Protein Engineering in Industrial Biotechnology

PROTEIN ENGINEERING IN INDUSTRIAL BIOTECHNOLOGY

Edited by

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harwood academic publishers
Australia • Canada • France • Germany • India • Japan
Luxembourg • Malaysia • The Netherlands • Russia • Singapore
Switzerland

Amsteldijk 166
1st Floor
1079 LH Amsterdam
The Netherlands

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library.

ISBN: 90-5702-412-8

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PREFACE

Protein engineering by its iterative rational design is a powerful tool to both test general theories of protein structure and activity, as well as to develop more useful catalysts to be used in biotechnological processes or products.

Protein Engineering in Industrial Biotechnology aims to present a series of examples in which the application of protein engineering has successfully solved problems as diverse as the purification of recombinant proteins or the development of target molecules for drug discovery.

This book is organized in three sections, respectively: protein engineering for bioseparation, for biocatalysis and for health care. The more relevant industrial enzymes are covered: lipases, proteases, carboxypeptidases, glucanases and glucosidases, pectinolytic enzymes and enzymes for the bio-remediation of recalcitrant compounds. The interplay of solvent engineering to modulate the structure–to–activity relations is also discussed. A chapter is devoted to the application of protein engineering to biosensors.

The large potential applications of protein engineering to health care are also covered, from the development of new safe vaccines to therapeutic proteins. Specific attention has been devoted to new protein engineering in the development of target molecules for drug discovery.

The chapters have been written by an international team of experts from Europe, USA and Japan who have made major contributions in the field.

The present book aims to attract the interest of students in industrial biotechnology at the undergraduate and graduate level as well as that of everybody interested in basic research in protein structure, molecular genetics, bio-organic chemistry, biochemistry, agrobiotechnology, pharmaceutical sciences and medicine.

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CONTENTS

1. PROTEIN ENGINEERING IN BASIC AND APPLIED BIOTECHNOLOGY: A REVIEW

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The major goal of protein engineering is the generation of novel molecules, intended as both proteins endowed with new functions by mutagenesis and completely novel molecules. This definition, which may sound broad and perhaps ambitious, in fact pinpoints one of the most promising developments in our ability to understand and control a protein's function. After the revolution introduced in protein science by the advent of genetic engineering, protein engineering can be considered as a second wave of innovation which is providing important breakthroughs in basic research and application, useful for studies on structure function relations and for exploitation in industry. Genetic engineering makes available unlimited amounts of purified proteins, whereas protein engineering produces tailor-made proteins redesigned such as to make them more suited to industrial requirements. On this basis it becomes evident that industrial biotechnology will enormously benefit of this possibility.

Natural targets of protein engineering are enzymes and several examples of modified catalysts have already been achieved and applied to industrial processes. However, protein engineering is not restricted to this field, since several non-enzyme proteins play important roles, for example, as drugs. Another fundamental area is that concerning antibodies, which may be planned both as specific carriers able to target drugs in the human body, as well as in the production of catalytic antibodies (abzymes) to be applied for reactions with non natural substrates.

Protein engineering is a complex and multidisciplinary field, where several different techniques and knowledge are applied in combination. The protein of interest needs to be first purified and characterized with regard to its functional properties, then to be cloned and overexpressed in a suitable host organism and subsequently to be modified so as to improve its performances. A variety of analytical as well as structural techniques will be then employed for its characterization, whereas fermentation technologies will support its large-scale production. Different enabling technologies contribute therefore to an interdisciplinary approach that is usually represented as the **protein engineering cycle** (Figure 1).

This chapter aims to outline the most innovative techniques as well as industrial applications of protein engineering, to provide the reader with a general, although necessarily non comprehensive, view on the field. More in general it attempts to "give the taste" of the opportunity provided by protein engineering to biotechnology, stressing the important and interactive linkage between basic science and application.

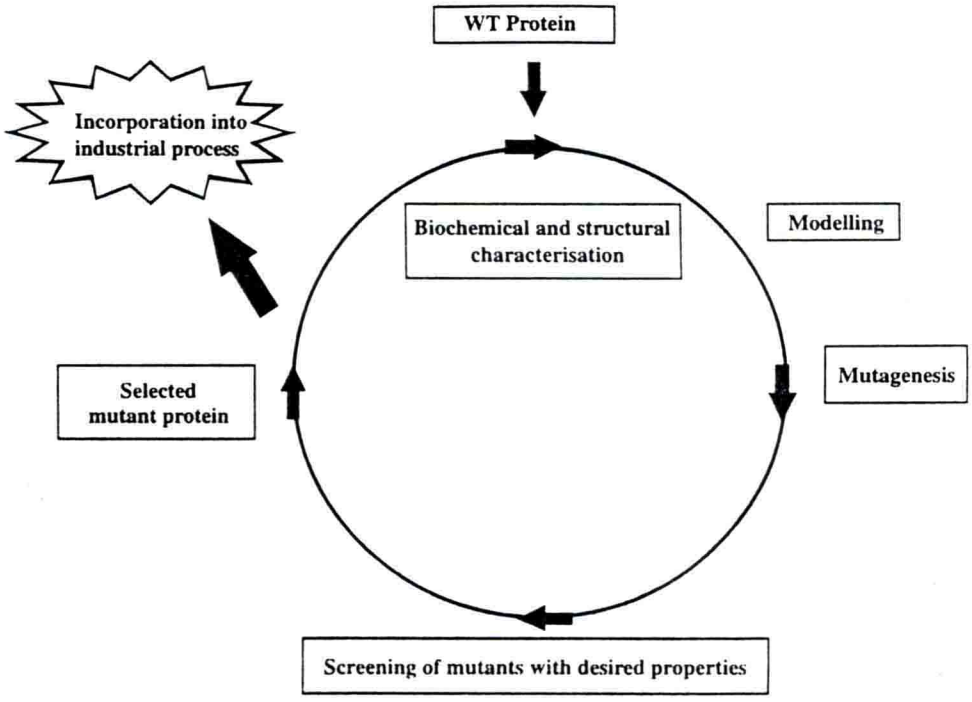

Figure 1 Cycle of protein engineering

ENABLING TECHNOLOGIES: HOW TO ENGINEER NEW PROTEIN FUNCTIONS

Diverse and complementary techniques form the knowledge and technological basis to engineer proteins and all of them must be mastered or at least be familiar to protein engineers. These basic techniques have been covered in recent and comprehensive books (Wrede and Schneider, 1994; Cleland and Craik, 1996;) and will therefore not be recalled hereafter. However, new techniques of mutagenesis/screening were recently introduced, that expanded the possibility of modifying a protein's properties and deserve a brief comment (Table 1). Other essential enabling technologies are also outlined in the following.

Techniques of Mutagenesis

The starting point for every step of mutagenesis is a DNA sequence cloned from the original source or synthesized based on the protein sequence of interest. One or several amino acids may be substituted/inserted/deleted using a wide variety of methods. Two main conceptual approaches can be followed: i) site-directed mutagenesis, i.e. substitutions of nucleotides in the correspondence of pre-selected positions in the gene (polypeptide) ii) random mutagenesis.

Table 1 Experimental approaches for protein engineering

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

Rational design

Site directed mutagenesis (SDM) is a classical approach for protein engineers, exemplified by a broad scientific literature and by several contributions of this book. It consists in introducing a change in one or more amino acids and evaluating the effect of these pre-selected substitutions in the mutated product. By definition, this strategy, also called "rational mutagenesis", requires a prior knowledge of the role played by specific residues or regions of the protein. This means availability of the protein 3D structure, if possible also in complex with substrates, ligands, regulation elements or at least availability of sequence of proteins with related but not identical activity for comparison. In fact, sequence alignments may also provide support in the selection of the positions to mutate, in particular when the protein of interest belongs to a large and well characterized family of proteins. On the other hand computer-assisted technology may help in predicting the functional effect of the planned substitutions.

Rational design is being very widely employed to engineer in proteins new functions or influence their regulation. Important goals have been achieved by rational design, as it will be briefly summarized in the following paragraphs.

Non-rational (random) design

Random mutagenesis on whole protein sequences or parts thereof is the method of choice in all those cases where knowledge about the structure and function of the protein of interest is not sufficient to support a rational design approach. The most innovative techniques involve the generation of repertoires (libraries) of mutated sequences and procedures of mutation/selection that mimic the processes followed by nature during evolution. In both cases, a very high number of mutant variants is produced, so that the development and availability of sensitive and fast procedures of screening is vital to manage the experimental work.

Molecular evolution. Very recently, protein engineering has been revolutionized by new methods that mimic evolution, of course in much shorter time, in that they allow for the accumulation and selection of mutations beneficial to the property of

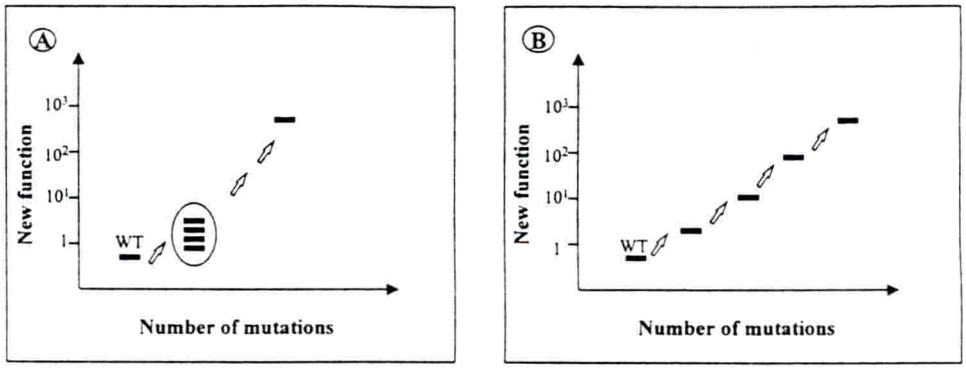

Figure 2 Comparison between two strategies for protein "evolution": DNA shuffling (A) and directed evolution (B) (modified from Arnold, 1996).

interest. These new approaches commonly referred to as *molecular evolution techniques*, allow to overcome the serious drawback represented by the fact that for several industrially important proteins the level of available knowledge is insufficient to rational design. Moreover, growing evidence suggests that many protein functions cannot be ascribed to single or few aminoacids but they rather depend on regions located far away from the active and regulatory sites, and therefore hard to be predicted *a priori*. Molecular evolution methods are based on the accumulation of beneficial mutations over several rounds of mutagenesis. Since the effect of every single mutation can be small, the availability of methods of screening sensitive and in addition applicable to large number of mutants, is an indispensable prerequisite to this goal. Evolutionary approaches can be grouped in two categories: DNA shuffling and directed evolution (Figure 2). Both methods rely on the well-established technology of polymerase-chain reaction (PCR), in its development known as "error-prone PCR", that allows for the introduction of a small preselected number of mutations in the DNA sequence.

DNA shuffling was developed by W. Stemmer in 1994 (Stemmer 1994 a and b). This technique is also known as "sexual PCR". It involves enzymatic fragmentation of DNA derived from different organisms previously randomly mutated as to introduce changes in the coding sequence. Fragmented DNA are then reassembled by error-prone PCR in the absence of primers, allowing their spontaneous reassemble and — at the same time — the introduction of new mutations. A final step of amplification with primers generates full-length products. This strategy has been applied to several cases. For example, the activity of β-lactamase towards the antibiotic cefotaxime was increased over 16,000 times through three cycles of sexual PCR, whereas the fluorescence of the green fluorescent protein was increased 45 fold (Crameri *et al.*, 1996). The ambitious goal of modifying multigene determinants of complex metabolic functions was also achieved enhancing the activity of an arsenate locus from *Staphylococcus aureus* composed by three genes. This results introduced DNA shuffling in the field of environmental bioremediation (Crameri *et al.*, 1997). In the same field, Kumamaru and colleagues recently evolved novel specificities in biphenyl dioxygenases (BPDox) by shuffling homologous genes from *Pseudomonas alcaligenes*