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学位の種類	博士（工学）
学位記番号	博甲第658号
学位授与の日付	平成16年3月25日
学位授与の要件	課程博士（学位規則第4条第1項）
学位授与の題目	Activation,Stabilization and Reaction Kinetic of Chromobacterium Viscosum Lipase in Organic Media: (1)AOT/Water/Isooctane reverse micelles,(2) Isooctane with low water（有機溶媒中のクロモバクテリウムリパーゼの活性化，安定性化とその反応機構:(1)AOT/水/イソオクタン逆相ミセル系，(2)微量水分を含むイソオクタン系）
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学位論文要旨

Summary

The research in this study was focused on enzyme activation, stabilization and kinetics in organic media: AOT/water/isooctane reverse micelles, isooctane with low water. The activation of lipase in reverse micelle was performed by the addition of additive and by pretreatment with acetone, and that in isooctane with low water content was done by the application of ultrasound generated by 4-waves (25.8, 26.2, 35.5, 36.5 kHz) ultrasonic generator.

The short chain polyethylene glycols (PEGs) or methoxy PEG (MPEGs) was effective to increase the lipase activity in AOT/isooctane reverse micelle. The molecular weight and the concentration of PEG or MPEG significantly influenced the lipase activity, and the PEG and MPEG of nominal molecular weight 400 and 550 respectively for a concentration 5-6 g dm^{-3} was most effective for lipase activation. The maximum activity of lipase in reverse micelle with PEG 400 or MPEG 550 was about 175% higher than that in simple AOT reverse micelle. The increase in lipase activity was attributed to the following reasons: the decrease in lipase-AOT interaction by burring charges at the micellar interface; the reduction of bound water, which inhibits combination of the lipase active site with substrate at micellar interface; the protection

of lipase by PEG 400 clouds formed around the lipase molecule; the conformational change of lipase entrapped in reverse micelles. The *C. viscossum* lipase entrapped in AOT/PEG 400 reverse micelles was very stable at 25℃, retaining over 75% of its initial activity after 60 days, whereas lipase half-life time in simple AOT reverse micelles was 38 days. The lipase stability was improved because of the increase in rigidity of lipase structure by cross-linking with PEG 400.

FTIR analysis suggests that the PEG 400 molecules strongly interacted with the head groups of anionic surfactant AOT, and reduced the fraction of bound water. The change in lipase conformation caused by the interaction with PEG 400 was confirmed by tryptophyl fluorescence spectroscopy of *C. viscosum* lipase. The presence of PEG 400 increased the exposure of hydrophobic residues around the active site, and thus enhanced and stabilized the interaction with olive oil at micellar interface.

A kinetic model was proposed here for better understanding the kinetic of activated lipase-catalyzed hydrolysis of olive oil in AOT reverse micelles. The activity value predicted by the model equation agreed well with the experimental data. The predicted kinetics parameters suggested that affinity of PEG 400 to lipase was higher than that of MPEG 550 but MPEG 550 reduced the interactions between lipase and AOT molecules more significantly than PEG 400. The enhancement of lipase activity either by PEG 400 or MPEG 550 was therefore almost the same. The kinetics parameters obtained by proposed model were further used to estimate the fraction of activated and native lipases.

The pretreatment of lipase with acetone was another alternative to activate the lipase in reverse micelle. The activation of lipase by pretreatment with acetone was attributed to the change in lipase conformation, which was confirmed by the results of tryptophyl fluorescence spectroscopy of treated lipase. The parameters relevant to the lipase pretreatment were optimized, and the activity of acetone treated lipase in AOT reverse micelles with and without PEG 400 was investigated. The maximum activity of acetone-treated lipase in AOT/PEG 400 reverse micelles was found to be 200% higher than that reported for the native lipase in simple AOT reverse micelles.

The activity of lipase in isooctane-water with ultrasound was investigated under various conditions and the results were compared with that in isooctane-water with magnetic stirring. The maximum activity in ultrasonicated system was 175% higher than that in stirred system. However, the lipase stability in ultrasonicated system was drastically decreased. PEG of different molecular weights was added to the ultrasonicated system to improve the lipase stability. A little deactivation (about 8%) of the lipase was found by the presence of PEG 1000 of concentration 5 g dm^{-3}, but the stability was greatly increased: the residual activity was constant after 10 hrs at which lipase retained its 80% initial activity, whereas residual activity of lipase without PEG 1000 was zero after 10 minutes.

Background

The industrial process for non-enzymatic hydrolysis of fats and oils requires high pressure and temperature. For the sake of energy conservation and minimizing thermal degradation of the products, researchers have set out to investigate the feasibility of the hydrolysis of fats and oils catalyzed by the lipase enzyme during the last decade. Enzymatic hydrolysis has the advantages such as mild conditions, high substrate specificity, less pollution to the environment than does traditional chemical reaction. Although enzymes are typically employed in aqueous media, there are several advantages of enzymatic hydrolysis of fats and oils in organic media. First, organic solvents solve the problem of dissolving oils or fats, which often causes a serious obstacle when reaction is carried out in water. Second, the use of organic solvents obviates the danger of bacterial contamination of technological equipment. Third, this system can effectively alleviate product inhibition by partitioning the product away from the enzymes into the organic phase.

One of the intrinsic features of the surface–active enzyme (e.g. lipase) is its activation by interface. Catalytically, the lipases act at or near the interface (Brockman HL, 1984; Khemelnitsky et. al., 1988). It is thus necessary to increase the oil-water interfacial area for rapid reaction rate of lipase-catalyzed hydrolysis of oils or fats. From this viewpoint, organic-aqueous biphasic systems with stirring have been reported to accelerate the enzymatic hydrolysis of oils or fats by using a suitable

amount of water-immiscible solvent (Tsai et. al., 1991; Mukataka et. al., 1987). The remarkable features of organic-aqueous biphasic system are the simple preparation, the easy regeneration of enzyme and the easy separation of reaction product. However such a system has the several drawbacks: the deterioration of the catalytic properties of the enzyme by direct contact with organic solvents and shear stress forces caused by high stirring speed; organic-aqueous interfacial area is not sufficiently enough even after extensive stirring; accurate measurement of surface area is still difficult. Biphasic systems containing solid phase with immobilized enzymes can partially overcome the problem of lipase deactivation but it has been reported that the hydrolytic activity of immobilized lipase was negatively affected by the mass transfer limitation in the pores of solid support (Lee and Swaisgood, 1997; Shaw et.al., 1990).

Reverse micelles, a micro heterogeneous reaction media, offer a unique possibility to overcome the problems caused by the macro heterogeneous medium, such as organic-aqueous biphasic medium with or without solid phase. The fact that reverse micelles can solubilize water and protein (i.e. enzyme) molecules in organic solvents and provides an enormous amount of interfacial area, which promotes contact between enzyme and substrate, has made it an important medium for lipase-catalyzed hydrolysis of oils and fats. In reverse micelle, the lipase is trapped in micellar water-pool, and is isolated from the organic solvent by a surfactant layer; this system therefore protects the lipase from detrimental effect of organic solvent. The other aspect is that the exchange process may take place between two micelles or between each micelle and the bulk organic solvent. Upon collision of two micelles their coalescence occurs through a transient dimmer, that permits a rapid exchange of material (Luisi PL., 1985). The optically transparence of reverse micellar systems provides the opportunity to spectroscopic investigation of lipase conformation when solubilized in a reverse micelle.

The anionic double-tailed surfactant AOT [sodium bis-(2-ethyl 1 hexyl) sulfosuccinate] is frequently used for the study of reverse micelles. The motivation to use AOT in micellar technology has been the large amount of published data on the physio-chemical properties of AOT/isooctane reverse micelle and easy formation of reverse micelle.

However activities of enzymes trapped in AOT reverse micelles are inhibited by the strong interactions with surfactant molecule, and the distinctive properties of micellar water [Hayes and Gulari, 1990; Walde et.al., 1993; Sarkar et.al., 1992].

To overcome this problem, a chemically modified AOT surfactant (Wu et. al., 2001) or a newly synthesized surfactant (Iskandar et. al., 1998) can be used to prepare reverse micelles. But such modification and synthesis of surfactant are laborious and time consuming. In addition, purification of modified AOT in a good yield is very difficult. Although chemical modification of the lipase has also been reported to overcome this problem, specific hydrolytic activities are significantly reduced by the chemical modification of lipase [Basri et al, 1995]. Another simple and easy alternative to reduce the interaction between lipase and head groups of AOT molecules is to use some additives, (Hosain et. al., 1999; Yamada et. al., 1993; Freeman et. al., 1998; Piera-Velazquez et. al, 2001). Recent work in the reverse micellar enzymology has thus centered upon the effect of additives on the catalytic properties of lipase entrapped in reverse micelles.

Objectives

Up to date, the inclusion of additives in AOT reverse micellar enzymology is restricted to reduce the interaction between lipase and head groups of AOT molecules through reducing the charges on micellar interface. There is no report on the use of additives, which attaches on the surface of lipase entrapped in reverse micelles and buries the charges on the micellar interface, thus strongly protects the lipase from unpleasant action of AOT molecules. Furthermore, although the various additives have been used to improve the lipase activity, their effect on the enzyme stability has been ignored. The main objective of this study is therefore to activate and stabilize the lipase by evaluating an additive, which is relative more suitable for lipase-catalyzed reaction than that reported in the literature.

Polymers like polyethylene glycols (PEGs) present attractive physical, chemical and biological properties such as very low toxicity, high polarity; furthermore PEGs are economic and easily available in variety of molecular weight. The terminal hydroxyl groups of the PEG molecule provide a ready site for non-covalent attachment on lipase surface

(Harris JM, 1992). In this study, the short chain PEGs or MPEGs was thus added to enhance the activity and stability of lipase entrapped in AOT/isooctane reverse micelles. The short chain PEGs or MPEGs are only soluble in the micellar water-pool, and attractive interactions between anionic surfactant and PEG or MPEG molecules, lead to PEGs or MPEGs adsorption at the interior surface of the micellar interface (Meier W, 1996) and thus charges on the interface are buried beneath the neutral PEG molecules, reducing the electrostatic interaction between lipase and head groups of AOT molecule.

Various kinetic models have been reported to explain enzyme activity in reverse micelles, but most of them are suitable for water-soluble substrates (Bru et al, 1990; Khmelnitsky et al, 1990; Oldfield C, 1990; Verhaert et al, 1990). For water-insoluble substrate, Hossain et al (1996) have reported a kinetic model considering the free substrate were in equilibrium with the substrate adsorbed on the surface of micellar surfactants. However the model could not satisfactorily explain the lipase activation induced by additives such as short chain PEGs or MPEGs. So another objective of this research work is to develop a kinetic model for enzymatic kinetics in reverse micelles involving water-insoluble substrates and additives.

The active site of lipolytic enzyme (e.g. *C. viscosum* lipase) locates in the hydrophobic amino acids region, and combined with the hydrophobic substrate (e.g. olive oil) at the micellar interface through hydrophobic interaction. The increase of the exposure of hydrophobic amino acids to the lipase surface therefore enhances and stabilizes the interaction with the substrate at the micellar interface. However, no reports are available to enhance the lipase activity in reverse micelles through increasing the exposure of hydrophobic residue around the active site to the lipase surface. In this regard, objective is also set to increase the activity of lipase by pretreatment with acetone. Pretreatment with organic solvent enhances the interaction between hydrophobic substrate and lipase active site by increasing the exposure of hydrophobic amino acid residues to the lipase surface that causes the change in lipase conformation from closed form (lid covering the active site is in closed state) to open form (lid covering the active site is in open state) at micellar interface (Chmorro et. al., 1998; Colton et. al., 1995; Radzicka et. al., 1992; Rue et. al., 1993).

The mixing, which causes the molecular contact between enzyme and substrates, and enhances the mass transport, is the prelude for a rapid enzymatic reaction in organic-aqueous biphasic system, especially for water insoluble substrate such as olive oil. The conventional method, such as, magnetic stirring, mechanical shacking, is usually used as a mixing device. Such methods are however less effective to overcome the mass transport limitation and to increase the molecular contact between lipase and olive oil. Therefore, the objective is extended to enhance the activity of lipase in isooctane-water by application of ultrasound. Ultrasound causes the transient cavitations resulting in high mixing energy: gas and vapor bubbles are formed and violently collapsed in a transient time. Ultrasound is thus used for forming the stable mico-emulsion characterized as a large interfacial area (James, 1988). It has been reported that ultrasound improved molecular contact between enzyme and substrate molecules (Bracey et al, 1998) and reduced the mass transfer limitation by increasing available surface area of the enzyme (Vulfson et al, 1991).

Structural organization of the thesis

The research carried out in this study was introduced in chapter one. Reverse micelles as a reaction media for lipases was described in chapter two. The fundamentals of reverse micellar were described and related to the lipase performance both in terms of activity and stability. The phase boundary between the one phase (reverse micelles) and two-phase (non micellar media) region in ternary system of AOT/water/isooctane was investigated and reported here. The W_0 values were corrected by measuring the water content in reverse micellar system by Karl Fischer titration. Some literature review of the structure, the size and the size distribution of reverse micelles and the lipase micro-encapsulation technique were also reported in this chapter.

Activation of *C. viscosum* lipase by short chain PEG or MPEG was described in chapter three. The influence of PEG or MPEG molecular weight and concentration was investigated, and the PEG 400 or MPEG 550 was found to be most effective. The lipase-catalyzed hydrolysis of olive oil in AOT/PEG 400 reverse micelles was characterized in terms of W_0, buffer pH, buffer ionic strength, surfactant concentration, substrate concentration, etc; the results were compared with those in simple AOT

reverse micelles. The most striking characteristics described in this chapter are the improvement of lipase activity at higher W_0 values and at higher AOT concentrations. The maximum activity in reverse micelles with PEG 400 or MPEG 550 was 175% higher than that in simple AOT reverse micelles.

The detail stability of *C. visocsum* lipase in AOT reverse micelles with or without PEG 400 was reported in chapter four. Two different approaches were considered: the determination of half-life time and the mechanistic analysis of deactivation kinetics. The *C. viscossum* lipase entrapped in AOT/PEG 400 reverse micelles was very stable at 25℃, retaining over 75% of its initial activity after 60 days (half-life time 136 days), whereas half-life time in simple AOT reverse micelles was 38 days. The deactivation rate constant (k_d) was decreased and thermodynamic parameter standard free energy (ΔG^o) of the lipase deactivation was increased by the addition of PEG 400.

The influence of PEG 400 on the microenvironment of *C. viscosum* lipase entrapped in reverse micelle was reported in chapter five. FTIR analysis suggests that PEG 400 molecules participated in the rearrangement of water inside the reverse micelles and strongly interacted with the head groups of anionic surfactant AOT. The fluorescence probing of reverse micelle indicated that viscosity and hydrophobicity of micellar water-pool were increased by the presence of PEG 400. The conformational change of lipase by the presence of PEG 400 was confirmed by tryptophyl fluorescence spectroscopy of *C. viscosum* lipase and was reported here.

The combine effect of Tween 85 (polyoxyethylene sorbitan trioleate) and MPEG 550 on lipase activation was reported in chapter six. The maximum activity in AOT/Tween 85/MPEG 550 reverse micelles was 220% higher than that in simple AOT reverse micelles. Reaction kinetic suggests that in contrast to Tween 85, MPEG 550 has no effect on the substrate adsorption at the micellar surfactant surface.

The proposed kinetic model for enzymatic reactions in reverse micellar systems involving water-insoluble substrate (i.e. olive oil) and enzyme activators such as PEG 400 or MPEG 550 was described in chapter seven. A comparison was made between the data predicted by

model equation and that found from the experimental results. The improvement of the utility of the proposed model and previous model (Hossain et al, 1996) was reported in chapter eight. The determination of activation energy for *C. viscosum* lipase-catalyzed hydrolysis olive oil in reverse micelles with and without PEG 400 by Arrhenius equation was reported in chapter nine. The activation energy was decreased from 7.6 to 4.8 kcal/mol by the addition of PEG 400.

The activation of lipase by pretreatment with acetone was described in chapter ten. The parameters relevant to the lipase pretreatment were optimized through measuring the activity of treated lipase-catalyzed hydrolysis of olive oil in AOT reverse micelles under a fixed set of reaction condition. In chapter eleven, the activity of acetone treated lipase in AOT reverse micelle was investigated varying the reaction conditions, such as W_0, pH, AOT concentrations, etc, and the results were compared with those for the native lipase. In contrast to native lipase, no sharp fall of the activity at higher W_0 values and at higher AOT concentration was observed, whereas the optimal pH and reaction temperature were the same with those for the native lipase. The effect of PEG 400 on the activity of treated lipase was investigated in chapter twelve. It was found that the PEG 400 further enhanced the activity of treated lipase and the maximum activity of treated lipase in AOT/PEG 400 reverse micelles was 200% higher than that of the native lipase in simple AOT reverse micelles.

The collisions between reverse micelles are the prelude for rapid enzymatic reaction. In order to increase the micellar collision, ultrasound generated by 4-waves (25.8, 26.2, 35.5, 36.5 kHz) ultrasonic generator was used, and the activity of lipase in ultrasonicated system was investigated in chapter thirteen. The remarkable feature of the ultrasonicated reverse micellar system was no sharp fall of lipase activity at higher W_0 values. The maximum activity obtained in reverse micelles with ultrasound was 120% higher than that in conventional magnetic stirred system. The result indicates that the effect of ultrasound on enhancement in micellar collision was insignificant because the reverse micelles are very dynamic and the rate of collision is high.

The application of ultrasound to increase the reaction rate was

further tested in non-micellar system (isooctane with low water) and was described in chapter fourteen. In contrast to reverse micellar system, the large activation of *C. viscosum* lipase in isooctane-water by ultrasound was obtained. The effect of ultrasonic intensity on lipase activity was investigated, and progressive deactivation of lipase was observed at higher intensity. The maximum activity obtained in ultrasonicated system was 175% higher than that in conventional magnetically stirred system. It should be noted that *C. viscosum* lipase activity in both the AOT/water/isooctane reverse micelle and isooctane-water with magnetic stirring was almost the same, and the result indicates that lipase activity in isooctane-water with ultrasound is higher than that in reverse micelle. Although the activity of lipase was significantly increased by the ultrasound at moderate intensity, the stability of lipase in isooctane-water with ultrasound (half life is 2-3 min) was relatively low with that in reverse micelles (half life is 38 days) or in isooctane-water (half life is 10 min) with magnetic stirring. The enhancement in lipase stability in ultrasoniicated system was thus very important considering the industrial application of lipase.

The stabilization of *C. viscosum* lipase in isooctane-water with ultrasound by the addition of PEG was reported in chapter fifteen. The influence of PEG molecular weight and concentration was investigated, and PEG 1000 was selected for lipase stabilization. The residual activity of lipase in PEG 1000 added system was constant after 10 hrs at which lipase retained its 80% initial activity, whereas residual activity of lipase without PEG was zero after 10 minutes.

The conclusions and the recommendations for future work were reported in chapter sixteen. The rest of the thesis consists of appendixes, abbreviations, literature citation and acknowledgement.

Conclusions

The activation and stabilization of *C. viscosum* lipase in organic media was carried out. The molecular weight and the concentration of PEG or MPEG influenced the lipase activity in reverse micelle, and the PEG or MPEG of nominal molecular weight 400 and 550 respectively for a concentration 5-6 g dm^{-3} was the most effective for lipase activation. The maximum activity of lipase in reverse micelle with PEG 400 or

MPEG 550 was about 175% higher than that in simple AOT reverse micelle.

The *C. viscosum* lipase entrapped in AOT/PEG 400 reverse micelles was very stable at 25℃, retaining over 75% of its initial activity after 60 days, whereas half-life time in simple AOT reverse micelles was 38 days.

FTIR analysis suggests that PEG 400 strongly interacted with the head groups of anionic surfactant AOT, and reduced the fraction of bound water, which inhibits the combination of lipase active site with olive oil at the micellar interface. The strong interaction with AOT head groups proved that charges on the micellar interface was buried beneath the neutral PEG 400, and the AOT-lipase interaction was thus reduced. The change in lipase conformation induced by the interaction with PEG 400 was confirmed by tryptophyl fluorescence spectroscopy of *C. viscosum* lipase.

Although, the substrate adsorption on micellar surfactant surface, was unaffected by PEG 400 or MPEG 550, it was significantly reduced by Tween 85. The activity of lipase in AOT/MPEG 550 reverse micelle was thus further increased by the presence of Tween 85, and the maximum activity in AOT/MPEG 550/Tween 85 was 220% higher than that in simple AOT reverse micelles.

The proposed kinetic model was very effective to predict the activity of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reverse micelles containing PEG 400 or MPEG 550, and the fraction of activated and native lipases were successfully estimated.

The pretreatment of lipase with acetone was another alternative to increase the lipase activity in AOT reverse micelle. The maximum activity of acetone-treated lipase in AOT/PEG 400 reverse micelles was 200% higher than that of native lipase in simple AOT reverse micelles.

Ultrasound enhanced the lipase activity in isooctane-water and the maximum activity in ultrasonicated system was 175% higher than that in stirred system. The lipase stability in isooctane-water with ultrasound was relatively lower than that in stirred system. The stability in ultrasonicated system was however greatly increased by the presence of PEG 1000: the residual activity, was constant after 10 hrs at which

lipase retained its 80% initial activity, whereas residual activity of lipase without PEG 1000 was found to be zero after 10 minutes.

学位論文審査結果の要旨

平成16年1月27日の口頭発表、同日開催の論文審査委員会における最終審査により、以下の通り判定した。

本申請論文では低分子量のポリエチレングリコール(PEG400)をAOT逆相ミセル系に添加すると、リパーゼ活性、並びに安定性が向上することを実験的に検証した。そしてFTIR及び蛍光光度計による解析により、ミセル内でのPEG400とリパーゼ酵素との親和性並びに酵素の立体構造変化を究明し、ナノ粒子内でのPEG400の分子特性を明らかにした。特に、アセトンで前処理したリパーゼ酵素を用いると、酵素の立体構造変化から更に酵素活性が向上することを見出している。また、界面活性剤によるミセル系を使用しなくとも、微量水分を含むイソオクタン系加水分解反応を超音波雰囲気で行うと酵素活性が向上すること、この系にPEG1000を添加することにより、酵素安定性が向上することを実験的に明らかにした。以上の結果に基づき、PEG400を添加したAOT逆相ミセル系酵素反応に係わる反応機構を解明した。これらの研究成果は、博士（工学）に値するものと認定する。