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Protein Engineering Methods and Applications

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1. Introduction

Protein engineering is the design of new enzymes or proteins with new or desirable functions. It is based on the use of recombinant DNA technology to change amino acid sequences. The first papers on protein engineering date back to early 1980ies: in a review by Ulmer (1983), the prospects for protein engineering, such as X-ray crystallography, chemical DNA synthesis, computer modelling of protein structure and folding were discussed and the combination of crystal structure and protein chemistry information with artificial gene synthesis was emphasized as a powerful approach to obtain proteins with desirable properties (Ulmer, 1983). In a later review in 1992, protein engineering was mentioned as a highly promising technique within the frame of biocatalyst engineering to improve enzyme stability and efficiency in low water systems (Gupta, 1992). Today, owing to the development in recombinant DNA technology and high-throughput screening techniques, protein engineering methods and applications are becoming increasingly important and widespread. In this Chapter, a chronological review of protein engineering methods and applications is provided.

2. Protein engineering methods

Many different protein engineering methods are available today, owing to the rapid development in biological sciences, more specifically, recombinant DNA technology. These methods are chronologically reviewed in this section, and summarized in Table 1.

The most classical method in protein engineering is the so-called "rational design" approach which involves "site-directed mutagenesis" of proteins (Arnold, 1993). Site-directed mutagenesis allows introduction of specific amino acids into a target gene. There are two common methods for site-directed mutagenesis. One is called the "overlap extension" method. This method involves two primer pairs, where one primer of each primer pair contains the mutant codon with a mismatched sequence. These four primers are used in the first polymerase chain reaction (PCR), where two PCRs take place, and two double-stranded DNA products are obtained. Upon denaturation and annealing of them, two heteroduplexes are formed, and each strand of the heteroduplex involves the desired mutagenic codon. DNA polymerase is then used to fill in the overlapping 3′ and 5′ ends of each heteroduplex and the second PCR takes place using the nonmutated primer set to amplify the mutagenic

DNA. The other site-directed mutagenesis method is called "whole plasmid single round PCR". This method forms the basis of the commercial "QuikChange Site-Directed Mutagenesis Kit" from Stratagene. It requires two oligonucleotide primers with the desired mutation(s) which are complementary to the opposite strands of a double-stranded DNA plasmid template. Using DNA polymerase PCR takes place, and both strands of the template are replicated without displacing the primers and a mutated plasmid is obtained with breaks that do not overlap. *Dpn*I methylase is then used for selective digestion to obtain a circular, nicked vector with the mutant gene. Upon transformation of the nicked vector into competent cells, the nick in the DNA is repaired, and a circular, mutated plasmid is obtained (Antikainen & Martin, 2005).

Rational design is an effective approach when the structure and mechanism of the protein of interest are well-known. In many cases of protein engineering, however, there is limited amount of information on the structure and mechanisms of the protein of interest. Thus, the use of "evolutionary methods" that involve "random mutagenesis and selection" for the desired protein properties was introduced as an alternative approach. Application of random mutagenesis could be an effective method, particularly when there is limited information on protein structure and mechanism. The only requirement here is the availability of a suitable selection scheme that favours the desired protein properties (Arnold, 1993). A simple and common technique for random mutagenesis is "saturation mutagenesis". It involves the replacement of a single amino acid within a protein with each of the natural amino acids, and provides all possible variations at that site. "Localized or region-specific random mutagenesis" is another technique which is a combination of rational and random approaches of protein engineering. It includes the simultaneous replacement of a few amino acid residues in a specific region, to obtain proteins with new specificities. This technique also makes use of overlap extension, and the whole-plasmid, single round PCR mutagenesis, as in the case of site-directed mutagenesis. However, the major difference here is that the codons for the selected amino acids are randomized, such that a mixture of 64 different forward and 64 different reverse primers are used, based on a statistical mixture of four bases and three nucleotides in a randomized codon (Antikainen & Martin, 2005).

In 1994, important fields for protein engineering were also discussed in a review article by Anthonsen and co-workers (Anthonsen *et al*., 1994). The challenge in protein sequence deduction from DNA sequence, resulting from post-transcriptional and post-translational modifications and splicing, was emphasized. Homology modelling of protein structures, NMR of large proteins, molecular dynamics simulations of protein structures, and simulation of electrostatic effects (such as pH-dependent effects) were mentioned as important scientific areas to provide additional key information to protein engineering studies.

Another important method that finds applications in protein engineering is "peptidomimetics". It involves mimicking or blocking the activity of enzymes or natural peptides upon design and synthesis of peptide analogs that are metabolically stable. Peptidomimetics is an important approach for bioorganic and medical chemistry. It includes a variety of synthesis methods such as the use of a common intermediate, solid phase synthesis and combinatorial approaches (Venkatesan & Kim, 2002).

"*In vitro* protein evolution systems" are also important methods in protein engineering. They are based on the hierarchical evolution principle of genes. It was suggested that modern genes developed from small genetic units upon hierarchical and combinatorial processes. An example is MolCraft, an *in silico* evolved microgene which was then tandemly polymerized, including insertion or deletion mutations at the junctions between microgene units. The junctional perturbations allowed molecular diversity and the formation of combinatorial peptide polymers, whereas the repetitiousness allowed the formation of ordered structures (Shiba, 2004).

In a review article by Antikainen and Martin, (2005), the major protein engineering methods were described in detail. These methods were classified as rational methods that involve site-directed mutagenesis, random methods including random mutagenesis and evolutionary methods which involve "DNA shuffling". In DNA shuffling method, a group of genes with a double-stranded DNA and similar sequences is obtained from various organisms or produced by error-prone PCR. Digestion of these genes with *DNase*I yields randomly cleaved small fragments, which are purified and reassembled by PCR, using an error-prone and thermostable DNA polymerase. The fragments themselves are used as PCR primers, which align and cross-prime each other. Thus, a hybrid DNA with parts from different parent genes is obtained. Variations of DNA shuffling method such as the use of a mixture of restriction endonucleases instead of *DNase*I, or the "staggered extension process" that does not require parental gene fragmentation were also discussed (Antikainen & Martin, 2005). Additionally, the development of efficient screening methods to screen large libraries of proteins/enzymes such as "cell surface libraries coupled with fluorescence activated cell sorting (FACS)", or "phage display technology" were discussed (Antikainen & Martin, 2005). The combination of cell surface libraries with FACS can be used to screen very large libraries. The system is based on a scissile bond of the substrate, such as an Arg-Val linkage, which can be cleaved by a surface-displayed enzyme or not. The scissile bond on the designed substrate links a fluorophore and a quencher. If the scissile bond of the substrate is not cleaved by the surface-displayed enzyme, the fluorophore emission is then quenched by the quenching fluorophore. Thus, no fluorescence emission occurs. However, if the enzyme cleaves the scissile bond of the substrate, the fluorophore and the quenching fluorophore are then separated, and fluorescence occurs. Fluorescence of the clones with cleavage of the scissile bond is then detected by FACS (Antikainen & Martin, 2005). Phage display technology is another powerful technique for screening large libraries of proteins. The method requires degenerate reverse primers to be used in a PCR for random mutagenesis of the starting cDNA throughout a target region. The PCR products are then subcloned into a bacteriophage vector coding for a phage coat protein. Each phage of the mutant pool expresses a different protein displayed on the coat protein of the phage surface. Elution experiments help screen and identify the variants that bind tightly to a substrate of interest. Thus, the identified mutants are purified and sequenced (Antikainen & Martin, 2005).

"Flow cytometry", a powerful method for single cell analysis, is also used in protein engineering studies. A variety of examples are available where the sorting was done according to ligand binding in antibody and peptide surface display studies, or enzyme engineering of intra- and extracellular enzymes (Mattanovich & Borth, 2006).

The advantages and disadvantages of random mutagenesis methods used in protein engineering were also determined and compared to each other in detail. Based on the nucleotide substitution method used, these random mutagenesis methods were divided into four major groups: enzyme-based methods, synthetic chemistry-based methods, whole cell methods and combined methods. Their comparison was made according to a variety of parameters such as controllable mutation frequency, technical robustness, cost-effectiveness, etc. (Wong *et al*., 2006).

"Cell-free translation systems" were also described as important tools for protein engineering and production. They are an alternative to *in vivo* protein expression. When template DNA or mRNA is added to a reaction mixture, proteins are produced upon incubation in the absence of cells. PCR products can be used, and proteins are synthesized from cDNA rapidly. Cell-free translation systems are based on the ribosomal protein system of cells, which is provided as a cell extract from *Escherichia coli* etc. obtained as a supernatant upon centrifugation at 30'000 g. This supernatant contains necessary compounds for protein synthesis, such as ribosomes, t-RNAs, translation factors and aminoacyl-tRNA synthetases. Potential applications involve production of biologically active proteins, synthesis of membrane proteins for minimal cells, and artificial proteins. With further development, cell-free translation systems could be a strong alternative to *in vivo* protein expression, due to their high level of controllability and simplicity. The limitations of recombinant protein expression in living cells, such as protein degradation and aggregation will also be avoided (Shimizu *et al*., 2006).

Green fluorescent protein (GFP) is a very important protein that is widely used for biological and medical research purposes. It is a 238-residue protein from the jellyfish *Aequorea victoria*. GFP has unique spectroscopic characteristics, undergoes an autocatalytic post-translational cyclization and oxidation of the polypeptide chain around Ser65, Tyr66, and Gly67 residues, to form an extended and rigidly encapsulated conjugated Π system, the chromophore, that emits green fluorescence. Additionally, no cofactors are required for the formation or the function of the chromophore. GFP has high structural stability and high fluorescence quantum yield, which are other important properties for its widespread use. GFP has been modified extensively to be used as a marker for gene expression, protein localization and protein-protein interactions, as well as a biosensor. The proper folding of GFP is critical for its functional efficiency. Thus, protein engineering methods such as random mutagenesis and screening, DNA shuffling, as well as computational methods and X-ray crystallography improved the folding of GFP and emphasized the importance of the use of different methods such as biophysical techniques in improving protein properties (Jackson *et al*., 2006).

"Designed divergent evolution" is also an important protein engineering method that is used in redesigning enzyme function. The method is based on the theories of divergent molecular evolution. According to these theories, firstly, enzymes with more specialized and active functions have evolved from those enzymes with promiscuous functions. Secondly, this process is driven by a few amino acid substitutions; and finally, the effects of double/multiple mutations are usually additive. Thus, the method allows the selection of combinations of mutations that would confer the desired functions and their introduction into the enzymes (Yoshikuni & Keasling, 2007).

"Stimulus-responsive peptide systems" are based on both naturally existing peptides and rationally engineered systems. These systems exploit the fact that the peptides and proteins are able to change their conformations as a response to external stimulants such as pH, temperature or some specific molecules. There is a broad range of applications of these systems in research fields such as biosensors, bioseparations, drug delivery, nanodevices and tissue engineering. Directed evolution of stimulus-responsive peptides, however, requires an appropriate selection or screening scheme. Thus, protein-based conformational change sensors (CCSs) were developed using immunofluorescence and recombinant DNA technology (Chockalingam *et al.,* 2007).

Avidin and streptavidin are proteins that are structurally and functionally analogous. Because of their ability to bind biotin very tightly, they are widely used in (strept)avidin-biotin binding technology that is a common tool in life sciences and nanotechnology. To further improve these protein tools and obtain genetically engineered (strept)avidins, protein engineering methods were applied including simple amino acid substitutions to change physico-chemical properties, or more complex changes, such as chimeric (strept)avidins, topology rearrangements and non-natural amino acid stitching into the active sites (Laitinen *et al*., 2007).

"Receptor-based QSAR methods" are also valuable for protein engineering studies. These methods are based on a computational combination of structure-activity relationship analysis and receptor structure-based design. They provide valuable pharmacological information on therapeutic targets. The Comparative Binding Energy (COMBINE) analysis, for example, probes bioactivity changes with respect to amino acid variations in a series of homologous protein receptors and with respect to conformational changes within a protein of interest (Lushington *et al*., 2007).

As mentioned previously, phage display technology is one of the most commonly known molecular display technologies which relates phenotypes with their corresponding genotypes. Phage display technique is used particularly in "synthetic binding protein engineering", where libraries of 'synthetic' binding proteins were developed with antigen-binding sites constructed from man-made diversity. It was suggested that the combination of phage display and synthetic combinatorial libraries will be preferred for synthetic binding protein engineering (Sidhu & Koide, 2007). Similar to phage display technology, "yeast surface display" is also a useful method for protein engineering and characterization. Using this method, many different proteins can be displayed on yeast surface, and the yeast secretory biosynthetic system promotes efficient N-linked glycosylation and oxidative protein folding. Rapid and quantitative library screening by FACS analysis and easy characterization of mutants without requiring their soluble expression and purification are among the major advantages of this method. Yeast surface display has recently been suggested as an important methodology for protein characterization, and identifying protein-protein interactions (Gai & Wittrup, 2007). In a later review article, library creation methods and display technologies related with enzyme evolution and protein engineering were also discussed in detail (Chaput *et al*., 2008).

An interesting protein tool that was obtained by protein engineering methods is "anticalin". It offers a variety of applications in biochemical research as well as in medical therapy as

potential drugs. Anticalins are a combination of antibodies and lipocalins. Lipocalins are a protein family with a binding site that has high structural plasticity. By applying protein engineering methods such as site-directed random mutagenesis and selection by phage display technology, artificial lipocalins with novel ligand specificities, i.e. "anticalins" were obtained. Anticalins have many advantages such as being significantly smaller than antibodies, not requiring post-translational modifications, having robust biophysical properties and the ability to be produced in microbial expression systems (Skerra, 2008).

Method name	**Reference(s)**
Rational design	(Arnold, 1993)
Site-directed mutagenesis	(Arnold, 1993), (Antikainen & Martin, 2005)
Evolutionary methods/directed evolution	(Arnold, 1993)
Random mutagenesis	(Antikainen & Martin, 2005), (Wong *et al*., 2006), (Jackson *et al*., 2006), (Labrou, 2010)
DNA shuffling	(Antikainen & Martin, 2005), (Jackson *et al*., 2006)
Molecular dynamics	(Anthonsen *et al*., 1994)
Homology modeling	(Anthonsen *et al*., 1994)
'MolCraft'*in vitro* protein evolution systems	(Shiba, 2004)
Computational methods (computational protein design)	(Jackson *et al*., 2006), (Van der Sloot *et al*., 2009), (Golynskiy & Seelig, 2010)
Receptor-based QSAR methods	(Lushington *et al*., 2007)
NMR	(Anthonsen *et al*., 1994)
X-ray crystallography	(Jackson *et al*., 2006)
Peptidomimetics	(Venkatesan & Kim, 2002)
Phage display technology	(Antikainen & Martin, 2005), (Sidhu & Koide, 2007), (Chaput *et al*., 2008)
Cell surface display technology	(Antikainen & Martin, 2005), (Gai & Wittrup, 2007), (Chaput *et al*., 2008)
Flow cytometry / Cell sorting	(Mattanovich & Borth, 2006)
Cell-free translation systems	(Shimizu *et al*., 2006)
Designed divergent evolution	(Yoshikuni & Keasling, 2007)
Stimulus-responsive peptide systems	(Chockalingam *et al*., 2007)
Mechanical engineering of elastomeric proteins	(Li, 2008)
Engineering extracellular matrix variants	(Carson & Barker, 2009)
Traceless Staudinger ligation	(Tam & Raines, 2009)
De novo enzyme engineering	(Golynskiy & Seelig, 2010)
mRNA display	(Golynskiy & Seelig, 2010)

Table 1. A summary of different methods used in protein engineering

In a recent review by Goodey and Benkovic (2008), the allosteric regulation of proteins was discussed in detail. Ligand binding or an amino acid mutation at an allosteric site can significantly change enzymatic activity or binding affinity at another site such as the active site. Thus, this site-to-site communication of allosteric regulation is an important concept to be considered for protein engineering studies. Particularly, if the allosteric mechanisms are well understood, new proteins with switch-like properties could be designed for drug delivery, etc. (Goodey & Benkovic, 2008).

Recently, engineering of elastomeric proteins has been discussed as a new approach to improve the mechanical properties for the construction of biomaterials. Elastomeric proteins are important in regulating the mechanical properties in biological machineries. Using a combination of protein engineering methods and single molecule atomic force microscopy, the molecular basis of the mechanical stability of elastomeric proteins could be understood, and the mechanical properties of elastomeric proteins could be further improved by their 'mechanical engineering' (Li, 2008).

Protein and catalytic promiscuity are also important concepts for protein engineering. Catalytic promiscuity is defined as the ability of a single active site to catalyse more than one chemical reaction (Kazlauskas, 2005). Understanding protein and catalytic promiscuity is important for optimizing protein engineering applications (Nobeli *et al*., 2009).

In a recent review, the advances in mammalian cell and protein evolution were discussed, which would have important applications in commercial mammalian cell biotechnology. As mutagenesis and selection of mammalian cells is quite elaborate, the improvement of mammalian protein evolution systems would be crucial for obtaining new diagnostic tools and designer polypeptides (Majors *et al*., 2009).

Another recent concept in protein engineering research is the "engineering of extracellular matrix variants" to direct cell behaviour, particularly differentiation, as a response to biomaterials, in regenerative medicine applications. Extracellular matrix-derived peptides, such as Arg-Gly-Asp, are useful in supporting cell adhesion and specific integrin-signalling scaffolds and growth factor-receptor signalling are required for directing cell phenotype. Thus, by making use of this information, engineering of extracellular matrix variants could be a promising protein engineering approach (Carson & Barker, 2009).

Manipulation of proteins in a controlled way is a key requirement for many protein engineering studies. To facilitate that, "the traceless Staudinger ligation" method was recently introduced. It is based on the Staudinger reaction, where a phosphine is used to reduce an azide to an amide. The reaction occurs by means of a stable intermediate, an iminophosphorane, that has a nucleophilic nitrogen which can be acylated in inter- and intramolecular ligations. In peptide synthesis, the Staudinger reaction is applied by using a phosphinothiol for uniting an azide and a thioester. This method allows convergent chemical synthesis of proteins, and can ligate peptides at noncysteine residues. Thus, it overcomes a limitation of other strategies, and can be used as a powerful method for protein engineering (Tam & Raines, 2009).

In addition to the traditional methods of protein engineering, such as 'classical' rational design and directed-evolution methods, computational protein design tools are becoming

increasingly important. In a recent review by Van der Sloot *et al.* (2009), "computational protein design principles and applications" were discussed. Computational protein design principles are based on the combination of a force field and a search algorithm to identify the amino acid sequence that is most compatible with a given protein three-dimensional backbone structure. At selected positions, the computational protein design algorithm 'mutates' or changes the original amino acid to all other natural amino acids and results in new conformations. The energy of the structure is determined after simultaneous optimization of the side-chain and/or backbone conformations of the substituted amino acid and the interacting amino acids. Thus, low energy substitutions which are favorable are retained (Van der Sloot *et al*., 2009). Another recent review article on random mutagenesis methods used in protein engineering/enzyme evolution also discussed different methods such as "error-prone" PCR mutagenesis, chemical mutagenesis, rolling circle error-prone PCR, saturation mutagenesis and novel methodologies (Labrou, 2010). The potential of "*de novo* enzyme engineering" method was also emphasized recently (Golynskiy & Seelig, 2010). *De novo* means that the enzymes are not based on a related parent protein regarding substrate or reaction mechanism. Obtaining *de novo* enzymes from scratch has been possible by i) *in silico* rational design; ii) utilizing the understanding of a reaction mechanism and the diversity of the immune system by means of catalytic antibodies; and iii) empirical search of large protein libraries by using mRNA display. mRNA display is a powerful new technique that can select *de novo* proteins from libraries that are several orders of magnitude larger than most other selection methods such as phage display and cell surface display. The proteins obtained by mRNA display method are covalently attached to the mRNA encoding them. Thus, each protein becomes directly amplifiable. The key feature of this method is the presence of the antibiotic puromycin that mimics a charged tRNA. Thus, puromycin is added into the growing polypeptide chain by the ribosome. The transcription of a synthetic DNA library into mRNA and its modification with puromycin is usually followed by *in vitro* translation, where a covalent link is made between each protein and the mRNA encoding that protein. This step is followed by reverse transcription of the library of mRNA-displayed proteins with a substrate-modified primer, and attachment of the substrate to the cDNA/RNA/protein complex. Proteins catalyzing the substrate reaction change their encoding cDNA with the product, and selected cDNA sequences are amplified by PCR and used for the next selection step (Golynskiy & Seelig, 2010). The future of protein engineering will definitely involve many new technologies and combinational use of existing methods.

3. Protein engineering applications

A variety of protein engineering applications have been reported in the literature. These applications range from biocatalysis for food and industry to environmental, medical and nanobiotechnology applications (as summarized in Table 2), and will be discussed in this section.

3.1 Food and detergent industry applications

Early reports on the importance of protein engineering methods to design new enzymes for enzyme biotechnological industries date back to 1993 (Wiseman, 1993). Particularly, the enzymes used in food industry were emphasized as an important group of enzymes, the

industrially important properties of which could be further improved by protein engineering. Those properties include thermostability, specificity and catalytic efficiency. Additionally, the design and production of new enzymes for food industry by using protein engineering was discussed to produce new food ingredients (James & Simpson, 1996). In a later review, new application areas of enzymes were discussed, resulting from significant developments in biotechnology, such as protein engineering and directed evolution. Successful combinations of rational protein engineering with directed evolution (Voigt *et al*., 2000; Altamirano *et al*., 2000) have also been mentioned and it was emphasized that the combined use of rational design, directed evolution and the diversity of the nature would be much more powerful than the use of a single technique (Kirk *et al*., 2002).

Application name	**Example reference(s)**
Food industry applications	(James & Simpson, 1996), (Kirk *et al*., 2002), (Akoh *et al*., 2008)
Detergent industry applications (proteases)	(Gupta *et al*., 2002)
Environmental applications	(Wiseman, 1993), (Cirino & Arnold, 2002), (Le Borgne & Quintero, 2003), (Ayala *et al*., 2008), (Cao *et al*., 2009)
Medical applications	(Buckel, 1996), (Filpula & McGuire, 1999), (Paques & Duchateau, 2007), (Nuttall & Walsh, 2008), (Liu *et al*., 2009), (Lam *et al*., 2003), (Zafir-Lavie *et al*., 2007), (Vazquez *et al*., 2009), (Olafsen & Wu, 2010)
Biopolymer production applications	(Chow *et al*., 2008), (Rehm, 2010), (Banta *et al*., 2010)
Nanobiotechnology applications	(Hamada *et al*., 2004), (Banta *et al*., 2007) (Sarikaya *et al*., 2003) (Tamerler *et al*., 2010)
Applications with redox proteins and enzymes	(Saab-Rincon & Valderrama, 2009), (Kumar, 2010)
Applications with various industrially important enzymes	(Martinkova & Kren, 2010), (Clapes *et al*., 2010), (Jordan & Wagschal, 2010), (Rao *et al*., 2009), (Marcaida *et al*., 2010).
Other new applications	(Lofblom *et al*., 2010), (Elleuche & Poggeler, 2010), (Klug, 2010), (Guven *et al*., 2010),(Nagahara *et al*., 2009), (Henriques & Craik, 2010)

Table 2. A general summary of selected protein engineering applications

An important application area of protein engineering regarding food industry is the wheat gluten proteins. Their heterologous expression and protein engineering has been studied using a variety of expression systems, such as *E.coli*, yeasts or cultured insect cells. Wild-type and mutant wheat gluten proteins were produced to compare them to each other for protein structure-function studies. Generally, *E.coli* expression systems were suggested as suitable systems for many applications, because of their availability, rapid and easy use, as well as high expression levels (Tamas & Shewry, 2006). Food industry makes use of a variety of food-processing enzymes, such as amylases and lipases, the properties of which

are improved using recombinant DNA technology and protein engineering. The deletion of native genes encoding extracellular proteases, for example, increased enzyme production yields of microbial hosts. In fungi, for example, the production of toxic secondary metabolites has been reduced to improve their productivity as enzyme-producing hosts (Olempska-Beer *et al*., 2006).

Some large groups of enzymes like proteases, amylases and lipases are important for both food and detergent industries, as they have a broad range of industrial applications. Proteases, for example, are used in several applications of food industry regarding low allergenic infant formulas, milk clotting and flavors. They are also important for detergent industry for removing protein stains (Kirk *et al*., 2002). The improvement of proteases for industry to have, for example, high activity at alkaline pH and low temperatures, or improved stability at high temperatures is a challenge for protein engineering. Microbial protease production is industrially suitable because of low costs, high production yields, and easy genetic manipulation. Microbial protease genes have also been investigated for protein engineering of the enzymatic properties, clarifying the role of proteases in pathogenicity, as well as for overproduction purposes (Rao *et al*., 1998). There are some protein engineering applications to improve proteases: cold adaptation of a mesophilic subtilisin-like protease was performed using laboratory evolution (Wintrode *et al*., 2000); and DNA shuffling was applied to isolate new proteases with improved properties from an initial material of 26 subtilisin proteases (Ness *et al*., 1999).

Among different proteases, bacterial alkaline proteases are a commercially important group. They are particularly important for detergent industry and commercial products include subtilisin Carlsberg, subtilisin BPN and Savinase. The use of protein engineering techniques resulted in improvement of their catalytic efficiency, stability against high temperatures, oxidation and changes in washing conditions. Site directed mutagenesis and/or random mutagenesis resulted in new alkaline proteases, such as Durazym, Maxapem and Purafect, whereas new subtilisin products with improved stability and specificity were also obtained by directed evolution. The recent "metagenomic" approaches to discover natural and molecular diversities were also suggested as new technologies to isolate new microbial sources with better alkaline protease activities (Gupta *et al*., 2002). Among many bacteria, *Bacillus* species play an important role in microbial commercial enzyme production. The fact that some *Bacillus* species are classified as GRAS (generally regarded as safe) organisms, and have the ability to produce and secrete high amounts of extracellular enzymes, makes them valuable hosts for industrial enzyme production. Classical mutation and selection techniques, as well as protein engineering methods resulted in high-efficiency production of new enzymes with improved properties (Schallmey *et al*., 2004).

Amylases are also important for both food and detergent industries. In food industry, they are used for liquefaction and saccharification of starch, as well as in adjustment of flour and bread softness and volume in baking. The detergent industry makes use of amylases in removal of starch stains (Kirk *et al*., 2002). Recently, the production of "functional foods" is becoming increasingly important for food industry. Particularly, the production of industrial products and functional foods from cheap and renewable raw agricultural materials is desirable. Conversion of starch to bioethanol or to functional ingredients like fructose, wine, glucose and trehalose, for example, has been studied. Such a conversion

requires microbial fermentation in the presence of biocatalysts such as amylases to liquefy and saccharify starch. To improve the industrially important properties of amylases, such as high activity, high thermo- and pH-stability, high productivity, etc.; recombinant enzyme technology, protein engineering and enzyme immobilization have been used. In a recent review article, rice was given as a typical example for biocatalytical production of useful industrial products and functional foods from cheap agricultural raw materials and transgenic plants (Akoh *et al*., 2008).

Another major group of enzymes utilized by food and detergent industries is constituted by lipases. They are used in many applications of food industry such as for the stability and conditioning of dough (as an *in situ* emulsifier), and in cheese flavor applications. Lipases are also crucial for the detergent industry, as they are used in removal of lipid stains (Kirk *et al*., 2002). As lipases are commonly used in food industrial applications, having toxicologically safe lipases is an important requirement of food industry. The commercial lipase isoform mixtures prepared from C*andida rugosa* meet this requirement. Obtaining pure and different *C. rugosa* lipase isoforms is possible by means of computer modelling of lipase isoforms, and protein engineering methods such as lid swapping and DNA shuffling (Akoh *et al*., 2004). A recent review on microbial lipases focused on non-aqueous microbial lipase catalysis and major factors affecting esterification/transesterification processes in organic media. Additionally, protein engineering, directed evolution, metagenomics and application of these strategies on lipase catalysis were discussed (Verma *et al*., 2008). Similarly, lipases from other organisms such as mammals and fishes were also reviewed (Kurtovic *et al*., 2009).

3.2 Environmental applications

Environmental applications of enzyme and protein engineering are also another important field. Early reports on enzyme and cell applications in industry and in environmental monitoring, such as environmental biosensors, date back to 1993 (Wiseman, 1993). One year later, recent genetic methods and strategies for designing microorganisms to eliminate environmental pollutants were discussed in detail. Those methods and strategies included gene expression regulation to provide high catalytic activity under environmental stress conditions, such as the presence of a toxic compound, rational changes introduced in regulatory proteins that control catabolic activities, creation of new metabolic routes and combinations thereof etc. (Timmis *et al*., 1994).

In a later review in 2000, the importance of microbial strains and their enzymes in bioremediation and biotransformation applications was discussed, pointing out the utilization of modern strategies such as protein engineering or pathway engineering to improve microbial processes. Molybdenum hydroxylases, enzymes that catalyze the initial bacterial hydroxylation of a N-heteroaromatic compound, and ring-opening 2,4-dioxygenases that play a role in the bacterial quinaldine degradation, were investigated in detail, to study and improve the enzymes involved in aerobic bacterial degradation of N-heteroaromatic compounds (Fetzner, 2000). Protein engineering of oxygenases, an important group of enzymes with high selectivity and specificity, which enable the microbial utilization and biodegradation of organic, toxic compounds, was also discussed. The potential application of oxygenases in chemical synthesis and bioremediation was also

emphasized (Cirino & Arnold, 2002). Apart from oxygenases, other oxidative enzymes such as peroxidases and laccases are also important for the treatment of organic pollutants. These enzymes have broad substrate specificities and can catalyze the oxidation of a wide range of toxic organic compounds. Many organic pollutants such as phenols, azo dyes, organophosphorus pesticides and polycyclic aromatic hydrocarbons can be detoxified using enzymatic oxidation. However, there are some limitations of enzymatic treatment which should be overcome. These include enzyme denaturation by the use of organic solvents used in enzymatic reactions, inhibition/stabilization of enzyme-substrate complexes, low reaction rates of laccases, toxicity of mediators, high costs and limited availability of the enzymes, etc. Chemical modification or protein engineering of oxidative enzymes to have robust enzymes with high activity was suggested (Torres *et al*., 2003). Another review article published in 2004 focused on the environmental applications with enzymes, such as the use of enzymes in waste management and pollution control. Protein engineering, rational enzyme design and recombinant DNA technology were mentioned as important research areas that would influence environmental enzyme applications. Utilization of new technologies such as gene shuffling, high throughput screening, and nanotechnology was suggested as future prospects of environmental enzyme applications (Ahuja *et al*., 2004).

Petroleum biorefining is also an important environmental application area, where new biocatalysts are required. Protein engineering, isolation and study of new extremophilic microorganisms, genetic engineering developments are all promising advances to develop new biocatalysts for petroleum refining. Petroleum biorefining applications such as fuel biodesulfurization, denitrogenation of fuels, heavy metal removal, depolymerisation of asphaltenes, etc. were discussed (Le Borgne & Quintero, 2003).

Microbial bioplastics, or polyhydroxyalkanoates (PHAs), are also an important research area in environmental biotechnology. They are storage polymers produced by many bacteria and archea, and their properties are similar to those of petroleum-derived plastics. PHAs are, however, biodegradable and thus, environment-friendly. Thus, microbial large-scale and low-cost production of PHAs is a challenge for biotechnologists. PHAs are deposited in cells as water-soluble, cytoplasmic granules of nano-size. Protein engineering of polyester synthases and phasins, the two proteins involved in PHA polyester formation, and structural issues, respectively, was used to understand the genetics and biochemistry of PHA granule self-assembly. This information would also be used for medical applications involving biocompatible and biodegradable biomaterials (Rehm, 2006). The biogenesis of microbial polyhydroxyalkanoate granules, and protein engineering of polyester synthases and phasins to functionalize the polyester particle surface allowed microbial and biocatalytic production of particles with controlled size, polyester care composition and surface functionality. This would allow a platform technology for the production of tailor-made bioparticles, particularly for medical applications (Rehm, 2007).

In a recent review, microbial surface display applications for environmental bioremediation and biofuels production were discussed. Yeast and bacterial cell systems where proteins or peptides are expected on the cell exterior were reported to be used as biocatalysts, biosorbents and biostimulants (Wu *et al*., 2008).

Another important environmental application of protein engineering involves fungal enzymes. Particularly peroxidases isolated from fungi can transform xenobiotics and many

pollutants. For the development of applications, the enzyme stability and availability need to be improved. Thus, many protein engineering strategies were identified such as improvement of hydrogen peroxide stability, increasing the redox potential to broaden the substrate range, heterologous expression and industrial production development (Ayala *et al*., 2008).

In recent reviews on environmental applications of protein engineering, recent 'omics' technologies have also been discussed. Metagenomic libraries, which identify and analyze genetic resources of complex microbial communities were suggested to help identify microbial enzymatic diversity, with implications in medicine, environmental issues, agriculture etc. Thus, contributions in renewable energy sources, decrease in pollutant burdens and process energies were expected with metagenomics applications in the future (Ferrer *et al*., 2009). Similarly, in a review on biodegradation of aromatic compounds such as benzene, toluene, ethylbenzene and xylene, the importance of metabolic engineering, protein engineering, and "omics" technologies were emphasized (Cao *et al*., 2009).

3.3 Medical applications

Medical applications of protein engineering are also diverse. The use of protein engineering for cancer treatment studies is a major area of interest. Pretargeted radioimmunotherapy has been discussed as a potential cancer treatment. By pretargeting, radiation toxicity is minimized by separating the rapidly cleared radionuclide and the long-circulating antibody. Advances in protein engineering and recombinant DNA technology were expected to increase the use of pretargeted radioimmunotherapy (Lam *et al*., 2003). The use of novel antibodies as anticancer agents is also an important field of application, where the ability of antibodies to select antigens specifically and with high affinity is exploited, and protein engineering methods are used to modify antibodies to target cancer cells for clinical applications (Zafir-Lavie *et al*., 2007). Recently, the term "modular protein engineering" has been introduced for emerging cancer therapies. Treatment strategies based on targeted nanoconjugates to be specifically directed against target cells are becoming increasingly important. Additionally, multifunctional and smart drug vehicles can be produced at the nanoscale, by protein engineering. These strategies could be combined to identify and select targets for protein-based drug delivery (Vazquez *et al*., 2009).

Protein engineering applications for therapeutic protein production is an important area, particularly for medicine. In 1996, recombinant protein production for therapeutic purposes was reviewed. It was stated that protein engineering resulted in a second generation of therapeutic protein products with application-specific properties obtained by mutation, deletion of fusion. The third generation of such products were mentioned as "gene therapy" protein products to be produced by the patients, upon gene transfer (Buckel, 1996). Other studies on therapeutic protein production include single-chain Fv designs for protein, cell and gene therapy (Filpula & McGuire, 1999). DNA shuffling and recursive genetic recombination studies to improve therapeutic proteins (Kurtzman *et al*., 2001); development of secreted proteins such as insulin, interferon, erythropoietin as biotherapeutics agents (Bonin-Debs *et al*., 2004), combinatorial protein biochemistry for therapeutics and proteomics (Lowe & Jermutus, 2004), meganucleases and DNA double-strand break-induced recombination for gene therapy (Paques & Duchateau, 2007), the use of protein cationization techniques for future drug discovery and development (Futami *et al*., 2007),

protein display scaffolds for protein engineering of new therapeutics (Nuttall & Walsh, 2008), and polymer-based therapeutics for drug delivery and tissue regeneration (Liu *et al*., 2009).

Protein engineering applications with antibodies are also diverse. Owing to advances in recombinant DNA technology, "antibody engineering" is possible. Improvements such as minimal recognition units and antigenized antibodies were described. Combinational approaches such as bacteriophage display libraries have been introduced as a strong alternative to hybridoma technology for antibody production with desired antigen binding characteristics (Sandhu, 1992). Studies on genetic manipulation of mouse monoclonals for producing humanized antibodies and bacteriophage display libraries for Ig repertoires have been reported (Zaccolo & Malavasi, 1993). Phage display has become a powerful technique in protein engineering, immunology, oncology, etc. Phage display of antibody fragments, particularly the production of artificial epitopes by phage antibodies is an important application (Pini & Bracci, 2000). "Antibody modeling" studies to engineer antibody-like molecules and increase their stability and specificity are also common, particularly for humanization of antibodies of animal origin (Morea *et al*., 2000). Recently, the use of antibodies as vectors for molecular imaging has become popular. Pharmacokinetic properties of antibodies have been improved by protein engineering and antibody variants of different size and antigen binding sites have been produced for the ultimate use as imaging probes specific to target tissues. A variety of examples include antibody fragments which have been conjugated to bioluminescence, fluorescence, quantum dots for optical imaging, as well as iron oxide nanoparticles for magnetic resonance imaging. It is obvious that molecular imaging tools based on antibodies will find more applications in the future regarding diagnosis and treatment of cancer and other complex diseases (Olafsen & Wu, 2010).

3.4 Applications for biopolymer production

Protein engineering applications for biopolymer production are also promising. Particularly, peptides are becoming increasingly important as biomaterials because of their specific physical, chemical and biological properties. Protein engineering and macromolecular self-assembly are utilized to produce peptide-based biomaterials, such as elastin-like polypeptides, silk-like polymers, etc. (Chow *et al*., 2008). Similarly, biosynthesis, modification and applications of bacterial polymers have also been discussed recently (Rehm, 2010).

The ability of protein engineering to create and improve protein domains can be utilized for producing new biomaterials for medical and engineering applications. One such example is the use of protein engineering to make new protein and peptide domains which enable advanced functional hydrogel formation. These domains include leucine zipper coiled-coil domains, the EF-band domains and elastin-like polypeptides (Banta *et al*., 2010).

3.5 Nanobiotechnology applications

Nanobiotechnology applications of protein engineering are becoming increasingly important. The synthesis and assembly of nanotechnological systems into functional structures and devices has been difficult and limiting their potential applications for a long

time. However, when biomaterials are investigated, it can be realized that they are highly organized from molecular to the nano- and macroscales, hierarchically. Biological macromolecules, such as proteins, carbohydrates and lipids are used in the synthesis of biological tissues in aqueous environments and mild physiological conditions, where this biosynthetic process is under genetic regulation. Particularly proteins are crucial elements of biological systems, based on their roles in transport, regulation of tissue formation, physical performance and biological functions. Thus, they are suitable components for controlled synthesis and assembly of nanotechnological systems. Combinatorial biology methods commonly applied in protein engineering studies, such as phage display and bacterial cell surface display technologies, are also used to select polypeptide sequences which selectively bind to inorganic compound surfaces, for ultimate applications of nanobiotechnology. Biopanning procedures that involve washing cycles of the phages or the cells to remove nonbinders from the surface reveal individual clones that strongly bind to a given inorganic surface. Those clones are then sequenced to identify the amino acid sequences of the polypeptides which bind strongly to the inorganic target compound surface, such as (noble) metals, semiconducting oxides and other important compounds for nanotechnology. The so-called "genetically engineered proteins for inorganics" (GEPIs) were suggested as important tools for the self-assembly of molecular systems in nanobiotechnology (Sarikaya *et al*., 2003). Since then, many genetically engineered peptides have been selected that specifically bind a variety of inorganic materials such as platinum, gold, and quartz; and their binding characteristics were investigated (Seker *et al*., 2009; Oren *et al*., 2010). Combining experimental approaches with computational tools allows engineering of the peptide binding and assembly characteristics. Thus, higher generation function-specific peptides can be obtained for applications in tissue engineering, therapeutics, and nanotechnology where inorganic, organic and biological materials are used (Tamerler *et al*., 2010). Engineering protein and peptide building blocks to be used as molecular motors, transducers, biosensors, and structural elements of nanodevices, and the importance of proteins and peptides for the development of biocompatible nanomaterials, as well as the impact of computational techniques in this field have been well recognized (Banta *et al*., 2007).

Another interesting nanotechnology application is the use of amyloid fibrils as structural templates for nanowire construction. This application is based on the fact that some proteins form well-ordered fibrillar aggregates that are called amyloid fibrils. As the self-organization and assembly of small molecules are crucial for nanotechnology, the self-association of well-ordered growth fibrils through noncovalent bonds under controlled conditions was suggested to have a high potential to be used for nanobiotechnology. The use of amyloid fibrils as structural templates for nanowire construction was explained as a typical example of potential applications (Hamada *et al*., 2004).

3.6 Applications with redox proteins and enzymes

Improvement of redox proteins and enzymes by protein engineering is also an important application field. Such proteins and enzymes can be modified to be used in nanodevices for biosensing, as well as for nanobiotechnology applications (Gilardi & Fantuzzi, 2001). The electrochemistry of redox proteins particularly draws attention for applications in biofuel cells, chemical synthesis and biosensors. Thus, protein engineering applications using rational design, directed evolution and combination thereof are found for bioelectrocatalysis

(Wong & Schwaneberg, 2003). A recent review on protein engineering of redox-active enzymes pointed out two emerging areas of protein engineering of redox-active enzymes: novel nucleic acid-based catalyst construction, and intra-molecular electron transfer network remodelling (Saab-Rincon & Valderrama, 2009). A variety of studies focused on cytochrome P450 superfamily of enzymes, such as heme monooxygenases which are involved in biosynthesis and biodegradation of metabolic compounds and in the oxidation of xenobiotics. Thus, protein engineering of P450 enzymes for degradation of xenobiotics is a biotechnological challenge (Wong *et al*., 1997). Additionally, the fact that heterologous expression of P450s in bacteria resulted in blue pigment formation required detailed studies of intermediary metabolism, toxicology, further protein engineering studies and suggested potential applications in dye industry (Gillam & Guengerich, 2001). More recently, review articles were published on cytochrome P450 monooxygenases (Urlacher & Eiben, 2006) and protein engineering of cytochrome P450 biocatalysts for medical, biotechnological and bioremediation applications (Kumar, 2010).

3.7 Applications with various industrially important enzymes

Protein engineering applications with a variety of industrially important enzymes can be found in the literature. These include nitrilases (Martinkova & Kren, 2010), aldolases (Clapes *et al*., 2010), microbial beta-D-xylosidases (Jordan & Wagschal, 2010) etc. Nitrilases are important enzymes for biotransformation, but the enzymatic reactions require improvement for higher industrial process efficiencies. For this purpose, new enzymes were screened from new isolates, medium and protein engineering methods were applied (Martinkova & Kren, 2010). Aldolases are also important enzymes for stereoselective synthesis reactions regarding carbon-carbon bond formation in synthetic organic chemistry. Protein engineering or screening methods improved aldolases for such synthesis reactions. *De novo* computational design of aldolases, aldolase ribozymes etc. are promising applications (Clapes *et al*., 2010). Microbial beta-D-xylosidases are also an industrially important group of enzymes, particularly for baking industry, animal feeding, D-xylose production for xylitol manufacturing and deinking of recycled paper. As they catalyse hydrolysis of non-reducing end xylose residues from xylooligosaccharides, they could be used for the hydrolysis of lignocellulosic biomass in biofuel fermentations to produce ethanol and butanol. Thus, improving the catalytic efficiency of beta-D-xylosidases is crucial for many industrial applications (Jordan & Wagschal, 2010). As the use of organic solvents is industrially suitable for enzymatic reactions, but has adverse effects on enzyme activity and/or stability, protein engineering of organic solvent tolerant enzymes (Gupta, 1992; Doukyu & Ogino, 2010) has become an important research area. Screening organic solvent-tolerant bacteria or extremophiles has been preferred to isolate and improve naturally solvent-stable enzymes (Gupta & Khare, 2009; Doukyu & Ogino, 2010). Other protein engineering examples with industrially and/or pharmacologically important enzymes include studies on cholesterol oxidase (Pollegioni *et al*., 2009), cyclodextrin glucanotransferases (Leemhuis *et al*., 2010), human butyrylcholinesterase (Masson *et al*., 2009), microbial glucoamylases (Kumar & Satyanarayana, 2009), lipases of different origins (Akoh *et al*., 2004; Verma *et al*., 2008; Kurtovic *et al*., 2009), phospholipases (Song *et al*., 2005; De Maria *et al*., 2007; Simockova & Griac, 2009) and phytases (Rao *et al*., 2009). Studies on extremozymes, enzymes isolated

from extremophilic species, revealed their different structural and functional characteristics which could be exploited for biotechnological applications and improved further by protein engineering (Bjarnason *et al*., 1993; Hough & Danson, 1999; Georlette *et al*., 2004). Homing endonucleases are another important group of enzymes with application potential in gene therapy of monogenic diseases. They are double-stranded *DNases* with extremely rare recognition sites, and are used as templates for engineering genetic tools to cleave DNA sequences different from the wild-type targets (Marcaida *et al*., 2010).

3.8 Other new applications

Recently, novel types of proteins have been developed, using combinatorial protein engineering techniques. These binding proteins of non-Ig origin are called "affibody binding proteins". With their high affinity, these proteins have been used in many different applications such as diagnostics, bioseparation, functional inhibition, viral targeting, and *in vivo* tumor imaging or therapy (Nygren, 2008). More recently, comprehensive reviews on engineered affinity proteins (Gronwall & Stahl, 2009), and affibody molecules (Lofblom *et al*., 2010) were published, where their therapeutic, diagnostic and biotechnological applications were discussed in detail.

Inteins are protein splicing elements that are involved in a variety of applications such as protein purification, protein semisynthesis, *in vivo* and *in vitro* protein modifications. The use of intein tags for protein purification in plants with high protein production could potentially enable industrial production of pharmaceutically important proteins (Evans *et al*., 2005). The proteolytic cleavage and ligation activities of inteins have been understood, which resulted in novel intein applications in protein engineering, enzymology, microarray production, target detection and transgene activation in plants. The conversion of inteins into molecular switches was introduced by intein-mediated protein attachment to solid supports for microarray and western blot studies and by linking nucleic acids to proteins and controlled splicing (Perler, 2005). Recent intein-mediated protein engineering applications like protein purification, ligation, cyclization and selenoprotein production have been discussed in detail lately (Elleuche & Poggeler, 2010).

"Insertional protein engineering" applications are also becoming important, particularly for biosensor studies. The applications of insertional protein engineering for analytical molecular sensing have been reviewed by Ferraz and coworkers (Ferraz *et al*., 2006).

"Zinc finger protein engineering" is another approach that has been used in gene regulation applications. The zinc finger design and principle is used to design DNA binding proteins to control gene expression. Examples include a three-finger protein to block the expression of an oncogene that was transformed into a mouse cell line. Fusion of zinc finger peptides to repression or activation domains allows selective gene switching off and on (Klug, 2010).

Applications of protein engineering in enzymatic biofuel cell design is also becoming increasingly important. Particularly, obtaining biofuels from lignocellulosic resources is a challenge, as the enzyme hydrolysis efficiency of lignocellulose is low which increases the costs of biofuels. Thus, protein engineering methods have been used to improve the performance of lignocellulose-degrading enzymes, and biofuels-synthesizing enzymes (Wen *et al*., 2009). Protein engineering is also applied to obtain an efficient electrical

communication between biocatalyst(s) and the electrode by rational design and directed evolution, within the frame of biocatalyst engineering (Guven *et al*., 2010).

"Virus engineering" is another emerging field, where the virus particles are modified by protein engineering. Viruses have many promising applications in medicine, biotechnology and nanotechnology. They could be used as new vaccines, gene therapy and targeted drug delivery vectors, molecular imaging agents and as building blocks for electronic nanodevices or nanomaterials construction. Thus, the improvement of the physical stability of viral particles is crucial for efficient applications with them. Protein engineering methods are employed to improve physical stability of viral particles (Mateu, 2011).

"Protein cysteine modifications" are also important protein engineering applications. As cysteine modifications in proteins cause diversities in protein functions, cysteine thiol chemistry has been applied for *in vitro* glycoprotein synthesis. This method could be potentially used for development of new protein-based drugs, improving their half-life, reducing their toxicity and preventing multidrug resistance development (Nagahara *et al*., 2009).

Cyclotides are important proteins that have recently been popular for protein engineering applications. They are plant proteins made up from small disulfide-rich peptides and are exceptionally stable to thermal, chemical or enzymatic degradation. This property of cyclotides makes them valuable molecular templates for many protein engineering and drug design applications (Craik *et al*., 2007; Daly *et al*., 2009; Henriques & Craik, 2010).

4. Conclusion

The modification of natural enzymes and proteins by protein engineering is an increasingly important scientific field. The well-known methods of rational design and directed evolution, as well as new techniques will enable efficient and easy modification of proteins. New technologies such as computational design, catalytic antibodies and mRNA display would be crucial for *de novo* engineering of enzymes and also for new areas of protein engineering.

Protein engineering applications cover a broad range, including biocatalysis for food and industry, as well as medical, environmental and nanobiotechnological applications. With advances in recombinant DNA technology tools, "omics" technologies and high-throughput screening facilities, improved methods for protein engineering will be available, which would enable easy modification or improvement of more proteins/enzymes for further specific applications.

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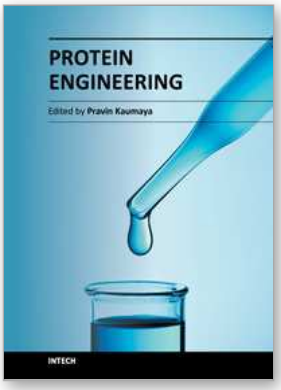

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A broad range of topics are covered by providing a solid foundation in protein engineering and supplies readers with knowledge essential to the design and production of proteins. This volume presents in-depth discussions of various methods for protein engineering featuring contributions from leading experts from different counties. A broad series of articles covering significant aspects of methods and applications in the design of novel proteins with different functions are presented. These include the use of non-natural amino acids, bioinformatics, molecular evolution, protein folding and structure-functional insight to develop useful proteins with enhanced properties.

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