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Characteristics of the Lipase from *Candida rugosa* Modified with Copolymers of Polyoxyethylene Derivative and Maleic Acid Anhydride

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Abstract−The hydrophilic copolymer, polyethylene oxide (PEO) allyl ester-maleic anhydride (MA), copolymer was used to modify the lipase from *Candida rugosa*. MA, in a functional group, reacts easily with amino acids of lipase. The degree of modification (DM) was varied by changing the weight ratio of copolymer to protein of lipase over the range of 10-120 (w/w). The specific activity decreased as DM increased. At the maximum modification degree of 35%, the modified lipase retained more than 65% of the unmodified native lipase activity. The modified lipase displayed a high stability of activity against temperature and pH. The remaining activity of modified lipase was about 2-4 fold of that of native lipase in the severe pH and temperature condition. Finally, it showed 20% greater reaction of substrate at 10 hr than in the case where native lipase was used.

Key words: Ester Hydrolysis, Modified Lipase, Maleylation, Copolymer, Stability of Enzyme, Two Phase Partition

INTRODUCTION

The effective catalytic properties of enzymes have led to their introduction into several industrial products and processes [Dordick, 1991; Koeller and Wong, 2001; Schmid et al., 2001; Rasor and Voss, 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 widely used in industry for hydrolysis of oils and fats, synthesis of fatty acid esters as cosmetics ingredients or surfactants, and production of intermediates for organic synthesis [Jeon et al., 1999; Guo et al, 2003; Jaeger and Reetz, 1998; Kontkanen, 2004; Borgstorm and Brockman, 1984; Benzamin and Pandey, 1998; Reetz, 2002; Wu et al., 1996; Bousquet et al., 1999; Wu and Song, 2002]. 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 [Longo and Combes, 1999; Noel and Combes, 2003; Matsumoto et al., 2001; Jensen, 1983], extreme pH range or in organic solvents. Numbers of strategies have been proposed to overcome such a limitation including the use of soluble additives, immobilization, protein engineering, and chemical modification [Lee et al., 2002; Chae et al., 1998; Kwon and Rhee, 1984; 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 were typically macroscopic catalysts that were retained in the reactor; therefore, continuous replacement of the enzyme is not necessary.

Modified enzymes were 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 were often more stable than enzymes in solution, and can be applied in a wide range of different reactors. 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 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.

In our previous studies, the 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, 2000, 2001, 2002; Park et al., 2002; Moon and Park, 1993].

In this paper, lipases were modified with copolymer and the characteristics of modified lipases were studied. First, lipases from *Candida rugosa* were modified by chemical reaction with water soluble copolymers such as AKM 0530 and AKM 2010. Then, the activity of native and modified lipases was determined and compared. Finally, stability of modified lipases was tested at various pH, temperature, and reaction time.

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 pow-

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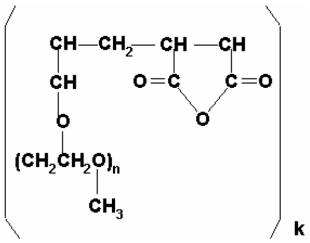
Table 1. The characteristics of synthetic copolymers

Product name of copolymer	n	k	Molecular weight
AKM0530	10	30	18,000
AKM2010	41	11	21,000



n: the number of ethylene oxide units in one PEO chain
k: the degree of copolymerization

der. Lipase OF shows non-regioselectivity and hydrolyzes triacylglycerols almost thoroughly into fatty acids and glycerol. The characteristics of synthetic copolymers used in this experiment are listed in Table 1. Modifier copolymers (AKM0530 and AKM2010), which consist of polyethylene oxide (PEO) and maleic anhydride (MA), were purchased from Nippon Oil & Fats (Tokyo, Japan). The characteristics of synthetic copolymers are given in Table 1. The 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.

2. Modification of Enzyme

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. 1) with the copolymer modifier was carried out as follows: Copolymer was added stepwise to the lipase solution, and the mixture was slowly stirred at 0-4 °C under pH 8.0-8.2, which was controlled with 0.2 M NaOH. This reaction occurred effectively under the condition of pH 8.0 and at low temperature. In the modification of lipase with pure MA, the amino acid groups chemically reacts with the maleic anhydride groups and form covalent bond between them (Fig. 1). 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 range of 10-120 (w/w).

3. Activity Analysis

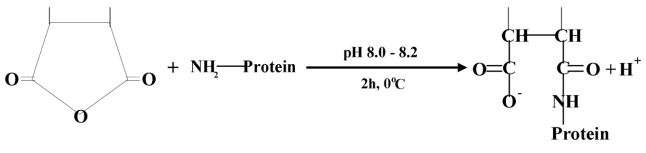


Fig. 1. The scheme of maleylation.

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. 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 at 37 °C and at pH 7 by substrate emulsion method using ethyl caprate as the substrate [Jansen, 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.

4. Two Phase Partition

Two phase partition is the method used to determine the moving ratio of modified and native lipase, respectively. First, dextran and AKM copolymer (AKM 0530, AKM 2010 each) were mixed and native/modified lipase (1 ml) was added. This mixture was then shaken at 300 rpm for 10 min and separated after centrifugation at 3,000 rpm for 10 min. 1 ml sample from top and bottom layers were then taken, and lipase activity was measured [Kim et al., 2004].

RESULTS AND DISCUSSION

1. Chemical Modification of the Lipases

Lipase was modified with pure MA and two types of copolymer. The DM with various weight ratios of copolymer to lipase is shown in Fig. 2. As the weight ratio of a copolymer to lipase increased, DM increased. However, in the case of modification with copolymers, even at high weight ratios, DM did not increase above 35%.

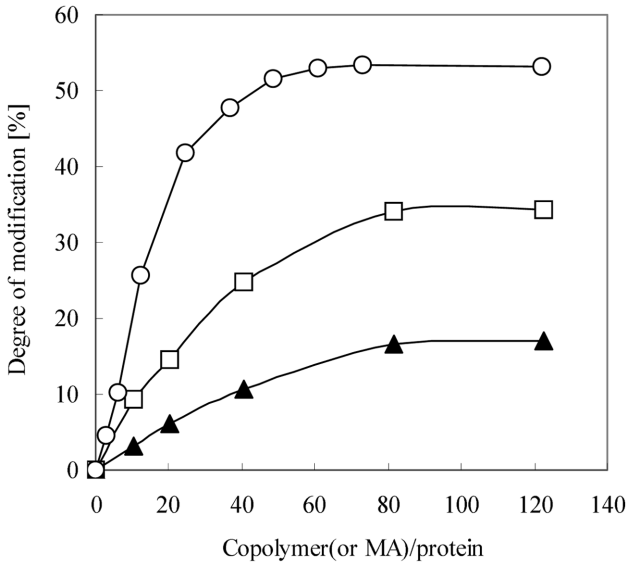


Fig. 2. Effect of weight ratio of polymer on the degree of modification of modified lipase: (○) pure MA; (□) AKM0530; (▲) AKM2010.

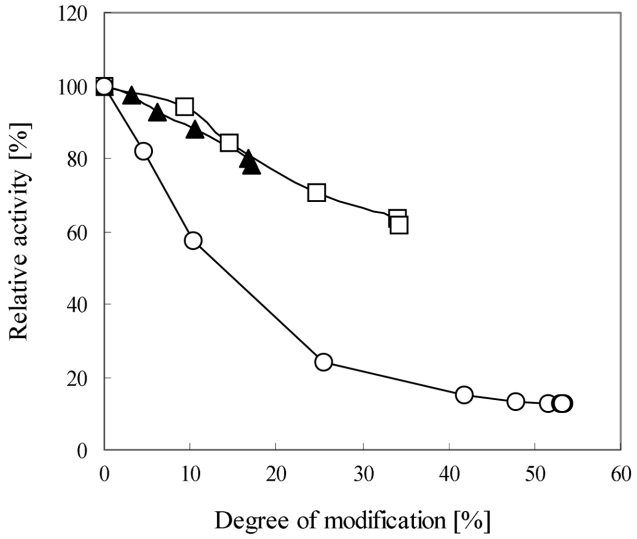


Fig. 3. The relative activity of modified lipase with pure MA and copolymers: A DM value of zero corresponds to native lipase. Modifiers: (○) pure MA; (□) AKM0530; (▲) AKM2010; Reaction condition: Substrate emulsion: ethyl caprate (20 mM)+sodium chloride (20 mM)+calcium chloride (1 mM)+ gum arabic (0.5%); lipase: 5 mg/ml; reaction temperature: 37 °C; reaction time: 15 min; reaction pH: 7.0.

In contrast, the maximum DM by MA was greater than that of synthetic copolymers. These results suggested that a polymer, which was of a higher molecular weight than MA, was more difficult than MA to react with amino groups of lipase due to the steric hindrance of the copolymer.

The relation between DM and relative activity is shown in Fig. 3. Relative activity was defined as the ratio of remaining activity of modified enzyme to native activity. As DM increased, the relative activities decreased. In the case of modification with AKM0530, maximum DM was 35% and more than 65% relative activities were retained. However, the relative activities of modified lipase with MA were less than that with copolymer at the same DM. DM also increased as n value of copolymer decreased. From these results, it was concluded that PEO chains of copolymer, which create a hydrophilic environment, played an important role in the buffering action against denaturation of the enzyme. When enzyme was modified with copolymer, activity reduced because of hindrance effect due to copolymer and enzyme. Enzyme is surrounded by the large branched copolymer, and absorption of substrate is difficult even though copolymer enhances enzymatic absorption. Thus, activity decreased when enzyme was modified. But modified enzyme was stabilized against environmental factor because of copolymer which besieges the enzyme. PEO chains of copolymer were used with buffering reagents. As lipase showed higher DM with AKM0530 copolymer, lipase modified with AKM0530 copolymer was selected to study the optimum reaction conditions for lipase activity.

2. Optimum Condition of Lipase Activity

Optimum reaction conditions of modified lipase with DM 14.6% and relative activity 84.1% with respect to native lipase at its optimum condition were determined. The relative activity according to reaction temperature and pH are shown in Fig. 4(A) and (B), respectively. In the case of temperature change, native and modified

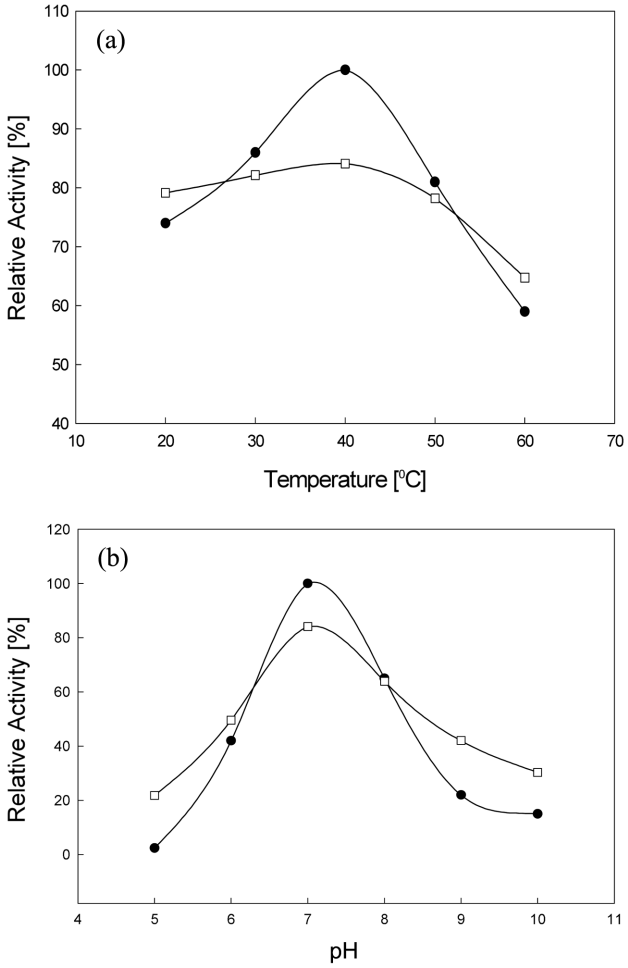


Fig. 4. The relative activity of lipase according to (a) reaction temperature and (b) pH: (●) native lipase; (□) AKM0530-modified lipase with 14.6% DM (84.1% relative activity).

lipase with AKM0530 showed similar patterns. As shown in Fig. 4(A), both the native and the modified activity showed optimum temperature at 37 °C. As the temperature varied from the optimum value, the activities decreased rapidly. However, decrease of activity was more severe for native lipase in the high temperature region. Thus, modified lipase showed higher reactivity than native lipase at temperature below 25 °C and above 50 °C. As shown in Fig. 4(B), a similar tendency was found between native and modified lipase. The optimum pH was around 7 for both, but the activity of modified lipase was less affected by pH change than that of the native one [Guo et al., 2003]. Thus, modified lipase showed higher activity than the native one at pH in a wide range. It was thought that modified enzyme could be adapted at any pH conditions even though the initial activity of modified enzyme was lower than that of native enzyme.

3. Stability of Modified Lipase Against Environment

To examine the stability of enzyme against temperature, the modified lipase with maximum degree of modification (35% DM and 65% relative activity) was incubated at pH 5.0 and 37 °C and the effect is shown in Fig. 5. Because the activity of native lipase decreased rapidly with time, only 10% of activity was remained after 3 hr incubation. On the other hand, the modified lipase was more

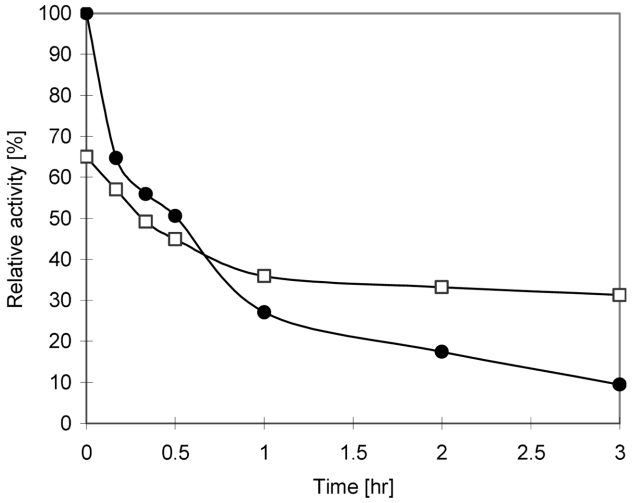


Fig. 5. Stability of native and modified lipase according to incubation time: (●) native lipase; (□) AKM0530-modified lipase with DM 35% and relative activity 65%.

stable and showed higher activity in spite of low initial activity during first 40 min. than native lipase. Modified lipase showed same relative activity at 40 min of incubation, and then it showed better reactivity after that time. The stability of enzyme against several pH was studied as well. After 40 min. of incubation time, modified lipase with a copolymer was more stable against pH than the native lipase because of the buffering action created by PEO chain of modifier copolymer (data not shown). When enzyme was modified with copolymer, the modified enzyme was showing both enzyme- and polymer-like properties. While MA was chemically bonded with amine groups of lipase, hydrophilic PEO chains were facing water.

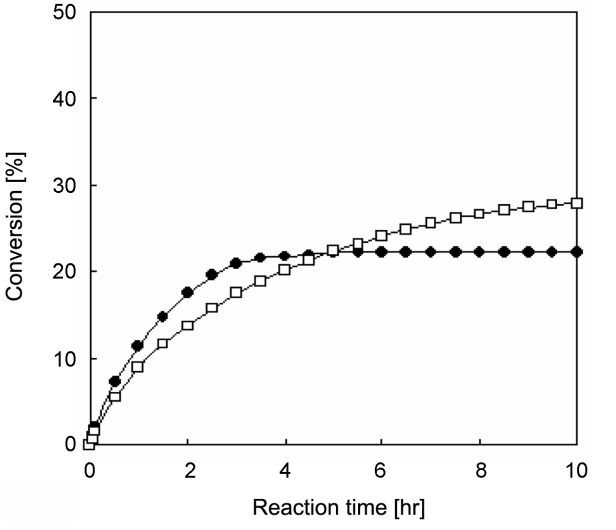


Fig. 6. Conversion rate according to incubation time: (●) native lipase; (□) AKM0530-modified lipase with DM 35% and relative activity 65%; Reaction condition: Substrate emulsion: ethyl caprate (20 mM)+sodium chloride (20 mM)+ calcium chloride (1 mM)+gum arabic (0.5%); lipase: 5 mg/ml; reaction temperature: 37 °C; reaction time: 15 min; reaction pH: 7.0.

Thus, copolymer containing PEO chain acted as cushion or buffering action to protect enzyme from the harsh environmental factors such as high temperature or pH.

4. Conversion Rate of the Hydrolysis Reaction

Fig. 6 shows effect of reaction time on the conversion of substrate. Native and modified lipases of the same initial enzyme concentration (10 g powder/L) were added to each reaction system. The substrate conversion increased almost linearly with time within the first 2 h, but the reaction began to slow afterwards. The native lipase, having higher initial activity than the modified lipase, showed greater conversion than modified one in the initial period of the reaction. But at 5 h, the conversions showed the same value, and after 10 h, the modified lipase showed 20% greater conversion than the native one. Native and modified enzyme showed almost same adsorption rate at the beginning (data not shown). However, native lipase showed stronger adsorption than the modified one, and initial conversion rate was higher. But strong absorption actually inhibits further reaction. For modified enzyme, it was easier to re-adsorb the substrate than the native one because of weak adsorption. As reaction proceeded, more modified enzyme was desorbed from the substrate than the native one, and more free enzymes were available for reaction. Thus, modified enzyme which showed relatively weak adsorption, actually showed better conversion than the native one as reaction time was increased. From these results it was found that modified lipase, which was stable against temperature and pH, also showed greater conversion than native lipase for reaction time.

5. Two Phase Partition of Native and Modified Lipase

Copolymer and dextran were mixed for the phase separation, and a phase partition curve of a mixture is shown in Fig. 7. These lines separate one/two phase region. The lower region of the line represents one phase layer where no separation is occurring. Several researchers have made use of the theory developed by Flory and Huggins to describe the thermodynamics that lead to phase separation. From their theory, 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

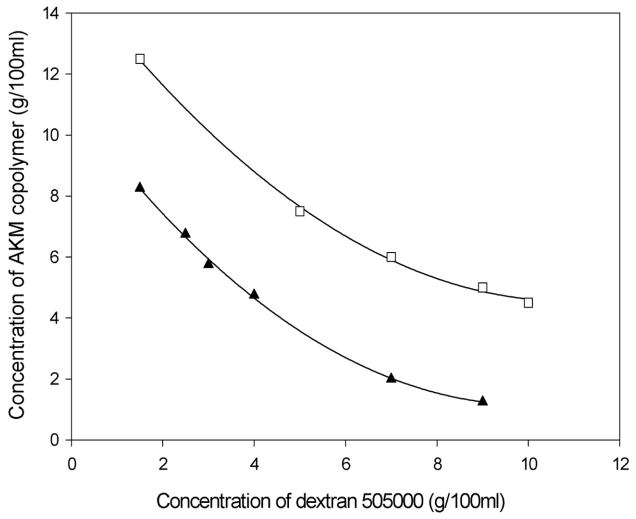


Fig. 7. Phase diagram of dextran 505000 and AKM copolymer: (□) AKM0530; (▲) AKM2010.

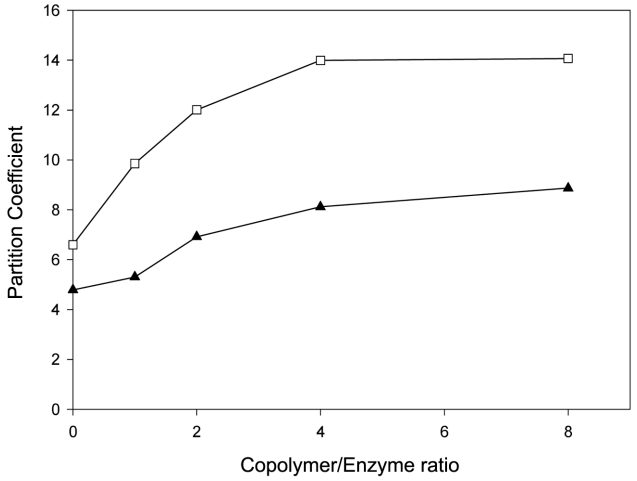


Fig. 8. Partition coefficients according to copolymer/enzyme ratio: (□) AKM0530; (▲) AKM2010.

concentration of polymer was also needed to obtain phase separation when copolymer with low n value was used.

Copolymer and dextran were used to separate the modified enzyme, and the partition coefficient curve is shown in Fig. 8. Partition coefficient (PC) was defined as the ratio of the glucose concentration of top phase to the bottom phase. Partition coefficient of modified enzyme increased as modification ratio of copolymer increased. Fig. 8 shows that the modified lipase with reactive copolymer moved to the upper phase, AKM copolymer phase. As a result, over 93% of enzyme, moved to copolymer phase. In case of AKM 0530, the partition coefficient of native enzyme was 5 point, but it was increased above double point for the modified one.

CONCLUSIONS

Lipase from *Candida rugosa* was modified with copolymers. Maleic acid functional groups of copolymer were covalently coupled with amino groups of the lipase surface. The degree of modification (DM) could be controlled by varying the weight ratio of copolymer to protein of lipase. As the DM increased, the relative activity of modified lipase decreased slightly, but more than 65% activity of modified lipase was maintained in spite of maximum DM of 35%. Modified lipase displayed high stability of activity against temperature, pH, and reaction time. In addition, various copolymers could be used in the modification reaction to achieve different molecular sizes as well as hydrophilicity. Partition coefficient of enzyme was greatly affected by the types of AKM copolymer in the reactive two phase separation. Partition coefficient of enzyme decreased as length of EO chain of polymer increased, and increased with increase of k. Thus, AKM-0530 copolymer was found to be enzyme selective and modified enzyme always showed better partitioning than that of native one. Thus, it has been found that copolymerization of native lipase with copolymer makes it more stable over a wide range of temperature and pH; and also it seems to be possible to make it applicable in different reaction environment by synthesizing copolymer by varying the degree of copolymerization (k) and the number of ethylene oxide units in one PEO chain (n). This would form the basis of our future paper.

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REFERENCES

Benjamin, S. and Pandey, A., "*Candida rugosa* Lipases: Molecular Biology and Versatility in Biotechnology," *Yeast*, **14**, 1069 (1998).

Borgstrom, B. and Brockman, H. L., *Lipases*, Elsevier, New York (1984).

Bousquet, M.-P., Willemot, R.-M., Monsan, P. and Boures, E., "Lipase-catalyzed α-Butylglucoside Lactate Synthesis in Organic Solvent for Dermo-cosmetic Application," *J. Biotechnol.*, **68**, 61 (1999).

Chae, H. J., In, M. J. and Kim, E. Y., "Optimization of Protease Immobilization by Covalent Binding Using Glutaraldehyde," *Appl. Biochem. Biotech.*, **69**, 53 (1998).

Charusheela, A. and Arvind, L., "Enzyme Catalyzed Hydrolysis of Esters Using Reversibly Soluble Polymer Conjugated Lipases," *Enzy. Mirob. Technol.*, **30**, 19 (2002).

Diamond, A. D. and Hsu, J. T., "Aqueous Two-phase Systems for Biomolecule Separation," *Advances in Biochem. Eng. Biotech.*, **49**, 89 (1992).

Dordick, J. S., *Biocatalysis for Industry*, Plenum Press, New York (1991).

Dossat, V., Combes, D. and Marty, A., "Efficient Lipase Catalyzed Production of a Lubricant and Surfactant Formulation Using a Continuous Solvent-free Process," *J. Biotechnol.*, **97**, 117 (2002).

Eijsink, V. G. H., Bjørk, A., Gåseidnes, S., Sirevåg, R., Synstad, B., Burg, B. van den and Vriend, G., "Rational Engineering of Enzyme Stability," *J. Biotechnol.*, **113**, 105 (2004).

Furukawa, M., Kodera, Y., Uemura, T., Hiroto, M., Matsushima, A., Kuno, H., Matsushita, H. and Inada, Y., "Alcoholysis of ε-Decalactone with Polyethylene Glycol-Modified Lipase in 1,1,1-Trichloroethane," *Biochem. and Biophys. Res. Commun.*, **199**, 41 (1996).

Goto, M., Kamiya, N., Miyata, M. and Nakashio, F., "Enzymatic Esterification by Surfactant-coated Lipase in Organic Media," *Biotechnol. Prog.*, **10**, 263 (1994).

Guo, Z. and Sun, Y., "Characteristics of Immobilized Lipase on Hydrophobic Superparamagnetic Microspheres to Catalyze Esterification," *Biotechnol Prog.*, **20**, 500 (2004).

Habeeb, A. F. S. F., "Determination of Free Amino Groups in Protein by Tri-nitrobenzene Sulfonic Acid," *Anal. Biochem.*, **14**, 328 (1966).

Jaeger, K. and Reetz, M., "Microbial Lipase Form Versatile Tools for Biotechnology," *Trends Biotechnol.*, **16**, 396 (1998).

Jeon, G. J., Hur, B. K. and Yang, J. W., "Hydrolysis of Castor Oil with Lipases and Organic Solvents," *Korean J. Biotechnol. Bioeng.*, **14**, 696 (1999).

Jensen, R. G., "Detection and Determination of Lipase (Acylglycerol Hydrolase) Activity from Various Sources," *Lipids*, **18**, 650 (1983).

Joo, H., Yoo, Y. J. and Dordick, J. S., "Polymers from Biocatalysts," *Korean J. Chem. Eng.*, **15**, 362 (1998).

Kim, B. G. and Choi, C. Y., "A Study on Ethanol Production by Immo-

bilized Cells of Zymomonas Mobilis," *Korean J. Chem. Eng.*, **1**, 13 (1984).

Kim, Y. S., Lee, H. J., Jang, S. K., Park, K. N., Park, J. W. and Maken, S., "Purification of Native and Modified Enzymes Using a Reactive Aqueous Two-phase System," *J. Ind. Eng. Chem.*, **10**, 384 (2004).

Koeller, K. M. and Wong, C.-H., "Enzymes for Chemical Synthesis," *Nature*, **409**, 232 (2001).

Kontkanen, H., Tenkanen, M., Fagerström, R. and Reinikainen, T., "Characterisation of Steryl Esterase Activities in Commercial Lipase Preparations," *J. Biotechnol.*, **108**, 51 (2004).

Kwon, D. Y. and Rhee, J. S., "Immobilization of Lipase for Fat Splitting," *Korean J. Chem. Eng.*, **1**, 153 (1984).

Lee, S. K., Park, S. W., Kim, Y. I., Chang, K. H., Hong, H. I. and Kim, S. W., "Immobilization of GL-7-ACA Acylase for the Production of 7-ACA," *Korean J. Chem. Eng.*, **19**, 261 (2002).

Longo, M. A. and Combes, D., "Thermostability of Modified Enzymes: A Detailed Study," *J. Chem. Technol. Biotechnol.*, **74**, 25 (1999).

Matsumoto, M., Kida, K. and Kondo, K., "Enhanced Activities of Lipase Pretreated with Organic Solvents," *J. Chem. Technol. Biotechnol.*, **76**, 1070 (2001).

Moon, H. Y. and Park, J. W., "Reactive Aqueous Two-phase Partition of Cellulose," *Korean J. Chem. Eng.*, **10**, 235 (1993).

Nishio, T., Takahashi, K., Tsuzuki, T., Yoshimoto, T., Kodera, Y., Matsushima, A., Saito, Y. and Inada, Y., "Ester Synthesis in Benzene by Polyethylene Glycol-modified Lipase from Pseudomonas Fragi 22.39B," *J. Biotechnol.*, **8**, 39 (1988).

Noel, M. and Combes, D., "Effects of Temperature and Pressure on *Rhizomucor miehei* Lipase Stability," *J. Biotechnol.*, **102**, 23 (2003).

Park, C. Y., Ryu, Y. W. and Kim, C., "Kinetics and Rate of Enzymatic Hydrolysis of Cellulose in Supercritical Carbon Dioxide," *Korean J. Chem. Eng.*, **18,** 475 (2001a).

Park, K. N. and Park, J. W., "Modification of Cellulose by Synthesized Copolymer with Polyethylene Oxide and Malic Acid Anhydride," *J. Applied Polymer Science*, **77**, 368 (2000).

Park, J. W. and Park, K. N., "Biological De-inking of Waste Paper Using Modified Cellulose with Polyoxyethylene," *Biotechnology Techniques.*, **13**, 49 (1999).

Park, J. W., "Chracteristics of Cellulase Modified with Amphiphilic Copolymer in Organic Solvent," *Korean J. Chem. Eng.*, **12**, 523 (1995).

Park, J. W. and Park, K. N., "Improvement of the Physical Properties of Reprocessed Paper by Using Biological Treatment with Modified Cellulose," *Bioresource Technol.*, **79**, 91 (2001).

Park, J. W., Park, K. N., Song, H. C. and Shin, H. C., "Saccharification and Adsoption Characteristics of Modified Cellulases with Hydrophilic/Hydrophobic Copolymers," *J. Biotechnol.*, **93**, 203 (2002).

Park, K. N. and Park, J. W., "Biological Reprocessing of Mixed Office Waste Using Modified Cellulase by Production of Functional Copolymer," *Korean J. Chem. Eng.*, **19**, 285 (2002).

Park, S. W., Kim, Y. I., Chang, K. S. and Kim, S. W., "Improvement of Stability of Immobilized GL-7-ACA Acylase through Modification Glutaraldehyde," *Process Biochem.*, **37/2**, 153 (2001b).

Reetz, M. T., "Lipases as Practical Biocatalysts," *Curr. Opin. Chem. Biol.*, **6**, 145 (2002).

Schmid, A., Dordick, J. S., Hauer, B., Wubbolts, M. and Witholt, B., "Industrial Biocatalysis Today and Tomorrow," *Nature*, **409**, 258 (2001).

Wu, J. C. and Song, B. D., "Esterification Reactions Catalyzed by Surfactant-coated *Candida rugosa* Lipase in Organic Solvents," *Process Biochem.*, **37**, 1229 (2002).

Wu, Y. X., Jaaskelainen, S. and Linko, Y. Y., "An Investigation of Crude Lipases for Hydrolysis, Esterification and Transesterification," *Enzyme Microb. Technol.*, **19**, 226 (1996).

Wu, Z. C., He, Z. M., Yao, C. Y. and Yu, K. T., "Increased Activity and Stability of *Candida rugosa* Lipase in Reverse Micelles Formed by Chemically Modified AOT in Isooctane," *J. Chem. Technol. Biotechnol.*, **76**, 949 (2001).