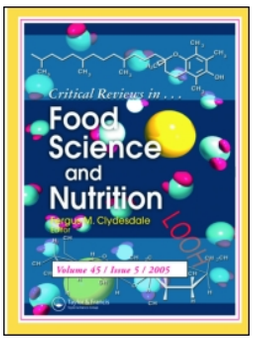

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Protein engineering and its applications in food industry

Swati Kapoor, Aasima Rafiq, and Savita Sharma

Department of Food Science and Technology, Punjab Agricultural University, Ludhiana, India

ABSTRACT

Protein engineering is a young discipline that has been branched out from the field of genetic engineering. Protein engineering is based on the available knowledge about the proteins structure/function(s), tools/instruments, software, bioinformatics database, available cloned gene, knowledge about available protein, vectors, recombinant strains and other materials that could lead to change in the protein backbone. Protein produced properly from genetic engineering process means a protein that is able to fold correctly and to do particular function(s) efficiently even after being subjected to engineering practices. Protein is modified through its gene or chemically. However, modification of protein through gene is easier. There is no specific limitation of Protein Engineering tools; any technique that can lead to change the protein constituent of amino acid and result in the modification of protein structure/function is in the frame of Protein Engineering. Meanwhile, there are some common tools used to reach a specific target. More active industrial and pharmaceutical based proteins have been invented by the field of Protein Engineering to introduce new function as well as to change its interaction with surrounding environment. 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. Successful combinations of various protein engineering methods had led to successful results in food industries and have created a scope to maintain the quality of finished product after processing.

KEYWORDS

Protein engineering; modification; stability; food applications

1. Introduction

Proteins are macromolecules, which participate in every process of different cells. Proteins work together to achieve a particular function, and they often associate to form stable complexes (Anthea et al., 1993). Insulin was the first protein to be sequenced. Insulin sequence has been solved by using protein amino acids sequencing rather than using insulin-related genes (Amara, 2013) explaining the potential use of protein engineering technique to fabricate novel products. Proteins are engineered to improve its quality in particular applications such as catalytic activities, stability, and selectivity. Protein engineering is a technique to change the amino acid sequence of proteins in order to improve their specific properties (Brussels, 2009). It is based on the use of recombinant DNA technology to change amino acid sequences.

Protein engineering has a very important role in Food industry. Protein engineering (PE) of enzymes is a faster, more controlled, more targeted, and more accurate method to optimize the properties of enzymes for a specific industrial application than the traditional method. Another important backbone for Protein engineering is organisms from extreme environments with significantly different properties and flourished the protein databases with amazing structures. These can be modified and their applications can be made in paper industry, detergent, drugs, degradation of different wastes, textile, food, pharmaceutical, leather, degumming of silk goods, manufacturing of liquid glue, cosmetics, meat tenderization, cheese production, growth promoters etc. PE can be used to increase the catalytic action of various enzymes like cellulases, can be used for increasing the production of enzymes and other important metabolites using micro-organisms.

Conventional technique such as recombinant DNA technology have been widely used in to cater the needs of food industry such as enhancement of food flavors, colors, and other improvements in food functionality but is often a time consuming and multi-step procedure of cloning. The process requires at least two to three days for the initial isolation of an organism, followed by the requirement for several days of additional confirmatory testing. However, the emerging concept of protein engineering has the potential to overcome these disadvantages by reducing the time required to fabricate novel functional proteins by faster screening methods.

All proteins, including enzymes, are based on the same 20 different amino acid building blocks arranged in different sequences. Enzyme proteins typically comprise sequences of several hundred amino acids folded in a unique three-dimensional structure. Only the sequence of these 20 building blocks determines the three-dimensional structure, which in turn determines all properties such as catalytic activity, specificity and stability. Engineering protein is a sensitive and systematic process that can get a breakdown if any mistake is committed. In case of unwanted addition or loss of one or more base pair

CONTACT Aasima Rafiq aasima20@gmail.com Department of Food Science and Technology, Punjab Agricultural University, Ludhiana, India.
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during the sequencing process or stepwise analysis will lead to DNA frame shift, causing a change in its amino acids sequences leading to undesirable functions in proteins (Amara, 2013). The properties of enzymes used for industrial purposes also require some adaptations in order to function more effectively in applications for which they were not designed by nature. Traditionally, such enzyme optimization is performed by screening naturally occurring microorganisms, followed by classical mutation and selection. The disadvantage of this method is that it takes a very long time until the enzyme with the desired properties is found.

Protein engineering of enzymes is a faster, more controlled, more targeted and more accurate method to optimize the properties of enzymes for a specific industrial application than the traditional method. Organisms from extreme environments are becoming an important source of new backbones for engineering proteins with significantly different properties and flourished the protein databases with amazing structures (Van den Burg, 2003). The use of amylases in food processing industry is an ideal example of protein engineering technology (Rubingh, 1997).

2. Protein engineering methods

Protein engineering is the design and construction of novel proteins, usually done by manipulation of their genes and is used to create enzymes with the desired properties. Protein engineering methods comprise three main strategies: Rational design, Directed evolution and Semi rational design (site saturation mutagenesis) that is the combination of both methods (Ordu and Karaguler, 2012). The choice of method used depends on the property of interest to be enhanced and on the existing structural and mechanistic knowledge along with practical considerations, such as the availability of a high-throughput screening or selection system (Singh et al., 2013). However, some of the other methods used in protein engineering include random mutagenesis (Antikainen and Martin, 2005; Labrou, 2010), DNA shuffling (Jackson et al., 2006), Molecular dynamics and homology modeling (Anthonsen et al., 1994), Peptidomimetics (Venkatesan and Kim, 2002), Phage display technology (Chaput et al., 2008; Sidhu and Koide, 2007), Cell surface display technology (Gai and Wittrup, 2007; Chaput et al., 2008), Flow cytometry/Cell sorting (Mattanovich and Borth, 2006), *De novo* enzyme engineering (Golynskiy and Seelig, 2010).

(i) Rational design

Rational design was the earliest approach to protein engineering. The strategy is based on relating structure to function, frequently via molecular modeling techniques. Rational design (computational design of proteins) requires the amino acid sequence, 3D structure, and function knowledge of the protein of interest (Fig. 1). This method provides controllable amino acid sequence changes such as insertion, deletion, or substitution. Controlled changes are necessary to determine the effect of individual change on the protein structure, folding, stability, or function.

The first step in rational design is the development of a molecular model by using an appropriate algorithm. X-ray crystallography provides a three-dimensional structure which can be presented graphically and mathematically on computer. 3D modeling is based on relating a structure to the possible expected functions of amino acids residues (homology modeling) and replacing them with other functions (molecular dynamics) in order to change their properties (Visegràdy et al., 2001). The computer model allows predictions to be made, for

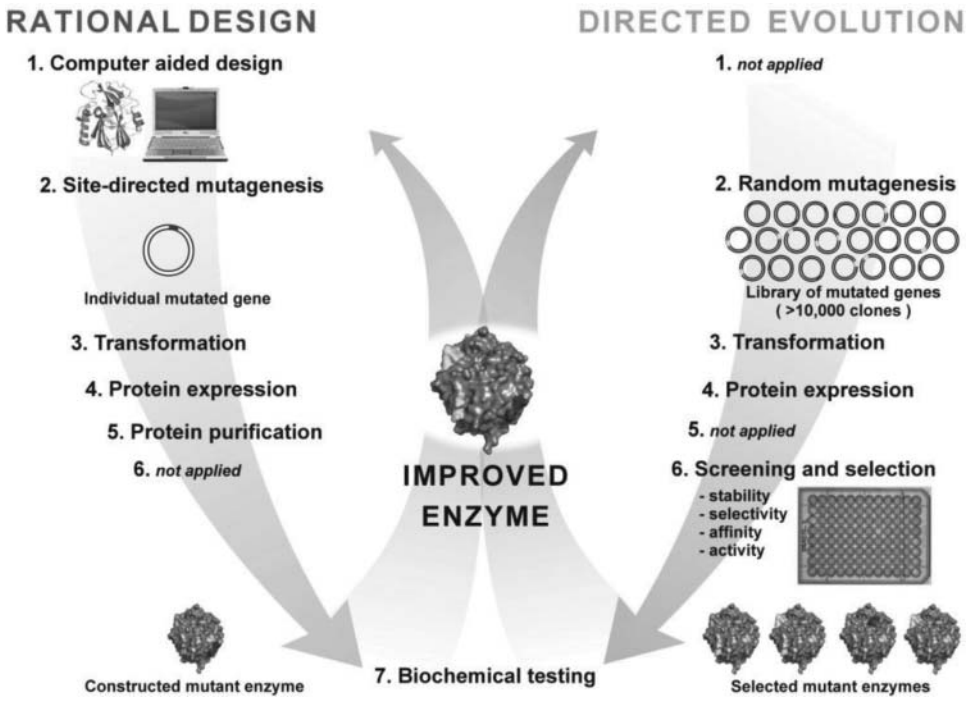

Figure 1. Rational design vs. Directed evolution design used for Protein engineering (Damborsky, 2007).

the effect of mutations on structure-based properties (Rubingh and Grayling, 1996). This is followed by experimental construction and analysis of the properties of the designed protein. Mechanisms for altering these properties include manipulation of the primary structure. Just a single point mutation may cause significant structural or functional changes in the protein.

There are many rational strategies to change protein characteristics such as introducing disulfide bridges, optimization of electrostatic interactions, improved core packing, shorter and/or tighter surface loops etc. The essential component of rational design is the ability to make variants of the native protein by recombinant rDNA techniques. The rDNA method of choice is site-directed mutagenesis in which one amino acid at a particular location is replaced with another. In this technique, mutations are created at computationally defined sites in the gene sequence via PCR using primers containing nucleic acids changes which correspond to the desired amino acid changes (Willemsen et al., 2008). Once made and purified, the new protein is evaluated to see if the desired property is achieved. If the desired property is not achieved, the information obtained in the evaluation is used in a second round of crystallography, modeling and mutagenesis.

(ii) Directed evolution

Directed evolution, also called molecular evolution, sexual PCR, and *in vitro* evolution. It is the technique of preparing protein variants by recombining gene fragments *in vitro*, using PCR or DNA shuffling (Stemmer et al., 1995), expressing the protein and then selecting or screening for those with improved properties (Fig. 1). The main advantage with directed evolution (*in vitro* evolution or random mutagenesis) technique is that it does not require any knowledge about sequence, structure or function of proteins. Directed evolution requires two essential steps; one is the generation of random genetic libraries and the other one is screening and selection of variant enzymes that possess the desired characteristics, for example increased catalytic activity, enhanced selectivity or improved stability. In order to select a target protein from a large pool of mutant proteins, an efficient screening strategy, such as high-throughput solid phase digital imaging, phage display and other different screening techniques, is the most important requirement for the success of this method. The disadvantage of this method is the time-consuming process of screening and the selection of desired mutants and generally it requires robotic equipment to screen large libraries of enzyme variants (Turner, 2003). Physical linkage between nucleic acid and protein is essential during protein engineering via directed evolution, thus, genotype and phenotype need to be coupled. It also requires high throughput screening and protein needs to be "displayed" in order to be assayed (i.e. tested). Difference between rational design and directed evolution has been explained in Fig. 1. Rational approach requires knowledge of protein structure to design mutants which are then prepared by site-directed mutagenesis. Thereafter, the protein is expressed after transformation in host organism and is purified and tested for desired characteristics. However, in directed evolution technique large library of mutant genes is prepared followed by transformation and expression. The mutants are then screened for desired properties and selected mutants are tested biochemically for confirmation of enhanced properties (Chen, 2001).

Screening techniques

(a) Phage display. It is the most widely used system for screening libraries produced during directed evolution technique. Filamentous phage is a virus that infects bacteria. Through recombinant technology, peptides or protein domains are fused to the gene III protein (gIIIp) of filamentous phage. Virus expresses the foreign protein on the surface (Fig. 2). Once the protein is displayed in the host, it can be tested for its activity, e.g. binding affinity, catalysis etc (Deperthes, 2002).

(b) Fluorescence assisted cell sorting (FACS). Fluorescence assisted cell sorting consists of cells or beads with proteins on the surface. Individual cells are fluorescently labeled using antibody. Substrate binding correlates with increased fluorescence i.e.—with increase in substrate binding, fluorescence increases proportionally. Laser is used that can inspect individual cells at high speed (>1,000 cells/sec) and sort them based on a combination of color and intensity. Sorted cells represent an "enriched" population and the average affinity for the substrate is higher compared to the presort population (Fig. 3). It works with bacteria, yeast, and mammalian cells, but not with phage (Bessette et al., 2004).

(iii) Semi-rational design

To overcome the time consuming screening and selection process of directed evolution and the necessity of amino acid sequence and three-dimensional information for rational design, a new approach has been developed. A combination of both strategies represents the new route to improve the properties and function of an enzyme (Bommarius et al., 2006). With

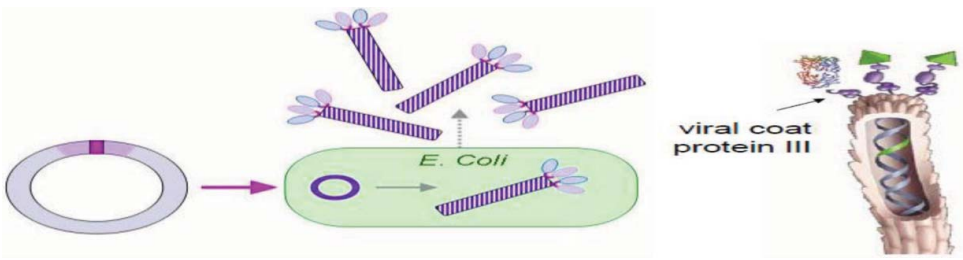

Figure 2. Phage display technique (Deperthes, 2002).

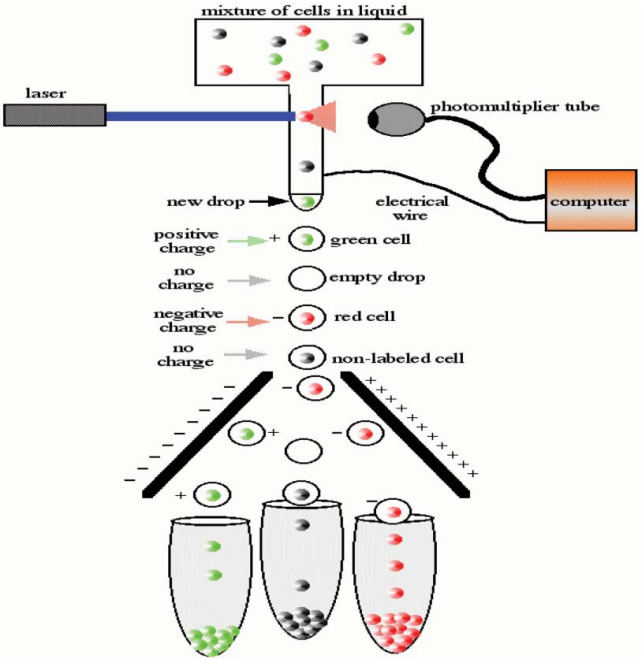

Figure 3. Use of FACS technique to screen mutant libraries (Bessette et al., 2004).

saturation mutagenesis, it is possible to create a library of mutants containing all possible combination of 22 different amino acids at one or more predetermined target positions in a gene. Saturation mutagenesis is an *in vitro* mutagenesis strategy wherein one tries to generate all (or most) possible mutations within a narrow region of a gene. Choice of the correct mutagenesis, positions that can be responsible for desired changes is determined by homology modeling which requires 3D information (Lehmann and Wyss, 2001).

Engineering method should be selected on the basis of the structural and mechanistic information and the feasibility of a high-throughput screening (HTS) system for screening or selection.

3. Protein engineering and immobilization

Enzymes found in nature have been exploited in industry due to their inherent catalytic properties in complex chemical processes under mild experimental and environmental conditions. Soluble enzymes are often immobilized onto solid insoluble supports to be reused in continuous processes and to facilitate the economical recovery of the enzyme after the reaction without any significant loss to its biochemical properties (Singh et al., 2013). Immobilization has been defined as "the biomolecules that are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" (Brena and Batista-Viera, 2006). Enzyme immobilization is a progressing field in industrial applications owing to its multitude functions such as reuse of enzymes for the same reaction, longer half lives, less degradation and prevention of substrate contamination with enzymes or other compounds (Abdelmajeed et al., 2012). Immobilization confers considerable stability towards temperature variations and organic solvents. However, recent developments in protein engineering have revolutionized the development of commercially available enzymes into better industrial catalysts than provided by immobilization. Protein engineering and immobilization techniques are sequential and compatible approaches for the improvement of enzyme properties. Enzymes are immobilized to enhance its stability in many industrial processes and immobilized enzymes can be easily separated from reaction mixture and used again, thus, leading to economy of the process.

Three most common methods of immobilization are—adsorption, entrapment, and crosslinking or covalently binding to a support. Proteins are immobilized either by physical adsorption to the surface of the nanoparticle or by covalent bonding to previously functionalized nanoparticles. Physical adsorption and covalent binding both reduce or avoid enzyme leaching, but binding to a planar surface can lead to decreased stability or even protein denaturation (Brode et al., 1996). Crosslinking of enzymes usually increases their stability at the expense of decreased activity. Microencapsulation into micelles or micellar polymers offers the highest potential to significantly increase enzyme lifetime and stop enzyme leaching, although mass transfer problems may occur. Covalent binding of an enzyme to a carrier has the advantage that the enzyme is tightly fixed. This is due to the fact that the formation of multiple covalent bonds between the enzyme and the carrier reduces conformational flexibility and thermal vibrations, thus preventing protein unfolding and denaturation (Hanefeld et al., 2009; Singh et al., 2010). Multipoint and multi subunit covalent attachments of enzymes on appropriately functionalized supports via linkers provide rigidity to the immobilized enzyme structure, ultimately resulting in improved enzyme stability. However, there are few disadvantages linked with enzyme immobilization such as enzyme leakage due to weak bonds formation between enzyme and the carrier owing to changes in temperatures, pH, ionic strength or even the mere presence of substrate (Dariush, 2003). Restricted diffusion of high molecular weight substrates (ribonuclease, trypsin, and dextranase) into entrapped enzymes (Sankaran et al., 1989) is another problem. Also, selection of conditions for immobilization by covalent binding is more difficult than in other carrier binding methods.

Protein engineering however, can counteract these disadvantages by altering amino acid composition of enzymes that comprise sequences of several hundred amino acids folded in a unique three-dimensional structure. Protein engineering is a faster, more controlled, targeted and more efficient technique then immobilization to optimize the properties of enzymes used in industrial applications and results in higher enzyme activity and stability. Other benefits associated with protein engineered enzymes include: reduced consumption of raw materials and energy, use of alternative and renewable raw materials, reduced CO_2, and other greenhouse gas emissions, improved performance of industrial processes, improved quality of foods and animal feeds and cost-effective production and sustainable development (amfep.org 2009).

4. Applications of protein engineering

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. Successful combinations of rational protein

engineering with directed evolution (Altamirano et al., 2000; Voigt et al., 2000) and combined use of rational design, directed evolution and the diversity of the nature have found to be much more powerful than the use of a single technique (Kirk et al., 2002).

(i) Protein engineered enzymes

Enzymes have been used in many important industrial products. Their applications can be made in paper industry, detergent, drugs, degradation of different wastes, textile, food, pharmaceutical, leather, degumming of silk goods, manufacturing of liquid glue, cosmetics, meat tenderization, cheese production, growth promoters etc (Leuschner, 1995; Rao et al., 1998)

Food industry makes use of a variety of food-processing enzymes, such as amylases, proteases and lipases, the properties of which are improved using recombinant DNA technology and protein engineering. The protein engineering is widely used to improve the properties of industrially important enzymes like thermostability, specificity and their catalytic efficiency. From both economical and engineering point of view, thermostability of enzyme is an important factor. Various factors effect thermostability of enzymes, such as temperature, pH, solvent, and the presence of surfactants. Among all possible deactivating factors, temperature is the best studied. Many enzymes tend to become (partly) unfolded and/or inactivated at elevated temperatures, meaning that they are no longer able to perform the desired tasks. Site-directed mutagenesis (SDM) and directed evolution have been successfully used in improving thermostability of enzymes. However, a combination of both strategies is becoming popular among researchers. Redesigning of enzymes by changing their amino acid sequence for enhancing their catalytic activity and stability in nonaqueous solvents has been successfully achieved. Directed evolution and rational design approaches are widely for altering functional properties of enzymes. Directed evolution approaches involving site-directed mutagenesis are more efficient when detailed structural information and the molecular basis for the property of interest are poorly understood.

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).

(a) Proteases

Proteases are used in several applications of food industry for example, in low allergenic infant formulas, milk clotting, meat tenderization, and flavors. They are also important for detergent industry for removing protein stains (Kirk et al., 2002). The improvement of proteases for industry to extreme conditions, like 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 for genetic manipulation.

Various protein engineering techniques have been successfully used to improve proteases. These mainly include cold adaptation of a mesophilic subtilisin-like protease using laboratory evolution techniques (Wintrode et al., 2000) with the aim that enzymes isolated from organisms native to cold environments generally exhibit higher catalytic efficiency at low temperatures and greater thermosensitivity than their mesophilic counterparts. Following steps were followed: First directed evolution method was used to convert a mesophilic subtilisin-like protease from Bacillus sphaericus, SSII, into its psychrophilic counterpart. Than single round of random mutagenesis followed by recombination of improved variants yielded a mutant, P3C9. Catalytic rate constant at 10°C was found to increase 6.6 than that of wild type. Then DNA shuffling was applied to isolate new proteases with improved properties from an initial material of 26 subtilisin proteases (Ness et al., 1999) such as Durazym, Maxapem, and Purafect.

Protein-engineered subtilisins are widely used in commercial products and are also called a stain cutter, for example, in laundry and dishwashing detergents, cosmetics, food processing, skin care ointments, contact lens cleaners, and for research in synthetic organic chemistry.

Other important application of protein engineering is in increasing stability of BPN from *Bacillus amyfoliquefaciens* in the chelating environment of the detergent by deleting the strong calcium-binding site (residues 75–83) and re-stabilizing the enzyme through interactions not involving metal-ion binding. Stability increases of greater than 1,000-fold in 10 mM EDTA have been reported for this protease (Strausberg et al., 1995; Rubingh, 1996).

(b) Amylases

Alpha amylases constitute a very diverse family of glycosyl hydrolases that cleave α,1–4 linkages in amyloze and related polymers. Amylases are used in a number of industrial processes for example-starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups; partial replacement for the expensive malt in the brewing industry; used as flour improvers in baking industry; produce modified starches for the paper industry; used as additives to detergents for both washing machines and automated dishwashers.

Each of these processes take place under physical and chemical conditions that are quite diverse, thus a single amylase will obviously not be able to meet the particular demands of every industrial process. Significant progress has, however, been made in the optimization of α-amylases using protein engineering for the use in the starch liquefaction process and detergent powders, especially in terms of thermo-stability and pH.

Alpha-amylases obtained from *Bacillus* have found widespread use in industrial processes, because of high thermo-stability. Calcium ions and some sodium ions where seen in α-amylases which increase their stability. Also several mammalian α-amylases have shown to contain a chloride ion in the active site, which has been shown to enhance the catalytic efficiency of the enzyme, presumably by elevating the pKa of the hydrogen-donating residue in the active site. Half-life of *Bascillus liicheniformis* α-amylase is increased ninefold at 90°C by

changing alanine to valine at position 209 and histidine to tyrosine at position 133 (Declerck et al., 1995).

Random mutagenesis of β-amylase from barley resulted in increased its thermostability (Okada et al., 1995). Also a glucoamylase with improved thermostability has been prepared by making glycine to alanine mutations within the α-helical secondary structures of the molecule. These mutations are thought to function by reducing helix flexibility (Chen et al., 1996).

(c) Cellulases

Most commercial cellulases are endoglucanases (promoting internal bond hydrolysis) and contain a catalytic functional region and a cellulose binding domain (CBD) connected by a linker region. Since cellulases have very poor activity against insoluble cellulose without the binding domain, significant effort has been made to increase activity of cellulases.

Linder et al.(1996) found that covalent linkage of two different CBDs resulted in much more tightly binding than the separate domains. It was found binding of one domain increases the effective concentration of the other domain. This makes the interaction of the second domain with the surface much more probable, which in turn can increase its affinity. A study of the role of Tyr169 in the *Trichodmna reesei* cellobiohydrolase II catalytic domain suggests that it plays an important role in distorting the glucose ring into a more reactive conformation (Koivula, 1996).

(d) Xylanases

Xylanases is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. Xylanases are used in the pulp and paper industry to reduce the quantity of chemicals required for bleaching. Modification of distance between the two catalytically active carboxyl groups at the active site of xylanase from *B. circulans* was varied and it was observed that the enzyme activity fall off more rapidly when the distance was increased than when it was shortened (Lawson et al., 1996).

Benefits of protein engineered enzymes. Protein engineering enables faster development of optimized enzymes, offering benefits for industry, agriculture, consumers and the environment, such as:

- Reduced consumption of raw materials and energy
- Use of alternative and renewable raw materials
- Reduced CO_2 and other greenhouse gas emissions
- Improved performance of industrial processes
- Improved quality of foods and animal feeds
- Cost-effective production and sustainable development

(ii) Gluten proteins

An important application area of protein engineering regarding food industry is the heterologous expression of wheat gluten i.e. production of gluten using micro-organisms. The three important things which are important for expression of gluten protein are expression system (*E. coli*, yeasts (*Saccharomyces cerevisiae, Pichia pastoris)* or cultured insect cells), choice of promoter (a strong promoter for high-level protein expression), plasmid stability and codon usage.

Generally, *E. coli* expression systems were suggested as suitable systems for many applications, because of following advantages (Tamas and Shewry, 2006). Inducible promoters (with expression being induced by either specific chemicals or thermal effects) allow cultures to grow to high density providing higher yield and minimising effects of toxic proteins. The T7-based promoter induced by IPTG (isopropyl-b-d-thiogalactopyranoside) is particularly popular for high-level expression.

Advantages and disadvantages of *E. coli* for heterologous expression systems for wheat gluten proteins

Advantages

- Cheap, quick, and easy to use.
- High yield
- Fusion technology is available.

Disadvantages

- Promoters are not completely down regulated under non-induced conditions.
- Protein often unfolds and precipitates in inclusion bodies.
- S–S bond formation occurs only in specific host strains.

(iii) Pharmaceutical applications

PE has been used to produce therapeutic pharmaceutical proteins with improved properties such as increased solubility and stability. The use of protein engineering for cancer treatment studies is a major area of interest. Protein engineering methods are used to modify antibodies to target cancer cells for clinical applications (Zafir-Lavie et al., 2007).

Insulin

Insulin was engineered through mutagenesis to create monomeric forms, which are fast acting. Conversely, another form of insulin (glargine) was created by mutagenesis to precipitate upon injection and give a sustained release of insulin. Whittingham et al. (1997) have reported a crystal structure of prolonged-acting insulin with albumin binding properties

(iv) Environmental applications

Genetic methods and strategies for designing microorganisms to eliminate environmental pollutants were studied. Protein engineering of oxygenases, an important group of enzymes with high selectivity and specificity, enable the microbial utilization and biodegradation of organic, toxic compounds, thus has a potential application in reducing environmental pollution.

Apart from oxygenases, other oxidative enzymes such as peroxidases and laccases are also important for the treatment of organic pollutants. 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. Rational enzyme design and recombinant DNA technology have been found to overcome problems of enzyme denaturation

Table 1. Protein engineered enzymes. Singh et al. (2013).

Enzyme	Organism	Method	Improved property	Application	Reference
Hydantoinase	*Arthrobacter* sp.	Saturation mutagenesis, screening	Enantioselective hydantoinase and 5-fold more productivity	Production of L-Met (L-amino acids)	May et al. (2000)
Cyclodextrin glucanotransferase	*Bacillus stearothermophilus* ET1	Site-directed mutagenesis	Modulation of cyclizing activity and thermostability	Bread industry	Lee et al. (2002)
Lipase B	*Candida antarctica*	epPCR	20-fold increase in half-life at 70°C	Resolution and desymmetrization of compound	Zhang et al. (2003)
Tagatose-1, 6-Bisphosphate aldolase	*E. coli*	DNA shuffling and screening	80-fold improvement in kcat/Km and 100-fold change in stereospecificity	Efficient syntheses of complex stereoisomeric products	Williams et al. (2003)
Xylose isomerase	*Thermotoga neapolitana*	Random Mutagenesis and screening	High activity on glucose at low temperature and low pH	Used in preparation of high fructose syrup	Sriprapundh et al. (2003)
Amylosucrase	*Neisseria polysaccharea*	Random mutagenesis, gene shuffling, and directed evolution	5-fold increased activity	Synthesis or the modification of polysaccharides	Van der Veen et al. (2004)
Galactose oxidase	*F. graminearum*	epPCR and screening	3.4–4.4 fold greater Vmax/Km and increased specificity	Derivatization of guar gum	Wilkinson et al. (2004)
Fructose bisphosphate aldolase	*E. coli*	DNA shuffling	Increased thermostablity and stability to treatment with organic solvent	Use in organic synthesis	Hao et al. (2004)
Lipase	*P. aeruginosa*	Random mutagenesis and screening	2-fold increase in amidase activity	Understanding lipase inability to hydrolyze amides	Fujii et al. (2005)
Protease BYA	*Bacillus* sp. Y	Site-directed mutagenesis	Specific activity1.5-fold higher	Detergents products	Tobe et al. (2006)
Xylose isomerase	*Thermotoga neapolitana*	Random mutagenesis	2.3-fold increases in catalytic efficiency	Production of high fructose corn syrup	Sriprapundh et al. (2003)
α-Amylase	*Bacillus* sp. TS-25	Directed evolution	10°C enhancement in thermal stability	Baking industry	Jones et al. (2008)
Endo-β-1,4-xylanase	*Bacillus subtilis*	Rational protein engineering	Acid stability	Degradation of hemicellulose	Belien et al. (2009)
Subtilase	*Bacillus* sp.	Directed evolution and site-directed mutagenesis	6-fold increase in caseinolytic activity at 15–25°C	Detergent additives and food processing	Zhong et al. (2009)
β-glucosidase	*Trichoderma reesei*	Site-directed mutagenesis	Enhanced kcat/K_m and k_{cat} values by 5.3- and 6.9-fold	Hydrolysis of cellobiose and cellodextrins	Lee et al. (2009)
Cyclodextrin Glucanotransferase	*Bacillus sp. G1*	Rational mutagenesis	Enhancement of thermostability	Starch is converted into cyclodextrins	Goh et al. (2012)
Cellobiose phosphorylase	*Clostridium thermocellum*	Combined rational and random approaches	Enhancement of thermostability	Phosphorolysis of cellobiose	Ye et al. (2012)
Pyranose 2-oxidase	*Trametes multicolor*	Designed triple mutant	Increase half life from 7.7 min to 10 h (at 60°C)	Food industry	Spadiut et al. (2009)
Superoxide dismutase	*Potentilla atrosanguinea*	Site-directed mutagenesis	Thermostability	Scavenging of O_2	Kumar et al. (2012)

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.

(v) Other applications

Biosensor studies- "Insertional protein engineering" applications are also becoming important, particularly for biosensor studies.

Control gene expression- "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.

Biofuels- Protein engineering methods have been used to improve the performance of lignocellulose-degrading enzymes, and biofuels-synthesizing enzymes (Wen et al., 2009).

Virus engineering -"Virus engineering" is another emerging field, where the virus particles are modified by protein engineering. 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.

5. Conclusion

Protein engineering is the technique successfully used for developing useful or valuable proteins. This involves the understanding of protein folding and recognition for protein design principles. Protein engineering mainly involves two general strategies, "rational" protein design and directed evolution. These techniques are not mutually exclusive and are applied together.

Protein engineering involves modification of protein genetically or chemically. However, modification of protein through gene is easier. There is no specific limitation of PE tools; any technique that can lead to change the protein constituent of amino acid and result in the modification of protein structure/function is in the frame of PE. 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. Successful combinations of various protein engineering methods had led to successful results.

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