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学位授与の題目	Enhanced Activity and Stability of Chromobacterium Viscosum Lipase in AOT Reverse Micelles by Enzyme Pretreatment and Media Engineering （AOT 逆相ミセル反応場における C.V.リパーゼの活性化と安定化－酵素修飾とミセル修飾－）
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1. BACKGROUND

Enzymatic catalysis performed in nonaqueous media has been the object of intensive basic and application-oriented research. The use of enzymes in nearly anhydrous organic media instead of the natural, aqueous media, has expanded their potential for using in syntheses including the ability to catalyze reactions impossible in water, enhanced thermostability and radically altered selectivity. However, one important drawback regarding the use of enzymes in organic media is the fact that they are usually less active in organic solvent-based systems than in aqueous solutions. This low activity may be attributed to several different phenomena including insolubility of enzymes in organic solvents, a drastic decrease in the polarity of the enzyme's microenvironment and less contact of enzyme with substrates due to insufficient interfacial area.

To counter these limitations, a number of different techniques have been introduced leading to improve the enzyme activity in organic solvents. For example, enzyme colyphilization with lyoprotectants and salts, immobilization via covalent attachment in solid supports and imprinting with substrates and substrate analogs represent strategies that have been successful for improving the catalytic activity of enzymes. However, such modification of enzymes is laborious and time consuming, and many cases, they have not significantly increased the enzyme activity. In addition to the enzyme modification, media engineering has also been undertaken to overcome the limitations, such as addition of water-mimicking agents and solubilization of enzymes. The advantage of solubility technique lies in the simplicity of the preparation of reaction media and placing the enzyme molecule into such microreactor. The solubility techniques involve formation of either biphasic mixtures where aqueous enzyme solution is emulsified in a water-immiscible solvent (isooctane or n-heptane or n-hexane) or reverse micelles where enzyme is dissolved in nanometer sized pools of water entrapped within surfactant molecules. The remarkable features of organic-aqueous biphasic systems are simple preparation, easy regeneration of enzyme and easy separation of the reaction product.

However, such a system faces several drawbacks, namely deterioration of the catalytic properties of the enzyme by direct contact with organic solvents, shear stress forces caused by high stirring speed and insufficient organic-aqueous interfacial area for enzyme activation even after extensive stirring. Most of these problems have successfully been overcome by forming self-assembled aggregation of surfactants, e.g., reverse micellar systems.

A reverse micelle is a self-organized aggregation of surfactant molecules formed in apolar solvent and has the ability to solubilize a relatively large amount of water in the polar core to form a nanometer-sized water pool. Enzyme molecules are entrapped in the inner water cavities of RMs, and are protected by a layer of surfactant shell, which protects the enzyme against the inactivation by the bulk organic phase. This system is especially interesting for lipases that are only activated at an oil-water interface, and reversed micelles offer such interfaces that are stabilized by a layer of surfactant molecules. In addition, the macroscopic homogeneity transparency of this system permits the use of continuous spectrosphotometric techniques for the investigation of enzyme conformation as well as micellar structure. In view of these advantageous features, reverse micellar systems have been extensively used to investigate enzymatic reactions containing water-insoluble substrates and/or products.The AOT [sodium bis (2-ethyl -1-hexyl) sulfosuccinate]/isooctane system is used as one of the most suitable systems since reverse micellar systems with this surfactant are easily obtained in the absence of cosurfactants, and are thermodynamically stable over a wide range of temperature and composition. However, Riter and coworkers [1,2] have reported that the interaction of water with AOT head groups eliminates bulk-like solvent dynamics, where water is essentially ice like in structure. The performances of enzymes hosted in AOT/isooctane/water reverse micelles are thus negatively affected by the distinctive properties of water, which inhibits the accessibility of enzyme active site to the micellar interface. In addition, ionic surfactants like AOT cause degradation of the activity of enzymes through electrostatic and hydrophobic interactions with solubilized enzyme molecules [3-5].

In order to improve the lipase activity entrapped in AOT reverse micelles, several strategies have been undertaken, such as chemical modification of AOT [6], modification of AOT reverse micelles through the use of additives [7,8] and formation of RMs with the aid of co-surfactants [9]. Our previous groups [10-12] have found that the addition of short chain methoxypolyethylene glycol, low molecular weight polyethylene glycol and Tween 85 to the AOT reverse micelles is very effective in enhancing lipase activity. The first two additives dissolve in the water pool whereas the third dissolves at the micellar interface, where it acts as a cosurfactant. The purification of modified AOT in a good yield in very difficult while the additive complicates the down stream separation. However, less attention was paid to investigation the effects of lipase pretreatment on its activity, kinetics and stability entrapped in AOT/isooctane/water reverse micellar systems. Although chemical modification of the enzyme has been reported to be effective in decreasing the interactions between lipase and

AOT head groups, specific hydrolytic activities of lipase are significantly reduced. To attain appreciable catalytic activity of lipase in RMs, therefore, a simple and effective pretreatment procedure for the lipase needs to be developed. In the first part of my thesis, I report on studies dealing with enhancing enzymatic performance in AOT reverse micelles by the pretreatment with various organic solvents.

To date, the majority of the studies in reverse micellar systems utilize water as the polar component. In comparison to the extensive information available about water in AOT reverse micelles, there exist few references to reverse micelles where the polar phase is anything other than water. It has been previously mentioned that the peculiar properties of micellar water, which are different in physicochemical properties from those of bulk water, is one of the main constraints in the development of micellar enzymology. It is reasonable to expect that if lipase–catalyzed reactions are carried out in RMs in which the polar interior is composed of a mixture of polar organic solvent and water other than only water, the reaction rate may be increased as a result of the largely overcoming the problems caused by micellar water. In addition, since low water favors the esterification reaction, polar organic solvents can be used as a water mimic in AOT reverse micellar systems. Therefore, our interest is to explore the formation of micellar systems in which water is partially replaced by polar solvents, having relatively high dielectric constants and being immiscible in hydrocarbon solvents.

In recent years, the effect of aprotic solvents (polar solvents) on the properties of AOT reverse micelles has been extensively reported [13,14]. Aprotic solvents like dimethyl sulfoxide (DMSO) are interesting molecules with a polar group S=O, and this oxygen atom provides a ready site for the formation of a hydrogen bond to other molecules such as enzymes and water.. However, these research focused only on reverse micelle formation, micellar size and phase behavior. No reports are available on the activity and stability of lipase-catalyzed hydrolysis/esterification/ transesterification in AOT reverse micelles. Such studies are important since aprotic solvents in the micellar interior could lead either to a denaturation or to an enhanced effect, relative to the systems in which the polar phase is only water.

2. Objectives

The major aim of the present study is to enhance the activity, stability and kinetics of lipase entrapped in AOT reverse micelles by pretreatment with organic solvents. Another aim is to investigate the lipase performance in a new AOT reverse micellar system in which micellar polar core is formed with a mixture of aprotic solvents and water other than only water. The following subsections describe the specific objectives of the research presented in this thesis.
The selection of an organic solvent and a particular pretreatment method of the lipase are the main tasks for the enzyme pretreatment. Polar organic solvent acetone is firstly selected as a pretreatment agent. Subsequently, hydrophobic organic solvents are also used in the

pretreatment process. The dependence of catalytic activity on the various parameters relevant to the pretreatment procedure such as organic solvent content, solvent hydrophobicity, water-solvent mixture pH, agitation time (i.e., duration of lipase treatment with organic solvent) and freeze-drying time are investigated in order to optimize pretreated lipase activity.

In bioprocesses, it is a very important factor to have high activities as well as to maintain these activities during the elapsed time. A detailed stability study of the treated lipase entrapped in RMs is conducted. The kinetics of the olive oil hydrolysis reaction with the pretreated lipase is rather important in this study. A kinetic model [15] that considers the free substrate in equilibrium with the substrate adsorbed on the micellar surface is used to better understand the activity enhancement due to the modification of lipase surface. Some intrinsic parameters, V_{max}, K_m and K_{ad} are determined for the treated lipase and compared with those for the untreated lipase.

The spectroscopic analysis can also be used to directly evaluate structural information of the treated lipase. This study is directly addressed to the investigation of lipase structure by a Fourier- transform infrared (FTIR) as well as fluorescence analysis. The fluorescence methods of treated and untreated lipases are undertaken in enzymes characterization because of the inherent sensitivity of this technique. Enzymes have tryptophan as intrinsic fluorophore, highly sensitive to microenvironment polarity. In addition, FTIR spectroscopic technique will also be carried out to give further insight on the conformational changes of the treated lipase.

In the present study, the activity and stability of *C. viscosum* lipase-catalyzed hydrolysis of olive oil and esterification of fatty acid in AOT/water/isooctane reverse micelles is studied when the polar phase is composed of a mixture of aprotic solvent and water (other than only water). Selection of a suitable aprotic solvent is the first task for the modification of micellar interior. For this purpose, the effects of different aprotic solvents of different concentrations on the enzyme activity are studied in the hydrolysis of olive oil.

The influence of aprotic solvents on the micellar properties was investigated by FTIR and fluorescence spectroscopy. The location of DMSO molecules in the micellar systems is identified, using two different fluorescent probes, anionic 1,8-anilinonaphthalenesulfonic acid (ANS) and cationic tris(2,2′-bipyridine) ruthenium dichloride hexahydrate ($Ru(bpy)_3^{2+}$), which reflect the microenvironment of RMs. In order to attain well understanding of the effect of DMSO on the stability and kinetics of lipase in reverse micelles, such studies are essential.

3. Experimental Methodology

The lipase activity (defined as the initial reaction rate, μ mole $dm^{-3}s^{-1}$) is a key parameter investigated in this study. The stability of lipase will be expressed as the residual activity, calculated as a percentage of the original activity (considered 100%), obtained at t = 0 min incubation. The half-life of lipase is also calculated directly from residual activity profiles. Fluorescence spectroscopy and Fourier transform infrared spectroscopy (FTIR) are used to detect the lipase conformational changes caused by pretreatment and to elucidate the effects of DMSO on the properties of AOT reverse micelles. Since lipase fluorescence emission is dominated by the tryptophyl contribution, steady state fluorescence emissions of this residue from untreated and treated lipases is characteristic. On the other hand, FTIR technique is used to detect the exposure of various groups such as amino and carboxylic groups in enzyme molecules, based on the principle that each type of group has a characteristic absorption wave length represented as a downward peak on charted spectrum (Williams and Fleming, 1985). This spectroscopic study will also be used to study the O-H stretching region in AOT reverse micelles with and without DMSO.

4. Summary of the Results

The activity and stability of lipase for hydrolysis of olive oil in AOT reverse micelles was significantly increased by the pretreatment with various organic solvents. Hydrophobic solvents were found to be superior to hydrophilic ones as pretreatment media. Among the organic solvents used, n-hexane was found to be the most effective. The enhancement in lipase activity was attributed to the change in lipase conformation from closed to open form through the exposure of hydrophobic residues to the lipase surface. Catalytic activity of treated lipase was highly dependent on the pretreatment conditions, such as solvent content and hydrophobicity, contact time of the lipase with solvents (agitation time) and freeze-drying time. It was evident that optimum agitation time shifted from 1 hr to 15 min when pretreatment solvent was changed from acetone to hexane. It was also observed that higher n-hexane content with shorter agitation time and vice versa had almost the same effect on the initial activity of lipase. The activity of treated lipase was further enhanced when encapsulated in PEG 400 modified AOT reverse micelles. For the treated lipase in AOT/PEG reverse micelles, besides the favorable effect of enzyme pretreatment, the coexistence of PEG 400 also plays an important role in enhancing enzyme activity by decreasing the interaction between enzyme and surfactants.

The two spectroscopic techniques, fluorescence and Fourier transform infrared (FTIR), of treated and untreated lipases were employed to detect possible conformational changes in the enzyme caused by the pretreatment. Fluorescence spectroscopy indicated that pretreatment with various organic solvents changed the lipase conformation from less hydrophobic close form to more hydrophobic open form through the exposure of hydrophobic residues to the lipase

surface. The more hydrophobic lipase surface favored the binding of hydrophobic substrate leading to an increase in reaction rate. FTIR analysis also suggested that the pretreatment altered the lipase conformation by the exposure of amino group (containing the active site) on the surface of lipase, which was obscured in the case of untreated lipase.

Detailed kinetic study of treated lipase-catalyzed hydrolysis of olive oil was conducted to better understand the activity enhancement due to the modification of lipase surface. A kinetic model that considers the free substrate in equilibrium with the substrate adsorbed on the micellar surface was successfully used to evaluate the intrinsic parameters, V_{max}, K_m and K_{ad}. The differences of K_m and K_{ad} for treated versus untreated lipase indicated that some alteration in the lipase intrinsic properties was caused by the pretreatment of lipase with organic solvents. The lower value of K_m and K_{ad} for treated lipase suggested that the pretreatment with organic solvents enhanced the affinity of lipase for substrate and reduced the interactions with AOT head groups. The activity values calculated by using the model agreed well with the experimental results.

Pretreated lipase exhibited higher stability than that of untreated lipase. For example, the acetone treated lipase entrapped in AOT reverse micelles was very stable, retaining over 83% of its initial activity after 40 days, whereas the half-life of untreated lipase was 38 days. Pretreatment with organic solvents enhances the enzyme surface with hydrophobic residues, which may stabilize the enzyme against denaturation in organic media. Such enhanced lipase operational stability makes the industrial use of the pretreated lipase attractive.

The influence of aprotic solvents upon the properties of the AOT/isooctane/water reverse micelles has been investigated by fluorescence spectroscopy, using two fluorescent probes, anionic 1,8-anilinonaphthalenesulfonic acid (ANS) and cationic tris(2,2′-bipyridine) ruthenium dichloride hexahydrate ($Ru(bpy)_3^{2+}$). Dimethyl sulfoxide (DMSO) improved the lipase activity significantly among the aprotic solvents used. DMSO located at the micellar interface, and concomitantly modified the properties of AOT reverse micelles, such as increasing micellar size, altering properties of micellar interface as well as micellar water, and interposing between lipases and AOT molecules. In this contribution, the interactions between lipase and AOT molecules might be reduced, and the diffusion of substrate to the lipase active site enhanced appreciably. A Fourier transform infrared study of the states of water in RMs was carried out at various DMSO contents. This revealed that DMSO altered the hydrogen bonding network of micellar water and reduced the fraction of bound water due to the strong interactions between DMSO and water molecules. Consequently, the active site of lipase could be oriented more easily toward the micellar interface and come into contact with substrate.
A comparison study was carried out between DMSO modified AOT and simple AOT reverse micellar systems by characterizing the various physicochemical parameters for both systems,

including pH, temperature, surfactant concentration and the water content (W_0, given by the ratio between water and surfactant concentration), using hydrolysis of olive oil and esterification of oleic acids. The maximum activity of lipase entrapped in modified systems was 200% higher than that of simple AOT reverse micelles for the esterification as compared to 160% for hydrolysis. The effect of temperature on reaction rate was studied, and apparent activation energies for the two systems were calculated according to the Arrhenius plot. The lower value for the system modified with DMSO suggested that the addition of DMSO reduced the enthalpy of the transition state or activated complex in AOT reverse micellar systems. In addition, a detailed stability study of lipase entrapped in reverse micelles was undertaken in the presence or absence of DMSO, indicating excellent lipase stability in the presence of DMSO. A simple first order deactivation model was considered to determine the deactivation rate constant. The thermodynamic stability of lipase in reverse micelles was also measured with Gibbs free energy. Improved half-life correlated with a decrease in deactivation rate constant and an increase in Gibbs free energy.

5. Conclusions

This thesis has presented the activation, stabilization and reaction kinetics of *Chromobacterium viscosum* lipase entrapped in AOT/isooctane/water reverse micellar systems. To improve the lipase performances, two techniques have been addressed in my research study: (i) pretreatment of lipase with various organic solvents and (ii) modifying the micellar properties (micellar size, micellar interface and micellar water bound to the interface) by the formation of micellar interior with a mixture of aprotic solvent and water other that only water.

Pretreatment of lipase with organic solvents is a new approach leading to improved enzymes catalytic performances in reverse micelles. This pretreatment of enzyme is a very simple and effective technique to improve its catalytic activity in RMs towards hydrolysis of olive oil. It has provided important insights in enzyme structures such as the amino acids involved in the active site of enzymes, and this may be useful in some applied conversions catalyzed by lipases in organic media A surprising dependence of catalytic activity of the treated lipase on the pretreatment conditions was observed.

The second part of this thesis focused upon the effect of aprotic solvents on the structure and behavior of AOT RMs. Structural alterations of RMs due to the inclusion of DMSO detected by FTIR and fluorescence spectroscopy were related to the lipase activity, kinetics and stability. Two types of lipase-catalyzed reactions, hydrolysis and esterification were considered as model reactions. Spectroscopic analysis suggested that modification of the micellar properties in such a way, as to enhance its effectiveness as media for hosting enzymatic reactions.

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学位論文審査結果の要旨

[審査経過]

平成 18 年 12 月 27 日に第 1 回審査委員会を行い、各委員による面接と諮問、並びに論文内容を調査した。平成 19 年 2 月 5 日に口頭発表(最終試験)を行い、終了後に開催した審査委員会において、以下のように判定した。

[審査結果]

本論文は、10nm スケールの逆相ミセルを反応場としたリパーゼ触媒による非水領域でのオイルの加水分解反応に関するものである。リパーゼ酵素の活性と、安定性を高めるために、これまで全く報告されていない有機溶媒による酵素修飾とミセル修飾を行ない、活性、並びに安定性が向上する原因を明らかにしている。研究成果は 6 編の学術雑誌に掲載されており、きわめて優れた内容である

本論文は、C.V.リパーゼ酵素を触媒とする酵素反応を、有機溶媒中に形成されるミセル内で温和な条件で、効率的に行わせるために有用な知見及び情報を提供しており、博士(学術)に値する。