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Identification of Dinitrotoluene Selective Peptides by Phage Display Cloning

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Biomolecules specific to explosives can be exploited as chemical sensors. Peptides specific to immobilized dinitrotoluene (DNT) were identified using a phage display library. A derivative of DNT that contained an extended amine group, 4-(2,4-dinitrophenyl)butan-1-amine, was synthesized and immobilized using a self-assembled monolayer surface on gold. Filamentous M13 phages displaying random sequences of 12-mer peptides specific to the immobilized DNT-derivate were isolated from the M13 phage library by biopanning. A common peptide sequence was identified from the isolated phages and the synthesized peptides showed selective binding to DNT. When the peptide was immobilized on a quartz crystal microbalance (QCM) chip, it showed a binding signal to DNT, while toluene barely showed significant binding to the QCM chip. These results demonstrate that peptides screened by biopanning against immobilized DNT can be useful for quick and accurate detection of DNT.

Key Words: Explosive, DNT, Sensor, Peptide, Phage display

Introduction

Trinitrotoluene (TNT) is one of the most widely used explosives and a major target molecule for explosive sensors. The ability to detect explosives in the air with a high level of sensitivity is a primary concern for those entrusted with the protection of civilians from the uncontrolled use of explosives. To protect both civilian and military populations, as well as to clean potentially contaminated areas, a sensitive, selective, and fast method of detecting TNT or dinitrotoluene (DNT), a major volatile contaminant of trinitrotoluene, in air or aqueous samples is required. Conventionally, explosives are detected using gas chromatography coupled with mass spectrometry, high-performance liquid chromatography (HPLC), or other analytical devices which are expensive, time-consuming, or lack sensitivity (1, 2). To detect TNT-based explosives, more sensitive and rapid methods need to be developed.

Various sensitive detection methods for DNT or TNT have been developed. Optical sensors for TNT using TNT-selective fluorescent labeled polymers (3) or gold nanoparticle-based surface enhanced Raman spectroscopy probes (4) have been applied to assay TNT in soil or wastewater samples. To enhance selective recognition of TNT, biopolymers such as RNA aptamer (5) have been developed. Polyclonal antibodies raised against DNT can be applied for detection of DNT in solution by enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance methods (6, 7). These techniques show selectivity against other compounds containing nitro groups, and detection limits of submicromolar concentrations of DNT or TNT. The biopolymers demonstrated high sensitivity in aqueous solutions and could be applied to detect TNT or DNT contamination in water or soil; however, they have limitations when it comes to direct detection of TNT or DNT in the air since RNA aptamers or antibodies are assumed to have limited stability in the gas phase.

Short peptides specific to DNT have been developed for direct detection of explosives in the gas phase since DNT has a much higher vapor pressure than TNT (8). Peptides with high affinity to solid DNT or TNT were isolated by a phage display screening method using solid DNT or TNT as bait. These peptides showed specific binding to TNT or DNT in solution as well as in the gas phase (9). Phage display screening has been successfully applied to identify peptides or proteins that bind specifically to a small molecule as well as biopolymer (10). Phage clones displaying specific peptides or proteins targeting small molecules are typically screened using immobilized surface target molecules. To identify a peptide with high affinity to a target molecule an optimized interaction between the target molecule and the peptide displayed on the phage surface is required. A derivative of DNT connected to a solid support with a linker would provide maximum interaction with the displayed peptide of the phage particle.

In this study, we immobilized a DNT derivative, which has a reactive amine group linked to a benzene ring on a gold surface, and identified a DNT-interacting peptide using a phage library displaying random 12mer peptides on the surface of a M13 phage. The identified peptides were synthesized and showed specific binding to DNT in the aqueous phase at micromolar concentrations.

Materials and Methods

Materials. Gold coated plates were obtained from KMAC (Korea). 2,5-Dioxopyrrolidin-1-yl 23-mercapto-3,6,9,12-tetraoxatricosan-1-oate was purchased from COS Biotech (Korea). The M13 phage library displaying random 12mer peptides at the N-terminus of pIII protein was purchased from New England Biolabs Inc. (USA). Gold-coated chips were obtained from Applied Biochem (Sweden). Peptides were purchased from Peptron Inc. (Korea). All other reagents were of reagent grade.

Synthesis of 4-(2,4-dinitrophenyl)butan-1-amine. An amine substitute of DNT, 4-(2,4-dinitrophenyl)butan-1-amine, was

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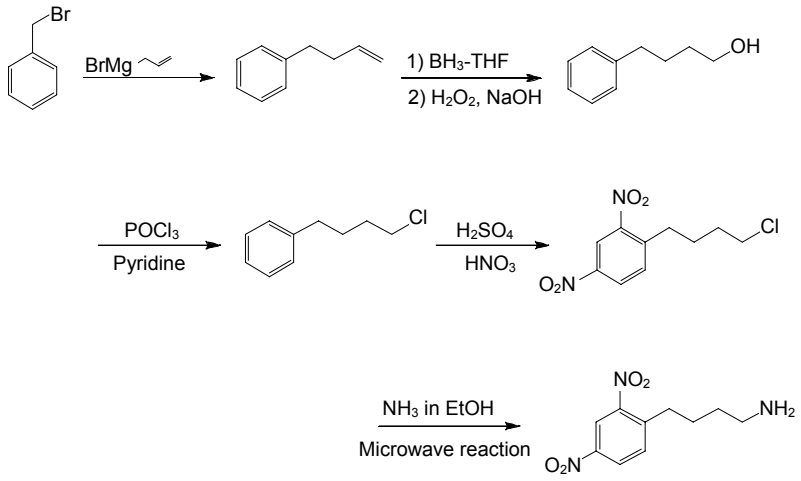

Figure 1. Synthetic scheme of 4-(2,4-dinitrophenyl)butan-1-amine from benzyl bromide.

synthesized from benzyl bromide (Fig. 1). Equal equivalents of benzyl bromide and allylmagnesium bromide were reacted in diethyl ether for 2 hr at 50°C. The product, 1-(but-3-enyl) benzene, was then reacted with BH_3-THF, NaOH, and H_2O_2 to produced 4-phenylbutan-1-ol. 1-Chloro-4-phenylbutane was synthesized by reacting 4-phenylbutan-1-ol with $POCl_3$ and pyridine, and then converted to 1-(4-chlorobutyl)-2,4-dinitrobenzene by reacting with 98% H_2SO_4 and 70% HNO_3. The 1-(4-chlorobutyl)-2,4-dinitrobenzene was then reacted with ammonia in water in a microwave reactor for 20 min at 120 °C.

Immobilization of a DNT derivative on a gold surface with biopanning. Synthesized 4-(2,4-dinitrophenyl)butan-1-amine was immobilized on a gold-coated surface self-assembled monolayer containing an amine reactive group as previously described (11). Briefly, the gold-coated surface was washed with ethanol and reacted with 2 mM of 2,5-dioxopyrrolidin-1-yl 23-mercapto-3,6,9,12-tetraoxatricosan-1-oate in ethanol for 14 hr at room temperature. After washing with ethanol, the gold surface was incubated with 1 mM of 4-(2,4-dinitrophenyl)butan-1-amine and 3 mM of glycine in 50 mM $NaHCO_3$ (pH 9.0) for 14 hr at room temperature. After washing the surface, the gold plate immobilized with DNT derivative was incubated with 100 μL of a M13 p12 phage display library (1.5×10^{12} pfu/mL) in Tris buffered saline (TBS) buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 hr at room temperature. After washing the gold surface 10 times with TBS buffer, the bound phage was eluted after a 10 min incubation with 0.1 mL of 0.2 M glycine solution (pH 2.2). Eluted phages were amplified following infection with *E. coli* strain ER2738 according to the manufacturer's instructions (New England Biolabs, USA). Amplified phages were applied once more to the gold surface immobilized with the DNT derivative for the second round of biopanning and repeated washing, elution, and amplification. Phages bound to the surface were washed with 0.2% Tween 20 in TBS buffer at the third to fifth round of biopanning.

DNA sequencing and peptide synthesis. After the fifth round, phages were infected to *E. coli* strain ER2738 and plated on agar plate. The phage DNA from individual phage plaques was purified using DNA isolation kits (Qiagen, USA). The sequence of isolated phage DNA was determined and the displayed peptide sequences subsequently deduced.

Isothermal titration calorimetry analysis. Isothermal titration calorimetry (ITC) experiments were performed using VP-ITC and ITC200 instruments (MicroCal LLC, USA) at 25°C (12), and the data analyzed using the Prism 5.02 program (GraphPad, USA). DNT and DNT-BP1C, prepared in 10% TBS buffer (100 mM Tris-HCl, 150 nM NaCl, pH 7.5) and 90% acetonitrile, were degassed at 25°C prior to measurements. Using a microsyringe 5 μL of 100 μM DNT solution was added at intervals of 150 s to the peptide solution in the cell with gentle stirring. Experimental raw data were corrected for dilution by subtracting the value for buffer alone and fit by a one-site binding model.

Quartz crystal microbalance analysis. Quartz crystal microbalance with dissipation monitoring (QCM-D) was used to measure the specific binding of DNT to the screened peptide DNT-BP1C (13). The QCM chip has a gold electrode layer sequentially coated with alkanethiol, oligo (ethylene glycol) (OEG): 4,7,10-trioxa-1,13-trideanedeamine), cross linker (GMBS: *N*-[γ-maleimidobutyryloxy]succinimide ester), and DNT-BP1C. A solution of 0.55 mM DNT and toluene was prepared using 5% dimethyl sulfoxide (DMSO) buffered with phosphate buffered saline (PBS). The flow rate was 0.1 mL/s and the QCM chamber temperature was 25°C.

Results and Discussion

Synthesis and immobilization of DNT derivatives. Explosive nitro compounds were characterized by a high nitro group content in the molecular structure. Peptides that can efficiently detect DNT should recognize the nitro groups attached to benzene ring structures. A derivative of DNT, 4-(2,4-dinitrophenyl) butan-1-amine, in which the methyl group was extended by four carbon units with an amine group was synthesized (Fig. 1). The structure of the synthesized DNT derivative was confirmed by nuclear magnetic resonance (NMR) spectrometry (data not shown). The amine group of the DNT derivative was covalently attached to the *N*-hydroxysuccinimide (NHS) group linked to the self-assembly monolayer (SAM) on a gold surface (Fig. 2).

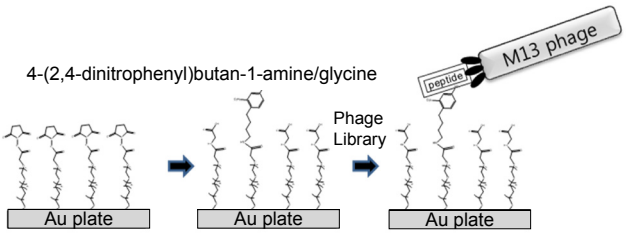

Figure 2. Schematic representation of immobilized 4-(2,4-dinitrophenyl)butan-1-amine on a gold plate and biopanning with a peptide display M13 phage library. The gold plate was incubated with NHS(*N*-hydroxysuccinimide)-thiol overnight, and 4-(2,4-dinitrophenyl)butan-1-amine and glycine at a 1:3 molar ratio were then incubated for 3 hr at pH 9.0.

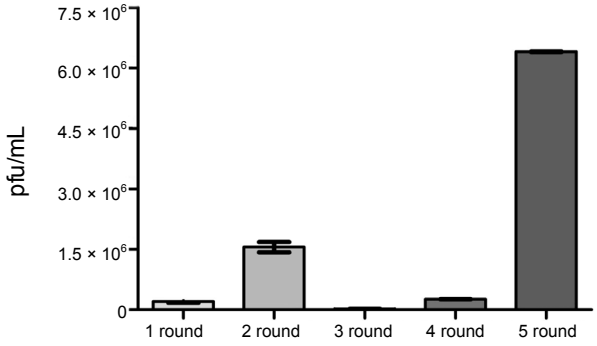

Figure 3. Amplification of phages during each round of biopanning. The phage titer was determined for each washing and eluted solution at each round.

Table 1. Sequences of identified peptides and synthetic peptide for analysis

Peptide	Sequence	Frequency[a]
DNT-BP1	H_2N-KMHTASLSQPLM-COOH	19/20
DNT-BP2	H_2N-TSDIKSRSPHHR-COOH	1/20
DNT-BP1C	CH_3ONH-KMHTASLSQPLMGC-$CONH_2$	
DNT-peptide[b]	H_2N-HPNFSKYILHQR-COOH	

[a]The number of identical peptides among the 20 phages isolated by biopanning. [b]The sequence of previously identified DNT-specific peptide (ref. 9).

The formation of a SAM and attachment of peptide on the SAM surface was confirmed by measuring the contact angle of the modified surface due to the attachment of hydrophilic peptides (data not shown).

Identification of DNT specific peptides by biopanning a phage display library. A M13 phage library displaying random sequence of 12-mer peptides on the N-terminus of pIII was screened for peptides binding specifically to the immobilized DNT derivative linked to a SAM on a gold surface. The phage titer of the eluted solutions increased from 10^5 pfu/mL after the first round to 6×10^6 pfu/mL at the fifth round of biopanning (Fig. 3). The phage titer decreased at the third round of biopanning when the surface was washed with high stringency buffer containing 0.2% Tween 20. However, the phage titer increased at the fourth and fifth round of biopanning indicating that a fraction of M13 phages were selectively amplified during the biopanning process. The phages eluted from the fifth round were infected to *E. coli* ER2537, and the phages from 20 plaques were isolated. The DNA sequences of the isolated phages were determined and the peptide sequences displayed on the phages were deduced. Among the 20 phages, the peptide sequences from 19 were identical (DNT-BP1) and the other phage showed a quite difference sequence (DNT-BP2) compare to DNT-BP1 (Table 1). The highly degenerated peptide sequence indicated that phages displaying DNT-specific peptides were selectively amplified and isolated. It should be noted that the identified sequences (DNT-BP1 and DNT-BP2) had a high percentage of positively charged residues such as histidine, lysine, or arginine. The sequences of these peptides lacked a sequence motif in common with the previously identified DNT-specific peptide (DNT-peptide, Table 1) screened against DNT in the solid state (9). However, a high percentage of positively charged and hydrophilic amino acids were commonly observed. The positively charged residues in these peptides might be involved in interaction with the nitro groups of DNT. These peptide characteristics suggest that the positive or hydrophilic residues of these peptides constitute a binding motif to DNT and a well-formed 3-dimensional structural motive.

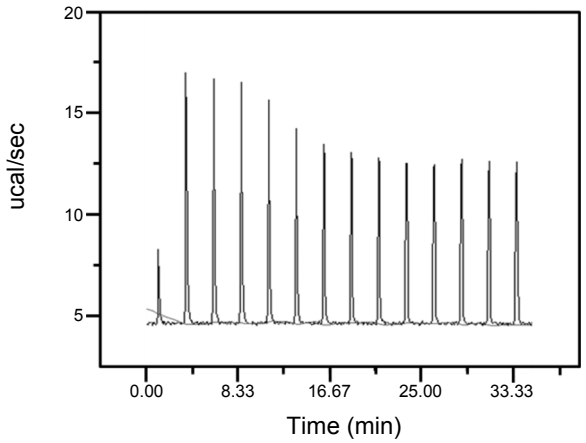

Figure 4. Isothermal titration calorimetry (ITC) analysis of the interaction between DNT-BP1C and DNT. Heat generated from the DNT-BP1C peptide in 90% acetonitrile solution was measured when 5 μL of 100 μM DNT solution was added at intervals of 150 s.

Characterization of the binding characteristics of DNT-BP1C to DNT. The peptide sequence identified from biopanning was synthesized to examine its interaction with DNT. A 14-mer peptide containing the identified sequence of DNT-BP1 with glycine and cysteine residues at the C-terminus (DNT-BP1C) was synthesized and purified using HPLC. The identity of the purified peptide was confirmed by mass spectrometry (data not shown). The affinity of the peptide was examined by ITC. The peptide showed binding isotherm when different concentrations of DNT were added to the DNT-BP1C peptide (Fig. 4). The apparent dissociation constant of DNT to the peptide was calculated as 1.5×10^{-6} M. The affinity of DNT-BP1C was about 20-fold lower than the affinity of a previously reported TNT-specific peptide against TNT (9). The selectivity

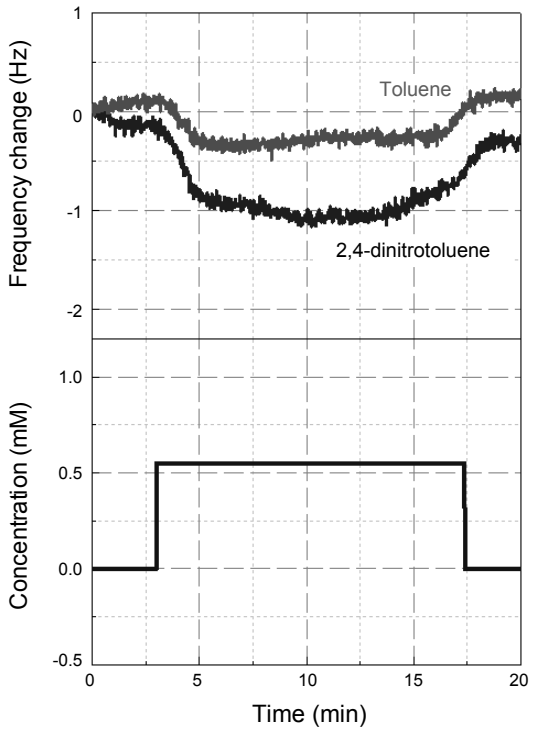

Figure 5. Quartz crystal microbalance (QCM) analysis of aqueous phase DNT and toluene using DNT-BP1C. DNT, 0.55mM (lower line), or toluene (upper line) solution in 5% DMSO was applied to the QCM chip immobilized with DNT-BP1C for 15 min and the frequency change measured. The period of sample application is indicated in the bottom panel.

of DNT-BP1C against DNT over toluene was also examined. The binding of DNT and toluene to surface immobilized DNT-BP1C was measured using QCM. The frequency shift for 0.55 mM DNT was measured at 1.0 Hz, and this frequency shift signal was recovered following the injection of purging solution (Fig. 5, lower line). In contrast, toluene produced only a marginal frequency shift (Fig. 5, upper line) indicating that DNT-BP1C has at least 3.3 times higher selectivity over toluene in the solution phase.

Conclusion

We have identified DNT-specific peptides from biopanning of phage displayed library using a derivative of DNT immobilized on gold surface. The identified peptides showed distinctive sequence compared to the peptides screened against solid DNT suggesting that these peptides have different mode of interaction with DNT. Further analysis of the specificity and detection limits of these identified peptides in the gas phase is required to verify the efficiency of this screening method and the potential application of these peptides as explosive sensors.

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