Anal. Chem. **2005,** *77,* 1588−1595

Self-Assembled Monolayer of a Pepstatin Fragment as a Sensing Element for Aspartyl Proteases

Hiromi Kitano,* Yoshinobu Makino, Hideaki Kawasaki, and Yusuke Sumi

Department of Chemical and Biochemical Engineering, Toyama University, Toyama 930-8555, Japan

A novel disulfide, which carried two pepstatin fragments at both ends, was prepared by the coupling of 11,11′-dithiobisundecanoic acid (DTUA) with a fragment (Val-Val-Sta) carrying a *n*-hexyl end (Pepsta(h)). The compound obtained (DTUA-Pepsta(h)) formed a self-assembled monolayer (SAM) on a gold electrode and vacuum-evaporated gold thin film as proven by cyclic voltammetry and reflection absorption infrared spectroscopy, respectively. When the SAM-modified gold electrode was incubated with a solution of aspartyl protease, pepsin, a decrease in both anodic and cathodic peak currents and an increase in potential difference were observed in the cyclic voltamogram of hydroquinone as a probe, whereas a coexistence of free pepstatin fragment inhibited these phenomena, indicating the specific binding of pepsin to the fragment at the exterior of the SAM. The binding rate of the enzyme to the SAM was largely dependent on the surface density of the fragment moiety in the SAM. Furthermore, when the SAM of DTUA-Pepsta(h) on a gold colloid array deposited on an amino group-modified glass plate was immersed in a pepsin solution, absorption of the glass plate at 550 nm corresponding to a localized surface plasmon resonance of the gold colloid abruptly increased and slightly red-shifted, and a further addition of pepstatin A gradually decreased the absorbance. From the increasing and decreasing profiles of absorbance, the association constant (K_{assoc}) for pepsin with the fragment on the SAM was determined. Similar phenomena were observed upon immersion of the fragment-modified SAM in a solution of HIV-1 protease, suggesting a usability of the pepstatin fragment SAM for the detection and removal of the enzyme from biological fluids.

Many kinds of proteins and peptides participate in many biological processes including specific recognition and response to external signals (i.e., functional mediator of information) on membrane surfaces.[1,2] To investigate diverse interactions on membrane surfaces, simple biomembrane mimetic systems such as monolayers,[3] liposomes,[4−6] and Langmuir−Blodgett films composed of natural lipids or synthetic lipid analogues have very often been used. We have been investigating molecular recognition processes of proteins and sugar derivatives on the surface of model cell membranes. For example, amphiphiles with many pendent sugar groups were prepared, and recognition of the sugar residues in the amphiphiles incorporated in liposomes by lectins and enzymes was examined.[7−13]

Alkyl or aromatic disulfides and thiols form a close-packed ordered monolayer, "self-assembled monolayer" (SAM), on gold or silver surfaces via chemisorptive S−Au or S−Ag bonds.[14−21] Di-*n*-alkyl sulfides form SAM on metal surfaces, too, though the stability of the SAM is not sufficient.[22,23] Because of their structural analogy to biomembranes, ease of preparation, and apparent stability, SAMs have extensively been studied as novel cell membrane mimetics in recent years.[24−31] For example, SAMs of

* To whom correspondence should be addressed. E-mail: kitano@eng.toyama-u.ac.jp.

(1) Gennis, R. B. *Biomembranes*: *Molecular Structure and Functions*; Springer-Verlag: New York, 1989.
(2) Eggens, I.; Fenderson, B.; Toyokuni, T.; Dean, B.; Stroud, M.; Hakomori, S. *J. Biol. Chem.* **1989**, *264*, 9476−9484.
(3) Kitano, H.; Kuwayama, M.; Kanayama, N.; Ohno, K. *Langmuir* **1998**, *14*, 165−170.
(4) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 114−158.
(5) Kakinoki, K.; Maeda, Y.; Hasegawa, K.; Kitano, H. *J. Colloid Interface Sci.* **1995**, *170*, 18−24.
(6) Takeuchi, H.; Ohtsuka, Y.; Harada, I. *J. Am. Chem. Soc.* **1992**, *114*, 5321−5328.
(7) Kitano, H.; Ohno, K. *Langmuir* **1994**, *10*, 4131−4135.
(8) Kitano, H.; Sohda, K.; Kosaka, A. *Bioconjugate Chem.* **1995**, *6*, 131−133.
(9) Ohno, K.; Kosaka, A.; Sohda, K.; Kitano, H. *Bioconjugate Chem.* **1995**, *6*, 361−366.
(10) Kawaguchi, T.; Tagawa, K.; Senda, F.; Matsunaga, T.; Kitano, H. *J. Colloid Interface Sci.* **1999**, *210*, 290−295.
(11) Tagawa, K.; Sendai, N.; Kawaguchi, T.; Ohno, K.; Kitano, H.; Matsunaga, T. *Bioconjugate Chem.* **1999**, *10*, 354−360.
(12) Kitano, H.; Sumi, Y.; Tagawa, K. *Bioconjugate Chem.* **2001**, *12*, 56−61.
(13) Kitano, H.; Ishino, Y.; Yabe, K. *Langmuir* **2001**, *17*, 2312−2316.
(14) Nuzzo, R. G.; Allara, D. L. *J. Am. Chem. Soc.* **1983**, *105*, 4481−4483.
(15) Nuzzo, R. G.; Fusco, F. A.; Allara, D. L. *J. Am. Chem. Soc.* **1987**, *109*, 2358−2368.
(16) Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G. *J. Am. Chem. Soc.* **1989**, *111*, 321−335.
(17) Chidsey, C. E. D.; Loiacono, D. N. *Langmuir* **1990**, *6*, 682−691.
(18) Laibinis, P. E.; Whitesides, G. M.; Allara, D. L.; Tao, Y.-T.; Parkih, A. N.; Nuzzo, R. G. *J. Am. Chem. Soc.* **1991**, *113*, 7152−7167.
(19) Dubois, L. H.; Nuzzo, R. G. *Ann. Rev. Phys. Chem.* **1992**, *43*, 437−463.
(20) Templeton, A. C.; Wuelfing, W. P.; Murray, R. W. *Acc. Chem. Res.* **2000**, *33*, 27−36.
(21) Schreiber, F. *Prog. Surf. Sci.* **2000**, *65*, 151−257.
(22) Troughton, E. B.; Bain, C. D.; Whitesides, G. M.; Allara, D. L.; Proper, M. D. *Langmuir* **1988**, *4*, 365−385.
(23) Katz, E.; Itzhak, N.; Willner, I. *J. Electroanal. Chem.* **1992**, *336*, 357−362.
(24) Spinke, J.; Lileym, M.; Guder, H.-J.; Angermaier, L.; Knoll, W. *Langmuir* **1993**, *9*, 1821−1825.
(25) Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714−10721.
(26) Lopez, G. P.; Albers, M. W.; Schreiber, S. L.; Carroll, R.; Peralta, E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 5877−5878.
(27) Schierbaum, K.-D.; Weiss, T.; van Velzen, E. U. T.; Engbersen, J. F. J.; Reinhoudt, D. N.; Göpel, W. *Science* **1994**, *265*, 1413−1415.
(28) Jeon, N. L.; Finnie, K.; Branshaw, K.; Nuzzo, R. G. *Langmuir* **1997**, *13*, 3382−3391.

10.1021/ac0488558 CCC: $30.25
Published on Web 02/09/2005

ω-mercaptoalkanoic acid[32] and phenylboronic acid,[33] and that of a polymer chain with many pendent glucose residues,[34,35] were constructed on silver colloids or gold electrode to analyze the interaction between proteins and biomembranes. Furthermore, thiolated cyclodextrin (CD) derivatives were fixed as a sensing device due to the molecular recognition properties of CD.[36−41]

Studies on SAM of substrates for enzymes have also been reported. However, it is very difficult for an enzyme to pursue catalysis to a substrate at an exterior surface of the SAM. To realize the specific adsorption of various substances including enzymes to the SAM-modified surface, it is important to construct the SAM with optimum performances; that is, it resists against nonspecific adsorption of the target molecule and affords a suitable environment for functional fragments (surface density, distance from the solid surface, etc).[42,43] In contrast with the substrate-carrying SAM, only a few reports concerning SAM of enzyme inhibitors have been made despite their importance in basic and practical research fields.[44,45] The enzyme inhibition has very often been examined in solution phase for determining concentrations of the inhibitors themselves, whereas it has not been adopted for determining concentrations of the corresponding enzymes so often.

Meanwhile, cyclic voltammetry (CV) is an electrochemical technique that monitors processes occurring at the interface of the electrode. The CV method involves measurement of current flow as a function of applied potential and gives a great deal of information about the redox activity of a compound and the stability and accessibility of its reduced and oxidized forms.[46] Using this technique, furthermore, it is possible to obtain valuable information about a microenvironment near the electrode surface. For that purpose, an electrochemically active compound such as ferricyanide ion[33−35,38] and hydroquinone[40,41] can be used as a probe. The fixation of an azurin (blue copper protein from *Pseudomonas aeruginosa*) to the gold electrode, for example, resulted in a strongly asymmetric CV profile of ferricyanide ion with a markedly smaller cathodic current and a large peak separation due to effective blocking of the faradaic process.

Recently a label-free localized surface plasmon resonance absorption spectroscopy (abbreviated as LSPR-AS) has drawn attention.[47−51] When a monolayer of gold colloid deposited on a glass plate was incubated with thiolated compound, absorption around 525 nm corresponding to a localized surface plasmon resonance of the gold colloid increased and red-shifted. An attachment of macromolecules to the SAM induced further increase in absorbance and red-shift. Using this technique, a binding process of streptavidin to a biotin-carrying SAM could easily be followed in situ.[47]

Aspartyl proteases including pepsin, chymosin, and HIV-1 protease are characterized by the presence of two active site aspartate residues whose carboxylate side chains are involved in catalysis. This family of enzymes is characterized by their inhibition with a low level of pepstatin (Pepstatin A, Iva-Val-Val-Sta-Ala-Sta) from *Streptomyces sp.*[52] The unit of statine (4-amino-3-hydroxy-6-methylheptanoic acid) in the pepstatin molecule resembles the tetrahedral intermediate in the hydrolysis of peptides, which results in the effective inhibition of catalyses by aspartyl proteases.[53]

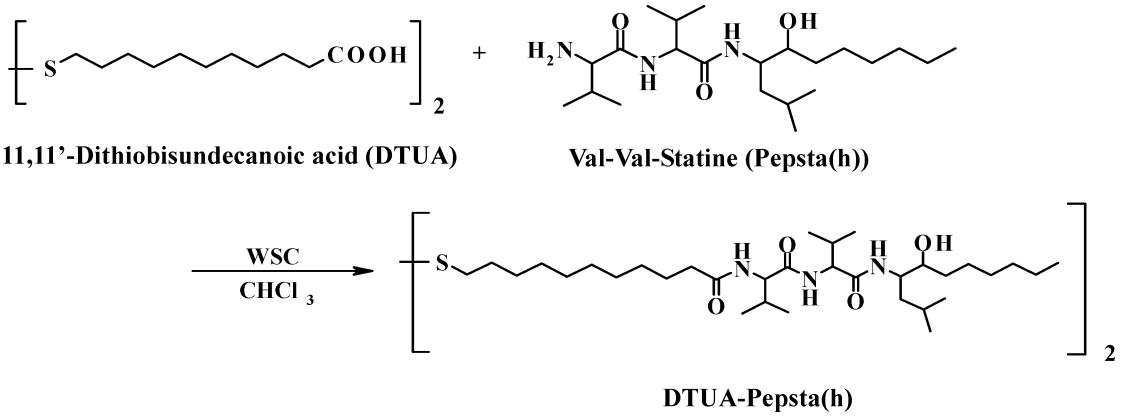
In this report, a SAM carrying a fragment of pepstatin was constructed on a gold surface, and recognition of the fragment at the SAM surface by proteolytic enzymes, pepsin and HIV-1 protease, was studied using both the LSPR-AS technique and the CV method with hydroquinone as a probe. The effect of the surface density of the inhibitor moiety in the SAM on the recognition by the enzyme was investigated. In addition, the effect of chain length at the end of SAM on the formation of enzyme−inhibitor complex was also examined. Furthermore, using the LSPR-AS technique, we could construct a sensor chip to follow association and dissociation processes of aspartyl proteases. This chip would be useful for a highly sensitive detection of the aspartyl proteases.

EXPERIMENTAL SECTION

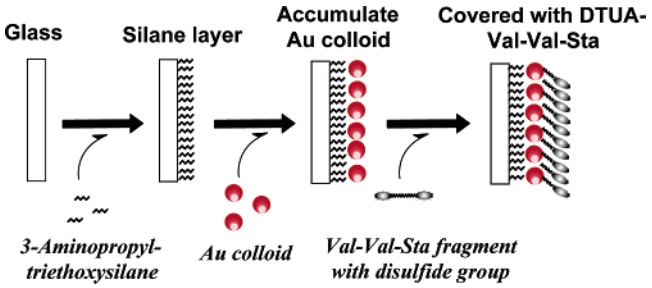
Material. Pepsin (porcine pancreas, three times recrystallized, 3000 units/mg), trypsin (porcine pancreas, three times recrystallized, 5600 units/mg), peroxidase (horseradish (HRP), 100 units/mg), and 2-hydroxyethyl disulfide (2-HEDS) were purchased from Wako Pure Chemicals (Osaka, Japan). Pepstatin A was purchased from Peptide Institute, Inc. (Osaka, Japan). HIV-1 protease (recombinant, expressed in *Escherichia coli*) was from Sigma (St. Louis, MO). 11,11′-Dithiobisundecanoic acid (DTUA) was prepared from 11-bromoundecanoic acid as previously reported.[54] Synthetic procedures of a conjugate of DTUA with a pepstatin fragment (Val-Val-Sta) carrying a *n*-hexyl end (DTUA-Pepsta(h), Scheme 1) and that carrying an ethyl end (DTUA-Pepsta(e))[55−57] are described in Supporting Information. Other reagents were commercially available. A Milli-Q grade water was used for preparation of sample solutions.

(29) Flink, S.; Boukamp, B. A.; van den Berg, A.; van Veggel, F. C. J. M.; Reinhoudt, D. N. *J. Am. Chem. Soc.* **1998**, *120*, 4652−4657.
(30) Gorman, C. B.; Miller, R. L.; Chen, K.-Y.; Bishop, A. R.; Haasch, R. T.; Nuzzo, R. G. *Langmuir* **1998**, *14*, 3312−3319.
(31) Flink, S.; van Veggel, F. C. J. M.; Reinhoudt, D. N. *Adv. Mater.* **2000**, *12*, 1315−1328.
(32) Maeda, Y.; Yamamoto, H.; Kitano, H. *J. Phys. Chem.* **1995**, *99*, 4837−4841.
(33) Kanayama, N.; Kitano, H. *Langmuir* **2000**, *16*, 577−583.
(34) Yoshizumi, A.; Kanayama, N.; Maehara, Y.; Ide, M.; Kitano, H. *Langmuir* **1999**, *15*, 482−488.
(35) Kitano, H.; Ohhori, K. *Langmuir* **2001**, *17*, 1878−1884.
(36) Maeda, Y.; Kitano, H. *J. Phys. Chem.* **1995**, *99*, 487−488.
(37) Yamamoto, H.; Maeda, Y.; Kitano, H. *J. Phys. Chem. B* **1997**, *101*, 6855−6860.
(38) Maeda, Y.; Fukuda, T.; Yamamoto, H.; Kitano, H. *Langmuir* **1997**, *13*, 4187−4189.
(39) Fukuda, T.; Maeda, Y.; Kitano, H. *Langmuir* **1999**, *15*, 1887−1890.
(40) Kitano, H.; Taira, Y.; Yamamoto, H. *Anal. Chem.* **2000**, *72*, 2976−2980.
(41) Kitano, H.; Taira, Y. *Langmuir* **2002**, *18*, 5835−5840.
(42) Mrksich, M.; Grunwell, J. R.; Whitesides, G. M. *J. Am. Chem. Soc.* **1995**, *117*, 12009−12010.
(43) Ruan, C.; Yang, F.; Lei, C.; Deng, J. *Anal. Chem.* **1998**, *70*, 1721−1725.
(44) Wang, J.; Chen, L. *Anal. Chem.* **1995**, *67*, 3824−3827.
(45) Smit, M. H.; Rechnitz, G. A. *Anal. Chem.* **1993**, *65*, 380−385.
(46) Kaifer, A. E. In *Comprehensive Supramolecular Chemistry*; Atwood, J. L., Davies, J. E. D., MacNicol, D. D., Vögtle, F., Eds.; Pergamon: Oxford, 1996; p 499.
(47) Nath, N.; Chilkoti, A. *Anal. Chem.* **2002**, *74*, 504−509.
(48) Okamoto, T.; Yamaguchi, I.; Kobayashi, T. *Opt. Lett.* **2000**, *25*, 372−374.
(49) Cheng, S.-F.; Chau, L.-K. *Anal. Chem.* **2003**, *75*, 16−21.
(50) Jung, L. S.; Nelson, K. E.; Stayton, P. S.; Campbell, C. T. *Langmuir* **2000**, *16*, 9421−9432.
(51) Grabar, K. C.; Freeman, R. G.; Hommmer, M. B.; Natan, M. J. *Anal. Chem.* **1995**, *67*, 735−743.
(52) Nakatani, H.; Tsuchiya, M.; Morita, T.; Ohki, S.; Hiromi, K. *Biochim. Biophys. Acta* **1980**, *614*, 144−150.
(53) Bugg, T. *An Introduction to Enzyme and Coenzyme Chemistry*; Blackwell Science, Oxford, 1997.
(54) Wallace, T. J. *J. Am. Chem. Soc.* **1964**, *86*, 2018−2021.

Scheme 1. Synthesis of DTUA-Pepsta(h)



Scheme 2. Construction of the Sensor Chip



Absorption Measurements. (a) Enzyme Reaction.[55,56,58,59] A procedure of kinetic measurements is described in Supporting Information in detail.

(b) Localized Surface Plasmon Resonance Absorption Spectroscopy.[47] Gold colloid was prepared by the conventional procedure using $HAuCl_4$ and citric acid.[33] The hydrodynamic diameter of the colloid was determined to be 19 nm on average using a dynamic light scattering technique (He−Ne laser at 632.8 nm, DLS-7000, Ohtsuka Electronics, Hirakata, Japan).

Glass plate (20 × 60 × 0.15 mm, Matsunami, Osaka, Japan) was rinsed with nitric acid and water. The glass plate was incubated with (3-aminopropyl)triethoxysilane (10 (v/v)% in ethanol) for 1 h at room temperature. The glass plate modified with aminopropyl groups was further incubated with the gold colloid, washed several times with water, and finally dried at 65 °C. The pink-colored glass plate was incubated with various compositions of DTUA-Pepsta(h) and 2-hydroxyethyl disulfide mixture (total concentration, 1 mM; solvent, chloroform) for 24 h at room temperature and washed with chloroform, methanol, and water several times (Scheme 2).

A solution of pepsin was incubated with the fragment-modified glass plate in a quartz cell, and the absorbance change around 550 nm was observed using a spectrophotometer (Lambda 19 UV/VIS/NIR spectrometer, Perkin-Elmer).

Electrochemical Measurements. CV measurements were performed with a potentiostat (HA-301, Hokuto-Denko, Tokyo, Japan) and function generator (HA-104, Hokuto-Denko). Outputs of the potentiostat were converted by an A/D converter and collected by a microcomputer (PC-486 SE, Epson, Suwa, Japan). Data analyses were performed with an in-house program. Gold and Pt electrodes and a KCl-saturated calomel electrode were used as working, counter, and reference electrodes, respectively. An electrochemical cell was thermostated at 25 °C by a circulating water bath (RM6, Lauda, Postfach, Germany).

The gold electrode (area, 0.023 cm^2) was incubated with various compositions of DTUA-Pepsta(h) and 2-hydroxyethyl disulfide mixture (total concentration, 1 mM; solvent, chloroform) for 24 h at room temperature and rinsed with chloroform, methanol, and Milli-Q grade water several times before the voltammetric measurement. The electroactive probe used was 1 mM hydroquinone in a 0.1 M Na_2SO_4−10 mM phosphate buffer (pH 7.0).[40,41] The scan rate was 10 mV/s.

The electrochemical reductive desorption of the SAM from the electrode was performed in a 0.5 M KOH solution by scanning from 0 to −1.2 V at a scan rate of 100 mV/s. The corresponding process is represented by the following equation:

$$R{-}S{-}Au + e^- = Au + R{-}S^- \quad (1)$$

Contact Angle Measurements. A method for contact angle measurements by sessile drop method was described in Supporting Information.

RESULTS AND DISCUSSION

Inhibitory Effect of Pepstatin Fragment. At first, an inhibitory effect of the pepstatin fragment on the catalysis by pepsin was examined. The addition of pepstatin fragment inhibited the pepsin-catalyzed hydrolysis of peroxidase, and consequently, the rate of absorbance change at 492 nm corresponding to the product of peroxidase-catalyzed reaction, quinoneimine dye, increased (indicated in Supporting Information).

Table 1 shows the concentration of 50% inhibition (IC_{50}) and inhibition constant (K_i) for two kinds of pepstatin fragments. The K_i value for Boc-Pepsta(h) was in good agreement with the literature value, showing the validity of experimental procedures adopted in this work. In addition, the side chain of the C-terminus represents a very effective part of the inhibitors, as described by Kratzel et al.[55,59] In general, it was said that the hydrophobic

(55) Kratzel, M.; Hiessbock, R.; Bernkop-Sohnürch, A. *J. Med. Chem.* **1998**, *41*, 2339−2344.
(56) Kratzel, M.; Schlichtner, B.; Kirchmayer, R.; Bernkop-Sohnürch, A. *J. Med. Chem.* **1999**, *42*, 2041−2045.
(57) Anwer, K. M.; Spatola, F. A. *Synthesis* **1980**, *11*, 929−932.
(58) Bernkop-Schnürch, A.; Dundalek, K. *Int. J. Pharm.* **1996**, *138*, 75−83.
(59) Kratzel, M.; Bernkop-Sohnürch, A. *Peptides* **2000**, *21*, 289−293.

Table 1. IC_{50} and K_i Values of Pepstatin Fragments for Pepsin

inhibitor	IC_{50} (μM)	K_i (μM)
Boc-Pepsta(h)	2.6 ± 0.3	0.4 ± 0.3[a]
Boc-Pepsta(e)	3.8 ± 0.5	1.7 ± 0.5

[a] Literature value, $(7.6 \pm 3.6) \times 10^{-7}$ M.[56]

Table 2. Γ Values for Various SAMs

disulfide	Γ (nm^2)
DTUA-Pepsta(h)	0.90
DTUA-Pepsta(h):2-HEDS = 9:1	0.82
2-HEDS	0.56 (0.59)[a]
DTUA	1.50

[a] Reference 66.

environment surrounding the statine unit does a potent role for inhibitors of aspartyl proteases, which was consistent with the results obtained in this work.

Cyclic Voltammetry of DTUA-Pepsta(h). Next, a characterization of SAM formed on a gold electrode was carried out using cyclic voltammetry with hydroquinone (HQ) as a probe. The oxidation of HQ gives *p*-benzoquinone via a two-step mechanism shown below, and HQ is used in electrochemical analysis in the vicinity of the electrode surface.

$$HQH_2 \leftrightarrow HQ^{2-} + 2H^+ \quad (2)$$

$$HQ^{2-} \leftrightarrow HQ + 2e^- \quad (3)$$

The deceleration of the diffusion at solution−electrode interfaces due to the adsorption of substrate brings about inhibition of the electron transfer on a working electrode. The potential difference (ΔE_p) between the oxidation (E_{pa}) and reduction peaks (E_{pc}) of the bare electrode was 98 mV. After incubation of the electrode with DTUA-Pepsta(h) and DTUA-Pepsta(e), the ΔE_p value between E_{pa} and E_{pc} values increased to 524 mV and 417 mV, respectively (Figure 1). Correspondingly, the anodic peak

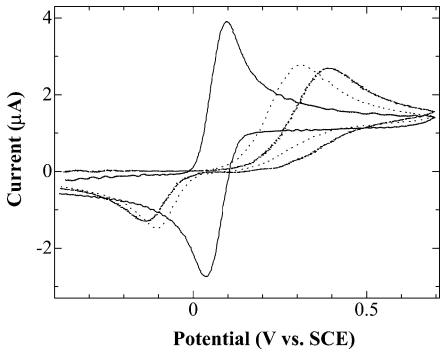


Figure 1. Cyclic voltammograms of various SAM-modified Au electrodes: bare Au (solid line), DTUA-Pepsta(h) (dashed line), and DTUA-Pepsta(e) (dotted line). Scan rate, 10 m/s. [HQ] = 1 mM, in a 0.1 M Na_2SO_4−10 mM phosphate buffer (pH 7.0) solution at 25 °C.

current (I_a) decreased from 3.97 to 2.58 and 2.78 μA, respectively. A quite similar tendency was observed in the decrease in cathodic peak current ($-\Delta I_c$), and therefore, we will discuss the $-\Delta I_a$ and ΔE_p values hereafter. The CV measurements at different scan rates showed that the anodic peak current was proportional to the square root of the scan rate (data not shown). Therefore, both the decrease in anodic peak current ($-\Delta I_a$) and the increase in ΔE_p value indicated that the SAM formed on the electrode surface decelerated the diffusion of the probe. Similarly, the ΔE_p values of the electrode incubated with a mixture of DTUA-Pepsta(h) and 2-HEDS (9:1) and that only with 2-HEDS were 411 and 347 mV, respectively. The resistance of the mixed SAM for HQ to give an electrode reaction was slightly larger than that of pure 2-HEDS SAM.

The gold electrode, which had been incubated with a solution of DTUA-Pepsta(h) beforehand, showed a peak at ~ −0.9 V by scanning from 0 to −1.2 V in a 0.5 M KOH solution (see Supporting Information Figure S-2). This peak has been attributed to the reductive desorption of thiolated compounds, which are chemisorbed to Au (eq 1),[60,61] which supports the fact that DTUA-Pepsta(h) was sorbed onto the gold electrode with the cleavage of the S−S bond and following Au−S bond formation. Using the peak area of reductive desorption curve, the molecular occupation area (abbreviated as "Γ" hereafter) for various SAMs formed on the electrode surface was determined (Table 2).

The table shows that the addition of 2-HEDS reduced the Γ value for the SAM (DTUA-Pepsta(h):2-HEDS = 9:1) in comparison with that for the pure DTUA-Pepsta(h) SAM, indicating that, by the coexistence of a disulfide of shorter molecular length, the density of SAM on the electrode surface was increased. The previous studies on alkanethiolates chemisorbed to Au (111) surfaces showed that the symmetry of sulfur atoms is hexagonal with an S−S spacing of 0.50 nm, and the calculated area per molecule is 0.20 nm^2,[62−64] which is much smaller than the Γ value for DTUA-Pepsta(h)/2-HEDS mixed SAM (9:1) in the table (0.82 nm^2).

When 2-HEDS was incubated solely with the gold electrode, the packing density of the SAM was the largest, probably due to a smaller volume of 2-HEDS moiety than that of DTUA-Pepsta(h) moiety. For the same reason, the Γ value for DTUA-Pepsta(h) SAM was larger than those for 2-HEDS SAM and DTUA-Pepsta(h)/2-HEDS mixed SAM.

Effect of Pepsin on the Cyclic Voltamograms. Upon immersion in a pepsin solution, the voltamogram of the electrode modified with DTUA-Pepsta SAM changed somewhat (Figure 2A,B). Compared with quartz crystal microbalance measurements, which have been used for the observation of various interfacial reactions,[65] it was previously shown that both the initial rate of decrease in anodic peak current ($d\Delta I_a/dt$) and that of increase in ΔE_p ($d\Delta E_p/dt$) were linearly correlated with the initial rate of increase in weight of the materials attached to the surface of

(60) Walczak, M. M.; Popenone, D. D.; Deinhammer, R. S.; Lamp, B. D.; Chung, C.; Porter, M. D. *Langmuir* **1991**, *7*, 2687−2693.
(61) Rojas, M. T.; Königer, R.; Stoddart, J. F.; Kaifer, A. E. *J. Am. Chem. Soc.* **1995**, *117*, 336−343.
(62) Strong, L.; Whitesides, G. M. *Langmuir* **1988**, *4*, 546−558.
(63) Lee, K. A. B. *Langmuir* **1990**, *6*, 709−712.
(64) Dubois, L. H.; Zegarski, B. R.; Nuzzo, R. G. *J. Chem. Phys.* **1993**, *98*, 678−688.
(65) Ebara, Y.; Okahata, Y. *J. Am. Chem. Soc.* **1994**, *116*, 11209−11212.

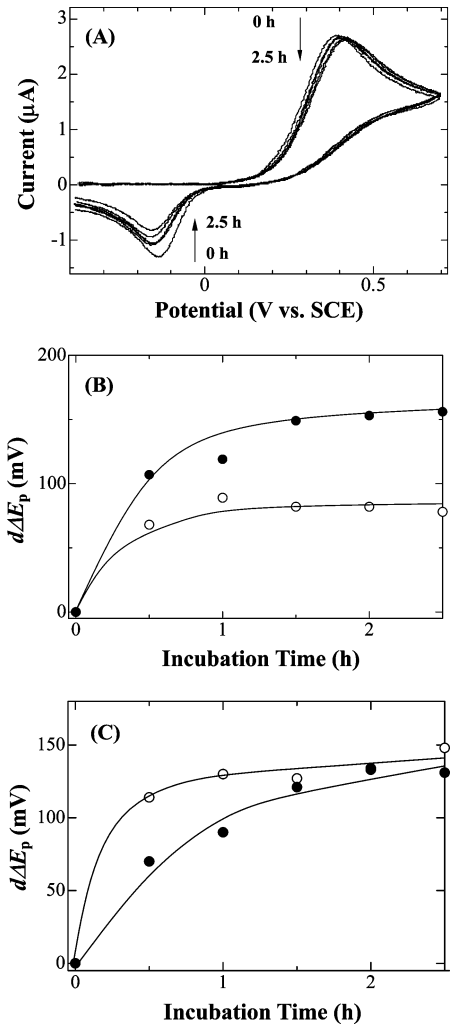


Figure 2. (A) Cyclic voltammograms of DTUA-Pepsta(h)-modified electrode after onset of the incubation with the pepsin solution (1 μM). (B) Effect of the concentration of pepsin on the potential difference of DTUA-Pepsta(h)-modified Au electrode. ○, [pepsin] = 1 μM. ●, 2 μM pepsin was dissolved in 0.05 N HCl. Scan rate, 10 mV/s. (C) Time dependences of the value of the increase in potential difference of DTUA-Pepsta(h)/2-HEDS (9:1)-modified electrode. [pepsin] = 1 μM, [pepstatin A] = 15 μM dissolved in 0.05 N HCl/methanol (3:1). ○, pepsin only. ●, pepsin + pepstatin A.

SAM.[66] Therefore, both the $d\Delta I_a/dt$ and $d\Delta E_p/dt$ values can be used as an index of the binding process at the solution−electrode interfaces. The figure indicated that the binding of pepsin to the SAM decelerated the diffusion of the probe at the electrode surface.

Aspartyl proteases have a specific affinity for pepstatin.[52] Therefore, the increase in ΔI_a and $d\Delta E_p$ values upon the incubation with pepsin could be attributed to the binding of enzyme to the pepstatin analogous groups in the SAM and concomitant increase in the average thickness of the adsorbed substance, which decelerated the diffusion of the probe, and consequently, increased the ΔI_a and $d\Delta E_p$ values (Scheme 3). The inhibitory effect of pepstatin A on the initial increase in ΔE_p value for the DTUA-Pepsta(h)/2-HEDS (9:1)-modified electrode (Figure 2C) suggested that the binding of pepsin to Pepsta(h) moieties was

(66) Kitano, H.; Saito, T.; Kanayama, N. *J. Colloid Interface Sci.* **2002**, *250*, 134−141.

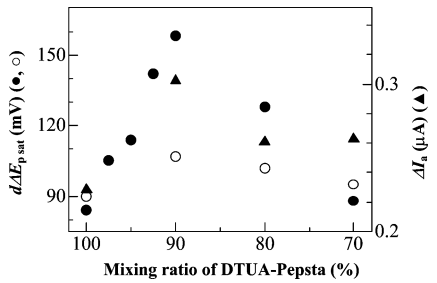
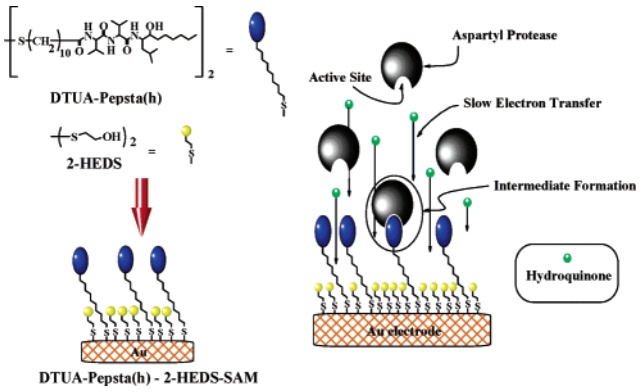


Figure 3. Effect of the mixing ratio of DTUA-Pepsta and 2-HEDS (total concentration, 1 mM) on the saturated values of the change in potential difference ($d\Delta E_{p\ sat}$) after incubation with pepsin (1 μM) dissolved in 0.05 N HCl solution for 2.5 h. DTUA-Pepsta: ●, *n*-hexyl. ○, ethyl; ▲, absolute value of the decrease in anodic peak current (ΔI_a) of DTUA-Pepsta(h)/2-HEDS mixed SAM-modified Au electrode.

Scheme 3. Susceptible Structure of the Mixed SAM of DTUA-Pepsta(h) and 2-HEDS on the Au Surface



specific. It should be mentioned here that, by the UV−visible absorption measurements, it was confirmed that 1 mM HQ did not affect the catalytic activity of pepsin in the hydrolysis of HRP.

Varying the mixing ratio of DTUA-Pepsta and 2-HEDS fed at the construction of SAM, the changes in ΔE_p and ΔI_a values 2.5 h after the incubation with pepsin solution were followed (Figure 3). The increase in potential difference ($d\Delta E_p$) and the absolute value of the decrease in anodic peak current (ΔI_a) were the largest at the ratio of DTUA-Pepsta(h):2-HEDS = 9:1, which might reflect the optimum conditions both in the distance between the fragment moieties and the flexibility of undecanoyl Pepsta(h) chain within the SAM. Below 80% in the mixing ratio of DTUA-Pepsta(h), the surface density of Pepsta(h) groups in the SAM might be insufficient, resulting in the decrease in both ΔI_a and $d\Delta E_p$ values. The same tendency was observed for DTUA-Pepsta(e) SAM. However, the $d\Delta E_p$ values for DTUA-Pepsta(h) and DTUA-Pepsta-(e) were different from each other, especially at the mixing ratio of 9:1, probably due to the difference in the amount of pepsin adsorbed to the exterior of SAM. As previously mentioned, the hydrophobic environment surrounding the statine unit has a potent role for the binding stability between pepstatin and aspartyl proteases.

Comparing the spectrum obtained by using IR-reflection absorption spectroscopy for the pure DTUA-Pepsta(h) SAM with that for the mixed SAM of DTUA-Pepsta(h) and 2-HEDS (9:1) (data not shown), it could be estimated that the occupation of DTUA-Pepsta(h) in the mixed SAM was 33%. This result indicated that it is relatively much easier for 2-HEDS to form a Au−S bond

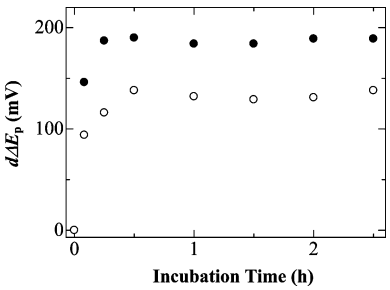


Figure 4. Time dependence of the change in potential difference of the DTUA-Pepsta/2-HEDS (9:1)-modified electrode after the incubation with HIV-1 protease solution (1 μM). ●, DTUA-Pepsta(h). ○, DTUA-Pepsta(e). [HQ] = 1 mM, in a 0.1 M Na_2SO_4−10 mM phosphate buffer (pH 7.0) solution at 25 °C. HIV-1 protease was dissolved in 0.05 N HCl. Scan rate, 10 mV/s.

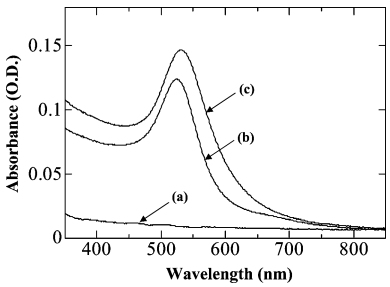


Figure 5. Absorption spectra of amino group-modified glass plates. (a) Amino group-modified glass plate. (b) After incubation with gold colloids. (c) After incubation with DTUA-Pepsta(h).

(in other words, SAM) than for DTUA-Pepsta(h). After incubation of the mixed SAM with pepsin solution, a large absorption increase around 1600−1700 cm^{-1}, which was ascribable to amide bonds in polypeptides, was clearly observed, supporting the fixation of pepsin to the SAM.

The contact angle of a water droplet for the SAM of various mixing ratios showed that wettability of the SAM was increased with a decrease in the mixing ratio of DTUA-Pepsta(h) (Supporting Information Table S-1). However, one should note that, since one drop of water has a much larger size than those of the disulfide molecules examined, the contact angle does not sensitively reflect the hydrophilicity of disulfides, but simply reflects the *average* wettability of the monolayer.

Furthermore, when the same CV experiments as Figure 3 were carried out for comparison using trypsin, which has no affinity for pepstatin,[67,68] only a very small increase in ΔE_P (50 mV in 2.5 h at DTUA-Pepsta(h):2-HEDS = 9:1) due to a nonspecific adsorption of trypsin on the SAM was observed. Various molecules including enzymes often adsorb to a hydrophobic interface (such as a bare Au surface, hydrocarbon surface, etc,) irreversibly (thermodynamically stable). However, each $d\Delta E_P$ value for DTUA-Pepsta(h and e)/2-HEDS mixed (9:1) SAM-modified electrode incubated in pepsin solution was larger than that of pure DTUA-Pepsta(h and e) SAM-modified electrode despite the smaller hydrophobicity of DTUA-Pepsta/2-HEDS mixed (9:1) SAM-modified surface than that of DTUA-Pepsta SAM-modified surface (by contact angle measurements, Supporting Information Table S-1). These results suggested that the binding of pepsin to the electrode could be attributed to the specific binding of the enzyme to the pepstatin fragment at the exterior of the SAM. This point will be discussed further in the following section using SPR absorption spectroscopy.

To examine whether other kinds of aspartyl proteases could be accumulated on the surface of SAM, HIV-1 protease was incubated with the SAM-modified electrode. Previously, a strong binding of pepstatin A to HIV protease was reported by Sarubbi et al.[69] As shown in Figure 4, the $d\Delta E_p$ value increased in a manner similar to pepsin. But the saturation occurred at much shorter incubation time than in the case of pepsin, probably due to the difference in molecular size of these enzymes (M_w of pepsin and HIV-1 protease, 34 and 11 kDa, respectively). These results definitely show that the Pepsta(h) group-carrying SAM on the gold electrode could be recognized and bound by aspartyl proteases such as pepsin and HIV-1 protease. Using the pepstatin fragment SAM system, therefore, it will be possible to effectively remove HIV-1 protease from biological fluids.

Localized Surface Plasmon Resonance Absorption Spectroscopy. When a dispersion of gold colloid was incubated with amino group-modified glass plate, the glass plate changed to pink (Figure 5b), showing that the surface of the glass was covered with the gold colloid. The gold colloid was so strongly bound to the glass plate that the colloid could not be desorbed even by an incubation with 3 M NaCl solution.[70] When a mixed solution of DTUA-Pepsta(h) and 2-HEDS (solvent, chloroform) was incubated with the gold colloid-modified glass plate, the absorption of the glass plate further changed, indicating that the SAM of the fragment was constructed on the gold colloid (Figure 5c). When a pepsin solution was incubated with the SAM-modified glass plate, moreover, the absorption around 550 nm further increased and the position of the absorbance peak (λ_{max}) made a red shift (Figure 6).

Figure 6 shows the gradual increase in absorbance of the glass chip at 550 nm upon immersion in pepsin solution. By the incubation of pepstatin A with the glass plate, the absorbance gradually decreased indicating the desorption of pepsin. From the relaxation times for the increase and decrease in absorbance, the binding and desorption rate constants (k_{assoc} and k_{diss}) were evaluated using equations shown below.[71]

$$\Delta Abs_{assoc} = Abs_t - Abs_o = \frac{[Pepsin]k_{assoc}\Delta Abs_{max}\{1 - \exp[-([Pepsin]k_{assoc} + k_{diss})t]\}}{[Pepsin]\,k_{assoc} + k_{diss}} \quad (4)$$

$$\Delta Abs_{diss} = Abs_t - Abs_{t\to\infty} = (Abs_o - Abs_{t\to\infty})\exp(-k_{diss}\,t) + Abs_{t\to\infty} \quad (5)$$

where Abs_o, Abs_t, and $Abs_{t\to\infty}$ are the absorbance at time 0, t, and infinity, respectively.

(67) Kunimoto, S.; Aoyagi, T.; Morishima, H.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1972**, *25*, 251−255.
(68) McConnell, R. M.; Frizzell, D.; Camp, A.; Evans, A.; Jones, W.; Cagle, C. *J. Med. Chem.* **1991**, *34*, 2298−2300.
(69) Sarubbi, E.; Seneci, P. F.; Angelastro, M. R.; Peet, N. P.; Denaro, M.; Islam, K. *FEBS Lett.* **1993**, *319*, 253−256.
(70) Morokoshi, S.; Ohhori, K.; Mizukami, K.; Kitano, H. *Langmuir* **2004**, *20*, 8897−8902.
(71) Lookene, A.; Chevreuile, O.; Østergaard, P.; Olivecrona, G. *Biochemistry* **1996**, *35*, 12155−12163.

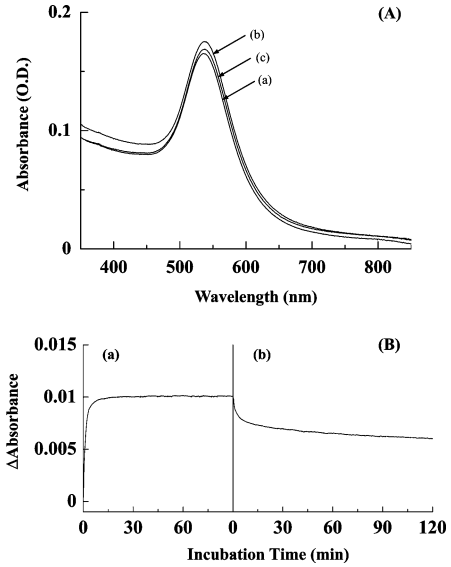


Figure 6. (A) Absorbance spectra of SAM-modified gold colloid on glass plate. (a) DTUA-Pepsta(h)-modified gold colloids. (b) after Incubation with a solution of pepsin. (c) After incubation with a solution of pepstatin A. (B) Time dependence of absorbance of gold colloids on a glass plate at 550 nm. (a) Incubation of DTUA-Pepsta(h)-modified gold colloids on a glass plate with pepsin. (b) After incubation with pepstatin A. [pepsin] = 1 μM dissolved in a 0.05 N HCl, [pepstatin A] = 3 μM in 0.05 N HCl and methanol (3:1).

The association constants ($K_{assoc} = k_{assoc}/k_{diss}$) for pepsin with the pepstatin fragment above the SAM of various compositions (Table 3) were comparable with the value for the free fragment pepstatin−pepsin system and had a maximum at the mixing ratio of 9:1 (DTUA-Pepsta(h):2-HEDS) at the construction of SAM, which was quite similar to the ΔE_p value at the CV measurements (Figure 3).

As indicated in Figure 6, the immersion of the sensor chip, which had been in contact with the pepsin solution beforehand, in the pepstatin A solution decreased the absorbance. In the case of bare chips, on the contrary, no absorbance decrease was noticeably observed upon the same treatment (data not shown). At first we considered that the incomplete desorption in Figure 6B would be attributable to the physical adsorption of enzyme to the alkyl end group of the SAM. To clarify this point, we evaluated the inadequate desorption using conventional surface plasmon resonance absorption spectroscopy (SPR-AS, continuous flow system; see Supporting Information). The complete desorption of adsorbed pepsin (Figure S-3) seemed to support a specific binding of pepsin on the SAM-modified surface.[42] We consider that HIV-1 protease, another kind of aspartyl protease, also has a similar tendency. Because of both high affinity of the pepstatin fragment for aspartyl protease and the nonflow assay system of CV and LSPR-AS, the complete desorption was not observed by these methods.

Table 3. K_{assoc} Values of Pepsin with Various Mixed SAMs

DTUA-Pepsta(h) (%)[a]	K_{assoc} ($\times 10^6$ M^{-1})[b]	R^2 [c]
100	2.8	0.90
90	5.8	0.94
80	4.7	0.94
70	2.8	0.94

[a] Mol % at the immersion with a gold colloid-modified cover glass. [b] $1/K_i$ value for Boc-Pepsta(h) fragment, 2.5×10^6 M^{-1}. [c] Coefficient of determination.

The addition of trypsin did not induce such absorption changes, whereas the incubation of HIV-1 protease induced the similar absorption increase and decrease due to the specific binding giving the K_{assoc} value of 1.8×10^5 M^{-1}.

Finally, the detectability of the sensor chip modified with DTUA-Pepsta(h)/2-HEDS (9:1) SAM was estimated using various concentrations of pepsin. Figure 7 shows that the maximum amount of pepsin bound to the surface in the steady state is directly related to the solution concentration, and an absorbance change at 550 nm could only be observed when the solution concentration was above 8 nM.

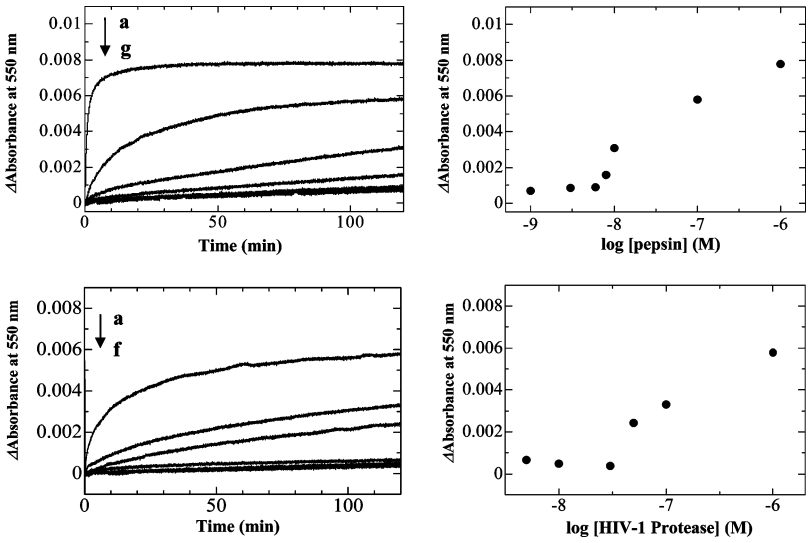


Figure 7. Time dependence of absorbance of DTUA-Pepsta(h)/2-HEDS (9:1) SAM-modified sensor chip at 550 nm after immersion in aspartyl protease solution, and absorbance change in 120 min after the immersion as a function of the protease concentration. Upper (a−g) [pepsin] = 1.0×10^{-6}, 1.0×10^{-7}, 1.0×10^{-8}, 8.0×10^{-9}, 6.0×10^{-9}, 3.0×10^{-9}, and 1.0×10^{-9} M. Lower, (a−f) [HIV-1 protease] = 1.0×10^{-6}, 1.0×10^{-7}, 5.0×10^{-8}, 3.0×10^{-8}, 1.0×10^{-8}, and 5.0×10^{-9} M.

In the case of HIV-1 protease, the detection limit was 50 nM, probably due to the much larger K_i value of pepstatin A for the HIV-1 protease-catalyzed reaction (5−35 nM)[72] than that for the pepsin-catalyzed one (0.046 nM) in the solution phase.[68] The very small detection limit of pepsin using the DTUA-Pepsta(h) SAM-carrying sensor chip indicates that this sensor chip has a very high relative sensitivity.

There have been some reports concerning biological reactions at the surface of SAM.[66,73,74] However, only a few kinetic studies have yet been made.[42] The results obtained in this work will be highly useful to develop electrochemical and spectrophotometric biosensors to detect enzymes in solution using a rate assay.

CONCLUSION

For the construction of a sensing device for aspartyl proteases, the pepstatin fragment-carrying SAM could easily be prepared on the metal surfaces, and the density of fragment in the SAM was adjusted by coexistence of a disulfide of short chain length. The cyclic voltammetry and localized surface plasmon resonance absorption spectroscopy showed that the fragment-carrying SAM could specifically be bound by pepsin to form a very stable complex. The binding rate of pepsin to the fragment moiety changed with the surface density of the fragment moiety in the SAM. HIV-1 protease, another kind of aspartyl protease, strongly bound to the fragment-modified electrode, too. The present results will be quite useful to further enlarge the usability of SAMs carrying enzyme inhibitor moieties as molecular sensing devices for enzymes.

(72) Richards, A. D.; Roberts, R.; Dunn, B. M.; Graves, M. C.; Kay, J. *FEBS Lett.* **1989**, *247*, 113−117.
(73) Yoshida, T.; Sato, M.; Ozawa, T.; Umezawa, Y. *Anal. Chem.* **2000**, *72*, 6−11.
(74) Ratner, D. M.; Adams, E. W.; Su, J.; O'Keefe, B. R.; Mrksich, M.; Seeberger, P. H. *Chembiochem* **2004**, *5*, 379−383.

ACKNOWLEDGMENT

This work was supported by the Grant-in-Aid for Scientific Research (16205015) from the Japan Society for the Promotion of Science. Presented at the 52nd Annual Meeting of the Society of Polymer Science, Japan, in Nagoya in May 2003.

SUPPORTING INFORMATION AVAILABLE

Syntheses of pepstatin fragment−DTUA conjugates (DTUA-Pepsta(h) and DTUA-Pepsta(e)). Data and additional descriptions for inhibitory effect of pepstatin fragment, voltammograms of the reductive desorption of various SAMs, contact angle measurement, and surface plasmon resonance absorption spectroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 4, 2004. Accepted December 16, 2004.

AC0488558