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Journal of Molecular Catalysis B: Enzymatic 10 (2000) 23–37

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Review

Biomolecular engineering: a new frontier in biotechnology

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Received 15 November 1999; accepted 1 March 2000

Abstract

The advances in high throughput screening technology for discovery of target molecules and the accumulation of functional genomics and proteomics data at an ever-accelerating rate will enable us to design and discover novel biomolecules and proteins on a rational basis in diverse areas of pharmaceutical, agricultural, industrial, and environmental applications. The biomolecular engineering will no doubt become one of the most important scientific disciplines in that it will enable us to comprehensively analyze gene expression patterns in both normal and diseased cells and to discover many new biologically active molecules rationally and systematically. As an applied molecular evolution technology, DNA shuffling will play a key role in biomolecular engineering. In contrast to the point mutation techniques, DNA shuffling exchanges large functional domains of sequences to search for the best candidate molecule, thus mimicking and accelerating the process of sexual recombination in the evolution of life. The phage-display system of combinatorial peptide libraries will be extensively exploited to design and create many more novel proteins, due to the relative ease of screening and identifying desirable proteins. Its application will be extended further into the science of protein–receptor or protein–ligand interactions. The bioinformatics including EST-based or SAGE-tag-based functional genomics and proteomics will continue to advance rapidly. Its biological knowledge base will expand the scope of biomolecular engineering, and the impact of well-coordinated biomolecular engineering research will be very significant on our understanding of gene expression, upregulation and downregulation, and posttranslational protein processing in healthy and diseased cells. The bioinformatics for genome and proteome analysis will contribute substantially toward ever more accelerated advances in pharmaceutical industry. When the functional genomics database, EST and SAGE techniques, microarray technique, and proteome analysis by 2-dimensional gel electrophoresis or capillary electrophoresis are all put to good use, the biomolecular engineering research will yield new drug discoveries, improved therapies, and new or significantly improved bioprocesses. With the advances in biomolecular engineering, the rate of finding new high-value peptides or proteins including antibodies, vaccines, enzymes, and therapeutic peptides will continue to be accelerated. The targets for rational design of biomolecules will be very broad, diverse, and complex, but many application goals can be achieved through the expansion of knowledge base on biomolecules of interest and their roles and functions in cells and tissues. In the near future, more therapeutic drugs and high-value biomolecules will be designed and produced for the treatment or prevention of not-so-easily-cured diseases such as cancers, genetic diseases, age-related diseases, and other metabolic diseases.

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1381-1177/00/$ - see front matter
PII: S1381-1177(00)00105-3

Also anticipated are many more industrial enzymes that will be engineered to confer desirable properties for the process improvement and manufacturing of many high-value biomolecular products. Many more new metabolites including novel antibiotics that are active against resistant strains will be also produced by recombinant organisms having de novo engineered biosynthetic pathway enzyme systems. The biomolecular engineering era is here and a great deal of benefits can be derived from this field of scientific research for many years to come if we are willing to put it to good use.

Keywords: Biomolecular engineering; Biotechnology; Bioprocess engineering; Genomics; Proteomics; Bioinformatics; Antibiotics; Enzymes; Vaccines; Antibodies; Therapeutic peptides; Metabolic engineering

1. Introduction

Biomolecular engineering is a new field of academic discipline and an industrial practice that deals with biomolecules and biomolecular processes to discover and/or create new high-value biomolecules and to develop enabling technology to produce them for improvement of quality of our life and environment. We can achieve this goal through the directed genome evolution, metabolic pathway engineering, protein engineering, analyses of functional genomics and proteomics, high throughput screening techniques, and development of enabling bioprocess technology for production of high value biomolecules of medical, agricultural, and economic importance.

For the rational design of novel biomolecules, the alteration and selection of desired biomolecules have to be approached based on structure–stability/structure–activity relationships. There has been significant progress in establishment of mutation and selection methodology. The DNA shuffling technology mimicking natural evolution is employed for artificial DNA recombination, and phage-displayed combinatorial peptide library offers rapid selection method for proteins expressed from mutated genes. Bioinformatics tools related to functional genomics and proteomics have also been developed for ready access to the information related to the protein function and genome protein, leading to the design and identification of new drug targets. Through the use of massive amount of bioinformatics data bases, many protein/peptide and metabolite molecules have been designed based on their structure–function information. Examples are monoclonal antibodies (Mabs), vaccines, enzymes, antibiotics, therapeutic peptides, and others.

The rapid progress in genetic engineering and the development of many more new recombinant DNA techniques have provided us with the capability of design, modification and engineering of the natural biomolecules. The major goal of biomolecular engineering is to design and create novel biomolecules and to develop bioprocesses required for production of high-value biomolecules. For industrial application, the engineered biomolecules have to be stable and active for a prolonged use under unusual bioprocess conditions, such as temperature, acidity and reaction environment. For instance, sustained activity and stability are required in the presence of strong solvents, highly reactive chemicals, or extremes of pH. On the other hand, the engineered biomolecules targeted for medical application often require activity and stability under physiological conditions in the presence of enzymes and natural inhibitory compounds, long-acting property in vivo system, and for their efficient delivery to the targeted cells or tissues.

Biomolecular engineering as a new field of scientific discipline is based on the following five areas of intellectual endeavor (Fig. 1). They include: (1) bioinformatics, including the functional genomics and proteomics, (2) protein chemistry and protein engineering dealing with protein structure–function and structure–activity relationships, structure-based design, prediction of designed protein structure, (3) recombinant techniques including random mutation, DNA shuffling, and phage-display technique,

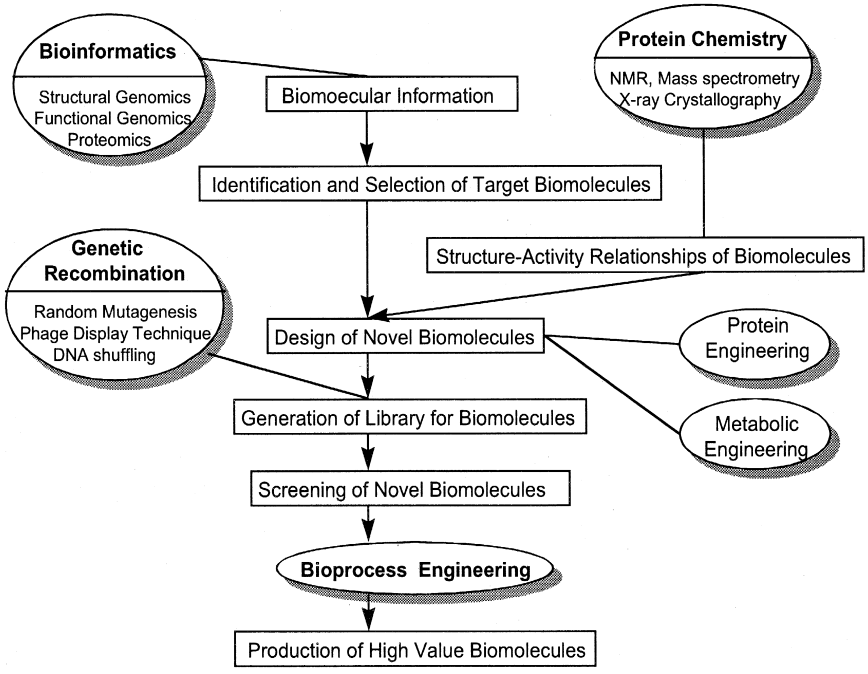

Fig. 1. Important knowledge bases and tools of biomolecular engineering discipline.

(4) metabolic pathway engineering including the metabolic flux analysis, and (5) bioprocess engineering which develops enabling technology to produce the desired high-value biomolecules. In this review, recent progress in key technologies and tools employed in biomolecular engineering research and the main target products of biomolecular engineering research are discussed.

2. What are the key technologies and tools employed in biomolecular engineering?

2.1. Oligonucleotide-directed mutagenesis

In late 1980s, the protein molecules have been altered by site-directed or site-specific mutagenesis of their genes. This opened an era of protein engineering [1–3]. This sequence-based design of biomolecules can be achieved after computer modeling of individual amino acid change or replacement, followed by site-directed mutagenesis of the corresponding DNA with predesigned oligonucleotide primer, and expression of the recombinant protein in mutants for testing and evaluation. Even though it provides prudent and rational design and modification of presumptive protein structure, it has three main limiting factors: limited knowledge on protein structure–function relationship, the approximate nature of computer modeling, and the astronomical numbers of relevant mutants to be studied and examined. Furthermore, this method is not appropriate to generate many novel biomolecules simultaneously in a single pot.

To enhance exploitation of biomolecular diversity, new strategies have been attempted for creating combinatorial libraries that have individual point mutations, namely the repeated cycle of ‘‘error-prone PCR’’ [4,5] or the repeated oligonucleotide-directed mutagenesis [6]. Error-prone PCR employs a low-fidelity replication step to introduce random point mutations at each round of amplification. This method has the advantage of simplicity and ease of use. However, the power of this method is also

limited due to small sizes of library relative to the sequence size, resulting in enormous exhaustive random screening and selections.

2.2. DNA shuffling

The technique of DNA shuffling came from mimicking natural recombination by allowing in vitro homologous recombination of DNA [7,8]. In this method, a population of related genes is randomly fragmented and subjected to denaturation and hybridization, followed by extension of 5'-overhang fragments by Taq DNA polymerase. As a result of repeated PCR cycles, the length of fragment becomes increased. The DNA recombination occurs when a fragment derived from one template primes a template with different sequences.

In contrast to the error-prone PCR mutagenesis, DNA shuffling allows more direct recombination of all beneficial mutations from any given round to generate multistep mutants with dramatically improved phenotypes. Nowadays, DNA shuffling technique combined with well-focused selection procedure allows one to rapidly develop genes for a wide variety of industrial applications. The DNA shuffling technique has been applied to the following areas of research with a reasonable success. They include the optimization of enzymes, such as protease, lipase, amylase and cellulase; the development of extended and/or altered metabolic pathways specialized to synthesize specialty chemicals; antibiotics and pharmaceutical proteins; plasmids and viruses for novel vaccines and gene therapy applications [9].

2.3. Phage-displayed peptide library screening

The idea for phage-displayed peptide library came from Smith [10] for the isolation of Mabs. Driven by the initial success, the field of phage-displayed library has been widely used in finding high-affinity antibodies within a random combinatorial library of antibody fragments [11,12].

The basic principles of this technique are to display the peptide libraries fused with the carboxy-terminal domain of the minor coat protein, gene III product, on the surface of filamentous phage [92] (Fig. 2). The expressed peptide libraries are then directly detected and screened using the target molecules.

The direct linkage of displayed peptide phenotype on the phage coat with encapsulated genotype via the phage surface gives two advantages. First, it allows the selection of particular clones from pools of millions, especially through amplification by infecting male *E. coli*. Second, the amino acid sequence of a peptide displayed on a phage can be readily ascertained by deciphering the DNA sequence of relevant section of the phage genome.

This phage-displayed peptide libraries are extensively employed in and applied to searching for a variety of biomolecules, including antibodies, receptors, and enzymes, as evidenced by many papers published in recent years [13].

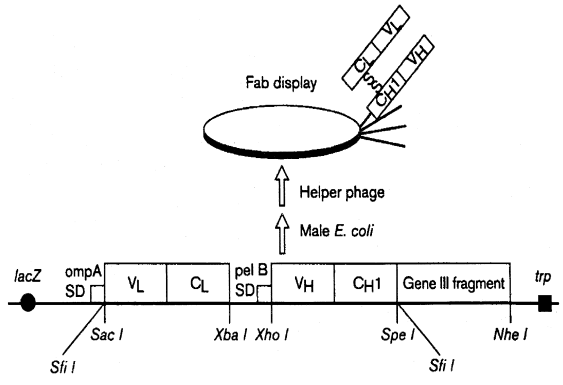

Fig. 2. Phage-displayed combinatorial antibody libraries. The phagemid vector is designed for the expression of antibody fragments on the phage surface or in soluble form. Generally, the antibody fragments are fused to the carboxy-terminal domain of the minor coat protein of filamentous phage, gene III protein, which is important for phage assembly. To generate a combinatorial antibody library, the *Sac*I–*Xba*I restriction fragment encoding light chain or *Xho*I–*Spe*I fragment for Fd fragments of heavy chain can be replaced with DNA fragments having random sequences [92].

2.4. Bioinformatics

Bioinformatics is a rapidly developing field of science that makes use of biological data and knowledge base stored in computer databases, complemented by computational methods and data analysis, to retrieve and/or derive new and useful value added biological information. It is a theoretical biology but firmly grounded on massive amount of comprehensive experimental data and facts. Based on the structural genomics, the bioinformatics was first established by mapping and sequencing the whole genomes of many living organisms, the comparative genomics followed next for the prediction of protein function by pair-wise sequence-to-sequence comparison and profile-to-profile comparison [14].

2.4.1. Functional genomics and microarray technique

Recently, bioinformatics has been making very significant and practical contributions to the new drug discovery, identification of target biomolecules, and to the design of the combinatorial libraries based on knowledge of one or more protein structures deduced from multiple genomic sequences. Functional genomics constitutes one of the key areas in bioinformatics. The expressed sequence tags (ESTs), the serial amplification of gene expression tags (SAGE tags), and the microarray technique with the oligonulceotide chip technology are the key techniques and tools for bioinformatics and are at the forefront of technological change in the biomedical research [15,16].

Microarray technique employs a large number of oligonucleotides gridded on a solid support, either as oligonucleotides chosen from genome analysis data or as PCR products of ESTs, which can be hybridized to fluorescence-tagged samples to provide qualitative and quantitative gene expression profiles [17] (Fig. 3). Thus, it is possible to analyze the expression patterns by hybridizing on microarray devices containing a multiplicity of oligonucleotides, each one of known sequence leading to specific hybridization patterns, called DNA chips [18]. Especially, ESTs and SAGE tags are widely used in identifying gene transcripts, finding missing gene family members, or monitoring complex gene expression patterns.

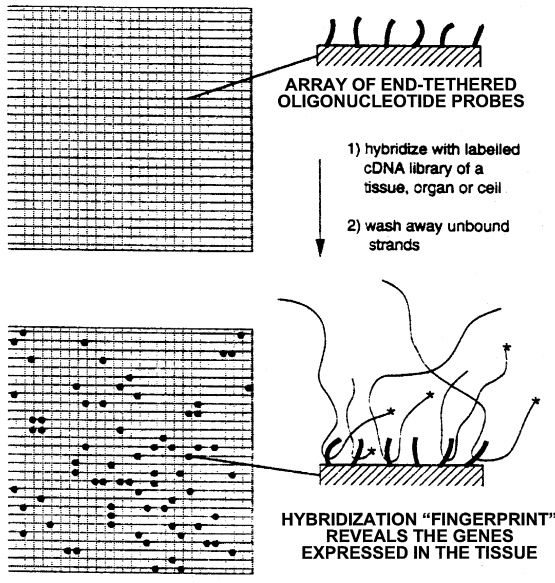

Fig. 3. Microarray technology. The DNA chip is divided into many separate grid segments, each containing an oligonucleotide or sequence tag with a DNA sequence specific for a single known gene. A cDNA library prepared from a cell, tissue or organ of interest is labeled by incorporation of fluorescence-labeled dNTPs or other signal-generating molecule and then hybridized to the chip. The expression level of each gene on the chip is quantitatively analyzed by the measurement of the intensity of fluorescence or other signal emitted by each segment of the chip [17].

With complex EST or SAGE-tag-based expression networks or linkages, functional genomics is in keeping with the shift from genetics to genomics for those applying RNA-based expression strategies rather than solely gene-based strategies for studying diseases and gene therapies. This functional genomics allows biology to move from single gene to multiple genes, or to even more complex epigenetics, for the understanding of disease processes and/or diseased cells and for the discovery of better disease treatments [15].

2.4.2. Proteomics

Another important area of bioinformatics established most recently is proteomics, which is the use of quantitative protein-level measurements of gene expression to characterize biological processes including the disease processes, cellular response to drugs, and deciphering the mechanisms of gene expression control [19]. This is obviously different from functional genomics in the following respects: protein expression levels are not perfectly correlated to mRNA expression levels, and the expressed proteins are dynamically processed through posttranslational modification which are not necessarily from gene sequence. Now proteomics becomes one of the most powerful bioinformatics tools because proteomes reflect dynamically and exactly the state of biological cell system, responding to growth stage, nutrition condition, temperature, stress, and pathological conditions [20]. Although there have been significant advances in proteome analysis with the aid of two-dimensional gel electrophoresis, capillary electrophoresis, and mass spectrometry, further improvements are needed for the automation and miniaturization to achieve high throughput screening as well as its simple use and accuracy [21].

Both the functional genomics and proteomics offer abundant information on gene expression patterns and phenotypes of biological system at different states of cells and cellular metabolism (Fig. 4). Thus, the bioinformatics may yield very important insights into many of the most common but not-easily-cured human diseases, such as diabetes, arthritis, cancers, and Alzheimer's disease, where multiple factors, including environmental and genetic, are attributable to [15].

3. What are the main targets of biomolecular engineering?

3.1. Engineered antibodies

Mabs have long been considered and accepted as good natural drugs, because they mimic their natural role in the body and they have no inherent toxicity. However, when rodent Mabs are readily generated, the widespread use as therapeutic agents has been hampered

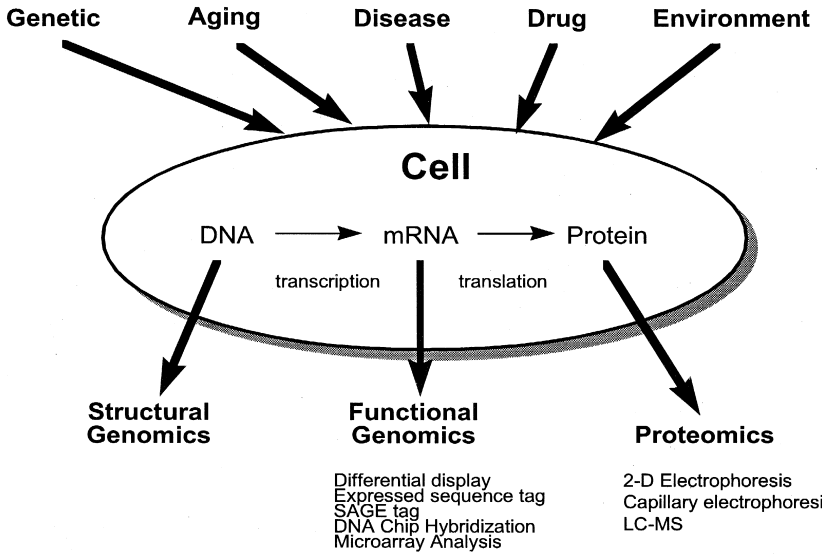

Fig. 4. An overview of the cellular response to various stimuli. The functional genomics and proteomics give a great deal of information on how the cellular metabolism and physiology are affected by genetic heredity, disease, aging, drug administration, metabolic stress, and other environmental factors.

because they are recognized as foreign by patients. The clinical Mabs, as human as possible, has been developed and called ''chimerized'' or ''humanized antibodies,'' by exchanging the constant region of rodent Mab with their human counterparts [22,23].

3.1.1. Humanized antibodies

The humanized antibodies are commonly created as rigid β-sheets, and the remaining V region amino acids known as ''framework residues'' act as scaffold to support these loops. The humanized antibodies are commonly prepared by transplanting the antigen binding segments from rodent antibodies to human antibodies. There are three hypervariable regions in sequence of antibody V domain, which is primarily responsive to antigen recognition. These residues referred to as complementarity determining region (CDR) form loops at the ends of immunoglobulins and are well conserved across species. It is possible to exchange the fragments containing effector functions [24] or to transplant CDRs from one antibody to another [25] by protein engineering in order to confer or transfer antigen-binding capability.

High-affinity human antibodies are also obtainable from transgenic mice containing human antibody genes and disrupted endogenous immunoglobulin loci. Immunization leads to the production of human antibodies, which can be recovered using standard hybridoma technology [26,27]. Even though the megabase size range of the human immunoglobulin loci together with the complexity of the segmented gene structure represents an obstacle to develop transgenic mice for the full human immunoglobulin gene repertoire, the transgenic mice containing a nearly complete set of diversity and joining segment can be created. Especially, mice carrying a more complete set of human V_κ (but not V_λ) and V_H genes closely resemble the processes of antibody production in humans, including gene rearrangement, diverse gene usage, and somatic mutation, to yield high affinity, fully human IgG_2 antibodies against a variety of antigens.

More than 20 humanized Mabs have now been subjected to clinical trials for a variety of treatments and indications. Among them, the first humanized Mab tested was CAMPATH-1H for the treatment of non-Hodgkins lymphoma and rheumatoid arthritis [28]. Although evidence of obvious benefit was observed, over 50% of rheumatoid arthritis patients developed human anti-mouse antibody (HAMA) responses to repeated therapy. On the other hand, another humanized Mab has shown negligible HAMA responses in patients, for example on multiple dosing with an anti-CD33 Mab in myeloid leukemia patients [29], and with an anti-Tac antibody in patients with graft-versus-host disease or undergoing renal transplantation [30]. In these cases, only one out of 38 patient developed an immune response. Another humanized antibody for the HER2/neu proto-oncogene product, Herceptin, has been approved by FDA for metastatic breast cancer (FDA License No. 1048, 09/25/98). It showed minimal side effects and no detectable immunogenicity [31].

3.1.2. Recombinant human antibody fragments

Although very often successful, humanization is rather laborious and is being superseded by the rapid and direct isolation of human antibodies from phage-display libraries and transgenic mice. For production of the phage-displayed human antibody fragment, V_H and V_L domain of human antibody are PCR-cloned in filamentous phage DNA and displayed on the surface coat of phage as single-chain Fv or Fab fragments (Fig. 5). Antigen-specific phage is then selected by in vitro panning against the target antigen of interest. The advantage of this methodology is on the isolation of high-affinity human antibodies (nanomolar range) to many different antigens from the huge antibody phage library ($> 10^{10}$ clones) within a few weeks. The recombinant ScFv, in which the V_H and V_L domains are linked together with a polypeptide

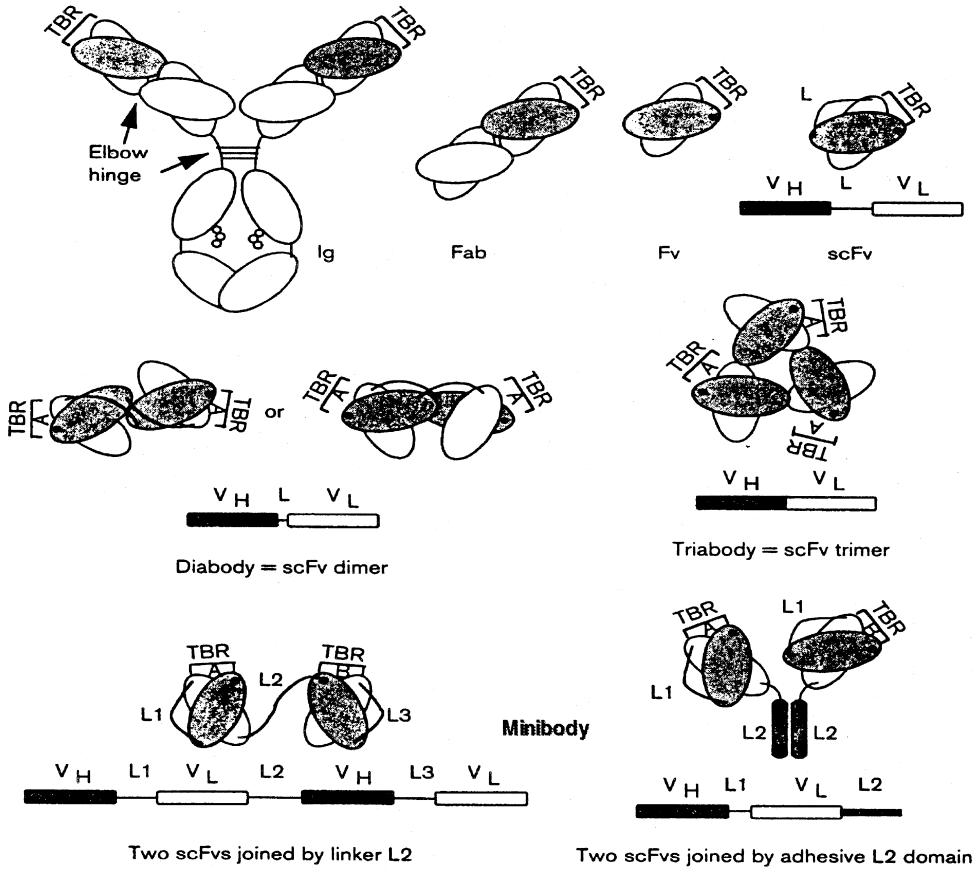

Fig. 5. Preparation of antibody fragments. Heavy chains (V_H) are shown in black and light chains (V_L) in white. Ig, immunoglobulin; Fab, fragment for antigen binding; Fv, variable fragment; scFv, single chain variable fragment; L1, L2, L3, linkers connecting the carboxy terminus of V_H with the amino terminus of V_L; TBR, target binding region [38].

bridge (about 15 hydrophilic amino acid residues), mimics the Fv domain of the antibody [32]. Since amino termini of both V_H and V_L are near to, but not part of, the antigen-binding interface, ScFvs generally retain the same affinity as the parent Fv module and express higher levels than Fabs in bacteria. Even though ScFv and Fab fragments provide effective and highly specific in vivo targeting reagents for tumor and cancer, these monovalent antibody fragments are cleared and removed rapidly from the blood due to their small size and the single binding site [33].

Because intact antibodies are generally polyvalent molecules, there have been many attempts to conjugate Fab or ScFv molecules into dimers or higher multimers [38] (Fig. 5) to produce highly functional reagents of 60–120 kDa in size, capable of rapid tumor penetration without fast kidney clearance. The simplest design for this is a diabody, in which a short 5-residue linker between V_H and V_L domains prevents formation of alignment of V domains into a functional Fv module and results in combination of the V domains of two ScFv molecules into a bivalent dimer [34]. The reduction of linkers to less than three residues prevents the formation of a diabody and directs three ScFv molecules to associate into a trimer, called triabody, with three antigen binding sites [35]. It has been revealed that the molecules of diabody and triabody are relatively flexible from

the orientation of antibody-binding sites [36], and that those showed the higher functional affinity with reduced kidney clearance rates [33]. Recently, another antibody fragment, called minibody, has been designed by joining a ScFv to a C_H3 domain via a linker [37]. This minibody is readily expressed in Sp2/0 cells and a prepared antibody forms a disulfide-linked dimer by virtue of the C_H3 domain and a cystein-containing linker. These minibodies have proved to localize very efficiently to a tumor xenograft in mice and show higher retention in tumor cells.

The ''human antibody fragments'' screened from phage-displayed antibody libraries or ''human antibodies'' produced in transgenic mice has been further optimized for therapeutic application. The recombinant antibodies and their fragments now represent about 30% of all biological proteins undergoing clinical trials and studies for FDA approval of engineered cancer therapeutic antibody [38]. The human antibody fragments derived from phage display have already entered phase II/III clinical trials for treatment of rheumatoid arthritis (anti-tumor necrosis factor-α) and ocular fibrosis (anti-transforming growth factor-β). These will be the second wave of products following the first humanized antibody product for Her2/neu oncogene (Herceptin) [23].

3.1.3. Immunotoxins

In the field of antibody engineering, another important development is to make immunotoxins or immunoconjugates by comprising a cell-binding moiety, such as an antibody or ligand, linked to a protein toxin or small toxic molecules. Over the past two decades, the problems raised in clinical evaluation of immunotoxins are their immunogenicity which often precludes multiple dosing and their toxicity that is sometimes life threatening. In spite of these problems, objective antitumor responses by an immunotoxin, anti-$Lewis^Y$-*Pseudomonas* exotoxin (PE), were recently reported for treatment of epithelial tumors [39]. This immunotoxin binds to the carbohydrate antigen, $Lewis^Y$, only found in many human tumors, and kills the cells by ADP-ribosylation of elongation factor 2 with PE, which inactivates protein synthesis. When human enzymes such as RNase are fused with an anti-transferrin receptor Fab'_2 or ScFv, it was found to be cytotoxic in vitro onto antigen positive, but not antigen-negative, cells [40].

3.2. Engineered vaccines

3.2.1. Polypeptide vaccines

Since humoral and cellular immune responses recognize and react with only specific regions of pathogens, it is possible to design vaccines based on subunits of pathogen, either naturally occurring immunogenic polypeptides or synthetic peptides that correspond to highly conserved regions required for the pathogen's function. The goal of this strategy is to vaccinate with the smallest possible structural fragment, consisting of a well-defined antigen, in order to stimulate an effective specific immune response, while avoiding potentially hazardous risks. In this strategy, there should be epitopes recognized by both the B cells and T cells, as well as the major histocompatibility complex (MHC) restriction of the T-cell response.

An influenza vaccine composed of a chimeric *Salmonella* flagellin and proteosome which carries three epitopes from the hemagglutinin and nucleoprotein was successfully used for nasal immunization of mice [41]. Carrying this approach one step further, an effort to construct a human vaccine is currently underway using the same approach [42].

In case of HIV, there are some difficulties in successful vaccination, due to the following problems. The virus attacks and destroys the T-helper lymphocyte, secondly the proviral DNA integrates into the host genome and remains unrecognizable, and finally the virus undergoes antigenic variation. An HIV-1 p17 synthetic peptide vaccine, HGP-30, designed from early regulatory protein in order to prevent the first virus–host interaction, was evaluated in phase I clinical trials and both cellular and

humoral responses were observed [43]. Similarly, four epitopes from the HIV-1 regulatory protein Rev [44] and V3-loop epitope of gp120 recognized by cytotoxic T cell [45] were tested for in vitro immunization of human lymphocytes. They were found to elicit specific cytotoxic T cells that could destroy the infected cells.

Using similar approaches, the T- and B-cell epitopes for measles virus, hepatitis B virus, respiratory syncytial virus, and poliovirus have been identified, and they are currently under investigation as candidates for peptide-based vaccines [46].

Since large-scale cultivation and vaccine preparation is impractical using the parasitic protozoan pathogens, the development of synthetic peptide vaccines is particularly suitable. For instance, a synthetic peptide vaccine, Spf66, designed to contain upto 40 repetitions of the circumsporozoit protein repeat sequence of malaria, was already subjected to large-scale clinical trials, and it has been proven to be effective in prevention with humoral and cellular responses [47]. Another peptide vaccine candidate consists of six epitopes of pf155/RESA, the blood stage antigen of malaria. When investigated in women from an endemic area, most reacted to at least one epitope, and only 23% of the vaccinated women failed to respond to any of those peptide epitope vaccines [48]. Another polypeptide epitope under evaluation is the 15-kDa carboxy-terminal region of the merozoit surface antigen, which induced protective immune responses in rodents [49]. Following similar approaches, the recombinant polypeptide vaccine for parasitic schistosomiasis, which afflicts about 200 million people mostly in developing countries, was also evaluated for mice and baboons [50]. It was found to elicit high antibody titers and significant resistance to challenged infection.

3.2.2. DNA vaccines

Rather than pathogenic polypeptide itself, naked DNA as a vaccine is under intensive study. When the plasmid DNA encoding the pathogenic polypeptide is injected intramuscularly, it is taken up by myocytes, transcribed into mRNA, and expressed as protein to induce humoral and cellular immune responses [51]. The major characteristic of DNA vaccines is low-level but long-lasting expression, which induces immune response and the vaccination effect by a continuous stimulation of the immune system and training of memory cells. The HIV*env* (gp120) DNA first entered clinical trials as a therapeutic and prophylactic vaccine for AIDS, and gave promising results to induce immune system including cytotoxic T-cell responses [52]. Other DNA vaccines being developed are against hepatitis B [53], influenza [54], rotavirus [55], herpesvirus-1 [56], dengue virus [57], tuberculosis [58], and malaria [59].

3.3. Engineered enzymes

In order to address industrially relevant problems involved in enzyme processes, attempts have been made to design enzymes that will confer or enhance useful properties by using biomolecular engineering techniques. Thus, the engineered enzyme will have such improved properties as pH stability, thermostability, increased activity, and the enhancement of proper interaction with surfaces.

Although rational design approach based on its structure–function relationship is widely used, directed evolution approach is generally favored for many industrial enzymes, owing to the difficulties of relating the desired application with the required properties. The direct evolution, also called molecular evolution, through DNA shuffling, is a technique of preparing protein variants by recombining gene fragments in vitro and expressing and selecting for those with improved properties.

There are three approaches to generate enzymes with improved properties: first, the specificity or catalytic properties of existing enzymes can be modulated by a change or replacement of single or a few amino acid residues; second,

exchange of functional domains to alter the enzyme specificity and catalytic properties; third, introduction of new active site into a small protein fragment scaffold of the enzyme which is devoid of its original active site (for example, scorpion toxin) [60]. The rationale behind this kind of ''hybrid enzyme'' is that enzymes catalyzing similar reactions have similar active site structures, and that the amino acid residues making up the active site domain in a given family of enzymes possess a specific geometric arrangement [61].

3.3.1. Mutated enzymes

Among many industrial enzymes protease used for laundry and cleaning products has been intensively studied. The stability of protease BPN from *Bacillus amyloliquefaciens* in the chelating environment of the detergent was improved by deleting the strong calcium-binding site (residues 75–83) and restabilizing through interactions not involving metal-ion binding [62]. The surface properties of protease BPN have also been engineered, and it was found that variants having negative charges in the active site region gave better laundry performance [63]. The thermostability of protease has been improved by replacing residues in a thermolysin-like protease from *B. stearothermophilus* at the equivalent amino acid positions [64].

For the amylases used in starch conversion process, the improvement of thermal stability is a main target because of the high temperature process requirement. It has been reported that point mutations at two amino acid positions increased the half-life of *B. licheniformis* α-amylase ninefold at 90°C [65]. Similarly, for β-amylase from barley [66] and glucoamylase from *Aspergillus awamori* [67], their thermostability has been improved by point mutations.

Most lipases used in cleaning application are activated at the oil–water interface by a conformational change in which a lid is shifted to expose the hydrophobic binding pocket of the enzyme. Two point mutations in this lid region have been reported to alter activity of *Humicola lanuginosa* lipase [68]. Another approach in designing variants that reduce the inhibition by surfactant and calcium sequestering agents is recently studied to achieve improved laundry performance [69].

3.3.2. Hybrid enzymes

Design of hybrid enzymes using highly homologous enzymes have been demonstrated by exchanging residues or structures of their homologous regions. For instance, hybrid of an *Agrobacterium tumefaciens* β-glucosidase (optimum at pH 7.2–7.4 and 60°C) and a *Cellvibrio gilvus* β-glucosidase (optimum at pH 6.2–6.4 and 35°C) was developed by exchanging the homologous regions, which showed the optimal activity at pH 6.6–7.0 and 45–50°C [70].

The exchange of active sites of enzymes alters their catalytic properties. In case of trypsin, the exchange of four residues in the active site and two nonstructural surface loops gave the substrate specificity of chymotrypsin [71]. Similarly, chymotrypsin has been transformed into trypsin [72]. Exchanging residues in the coenzyme-binding domains of glutathion reductase [73] and lipoamide dehydrogenase [74] successfully altered their cofactor preferences from NADP to NAD and NAD to NADP, respectively.

The creation of restriction enzymes with novel specificities through a fusion of a specific DNA-binding domain with the catalytic machinery of a restriction enzyme has been also attempted. A hybrid enzyme, made by fusing the cleavage domain of the type II restriction enzyme *Fok*I with the DNA-binding motif from the *Ubx* homeodomain of *Drosophila* and with consensus zinc-finger protein, was able to recognize and cleave specific DNA target sequences [75].

3.4. Engineered antibiotics

During the last two decades, the gene clusters for the production of secondary metabolites, especially antibiotics, have been identified, and

discovered from many industrial microorganisms by employing the recombinant DNA techniques. The major enzymes involved in antibiotic biosynthesis are nonribosomal peptide synthetase (NRPS) and polyketidesynthase (PKS) in repeated condensation of acetyl and malonyl units. Since these enzymes are comprised of multiple modules each having an active domain, they are amenable to construction of hybrid enzymes for the production of novel secondary metabolites.

3.4.1. Engineering of peptide antibiotics

Nonribosomal synthesis of peptide antibiotics is catalyzed by multifunctional peptide synthetase whose structural domains are responsible for specific amino acid activation and modification (adenylation and thionylation domain) and peptide-bond formation (elongation domain). The arrangement of these domains determines the number and order of amino acids in the peptide. Thus, it is possible to synthesize any defined peptide secondary metabolites by the correct number and arrangement of activating domains. Using this building-block approach, a new hybrid antibiotic has been produced by individually exchanging a Leu-activating domain of *B. subtilis* surfactin synthetase with Phe-, Orn- and Leu-activating domains from *B. brevis* and Cys- and Val-activating domains from *Penicillium chrysogenum* [76]. It was also reported that recombinant enzyme obtained by repositioning the thioesterase domain of surfactin synthetase, which is thought to release the peptide chain from the enzyme complex, synthesized the peptides of reduced length [77].

3.4.2. Engineering of polyketide antibiotics

The modular PKS gene cluster encodes several large polyketide chains, which can be divided into three domains. They include ''loading domain'' consisting of an acyl transferase and an acyl carrier protein, ''multiple modules'' containing a ketosynthetase, an acyl transferase, and an acyl carrier protein, and terminal ''releasing domain'' having a thioesterase and often a cyclase activity. Owing to the modularity of PKSs, it is also possible to produce novel polyketide structures by manipulating PKS genes. Gene manipulation of 6-deoxy-erythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*, a PKS for biosynthesis of erythromycin aglycon provided novel polyketide lactones of the shortened length, by deleting modules late in the biosynthetic pathway except for natural thioesterase domain [78]. The production of a novel macrolactone via internal acyl transferase domain has been also attempted. When substituting the acyl transferase domain from DEBS module 2 with an acyl transferase domain of module 2 from rapamycin PKS, modified 2-norerythromycins with antibacterial activity was synthesized in *Streptomyces erythrea* [79]. Similar approach has been used for production of novel norerythromycin derivatives, 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A, by exchanging acyl transferase domain of DEBS modules 1 and 2 with three heterologous acyl transferase domains specific for malonyl CoA [80]. The loading domain of DEBS has been replaced with that from avermectin PKS, which has a broader specificity for branched-chain carboxylic acids [81]. From the fermentation broth, a mixture of erythromycins D, B, and A was detected along with corresponding analogs having isopropyl and isobutyl at the C-13 position.

The module exchanges for β-modifying enzymes such as ketoreductase to reduce the ketone to an alcohol, dehydratase to dehydrate the alcohol to a double bond, and enoyl reductase to convert the double bond to a saturated single bond are under investigation. Similarly, attempts are made to change the stereochemical configuration of secondary metabolites by using the module exchange method [82].

3.5. Therapeutic peptides and peptidomimetics

One challenge in the development of therapeutic compounds is to find a small molecule

capable of mediating a desired biological effect. Transition pathway from a large protein to a small molecule to bring in a form of peptide-based compound is called ''peptidomimetics''. The information obtained from the study of structure–activity relationships and the conformational properties of peptide structures will enable us to advance the peptidomimetics. Furthermore, the combined knowledge base of molecular biology and medicinal chemistry will enable us to speed up the process of drug discovery through exploitation of the molecular diversity, combinatorially synthesized peptide library, or phage-displayed peptide library.

These peptide libraries are now beginning to be exploited extensively for new drug discovery and cancer therapy, either as an antagonist or an agonist of a natural ligand–receptor interaction [13]. Recently, many examples of the peptide ligands for receptors such as erythropoietin [83], thrombopoietin [84], angiogenin [85], α-bungarotoxin [86], melanocortin [87], and CD80 [88] have been found.

Other peptidomimetic ligands have been also developed based on the sequence of target proteins for SH2 and SH3 domains, which is involved in initiation of signal transduction of activated tyrosine kinase [89]. These classes of compounds will be most likely applicable to the treatment of acute lymphocytic leukemias and HER-2/neu in breast and ovarian cancers. Two types of inhibitors for Ras protein product, p21, have also been developed: benzodiazepine peptidomimetics and a CAAX tetrapeptide, which blocks the farnesylation of p21 thereby affecting *Ras*-transforming properties [90]. The development of small molecule inhibitor for thrombin active site is also an area of intensive research [91].

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