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Review

Bioconjugates of proteins and polyethylene glycol: potent tools in biotechnological processes

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Abstract

Chemical modification of enzymes and other bioactive molecules with polyethylene glycol derivatives, activated PEG and PM, can eliminate some of the drawbacks of the biomolecules and/or give them new functions in biotechnological processes. PEG- or PM-lipase becomes soluble and active in organic solvents so that the reverse reactions of hydrolysis proceed effectively, not only in organic media but also in straight substrates without any solvent. These include ester synthesis and ester exchange reactions including lactone synthesis and optical resolution. Enzymes such as lipase and asparaginase modified with activated PMs gain stabilization towards heat and urea denaturation and, for asparaginase in vivo, prolongation of clearance time. Photostabilization of natural pigments, magnetization of enzymes and effective affinity partitioning are achieved by modification with PEG derivatives.

Keywords: Bioconjugate; Polyethylene glycol; Chemical modification

1. Introduction

Chemical modification of proteins became commonplace in the late 1950s: the techniques were originally developed to aid in the structural analysis of protein molecules. The purpose of such modifications was to investigate the physicochemical states of various amino acid residues in a protein molecule and to identify the amino acid residues participating in a particular protein function. Since the late 1970s, many papers on the chemical modification of proteins with synthetic macromolecules have been published. The purpose of these studies was to eliminate disadvantageous properties and/or to confer new functions on native proteins. Among such studies, chemical modification of proteins with polyethylene glycol (PEG), a linear, non-toxic, non-immunogenic and amphipathic polymer has been extensively studied as a potential means to add unique properties to proteins. In biomedical fields [1–8], exogenous enzymes, L-asparaginase with anti-tumor activity and adenosine deaminase (ADA) that ameliorates the severe combined immunodeficiency of ADA-defi-

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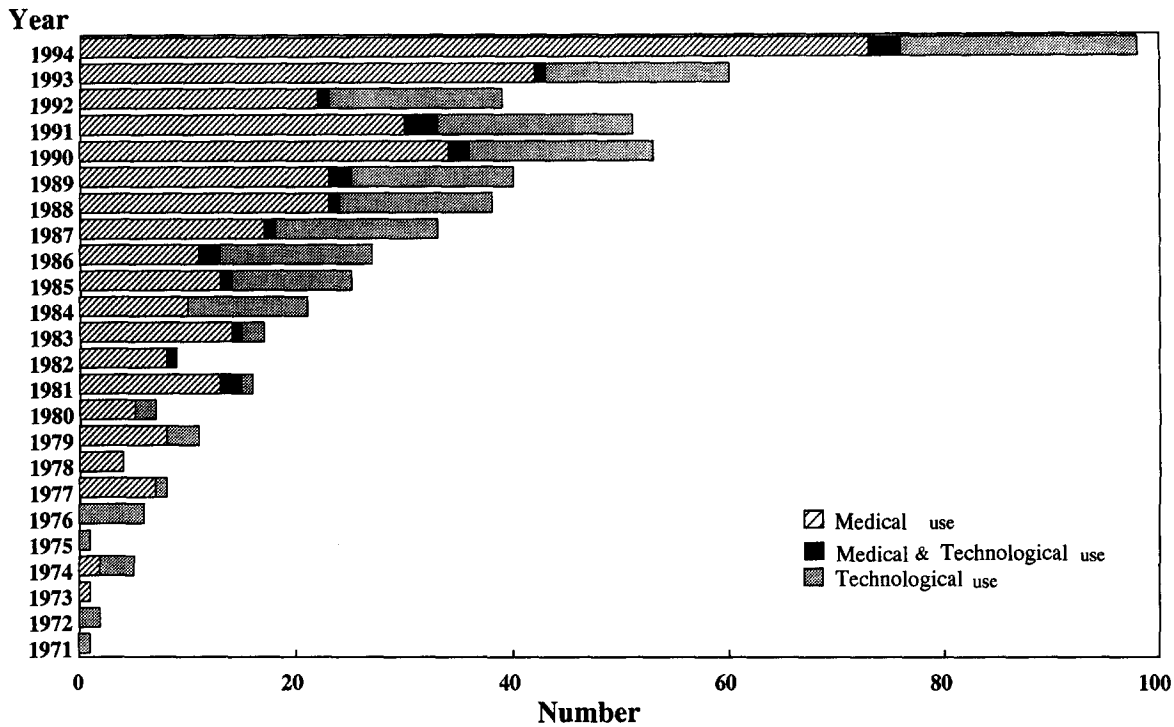

Fig. 1. The number of reports that have appeared by year on the chemical modification of proteins with PEG.

cient patients, were successfully modified with PEG to reduce their immunoreactivity and immunogenicity. The plasma half-lives of these enzymes were markedly prolonged by the modification when the enzymes were administered into the circulation system of animals or patients [5]. In biotechnological fields [1–4,7–9], PEG-modified hydrolases became soluble and active in hydrophobic media and catalyzed the reverse reactions of hydrolysis, ester synthesis and ester exchange reactions in organic solvents. The number of reports on chemical modification of proteins with PEG has been increasing yearly as is shown in Fig. 1.

This review deals with recent studies on the synthesis of PEG derivatives suitable for the modification of enzymes as well as potential application of PEG–enzymes in biotechnological processes.

2. Synthesis of polyethylene glycol derivatives [3]

Proteins can be modified with activated polyethylene glycol derivatives which are usually synthesized from monomethoxypolyethylene glycol with one hydroxyl group at the end of the molecule as a handhold for the derivatization. Syntheses of various PEG derivatives conjugating with primary amino groups or sulfhydryl groups were summarized by Harris [9]. In this review, the syntheses of two types of modifiers (Fig. 2) suitable for the preparation of PEG–protein conjugates used in biotechnological as well as biomedical applications are described. One is of a chain-shaped 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine-activated PEG_2 (Fig. 2a); and another is of a comb-shaped copolymer of maleic anhydride and a monomethoxypolyethylene glycol derivative,

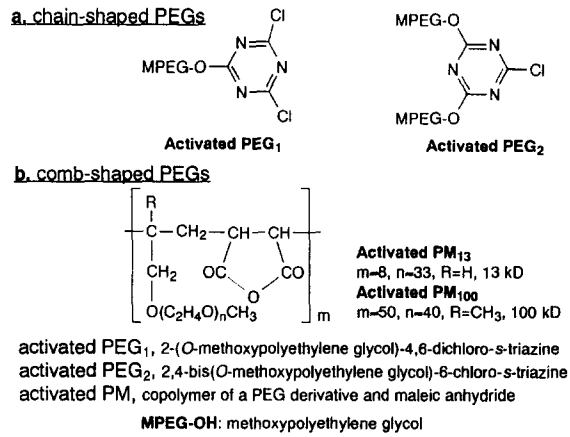

Fig. 2. Two types of modifiers: (a) chain-shaped activated PEG_1 and activated PEG_2; (b) comb-shaped activated PM_{13} and activated PM_{100}.

activated PM (Fig. 2b). Each modifier reacts with mainly the ϵ-amino group of lysine residues and/or N-terminal amino group(s) in a protein molecule.

2.1. Synthesis of activated PEG_2

Activated PEG_2, 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine, had been synthesized by Matsushima et al. [10] from monomethoxypolyethylene glycol and cyanuric chloride in benzene using sodium carbonate as a catalyst. Recently, Ono et al. [11] obtained the activated PEG_2 in homogeneous state by using zinc oxide as a catalyst. This modifier does not contain any by-products such as 2-(*O*-methoxypolyethylene glycol)-4,6-dichloro-*s*-triazine (activated PEG_1) (Fig. 2a) and its polymerized macromolecules. The time-course of activated PEG_2 synthesis using zinc oxide is shown in Fig. 3. Because the activated PEG_2 has two polyethylene glycol chains in the molecule, two PEG chains can be introduced to one amino group in a protein. Therefore, with activated PEG_2, effective

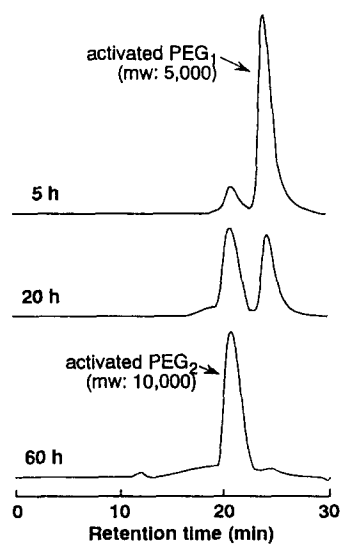

Fig. 3. Time-course of activated PEG_2 synthesis using zinc oxide. The product was analyzed by gel filtration HPLC equipped with a TSKgel G3000SW column.

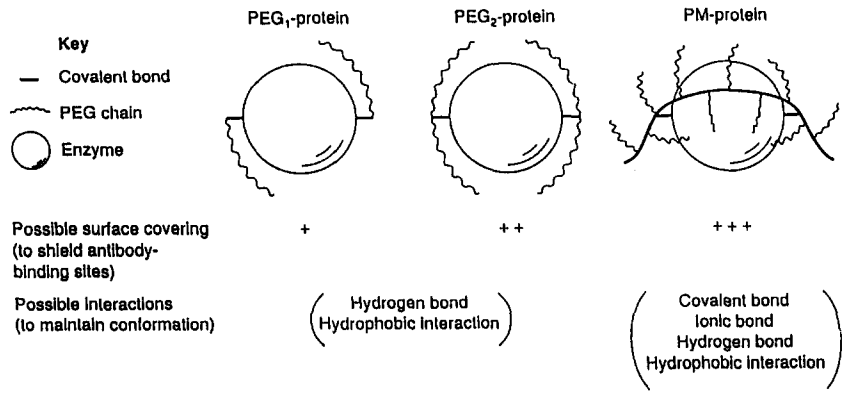

Fig. 4. Putative models of the structural relationship between the modifiers and the protein (reproduced from [5]).

modification of a protein is achieved in comparison with activated PEG_1, which has one PEG chain in the molecule [12].

2.2. *Synthesis of activated PM:*

Activated PM, a copolymer of maleic anhydride and PEG derivative, has a comb-shaped form with multi-valent reactive groups [13]. There are two kinds of activated PM (Fig. 2b): activated PM_{13} (MW:13,000, $m \approx 8$, $n \approx 33$, R = H) and activated PM_{100} (MW:100,000, $m \approx 50$, $n \approx 40$, $R = CH_3$). Amino groups in a protein are directly coupled with maleic anhydride in the PM modifier through acid–amide bonds. These comb-shaped modifiers have a unique structure, covering the surface of a protein molecule and endowing anionic groups ($-COO^-$) to the surface of a protein molecule (Fig. 2b and Fig. 4).

3. Biotechnological application of PEG–enzymes

In biotechnological process, hydrolases such as esterase, lipase and protease have profound potential for applications in organic synthesis [14]. To accomplish the reaction conditions that favor ester or amide formation rather than hydrolysis, investigators have explored several reaction systems in which enzymes are suspended in organic media [15], or in which lipids are complexed (emulsified) with enzymes [16]. However, as reactions proceed in non-homogeneous states, catalytic reactions in these systems are usually difficult to be controlled. During the course of studies on enzyme-catalyzed reaction in organic solvents, we find that enzymes modified with PEG become soluble and retain the enzymic activity in organic solvents such as benzene, toluene or chlorinated hydrocarbons [8,17–19]. Since PEG is an amphipathic macromolecule, its hydrophilic nature makes it possible to modify enzymes in aqueous solutions, and its hydrophobic nature makes the modified enzymes soluble in a hydrophobic environment.

Application of PEG-modified hydrolases to biotechnological processes may have the following advantages:

1. Synthesis of esters (amides) from carboxylic acids and alcohols (amines) can be catalyzed by PEG–enzymes in a homogeneous organic solvent.

2. Ester synthesis and ester exchange reactions can often be catalyzed without the use of hazardous organic solvents (the substrate needs to be a liquid).

3. Hydrolytic activity of PEG–enzymes towards non-polar esters (amides) is often higher than the unmodified enzymes.

4. Stereospecific ester synthesis or optical resolution of racemic esters can be catalyzed in hydrophobic conditions.

5. Esters (amides) of unstable compounds can be synthesized in mild conditions (at room temperature).

6. PEG–enzymes show increased thermostability in both hydrophobic and aqueous media.

The question has arisen whether unmodified hydrolases catalyzes effectively the reverse reactions of hydrolysis in an organic solvent or in pure substrates. Klibanov [87] reported that porcine pancreatic lipase catalyzes the transesterification reaction (between tributyrin and various primary and secondary alcohols) in a 99% organic medium. Its reaction system consist of 10 ml of alcohol containing 100 mg of lipase powder in the presence of 0.015% water. The reverse reaction of hydrolysis with PEG–lipase proceeds with 0.1–4 mg/10 ml of solvent. Furthermore, alcoholysis of ϵ-decalactone with PEG–lipase proceeded 20 times faster than that with unmodified lipase (see Fig. 7 c).

The main disadvantage is that the enzymic activity of PEG–enzyme is markedly reduced using a substrate with a high molecular weight. In fact, PEG–thrombin has little activity towards its natural substrate, fibrinogen with molecular weight of 340,000. It may be explained that the active site of thrombin is covered with PEG molecules which disturbs the formation of the enzyme–substrate complex. In addition, the modified enzyme is not always homogeneous especially in the number and in amino acid residues of the PEG attachment. Genetic engineering may be a good tool to resolve the non-homogeneity.

Generally, the success in enzyme modification with PEG derivatives depends upon the degree of modification of the amino groups in each enzyme molecule. Too low a degree of modification would not bring about sufficient solubilization of proteins in organic solvents, while too high a degree of modification would cause needless reduction of enzymic activity because of distortion of the protein conformation. The degree of modification of amino groups in a protein molecule can be adjusted by changing the amount of the modifier added to the enzyme solution. PEG–enzymes thus prepared are summarized in Table 1.

Table 1
PEG–enzymes for biotechnological use

1.	**Oxidoreductase** catalase [4,20], dehydrogenases [21,22], glucose oxidase [23], peroxidase [23,24]
2a	**Esterase** lipase [25–33], phospholipase [34,35]
2b	**Protease** alkaline proteinase [36], chymotrypsin [37–47], papain [41,42,46,48–53], pepsin [42], subtilisin [54], thermolysin [42,46], trypsin [41,42,55–58]
2c	**Glycosidase** cellulase [59], β-galactosidase [60]
3	**Miscellaneous** NAD(P) [21,22,61–66], lignin [67]

3.1. PEG–lipase

3.1.1. Properties of PEG_2–lipase

Modified hydrolytic enzymes such as lipase (*Pseudomonas fluorescens*) [68] effectively catalyze the reverse reaction of hydrolysis, ester synthesis and ester exchange reactions in water-immiscible organic solvents such as benzene, toluene, chloroform and 1,1,1-trichloroethane as shown below.

$$R_1COOH + R_2OH \rightarrow R_1COOR_2 + H_2O$$
$$R_1COOR_2 + R_3COOR_4 \rightarrow R_1COOR_4 + R_3COOR_2$$

The ester synthetic activity of PEG_2–lipase (*P. fluorescens*) in 1,1,1-trichloroethane was found to be almost 3.6 times higher than that in benzene or toluene [69]. However, a trace amount of water in the water-immiscible organic solvents was needed to achieve these activities. Little activity was detected in water-miscible solvents such as acetone and *N*,*N*-dimethylformamide [70]. The water molecules may be absorbed onto the surface of the protein molecule, so that the protein conformation could be maintained. Therefore, organic solvents saturated with water are recommended in reverse reactions of hydrolysis with PEG_2–lipase. PEG_2–lipase also catalyzes ester exchange in straight substrates without solvents, when substrates are liquid at the reaction temperature.

PEG_2–lipase exhibits high stability in benzene. Thus PEG_2–lipase still retained about 50% of its original activity for amyl laurate synthesis after storage at room temperature for 3 months, and about 40% activity even after 140 days [71]. PEG_2–lipase can be recovered from the reaction mixture as a precipitate by addition of *n*-hexane or petroleum ether [71].

Recent reports on PEG– or PM–lipases as well as PEG–proteases are listed in Table 2.

3.1.2. Optical resolution

From a kinetic study of the esterification of chiral secondary alcohols with a fatty acid, PEG_2–lipase from *Pseudomonas fragi* 22.39B was found to catalyze the acylation of (*R*)-isomers preferentially [25]. As is shown in Table 3, K_m and V_{max} values for the (*R*)-isomers are relatively constant even upon changing the number of carbon atoms (C_4–C_9) in the secondary alcohol. On the other hand, the K_m value for the (*S*)-isomers is increased, and the V_{max} value is lowered by increasing the number of carbon atoms in the alcohol. Each (*R*)-isomer displays a smaller K_m value and a higher V_{max} value than the corresponding (*S*)-isomer. In Fig. 5 is shown the result of a similar study on the optical resolution of (*RS*)-α-phenylethanol with dodecanoic acid and PEG_2–lipase in 1,1,1-trichloroethane. Only the (*R*)-isomer is esterified with PEG_2–lipase. The optical purity of the unreacted alcohol, (*S*)-α-phenylethanol, reaches a 99% enantiomeric excess in 7 h.

3.1.3. Lactone synthesis and optical resolution of lactones

Lactones are bioactive substances widely utilized as components of fragrances, macrolide antibiotics and pharmaceuticals. PEG_2–lipase from *Pseudomonas cepacia* catalyzes lactone synthesis from 16-hydroxyhexadecanoic acid ethyl ester in 1,1,1-trichloroethane to form a lactone, 16-hexadecanolide (Fig. 6a and b) [31]. The optimum temperature for yielding the lactone is 65–70°C. Because lactone synthesis occurs by intramolecular transesterification, the efficiency of the reaction depends on the substrate concentration. At 1 mM concentration, 92% of the substrate was converted into the lactone (Fig. 6c). As the substrate concentration is increased, the yield of lactone is reduced, while the concentration of lactone synthesized is markedly increased. Synthesized lactone concentrations are 4.8 mM (48% yield) and 11 mM (11% yield) when 10 and 100 mM substrate are used, respectively.

Table 2
Application of modified hydrolases (lipase and protease) with PEG derivatives to biotechnological processes

Modified enzyme	Reaction	Solvent [a]	Purpose	Ref.
PM_{13}–lipase (Pf)	lauryl alcohol + stearic acid → lauryl stearate + H_2O	trichloroethane	stability	[30]
PEG_2–lipase (Pc)	16-hydroxyhexadecanoic acid ethyl ester → 16-hexadecanolide + EtOH	trichloroethane	lactonization, fragrances, antibiotics	[31]
PEG_2–lipase (Pb)	(*RS*)-α-phenylethanol + dodecanoic acid → (*R*)-α-phenylethyldecanate + (*S*)-α-phenylethanol	trichloroethane	optical resolution	[25]
PEG_2–lipase (Pc)	(*RS*)-ε-decalactone + ethanol → (*R*)-hydroxy-decanoic acid ethyl ester + (*S*)-ε-decalactone	trichloroethane	optical resolution	[32]
PEG_2–lipase (Pf)	trilaurin + triolein → monooleoyl dilauroyl glycerol + monolauroyl dioleoyl glycerol	straight substrates	reformation of oils and fats	[73]
PEG_2–lipase (Pc)	(*RS*)-δ-decalactone + decanol → (*R*)-hydroxydecanoic acid decyl ester + (*S*)-δ-decalactone	decanol (substrate)	optical resolution	[33]
PEG_2–lipase (Pb)	farnesylacetic acid + geraniol → gefarnate + H_2O	trichloroethane	anti-peptic-ulcer drug	[26]
PEG_2–lipase (C)	methanol + benzoic acid → methyl benzoate + H_2O	benzene	substrate specificity	[72]
PEG_2–lipase (C)	methanol + retinoic acid → methyl retinoate + H_2O	benzene	substrate specificity	[72]
PEG_2–papain	Z-Orn(Z)-OEt + β-Ala–OBzl → Z-Orn(Z)–β-Ala–OBzl + EtOH	trichloroethane	salty peptide	[49]
PEG_2–proteases papain, chymotrypsin, thermolysin, trypsin, or pepsin	H–Arg–Gly–Phe–OH	dimethylformamide	solid phase synthesis	[42]
	H–Leu–Phe–Gly–OH			
	H–Lys–Phe–Phe–OH			
	H–Ala–Ala–Ala–OH			
	Z–Gly–Phe–Gly–OH			
	Z–Phe–His–Leu–OH			
	Z–Val–Phe–Gly–OH			
	Z–PHe–Leu–Gly–OH			

Pf: *Pseudomonas fluorescens*, Pc: *P. cepacia*, Pb: *P. fragi* 22.39B, C: *Candida cylindracea*.
[a] Reactions carried out in water-saturated solvents or straight substrates.

Table 3
K_m and V_{max} of the esterification of secondary alcohols by PEG_2–lipase in benzene [a]

	K_m (M)		V_{max} (μmol/min/mg)		V_{max}/K_m	
	R	*S*	*R*	*S*	*R*	*S*
2-butanol	0.43	0.43	1.18	1.23	2.7	2.9
2-pentanol	0.43	1.34	1.25	0.45	2.9	0.34
2-octanol	0.54	1.50	1.68	0.42	3.1	0.28
2-nonanol	0.50	1.66	1.37	0.30	2.7	0.18
α-phenylethanol	0.47	– [b]	0.90	– [b]	1.9	– [b]

[a] Reactions were conducted using 0.5 M dodecanoic acid in benzene with PEG_2–lipase from *P. fragi* 22.39B.
[b] Did not serve as a substrate.

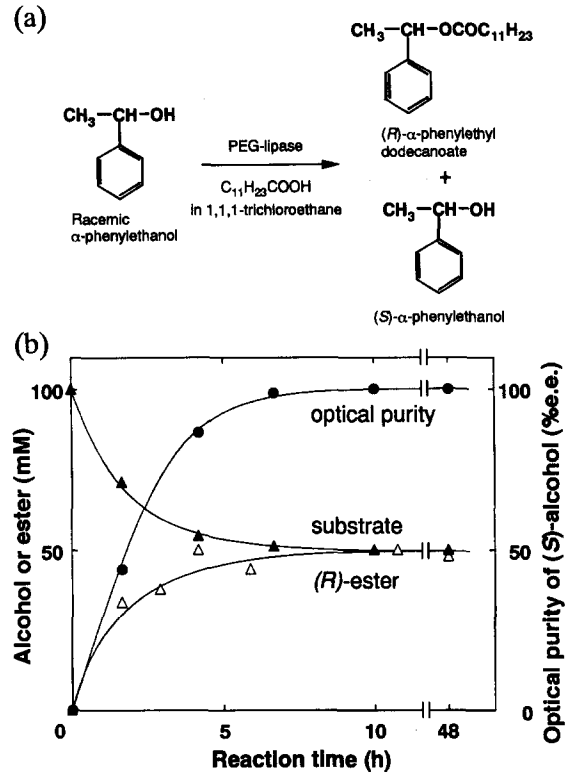

Fig. 5. Esterification of chiral α-phenylethanol with dodecanoic acid by PEG_2–lipase in 1,1,1-trichloroethane: (a) reaction scheme; (b) time-course of the optical resolution of α-phenylethanol by the esterification with PEG_2–lipase. In the figure, optical purity is expressed as enantiomeric excess (% ee) of unreacted alcohol ((*S*)-alcohol).

Natural lactones which are components of fragrances are known to be optically-active compounds with (*R*)- or (*S*)-form. Although racemic lactones can be synthesized, chiral lactones are rarely obtained by conventional organic synthesis. In this section, we deal with the optical resolution of racemic lactones by alcoholysis with PEG_2–lipase.

PEG_2–lipase from *P. cepacia* was found to catalyze the alcoholysis of racemic ϵ-decalactone with ethanol in 1,1,1-trichloroethane to form (*R*)-hydroxydecanoic acid ethyl ester, no alcoholysis of (*S*)-decalactone took place with the modified lipase as is shown in Fig. 7a [32]. The time-course of the reaction is demonstrated in Fig. 7b. The amount of (*S*)-decalactone was not reduced during a reaction time of 72 h, while that of (*R*)-lactone was sharply decreased with reaction time and tended to approach a constant level. The reaction product, (*R*)-6-hydroxydecanoic acid ethyl ester, appeared with time and approached a constant level of 100%. Furthermore, PEG_2–lipase recognizes the carbon number of alcohol substrates; increasing the carbon number of alcohol (C_2–C_{16}) gave rise to a high yield of the product in the alcoholysis in 1,1,1-trichloroethane. The alcoholysis activity of unmodified lipase in 1,1,1-trichloroethane was found to be one-twentieth of that of PEG_2–lipase (Fig. 7c) [32].

3.1.4. Enzymic reaction in straight substrates

It was found that PEG_2–lipase (*P. fluorescens*) can catalyze the ester exchange reaction not only in organic solvents but also in straight hydrophobic substrates [73]. In the presence of PEG_2–lipase, a mixture of two substrates, trilauroylglycerol and trioleoylglycerol, is converted by ester exchange

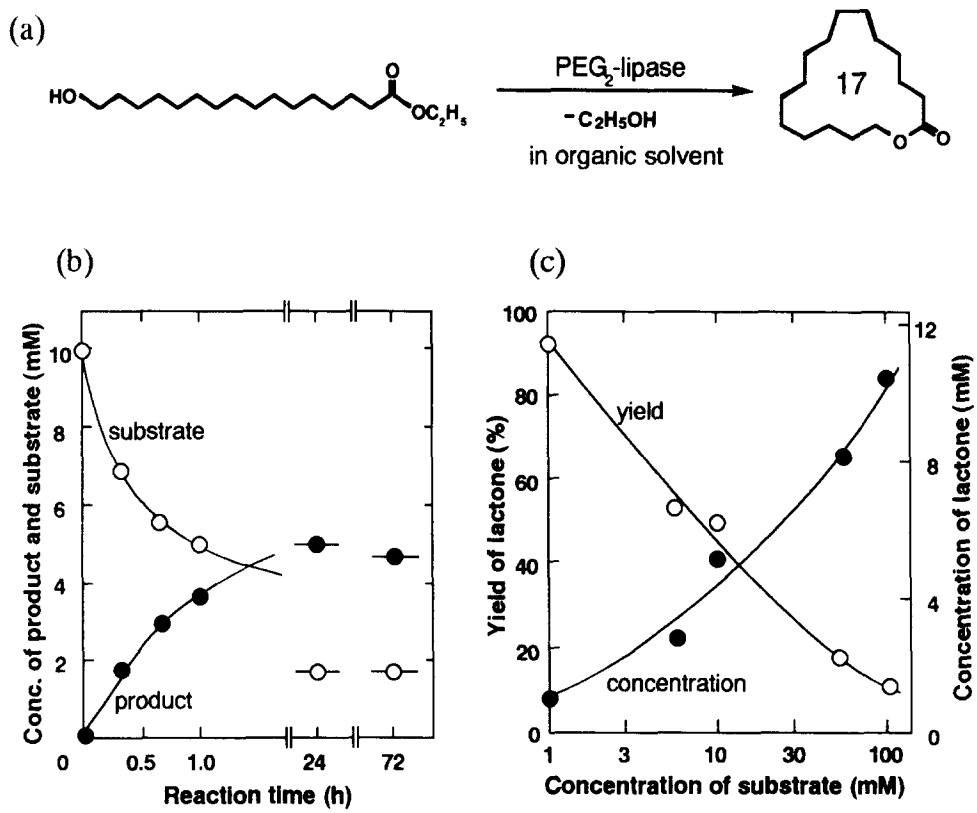

Fig. 6. Lactone synthesis from 16-hydroxyhexadecanoic acid ethyl ester in organic solvents with PEG_2–lipase: (a) reaction scheme; (b) time-course of lactone synthesis; (c) effect of substrate concentration on lactone synthesis.

reactions at 58°C without any solvents into another mixture of esters, trilauroyl glycerol, dilauroyl monooleoyl glycerol, monolauroyl dioleoyl glycerol and trioleoyl glycerol. Consequently, the melting temperature of the mixture is decreased from 33–36°C to 11–13°C [73]. Similar ester exchange reactions take place between fats and oils composed of triglycerides, accompanied by a decrease in the melting temperature of the reaction mixture. This technique may be available for the improvement of the melting temperature of oils and fats [73].

PEG_2–lipase (*P. cepacia*) catalyzes the asymmetric alcoholysis of racemic δ-decalactone in the presence of a *n*-alcohol to form (*R*)-5-hydroxydecanoic acid alkyl ester in straight hydrophobic substrates without the use of solvents (Fig. 8a) [33]. In decanol at 50°C, the yield was 69% with 83% enantiomer excess after 3 h reaction (Fig. 8b). Effective alcoholysis of δ-decalactone depends upon the carbon number of alcohols, and PEG_2–lipase catalyzes alcoholysis preferentially in *n*-alcohols with long-chain ($> C_6$) (Fig. 8c). In comparison a little alcoholysis of δ-decalactone takes place with decanol in 1,1,1-trichloroethane solvent using PEG_2–lipase.

The advantages of this technique are as follows: 1. PEG_2–lipase recognizes the chirality of a lactone and catalyzes the alcoholysis under mild conditions. 2. The reaction proceeds with two straight substrates without the use of any harmful organic solvents such as benzene and 1,1,1-trichloroethane. 3. The products may be easily obtained by fractional distillation under low pressure. 4. Greater amounts of the product can be obtained from a definite volume of the reaction system. This finding may lead to a new avenue to resolving racemic lactones.

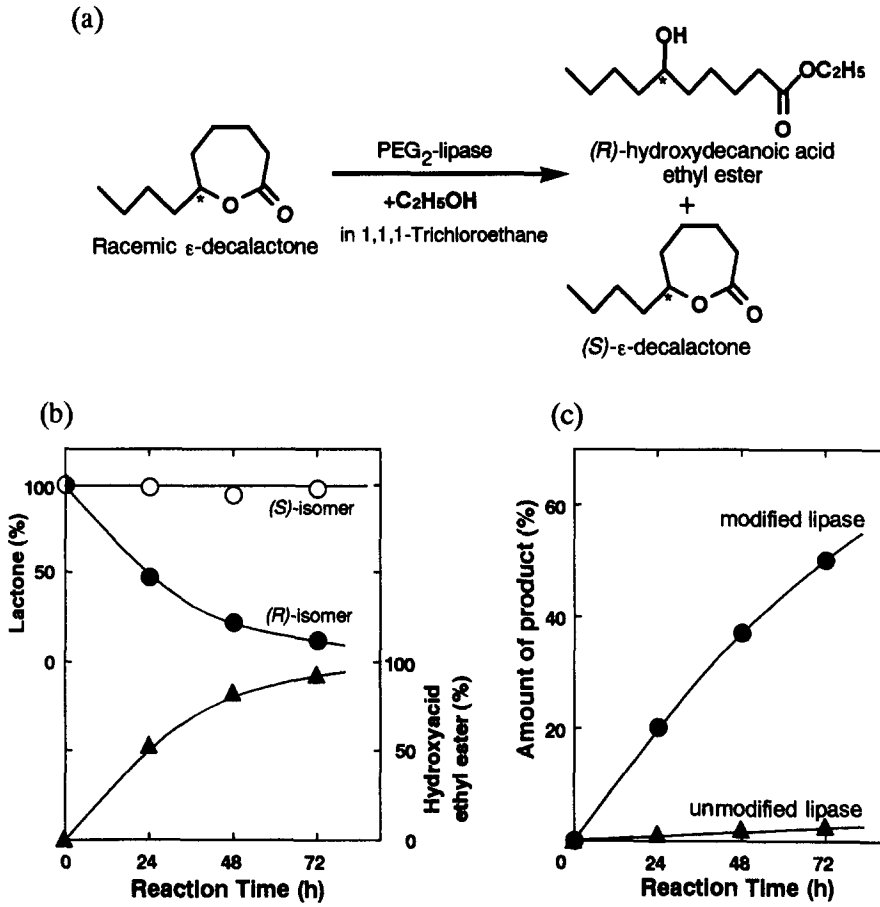

Fig. 7. Asymmetric alcoholysis of ε-decalactone with PEG_2–lipase in 1,1,1-trichloroethane; (a) scheme of the reaction; (b) time-course of alcoholysis at 65°C; (c) comparison between PEG_2–lipase and unmodified lipase on the alcoholysis at 35°C.

3.2. PM–lipase

Lipase from *P. fluorescens* was coupled with activated PM_{13} (Fig. 2b) to give PM_{13}–lipase [30]. Fig. 9 shows the esterase activity in an aqueous system and the ester synthesis activity in benzene of PM_{13}–lipases with varies degrees of modification of amine groups. The degree of modification was increased by increasing, in the coupling reaction, the molar ratio of activated PM_{13} per amine group in the lipase, and tended to approach a constant level of 60%. The esterase activity in an emulsified aqueous system of PM_{13}–lipase was 1.3 times higher than that of unmodified lipase. PM_{13}–lipase also becomes soluble in organic solvents and in benzene catalyzes the reverse reactions of hydrolysis, viz. ester synthesis reactions. Thus, lauryl stearate was synthesized with PM_{13}–lipase in a clear benzene solution containing lauryl alcohol and stearic acid. The ester synthesis activity of PM_{13}–lipase was enhanced by increasing the degree of modification and tended to approach a constant level (4.7 $\mu mol\ min^{-1}\ mg^{-1}$). In this reaction system, secondary carboxyl groups in the PM molecule do not serve as a substrate of ester synthetic reaction in organic solvents. This result is compatible with the substrate specificity of PEG_2–lipase from *P. fluorescens* in an organic solvent [68].

4. Stabilization of enzymes by modification with PM

Lipase from *P. fluorescens* is stabilized toward heat denaturation by the modification with activated PM [30]. Although the esterase activity of unmodified lipase in an emulsified system was completely lost in 150 min with incubation at 55°C, 60% of the activity of PM_{13}–lipase is retained after 150 min. Furthermore, 50% of the ester synthesis activity of PM_{13}–lipase in benzene is retained in the same incubation time.

L-Asparaginase from *Escherichia coli* was coupled with comb-shaped modifiers, activated PM_{13} and activated PM_{100}, and the modified asparaginases were found to be stable both in vivo and in vitro [5]. After a single intravenous injection of PM_{100}–asparaginase into rats, the enzymic activity was retained for at least 11 days in blood circulation, and the serum L-asparagine concentration remained undetectable for 27 days (Fig. 10). In the case of unmodified asparaginase, its enzymic activity was lost after less than one day. The half-lives of PM_{100}–asparaginase and unmodified asparaginase were 50 and 1.5 h, respectively. L-Asparaginase was also made resistant against heat, urea and acidity by modification with activated PM_{13} and PM_{100} (Fig. 11). In the heat-stability test at 65°C (Fig. 11a), the enzymic activity of unmodified asparaginase was completely lost after 30 min incubation. On the other hand, PM_{13}– and PM_{100}–asparaginases retained 35% and 90% of their enzymic activities under

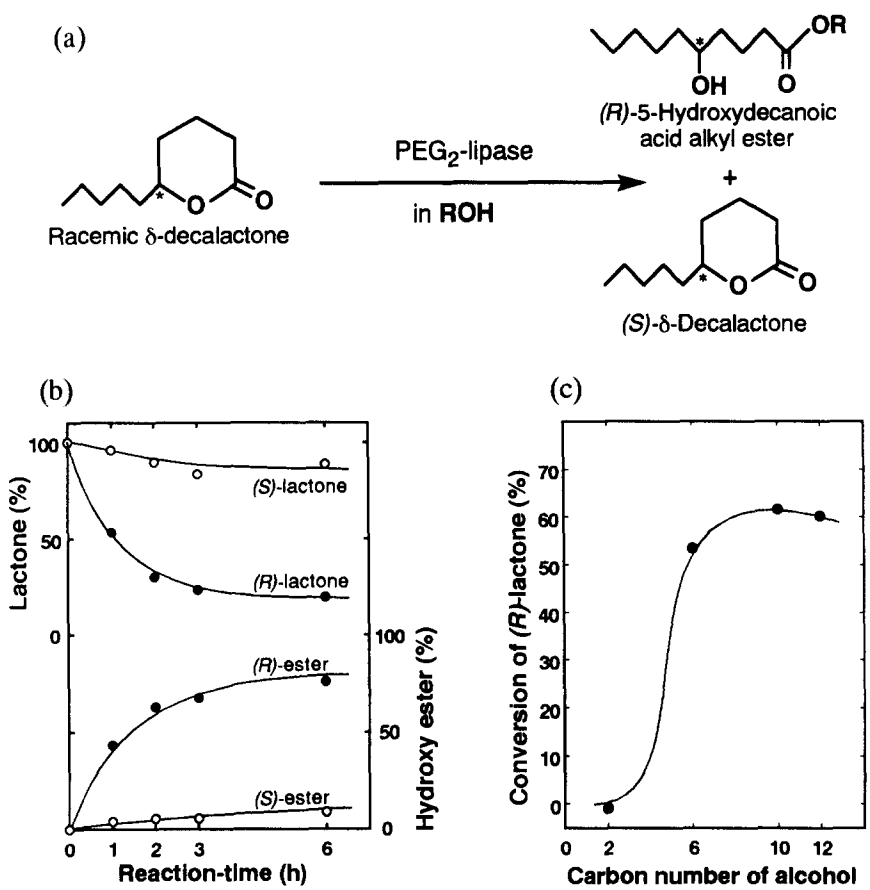

Fig. 8. Asymmetric alcoholysis of δ-decalactone with PEG_2–lipase in a straight substrate; (a) reaction scheme; (b) time-course of alcoholysis at 50°C; (c) effect of carbon number of alcohol on alcoholysis at 50°C for 3 h.

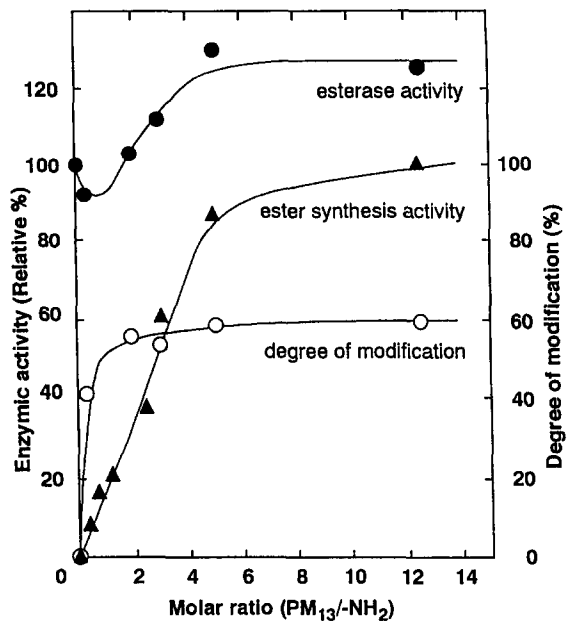

Fig. 9. Degree of modification of PM_{13}–lipase together with esterase activity in an emulsified aqueous system and ester synthesis activity in benzene. Various amounts of PM_{13} per single amino group in lipase (the total amino groups in the lipase molecule = 8) were added to the modification system at 4°C for 1 h.

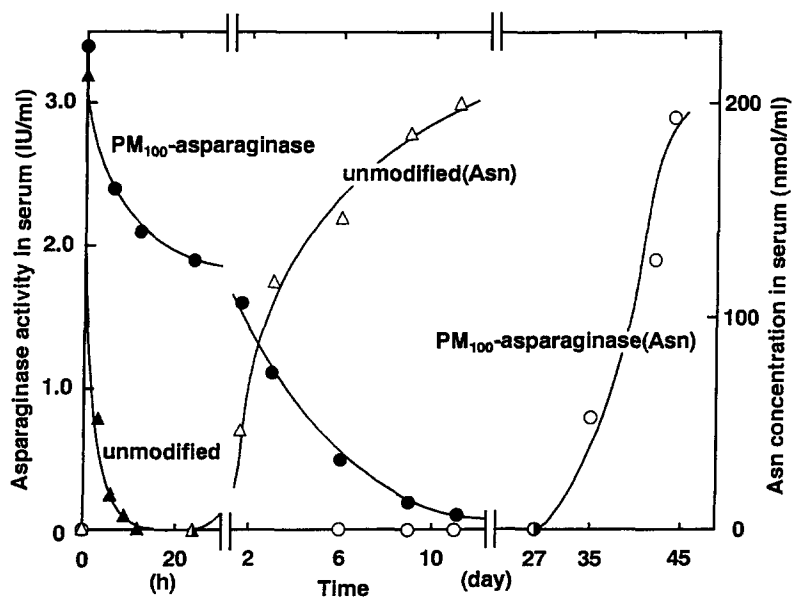

Fig. 10. Comparison of clearance-time of PM_{100}–asparaginase and unmodified asparaginase. PM_{100}–asparaginase and unmodified asparaginase were injected once intravenously at 100 IU/kg into male albino rats (Wistar strain).

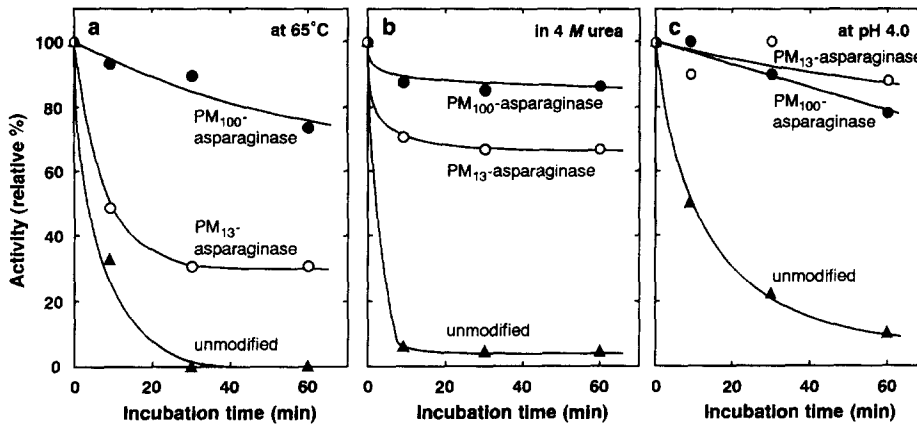

Fig. 11. Stabilization of L-asparaginase modified with activated PMs toward heat at 65°C (a), 4.0 M urea (b), and acidity of pH 4.0 (c).

the same experimental conditions, respectively. Although unmodified asparaginase almost completely lost its enzymic activity with 4 M urea in 10 min, PM_{13}– and PM_{100}–asparaginases retained 70% and 85% of the initial activities, respectively (Fig. 11b). A similar stabilization was also observed at pH 4.0 (Fig. 11c). PM_{100}–asparaginase was shown to retain high enzymic activity toward heat or urea, compared with PM_{13}–asparaginase. It was suggested that these comb-shaped modifiers with multivalent reactive sites may cover the whole surface of the asparaginase molecule and stabilize its conformation, possibly through multiple covalent bindings and through various non-covalent interactions (Fig. 4).

Similar stabilization towards heat and urea denaturation were observed for trypsin modified with activated PM_{13} or PM_{100} [58]. By incubation at 60°C for 1 h, only 30% of the original activities of PM_{13}– and PM_{100}–trypsins were lost; on the other hand, almost all of the activity of unmodified trypsin was lost. In 4 M urea at 37°C, PM_{13}– and PM_{100}–trypsins retained 75–80% of the original activities; however, only 5% of the original activity was retained for unmodified trypsin. Modification of trypsin with low molecular weight modifier such as succinic anhydride did not bring about stabilization towards heat or urea denaturation.

5. Other conjugates

Some bioactive compounds other than proteins and enzymes have also been coupled with PEG derivatives for the purpose of biomedical application. Low molecular weight drugs such as antibiotics and nucleotide analogues have been coupled with PEG derivatives with the aim of improving drug delivery [74,75]. Moreover, liposome and polymer matrixes were also modified with PEG derivatives for the purpose of effective delivery of drugs [76,77] and an artificial organ [78].

Since these bioactive conjugates also have attractive properties in the field of biotechnology, some of them are described below.

5.1. PEG–natural pigment

Utilization of natural pigments (or their derivatives) in biotechnological and biomedical fields are restricted by lability in light and limited solubility. Porphyrin compounds such as hemin, hematopor-

phyrin and chlorophyllin became soluble in both aqueous media and organic solvents by modification with PEG derivatives [79–81]. Furthermore, PEG–hemin and PEG–hematoporphyrin were found to catalyze peroxidase-like [79] and photooxidation [80] reactions, respectively. Recently, PEG–chlorophyllin was found to be more stable toward light than unmodified chlorophyllin and to possess potent photosensitizing activity to produce superoxide anion during the illumination. In contrast, unmodified chlorophyllin lost its activity rapidly during illumination [81]. Melanin, an insoluble dermal pigment, also became soluble in aqueous solution and in organic solvents [82] upon coupling with a PEG derivative which is expected to exhibit photo-protective and/or radical scavenging properties.

5.2. *Magnetic PEG–enzyme and PEG–coenzyme*

Using enzymes in a bioconversion system, it is important to recover the enzymes intact as a catalyst. To cut cost, enzymes are required to be immobilized onto insoluble beads, to be separated

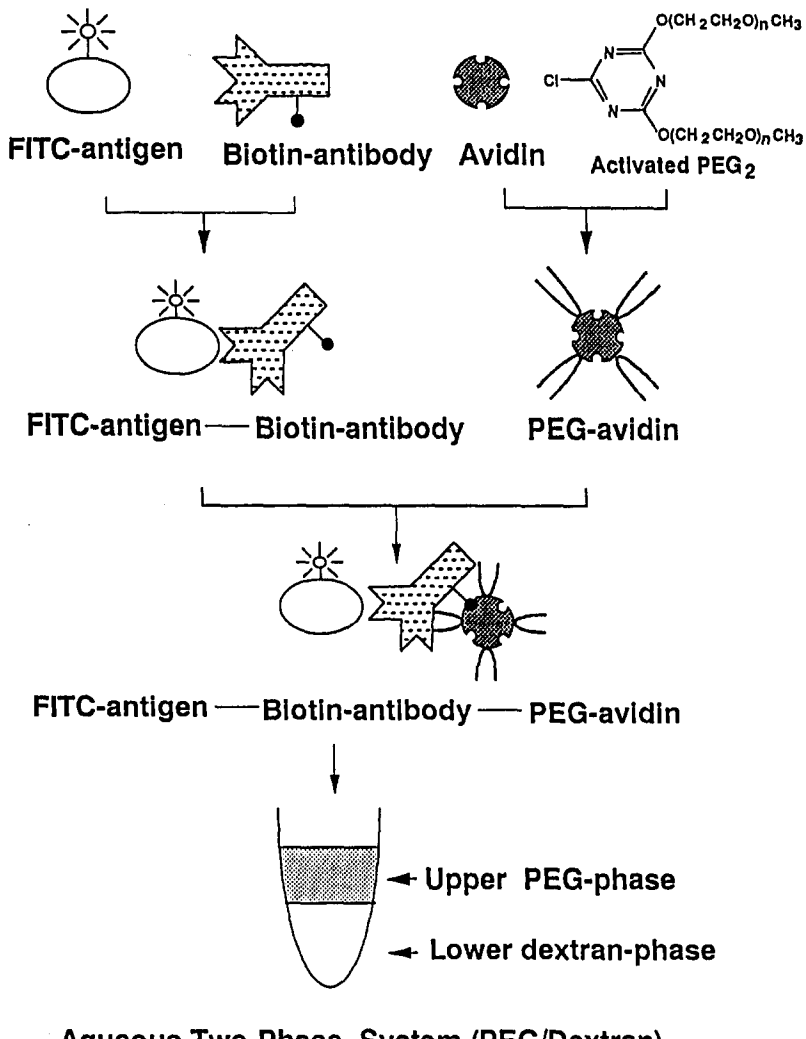

Fig. 12. PEG–avidin as a novel agent for selective extraction of antigen–antibody complex in an aqueous two-phase system. FITC-antigen, antigen molecule labeled with fluorescein; Biotin antibody, biotinylated antibody; PEG–avidin, chicken avidin modified with activated PEG_2.

from the products by an ultrafiltration membrane. PEG–lipase (*P. fluorescens*) described in a previous section can be easily collected as a precipitate by adding hexane without any loss of activity [71]. We also tried to prepare magnetic enzymes to recover enzymes by applying a magnetic field [19]. A PEG–enzyme is conjugated to magnetite (Fe_3O_4) to form a magnetic PEG–enzyme. The magnetic enzymes disperse stably in both organic solvents and aqueous solutions. Magnetic lipase (*P. flagi* 22.39B) thus prepared can catalyze ester synthesis reaction in organic solvents and can be readily recovered by magnetic field without loss of enzymic activity. This approach could have a great practical potential in enzyme technology.

A similar line of work has been done for recovering coenzymes. NAD(P) coupled with PEG derivative can be easily separated from a reactor by the ultrafiltration membrane [83]. Moreover, dehydrogenases covalently attached with NAD through bifunctional PEG chain as a spacer successfully enhanced the reaction rate [21,22].

5.3. Affinity partitioning with PEG–ligand

An aqueous two-phase system composed of aqueous solutions of PEG and dextran has long been studied as a potent tool to separate a variety of macromolecules. In the system, a hydrophobic protein is preferentially partitioned into the upper PEG phase, and the partition coefficient can be controlled by varying ionic strength and pH values. In the PEG/dextran aqueous two-phase system, PEG-dyes such as Chibacron Blue were utilized as affinity ligands to purify a series of dehydrogenases [84]. To ensure more specific and varied partitioning, various PEG ligands such as PEG-modified antibodies and enzyme substrates have been proposed [85]. Recently, we reported that PEG_2–avidin is an effective tool to extract antigen by complexing with biotin-labeled antibody in the aqueous two-phase system [86]. Using PEG_2–avidin, the immune complex formed between biotinylated anti-mouse IgG and its antigen IgG (mouse) molecules was successfully transferred into the PEG phase (Fig. 12). This technique may be successfully used not only in affinity partitioning in combination with counter-current chromatography, but also in affinity immunoassay with minimized nonspecific adsorption problems.

In this paper, we reviewed the recent fruits of chemical modification of proteins and biological substances. We defined 'bio-hybrid' conjugate as a biomaterial composed of two or more components such as proteins, nucleic acids and biological substances together with synthetic polymers and/or inorganic substances. As these hybrid materials have potent properties other than that of each original component, numerous world-wide investigations have been in progress.

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