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# Difference in Catalytic Activities of Subtilisin Carlsberg and Subtilisin BPN′ and Immobilization–Activation for Ester Synthesis and Transesterification in Ethanol[1]

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Free subtilisin Carlsberg (STC) catalyzes the ester formation reaction from *N*-acetylated aromatic amino acids in alcohols containing small amounts of water. The reaction rate and ester yield are strongly dependent on the water concentration, and the maximum rate and ester yield were obtained at around 2% water. The stereo- and substrate specificities of STC are retained in these reaction media. STC is highly stable in ethanol and activity for ester synthesis was mtaintained for at least 5 weeks at 4°C. In contrast to STC, free subtilisin BPN′ (STB) is inactive for ester synthesis in alcohols, but is markedly activated by immobilization (complexation) to poly(vinyl alcohol) or polysaccharides, such as chitin or chitosan. STC is also an effective catalyst for transesterification of *N*-acetyl-L-tyrosine methyl ester to ethyl ester. The immobilization–activation of STB was also realized for transesterification. The change in binding of substrates to STC and the activity of the enzyme for esterification, transesterification, and hydrolysis are discussed on the basis of kinetic measurements.


## INTRODUCTION

It is known that proteases catalyze the synthetic reactions of peptides or esters, which are reverse processes of the hydrolyses of these compounds. In order to shift the equilibrium of the reactions to the synthesis, organic solvents, either miscible (*1–8*) or immiscible (*9–14*) with water, have been employed with minimum amounts of water. Alternatively, "powdered" enzymes dispersed in organic solvents have been used (*15–17*). In most of these reaction systems, enzymes are insoluble or insolubilized by immobilization to solid supports. Generally, the solubilities of enzymes in organic solvents are low, and solubilized enzymes are liable to lose catalytic activity due to unfolding of the peptide chains.

In the course of studies on enzymatic reactions in hydrophilic (water-miscible) organic solvents, it was found that free subtilisin Carlsberg (STC) is soluble but exhibits high catalytic activity for ester synthesis and transesterification of aromatic amino acid derivatives in high concentrations of ethanol. In contrast to STC, however, the catalytic activity of subtilisin BPN′ (STB) is very low but dramatically increases by immobilization to certain solid supports. This article

[1] Enzymatic Reactions in Aqueous–Organic Media, Part IX (*1*).



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describes the details of the catalysis of STC and STB for ester synthesis and transesterification, as well as the effects of immobilization in ethanol containing small amounts of water.

## EXPERIMENTAL

### *Materials*

Subtilisin Carlsberg (type VIII) and subtilisin BPN′ (Nagarse, type XXVII) having specific proteinase activities, with casein, of 11.6 and 6.8 units ($\mu$mol $min^{-1}$ $mg^{-1}$), respectively, were purchased from Sigma Chemical Co. *N*-Acetyl-L-tyrosine (Ac-Tyr-OH) and other *N*-acetylated amino acids were also purchased from Sigma. *N*-Acetyl-L-tyrosine methyl ester was prepared from Ac-Tyr-OH and methanol by the catalysis of hydrogen chloride and recrystallized from ethyl acetate. Alcohols of guaranteed grade were obtained from Nakarai Chemical Co. and dried on 3-Å molecular sieves. Poly(vinyl alcohol) (PVA, degree of polymerization 1700) was purchased from Wako Pure Chemical Co. Chitin and chitosan were products and generous gifts of Katokichi Co. Other support materials were obtained from Nakarai.

### *Reactions and Measurements*

A typical synthetic reaction of Ac-Tyr-OEt was carried out as follows: To a solution of STC (5 mg) in water (0.5 ml) was added a solution of Ac-Tyr-OH (22.3 mg, 10 mM) and acetanilide (25 mg), which was an internal standard for HPLC analysis, in 10 ml of ethanol. The solution was incubated with constant reciprocal shaking (about 150 cycles per minute) at 30°C. The amounts of Ac-Tyr-OH and its ethyl ester (Ac-Tyr-OEt) were determined with an HPLC (JASCO Tri Rotar SR-1) using a JASCO Finepak SIL C18 column eluted with water–acetonitrile (50/50 by volume). The reaction rate was calculated from the amount of Ac-Tyr-OEt formed after 10–60 min reaction. Transesterification was carried out in a manner similar to that of the esterification using Ac-Tyr-OMe as a substrate in ethanol.

The immobilization of STB was carried out by mixing a support material with an aqueous STB solution prior to the addition of a substrate solution. The stability of STC in solutions was studied as follows: 5 mg of STC was dissolved in 0.25 ml of water and kept standing at 4°C for 2 or 5 weeks. Then a solution of Ac-Tyr-OH in ethanol was added, and the solution was incubated as above (method A). Alternatively, 5 mg of STC was dissolved in 0.25 ml of water and then 10 ml of ethanol was added. After 2 or 5 weeks at 4°C, Ac-Tyr-OH was added and the solution was incubated as above (method B).

## RESULTS AND DISCUSSION

### *Esterification of Ac-Tyr-OH by STC*

STC is a unique enzyme in that it is soluble in high concentrations (up to 98%) of ethanol and catalyzes esterification of Ac-Tyr-OH. In general, enzymes lose cata-

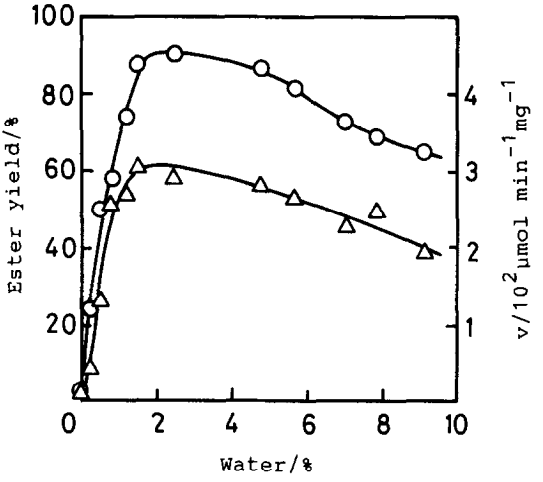


FIG. 1. Effect of water content on rate of ester formation ($v$) and yield of the ester from Ac-Tyr-OH and ethanol. Ac-Tyr-OH, 10 mM; ethanol, 10 ml; STC, 5 mg; 30°C. ○, Ester yield; △, reaction rate.

lytic activity in solutions of organic solvents, probably due to unfolding of the peptide chains. The catalysis of STC for esterification of Ac-Tyr-OH was first reported in ethanol–glycerol by Ingalls *et al.* (*2*). Glycerol was required for the solubilization and stabilization of STC, and the catalytic activity of the enzyme was moderate (the highest ester yield after 70 h was less than 50% at a water content of 10%). In the present study, as Fig. 1 shows, at optimum water contents of around 2%, the yield of the ester, Ac-Tyr-OEt, reached 90% after 24 h reaction in ethanol. Propanols and butanols, in which STC is less soluble and the reaction mixtures were turbid, also gave the corresponding esters (Table 1).

The results of esterification of several *N*-acetylated amino acids are summarized in Table 1. It is obvious that the reactions are highly specific for L-aromatic amino acid derivatives. *N*-Acetyl-L-alanine and -leucine are less reactive, and D-amino acid derivatives and pseudo substrates, HPAA and HPPA, are absolutely inactive. These substrate and stereo-specificities are quite similar to the $S_1$ specificities of STC in hydrolytic reactions. The results suggest that STC strictly maintains its native conformation in this highly water-restricted medium. Interestingly, recent studies showed that the substrate specificities of free STC in octane (*18*) and PEG-modified STC in benzene (*19*) are different from those in water. It must be taken into consideration that the microenvironments around the enzyme in these three reaction systems are different, and this could lead to different substrate specificities due to different partitions of the substrates to the enzyme-binding sites as well as to changes in enzyme–substrate interactions.

As Fig. 1. shows, in the absence of water, where STC is insoluble, the enzyme loses catalytic activity, suggesting that hydration of the enzyme is essential for the catalysis. A similar result was obtained in the catalysis of STC for peptide synthesis in acetonitrile (*1*). The reaction rate increases rapidly with the water concentration up to around 2% (Fig. 1). In this case, the solubility of the enzyme in-

TABLE 1

Ester Synthesis by Subtilisin Carlsberg (STC)[a]

| Substrate | Alcohol | $H_2O$ (%) | Ester yield (%) |
|---|---|---|---|
| Ac-L-Tyr-OH | Ethanol | 2.44 | 90 |
| Ac-L-Trp-OH | | | 82 |
| Ac-D-Trp-OH | | | 0 |
| Ac-L-Phe-OH | | | 80 |
| Ac-D-Phe-OH | | | 0 |
| Ac-L-Ala-OH | | | 23 |
| Ac-L-Leu-OH | | | 53 |
| Ac-L-His-OH | | | 0 |
| Ac-L-Arg-OH | | | 0 |
| Ac-L-Pro-OH | | | 0 |
| HPAA[b] | | 3.85 | 0 |
| HPPA[c] | | | 0 |
| Ac-Tyr-OH | 1-Propanol | 4.76 | 73 (87) |
| | 2-Propanol | | 58 (83) |
| | 1-Butanol | | 34 (82) |
| | 2-Butanol | | 17 (59) |

[a] Substrate, 10 mM; alcohol, 10 ml; STC, 5 mg; 30°C; 24 h. Yields after 96 h are in parentheses.

[b] *p*-Hydroxphenylacetic acid.

[c] 3-(*p*-Hydroxyphenyl)propionic acid.

creases with the water concentration and the reaction mixtures are clear homogeneous solutions at above 2% water. Therefore, it seems that the activity of the enzyme is closely related to its solubility as well as to its degree of hydration. However, recent studies on STC catalysis in heterogeneous systems revealed that solid (dispersed) STC exhibits significant catalytic activity for transesterification (*16*) and acylation of amines (*17*). Thus, whether dissolution of STC is important for its catalysis is still open to question, but at least it has the advantage of eliminating the diffusion limitations of substrates which could be a controlling factor in heterogeneous systems (*20*). Figure 1 indicates that excess water retards the reaction. Since there is evidence that hydrolysis of Ac-Tyr-OEt occurs under these conditions, at least part of the rate reduction at higher water concentrations may be ascribed to the hydrolysis of the product.

Homogeneous reaction systems are suitable for kinetic studies of enzymatic reactions, especially the effects of organic solvents on enzyme catalysis, since there are no diffusion limitations as mentioned above and no differences in local substrate concentration. As shown in Fig. 2, the reaction rate exhibits Michaelis–Menten type dependency on substrate concentration. The value of $k_{cat}/K_m$ obtained by a Lineweaver–Burk plot is 4.5 $M^{-1}$ $s^{-1}$ at water content of 4.8% and 30°C (Table 2). The yield of the ester after 24 h reaction decreases with substrate concentration, but after 7 days, equilibrium yields of around 90% were obtained at all the substrate concentrations examined (Fig. 2).

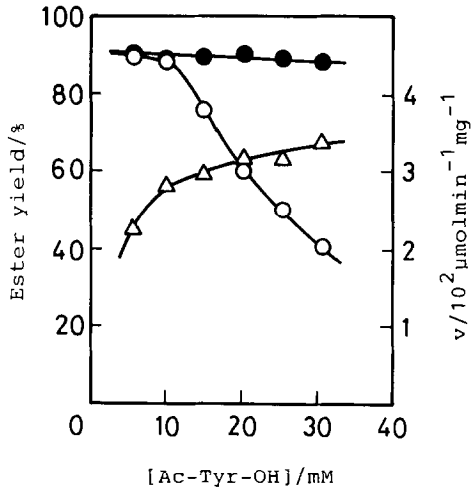


FIG. 2. Plots of reaction rate and ester yield against Ac-Tyr-OH concentration. Ethanol, 10 ml; water, 4.8%; STC, 5 mg, 30°C. ○, Yield after 24 h; ●, yield after 7 days; △, reaction rate.

The stability of STC in ethanol was investigated by examining the catalytic activity of STC after it had been kept in ethanol for 2 and 5 weeks at 4°C. As Table 3 shows, STC exhibited high catalytic activity even after 5 weeks in ethanol, whereas in water the activity dropped under the same conditions.

Figure 3 shows plots of the reaction rate and ester yield against temperature for the reaction of Ac-Tyr-OH and ethanol. The maximum reaction rate was obtained at around 40°C, whereas optimum temperatures for ester yield were between 25 and 35°C. This result suggests that STC deactivates gradually above 40°C; thermal stability in ethanol is much lower than that in water, where the optimum temperature for peptide hydrolysis is around 60°C.

### *Esterification of Ac-Tyr-OH by STB and Effect of Immobilization*

The peptide sequences and three-dimensional structures of STB (*21, 22*) and STC (*23, 24*) have been determined. STB consists of 275 amino acid residues

TABLE 2

Kinetic Parameters for Esterification and Transesterification[a]

| Reaction | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| Esterification | 3.8 | 0.017 | 4.5 |
| Transesterification | 10 | 0.53 | 53 |
| Hydrolysis[b] | 70 | 1930 | 27,600 |

[a] Substrates, Ac-Tyr-OH or Ac-Tyr-OMe, 12–70 mM; STC, 5 mg; EtOH, 10 ml; water, 0.5 ml; 30°C.

[b] In aqueous solutions at pH 8.0 and 37°C (*25*).

TABLE 3

Stability of STC in Water and Ethanol[a]

| Method[b] | Storage time (weeks) | Ac-Tyr-OEt yield (%) |
|---|---|---|
| A | 2 | 80 |
| | 5 | 38 |
| B | 2 | 88 |
| | 5 | 86 |

[a] Reaction conditions: Ac-Tyr-OH, 10 mM; ethanol, 10 ml; water, 2.44%; STC, 5 mg; 30°C; 24 h.
[b] See Experimental.

which differ from STC at 84 positions and by one addition assigned to Pro56. However, the peptide fold (*24*) and enzymatic behavior in aqueous solutions are similar for both subtilisins; the activity of STB for peptide hydrolysis is comparable to that of STC (*25, 26*).

In sharp contrast to STC, STB was found to be almost insoluble in hydrous ethanol and inactive for esterification of Ac-Tyr-OH. The difference in solubility of the two subtilisins in ethanol may be a consequence of the fact that out of 84 different amino acid residues 75 are pointing outward (*22*); that is, despite the similarity in the molecular topology on the surface (*24*), the hydrophilic–hydrophobic properties of the surfaces of the two subtilisins might be different.

It was found, however, that immobilization to PVA or addition of certain polysaccharides, such as chitin or chitosan, leads to marked increases in the activity of STB in ethanol. In the latter case, it was assumed that only a part of STB was

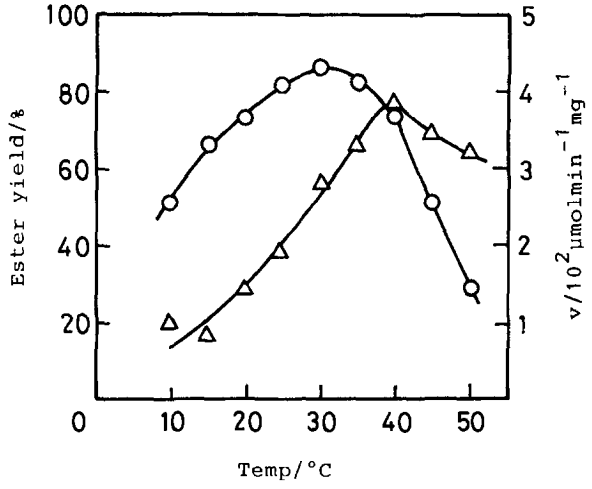


FIG. 3. Effect of temperature on ester synthesis. Ac-Tyr-OH, 10 mM; ethanol, 10 ml; water, 4.8%; STC, 5 mg. ○, Yield after 24 h; △, reaction rate.

TABLE 4

Esterification of Ac-Tyr-OH and Transesterification of Ac-Tyr-OMe by Immobilized STB[a]

| Support | Ac-Tyr-OEt yield (%) | | |
|---|---|---|---|
| | Esterification | | Transesterification |
| | 24 h | 96 h | 3 h |
| — | 0 | 7 | 21 |
| PVA | 21 | 66 | 39 |
| Xylan | 38 | 70 | 64 |
| Chitin | 62 | 90 | 81 |
| Chitosan | 71 | 77 | 83 |
| Sephadex G25 | 16 | 54 | 60 |
| Sephadex LH20 | 0 | 9 | 18 |

[a] Ac-Tyr-OH or Ac-Tyr-OMe, 10 mM; STB, 5 mg; ethanol, 10 ml; water, 4.8%; 30°C.

bound to the support material, and both free and immobilized STB exist in the reaction mixtures. Attempts to separate these two STB were unsuccessful because of the insolubility of STB in these systems. The results of the reactions are summarized in Table 4. Considering that the effective support materials are mostly hydrophilic, one may attribute the activation of STB by immobilization to the increase in the local concentration of water around the enzyme. However, this is not the case, since free STB could not be activated by the increase in water concentration up to 10%. Furthermore, the molecular structure of the support materials seems to affect the activity of the enzyme: cellulose and aminoethyl cellulose are ineffective. Therefore, it may be assumed that STB is activated through specific molecular interactions with the supports.

*Transesterification of Ac-Tyr-OMe to Ac-Tyr-OEt*

Transesterification of Ac-Tyr-OMe to Ac-Tyr-OEt in ethanol is much more rapid than esterification of Ac-Tyr-OH described above. The time courses of the reactions by STC and STB, as well as by α-chymotrypsin (CT), are shown in Fig. 4. The order of the reaction rate was STC ≈ CT > STB, and the results indicate again that STC is highly active in the solubilized form. Interestingly, free STB has certain activity for transesterification. This means that STB is not destroyed in this reaction medium, and the lack of activity of free STB for the esterification mentioned above is the consequence of the low specificity of the enzyme toward the acid substrate.

The values of $K_m$ and $k_{cat}$ obtained by a Lineweaver–Burk plot are listed in Table 2. As shown in Scheme 1, esterification of Ac-Tyr-OH and transesterification of Ac-Tyr-OMe proceed via a common intermediate, Ac-Tyr-enzyme, and the deacylation steps ($k_3$) of the two reactions are identical. Therefore, the differ-

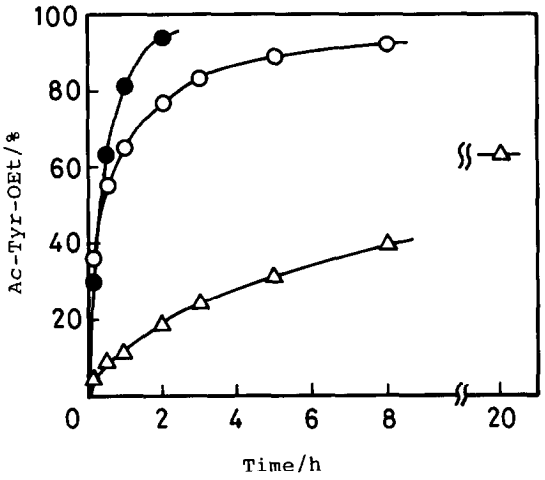


FIG. 4. Time courses of transesterification of Ac-Tyr-OMe to Ac-Tyr-OEt. Ac-Tyr-OMe, 10 mM; enzyme, 5 mg; ethanol, 10 ml; water, 4.8%; 30°C. ●, STC; ○, CT; △, STB.

ence in $k_{cat}$ is attributed to the difference in $k_2$ (*27*); that is, acylation of STC by Ac-Tyr-OH is slower than that by Ac-Tyr-OMe and rate determining in esterification. Unexpectedly, the values of the Michaelis constant $K_m$ for esterification and transesterification suggest that the acid substrate binds more strongly to STC than the ester substrate. For serine proteases, the binding of a substrate to an enzyme is considered electrostatic and hydrophobic, and the relative importance of the two factors would change going from aqueous to organic solvents. The above result may be interpreted as a reduction in the hydrophobic interaction in ethanol relative to the electrostatic one between the enzyme and the substrate.

In a comparison of the transesterification and the hydrolysis of Ac-Tyr-OMe, the large difference in the $k_{cat}$ values of the two reactions can be attributed to the difference in the $k_3$ values, provided that the rates of acylation of STC by Ac-Tyr-OMe in ethanol and water are almost identical. It is likely, therefore, that the deacylation step is rate determining in transesterification. Furthermore, the large difference in $k_{cat}/K_m$, which is equal to $k_2/K_s$, implies a much weaker binding of

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Ac-Tyr-E} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{Ac-Tyr-OEt} + E$$

E: STC; S: Ac-Tyr-OH or Ac-Tyr-OMe

$$K_m = K_s \frac{k_3}{k_2+k_3};\quad k_{cat} = \frac{k_2 k_3}{k_2+k_3};\quad K_s = \frac{k_{-1}}{k_1}$$

SCHEME 1. Enzymatic esterification and transesterification.

Ac-Tyr-OMe to STC (larger value of $K_s$) in ethanol than in water. As mentioned above, hydrophobic interaction should be minimized in organic solvents, and this may explain why the $K_s$ value increases in ethanol. The changes in substrate specificity and inhibitor efficiency for PEG-modified chymotrypsin and subtilisin in benzene also suggest a large reduction in the hydrophobic interaction between a substrate and the enzyme specificity sites (*19*).

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