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Journal of Molecular Catalysis B: Enzymatic 9 (2000) 259–267

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Catalytic activity of Teflon particle-immobilized protease in aqueous solution

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Received 18 August 1999; received in revised form 20 October 1999; accepted 21 October 1999

Abstract

α-Chymotrypsin (Chy) was entrapped in polytetrafluoroethylene (PTFE) particles. The entrapped enzyme showed twofold catalytic activity for amino acid ester hydrolysis in aqueous solution than free enzyme. The Chy/PTFE particles also catalyzed the peptide synthesis in aqueous solution with a yield of 14%. Both the synthetic and the hydrolytic activities of the entrapped enzyme were enhanced as compared with the free enzyme. The PTFE matrix should provide the enzyme molecules by creating a hydrophobic environment which results in enhanced peptide synthesis in aqueous solution.

Keywords: Teflon; Protease; Immobilization; Peptide synthesis

1. Introduction

Proteinases are commonly known to catalyze either hydrolysis or synthesis of protein. This group of enzymes has a wide range of specificity toward the different amino acids and can be used to synthesize peptide by reversing the catalytic process toward synthesis in hydrophobic environment [1–7]. Useful peptides are synthesized by chemical methods, enzymatic methods using proteinases, and genetic engineering. Peptide bond formation catalyzed by papain and α-chymotrypsin (Chy) was first reported by Bergmann and Fraenkel-Conrat [8] in 1937 and Bergmann and Fruton [9] in 1938, respectively. Proteases can be immobilized without loss of function, and the potential of immobilized proteolytic enzymes for peptide synthesis has been demonstrated. The simplified work-up procedure that becomes possible when immobilized proteases are used, the long-term stability of the immobilized enzyme preparations, and the successful reutilization experiments are among the advantages to such an approach.

Optimization of protease-catalyzed peptide synthesis has been investigated recently [10,11]. Among these enzymes, α-Chy is one of the most studied potential enzymes. The main attraction of protease-mediated peptide synthesis has the advantages of: (a) their capacity to affect peptide bond formation stereospecifically

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1381-1177/00/$ - see front matter
PII: S1381-1177(99)00104-6

without the need for side-chain protection; (b) the enzymatic specificity prevents the formation of undesired by products; (c) enzyme immobilization allows for the recovery of catalytic activity, giving these techniques considerable economic advantage over chemical, stoichiometric procedures.

There has been a revival of interest in the ability of proteolytic enzyme to catalyze the formation of peptide bonds for the synthesis of biological active peptides and semi-synthesis of proteins. Recently, attempts have been made to extend the method to the preparation of longer peptides and even small proteins [12]. Protease-catalyzed peptide bond formation represents a promising alternative to chemical synthesis procedures.

The main drawback of enzymatic peptide synthesis in water–organic mixed solvent is the susceptibility of enzymes to denature which may cause very low enzyme activity. To overcome these, a novel approach for peptide synthesis in an aqueous solution has been performed by taking advantages of hydrophobic environment in the polytetrafluoroethylene (PTFE) matrix wherein Chy is entrapped.

This is the first report to introduce the hydrophobic polymer, PTFE, to immobilize proteases while retaining their catalytic activity. We have chosen the well-studied protease, α-Chy, since the mechanism of Chy has been clarified in details, and it is one of the enzymes of commercial importance.

In general, the methods of protease-mediated peptide bond formation can be classified into two basic strategies according to the type of carboxyl component used. In the first strategy, this component has a free carboxyl terminus, and the formation of peptide bond occurs with thermodynamic control as the reverse of peptide hydrolysis. In the second strategy, the carboxyl component is employed in an activated form, mainly as an alkyl ester, and the synthesis occurs with kinetic control by competitive partitioning of a rapidly formed acyl–enzyme intermediate between the nucleophile and water.

Serine proteinases like subtilisin and Chy turned out to be especially efficient for kinetically controlled synthesis by condensation of peptide esters and peptide derivatives with free amino groups [13,14].

The choice of an enzyme suitable for the synthesis depends upon several factors. Its specificity is of importance; although being not too stringent, it rarely represents a crucial factor, so far as rather large amount of the enzyme is usually introduced into the reaction and the duration of the latter is as a rule long enough. Both these factors allow to overcome the kinetic problems. Therefore, the enzyme can be used to catalyze the formation of peptide bonds that can hardly be considered as favorable ones for its specificity requirements.

To demonstrate the peptide synthesis, we have selected a peptide synthesis process by taking Ac-Phe-OEt and Ala-NH_2 as an acyl donor ester and a nucleophile acceptor, respectively, to synthesize Ac-Phe-Ala-NH_2 as reported by Nilsson and Moshbach [1]. We introduce a new hydrophobic interface, PTFE matrix, for enzyme immobilization as well as modulating the reaction environment.

2. Experimental

2.1. Materials

α-Chy (Type II: from bovine pancreas, 3 × crystallized 39 U/mg solid), *N*-acetylphenylalanine ethyl ester (Ac-Phe-OEt), and alaninamide (Ala-NH_2) were purchased from Sigma (St. Louis, MO 63178, USA). Teflon (PTFE) aerosol (TFE Coat FC-102) were purchased from Fine Chemicals (Tokyo, Japan). Acetonitrile (ACN) and trifluoroacetic acid (TFA) of high-performance liquid chromatography (HPLC)-grade were purchased from Wako (Osaka, Japan). Other chemicals were guaranteed reagent-grade.

2.2. Preparation of α-Chy-immobilized PTFE particle

Chy (40 mg) was mixed with 2 ml Teflon-containing solvent and then allowed to evaporate of dryness to remove the undesired organic solvents. Then the enzyme-immobilized PTFE particles were washed several times with water and buffer successively. After thorough washing, the Chy/PTFE particle was dried in a vacuum desiccator and then used in experiment. The amount of the free enzyme was measured by the protein dry weight method.

2.3. Enzyme activity of Chy / PTFE particle

The enzyme activities of various Chy/PTFE particles were determined by the rate of hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) with 2 M calcium chloride solution, 1 mM hydrochloric acid and 80 mM Tris–HCl buffer (pH 7.8) as determined through the change in absorbance at 256 nm at 25°C. One unit of Chy activity is defined as that amount of enzyme which converts 1.0 μmol of BTEE to *N*-benzoyl-L-tyrosine per minute at 25°C.

2.4. Determination of kinetics parameters

The rate of BTEE hydrolysis was measured at substrate concentrations ranging from 1.00 to 2.00 mM for the free and for the immobilized Chy. The V_{max} and Michaelis–Menten constant, K_m, were calculated from the Lineweaver–Burk reciprocal plot.

2.5. Reactions catalyzed by Chy / PTFE particle

Two different kinds of mechanisms have been followed for Chy/PTFE-catalyzed peptide synthesis.

In the case of the first reaction mechanism, the reaction mixture was composed of 20 mM acyl donor ester (Ac-Phe-OEt) and 40 mM nucleophilic acceptor ($Ala\text{-}NH_2$) in 0.1 M $Na_2CO_3/NaHCO_3$ buffer with a pH 9 which contains 2% acetonitrile for the solubilization of ester substrates. A mixed solvent of acetonitrile and water with a mixing ratio of 1:1 was used to dissolve the ester substrates. Finally, the solution was mixed with an aqueous solution of nucleophilic acceptor and then diluted with the carbonate buffer solution to make the final concentration. The volume for the synthesis reaction was 2 ml. The reaction was performed by the Chy/PTFE particle for 6 h. In this system, the reaction mechanism is illustrated in Fig. 1.

In the case of the second reaction mechanism, the reaction mixture contained 200 mM acyl donor ester (Phe-OEt) in 1 M carbonate buffer solution. The pH of the reaction mixture was adjusted with few drops of NaOH (2 M) to 10.5–11.0 before the addition of Chy/PTFE particle. The typical volume of the reaction mixture was 1 ml. The solution was stirred vigorously with a magnetic stirrer in a glass

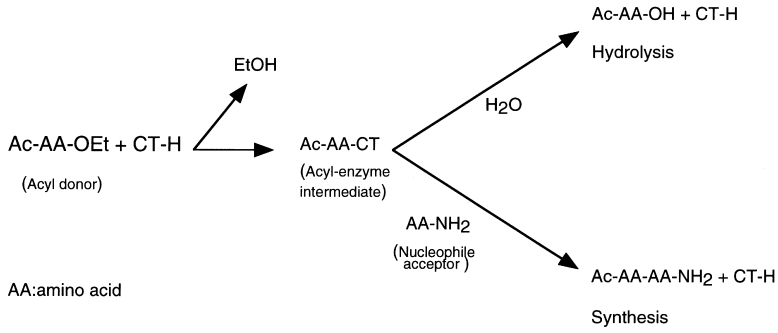

Fig. 1. Reaction mechanism of α-Chy-catalyzed peptide synthesis.

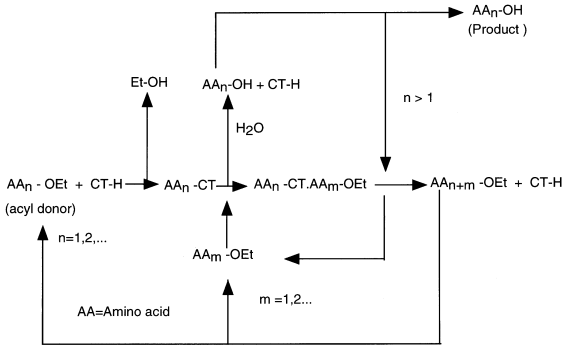

Fig. 2. Chy-catalyzed coupling reaction for oligopeptide synthesis.

vial. After 3–6 h, 0.5–5 ml of acetic acid was added to terminate the enzymatic reaction. Then an aliquot of the reaction mixture was injected to the HPLC for analysis. The reaction mechanism is illustrated in Fig. 2.

2.6. HPLC analysis

The products were analyzed using a reverse-phase HPLC column of Wakosil-$^5C_{18}$ (Wako, Osaka, Japan). A linear gradient flow consisting of A (0.1% trifluoroacetic acid in 100% water) and B (0.08% trifluoroacetic acid in 100% acetonitrile) was employed with a flow rate of 1 ml/min at 30°C. Every component was monitored spectrophotometrically at 260 nm. Ester substrate, acidic and peptide product were baseline-separated in all cases. The product yield was calculated from the ratio of the peak area compared with pure reagent.

2.7. Apparatus

The preparation of Chy/PTFE particles was performed with a vacuum-rotatory evaporator (EYLA, Tokyo, Japan). The enzyme activity and the kinetic parameters were determined through a JASCO UV detector (JASCO, Tokyo, Japan). All the analytical procedures were monitored through JASCO computerized intelligent HPLC integrator equipment (JASCO, Tokyo, Japan).

3. Results and discussion

3.1. Biocatalysts preparation

Chy was used as a model enzyme because of its well-known reactivity in both aqueous and organic solvents as well as ease of solubilization in organic solvents through hydrophobic ion pairing. PTFE was used for the enzyme immobilization. Initial experiments involved contacting the PTFE with varying concentrations of Chy (10–50 mg/ml) in order to determine the maximum amount of protein which could be entrapped. Different amounts of enzyme (10–50 mg) were mixed directly to the 1 ml of Teflon (PTFE) aerosol separately and the morphological character of each type of particles has been investigated as presented in Table 1.

The amount of the protein bound to the Teflon is hard to quantitate directly due to the insolubility of Teflon in aqueous solvent. So it is determined through the amount of free enzyme obtained from the Chy/PTFE particle after complete washing. Approximately 37.5% (15 mg free enzyme from 40 mg Chy/PTFE particle) of the free enzyme was obtained from Chy/PTFE particle, which indicates that a consistently high percentage of Chy was bound to the PTFE.

Scanning fluorescence microscopy has also done using the fluoresceine isothiocyanate (FITC) fluorescence probe. Two slides were prepared: (a) a slide containing Teflon resin and (b) a slide containing Teflon resin with embed-

Table 1
Morphological characteristics of various types of Chy/PTFE particles

Amount of enzyme (mg/ml)	Apparent enzyme activity (U/ml)	Physical conditions of Chy/PTFE particles
20	15.96	highly sticky
30	27.07	sticky
40	28.10	less sticky

ded enzyme. These two slides were labeled with FITC. According to the scanning fluorescence microphotograph (Fig. 3), the one containing enzyme shows more staining that the one with-

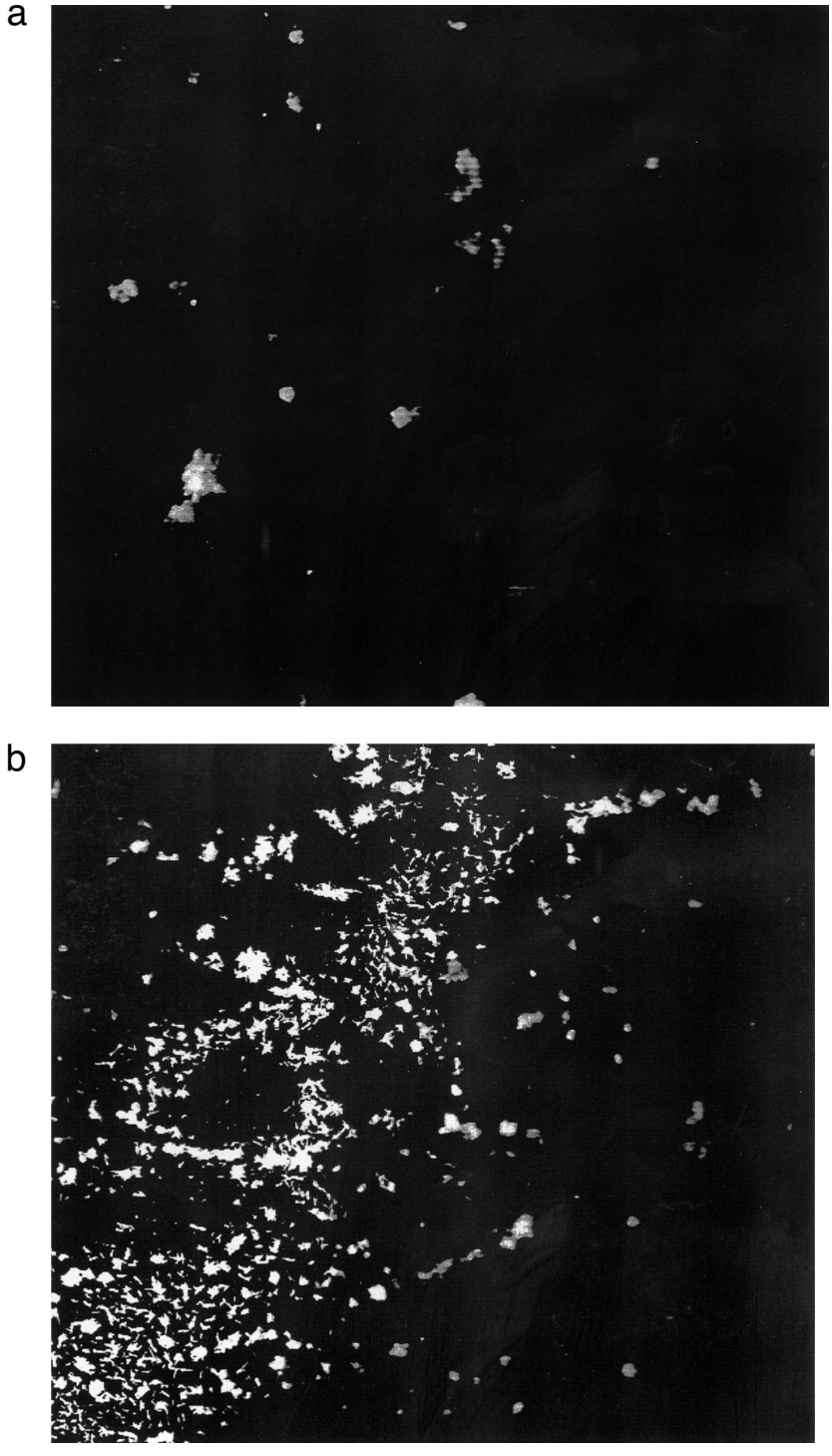

Fig. 3. Scanning fluorescence micrograph of FITC-labeled Teflon slide: (a) without enzyme; (b) with enzyme entrapped in the PTFE matrix.

out enzyme. So we can say that there is an evidence of entrapment of enzyme to the PTFE.

3.2. Catalytic properties of Chy / PTFE particle

Various types of Chy/PTFE particle, where several different amounts of enzyme were loaded against a fixed amount of Teflon, were active in aqueous buffer for the hydrolysis of BTEE. The initial rate of hydrolysis of BTEE catalyzed by Chy/PTFE particles (40 mg/1 ml) in aqueous buffer was about twofold higher than that catalyzed by the native Chy. It has been seen that the higher the enzyme concentration, the better the enzymatic activity obtained. In the present study, the optimum enzyme concentration in the Chy/PTFE particle was found to be 25 mg/ml. In the case of this Chy/PTFE particle, the enzyme activity is about 28.10 U/ml. Whereas, the native enzyme shows the activity about 12.50 U/ml. Immobilized enzyme activity for each of the Chy/PTFE particles was expressed as units per milliliter activity. Considering the immobilization of Chy onto the PTFE, the result of increasing enzyme concentration in the immobilization mixture was to increase the enzyme activity (Fig. 4). But higher than 40 mg of enzyme per milliliter of Teflon allowed suspension condition. The highest apparent enzyme activity was obtained from the Chy/PTFE particle where 40 mg of enzyme was used against 1 ml of Teflon (after removing the free enzyme from the Chy/PTFE particle, the actual enzyme concentration becomes about 25 mg/ml).

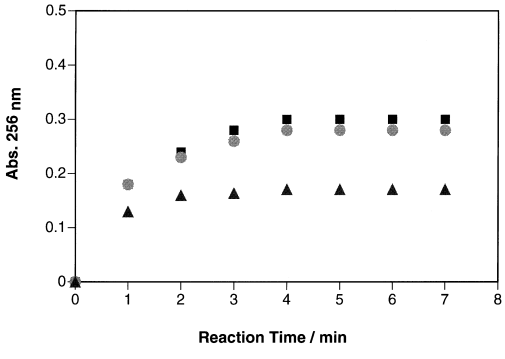

Fig. 4. The rate of hydrolysis of BTEE by Chy/PTFE particle, three different amounts of the enzyme were loaded to PTFE: 40 mg/ml (■); 30 mg/ml (●); 20 mg/ml (▲).

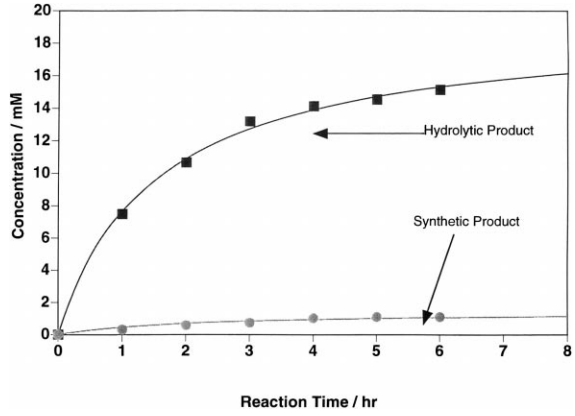

Fig. 5. Time course of Chy/PTFE-particle-catalyzed product formation where 20 mM of *N*-Ac-Phe-OEt and 40 mM of Ala-NH_2 were used as an acyl donor and nucleophile, respectively. Hydrolytic product (■) and synthetic product (●).

3.3. Reactions catalyzed by Chy / PTFE particle

Both the synthetic and the hydrolytic activities of free and immobilized Chy were investigated.

To examine the hydrophobic effect of PTFE on the activity of protease immobilized into PTFE, peptide synthesis was performed in 2% ACN containing aqueous solution with 20 mM acyl donor and 40 mM nucleophilic acceptor. About 2–3 mg of Chy/PTFE particle was used in that reaction mixture. Acetonitrile was mixed with 0.1 M CBS with pH 9.0. The reaction was performed for 6 h and was terminated by the addition of 100 μl of acetic acid. The result is presented in Fig. 5. An adequate amount of sample was periodically withdrawn from the reaction mixture from the initial time of the reaction up to the end of the reaction and then filtrated and finally injected to the HPLC for analysis. The reaction process was proceeded through the disappearance of the substrates to the products.

The chromatogram behavior of the reaction catalyzed by the Chy/PTFE particle shows that

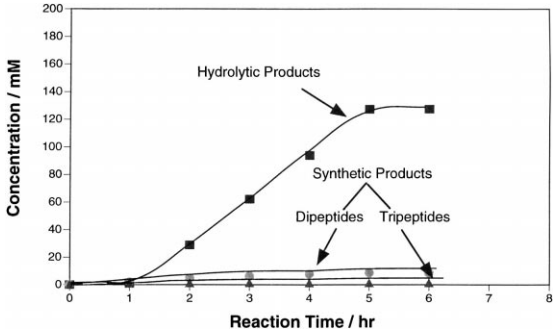

Fig. 6. Time course of Chy/PTFE-particle-catalyzed product formation where 200 mM Phe-OEt was used as a reaction substrate. Hydrolytic product (■), dipeptide (●) and tripeptide (▲).

the main products peak with the retention time which are very similar to the retention time of the standard samples of phenylalanine ethyl ester (Phe-OEt), phenylalanine amino acid, acyl-phenylalanine ethyl ester (Ac-Phe-OEt) and dipeptide of phenylalanine (Phe–Phe). But in the case of the reaction catalyzed by the native enzyme, the peak for the dipeptide is absent. This means that the hydrolysis rection is prominent in that case. So, this result suggests that the equilibrium of the Chy/PTFE- catalyzed reaction shifts to the synthesis in the presence of the hydrophobic PTFE environment even in aqueous solution, though the yield of the peptide is negligible. The PTFE matrix provides hydrophobic environment around the entrapped enzyme which may have some effects on the reaction medium. Therefore, PTFE matrix is very useful to enhance the hydrophobicity for the synthesis of peptide in an aqueous solution.

During the synthesis of dipeptides, amino acid ester with a hydrophobic residue was used as a nucleophile. But it was observed that these esters are not good nucleophile, they are rather good hydrolytic substrates of Chy. At highly basic pH such as 10–11, Chy was found to catalyze the synthesis of oligopeptides of Phe with Phe-ester in which one Phe-ester unit acted as an acyl donor while other Phe-ester units acted as nucleophilic acceptor [15]. At higher pH, due to the slow hydrolysis of amino acid ester, the poor nucleophile such as amino acid ester gets more time to react with acyl–enzyme intermediate favoring the peptide synthesis. Therefore, at higher pH, by increasing Phe-ester concentration, the equilibrium of the catalytic reaction can be shifted substantially toward the repeated coupling of Phe, resulting in the synthesis of oligopeptides. Aizawa et al. have developed a new method to synthesize oligopeptides from amino acid esters. By this repeated coupling, oligomer ranging from dimer to octamer was obtained in aqueous solution which was investigated in our laboratory previously by Aizawa et al. To achieve the same aim, we performed the peptide synthesis reaction using Chy/PTFE with amino acid ester in highly alkaline conditions. The chromatogram behavior of the reaction catalyzed by the native enzyme has the evidence of oligopeptide synthesis; but in the case of Chy/PTFE-catalyzed reaction, though the peak of the dipeptide and tripeptide has appeared, the peak for higher oligomer synthesis is not clear yet (Fig. 6). From these observations, we speculate that: (1) the enzyme loading is high to PTFE but due to the absence of well arrangement of the Chy/PTFE particle, most of the enzyme molecules could not come in contact to the substrate in the reaction medium, causing the prohibition of synthesis reaction; (2) the hydrophobic surface of the PTFE may distort the conformation of the enzyme, thus rendering it inactive; (3) obscuration of the enzyme active site. The total reaction products achieved from these two different kinds of mechanism catalyzed by Chy/PTFE are given in Table 2.

Table 2
Total yield of the reaction products catalyzed by Chy/PTFE particles

Esters	Yield of hydrolysis products (%)	Yield of synthetic products (%)
(a) 20 mM *N*-Ac-Phe-OEt 40 mM Ala-NH_2	75.70	10.97
(b) 200 mM Phe-OEt	63.65	14.00

3.4. Organic solvent dependency of peptide synthesis with free and PTFE-entrapped enzyme

The synthetic activities for the free and PTFE-entrapped Chy were investigated in a series of water–acetonitrile mixed solution containing 20 mM acyl donor and 40 mM nucleophilic acceptor. The result is presented in Fig. 7. Up to 20% content of ACN, the donor ester substrate, completely disappeared. Above this content, the product formation for synthetic product in the case of free enzyme dropped remarkably when the ACN content increased from 30% to 40%, and then increased sharply when the content of the ACN was 60% and then again decreased with the increase of ACN content. With the increase of ACN content up to 60%, the yield of synthetic product increased. This result suggests that due to the increase of hydrophobic environment, the equilibrium of the corresponding reaction shifts to synthesis. The equilibrium shift from hydrolysis to synthesis is closely related to the hydrophobic environment. Around 35–45% of ACN, both the synthetic and hydrolytic yields were exceptionally low probably due to the inactivation of the enzyme. Up to 50% content of ACN the enzyme is completely soluble, accompanying enzyme denaturation. On the contrary, when the acetonitrile content was above calculated 60%, the enzyme commenced to reach suspended state. In this suspended state, the enzyme retained its activity as reported by Dastoli et al. [16] and Dastoli and Price [17,18].

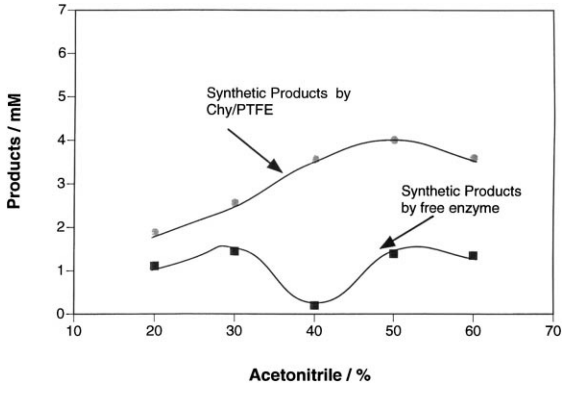

Fig. 7. Organic solvent dependence on the peptide synthesis by the free and the entrapped enzyme. Acetonitrile was mixed with 0.1 M CBS of pH 9.0 before used. The reactions were performed for 2 h and were terminated by the addition of 100 μl acetic acid.

The effect of the acetonitrile content on the synthesis of Ac-Phe–Ala-NH_2 catalyzed by Chy/PTFE particle is compared with the synthesis catalyzed by the free enzyme. The synthetic activity of the Chy/PTFE particle was found to be active as long as the acetonitrile content was about 2%. The formation of the synthetic product increased with the increase of the ACN content. The maximum yield of the synthetic product was obtained when the ACN content was 50%. After that,the formation of the synthetic product decreases with the increase of the ACN content to the reaction medium. The main reason of this low activity seems to be the deactivation of the enzyme due to the hydrophobic environment caused by the organic solvent, though the inactivation of the enzyme was not 100% irreversible. At least 5% of the synthetic yield was achieved at the 80% content of ACN. At higher ACN content, the synthetic yield of the Chy/PTFE particle was higher than that of free enzyme. There is no evidence of formation of synthetic product by the native enzyme when the ACN content is 2% in the reaction medium. On the other hand, the formation of the synthetic product was achieved by the Chy/PTFE particle in 2% of ACN content reaction medium. Therefore, the Chy/PTFE-particle-catalyzed reaction should be performed in aqueous solution.

3.5. Reaction kinetics

The performances of free and immobilized Chy in terms of the rate of the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were compared to check which one of the apparent Michaelis constants was affected by immobilization. For the free Chy, the plot of the rate of BTEE hydrolysis vs. substrate concentration was

Table 3
Kinetic constants for BTEE hydrolysis by free Chy and immobilized Chy

Conditions	V_{max} (μmol/min)	K_m (mM)
Free Chy	0.45	0.36
Immobilized Chy	0.34	0.27

hyperbolic, suggesting Michaelis–Menten kinetics.

For the immobilized Chy, the first part of the curve was hyperbolic, but the activity decreased for higher substrate concentration. This inactivation may result from substrate inhibition. The apparent V_{max} and Michaelis constant, K_m, for the hydrolysis of BTEE by free and immobilized Chy are given in Table 3.

4. Conclusion

We have introduced hydrophobic PTFE as a new material where protease-like enzyme can be immobilized. The enzyme entrapped to the PTFE remains its activity. This is the first report using Teflon polymer for enzyme immobilization. We also try to investigate the hydrophobic effect of PTFE in the reaction system for the ultimate goal of peptide synthesis. Though the presence of the PTFE in the reaction system does not produce any drastic change, due to its hydrophobicity in the reaction medium, it is also capable of catalyzing the synthesis of peptides. This suggests the dual roles of PTFE. It is able to immobilize enzyme as well as create a new environment for the reaction system.

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