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Direct Triazine Herbicide Detection Using a Self-Assembled Photosynthetic Reaction Center from Purple Bacterium

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Abstract In this study, a direct detection system for triazine derivative herbicides was developed using the photosynthetic reaction center (RC) from the purple bacterium, *Rhodobacter sphaeroides*, and surface plasmon resonance (SPR) apparatus. The histidine-tagged RCs were immobilized on an SPR gold chip using nickel-nitrilotriacetic acid groups as a binder for one of the triazine herbicide, atrazine. The SPR responses were proportional to the sample concentrations of atrazine in the range 0.1-1 μg/mL. The sensitivity of the direct detection of atrazine using the RC-assembled sensor chip was higher than that using the antibody-immobilized chip. The other types of herbicides, DCMU or MCPP, were not detected with such high sensitivity. The results indicated the high binding selectivity of the RC complex.

Keywords: photosynthetic reaction center, herbicide, triazine derivative, surface plasmon resonance, biosensor, affinity binding

INTRODUCTION

Herbicide contamination in the environment is a very serious problem. Although various types of herbicides are used in agriculture, some of them are feared because of their serious effects on the ecosystem and human life, *e.g.* carcinogenic activity or endocrine disrupting activity. Recently many risk characterizations or epidemiological studies for triazine herbicides (*i.e.* atrazine, simazine, cyanazine) have been reported including the clastogenic potentials of atrazine and simazine on Chinese hamster ovary cells [1] or their carcinogenicity [2, 3]. Moreover it has been confirmed that the chlorotriazine herbicides disrupt hormonal control [4]. In Japan, under the basic environmental law, the environmental quality standard for water pollutants was established by the environment agency of Japan, with the standard for simazine set at 3 ng/mL. Triazine herbicide concentration in water is also strictly regulated worldwide and is equal to or lower than ppb level. To monitor such low residual herbicide levels a need has arisen for a fast, sensitive and inexpensive detection method.

Of course, many detection methods for triazine herbicides have been developed realizing a sub-ppb level of detection during the last few years. Most of them are enzyme-linked immunosorbent assays (ELISA) [5-8] or methods using a competitive immuno-reaction [9-11] which needs some conjugate or special complex because of detection difficulties due to the small molecular size of the target chlorotriazines. Therefore, a simpler direct measurement method is required.

The triazine herbicides inhibit the light reactions in photosynthesis by binding to the photosystem II (PSII) complex [12]. The herbicides replace the secondary quinone (Q_B) binding site on the D1 protein of the PSII complex. Herbicide detection methods using D1 proteins [13,14], PSII particles [15] and thylakoid membranes [16] have been reported. Koblizek *et al.* and Piletskaya *et al.* demonstrated direct detection methods by measuring the oxygen evolution activity of PSII and a current through a cast film of D1 protein, respectively [13,15]. However not only triazine herbicides but also other categories of herbicides bound to the D1 protein (*i.e.* DCMU, ioxynil, bromoxynil and dinoseb) were detected. Therefore, the D1 protein is not a suitable binding material when the detection target was defined specifically as triazine herbicides. Purple bacterial reaction centers (RCs) reveal a structural and functional homology to the D1 proteins of plants [12,17]. The bacterial RC also has a Q_B binding pocket (see Fig. 1(a)). Recently, the three-dimensional structure of atrazine-bound RC of *Rhodopseudomonas viridis* was elucidated using X-ray crystallography [18]. The triazine herbicide binds to the RC more strongly than the other herbicides [12,19]. On this account, bacterial RC is suitable for triazine herbicide detection.

RCs from some purple bacteria have been used in our laboratory to construct a photo-generating electric device [20-22]. Orientation control and dense packing of RCs are essential requirements for efficient current generation. A complex, RC from *Rhodobacter sphaeroides* with a six-histidine tagged heavy (H) subunit was syn-

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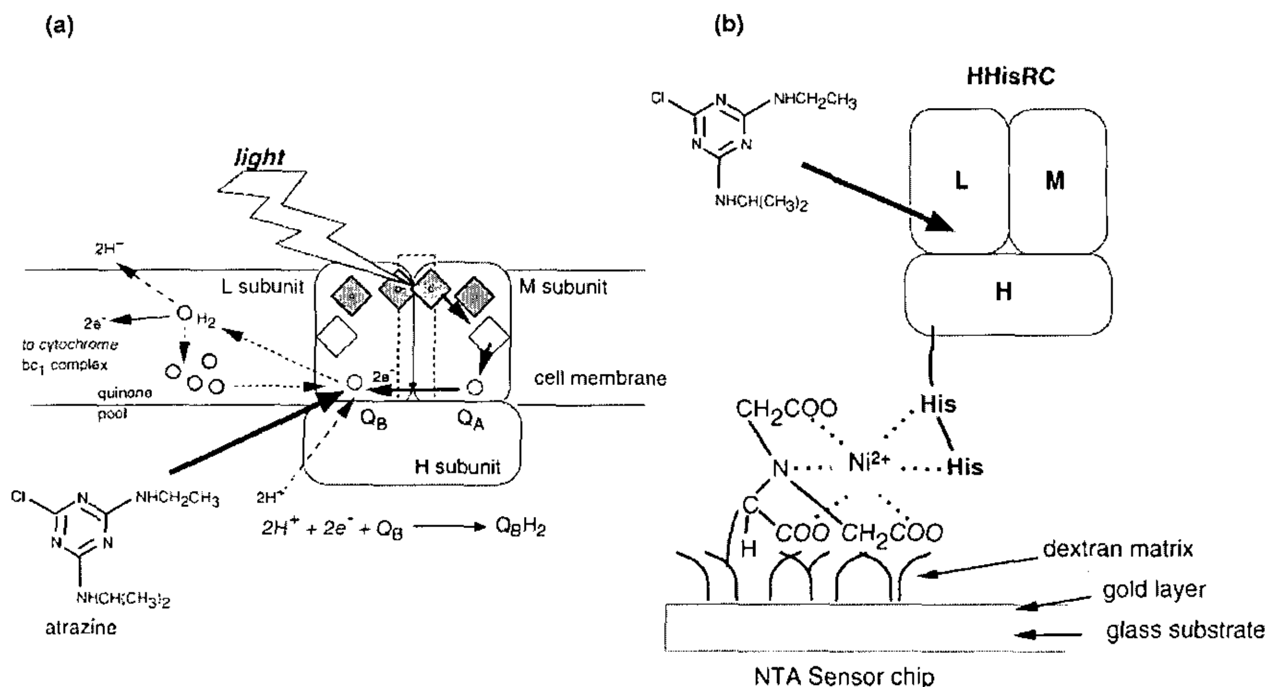

Fig. 1. Schematic of atrazine binding to RC of *Rhodobacter sphaeroides* (a) and histidine-tagged RCs on an Ni-NTA chip (b). Light-induced charge separation occurs at the chlorophyll special pair, the electron is then transferred to Q_A through pheophytin. The final electron acceptor Q_B in *R. sphaeroides* is ubiquinone Q-10. A protonated quinone is released from Q_B site and forms a proton gradient. Atrazine shifts to the Q_B site on the L subunit of RC resulting in inhibition of electron transfer in the photosynthetic reaction. The binding constant of HHisRC to Ni-NTA chip was 1.6×10^8 M^{-1} [25].

thesized by modified gene expression using the H-subunit-defective mutant, PUHA1 [23], to enable self-assembly and orientation control of RC on the substrate [24,25]. In this study the H-subunit-histidine-tagged RC (HHisRC) was used in the surface plasmon resonance (SPR) system to detect triazine herbicide. In SPR analysis the capturing molecule is immobilized on a sensor chip, if a sample containing a molecule that binds to the capturing molecule is passed over the chip, the binding event is detected by the SPR instrument. The kinetics of molecular binding can be also measured by the SPR system. The ability of direct detection of atrazine by RC was estimated, and compared to the anti-atrazine antibody.

MATERIALS AND METHODS

Reagents

Atrazine, DCMU and MCPP were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). The other reagents used in this study were analytical grade. A rabbit anti-atrazine polyclonal antibody was purchased from Chemicon International Inc. (Temecula, USA). The immunoglobulin G (IgG) was fractionated from the serum using a HiTrap ProteinG column (Amersham Pharmacia Biotech Ltd., Amersham Place, UK).

Bacterial Strain and RC Preparation

The HHisRC was expressed in the recombinant strain of PUHA1 harboring pCHT919 as described in our previous report [24]. The recombinant strain was grown microaerobically on ASY medium containing 1 μg/mL of tetracycline and 25 μg/mL of kanamycin at 30 °C under 100 W/m^2 of light irradiation. HHisRCs were purified by the previously reported method [24,26].

Immobilization of RC or IgG on SPR Chip

An SPR apparatus, BIACORE X (Biacore International AB, Uppsala, Sweden) was used to detect atrazine in this study. BIACORE X has a flow-injection system to enable a real time measurement of molecular binding. There are two flow cells, one flow cell was used for capturing molecule immobilization and the other was used as a reference cell. NTA sensor chip and CM5 sensor chip (Biacore International AB) were used for the SPR measurements using HHisRC and anti-atrazine IgG, respectively. The NTA and CM5 chip have the same basic surface composition, a glass substrate, a 50-nm-thick gold layer, and a 100-nm-thick dextran matrix (Fig. 1(b)). The NTA chip is an NTA-group-immobilized CM5 chip. A nickel chloride solution (500 μM) was placed on the NTA chip to coordinate Ni^{2+} ions. Subsequently, HHisRC (3.3 μM) was dissolved in

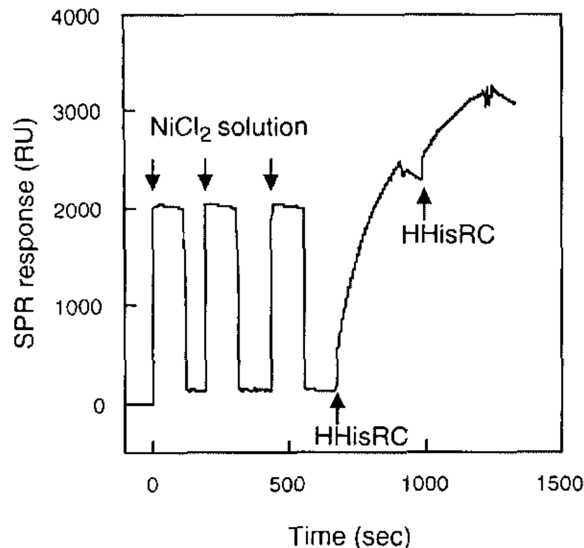

Fig. 2. Sensorgram for the immobilization of HHisRC on an Ni-NTA chip. Nickel ions were introduced into a flow cell for coordinating NTA groups on the chip. After injecting Ni^{2+} three times, 3.3 μM of HHisRC was injected.

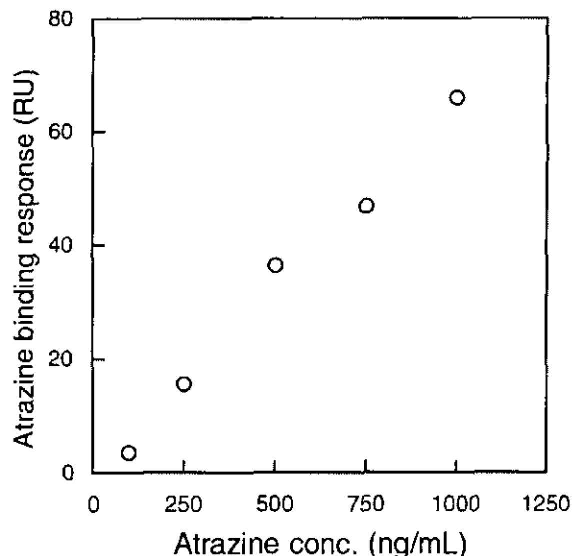

Fig. 3. Calibration graph of atrazine detection using HHisRC immobilized on the Ni-NTA chip.

50 mM phosphate buffer (pH of 8.0) containing 50 mM NaCl, 0.2% Triton X-100 and was then injected into the flow cell for immobilization (see Fig. 1(b)). Using an amine coupling method IgG was immobilized on the sensor chip CM5, which has a carboxylated dextran layer. A solution containing 50 mM N-hydroxysuccinimide and 20 mM N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide was injected into the flow cell to activate the carboxyl groups. Then, the anti-atrazine IgG (0.2 mg/mL) dissolved in HBS-EP buffer (10 mM Hepes with 150 mM NaCl, 3 mM EDTA, and 0.005% tween 20 at pH 7.4) was loaded on the chip for immobilization. Finally, a solution containing ethanolamine was injected to block excess reactive groups. The same treatment was applied to the reference flow cell, without antibody. For BIACORE X, 0.1 milli-degree of SPR angle change corresponds to 1 resonance unit (RU). An SPR signal of 1000 RU corresponds to about 1.0 ng/mm^2 of bound protein [27], and the area of the flow cell was 1.2 mm^2.

Herbicide Measurement by SPR

Atrazine or the other herbicides were dissolved in 50 mM phosphate buffer (pH of 8.0) containing 50 mM NaCl (10 mM for IgG chip), 0.2% Triton X-100 and 0.1% ethanol at various concentrations. Herbicide solutions were injected into the flow cell docked to the HHisRC-immobilized chip or the IgG-immobilized chip at a flow rate of 20 μL/min for 3 min at 25°C. Atrazine binding was monitored as an SPR signal change. Data for the signal from the capturing molecule immobilized flow cell minus that of the reference flow cell was obtained.

RESULTS AND DISCUSSION

Fig. 2 shows an SPR sensorgram of HHisRC immobilization onto the Ni-NTA sensor chip following two injections of HHisRC. The triplicate rectangular signals represent the injection of the nickel solution. The HHisRC-immobilized chip was prepared with approximately. 3000 RU of SPR signal increment corresponding to the surface density 3 ng/mm^2 (30 fmol/mm^2). On the other hand for anti-atrazine-antibody, the sensor signal increased up to about 12000 RU. Thus, the estimated surface density of antibody is about 12 ng/mm^2 (80 fmol/ mm^2 based on rough calculation). The functional binding titer of the immobilized IgG was unknown since the antibody solution used was a polyclonal IgG fraction and the immobilization site of IgG could not be restricted to avoid functional domain amine coupling.

The direct detection of the low-molecular weight herbicides, atrazine (215.69), MCPP (214.65) and DCMU (233.10) was carried out using the HHisRC or IgG immobilized SPR chip. Fig. 3 shows calibration graph of sensor for various concentrations of atrazine detection using the HHisRC immobilized chip. The plotted data, SPR response, represents the maximum SPR signal changes caused by injection of each concentration of atrazine solution. A linear correlation was obtained in the range from 100 ng/mL to 1 μg/mL. The immobilized RC can be used for direct detection of atrazine at sub-ppm levels in the SPR system. For DCMU, a lower sensor response compared to that for atrazine was observed at over 10 μg/mL (about 70% of the cross reactivity of atrazine). DCMU is also categorized in quinone binding inhibitors as a triazine herbicide. The difference in binding affinity between the herbicides and RC was reflected in the SPR responses, however, the difference was not as large as we expected. In any

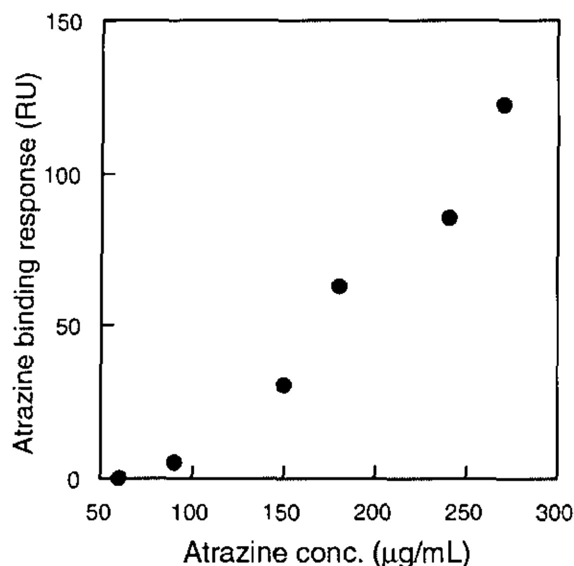

Fig. 4. Calibration graph of atrazine detection using anti-atrazine IgG immobilized on the CM5 chip.

case, triazine- herbicide specific detection would be possible for concentrations less than 1 μg/mL. On the other hand, for MCPP, no obvious sensor response was observed even at high concentrations (10 μg/mL). Because of a difference in the functioning mechanism MCPP, a plant hormone disrupter, could not be bound to the immobilized RC even when present in a high concentration. If any quinone binding herbicides (DCMU, ioxynil, bromoxynil, dinoseb, *etc.*) were mixed with a sample at high concentration, over 10 μg/mL, it would produce noise for triazine-herbicide detection using this system. The calibration graph obtained using the antibody-immobilized chip is also represented in Fig. 4. Although a linear correlation between atrazine concentration and SPR signal was observed from 90 μg/mL to 300 μg/mL, no SPR response appeared absolutely for 60 μg/mL of atrazine injection. The detection limit of the RC chip was 900 times lower than that of the IgG chip.

The RC and SPR system of this work was not sensitive compared to the method of Sasaki *et al.* using a monoclonal antibody against atrazine and SPR system which realized a detection limit of 50 pg/mL atrazine [11]. However, the method needs an atrazine-protein conjugate because direct monitoring of low-molecular-weight chemicals by SPR apparatus is very difficult even using a monoclonal antibody. The direct detection of a 200 Da target is not applicable for BIACORE X [28]. In other words, the construction of a direct and triazine-herbicide specific detection system using a biologically sorbent material is a challenging trial. Why does the SPR signal appear from direct binding of low-molecular-weight atrazine even at low concentrations? SPR signal change is affected by permittivity changes. We suppose that the herbicide binding to the Q_B site of RC caused an increment of permittivity resulting in a greater SPR signal change. The electrochemical property change caused by atrazine binding was observed in the D1 protein and it was applied to atrazine detection [13].

From SPR measurements, the calculated dissociation constant of atrazine with HHisRC was about 230 μM which is more than 2,000 times higher than the previously reported value, 0.08 μM for terbutryn binding to Q_B site of *Rb. sphaeroides* [29]. The result suggests that binding conditions in SPR measurement, *e.g.* concentrations of salt, detergent and organic solvent (ethanol in this study), could be improved by optimization for higher affinity binding of atrazine to RC and the detection sensitivity would improve after further optimization. On the viewpoint of measurement under optimized conditions, direct injection onto SPR chip is difficult for natural samples, pond water, groundwater or blackish water and so on, pre-treatment would be needed. Furthermore, application of the sensitization method (*e.g.* conjugate competitive method) is expected to improve the sensitivity by over one thousand times.

CONCLUSION

The H-subunit-histidine-tagged RC was immobilized on an SPR gold chip with Ni-NTA groups and was used for direct atrazine detection in the Biacore system. Atrazine was detected in the range from 100 ng/mL to 1 μg/mL. The sensitivity was 900 times higher than that of antibody-immobilized chip. For DCMU and MCPP levels of less than 1 μg/mL, no obvious sensor response was observed suggesting binding specificity of RC. There are further possibilities to improve the detection sensitivity of this system, if the atrazine binding conditions in SPR measurement could be optimized for higher affinities and sensitization method could be applied.

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NOMENCLATURE

DCMU : *N'*-(3,4-Dichlorophenyl)-*N,N*-dimethylurea
ELISA : Enzyme-linked immunosorbent assay
HHisRC : Heavy-subunit-histidine-tagged RC
IgG : Immunoglobulin G
MCPP : 2-(2-Methyl-4-chlorophenoxy) propionic acid
Ni-NTA : Nickel-nitrilotriacetic acid
PSII : Photosystem II
Q_B : Secondary quinone of PSII or RC
RC : Photosynthetic reaction center
SPR : Surface plasmon resonance

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