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AN *IN SILICO* STUDY OF ACTIVITY OF HYDROPHOBIC AMINO ACIDS AS POSSIBLE MOTIF STRUCTURES IN ANTIMICROBIAL PEPTIDES OF DIVERGENT ORIGINS

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Abstract
Antimicrobial peptides (AMPs) are widespread in nature and are being employed by a diverse group of organisms, from bacteria to humans for exterminating microbial pathogens. Although having diverse origins, different structures and unique modes of action, all the AMPs share a common feature and that is, till date there are almost no reports suggesting the development of microbial resistance against these peptides. The present study uses a mathematical graph theoretical approach and the information from an updated AMP database-APD2 (http://aps.unmc.edu/AP/main.php)-to demonstrate the recurrence of some hydrophobic amino acid residues (motifs) within different classes of AMPs.
Key words: Sequences; Protein motifs; databases; biochemical networks

Introduction:

Exterminating microbial pathogens by utilizing specially designed antimicrobial peptides (AMPs) is an interesting strategy employed by nature. Extremely widespread production of these peptides throughout bacterial, fungal, plant and animal kingdom could be taken as a proof of their success.

Depending upon their primary sequence homology the AMPs are divided into several different classes. Most of the members belonging to one class have similar origins with some minor exceptions. But strikingly, all the AMPs across the various different classes share a common feature and that is, till date there are almost no reports suggesting the development of microbial resistance against these peptides.

In the present work, we demonstrate by using BLAST P, the protein sequence alignment tool, that in spite of belonging to different classes many of these AMPs do show a remarkable homology in the content of a particular category of their amino acid residues. These amino acids are typically hydrophobic in nature, and might be present as a block of two or as distantly interacting single residues at any position in the peptide sequence. Their presence was consistently observed within different classes of AMPs. Some of the amino acid combinations that were studied and scanned for were TG, HV, LC, AL, GL, etc. in increasing frequency of occurrence. Moreover, all the AMPs showing the presence of these amino acids had distinctly divergent origins which included peptides from bacteria, fungi, invertebrates, amphibians, plants and mammals.

We particularly focus on and address the issue of presence as well as frequency of appearance of some identifiable blocks of hydrophobic amino acid as pairs or as singleton in the linear sequence of amino acids that form the primary structure of proteins. We scan the retrieved sequences for the appearance of either the amino acid pairs, or even solitary ones of the hydrophobic residues identified in the course of this work, and postulate that some of the characters of the AMPs in which these appear, are induced due to the presence of such special blocks that assume importance enough to be labelled as one of the fundamental building blocks of the amino acid sequence.

Most of the AMPs essentially have hydrophobic amino acids which are responsible for their antimicrobial activity. The peptides selected for the present study preferentially lack the secondary structure which might otherwise require polar/charged-polar amino acids at specific positions. These polar amino acids are incorporated so that they could be modified or crosslinked to provide the peptide with a specific secondary structure essential for its activity.

For our present work, we have selected some hydrophobic amino acids whose mere presence irrespective of their position and hence independent of peptide secondary structure, is enough for the peptides' activity. The amino acids selected for our study were Alanine (A), Leucine (L), Valine (V), Isoleucine (I). These four amino acids have similar degree of hydrophobicity. In addition to these Glycine (G) which is neither hydrophilic nor hydrophobic was also considered as a special case. Amino acids Cystein (C), Histidine (H) and Threonine (T) belonging to less polar category, but still somewhat capable of undergoing hydrophobic interactions were also included for screening.

APD2, the updated AMP database was used for scanning of the antimicrobial peptides

which have the above amino acids, for the present study. The availability of detailed molecular data via high throughput technologies allows us a reconstruction of the amino acid sequence structure at the system level. We apply graph theory for modelling the system constituting the protein primary sequence, and try to gain and insight into the significance of the presence and frequency of the selected hydrophobic amino acid blocks within the AMP sequences.

Graph theory

Various cellular functions of an organism are carried out through a complex network of interactions among the specific cellular components and factors designed and responsible for a given process [1]. Networks are the topological aspects of a graph, and have been used in the literature by scholars to study and describe processes such as protein - protein interaction, prediction of conditional gene essentiality, transcriptional regulatory networks and metabolic pathways, to mention few of the important issues current in the scholarship [2, 3,4,5,6,7,8,9].

Network motifs are patterns of interactions occurring in complex networks, which occur with a remarkably high frequency compared to a randomized network. These motifs could be viewed as the vertices of a graph, with the interactions among them being mediated by the edges, thus constituting the basic building blocks of networks [10, 11, 12].

In this paper we consider the undirected graph G comprising the triple $G=(V,E,\gamma)$, where the amino acids constituting the peptide sequence are represented as vertices v_i and comprise the set V while the interaction between any two amino acids is represented by an edge e_i and comprise the set E, with $E\bigcap V=\phi$. The mapping $\gamma: E\to V\times V$, assigns an edge to any two specific vertices by the definition $e_i \mapsto \gamma(e_i)=\{v_i,v_j\}$. The vertices v_i and v_j share an edge e_i and are adjacent vertices. The set $E=\{e_1,e_2,....,e_n\}, n\in Z_+$ is a finite collection of interactions between the amino acids in pairs, and therefore the set of edges $E\subseteq [V]^2$, a two-element subset of the set of vertices V. A graph G is connected if there exist paths (a sequence of adjacent vertices and edges connecting these) in G between any two vertices $\in V$. $G'=(V',E',\gamma')$ with $V'\subseteq V, E'\subseteq E,$ and γ' a restriction on γ to E' is a subgraph of graph G.

A motif is a connected subgraph of the graph G that represents the primary (linear) sequence structure of the amino acids forming a protein or a peptide. A motif, in the present context, could thus be understood as a basic graph template of biologically interacting amino acid vertices that acts as a fundamental building block for the peptide sequence in conferring the protein/peptide its functional characteristics [13, 14, 15].

In the present work, we focus on the two-vertex graphs formed by pairs of amino acids, in the primary sequence of AMPs. Irrespective of the actual locus of these amino acids in the peptide sequence, the mutual interactions between any two of them or the interactions of a lone amino acid with its neighbours in the sequence imply the existence of edges between as well as to and from these vertices. Our principal objective in this work is to establish the importance of these motifs within the AMPs studied, by contextualizing the presence of these graphs vis-a-vis the lethality of the AMPs.

A match of a motif (amino acid(s)) G' within a target graph (the protein) G is a graph G'' which is also a subgraph of G, and is isomorphic to the motif G'. The frequency of a motif is the number of its matches in the target graph [16].

Procedure

The present work is based on *in silico* data, retrieved from the APD2 database http://aps.unmc.edu/AP/main.php.

The following is the tabulation of the frequencies of amino acid motifs occurring as pairs as well as split single residues in the selected AMPs:

It should be noted that in Table 1 the total number of antibacterial peptides (1179) available in the database was used to calculate the match percentage. If the number of antifungal and antiviral peptides is also added the total becomes 1731 however this number is not considered because most of the peptides belonging to antifungal and antiviral class also exhibit antibacterial activity.

Table 1 shows an appreciably higher frequency of match for the hydrophobic amino

acid blocks, both as two-vertex as well as one-vertex motifs. It is noteworthy to focus on the GL motif, which is seen to have the highest frequency of match within the peptide sequences. The frequency of this graph when split into two separate amino acid vertices G-L (single-vertex motifs) is still significantly higher at 81.5%. The frequencies for some other motifs with similar degree of hydrophobicity as GL are also seen to be appreciably high, particularly as one-vertex motifs in the selected AMPs. Moreover when the interaction of selected amino acids within themselves was determined in the peptide sequence as single vertex motifs the frequency recorded was even higher (eg. G-G etc.).

The peptide sequences obtained from the database were further short listed on the basis of their content of the four hydrophobic amino acids (and/or Glycine) identified above, either as two-vertex or one-vertex motifs. The objective was to find those amino acid residues which were common within every scanned result, as well as those which were unique to a single query.

From these peptides few of the shortest sequences were selected for our work, because being the shortest, these peptides would possess the minimal essential amino acids to retain their antimicrobial activity, as also would show low propensity for folding. While selecting the short peptides, those belonging to different and distant origins were preferred in order to invoke a genetic diversity in the selection, and then to identify possible motifs in these peptides from diverse origins. The selected peptides with their origins in parentheses are listed below:

1. **GLLKRIKTLL-NH_2** Anoplin (insect, invertebrates, animals),
2. **PFKLSLHL-NH_2** Jelleine-I (honeybees, insect, invertebrates, animals),
3. **RLCRIVVIRVCR** Bactenecin (Cyclic dodecapeptide, cow cathelicidin, animals),
4. **GLLDIVKKVVGAFGSL-NH_2** Aurein 2.1 (frog, amphibians, animals),
5. **SVAGRAQGM** Cn-AMP1 (Cocos nucifera L. antimicrobial peptide 1, plants).

As can be seen from their amino acid sequence not all the selected peptides have all the hydrophobic amino acids of interest. Anoplin and Jelleine are from insect origin, Bactenecin from mammalian origin, Aurein from amphibian while Cn-AMP1 from plant origin (and a few more are given in the table 2). Although from diverse origins, the hydrophobic amino acid motifs comprise at least around 50% of the constitution of each of the above chosen peptide sequences.

Discussion and Inference:

The amino acid substitution studies within all the above selected peptides have conclusively proved the importance of amino acid motifs in the peptides in terms of their lethality (refer table 2 for the entire discussion hereafter). Anoplin is a decapeptide amide, GLLKRIKTLL-NH_2 derived from the venom sac of the solitary spider wasp, *Anoplius samariensis*. Complete alanine scan of this peptide has proved that the replacement of any polar amino acid by "A" within its sequence increases the activity of the resulting peptide in most of the cases. Moreover, it is also reported that the replacement of the 5th amino acid arginine (R) with any of the hydrophobic amino acid remarkably enhances the activity of the peptide. Interestingly, however, replacement of any previously existing hydrophobic amino acid with any other polar amino acid markedly reduces the activity of the peptide[17]. This similar observation is also recorded for the next peptide Jelleine-I which along with 3 other homologous AMPs forms an integral part of the "royal jelly" produced by honey bees, which is the principle food of the queen honey bee. The other three Jelleine peptides differ only at their first amino acid from Jelleine-I where they have an extra amino acid, otherwise the sequence is exactly same as Jelleine-I. The exception here is of Jelleine-IV which lacks the last Leucine residue and thus is reduced to the same size as that of Jelleine-I (8 amino acids). But this loss of one terminal leucine residue results in a lethal loss of activity within Jelleine-IV. This observation clearly demonstrates the importance of the terminal leucine residue within the Jellein peptides[18].

Bactenecin (also called bovine dodecapeptide) from bovine neutrophils is a cyclic dodecapeptide. However in addition to its naturally occurring cyclic form several linear derivatives were synthesized. Both within cyclic and linear derivatives those peptides with A, L or V substitution for polar amino acids proved to be beneficial for the peptide's activity, while the reverse substitutions lead to loss of activity in most of the situations[19]. Similar observations were recorded for the other two study peptides-Aureins and Cn-AMP1. Aurein peptides are amphipathic and α-helical peptides; Aurein1.2 with only 13 amino acid residues, it is one of the smallest amphibian peptides so far reported.

Although Aureins are amphipathic it is their hydrophobicity that is responsible for their antimicrobial activity[20-21]. Same is true for the green coconut water peptide Cn-AMP1 which is the most active peptide within the collection of three AMPs from the same source, named Cn-AMP2 and 3 and which differs from Cn-AMP1 just in their proportion of hydrophobic amino acids[22]. One of the most important evidences about the vitality of our selected hydrophobic amino acids in imparting microbicidal property to AMPs comes from the example of another amphibian peptide uperin 3.6 isolated from the Australian toadlet *Uperoleia mjobergii*. With only 17 amino acids it is one of the smallest of known amphibian AMPs. Although small it has been reported to be very potent against even the most deadly of all bacterial pathogens including methicillin-resistant staphylococci, and vancomycin-resistant enterococci[23]. Interesting feature of this peptide is its amino acid content. Ten of its amino acids are hydrophobic out of which only one is terminal phenylalanine which is aminated, while others include V,I,L,A and in addition to these there is one G at the N-terminal and six other polar amino acids[23]. With this amino acid composition it could be very well stated that the secret of uperin 3.6's potency is largely because of its hydrophobic amino acid composition.

From the above discussion, we infer that since the selected amino acid motifs appear in AMPs drawn from diverse origins, it is possible that there may exist an evolutionary pressure to maintain these specific motifs for the significant activity levels of the peptides. These motifs then should be evolutionarily conserved should have identifiable orthologs across the diverse organisms.

Table 1: Occurrence frequency of selected amino acids as single or two vertex motifs.

Amino acid motif	Nature	Match frequency (out of 1179)	Match %
TG	Partially hydrophobic	134	11.4
GT	Partially hydrophobic	132	11.2
LC	hydrophobic & polar	113	9.6
CL	Polar & hydrophobic	90	7.6
HV	polar & hydrophobic	71	6.0
VH	Hydrophobic & polar	30	2.5
AL	Hydrophobic	215	18.2
LA	Hydrophobic	236	20.0
AV	Hydrophobic	133	11.3
VA	Hydrophobic	180	15.3
AI	Hydrophobic	192	16.3
IA	Hydrophobic	198	16.8
VL	Hydrophobic	187	15.9
LV	Hydrophobic	143	12.1
IL	Hydrophobic	173	14.7
LI	Hydrophobic	132	11.2
IV	Hydrophobic	110	9.3
VI	Hydrophobic	112	9.5
GL	Hydrophobic	395	33.5
LG	Hydrophobic	290	24.6
GA	Hydrophobic	251	21.3
AG	Hydrophobic	325	27.6
GI	Hydrophobic	245	20.8
IG	Hydrophobic	276	23.4
GV	Hydrophobic	248	21.0
VG	Hydrophobic	264	22.4
T-G/G-T	Hydrophobic	655	55.5
L-C/C-L	Hydrophobic	470	39.9
H-V/V-H	Hydrophobic	364	32.6
L-A/A-L	Hydrophobic	846	71.8
A-V/V-A	Hydrophobic	743	63.0
A-I/I-A	Hydrophobic	786	66.7
V-L/L-V	Hydrophobic	822	69.7
I-L/L-I	Hydrophobic	890	75.5
I-V/V-I	Hydrophobic	781	66.2
G-L/L-G	Hydrophobic	961	81.5
G-I/I-G	Hydrophobic	904	76.6
G-A/A-G	Hydrophobic	860	72.9

G-V/V-G	Hydrophobic	860	72.9
G-G	Hydrophobic	1078	91.4
L-L	Hydrophobic	1046	88.7
I-I	Hydrophobic	969	82.2
A-A	Hydrophobic	921	78.1
V-V	Hydrophobic	916	77.7

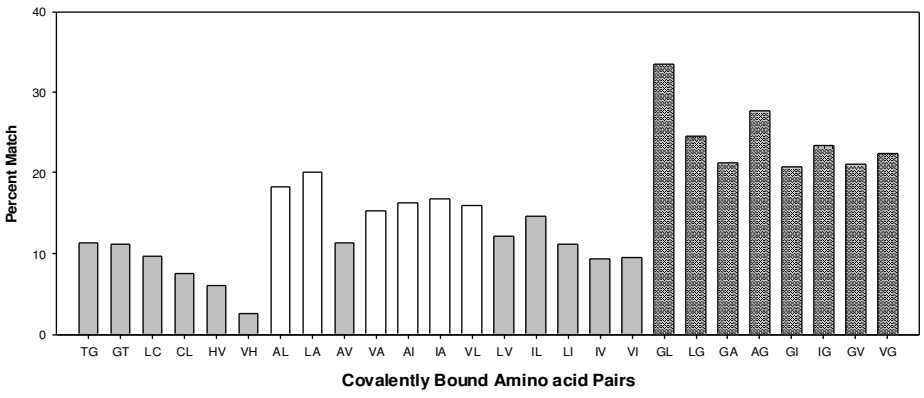
Table 2: Amino acid sequences of different selected study group peptides and there derivatives with their respective Minimal Inhibition Concentration (MIC) values. Higher the MIC lower is the potency except for Ponericins where activity is represented in terms of zone of inhibition, and so, higher the value more is the potency.

S.No.	Name of Peptide	Sequence	MIC (µg/ml)	
I.	**Anoplin** (insect, invertebrates, animals)		S.aureus	E.coli
1.	Anoplin	GLLKRIKTLL- NH_2	13	26
2.	ano-A1	**A**LLKRIKTLL- NH_2	>21	>21
3.	ano-A2	G**A**LKRIKTLL- NH_2	>43	>43
4.	ano-A3	GL**A**KRIKTLL- NH_2	>38	>38
5.	ano-A4	GLL**A**RIKTLL- NH_2	28	16
6.	ano-A5	GLLK**A**IKTLL- NH_2	10	5
7.	ano-A6	GLLKR**A**KTLL- NH_2	>41	>41
8.	ano-A7	GLLKRI**A**TLL- NH_2	11	11
9.	ano-A8	GLLKRIK**A**LL- NH_2	8	16
10.	ano-A9	GLLKRIKT**A**L- NH_2	>31	49
11.	ano-A10	GLLKRIKTL**A**- NH_2	>48	>48
II.	**Jelleine** (honeybees, insect, invertebrates, animals)			
12.	Jelleine-I	PFKISIHL- NH_2	10	2.5
13.	Jelleine-II	**T**PFKISIHL- NH_2	15	15
14.	Jelleine-III	**E**PFKISIHL- NH_2	30	15
15.	Jelleine-IV	**T**PFKISIH- NH_2	R	R
III.	**Bactenectin** (cow cathelicidin, animals) Cyclic peptides			
16.	Bactenecin	RLCRIVVIRVCR	32-64	8
17.	Bac R	***R***RLCRIVVIRVCR***R***	64	2
18.	Bac P3R	***R***RR**C**PIVVIRVCR***R***	>64	2
19.	Bac P3R-V	***RR***RLC**P**IV_IRVCR***R***	>64	2
20.	Bac 2I-NH_2	R**I**CRIVVIR_C***I***R- NH_2	32	4
21.	Bac P2R- NH_2	RLC**PR**V**R**IRVCR- NH_2	>32	4
22.	Bac P1	RLCRIV***P***VIRVCR	64	32
23.	Bac W	RLCRIV***W***VIRVCR	4	8
24.	Bac W2R	***R***RLCRIV***W***VIRVCR***R***	2	2
	Linear peptides			
25.	Linear (reduced)Bactenecin	RLCRIVVIRVCR	>64	64
26.	Lin Bac 2S- NH_2	RL**S**RIVVIRV**S**R- NH_2	4	2
27.	Lin Bac 1S- NH_2	RL**S**RIVVIRVCR- NH_2	16	4
28.	Lin Bac 2A- NH_2	RL**A**RIVVIRV**A**R- NH_2	4	4
29.	Lin Bac P3R	***R***RR**C**PIVVIRVCR***R***	>64	8
30.	Lin Bac P3R-V	***RR***RLC**P**IV_IRVCR***R***	>64	4
31.	Lin Bac P1	RLCRIV***P***VIRVCR	>64	16
32.	Lin Bac W	RLCRIV***W***VIRVCR	>64	>64
33.	Lin Bac W2R	***R***RLCRIV***W***VIRVCR***R***	>64	>64
IV.	**Cn-AMP1** (Cocos nucifera L. antimicrobial peptide 1, plants)			
34.	Cn-AMP1	SVAGRAQGM	80	82
35.	Cn-AMP2	***TE***S**YFVFSV**GM	170	170
36.	Cn-AMP3	YCSYTMEA	302	274
V.	**Aurein** (frog, amphibians, animals)			
37.	Aurein 1.2[24]	GLFDIIKKIAESF	8[a] 32	256
38.	Aurein 2.1	GLLDIVKKVVGAFGSL	-	-
39.	Aurein 2.2	GLFDI**V**KK**VVGAL*GSL***-$CONH_2$	15	-
40.	Aurein 2.3	GLFDI**V**KK**VVGAIG*SL***-$CONH_2$	25	-

41.	Aurein 2.4	GLFDIVKKVVGTLAGL	-	-
42.	Aurein 2.5	GLFDI**V**KK**VVGA**F***GSL***-NH_2	206.25	49.5
43.	Aurein 3.1	GLFDIVKKIAGHIAGSI	-	-
44.	Aurein 3.2	GLFDIVKKIAGHIASSI	-	-
45.	Aurein 3.3	GLFDIVKKIAGHIVSSI	-	-
VI.	**Uperin** (amphibians, toad, animals)			
46.	Uperin 2.1	GIVDFAKKVVGGIRNALGI-OH	>100	>100
47.	Uperin 2.5	GIVDFAK**G**V**L**G**K**I**K**N**V**LGI-OH	-	-
48.	Uperin 2.8	GI**L**D**V**AK**TL**VG**KL**RN**V**LGI-OH	>100	>100
49.	Uperin 3.5	G**VG**D**LIR**K**A**V**SV**I**K**N**IV**__**-NH_2	50	>100
50.	Uperin 3.6	G**VI**D**A**AKKVV**NVLK**N**LF**-NH_2	8[a] 16	128
VII.	**Ponericin** (ants, insects, invertebrates, animals)		Zone of Inhibition (mm) Test Conc. 0.4-0.5mM	
51.	Ponericin G1[25]	GWKDWAKKAGGWLKKKGPGMAKAALKAAMQ	11.5	9
52.	Ponericin G2	GWKDW**L**KK**GKE**WLK**A**KGPG**IV**KAAL**Q**AA**T**Q	-	-
53.	Ponericin G3	GWKDW**LN**K**GKE**WLKKKGPG**IM**KAALKAA**T**Q	7.5	8
54.	Ponericin G4	**DF**KDW**M**K**T**AG**E**WLKKKGPG**IL**KAA**MA**AA**T**_	-	-
55.	Ponericin G5	G**L**KDW**V**K**I**AGGWLKKKGPG**IL**KAA**MA**A A**T**Q	-	-
56.	Ponericin G6	G**LV**D**VLG**K**V**GG**LI**KK**LL**P-NH_2-_____	10	-
57.	Ponericin G7	G**LV**D**VLG**K**V**GG**LI**KK**LL**PG_____	-	-
58.	Ponericin L1	LLKELWTKMKGAGKAVLGKIKGLL	-	-
59.	Ponericin L2	LLKELWTK**I**KGAGKAVLGKIKGLL	4.5	4
60.	Ponericin W1	WLGSALKIGAKLLPSVVGLFKKKKQ	10	5
61.	Ponericin W2	WLGSALKIGAKLLPSVVGLFQKKKK_	-	-
62.	Ponericin W3 des K	***GI***W**GTLA**KIG**I**K**AV**P**R**V**IS**ML**KKK_Q	13	7
63.	Ponericin W4	***GI***W**GT**ALK**W**G**V**KLLP**KL**VG**MA**Q**T**KK_Q	11.5	6
64.	Ponericin W5	***F***W**GALI**K**GA**AKL**I**PSVVGLF__KKKQ	7.5	2
65.	Ponericin W6	**FI**G**T**AL**G**I_A**SAI**P**AI**V**K**LF_K	7	-

It should be noted that in Table 2 Significance of letters in the peptide sequence is as follows:
Bold – Amino acid substitutions in the original sequence
Bold Italics – Additional amino acids in the original sequence
Underscore _- Missing/deleted amino acids from the original sequence.
And the significance of the superscript characters is as follows:
[a] – Methicillin Resistant S. aureus (MRSA)

Figure 1: Representation of covalently linked amino acid pair frequency in AMPs. The grey, blue and red bars indicate amino acid pairs with <15%, 15-20% and >20% occurrence frequencies in AMPs respectively.



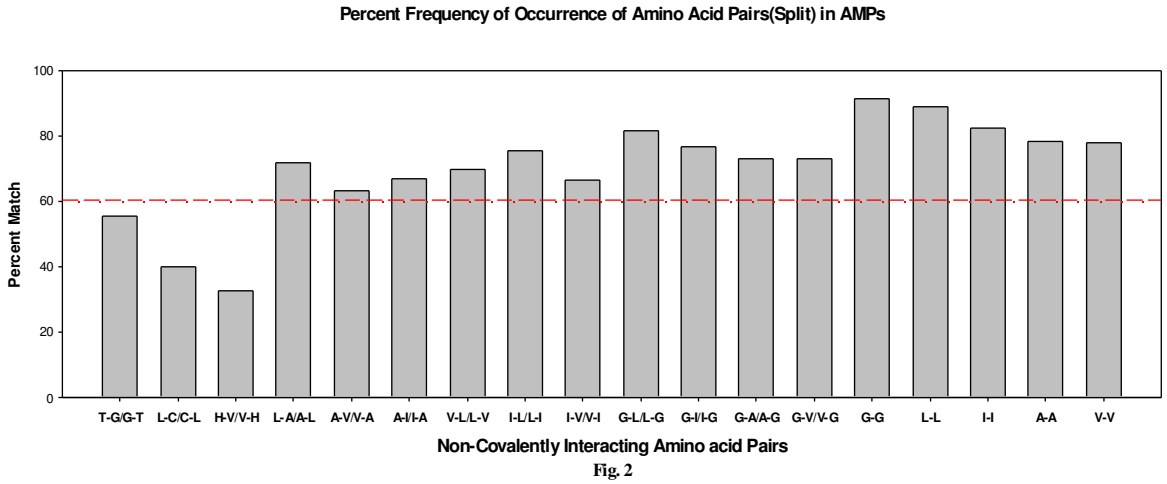


Figure 2: Representation of frequency of non-covalently linked amino acid pairs interacting through hydrophobic interactions in AMPs. The dashed red line indicates the threshold of 60% occurrence frequency in AMPs.

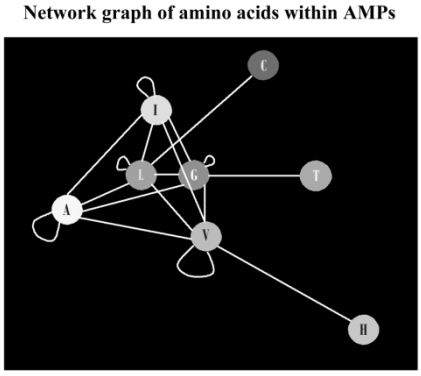


Figure 3: Network graph of frequently appearing amino acids within the primary sequence of AMPs. The amino acids (vertices) are denoted by their respective single letter codes and the connections (edges) between them denote their interaction. The distance between each amino acid pair is inversely proportional to its occurrence frequency within AMPs.

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