph (blood), from where they can easily diffuse to act throughout the whole animal. Most of the AMPs are produced quite massively, many of them reaching high micromolar concentrations (i.e. mg/L). In contrast, in insects with incomplete metamorphosis, AMPs are produced by hemocytes (blood cells) in the healthy animal and secreted into the hemolymph upon infection.[18] Insects such as cockroaches represent a plentiful and untapped potential source of few antimicrobial drugs prompting us to investigate the antibacterial activity of their various tissues.[19,20] The studies have shown that cockroach and locust brain tissues have powerful antibiotic properties and may serve as potential sources of antimicrobials in the future.[19,20,21,22] Many proteins are known to have more than one activity in a single domain. The property of proteins having multiple activities is sometimes referred to as moonlighting.[23,24] Moonlighting proteins and peptides presenting more than one activity in a single domain differ from proteins that have multiple activities in multiple domains.[25] Several moonlight proteins are identified in *Drosophila melanogaster* like transmembrane protein 16 (TMEM16)[26], NON3[27], RACK1[28], mRNA decapping protein (DCPs)[29] etc. also two such proteins have been identified from fly.[30] According to literature drosophila and fly are probably the only insect in which moonlight proteins have been identified. Protein identification can be best done by Mass spectrometer (MS) techniques. Recently, several combinations of MS techniques are used for better protein separation and identification like Liquid chromatography (LC), Linear Trap Quadrupole (LTQ) Orbitrap, Time of flight (TOF), Matrix-Assisted Laser Desorption and Ionisation - Time of Flight (MALDI-TOF), etc. The variable pI range of proteins, their relative abundance, hydrophobicity, and solubility makes them difficult to separate through the classical 2-Dimensional Electrophoresis. The liquid chromatography technique connected with MS (LC-MS/MS) can be used as an alternative separation method.[31] In the Orbitrap, ions are trapped and orbit around a central spindle-like electrode and oscillate harmonically along its axis with a frequency characteristic of their m/z values, inducing an image currently in the outer electrodes that is Fourier transformed into the time domain producing mass spectra.[32] It consists of an LTQ coupled to a C trap and the Orbitrap. It combines the robustness, sensitivity, and MS/MS capability of the LTQ with very high mass accuracy and high-resolution capabilities of the Orbitrap, and has become a powerful tool in proteomics. The LTQ-Orbitrap was used firstly for peptide analysis of a digested human saliva sample in a shotgun bottom-up fashion.[33] LC-MS/MS technique is used for identification of targeted sample while high resolution LTQ orbitrap is used for identification of untargeted sample.[34] The identified peptides of master protein can be analyzed using the UniProt and NCBInr database. This protein can be checked for its possible structure homology using several *in silico* online servers like SWISS-MODEL[35], RaptorX[36], etc. This will further help in identifying the active sites of antibacterial protein and develop the drug-using drug designing technique. Current study deals with identification and characterisation of two proteins isolated from the brain tissue of cockroach. Their identification will help in bringing better understanding and assess to their future course of action. Mass spectrometer (MS) technique combined with high-resolution Q-Exactive orbitrap and liquid chromatography (LC) were considered for identification of protein in crude tissue lysate samples that were separated in the polyacrylamide gel.

2. MATERIALS AND METHODS

2.1. Sample preparation for mass spectrometer

2.1.1. Q-TOF HRLC-MS/MS

Protein samples were isolated and purified using 1D Native Polyacrylamide gel electrophoresis of the crude brain extract. The gel pieces of protein bands[20] were rinsed with 100 mL of 25 mM ammonium Bicarbonate (ABC) and dehydrated with 100 ml of solution [A] ([A] = 2:1 mixture of acetonitrile (ACN): 50mM ABC) for 5 min. 100 ml of 25 mM ABC was added to supernatant for 5 min. The process was repeated to concentrate protein. Then the gel slice was rehydrated in 50 ml of 10 mM Dithiothreitol (DTT) and placed at 56 ^{0}C for 1 hour. DTT was then removed and the slice was rinsed with 100ml 25mM ABC. Later, 50ml of 100 mM Iodoacetamide (IAA) was added and incubated at RT in dark for 30 min and then rinsed with 100 ml ABC. Preparation of trypsin was carried out by adding 1ml of ice-cold 25 mM ABC to a standard 20 mg trypsin vial (Promega

sequencing Grade trypsin) and stored on ice. Gel slice was rehydrated with trypsin (about 10-20 ml) at 0□C on ice and incubated on ice for 20-30 min. until trypsin is absorbed. Once slice is completely rehydrated, just enough (50 ml) 25 mm ABC was added to cover the gel slice in the tube and incubated overnight at 37 °C. Supernatant was removed and slice was vortexed with 100 ml of extraction buffer (having 50% ACN and 0.1% TFA) for 10 min and pool into single tube. Process was repeated (having 60% ACN and 0.1% TFA in the extraction buffer) and sonicated for 1 min (2 sec pulse, 1 sec gap, 25% amplitude) and by vortexing for 10 min supernatant was added to the previously pooled sample. Extraction was repeated with 80% ACN and 0.1% TFA and vortex for 10 min and the supernatant was collected. This solution was used for mass spectrometer after ZIPTip.[37]

2.1.2 Q-Exactive orbitrap LC - MS

Brain tissue of American cockroach was minced under sterile conditions and placed directly into a 2ml microtube containing 160 µl of freshly prepared 7M urea, 2M thiourea, and 1M ammonium bicarbonate at 95°C for 20 min followed by 60°C for 2 hours. The tissue lysate was centrifuged at 8,000 rpm/15 min and supernatant was considered for trypsin digestion. Lysate was digested with Mass spectrometry grade trypsin (1: 20 w/w) at 37°C overnight.[38]

2.2. Protein identification by Orbitrap LC-MS

Orbitrap LC-MS was used for identification of overall protein content present in the brain tissue lysate of cockroaches. Liquid chromatography was performed using Thermo EASY-nLC instrument. The sample flow was maintained at 4.0 µl / min, total volume in pre column equilibrium was 10 µl and that in analytical equilibrium was 3 µl at a constant pressure maintained at 850 Bar respectively. The total flow was maintained at 300 nl/min for around 60 min. The analytical column used was PepMap RSLC C18 2um, 100A x 50 cm and Pre-column was Acclaim PepMap 100, 100um x 2cm nanoviper. The Mobile Phase consisted of solvent A (0.1% FA in milliq water) and solvent B [80:20 (ACN:milliq water) + 0.1% FA].The mixture B was 5% for beginning, 10% at 5 min, 50% at 50 min, 95% at 55 and 60 min.[39] The scan was collected in orbitrap at resolution of 70,000 in m/z range of 350 – 2000 amu. Q Exactive Plus - Orbitrap MS instrument was used for performing orbitrap analysis of crude protein samples. The sample was run for 60 min with constant flow rate of 3.0 µl/min, inner instrumental diameter was 2.303 mm. The data obtained (Graph 1) was identified using PDB - Uniprot data.[14,39]

2.3. Protein identification based on peptide sequencing by Q-TOF HRLC-MS/MS

The proteins in band 2 and 4, obtained on native PAGE[20] were identified using high resolution LC-MS/MS. HRLC-MS/MS was performed on Agilent's 1260 series Nano HPLC system with Chipcube for ionization, coupled to an Agilent's 6550 Q-TOF system. After in-gel trypsin digestion, peptides were eluted into the nano pump at the flow rate of 0.3 µL/min. Peptides were separated using the mobile phase gradient solvent A and Solvent B viz. Water and Acetonitrile respectively. Separation along mobile phase was in ratio of A 80% and B 20% for first 2 min, A 2% B 98% for next 15 min, A 2% B 98% for next 20 min, A 97% B 3% for next 25min , A 97% B3% for next 35 min. The m/z range of 300 – 3200 with MS scan rate of 5.0 spectra/sec and MS/MS scan rate of 3.0 spectra/sec was considered. LC-MS/MS protein data acquired was identified using NCBInr data.[39]

2.4. Protein structure homology modelling using SWISS MODEL and RaptorX

The FASTA sequence of identified proteins was obtained from BLASTp using their accession number. The FASTA sequence was then entered in SWISS MODEL server and RaptorX for structure homology modelling. The quality of model is estimated based on QMEAN value on SWISS MODEL (https://swissmodel.expasy.org/) whereas RaptorX (http://raptorx.uchicago.edu/) shows approximately 80% of structural accuracy.[35,36]

3. RESULTS

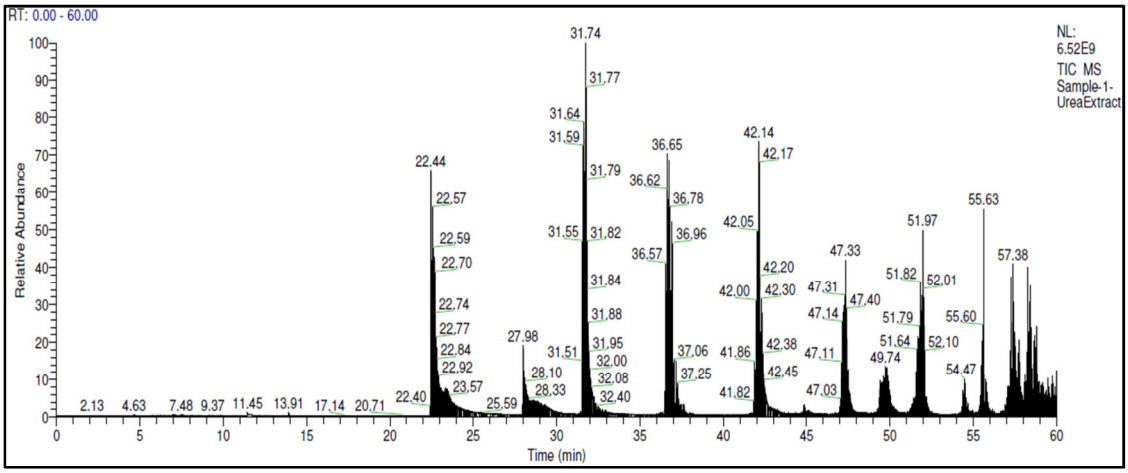
3.1. Protein Peptide Identification

The brain tissue lysate of *Periplaneta americana* was first analysed using Q Exactive orbitrap LC-MS technique which showed presence of around 79 different protein peptides some of them are shown in Table 1. The graph 1 represents peaks of protein peptides obtained by orbitrap LC-MS analysis performed for identifying complete protein content present in brain tissue lysate.

Table 1. Q Exactive orbitrap LC-MS protein analysis data of brain tissue of cockroach

Sr. no.	Protein	Accession number	M.W (kDa)	Amino acid	pI
1	ATP synthase subunit beta	A0A481SN44	38.2	353	5.19
2	Putative Per a allergen	A0A2P0XJ16	102.9	882	5.59
3	Ubiquitin (Fragment)	A1E2I6	8.5	76	7.25
4	Putative Per a 8 allergen variant	A0A2P0XIZ1	22.6	208	4.81
5	Actin	A0A2R4CIH4	41.8	376	5.48
6	Putative Per a 6 iso allergen	A0A2P0XIY8	16.9	150	4.13
7	**Glyceraldehyde-3-phosphate dehydrogenase**	**A0A2P0XIG5**	**35.4**	**332**	**7.47**
8	Troponin T	Q9XZ71	45.9	384	4.98
9	Putative Per a 7 iso allergen	A0A2P0XIF1	32.4	284	4.84
10	Myosin light chain variant 1	G8XWV3	17	151	4.77
11	Allergen (Fragment)	Q94643	75.5	631	7.09
12	Tropomyosin Per a 7.0102	P0DSM7	32.8	284	4.77
13	Arginine kinase	A1KY39	39.7	356	5.87
14	p10	O17447	15	130	6.76

15	Putative Per a allergen	A0A2P0XJ03	36	341	9.13
16	Fructose-bisphosphate aldolase	A0A2P0XIZ4	39.5	364	7.39
17	Putative Per a 6 iso allergen	A0A2P0XIE8	17.2	151	4.21
18	Glucose-6-phosphate isomerase (Fragment)	D0UMY8	24.8	222	7.78
19	MPA13 allergen	Q1M0X8	14.7	131	6.7
20	Cytochrome c oxidase subunit 2	H6S3P0	26.2	228	5.14
21	CRF-DH (Fragment)	A0A0U2JZ21	13	114	6.83
22	Aminopeptidase (Fragment)	A0A059WIM4	25.3	238	8.43
23	Putative Per a 6 allergen variant	A0A2P0XIE3	17.1	151	3.99
24	Per a 16 allergen	A0A481SQK2	21.5	190	5.36
25	Formyl-tetrahydrofolate synthetase (Fragment)	D8X0L9	38.2	353	6.24
26	Alpha-spectrin (Fragment)	D0UNA0	22.2	198	5.34
27	Malonyl CoA-acyl carrier protein transacylase	D0J8R4	31.8	287	9.09
28	Homeobox protein engrailed-like	Q9Y071	37.2	333	10.24
29	Putative Per a allergen	A0A2P0XIF0	47.2	433	6.33
30	Putative Per a allergen	A0A2P0XJ09	11.9	108	9.57
31	SH2 domain binding protein (Fragment)	D0UMV2	19.9	178	9.13
32	Transketolase, N-terminal subunit	D0J945	32.1	287	6.54
33	Prephenate dehydrogenase	D0J9A1	31.9	281	9.32
34	Glutathione S transferase class delta variant 1	G8XWU4	24.6	216	6.9
35	DNA-directed RNA polymerase subunit alpha	D0J9G7	38	332	9.06
36	30S ribosomal protein S3	D0J9I6	27	235	10.43
37	Putative Per a 3 allergen variant	A0A2P0XIG9	72.9	608	6.86
38	Histone H3 (Fragment)	Q95W47	11.8	104	10.62
39	Per a 3 allergen	D3YP11	81.1	685	6.74
40	Elongation factor Tu	D0J9Q4	43.8	395	6.05
41	Formyl-tetrahydrofolate synthetase (Fragment)	D8X0H9	37.8	352	6.74
42	4-hydroxy-tetrahydrodipicolinate synthase	D0J9L9	32.8	294	8.94
43	Cytochrome b	C6F3V9	42.9	377	8.38
44	Formyl-tetrahydrofolate synthetase (Fragment)	D8X0N4	38	352	5.9
45	Ala-tRNA synthetase (Fragment)	D0UKL7	26.2	235	6.93
46	Melatonin receptor	A0A0U5AH58	39.3	350	8.59
47	Molybdopterin oxidoreductase membrane subunit	D0J8M4	53.4	465	9.33
48	Major allergen Cr-PI	D3JUE9	82.1	688	7.05
49	GTPase Der	D0J980	50.8	439	9.82
50	RNA helicase (Fragment)	D0UKH2	27.6	248	8.9
51	Elongation factor 1-alpha (Fragment)	O02460	39.8	364	7.75
52	Triosephosphate isomerase	A0A2P0XIH0	26.8	247	6.55
53	Putative type II NADH dehydrogenase	D0J9N5	49.3	429	9.76
54	Cystathionine gamma-synthase	D0J8N6	42.5	383	8.07
55	Formyl-tetrahydrofolate synthetase (Fragment)	D8X0I5	38	352	7.74
56	Homeobox protein engrailed-like	Q9Y070	47.1	427	7.75
57	**Transferrin**	**H2F490**	**78.4**	**714**	**5.72**



Graph 1: Q-Exactive orbitrap LC-MS graph obtained for brain tissue lysate of *Periplaneta americana*.

The protein concentration in crude brain lysate was estimated using Bradford's test and was found to be 250 µg / ml. Several protein bands ranging from 97.4 kDa to 14.3kDa were found on native PAGE. The protein in band 2 and band

4 showed control over maximum bacteria like MRSA, MRSS, *E.coli, P.aeruginosa,* etc. *S.typhi* and *C.diphtheriae* were found to be susceptible to band 2, while *E.coli* was susceptible to band 4[20]. The Q-TOF - LC-MS/MS technique was selected for the identification of protein present in these two bands. The results were compared with the data of orbitrap reports, these are identified to be transferrin (MW: 79.91 kDa) and glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH) (MW: 35.58 kDa) enzyme respectively (Table 2). These enzymes are constitutively secreted in our cell due to their irreplaceable role in iron transport and glucose metabolism respectively. The antibacterial activity of these two enzymes is proposed to be one of its many moonlight properties in cells.

Table 2. Identification of protein using Q–TOF LC-MS/MS analysis method.

Band no.	MW (Da)	pI	Species	Database	Accession no.	Entry name
2	79914.4	5.43	Periplaneta americana	NCBInr	372292427	Transferrin
4	35585.1	6.98	Periplaneta americana	NCBInr	343965965	Glyceraldehyde-3-phosphate dehydrogenase

Table 3: LC-MS/MS analysis data of several peptides of Transferrin.

Score	Peak Intensity	Total Intensity	Sequence	Entry Name
24.3	93.7	9.79E+04	(R)YPNLCALCEHPEQCDYPDKYSGYDGALR(C)	transferrin
22.41	98.5	6.59E+04	(K)HFGLPIGAGEAVPTGEDPDNYAFLCPDGTK(K)	transferrin
22.19	92.5	4.40E+04	(R)SQISLADNIGETEHAAWLSK(V)	transferrin
19.77	89.7	1.51E+05	(R)DIRPAFDCVQESTNQDCMATVR(D)	transferrin
19.3	85.7	2.54E+04	(K)IQHHDADFVPVDPEDIFLASK(I)	transferrin
19.02	87.4	1.08E+05	(R)DIRPAFDCVQESTNQDCMATVR(D)	transferrin
19.01	90	4.89E+05	(R)SQISLADNIGETEHAAWLSK(V)	transferrin
18.98	93.5	5.00E+03	(R)DNGADVITLDGGDVFTAMR(E)	transferrin
18.88	88.4	6.87E+03	(K)KHFGLPIGAGEAVPTGEDPDNYAFLCPDGTK(K)	transferrin
18.65	88.9	2.70E+04	(R)CHLAEVPPHMVVTSNDKSDNVLNEIR(H)	transferrin
17.9	93.9	2.68E+05	(R)AVDTGTPVMQHYTEMLDVIR(T)	transferrin
17.31	83.1	1.38E+05	(K)LFGDFDGTKDLLFK(N)	transferrin
17.1	92.2	6.25E+04	(R)KMGVLTNLNDPDMTPR(E)	transferrin
16.43	95.5	1.55E+05	(R)DNGADVITLDGGDVFTAMR(E)	transferrin
16.31	73.5	1.50E+04	(R)EYNLKPIIAEQYGEHGSMYYAVAVVKK(S)	transferrin
15.98	85.7	9.31E+04	(K)MGVLTNLNDPDMTPR(E)	transferrin
15.87	83	9.62E+04	(R)TKEEPDEEFRYEAVAVIHK(N)	transferrin
15.68	89.1	3.64E+04	(R)TACLDKIQHHDADFVPVDPEDIFLASK(I)	transferrin
15.55	85.2	2.72E+05	(R)CLASGAGDVAFVK(H)	transferrin
15.27	92.2	4.27E+04	(R)AVDTGTPVMQHYTEMLDVIR(T)	transferrin
15.22	77.3	8.33E+03	(R)FCVTSDTELEKCHVLR(R)	transferrin
14.46	75.7	1.76E+04	(R)TACLDKIQHHDADFVPVDPEDIFLASK(I)	transferrin
14.14	76.5	1.65E+05	(R)ENELHALSQLFSK(A)	transferrin
13.72	65.3	6.66E+04	(R)DNGADVITLDGGDVFTAMR(E)	transferrin
13.49	61.5	4.14E+03	(R)FCVTSDTELEKCHVLR(R)	transferrin
13.44	83.5	2.49E+04	(R)AVDTGTPVMQHYTEMLDVIR(T)	transferrin
13.4	66.5	1.47E+05	(R)ENELHALSQLFSK(A)	transferrin
13.06	83.1	3.15E+04	(R)AVDTGTPVMQHYTEMLDVIRTCENQTPAQE(-)	transferrin
12.55	81	1.73E+04	(R)CHLAEVPPHMVVTSNDKSDNVLNEIR(H)	transferrin
12.09	85.2	1.02E+04	(R)AVDTGTPVMQHYTEMLDVIRTCENQTPAQE(-)	transferrin
11.89	56.7	3.37E+04	(K)MGVLTNLNDPDMTPR(E)	transferrin
11.76	63.4	2.70E+04	(R)TKEEPDEEFRYEAVAVIHK(N)	transferrin
9.87	53.6	7.81E+03	(K)ACLVGKWAPDPAQNQALK(E)	transferrin
9.54	54	1.26E+04	(R)EYNLKPIIAEQYGEHGSMYYAVAVVKK(S)	transferrin

Trypsin digestion of transferrin led to the production of multiple peptides. Those detected by mass spectrometry coupled with liquid chromatography are listed in table 3. The score of a peptide (R) YPNLCALCEHPEQCDYPDKYSGYDGALR(C) is maximum i.e 24.3 defining high concentration of this peptide in a sample with peak intensity of 93.7. However, (K) HFGLPIGAGEAVPTGEDPDNYAFLCPDGTK (K) peptide showed a maximum peak intensity of 98.5.

Table 4: LC-MS/MS analysis data of several peptides of GAPDH

Score	Peak Intensity	Total Intensity	Sequence	Entry Name
23.18	94.8	3.68E+05	(K)AGADYVVESTGVFTTIDKASAHLEGGAK(K)	glyceraldehyde-3-phosphate dehydrogenase
22.81	98.4	9.17E+04	(K)LTGMAFRVPVPNVSVVDLTVR(L)	glyceraldehyde-3-phosphate dehydrogenase
20.46	91.6	1.52E+05	(K)LTGMAFRVPVPNVSVVDLTVR(L)	glyceraldehyde-3-phosphate dehydrogenase
20.08	89.6	1.26E+06	(R)VPVPNVSVVDLTVR(L)	glyceraldehyde-3-phosphate dehydrogenase
17.76	84.1	9.38E+04	(K)AGADYVVESTGVFTTIDK(A)	glyceraldehyde-3-phosphate dehydrogenase
16.65	86.3	1.04E+05	(K)AGADYVVESTGVFTTIDKASAHLEGGAK(K)	glyceraldehyde-3-phosphate dehydrogenase
16.52	78.1	1.04E+05	(K)AGADYVVESTGVFTTIDKASAHLEGGAKK(V)	glyceraldehyde-3-phosphate dehydrogenase
15.53	53.8	1.37E+04	(K)VIHDNFEIVEGLMTTVHAVTATQK(T)	glyceraldehyde-3-phosphate dehydrogenase
14.44	89.7	4.66E+04	(K)AGIPLNNNFVK(L)	glyceraldehyde-3-phosphate dehydrogenase
14.11	66.2	6.57E+04	(K)GILDYTEDDVVSSDFISDTHSSIFDAK(A)	glyceraldehyde-3-phosphate dehydrogenase
13.99	83	1.53E+05	(R)VPVPNVSVVDLTVR(L)	glyceraldehyde-3-phosphate dehydrogenase
13.55	69.9	2.38E+04	(K)AGADYVVESTGVFTTIDKASAHLEGGAKK(V)	glyceraldehyde-3-phosphate dehydrogenase
11.44	60.5	1.12E+04	(K)VIHDNFEIVEGLMTTVHAVTATQK(T)	glyceraldehyde-3-phosphate dehydrogenase
9.63	56.6	2.88E+04	(K)VIISAPSADAPMFVVGVNLEAYDPSLK(I)	glyceraldehyde-3-phosphate dehydrogenase

Trypsin digestion of GAPDH led to the production of multiple peptides. Those detected by mass spectrometry coupled with liquid chromatography are listed in table 4. The score of a peptide (K) AGADYVVESTGVFTTIDKASAHLEGGAK (K) is the maximum i.e 23.18 defining high concentration of this peptide in the solution with peak intensity of 94.8. However, (K) LTGMAFRVPVPNVSVVDLTVR (L) peptide showed a maximum peak intensity of 98.4.

3.2. Protein structure homology modelling

The protein structure homology model using SWISS MODEL is considered to be reliable if the QMEAN value is closest to 1. The structure homology model of Transferrin and GAPDH were built and their analysis report (figure 1 and figure 2 respectively) suggests that the GAPDH model is reliable as compared to transferrin based on their QMEAN value. Since we couldn't build a reliable model of transferrin using SWISS-MODEL, RaptorX was used for building a structural homology model. The uGDT (un-normalized Global Distance Test) value of structure was 57 (i.e >50) which is counted to have 98% reliability. The P-Value of the structure is 1.92e-17 (figure 3)

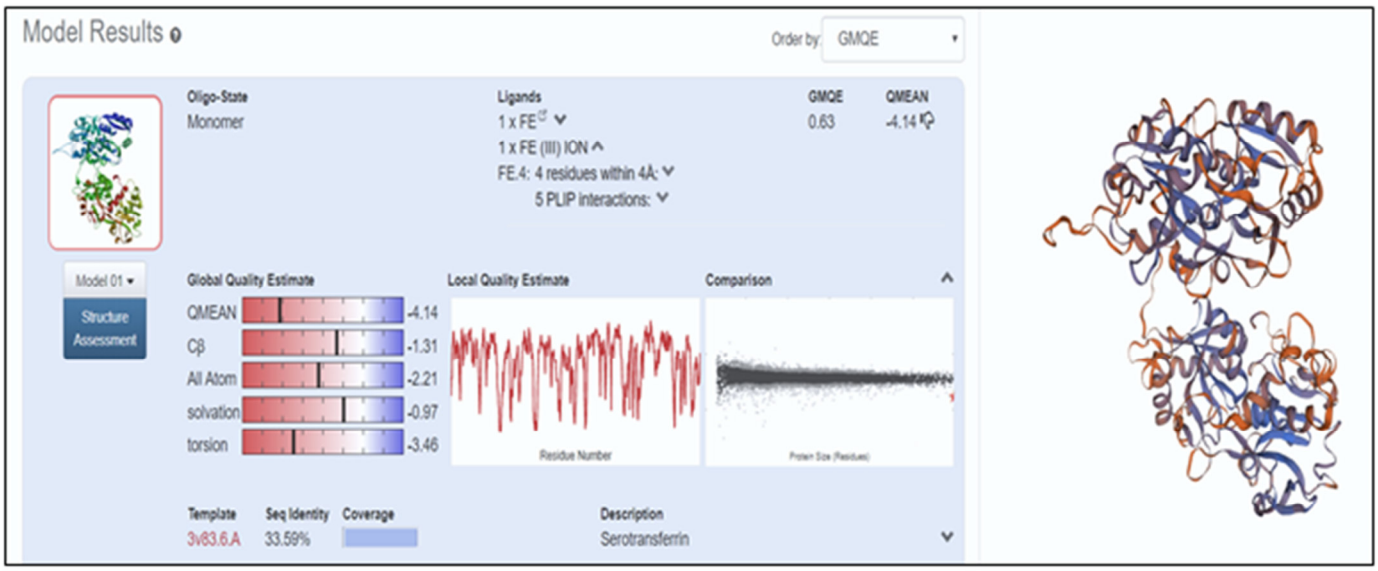


Fig 1: SWISS MODEL results of Transferrin structure homology modelling.

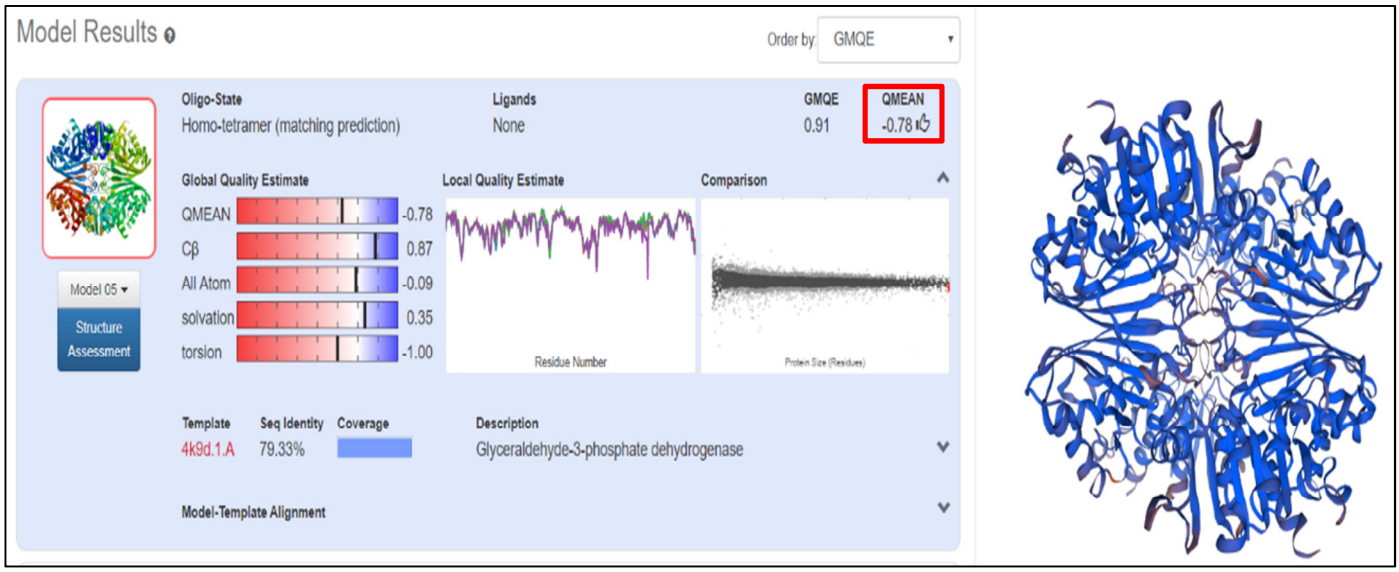


Fig 2: SWISS MODEL results of GAPDH structure homology modelling.

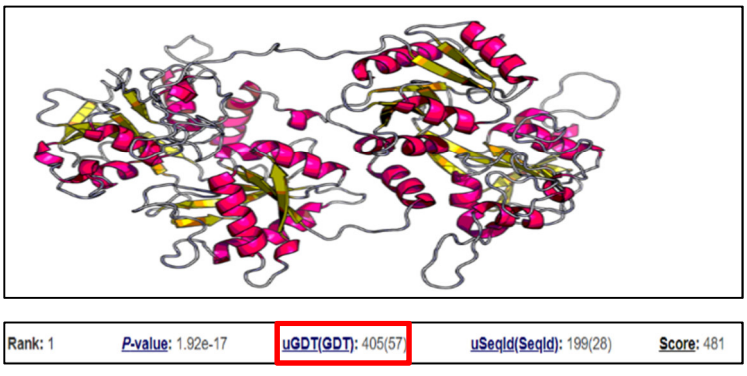


Fig 3: Structure prediction result of Transferrin using RaptorX

4. DISCUSSION

Antibacterial peptides are recorded to be a novel solution for the control of several pathogenic and drug-resistant bacteria and fungi. Natural products have led to discovery of numerous medical breakthroughs and lifesaving drugs particularly within the treatment of infectious diseases, cancer, hyper-cholesterolemia, and immune dysfunction. During the year 2003– 2008, 21 drugs originating from natural products (mainly from an actinomycete, bacterial, or fungal sources) were approved for marketing.[40] Glyceraldehyde-3-phosphate dehydrogenase a glycolytic enzyme is among the first identified moonlight protein. These are noted to play completely different functions in different parts of the cell.[41] GAPDH acts as a surface receptor for transferrin protein[42] on the membrane of *Staphylococcus*

aureus.[43] It plays role in/as adhesive protein[44], plasminogen binding protein[45], a surface receptor on human epithelial cells for *Mycobacterium avium* complex[46], pathology of human disease[47], etc. GAPDH of the human cell shows its antibacterial activity against different strains of *Candida albicans.*[48] Likewise, Iron-binding proteins like transferrin and lactoferrin express their antibacterial property by damaging the outer membrane of gram-negative and alter bacterial outer membrane permeability for the entry of iron.[49] Transferrin is known to play an important role in the transfer of iron in the bacterial cell.[50] It is an important component of bacterial proliferation. Several studies indicate the antibacterial effect of transferrin starvation.[51] Similarly, the antibacterial activity of both GAPDH and transferrin make them an eligible contender for classification as first moonlight protein identified from the brain tissue of *Periplaneta americana*. MOONPROT is a database compiled by Jeffery C. that stores information about moonlighting proteins that there exists biochemical or biophysical evidence. It contains 291 proteins.[52] MoonDB contains human moonlighting proteins recovered from the literature and candidates predicted by a protein-protein network-based approach. These databases provide platforms for systematic analysis of moonlighting proteins.[53] Several shotgun techniques have been evolved for the identification and characterization of such proteins from a variety of sources. The mass spectrometer is amongst the most advanced methods in protein studies. A detailed study of the antimicrobial protein profile of *Piper nigrum* was done using LTQ orbitrap.[54] Q-Exactive orbitrap is one of the most sensitive MS techniques which is capable of identifying proteins with higher resolution and better accuracies. [55,56] The sample preparation has a very important role played for the results of Q-Exactive orbitrap LC-MS. HRLC-MS/MS helps in the identification of target protein using a standard protein for reference. Protein identification by LC-MS/MS is based on independent sequencing of peptides. It is often possible to confidently identify a protein required based on MS/MS sequence of only one peptide, in contrast to 10-20 peptides required for MALDI-TOF fingerprinting. The identification of peptide using LC helps in ruling out several shortcomings of gel-based protein isolation.[57] Coupling of MS technique with LC is proposed to give much better findings than GC.[58,59,60] The data obtained from Q Exactive Orbitrap were selected based on the high confidence in Sequest HT search and Xcorr value above 2.[61] Peptide mass fingerprinting (PMF) is a technique best used for identification of protein. GAPDH and Transferrin were identified by a similar technique. The peak intensity and score values in table 3 and 4 explains the abundance of respective peptides in the sample. The Data obtained after Q TOF LC- MS/MS (as shown in Table 3 and 4) showed highest matching score for Transferrin and GAPDH with the score value of 374.84 and 188.27 respectively using Mascot PMF search engine. This Score value is related to the probability that the match is real than purely random making it more reliable for consideration.[62] Modelling the structure of an identified protein can provide an ease in future drug designing.[63] The structure homology modelled using SWISS MODEL provides QMEAN value which is considered to be good if its value is close to 0 making the selected GAPDH model fit for consideration whereas reject Transferrin model. RaptorX is another online server that is used for structure modelling. The excellence of modelled structure is defined by uGDT value higher than 50.[36] Finding out the interaction of this antibacterial protein with bacterial protein can help in gaining a better understanding of its action.

5. CONCLUSION

The American cockroach can be an alternative source of antibacterial protein. For untargeted protein identification Q-Exactive orbitrap LC-MS is the best technique of the current generation whereas HRLC-MS/MS is a reliable and accurate technique to be used for targeted protein identification. It is able to analyze very complex mixtures since each peptide is independently sequenced. The identified proteins using both the above mentioned techniques were glyceraldehyde-3-phosphate dehydrogenase and transferrin. They are constitutively expressed within the cell for glucose metabolism and iron transport respectively. Their antibacterial properties along with their assigned role in cells make them to be classified as moonlight protein. The structure developed using online homology modelling servers can be used for detection of their target on/in cell.

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7. AUTHORS CONTRIBUTION STATEMENT

Mr.Siddharth Sagar has taken a role in conceptualizing and gathering data; as well as preparation of manuscript. Dr. Jayaprada Rao Chunduri guided Mr. Siddharth Sagar in analysis and identifying the necessary inputs while designing the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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