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Unusual Enhancement of Protease Activity in Organic Solvents by Amines

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The catalytic activities of subtilisin BPN' and α-chymotrypsin for transesterification of N-acetyl-L-tyrosine methyl ester in organic solvents were dramatically increased by addition of tertiary amines. The effects may be ascribed to the change in dissociation state of polar groups on the surface of the enzymes.

There is a growing interest in enzymatic reactions in organic solvents.[1-4] Use of organic solvents for enzymatic reactions has several advantages such as high solubilities of nonpolar substrates, suppression of undesirable reactions caused by water, and easy separation of reaction products. However, it is known that activities of enzymes in organic solvents are much lower than those in aqueous solutions. One of the possible reasons for this is the changes in enzyme structures caused by interactions between enzymes and organic solvents. Also, dissociation state of polar groups on the surface of an enzyme may be different in aqueous and organic media. Especially, it may be reasonably assumed that dissociation of polar groups on enzymes may be greatly suppressed in organic solvents as compared to those in aqueous solutions due to lower dielectric constants of organic solvents. Furthermore, organic solutions are usually not buffered, and therefore, structures and properties of enzymes are considered to be highly sensitive to acidic or basic compounds present in reaction mixtures.

The above consideration led us to examine the effects of amines on the catalytic activity of proteases in organic solvents. It was found that tertiary amines significantly enhance the activity of subtilisin BPN' (STB) and α-chymotrypsin (CT) for transesterification of N-acetyl-L-tyrosine methyl ester (ATME) in organic solvents.

Transesterification of ATME to its ethyl ester (ATEE) was carried out as follows:[5] an aqueous solution (0.5 ml) of an enzyme (1 mg) was added to a solution of ATME (0.1 mmol), an amine (5 mmol), and acetanilide (25 mg) which was an internal standard for HPLC analysis, in 10 ml of ethanol. The reaction mixture was incubated with constant reciprocal shaking (about 150 cycles per min) at 30° C. Aliquots of the reaction mixture were taken at intervals and filtered by polytetrafluoroethylene membrane filters and injected into HPLC (Shimadzu LC-6A). A Shim-pack CLC-ODS column (0.15 m x 6.0 mm) was used for analysis and the column was eluted with water-acetonitrile (50/50 by volume). The reaction rates were calculated from the initial increase in the amounts of ATEE.

Table 1 summarizes the results of transesterification of ATME to ATEE by the catalysis of STB. It can be clearly seen that in the absence of amines, the reaction was slow and the yield of ATEE was below 20% after 24 h reaction. However, by addition of 5 mmol of triethylamine under the same reaction conditions, the reaction rate increased 19 fold and the yield of ATEE reached 82%. Three other tertiary amines which differ in carbon chain length (C_3, C_4, and C_8) exhibited similar effects on the reaction rate and the yield of ATEE. Therefore, the result may be ascribed to change in surface charge of the enzyme rather than hydrophobic interactions between the amines and the enzyme. Probably, addition of an amine increases the net negative charge on the surface of the enzyme by elimination of protons from ammonium groups of basic amino acid residues. As a consequence, change in enzyme structure may occur which would lead to the enhancement of catalytic activity of the enzyme.

Interestingly, effects of tertiary amines on catalytic activity of subtilisin Carlsberg (STC) for transesterification were much smaller than those for STB as shown in Table 2. The acceleration was less than 2 fold, and the yield of ATEE decreased by addition of the amines. It is known that the folding structures of the peptide chains and enzymatic behaviors of STB and STC are very similar in aqueous solutions.[6] The activities of both subtilisins for peptide hydrolysis are comparable.[7,8] However, these enzymes differ in amino acid residues at 84 positions, out of which 75 residues are pointing outward.[9] Furthermore, the fact that STC is more soluble in ethanol-water than STB is indicative of different surface properties of these enzymes.[10] Perhaps the surface of STB is more polar than STC and sensitive to amines due to acid-base interactions.

The transesterification by CT was also accelerated by tertiary amines as seen in Table 3. About 10 fold increase in reaction rate was observed by addition of tri-n-propyl, -butyl, and -octylamines. It should be noticed that the product yields after 24 h reactions are very low as compared to that for the reaction without amines. It is known that transesterification by a serine protease is competitive to hydrolysis. The reactions proceed via a common intermediate, acyl-enzyme, and nucleophilic attack of ethanol and water on the intermediate gives ATEE and a hydrolysis product N-acetyl-L-tyrosine (AT), respectively. The low yields of ATEE by CT may be ascribed to secondary hydrolysis of ATEE followed by formation of stable quaternary ammonium salts between the amines and AT. This has been suggested by the observation of the time course of the reaction; the amount of ATEE increased at an early stage of the reaction, then it decreased with increasing reaction time, giving maximum yield of 76% after 1.5 h.

The kinetic parameters obtained by Lineweaver-Burk plots for transesterification by CT are listed in Table 4. Interestingly, both K_m and k_{cat} increased markedly by addition of triethylamine. However, in spite of a large increase in K_m, the secondary rate constant k_{cat}/K_m is about 50 fold of that in the absence of triethylamine suggesting that the rate increase is due mainly to acceleration of deacylation step, that is nucleophilic reaction of ethanol with the acyl-enzyme intermediate.

It has been reported that the activities of several proteases and lipases in nonpolar organic solvents were enhance by N-acetyl-L-phenylalaninamide or sugar alcohols.[11,12] Furthermore, activity of proteases in anhydrous organic solvents can be

Table 1. Effect of amines on transesterification of ATME by STB in ethanol[a)]

Amine	Relative rate			Yield(24 h)/%		
	ATEE	AT	ATEE/AT	ATEE	AT	ATEE/AT
-	1.0	0.02	50.0	17.7	1.9	9.3
Triethylamine	19.0	2.71	7.0	82.3	17.7	4.6
Tri-n-propylamine	22.3	2.86	7.8	76.6	23.4	3.3
Tri-n-butylamine	20.4	3.14	6.5	79.6	20.4	3.9
Tri-n-octylamine	23.3	2.71	8.6	83.2	16.8	3.9

a) Relative rate of 1.00 corresponds to 0.07 μ mol · $min^{-1}mg^{-1}$. ATME 0.1 mmol, STB 1 mg, ethanol 10 ml, water 0.5 ml, amine 5 mmol, 30 ℃ .

Table 2. Effect of amines on transesterification of ATME by STC in ethanol[a)]

Amine	Relative rate			Yield(24 h)/%		
	ATEE	AT	ATEE/AT	ATEE	AT	ATEE/AT
-	1.00	0.13	7.7	95.2	4.8	19.8
Triethylamine	1.66	0.24	6.9	74.9	25.1	3.0
Tri-n-propylamine	1.78	0.24	7.4	74.5	25.5	2.9
Tri-n-butylamine	1.91	0.39	4.9	71.7	28.3	2.5
Tri-n-octylamine	1.84	0.30	6.1	72.9	27.1	2.7

a) Relative rate of 1.00 corresponds to 0.80 μ mol · $min^{-1}mg^{-1}$. ATME 0.1 mmol, STC 1 mg, ethanol 10 ml, water 0.5 ml, amine 5 mmol, 30 ℃ .

Table 3. Effect of amines on transesterification of ATME by CT in ethanol[a)]

Amine	Relative rate			Yield(24 h)/%		
	ATEE	AT	ATEE/AT	ATEE	AT	ATEE/AT
-	1.00	0.29	15.0	88.4	7.8	11.3
Triethylamine	5.67	1.00	5.7	33.8	66.2	0.5
Tri-n-propylamine	10.3	1.61	6.4	8.9	91.1	0.1
Tri-n-butylamine	10.0	2.18	4.6	5.1	94.9	0.05
Tri-n-octylamine	10.6	1.92	5.5	2.6	97.4	0.03

a) Relative rate of 1.00 corresponds to 1.35 μ mol · $min^{-1}mg^{-1}$. ATME 0.1 mmol, CT 1 mg, ethanol 10 ml, water 0.5 ml, amine 5 mmol, 30 ℃ .

Table 4. Kinetic parameters for transesterification of ATME by CT in acetonitrile[a)]

Additive	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
-	20.2	0.2	9.9
Triethylamine	538	275	511

a) CT 1 mg, ethanol 1 ml, water 0.3 ml, triethylamine 5 mmol, total volume 10 ml, 30 °C .

affected by the pH of the aqueous solutions from which the enzymes were liophilized,[13] although in the present reactions the activity of CT was found to be strongly affected by the nature of buffer components rather than pH value.[14] In aqueous solutions, activity of lipases was enantioselectively enhanced or retarded by alkaloids[15] or methioninol.[16]

These and the present results indicate that the catalytic activity of enzymes in organic solvents can be largely altered by small amounts of additives probably due to change of surface properties of the enzymes. For enzymatic reactions in organic solvents, effects of these substances should be carefully taken into consideration to obtain maximum activities of the enzymes, and more comprehensive studies are required with respect to the molecular mechanism of the enzyme activation.

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