10
Bioconjugation for Enzyme Technology

OH HYEONG KWON[1] AND YOSHIHIRO ITO[2*]

[1]*Department of Polymer Science and Engineering, Kumoh National University of Technology, 188 Shinpyung-dong, Kumi, Kyungbuk 730-701, Korea and* [2]*Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima 770-8506, Japan*

Introduction

Enzymes play important roles in all living cells. They possess remarkable catalytic properties in terms of high catalytic activity, exclusion of undesirable side-reactions such as racemization, and operations under mild conditions. The most specific features of enzyme function are the high substrate specificity including regioselectivity and stereospecificity. As a consequence, enzymes have been used in various industrial and medical fields. Bioconjugation has expanded the possibility of application of enzymes towards bioreactor catalyst, bioreactor sensor and medical drug technologies. Immobilization of enzymes onto solid matrices has enabled the recyclization of enzymes: immobilization of enzymes onto sensing devices including electrodes and optodes has also provided the basis for biochemical sensors. Bioconjugation of medical enzymes extracted from animals with an amphiphilic polymer, polyethylene glycol, has been shown to reduce the immuno-reaction induced by the application of sensor or therapeutic enzymes into the human body. Various bioconjugations of biological molecules are very important in the biomedical fields (Aslam and Dent, 1998).

Bioconjugation is divided into two categories in the present review. One is gene-engineered bioconjugation, and the other chemically engineered bioconjugation (*Figure 10.1*). Gene-engineered modification has been used for the alteration of enzymatic activity, such as thermal stabilization (Bryan *et al.*, 1986), alteration of

*To whom correspondence may be addressed (ito@bio.tokushima-u.ac.jp)

Abbreviations: PEG, polyethylene glycol; *M. luteus*, *Micrococcus luteus*; *B. subtilis*, *Bacillus subtilis*; *E. coli*, *Escherichia coli*; k_{cat}, turnover constant; K_m, Michaelis constant; CD, circular dichroism; CF, carboxyfluorescene; diS-C_3-(5), 3,3'-dipropylthiadicarbocyanine iodide; AOT, aerosol-OT; PEG-Sub, PEG-modified subtilisin Carlsberg; AOT-Sub, AOT-modified subtilisin Carlsberg; FT-IR, Fourier-transform infrared; APEE, *N*-acetyl-L-phenylalanine ethyl ester; *N*-Ac-L-Phe, *N*-acetyl-L-phenylalanine; PNIPAAm, poly(*N*-isopropylacrylamide); LCST, lower critical solution temperature; PSP, photoresponsive copolymer carries the spiropyran and carboxyl groups; PSP-Sub, PSP-subtilisin Carlsberg; UV, ultraviolet.

Biotechnology and Genetic Engineering Reviews – Vol. 18, July 2001
0264–8725/01/18/237–263 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, U.K.

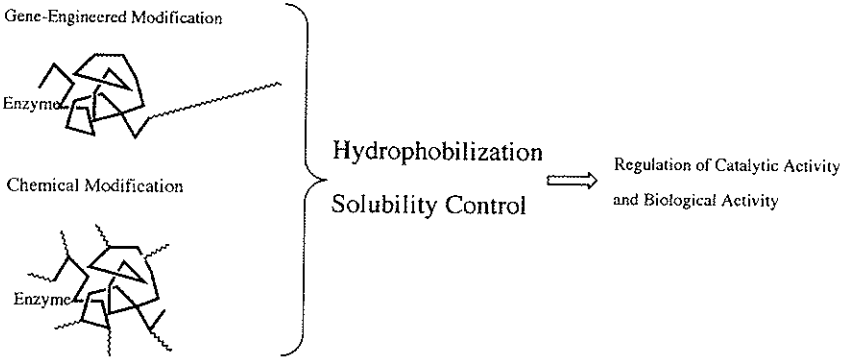

Figure 10.1. Modification of enzyme by genetic engineering and chemical methods.

substrate specificity (Sears *et al.,* 1994), and use in organic synthesis (Wangikar *et al.,* 1993, 1995). The gene-engineered modification of enzymes is laborious and modification sites are limited, but such technologies can strictly regulate the structure and the molecular weight of the modified enzyme.

The chemical bioconjugation modification strategy includes attachment of lipid molecules or synthetic polymers to enzymes. Y. Inada and his colleagues have synthesized PEG-attached enzymes for immuno-isolated drugs and for catalytic reactions which are unable to occur in aqueous solution (see Matsushima *et al.,* 1984; Inada *et al.,* 1995). Okahata *et al.* (1995) and Dordick and his co-workers (see Paradkar and Dordick, 1994; Wangikar *et al.,* 1997) have modified enzymes with lipid molecules or surfactant molecules to render the enzymes soluble in organic media. Ito *et al.* (1992, 1993, 1994) have connected hydrophobic synthetic polymers to enzymes. More recently, Wang *et al.* (1997) have synthesized biocatalytic plastics by the coupling of enzymes with acryloyl chloride to provide a polymerizable functionality, subsequent extraction of acryloylated enzymes with Aerosol-OT (AOT) into the organic phase, and followed finally by polymerization with hydrophobic monomer and crosslinker. In general, the chemical modification of enzymes can be performed under facile conditions and the physical properties can be drastically altered, although precise control of the chemical reaction can be difficult.

In the present review, we consider the above two methods for enzyme modification. The *genetic engineering approach* has been used for hydrophobilization of enzymes: hydrophobilization has been shown to affect the hydrolytic activity or bactericidal activity of enzymes. *The chemical modification approach* has been performed on enzymes by conjugation with various synthetic polymers: solubility was controlled by the conjugation. When a stimuli-responsive polymer was conjugated, the solubility of the conjugated enzyme was subsequently regulated by stimulation.

Genetically engineered bioconjugation

HYDROPHOBILIZATION BY GENETIC ENGINEERING

Genetic engineering can control the functions of enzymes and using this technology

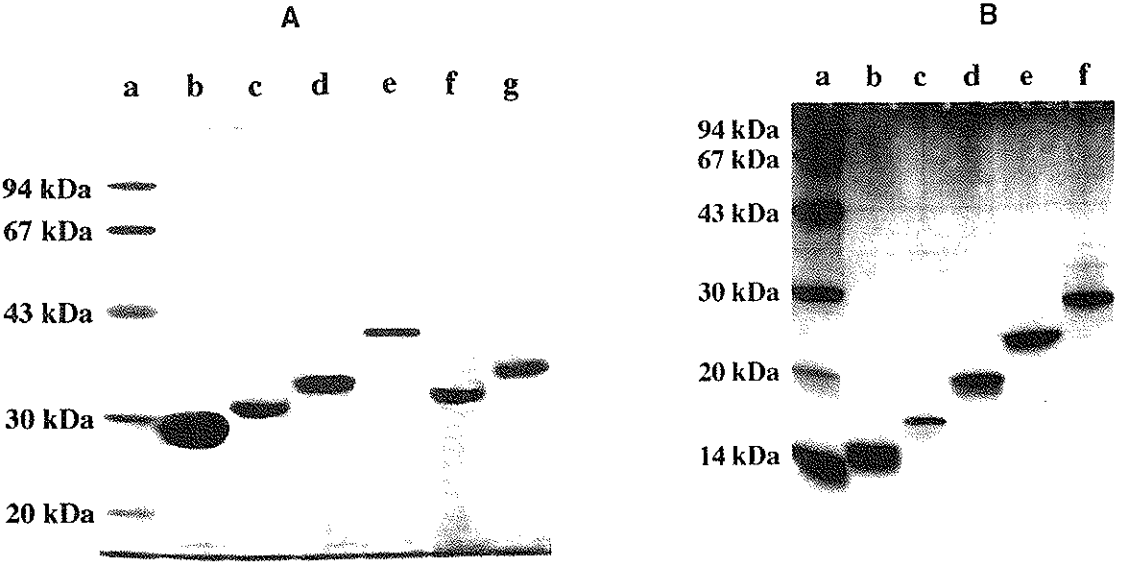

Figure 10.2. Analysis of proteins by SDS-polyacrylamide gel electrophoresis. **A:** Esterases. a) marker proteins, b) the native esterase, c) esterase carrying 10 proline residues, d) esterase carrying 20 proline residues, e) esterase carrying 40 proline residues, f) esterase carrying 10 tyrosine residues, and g) esterase carrying 20 tyrosine residues. **B:** Lysozymes. a) marker proteins, b) the native lysozyme, c) lysozyme carrying 10 proline residues, d) lysozyme carrying 20 proline residues, e) lysozyme carrying 30 proline residues, and f) lysozyme carrying 40 proline residues.

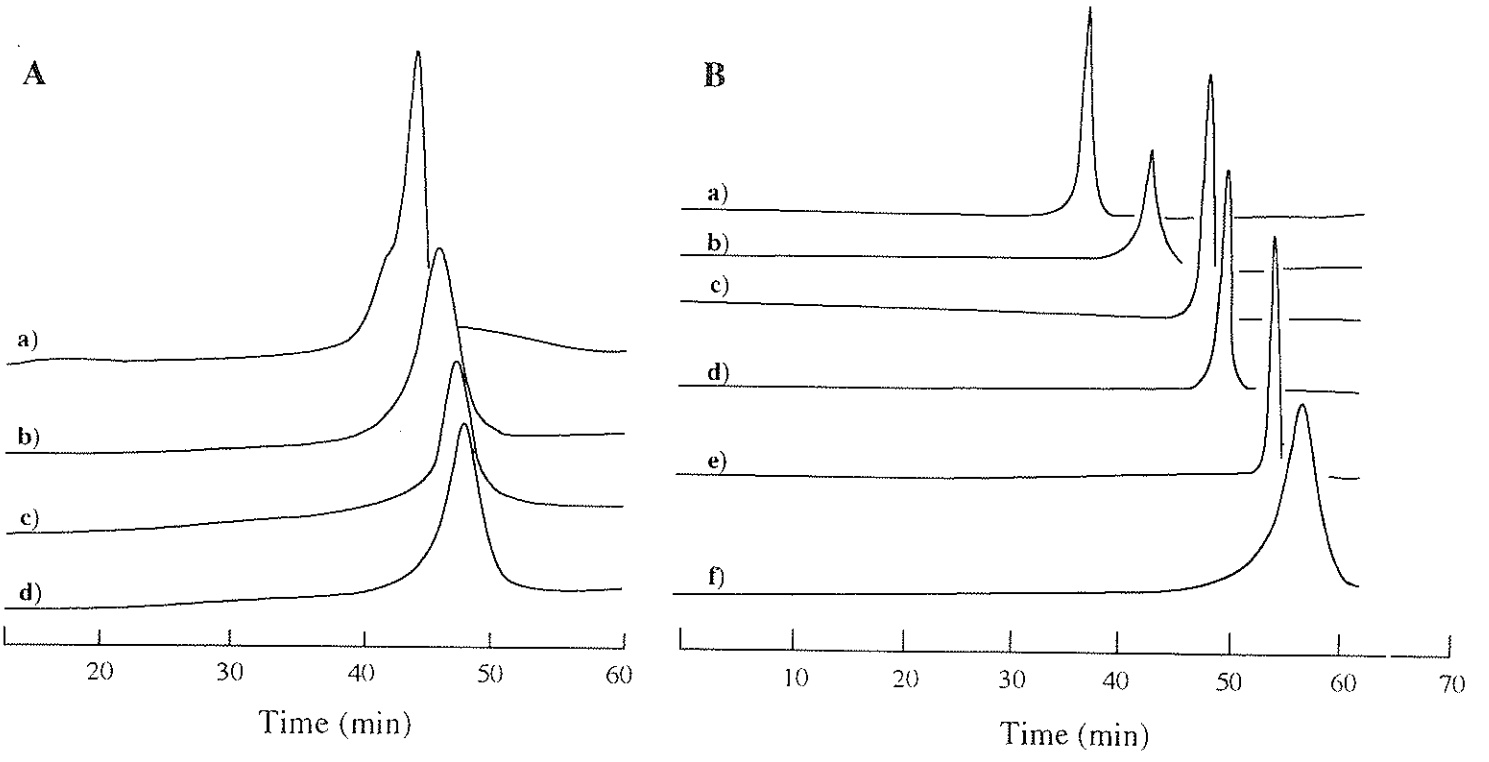

Figure 10.3. Elution profiles from hydrophobic chromatography using TSKgel phenyl-5PW (Tosoh, Japan) (a linear gradient from 0.5 M phosphate-buffered solution (pH 7.0) + 2.0 M ammonium sulphate solution to 0.5 M phosphate buffered solution (pH 7.0) over 60 min at the rate of 1.0 ml/min). **A:** a) the native esterase, b) esterase carrying 20 proline residues, c) esterase carrying 40 proline residues, and d) polyproline. **B:** a) the native lysozyme, b) lysozyme carrying 10 proline residues, c) lysozyme carrying 20 proline residues, d) lysozyme carrying 30 proline residues, e) lysozyme carrying 40 proline residues, and f) polyproline.

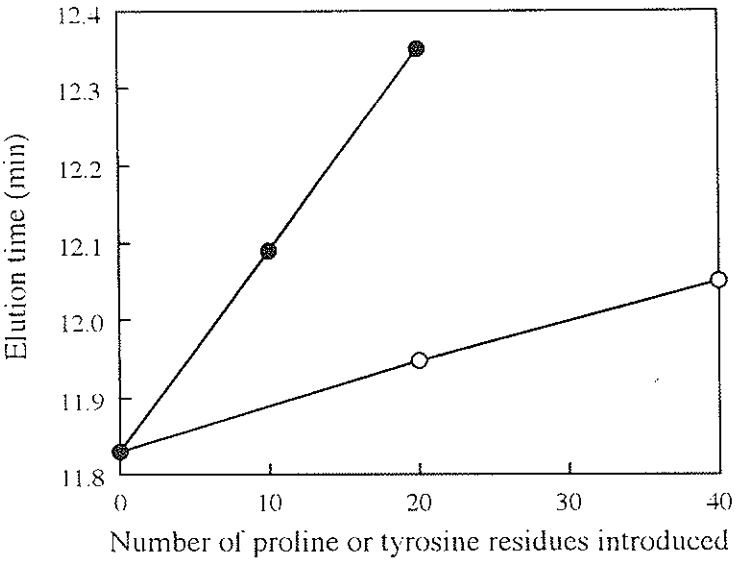

Figure 10.4. Analysis of hydrophobicity of the fusion esterases in terms of elution time of a COSMOSIL $5C_{18}$-AR-300 (Nacalai Tesque, Japan) reversed phase chromatography experiment. A linear gradient from 20% acetonitrile containing 0.05% trifluoroacetic acid to acetonitrile containing 0.05% trifluoroacetic acid over 30 min at the rate of 1.0 ml/min was employed: ○, the fusion esterases carrying polyproline; ●, the fusion esterases carrying polytyrosine.

the utility of enzymes has been extended. Genetic engineering based on recombinant DNA technology has been successfully applied to aqueous enzyme systems to increase their thermal stability, to alter their substrate specificity or the optimum pH, or to facilitate their use in organic solvents. In addition, genetic engineering has yielded various types of proteinous materials or fusion proteins. In early work, Chen and Arnold (1993) adapted the activity of subtilisin for catalysis in dimethylformamide. The addition of polyhistidine terminal extensions, '*His tagging*' designed to allow the easy purification of the target protein on a metal-chelating gel, has now become a standard practice in molecular biology (Ledent *et al.*, 1997; Chen and Chen, 2000). Stempfer *et al.* (1996) have designed a new method for enzyme immobilization using a fusion protein of yeast α-glucosidase containing a polycationic hexa-arginine fusion peptide at its C-terminus. This fusion protein was directly adsorbed from crude cell extracts on polyanionic matrices in a specific, oriented fashion. It was demonstrated that the non-covalent coupling did not influence the specific activity, the Michaelis constant, nor the activation energy of the enzymatic reaction.

Genetic engineering has also been used in our laboratories for the hydrophobilization of enzymes, and in the paragraphs that follow we now review this work. Esterase and lysozyme have been hydrophobilized by connection of polytyrosine or polyproline chain of various lengths to the carboxyl end of the enzymes, and the catalytic activity as well as biological activity of the mutant enzyme was investigated.

The construction method of mutant plasmid coding mutant esterase and lysozyme carrying polytyrosine or polyproline at the carboxyl terminal has been described by Kwon *et al.* (1998a,b) and Ito *et al.* (1997). The purified esterases and lysozymes carrying polytyrosine or polyproline chains of various lengths at the carboxyl terminus

were analysed by SDS-polyacrylamide gel electrophoresis (*Figure 10.2*). The band positions of mutant enzymes were found to be higher than those expected. However, measurement by mass spectroscopy or gel permeation chromatography demonstrated that the molecular weight of the mutant enzyme was the same as the calculated value. It has been reported that hydrophobic proteins show higher affinity for SDS than usual, because SDS–protein interaction forces are mainly hydrophobic. The same explanation should be applied to the present cases: SDS binding to the fusion enzymes is responsible for the anomalously slower than expected electrophoretic migration.

The hydrophobicity of such mutant enzymes has been investigated by hydrophobic chromatography (*Figure 10.3*). The measured elution time was found to increase with increasing length of the connected polyproline chain and approached that of pure polyproline. *Figure 10.4* shows the relationship between the elution time and the chain length of the insert. The fusion esterase carrying polytyrosine was more hydrophobic than that carrying polyproline.

Figure 10.5 shows the CD spectrum of mutant enzyme carrying polytyrosine or polyproline. The CD spectrum of polyproline-fused enzyme was almost the same as the addition spectrum of the wild-type enzymes and polyproline having a type II helix structure. This means that the connection of polyproline did not induce a remarkable conformational change. However, polytyrosine introduction caused a significant decrease of α-helix structure and the spectrum was changed to a typical β-structure (*Figure 10.5A*). The conformational change of esterase was found to be more significant with increasing length of the polytyrosine chain insert. It was considered that the amphiphilic polyproline chain extends beyond the surface of the enzyme molecule, whilst the hydrophobic polytyrosine chain penetrates the interior of the enzyme molecule by hydrophobic interactions, causing a drastic conformational change. From these measurements, an expected polyproline-fusion lysozyme structure is illustrated in *Figure 10.6*.

BIOLOGICAL ACTIVITIES OF HYDROPHOBILIZED ENZYMES

The hydrolytic activity of esterase has been shown by our laboratories to be affected by the fusion with polytyrosine or polyproline to the carboxyl terminus (*Table 10.1*). The rate of hydrolysis of *p*-nitrophenyl propionate was reduced by the introduction of polyproline. The K_m value was not so influenced by connection of polyproline chain to the enzyme, whereas the k_{cat} value significantly decreased. The decrease of k_{cat}/K_m

Table 10.1 Kinetic parameters for hydrolysis of *p*-nitrophenyl, pentanoate and hexanoate by the wild type and fusion esterases

Substrate	Wild-type esterase			Esterase-Pro40			Esterase-Tyr10	Esterase-Tyr20	Turbidity of aqueous substrate solution (A_{450})
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat}/K_m ($M^{-1}s^{-1}$)	
propionate	0.039	1.85×10^4	7.9×10^6	0.036	1.29×10^4	6.0×10^6	~0	~0	0.007
pentanoate	0.037	0.86×10^4	3.9×10^6	0.023	1.10×10^4	7.6×10^6	~0	~0	0.018
hexanoate	0.035	0.25×10^4	1.2×10^6	0.011	3.72×10^3	5.6×10^6	~0	~0	0.026

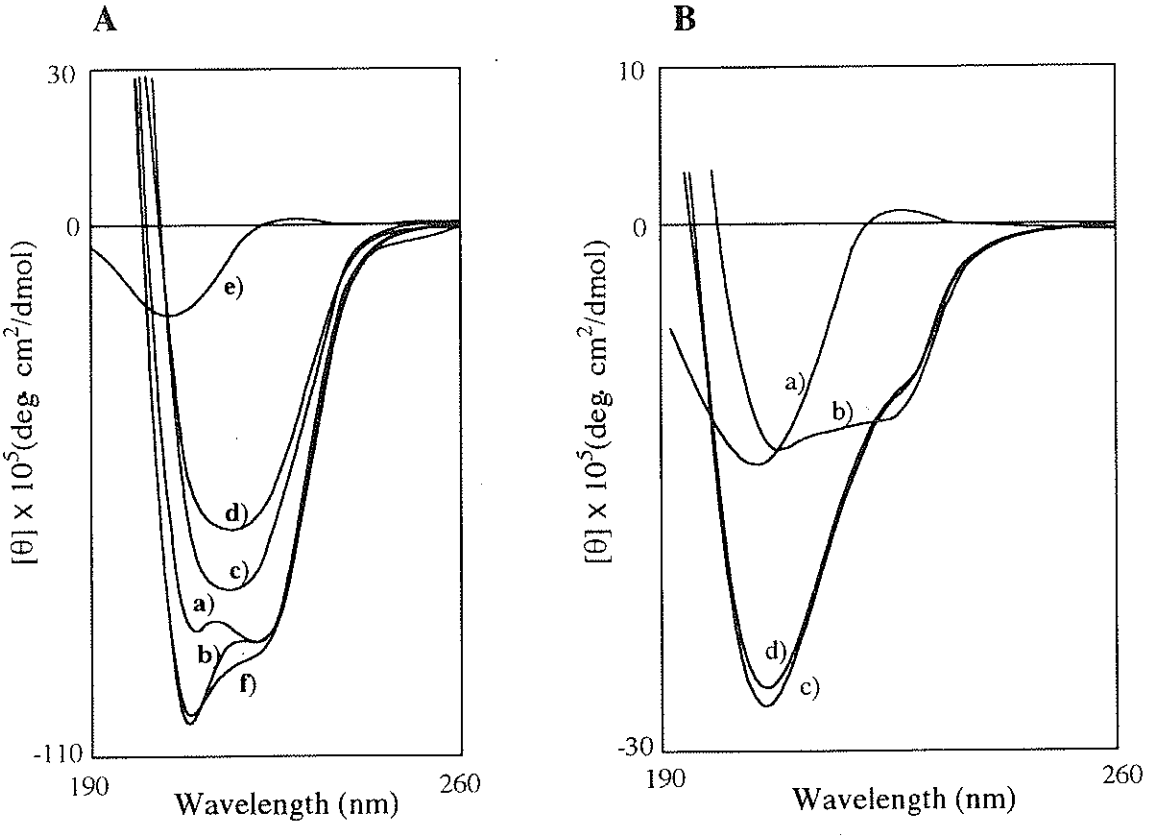

Figure 10.5. Circular dichroism (CD) spectra. **A:** a) the native esterase, b) esterase carrying 40 proline residues, c) esterase carrying 10 tyrosine residues, d) esterase carrying 20 tyrosine residues, e) polyproline, and f) the addition spectrum of a) and e). **B:** a) polyproline, b) the native lysozyme, c) lysozyme carrying 40 proline residues, and d) the addition spectrum of a) and b).

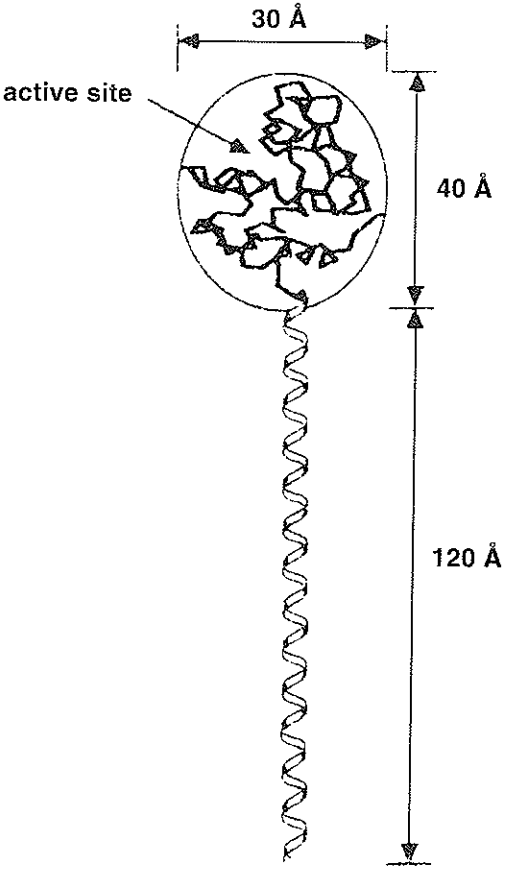

Figure 10.6. Schematic drawing of the structure of lysozyme carrying 40 proline residues at the carboxyl terminal.

caused by the introduced polyproline was considered to be due to a subtle conformational change that may not have been detected by CD spectra.

On the other hand, in spite of several efforts to renature the inclusion bodies, esterase fusion carrying polytyrosine had a negligible activity. The drastic conformational change of the esterase induced by the connection of polytyrosine must be a reason for the extremely low activity.

The solubility of substrate in water decreased with increasing length of acyl chain, as shown in the last column (turbidity of aqueous substrate solution) of *Table 10.1*. The k_{cat}/K_m values of the wild-type esterase were found to decrease with increasing acyl chain length of the substrate, i.e. with increasing hydrophobicity of substrate. On the other hand, the acyl chain length of substrate did not significantly affect the k_{cat}/K_m values of polyproline-fused esterases. Polytyrosine was found to be so hydrophobic that the active conformation of fusion esterase was seriously disrupted so as to disable the enzymatic activity. By contrast, enzyme modification with polyproline did not seriously affect the secondary structure of the enzyme, and enhanced the affinity to hydrophobic substrate in water due to the increased hydrophobicity.

Table 10.2. Hydrolysis of *p*-nitrophenyl tri-*N*-acetyl-β-chitotrioside (Tri) and *p*-nitrophenyl penta-*n*-acetyl-β-chitopentaoside (Penta) by various human mutant lysozymes

Enzyme	Number of proline residues at the C-terminal	Tri			Penta
		Relative activity (%)	K_m (mM)	k_{cat} (min^{-1})	Relative activity (%)
Native	0	100	2.27	2.31	100
Mutant	10	81	2.26	1.82	–
	20	37	2.27	0.77	–
	30	11	2.20	0.23	21
	40	9	2.00	0.19	–

Secondly, the hydrophobilization effect on lysozyme was investigated. The hydrolytic activity of lysozyme was reduced by connection of a polyproline chain to the carboxyl terminus (*Table 10.2*). Although K_m was not significantly affected by the polyproline connection, k_{cat} decreased with increasing length of the polyproline tail. These results indicate that the polyproline tail is not a barrier against the access of substrate, but that it induces some form of conformational change around the active site of lysozyme. The reduction of hydrolytic activity of lysozyme by the polyproline connection depended on the hydrophobicity of the substrate. The relative hydrolytic activity of a mutant lysozyme carrying 30 proline residues to the wild-type lysozyme was higher with penta-*N*-acetyl-β-chitopentaoside (21%) than with tri-*N*-acetyl-β-chitotrioside (11%). The hydrophobic mutant lysozyme was thus considered to easily access the hydrophobic substrate, penta-*N*-acetyl-β-chitopentaoside.

It is well known that lysozyme is an important, naturally occurring antibacterial protein. The bactericidal action of lysozyme is strong against Gram-positive bacteria but much weaker against Gram-negative bacteria. This difference is due to the unique cell envelopes of the latter, which consists of an outer membrane (Wilkinson *et al.*, 1972), a peptidoglycan layer, and an inner membrane (Murry *et al.*, 1965). Lysozyme catalyzes the hydrolysis of the β(1,4)-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine involved in the peptidoglycan of bacterial cell wall (Jollès and Jollès, 1984). It interacts with the lipopolysaccharide (LPS) layer in the outer membrane, and hydrolyses the peptidoglycan layer. Despite its action, the wild-type lysozyme does not affect the inner membrane and the viability of Gram-negative bacteria, while the same actions are lethal to Gram-positive bacteria.

Ibrahim *et al.* (1994) reported that the covalent attachment of palmitic acid to the lysyl residues or fusion with a hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro) switched the bactericidal activity of lysozyme from a Gram-positive bacterium, *Micrococcus luteus* (*M. luteus*) to a Gram-negative bacterium, *Escherichia coli* (*E. coli*). They suggested that the hydrophobic domains promoted the penetration of the cationic molecules into the inner membrane and this dissipates electrochemical potential across the cell membrane.

In our laboratories, the bactericidal activity of genetically hydrophobilized lysozyme against Gram-positive and Gram-negative bacteria has been investigated. *Figure 10.7A* shows that the bactericidal activity of lysozyme on the Gram-positive bacteria (*M. luteus* and *Bacillus subtilis*) decreased with increasing length of the polyproline tail.

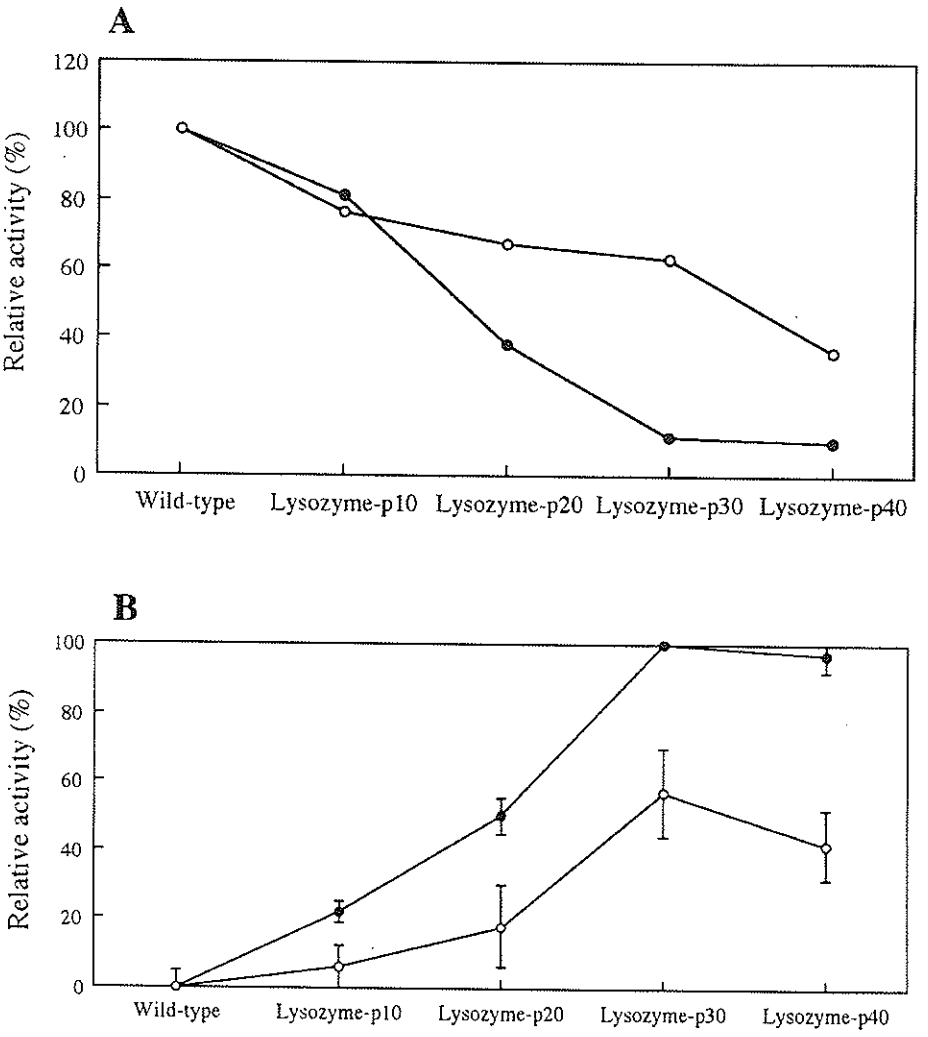

Figure 10.7. **A:** Bactericidal activity of mutant lysozymes against *Bacillus subtilis* (○) and *Micrococcus luteus* (●) at I=0.04. The activity of native lysozyme was taken as 100%. **B:** Bactericidal activity of mutant lysozymes against *Escherichia coli* (○) and *Pseudomonas aeruginosa* (●). When all of the bacterial cells were dead, the relative bactericidal activity was taken as 100%.

Figure 10.7B shows that the activity against Gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) increased with increasing length of polyproline chain connected. The increasing hydrophobicity thus seems to increase the lysozyme activity against Gram-negative bacteria.

In order to investigate the relationship between bactericidal activity and hydrophobicity of lysozyme, the interaction of mutant lysozymes with a liposome made of *E. coli* extracts has been investigated by our laboratories. The method is to measure the release of encapsulated carboxyfluorescein (CF) from the *E. coli* liposome. The addition of mutant lysozyme to the liposomal suspension induced CF leakage, while addition of the wild-type lysozyme at the same concentration did not

induce the release. The increasing length of polyproline tail enhanced the rate of CF release. Taking into consideration that neither polyproline nor lysozyme alone affected the CF release, the combination of lysozyme with hydrophobic polyproline appears indispensable for membrane perturbation. In addition to the CF leakage experiment, the effect of mutant human lysozymes on the fluorescence quenching of diS-C_3-(5) by valinomycin in the *E. coli* phospholipid liposome has also been investigated by our laboratories. The addition of mutant lysozyme to liposomes hyperpolarized by valinomycin reversed the fluorescence quenching, but the addition of wild-type human lysozyme at the same concentration had no affect on the signal (Ito *et al.,* 2000). The reversal of the fluorescence quenching by mutant human lysozymes may reflect their ability to disrupt the electrochemical potential.

It was also shown that polyproline chain connected to the carboxyl terminal of lysozyme takes a polyproline II-type helix structure, resulting in increased hydrophobicity. Considering that the *p*Ka of human lysozyme is ~11, the protein is positively charged under neutral conditions. A perturbation of membrane potential by the mutant lysozymes could occur either by the hydrophobic interaction of the polyproline tail with lipid membrane or by electrostatic interaction between the positively charged lysozyme and the anionic polar head groups of phospholipids. It has been concluded from these observations that the physico-chemical interaction switches the bactericidal action of lysozyme from Gram-positive bacteria to Gram-negative bacteria.

Chemically engineered bioconjugation

COMPARISON OF CHEMICAL BIOCONJUGATION

Enzymes are conventionally used in organic synthesis (Wong and Whitesides, 1994; Koskinen and Klibanov, 1996) and 'enzymatic polymerization' has become a new field of precision polymerization (Ikeda *et al.,* 1996; Kobayashi *et al.,* 1996). Enzymes catalyze various organic reactions in organic solvents. However, the catalytic activity of enzymes in organic solvents is, in general, orders of magnitude lower than that in an aqueous environment (Dordick, 1992; Klibanov, 1997). The reasons for this low catalytic activity are limited diffusion of enzymes (Yamamoto and Kise, 1993), the partial denaturation of enzymes during lyophilization (Mishra *et al.,* 1996), and the reduced flexibility of proteins (Affleck *et al.,* 1992; Suzawa *et al.,* 1995) in anhydrous solvents.

To overcome these problems, in addition to the employment of lyoprotectants such as sugars (Dabulis and Klibanov, 1993), salts (Khmelnitsky *et al.,* 1994), substrate-resembling ligands (Russell and Klibanov, 1988; Dabulis and Klibanov, 1993), and crown ethers in the process of enzyme preparation, the use of enzymes encapsulated in reverse micelles (Bommarius *et al.,* 1995), coated with surfactant (Paradkar and Dordick, 1994) and fatty-acid (Goto *et al.,* 1994; Okahata *et al.,* 1995), solubilized in organic solvents by the attachment of an amphiphilic polymer (Inada *et al.,* 1995) or hydrophobic polymers (Ito *et al.,* 1992, 1993, 1994), and coupled with crosslinked polymers (Yang *et al.,* 1995; Wang *et al.,* 1997) has been proposed.

PEG has been widely studied for protein modification, increasing solubility on organic solvents (Inada *et al.,* 1995). Koops *et al.* (1999) modified lipase with tresyl-

activated PEG with the aim to improve their catalytic properties in organic solvent. The activated PEG was covalently linked to lysine residues at the surface of the enzyme leading to varying surface hydrophobicity. Bioconjugation of tresyl-activated PEG with lipase increased transesterification activity up to 27-fold in organic solvent.

Bioconjugation of enzymes by means of covalent coupling using soluble polymers results in enzymes that retain high biological activity and display resistance to denaturants and high temperature. Sundaram and Venkatesh (1998) recently reported that α-chymotrypsin covalently modified by reductive alkylation using polymeric sucrose, dextran, and carboxymethyl cellulose retained around 50–80% activity depending on the polymer used and the extent of modification, and the bioconjugated α-chymotrypsin displayed better thermo-tolerance than their native counterpart at 4–14°C. Dordick and co-workers have developed enzyme-containing polymeric materials which have high activity and stability in both aqueous and organic media (Wang *et al.*, 1997; Novick and Dordick, 2000). They synthesized biocatalytic plastics by the coupling of enzymes with acryloyl chloride to provide a polymerizable functionality, subsequently solubilized into organic solvent via hydrophobic ion pairing with surfactant molecules, and followed by polymerization with hydrophobic monomer and crosslinker.

Although many bioconjugation procedures have been devised, the procedures have not been directly compared. Therefore in our laboratories, we have investigated the solubility and secondary structure of subtilisin modified with PEG or the anionic surfactant, aerosol-OT (AOT) in various organic solvents in relation to enzymatic activity. Subtilisin (Carlsberg) modified with PEG (PEG-Sub) (Kwon *et al.*, 1999) or AOT (AOT-Sub) (Wangikar *et al.*, 1997) was synthesized so as to be soluble in organic solvents. About half of the amino acid residues residing on the surface of the subtilisin molecule were modified with PEG: in this 'PEG-Sub' a subtilisin molecule carries about five chains of PEG. The corresponding AOT conjugated subtilisin, 'AOT-Sub' contained about 61 molecules of AOT, a result which indicated that about 30% of the surface of such a subtilisin molecule is covered with AOT.

The solubilities of these modified subtilisins in various organic solvents are summarized in *Table 10.3*. PEG-Sub has been found to be soluble in water and some organic solvents other than alcohol and isooctane. The solubility of PEG-Sub was less than 5 mg/ml in the organic solvents. Because the solubility of PEG depends on the nature of solvents, the solubility of PEG-Sub also depends on the nature of the solvents. The solubility trend of modified subtilisin was the same as that of the modifier, PEG. AOT-Sub, by contrast, was found to be soluble in many kinds of organic solvents (except for acetonitrile) irrespective of solvent polarity. PEG-Sub is less soluble in organic solvent than AOT-Sub. The extent of surface coverage by modifier was found to affect significantly the solubility of subtilisin.

The secondary structures of native and modified subtilisins have been determined by Fourier-transform infrared (FT-IR). To calculate the α-helix content, all FT-IR spectra in the amide I region (1600–1700 cm^{-1}) were resolution-enhanced by Fourier self-deconvolution and followed by Gaussian curve-fitting analysis. The assignment of absorption in the amide I region is based on that reported in previous studies (Jackson and Mantsch, 1991; Görne-Tschelnokow *et al.*, 1993; Dong *et al.*, 1995; Griebenow and Klibanov, 1995, 1997). The secondary structure of both modified subtilisins depended strongly on the solvent used (*Table 10.4*). The conformational

Table 10.3. The solubility of native subtilisin and modified subtilisin with PEG or AOT in various organic solvents

Solvent	Solubility (mg/ml)			Dielectric constant (ε)[c]	Partition coefficient (Log*P*)[d]
	Subtilisin	PEG-Sub[a]	AOT-Sub[b]		
isooctane	~0	0.03	12.2	1.95	4.5
benzene	~0	3.55	11.5	2.27	2.0
tetrahydrofuran	~0	4.15	12.0	7.58	0.49
dichloromethane	~0	2.86	11.8	8.93	1.25
3-methylbutanol	~0	~0	11.3	15.19	–
propanol	~0	~0	11.9	20.45	0.28
acetonitrile	~0	4.49	~0[e]	35.94	−0.33
dimethylsulphoxide	~0	4.77	13.2	46.45	−1.30

[a]The solubility of PEG-Sub was determined by the Coomassie brilliant blue assay (Bradford, 1976).
[b]The solubility of AOT-Sub was determined by the Lowry method as reported by Chin *et al.* (1994).
[c]The dielectric constants were taken from the monograph of Reichardt (1988).
[d]The partition coefficients were taken from the reports of Chin *et al.* (1994) and Chaudhary *et al.* (1996).
[e]In a solvent-swollen state.

Table 10.4. The content (%) of secondary structures of native subtilisin, PEG-Sub, and AOT-Sub in various organic solvents

State and solvents		Native subtilisin			PEG-Sub			AOT-Sub		
		α-Helix	β-Sheet	Disordered	α-Helix	β-Sheet	Disordered	α-Helix	β-Sheet	Disordered
	powder	28± 2	22± 4	50± 3	32± 3	22± 3	46± 3	30± 3	23± 2	47± 3
Water-immiscible	isooctane	29± 2	25± 3	44± 3	31± 2	25± 3	44± 3	33± 3	22± 2	45± 2
	benzene	29± 3	26± 3	45± 4	33± 1	24± 1	43± 2	31± 3	23± 2	46± 2
	dichloromethane	29± 3	25± 2	46± 3	28± 3	25± 4	47± 4	33± 3	18± 2	49± 4
Water-miscible	tetrahydrofuran	29± 3	22± 2	49± 4	25± 2	25± 4	51± 3	21± 2	28± 4	51± 4
	3-methylbutanol	30± 2	27± 4	43± 4	30± 3	26± 4	44± 3	23± 4	26± 2	51± 4
	propanol	30± 1	24± 3	46± 3	34± 1	22± 1	44± 2	23± 3	25± 2	52± 3
	acetonitrile	28± 2	34± 4	38± 4	19± 2	28± 4	55± 4	15± 2	39± 4	44± 5
	dimethylsulphoxide	8± 3	23± 3	69± 4	4± 3	21± 3	75± 4	7± 3	19± 3	74± 4

change did not occur in the water-immiscible nonpolar solvents isooctane, benzene, and dichloromethane. The α-helix contents of PEG-Sub and AOT-Sub were all found to be reduced in tetrahydrofuran, acetonitrile, and dimethylsulphoxide, which are miscible with water. The α-helix content of AOT-Sub in 3-methylbutanol and propanol was also reduced, although the content was not reduced in alcoholic solvents. Considering that the PEG-Sub was insoluble in alcoholic solvents, the conformational change was caused by the solubilization of subtilisin. In addition, the reason that the α-helix content of AOT-Sub was reduced in acetonitrile in spite of the insolubility was considered to be the swelling of AOT-Sub. The α-helix content of native subtilisin, PEG-Sub, and AOT-Sub in dimethylsulphoxide was markedly reduced to 8, 4, and 7%, respectively. This indicates protein unfolding occurring in dimethylsulphoxide to an extent resulting in virtually complete disruption of any secondary structure.

The catalytic activity of the native subtilisin and subtilisins modified by the transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with 1-propanol are shown in *Table 10.5*. Both of the modified subtilisins were more active

Table 10.5. Kinetic parameters of transesterification reaction catalyzed by the native subtilisin, PEG-Sub and AOT-Sub in various organic solvents

Solvent	Subtilisin (suspended)				PEG-Sub (soluble)				AOT-Sub (soluble)			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Active enzyme fraction (%)[a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Active enzyme fraction (%)[a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Active enzyme fraction (%)[a]
isooctane[b]	50	1.20	24 ± 3	30	52	1.4	26± 4[c]	27	30	15.5	517±20	24
benzene	61	0.11	1.8± 0.06	28	35	12.3	352±11	25	37	12.0	324±13	19
dichloromethane	63	0.11	1.7± 0.2	23	42	9.1	216±10	19	60	3.3	55± 5	13
tetrahydrofuran	60	0.21	3.5±0.12	15	70	3.9	56± 4	12	85	0.7	8± 1	9
3-methylbutanol[d]			–				–				–	
propanol[d]			–				–				–	
acetonitrile	58	0.24	4.1±0.1	11	76	2.4	31±3	7	89	0.2	2±0.2[c]	6
dimethylsulphoxide			~0				~0				~0	

[a]Catalytically active fraction of each enzyme preparation determined by active site titration.
[b]Because the solubility of APEE in isooctane is low, 2 M of 1-propanol was added into the reaction.
[c]Suspended state.
[d]The 3-methylbutanol and propanol act as substrates.
[e]In a swollen state.

than native subtilisin in organic solvents. In addition, in the case of modified subtilisins, the catalytic activity was more active in the nonpolar organic solvents than in the polar organic solvents (except for PEG-Sub having very low solubility in isooctane). It was considered that the high activity of modified subtilisin in the water-immiscible nonpolar organic solvent is due to enhanced solubility and to the maintenance of the active conformation of the enzyme. When dimethylsulphoxide was used as a solvent, the catalytic activities of PEG-Sub and AOT-Sub were extremely low, presumably because of the marked conformational change: the reason was considered to be the same as that for native subtilisin.

Although PEG-Sub was found to be less soluble in organic solvents than AOT-Sub, the transesterification activity of PEG-Sub in some solvents (except for isooctane not dissolving PEG-Sub) was found to be higher than that of AOT-Sub. It is conceivable that the AOT modification increases the solubility in organic solvents and allows the water-miscible organic solvent to alter the conformation of AOT-Sub more significantly than that of PEG-Sub. The catalytic activity of enzymes depended both on the solubility and the conformational stability.

In conclusion, the experimental data shown in *Tables 10.3–10.5* reveal that the solubility and catalytic activity of subtilisin have been significantly affected by the chemical modification. Moreover, these data indicate that those of modified subtilisin depended on the nature of the solvent, whereas those of native subtilisin suspended in an organic solvent were not markedly affected.

EFFECT OF PREPARATION CONDITIONS ON BIOCONJUGATED ENZYME

It has been demonstrated that the activity of enzymes in organic solvents depends on the conditions of lyophilization during the preparation. For example, the activity of enzymes lyophilized in organic solvents depends on the pH of the aqueous solution in which these enzymes had previously been dissolved (Zaks and Klibanov, 1988; Yang *et al.*, 1993). Khmelnitsky *et al.* (1994) and Ru *et al.* (2000) have reported that the dramatic enhancement of enzymatic activity in the presence of organic solvents is brought about by the presence of KCl in the lyophilized enzyme. In addition, lyophilization of enzymes from aqueous solutions containing inhibitor or substrate analogue, followed by their removal, has been shown to cause significant enhancement of enzyme activity and substrate selectivity (Russell and Klibanov, 1988; Braco *et al.*, 1990; Ståhl *et al.*, 1991; Dabulis and Klibanov, 1992; Rich and Dordick, 1997). The lyophilization of enzyme usually induces conformational change (Mishra *et al.*, 1996). However, lyoprotectants, such as sorbitol, sucrose, or polyethylene glycol, have been shown to significantly enhance the activity of enzymes in organic media due to the inhibition of conformational change and protection of enzyme molecules from solvent (Dabulis and Klibanov, 1993).

Enzymes that have been chemically modified to be soluble in organic solvents are useful as catalysts of organic reactions. However, the effect of lyophilization conditions on the activity of solubilized enzymes has not been investigated. Therefore our laboratories have focused on the effects of lyophilization conditions such as pH, salt and molecular imprinting on the activity of organic solvent-solubilized subtilisin (PEG-Sub) in benzene in relation to conformational change (Kwon *et al.*, 2000).

In studies by our laboratories, the native subtilisin or PEG-Sub was dissolved in

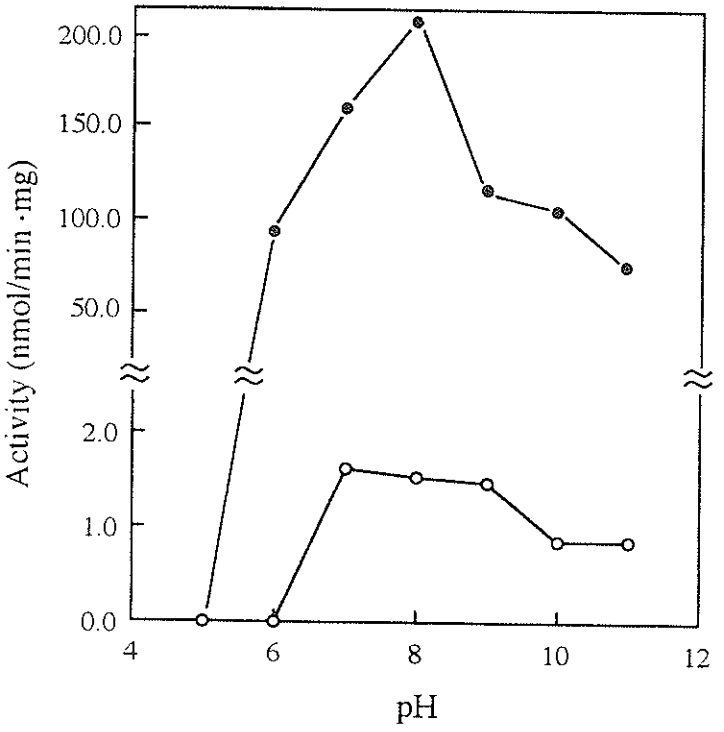

Figure 10.8. Transesterification activity in benzene of the native subtilisin (○) and PEG-Sub (●) lyophilized at different pHs.

aqueous solution of different pH and was subsequently lyophilized: the transesterification activity in benzene was then investigated (*Figure 10.8*). The catalytic activity was found to be profoundly affected by the pH of the aqueous solution from which the subtilisin had been lyophilized. Subtilisin showed the so-called 'pH memory' which has been reported previously (Yang *et al.*, 1993). PEG-Sub was more active than subtilisin and also shows this pH memory. In addition, because the PEG chain enhances the solubility of the enzyme in benzene, the rate of the transesterification reaction was significantly enhanced by the modification with PEG. PEG-Sub lyophilized from acidic solutions retained higher activity than native subtilisin. This result may be due to the fact that a half of amino groups on the surface of the subtilisin molecule were modified with PEG and the net charge of enzyme was thereby changed.

In order to investigate the salt effect on enzymatic activity, the native subtilisin and PEG-Sub were lyophilized in the presence of 95% KCl. The activity of native subtilisin for transesterification reaction of APEE with 1-propanol was found to be dramatically increased by the presence of KCl. The activity of PEG-Sub was also increased by the addition of KCl. The kinetic parameters for the transesterification reaction by the native subtilisin and PEG-Sub lyophilized from 95% aqueous KCl solution or 37.5% of PEG have also been determined (*Table 10.6*). The increase of enzymatic activities by the addition of KCl during lyophilization of the native subtilisin and PEG-Sub appears to be a result of increasing k_{cat} rather than decreasing

Table 10.6. The effect of additives in an aqueous solution on the catalytic parameters of the lyophilized native subtilisin and PEG-Sub in benzene

Content of lyophilized powder (wt%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
100% subtilisin	61 ± 4	0.11 ± 0.01	1.8 ± 0.1
62.5% subtilisin + 37.5% PEG	35 ± 5	0.70 ± 0.09	20.0 ± 0.5
5% subtilisin + 95% of KCl	30 ± 3	3.3 ± 0.1	110 ± 7
5% subtilisin + 2% PEG + 93% KCl	30 ± 6	3.6 ± 0.4	121 ± 12
100% PEG-Sub	35 ± 4	12.3 ± 0.3	351 ± 11
5% PEG-Sub + 95% of KCl	19 ± 2	40.2 ± 0.9	2120 ± 42

K_m. The presence of uncoupled PEG itself enhanced the catalytic activity by 11-fold, but the KCl effect was found to be higher than uncoupled PEG (100-fold). The covalent coupling of PEG enhanced the activity by 10^2 times and the effect was more enhanced by KCl (1000-fold).

The mechanism of salt-induced activation of enzyme in benzene has been explained in two different ways: the salt may protect the enzyme from direct inactivation by the organic solvent, a phenomenon called the 'salt matrix effect' (Khmelnitsky *et al.*, 1994), or the salt may help to maintain the enzyme's native structure during lyophilization – the 'lyoprotectant effect' (Griebenow and Klibanov, 1997). Our laboratories have found that KCl suppressed a decrease of α-helix content of the native subtilisin during lyophilization. PEG also had the same effect on the native subtilisin. However, KCl was more effective in enhancing the catalytic activity of subtilisin than PEG. Therefore, it was considered that the effect of KCl was due to both the salt matrix effect and the lyoprotectant effect.

In addition, KCl was also shown to be effective on PEG-Sub, which is soluble in benzene. When the reaction suspension was separated into soluble and precipitated parts, 98% of PEG-Sub and 5% of KCl were found in the benzene phase and high activity was observed in this phase. The precipitate was almost totally salt and the catalytic activity present derived from the soluble enzyme. Therefore, it was difficult to explain the salt effect on soluble enzyme by the above-mentioned salt matrix effect. It is known that subtilisin catalyzes the transesterification reaction through a charged and highly polar transition state, and that a polar environment stabilized the transition state. Therefore, the increased enzymatic activity of PEG-Sub by KCl may be explained by the formation of a polar environment provided by salts adsorbed on soluble PEG-Sub to favour the polar transition state of the enzymatic reaction, in addition to the lyoprotectant effect.

Transesterification activity of the native subtilisin and PEG-Sub imprinted with *N*-acetyl-L-phenylalanine (*N*-Ac-L-Phe) has also been investigated. Ståhl *et al.* (1991) and Braco *et al.* (1990) have reported that the substrate analogue molecule bound to the active site of enzyme induced a molecular imprinting effect. *Table 10.7* shows that the enzymatic activity of the native subtilisin imprinted with *N*-Ac-L-Phe is 70 times as high as that of non-imprinted counterpart. The effect was found to be stronger than that for PEG or KCl. The presence of both analogue and PEG further enhanced the activity. The increase of enzymatic activity of the imprinted subtilisin is a result of significantly increased k_{cat} plus slightly decreased K_m.

The activity of PEG-Sub has also been found to be enhanced by the presence of

Table 10.7. The catalytic parameters for transesterification reaction catalyzed by the native subtilisin, PEG-Sub, imprinted subtilisin, imprinted subtilisin containing PEG, and imprinted PEG-Sub

Content of lyophilized powder (wt%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
100% subtilisin	61 ± 4	0.11 ± 0.01	1.8 ± 0.1
100% imprinted subtilisin[a]	49 ± 5	6.3 ± 0.2	129 ± 7
62.5% imprinted subtilisin[a] + 37.5% PEG	51 ± 6	9.4 ± 0.6	184 ± 9
100% PEG-Sub	35 ± 4	12.3 ± 0.3	351 ± 11
100% imprinting PEG-Sub[a]	27 ± 5	31.3 ± 0.7	1160 ± 13

[a]The concentration of imprinted molecule, *N*-Ac-L-Phe, was 40 mM.

N-Ac-L-Phe during lyophilization. The increased enzymatic activity comes primarily from increasing k_{cat} and secondarily from decreasing K_m. This result is similar to that of the native subtilisin. It has also been found that imprinting of enzyme with substrate analogue suppressed a decrease of α-helix content of the native subtilisin and PEG-Sub during lyophilization. Griebenow and Klibanov (1997) reported that the effects of the ligands and of lyoprotectants were non-additive, and these workers have suggested the same mechanism of action. It is difficult to explain the molecular mechanism of activity enhancement by the substrate analogue. However, this study clearly indicated that the ligand effect could be exerted on organic solvent-soluble enzymes. These results have demonstrated that the high catalytic activity of organic solvent-soluble enzyme could be further enhanced by appropriate conditions in the lyophilization, similar to that of insoluble native enzyme.

SOLUBILITY CONTROL

Solubilization of enzymes by bioconjugation with polymers is believed to result in more efficient organic catalysis because the diffusion-determining reaction speed in the homogeneous state is higher than that in the heterogeneous state. However, it is costly to recover and re-use solubilized enzyme from a reaction mixture at the completion of the reactions. The stimuli-sensitive polymer-conjugated enzymes can efficiently catalyze various chemical reactions in the soluble state and can be recovered by precipitation in response to the stimulation (Ito, 1999). Some methods of chemical modifications with pH-, ionic strength-, temperature-sensitive polymers for controlling enzyme solubility in aqueous solution have been reported.

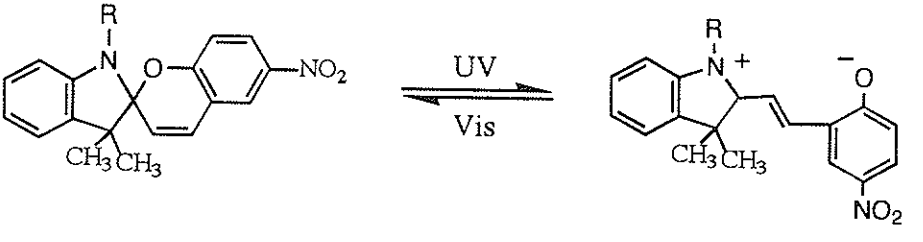

Figure 10.9. Photo-isomerization of spiropyran in response to UV/visible light irradiation.

To regulate the solubility of enzymes by solution pH, Charles *et al.* (1974) have used a biomacromolecule, the polysaccharide alginate, whose solubility depends on pH, for conjugation. A polyelectrolyte complex of poly(methacrylic acid) and poly(4-vinyl-*N*-ethylpyridine) has been used for conjugation with the enzymes (Margolin *et al.*, 1982). The solubility of the complex was shown to depend on the pH or ionic strength of the medium. A poly(methacrylic acid-*co*-methyl acrylate-*co*-methyl methacrylate) was used to prepare hybrid papain and chymotrypsin which was soluble above a pH 5.8 and insoluble below pH 4.8.

Poly(*N*-isopropylacrylamide) (PNIPAAm) exhibits a soluble hydrophilic state below its lower critical solution temperature (LCST) at 32°C in aqueous solution, but it adopts an insoluble hydrophobic state above this LCST. The main mechanism of PNIPAAm's aqueous phase separation is the thermally induced release of water molecules bound to polymer isopropyl side groups, resulting in increasing intra- and intermolecular hydrophobic interactions between isopropyl groups above the LCST. A requisite property for these reversible water soluble–insoluble transitions is a balance of hydrophobic and hydrophilic groups. Thus, the LCST of NIPAAm copolymers can be controlled over a broad temperature range by copolymer compositions, making these polymers very interesting for biotechnological applications.

Several groups (Nguyen and Luong, 1989; Park and Hoffman, 1993) have reported the synthesis and characterization of the temperature-sensitive PNIPAAm-conjugated enzymes. They used copolymer composed of NIPAAm and carboxylic acid-containing vinyl monomer as the temperature-sensitive polymer. Therefore the enzyme is coupled to the polymer through multi-point. On the other hand, the Okano group (Takei *et al.*, 1993; Matsukata *et al.*, 1994) synthesized the polymer–enzyme conjugate consisting of oligo(NIPAAm-co-*N,N*-dimethylacrylamide) copolymer with carboxyl end groups by telomerization and lipase coupled via simple condensation reactions. Those researchers reported that grafting of semi-telechelic oligomers, remaining free at one end, yielded the advantages of high polymer chain mobility and rapid response to temperature changes compared to multi-point conjugation. Chen and Hoffman (1995) have synthesized a polymer conjugate that undergoes marked solubility changes in water in response to temperature and/or pH changes by grafting PNIPAAm chains on to a pH-sensitive backbone.

Stayton *et al.* (1995) have reported that the temperature-sensitive polymer can be used for regulation of enzyme activity without macroscopic solubility change. They controlled the enzyme activity by blocking the active site by using temperature-sensitive polymer grafted near the active site. Ito *et al.* (1992, 1993, 1994) developed another novel method to conjugate a wide range of vinyl polymers with enzymes. They synthesized azo-groups containing enzymes by using carbodiimide, and used as an initiator for graft polymerization of monomers from the enzyme surfaces.

While some methods of chemical modifications for controlling enzyme solubility in aqueous solution have been reported, until now, there have been no reports of modifications to allow solubility control of enzymes in organic media. We considered that photo-irradiation, among many stimuli, is the best method for controlling enzyme solubility in organic solvents, and developed a novel enzyme conjugated with a photoresponsive copolymer to modulate enzyme solubility in organic media (Ito *et al.*, 1999). It is well known that the spiropyran group (nonpolar) is reversibly

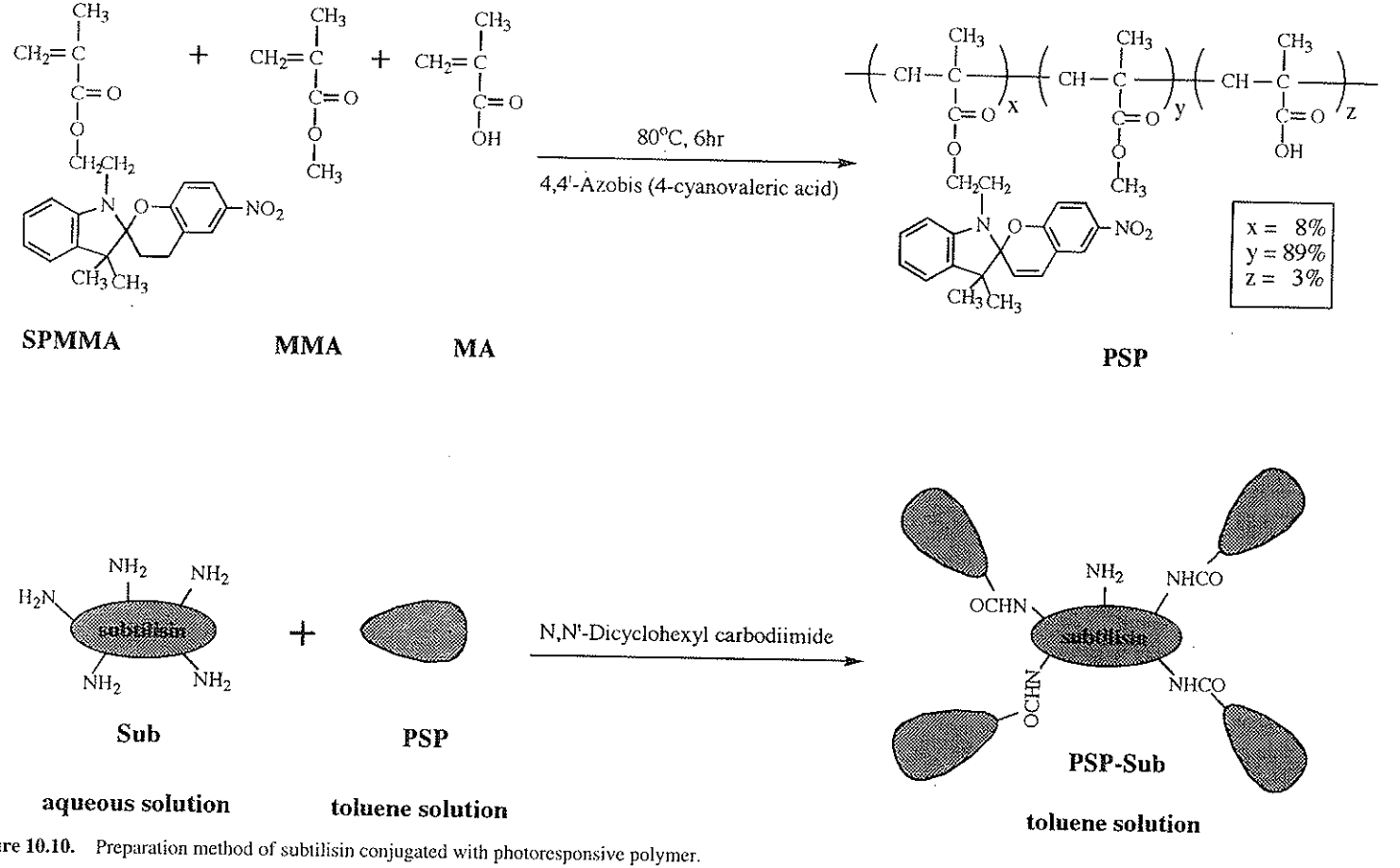

Figure 10.10. Preparation method of subtilisin conjugated with photoresponsive polymer.

converted to the merocyanine form (zwitterion, polar) by ultraviolet and visible light irradiations (*Figure 10.9*).

It has previously been shown that polymers carrying photoresponsive spiropyran groups allow the manipulation of their solubility in organic solvent due to the change in polarity (Chung *et al.*, 1994; Park *et al.*, 1998). The polymer has been used to modify an industrially useful enzyme, subtilisin. Subtilisin was covalently bonded to the photoresponsive terpolymer (PSP) consisting of 1-(β-methacryloxyethyl)-6'-nitro-3,3'-dimethyl-spiro[2H-1-benzopyran]-2,2'-indiline, methyl methacrylate, and methacrylic acid moieties that carry the spiropyran groups and carboxyl groups in its pendant groups as shown in *Figure 10.10*. The enzyme in water and the PSP in toluene were mixed and shaken vigorously in the presence of a coupling agent, *N,N*-dicyclohexylcarbodiimide. After the reaction, the toluene-soluble fraction was recovered. It was confirmed from elution profiles of gel permeation chromatography that most of the PSP in toluene was conjugated with subtilisin to form PSP-Sub.

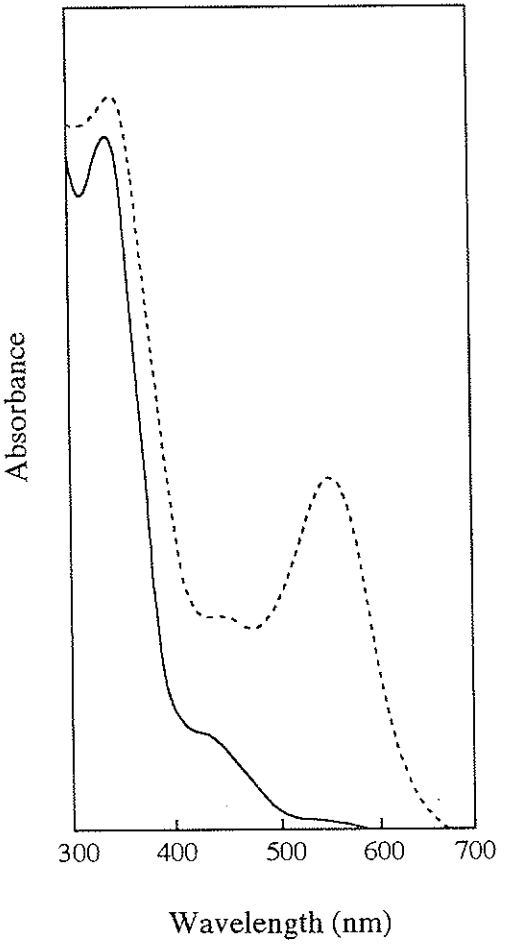

Figure 10.11. Ultraviolet absorbance of PSP-Sub before (solid line) and after (dotted line) UV light irradiation.

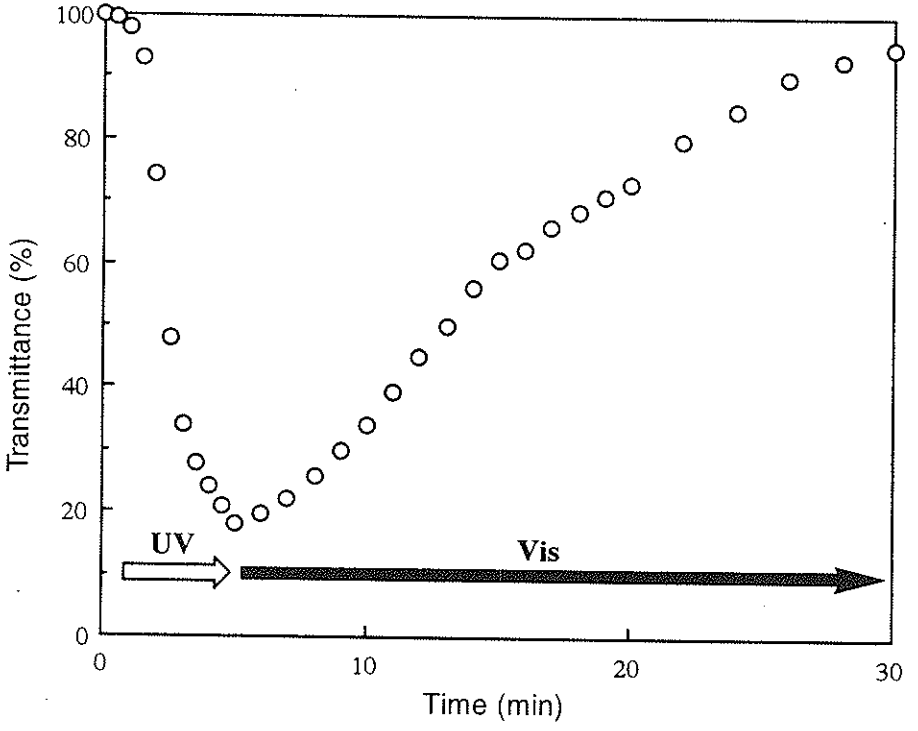

Figure 10.12. Time course of the solubility change of PSP-Sub after UV or visual light irradiation.

The ultraviolet (UV) absorbance of PSP-Sub has been found to be altered by photo-irradiation (*Figure 10.11*). The spiropyran groups were responsible for the peak at 350 nm before UV irradiation. After irradiation, a new peak due to the polar merocyanine form appears at 570 nm, and the PSP-Sub protein becomes insoluble. The appearance of the peak at longer wavelengths is due to electron delocalization (*Figure 10.9*). Visible light irradiation converts the merocyanine form to the spiropyran form, restoring the original spectrum, and solubility in toluene. Precipitated PSP-Sub was quantitatively recovered by centrifugation, and the modified enzyme could be repeatedly and reproducibly cycled through solubilization and precipitation steps.

The time course of solubility change was monitored by measuring turbidity (*Figure 10.12*). Precipitation after UV irradiation took about 5 min, and the visible light-irradiation increased the transmittance associated with solubilization during about 30 min. It was considered that a longer time was required for light to be transmitted through the suspension (precipitate) than through transparent solution (solubilized state).

PSP-Sub, which is soluble in toluene, has been shown to have a higher catalytic activity in the solvent than native subtilisin, which is insoluble (*Table 10.8*). The catalytic activity of PSP-Sub was compared with that of other toluene-soluble subtilisins modified with PEG or AOT. The activity of PSP-Sub was found to be slightly lower than that of PEG-Sub and nearly the same as that of AOT-Sub. The activities of all soluble subtilisins were shown to be more than 100 times that of native subtilisin by reducing the diffusional limitations. The precipitation and solubilization

Table 10.8. Initial transesterification reaction rate for *N*-acetyl-L-phenylalanine ethyl ester (APEE) with 1-propanol by the native subtilisin, PEG-Sub, AOT-Sub, and PSP-Sub in toluene

Enzyme	Repeated use	Initial reaction rate (nmol/mg min)
native subtilisin	–	1.2
PEG-Sub	–	2.1×10^2
AOT-Sub	–	1.7×10^2
PSP-Sub	0	1.3×10^2
	1	1.6×10^2
	2	1.5×10^2
	3	1.4×10^2

cycle of PSP-Sub was repeated, after which the activity was measured. Even after three such cycles, the catalytic activity of PSP-Sub was found not to be reduced. Manipulating their solubility in organic solvents via bioconjugation with stimulus-responsive copolymers will be a general method to extend the use of enzymes in organic synthesis.

Conclusion

In the present review, two methodologies to modify enzymes have been considered: bioconjugation by genetic engineering and bioconjugation by chemical engineering. The genetic engineering strategy is successful in changing the physico-chemical properties of enzymes and hydrophobilization, changes which facilitate regulation of catalytic activity and biological activity of enzymes. For the chemical bioconjugations strategy, conjugation with PEG and AOT modifiers have been compared. The modifier significantly affected the solubility and the catalytic activity of enzymes in organic solvents. In addition, the specific preparation conditions during enzyme modification also affected their activities. Finally, we considered the conjugation of stimuli-responsive polymer to an enzyme to control the solubility in response to stimulation.

The genetically engineered and chemical bioconjugation of enzymes will expand the utility of enzyme technology such as drug design, design of novel catalysts and enzyme biosensor devices.

References

AFFLECK, R., XU, Z-F., SUZAWA, V.M., FOCHT, K., CLARK, D.S. AND DORDICK, J.S. (1992). Enzymatic catalysis and dynamics in low-water environments. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 1100–1104.

ASLAM, M. AND DENT, A. (1998). *Bioconjugation. Protein coupling techniques for the biomedical sciences*. London: Macmillan Reference Ltd.

BOMMARIUS, A.S., HATTON, T.A. AND WANG, D.I.C. (1995). Xanthine oxidase reactivity in reversed micellar system: a contribution to the prediction of enzymatic activity in organized media. *Journal of American Chemical Society* **117**, 4515–4523.

BRACO, L., DABULIS, K. AND KLIBANOV, A.M. (1990). Design of novel receptors by molecular imprinting of proteins. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 274–277.

BRADFORD, N.M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

BRYAN, P.N., ROLLENCE, M.L., PANTOLIANO, M.W., WOOD, J., FINZEL, B.C., GILLILAND, G.L., HOWARD, A.J. AND POULOS, T.L. (1986). Proteases of enhanced stability: characterization of a thermostable variant of subtilisin. *Proteins* **1**, 326–334.

CHARLES, M., COUGHLIN, R.W. AND HASSELBERGER, F.X. (1974). Soluble-insoluble enzyme catalysts. *Biotechnology and Bioengineering* **16**, 1553–1556.

CHAUDHARY, A.K., KAMAT, S.V., BECKMAN, E.J., NUROK, D., KLEYLE, R.M., HAJDU, P. AND RUSSELL, A.J. (1996). Control of subtilisin substrate specificity by solvent engineering in organic solvents and supercritical fluoroform. *The Journal of American Chemical Society* **118**, 12891–12901.

CHEN, G. AND HOFFMAN, A.S. (1995). Graft copolymers that exhibit temperature-induced phase transitions over a wide range of pH. *Nature* **373**, 49–52.

CHEN, H-M. AND CHEN, K-T. (2000). Production and characterization of glutathione-S-transferase fused with a poly-histidine tag. *Enzyme and Microbial Technology* **27**, 219–226.

CHEN, K. AND ARNOLD, F.H. (1993). Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 5618–5622.

CHIN, J.T., WHEELER, S.L. AND KLIBANOV, A.M. (1994). On protein solubility in organic solvents. *Biotechnology and Bioengineering* **44**, 140–145.

CHUNG, D.-J., ITO, Y. AND IMANISHI, Y. (1994). Preparation of porous membranes grafted with poly(spiropyran-containing methacrylate) and photocontrol of permeability. *Journal of Applied Polymer Science* **51**, 2027–2033.

DABULIS, K. AND KLIBANOV, A.M. (1992). Molecular imprinting of proteins and other macromolecules resulting in new adsorbents. *Biotechnology and Bioengineering* **39**, 176–185.

DABULIS, K. AND KLIBANOV, A.M. (1993). Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants. *Biotechnology and Bioengineering* **41**, 566–571.

DONG, A., PRESTRLSKI, S.J., ALLISON, S.D. AND CARPENTER, J.F. (1995). Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. *Journal of Pharmaceutical Science* **84**, 415–424.

DORDICK, J.S. (1992). Designing enzymes for use in organic solvents. *Biotechnology Progress* **8**, 259–267.

GÖRNE-TSCHELNOKOW, U., NAUMANN, D., WEISE, C. AND HUCHO, F. (1993). Secondary structure and temperature behaviour of acetylcholinesterase studies by Fourier-transform infrared spectroscopy. *European Journal of Biochemistry* **213**, 1235–1242.

GOTO, M., KAMIYA, N., MIYATA, M. AND NAKASHIO, F. (1994). Enzymatic esterification by surfactant-coated lipase in organic media. *Biotechnology Progress* **10**, 263–268.

GRIEBENOW, K. AND KLIBANOV, A.M. (1995). Lyophilization-induced reversible changes in the secondary structure of proteins. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 10969–10976.

GRIEBENOW, K. AND KLIBANOV, A.M. (1997). Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin Carlsberg in organic solvents? *Biotechnology and Bioengineering* **53**, 351–362.

IBRAHIM, H.R., YAMADA, M., MATSUSHITA, K., KOBAYASHI, K. AND KATO, A. (1994). Enhanced bactericidal action of lysozyme to *Escherichia coli* by inserting a hydrophobic pentapeptide into its C terminus. *Journal of Biological Chemistry* **269**, 5059–5063.

IKEDA, R., SUGIHARA, J., UYAMA, H. AND KOBAYASHI, S. (1996). Enzymatic oxidation polymerization of 2,6-dimethylphenol. *Macromolecules* **29**, 8702–8705.

INADA, Y., FURUKAWA, M., SASAKI, H., KODARA, Y., HIROTO, M., NISHIMURA, H. AND MATSUSHIMA, A. (1995). Biomedical and Biotechnological applications of PEG- and PM-modified proteins. *TIBTECH* **13**, 86–91.

ITO, Y. (1999). Solubility control of enzyme by conjugation with stimuli-responsive polymers.

Journal of Biomaterials Science. Polymer Edition **10**, 1237–1250.

ITO, Y., FUJII, H. AND IMANISHI, Y. (1992). Lipase modification by various synthetic polymers for use in chloroform. *Biotechnology Letters* **14**, 1149–1152.

ITO, Y., FUJII, H. AND IMANISHI, Y. (1993). Catalytic peptide synthesis by trypsin hybridized with polystyrene in chloroform. *Biotechnology Progress* **9**, 128–130.

ITO, Y., FUJII, H. AND IMANISHI, Y. (1994). Modification of lipase with various synthetic polymers and their catalytic activities in organic solvent. *Biotechnology Progress* **10**, 398–402.

ITO, Y., KWON, O.H., UEDA, M., TANAKA, A. AND IMANISHI, Y. (1997). Bactericidal activity of human lysozymes carrying various lengths of polyproline chain at the C-terminus. *FEBS Letters* **415**, 285–288.

ITO, Y., SUGIMURA, N., KWON, O.H. AND IMANISHI, Y. (1999). Enzyme modification by polymers with solubilities that change in response to photoirradiation in organic media. *Nature Biotechnology* **17**, 73–75.

ITO, Y., KWON, O.H., UEDA, M., TANAKA, A. AND IMANISHI Y. (2000). Switching in bactericidal activity of lysozyme by gene-engineered hydrophobilization. *Journal of Biocompatible and Active Polymers* **15**, 376–395.

JACKSON, M. AND MANTSCH, H.H. (1991). Beware of proteins in DMSO. *Biochimica et Biophysica Acta* **1078**, 231–235.

JOLLÈS, P. AND JOLLÈS, J. (1984). What's new in lysozyme research? *Molecular and Cellular Biochemistry* **63**, 165–198.

KHMELNITSKY, Y.L., WELCH, S.H., CLARK, D.S. AND DORDICK, J.S. (1994). Salts dramatically enhance activity of enzymes suspended in organic solvents. *Journal of American Chemical Society* **116**, 2647–2648.

KLIBANOV, A.M. (1997). Why are enzymes less active in organic solvents than in water? *Trends in Biochemical Science* **15**, 97–101.

KOBAYASHI, S., KIYOSADA T. AND SHODA, S. (1996). Synthesis of artificial chitin: Irreversible catalytic behavior of a glycosyl hydrolase through a transition state analogue substrate. *Journal of American Chemical Society* **118**, 13113–13114.

KOOPS, B.C., VERHEIJ, H.M., SLOTBOOM, A.J. AND EGMOND, M.R. (1999). Effect of chemical modification on the activity of lipases in organic solvents. *Enzyme and Microbial Technology* **25**, 622–631.

KOSKINEN, A.M.P. AND KLIBANOV, A.M. (1996). *Enzymatic reactions in organic media.* London: Blackie.

KWON, O.H., ITO, Y., UEDA, M., TANAKA, A. AND IMANISHI, Y. (1998a). Hydrophobilization of an esterase by genetic combination with polyproline at the carboxyl terminal. *Biotechnology and Bioengineering* **59**, 583–586.

KWON, O.H., ITO, Y., UEDA, M., TANAKA, A. AND IMANISHI, Y. (1998b). Hydrophobilization of esterase by genetic combination with polyproline or polytyrosine at the carboxyl terminal. *Biochimica et Biophysica Acta* **1388**, 239–246.

KWON, O.H., IMANISHI, Y. AND ITO, Y. (1999). Catalytic activity and conformation of chemically modified subtilisin Carlsberg in organic media. *Biotechnology and Bioengineering* **66**, 265–270.

KWON, O.H., IMANISHI, Y. AND ITO, Y. (2000). Enhancement of catalytic activity of chemically modified subtilisin Carlsberg in benzene by adjustment of lyophilization conditions. *Bulletin of Chemical Society, Japan* **73**, 1277–1282.

LEDENT, P., DUEZ, C., VANHOVE, M., LEJEUNE, A., FONZÉ, E., CHARLIER, P., RHAZI-FILALI, F., THAMM, I., GUILLAUME, G., SAMYN, B., DEVREESE, B., VAN BEEUMEN, J., LAMOTTE-BRASSEUR, J. AND FRÈRE, J-M. (1997). Unexpected influence of a C-terminal-fused His-tag on the processing of an enzyme and on the kinetic and folding parameters. *FEBS Letters* **413**, 194–196.

MARGOLIN, A.L., IZUMRUDOV, V.A., SVEDAS, V.K. AND ZEZIN, A.B. (1982). Soluble-insoluble immobilized enzymes. *Biotechnology and Bioengineering* **24**, 237–240.

MATSUKATA, M., TAKEI, Y., AOKI, T., SANUI, K., OGATA, T., SAKURAI, Y. AND OKANO, T. (1994). Temperature modulated solubility-activity alterations for poly(*N*-isopropylacrylamide)-lipase conjugates. *Journal of Biochemistry* **116**, 682–686.

MATSUSHIMA, A., OKADA, M. AND INADA, Y. (1984). Chymotrypsin modified with polyethylene glycol catalyzes peptide synthesis reaction in benzene. *FEBS Letters* **178**, 275–277.

MISHRA, P., GRIEBENOW, K. AND KLIBANOV, A.M. (1996). Structural basis for the molecular memory of imprinted proteins in anhydrous media. *Biotechnology and Bioengineering* **52**, 609–614.

MURRY, R.G.E., STEED, P. AND ELSON, H.H. (1965). The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other Gram-negative bacteria. *Canadian Journal of Microbiology* **11**, 547–560.

NGUYEN, A.L. AND LUONG, J.H.T. (1989). Synthesis and applications of water-soluble reactive polymers for purification and immobilization of biomolecules. *Biotechnology and Bioengineering* **34**, 1186–1190.

NOVICK, S.J. AND DORDICK, J.S. (2000). Investigation of the effects of polymer chemistry on activity of biocatalytic plastic materials. *Biotechnology and Bioengineering* **68**, 655–671.

OKAHATA, Y., FUJIMOTO, Y. AND IJIRO, K. (1995). A lipid-coated lipase as an enantioselective ester synthesis catalyst in homogeneous organic solvents. *Journal of Organic Chemistry* **60**, 2244–2250.

PARADKAR, V.M. AND DORDICK, J.S. (1994). Mechanism of extraction of chymotrypsin into isooctane at very low concentrations of aerosol OT in the absence of reversed micelles. *Biotechnology and Bioengineering* **43**, 529–540.

PARK, T.G. AND HOFFMAN, A.S. (1993). Synthesis and characterization of a soluble temperature-sensitive polymer-conjugated enzyme. *Journal of Biomaterials Science. Polymer Edition* **4**, 483–504.

PARK, Y.S., ITO, Y. AND IMANISHI, Y. (1998). Photo-controlled gating by polymer brushes grafted on porous glass filter. *Macromolecules* **18**, 2418–2422.

REICHARDT, C. (1988). *Solvents and solvent effects in organic chemistry*, 2nd ed. Weinheim: VCH.

RICH, J.O. AND DORDICK, J.S. (1997). Controlling subtilisin activity and selectivity in organic media by imprinting with nucleophilic substrates. *Journal of American Chemical Society* **119**, 3245–3252.

RU, M.T., HIROKANE, S.Y., LO, A.S., DORDICK, J.S., REIMER, J.A. AND CLARK, D.S. (2000). On the salt-induced activation of lyophilized enzymes in organic solvents: Effect of salt kosmotropicity on enzyme activity. *Journal of American Chemical Society* **122**, 1565–1571.

RUSSELL, A.J. AND KLIBANOV, A.M. (1988). Inhibitor-induced enzyme activation in organic solvents. *Journal of Biological Chemistry* **263**, 11624–11626.

SEARS, P., SCHUSTER, M., WANG, P., WITTE, K. AND WONG, C-H. (1994). Engineering subtilisin for peptide coupling: Studies on the effects of counterions and site-specific modifications on the stability and specificity of the enzyme. *Journal of American Chemical Society* **116**, 6521–6530.

STÅHL, M., JEPPSSON-WISTRAND, U., MÅNSSON, M. AND MOSBACH, K. (1991). Induced-stereoselectivity and substrate selectivity of bio-imprinted α-chymotrypsin in anhydrous organic media. *Journal of American Chemical Society* **113**, 9366–9368.

STAYTON, P.S., SHIMBOJI, T., LONG, C., CHILKOTI, A., CHEN, G., HARRIS, J.M. AND HOFFMAN, A.S. (1995). Control of protein-ligand recognition using a stimuli-responsive polymer. *Nature* **378**, 472–474.

STEMPFER, G., HÖLL-NEUGEBAUER, B., KOPETZKI, E. AND RUDOLPH, R. (1996). A fusion protein designed for noncovalently immobilization: stability, enzymatic activity, and use in an enzyme reactor. *Nature Biotechnology* **14**, 481–484.

SUNDARAM, P.V. AND VENKATESH, R. (1998). Retardation of thermal and urea induced inactivation of alpha-chymotrypsin by modification with carbohydrate polymers. *Protein Engineering* **11**, 699–705.

SUZAWA, V.M., KHMELNITSKY, Y.L., GIARTO, L., DORDICK, J.S. AND CLARK, D.S. (1995). Suspended and immobilized chymotrypsin in organic media: structure–function relationships by electron spin resonance spectroscopy. *Journal of American Chemical Society* **117**, 8435–8440.

TAKEI, Y.G., AOKI, T., SANUI, K., OGATA, N., OKANO, T. AND SAKURAI, Y. (1993). Temperature-

responsive bioconjugates. 1. Synthesis of temperature responsive oligomers with reactive end groups and their coupling to biomolecules. *Bioconjugated Chemistry* **4**, 42–46.

WANG, P., SERGEEVA, M.V., LIM, L. AND DORDICK, J.S. (1997). Biocatalytic plastics as active and stable materials for biotransformations. *Nature Biotechnology* **15**, 789–793.

WANGIKAR, P.P., GRAYCAR, T.P., ESTELL, D.A., CLARK, D.S. AND DORDICK, J.S. (1993). Protein and solvent engineering of subtilisin BPN' in nearly anhydrous organic media. *Journal of American Chemical Society* **115**, 12231–12237.

WANGIKAR, P.P., RICH, J.O., CLARK, D.S. AND DORDICK, J.S. (1995). Probing enzymatic transition state hydrophobicities. *Biochemistry* **34**, 12302–12310.

WANGIKAR, P.P., MICHELS, P.C., CLARK, D.S. AND DORDICK, J.S. (1997). Structure and function of subtilisin BPN' solubilized in organic solvents. *Journal of American Chemical Society* **119**, 70–76.

WILKINSON, R.G., GEMSKI, P. AND STOCKER, B.A.D. (1972). Non-smooth mutants of *Salmonella typhimurium*: Differentiation by phase sensitive and genetic mapping. *Journal of Genetic Microbiology* **70**, 527–554.

WONG, C-H. AND WHITESIDES, G.M. (1994). *Enzymes in synthetic organic chemistry*. Oxford; Pergamon.

YAMAMOTO, Y. AND KISE, H. (1993). Catalysis of enzyme aggregates in organic solvents: an attempt at evaluation of intrinsic activity of proteases in ethanol. *Biotechnology Letters* **15**, 647–652.

YANG, Z., ZACHERL, D. AND RUSSELL, A.J. (1993). pH dependence of subtilisin dispersed in organic solvents. *Journal of American Chemical Society* **115**, 12251–12257.

YANG, Z., MESIANO, A.J., VENKATASUBRAMANIAN, S., GROSS, S.H., HARRIS, J.M. AND RUSSELL, A.J. (1995). Activity and stability of enzymes incorporated into acrylic polymers. *Journal of American Chemical Society* **117**, 4843–4850.

ZAKS, A. AND KLIBANOV, A.M. (1988). Enzymatic catalysis in aqueous solvents. *Journal of Biological Chemistry* **263**, 3194–3201.