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Signal amplification in electrochemical detection of buckwheat allergenic protein using field effect transistor biosensor by introduction of anionic surfactant

Sho Hideshima [a], Keisuke Fujita [b], Yoshitaka Harada [c], Mika Tsuna [c], Yasuhiro Seto [c], Satoshi Sekiguchi [c], Shigeki Kuroiwa [a], Takuya Nakanishi [a], Tetsuya Osaka [a,b,*]

[a] Research Organization for Nano & Life Innovation, Waseda University, 513 Waseda-tsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan
[b] Graduate School of Advanced Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan
[c] Central Laboratory, Nippon Flour Mills Co. Ltd., 5-1-3 Midorigaoka, Astugi-shi, Kanagawa 243-0041, Japan

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ABSTRACT

Food allergens, especially buckwheat proteins, sometimes induce anaphylactic shock in patients after ingestion. Development of a simple and rapid screening method based on a field effect transistor (FET) biosensor for food allergens in food facilities or products is in demand. In this study, we achieved the FET detection of a buckwheat allergenic protein (BWp16), which is not charged enough to be electrically detected by FET biosensors, by introducing additional negative charges from anionic surfactants to the target proteins. A change in the FET characteristics reflecting surface potential caused by the adsorption of target charged proteins was observed when the target sample was coupled with the anionic surfactant (sodium dodecyl sulfate; SDS), while no significant response was detected without any surfactant treatment. It was suggested that the surfactant conjugated with the protein could be useful for the charge amplification of the target proteins. The surface plasmon resonance analysis revealed that the SDS-coupled proteins were successfully captured by the receptors immobilized on the sensing surface. Additionally, we obtained the FET responses at various concentrations of BWp16 ranging from 1 ng/mL to 10 μg/mL. These results suggest that a signal amplification method for FET biosensing is useful for allergen detection in the food industry.

1. Introduction

People all over the world, irrespective of age or sex, suffer from food allergies. From the standpoint of food manufacturers, the management of allergenic substances in foods is important because food allergies cause many kinds of skin, respiratory, and digestive symptoms [1]. In a serious case, an immediate hypersensitivity reaction including anaphylaxis may cause a reduction in blood pressure or unconsciousness, resulting in a life-threatening condition. Although buckwheat flour products are familiar to people in many countries especially East Asia and Europe, the buckwheat protein, BWp16, can cause an acute allergic reaction [2]. BWp16 is known as a major allergen leading to anaphylaxis because it shows pepsin resistance [3,4]. The protein remains in food even after cooking, thus determination of a trace amount of the protein is of great importance in the manufacturing process. In Japan, Food Allergen Labeling Regulations require mandatory labeling of seven allergens including buckwheat proteins contained in foods 10 μg/g or more [5], thus the manufacturing control of allergens has strict regulations to ensure food safety.

Screening tests that evaluate if manufacturing lines have low concentrations of allergen proteins remaining have been widely applied in the food manufacturing process [6]. However, an existing screening test based on a highly sensitive enzyme linked immunosorbent assay (ELISA) is limited by its requirement for multiple steps, an appropriately labeled secondary antibody and the necessary optical equipment [7]. To overcome these drawbacks, a simple label-free allergen detection technique for food manufacturing lines is desirable. A field effect transistor (FET) biosensor is a promising platform because it can be used in mass production and for large-scale integration. FET biosensors may directly detect the intrinsic charge of proteins captured by immobilized receptors [8,9], suggesting that it has the potential to eliminate some of the steps involved in conventional allergen detection processes. We developed an FET biosensor with high chemical durability by modifying its surface with self-assembled monolayers [10], and adapted the sensors for use in various healthcare fields by employing different probe molecules [11–13].

* Corresponding author at: Waseda University, Okubo 3-4-1, Shinjuku-ku, Tokyo 169-8555, Japan.
E-mail address: osakatets@waseda.jp (T. Osaka).

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The magnitude of the FET response primarily depends on the total amount of protein charges, thus introduction of extra charges by supplementation of additives is an effective procedure to amplify the FET signals for sensitive detection [14,15]. However, these methods are limited by the synthesis or preparation of sophisticated nano-amplifiers. An anionic surfactant, sodium dodecyl sulfate (SDS), which is widely used as a chemical reagent for polyacrylamide gel electrophoresis (PAGE), is known to couple at a constant rate with soluble proteins, suggesting that the additional charges can be successfully introduced to target proteins regardless of their charge properties. In this study, we propose the use of a charge-amplification technique using SDS surfactant to cover target proteins with anionic charges, for sensitive detection by FET of buckwheat allergenic protein (Fig. 1a).

2. Materials and methods

2.1. Materials

The recombinant buckwheat protein BWp16 was prepared using an *Escherichia coli* strain carrying the cDNA of the structural gene [16]. The anti-BWp16 monoclonal antibody was produced with a hybridoma clone, which was prepared from a mouse immunized with the BWp16 protein. The BWp16 protein and anti-BWp16 antibody were prepared by diluting the stock solution in phosphate buffer saline (PBS). The self-assembled monolayer (SAM) reagent, 3-aminopropyltriethoxysilane (APTES), was purchased from Sigma-Aldrich. The capping reagent, ethanolamine, was purchased from Tokyo Chemical Industry Co. (Japan). Albumin from chicken egg white (OVA) and sodium dodecyl sulfate (SDS) were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Pierce™ Mouse IgG1 Fab and F(ab')$_2$ Preparation Kit and SDS-Out SDS Precipitation Kit were purchased from Thermo Scientific (US). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) was purchased from GE Healthcare (Sweden). The Biotin Labeling Kit-NH_2 and ExtrAvidin®-Peroxidase was from Dojindo Molecular Technologies (Japan) and Sigma-Aldrich Co. LLC (USA), respectively. Tris(hydroxymethyl)-aminomethane (Tris) was purchased from Nacalai Tesque, Inc. (Japan). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), disodium salt, dihydrate were purchased from Wako Pure Chemical Industries, Ltd. (Japan). All other materials were purchased from Kanto Chemical Co. (Japan). All the chemicals were of analytical reagent grade and used as received.

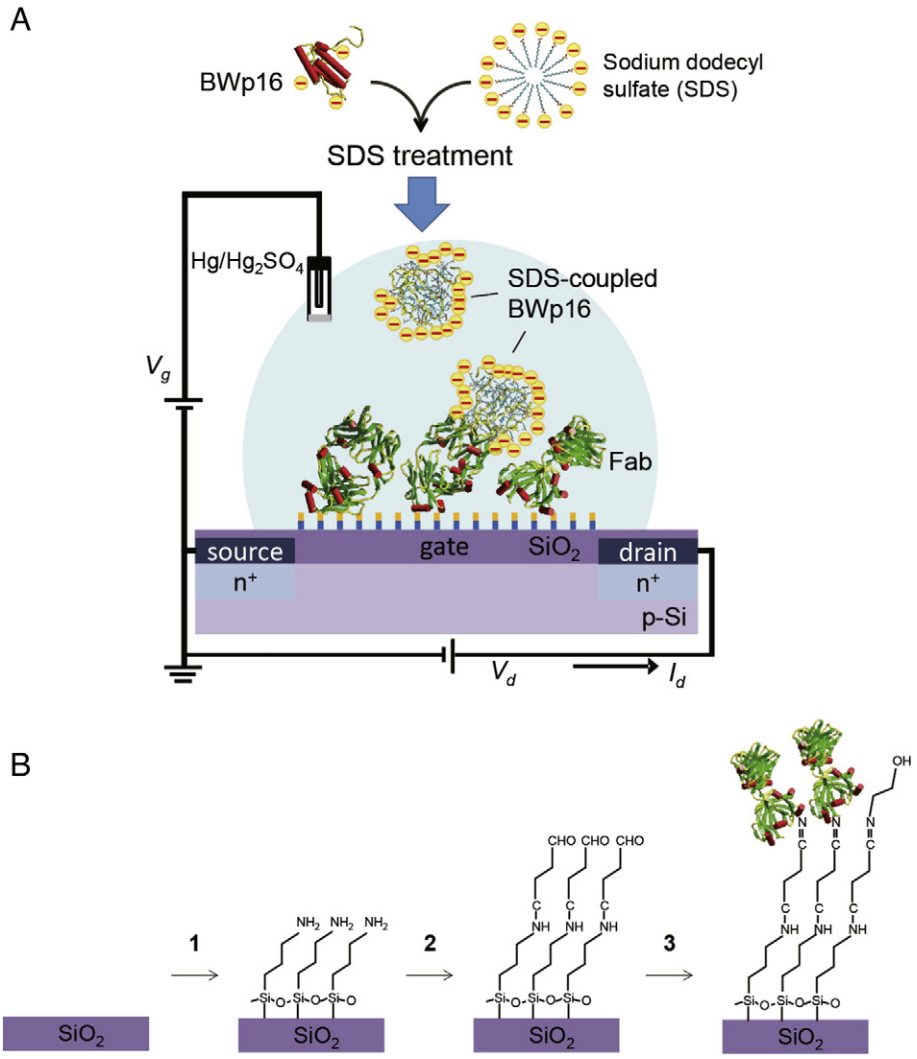

Fig. 1. Schematic illustration of the FET sensing. (a) Signal amplification for FET detection by coupling the anionic surfactant with the target buckwheat protein. The negative charges derived from the anionic surfactant, SDS, enhanced the intrinsic negative charges of the buckwheat protein BWp16. (b) Preparation process for the FET biosensor. Silanization with aminopropylsilane (1), modification with glutaraldehyde and reduction of Schiff base (2), and Fab immobilization and ethanolamine-capping treatment (3).

2.2. Detection of surfactant-coupled buckwheat proteins by using Fab-immobilized FET biosensor

The preparation procedure of the ethanolamine-capped Fab-immobilized FET biosensors and the measurement conditions used in this study were the same as described in our previous paper (Fig. 1b) [17]. The Fab was prepared by digesting an anti-BWp16 monoclonal antibody using the Pierce™ Mouse IgG1 Fab and $F(ab')_2$ Micro Preparing Kit, and then covalently linked to the amino-terminated SAM. The Fab-immobilized FETs were used without their preservation. To amplify the response of FET, the samples were mixed and treated with 1% SDS solution for 5 min in a hot water bath at 100 °C. After the treatment, free SDS molecules were removed by using the SDS-Out SDS Precipitation Kit. The final protein recovery was 95%. The Fab-immobilized FETs were immersed in the SDS-coupled protein solutions for 60 min. After the immersion, the residue of the protein solution was washed with 0.01 × PBS. The characteristics of V_g–I_d relation of the protein-reacted FET was measured in 0.01 × PBS and compared with those of the as-prepared Fab-immobilized FET. The threshold voltage shift (ΔV_g) was then calculated.

2.3. Measurement of the molecular interaction by SPR and ELISA

SPR analysis was performed with SR7000DC (Reichert Inc.) by analyzing the interaction between the target proteins and the Fab molecules on the sensor chip. The Fab molecules were covalently linked to the sensor chip (500,000 Da carboxymethyl dextran, Reichert Inc.) through an amide linkage. The gold sensor chip was activated with 200 mM EDC followed by 50 mM NHS treatment before the immobilization of the Fab molecule. Unreacted activated carboxyl residues on the sensor chip were capped with 2 M Tris–HCl (pH 8.0). The BWp16 proteins treated with SDS and the untreated control (each of which was prepared at 0.5, 1.0 and 2.0 μM with HBS-EP; 10 mM HEPES, 150 mM sodium chloride, 3 mM EDTA, and 0.05% Surfactant P20) were subjected to SPR measurement to evaluate K_{on} and K_{off} followed by removal of these antigens by washing with 10 mM glycine–HCl pH 1.5. The K_D value (equilibrium dissociation constant) of each interaction was calculated using Scrubber 2 software (BioLogic). For the ELISA measurement, the BWp16 protein was captured by the Fab molecules on a microplate and detected using the biotinylated secondary antibody followed by detection with avidin–peroxidase [18].

3. Results and discussion

3.1. Signal amplification of an allergenic protein of buckwheat by introduction of an ionic surfactant

First, we investigated the effect of the anionic surfactant, SDS, on the signal amplification of the FET detection. The response of FET to the recombinant buckwheat protein, BWp16, in the absence or presence of SDS was evaluated (Fig. 2a). No significant shifts in the signals were observed when the FETs were immersed into the protein BWp16 solution (10 μg/mL) without the additive. The molecular weight and isoelectric point of the BWp16 protein was 15,106 and 5.25, respectively, suggesting that the protein does not possess enough intrinsic net charge in the pH 7.4 buffer to be detected by the sensor in view of its low molecular

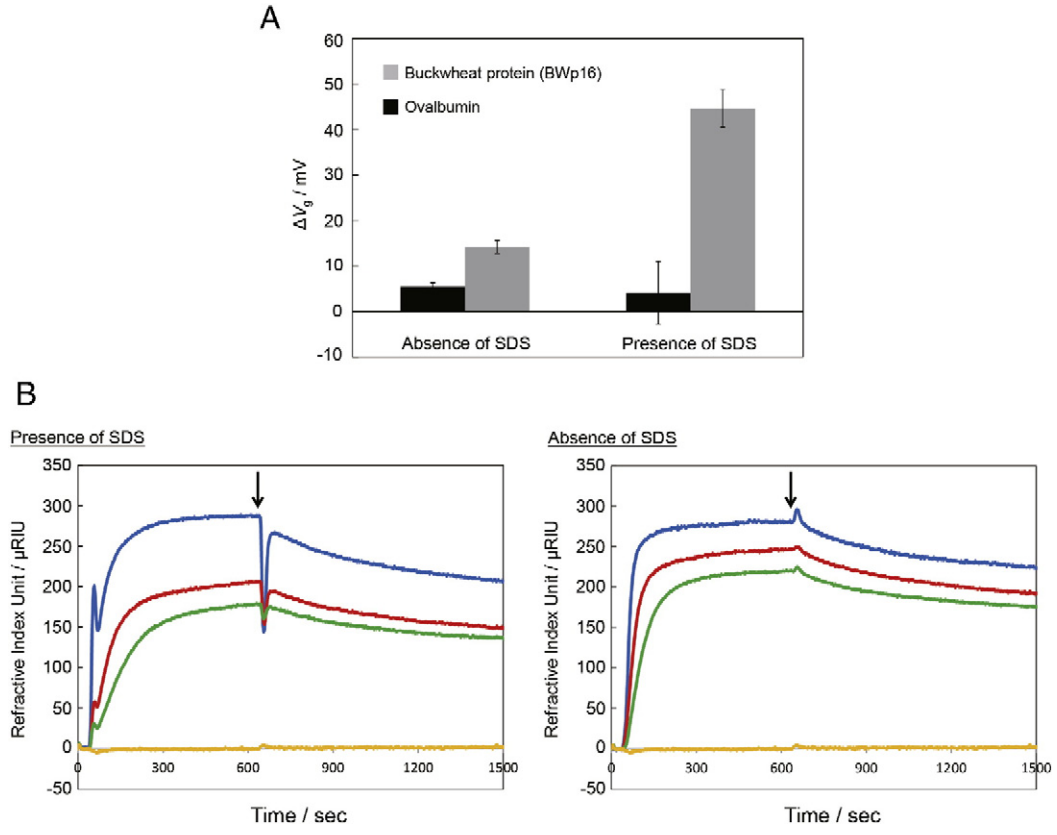

Fig. 2. Signal amplification of the FET response by introduction of the surfactant. (a) Effect of the additional anionic surfactant charge on the FET response (n = 3). SDS was used as an anionic surfactant. The proteins were ovalbumin (5 mg/mL, negative control) and BWp16 (10 μg/mL, positive control). The response for the blank test (PBS) was 3.1 mV (absence of SDS) and 4.8 mV (presence of SDS). (b) Evaluation of the binding affinity of SDS-coupled protein to the Fab-immobilized surface by surface plasmon resonance measurement. The target injection occurred at t = 0, while the HBS-EP injection is indicated by the arrow. The concentration of the injected proteins (BWp16) was 0 μM (yellow), 0.5 μM (7.5 μg/mL) (green), 1 μM (15 μg/mL) (red), and 2 μM (30 μg/mL) (blue).

weight and isoelectric point. The charge number of the BWp16 protein in the solution at pH 7.4 is calculated to be −3.67 from its amino acid sequence [19], which suggests that the FET sensing system for the BWp16 protein detection might not achieve the desired concentration (10 ng/mL) without any signal amplification methods. As expected, when BWp16 was incubated in the presence of the anionic surfactant, a significant FET response to the BWp16 protein was observed. In this case, by covering the surface of the protein with SDS, the SDS-coupled BWp16 protein possessed enough negative charge to be detected by the sensor. Additionally, almost no shift was observed when the 5 mg/mL OVA solution treated with SDS was used instead of the BWp16 solution. The surface of the SDS-coupled OVA protein became hydrophilic and the nonspecific adsorption of the protein via hydrophobic interactions was successfully suppressed. It should be noted that the residues of free SDS molecules were removed by using SDS-out columns after the coupling of the proteins to avoid affecting the sensor response. Thus the treatment of target proteins with the anionic surfactant helps to amplify the electrical signal for FET detection. In the present study, the availability of other surfactants (anionic, cationic, and nonionic) was examined for further amplification of the FET detection.

3.2. Analysis of the molecular binding ability of the surfactant-conjugated protein by using surface plasmon resonance measurement

We investigated the binding ability of the SDS-coupled protein to immobilized Fab molecules by SPR measurement. The SPR responses were obtained when the protein solution (0.5 μM, 1 μM, and 2 μM) was injected onto the Fab-immobilized sensor chip (Fig. 2b), while bovine serum albumin (at a concentration of 2.0 μM) treated with SDS showed no interaction with the Fab molecule (data not shown). The K_D values for the association between the protein and the Fab were 7.8×10^{-8} M for the SDS-coupled BWp16 and 1.4×10^{-8} M for BWp16, respectively. Additionally, the ELISA measurement also showed that the binding ability of the SDS-coupled protein was similar to that of the as-prepared protein (data not shown). These results showed that the binding ability of the SDS-coupled protein to the Fab molecule was somewhat lowered but still retained, suggesting that the proposed method is effective to amplify the response of FET without any major changes in the affinity of the target protein. According to a previous report from Pittrive and Impiomba, the SDS/protein coupling ratio without reduction of the protein disulfide groups is known to be approximately 0.9, while that with reduction is 1.4 [20]. In this study, no reagents were added for the reduction of disulfide groups because it is possible that the reductant may damage the structure of the Fab molecule immobilized on the sensing surface. A single molecule of BWp16 protein is estimated to bind 47 molecules of SDS, expecting that the increase in the amount of negative charges amplifies the FET signal. In this study, although the number of captured proteins might decrease because of the surfactant coverage, the intrinsic charge of the protein was successfully enhanced by binding the anionic surfactant, resulting in successful signal amplification for FET detection.

3.3. Determination of buckwheat allergenic protein

To determine the sensitivity of the method for protein detection, we examined the response of the FET biosensor to the allergenic protein solution ranging from 1 ng/mL to 10 μg/mL in buffer. Fig. 3 shows the sensor responses from 3 different FET devices displaying Fab receptors with respect to BWp16 concentration. Increasing the concentration of BWp16 molecules increased the negative charge of the adsorbed proteins within the Debye length, resulting in the enhancement of the magnitude of ΔV_g. This result suggests that each target protein successfully formed an anionic micelle with SDS molecules, and the SDS-coupled proteins were specifically captured by immobilized receptors on the sensing surface. Thus we achieved FET detection when the level of the target protein was at least 10 ng/mL, which is the same degree of sensitivity as the conventional allergy test. In the present study, allergens in food extracts such as buckwheat, wheat, egg, and soybean were detected by the developed sensing system. With further modification, this method should prove useful toward the practical application of FET biosensors in the food industry.

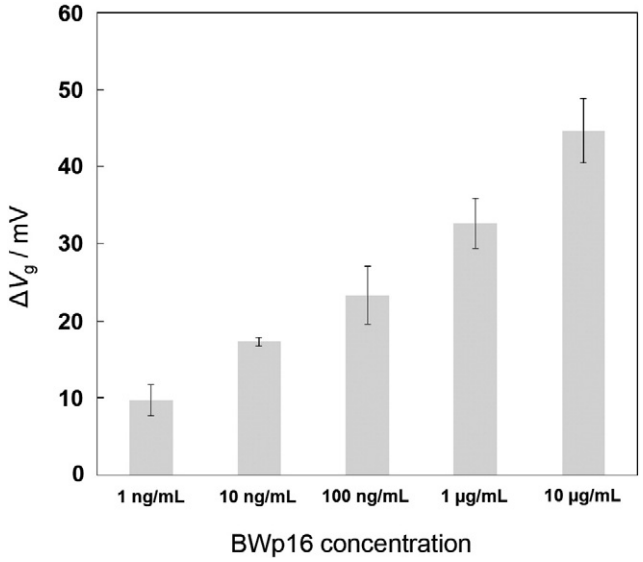

Fig. 3. Relationship between the concentration of recombinant buckwheat protein BWp16 coupled with the anionic surfactant SDS and the magnitude of ΔV_g (n = 3).

4. Conclusion

An FET-based method for the sensitive detection of the buckwheat protein, BWp16, was developed by introducing additional charge via the anionic surfactant, SDS, to the target protein. The target proteins coupled to the SDS molecules still retained their ability to recognize the immobilized Fab and yields a higher FET response than as-prepared protein. The proposed method is expected to be applicable to various kinds of proteins because all proteins contain hydrophobic units that can bind with the surfactants. This simple FET sensing system achieved the desired detection level of 10 ng/mL of allergen evaluation, suggesting that it would be an easy alternate method to incorporate in the manufacturing lines of food factories.

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