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Antiviral activity of hexapeptides derived from conserved regions of bacterial proteases against HCV NS3 protease

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Abstract: The main cause of hepatitis C is hepatitis C virus or HCV and for the cure of hepatitis C, NS3/4A protease has been found an important and emerging target. A number of HCV NS3/4A protease inhibitors have been discovered which have shown subsequent reduction in reducing the viral load leading to this infection however they are still undergoing clinical trials for improvement. Bacterial proteases are of great pharmaceutical importance and have a key role in various biological processes and in life cycle of several pathogens. The current study was planned to explore hexapeptides derived from conserved regions of bacterial proteases for their potential in blocking the NS3 protease activity of HCV which would finally inhibit HCV multiplication. For this, a novel protease gene *npr*B was isolated from a thermophilic bacterium *Streptomyces thermovulgaris* and bioinformatics analyses were performed. PCR amplification and sequencing of *npr*B gene indicated an open reading frame of 178 aa (20191.18 Dalton).The peptide GGVHIN was the top ranked with minimum S-score of -17.21) followed by hexapeptides VDAHAN, GVGREA, GALNES and VHINSS with their S-scores of -14.73, -13.78, -10.72 and -10.70, respectively. A phylogram was also reconstructed to reveal evolutionary relationships of nprB with its various homologs. The provided data will serve as a background to further reveal pharmaceutical and biotechnological importance of this novel protease gene from *S. thermovulgaris* in future.

Keywords: Hepatitis C virus, NS3/4A protease, molecular docking, hexapeptides, *Streptomyces thermovulgaris.*

INTRODUCTION

The hepatitis C virus (HCV) is a health threat worldwide and the infection has been found in about 10 million people in Pakistan. HCV infection causes chronic hepatitis in 50-80% of people and due to this some of the patients get severe liver diseases including cirrhosis and hepatocellular carcinoma (Wadood *et al*., 2013). The NS3/4A protease has emerged as an important target for the treatment of hepatitis C (Ezat *et al*., 2019). Two domains (i.e. N-terminal protease domain and a C-terminal helicase domain) are present in NS3 protease and both domains perform different functions. The protease domain is involved in the processing of polyprotein while helicase domain is involved in the replication of RNA. The protease domain has also been found to be involved in increasing helicase activity and the activity of protease gets increased by helicase (Wadood *et al*., 2013).

The HCV replicates independently in the host and is a positive strain RNA virus. A replicative intermediate and negative-strand RNA is produced during this sustained replication. The replication also plays a role in the stimulation of lipid metabolism that is further involved in the accumulation of lipid vesicles and the process helps in the assembly and maturation of the virus (Mustafa *et al*., 2020). The fusion of structural protein in the form of viral envelope takes place to the endosomal membrane to release viral RNA into the cytoplasm. For the binding of RNA the ribosomal internal sites are responsible. One possible reason for frequent mutations in the HCV RNA genome might be due to the lack of a proofreading enzyme by the viral RNA polymerase (Andres, 2020).

A wide variety of organisms (e.g. invertebrates, vertebrates, fungi, plants and microorganisms) are employed to produce different products of pharmaceutical importance (Arif *et al*., 2020; Bukhari *et al*., 2020; Munir *et al*., 2020). About two third commercial productions of proteases worldwide is contributed by the microorganisms and according to an estimate around 40% of the total sale in the world enzyme market is constituted by microbial proteases (Ibrahim *et al*., 2015). Because microbial sources are not vulnerable to temperature and pH fluctuations and they are also tolerant of various organic solvents and detergents therefore they are predominately commercialized (Kousar *et al*., 2013; Mustafa *et al*., 2016). Streptomyces is an actinomycete and is important for having abilities to produce various molecules and to grow in a variety of habitats with different environmental conditions including temperature and pH (Prasad *et al*., 2013; Mustafa and Jamil, 2017). The reports on

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thermophilic species which are capable to produce proteases with pharmaceutical importance are however lacking in the literature. Therefore the purpose of this study was to explore hexapeptides derived from bacterial proteases as ligands which could bind and inhibit the processing of NS3 polyprotein and finally the replication of hepatitis C virus.

MATERIALS AND METHODS

Protease gene amplification

The strain NBRC 12383 of *Streptomyces thermovulgaris* was grown on Bennett's agar medium and DNA was extracted as described previously (Mushtaq *et al*., 2020). The protease gene was amplified from the genomic DNA using two pairs of primers (i.e. pair1-F 5/-GATCGGCTGGCCAGAATAG-3/ and pair1-R 5/-AGGCGAGATCATATTCACCG-3/, and pair2-F 5/-GCAAAGAAATAGTCAGCGGC-3/ and pair2-R 5/-CATGCCGGTCCAGGTACA-3/). The PCR cycling conditions used were: 30 cycles of repeated denaturation for 30 sec at 94°C, annealing for 1 min at 56°C, extension for 1 min at 72°C followed by one step of extension for 10 min at 72°C.

Sequencing and translation of nprB

The services of Centre for Applied Molecular Biology (CAMB), University of the Punjab, Lahore, Pakistan were used to sequence the amplified protease gene. The nucleotide sequence of *nprB* was translated into protein sequence using Translator tool of ExPASY (https://web.expasy.org/translate/). The protein sequence of nprB was further used to reveal conserved regions in the sequence.

Molecular docking study

The molecular docking study was proposed to check peptides which were derived from conserved regions of bacterial proteases against HCV NS3/4A protein using Molecular Operating Environment (MOE) software.

Devising hexapeptides as ligands

Three motifs were discovered from ten selected homologs of nprB protein from *S. thermovulgaris* using the MEME Suite an online tool (Bailey *et al*., 2009). The discovered motifs served as conserved regions of bacterial proteases and were used to drive ten hexapeptides to be docked against HCV NS3/4A. The chemical structures of all derived hexapeptides were generated using Chem Sketch software (ACD/ChemSketch, 2012). The peptides were energy minimized using MOE and saved as .mol2 format. A database containing all minimized peptides was constructed and saved in .mdb format and used for docking as an input file.

Optimization of receptor protein

Protein databank (PDB) was used to retrieve 3D structure of HCV NS3/4A receptor protein with PDB ID: 4A92. Before docking, the solvent molecules were removed and hydrogen atoms were added. 3D protonation was done and energy was minimized for receptor optimization using MOE. The optimized structure was used for molecular docking.

The ligand database was docked against HCV NS3/4A receptor protein through pocket selection from the receptor and docking parameters setting by MOE. The output file was stored in .mdb format. All interactions were checked for their S-scores to find the best ones with the active sites.

Phylogenetic analysis

The protein sequence of nprB from *S. thermovulgaris* was analysed using BLASTX (Altschul *et al*., 1990) available on the NCBI. Along with nprB protein sequences of *S. thermovulgaris* thirty five homologous sequences from different bacterial species were also retrieved from protein database for phylogenetic analysis. All sequences were aligned using MUSCLE and imported into the MEGA5 program (Tamura *et al*., 2011) for manual alignment. A phylogenetic tree was reconstructed through Maximum Likelihood (ML) method using MEGA5 with 100 bootstrap replicates.

RESULTS

nprB gene manipulation

The nprB gene was successfully amplified (using the primer pairs given in Methods) and sequenced. The nucleotide sequence of nprB gene was found to be 792 base pairs which was deposited in GenBank of National Centre for Biotechnology Information (NCBI) and allotted an accession number (i.e. GenBank: KX879552.1).

Deriving hexapeptides from bacterial proteases

The MEME Suite was used to explore three motifs in ten selected homologs of nprB protein from *S. thermovulgaris*. The predicted motifs have been shown in fig. 1. The motifs were used to derive ten hexapeptides to be docked as ligands against HCV NS3/4A protease and the sequences and structures of these hexapeptides have been given in table 1.

Molecular docking

After docking, all conformations were stored on the basis of their docking score as a parameter. The peptide GGVHIN was found to be the most excellent conformation as it has the minimum S-score (i.e. -17.21) and ranked at top followed by the peptides VDAHAN, GVGREA, GALNES and VHINSS with their S-scores -14.73, -13.78, -10.72 and -10.70, respectively in table 2.

The conformations of top five peptides were investigated to find out the dynamics of their interactions with HCV

Table 1: Sequence and structures of devised hexapeptides from bacterial proteases

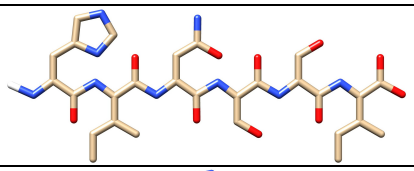
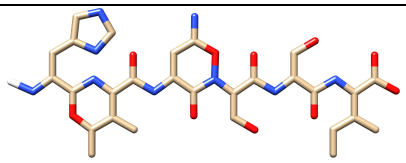
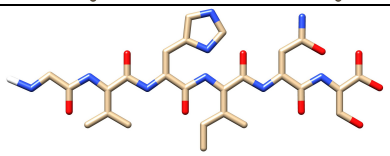
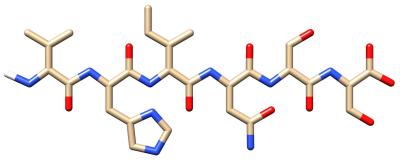
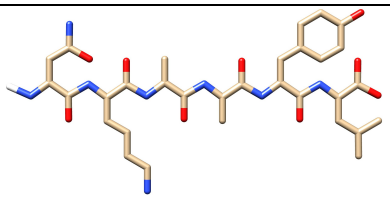
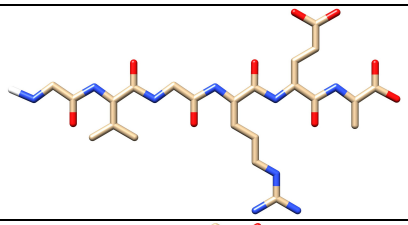
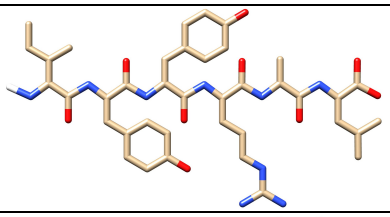
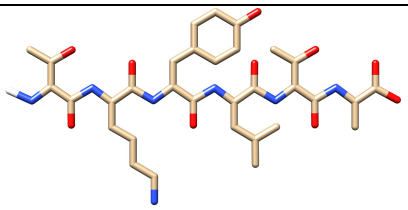
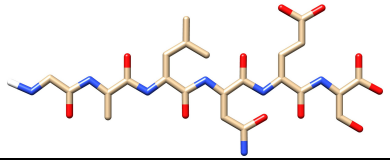
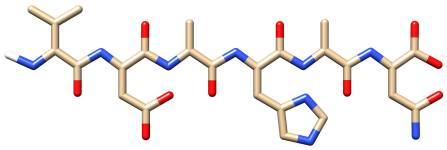
Peptide	Structure	Peptide	Structure
GGVHIN		HINSSI	
GVHINS		VHINSS	
NKAAYL		GVGREA	
IYYRAL		TKYLTA	
GALNES		VDAHAN	

Table 2: The interactions of devised hexapeptides with HCV NS3/4A protein

S No.	Peptide	S-score	Interacting residues of NS3/4A
1	GGVHIN	-17.21	Thr295, Asp412, Asp454, Ser457, Arg481, Pro482, Met485
2	VDAHAN	-14.73	Ser294, Thr295
3	GVGREA	-13.78	Thr295, Thr433, Gln434, Arg481
4	GALNES	-10.72	Arg464, Arg467
5	VHINSS	-10.70	Arg481, Pro482
6	HINSSI	-10.23	His293, Pro482, Met485
7	TKYLTA	-9.53	Thr433, Met485
8	NKAAYL	-8.81	Thr419, Arg467
9	GVHINS	-7.89	Met485
10	IYYRAL	-7.89	Gln460

NS3/4A. The interacting residues of receptor protein are also given in table 2. The interactions and binding patterns of top five hexapeptides with HCV NS3/4A have been shown in fig. 2.

Protease phylogeny

For the identification of similar local regions and statistically significant protein sequences of protease the BLASTX was used. Multiple sequence alignment was also performed through Geneious (Kearse *et al*., 2012). The truncated sequences were deleted during multiple sequence alignment and the longer sequences were shortened in order to make all the sequences equal in length. Molecular phylogenetic analysis was performed to show evolutionary relationships of protease from selected organisms (fig. 3). A protease from *Vibrio cholerae* was used to root the ML tree.

Phylogenetic tree was divided into four different monophyletic groups (MPG-I to MPG-IV) (fig. 3). The protein sequence of protease from *S. thermovulgaris* appeared in MPG-IV and showed its evolutionary

Fig. 1: Predicted motifs used to derive ten hexapeptides. Red rectangles are showing highly conserved regions from where peptide sequences were picked.

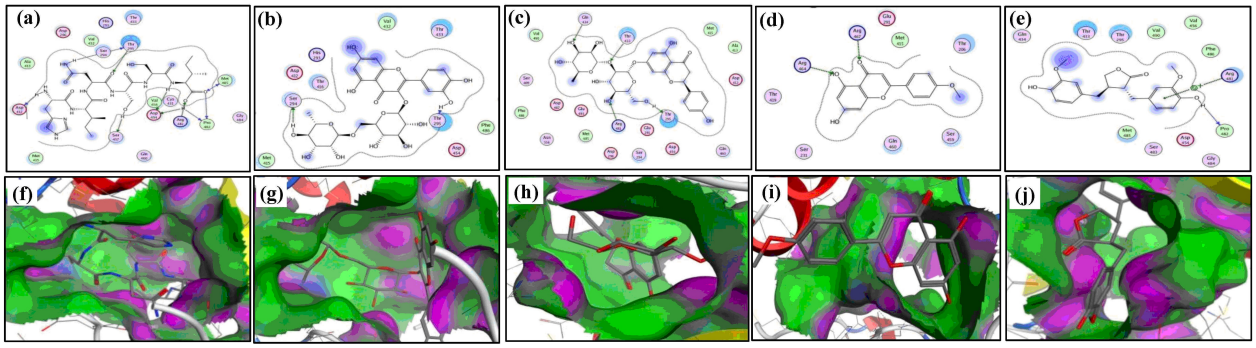


Fig. 2: Interactions (a to e) and binding patterns (f to j) of top five hexapeptides with HCV NS3/4A protease as target protein. (a, f) GGVHIN, (b, g) VDAHAN, (c, h) GVGREA, (d, i) GALNES and (e, j) VHINSS

closeness with *B. subtilis*, *B. halotolerans*, *B. mojavensis* and *Streptococcus pneumoniae*. One unclassified *Bacillus* species was also appeared in this group. All the taxa appeared in MPG-I belong to *Bacillus* species whereas two *Bacillus* species (i.e. *B. panaciterrae* and *B. megaterium*) joined MPG-II along with different bacterial species. The presence of three *Oceanobacillus* species in MPG-III with *Lentibacillus populi* and *Virgibacillus dakarensis* showed their close evolutionary relationships. The protease of *Bacillus koreensis* was not joined to any monophyletic group and appeared as a separate branch showing that it is very distantly related with other bacterial species.

DISCUSSION

Recently, the NS3 protease has been found an attractive and effective target for targeting and developing new antiviral drugs. Numerous efforts have been done to target helicase domain to develop and/or design some novel compounds with inhibitory properties. The non-structural protein NS3 has been found to be constituted by serine protease at N-terminus while by helicase at C-terminus. It has been revealed by *in vitro* and in silico studies that at N-terminus of the protease domain of NS3 the 181-residue forms a heterodimer with a small 54-residue NS4A peptide cofactor which leads in subsequent HCV polyprotein down-stream cleavage at junctions between viral proteins (Naika *et al*., 2015). This is why drug designing has been significantly investigated targeting the NS3/4A for blocking viral replication and restoring hepatocyte innate immune control of HCV replication.

In this study, ten hexapeptides were explored for their antiviral activity against HCV NS3/4A using molecular docking approach and top five peptides were picked on the basis of their S-scores and binding interactions. The top ranked peptide (i.e. GGVHIN) showed binding patterns with Thr295, Asp412, Asp454, Ser457, Arg481, Pro482 and Met485 of HCV NS3/4A. In VDAHAN and HCV NS3/4A interaction, Ser294 and Thr295 were acted as side-chain and backbone acceptors respectively. In the interaction of peptide GVGREA and receptor protein, Thr295 was acted as side-chain acceptor while Thr433, Gln434 and Arg481 were acted as side-chain donors. Similarly, in GALNES and HCV NS3/4A interaction, Arg464 and Arg467 were acted as side-chain donors. In the interaction between VHINSS and NS3/4A, the Arg481 showed a strong receptor contact and Pro482 was acted as backbone acceptor.

A phylogenetic tree of bacterial proteases based on their protein sequences was also generated in this study. All the taxa showed that they had originated from a common

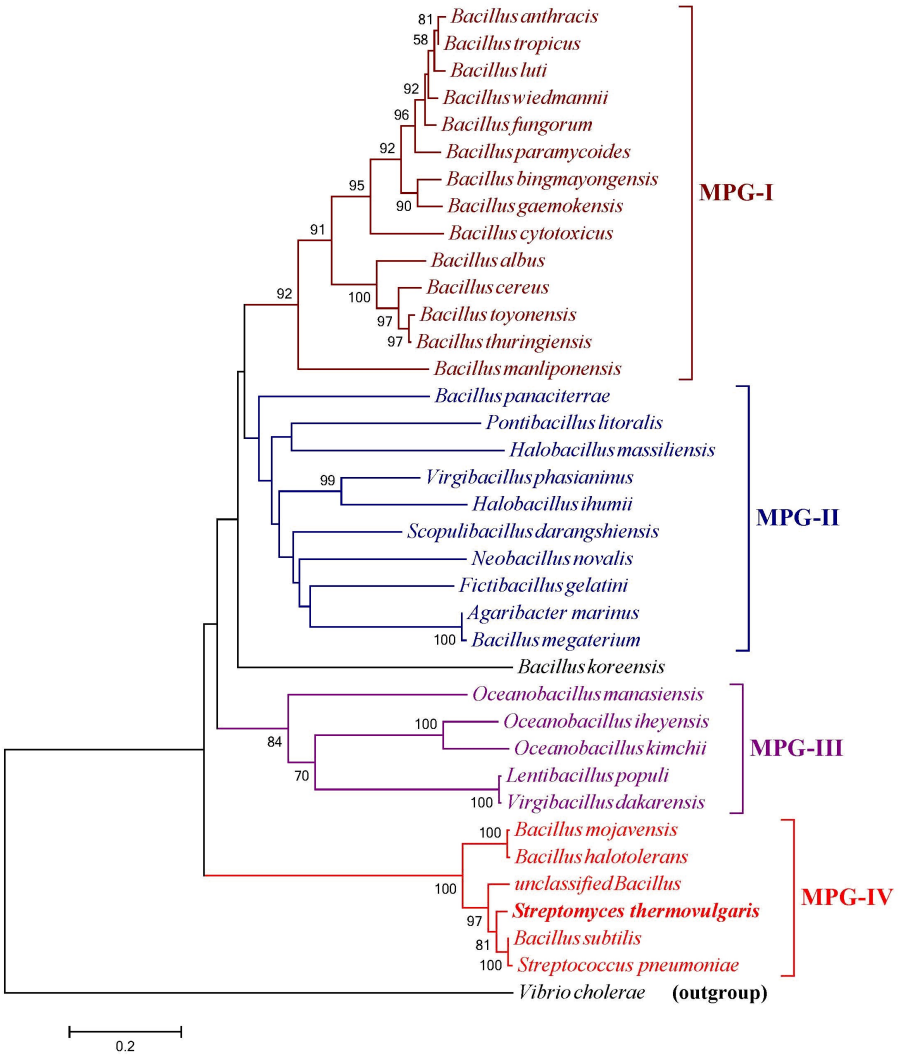


Fig. 3: Phylogeny of proteases from selected organisms using Maximum Likelihood method. Poisson correction model was used to generate the phylogram. To represent evolutionary history of selected taxa, the bootstrap consensus phylogram has been inferred from 100 replicates. All the branches corresponding to partitions which replicated less than 50% were collapsed. Bootstrap values (100 replicates) have been shown above the nodes. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. Total 37 amino acid sequences were taken for analysis in which all positions that contain gaps and missing data have been removed. The analysis was done in MEGA5 software.

ancestor and divided into four monophyletic groups. Jabalia *et al*. (2015) also reported that the sequences of papain family of cysteine proteases were evolved from a common ancestor and during the evolution they were divided into various sub groups. A phylogenetic tree of protease-producing bacteria was also reconstructed by Zhou *et al*. (2013). They observed five branches in the phylogenetic tree. *Bacillus* species were appeared in branch 1, strains similar to *Pseudoalteromonas arctica* were appeared in branch 2, *Flavobacterium* strains similar to *Flavobacterium degerlachei* were appeared in branch 3, strains similar to *Psychroserpens* sp. were appeared in branch 4 and *Lacinutrix* strains similar to *Lacinutrix* sp. were appeared in branch 5. Similarly, in a phylogenetic tree constructed using 16S rRNA gene sequences of 121 protease-producing bacteria suggested that the isolates belonged to 17 genera of 4 phyla (i.e. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria). The phylogram was dominated by the genera *Pseudoalteromonas* (40.5%) followed by *Bacillus* (36.3%) and *Photobacterium* (5.8%) (Li *et al*., 2017).

To comparative analyses based on DNA and/or protein sequences with rapid accumulation of molecular sequences the phylogenetic methods are getting more and more attention (Jabbir *et al*., 2019). Different phylogenetic methods are used to estimate evolutionary rates of various genes and proteins for following footprints of natural selection (Mustafa and Jamil, 2013; Mustafa *et al*., 2014; Aslam *et al*., 2018).

A wide variety of enzymes of pharmaceutical and industrial importance have been produced from different organisms including algae (Bukhari *et al*., 2018), fungi (Shaheen *et al*., 2017; Mustafa *et al*., 2018) and bacteria (Zafar *et al*., 2014; Liu *et al*., 2019). Proteases have wide range of uses in medical fields. Various formulas of alkaline proteases (e.g. non-woven tissues, gauze and ointment composition) have showed promising therapeutic properties. The oral administration of alkaline proteases has been used to aid deficiency of certain lytic enzymes (Awad *et al*., 2013). Fibrinolytic proteases are used for fibrin degradation which suggests their future applications as anticancer drugs and also in thrombolytic therapy. Proteases are also employed in collagen hydrolysis which liberates peptides of low molecular weights without releasing any amino acids. Likewise, proteases are also used to treat different diseases such as carbuncles, furuncles, burns, wounds and to prepare elastoterase which is immobilized on the bandage (Razzaq *et al*., 2019).

CONCLUSION

Ten hexapeptides were derived from conserved regions of bacterial proteases and molecular docking of these peptides was carried out against NS3 protease to explore their antiviral activities. The hexapeptide GGVHIN was ranked at the top on the basis of its docking score and binding interaction with NS3/4A protease. These findings have exhibited potential binding interactions of these hexapeptides with the NS3 protease active sites. The phylogenetic tree reconstructed in this study explored that various bacterial proteases are evolved from a common ancestor and they all are evolutionary related proteins. The properties of this enzyme make it promising for basic research and pharmaceutical and industrial applications.

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