Korean J. Chem. Eng., **23**(4), 601-606 (2006)

Modification of lipase from *Candida rugosa* with poly(ethylene oxide-co-maleic anhydride) and its separation using aqueous two-phase partition system

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(*Received 14 February 2005 • accepted 27 February 2006*)

Abstract−A copolymer was synthesized from polyethylene oxide (PEO) and maleic acid anhydride (MA). Number of ethylene oxide units was varied from 10 to 40. Lipase from *Candida rugosa* was modified through chemical bonding of MA with amino group of lipase. Degree of modification increased with a decrease in EO unit and increase in copolymer/enzyme ratio. The relative activity of modified enzyme increased with increase in EO unit. It was more than native lipase (100%) when copolymer/enzyme weight ratio was less than 3 for all copolymers. It might be due the conformation change of the lipase molecules on modification that would have exposed the catalytic sites making them more easily accessible. At the highest DM (39%), modified lipase retained more than 50% relative activity. Partitioning of native and modified lipase was also studied by using aqueous two phase synthesized copolymer/dextran system: modified lipase (with EO 30 and 40) showed better separation than the native one. Partition coefficient increased with increase in copolymer/enzyme weight ratio.

Key words: Synthesized Copolymer, Polyethylene Oxide, Maleic Acid Anhydride, Modified Lipase, Maleylation, Two Phase Partition

INTRODUCTION

The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes [Dordick, 1991; Koeller and Wong, 2001; Schmid et al., 2001; Park et al., 2001a]. Recent developments in biotechnology, particularly in areas such as protein engineering [Kim and Choi, 1984; Joo et al., 1998; Eijsink et al., 2004] and directed evolution, have provided important tools for the efficient development of new enzymes. Lipases are the most widely used class of enzymes in the chemical industry [Guo and Sun, 2004; Reetz, 2002]. They catalyze not only the hydrolysis but also the synthesis of long-chain acylglycerols [Jeon et al., 1999; Kontkanen et al., 2004; Wu and Song, 2002]. Important uses in biotechnology include their applications in the detergent, food, flavors industry, biocatalytic resolution of pharmaceuticals [Lee et al., in press; Young et al., 2004], paper and pulp [Bajpai, 1999; Park and Park, 1999, 2001, 2002], esters and amino acid derivatives [Jeong et al., in press; Yadav and Lathi, 2003], making of fine chemicals, agrochemicals, use as biosensor, bioremediation and cosmetics and perfumery [Salis et al., 2005; Borgstorm and Brockman, 1984].

Although the application of lipases as industrial catalyst has been utilized, there are several disadvantages for industrial application of biocatalyst. Enzymes are easily deactivated when they are subjected to the action of heat, extreme pH range or in organic solvents [Longo and Combes, 1999; Noel and Combes, 2003; Matsumoto et al., 2001; Jensen, 1983]. Numerous strategies have been proposed to overcome such a limitation including use of soluble additives, immobilization, protein engineering, and chemical modification [Kwon and Rhee, 1984; Cho and Rhee, 1993; Won et al., 2005; Lee et al., 2002; Chae et al., 1998; Park et al., 2001b]. The modification of protein surface with modifiers by chemical binding appears to be a good strategy to improve biocatalyst performance. Modified enzymes are typically macroscopic catalysts that were retained in the reactor; therefore, continuous replacement of the enzyme is not necessary. Modified enzymes are attached to or entrapped within a macroscopic support matrix, so that resulting catalyst can be reused and offer several potential advantages over soluble enzymes. Modified enzymes are often more stable than enzymes in solution, and can be applied in a wide range of different reaction environments. Polysaccharides and polyethylene glycol (PEG) showed excellent improvements in enzyme functionality among the investigated modifiers [Furukawa et al., 1996; Goto et al., 1994; Wu et al., 2001; Wu and Song, 2002; Charusheela and Arvind, 2002]. Nishio et al. [1988] have modified enzymes such as lipase, catalase, chymotrypsin and peroxidase with a copolymer of monomethoxy polyethylene glycol and cyanuric chloride. Hybridization of enzyme with copolymer, i.e., immobilization into a biopolymer by multipoint covalent attachment affords a straightforward, fast and convenient method for preparation of immobilized enzymes. This increases enzyme stability to heat, pH, organic solvents, and peroxides while retaining activity for a much longer period of time. Enzymes prepared in this way have been found to exhibit remarkable stability under normally denaturing conditions. Moreover, the covalent binding provides protein retention in the copolymer matrix so that the advantages of immobilization can be maximized [Hwang et al., 2004].

In our previous studies, cellulase was combined with a synthetic polymer such as a polyethylene glycol (PEG) derivative and the modified cellulase showed additional properties of the nonionic surfactant and/or synthetic polymer [Park, 1995; Park and Park, 1999, 2001, 2002; Park et al., 2002; Moon and Park, 1993]. Also, in our earlier work, lipases from *Candida rugosa* were modified by chemical reaction with water soluble copolymers such as AKM 0530

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and AKM 2010, and it was found that copolymerization of native lipase with copolymer makes it more stable over a wide range of temperature, pH and for longer reaction time. In this paper, a copolymer having maleic acid anhydride (MA) and polyethylene oxide (PEO) of varying number of ethylene oxide groups in one PEO chain was synthesized. Then lipases were modified with these synthesized copolymers to make them applicable in different reaction environments and their partitioning in aqueous two phase copolymer/dextran systems was investigated.

MATERIALS AND METHODS

1. Materials

The lipase from *Candida rugosa* was used throughout the experiments. The commercial name of lipase was lipase OF (produced by Meito Sangyo Co. Ltd.) and was in the form of dried white powder. Lipase OF shows non-regioselectivity and hydrolyzes triacylglycerol almost thoroughly into fatty acid and glycerol. The maleic acid anhydride (MA) groups can react with the amino groups of lipase. Ethyl caprate (Sigma Aldrich Co.) was used as the standard substrate in the hydrolysis reaction. All remaining reagents were of analytical grade and used without further purification.

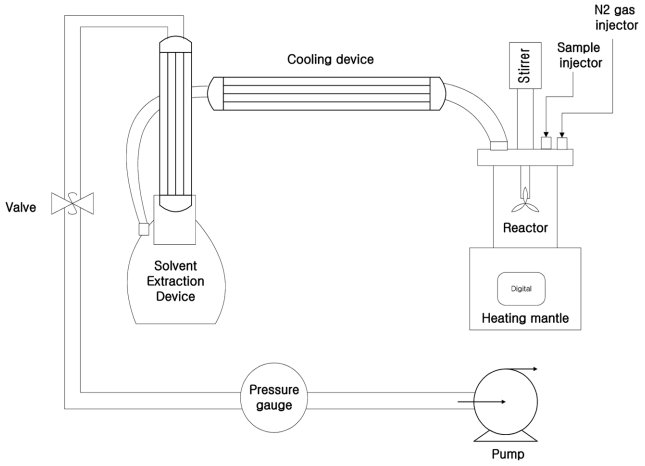

Fig. 1. Copolymer synthesis apparatus.

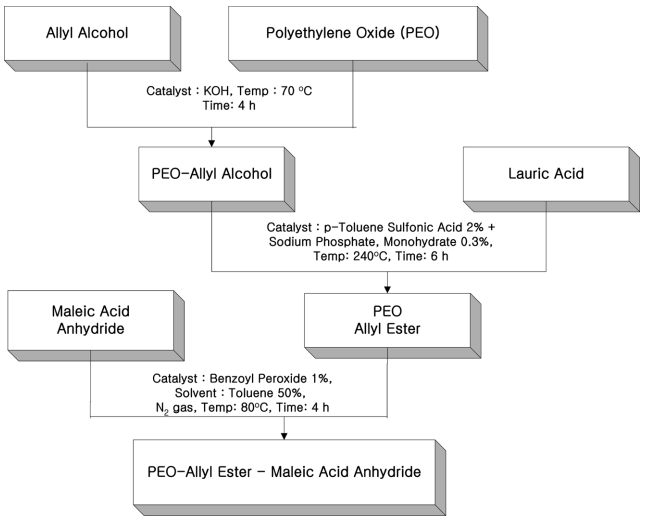

Fig. 2. Synthesis of copolymer.

2. Copolymer Synthesis

The copolymer, which consists of polyethylene oxide (PEO) and maleic acid anhydride (MA), was used as a modifier, and it was synthesized using the apparatus shown in Fig. 1; a schematic of the process is shown in Fig. 2. First, allyl alcohol was made to react with PEO at 70 °C for 4 h in an autoclave in presence of KOH as a catalyst. The number of EO units in one PEO chain was varied from 10 to 40 in order to observe the effect of EO chain length. Then, PEO-allyl alcohol was combined with lauric acid to form an ester. The mixture of PEO-allyl alcohol and lauric acid was reacted with for 6 h at a temperature 240 °C in presence of 2% *p*-toluene sulfonic acid and 0.3% sodium phosphate as catalysts. The conversion was calculated by the acid value, which was defined as the mg of KOH required to neutralize 1 g of the acid sample. Acid value was determined by acid-base titration. Finally, MA was added to the ester to form the desired copolymer compound. PEO makes the copolymer soluble in water, while MA gives the functional group necessary to bind with the amino acid group of the enzyme. In the copolymer synthesis, benzene and toluene remained in the product, and removal process of solvent or catalyst was essential. Therefore, vacuum process was carried out at 120 °C for 30 min to remove toluene. Reacting material was purified to enhance the yield of formation of product and was separated from impurities for further application. Finally, the structures of these products were analyzed with IR (IR-700, JASCO, Japan) and FTNMR (Bruker 250 spectrophotometer).

3. Modification of Lipase with Synthesized Copolymer

Amino acids are suitable for participation in covalent bond formation, chemical modification, with various organic acids. Maleylation is one of the chemical modifications of protein with MA. Modification of lipase (Fig. 3) with the copolymer modifier was carried out as follows: copolymer was added stepwise to the lipase solution, and the mixture was slowly stirred at 0 °C under pH 8.0-8.2, which was controlled with the addition of 0.2 M NaOH. This reaction occurred effectively under the condition of pH 8.0 and at low temperature (0 °C). In the modification of lipase with pure MA, the amino acid groups chemically react with the maleic anhydride groups and form covalent bond between them (Fig. 3). However, it was thus necessary to control pH value with a base because pH decreased due to the production of carboxylic acid as the reaction proceeded. In the case of modification with copolymer, lipase reacted easily with MA group of copolymer. The degree of modification (DM) of lipase with modifiers was defined as the ratio of modified amino groups of the lipase to the total amino groups of the native lipase. Amino groups of the lipase were determined with the trinitrobenzenesulfonic acid (TNBS) method [Habeeb, 1966]. The DM was varied by changing the weight ratio of copolymer to lipase over the

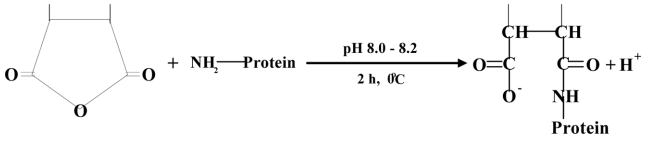

Fig. 3. The scheme of maleylation.

range of 10-80 (w/w). The DM of modified lipase is defined as

DM=1−(Unmodified-NH_2 of modified lipase /Total-NH_2 of native lipase).

4. Analysis of Lipase Activity

Activity changes with reaction environment such as reaction time, temperature, pH, and with activity measurement conditions. Thus, activity measurement conditions must be identical for each enzyme - native as well as modified. In this experiment, substrates were chosen to match with specific enzyme, and activity was measured at the optimal condition of each enzyme. Hydrolytic activity of lipase was determined by titrimetric method with auto titrator (718 STAT Titrino, Metrohm Swiss made) at 37 °C and at pH 7 by substrate emulsion method using ethyl caprate as the substrate [Jensen, 1983]. The hydrolytic activity was measured by the initial reaction rate. The enzyme was dissolved in a phosphate buffer solution. The following compounds in a thermostatic flask at 40 °C were then added: a 1 ml sample containing the enzyme and 29 ml substrate emulsion which was composed of 20 mM sodium chloride, 1 mM calcium chloride, 20 mM ethyl caprate, and 0.5% gum arabic as a surfactant. Activity was expressed in lipase units. One unit of lipase activity corresponds to the release of one micro-equivalent of fatty acid per minute under standard assay conditions.

5. Recovery of Enzyme using Aqueous Two Phase System

Two phase partition is the method used to determine the moving ratio of native/modified lipase in aqueous two phase system. A schematic diagram of the experimental procedure is shown in Fig. 4. First, dextran (MW=505,000) and copolymers (EO(10), EO(20), EO(30), or EO(40)) (20 wt% each) were mixed and native/modified lipase (1 ml) was added. This mixture was then shaken at 300 rpm for 10 min for proper mixing and became separated after centrifugation at 3,000 rpm for 10 min. Because of difference in densities of dextran and copolymers, native/modified lipase was partitioned into two phases of dextran and copolymer. Finally, 1 ml samples from top and bottom layers were then taken, and lipase activity was measured [Kim et al., 2004].

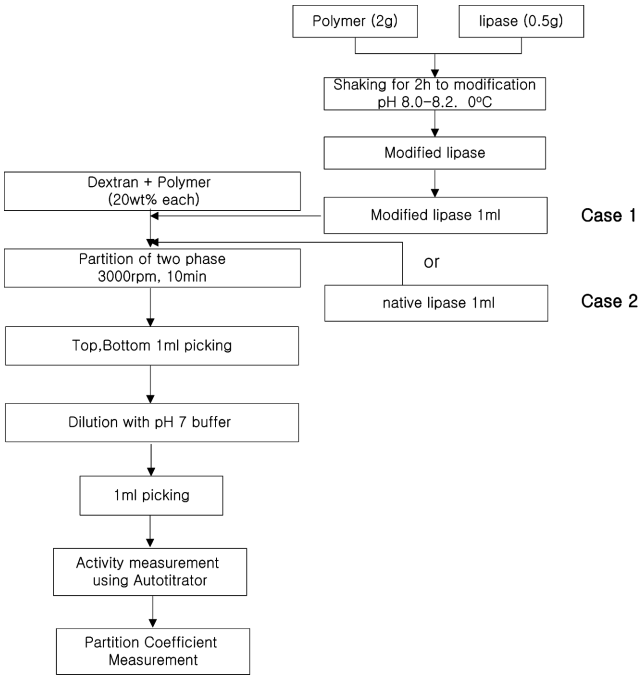

Fig. 4. Aqueous two phase system (ATPS).

RESULTS AND DISCUSSION

1. Copolymers Synthesis

Reaction mechanism of the copolymerization process is shown in Fig. 5. First, polyethylene oxide (PEO) was added to oxygen of the OH group of allyl alcohol. Then, PEO-allyl alcohol underwent an esterification reaction with lauric acid. This step was necessary to prevent a side reaction for the MA addition. The hydroxyl group in the allyl alcohol was neutralized with hydrogen ion from lauric

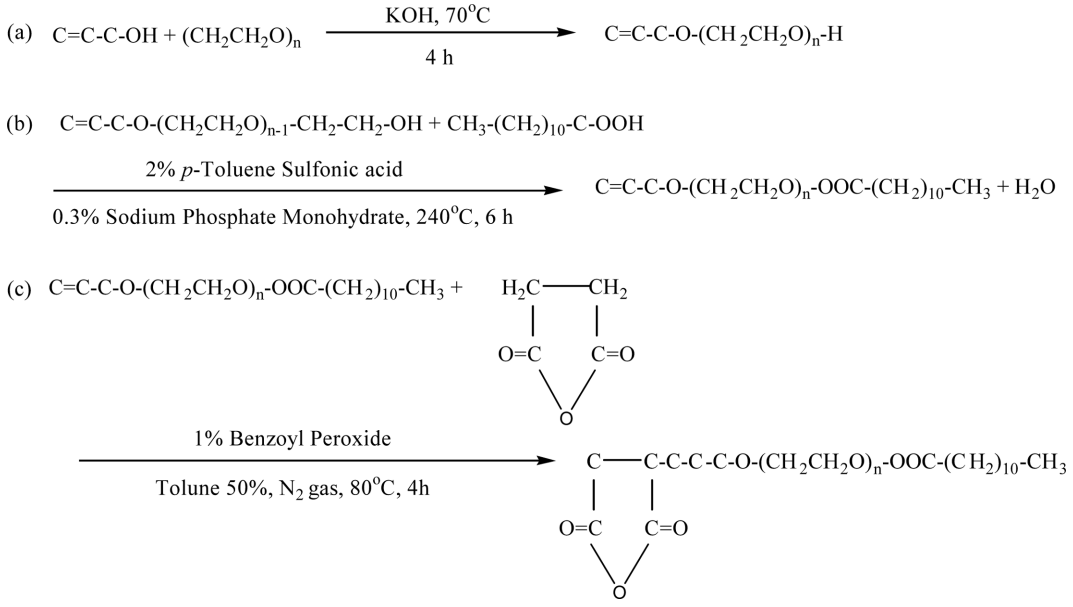

Fig. 5. Mechanism of copolymerization.

Table 1. Characteristics of synthesized copolymers

Property	PEO-Allyl alcohol			
Number of EO unit	10	20	30	40
EO additive mol number (Experimental)	11.0	21.87	30.81	39.55
Molecular weight	544	1020	1413	1798
Hydroxyl value	103.0	54.97	39.68	31.2
pH	6.31	6.20	6.00	5.73

acid to produce water (H_2O), while the carboxylate group of the lauric acid underwent esterification. Finally, MA was added to the PEO-allyl ester to make the final product. The carbon double bond present in the allyl ester was broken down into a carbon single bond and MA was added at the position of double bond of the PEO-allyl ester by breaking it into a carbon single bond.

The characteristics of PEO-allyl alcohol are shown in Table 1. The product was in a liquid state when the PEO with 10 EO units was used; while the products were in the paste forms when PEO of more than 10 EO units was used. The hydroxyl value decreased as the number of EO units of PEO increased. The hydroxyl value is the amount of KOH needed to neutralize H^+ from allyl alcohol. Allyl alcohol loses a hydrogen ion when it reacts with a PEO chain. PEO-allyl alcohol was transformed into its ester form in order to combine with MA without opening its ring structure. PEO-allyl alcohol was combined with lauric acid to form an ester, and the conversion could be calculated by measuring the acid value of the ester product. The acid value in case of the PEO with 10 EO units is shown in Fig. 6. As the reaction temperature increased, consumption of acid by the reaction with alcohol increased, and thus acid value of the reactant decreased while conversion increased. Acid value and conversion were 3.3 and 95.8%, respectively, at 240 °C. Allyl esters containing different numbers of EO units were synthesized and are shown in Table 1. As the EO number increased, the saponification value at the completion of the reaction decreased. Conversion values for each EO at unit lengths of 10, 20, 30, and 40 were 95.8, 93.2, 87.1, and 85.3%, respectively. PEO-allyl ester was combined with MA, and the product was analyzed with IR and FTNMR for its structure. The IR showed functional groups such as ester (1,775, 1,745, and 1,054 cm^{-1}), MA (1,848, 1,775, and 1,745 cm^{-1}), ethylene oxide (1,848, 1,775, 1,745, and 1,054 cm^{-1}), and benzene (3,082, 1,465, 948, 888, 848, and 722 cm^{-1}), and these coincided with the results from FTNMR. From the FTNMR analysis, 75% of the carbon double bonds in PEO-allyl ester (7.07-7.27, 7.26 ppm) were found to be converted into the carbon single bonds (2.32 ppm). We thought that the synthesis of the copolymer with the MA functional group was successfully synthesized with 75% completion.

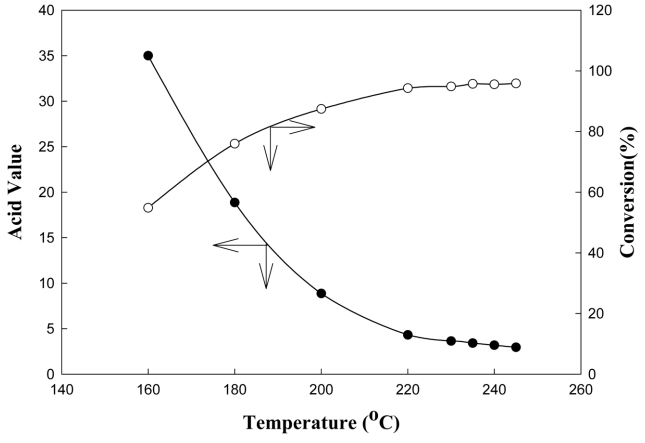

Fig. 6. Acid value and conversion of allyl ester with EO(10) at various temperatures.

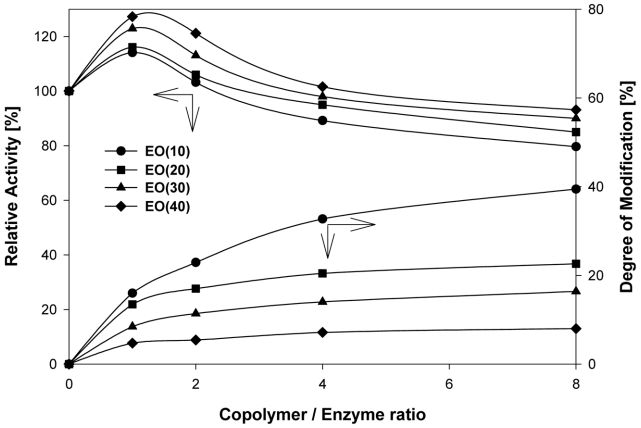

Fig. 7. Degree of modification (DM) and relative activity of modified lipase with hydrophilic copolymer.

2. Modified Lipase with Synthesized Copolymer

The degree of modification (DM) and the relative activity according to the copolymer/enzyme (native or modified) weight ratio are shown in Fig. 7. Relative activity is defined as the ratio of the activity of modified lipase to that of native lipase. As the weight ratio of a copolymer/enzyme increased, the DM increased and there was almost no change in DM for a weight ratio more than 4 except for EO (10). With different EO number, the DM decreased as the number of EO units increased (Fig. 7). The relative activity showed (Fig. 7) an unexpected behavior, first increased beyond 100% for all copolymers (having different EO number) and was maximum for copolymer/enzyme ratio ~0.15 and then started decreasing and became less then 100% for copolymer/enzyme ratio greater than 4 : 1 (for EO 30 and 40) and 3 : 1 (for EO 10 and 20). With a different EO number, the relative activity increased as the number of EO units increased, but DM decreased. It was clearly shown in the Fig. 7 that more than 50% of relative activity was retained even at the highest degree of modification (39%). It was thought that PEO chains can be chemically attached to lipase without great loss of activity, and modified lipase will have additional properties from the supporting materials.

To further confirm this abnormal behavior of relative activity (i.e., above 100%), substrate specificity of native as well as modified lipase was also studied for different substrate. This also showed that modified lipase had better conversion for all ester studied as shown in Fig. 8. The higher relative activity for modified lipase was also reported in literature [Brady et al., 1988; Guo et al., 2003; Wang and Hsieh, 2004]. It might be due to the conformation change of the lipase molecules on modification that would have exposed the catalytic sites making them more easily accessible.

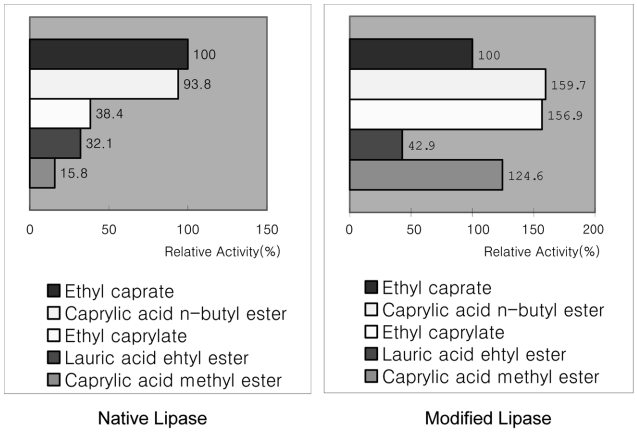

Fig. 8. Substrate specificity of native and modified lipase.

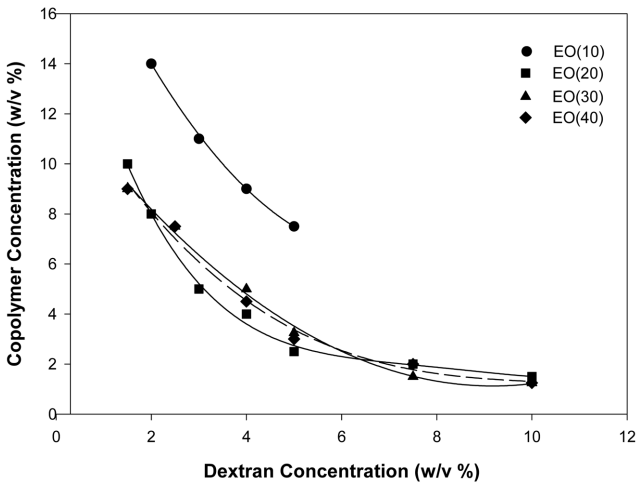

Fig. 9. Phase diagram of dextran (MW=505,000) and synthesized copolymer.

3. Phase Diagram and Phase Separation of Native and Modified Lipase

The synthesized copolymer with different EO unit (10, 20, 30, and 40) and dextran were mixed for the phase separation study, and phase partition curves for these mixtures are shown in Fig. 9. These lines separate one/two phase region. The lower region of the curve represents a single phase where no separation was observed. The upper region represents the two phase. According to Flory and Huggins theory [Flory, 1941, 1953; Huggins, 1942] that describe the thermodynamics of phase separation, water does not play a key role in determining the phase separation; rather, it is the polymer interaction that controls the phase separation. Thus, the separation occurred more clearly at higher polymer concentration [Diamond and Hsu, 1992]. High concentration of polymer was also needed to obtain phase separation when copolymer with low EO value was used.

An aqueous two phase system containing synthesized copolymer and dextran was used to separate the native/modified enzyme, and the partition coefficients are shown as a function of EO value in Fig. 10. It was found that partitioned coefficient of native/modified enzyme increased with increase in number of EO units of copolymer. Also, two phase systems with copolymer EO (30) and EO (40)

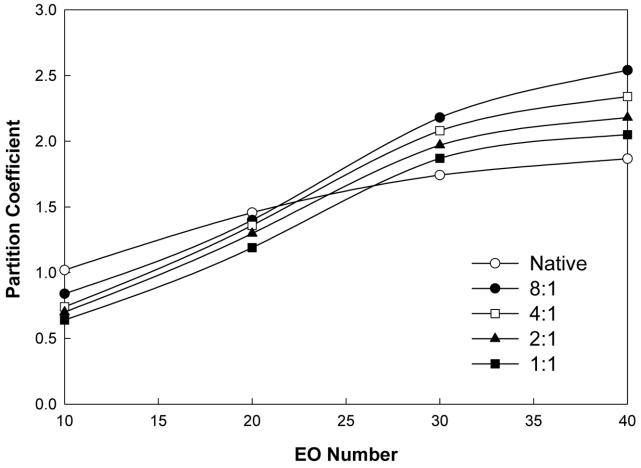

Fig. 10. Partition coefficient of native and modified lipase in aqueous two phase system as a function of EO number for different weight ratio of copolymer : enzyme.

showed better partitioning of modified enzyme than native enzyme. Partition coefficient of modified enzyme increased as the ratio of copolymer to enzyme increased. Fig. 10 shows that the modified lipase with reactive copolymer moves to the upper phase (copolymer phase). As a result, about 63% of enzyme moved to copolymer phase (in case of copolymer with EO (40)) when the copolymer to enzyme ratio was 8 : 1.

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