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Preparation and characterization of a polyclonal antibody from rabbit for detection of trinitrotoluene by a surface plasmon resonance biosensor

Kiyoshi Matsumoto [a,*], Akira Torimaru [a], Sachiko Ishitobi [a], Takatoshi Sakai [a], Hiroya Ishikawa [a], Kiyoshi Toko [b], Norio Miura [c], Toshihiko Imato [d]

[a] *Graduate School of Agriculture, Kyushu University, Fukuoka 812-8581, Japan*
[b] *Graduate School of Information Science and Electrical Engineering, Kyushu University, Fukuoka 812-8581, Japan*
[c] *Art, Science and Technology Center for Cooperative Research, Kyushu University, Kasuga-shi, Fukuoka 816-8580, Japan*
[d] *Graduate School of Engineering, Kyushu University, Fukuoka 812-8581, Japan*

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Abstract

A polyclonal antibody against trinitrophenyl (TNP) derivatives was raised in rabbit, and the antibody was applied to detection of trinitrotoluene (TNT) using a surface plasmon resonance (SPR) biosensor. TNP-keyhole limpet hemocyanine (TNP-KLH) conjugate was injected into a rabbit, and a polyclonal anti-TNP antibody was realized after purification of the sera using protein G. Aspects of the anti-TNP antibody against various nitroaromatic compounds, such as cross-reactivities and affinities, were characterized. The temperature dependence of the affinity between the anti-TNP antibody and TNT was also evaluated. The quantification of TNT was based on the principle of indirect competitive immunoassay, in which the immunoreaction between the TNP-β-alanine-ovalbumin (TNP-β-ala-OVA) and anti-TNP antibody was inhibited in the presence of free TNT in solution. TNP-β-ala-OVA was immobilized to the dextran matrix on the Au surface by amine coupling. The addition of a mixture of free TNT to the anti-TNP antibody was found to decrease the incidence angle shift due to the inhibitory effect of TNT. The immunoassay exhibited excellent sensitivity for the detection of TNT in the concentration range of 3×10^{-11} to 3×10^{-7} g/ml. To increase the sensitivity of the sensor, anti-rabbit IgG antibody was used. After flowing the mixture of free TNT and anti-TNP antibody, anti-rabbit IgG antibody was injected, and the incidence angle shift was measured. Amplification of the signal was observed and the detection limit was improved to 1×10^{-11} g/ml.

Keywords: Trinitrotoluene; Immunoassay; Surface plasmon resonance sensor; Anti-trinitrophenyl-protein antibody; Landmine detection

1. Introduction

There are more than 100 million landmines buried throughout the world, and 3 million new landmines are buried every year. Antipersonnel landmines cause extensive injury to more than 20,000 people a year. However, only 100,000 landmines are removed each year [1].

It is very difficult to detect a buried landmine. Dogs are used as on-site detectors. Dogs, however, need extensive training, and they cannot concentrate on detection for more than two hours [2]. Thus, it is very important to develop a novel technology for detecting landmines effectively. In recent years, various kinds of detection technologies based on physical sensors, such as ground-penetrating radar (GPR) have been developed [3,4]; however, it is difficult to detect landmines using only a physical sensor.

TNT detection methods using fluorescence quenching, capillary electrochromatography and HPLC have been reported [5–7], and detection methods using an antigen–antibody reaction, such as enzyme-linked immunosorbent assay (ELISA) and fluorescent and chemiluminescent immunosensors, are more useful and are able to detect parts per billion (ppb) levels of TNT [8–10]. These methods using antigen–antibody reactions are sensitive, but are they not suitable for on-site detection.

On the other hand, a surface plasmon resonance (SPR) sensor is small and portable, hence suitable for on-site detection. One of the most sensitive and promising sensors for detecting landmines is an immunosensor using a portable SPR sensor [11,12]. It might be possible to achieve highly sensitive on-site detection of TNT and its related compounds using this method. One

* Corresponding author. Fax: +81 92 642 3011.
E-mail address: kmatsu@agr.kyushu-u.ac.jp (K. Matsumoto).

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of the key components of an immunosensor is the high affinity antigen–antibody immunoreaction, which in combination with different physical transducers, provides both sensitivity and selectivity.

In recent years, we have focused on the development of highly sensitive SPR sensing methods for the detection of TNT using an antigen–antibody reaction [13–15]. In general, the detection of small molecules by direct SPR detection is difficult because they have insufficient mass to effect a measurable change in the refractive index. Therefore, we proposed SPR immunoassays for the detection of TNT based on the principle of indirect competitive immunoreaction [13–15]. In an indirect competitive immunoreaction, the affinity characteristics among the antigen (analyte), antibody and solid-phase antigen are very important to achieve highly sensitive detection. In a previous paper, we compared the efficiency of the immunoreactions between a commercial antibody (from goat) and a laboratory-prepared antibody (from rabbit) that was raised with differently designed immunogens [15].

In the present study, we investigated the factors of temperature, solid-phase antigens, response time and the regeneration method for the detection of TNT, and also evaluated the affinity constant between the antibody and antigen (analyte) and/or solid-phase antigen. Moreover, we tried to amplify the SPR response by using anti-rabbit IgG.

2. Experimental

2.1. Materials

Alkaline phosphatase (ALP) was obtained from Roche Diagnostics Japan Co. Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), *p*-nitrophenyl phosphate disodium salt (*p*-NPP), 2,4,6-trinitrobenzene sulfonate sodium salt (TNBS), sodium sulfate, *N*-hydroxysuccinimide (NHS) and gelatin were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Ovalbumin (OVA), keyhole limpet hemocyanin (KLH), ALP-labeled anti-rabbit IgG, anti-rabbit IgG, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), methylamine, ethylamine and butylamine were obtained from Sigma (St. Louis, MO, USA). 2,4,6-Trinitrotoluene (TNT) was supplied by Chugoku Kayaku Co. Ltd. as a 10.7 ppm aqueous solution. 2,4,6-Trinitrophenol (TNP-OH) and 1,3-dinitrobenzene (DNB) were purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). 2,4-Dinitrotoluene (DNT) and *bis*(2-methoxyethyl) ether were obtained from Wako Pure Chemicals Ind. Ltd. (Osaka, Japan). 2-Amino-4,6-dinitrotoluene (2-amino-DNT) and 4-amino-2,6-dinitrotoluene (4-amino-DNT) were purchased from Supelco (PA, USA). 2,4,6-Trinitrophenylglycine (TNP-gly) and 2,4,6-trinitrophenyl-β-alanine were obtained from Cosmo Bio Co. Ltd. (Tokyo, Japan). Freund's complete adjuvant was obtained from Difco (Detroit, MI, USA). Rabbits (6 weeks old, female) were purchased from Charles River (Yokohama, Japan). The Hi Trap Protein G column and PD-10 column were purchased from Amersham Bioscience (Piscataway, NJ, USA). All other reagents were of analytical-reagent grade. All buffer solutions were prepared using water purified with a Milli-Q filter (Millipore, Bedford, MA, USA) system.

2.2. Apparatus

SPR measurements were performed using the Biacore J-2000 surface plasmon resonance biosensor (Uppsala, Sweden). The sensor chip used was a CM5 (Biacore). ELISA measurements were performed using 96-well immunoplates (NUNC, No. 446612, Roskilde, Denmark) and a microplate reader (Spectra 1, Wako, Osaka, Japan). Spectrophotometric measurements were performed by using a Shimadzu Multi Spec 1500 (Kyoto, Japan).

2.3. Preparation of TNP-OVA, TNP-glycine-OVA, and TNP-β-alanine-OVA conjugates

TNBS (1 mg/ml H_2O, 1 ml) was reacted with 1 ml of 480 mM $NaHCO_3$ solution (pH 8.5) containing 10 mg OVA for 2 h at 40 °C. After the reaction, the preparation was dialyzed against five changes of H_2O at 4 °C for 3 days, and then lyophilized to produce TNP-OVA conjugate (9.4 mg yield). The molar ratio of the combined TNP moieties to OVA was estimated to be approximately 13.8 mol-TNP/mol-OVA using the molar adsorption coefficient of TNP-NH (1.1×10^4 cm^{-1} M^{-1}) [16].

TNP-glycine-OVA and TNP-β-alanine-OVA conjugates were prepared as follows. In 1 ml of *bis*(2-methoxyethyl) ether, 25 mg (88 μmol) of TNP-glycine or 26 mg (91 μmol) of TNP-β-alanine and 13 mg (111 μmol) of NHS were dissolved. Fifty milligrams (352 μmol) of sodium sulfate were added, and the mixture was cooled to 0 °C. To the mixture were added 34 mg (177 μmol) of EDC, and the reaction mixture was allowed to warm to room temperature and stirred overnight. Ten mg of OVA were dissolved in 1 ml of 25 mM borate buffer (pH 8.0). At intervals of 30 min, three portions of the NHS-ester solution (each 62 μl) were added slowly to the reaction mixture under intense stirring. After the reaction, the mixture was dialyzed against three changes of H_2O at 4 °C, and then lyophilized to produce TNP-glycine-OVA conjugate (TNP-gly-OVA) or TNP-β-alanine-OVA (TNP-β-ala-OVA).

2.4. Preparation of TNP-KLH conjugate

TNBS (1 mg/ml H_2O, 1 ml) was reacted with 1 ml of 480 mM $NaHCO_3$ solution (pH 8.5) containing 10 mg KLH for 2 h at 40 °C. After the reaction, the preparation was dialyzed against four changes of H_2O at 4 °C for 2 days, and then lyophilized to produce TNP-KLH conjugate.

2.5. Synthesis of TNP-derivatives

TNP-derivatives were synthesized according to our previous report [17].

2.6. Immunization

A rabbit was immunized with TNP-KLH conjugates according to the following procedure. Conjugates dissolved in PBS (0.3 mg/ml) were emulsified with an equal volume of Freund's complete adjuvant. On days 0, 14, 28, 42 and 56, 2 ml of the