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Potentiometric and Spectroscopic Studies of the Reaction between Trypsin and its Inhibitors on Chemically Modified Solid Surfaces

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Abstract

The potential of a titanium metal electrode modified with trypsin changes as a result of the complex formation reaction between trypsin and its inhibitor, aprotinin, dissolved in the solution. A similar potential change in the opposite direction occurs by the reaction between aprotinin-modified electrode and trypsin in the solution. The induced changes in both cases depend on the pH of the solution, showing the maximum change at pH = 9.5. The potentiometric response of the trypsin-modified electrode for the consecutive addition of aprotinin and proflavine proves that trypsin bound on the solid surfaces reacts with aprotinin much more strongly than with proflavine. This result is fully consistent with the spectroscopically observed behavior of a trypsin-modified quartz plate against these inhibitors. The surface coverage of trypsin on the quartz plate is also determined by a near-ultraviolet absorption measurement.

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Index Entries: Potentiometry, of a trypsin-modified Ti electrode; spectroscopy, of a trypsin-modified Ti electrode; trypsin, on a Ti electrode; aprotinin, on a Ti electrode; electrode, trypsin-modified Ti; titanium, trypsin-modified electrode of.

Introduction

Chemically modified electrodes have attracted considerable attention and a variety of attachment schemes have been proposed, e.g., irreversible adsorption (*1*) and covalent bonding (*2*). This chemical modification technique has been successfully applied to the field of chromatography for the isolation or purification of biological substances such as enzymes, antigens, and antibodies (*3*). It will find wider area of applications to electrochemistry, biomedical engineering, solar energy conversion, etc. It will become more versatile if better methods of tight and stable chemical modification are found.

We reported an electrical method for the detection of biological substances in which the potential of electrodes chemically modified with an antibody is changed by a solution containing its corresponding antigen (*4*, *5*). It can be concluded that the change in the electrode potential results from antigen–antibody complex formation at the electrode–solution interface. Similar potential changes were observed for the case of the complex formation between trypsin and aprotinin, the latter being an inhibitor of trypsin.

In this paper, the results of potentiometric studies are reported on the trypsin modified electrode. It is shown that these results are consistent with the results obtained from the ultraviolet–visible absorption measurement for a trypsin-modified quartz plate.

Experimental

Materials

Lyophilized trypsin was obtained from Worthington Biochemical Co., and lyophilized aprotinin (*6*) from Teikoku Hormone Mfg. Co. was used. The molecular weight of trysin is 23,800 and that of aprotinin is 6,500. 3,6-Diaminoacridium monohydrogen sulfate, proflavine hemisulfate, from Tokyo Kasei Co. was used without further purification.

Methods

The chemical modification of a titanium electrode was performed according to a method similar to that described in a previous paper (*4*). The tip of a titanium wire, 99.5% pure and 1 mm in diameter, was heated at 1000°C to form a thin oxide film on its surface. The wire was then extruded through a glass tube and cemented with epoxy resin in a way shown in Fig. 1 (a). The tip was chemically activated in a stirred aqueous solution of cyanogen bromide (*7*) at a pH between 10 and 11 for 15

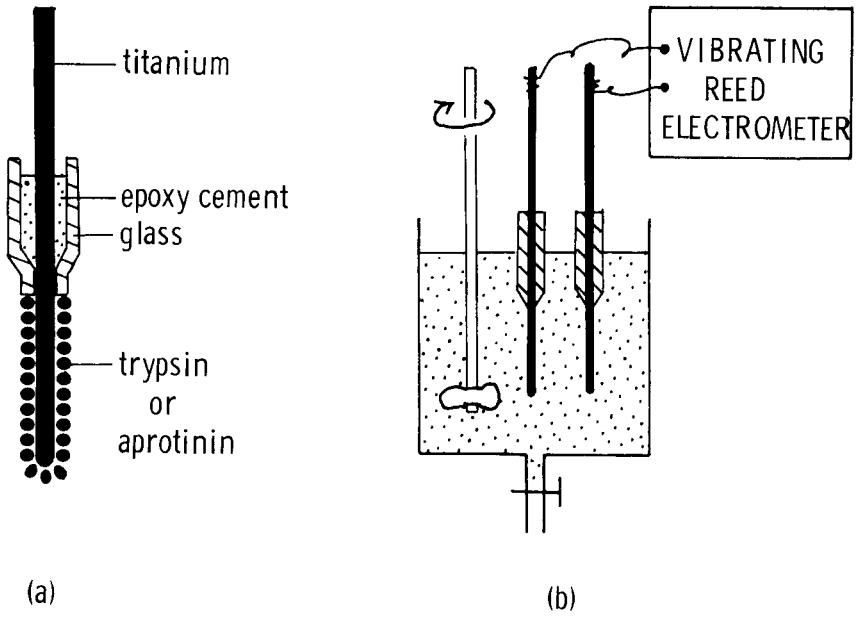

Fig. 1. Schematic drawings of the chemically modified electrode (a) and the experimental setup for potentiometric measurement (b).

min, then immersed in a 0.1*M* sodium bicarbonate solution (pH 8.4) containing 0.01 wt% of trypsin for 20 min. After that, the tip was immersed into a 1.0*M* urea solution for 20 min in order to deactivate the unreacted active spots on the titanium surface.

Modification of a titanium electrode with aprotinin was carried out in a similar way using a 0.01 wt% aprotinin solution instead of the trypsin solution. A urea electrode, used as the reference electrode, was prepared by chemical treatment with cyanogen bromide and urea in the same manner as described above.

Electric potential of a trypsin-modified electrode (or an aprotinin-modified electrode) against a urea reference electrode was measured in a 0.05*M* veronal buffer solution of 6 mL at pH 8.6 using a Takedariken TR 84M vibrating reed electrometer at 30°C [Fig. 1 (b)] and then, for example, the change in potential was followed by addition of aprotinin (or trypsin) into the solution. The pH dependence of the potentiometric response of the modified electrodes was followed using 0.05*M* acetate buffer solutions for pH < 6, 0.05*M* veronal buffer solutions in the pH region from 6 to 10, and a 0.05*M* glycine buffer solution for pH > 10. Throughout this paper, the concentrations are given as the final values taken after addition of the reactant solution.

An optically flat Suprasil plate of 20 $\times$ 50 mm, used for the spectroscopic measurement, was cleaned with commercially available anionic detergent and then immersed in a chromic acid mixture overnight. After washing with distilled water in an ultrasonic bath, one half of the surface was chemically modified with trypsin by the same method as used for the preparation of the trypsin-modified electrode, while the other half was left as a reference for the absorption spectral measurement.

The absorption spectra were measured by use of an ultrasensitive UV–visible spectrophotometer (*8*) having single-beam type optics and a photon counting system.

Results and Discussion

Potentiometric Measurement

Figure 2 (a) shows a typical change in potential of the trypsin-modified electrode observed. The potential of the trypsin electrode shifts gradually to negative when a small amount of aprotinin solution is added into the veronal buffer solution. The shifting is stopped by exchanging the aprotinin-containing solution with a simple buffer solution. This result indicates that the trypsin bound on the electrode forms a strong complex with aprotinin in solution, which does not dissociate even if the solution is replaced. In other words, the complex formation is essentially an irreversible reaction in the buffer solution.

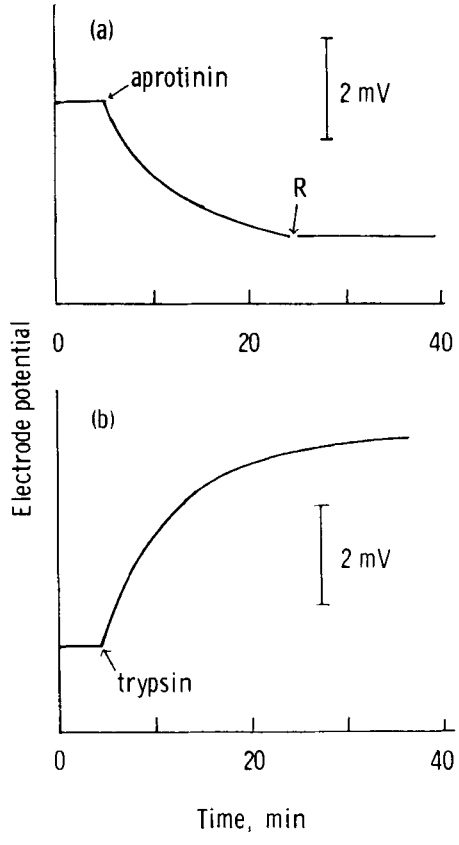

Fig. 2. Changes in electrode potential induced by the electrode surface reactions (a) for trypsin-modified electrode with addition of aprotinin to a final concentration of $1.3 \times 10^{-6}M$ aprotinin, and (b) for aprotinin-modified electrode with addition of $3.5 \times 10^{-7}M$ trypsin in final concentration. The solution was replaced with the simple buffer solution at time R.

A similar change in potential is found for the aprotinin-modified electrode by the addition of trypsin, but the direction of the shift is to positive [Fig. 2 (b)]. Such potential shifts are considered to result from the electric double layers caused by complex formation at the electrode surfaces (*4*). Under some assumptions, the potential, $U(t)$, observed at a reaction time t can be expressed by the following equation with the concentration, c, of the additive as follows (*4*):

$$U(t) = (U_f - U_i) \exp(-kct) + U_i \quad (1)$$

where U_i and U_f are the initial and the final potentials on the surface reaction and k is the rate constant of the complex formation.

The pH dependence of the change in potential was measured for the trypsin electrode or for the aprotinin electrode as shown in Fig. 3. The $| U_f - U_i |$ values, obtained from the analysis of potential vs reaction time curve in the same manner using Eq. (1) as previously described (*4*), are plotted against pH. The results for both electrodes indicate that they have the maxima of $| U_f - U_i |$ at around pH 9.5, and are insensitive below pH 5.

It is known that the complex between trypsin and aprotinin is most stable in a neutral solution (*9, 10*). Vincent and Lazdunski reported that its dissociation constant K_{diss} is as low as $6 \times 10^{-14} M$ at pH 8.0, and, in acidic solutions below pH 3, no complex is practically formed (*10*). The value of K_{diss} also becomes high in

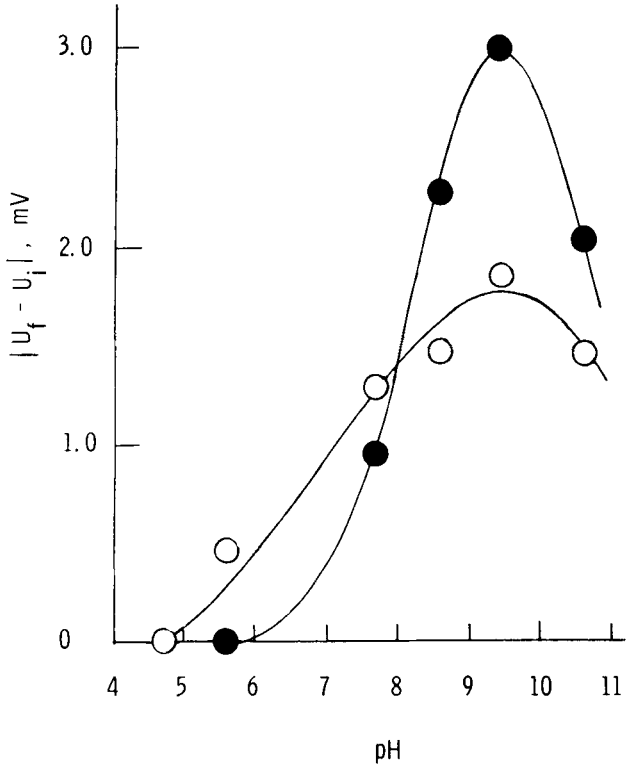

Fig. 3. The pH dependence of the electric response, $| U_f - U_i |$, for trypsin electrode (○) and for aprotinin electrode (●).

the pH region above 10.5 either because of alkaline denaturation of trypsin or deprotonation of the inhibitor (*10*). The pH dependence of the electrode response observed here is qualitatively in agreement with such behavior in solution, showing that chemical immobilization does not affect the characteristic properties of these proteins.

It is known that proflavine forms a 1 : 1 stoichiometric complex with trypsin, inhibiting the specific substrate reaction with trypsin (*11, 12*). Figure 4 shows the electrical potential behavior of a trypsin electrode in contact with solutions of aprotinin and proflavine. Firstly, the addition of aprotinin induces a large negative change in potential, resulting in the complex formation between trypsin and aprotinin [Fig. 4 (a)]. After replacing the aprotinin solution with a simple buffer solution, a small amount of proflavine solution is added into the measuring vessel. This second addition gives only very small change in potential. On the contrary, the potential changes to positive when proflavine is initially added into the solution [Fig. 4 (b)], and the subsequent exchange with a simple buffer followed by addition of aprotinin largely shifts the potential to the negative. The trypsin electrode shows no appreciable shift by the further addition of proflavine.

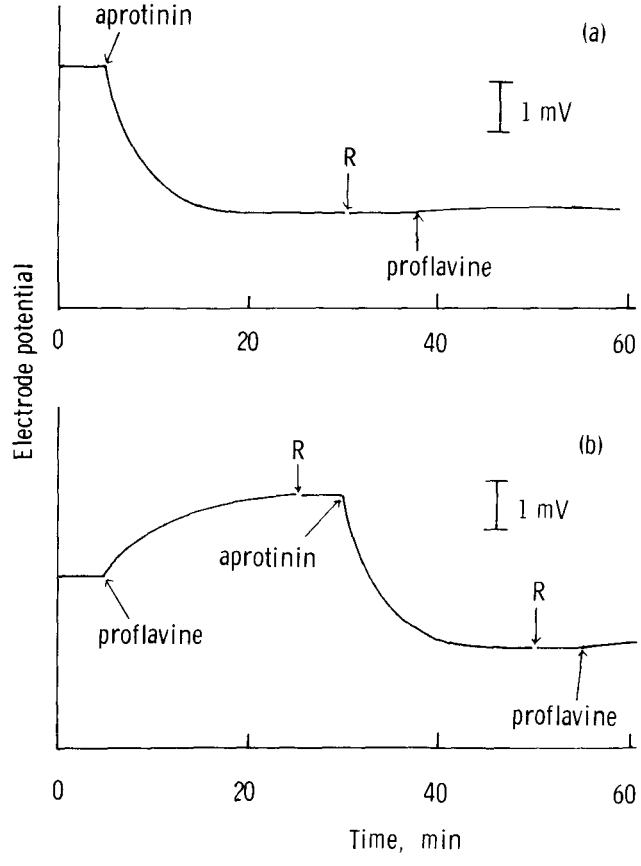

Fig. 4. Changes in potential of trypsin electrode induced by the additions of $6.2 \times 10^{-7}M$ aprotinin and $1.9 \times 10^{-6}M$ proflavine in final concentration. The replacement of solution is marked with an R.

These results are explained well by taking account of the difference in dissociation constant between these complexes. The dissociation constant of the trypsin–proflavine complex is $4.2 \times 10^{-5}M$ in solution of pH 6.0 (*12*), much higher than the value of $6 \times 10^{-14}M$ for the trypsin–aprotinin complex. In the case of Fig. 4 (a), therefore, the aprotinin molecules bound to the electrode-bound trypsin are not displaced by proflavine. In the case of Fig. 4 (b), the aprotinin easily expells proflavine from the binding site and binds itself with trypsin on the electrode surface, leading to the shift in potential as shown in Fig. 4 (b).

Trypsin is believed to have a binding pocket, which gives substrate specificity, with the carboxylate group of Asp-189 at the interior of the pocket specifically interacting with an ammonium group, forming a salt (*13*). It can be, therefore, thought that the induction of positive potential change results from the salt formation of the carboxylate group of Asp-189 and the ammonium group of proflavine neutralizing the electrostatic potential gradient. For the trypsin–aprotinin complex, the induced potential cannot be explained by such a simple electrostatic model because the bonding is considerably complicated (*14*). The negative change in potential in this case suggests that hydrogen bonding and other weak charge-transfer interactions between these macromolecules predominantly contribute to the surface potential.

Spectroscopic measurement

Figure 5 shows the absorption spectrum of a quartz (Suprasil) plate modified with trypsin. The near UV absorption consists of two bands at 280 and 260 nm, considerably broader than the 280 nm band of trypsin taken in solution [Fig. 6 (a)]. In spite of this, the near UV absorption found for the modified plate is mostly attribu-

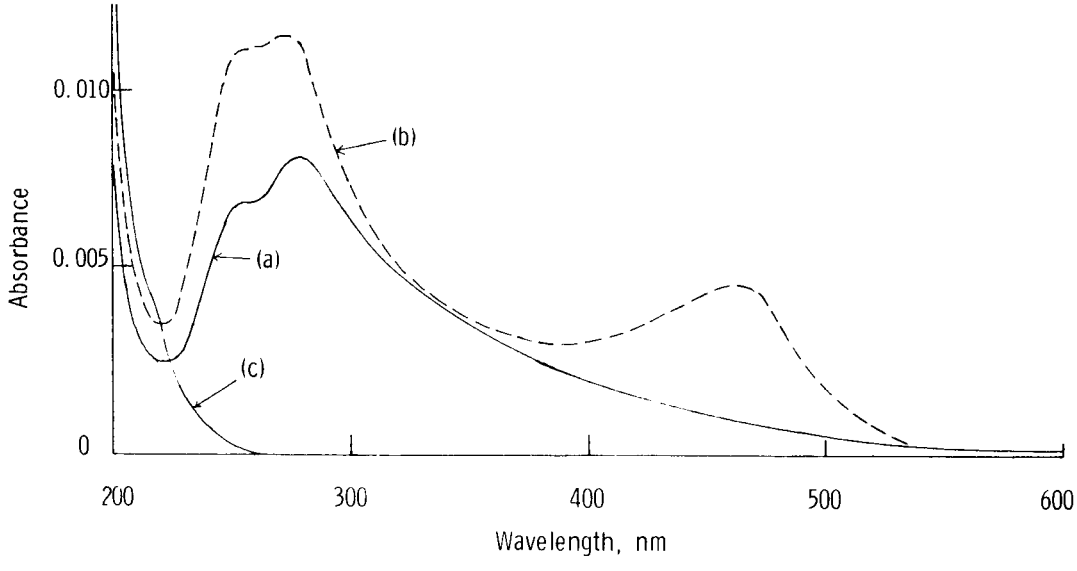

Fig. 5. Absorption spectra of a trypsin-modified quartz plate (a) and the trypsin-modified plate after immersion in a $3.7 \times 10^{-5}M$ proflavine solution for 30 min (b), and that of a urea modified quartz plate (c).

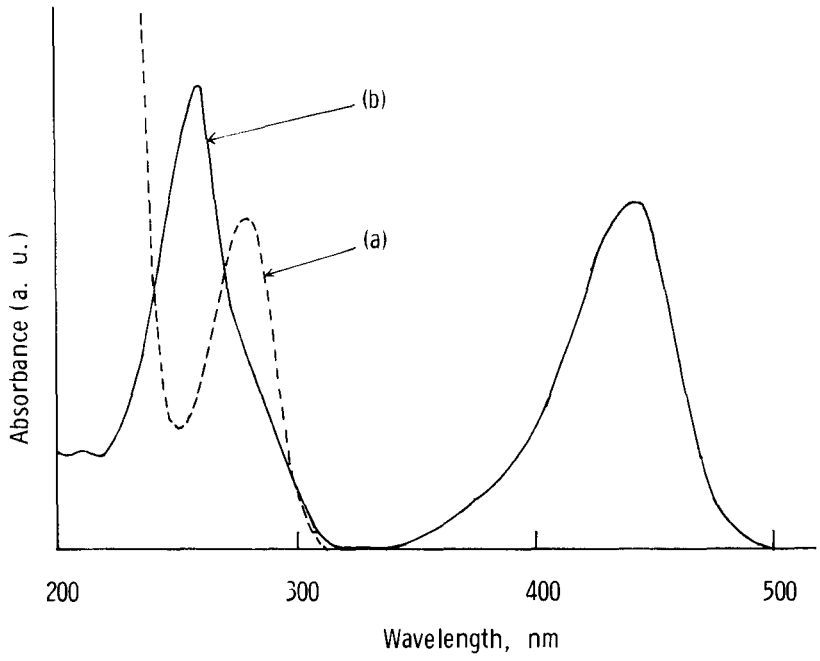

Fig. 6. Absorption spectra of trypsin (a) and proflavine (b) in dilute aqueous solutions.

table to aromatic amine residues in the trypsin. The tail of the π–π^* band below 250 nm in curves (a) and (b), characteristic of peptides, is largely blue-shifted from those in solution. This change may reasonably be explained by the environmental change from that in a solution to that on a solid surface. It was confirmed from the absorption measurement that the chemically bound trypsin could not be detached by washing with water in the ultrasonic bath for 5 min.

Curve (b) in Fig. 5 shows the absorption spectrum of the trypsin-modified quartz in air that was immersed in a proflavine solution. The spectrum shows a new absorption band in the visible region, and an increase in the near UV region. Free proflavine in solution has absorption bands at 444 and at 260 nm [Fig. 6 (b)]. It is reported that the visible band red-shifts by 25 nm on the complex formation with trypsin in solution (*11*). The new absorption bands appearing in Fig. 5 (b) undoubtedly show the contribution to the absorption of proflavine captured by the bound trypsin.

Generally, the absorbance, A, of a substance in a solution is defined as follows.

$$A = \varepsilon cL \tag{2}$$

where ε is the molar absorptivity of the solute in L mol^{-1} cm^{-1}, c is the concentration of the solute in mol L^{-1} and L is the optical path length in cm. In the case where the absorbing molecules are fixed on a flat surface, the absorbance is expressed by modifying Eq. (2) as follows, taking the same molar absorptivity as above.

$$A = 10^{-1}\,\varepsilon\,\Gamma \tag{3}$$

where Γ is the surface density of the molecules in units of mol m^{-2}. Assuming that ε at 280 nm for the bound trypsin is 3.42×10^4, equal to that at the near UV band

maximum of free trypsin in solution (*15*), Γ is calculated to be 9.2×10^{-7} mol m^{-2} for the bound trypsin. The Γ value of proflavine is derived as 4.8×10^{-7} mol m^{-2} in a similar way to the result shown in Fig. 5 (b), taking ε of 3.3×10^4 at the visible band maximum of proflavine bound to trypsin on the quartz as equal to that of proflavine in its complex with trypsin in solution (*11*). The estimated Γ value for proflavine is about one half that for trypsin, suggesting the possibility that some of the bound trypsin is unable to react with the inhibitor, if the 1 : 1 stoichiometric complex is considered.

Figure 7 (b) shows the absorption spectrum of a trypsin modified plate in air that was immersed in an aprotinin solution. In solution, the molar absorptivity at around 280 nm for aprotinin is six times as small as that of trypsin (*6*). The increase observed at 280 nm is about one-eighth of the absorbance measured for the trypsin-bound plate [Fig. 7 (b)], suggesting a 1 : 1 complex between trypsin and aprotinin. When a trypsin-modified plate was immersed first in a proflavine solution and then in an aprotinin solution, the absorption spectrum measured was essentially the same as Fig. 7 (b). This result indicates that proflavine cannot drive out aprotinin complexed with trypsin, in agreement with the behavior of the electrical potential investigated [Fig. 4 (b)].

In order to rule out any contribution of physical adsorption of proteins, a control spectrum was obtained by measuring the spectrum of a quartz plate derivatized with urea by the BrCN method. Curve (c) in Fig. 5 shows the absorption spectrum of such a urea plate that was not exposed to any protein. The absorption tail below 230 nm clearly indicates the existence of bound urea. After immersion in a trypsin or an aprotinin solution, the plate was washed in an ultrasonic bath for 5 min, dried, and then its spectrum was measured. The plate gave exactly the same curve

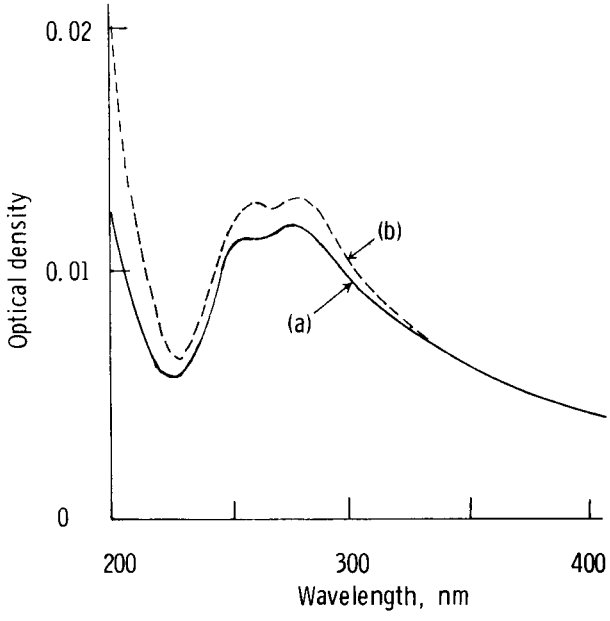

Fig. 7. Absorption spectra of a trypsin-modified quartz plate (a) and of a trypsin-modified plate after immersion into a 5.1×10^{-6} *M* aprotinin solution (b).

as curve (c) in Fig. 5. The result implies that physical adsorption is not important in our spectroscopic study of the trypsin-modified plate.

In conclusion, we have obtained compatible evidences for the chemical modification and enzyme–inhibitor interaction from the potentiometric and spectroscopic measurements. The chemical modification of the solid surface and the specific responses of the potentials of the modified electrodes by the reaction between biological substances on the electrode have been ascertained.

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