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Fluorometric Assay Protocol for Protease-Catalyzed Transesterification Reactions in Organic Solvents

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Abstract: A flourometric assay protocol for a subtilisin-catalyzed transesterification reaction in *n*-hexane has been developed. The method makes use of a Michael acceptor that forms a fluorescent adduct with thiophenol, one of the products generated in the transesterification reaction. The method may be employed for screening a biocatalyst useful for transesterification reactions in organic solvents and for optimizing the transesterification reaction conditions.

Enzymatic reactions in nonaqueous media have received much attention as a tool for organic synthesis.[1] One of important advantages of using organic solvents in enzymic reactions is that substrate specificity may be regulated by solvent selection,[1] although the catalytic activities in organic solvents are often reduced by orders of magnitude compared with those in aqueous solutions.[1] However, it has been demonstrated that the enzymic activities in organic solvents can be enhanced significantly by the incorporation of carbohydrates,[2] polymers,[3] organic buffers, or inorganic salts into dry enzyme,[4] and cross-linking,[5] thus overcoming the problem associated with the catalytic activity.

In using enzymes in organic synthesis, identification of enzymes that can be used for a specific reaction requires assaying over several dozens of potential enzymes, and once identified, one still needs to perform a large number of preliminary experiments with the identified enzyme to find optimum reaction conditions for the desired transformation. In these exploratory experiments, data collections usually relied on GC or HPLC, which is laborious and time-consuming. Accordingly, it is highly desirable to develop alternative approachs that can circumvent the inconvenience of the chromatographic method of product analysis. Recently, there have been reported a variety of alternative protocols that make use of FTIR spectroscopy,[6] IR thermography,[7] UV−visible spectroscopy,[8] NMR,[9] capillary array electrophoresis,[10] circular dichroism,[11] pH indicator,[12] mass spectrometry,[13] DNA microarray,[14] immunoassay,[15] and chemosensor,[16] and some of them are employed for high-throughput screening of lipase-catalyzed kinetic resolution of racemic esters.

We wish to describe herein a fluorometric method for monitoring protease-catalyzed transesterification reactions in organic medium. The protocol takes advantage of the facile Michael acceptor property of a commercially available dye, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM), which is known to undergo a rapid addition reaction with a thiol to generate a highly fluoresencent product.[17] It was envisioned that the transesterification may conveniently be probed fluorometrically, if the enzymic reaction is carried out in the presence of CPM using a thioester as the substrate, for the thiol that is generated in the reaction would undergo an addition reaction with CPM to form a fluorescent product (Figure 1). The protocol has been evaluated with a prototypical protease, subtilisin Carlsberg,[18] a much used protease[19] in transesterification reactions.[20]

Four thioesters (**1a**−**d**) were evaluated as the substrate for the enzymic transesterification reaction to find that **1d**[21] serves most satisfactorily. An approximately 130-fold enhancement of fluorescence intensity was measured when **1d** is used as substrate in the enzyme-catalyzed transesterification with 1-propanol using lyophilized KCl-activated subtilisin Carlsberg[22] (Figure 2). In a typical experiment, the biocatalyst (20 mg) was added to a mixture of *n*-hexane (5 mL) containing **1d** (2 mM),

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(21) Experimental details are found in the Supporting Information.

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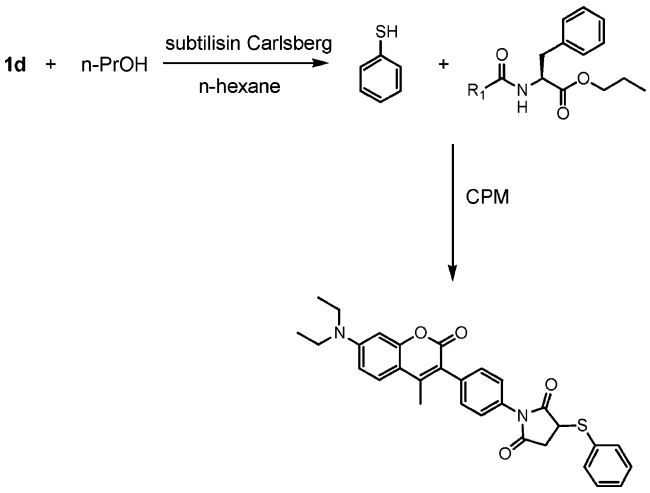
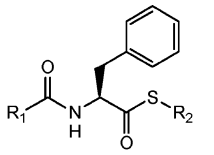


FIGURE 1. Schematic representation illustrating the fluorometric assay protocol for the subtilisin-catalyzed transesterification reaction in an organic solvent.



1a : R_1 = OBn R_2 = Ethyl
1b : R_1 = methyl R_2 = Ethyl
1c : R_1 = OBn R_2 = Ph
1d : R_1 = methyl R_2 = Ph

1-propanol (0.85 M) and CPM (10 μM), and the resulting mixture was shaken at 250 rpm in a constant temperature (30 °C) incubator for 10 min, then filtered, and the fluorescence of the filtrate was measured to observe a strong fluorescence emission at λ = 450 nm (λ_{ex} = 385 nm), as can be seen in Figure 2. No appreciable change in fluorescence was observed when the reaction mixture devoid of **1d** was treated under the parallel conditions, indicating that 1-propranol does not undergo the addition reaction with CPM to any appreciable extent, and that the fluorescence observed in the above transesterification reaction of **1d** is due to the Michael adduct formed with the thiophenol generated in the reaction.

It has been known that the catalytic activity of subtilisin Carlsberg in organic solvents is strongly dependent on the KCl content in the lyophilized preparation of the enzyme. Khmelnitsky et al. have established by GC analysis of the transesterification products that the maximum catalytic activity of subtilisin Carlsberg is obtained with the enzyme containing 98% (w/w) KCl.[22] We have reinvestigated the effect of KCl on the subtilisin-catalyzed transesterification reaction in *n*-hexane by the fluorometric method outlined above. Substrate **1d** was subjected to the transesterification reaction with 1-propanol in the presence of CPM and the subtilisin Carlsberg[23] containing various amounts of KCl, and the catalytic activity was monitored by the fluorescence at

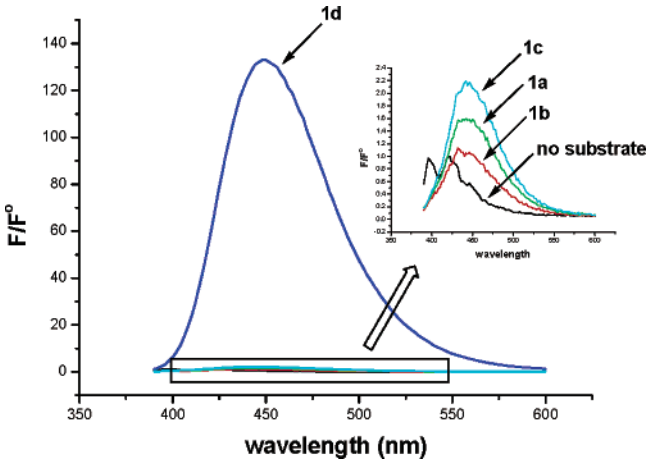


FIGURE 2. Subtilisin-catalyzed transesterification reactions of **1a**−**d** with 1-propanol in *n*-hexane in the presence of CPM. The reaction mixture of the substrate (2 mM), 1-propanol (0.85 M), CPM (10 μM), the biocatalyst (20 mg; subtilisin Carlsberg, 1% w/w + phosphate buffer salt, 1% w/w + KCl, 98% w/w), and *n*-hexane (5 mL) in a 20-mL glass scintillation vial with a Teflon-lined screw cap was shaken at 30 °C and 250 rpm for 10 min, and filtered. Fluorescence measurement was made on the filtrate at 450 nm (λ_{ex} = 385 nm).

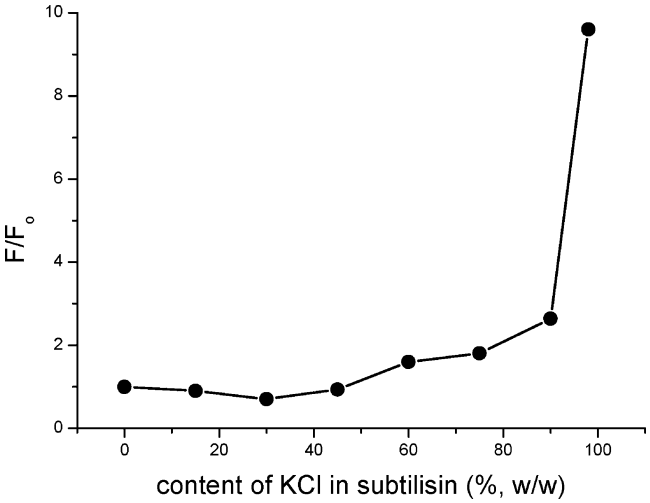


FIGURE 3. Relationship between transesterification activities in terms of F/F_o in the reactions of **1d** with 1-propanol and the content of KCl in subtilisin Carlsberg.

450 nm. Figure 3 shows that the enzymatic activity expressed in terms of the fluorescence intensity increases sharply when the salt content approaches 98%, which is in excellent agreement with the result obtained by Khmelnitsky et al.,[22] demonstrating that the present method is a viable alternative to the conventional method in identifying a useable biocatalyst for a desired transesterification reaction.

The plot of fluorescence changes vs the amount of biocatalyst gives a straight line (Figure 4) up to the subtilisin concentration of 3.37×10^{-6} M. We have also studied the transesterification reaction catalyzed by activated α-chymotrypsin[22] and obtained a similar result,[21] demonstrating that the fluorometric protocol can be applied to the transesterification reaction catalyzed by α-chymotrypsin, a prototypical serine protease. Figure

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(23) Subtilisin Carlsberg containing KCl was prepared as described in ref 22.

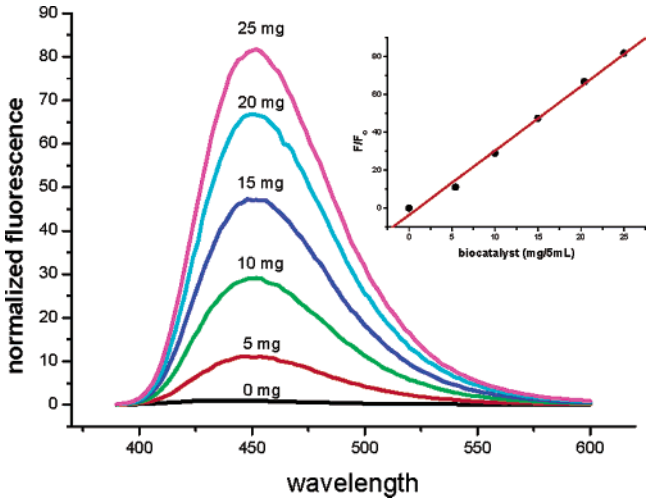

FIGURE 4. Reaction mixtures containing various amounts of the biocatalyst (subtilisin Carlsberg, 1% w/w + phosphate buffer salt, 1% w/w + KCl, 98% w/w) (0, 5, 10, 15, 20, and 25 mg), 1-propanol (0.85 M), **1d** (2 mM), and CPM (10 μM) in *n*-hexane (5 mL) were shaken at 250 rpm in a constant temperature (30 °C) incubator for 10 min, then fluorescence measurements were performed after removing insolubles by filtration at 450 nm (λ_{ex} = 385 nm). Inset: Replot of the normalized fluorescence against the amount of biocatalyst.

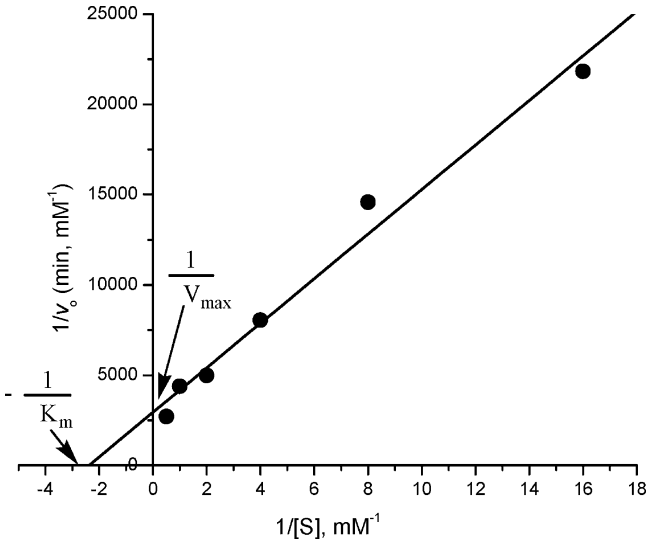

FIGURE 5. The Lineweaver−Burk plot for the transesterification reaction of **1d** with 1-propanol in *n*-hexane in the presence of biocatalyst (subtilisin Carlsberg, 1% w/w + phosphate buffer salt, 1% w/w + KCl, 98% w/w). In a typical experiment, 10 mg of the biocatalyst was added to 5 mL of *n*-hexane containing various amounts of **1d**, 0.85 M of 1-propanol, and 10 μM of CPM. The reaction was carried out at 30 °C. Fluorescence changes at 450 nm (λ_{ex} = 385 nm) were monitored. In obtaining the v_o value for the transesterification reaction at the specified **1d** concentration, the concentration of the fluorescent product is obtained from the following equation: 10 μM × (F_{obs}/F_{max}), in which F_{max} represents the maximum fluorescence intensity exhibited by 10 μM of the Michael adduct and F_{obs} represents the fluorescence intensity exhibited by the adduct when a specified amount of **1d** is used.

4 suggests further that at the initial stage of the reaction the formation of the Michael adduct may not be the rate-determining step in the overall process and thus enables one to perform kinetic analysis of the transesterification reaction. We have performed the kinetic study for the subtilisin-catalyzed transesterification reaction as described by Nieuwenhuizen et al.[24] but making use of the calibration curve in Figure 4. Figure 5 represents the Lineweaver−Burk plot[25] from which the values of K_m and k_{cat} for the reaction were calculated to be 4.2 × 10^{-4} M and 3.4 × 10^{-7} M min^{-1}, respectively. The k_{cat} value was inferred from the V_{max} value to be 9.06 × 10 min^{-1}.

In summary, we have developed a highly efficient fluorometric assay protocol for protease-catalyzed transesterification reactions in organic solvents. In this protocol, the thiophenol that is generated from the enzyme-catalyzed transesterification reaction undergoes in situ Michael addition reaction with CPM to form a highly fluorescent adduct, and thus the reaction progress can be monitored by the highly sensitive fluorometric method. We believe that the present assay protocol is much more convenient than conventional methods that rely on HPLC or GC in product analysis because the protocol is easy to employ, less time-consuming, highly sensitive, and allows simultaneous monitoring of parallel reactions in a high-throughput format. The kinetic constants for the subtilisin-catalyzed transesterification reaction in *n*-hexane were determined. Although there have been reported a variety of fast assaying methods for catalytic activities of liapases, no such method is known for transesterification catalyzed by serine proteases such as subtilisin Carlsberg and α-chymotrypsin. Since thioesters are generally less reactive toward enzyme-catalyzed transesterification reactions than esters, the reaction conditions determined by using the thioester as substrate can be transferred to transesterification reaction of the corresponding ester substrate.

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Supporting Information Available: Experimental procedures for the preparation of **1a**−**d** and fluorometric monitoring of α-chymotrypsin-catalyzed transesterificaton of **1d** with 1-propanol in *n*-hexane. This material is available free of charge via the Internet at http://pubs.acs.org.

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