Reversible Control of Enzymatic Transglycosylations in Supercritical Fluoroform using a Lipid-Coated _-D-Galactosidase

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We have reported that lipid-coated hydrolytic enzymes, in which hydrophilic head groups of lipids interact with the enzyme surface and lipophilic two long alkyl-chains extend away from its surface that solubilize the enzyme in organic solvents, act as an efficient catalyst for the reverse hydrolysis reactions such as esterification by lipase and transglycosylation by glycosidase.[1] The catalytic activities of the lipid-coated enzymes were 2-100 times higher than that of other systems, including enzymes in organic/aqueous emulsion,[2] dispersed powdered enzymes[3] and poly(ethylene glycol)-grafted lipase in organic solvents.[4] However, the activity and stability of the lipid-coated enzymes in organic media largely depend on their polarity, as well as other enzyme systems. Thus, the esterification rate of the lipid-coated lipase was high in nonpolar isooctane or benzene, but denatured in the polar media such as chloroform and tetrahydrofuran.[1] This is a large disadvantage of enzyme reactions in organic media, because the polar solvents are attractive for the high solubility of substrates.

Recently, supercritical fluids (scFs) became interested as a new media for chemical reactions including enzymatic reactions, since their physical properties (*e.g.* polarity, diffusion, and viscosity) are intermediate between those of gases and liquids, and they can be manipulated by small changes in pressure or temperature.[5-7] We have reported that the lipid-coated enzymes are soluble and show the high activity in supercritical carbon dioxide ($scCO_2$) as well as organic media.[7]

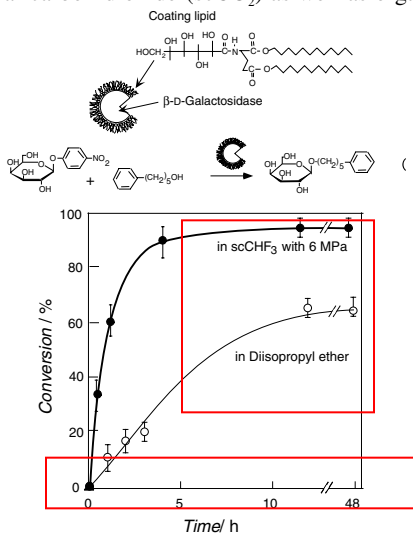

Figure 1. A schematic illustration of a lipid-coated β-D-galactosidase and time-courses of transgalactosylation from *p*-nitrophenyl-β-D-galactsopylanoside (10 mM) to 5-phenyl-1-pentanol (100 mM) at 37 °C catalyzed by the lipid-coated β-D-galactosidase (1 mg of protein) in 10 mL of $scCHF_3$ with 6 Mpa (●) or atomospheric diisopropyl ether (❍).

In this communication, we report that the transgalactosylation rate catalyzed by the lipid-coated β-D-galactosidase in supercritical fluoroform ($scCHF_3$) can be reversibly controlled by changing temperature or pressure (reflecting polarity changes) without damaging enzymes. The reason fluoroform was chosen as the scF is that the dielectric constants (ε) of $scCHF_3$ can be changed from 1 to 7 (corresponding to ε value of hexane to tetrahydrofuran, THF as organic media) by manipulating either temperature or pressure of scFs.

A lipid-coated β-D-galactosidase (from *Bacillus circulans*) was prepared by mixing aqueous solutions of enzyme and lipid molecules in the same way as reported previously.[1,7] ~~It was confirmed from elemental analysis, UV absorption, and gel chromatography in organic media that one enzyme is covered by about 200 ± 50 lipid molecules as a monolayer and~~ that the protein content in the complex is 7 ± 1 wt%.[1,7] The lipid-coated enzyme was found to be soluble (*ca.* 1 mg/10 mL) in $scCHF_3$ in the range of 30-60 °C and 5-25 MPa.

Transgalactosylation reactions were carried out as follows.[1c,7b] In a stainless steel vessel with pressure-resistant glasses both substrates of *p*-nitrophenyl-β-D-galactopyranoside and 5-phenyl-1-pentanol and a lipid-coated β-D-galactosidase were added, then liquid CHF_3 was injected at 5-25 MPa from a LC pump (Jasco PU-980 HPLC pump) connected to a CHF_3 gas cylinder. The vessel was warmed under stirring magnetically above 25 °C to create supercritical state, and the pressure was kept constant (± 0.1 MPa) by a backpressure regulator. At the every appointed time, the vessel was degassed carefully under cooling at 0 °C. The residual powder was solubilized in CH_3CN and analyzed by a HPLC.

Figure 1 shows typical time courses of the transgalactosylation from *p*-nitrophenyl-β-D-galactopyranoside (10 mM) to 5-phenyl-1-pentanol (100 mM) catalyzed by a lipid-coated β-D-galactosidase (1 mg of protein) at 37 °C both in $scCHF_3$ at 6 MPa and in atmospheric diisopropyl ether. In $scCHF_3$, 5-phenyl-1-(β-D-galactopyranosyl)pentanol was obtained as the only transgalactosylated product in 90-95% yield after 5 h. The transgalactosylation in $scCHF_3$ was 20-fold faster than in the atomospheric diisopropyl ether. The rate increase in $scCHF_3$ may be due to the decrease of viscosity or the degree of solvation to substrates, as compared with the conventional solvents. ~~Transgalactosylations also rapidly occurred with acceptor alcohols having a large alkyl group such as 1,2-didodecyl glycerol (conversion: 45 %) and cholesterol (conversion: 15 %).~~ When the native β-D-galactosidase was employed in $scCHF_3$ instead of the lipid-coated one, the reaction hardly proceeded because the native enzymes were insoluble and existed as powders (Curve c, Figure 1). Sentences were added.

One of the futures of scF as reaction media is that the physicochemical properties such as dielectric constant (ε) of media can be continuously changed by varying temperature or pressure in scF state.[5] Effects of continuous changes of temperature or pressure on both the initial rates and conversion of the transgalactosylation catalyzed by a lipid-coated enzyme are shown in Figure 2. When pressures were continuously changed at the constant temperature of 37 °C (Figure 2A), galactosylations were very slow below P_c = 48 °C, where the medium exists as gaseous CHF_3 and the lipid-coated enzyme exist as powders. Reaction rates were drastically increased above 4.8 MPa and then decreased drastically above 10 MPa,

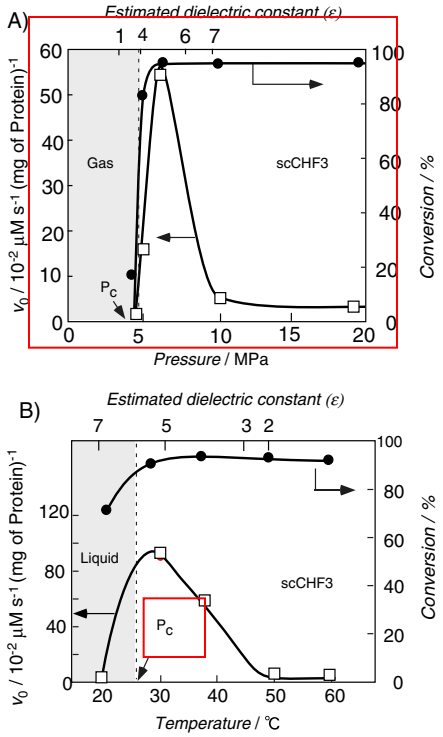

Figure 2. Effect of (A) pressure changes at 37 ℃ and (B) temperature changes with 6 MPa on the initial rates (□) and the conversion (●) of transgalactosylation catalyzed by the lipid-coated β-D-galactosidase in sc CHF_3.

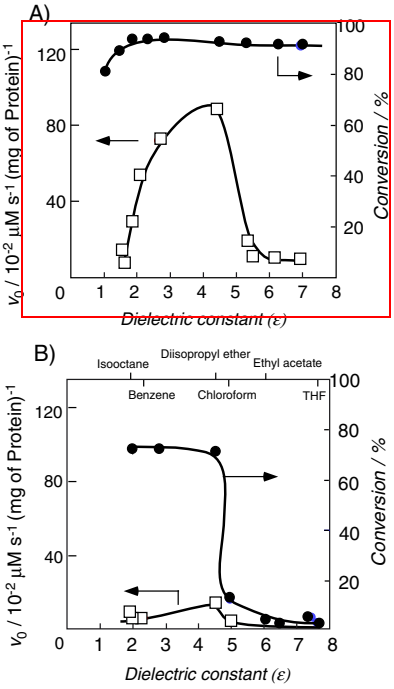

Figure 3. The initial rates (□) and conversion (●) of transgalactosylation were plotted against dielectric constants (ε). (A) In the $scCHF_3$ obtained by pressure and temperature changes, and (B) in the atomospheric organic solvents by changing media.

although where the lipid-coated enzyme is homogeneously solubilized in sc CHF_3. However, the conversions were constant to be 95% in the range of 6 to 20 MPa. The similar tendency was observed when temperatures were changed from 20 to 60 ℃ at the constant pressure of 6 MPa; the initial rate showed a bell shape behavior, although the conversion was constantly high as 90-95% (Figure 2B). Sentence was added. Since the β-D-galactosidase activity in aqueous buffer solution increased gradually with increasing temperature from 20 to 60 ℃, these activity changes are explained by special physical property changes of $scCHF_3$. When the temperature or pressure of $scCHF_3$ was changed continuously, it is well known that dielectric constants (ε) of the media altered continuously[5] and these estimated apparent ε values are shown at the upper x-axis of Figure 2.

In Figure 3A, the initial rare and conversion are plotted against apparent ε values in different pressures (5-20 MPa) and temperatures (20-60 ℃) of $scCHF_3$ shown in Figure 2. The initial rates showed a bell-shaped curve and the conversion was constantly high independent of ε values of $scCHF_3$. When the lipid-coated β-D-galactosidase was employed in various organic solvents as a catalyst for the same transglycosylation of eq. (1) of Figure 1, both the initial rate and conversion were drastically decreased in the polar solvents of chloroform, ethyl acetate, and tetrahydrofuran (Figure 3B).[1c] The similar denature of enzymes in the polar organic solvents has been shown in the esterification catalyzed by a lipid-coated lipase.[1b] Thus, the enzyme is active in the none-polar solvents such as isooctane, benzene, and diisopropyl ether, however, it easily denatured in the polar solvent and did not revert the reactivity. When $scCHF_3$ was employed as the medium, the polarity could be altered continuously by manipulating pressure or temperature without changing media, and the reaction rate could be controlled reversibly by keeping the high conversion. Thus, the enzyme can be kept inactive at 20 ℃ with 5 MPa or 50 ℃ with 15 MPa, and revert to the active at 30 ℃ with 7.5 MPa in $scCHF_3$. This could be controlled reversibly at least 10 cycles.

In conclusion, the lipid-coated β-D-galactosidase is soluble and can catalyze transgalactosylation 2-20 times faster in supercritical CHF_3 than in the conventional organic media. The reaction rate could switch on and off by adjusting pressure or temperature of $scCHF_3$. Since CHF_3 has no chloride or bromide atoms, it is not included in ozone-depletion fluorocarbons and can be practically used by recovering and recycling. We believe that the combination of the lipid-coated enzyme and $scCHF_3$ reaction media will become a new system as biotransformation studies.

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