

Design and Production of Specifically High Affinity Reacting Peptides (SHARP®-s)

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Abstract: *Background:* A partially random *target* selection method was developed to design and produce affinity reagents (*target*) to any protein *query*. It is based on the recent concept of *Proteomic Code* (for review see Biro, 2007 [1]) which suggests that significant number of amino acids in specifically interacting proteins are coded by partially complementary codons. It means that the 1st and 3rd residues of codons coding many co-locating amino acids are complementary but the 2nd may but not necessarily be complementary: like 5'-AXG-3'/3'-CXT-5' codon pair, where X is any nucleotide.

Results: A mixture of 45 residue long, reverse, partially complementary oligonucleotide sequences (*target* pool) was synthesized to selected epitopes of *query* mRNA sequences. The 2nd codon residues were randomized. The *target* oligonucleotide pool was inserted into vectors, expressed and the protein products were screened for affinity to the *query* in Bacterial Two-Hybrid System. The best clones were used for larger-scale protein syntheses and characterization. It was possible to design and produce specific high affinity reacting (Kd: ~100 nM) oligopeptide reagents to GAL4 *query* oligopeptides.

Conclusions: Second codon residue randomization is a promising method to design and produce affinity peptides to any protein sequences. The method has the potential to be a rapid, inexpensive, high throughput, *non-immunoglobulin* based alternative to recent *in vivo* antibody generating procedures.

Key Words: Combinatorial engineering, protein interaction, proteomic code, peptide design, methods.

INTRODUCTION

According to the Human Genome Project, the estimate for the total number of genes in the human genome has been revised down from 30,000 – 35,000 to 20,000 – 25,000 [2].

Despite the fairly low number of genes, the best estimates for the total number of proteins encoded by the human genome (the proteome) remain anywhere from 300,000 to 1 million. Ongoing efforts to study the proteome have kept the research antibody industry flourishing. Also, reaping financial benefits are motivating companies that provide reagents and devices required for antibody-related protocols. With basic science, drug developing researchers are creating a tide of demand, and the revenue stream from antibody-led protein hunts won't be drying up soon [3].

Revenue from antibodies for therapeutic and diagnostic use is expected to grow at an average annual growth rate of 11.5%, according to a 2005 report, "Dynamic Antibody Industry," published by the Business Communications Company [4]. With an estimated market of \$15 billion in 2005, revenues should reach \$26 billion by 2010.

The original procedure to induce antibodies (Ab) is the *in vivo* immunization. The success of this polyclonal antibody production in animals is variable, often not predictable, takes several weeks, require the use of well purified antigens [5]. The monoclonal antibody production uses animals only as

the source of biological machinery which is responsible for the immune response (B lymphocytes). The Ab production itself takes place *in vitro* [6]. It makes the monoclonal Ab production cheaper, faster and more reliable. In fact, monoclonal antibodies produced by animal immunization remain the 'gold standard' of affinity reagents. They are relatively renewable, can usually be made with high specificity and affinity for their target and can be used in common biochemical assays such as Western blotting [7], ELISA [8] and different branches of immunochemistry [9]. But the traditional monoclonal antibody has its drawbacks. Its production can be challenging, time-consuming and costly.

Additional concerns associated with Ab-s which are seriously limiting their therapeutic applications are their size and their origin. Antibodies are large and complex proteins (>150 KDaltons) which are much larger than necessary for antigen recognition and binding. They are antigenic themselves and are carrying the species characteristics of their origin. Size reduction, like single-chain variable (scFv) antibody fragments [10] and "humanization" [11] helps to solve these problems.

A moderate discomfort, even if not a ban, of Ab production is that we still not know exactly how antibodies are made by the immune system and therefore it is not possible to reproduce it *de novo*, without "borrowing" the technique of a living organism.

So there is a lot of interest in identifying novel affinity reagents that would be less expensive and quicker to produce.

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Affibodies [12] were among the first non-immunoglobulin-based affinity reagents. These small molecules are based on a bacterial receptor (Staphylococcus aureus protein A), and use combinatorial protein engineering to introduce random mutations in the affinity region [13]. Protein Z is a 58-residue three-helix bundle domain derived from staphylococcal protein A (SPA), which binds to the Fc portion of IgG from different species. By simultaneously randomizing 13 amino acid positions located at the two helices making up the Fc-binding face of protein Z, binding proteins (*affibodies*) capable of binding to desired targets have been selected by using phage display technology.

Another non-immunoglobulin-based affinity reagent that is becoming more widely used is the *aptamer*. Made of DNA, RNA or modified nucleic acids and typically 15–40 bases in length, aptamers have a stable tertiary structure that permits protein binding through van der Waals forces, hydrogen bonding and electrostatic interactions. Early studies showed that aptamers can be highly specific for target proteins, with the ability to distinguish between related members of a protein family [14].

No one of the above mentioned methods is able to satisfy the emerging need to produce these reagents at a truly *high-throughput* scale. The estimated number of affinity reagents (antibodies or else) needed to monitor the human proteome is probably not less than the number of different proteins (including splicing variations, but not the unknown number of folding variations), which is around 0.3-1.0 millions.

A completely different approach to understand and utilize the nature of specific protein-protein interactions is the concept of the *Proteomic Code*. The Proteomic Code is a set of rules by which information in genetic material is transferred into the physico-chemical properties of amino acids. It determines how individual amino acids interact with each other during folding and in specific protein-protein interactions. The Proteomic Code is part of the redundant Genetic Code [1]. This, 25-years-old theory states, that significant number of amino acids co-located on the specifically interacting protein-protein interfaces are coded by complementary codons.

The original concept expected a perfect complementarity coding of co-locating amino acids. There are hundreds of experiments from reliable laboratories, suggesting the validity of this expectation (for review see [1, 15]). However there are many (to many) exceptions.

Recently, bioinformatical studies suggested that co-locating amino acids are coded by partially complementary codons, where the 1st and 3rd codon residues are complementary in reverse orientation, but the 2nd codon residue is not necessarily complementary. This second generation *Proteomic Code* may be described by the 3'-NXN-5'/5'-nXn -3' formula, where N and n denote complementary base pairs, while X indicates any nucleotide. A method (The Method in this text) to *Design and Production of Specifically and with High Affinity Reacting Peptides (SHARP®-s)* is built on this formula [16].

GENERAL ASPECTS OF THE METHOD

The protein which is used to generate affinity peptides is called *query (Q)* in this description and it is an analog to the

terms *ligand, antigen or bait*. Query is one protein sequence that the target protein, designed and produced with the Method, will specifically interact with.

The generated affinity peptide is called *target (T)*, and it is an analog to the names *receptor, antibody or hit*. Target proteins are protein sequences which are designed by the Method to specifically interact with the query protein sequence.

The query and target are expected to react with each other specifically (able to distinguish between related but not identical peptide sequences) and with high affinity (Kd is at least in microM range).

Target Oligo-Nucleotide Pool (TONP) is designed by using a *Target Oligo-nucleotide Template (TONT)* which is a nucleic acid sequence containing 2/3 defined and 1/3 undefined (any) nucleotides (X). A TONT, which contains 15 undefined nucleic acid residues, (defining a pool of $4^{15}=10^9$ different oligo-nucleotides, TONP) will be translated into the corresponding number of oligopeptides.

Expression of TONT will result in the syntheses of a large number of different oligo-peptides, called Target Oligo-Peptide Pool (TOPP). Those oligopeptides which satisfy the criteria for specific, high affinity reactions with the query protein are called SHARP®-s.

The flow of SHARP® production may comprise the following main steps (Fig. 1).

1. Query Selection:

- The length of query peptide is limited to about 15 amino acids, depending on the available screening method (see below). Expression library utilizing phages [17] may contain maximum 10^9 different clones (which is $\sim 4^{15}$) while the recent maximal capacity of yeast based libraries [18] is much less, about 10^7 (which is $\sim 4^{12}$). This limits the number of variable bases to 15 and 12, respectively.
- Query may be part of a much larger protein. In these cases physicochemical, structural considerations might guide to select the most “promising” query sequences.
- The concept of selecting a particular sequence of a protein as a target to selected complementary peptide or vice-versa is a formidable challenge. Epitopes exist as continuous or discontinuous and as such are complex when antibodies are raised against them. To have an alternative system where protein-protein or protein-peptide interactions are involved the spatial conformation have to be taken into account.
- It is necessary to know the correct coding sequence of the query. A nucleic acid sequence derived by back-translation, using any Codon Usage Frequency Table, is not satisfactory.

2. Design the Target CDS:

- The CDS of target oligopeptides (TONP) is derived from the CDS of the query, using the 3'-NXN-5'/5'-nXn-3' formula [1] or

3'-_NNN_NNN_NNN_-5' QUERY

5'-_nXn_nXn_nXn_-3' TARGET OLIGO-NUCLEOTIDE TEMPLAT (TONT)

- Detect the signal protein (if it's CDS was added to the TONT). This is a further indication that translation is correct, no frame shift occurs and the target oligo-peptides (TOPP) are correctly expressed.

8. Prepare the Query Protein:

Some screening systems (two-hybrid yeast or bacterial systems) request the expression of the query too. In these cases prepare the query accordingly to the systems requirements and test the correctness of query expression.

9. Expression/Cloning Library Construction:

The TOPP might be expressed and multiplied in yeast [24], bacteria (using plasmid or phage vectors) [25], which are providing the TOPP expression library. These libraries are expected to contain and express about 10^6 - 10^9 , more or less different, oligopeptides. Target Oligopeptide Library is a partially (33%) random library, because the central residues of the codons are randomly selected.

10. Screening of the TOPP Expression Library:

There are at least two different methods which are suitable for screening the clones:

- a. protein-fragment complementation assays (PCA) [24]

This assay is utilized by the bacterial and yeast two hybrid methods (Fig. 2).

In these types of assays an active enzyme is dissected into inactive fragments and the fragments are fused to the test proteins. Interaction between the test proteins brings the inactive enzyme fractions together and restores the original function of the enzyme. This enzyme function is then detected by a simple assay (colorimetry, fluorimetry, colony survival).

- b. phage display technique (for review see [25], Fig. 3).

In these assays one of the test proteins (target) is fused to phage proteins and expressed on the surface of phages. The

Protein-fragment Complementation Assay (PCA)

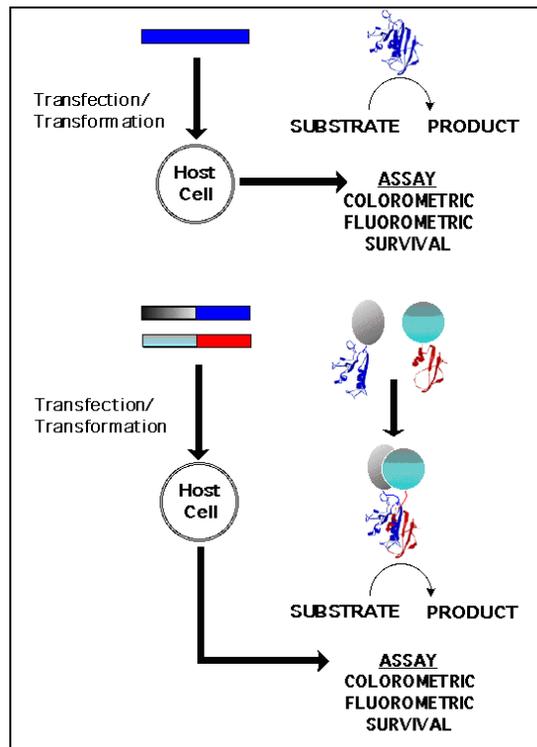


Fig. (2). General description of a PCA [26]. The gene for a protein or enzyme is rationally dissected into two or more fragments. Using molecular biology techniques, the chosen fragments are sub cloned, and to the ends of each, proteins that either are known or thought to interact are fused. Co-transfection or transformation of these DNA constructs into cells is then carried out. Reassembly of the probe protein or enzyme from its fragments is catalyzed by the binding of the test proteins to each other, and reconstitution is observed with some assay.

Scheme of Phage Display

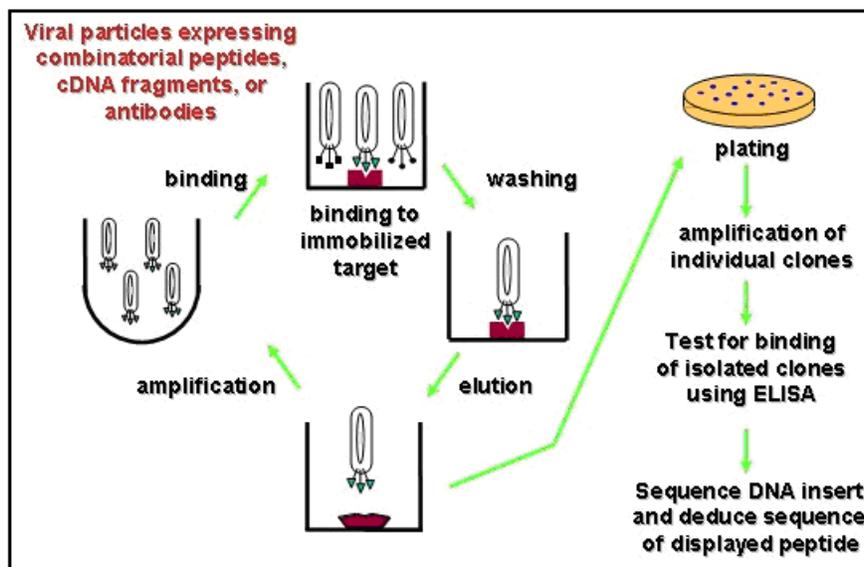


Fig. (3). Scheme of phage display [27].

other test protein (query) is attached to some solid surface. Interaction between test proteins will attach the phase to the solid surface and select the phages which are producing the interacting test protein.

11. Select the best clones and continue the cloning until monoclonal stadium. Save the best clones. The “best” clones are those which are displaying the largest number of target proteins with highest affinity to the query protein. Numerous methods are available for manual or automated library handling and screening of surface displayed interacting (binding) gene products [28-31].

12. The best clones should be further propagated to obtain larger quantity of the desired SHARP®-s for purification and **testing for physicochemical properties** (binding specificity and Kd).

- Large number of methods is known for Kd determination. Label-free real-time interaction analyses **using surface plasmon resonance and a sensor chip technology**, is one of the latest technologies [32, 33]. These methods work on crude bacterial extracts, target protein purification is not necessary.

13. Extract and **re-sequence the target CDSs** producing the best targets.

14. Re-sequence the Best Target Oligo-Peptides and Their mRNA. This step is not absolutely necessary. Finding the proper SHARP® might be satisfactory. However, re-sequencing is the source of important information for further experiments. For example

- re-sequencing is the final confirmation, that the target peptides are found by design and not only by chance.
- re-sequencing and analyzing the query/target sequences might be the source of valuable knowledge about the further rules of protein-protein interactions.

Therefore re-sequencing is highly recommended.

15. Visualization of Query/Target Complex (NMR) may be an additional step to gain further knowledge about the protein folding and interactions. NMR analyses for protein-protein interactions in solution are very important methods in understanding specific, macromolecular interactions [34-35]. However, the huge complexity of NMR investigation techniques shouldn't be underestimated.

16. Iterate the procedure 1-15 using another query.

EXAMPLE FOR APPLICATION OF THE METHOD

BacterioMatch II Two-Hybrid System

The system chosen was the BacterioMatch II two-hybrid system commercially available from Stratagene [36]. This system utilizes a *bait vector* (pBT) and a *target vector* (pTRG). The bait vector contains a portion of the λ cI gene and the target vector contains a portion of the alpha subunit of RNA polymerase. The bait and target peptides (or proteins) are genetically fused to the cI gene in pBT and the RNA polymerase subunit in pTRG, respectively. Inside a cotransfected bacterial cell, the λ cI gene product binds to a λ operator on a reporter plasmid. The bait portion of the construct is available to interact with the target portion of the target vector. When an interaction occurs (the bait and target

bind to each other), the RNA polymerase subunit is in close proximity to a weak RNA polymerase binding site (from the *lacZ* promoter) on the reporter plasmid. This binding allows the RNA polymerase to transcribe a pair of reporter genes, HIS3 and *aadA*. The HIS3 gene allows the bacterial cells to grow on medium lacking histidine (or more accurately containing the histidine antagonist 3-amino-1, 2, 4-triazole or 3AT). If the binding interaction is strong enough, the *aadA* gene is also transcribed conferring streptomycin resistance on the cells. Double selection on 3AT and streptomycin containing plates reduces the number of false positives. The BacterioMatch II kit is supplied with a pair of control vectors containing portions of the yeast *GAL4* and *GAL11* genes. The interaction of these gene products is both highly specific and tight. Cells from a transfection using both of these controls produce many colonies on doubly selective plates (Fig. 4).

Outline of Experimental Design

We designed a series of target peptide gene sequences that could potentially bind to the control vector pBT-LGF2 (containing the λ cI gene). These target oligonucleotides (the Target Oligo Nucleotide Pool) were synthesized by Retrogen [37], digested with appropriate restriction enzymes, and ligated into similarly digested pTRG. The pBT-LGF2 and pTRG-TONP were cotransfected into *E. coli*.

MATERIALS AND METHODS

Analytical grade chemicals were used.

The sequence of the GAL4 portion of the pBT-GAL4 obtained from the Stratagene website was inconsistent with the size of the GAL4 protein as described in the Stratagene literature. Forward and reverse oligonucleotide primers for sequencing pBT were synthesized (Retrogen) and the nucleotide sequence of the GAL4 protein was determined. This sequence was then matched by the published sequence to establish the exact portion of the GAL4 protein contained in the pBT-LGF2 vector.

TONTs were designed to match the protein sequence predicted from the newly determined nucleic acid sequence of GAL4. The sequences in TONP included the restriction sites for *Bam*HI and *Not*I (Fig. 5). One strand of the sequences in TONPs was chemically synthesized using multiple bases at various positions (denoted with an X in Fig. 5). The second strand was produced by a primer extension reaction; the products of this reaction were analyzed by gel electrophoresis (20% PAGEgel, 0.25M Tris acetate buffer at pH 7.6). After the primer extension reaction, the TONP were desalted (Sephadex G-50 Micro Columns), digested with *Bam*HI and *Not*I, heated to inactivate the restriction enzymes, and desalted again.

pTRG was digested with *Bam*HI and *Not*I. Approximately 160 ng digested vector (1 μ L), 6 to 10 μ L digested TONP, and 10 X ligase buffer, ATP, and water were placed in a final volume of 19 μ L along with 1 μ L of T4 DNA ligase. The reaction mix was incubated overnight at 4° C. XL1-Blue MRF' Kan cells (Stratagene) were transfected with the ligation mixes and plated on non-selective medium containing kanamycin and tetracycline. Several colonies were picked and plasmid DNA was prepared using Wizard

The BacterioMatch (TM) Two-Hybrid System

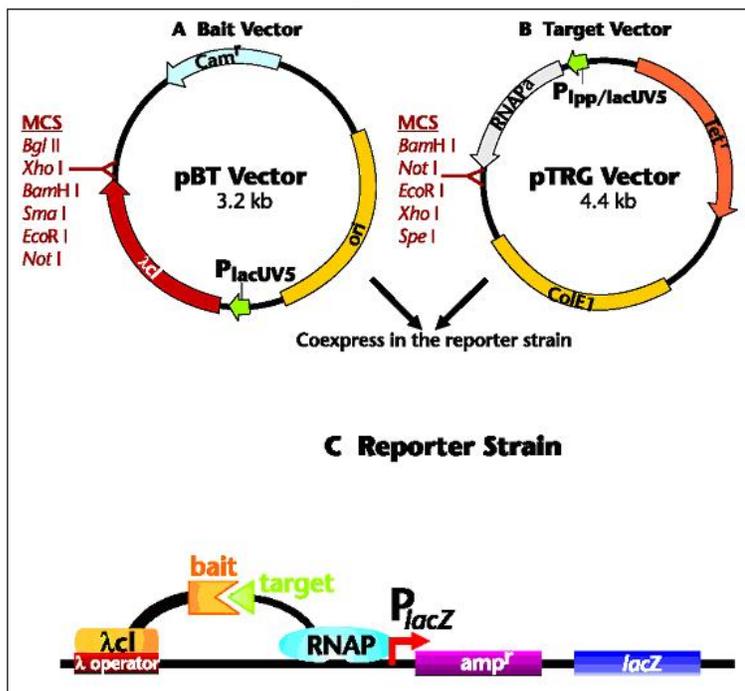


Fig. (4). The BacterioMatch™. Two-hybrid system [36].

Design of SHARP®s

A. Query Selection

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>1HBW:A REGULATORY PROTEIN GAL4
1      9      17     23     31                               57
.      .      .      .      .
TRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLRDIEALLTGFLVQDNVNKDA
      HHHHHHHHHHHHHH SSSSSS      HHHHHHTTHHHHHHHHHHH S SS

>K01486_SCGAL4_DIMDOM | ND, 171 bases, 1D18 checksum.
5'-- ACTAGGCGCATCTGACAGAACTGGAAATCAAGGCTAGAAGACTGGAACAGCTATTTCTACTGATTTTTCCTCGAAGACCTTGACATGATT
H2N- T R A H L T E V E S R L E R L E Q L F L L I F P R E D L D M I L

>Query 1_ESRLERLEQLFLLIF (GAL4 09-23AA)
5'-- GAAATCAAGGCTAGAAGACTGGAACAGCTATTTCTACTGATTTTT
H2N- E S R L E R L E Q L F L L I F

>Query 2_QLFLLIFPREDLDMI (GAL4 17-31AA)
5'-- CAGCTATTTCTACTGATTTTCTCGAAGACCTTGACATGATT
H2N- Q L F L L I F P R E D L D M I
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B. Target Oligo-Nucleotide Template (TONT) Design

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>TARGET TEMPLATE to Query 1_ESRLERLEQLFLLIF (GAL4 09-23AA)-ssDNA-sense
5'--GGATCC-[AxAAxT CxGTxGAxATxG CxGTxCxGTxT TxCTxG CxT TxATxG]-CGGCCGC-3'
|--BamHI--[ VARIABLE SEQUENCE, TONT 45 NA ]-NotI----|

>TARGET TEMPLATE to Query 2_QLFLLIFPREDLDMI (GAL4 17-31AA)-ssDNA-sense
5'--GGATCC-[AxTCxTGxCxGxCTxCTxGxGxAAxT CxGTxGAxATxG CxG]-CGGCCGC-3'
|--BamHI--[ VARIABLE SEQUENCE, TONT 45 NA ]-NotI----|
```

Fig. (5). Design of SHARP®s. Two 15 amino acid (AA) long oligopeptides (Query 1 & 2) were selected from the GAL4 sequence (underlined). Helices (H) and sheets (S) are indicated below the primary GAL4 sequence. Coding sequences of these queries were used to design TONT. Codons are emphasized by yellow boxes. Restriction enzyme cut sites are indicated by blue boxes.

Forms of Peptide to Peptide Interactions

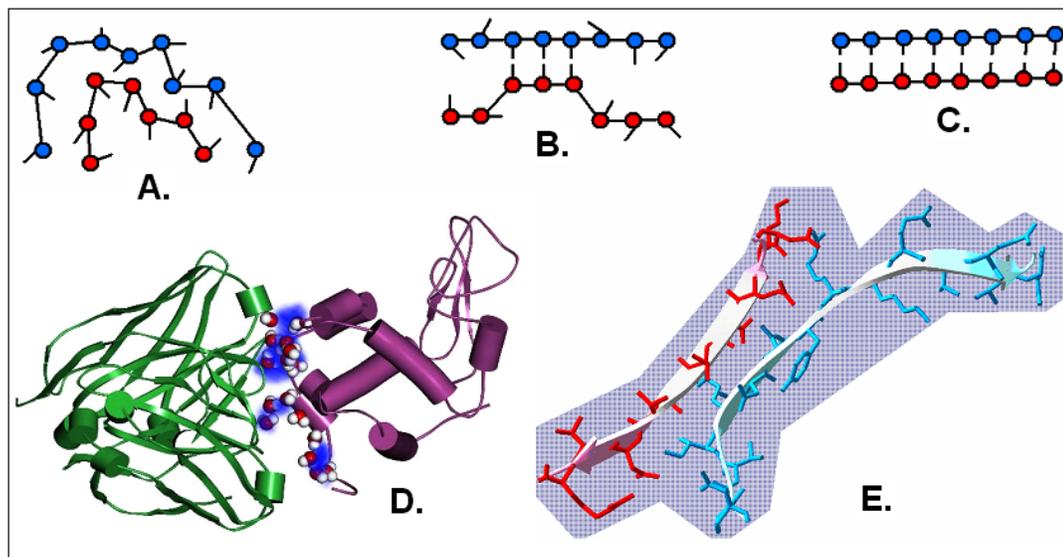


Fig. (7). Forms of peptide to peptide interactions. The specificity of interactions between two peptides might be explained in two ways. First, many amino acids collectively form larger configurations (protrusions and cavities, charge and hydrophathy fields) which fit each other (A and D). Second, the physico-chemical properties (size, charge, and hydrophathy) of individual amino acids fit each other like “lock and key” (C and E). There are even intermediate forms (B).

ruses, toxins, etc. in air, water and food supplies.

The idea of Proteomic Code is not new and it was successfully implemented in numerous cases (for review see [1]). The recent “second generation concept” is a new significant improvement compared to the original concept. However it is clear that, as in the case of any new methods, a large number of data and evidence (synthetic peptides, western blots, SELDI, SPR etc) are needed to prove the general validity of this approach.

What about if the concept of Proteomic Code is wrong? The SHARP® design Method still remains a novel variant of combinatorial protein engineering, an approach proved to be successful for affinity peptide design and production.

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