CHEMCOMM Communication
www.rsc.org/chemcomm

Optimum conformational flexibility of subtilisin to maximize the enantioselectivity for subtilisin-catalysed transesterification in an organic solvent with an addition of dimethyl sulfoxide

Keiichi Watanabe,[a] Takashi Yoshida[b] and Shin-ichi Ueji*[ab]

[a] *The Graduate School of Science and Technology, Kobe University, Nada, Kobe 657-8501, Japan*
[b] *Division of Natural Environment and Bioorganic Chemistry, Faculty of Human Development and Sciences, Kobe University, Nada, Kobe 657-8501, Japan. E-mail: ueji@kobe-u.ac.jp; Fax: +81-78-803-7761*

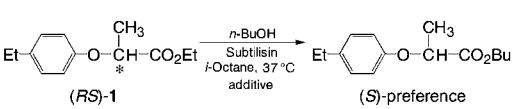
Received (in Cambridge, UK) 8th February 2001, Accepted 21st May 2001
First published as an Advance Article on the web 20th June 2001

For subtilisin-catalysed transesterification of the racemic esters in *i*-octane containing dimethyl sulfoxide as an additive, the relationship between the enantioselectivity and the conformational flexibility of subtilisin estimated from the ESR spectroscopic study provides the first experimental evidence that the enzyme has the optimum flexibility to produce the maximal enantioselectivity toward the given substrates.

The ability of enzymes to discriminate between enantiomers has been applied to the optical resolution of racemates in organic syntheses.[1] Among the strategies to improve the enzyme's enantioselectivity, organic chemists and enzymologists often employ various additives for enzyme-catalysed reactions in organic solvents,[2] because of their simplicity of use. Although the variation of the enzyme's flexibility caused by an additive, such as a small amount of water in organic solvents, is anticipated to control its enantioselectivity,[3] definite information is lacking as to the relationship between the enzyme's flexibility and its enantioselectivity for enzyme-catalysed reactions. According to research done so far, the only reported observation is that the effect on enantioselectivity by the variation of organic solvents is related to the enzyme's flexibility, estimated from time-resolved fluorescence anisotropic study.[4]

In this communication, for subtilisin-catalysed transesterification in *i*-octane, we wish to report the mechanism of enantioselectivity enhancement caused by addition of dimethyl sulfoxide on the basis of the relationship between the initial rates for each enantiomer of the substrates used here and the conformational flexibility of subtilisin estimated from ESR spectroscopic study. Furthermore, the relationship established reveals that the optimum conformational flexibility of subtilisin gives the maximal enantioselectivity.

For subtilisin-catalysed transesterification of ethyl (*R*)- or (*S*)-2-(4-ethylphenoxy)propionate **1** with *n*-butyl alcohol in *i*-octane, we investigated the behaviour of the initial rates for each enantiomer of **1** caused by addition of a small amount of various additives such as water or polar organic solvents (Scheme 1), because this method of enantioselectivity improvement by additives is the simplest one. In a typical subtilisin-catalysed transesterification, the substrate **1** (0.025 mmol) and *n*-butyl alcohol (0.15 mmol) were added to dry *i*-octane (2 ml) containing the additives (0–0.60 vol%), followed by ultrasonic dispersion, and then addition of subtilisin (10 mg).† The reaction mixture was shaken (170 strokes min^{-1}) at 37 °C.



(*RS*)-**1** (*S*)-preference

Scheme 1 Subtilisin-catalysed transesterification of ethyl 2-(4-ethylphenoxy)propionate **1** with *n*-butyl alcohol in *i*-octane.

The effects of the additives (0.45 vol%) on the initial rates as a measure of the enzymatic activity for subtilisin-catalysed transesterification of **1** in *i*-octane were investigated. When a small amount of DMSO (0.45 vol%) was added to the reaction medium, the initial rate for the correctly binding *S* enantiomer was found to be dramatically enhanced (V_S = 20 nmol h^{-1} mg^{-1}), as compared with those for the addition of other general additives such as water (V_S = 0.21 nmol h^{-1} mol^{-1}) or polar organic solvents (DMF, THF, acetone and acetonitrile), although the reaction for no additive conditions did not proceed at all. The effect of DMSO on the enantioselectivity enhancement was also observed for subtilisin-catalysed hydrolysis of **1** in aqueous buffer containing DMSO.[5]

In order to elucidate the optimum amount of added DMSO, the initial rates for each enantiomer of **1** for subtilisin-catalysed transesterification in *i*-octane were determined for a range of added DMSO (0–0.60 vol%). As is seen in Table 1, the initial rate for the correctly binding *S* enantiomer was dramatically enhanced by DMSO added to the reaction medium, as compared with that for the incorrectly binding *R* enantiomer. In particular, upon the addition of 0.45 vol% of DMSO, subtilisin displayed the largest initial rate for the *S* enantiomer, thus resulting in the maximal enantioselectivity (V_S/V_R = 9.6). A serious decrease in the enzymatic activity, however, was produced by addition of an excess amount of DMSO (0.60 vol%). This is probably because the increased flexibility of subtilisin caused by the excess addition of DMSO does not contribute to accommodating the substrate **1** into the subtilisin's binding site, thus leading to the decrease of the enzymatic activity accompanying the loss of the enantioselectivity. This assumption is also supported by the discussion below on the basis of the results obtained from the ESR spectroscopic study.

Furthermore, in order to investigate the DMSO effect on the other substrate, methyl mandelate **2** was submitted to the model reaction. For subtilisin-catalysed transesterification in *i*-octane, subtilisin preferentially catalysed the *R* enantiomer of **2**. The maximal enantioselectivity (V_R/V_S = 7.0) with an increase of

Table 1 Effects of DMSO on the enantioselectivity (V_S/V_R) and the initial rates for each enantiomer of ethyl 2-(4-ethylphenoxy)propionate **1** for subtilisin-catalysed transesterification in *i*-octane

	Initial rate (10^{-1} nmol h^{-1} mg^{-1})		
Amount (vol%)	V_S	V_R	V_S/V_R
0	0	0	—
0.150	0	0	—
0.300	17	9.9	1.7
0.375	105	26	4.0
0.450	201	21	9.6
0.500	65	19	3.4
0.600	14	10	1.4

 DOI: 10.1039/b101269j

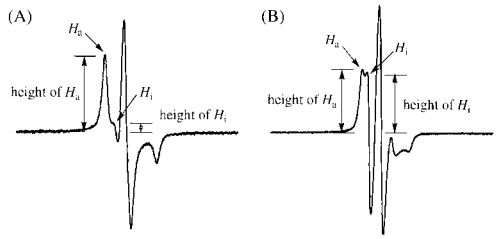


Fig. 1 Typical ESR spectrum of the spin-labeled subtilisin in *i*-octane; (A) no additive condition, (B) 0.45 vol% DMSO.

the enzymatic activity was produced by addition of 0.375 vol% of DMSO. Therefore, our approach using DMSO as the additive was found to be valid for the improvement of the enantioselectivity for subtilisin-catalysed reaction in an organic solvent, although there is the difference in the optimum amount of DMSO between **1** and **2** to obtain the maximal enantioselectivity.

The remarkable enhancement of the enantioselectivity observed is anticipated to be strongly affected by the change of the subtilisin's conformational flexibility caused by addition of DMSO. This view promoted us to investigate the relationship between the initial rates of each enantiomer of **1** and **2**, and the subtilisin's conformational flexibility estimated from ESR spectroscopy. The ESR measurement was carried out under the same conditions as that for the subtilisin-catalyzed transesterification of **1** and **2**, using a spin-labeled subtilisin with 1-oxy-2,2,6,6,-tetramethyl-4-piperidinyl ethoxyphosphorofluoridate prepared by the known method.[6] The spin-labeled subtilisin was indicated by MALDI-TOF MS, in which a fragment (27543) was found which almost corresponded to the sequence of subtilisin (27287) plus the weight of a spin-label (282) less the weight of F (19) and H (1). The spin-labeled subtilisin showed a decrease of enzymatic activity for our model reaction, due to the inhibition by the spin label attached to the active site serine. Fig. 1 shows a typical ESR spectrum, in which two parts of the spectrum are arbitrarily labeled H_a and H_i, respectively. The degree of the subtilisin's conformational flexibility can be monitored roughly by the change in the ratio of the peak height of H_i to $(H_a + H_i)$,[7] because each peak of H_a and H_i represents the anisotropy and the isotropy of the subtilisin's spin-label, respectively.[8] Thus, the increase of the $H_i/(H_a + H_i)$ value reflects that the subtilisin's conformation becomes more flexible. Fig. 2 shows the variation of the $H_i/(H_a + H_i)$ value estimated from the ESR spectra in *i*-octane as a function of the amount of DMSO, in which the increased amount of DMSO in *i*-octane is found to increase the conformational flexibility of subtilisin. In addition, under other additive conditions that give poor enantioselectivity and low enzymatic activity, the ESR signal showed the characteristics of a conformationally rigid enzyme ($H_i/(H_a + H_i)$ = 0.10–0.27).

The plots of the initial rates for each enantiomer of **1** for subtilisin-catalysed transesterification in *i*-octane containing a small amount of DMSO as a function of the $H_i/(H_a + H_i)$ value

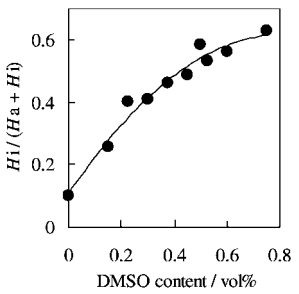


Fig. 2 The variation of the $H_i/(H_a + H_i)$ value estimated from the ESR spectra in *i*-octane as a function of the amount of DMSO.

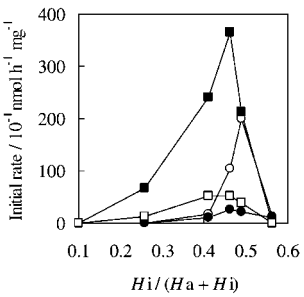


Fig. 3 The relationship between the $H_i/(H_a + H_i)$ value and the initial rates for subtilisin-catalysed transesterifications of **1** and **2** in *i*-octane, ●: **1**-(*R*), ○: **1**-(*S*), ■: **2**-(*R*), □: **2**-(*S*).

are depicted in Fig. 3. The initial rate for the correctly binding *S* enantiomer was significantly enhanced by an increase of the $H_i/(H_a + H_i)$ value. On the other hand, for the incorrectly binding *R* enantiomer, the initial rate is almost unchanged, in spite of the increase of the $H_i/(H_a + H_i)$ value. For subtilisin-catalysed transesterification, the variation of the subtilisin's conformational flexibility caused by addition of DMSO is found to be ascribed to the acceleration of the initial rate for the correctly binding *S* enantiomer, as compared with that for the incorrectly binding *R* enantiomer. Thus, the larger value of the ratio of the initial rates, arising from the marked difference in the flexibility effect on the initial rates for each enantiomer, is significantly responsible for the enhancement of the subtilisin's enantioselectivity.

A serious drop in the initial rates, however, was produced by a small increase of the $H_i/(H_a + H_i)$ value from the optimum flexibility to produce the largest initial rate (Fig. 3). This result is explained by assuming that the subtilisin's flexibility caused by the excess addition of DMSO does not induce the stable association between the substrate **1** and the subtilisin's binding site. Thus, subtilisin is found to display the optimum flexibility to produce the maximal enantioselectivity and the enzymatic activity toward the substrates used here. Furthermore, as is seen in Fig. 3, the change of the substrate from **1** to **2** shows the difference in the optimum flexibility to maximize the enantioselectivity [$(H_i/(H_a + H_i)$ for the largest V_S/V_R value of **1**] > [$H_i/(H_a + H_i)$ for the largest V_R/V_S of **2**], which suggests that the optimum conformational flexibility is responsible for the substrate's structure. Our first observation offers an important insight into the mechanism of the enantioselectivity enhancement for the enzyme-catalysed reactions under the various reaction conditions.

Notes and references

† Because the presence of water is important in the activity of enzymes in non-polar solvents, *i*-octane, *n*-butyl alcohol, and additives used here were dried over Molecular Sieves 4 Å. For our model reactions, however, the enzymatic activity was insensitive to the addition of water (0–0.9 vol%) into *i*-octane.

1 G. Carrea and S. Riva, *Angew. Chem., Int. Ed.*, 2000, **39**, 2226; T. Sugai, *Curr. Org. Chem.*, 1999, **3**, 373; S. M. Roberts, *J. Chem. Soc., Perkin Trans. 1*, 1999, 1; H. Stecher and K. Faber, *Synthesis*, 1999, 1.
2 F. Theil, *Tetrahedron*, 2000, **56**, 2905.
3 T. Okamoto and S. Ueji, *Chem. Commun.*, 1999, 939; K. Kawashiro, H. Sugahara, S. Sugiyama and H. Hayashi, *Biotechnol. Lett.*, 1995, **17**, 1161; H. Kitaguchi, I. Itoh and M. Ono, *Chem. Lett.*, 1990, 1203.
4 J. Broos, A. J. W. G. Visser, J. F. J. Engbersen, W. Verboom, A. Hoek and D. N. Reinhoudt, *J. Am. Chem. Soc.*, 1995, **117**, 12657.
5 K. Watanabe and S. Ueji, *Biotechnol. Lett.*, 2000, **22**, 599.
6 J. D. Morrisett and C. A. Broomfield, *J. Biol. Chem.*, 1972, **247**, 7224.
7 L. J. Berliner, *Biochemistry*, 1972, **11**, 2921.
8 P. P. Wangikar, P. C. Michels, D. S. Clark and J. S. Dordick, *J. Am. Chem. Soc.*, 1997, **119**, 70; R. Affleck, Z.-F. Xu, V. Suzawa, K. Focht, D. S. Clark and J. S. Dordick, *Proc. Natl. Acad. Sci. U.S.A.*, 1992, **89**, 1100.