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In Silico Investigations of Silk Fibroin Peptides Highlight their Therapeutic Values towards Wound Healing

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Abstract: *This work validates an in vitro observation that silk peptides derived from the α-chymotrypsin digestion of fibroin heavy chain promote wound healing properties. A molecular level evidence of binding of these peptide fragments to growth factors, their receptors and the corresponding ligand-receptor complexes has been provided. The strengths of interactions and possible conformational changes caused by binding of the select three fibroin peptide fragments to various (fifteen) target combinations have been evaluated. The analysis indicates that a number of target residues have been found to be involved in forming convincing hydrogen bonds and hydrophobic interactions with the three fibroin peptides, thus facilitating the wound healing process. Though very few of them were also involved in interactions with the amino acids at the ligand-ligand, receptor-receptor and ligand-receptor interfaces, it is proposed that the binding does not hinder the ligand-receptor interactions. In the first two peptides, the polar amino acids play a significant role in interacting with the targets. However, in case of the third peptide, due to its higher non-polar composition, the overall interactions are stabilized by hydrophobic interactions. This molecular level appreciation strengthens the fact that silk fibroin peptides could be exploited towards creating medicaments for wound healing and cosmetic surgeries.*

Keywords: *Silk fibroin fragments, peptide-growth factors interactions, hydrogen bonds, hydrophobic contacts, wound healing properties*

1. INTRODUCTION

Silk from *Bombyx mori* has been extensively used as a biomaterial and as a substitute for conventional materials in the fields of textiles, cosmetics, medicine, microbial technology and tissue engineering [1]. Silk fibroin provides an imperative set of material options as a biomaterial in biomedical applications because of its high tensile strength, controllable biodegradability, haemostatic properties, non-cytotoxicity, low antigenicity and non-inflammatory characteristics [2]. Many workers have previously demonstrated the therapeutic applications of silk, and have achieved promising results in the use of silk for its antibacterial activity [3,4], anti-tumour activity [5], as a matrix for cell growth [6], tissue engineering [7,8] and for cosmetic uses as a moisturizing agent [9]. The need for new therapeutics for wound healing has encouraged the drive to examine the nature and value of silk and its derivatives for the same.

A wound is defined as a damage or disruption to the normal anatomical structure and function [10]. Wound healing is a complex process involving coordinated interactions between diverse immunological and biological systems. It is a cascade of carefully and precisely regulated steps and events which fall into four distinct integrated and overlapping phases namely haemostasis, inflammation, proliferation, and tissue remodelling [11, 12]. Table 1 details the various factors, cell types involved in the four stages of wound healing process.

Silk is considered to have very high wound healing property. It has been shown to have good adhesive property and affinity to keratin. Silk threads are used for making non-degradable surgical sutures and silk sericin membranes are good bandage materials since the film has adequate flexibility and tensile strength [13]. It has been suggested that the healing property of

silk protein film is due to epithelial proliferative action. Some authors opine that wound-healing property of silk protein might be also due to the stimulation of the expression of basic fibroblast growth factor (bFGF) [13]. In addition, the silk protein film is a safe biomaterial without any skin-sensitizing and skin-irritating potential and with no effects on serum biochemical profile when applied to skin. In wounds treated with silk film, there was greater collagen regeneration, less inflammation and less lymphocyte infiltration [14]. Because of its good biocompatibility and infection-resistant nature, it is considered a novel wound-coagulant material [15].

Table 1. *Events, Cells and Factors in Normal Wound-healing Process [12]*

Phase	Cellular And Physiological Events	Factors Involved	Cells Involved
Haemostasis	a. Vascular constriction b. Platelet aggregation, degranulation, and fibrin formation (thrombus)	Cytokines: PDGF, TGF-β, EGF, IGF, serotonin	Endothelial Cells, Thrombocytes
Inflammation	a. Neutrophil infiltration b. Monocyte infiltration and differentiation to macrophages c. Lymphocyte infiltration	TGF-β, TGF-α, complement: C3a, C5a, integrins, HB-EGF, FGF, collagenase	Neutrophils, Macrophages, Monocytes Lymphocytes Keratinocytes, Fibroblasts, Endothelial Cells
Proliferation	a. Re-epithelialization b. Angiogenesis c. Collagen synthesis d. Formation of extracellular matrix	FGF, VEGF, PDGF, angiogenin, TGF-α, TGF-β	Fibroblasts
Remodelling	a. Collagen remodelling b. Vascular maturation and regression	Matrix metalloproteases, PDGF,TGF-β, FGF	Macrophages, Fibroblasts

Tsubouchi et. al. (2003) have shown that native molecular fibroin enhances the proliferation of cultured skin fibroblasts by 300% and also demonstrated that the enhancement was nullified by alkali-mediated degradation of fibroin, thereby establishing the role of silk fibroin in fibroblast division [16]. α-chymotrypsin mediated fragmentation followed by an assay of the resulting peptides revealed that two peptides fragments NINDFDED and VITTDSDGNE were indeed responsible for enhancement of fibroblast division by 150% [17]. Similar results have been reported by Tsubouchi et al. in their patent application [18], where they have explored the action of a 15 residue peptide fragment- GSSGFGPYVANGGYS.

Though all of these *in vitro* investigations highlight the potential applications of silk fibroin fragments, molecular level interactions with respective factors are not explained till date. Hence, the current investigation attempts to provide due appreciation of the molecular picture and the extent and nature of binding of these fragments to respective receptors and factors, involved in the wound healing process.

2. METHODS

Previous experiments have indicated that in impaired wound healing (in diabetic mice), exogenous application of FGF has been beneficial for a rapid re-epithelialization [26]. Also, application of EGF accelerates the rate of healing of partial-thickness skin wounds [27].VEGF is unique for its effects on multiple components of the wound healing cascade. An angiogenic growth factor may promote closure of chronic wounds exhibiting hypoxia and compromised vascularity. Hence, VEGF can be used on patients with non-healing wounds [28].

In light of this, the growth factors, their receptors and corresponding factor-receptor complexes (Table 2), have been considered as potential targets for exploring the binding potential of the

select three peptides, as agents for enhancing wound healing processes. Further, docking studies were performed using ClusPro [22, 23, 24a, b]. The hydrophobic interactions and H-bonds formed within a sphere of 3.6Å between the targets and peptides were evaluated.
The protocol followed in the study is detailed in Fig. 1.

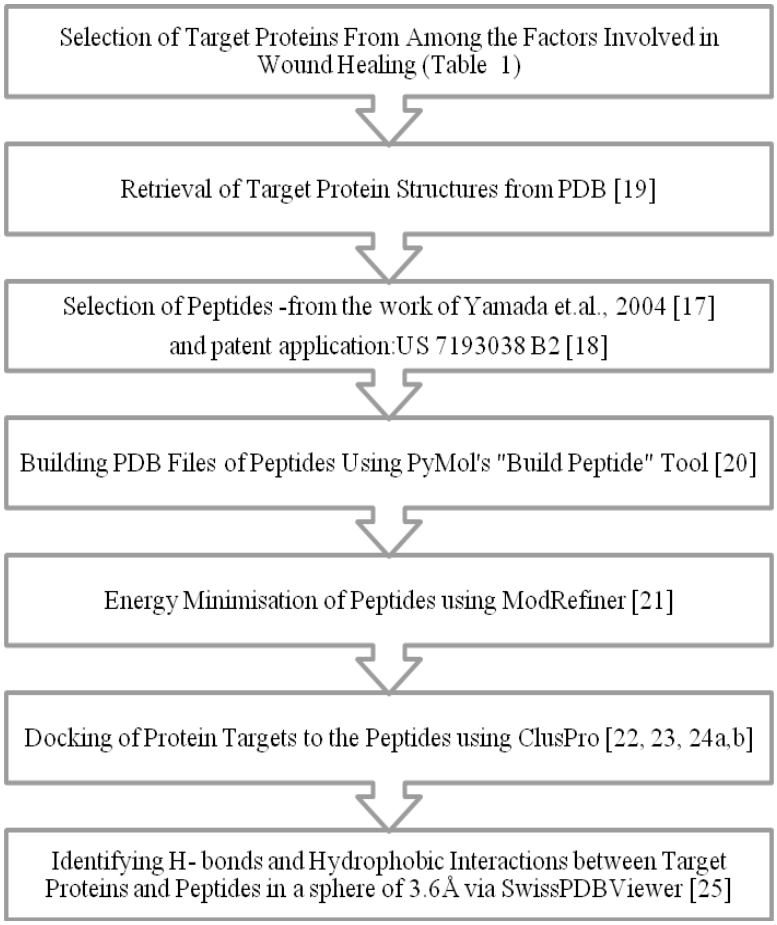

Figure 1. *Protocol followed in the current study*

Table 2. *Fifteen combinations of target proteins (ligands, receptors) used for docking with fibroin derived peptides: NINDFDED, VITTDSDGNE and GSSGFGPYVANGGYS respectively.*

Factor	Receptor	Complex (Active)	Complex (Inactive)
EGF – EGFR			
EGF (2KV4)	EGFR1 (3NJP)	EGF +EGFR (1IVO)	EGF +EGFR (1NQL)
FGF – FGFR			
FGF 1(2AXM)	FGFR 2 (1E0O)	FGF1 + FGFR2 (1E0O)	FGF1 + FGFR2 (1DJS)
FGF 2 (1BAS)	FGFR 1 (1FQ9)	FGF2 + FGFR1 (1FQ9)	FGF2 + FGFR1 (1CVS)
VEGF – VEGFR			
VEGFA (1VPF)	VEGFR1 (1QSZ)		VEGFA + VEGFR1 (1FLT)

The entries in the brackets correspond to the IDs of the molecules / complexes as in PDB [19].

3. RESULTS AND DISCUSSIONS

The molecular level interactions of the three peptides with the 15 possible targets combinations (ligands, receptors, ligand-receptor complexes) were looked into via docking process, to investigate the nature and efficacy of binding of the peptides, which exhibit enhanced wound healing properties as per the *in vitro* experimental studies [18]. The docking results are highlighted in the Table 3, which provides the number of interacting residues of respective targets with each of the three peptides.

Table 3. *Number of target residues binding to the peptide*

Target	Residues In	Number Of Residues Involved	Peptide 1 : NINDFDED		Peptide 2: VITTDSDGNE		Peptide 3: GSSGFGPYVANGGYS	
			A	B	A	B	A	B
EGF	LRI	11	4	2	4	1	3	-
EGFR	LRI; Dimer	25	3	2	2	5	1	1
FGF1	(HBS)	11	4	3	1	3	1	-
	LRI	10	-	-	2	-	1	-
FGFR2	HBS	5	-	1	-	1	-	-
FGF2	HBS	6	1	-	2	-	-	-
	LRI	11	-	-	1	-	1	-
FGFR1	HBS	8	1	4	1	3	-	-
VEGFA	LRI	5	3	1	-	-	1	2
	Dimer	2	-	-	-	2	-	-

A: number of target residues forming H-bonds with the peptide; B: number of target residues interacting hydrophobically with the peptide. [LRI: Ligand-Receptor Interface; HBS: Heparin Binding Site; Dimer: Dimerisation interface]

The number of amino acids involved in hydrophobic and H-bond interactions is tabulated in Table 4 given below. Also, the number of H-bonds formed between the fibroin peptides and target proteins, for each combination have been indicated. Additionally, for the purpose of comparison, the numbers in the absence of the fibroin peptide fragments is also highlighted in Table 4.

Table 4. *Number of residues of targets involved in forming H-bonds and hydrophobic interactions between ligand and receptor without the peptides and with each peptide*

Fibroin Peptides → Ligand+Receptor (Target) ↓	No Peptide		Peptide 1 : NINDFDED		Peptide 2: VITTDSDGNE		Peptide 3: GSSGFGPYVANGGYS	
	A	B	A	B	A	B	A	B
EGF → EGFR	3 (11)	6	7 (14)	11	11 (15)	16	7 (16)	11
EGFR → EGF	4 (11)	6	5 (17)	9	4 (16)	7	6 (18)	9
FGF1 → FGFR2	5 (12)	9	6 (14)	11	7 (13)	13	4 (12)	6
FGFR2 → FGF1	6 (12)	9	7(11)	12	8 (14)	14	8 (12)	13
FGF2 → FGFR1	8 (10)	11	4(10)	5	8 (11)	11	6 (9)	11
FGFR1 → FGF2	9 (10)	11	5(11)	8	5 (10)	7	3 (11)	5
VEGFA → VEGFR1	3 (6)	3	4 (11)	5	3 (9)	4	3 (8)	3
VEGFR1 → VEGF-A	3 (6)	3	5 (10)	5	5 (7)	5	3 (10)	4

A: Number of target residues involved in forming H-bonds, number in brackets indicates number of H-bonds between ligand and receptor; B: Number of target residues involved in hydrophobic interactions

The RMSD values of the binding site residues for every target vs target+peptide combination were calculated so as to reflect the changes in the conformation of the molecules due to peptide binding. These values are tabulated in Table 5. For this calculation, all atoms of the targets in their ligand-receptor, receptor- receptor and ligand- ligand interface residues were considered. In addition to the interface residues, in the case of FGF family (FGF1, FGF2, FGFR1, FGFR2 and

their complexes), all atoms of the amino acids in their heparin binding site (HBS) were also taken into account.

Table 5. *RMSD values of each target, with the number of amino acids and atoms involved.*

Peptide→ Target ↓	# amino acids	# atoms	Peptide 1 : NINDFDED	Peptide 2: VITTDSDGNE	Peptide 3: GSSGFGPYVANGGYS
EGF	11	100	1.03	0.88	0.78
EGFR	44	412	0.03	0.07	0.05
EGF-EGFR Inactive Complex	33	306	0.88	0.83	0.86
EGF-EGFR Active Complex	66	612	0.5	0.48	0.5
FGF1	42	392	0.71	0.84	0.87
FGFR2 (dimer including HBS)	30	254	0.97	0.97	0.94
FGF1+FGFR2 Inactive Complex	36	323	0.77	0.74	0.77
FGF1+FGFR2 Active Complex	62	646	0.81	0.82	0.81
FGF2	14	110	0.66	0.71	0.62
FGFR1	40	324	0.92	1.16	1.11
FGF2+FGFR1 Inactive Complex	70	594	1.15	1.19	1.22
FGF2+FGFR1 Active Complex	70	594	0.76	0.84	0.87
VEGF A	14	122	0.99	0.88	0.92
VEGFR1	12	110	0.32	0.40	0.43
VEGFA+VEGFR1	25	140	0.67	0.66	0.65

As reflected in Table 5, almost all of the RMSD values are lesser than or very close to 1Å, indicating that the binding of the peptide to the targets does not cause significant structural deviations of the target atoms. Further, it is also clear from Table 4 that the presence of the peptides has enhanced the H-bonds and hydrophobic interactions between the receptors and ligands, suggesting that the peptides do appear to facilitate receptor-ligand interactions, and aid in the enhancement of wound healing properties, via better signalling of the growth factors involved therein.

Bar graphs depicting the residue wise interactions of the respective peptides with the 15 target combinations are provided in Fig. 2a, 2b and 2c respectively. Although a large number of amino acids in the targets interact with the peptide 1- NINDFDED, only a few of the interface and dimerisation residues are involved in binding to the peptide (refer Tables 3, 4). The peptide appears to bind close to the targets at the ligand-receptor or dimer interface, and facilitates the ligand- receptor binding as can be seen from the increased number of hydrophobic and H-bonds formed between the ligands and receptors (refer Table 4).

Heparin is essential for mediating FGF-FGFR interaction and FGFR dimerisation [29]. It is interesting to observe that the peptide is not competing with the binding of heparin to the receptor. The residue Arg209 of FGFR1, which is a part of the 8 amino acid-long heparin binding site, forms hydrogen bonds with Asp5 of the peptide, suggesting that peptide binds in the proximity of, but not in, the heparin binding site. Thus, it can be inferred that the heparin binding site is unaffected, facilitating the process of wound healing.

Clearly, as illustrated in Fig 2a, the polar and charged residues of the peptide show a greater degree of interactions when compared to the non-polar residues of the peptide. All of the six polar amino acids in the peptide NINDFDED form H-bonds with at least eight (~50%) of the fifteen targets (ligands, receptors and the ligand-receptor complexes). But, only the amino acid (Ile) of the two non-polar amino acids interacts with more than 8 targets. However, all the residues of the peptide show a good number of hydrophobic interactions with all the targets. Since the amino acid

Phe in the peptide is the one which is exhibiting poorest number of interactions (in terms of both H-bonds hydrophobic interactions), the possibility of its mutation to a polar amino acid needs to be explored, with the objective of enriching the overall binding properties and wound healing functionalities.

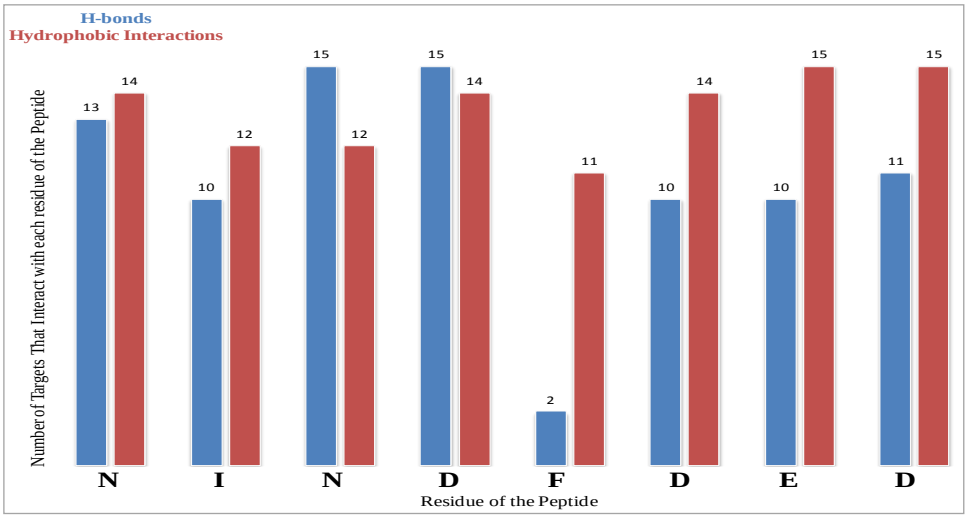

Figure 2a. *Of the 15 possible combinations, the number of targets binding to each amino acid of the 1st peptide: NINDFDED (H-bonds: Blue; Hydrophobic interactions: Red)*

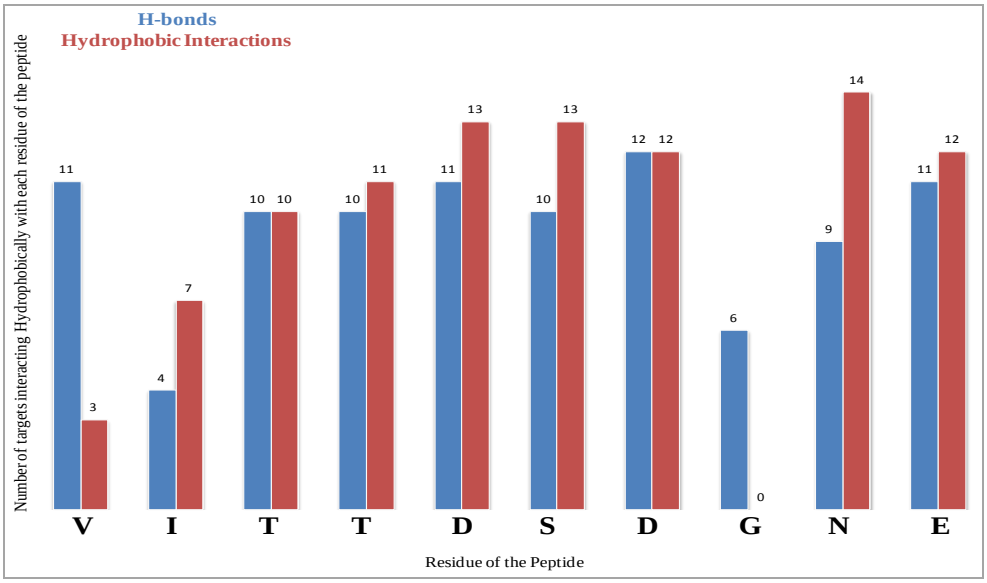

Figure 2b. *Of the 15 possible combinations, the number of targets binding to each amino acid of the 2nd peptide: VITTDSDGNE (H-bonds: Blue; Hydrophobic interactions: Red)*

Similarly, as reflected in Fig. 2b, although the number of interactions between the second peptide VITTDSDGNE and target proteins are high; only a few of the interface and dimerisation residues are actually involved in binding to the peptide (refer Table 3). The overall number of amino acids of the target proteins involved in forming both H-bonds and hydrophobic interactions with its counterpart increased greatly in the presence of the second peptide.

Clearly, the polar and charged residues show a greater degree of interactions when compared to the non-polar residues of the peptide. of the seven polar amino acids in the peptide VITTDSDGNE, all of them form H-bonds with at least eight (~50%) of the fifteen target combinations; but of the three non-polar amino acids in this peptide, only one residue, namely Valine, forms H-bonds (through its main chain atoms) with more than 8 targets. With the exception of Val, the number of hydrophobic interactions formed by the amino acids in the peptide is almost equal to or greater than the number of H-bonds formed by each of these amino acids. However, the amino acid Gly shows no hydrophobic interactions with any of the target

residues, and thus it can be suggested that this could be replaced with a polar amino acid to facilitate better interactions with the target molecule, hopefully conferring enhanced wound healing nature to the peptide.

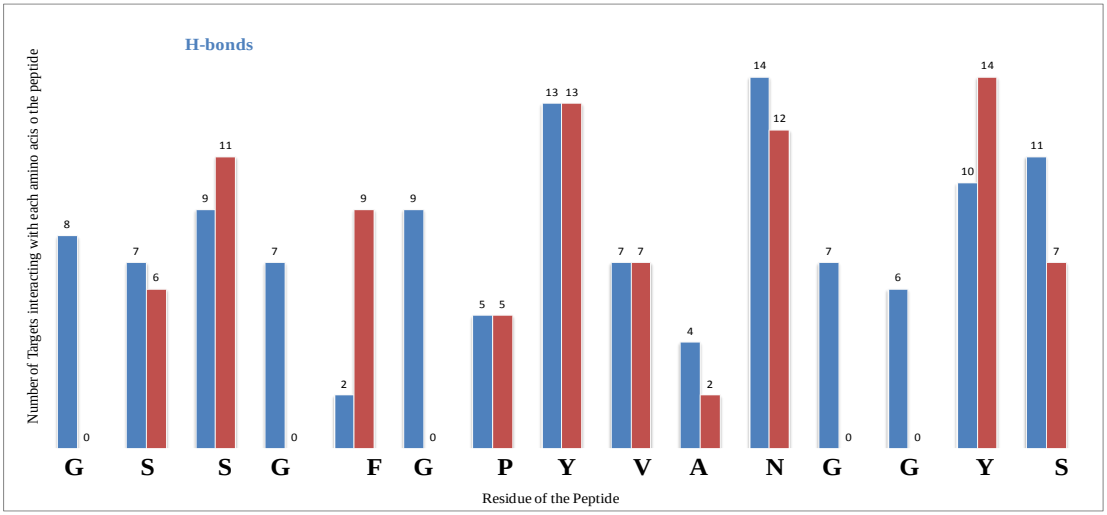

Figure 2c. *Of the 15 possible combinations, the number of targets binding to each amino acid of 3rd peptide: GSSGFGPYVANGGYS (H-bonds: Blue; Hydrophobic interactions: Red)*

Likewise, with regard to the interaction geometries of the third peptide (refer Fig. 2c), which differs from the previous two peptides (in that the majority of the amino acids are non-polar and only one amino acid is charged), it depicts the least number of H-bond and hydrophobic interactions with the target residues. As observed previously, here too, the polar amino acids of the peptide form a greater number of H-bonds with the targets than non polar ones. Although the number of interactions between the peptide and targets are not negligible, very few of the interface and dimerisation residues are actually involved in binding to the peptide (refer Table 4). The lower number of H-bonds formed by this peptide to the targets can be attributed to the presence of a large number of non-polar and polar uncharged amino acids. Tyrosine interacts with a majority (13) of the 15 target proteins on account of the -OH group in its side chain, while the amino acids Gly and Phe (non-polar) interact with the least number of targets. Of the four polar amino acids in the peptide GSSGFGPYVANGGYS, three of them interact with at least eight (~50%) of the fifteen target proteins; and only four of the eleven non-polar amino acids interacts with more than 8 targets. However, the second Serine in the peptide interacts with seven of the 15 targets. Although, the number of interactions between this peptide (GSSGFGPYVANGGYS) and the targets is far fewer than those of the other two peptides, the enhancement of ligand-receptor binding with reference to the no-peptide form, was nearly the same (refer Table 4). In order to propose better binding characteristics, the amino acids Ala and Gly could be replaced by suitable polar moieties, with the hope that this would improve wound healing properties of the peptide.

Further, in order to explore the probable sequence level similarities in the target proteins involved in wound healing process, a phylogenetic tree was derived using the tool Clustal Omega [30, 31]. It is clear from their phylograms (refer Figs. 3a, 3b and 4a, 4b) that though these sequences do exhibit diversity at the molecular level, the overall sequence level similarity between most of them is significantly above 65% (as indicated in Tables 6a, 6b). However, in order to confirm the actual conservation of the binding-site moieties, similar exercise by selecting the peptide-interacting residues needs to be performed.

Figure 3a. *Phylogram of the 4 growth factors considered in our study*

Figure 3b. *Phylogram of the 4 growth factor receptors considered in our study*

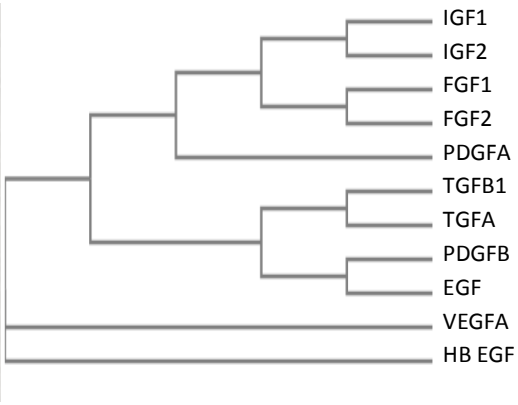

Figure 4a. *Phylogram of all the growth factors involved in wound healing*

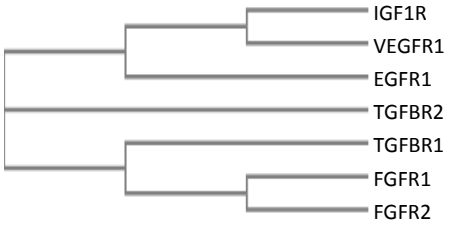

Figure 4b. *Phylogram of all the growth factor receptors involved in wound healing*

Table 6a. *% sequence similarities between the growth factors involved in wound healing (refer Table 1)*

	FGF1	FGF2	VEGFA	TGFA	TGFB	PDGFA	PDGFB	IGF1	IGF2	HBEGF
EGF	67.6	63.2	48.8	68.2	66.7	60	85.7	77.8	75	60.5
FGF1		80.3	56.5	50	52	57.1	57.1	48.1	51.3	51.3
FGF2			55.2	56.7	52.2	61.1	71.4	54.5	65.4	51.7
VEGFA				47.1	60.5	60.0	67.2	70.0	52.0	68.6
TGFA					70.6	48.6	80.0	50.0	66.7	67.4
TGFB1						70.8	47.5	66.7	83.3	62.5
PDGFA							80.0	45.2	50.0	72.2
PDGFB								54.2	66.7	60.0
IGF1									80.6	38.1
IGF2										71.4

Calculated using PIR's Pairwise Alignment tool [32]

Table 6b. *% sequence similarities between the growth factor receptors' extracellular domains*

	EGFR1	FGFR1	FGFR2	TGFBR1	TGFBR2	VEGFR1	IGF1R
EGFR1		52.2	62.5	54.3	47.8	75.0	50.0
FGFR1			82.6	60.7	50.8	58.9	54.2

FGFR2				62.2	75.0	62.2	52.9
TGFBR1					54.1	56.1	83.3
TGFBR2						48.1	68.2
VEGFR1							58.3

Calculated using PIR's Pairwise Alignment tool [32]

4. CONCLUSION

The binding mode of interactions between the target proteins and the select fibroin peptides have been assessed by docking studies. Through this study, molecular (atomic interactions) level information regarding the mechanism of peptides binding to the target proteins has surfaced. Many of the target proteins' residues interact hydrophobically and H-bond with the peptide, but only a few of them are part of the receptor-ligand/ ligand - ligand/ receptor-receptor interface. This indicates that the binding of the factors to their receptors or vice versa, upon peptide addition, is not hindered. Instead, the number of hydrophobic and H-bond interactions between the receptors and ligands increase upon binding of the peptide. This is synchronous with the observations of the *in vitro* experiments that the peptides enhance wound healing and growth/ division of cells in culture. The first two peptides (NINDFDED, VITTDSDGNE), which contains higher degree of charged/polar amino acid, does form a greater number of H-bonds with the targets when compared to the uncharged peptide (GSSGFGPYVANGGYS). The non-polar amino acids of the peptides (with the exception of Gly and Ala in the third peptide) play an important role in forming a large number of hydrophobic interactions with the targets. Therefore, the study unravels the fact that, fruitful experiments could be designed by replacing the not so critical residues (like Glycine and Alanine) with suitable polar amino acids, so as to obtain better therapeutic peptide with best wound healing properties.

FUTURE WORK

In light of the above work, there is a clear need to investigate the binding of peptides to other growth factors and their receptors involved in different stages of wound healing like PDGF, angiogenin, TGF-α, TGF-β, IGF, serotonin etc. Also, plausible mutational studies with the peptides, to observe enhanced binding properties need to be systematically designed.

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