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Electrochemical assay of plasmin activity and its kinetic analysis

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ABSTRACT

A sensitive and convenient electrochemical assay of plasmin activity and its kinetic analysis are described. Thus, a ferrocenyl peptide substrate (FcPS) having a plasmin-specific substrate sequence, Lys-Thr-Phe-Lys, and a Cys residue was prepared and immobilized on a gold electrode through the sulfur–gold linkage. The obtained electrode showed a redox signal based on the ferrocene moiety, suggesting the immobilization of FcPS on the electrode. After treatment of this electrode with plasmin, its electrochemical signal was decreased in proportion to an increase of the amount of plasmin. The detection limit for plasmin in this assay system was as low as 50 ng/ml or 0.15 mU/ml. Real-time monitoring of plasmin reaction on the electrode could also be achieved, and the kinetic parameters of this enzymatic reaction could be determined; for example, the k_{cat}/K_m value was 0.063 μM^{-1} s^{-1}. Furthermore, a quantitative assay for streptokinase as a plasminogen activator was also demonstrated by using this system.

Plasmin (EC 3.4.21.7) is a trypsin-like serine protease generated through a limited proteolytic cleavage of the zymogen plasminogen by the action of plasminogen activators [1–3]. This protease is responsible for the degradation of fibrin clots into soluble products and, thus, plays a critical role in blood hemostasis and fibrinolysis. Plasmin, together with the plasminogen activators and their inhibitors, also plays an important role in other many biological events such as tissue proliferation, cellular adhesion, pathogen invasion, cancer invasion, and metastasis [3–5] and has been suggested to be involved in coronary artery disease [6] and type 2 diabetes mellitus [7]. Considering the biological importance of plasmin and related proteases and inhibitors and their therapeutic uses [8], it is essential to develop a sensitive and simple detection system of plasmin activity. For the measurement of plasmin activity, a spectroscopic method using chromogenic or fluorogenic peptide substrates for plasmin has been most commonly employed [1,7,9–13]. However, these substrates are not readily applicable to protease assays in complex colored or turbid media such as blood and are moderately sensitive [14]. Recently, an electrochemical detection technology of biomolecules such as DNA [15,16] and glucose in blood [17] has attracted much attention because it enables direct electronic readout and miniaturization with good cost performance and potentially high sensitivity. In fact, Meyerhoff and coworkers succeeded in the electrochemical detection of plasmin at levels approaching 2 mU/ml by using a complex of protamine and pentosan polysulfate and a polyanion-sensitive membrane electrode [18].

In this study, we intended to develop an electrochemical plasmin assay method as a rapid, simple, and more sensitive one with an inexpensive and compact instrument by using an electrochemically active ferrocenyl peptide substrate (FcPS)[1] for plasmin immobilized on a gold electrode. The ferrocene (Fc) was adopted as a suitable electrochemically active reporter for the assay because Fc peptide-immobilized electrodes have been successfully used for an electrochemical analysis of proteases [19–21] and a tetrapeptide sequence, Lys-Thr-Phe-Lys, was selected as an optimal peptide substrate specific for plasmin [22]. The concept of our electrochemical plasmin assay method is illustrated in Fig. 1. Thus, the FcPS (Fig. 1A) with a 6-metric Gly spacer is immobilized on a gold electrode through the chemical adsorption of the thiol group of the terminal Cys residue (Fig. 1B) [21,23,24]. When the electrode is treated with plasmin, a peptide fragment containing Fc moiety would be cleaved and removed from the electrode (Fig. 1C and D). This should cause a decreased electrochemical response depending on the amount of plasmin (Fig. 1E).

In this article, we report the successful construction of a sensitive electrochemical assay system for plasmin activity and its kinetic analysis. We also demonstrate the feasibility of this detection system for a quantitative assay of streptokinase [25,26] as a plasmin activator in the presence of plasminogen.

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[1] *Abbreviations used:* FcPS, ferrocenyl peptide substrate; Fc, ferrocene; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; UV–vis, ultraviolet–visible; TFA, trifluoroacetic acid; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; DPV, differential pulse voltammetry; CV, cyclic voltammetry; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

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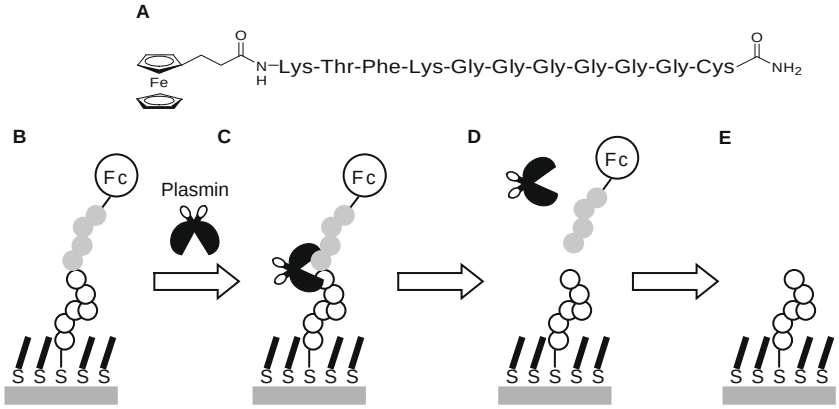

Fig. 1. Concept of electrochemical plasmin assay. (A) Structure of ferrocene-labeled plasmin substrate peptide (FcPS). (B) Electrode immobilized with FcPS. (C) Digestion of FcPS on the electrode by plasmin. (D) Release of the short peptide fragment carrying Fc moiety from the electrode by the plasmin digestion. (E) Gold electrode after washing with Milli-Q water. S, circle, Fc, and solid bar refer to sulfur atom, amino acid, ferrocene, and mercaptohexanol as a masking reagent, respectively.

Materials and methods

Materials

Reagents and solvents for peptide synthesis, 9-fluorenylmethoxycarbonyl (Fmoc) amino acids, Fmoc–NH–SAL resin, 1-hydroxybenzotriazole, 2-(1*H*-benzotriazole-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate, piperidine, *N*-methylpyrrolidone, dichloromethane, and *N,N*-dimethylformamide, were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Boc-Glu-Lys-Lys 4-methylcoumaryl-7-amide was obtained from Peptide Institute Inc. (Osaka, Japan). Ferrocenyl propanoic acid was prepared as described previously [27]. Plasmin (human), plasminogen (human), and streptokinase were purchased from Sigma–Aldrich (St. Louis, MO, USA). A gold electrode was obtained from Bioanalytical Systems (BAS, Tokyo, Japan).

General

The high-performance liquid chromatography (HPLC) system used in this experiment was composed of the following components: Hitachi L-4200 ultraviolet–visible (UV–vis) detector, L-6200 intelligent or L-6000 pump, and D-2600 chromato-integrator (Tokyo, Japan). Reversed phase HPLC was run using an Inertsil ODS-3 column (4.6 × 250 mm, GL Science, Tokyo, Japan) with the gradient conditions where acetonitrile content in 0.1% trifluoroacetic acid (TFA) was linearly changed from 3.5% to 45.5% over 60 min at a flow rate of 1.0 ml/min with detection at 210 nm. Synthesized peptide derivatives were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS, Voyager Linear-SA, PerSeptive Biosystems, Foster City, CA, USA) measurement of the products separated by HPLC. They were dissolved in a solution of saturated α-cyano-4-hydroxycinnamic acid in 0.1% TFA/acetonitrile (1:1) and dried. Mass spectra were measured in the positive mode. Fluorometric measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer.

Synthesis of plasmin-specific FcPS

FcPS was assembled by the Fmoc solid phase method [28] on an Applied Biosystems model 433A peptide synthesizer (Foster City, CA, USA) using the FastMoc protocol as described previously [29,30]. Ferrocenyl propanoic acid was coupled to the N terminus of the peptide resin for the incorporation of the Fc group. The peptide resin (500 mg) obtained was suspended in a mixture of TFA (9.45 ml), triisopropylsilane (100 μl), ethanedithiol (250 μl), and water (250 μl) and was stirred for 2 h at room temperature. After removing the resin by filtration, the crude product was obtained as light yellow powder from the filtrate by the addition of ether (200 ml). The crude peptide was purified by the Hitachi HPLC system to provide the desired peptide, Fc-Lys-Thr-Phe-Lys-Gly-Gly-Gly-Gly-Gly-Gly-Cys-NH_2 (FcPS). Its structure was identified by MALDI–TOF MS: found *m*/*z* 1207.47 $[M+H]^+$, calcd 1208.16 $[M+H]^+$ for $C_{53}H_{79}FeN_{14}O_{13}S$.

Preparation of FcPS-immobilized gold electrode

A gold electrode (2.0 mm^2 in area) was polished with 6 μm of diamond slurry, 1 μm of diamond slurry, and 0.05 μm of alumina slurry in that order and was washed with Milli-Q water. FcPS was immobilized onto the electrode by dipping with 2 μl of an aqueous solution containing FcPS at room temperature overnight. As a first trial, the aqueous solution containing 10, 50, 100, 250, 500, 750, or 1000 μM FcPS was used and an optimum concentration of the FcPS solution was settled as 250 μM. Subsequent experiments were performed under the settled conditions. After washing with Milli-Q water, 2 μl of 1 mM 6-mercapto-1-hexanol was placed on the electrode and kept for 1 h at 45 °C to mask the surface. Finally, the electrode was washed with 10 mM phosphate buffer (pH 7.4) containing 0.05% Tween 20. Prolonged stability of the FcPS-immobilized electrode was tested, and these electrodes were stable for at least 2 days (see Fig. S1 in Supplementary material).

Electrochemical measurement

Electrochemical measurement was made on an ALS model 630A electrochemical analyzer (CH Instruments, Austin, TX, USA). The cell was furnished with a three-electrode configuration consisting of an Ag/AgCl reference electrode, a Pt counter electrode, and an FcPS-immobilized electrode as a working electrode. The redox behavior of the FcPS-immobilized electrode was monitored by the differential pulse voltammetry (DPV) in 0.2 M $HClO_4$ and by the cyclic voltammetry (CV) in 50 mM Tris–HCl (pH 8.0). The conditions for DPV measurement were as follows: initial potential, 0 mV; increasing potential, 5 mV; pulse amplitude, 50 mV; pulse period, 0.05 s.

Reaction of plasmin on the FcPS-immobilized electrode and electrochemical measurement

Plasmin reaction was carried out by immersing the FcPS-immobilized electrode in 80 μl of a solution of 50 mM Tris–HCl (pH 8.0),