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Electrochemically monitoring the binding of concanavalin A and ovalbumin

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ABSTRACT

To evaluate protein–protein interactions, a new voltammetric method was developed using a protein labeled with an electroactive compound. Concanavalin A (ConA), which is a lectin, recognizes α-mannose residues. Because the ConA was to be bound to ovalbumin (OVA), which has a high-mannose sugar chain, ConA labeled with daunomycin was prepared as the probe to monitor the binding. The binding to OVA was caused by the label modification of the ConA. As a result, the electrode response of the labeled ConA decreased as the OVA concentration increased. The electrode response of the labeled ConA was linearly over the range of 1.5×10^{-10} and 1.5×10^{-9} M OVA. The relative standard deviation of 1.5×10^{-8} M labeled ConA and 1.5×10^{-10} M OVA was 6.9% ($n = 5$). The labeled ConA–OVA binding could then be conveniently monitored based on the change in response. In contrast, interactions between the labeled ConA and a protein with no specific sugar chain also were investigated. Incubation scarcely influenced the peak current of the labeled ConA. When several concentrations of OVA were added to a serum, good recovery determined it. Consequently, this method could be applied to the measurement of protein–protein interactions.

1. Introduction

In living organisms, a number of proteins have various interactions with DNA, carbohydrates, lipids and other proteins. As one of the interactions, DNA-bound protein plays the role of a transcription switcher [1,2]. Cellular adhesion and cellular recognition are caused by protein–carbohydrate binding [3,4], and the production of membrane protein is due to protein–lipid interactions [5]. Protein–protein interactions make up the core of the interactomics. These interactions contribute to the organization of these biomolecules [6,7]. In addition, protein complexes are relative to metabolism [8] and to signaling [9]. Therefore, an evaluation of the interactions has been carried out. When different proteins combine, the functions of the protein complex can be analyzed by monitoring the complex formation. A new function may be found by using multilateral measurements of the known functions of the proteins. To predict new protein functions, researchers must find the molecule that will bind to the protein.

To measure protein–protein interactions, Blagoev et al. reported a proteomics strategy that would elucidate functional interactions when applied to EGF signaling [10]. Green fluorescent protein has been used as a powerful probe for monitoring interactions in bacteria [11]. Spectrometry has been used to obtain information about the protein of three-dimensional structures, and identification of the interaction sites with the binding partners has been performed [12]. The mapping of protein interactions in solution has been attempted using NMR spectroscopy [13]. The structural analysis of G-protein-coupled receptors for hormones and neurotransmitters has been carried out using X-ray diffractometry [14].

Electrochemical procedures have been used to investigate the protein–protein interactions of sulfite oxidase and cytochrome *c* catalyzing the oxidation of sulfite for an Au electrode [15].

In the present study, the voltammetric measurement of protein–protein interactions was developed using a glassy carbon electrode. Ovalbumin (OVA), MW: 43,000, which is a glycoprotein from egg white, has a high-mannose sugar chain [16]. OVA is an allergen of the immediate reactivity, and it became the subject of discussion as an antigen causing a food allergy for infants. The food allergy is related to IgE-mediated food hypersensitivity from the binding between OVA and egg-specific antibodies [17]. Concanavalin A (ConA), MW: 26,000, is a simple protein from the jack bean that recognizes an α-mannose residue [18]. Because protein–protein interaction selectively occurs in solution, we decided that the measurement of OVA should be carried out using ConA instead of IgE. To monitor the binding between ConA and OVA, we introduced an electroactive compound as a label for ConA through a cross-linking agent (Fig. 1). When the labeled ConA recognized the OVA sugar chain, a labeled ConA–OVA complex was formed in the solution. To simplify the model of the complexation, the number of labels that combined with the dimer ConA comprised

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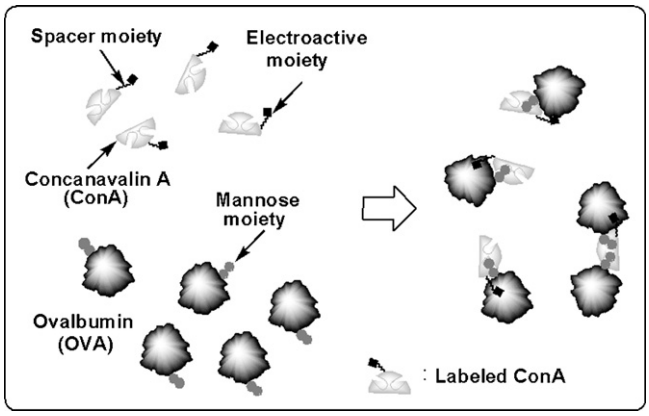

Fig. 1. Voltammetric evaluation of labeled ConA–OVA binding.

one molecule. Based on the interaction, the label bound to ConA was covered with OVA. As a result, the peak current of the labeled ConA was decreased due to the interaction. Accordingly, the interaction was evaluated by the monitoring of the peak currents. Therefore, this method could be applied to the detection of OVA.

We selected daunomycin that is one of the anticancer drugs as an electroactive label. In the previous study, the binding between avidin and biotin was evaluated using biotin labeled with the reagent [19]. As a result, the sensitive binding assay was achieved using accumulation voltammetry, due to the hydrophobic property of the daunomycin. The merits of the reagent were as follows. Because it is not present in natural products, the influence of contamination in measurements was low. The daunomycin, which has an amino group in its sugar, could react easily with a biotinylation reagent possessing a succinimidyl ester. Therefore, the labeled biotin was conveniently separated using only TLC. In addition, the peak potential and spectra of daunomycin was not changed by the labeling since the aromatic moiety was far from the sugar moiety. The labeling of ConA using the daunomycin was carried out because of these factors. It was expected that ConA combined with a cross-linking reagent containing a sulfosuccinimidyl ester.

Since this procedure does not require the separation of the free-labeled ConA from the OVA from that which was bound prior to the measurement, it becomes a convenient method for the evaluation of the interaction. Moreover, the effect of a cross-linking agent to introduce an electroactive compound to ConA was investigated. This was because the labeled ConA–OVA interaction was influenced by the length and type of the alkyl chain.

2. Experimental

2.1. Apparatus

A CV-50W analyzer (Bioanalytical Systems Inc. (BAS)) was used for the voltammetric measurements. A glassy carbon electrode (3 mm in diameter, Model No. 2012, BAS) was used as the working electrode. The electrode was polished using 1.0-, 0.3-, and 0.05-μm alumina (Baikowski International Corp., Charlotte, NC). A platinum wire was used as the counter electrode, and an Ag/AgCl (sat. NaCl, Model No. 11-2020, BAS) electrode served as the reference. All potentials were measured against the Ag/AgCl electrode. A mass spectrum in sinapic acid as a matrix was obtained using a Voyager DE-STR Workstation (Applied Biosystems).

2.2. Reagents

The ethylene glycolbis (sulfosuccinimidyl succinate) (Sulfo-EGS) and Bis[sulfosuccinimidyl] suberate (BS^3) were purchased from Thermo Fisher Scientific Inc. The OVA, ConA and fetal bovine serum were obtained from Sigma–Aldrich. The daunomycin was supplied from LKT Laboratories, Inc. A phosphate buffer (0.1 M) with KH_2PO_4 (0.1 M) and NaOH (0.1 M) was used for the reaction. The labeled ConA was prepared in a 0.1 M phosphate buffer (pH 8.5). The pH of the solution used for the electrochemical measurements was 5.6. Prior to use, the supporting electrolyte was deaerated using high-quality nitrogen. All reagents used were of analytical reagent grade.

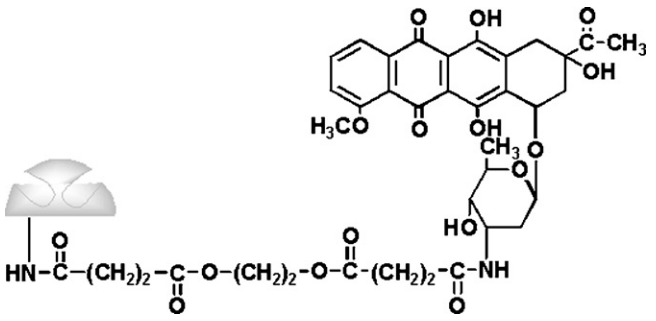

Fig. 2. Structure of ConA labeled with daunomycin.

2.3. Preparation of labeled ConA

To prepare ConA labeled with daunomycin, two-step reactions were used. First, ethylene glycolbis (sulfosuccinimidyl succinate) (10 mM), as a cross-linking agent, and daunomycin (10 mM) in 0.1 M phosphate buffer (pH 8.5) were mixed at 4 °C for 4 h. The product, for which daunomycin was bound at one edge of the cross-linking agent, was separated on a TLC alumina sheet coated with silica gel (Merck) and was developed at a ration of chloroform:methanol = 4:1 v/v%. When the reaction was performed at 25 °C, by-product for which daunomycin was bound at both edges of the cross-linking agent was observed. The temperature of the reaction was set at 4 °C because of the yield improvement.

The product was chipped away and extracted in ethanol and pure water. Next, the labeled ConA was obtained by using a column loaded with sephadex G-50 (GE Healthcare) after the product (0.4 mM) was reacted with ConA (0.01 mM) for 4 h in 0.1 M of phosphate buffer (pH 8.5). The separation of the labeled ConA was carried out with 0.1 M NaCl. To simplify the structure of the label, the daunomycin moiety of the ConA dimer was comprised of one molecule (Fig. 2). As described below, the voltammetric measurements were carried out in 0.1 M phosphate buffer (pH 5.6). At pH 5.0, Geoffrey et al. reported ConA dimer existed as a mixture of 2 types [20]. One group was homologous dimers of 2 intact monomer units and the other group was 2 fragmented monomer units. The species had 1, rather than 2, binding sites per molecule, and they showed a similar affinity for a ligand. There was a protein that was a noninteracting mixture of dimer and tetramer at pH 7.0. Under the measurement conditions, it was expected that the main species were a mixture of the ConA dimmer, such as the labeled ConA dimer combined with OVA. The molar absorption coefficient of the ConA dimer at 280 nm was about 31,000 in 0.1 M of phosphate buffer (pH 5.6). The concentration of labeled ConA was estimated from a molar absorption coefficient of daunomycin (11,000) at 490 nm and at a ratio of absorbance (280 nm/490 nm) = 0.48.

2.4. Procedure for the voltammetric measurements of labeled ConA

When OVA was added to a 0.1 M phosphate buffer (pH 5.6) with labeled ConA, the solution was stirred for 1 h. To measure an oxi-

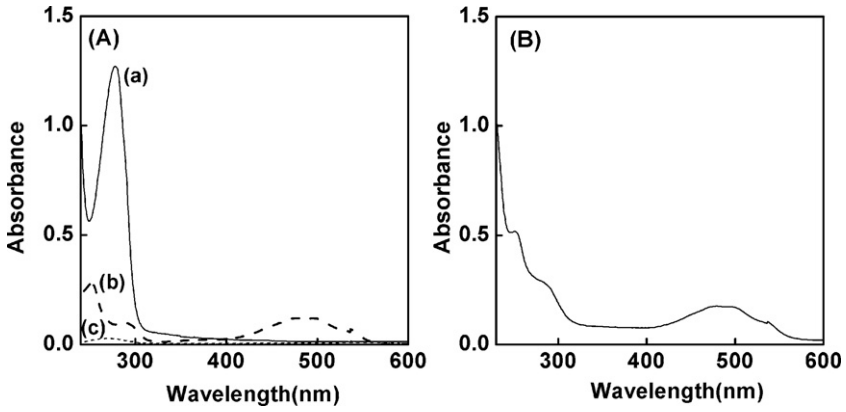

Fig. 3. Absorption spectra of ConA, daunomycin, Sulfo-EGS and labeled ConA. (A): (a) ConA, (b) daunomycin, (c) Sulfo-EGS, (B): labeled ConA. Measurements were carried out in 0.1 M phosphate buffer (pH 5.6) as dimer of ConA.

dation peak of the labeled ConA, a potential at −1.0 V was applied to the electrode for 5 min with stirring. The potential was scanned in the positive direction with differential pulse voltammetry (scan rate, 5 mV s^{-1}; pulse amplitude, 50 mV; sample width, 2 ms; pulse width, 50 ms; and, pulse period, 200 ms).

3. Results and discussion

3.1. Ratio of daunomycin moieties to ConA using spectrophotometry

To examine the ratio of daunomycin moieties to ConA, the absorbance spectra of ConA, daunomycin, EGS and labeled ConA were measured (Fig. 3). When the spectrum of the 1.0×10^{-5} M ConA (dimer) was recorded in 0.1 M of phosphate buffer (pH 5.6), the spectrum peak of ConA appeared at 280 nm. The peaks of 1.0×10^{-5} M daunomycin were observed at 250 and 490 nm. However, Sulfo-EGS as a cross-linking agent was scarcely absorbed in the same concentration. Based on the spectrum of the labeled ConA, the ConA concentration was estimated from molar absorption coefficients at 280 nm. The concentration of daunomycin moieties modified to ConA was also calculated using the molar absorption coefficients at 490 nm. As a result, the concentrations of ConA and daunomycin moieties were 1.3×10^{-6} M and 16.7×10^{-6} M, respectively. Because the ratio of the molar concentration was 12.8 for the dimer, the daunomycin moieties were bound to ca. 6.4 molecules per monomer of ConA.

3.2. Characterizations of ConA and labeled ConA using MALDI-TOF MS

To investigate whether daunomycin moieties were introduced to ConA through a cross-linking agent, the mass spectra of native ConA and labeled ConA containing native ConA were measured using MALDI-TOF MS. The signals of the monomer and the dimer produced from native ConA were m/z 25773.22 and m/z 51544.88, respectively (Fig. 4). For native ConA containing the labeled ConA, the peaks due to the monomer and the dimer were observed at m/z 25802.14 and m/z 51557.41. On the other hand, a new signal at m/z 30777.06 was expected from the labeled ConA. The difference of the mass number between ConA and labeled ConA was 4974.92

The value was divided by 754.7, and the mass number of the daunomycin moiety was added to that of the spacer in Sulfo-EGS. The reproducibilities of the labeling using spectrometry and mass spectrometry were calculated from quintuple experiments. As a result, there were 6.4 ± 0.3 daunomycin moieties per monomer of ConA obtained by spectrometry. The number of the label found using mass spectrometry was 6.6 ± 0.2. Accordingly, those values were nearly identical.

3.3. Voltammograms of daunomycin and labeled ConA

Fig. 5 shows the voltammograms of daunomycin and labeled ConA using a glassy carbon electrode in 0.1 M of phosphate buffer (pH 5.6). In a solution, two redox pairs were observed due to the daunomycin moiety [19]. The peaks at the more positive side depend on two hydroxyl groups, and the peaks at the other side are a redox from the quinone moiety of the molecule. When the spectrometric measurement of labeled ConA was performed, maximum absorbance of the daunomycin moiety was the same as that of only daunomycin. It was confirmed that the redox potential also did not shift from labeling. With this method, the oxidation peak on the negative side was used because of high sensitivity and reproducibility. A measurement of 2.0×10^{-7} M daunomycin was carried out using differential pulse voltammetry, after a potential at −1.0 V for 5 min was applied to the electrode. As a result, a peak at −0.60 V was observed by scanning the potential in a positive direction. When 2.0×10^{-7} M daunomycin and 1.5×10^{-8} M ConA were mixed for 1 h in solution, the peak current of the daunomycin was similar to that without OVA.

By contrast, the peak current of 1.5×10^{-8} M labeled ConA was measured in a solution. The concentration of the labeled ConA corresponded to the concentration of 1.9×10^{-7} M daunomycin. The number of daunomycin moieties per ConA dimer was 12.8, as estimated from MALDI-TOF MS. With the addition of 2.0×10^{-7} M OVA to the solution, the peak current of the labeled ConA was drastically decreased, which compared only to that of labeled ConA. This was because several daunomycin moieties of labeled ConA were covered with OVA. In addition, the diffusion constant of labeled ConA with OVA may decrease in solution. On the other hand, it was expected that the length of the spacer bound to ConA influenced the binding. We believe that a decrease in the peak current was mainly due to labeled ConA–OVA binding.

Consequently, the binding was rapidly evaluated by voltammetric measurement. Thus, daunomycin was suitable for the labeling of a protein. When the measurement was carried out using the electrode, the linear range of the calibration curve for OVA was between 1.5×10^{-10} and 1.5×10^{-9} M. The R.S.D. of 1.5×10^{-8} M labeled ConA and 1.5×10^{-10} M OVA was 6.9% ($n = 5$). The detection limit of OVA was 9.0×10^{-11} M estimated at 3-fold the standard deviation (3σ). Husby et al. measured the concentration of OVA by means of ELISA [21]. When 2.3×10^{-6} M of OVA was administered to clinical research volunteers, the concentration in the blood was from 1.2×10^{-10} to 2.4×10^{-9} M. Generally, ELISA with anti-ovalbumin

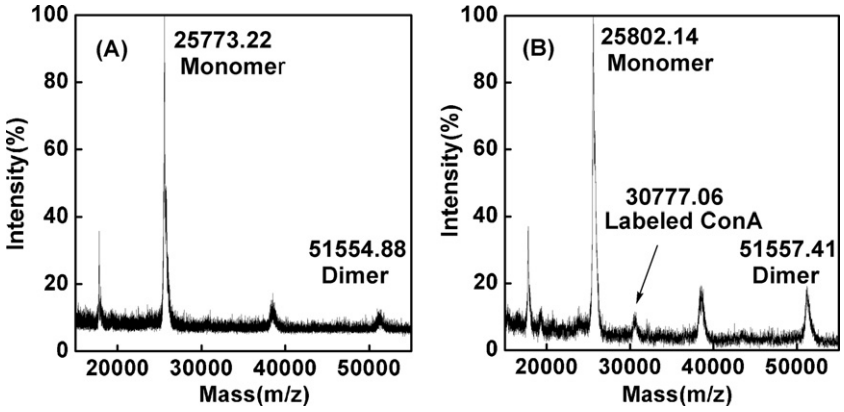

Fig. 4. Characterization of ConA and labeled ConA using MALDI-TOF MS. (A) Native ConA, (B) native ConA + labeled ConA.

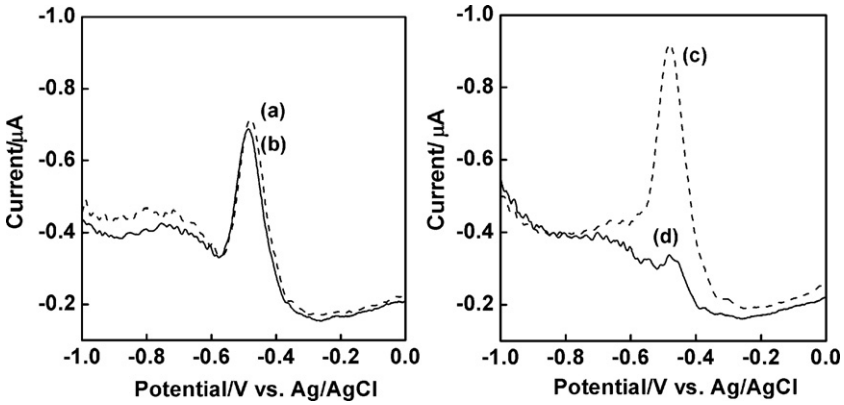

Fig. 5. Voltammograms of daunomycin and labeled ConA using a glassy carbon electrode. (a) 1.5×10^{-8} M ConA + 2.0×10^{-7} M daunomycin, (b) 1.5×10^{-8} M ConA + 2.0×10^{-7} M daunomycin + 2.0×10^{-7} M OVA, (c) 1.5×10^{-8} M labeled ConA, (d) 1.5×10^{-8} M labeled ConA + 2.0×10^{-7} M OVA. After incubation with stirring was carried out for 1 h in 0.1 phosphate buffer (pH 5.6), a potential at −1.0 V was applied to the electrode for 5 min. Measurements were carried out using differential pulse voltammetry.

antibody has been used to measure OVA *in vivo*. The determination of OVA was in the range of 1.8×10^{-10} M and 1.8×10^{-8} M (DS Pharma Biomedical Co., Ltd.). Considering the concentrations, our method could be applied to the determination of OVA. Therefore, it is expected that the electrochemical procedure will contribute to the screening of egg-allergic patients.

3.4. Effect of a cross-liking agent and labeled OVA with Sulfo-EGS

The binding of ConA to OVA was influenced by the length and type of spacer between the ConA and the daunomycin moiety. BS^3 with a spacer consisting of an ethylene chain was selected as the cross-linking agent. The spacer of BS^3 was 0.5 nm shorter than that of Sulfo-EGS. Labeled ConA with BS^3 was prepared using the same procedure as the labeled ConA with Sulfo-EGS. The ratio of daunomycin moieties to monomer ConA was 6.4. The value was determined using the MS mentioned above. The peak currents of labeled ConA with Sulfo-EGS and BS^3 exponentially decreased as the concentration of OVA increased (Fig. 6). The change of the labeled ConA with BS^3 was smaller than that of the labeled ConA with Sulfo-EGS. Therefore, it was clear that the peak current was influenced by the length and functional groups of the spacer.

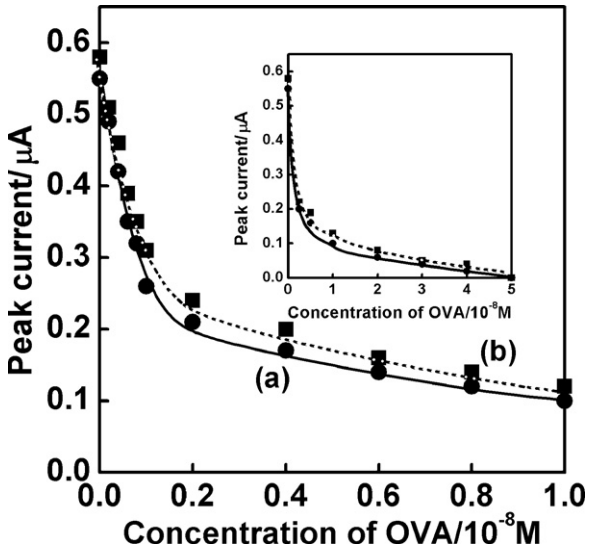

Fig. 6. Effect of a cross-liking agent for the labeling of ConA. (a) Labeled ConA with Sulfo-EGS, (b) labeled ConA with BS^3. After 1.5×10^{-8} M labeled ConA and OVA were mixed for 1 h in 0.1 M phosphate buffer (pH 5.6), a potential at −1.0 V was applied to a glassy carbon electrode for 5 min. Measurements were carried out using differential pulse voltammetry.

3.5. ConA influences of proteins with no sugar chain

To evaluate whether the binding between labeled ConA and OVA took place, a protein without a sugar chain to ConA and labeled ConA were incubated in 0.1 M phosphate buffer (pH 5.6). The voltammetric measurements mentioned above were carried out after 1.5×10^{-8} M of labeled ConA and the protein were stirred for 1 h. Table 1 lists the concentration limits at which the protein gave a relative standard deviation of less than 10% in the peak current of 1.5×10^{-8} M labeled ConA obtained after the applied

Table 1
Concentrations at which other proteins can be present without causing interference.

Concentration of protein	10^{-7} M
Bovine serum albumin	0.2
Streptavidin	1.0
Soybean agglutinin	1.0
Wheat germ agglutinin	1.0

Measurement of 1.5×10^{-8} M labeled ConA using a glassy carbon electrode for 5 min in 0.1 M phosphate buffer (pH 5.6).

Table 2
Determination of OVA in fetal bovine serum.

Sample	OVA added (nM)	Found (nM)	Recovery[a] (%)	R.S.D. (%)
1	0.50	0.49	98	6.6
2	1.00	1.01	101	6.1
3	1.50	1.48	99	5.7

[a] Average of five determinations at optimum conditions. 1:10,000 dilution with 0.1 M phosphate buffer (pH 5.6).

potential at −1.0 V for 5 min. Because BSA was non-selectively adsorbed onto the electrode, the permissible concentration of BSA was 2.0×10^{-7} M. For proteins such as streptavidin, soybean agglutinin and wheat germ agglutinin, the peak current of the labeled ConA was not influenced by the existence of 5.0×10^{-7} M protein. Accordingly, the labeled ConA was selectively combined with the OVA.

3.6. Voltammetric measurements of OVA with fetal bovine serum

To examine whether OVA can be measured using this method, we attempted voltammetric measurements of OVA with the serum. After several concentrations of OVA were added to the serum diluted with 0.1 M phosphate buffer (pH 5.6), 1.5×10^{-8} M labeled ConA was incubated for 1 h in the solution. The concentration of total protein in the serum was 0.0038 g/L. Then, the measurements were carried out under the conditions mentioned above. The results are summarized in Table 2. Because the recoveries of OVA were about 100%, influences from matrix were hardly observed up to 0.01% of the fetal bovine serum. This method could be applied to the direct determination of the OVA, judging from the good recovery in the serum.

4. Conclusion

To monitor the interactions between ConA and OVA, a probe in which electroactive daunomycin was introduced to ConA through a cross-linking agent was prepared. When the probe was applied to measure the protein–protein interaction, the binding was conveniently evaluated using the change in electrode response. The detection limit of this method was at the 10^{-10} M level and was similar to that of ELISA. In the presence of OVA in a fetal bovine serum, the recoveries were sufficient for determination.

The proposed method could be used to track biomolecular interactions and to develop a device for the diagnosis of disease. Furthermore, this method would be effective in the study of glycobiology and glycotechnology because glycoproteins are present on the surface of cells and outside cell walls.

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