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Predominant Transesterification Reaction Catalyzed by Immobilized Trypsin

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Immobilized trypsin covalently attached to cross-linked dextran was prepared. In the presence of high concentrations of primary and secondary alcohols, the transesterification of L-lysine esters catalyzed by the immobilized trypsin was observed to be predominant. In contrast to this, no transesterified product was detected with tertiary butyl alcohol. These reactions were compared with the acid-catalyzed transesterification reactions and the characteristics of the enzymatic reaction are discussed.

Keywords——immobilized trypsin; kinetic behavior; transesterification; hydrolytic enzyme; enzyme mechanism; enzyme catalysis; immobilized enzyme catalysis

Many reviews[1-4] have appeared on immobilized enzymes and their applications. It is generally assumed that immobilized enzymes, as distinct from enzymes in solution, are incapable of aggregation in the presence of organic solvents. This assumption prompted a search for an immobilized enzyme that could be used in an organic solvent. Chymotrypsin attached to porous glass was found to be operative as a catalyst even in a high concentration of organic solvent and was also useful for mechanistic studies of chymotrypsin.[5] As reported in this paper, the acylation step of the chymotrypsin-catalyzed reaction was shown to be almost independent of the water concentration, whereas the deacylation step was strongly dependent on it. These observations are in good accord with the widely accepted scheme for hydrolytic enzymes proceeding *via* acyl enzyme intermediates, and extended the previous scheme by introducing a water molecule as a reactant in the deacylation stage as shown below.

$$E+S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E+P$$
$$\qquad\qquad\qquad\qquad\quad + \quad H_2O$$
$$\qquad\qquad\qquad\qquad\quad P'$$

It may be reasonably considered from these results that if certain nucleophiles other than water are applied as a solvent for the immobilized enzyme-catalyzed reaction, the substitution reaction conducted by the nucleophile might predominate without the occurrence of enzyme aggregation.

Transesterification and transamidation reactions have been widely observed in the course of enzymatic hydrolyses.[6,7] Glazer[8] studied trypsin-catalyzed transesterification and demonstrated that the transesterified products are metastable intermediates appearing in the course of hydrolysis.

Experimental

Materials——Trypsin (TRL-53N635, lyophilized, twice-crystallized, salt-free) was obtained from Worthington Biochemical Corp. Sephadex G-200 (40—120 μ) was a product of Pharmacia Fine Chemicals.

Preparation of Trypsin Coupled to Sephadex——Sephadex G-200 was activated by treatment with cyanogen

bromide at pH 10 as described elsewhere.[9] After being washed, the activated gel was coupled to trypsin. The procedure was essentially the same as that described previously.[9] The gel (2 g) was suspended in 6 ml of 0.1 M sodium bicarbonate and trypsin (500 mg) was added. The reaction mixture was slowly rotated in a vial at 4 °C for 14 h. The gel was transferred to a small column and washed with 0.1 M sodium bicarbonate (250 ml), 1 mM HCl (10 ml), 0.5 M NaCl (250 ml) and finally water (30 ml). The product was treated with cold water–acetone mixtures of increasing acetone concentration and finally with acetone only, then dried *in vacuo*.

Amino Acid Analysis——Sephadex coupled with trypsin was hydrolyzed in 6 N HCl at 110 °C for 18 h in an evacuated, sealed tube and the hydrolysate was subjected to quantitative amino acid analysis. Analysis was performed with a Hitachi KLA-3B amino acid analyzer by the method of Spackman *et al.*[10]

Synthesis of L-Lysine Ester Hydrochloride——L-Lysine methyl, ethyl, *n*-propyl and isopropyl esters were synthesized following the general procedure described by Greenstein and Winitz,[11] *i.e.*, dry hydrogen chloride gas was passed through a suspension of L-lysine monohydrochloride in a large excess of the pertinent alcohol. These esters were recrystallized from the parent alcohol and ether. All melting points were in accordance with literature values.

Transesterification Reaction Catalyzed by Immobilized Trypsin——The reactions were carried out in a small vessel with stirring at 25 °C in an alcoholic medium containing 5% (v/v) 0.1 M acetate buffer, pH 6.2. The reactions were followed by removing aliquots at suitable time intervals and carrying out chromatographic separation.

Transesterification Reaction Catalyzed by Hydrochloric Acid——The procedure was the same as that for immobilized trypsin except for the reaction temperature and catalyst. Instead of immobilized trypsin, 2% hydrochloric acid (final concentration) was used as the catalyst, and the reaction mixture was heated on a steam bath.

Chromatographic Separation and Quantification——Excellent chromatographic separation was achieved by the method of Glazer.[8] Each aliquot was subjected to one-dimensional chromatography on Toyo Roshi No. 51 paper in *n*-butyl alcohol–pyridine–acetic acid–water (15 : 10 : 3 : 12). The chromatograms were dried and developed with ninhydrin reagent. The spots were cut out and eluted with 90% aqueous alcohol. The amount of each component was determined by the procedure of Atfield and Morris.[12]

Results

The quantity of trypsin coupled to Sephadex was determined to be 35 mg per gram of the dry gel by amino acid analysis. The catalytic activities of the coupled trypsin were similar to those reported,[9] *i.e.*, 13 and 2% of those of free trypsin toward tosylarginine methyl ester and casein as substrates, respectively.

The use of an immobilized enzyme and a high concentration of organic solvent enable the

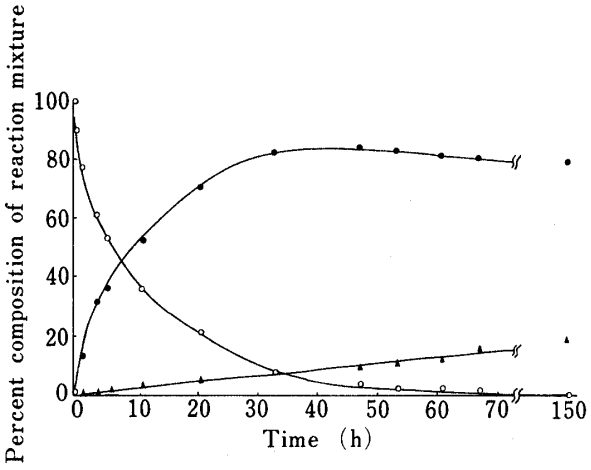

Fig. 1. Time Course of Immobilized Trypsin-Catalyzed Reaction of L-Lysine *n*-Propyl Ester in Aq. Ethanol

The reaction was carried out in ethanolic medium containing 5% (v/v) 0.1 M acetate, pH 6.2, at 25 °C. Substrate concentration was 0.125 M. The concentration of trypsin was calculated to be 0.35 mg per milliliter of medium based on amino acid analysis of the gel. ●, L-lysine ethyl ester; ○, L-lysine *n*-propyl ester; ▲, L-lysine.

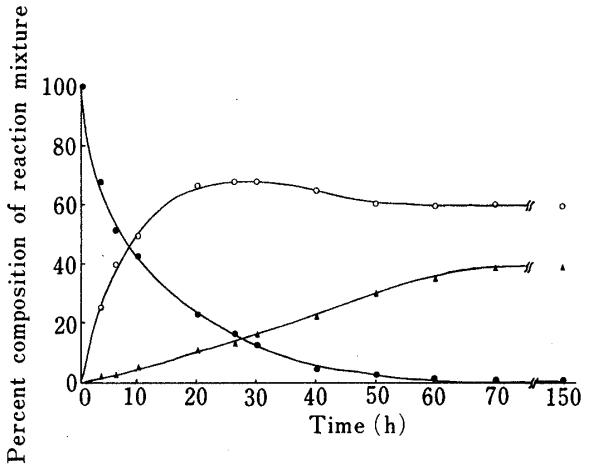

Fig. 2. Time Course of Immobilized Trypsin-Catalyzed Reaction of L-Lysine Ethyl Ester in Aq. *n*-Propyl Alcohol

The reaction was carried out in *n*-propyl alcohol containing 5% (v/v) 0.1 M acetate, pH 6.2, at 25 °C. Substrate and enzyme concentrations were the same as those of Fig. 2. ○, L-lysine *n*-propyl ester; ●, L-lysine ethyl ester; ▲, L-lysine.

Table I. Immobilized Trypsin-Catalyzed Conversion of L-Lysine Esters to Ethyl Ester

Starting ester	Ethyl ester formed (%)
Methyl	78
n-Propyl	80
n-Butyl	74

The reactions were carried out in ethanolic medium containing 5% 0.1 M acetate, pH 6.2 at 25 °C.
Concentrations of enzyme and substrate were the same as those of Fig. 1.

Table II. Immobilized Trypsin-Catalyzed Conversion of L-Lysine Ethyl Ester to Methyl, *n*-Butyl and Isopropyl Esters

Product ester	Percent yield (%)
Methyl	51
n-Butyl	81
Isopropyl	56

transesterification reaction to predominate over hydrolysis, as shown in Fig. 1. L-Lysine propyl ester was converted to the ethyl ester in 80% yield in 95% aqueous ethyl alcohol.

Conversion of ethyl to propyl ester was also tested using L-lysine ethyl ester as a substrate in 95% aqueous propyl alcohol (Fig. 2). Although no significant difference in time course between these two reactions was observed, the percentage conversions (after 50 h) were significantly different. It can be seen from the figures that the fractional ratios of each reaction product reached a stationary state after 50 h under the conditions employed. This suggests that the difference of equilibrium constants for the hydrolyses of ethyl and propyl esters is probably responsible for the different percentages of conversion shown in Figs. 1 and 2. This assumption that the final yield of products might be governed by the type of alcohol employed was confirmed by comparing the following reactions. Using propyl, methyl and *n*-butyl esters as substrates in a common medium, aqueous ethyl alcohol for example, the final yields of ethyl ester are the same in each case. These results are summarized in Table I.

All primary and secondary alcohols served as nucleophiles, giving transesterified products in high yields, as shown in Table II. The final yields of each ester differed significantly, although a common substrate, ethyl ester, was used for each case. In methyl alcohol, the methyl ester was shown to be equilibrated with lysine in a 1 : 1 ratio under the conditions used, and the *n*-butyl ester was shown to be the predominant species in the equilibrium with *n*-butyl alcohol. No transesterification reactions occurred with Sephadex itself or cyanogen bromide-treated Sephadex.

Transesterification reactions were also carried out by replacing the immobilized trypsin with hydrochloric acid as a catalyst. Starting from ethyl ester, 84% of *n*-butyl ester was observed at the equilibrium state, and 78% of ethyl ester from *n*-butyl ester. These values are in good agreement with those of the immobilized trypsin-catalyzed reactions.

Discussion

Glazer reported the transesterification reaction catalyzed by unbound trypsin in media containing 10—20% of alcohol.[8] He observed 40—65% of transesterified products as intermediates which disappeared within a certain period. In our study, the transesterified

product occurs as an equilibrated component. As shown in Figs. 1 and 2, the percentage of the product showed a slight peak in the early stages of the reaction. This phenomenon is commonly seen in enzymatic transesterification and transamidation reactions, and can be explained by the ease of alcoholysis or aminolysis as compared with hydrolysis, followed by hydrolysis of the transferred products. Bender and Glasson[13] studied the relative rates of chymotrypsin-catalyzed hydrolysis and methanolysis of acetyl-L-phenylalanine methyl ester and found that the rate constant for the methanolysis was 8.3 times that for the hydrolysis. If such a situation did not occur, no transferred products would have been observed as intermediates in the investigation by Glazer.[8] In other words, the intermediates formed at a low concentration of alcohol are kinetically controlled products. However, under the conditions used in this study, an equilibrium state is reached in which the transesterified product predominates. In the reaction with hydrochloric acid, the percentage of the transesterified product increased smoothly to the equilibrium value. The mechanism of the enzyme reaction is distinct from that of the low molecular weight catalyst, hydrochloric acid.

Tertiary butyl alcohol was tested as a nucleophile, but no component other than starting material and hydrolyzed lysine was observed on the chromatogram. As proposed by Bender *et al.*,[14] nucleophilic substitution at the carbonyl carbon of an acyl enzyme must be an essential step of the deacylation. Unlike primary and secondary alcohols, tertiary alcohols are definitely unsuitable as nucleophiles, presumably due to steric hindrance to the interaction with the enzyme active site. Furthermore, it is a well known fact that the formation process of a tertiary alcohol ester itself is thermodynamically less favored than its decomposition process in the equilibrium state.

This study has demonstrated that efficient transesterification reactions can be achieved with immobilized enzymes which are tolerant of organic solvents. This reaction should be appricable to the separation of lysine and arginine from amino acid mixtures, and also to the resolution of their DL-isomers.

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References

1) L. Goldstein, "Methods in Enzymology," Vol. 19, ed. by G. E. Perlmann and L. Lorand, Academic Press, New York, 1970, pp. 935—962.
2) E. Katchalski, I. H. Silman and L. Goldstein, "Advances in Enzymology," Vol. 34, ed. by F. F. Nord, Interscience Publishers, New York, 1971, pp. 445—536.
3) O. R. Zaborsky, "Immobilized Enzymes," CRC Press, Cleveland, 1973.
4) I. Chibata, "Immobilized Enzyme," Kodansha Ltd., Tokyo, 1978.
5) K. Tanizawa and M. L. Bender, *J. Biol. Chem.*, **249**, 2130 (1974).
6) C. E. McDonald and A. K. Ball, *J. Biol. Chem.*, **221**, 993 (1956).
7) T. Oka and K. Morihara, *J. Biochem.* (Tokyo), **82**, 1055 (1977).
8) A. N. Glazer, *J. Biol. Chem.*, **240**, 1135 (1965).
9) A. Axen and S. Ernback, *Eur. J. Biochem.*, **18**, 351 (1971).
10) D. Spackman, W. H. Stein and S. More, *Anal. Chem.*, **30**, 1190 (1958).
11) J. P. Greenstein and M. Winitz, "Chemistry of Amino Acids," Vol. 2, John Wiley and Sons, New York, 1961, p. 925.
12) G. N. Atfield and C. J. O. R. Morris, *Biochem. J.*, **81**, 606 (1961).
13) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960).
14) M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).