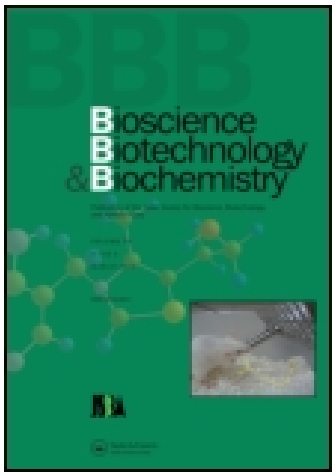

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Increase of Catalytic Activity of α-Chymotrypsin by Metal Salts for Transesterification of an Amino Acid Ester in Ethanol

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Note

Increase of Catalytic Activity of α-Chymotrypsin by Metal Salts for Transesterification of an Amino Acid Ester in Ethanol

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α-Chymotrypsin-catalyzed transesterification of *N*-acetyl-L-tyrosine methyl ester in ethanol was markedly accelerated by addition of small amounts of divalent metal salts. The reaction rate depended not only on the nature of metal ions but also on the nature of anionic counter ions. Calcium acetate was the most effective among the metal salts used. The reaction followed Michaelis–Menten kinetics, and it was found that the reaction increase is due to the increase in k_{cat}.

Key words: α-chymotrypsin; transesterification; metal salt; ethanol

Enzyme reactions in organic solvents are becoming common in application to organic synthesis and optical resolution.[1] In general, enzyme activity in an organic solvent is a complex function of the nature and concentration of the solvent. Furthermore, recent studies have shown that activity of enzymes in organic solvents can be greatly increased by organic additives such as amides or amines.[2–4] However, effects of metal salts on enzyme activity in organic solvents have rarely been investigated. Recently, it was found that calcium ion greatly increased the catalytic activity of α-chymotrypsin (CT) for esterification, transesterification, and hydrolysis in organic solvents.[5] In this paper, we report the effects of several divalent metal salts on the activity of CT in ethanol.

CT from bovine pancreas was purchased from Sigma Chem. Co. (type II, three times recrystallized and essentially salt-free). The purity of the enzyme preparation was 85% by spectroscopic active site titration.[6] *N*-Acetyl-L-tyrosine methyl ester (ATME) was prepared by the reaction of *N*-acetyl-L-tyrosine with methanol in the presence of thionyl chloride. Ethanol was dried before use on molecular sieves 3A. Metal salts of guaranteed grade were obtained from Wako Pure Chem. Ind. Ltd., and they were used without further purification.

Transesterification of ATME to *N*-acetyl-L-tyrosine ethyl ester (ATEE) was done as follows: CT (1 mg) was dissolved in 0.5 ml of an aqueous solution of a metal salt, and the solution was kept standing for 10 min. Then, the solution was added to 9.5 ml of a solution of ATME and acetanilide in ethanol. Acetanilide was used as an internal standard for HPLC analysis. The total volume of reaction mixture was 10 ml, and the concentration of ATME was 10 mM. In this paper, the concentration of ATME and metal salts is expressed as the molar concentration based on the total reaction volume. The reaction mixture was an apparently homogeneous (transparent) solution, and it was incubated with constant reciprocal shaking (about 150 cycles per min) at 30°C. Samples of the reaction mixture were taken at intervals and filtered by poly(tetrafluoroethylene) membrane filters. The filtrate was injected into an HPLC (Shimadzu LC-6A) with a column packed with Shim-pack CLC-ODS, which was eluted by water–acetonitrile (50 : 50 by volume). The rate of transesterification was calculated from the initial increase in the amount of ATEE.

The catalytic activity of CT in ethanol has been well documented for esterification and transesterification of amino acids.[7–9] A typical reaction is the transesterification of ATME to ATEE in which ethanol acts as a nucleophile toward the acyl–enzyme intermediate, *N*-acetyl-L-tyrosyl chymotrypsin. This reaction corresponds to hydrolysis of ATME in aqueous solutions in which water acts as a nucleophile to attack the acyl-enzyme intermediate. Considering that the catalytic activity of CT is usually estimated by the hydrolysis rate of amino acid esters in water, we used this transesterification for estimation of the catalytic activity of CT in ethanol.

First, the reaction rate was measured in the presence of several divalent metal chlorides (MCl_2, M = Ca, Ba, Sr, or Mg) to investigate the effects of metal ions on enzyme activity. The results are shown in Fig. 1. By addition of about 50 μM $CaCl_2$, the reaction rate increased about 4-fold. Addition of larger amounts of $CaCl_2$ lowered the reaction rate. Chlorides of Ba, Sr, and Mg also accelerated the reaction, but the magnitude of rate increase was smaller than that by $CaCl_2$ (Fig. 1). The conversion of ATME to ATEE after 24 h of reaction was about 80% irrespective of the nature and concentration of metal chlorides. This indicates that metal chlorides do not affect the equilibrium of the reaction, and this suggests that the reaction components are not strongly associated with M^{2+} or Cl^-.

The effects of Ca salts of different counter anions are shown in Fig. 2. It can be seen that all the Ca salts used accelerated the

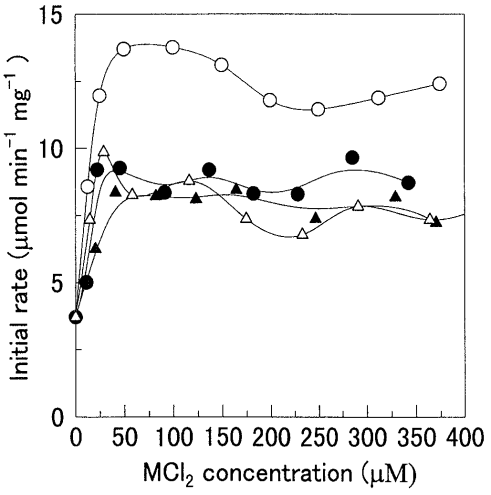

Fig. 1. Effects of Metal Chlorides on the Transesterification of ATME to ATEE.

See experimental section. The initial reaction rate is expressed as μmol of ATEE formed per min per mg of protein. The value of the reaction rate is not corrected for the purity of CT. ○, $CaCl_2$; ●, $BaCl_2$; △, $SrCl_2$; ▲, $MgCl_2$.

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Abbreviations: CT, α-chymotrypsin; ATME, *N*-acetyl-L-tyrosine methyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester.

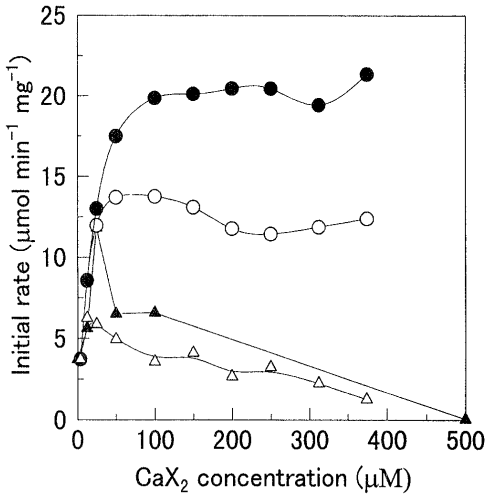

Fig. 2. Effects of Ca Salts with Different Counter Anions.

See experimental section and legend for Fig. 1. ○, $CaCl_2$; ●, $Ca(OCOCH_3)_2$; △, $Ca(H_2PO_4)_2$; ▲, $Ca(OH)_2$.

reaction, but the magnitude of rate increase was different for different anionic species. $Ca(OCOCH_3)_2$ was the most effective, followed by $CaCl_2$, $Ca(OH)_2$, and $Ca(H_2PO_4)_2$.

These results suggest that both cationic and anionic ions from metal salts strongly influence the rate of transesterification in ethanol. It should be mentioned that the optimal concentrations of metal salts are very low (1/100 (mol/mol) or less than that of ATME), and therefore, these results may be due to the change in catalytic activity of CT rather than a change in the reactivity of the substrate.

The reaction followed Michaelis–Menten kinetics, and the reaction parameters were obtained by Lineweaver–Burk plots. The results for $CaCl_2$ and $Ca(OCOCH_3)_2$ are listed in the Table. It can be seen that the acceleration of the reaction is due to the increase of k_{cat}, which overcomes the increase in K_m, resulting in the increase in k_{cat}/K_m. This suggests that metal salts primarily affect the acylation of CT by ATME and/or deacylation of *N*-acetyl-L-tyrosyl CT rather than binding of ATME to the enzyme. CT is not what is called a metalloenzyme, and it does not have

Table Reaction Parameters for Transesterification of ATME to ATEE by CT

Ca salt	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
—	16	3.4	210
$CaCl_2$	29	17	590
$Ca(OCOCH_3)_2$	35	40	1140

ATME was transesterified to ATEE in ethanol (9.5 ml)/water (0.5 ml) by CT (1 mg) at 30°C. The enzyme parameters were calculated on the basis of the enzyme purity of 85%, which was measured by active site titration (see Experimental section). The concentration of Ca salts was 100 μM.

specific binding sites for metal ions. Therefore, this result may be the consequence of a unique interaction between CT and ionic species in organic solvents, where ion–dipole or ion–ion interactions would be increased due to the lower polarity of ethanol as compared to that of water. No significant effects of metal salts were observed on hydrolysis of ATEE in water. These results suggest that selection of metal salts is important when they are used for enzyme reactions in organic solvents.

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