

# Voltage-Assisted Peptide Synthesis in Aqueous Solution by $\alpha$ -Chymotrypsin Immobilized in Polypyrrole Matrix

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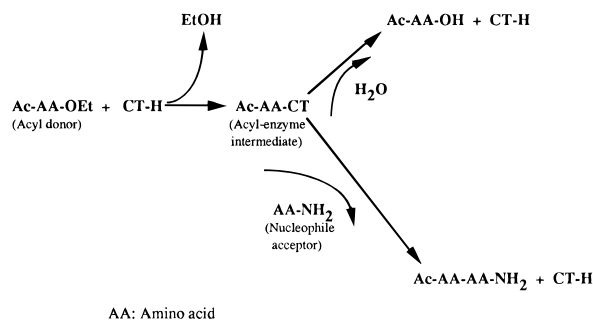
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**Abstract:** Potential-assisted enzymatic peptide synthesis in aqueous solution has been performed by  $\alpha$ -chymotrypsin immobilized in conducting polymer polypyrrole matrix. Chymotrypsin is electrochemically adsorbed on a Pt electrode, and then a thin polypyrrole membrane is electrochemically prepared on the enzyme layer. The enzyme/polypyrrole on an electrode retains chymotrypsin activity and the equilibrium of the enzymatic reaction shifts to the synthesis by ca. 25% due to the hydrophobic environment caused by polypyrrole matrix. Here, Ac-Phe-OEt and Ala-NH<sub>2</sub> are used as model substrates of acyl donor ester and a nucleophile acceptor, respectively, to synthesize Ac-Phe-Ala-NH<sub>2</sub>. Both the synthetic and the hydrolytic activity of the immobilized chymotrypsin are found to be enhanced several times when a positive potential (0.4–0.8 V vs Ag/AgCl) is applied to the electrode. In addition, the equilibrium of the enzymatic reaction shifts further to synthesis. About 73% of synthetic yield of Ac-Phe-Ala-NH<sub>2</sub> is obtained from equimolar concentration (200 mM) of Ac-Phe-OEt and Ala-NH<sub>2</sub> in aqueous solution. The effects of solution pH, temperature, organic solvent concentration, and substrates concentration on the peptide synthesis are also described. An explanation of possible effects of the applied voltage which causes the remarkable increase of catalytic activity is also presented.

## Introduction

Proteases are well-known to work for hydrolyzing peptide bond. However, the reverse reaction, i.e., peptide synthesis can also be catalyzed by proteases, which was first reported by Bergmann and Fraenkel-Conrat<sup>1</sup> in 1937. They reported the papain-catalyzed amide bond synthesis. Since then, many reports on the reverse reaction of proteases<sup>2–18</sup> have appeared. Although all proteases are theoretically capable of catalyzing their synthetic and hydrolytic reactions, the unfavorable equilibrium in aqueous solution, where water is not only a solvent but is also an excessive reactant, rules out significant function in the reverse synthesis for most of these enzymes. However, extensive reversal of enzymatic hydrolysis should be possible

**Scheme 1.** Reaction Mechanism of  $\alpha$ -Chymotrypsin-Catalyzed Peptide Synthesis



by decreasing the water content in a mixed solvent, which results in shifting of the equilibrium of a hydrolytic reaction to the synthesis of peptides.

The reaction catalyzed by  $\alpha$ -chymotrypsin (serine protease, EC.3.4.21.1) proceeds in two steps<sup>2</sup> as illustrated in Scheme 1. The first step is the coupling of an acyl donor to the serine-195 residue of the enzyme, and the second is a subsequent competitive deacylation with water or with a nucleophilic acceptor, such as peptide and C-protected amino acid. In the former case a hydrolytic product is obtained, whereas in the later case a peptide is produced. Therefore, peptide synthesis is favorable in a less aqueous environment at high concentration of nucleophilic acceptors from the viewpoint of equilibrium of the reaction. The main drawbacks of the enzymatic peptide synthesis in water-organic mixed solvent is the susceptibility of enzymes to denature which may cause very low enzyme activity. In addition, for the convenient separation of the products the enzyme catalyst should be immobilized to a solid matrix for repeated used.

To overcome these, a novel approach for peptide synthesis in an aqueous solution has been performed by taking advantages of hydrophobic environment in the polypyrrole matrix wherein chymotrypsin is entrapped. We have reported earlier that the

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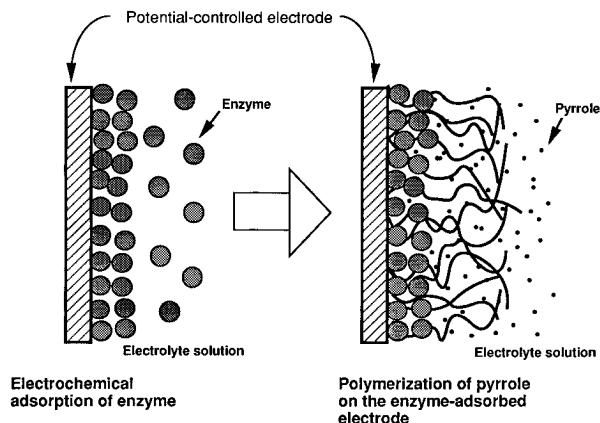


Figure 1. Preparation of CT-immobilized PP (PP/CT/Pt) electrode.

activity of flavoenzyme,<sup>19</sup> PQQ enzyme,<sup>20</sup> and NAD enzyme<sup>21</sup> immobilized into polypyrrole matrix can be controlled electrochemically. These enzymes have electroactive prosthetic groups and coenzyme. Potential-dependent controllability of these oxidoreductases's activity promotes us to study the effects of potential application on enzymes which have neither electron transfer path nor electrochemically active cofactor. We have chosen the well studied protease,  $\alpha$ -chymotrypsin, since the mechanism of chymotrypsin has been clarified in details, and it is one of enzymes of commercial importance.

To demonstrate the potential-assisted peptide synthesis, we have selected a peptide synthesis process by taking Ac-Phe-OEt and Ala-NH<sub>2</sub> as an acyl donor ester and a nucleophile acceptor, respectively, to synthesis Ac-Phe-Ala-NH<sub>2</sub> as reported by Nilsson and Mosbach.<sup>6</sup> We introduced a new hydrophobic interface, polypyrrole matrix, for the enzyme immobilization. Here, polypyrrole plays dual roles as a hydrophobic matrix and a conductive interface as illustrated in Figure 1. If the hydrophobicity of the interface material is electrochemically controlled, one can expect that the chymotrypsin-catalyzed peptide synthesis is remarkably enhanced. In addition, we speculate that not only the hydrophobicity can be controlled by the application of a potential but also the surrounding of the enzyme and its active center is controllable. The potential application may change the morphology of PP matrix which may change substrates diffusion and the change of the matrix pH which certainly have some effects on the enzyme activity.

## Experimental Section

**Materials.**  $\alpha$ -Chymotrypsin (Type II; from bovine pancreas, 3X crystallized, 39 U/mg solid), *N*-acetylphenylalanine ethyl ester (Ac-Phe-OEt), and alaninamide (Ala-NH<sub>2</sub>) were purchased from Sigma. Sodium *p*-toluenesulfonate (STS), sodium *p*-toluenesulfonic acid (STA), naphthalene-1,5-disulfonic acid disodium salt (NDADS), and 8-anilino-1-naphthalene sulfonic acid (8-ANS) were from Kanto Chemical Co. Pyrrole was purchased from Tokyo Kasei (Tokyo) and was further distilled before use. Acetonitrile (ACN) and methanol of HPLC grade were purchased from Wako Pure Chemical. Other chemicals were guaranteed reagents grade.

**Preparation of  $\alpha$ -Chymotrypsin-Immobilized Polypyrrole Matrix.** Chymotrypsin (40 mg) was dissolved in 2 mL of phosphate buffer (20 mM) solution of pH 7.0. A conventional three electrodes system consisting of a 0.5 cm  $\times$  0.5 cm Pt plate working electrode (electrochemically cleaned), a Pt plate counter electrode, and a Ag/AgCl reference electrode was inserted in this chymotrypsin solution.

A positive potential of 0.55 V was applied for electrochemical adsorption of the enzyme for 10 min. Then, the enzyme adsorbed electrode was transferred to another solution containing 0.1 M pyrrole in a deoxygenated electrolyte whose pH was adjusted beforehand to 5–6 with STA. The pH was not adjusted when KCl was used as an electrolyte. After 2 min, a potential of 0.6–0.7 V was applied to polymerize pyrrole on the enzyme adsorbed electrode. The polymerization was terminated after a defined amount of electricity was passed. Then, the enzyme immobilized electrode (PP/CT/Pt) was thoroughly washed with water and kept in water until further experiment.

### Peptide Synthesis of Chymotrypsin-Immobilized PP Electrode.

Unless otherwise specified, the reaction mixture was composed of 8 mM acyl donor ester (Ac-Phe-OEt) and 40 mM nucleophilic acceptor (Ala-NH<sub>2</sub>) in 0.1 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer which contained 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 the nucleophilic acceptor (1 M) and then diluted with the carbonate buffer solution to make the final concentration. The volume for the potential-assisted synthesis was 2 mL. All the experiments were carried out at room temperature (22  $\pm$  2  $^{\circ}$ C). The potential assisted synthesis was performed for 60 min.

In the case of peptide synthesis at a higher ester concentration, Ac-Phe-OEt (4 M) and Ala-NH<sub>2</sub> (2 M) were separately dissolved in 100% ACN and a carbonate buffer of pH 10, respectively. These two substrates were then mixed in the same buffer solution. Ac-Phe-OEt was hardly dissolved in the solution, when the concentration is over ca. 50 mM. Therefore, peptide synthesis was performed in an ester suspended solution.

A three electrodes system consisting of a PP/CT/Pt working electrode, a Pt plate counter electrode, and a Ag/AgCl reference electrode was set in a 10 mL test tube containing the reaction mixture. Then, a potential was applied for a desired time under magnetically stirred solution. After or during potential application a 25  $\mu$ L sample was directly injected to HPLC for the product analysis. All datum are the mean of 3–5 experiments.

### Assessment of the Hydrophobicity of the Polypyrrole Matrix.

The hydrophobicity in the polypyrrole matrix was assessed from the fluorescence change of 8-ANS.<sup>22</sup> 8-ANS was entrapped in PP matrix during polymerization of pyrrole. 8-ANS (20 mM) was dissolved in a deoxygenated aqueous solution containing 0.1 M NDADS and 0.1 M pyrrole, and polymerization was performed on a 1.4 cm  $\times$  3 cm Pt plate electrode at 0.7 V with a polymerization charge of 50 mC/sq-cm. After thorough washing with distilled water, the PP/ANS/Pt electrode was placed into a 1 cm  $\times$  1 cm cuvette containing a carbonate buffer solution (CBS) of pH 9.0. The electrode was positioned so as to excite the fluorescent probe at an angle of 45 $^{\circ}$ . The resulting light emission was then detected without changing the position of the photomultiplier. A wire type counter electrode and a reference electrode connected with a salt bridge were set the other side of the PP/ANS/Pt electrode. Fluorescent change was measured by changing applied potential.

**HPLC Analysis.** The products were analyzed using a reverse phase HPLC column of Wakosil-<sup>5</sup>C<sub>18</sub>. The eluent, a mixed solvent of phosphate buffer solution of pH 2.0 (NaH<sub>2</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub>; 20 mM), acetonitrile, and methanol with a ratio of 65:25:10 was pumped through the column at a flow rate of 1 mL/min at 30  $^{\circ}$ C. Every component was monitored spectrophotometrically at 260 nm. Ester substrate, acidic, and peptide product were baseline separated in all cases. The peptide yield was calculated from the ratio of the peak area compared with pure reagent.

**Apparatus.** Chymotrypsin adsorption, pyrrole polymerization, and potential-assisted synthesis were performed with a HA-301 potentiostat/galvanostat of Hokuto Denko Co. (Tokyo), and the electricity for polymerization was controlled with a HF-201 Coulomb/ampere meter of Hokuto Denko Co.

## Results

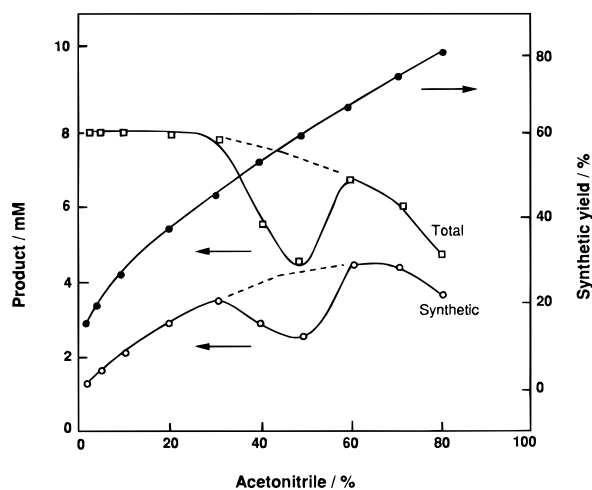
### Chymotrypsin-Catalyzed Peptide Synthesis in Solution.

Both the synthetic and the hydrolytic activities of free chymotrypsin were investigated in a series of water–acetonitrile mixed

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**Figure 2.** Organic solvent dependent peptide synthesis by free enzyme. The concentration of CT was 40  $\mu$ M. 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  $\mu$ L of acetic acid: (○) synthetic product, (□) total product, (●) percentage of synthetic yield.

solution containing 8 mM acyl donor and 40 mM nucleophilic acceptor. The concentration of CT was 40  $\mu$ M. Acetonitrile was mixed with 0.1 M CBS of pH 9.0 before used. The reaction was performed for 2 h and were terminated by the addition of 100  $\mu$ L of acetic acid. The result is presented in Figure 2. Up to 20% of ACN, the conversion of the donor ester to products was completed. Above this content yield gradually dropped to nearly 50% in a mixed solvent of 80% ACN. Around the 40%–50% of ACN both the synthetic, and the hydrolytic yield were exceptionally low probably due to the inactivation of the enzyme. During the experiment it was observed that the enzyme was completely soluble in the water/ACN mixture up to 50% of ACN, which might caused the enzyme inactivation. On the contrary, when the acetonitrile content was above ca. 60%, the enzyme commenced to be a suspended state. In this suspended state the enzyme retained its activity as reported by Dastoli et al.<sup>30–32</sup> Therefore, chymotrypsin activity again increased at around 60% of ACN due to the suspended active enzyme. With the increase of ACN content the percentage yield of synthetic product (the ratio of synthetic product to total products) increased gradually from 15 at 2% of ACN to 81 at 80% of ACN. This result suggests that the equilibrium of the chymotrypsin catalyzed reaction shifts from the hydrolysis to the synthesis with the increase of the hydrophobic environment, i.e., with the decrease of water concentration.

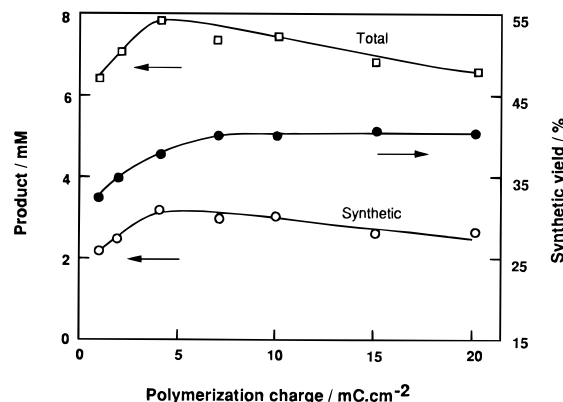
**Enzyme Activity of Immobilized Chymotrypsin.** In the previous study,<sup>20,23</sup> it was observed that application of a positive potential (0.5 V) favored very fast adsorption of fructose dehydrogenase on a Pt electrode. Here, we followed the same technique of electrochemical adsorption of chymotrypsin on Pt electrode. We observed that the optimum potential of chymotrypsin adsorption was 0.55 V in a phosphate buffer solution of pH 7.0. The amount of chymotrypsin adsorbed on the electrode and the hydrolytic activity of the adsorbed chymotrypsin (measured by the hydrolysis of benzoyl-L-tyrosine ethyl ester at pH 7.8 at 25 °C) were determined to be  $3.5 \pm 0.5 \mu\text{g}/\text{cm}^2$  and  $45 \pm 5 \text{ mU}/\text{cm}^2$ , respectively. After adsorption, the enzyme-adsorbed electrode was transferred to an electrolyte solution which contained pyrrole and electrolyte. And then, a very thin PP matrix was prepared to cover the adsorbed enzyme as shown in Figure 1.

The activity of this enzyme electrode depends on the morphology and the thickness of polypyrrole membrane. The

**Table 1.** Effect of Electrolyte on the Activity of  $\alpha$ -Chymotrypsin Immobilized in PP Matrix<sup>a</sup>

| electrolyte | polymerization potential V vs Ag/AgCl | synthetic yield, mM | total yield, mM | %age of synthetic yield |
|-------------|---------------------------------------|---------------------|-----------------|-------------------------|
| KCl         | 0.7                                   | 2.40                | 6.67            | 36                      |
| STS         | 0.6                                   | 3.00                | 7.82            | 38                      |
| NDADS       | 0.6                                   | 2.98                | 7.61            | 39                      |

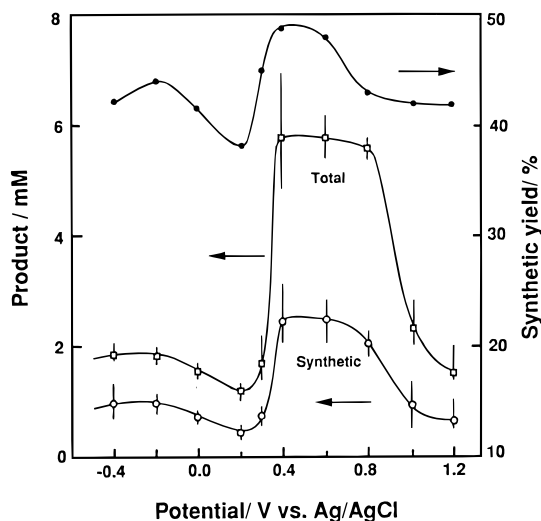
<sup>a</sup> All the reactions were conducted in carbonate buffer of pH 9.0 for 18 h. Reaction volume was 2 mL, and the polymerization charge was 10  $\text{mC}/\text{cm}^2$ .



**Figure 3.** Effect of PP membrane thickness on the activity of the PP/CT/Pt electrode. The reactions were conducted in CBS of pH 9.0 for 18 h. Reactions volume was 2 mL: (○) synthetic product, (□) total product, (●) percentage of synthetic yield.

morphology of the polypyrrole membrane changes considerably when the molecular size of the electrolyte and the polymerization potential is changed: the electrolyte of larger molecular size is favorable for the polymerization of loose polymer and polymerization at higher potential also produces loose polymer. Since the biocatalyst is involved, it is preferable to apply a lower potential to cause less damage to the enzyme. In the case of the enzyme entrapment the choice of electrolyte is the key step for controlling matrix morphology. Table 1 shows the effect of three electrolytes on the PP/CT/Pt electrode activity. In the case of KCl polymerization was performed at 0.7 V, because it was the lowest potential at which electropolymerization was most smoothly performed. As the molecular size of NDADS and STS and bulkier than KCl, less dense polymer was prepared. Therefore, the substrate diffusion becomes easier, causing higher enzyme activity. By taking advantages of bulkier electrolyte not only easily diffusible polymer was formed but less extreme potential could be applied for the polymerization, which was indispensable for the electrochemical immobilization of enzymes. Although, both STS and NDADS showed almost same activity, we employed NDADS as an electrolyte for the following study.

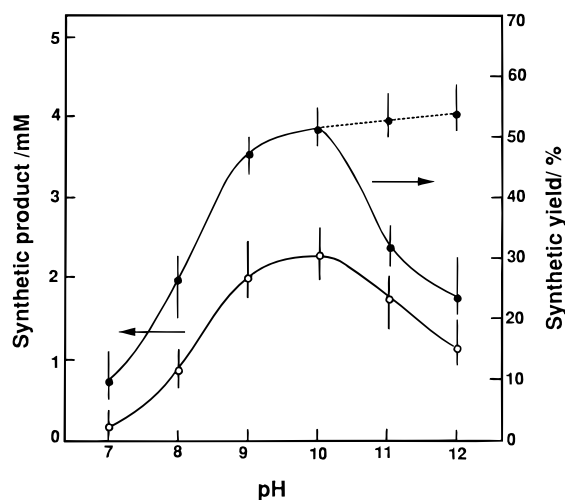
Figure 3 shows the relation of the membrane thickness to the enzyme activity. In general, polypyrrole membrane thickness is proportional to polymerization charge. With the increase in polymerization charge, both the synthesis and the hydrolysis activities increased up to a polymerization charge of 4–5  $\text{mC}/\text{cm}^2$  and then gradually decreased. It seems likely that initial increase of activity was caused by the physical stabilization of adsorbed chymotrypsin with PP coating. Therefore, at least 4–5  $\text{mC}/\text{cm}^2$  polymerization charge was necessary to prepare a membrane thick enough to avoid enzyme leaking out. The PP membrane thickness prepared by passing a charge of 4–5  $\text{mC}/\text{cm}^2$  corresponds roughly to 10–12 nm. Further increase of PP thickness the enzyme activity gradually decreased due to the increasing diffusion barrier for the substrates. On the other



**Figure 4.** Potential dependence on the activity of the PP/CT/Pt electrode: (○) synthetic product, (□) total product, (●) percentage of synthetic yield.

hand, the percentage of synthetic yield increased with the increase in polymerization charge up to 10–12 mC, and, finally became saturated. This fact suggests that the polypyrrole matrix provides hydrophobic environment around the entrapped enzyme. In the case of soluble chymotrypsin the synthesis was only 15% at 2% of ACN solution as shown in the Figure 2. Because of the immobilization of the enzyme in PP matrix the synthesis increased to ca. 40% in the same 2% of ACN solution. Therefore, PP matrix is very useful to enhance hydrophobicity for peptide synthesis in an aqueous solution. In the following experiments, a polymerization charge 10 mC/cm<sup>2</sup> was passed for immobilizing chymotrypsin.

**Potential-Assisted Peptide Synthesis.** Figure 4 shows the potential dependence on the synthetic and total yield of the PP/CT/Pt electrode catalyzed reaction. The rest potential of the PP/CT/Pt electrode in a buffer solution of pH 9.0 was around 0.2 V. At the rest potential, the activity of the PP/CT/Pt electrode was the minimum. The increase of potential in negative direction caused a gradual increase of both the synthetic and the hydrolytic yield up to -0.4 V, and then very slow decrease was observed. At around -0.4 V both the activities increased nearly twofold as compares to those of the rest potential. However, the synthesis as well as the hydrolysis sharply increased due to the application of a positive potential and attained the maximum value at around 0.4 V. This maximum value which is several fold (6–8) higher than that of at the rest potential continued with a very slow decrease up to 0.8 V, and then a sharp drop of activity was observed at a potential higher than 1.0 V. At an extreme potential like 1.0 V the enzyme might possibly be denatured; therefore, activity was dropped. The synthetic yield was about 38% at the rest potential. At a negative potential, the maximum synthesis of ca. 44% was obtained at -0.4 V, whereas at a positive potential it was ca. 50% at 0.4 V and decreased to 44% at 0.8 V. Therefore, application of potential, especially positive potential between 0.4 V to 0.8 V caused not only the increase of chymotrypsin activity several times but also caused the shift of the thermodynamic equilibrium of the enzymatic reaction toward synthesis. Although the maximum activity and the synthetic yield (%) was obtained at the potential of 0.4 V, we used 0.6 V as a most effective potential for peptide synthesis in the following experiments, because, the standard deviation of yield was higher at 0.4 V.



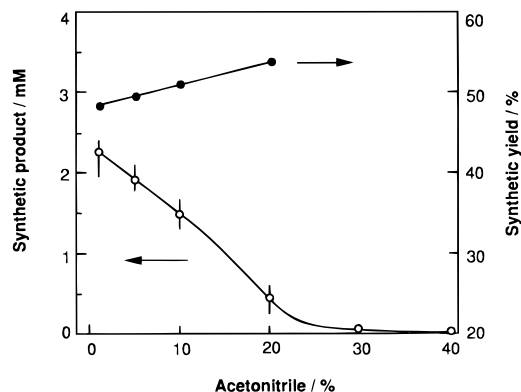
**Figure 5.** Effect of pH on the potential-assisted peptide synthesis by the PP/CT/Pt electrode: pH 7–8, phosphate buffer; pH 9–12, CBS; applied potential, 0.6 V; (○) synthetic product and (●) percentage of synthetic yield.

#### Optimization of the Potential-Assisted Peptide Synthesis.

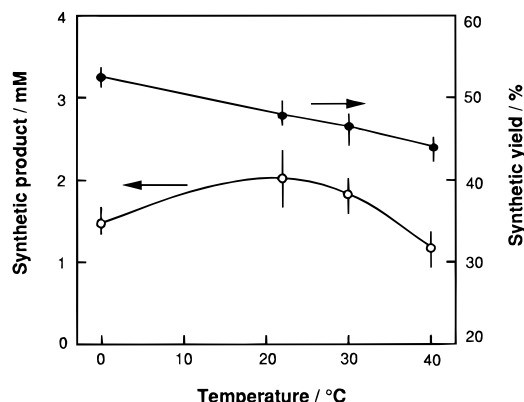
It is well-known that enzymatic peptide synthesis by CT effectively proceeds in an alkaline solution. The extent of pH is also dependent on the type of acyl donor substrate. For example, in the case of N-protected acid as an acyl donor, the optimum pH for the peptide synthesis is around 7.<sup>17</sup> However, in the case of ester substrate the synthesis is favored at higher pH in the range of pH 9–11.<sup>6,7,15</sup> Most of the ester substrate is highly unstable at a pH higher than 10 due to the autohydrolysis of ester. So, there is a limitation of using the higher pH buffer for peptide synthesis.

Figure 5 shows the pH dependence on the potential-assisted synthesis of Ac-Phe-Ala-NH<sub>2</sub>. With the increase of pH of the reaction mixture the synthetic yield increased up to pH 10 and then decreased. Ac-Phe-OEt is an unstable ester due to the autohydrolysis to Ac-Phe-OH at a pH higher than 10. Therefore, the drop of synthesis product at a pH higher than 10 was mainly due to the decrease of ester concentration. The percentage of synthetic yield also increased with the increase of pH from 10% at pH 7 to 53% at pH 10 and then decreased sharply due to the autohydrolysis of the ester to Ac-Phe-OH. If the autohydrolyzed AC-Phe-OH (determined by separate experiment) was deducted from the product, the percentage of synthesis yield became rather saturated (dotted line). These results have a good agreement with the reported results in literature.<sup>5,6</sup> From Figure 5, it is understood that the optimum pH for Ac-Phe-Ala-NH<sub>2</sub> synthesis is pH 10. In the following experiments pH 10 was used.

The effect of the concentration of acetonitrile on the synthesis of Ac-Phe-Ala-NH<sub>2</sub> is presented in Figure 6. The activity of the PP/CT/Pt electrode was observed to be limited in aqueous solution (<20%). At higher ACN content synthesis as well as hydrolysis decreased to nearly negligible at 30% of ACN. Therefore, the effect of organic solvent on the potential-assisted synthesis was very severe. The main reason of this lower activity seems to be the deactivation of the enzyme due to the combined hydrophobic environment of the organic solvent and the PP matrix. The inactivation of the enzyme (CT) was not totally irreversible. At least 50% of its activity was found to be regained when the electrode was transferred to an aqueous solution after 2 h incubation in 30% of ACN solution. On the other hand, the percentage of synthetic yield increased with the increase of ACN concentration, although the increase rate was much slower than the free enzyme (Figure 2). Despite the



**Figure 6.** Effect of organic solvent on the potential-assisted peptide synthesis by the PP/CT/Pt electrode: ACN was mixed with 0.1 M CBS of pH 10 before used; applied potential, 0.6 V; (○) synthetic product and (●) percentage of synthetic yield.



**Figure 7.** Effect of temperature on the potential-assisted peptide synthesis by the PP/CT/Pt electrode: (○) synthetic product and (●) percentage of synthetic yield.

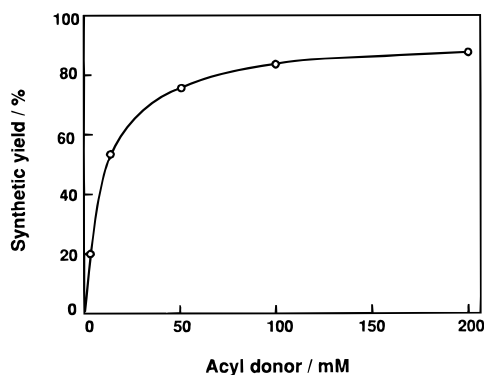
higher synthetic yield (%) at the higher ACN content, the potential-assisted synthesis should be performed in aqueous solution.

Figure 7 shows the effect of temperature on the potential assisted peptide synthesis. The maximum synthetic yield was obtained at a temperature between 20 and 25 °C, i.e., at room temperature. At a high temperature (over 30 °C) the yield became very low. In addition, the percentage of the synthetic yield decreased gradually with the increase of temperature. Therefore, the optimum temperature for peptide synthesis is at lower temperature between 10 and 25 °C.

The percentage of the synthetic yield increased sharply with the increase of substrates concentration and became nearly saturated over a concentration of 100 mM of acyl donor as shown in Figure 8. Eighty-six percent of synthetic yield was obtained at a concentration of 200 mM of acyl donor. The shift of the thermodynamic equilibrium toward synthesis was due mainly to the increase of nucleophilic acceptor. In this experiment, fivefold excess of nucleophilic acceptor was used. However, we also performed the experiment with equimolar concentration of both of the substrates (not shown here). A significant synthesis yield of 73% was observed at a concentration of 200 mM. These results indicate a great improvement in peptide synthesis, because most of the reports claimed that a several fold excess of nucleophilic acceptor is necessary for the sufficient synthesis.<sup>6,7,17,18</sup>

## Discussion

Due to the absence of redox cofactor in the active center there is no direct effect on the potential on the activity of chymot-

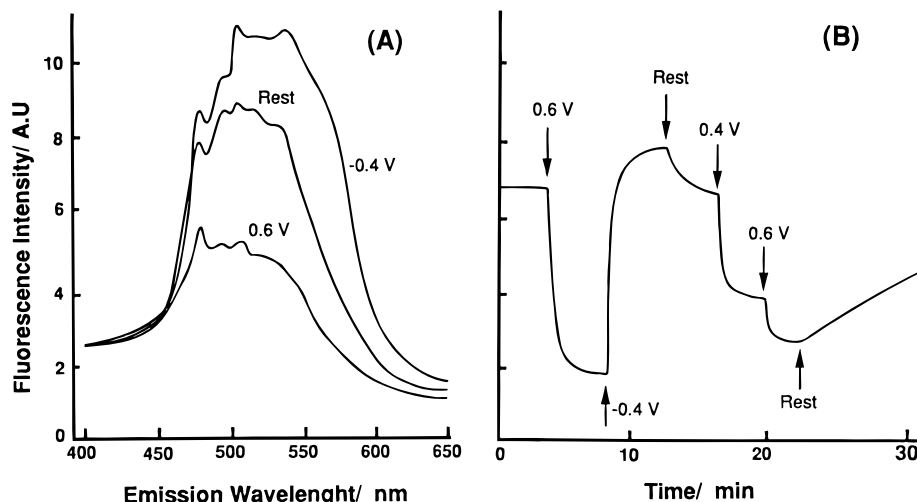


**Figure 8.** Effect of substrates concentration on the potential-assisted peptide synthesis by the PP/CT/Pt electrode. The concentration of the nucleophilic acceptor was 5 times more than an acyl donor.

rypsin. However, we believe that the application of potential changes the surrounding of the enzyme and its active site. We can summarize all the possible changes caused by the application of a potential as follows: (1) the hydrophobicity of the PP matrix is changed, (2) pH inside the matrix is changed which may alter the enzyme conformation to a favorable configuration, (3) PP matrix is swollen due to the charge interaction, and thus pore size is increased which favors substrates diffusion, and (4) relative permeability of the less hydrophobic nucleophilic acceptor over a more hydrophobic acyl donor into the matrix is enhanced. We speculate that all of these changes in the surrounding have some effects on the enzyme activity. To clarify these effects, we performed a couple of experiments.

We investigated the relationship between the hydrophobicity of polypyrrole matrix and the applied potential by using a hydrophobic fluorescence probe, 8-ANS. The highest fluorescence intensity of the probe was observed at a negative potential of  $-0.4$  V and the lowest one at positive potential of  $0.6$  V. In every case a significant fluorescence intensity was observed as shown in Figure 9A, which indicates that whatever the potential the environment in the PP matrix is hydrophobic, and the application of the potential changes the matrix hydrophobicity. Therefore, it can be concluded that the hydrophobicity inside the polypyrrole matrix depends on the doping state. The doped anions may have some influence on the change of hydrophobicity; however, in the present study, we did not examine the effect of anions on the change of hydrophobicity. At the undoped state ( $< -0.4$  V) the hydrophobicity increases due to the expulsion of the doped anion. At the doped state ( $> 0.4$  V) the matrix becomes less hydrophobic due to the increase of anion concentration. Therefore, the potential application caused the drastic change in fluorescence, depending on an applied potential as shown in Figure 9B. The initial fluorescent intensity was gradually restored when the applied potential was terminated. Although the hydrophobicity of the PP matrix can be controlled by the potential application, the positive potential is not necessarily favorable for the peptide synthesis. However, the peptide synthesis was mostly promoted at potential from  $0.4$  to  $0.8$  V. This contradiction indicates that other factors including hydrophobicity should be considered to clarify the enhancement the peptide synthesis.

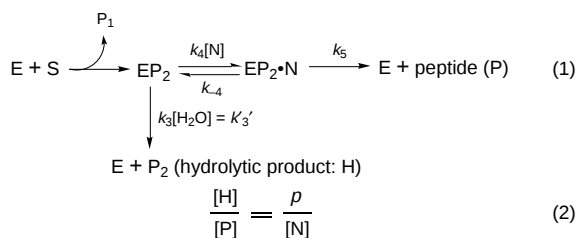
There is no direct experimental evidence that application of potential causes the change of pH inside the PP matrix. However, it can be easily understood that at a positive potential as anions such as  $\text{OH}^-$  and  $(\text{CO}_3)^{2-}$  doped into the matrix, the pH is certainly increased. The reverse phenomenon was observed by Sawai et al.<sup>24</sup> just outside the polyaniline matrix. We think that this change of pH around the enzyme certainly has some effects on the conformation of the enzyme active site,



**Figure 9.** Potential dependence on the hydrophobicity of the PP matrix in terms of fluorescence intensity: (A) fluorescence spectrum at Ex-315 nm and (B) time response at Ex-315 nm and Em-515 nm.

which may lead to the enhancement of synthetic yield as many groups reported that the synthesis is enhanced at alkaline pH.<sup>6,7,15,17</sup>

To investigate the kinetics of the enzyme catalyzed peptide synthesis, a technique for quantifying the efficiency of nucleophiles in a kinetic approach is used to examine the reaction of acyl-enzyme intermediate with nucleophiles<sup>25</sup> as presented in eq 1. The efficiency of a nucleophile is determined by as shown in eq 2 where [H] and [P] are the final concentrations of the hydrolysis product and peptide product, respectively, and [N] is the nucleophile concentration. Determination of *p* for a given nucleophile at various concentrations allows for the calculation of a partition ratio for nucleophile as well as an affinity constant of the nucleophile for the enzyme. Knowing these parameters of a nucleophile for the free enzyme and for the immobilized enzyme, the changes in active-site geometries, which may have direct responsibility on the synthesis reaction, can be examined.



In eq 1, the substrate S is acetyl-L-phenylalanine ethyl ester, the first product P<sub>1</sub> is EtOH, and the second product P<sub>2</sub> is acetyl-L-phenylalanine. The acyl-enzyme intermediate is EP<sub>2</sub>, in which it is believed that the oxygen of Ser-195 is linked to the carboxyl carbon of acetyl-L-phenylalanine. In eq 1, *k*<sub>1</sub>, *k*<sub>2</sub>, *k*<sub>3</sub>, and *k*<sub>5</sub> are the rate constants pertaining to the liberation of P<sub>1</sub>, P<sub>2</sub>, and peptide, respectively.

Gutfreund and Sturtevant<sup>26,27</sup> suggested that during the chymotrypsin-catalyzed hydrolysis of specific substrate esters the deacylation of acyl-enzyme intermediate (EP<sub>2</sub>) is the rate limiting step (*k*<sub>2</sub> ≫ *k*<sub>3</sub>). Same results was also published by Zerner et al.<sup>28</sup> Hinoe et al.<sup>29</sup> have succeeded to measure the value of *k*<sub>2</sub> and *k*<sub>3</sub> as 13/s and 2.2/s in a buffer solution of pH

5.0. The deacylation of EP<sub>2</sub> would be certainly slower than the above value in a solution of pH 9–10. Therefore, the rate limiting deacylation of EP<sub>2</sub> can be accelerated simply by increasing the concentration of nucleophilic acceptor. Results are shown in Figure 10.

The ratio of the acid to peptide product can be expressed as the following:<sup>30</sup>

$$\frac{[\text{H}]}{[\text{P}]} = \frac{k_3'[\text{N}] + k_3'K_N}{k_5[\text{N}]} = \frac{p}{[\text{N}]} \quad (3)$$

This equation can be rewritten as in eq 4

$$p = \frac{k_3'[\text{N}]}{k_5} + \frac{k_3'K_N}{k_5} \quad (4)$$

A plot of *p* vs [N] will yield a line of slope *k*<sub>3</sub>'/*k*<sub>5</sub> and a *y* intercept of *k*<sub>3</sub>'*K*<sub>N</sub>/*k*<sub>5</sub>, where *K*<sub>N</sub> is very similar to a *K*<sub>m</sub> for a normal enzyme reaction. When *k*<sub>5</sub> ≪ *k*<sub>-4</sub>, *K*<sub>N</sub> is roughly equal to *k*<sub>-4</sub>/*k*<sub>4</sub>, the dissociation constant for the EP<sub>2</sub>·N complex. Therefore, *K*<sub>N</sub> value is a good parameter to use for comparison enzyme affinity for nucleophile and for comparison of the geometries of the S<sub>1</sub>' binding site, which in kinetic controlled synthesis is the most important factor influencing the yield of peptide.<sup>31</sup> In this study, *K*<sub>N</sub> value for the free enzyme and for the immobilized enzyme were calculated to 48 and 214 mM, respectively. Which strongly suggests that a significant change in the shape of the S<sub>1</sub>' binding site occur due the immobilization of the enzyme in PP matrix. *K*<sub>N</sub> value for the potential-assisted synthesis could not be calculated as experimental data did not yielded a straight line rather yielded a parabolic line. We speculate that the concentration of the substrates in the PP matrix is changed when a potential is applied.

Figure 10 shows the effect of the nucleophilic acceptor on the synthetic and the hydrolytic yields of the three different systems: (A) free enzyme, (B) PP/CT/Pt electrode without

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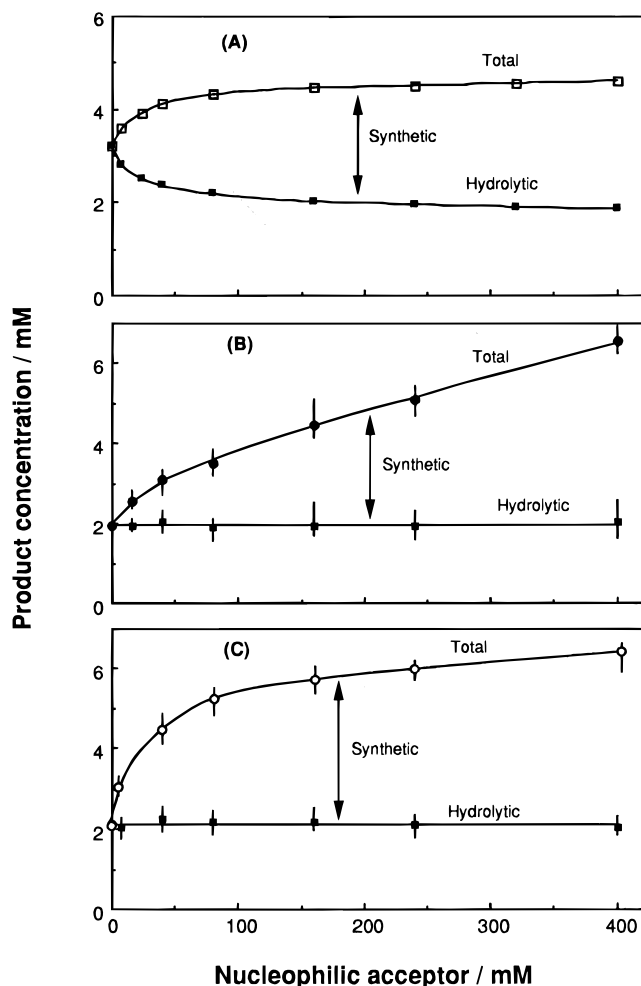
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**Figure 10.** Effect on nucleophilic and acceptor: (A) free enzyme, (B) the PP/CT/Pt electrode at without potential, and (C) the PP/CT/Pt electrode at 0.6 V. In all the cases, the concentration of acyl donor (Ac-Phe-OEt) was 8 mM in pH 9.0 CBS. [(A) CT 0.22  $\mu$ M; time, 5 min; (B) time, 2 h; reaction volume; 1 mL; (C) time, 60 min; reaction volume; 2 mL].

potential application, and (C) PP/CT/Pt electrode at 0.6 V. In the case of free enzyme [Figure 10A], the synthetic yield increased sharply at the lower nucleophile concentration and then continued slow increase with the increase of nucleophile. While the hydrolytic yield decreased. Total yield increased at the lower nucleophile concentration and trended to the saturation at higher concentration. This is quite in agreement with its  $K_N$  of 48 mM.

However, the effect of nucleophilic acceptor on the activity of PP/CT/Pt electrode is different [Figure 10B]. It shows that with the increase of nucleophilic acceptor the synthetic yield increased continuously, while the hydrolytic yield remained unaffected. This is to be expected for reactions in which the acylation step ( $k_2$ ) is extraordinarily fast and the product yield, product nature, etc. is determined by the deacylation step, i.e.,

the reaction of acyl-enzyme intermediate with water or nucleophilic acceptor. In such a situation, it is expected that the synthesis is enhanced due to the increase of nucleophile concentration.

When a potential, especially a positive potential, is applied a significantly higher synthesis is observed [Figure 10C] at a lower nucleophile concentration and the synthesis becomes relatively saturated at higher nucleophile concentration as compare to the synthesis at without potential [Figure 10B]. These results suggest that the potential is most effective at lower concentration of nucleophile which indirectly indicates that the application of potential causes an enhanced diffusion of relatively hydrophilic nucleophile. We believe that the ratio of nucleophile to acyl donor in the PP matrix is higher than the bulk solution when a positive potential is applied. For this reason, the potential assisted system did not yield a straight line when  $p$  vs  $[N]$  was plotted. Based on the above discussion it can be concluded that all the four points mentioned earlier contributed to the enhancement of the peptide synthesis.

## Conclusion

A significant percentage (ca. 86%) of synthesis yield and a remarkable enhancement of activity was obtained in aqueous solution by application of a positive potential (0.4–0.8 V). Authors believe that this finding will open a new era in enzymatic peptide synthesis especially in designing an activity controllable bioreactor for the synthesis of physiologically and commercially important peptides because it has overcome several problems for peptide synthesis. The advantage of this developed system are as follows: (1) it works in aqueous solution, (2) the enzyme immobilized in PP matrix is more stable at higher pH, (3) the enzyme-electrode can be repeatedly used, (4) the activity enhances several times due to the application of potential, (5) it works with equimolar concentration of acyl donor and nucleophilic acceptor and (6) catalytic activity can be controlled by electrochemical means. Solubility of most of the ester substrate is very low in aqueous solution which is the main limitation of this system. This problem can be partially overcome by conducting synthesis in suspended solution.

We have explained the mechanism of potential-assisted peptide synthesis. We have shown that due to the immobilization of CT in PP matrix the reaction mechanism is changed. The acylation step becomes extraordinarily fast when the deacylation step becomes a solely rate determining step.  $K_N$  value increases from 48 mM for free enzyme to 214 mM for the immobilized enzyme which indicates that the geometry of the nucleophile binding site is changed to have higher affinity for nucleophile. The potential application changes the enzyme surrounding. It causes drastic change in hydrophobicity of the PP matrix. It increases the diffusion of both substrates and, especially, the nucleophilic one as the matrix pore size increases and the hydrophobicity decreases. As a result, the synthesis increases several times due to the combined effect.

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