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Development and Engineering of CSαβ Motif for Biomedical Application

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1. Introduction

Protein engineering is a process that modifies/creates functions or increases stabilities of proteins through artificial selection and evolution (Angeletti, 1998). For the fast development of molecular biology techniques, numbers of proteins have been successfully engineered to equip noval functions in the past several decades (Alahuhta et al., 2008;Bottcher & Bornscheuer, ;Ehren et al., 2008;Huston et al., 1988;Leta Aboye et al., 2008). These engineered proteins are developed either for academic research purposes or biomedical applications. The core of protein engineering is an appropriate protein scaffold (Hey et al., 2005;Pessi et al., 1993;Skerra, 2007). An excellent protein scaffold not only provides a platform for developing noval functions but also has many benefits, such as cost reduction during development/production or lasting efficacy of protein. An suitable protein scaffold should equip several characteristics (Hey et al., 2005;Pessi et al., 1993;Skerra, 2007). To introduce tailored functions into protein scaffolds is a major challenge and has a unique significance in protein design (Hey et al., 2005;Pessi et al., 1993).

A protein scaffold is a peptide framework that exhibits a high tolerance of its fold for modifications (Hey et al., 2005). Candidates for suitable protein scaffolds should exhibit a compact and structurally rigid core. The folding properties of the protein scaffolds should not be significantly changed when the side chains in a contiguous surface region are replaced or loops of varying sequence and length are presented (Skerra, 2007). Several additional priorities have to be considered if the scaffold is used in biomedical applications. The scaffold must display extra stabilities to environments, such as low pH, high concentration of chaotropic agents and high temperatures. Molecular weight of the scaffold should be low and small molecules have advantages in passing tissue barrier (Baines & Colas, 2006). The scaffold should highly resist protease degradation and this ensures the engineered proteins can be safe in the gastrointestinal tract and not degraded (Aharoni et al., 2005). Low immunogenecity is important to reduce unexpected side effects and damage of healthy tissue (Van Walle et al., 2007). Post-translational modification of protein is an important issue, too. Most of eukarytic proteins require post-translationally modified then gain their functions. These modifications includ glycosylation, cleavage of pre-peptide, formation of disulphide bridge and association of multiple peptides. Appropriate glycosylation also could reduce immunogenicity of proteins (Kosloski et al., 2009;Wang et al., 2010;Wu et al.). Currently, bacterial cells are the

most conventional and convenious tools for mass production of recombinant proteins, but it is not easy for bacterial cells to undergo post-translational modication of eukaryotic proteins (Jacobs & Callewaert, 2009;Muir et al., 2009). Even expressed in eukaryotic cells, glycosylation of proteins is not exactly the same among difference specises (Perego et al., 2010). Unappropriatly modified recombinant proteins might lead to unexpected immune respones, if the proteins are used for medical purpose (Kosloski et al., 2009; Li & d'Anjou, 2009).

Protein designing challenges our understanding of the principles underlying protein structure and is also a good method to access our understanding of sequence-structure and structure-function relationship (Nikkhah et al., 2006). Rational design of proteins requires detailed knowledge of protein folding, structure, function, and dynamics (Chen et al., 2005). To build expression libraries, it is necessary to understand a protein scaffold in detail to amino acids usage on each residue position. This would reveal the key elements that affect functions and stabilities of a protein scaffold.

The appearance of new intellectual property, the breakthroughs in technology, or the increase in a market need are three major impacts to biopharma industry. Protein engineering is a branch of the biopharm industry, where intellectual property rights apparently play the most important role in the development and commercialization of final products. The intellectual property strategy to protect inventions of biopharma industry is to patent and to license them on an exclusive basis. The intellectual property also must be included in the linkage of research/development and business to ensure the commercial viability of biopharma products and to cope with the rapid changes of market. Currently, the intellectual property situation of biopharma industry is too complex and hampers the generation and production of recombinant protein/peptide drugs. Pantent analysis and patent map are necessary and helpful while planing the research and development and marketing. A well planed intellectual property strategy can not only protect the output of research and development but also defend market. A protein scaffold with simple legal situation will avoid knotty lawsuits. In recent years, shouts for alternative protein scaffolds is urgent and alternative protein scaffolds usually provide a favourable intellectual property situation. In this chapter, we will focus on a protein scaffold, cysteine-stabilized α/β (CSαβ) motif, and discuss protein engineering based on the scaffold.

2. Cysteine-stabilized α/β motif

2.1 Specific pattern and structural feature

CSαβ motif is a very unique protien scaffold. Proteins with a CSαβ motif express a high diversity in their protein primary structures (Figure 1) but share a common core sturcture that consists of a α-helix and an anti-parallel triple-stranded β-sheet (Figure 2 a to d). This is especially interesting not only for academic research but also very useful for applied utilities. From the protein sequence alignment analysis, six cysteines and one glycine (-C-X_l-CXXXC-X_m-GXC-X_n-CXC-) are exzactly conserved in all proteins containing a CSαβ motif (Carvalho & Gomes, 2009;Lay & Anderson, 2005). The cysteines form a framework and tightly connect the intramolecular structures. The most significant feature of the framework is two disulphide bonds which are formed with a patten of (*i, i+4*) and (*j, j+1*). The cysteines of (*i, i+4*) and (*j, j+1*) are located in the α-helix and the β3 strand of the β sheet; respectively (Figure 1). The helix and the sheet are connected by the disulphide bonds pairing with the

pattern (*i, i+4*) and (*j, j+1*) (Assadi-Porter et al., 2000;Fant et al., 1999;Sun et al., 2002;Zasloff, 2002;Zhu et al., 2005). The third disulphide bond connects loop 1 and and β2 strand. In the β2 strand one glycin is conserved and locates in the central region of the motif. In plant defensins, there is usually a forth disulphide bond that seal the N- and C- terminee. The positively charged α-helix is another character. The positively charged residues are especially concentrated in the helix. β-sheet of the motif is formed by a hydrophobic amino acid clust and the sheet is composed by two or three anti-parallal strands. Loop regions of the mofit are highly diversed in lenght among proteins. There is a small turn that connects the helix and β2 strand.

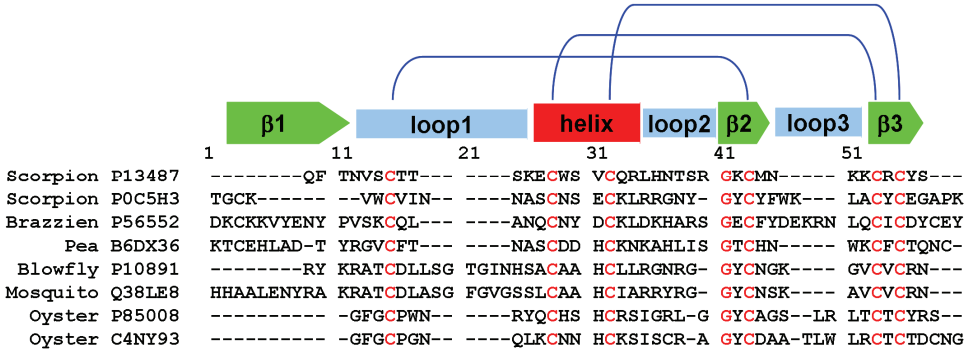
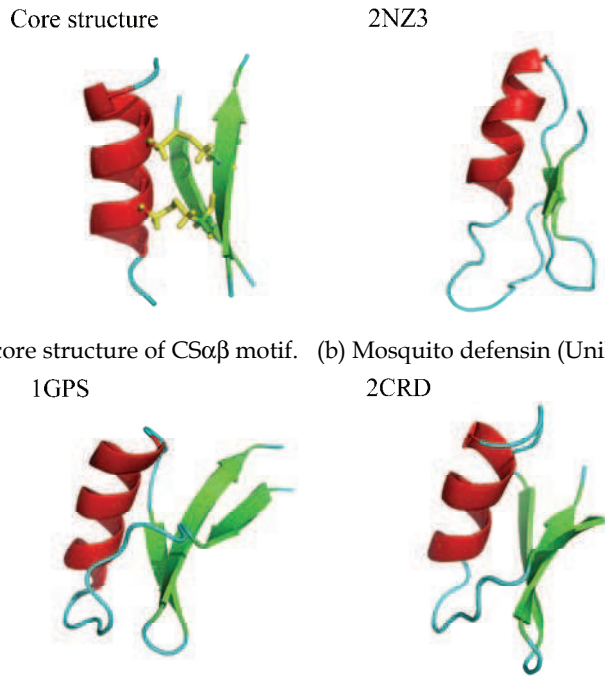


Fig. 1. The sequence alignment of proteins containing CSαβ motif. Sequences of two scorpion toxins (UniProt P13487, P0C5H3), two plant defensins (UniProt P56552, B6DX36), two insect defensins (UniProt P10891, Q38LE8) and two mollusk defensins (UniProt P85008, C4NY93) are aligned. The restrictly conservered residures, include six cysteins and one glycin, are high lighted in red. The green arrow box, red box and cyan boxs above the alignment represent β strands, α-helix and loops; respectively. The blue lines above on the top indcate the disulphide bond bwteen cysteines.

2.2 Biological distribution and functions

To date, there are at least four hundred proteins with a CSαβ motif have been discovered and deposited in databases. Proteins with the motif are widely distributed among plants, insects, arachnidia and mollusca (Sun et al., 2002;Zhu et al., 2005). They exhibit a wide spectrum of biological activities, including antimicrobial activity, enzyme inhibitory function, inhibition of protein translation and sweet taste (Assadi-Porter et al., 2000;Chen et al., 2005;Clauss & Mitchell-Olds, 2004;Spelbrink et al., 2004;Stec., 2006;Wong & Ng, 2005;Zhu et al., 2002). Proteins with the CSαβ motif usually serve a common function as defenders of their hosts (Lobo et al., 2007;Song et al., 2005;Zasloff, 2002).

Before designing a unique function into a protein scaffold, it is important to understand the relationship between each part of the scaffold and its biological function. Based on the three-dimenional structure, protein scaffold containing a CSαβ motif can be devided into three parts: one α-helix, one β-sheet and three loops. It is well known that the three parts bearing different biological functions (Liu et al., 2006;Thevissen et al., 1996;Wong & Ng, 2005;Zhao et al., 2002).



(a) The core structure of CSαβ motif. (b) Mosquito defensin (UniProt ID: Q17027)

(c) Wheat defensin (UniProt ID: P20158). (d) Scorpion toxin (UniProt ID: P13487)

Fig. 2. The core structure and three dimensional structrues of different specices. The structures are presented in color ribbon. Red: α-helix, green: β-sheet, and cyan: loop. Protein structures are retreved from Protein Data Bank and visualized with PyMol 0.99rc6.

The α-helix is related to antimicrobial ability. As described previously, the helix forms a positively charged cluster. When the positively charged residues were replaced with null or negatively charged amino acids, the anti-microbial ability of the proten is also changed. For its net positive charge, it is believed that proteins with the motif could interact with negatively charged cell membrane (Thevissen et al., 1996;Thomma et al., 2003). Several studies have demostrated that plant defensins are able to pass artificial membranes and lead to ions leaking from one side of the membrane. The mechanism about how plant defensin passing membrane is not revealed, yet.

Role of the β-sheet is less studied and disscussed. The direct mutagensis studies showed when the hydrophobic residues in the β-sheet are alanine substituted, biological function of mutated proteins are dromatically reduced (Walters et al., 2009;Yang et al., 2009). The maximal effects of mutated proteins are only 30-40% maximal effects of the wild type, even at a high protein concentration. Protein-protein docking model showed that the β-sheet could form interaction interface with their counterpart. These data imply that, the distribution of the hydrophobilc residues in the β-sheet plays a role in protein-protein interactions and β-sheet could relate to the protein-protein interaction specificity to their targets.

The length of loop regions are different from protein to protein and the loops connecting CSαβ motif can serve as functional epitopes (Figure 3) (Lay & Anderson, 2005;Wijaya et al., 2000;Zhao et al., 2002). For example, loop 1 of the *Arabidopsis thaliana* trypsin inhibitor (ATT_p) and loop 2 of cowpea thionine are the functional epitopes for trypsin inhibition (Wijaya et al., 2000;Zhao et al., 2002). The loop 3 of *Vigna radiate* defensin 1 (VrD1) is the functional loop for insect α-amylase inhibition (Lin et al., 2007;Liu et al., 2006).

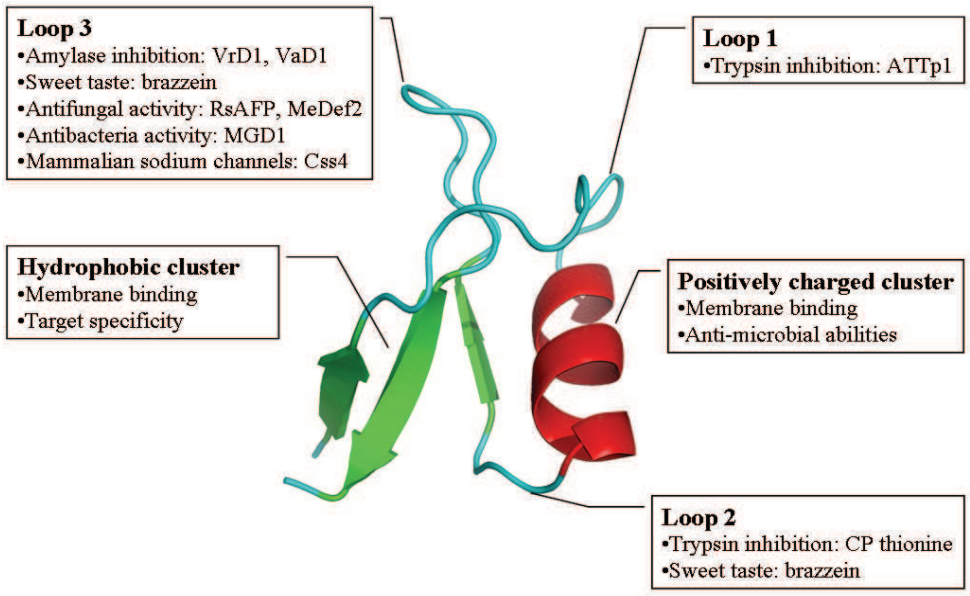


Fig. 3. Structure-function relationship of peptide with a CSαβ motif. Red: α-helix, green: β strands, and cyan: loops.

2.3 Structural ultra-stability

A protein scaffold with ultra high stability can maintain its three dimensional structure in extreme environments and its functions can be preserved. Therefore, when a protein scaffold applied in biomedical applications, its structural stabilities must be considered. The scaffold should be able to pass a low pH environment in the stomach, resist protease digestion, endure chaotropic agents at high concentrations and so on. These criteria are to ensure the engineered proteins could reach their target sites and perform functions inside body, and are not destroyed. It has been reported that proteins with a CSαβ motif equip ultra stabilities to several extreme environments such as urea at a concentration of 6M, a temperature over than 95°C, and resist protease digestion without changing its structure (Malavasic et al., 1996;Yang et al., 2009). In Table 1, the properties of CSαβ motif and single-domain antibody are listed and compared (Holt et al., 2003;Skerra, 2007;Yang et al., 2009;Yang & Lyu, 2008). For their advantages, such as high specificity and affinity, antibodies still are the most popular protein scaffold for engineering. Antibodies are widely

used both in routine laboratorial experiments and clinical diagnosis. In spite of their significant clinical success, several disadvantages, including high cost in manufacturing, large in size, undesired effector functions and complex intellectual property situations, obstruct their development and applications (Jones et al., 2008). Single-domain antibody is only constituted of antibody variable regions and does not have constant regions (Holt et al., 2003). Compared with single-domain antibodies, the proteins with a CSαβ motif have smaller molecular weights and higher structural stabilities (Holt et al., 2003;Skerra, 2007;Yang et al., 2009). As previously described, proteins with a CSαβ motif share low sequence identity but high structural similarity (Lin et al., 2007). Their functions are highly varied and sturctures are ultra-stable. There is a possibility to utilize CSαβ motif as an engineering scaffold for biomedical applications (Yang & Lyu, 2008).

Item	Molecule	
	Single-domain antibody	CSαβ motif protein
Molecular weight	11-15 kDa	5-7 kDa
Generation of expression library	B cell mRNA	Synthetic
Water solubility	Less	High
Ultra stability	Unfolded 6M Gdn-HCl T_m 60-78°C	Ultra stable 6M Gdn-HCl T_m > 96°C
Post-translational modification	Glycosylation	Disulfide bridge
High functional diversity	Yes	Yes
Tolerate to amino acid substitution	Loop regions	Structural and loop regions
Enzyme inhibition	Not certain	Direct inhibition
Membrane binding	Not certain	Direct binding
Legal problem	Very complex	Simple

Table 1. Comparison of properties bwtween CSαβ motif and single-domain antibody.

2.4 Amino acid usage of CSαβ motif

To understand the amino acid usage of a protein scaffold can reveal relationships among structures, functions and sequence residues (Kristensen et al., 1997;Yang et al., 2009). To completely understand the relationships could be an approach through extensive amino acid substitution and analysis of protein sequences (Corzo et al., 2007;Wang et al., 2006). Amino acid substitution have been performed in plant defensins, brazzein of *Pentadiplandra brazzeana* and VrD1 of *Vigna radiate*, and some key residue positions are discovered (Assadi-Porter et al., 2010;Yang et al., 2009). In both cases, amino acid substitution does not lead to the structure significantly being changed in all positions along the sequence but the replacement in some positions have effects on biochemical function (Assadi-Porter et al., 2010;Yang et al., 2009).

It has been noted that certain amino acids have preference to fold into a given secondary structure (Chan et al., 1995;Zhong & Johnson, 1992). Comprehending preference of amino acids usage will be really helpful to protein engineering and can be as a fundment for designing innovative peptides. The two major classes of CSαβ motif protein are plant defensins and scorpion toxins (Zhu et al., 2005). Currently, there are at least 140 sequences of scorpion toxin and 180 sequences of plant defensin deposited in the SwissProt database and the numbers are continuely increased. The peptide sequences of the scorpion toxin and

plant defensin peptides deposited in the SwissProt database are retrieved and amino acid usage perferacnes are separately analyzed (Table 2 and Table 3).

In Table 2 and Table 3, the twenty amino acids are listed on the top row, the secondary structures and residue positions are listed on the left two columns. Use of amino acids in each position is counted and the usage frequency is calculated (form 0.00 to 1.00). Amino acids with a high usage frequency (> 0.20) are listed on the right column. The results are interesting and different between scorpion toxins and plant defensins. In scorpion toxins, peptides tend to employ similar amino acids in the same position. Therefore, sequences of scorpion toxins are less diverse and more uniformed. In plant defensins, sequences of peptides are more diverse. The major targets of scorpion toxins are ion channels on the neuron cell surface but the targets of plant defensins are various form peptide to peptide (Zhu et al., 2005). This might be the reason for the amino acid usage difference between the two classes of CSαβ motif peptides. In the helix region, scorpion toxins prefer using charged residues and plant defensins are more inclusive of different type of amino acids. The most interesting is the two residues flanking the exzactly conserved glycine on $\beta_2$2. In scorpion toxins, two tyrosins are prefered on $\beta_2$1 and $\beta_2$3, and the rates are 0.44 and 0.75; respectively. In plant defensins, a preceded glycine ($\beta_2$1) is prefered to the glycine on $\beta_2$2 and the frequency is 0.35. It is worth to clarify the role of the two tyrosins surrounding the glycine on $\beta_2$2 of scorpion toxins. Although the frequnectly used amino acids are listed, it just provides a reference for protein designing and there is no necessarity that combination of these amino acids forms a universal sequence.

	Position	Amino acids																			High frequency[a]
		A	G	V	L	I	F	Y	W	C	S	T	D	E	N	Q	R	H	K	M	P	
Strand 1	S1		0.81												0.12		0.03					G
	S2 (C)									1.00												C
	S3	0.05		0.03		0.01						0.03					0.02		0.85			K
	S4			0.34	0.13	0.10	0.03	0.22					0.02	0.01	0.02		0.10			0.03		V Y
	S5	0.05	0.07	0.02	0.00	0.06			0.04		0.51	0.06	0.03	0.03	0.02			0.02	0.05		0.01	S
Helix	H1	0.04	0.16	0.08							0.04	0.03	0.05	0.20	0.01	0.06	0.03	0.06	0.16		0.07	E
	H2		0.13		0.01	0.00	0.16	0.37	0.03		0.10		0.03	0.03	0.05	0.08		0.01				Y
	H3 (C)									1.00												C
	H4	0.09	0.05	0.03	0.05	0.10		0.01				0.01	0.25	0.06	0.22	0.03	0.03		0.03	0.01	0.01	D E
	H5	0.01	0.01	0.01	0.01	0.01					0.09	0.09	0.14	0.03	0.17	0.04	0.07	0.01	0.30		0.01	K
	H6	0.01		0.09	0.08	0.07	0.01			0.03	0.01	0.01	0.01	0.43	0.02	0.02		0.13	0.01	0.02	0.07	E
	H7 (C)									1.00												C
	H8	0.03	0.03	0.01	0.01						0.09	0.02		0.03	0.01	0.07	0.11	0.01	0.57			K
	H9	0.22	0.05	0.03	0.11	0.03	0.01	0.02			0.06	0.01	0.14	0.04	0.01		0.09		0.16	0.01		A
β_2 strand	$\beta_2$1	0.01	0.04	0.01		0.01	0.09	0.44			0.13	0.02	0.15	0.02	0.01			0.01	0.05			Y
	$\beta_2$2 (G)		1.00																			G
	$\beta_2$3					0.01	0.02	0.75			0.01	0.03	0.01	0.02		0.01	0.01	0.01	0.11			Y
	$\beta_2$4 (C)									1.00												C
	$\beta_2$5	0.01		0.01		0.01	0.02	0.34	0.26	0.00	0.03	0.01	0.03	0.01	0.00	0.01		0.10	0.01	0.07		Y W
β_3 strand	$\beta_3$1	0.49	0.05		0.03	0.00	0.01	0.01			0.05	0.01	0.01	0.02	0.00	0.04	0.02	0.01	0.25			A K
	$\beta_3$2 (C)									1.00												C
	$\beta_3$3	0.01			0.01	0.01	0.01	0.28	0.45				0.02		0.01	0.01		0.05	0.14	0.01		W Y
	$\beta_3$4 (C)									1.00												C
	$\beta_3$5	0.04	0.07		0.01	0.03	0.01	0.22	0.00		0.02	0.07	0.01	0.42	0.01	0.05			0.05	0.01		E Y

Table 2. The high frequent amino acids in the structural regions of scorpion β toxin.

	Position	Amino acids																					High frequency[a]
		A	G	V	L	I	F	Y	W	C	S	T	D	E	N	Q	R	H	K	M	P		
Strand 1	S1		0.02	0.08	0.20	0.07	0.01	0.03			0.01	0.27	0.02	0.08	0.01	0.02	0.07	0.01	0.04	0.04	0.02		T L
	S2 (C)									1.00													C
	S3	0.01		0.04	0.07	0.02	0.02	0.02	0.01		0.05	0.03	0.05	0.34	0.01	0.04	0.08	0.04	0.11	0.07	0.01		E
	S4	0.06	0.02	0.07	0.05	0.03					0.18	0.10	0.04	0.07	0.08	0.02	0.13	0.03	0.12		0.01		
	S5	0.05	0.04	0.05	0.12	0.05	0.01	0.04			0.06	0.05	0.03	0.07	0.00	0.12	0.08	0.01	0.08	0.01	0.13		
Helix	H1	0.07	0.10	0.02	0.01	0.02	0.01	0.01			0.15	0.15	0.05	0.03	0.14	0.05	0.06	0.04	0.08		0.04		
	H2	0.12	0.04	0.01	0.08	0.02	0.02	0.00	0.02		0.07	0.05	0.11	0.05	0.27	0.02	0.02	0.03	0.06	0.01	0.03		N
	H3 (C)									1.00													C
	H4	0.24	0.03	0.04	0.02	0.02	0.00	0.03			0.06	0.02	0.17	0.03	0.08	0.02	0.09		0.19				A
	H5	0.06	0.01	0.02	0.04	0.02	0.03		0.01		0.11	0.08	0.09	0.06	0.23	0.09	0.03	0.04	0.10		0.01		N
	H6	0.05	0.01	0.25	0.06	0.01	0.01	0.04		0.02	0.08	0.05	0.01	0.05	0.05	0.16	0.01	0.11	0.02	0.03	0.02		V
	H7 (C)									1.00													C
	H8	0.03	0.01	0.04	0.11	0.18	0.02	0.03	0.01		0.01	0.04	0.01	0.01	0.02	0.09	0.18	0.05	0.16	0.03	0.02		
	H9	0.06	0.02		0.01		0.01	0.01	0.01		0.09	0.21	0.02	0.07	0.19	0.11	0.08	0.01	0.10	0.01	0.02		T
β_2 strand	$\beta_2$1	0.05	0.35	0.05	0.01	0.02	0.01	0.01		0.01	0.11	0.03	0.08	0.02	0.05	0.01	0.05	0.14	0.05				G
	$\beta_2$2 (G)		1.00																				G
	$\beta_2$3	0.04	0.02	0.05	0.01	0.01	0.05	0.06			0.18	0.07	0.05	0.06	0.07	0.03	0.11	0.06	0.17	0.01			
	$\beta_2$4 (C)									1.00													C
	$\beta_2$5	0.02	0.01	0.07	0.05	0.06	0.04	0.01	0.01		0.06	0.02	0.14	0.04	0.17	0.03	0.16	0.09	0.06	0.01	0.01		
β_3 strand	$\beta_3$1	0.05		0.02	0.02	0.07	0.02	0.02			0.04	0.05	0.01	0.02	0.04	0.06	0.30	0.04	0.23	0.05			K
	$\beta_3$2 (C)									1.00													C
	$\beta_3$3	0.01	0.02	0.07	0.12	0.15	0.19	0.13	0.07			0.02	0.02	0.03	0.01	0.01	0.03	0.05	0.05	0.05			
	$\beta_3$4 (C)									1.00													C
	$\beta_3$5	0.02	0.02	0.03	0.04	0.06	0.02	0.22		0.04	0.07	0.30	0.03	0.02	0.03	0.04	0.04	0.01	0.04	0.02			Y

Table 3. The high frequent amino acids in the structural regions of plant defensin.

3. Applications of CSαβ motif peptides

Human beings have been using natural products containing CSαβ motif peptides for several hundreds years. The most well known natural products are sweet peptides of plants and scorpion venom. For their anti-microbial and pesticide abilities, in many cases, peptides with CSαβ motif are transfected into industrial crops to protect these transgenic plant from pathogens. In recent years, some of plant defensins are also screened and selected as antibiotics for therapeutic utilities. In this section, some peptides with therapeutic potential are selected and discussed.

3.1 Sweet peptide: Brazzein

Brazzein is isolated from *Pentadiplandra brazzeana*, a climbing plant plant of the West Africa. Berry fruits of *Pentadiplandra brazzeana* have sweetness and are traditional foods of African natives (Assadi-Porter et al., 2008;Yang & Lyu, 2008). For a long time, the sweetness of the berry fruits is a secret. Ding and Hellekant at University Wisconsin Madison isolated a small peptide with sweetness from *Pentadiplandra brazzeana* and named it as brazzein 1994 (Ming & Hellekant, 1994). Peptide sequence analysis shows that brazzein contains a set of (*i, i+4*) and (*j, j+1*) cysteine pattern. Its NMR structure is solved and confirms it as a CSαβ motif peptide 1998 (Caldwell et al., 1998). Brazzein is the smallest, most heat-stable and pH-stable protein known to have an intrinsic sweetness (Jin et al., 2006). Brazzein is 200 times sweeter than sugar and it can be used both in cold and hot drinks/foods without change its sweet

taste (Jin et al., 2006). Brazzein is widely recurited as sugar substitute in low caloric dietary formulas or for diabetes patients. Brazzein is a food additive for several years and no side effects have been reported (Yang & Lyu, 2008).

3.2 Scorpion venom: Traditional remedies

Scorpion is therapeutically used in traditional Chinese medicine for hundreds of years. Figure 4 shows the therapeutic instruction of using scorpion described in the *Compendium of Materia Medica*, an ancient Chinese pharmacopoeia compiled in the 1500s AD. In traditional Chinese medicine, scorpion is recruited as one of the major treatments for stroke, rheumatism, epilepsy, hemiplegia, convulsions, cramps, twitchs, headaches, tetanus and scrofula. *Buthus martensii Karsch* is the most common species of scorpion that are processed as traditional Chinese medicine. Captured scorpions have to be preserved in salt. The preserved scorpions are usually boiled with herbs, such as licorice, to reduce toxicity after washing off the salt. Patients take the boiled extracts twice daily and the amount of scorpions should be controlled to under 10 grams for an adult daily. Most of proteins will be denatured and loss functions after being exposed to salt at a high concentration and water boiling temperature. The major effective composition of scorpion is scorpion venom. It has been demonstrated the venom can block cation channel of insects (Li et al., 2000;Wang et al., 2001). Several peptides containing CSαβ motif have been isolated from scorpion venom. The peptides can keep their structures and functions after be exposed to water boiling temperature and solvents of high salt concentration. BmK AEP, a peptide containing a CSαβ motif isolated from *Buthus martensii Karsch* venom, is the first anticonvulsant purified in the venom (Wang et al., 2001). BmK AEP has inhibitory effect to coriaria lactone-induced epilepsy model in rodents. It has a low toxicity to mice in doses up to 20 mg/animal (Rajendra et al., 2004). There is nearly no effect on heart rate, electroencephalogram, breathing rate and blood pressure (Rajendra et al., 2004;Wang et al., 2001). CII9, another peptide with a CSαβ motif isolated from *Centruroides limpidus Karsch*, can partially inhibit

Fig. 4. The therapeutic instruction of scorpion described in the ancient Chinese pharmacopoeia: *Compendium of Materia Medica.*

sodium ion currents from superior cervical ganglion neurons of rats (Dehesa-Davila et al., 1996;Possani et al., 1999). In the rat model, CII9 also exerts a depressant effect on the behavior and electroencephalographic activity and antagonizes the epileptiform activity induced by penicillin (Dehesa-Davila et al., 1996;Gazarian et al., 2005;Possani et al., 1999).

3.3 Peptide antibiotics: Plectasin

Peptides with a CSαβ motif act as primary defenders for their host. Microbe-killing abilities of plant defensins have been demonstrated and transgenic industrial crops have been bred to carry specific defensins to increase their tolerance to plant pathogens. For directly binding to membranes or cell walls, target pathogens do not easily resist peptides with a CSαβ motif as they do to protein-targeting antibiotics. Pathogenic microbes would escape attacking from conventional antibiotics by mutating proteins but it is never easy to change the properties of cell membranes or cell walls. Scaffold of a CSαβ motif could be as a suitable platform for developing peptide antibiotics. Some plant defensins are tested and show activities againt human pathogenic microbes. For example, Rs-AFP2, a defensin isolated from seeds of *Raphanus sativus*, has an inhibitory activity against *Candida albicans* (Landon et al., 2004;Terras et al., 1992;Thomma et al., 2003). Scientists of Novozymes, a Dnaish biotech company, isolated a novel plant defensin, plectasin, with excellent antibiotics ability.

Plectasin is a 40-amino acid residue peptide isolated from saprophytic ascomycete, *Pseudoplectania nigrella*, and might be the first plant defensin with a CSαβ motif isolated from fungus (Mygind et al., 2005). Plectasin expresses anti-bacterial activities against a broad Gram-positive bacteria and has an inhibitory effect at a low concentration of 0.25 μg/ml to growth of *Streptococcus pneumoniae* (Mygind et al., 2005). Plectasin also has a comparable killing rate to *Streptococcus pneumoniae* as do conventional antibiotics (Mygind et al., 2005). *Streptococcus pneumoniae* is a major pathogenic bacteria and the most common cause of hospital/community-acquired pneumonia, bacterial meningitis, bacteremia, sinusitis, septic arthritis, osteomyelitis, peritonitis, and endocarditis (Whitney et al., 2000). Currently, antibiotics are major treatments to patients with infection of *Streptococcus pneumoniae*. In recent years, *Streptococcus pneumoniae* is more and more resistant to antibiotics and the demand for new drugs to cure *Streptococcus pneumoniae* infection is urgent (Baquero et al., 1991;Whitney et al., 2000). Another challenge of theraping *Streptococcus* infection is that *Streptococcus* can be an intracellular pathogen and avoids targeting by the immune system or drugs (Gordon et al., 2000;Talbot et al., 1996). Different from the conventional antibiotics, plectasin can directly act on the cell wall precursor lipid II of *Streptococcus* (Mygind et al., 2005). Studies also showed that plectasin has an intracellular activity against *Streptococcus aureus* both in human monocytes and in mouse peritonitis model without effecting the cells viability or inducing IL-8 production (Brinch et al., 2009;Hara et al., 2008).

4. Protein engineering based on CSαβ motif scaffold

Based on the scaffold, some CSαβ proteins have been engineered to exhibit new functions or changing of antimicrobial activities (Lin et al., 2007;Vita et al., 1999;Vita et al., 1995). Different approaches, including minimal residue substitution, functional epitope exchange, structural based modification and combinatorial chemistry have been employed to engineer the scaffold to exhibit new functions (Lin et al., 2007;Thevissen et al., 2007;Van Gaal et al.,

2004;Vita et al., 1999;Vita et al., 1995;Zhao et al., 2004). Protein structure is important to the rational design of a protein scaffold. It does not only reveal the shape of a protein molecule but sidechain orientation of each residue. This information is crucial for investigating possible interactions between designed protein and its target.

4.1 Site-direct mutagenesis

In some studies, researchers focus on the relationships of structures, functions and each residue of peptides with a CSαβ motif. Extensive residue substitution is usually performed on the peptides and the changes of structures and functions are observed. The process is time-consuming and labor intensive but it is required for collecting basic information about the scaffold. Amino acid substitution has been extensively performed on two peptides with a CSαβ motif, VrD1 and brazzein (Assadi-Porter et al., 2010;Yang et al., 2009). In the two studies, molecular docking models are also established to investigate the interactions between receptors and ligands.

In both cases, similar positions along the structures are discovered that are crucial to functions of both peptides (Figure 5). These sites are widely distributed on β1 strand, loop 1, loop 2 and loop 3 (Assadi-Porter et al., 2000;Walters et al., 2009;Yang et al., 2009). From the molecular docking models, these sites either directly interact with their targets or play as functional epitopes and insert into the active site of their targets (Assadi-Porter et al., 2010;Liu et al., 2006;Yang et al., 2009). It is interesting that, when two negatively charged residues, D29 and E41, of brazzein are replaced by a positively charged amino acid, the sweetness of brazzein is greatly improved (Assadi-Porter et al., 2010). The mutants should be with high interests to industrial utilities. In the case of VrD1, there is no functional improvement observed on its mutants (Yang et al., 2009). Comparing conformations of the wild-type proteins and their mutations, there are only minimal shifts measured (Assadi-Porter et al., 2010). This implies that structure of peptide with a CSαβ motif is relatively stable and has high tolerance to amino acid substitution. Side chains of these residues on the interactive surface are crucial to biological functions of the peptides.

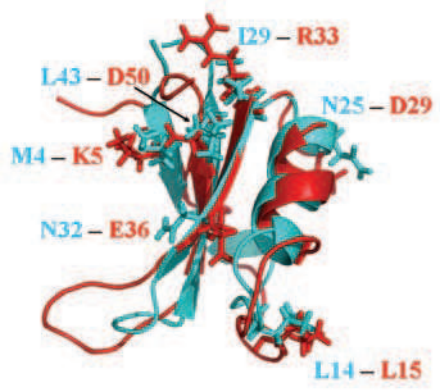


Fig. 5. Superposition of brazzein and VrD1. Some critical residues are overlapping between brazzein and VrD1. Red: brazzein, cyan: VrD1. Red and cyan labeled residues: critical residues of brazzein and VrD1; respectively. The two structures are aligned with SARST (http://sarst.life.nthu.edu.tw/iSARST/).

Multiple mutation also has been performed on the scaffold. Vital *et al* performed minimal residue substitutions on the charybodotxin (Chtx), an scorpion toxin containing CSαβ motif, to equip metal ion binding ability (Vita et al., 1995). Three residues, K27, M28 and R34, on the β sheet are substituted with histidines. The modified protein exhibts a chelatine property but has the same circular dichroism spectrum profile as the native Chtx does.

4.2 Functional epitope exchange

Grafting epitopes with known function is a straightforward stratage for a protein to gain new function. Functional epitopes could be exchanged between proteins have a high sequence identity or share a high structural similarity. Based on the structural homologous, the β2-β3 hairpin of scyllatoxin, a scorpion toxin containing the CSαβ motif, is replaced by the CDR2-like loop of human CD4. The chimeric protein can bind to the HIV-1 envelope glycoprotein and the affinity is increased 100 fold as compared with the native scyllatoxin (Vita et al., 1999). In another case, VrD1 and VrD2, two defensins of *Vigna radiate*, share 80% sequence identities. The major difference of sequences concentrats on the loop 3. When the loop 3 of VrD1 is grafted to VrD2, the chimeric peptide exhibits the enzyme inhibitory function as VrD1 does (Lin et al., 2007).

4.3 Combinatorial chemistry approach

Combinatorial chemistry approach are powerful in screening and selecting binders with high affinity and high specificity (Hosse et al., 2006). It has been widely applied in antibody scaffold and thousands of antibodies are generated through the technology. Combinatorial chemistry approach also has been recruited to develop and isolate artificial proteins with new functions (Zhao et al., 2004). The approach could accelerate protein engineering based on a CSαβ motif to develop novel peptides with biomedical interesting (Thevissen et al., 2007;Van Gaal et al., 2004). Based on scaffold of insect defensin A, an expression library of peptides with 29 residues is constructed and used in screeening novel binders to targets. The expression library is artificially synthesized and amino acid of seven positions on the loops are randomized (Zhao et al., 2004). Tumor necrosis factor α (TNF-α), TNF receptor 1, TNF receptor 2 and antibody against BMP-2 are selected as targets and the screening results show significant enrichment in all cases.

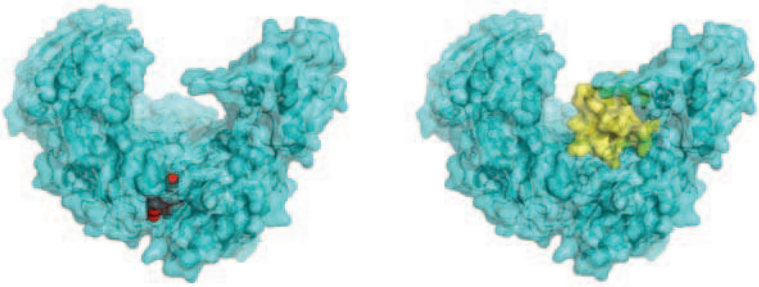
5. The challenges of engineering of CSαβ motif

Although several successful engineered peptides based on the CSαβ motif scaffold have been reported, to design new functions into the scaffold does perplex us. In this section, we would like to discuss challenges in engineering a CSαβ motif to equip desired functions.

5.1 Lacking co-structural model

To introduce tailored functions into a protein scaffold is never an easy task. For its ultra stability and high diversity of function, there is no doubt that peptide with a CSαβ motif is a suitable scaffold to be engineered for biomedical applications. To date, there are only a few of successful cases are reported. Many structures of peptides with CSαβ motif have been solved and deposited in database, but knowledge about the motif is poor. In spite of hundreds of peptides with a CSαβ motif deposited in the database, it is hard to make conclusions relating to structures and biochemical functions of the motif. Key residues are

revealed in some peptides with a CSαβ motif and molecular docking models provide reasonable explainations (Figure 6). The real interaction is not clarified, for lacking of a co-structural model. A co-structural model of the motif and its target will provide information about the dynamic interactions of the two protein molecules (Dumas et al., 2004;Thioulouse & Lobry, 1995). Structures of the complexes of peptides with a CSαβ motif and its counterparts will provide the situation of protein-protein interaction and have a great benefit to engineering of the scaffold.



(a) HIV reverse trascriptase with inhibitor (b) Docking model

Fig. 6. Docking model of plant defensin to HIV reverse transcriptase. Structures of HIV reverse transcriptase (3DRP, HIV RTase) and VrD2 (2GL1) are retrieved from the Protein data bank. Molecular docking is performed with PatchDock (http://bioinfo3d.cs.tau.ac.il/PatchDock/). (a) The crystal structure of HIV RTase with inhibitor. (b) Molecular docking model of VrD2 and HIV RTase. Cyan: HIV RTase, yellow: VrD2 and red: HIV RTase inhibitor.

5.2 Generation of gene library and construction of expression system

The advantages of combinatorial chemistry approach to protein engineering is its ability to associate every protein with its genetic material (Weng & DeLisi, 2002). There are two core parts about applying combinatorial chemistry approach in protein engineering: display techniques and gene expression library. These two core parts are equally important and affect each other. To date, several display techniques have been developed (Table 4) (Daugherty, 2007;Gronwall & Stahl, 2009). Natural properties of peptides with a CSαβ motif are as defenders for their native hosts and most of the peptides express anti-bacterial abilities, virus inhibitory abilities and inhibition of protein translation. How to design a expression library based on a CSαβ motif scaffold is a tough task.

Technique	Library limitation	Expression system	Disulphide bond
Bacterial display	10^{11}	Circular	No
Phage display	10^{11}	Circular	No
Ribosome display	10^{14}	Circular/Linear	Yes

Table 4. Comparison of three display techniques.

Peptides with a CSαβ motif are tightly held by disulphide bridges and may be not easily folded into appropriate structures inside bacterial cells (Villemagne et al., 2006). Ribosomal display technique can overcome the problems (Figure 7), but it requirs an ultra clean

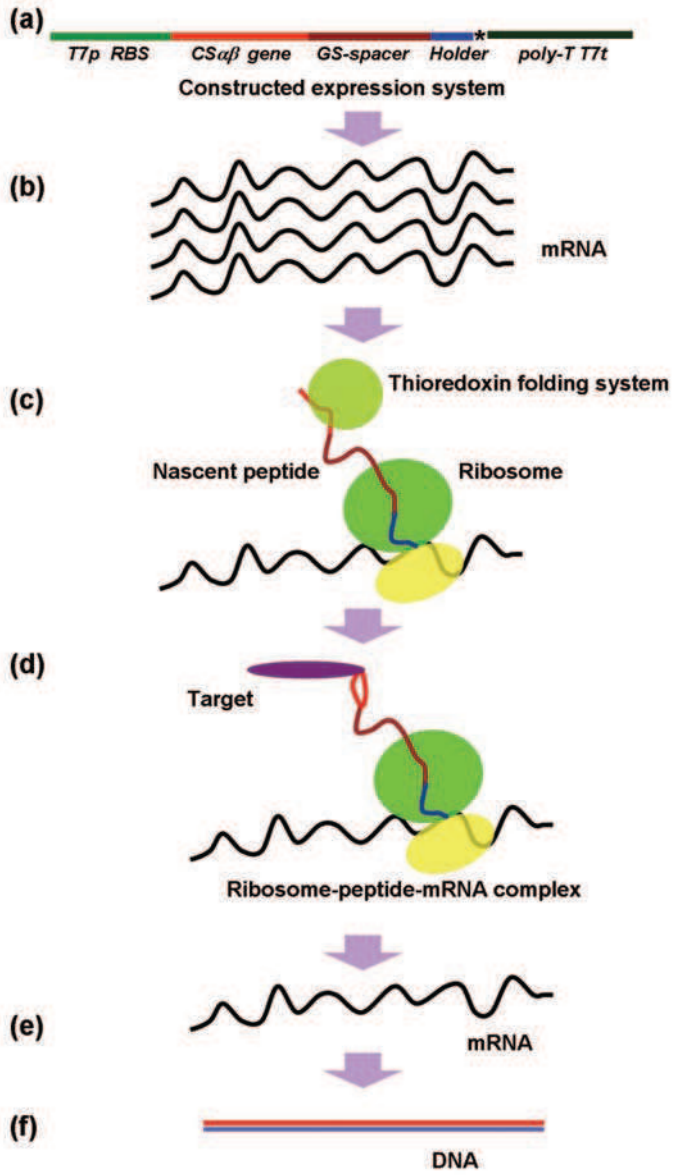


Fig. 7. Concept of ribosome display techniques. (a) expression system, (b) & (c) transcription, translation and folding while translation, (d) binding and selection, (e) isolation of mRNA from the complex, (f) reverse transcription and construction of expression system for next cycle of selection. A specific holder gene usually is constructed to the expression system. The holder can arrest protein translation and the nascent peptide can form a stable complex with ribomsome.

environment to protect mRNA from degradation (Fennell et al., 2010;Villemagne et al., 2006). The gene library of a CSαβ motif has to be artificially synthesized then constructed to the expression system. There are two key issues of the synthesized gene library have to be considered, one is the size of library the other one is how many random residue positions it should have (Gronwall & Stahl, 2009;Michel-Reydellet et al., 2005). These two questions are hard to answer. A large library with more randomized residue positions could have more genotypes/phenotypes but it also has a high possibility to generate nonsense genes for the unvoided stop codon on unexpected positions. A library with a randomized loop 3 of a CSαβ motif scaffold has been constructed and successfully used in a noval binder to human TNF-α (Zhao et al., 2004). It also has been reported that all three loops of peptides with a CSαβ motif can equip unique functions and the structural regions also fine regulate the functions of the peptides (Assadi-Porter et al., 2010;Liu et al., 2006;Yang et al., 2009;Zhao et al., 2002). For a library of long length artificial genes, several cycles of PCR overlapping extensions are required and may reduce quality of the library. A circular or a linear expression systems can be recruited (Fennell et al., 2010;Shimizu et al., 2001;Villemagne et al., 2006). To construct the linear library, all required DNA elements, as promotor, ribosomal binding site and poly T tail, are synthesized or copied and constructed with the desired gene library through PCR (Katzen et al., 2005;Shimizu et al., 2001) . In the linear expression system, the library can have a maximal size up to 10^{13} (Table 4) (Gronwall & Stahl, 2009).When a linear expression system is recruited, the qualities of final PCR products should be well controlled. It is better for the library to be freshly constructed everytime the protein display is performed. The size of a library constructed with a circular expression system is much smaller for the genotype lost during construction, however, the library can be stably stored in a good condition after construction.

5.3 Mass production and folding of proteins

To screening possible funtions of a protein, it requires a large amount of the protein. The amount of proteins with a CSαβ motif in their native host is rare and to obtain eounght of the native proteins for assays requires a lot of raw materials. To produce recombinant proteins of a CSαβ motif in bacteria cells, the proteins have to be fused with a large tag to reduce their toxicity to bacteria. Protein folding is also another problem for apporpriately forming the disulphide bonds of a CSαβ motif. To overcome the problems, peptides with CSαβ motif can be fused with a thioredoxin tag and a cleavagable site is placed between the peptide and the tag. The recombinant proteins are expressed in *E. Coli* and purified. After the thioredoxin tag is cut off, the desired proteins are further purified. A simple method to cut off the thioredoxin tag is to place a acidohydrolysis site, -Asp-Pro-, between the peptide and the tag (Liu et al., 2006). A large amount of reconbinant proteins can be obtained by following the procedures and the native properties and functions of the proteins are preserved. The acidohydrolysis process is not suitable for proteins with a CSαβ motif containing proline for the proteins would be unstable in a heat and acidic environment (Cunningham & O'Connor, 1997;LeBlanc & London, 1997;Wilce et al., 1998). To resolve the problem, an alternative enzyme cleavage site can be introduced to replace the acidohydrolysis site.

5.4 Unpredictable biochemical function

Enzyme inhibitory function and microbial killing ability are the two greatest advantages of peptide with a CSαβ motif. The conventional bacterial/fungal killing assay is time

consuming and it is possible for a performer to be exposed to pathogens during the operation. The peptides with a CSαβ motif have been shown to form pores on an artificial membrane, but the artificial membrane does not represent the membrane of different organisms. A new high throughput screening recruiting membrane of different pathogens will be a great help in resolving the problem (Yang & Lyu, 2008).

Not simple as binding assay, enzymes act on specific substrates and it is extremely difficult to unify experimental conditions for a broad spectrum of enzymes. *In vitro* enzyme inhibitory assays are costly and unable to represent the actually physiological conditions. Computer aided drug screening methods have been established to screen drugs with small molecule weights that can fit into active sites of target proteins (Barrons, 2004;Weideman et al., 1999). Drug dynamics also can be *in silico* simulated (Sinek et al., 2009;Zunino et al., 2009). An antibody can be as a simple binder and its binding targets usually are short fragments exposed to the surface of the proteins. The binding fragments are predicable for a huge amount of knowledge accumulated in the last several decades (Blythe & Flower, 2005;Kulkarni-Kale et al., ;Odorico & Pellequer, 2003). Currently, we do not well understand the interface between peptides with a CSαβ motif and their targets. How functional loops access active sites of the targets is needed to be decrypted. We know too little about the scaffold and it is to difficult to engineer it. It is hard to develop an algorithm to predict the biochemical functions of the peptides with a CSαβ motif. For the complexity of protein-protein interaction, it is a long way to screen peptide drugs *in silico*.

6. Business issue

To biopharma companies, protein engineering/evolution platforms must provide solutions to improve the throughput, safety, biodistribution, activity and frequency of dosing of designed protein, evading intellectual property issues and reducing manufacturing costs, especially in the late process of development and production stages. Intellectual property rights are definite issues, the intellectual property situations of antibodies are obscure as previously described. An alternative scaffold equips the same abilities as antibody does is a valuable prospect and desired to avoid the complex intellectual property landscape of antibody. A protein scaffold can access to a cardinal target and circumvent intellectual property issues that does not mean that it is necessarily a platform worth investing in. The successfully designed protein must equip an improved performance and provide solutions to the concerned points described above.

7. Conclusion

The knowledge about the scaffold is not enough to establish rules to predict its biochemical functions. There are still some technical limitations and the development of the scaffold is confined. Peptides with a CSαβ motif bear many excellent properties and can serve as an alternative protein scaffold for biomedical application. Natural products containing peptides with a CSαβ motif have been therapeutically used for hundreds of years. Some of the purified peptides are tested and have low toxicities to mammalian species. Prudential is needed when recruiting new proteins/peptides as treatments for diseases. The intellectual property situation of peptides with a CSαβ motif is not as complex as antibody, especially in biomedical application (Yang & Lyu, 2008). For its original expression in plant, the engineered proteins with medicinal effect can be expressed in transgenic crops and diseases

can be controlled through foods. To predict the biochemical functions of peptides with a CSαβ motif is difficult, some prediction tools have been developed to predict functions of protein from the primary structure, but how proteins interact with their counterparts has to be considered. Folding and post-translational modification of proteins has to be thought over. We believe that peptides with a CSαβ motif will be an alternative scaffold and can be widely applied in biomedical utilities.

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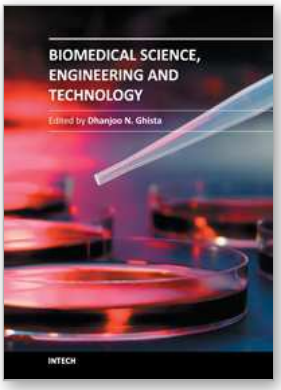

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This innovative book integrates the disciplines of biomedical science, biomedical engineering, biotechnology, physiological engineering, and hospital management technology. Herein, Biomedical science covers topics on disease pathways, models and treatment mechanisms, and the roles of red palm oil and phytomedicinal plants in reducing HIV and diabetes complications by enhancing antioxidant activity. Biomedical engineering coves topics of biomaterials (biodegradable polymers and magnetic nanomaterials), coronary stents, contact lenses, modelling of flows through tubes of varying cross-section, heart rate variability analysis of diabetic neuropathy, and EEG analysis in brain function assessment. Biotechnology covers the topics of hydrophobic interaction chromatography, protein scaffolds engineering, liposomes for construction of vaccines, induced pluripotent stem cells to fix genetic diseases by regenerative approaches, polymeric drug conjugates for improving the efficacy of anticancer drugs, and genetic modification of animals for agricultural use. Physiological engineering deals with mathematical modelling of physiological (cardiac, lung ventilation, glucose regulation) systems and formulation of indices for medical assessment (such as cardiac contractility, lung disease status, and diabetes risk). Finally, Hospital management science and technology involves the application of both biomedical engineering and industrial engineering for cost-effective operation of a hospital.

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