DIFFERENT APPROACHES FOR PROTEIN ENGINEERING IN INDUSTRIAL BIOTECHNOLOGY

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Abstract

Protein engineering is the novel field which has wide applications from pharmaceutics, industry, commercial, laundry and research. It may apply rational design or non rational design or both. Site directed mutagenesis is a classical approach involving the protein folding principles and as such different techniques involving multidisciplinary research and broad knowledge is required involving biocomputing of complex data obtained from various sequencing projects and prediction of the future protein structure either chemically or genetically modified. Non rational mutagenesis or directed evolution involves random mutations in the gene encoding protein or shuffling the genes encoding different domains producing a random set of numerous large libraries of mutant proteins, using advanced technology the desired protein can be selected but the exact structure or changes may remain unnoticed.

Introduction

Protein engineering aims at modifying the sequence of a protein, to produce novel molecules either having some new functions by doing mutagenesis or creating completely novel protein molecules, in order to get a better understanding of its structure- activity relationship, and control a protein's function by obtaining a desired change in its activity. Protein engineering is considered as a second wave of

innovation after genetic engineering. It is useful for studies of basic research and application, in structure-function relations and for exploitation in industry. Genetic engineering provides proteins in bulk which are processed and redesigned to make them suitable for pharmaceutical and industrial requirements (Protein engineering in Albherghina, 2000). Protein engineering is a coordinated application of a number of disciplines usually referred to as the cycle of protein engineering (fig. 1) like determination of prediction of protein conformation (secondary and tertiary), experimental studies of protein folding, chemical modification, mutagenesis (general and site specific) and physical/biochemical methods for structure-activity relationships.

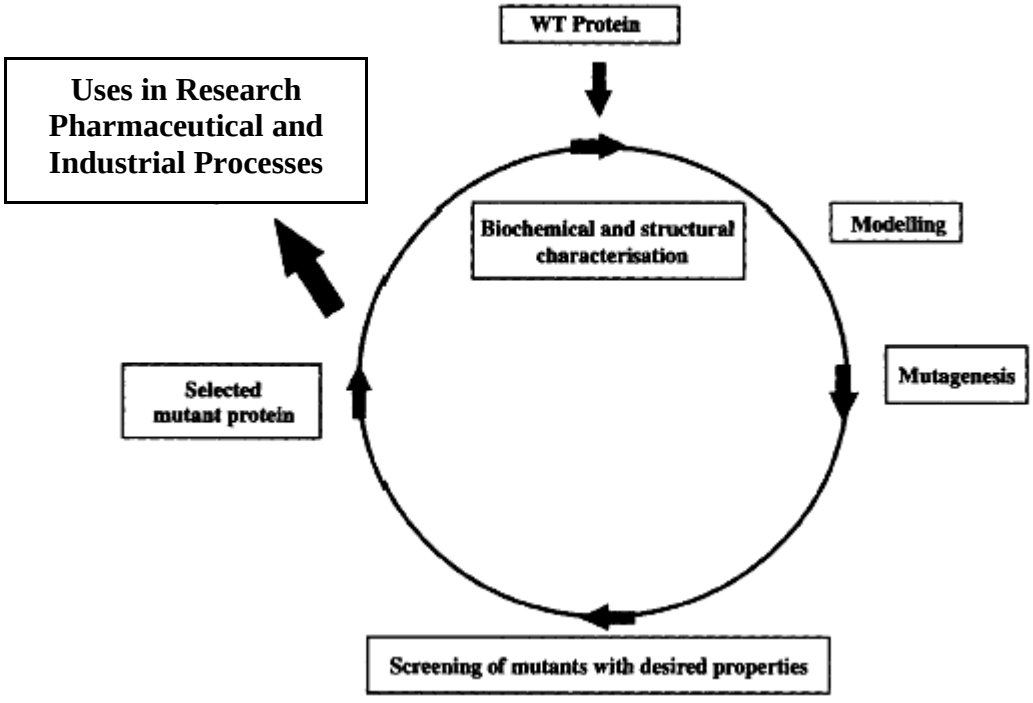

Fig 1: The Cycle of Protein Engineering

The first step in protein engineering is mutagenesis, which requires DNA sequences cloned from original source or DNA synthesized on the protein sequence

of interest. There are two main methods of mutagenesis. (a) Site directed mutagenesis and (b) Random mutagenesis.

Experimental approaches for protein engineering

The experimental approaches for protein engineering are;(Table 1).

(i) Rational design

(ii) Molecular evolution

(iii) Generation of random libraries

(iv) De novo protein design

Table 1 Experimental approaches for protein engineering

Rational design Allows for the introduction of mutations targeted to specific protein sites. Requires a detailed knowledge of the protein structure and of structure-function relationships.
Molecular evolution Does not require any knowledge on the protein structure and mechanism of action. It is based on the random generation of a vast number of mutants followed by screening for the desired functions
Generation of random libraries Production of large collections of proteins, peptides of region thereof. Is often coupled with surface display to ease screening of the mutants
***De novo* protein design** Generates novel structural scaffolds able to accommodate active sites or other protein functions

(i) **Rational Design/ Rational Mutagenesis/ Site Directed Mutagenesis (SDM):**

Chemical or synthetic modification of protein

Proteins play many roles in cellular physiology, serving as structural building blocks, catalyzing biochemical reactions, performing regulatory functions and, in combination with other proteins, self-assembling into complex molecular machines, such as the contractile fibres that are involved in cell motility or the channels that control ion flow across cell membranes. Protein engineering, which first emerged in

the early 1980s, involves the use of chemical or genetic techniques to modify the structure of a protein, thereby increasing its stability or altering its physiological function or activity In protein engineering, using x-ray crystallography, chemical synthesis of DNA, and computer modelling of protein structure and folding has made it possible to attempt to modify many different properties of proteins by combining information on crystal structure and protein chemistry with artificial gene synthesis. Such techniques offer the potential for altering protein structure and function in ways not possible by any other method (Carter, 1986).

Site directed mutagenesis

Site directed mutagenesis is a classical approach, introducing a change in one/more amino acids and evaluate the effect in mutated product e.g.; Influencing the regulation of a protein. This method required a prior knowledge of the protein or role played by specific residues or regions of the protein, availability of the protein 3D-structure if possible along substrate, ligands regulation elements or at least another related protein for comparison, in order to make the desired changes. It may need sequence alignment to support the selection of positions to mutate, particularly when the protein belongs to large well characterized family of proteins and then predict the function effects of planed substitutions. This method has achieved important goals. It is easy, in expensive and well developed. But it is not easy to get the detailed knowledge of the protein or predict the effect of various mutations.

The nucleotide sequence of a cloned DNA fragment may be changed at will by site-directed mutagenesis using synthetic oligonucleotides (Smith, 1985).

(ii) Non-rational design/ random design/ directed evolution

Random mutagenesis on whole protein sequences or parts thereof is the method of choice in all those cases where knowledge about the structure and function of the protein of interest is not sufficient to support a rational design approach. The most innovative techniques involve the

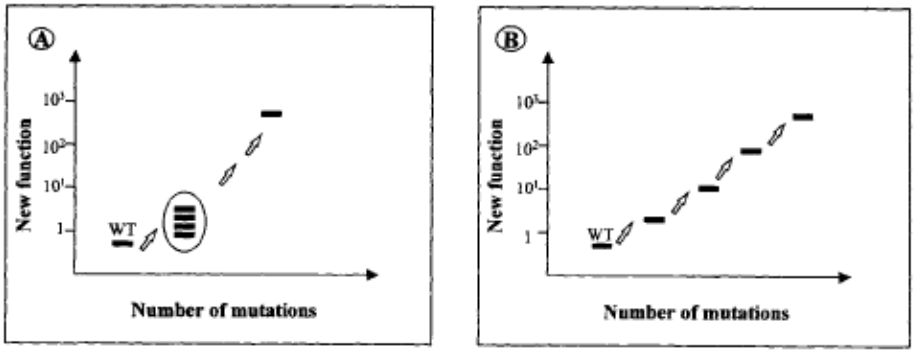

Figure 2 Comparison between two strategies for protein "evolution": DNA shuffling (A) and directed evolution (B) (modified from Arnold, 1996).

generation of repertoires (libraries) of mutated sequences and procedures of mutation/selection that mimic the processes followed by nature during evolution. In both cases, a very high number of mutant variants is produced, so that the development and availability of sensitive and fast procedures of screening is vital to manage the experimental work.

(A) DNA shuffling

*DNA shuffling (*Stemmer., 1994a and b). This technique is also known as "sexual PCR". A final step of amplification with primers generates full-length products. The activity of β-lactamase towards the antibiotic cefotaxime was

increased over 16,000 times through three cycles of sexual PCR, whereas the fluorescence of the green fluorescent protein was increased 45 fold (Crameri *et al.,* 1996).

(B) Directed evolution

In vitro directed evolution is another powerful approach, developed by F.Arnold at Caltech, which, with repetitive cycles of mutagenesis/screening forces the evolution of the selected sequence towards the desired functions (Arnold, 1996). Differently from DNA shuffling, in this approach after every cycle of random mutagenesis, only a single variant showing functional improvements is selected and used as the parent for the next generation of mutants. An esterase from *Bacillus subtilisvtas* modified as to become able to hydrolyze pnitrobenzyl ester bonds in the presence of organic solvents, a reaction of interest for the pharmaceutical industry to remove protecting groups introduced during the synthesis of antibiotics of the class of cephalosporins. Specific activity was increased of 30-folds through four generations of random mutagenesis followed by DNA recombination (Moore and Arnold, 1996). Even the specific activity of subtilisin E in polar solvent was enhanced through the same approach (Chen and Arnold, 1993). Another property of paramount importance in biotechnology—enantioselectivity— was introduced by directed evolution in a lipase from *Pseudomonas aeruginosa* (Reetz *et al.,* 1997).

Structures and modelling

Besides developments in molecular biology and biochemistry, biocomputing and methods for the determination of 3D structures, are indispensable tools in this

field. Since the three-dimensional structure of a protein (enzyme, hormone, receptor) determines its biological function, it becomes important to have available a high resolution 3D structure, especially when the protein engineer aims to redesigning the protein function.

Medium engineering and bioimprinting

Often enzymes exposed to particular pH or ligands before liophylization and subsequent dissolution in organic solvents, prove to be able to retain specific properties, i.e affinity for the ligand, a property referred to as “memory”. Based on this observation, a strategy for the activation of proteins has been developed which was called “molecular (bio) imprinting” and can be adapted to particular conditions where the reaction of interest has to take place. The ability to use proteins in unusual or nonnatural environments greatly expands their potential applications in biotechnology (Arnold, 1993).

Engineering specificity

Rational design, random mutagenesis as well as molecular evolution techniques, have all been applied in the effort of controlling protein specificity. A common approach towards redesigning enzyme specificity is that of using existing protein (Shao and Arnold, 1996).

Engineering feature to ease protein purification

Tags are introduced to obtain enzymes in poure form specially in industry. Two contribution of this volume specifically focus on the introduction of “tags” allowing purification and folding of the protein of interest. Hober and Uhlen describe the use of fusion proteins and co-expression strategies to ease folding and recovery

of recombinant proteins. Schmidt and Skerra developed by molecular repertoire techniques a novel tag able to bind with high affinity to streptavidin from where it can be eluted by competition with biotin analogues.

Artificial non antibody binding proteins (Affibodies) Combinatorial proteins/fusion proteins for pharmaceutical and industrial applications

Using combinatorial chemistry to generate novel non antibody binding molecules based on protein frameworks ('scaffolds') is a concept that has been strongly promoted during the past five years in both academia and industry and attempts are made to engineer affinity to perfection. Nonantibody recognition proteins derive from different structural families and mimic the binding principle of immunoglobulins to varying degrees. In addition to the specific binding of a pre-defined target, these proteins provide favourable characteristics such as robustness, ease of modification and cost-efficient production. The broad spectrum of potential applications, including research tools, separomics, diagnostics and therapy, has led to the commercial exploitation of this technology by various small- and medium-sized companies. It is predicted that scaffold-based affinity reagents will broaden and complement applications that are presently covered by natural or recombinant antibodies. (Thomas *et al*., 2005).

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