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Development of a herbicide biosensor using a peptide receptor screened from a combinatorial library

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Abstract

The purpose of this study was to screen for peptides that bind herbicides with a chlorinated aniline chemical structure. A tetrapeptide library was constructed using a solid phase split synthesis approach. Peptide beads were suspended in a buffer containing fluorescent-labeled dichloroaniline (DCA) as the bait. Eighteen fluorescent peptide beads were selected which bound to the bait after two rounds of staining screenings. The beads were then stained and suspended in a solution containing an excess of DCA and five quenched peptide beads were subsequently selected that recognized the DCA moiety. The screened peptides had many sequence similarities. The binding affinity of the screened peptides to herbicides was analyzed using surface plasmon resonance (SPR). *N′*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea [3-(3,4-dichlorophenyl)-1,1-dimethylurea] solution was injected over the peptide immobilized SPR chip. The SPR signal was found to increase in proportion to the DCMU concentration, whereas no signal was obtained from the negative control, 2-(2-methyl-4-chlorophenoxy) propionic acid (MCPP). From these results it is suggested that the screened peptide selectively recognizes the chemical structure of DCA.
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1. Introduction

The herbicides diuron (DCMU) [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine) are widely used for crop protection. However, due to their widespread use they largely contribute to water pollution on a worldwide scale [1,2]. In Japan, usage of these chemicals is controlled by several laws and although the use of atrazine is decreasing, production and consumption of DCMU is increasing. The structures of these chemicals include a partial dichloro- or monochloro-aniline structure, which is similar to the structure of endocrine-disrupting polychloro-hydrocarbon molecules. Indeed, it has been reported that atrazine causes a significant decrease in the testosterone levels of male African clawed frogs (*Xenopus laevis*) [3]. Due to the potential problems associated with these trace organic chemicals, excess quantities in field runoff may have an adverse effect on the environment and thus a need exists to develop a system which can detect such chemicals in the environment.

We have previously tried to construct systems that can detect trace organic chemicals using proteins or peptides [4]. In a previous study, the photosynthetic reaction center of the purple bacterium was used to detect herbicides using surface plasmon resonance (SPR). Although the system was able to assess the specific concentration of the herbicides, the sensing material proved to be complicated and expensive to use. We have therefore in this current study tried to develop an alternative system using polypeptides that specifically bind to herbicides.

Recently, the introduction of combinatorial or bio-combinatorial screening methods has offered a rapid means for screening peptide sequences with desired functions [5–7]. Various approaches have been refined and sophisticated methods have been used to enable the identification and isolation of peptides with specific functions from a large

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pool of synthesized peptides. We anticipate that the use of such methods might provide a peptide sequence that can bind to specific herbicides and ultimately be incorporated into a biosensor. Since herbicides and our intending target are likely to be hydrophobic, organic solvents may be necessary to solubilize the target molecules during the screening and washing stages. For this reason we have adopted a peptide library that is chemically synthesized on a solid-phase. In bio-combinatorial screening, such as phage-display, physiological conditions must be used since polypeptide synthesis relies on the activity of the phage. By using chemically synthesized beads during the screening process, we can fine-tune the screening conditions by controlling the organic solvent content at the screening stage as well as using suitable organic solvents for solubilization of the ligand. Taking into account the balance between library diversity and efficiency of isolation, we believe a practical peptide length of less than six residues is required for a combinatorial random library with 19 amino acids. A combinatorial library with such short peptides can be synthesized rapidly and inexpensively compared to proteins or protein complexes. In this study, we have screened peptides that bind dichloroaniline groups and have analyzed the binding of herbicides to the peptides by SPR.

2. Experimental

2.1. Materials

Peptide resins as well as coupling reagents were purchased from Shimadzu. Fmoc-amino acids were purchased from PE biosystems. Herbicides were purchased from Wako chemicals. Other reagents and solvents were purchased from Wako chemicals and Nakalai tesque. The BIAcoreX instrument, sensor chip CM5, HBS buffer and amine-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (1-ethyl-3-dimethyl aminopropylcarbodiimide), ethanolamine hydrochloride, 2-(2-pyridinyldithio) ethaneamine (PDEA) were obtained from Biacore AB.

2.2. Preparation of tetrapeptide library

The peptide resins were synthesized using a 'split-and-mix' method using 19 separated vessels for each amino acid without a Cys. The reactions were based on Fmoc solid-phase peptide synthesis (Fmoc-SPPS) [8]. One hundred milligrams of resin per vessel were subjected to synthesis such that the number of peptide beads surpassed the sequence number (1.3×10^5). The resin was reacted with 20% piperidine/dimethylformamide (DMF) solution for deprotection of the Fmoc group following reaction with Fmoc-amino acid/PyBOP/HOBt for coupling of the amino acids (PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; HOBt, 1-hydroxy-1-H-benzotriazole). After washing, all the resins were mixed and divided into the 19 vessels to continue with the next stage of the coupling. After the final coupling stage, the resins were kept separated to enable identification of the N-terminal amino acid of each vessel. The peptide resins were deprotected with trifluoroacetic acid (TFA)/thioanisole/H_2O/ethanedithiol/ethylmethyl sulfide/phenol (82:5:5:3:2:3) and the resins were washed with TFA, diethylether and DMF.

2.3. Synthesis of dye-target conjugate, DCA–NBD

4-Amino-*n*-butyric acid (1 mmol) in 50 mM boric acid buffer (pH 8.0) was mixed with 4-fluoro-7-nitrobenzofurazan (NBD-F, 0.1 mmol) in acetonitrile and reacted for 1 min at 60 °C. The resulting compound was purified by reverse-phase-HPLC (RP-HPLC) using and identified by ESI-MS to give an expected ion peak at *m*/*z* 249.1. The carboxylate group of the compound was activated by using NHS (0.1 mmol) and EDC (0.1 mmol) in 50 mM boric acid buffer (pH 6.5). The activated compound was then reacted with 3,4-dichloroaniline (DCA) overnight. The resulting DCA–NBD conjugate was purified by RP-HPLC and identified by ESI-MS to give an ion peak at *m*/*z* 412.1.

2.4. Screening of DCA binding peptides

(i) *First screening*: The peptide beads were swollen for 1 h with the screening buffer 10 mM phosphate/100 mM NaCl (pH 7.0). The beads were then suspended in 1 μM DCA–NBD for 3 h at room temperature. After removal of supernatant, the fluorescent beads were extracted by capillary action under observation using a fluorescent inverted microscope IX-70 (Olympus) equipped with filter cube, U-MWB (BP450-480, DM500 and BA515). The fluorescence of the beads were not quantified but distinguished by the naked eye. The obtained peptide beads were then washed with ethanol. After washing, the beads that still had fluorescence were excluded since they bound DCA–NBD irreversibly.

(ii) *Second screening*: The obtained beads were suspended in the screening buffer containing 5% ethanol. The fluorescent beads were extracted in the same manner as for the first screening.

(iii) *Third screening*: The stained beads were subjected to a competitive reaction in order to select only the peptides binding to the DCA moiety of the DCA–NBD. The beads stained with DCA–NBD were suspended in 5 mM DCA solution for 3 h and those beads which showed decreased fluorescence were selected. The sequences of four residues from the N-terminus were determined using an automatic standard Edman degradation in a PPSQ-23 sequencer from Shimadzu.

2.5. SPR experiments

A running buffer (HBS-EP; 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate20) was

used to dilute all the test species in the BIACORE experiments. Five to 20 mM herbicide stock solutions in ethanol were diluted from 1 to 0.25 mM in 5% ethanol prior to injection.

2.6. Immobilization of peptide D1C–D5C on the sensor surface

The peptides, D1C–D5C were synthesized using Fmoc-SPPS with Fmoc-Cys (Trt)-PEG-PS on an automatic peptide synthesizer (PSSM8, Shimadzu). After deprotection and cleavage from the resin, the peptides were solidified with cold diethylether and dried in vacuum. The resulting crude peptides were purified by RP-HPLC. The peptides were covalently immobilized on the surfaces of the CM5 sensor chips using a standard ligand–thiol coupling procedure and the flow rate was controlled at 5 μl/min. Specifically, surface carboxylic acid groups were activated by injection of 40 μl of a mixture containing 0.05 M NHS and 0.2 M EDC, followed by PDEA injection (80 μl of 80 mM PDEA in 100 mM boric acid, pH 8.5). The peptides (2 mM, 100 μl) were injected under manual control until the desired amount of peptide was coupled. Finally, freshly prepared 0.05 M L-Cys (80 μl, in 1 M NaCl, 0.1 M sodium formate, pH 4.3) was injected to block any remaining activated sites on the sensor chip surface. Control surfaces were modified in a similar manner, except that the running buffer was injected instead of the peptides.

2.7. BIACORE kinetic assays

All kinetic experiments were carried out at 25 °C with a flow rate of 20 μl/min. Different concentrations of the herbicides were injected over the peptide surface as well as over the control surface (60 μl injections, 3 min analyses) following replacement of the analyte solution with buffer (3 min analyses) and the response signals measured. The same measurements were repeated three times. The response signals in Figs. 2B and 4A were divided by the amount of immobilized peptide to compare the response between the different peptides. The amount of each immobilized peptide was estimated from the increase in response signal after injection of the peptides (1200–1800 RU). The sensor chip was regenerated by injecting 0.5% SDS after each injection. The data were analyzed using BIAevalution software (BIAcore AB). Kinetic analysis of each ligand receptor interaction was obtained by fitting the response data to a reversible 1:1 bi-molecular interaction model. The association constant (K_a) was calculated from the ratio of the dissociation rate (k_d) and association rate (k_a). Constants reported in Figs. 3 and 4 represent the average of three analyses at least for each peptide/ligand interaction by using the identical chip.

3. Results and discussion

In this study, we have screened for peptides that recognize and bind to a partial structure of DCMU from a combinatorial library of tetrapeptides on a solid-phase. We used the dichloroaniline (DCA) group as bait after consideration of the chemical structure of DCMU and its synthesis simplicity. In order to screen peptides that bind the DCA group, a fluorescent-labeled dichloroaniline molecule (DCA–NBD) was synthesized by conjugating a dye with dichloroaniline through a linker (Fig. 1). We selected a NBD moiety for the dye because of its small chemical structure and large Stokes shift.

Scheme 1 illustrates the procedure for screening the DCA-binding peptides from the tetrapeptide library. The library was constructed by standard Fmoc-SPPS with a 'split-and-mix' procedure. According to this method, all possible 19^4 (1.3×10^5) sequences can be synthesized on 4.3×10^5 beads. After a final elongation with Fmoc-amino acids, the peptide beads were deprotected, washed carefully and dried. At first, the peptide-beads were stained with DCA–NBD by suspending in a screening buffer ([DCA–NBD] $= 0.18\,\mu M$) for 3 h. Three hundred and ninety nine fluorescent beads were selected from the peptide library which bound to the DCA–NBD conjugate. The screened beads were then washed and subjected to another round of screening using screening buffer containing 5% ethanol in order to select for peptide beads that bound the DCA–NBD. In this second round of screening, the number of positive beads reduced to 18. Finally, a competitive reaction was conducted to exclude peptide sequences that bound the fluorescent label, NBD. The 18 beads stained with DCA–NBD were suspended in buffer containing 5 mM

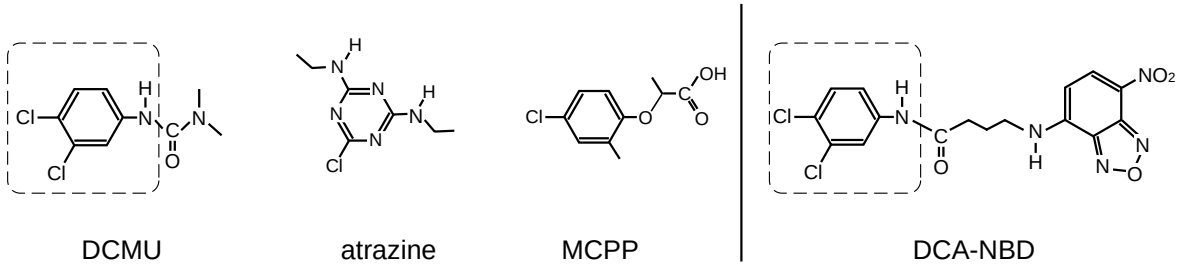
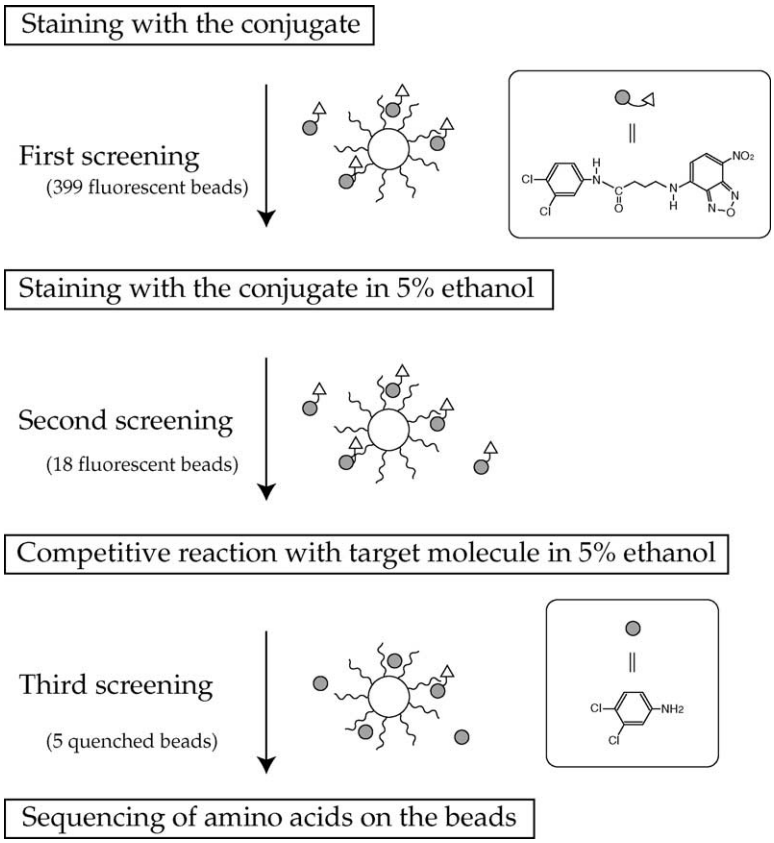


Fig. 1. Chemical structures of the herbicides and fluorescent-labeled dichloroaniline as the bait.



Scheme 1. Schematic representation of staining and competitive reaction of the peptide beads during the screening procedures.

DCA. Of these beads only five showed fluorescence quenching, suggesting that the fluorescent DCA–NBD molecules that were retained on these five beads dissociated under equilibrium conditions in the presence of DCA.

The sequences of the screened peptide beads were analyzed on an automated peptide sequencer. Table 1 shows the sequences of the screened peptides. Interestingly, all sequences contained an Asp residue at the N-terminus but had no further charged residues throughout the rest of the sequence. All the sequences were found to have a negative charge at the side-chain of the Asp residue and a positive charge at the N-terminus, giving a net charge of zero. Their hydrophobicities varied largely and ranged from −0.43 (D1) to 2.64 (D4) according to a conventional hydrophobic scale [9]. These results suggest that the hydrophobicity of the overall polypeptide chain is not the sole factor in determining the binding ability of the peptides. The second amino acid showed un-charged residues, including the hydrophilic residues in D1 (Thr2), D3 (Asn2) and D5 (Gln2). These residues might play a role in forming hydrogen bonds between the side-chain and the ligand or between the side-chain and the main-chain of the peptides. The sequences also appeared to have remarkable similarities with regards to three residues at the C-terminus, in that one or two aromatic residues, such as Tyr or Phe, appeared at the C-terminal region of D1 (-Tyr3-Tyr4-), D2 (-Phe2-Tyr3-), D3 (-Tyr4-) and D5 (-Phe3-). Aliphatic residues also appeared in the region of D3 (-Ile3-), D4 (-Val2-Ile3-Val4-) and D5 (-Leu4-). Since the target molecule has an aromatic and hydrophobic dichlorobenzene group, it might interact with the hydrophobic residues around the C-terminus.

Table 1
Peptide sequences of the selected beads D1–D5 and sequences of the designed peptides E1 and N1

Peptide	Sequence
D1	Asp-Thr-Tyr-Tyr-
D2	Asp-Phe-Tyr-Ala-
D3	Asp-Asn-Ile-Tyr-
D4	Asp-Val-Ile-Val-
D5	Asp-Gln-Phe-Leu-
E1	Glu-Thr-Tyr-Tyr-
N1	Asn-Thr-Tyr-Tyr-

For the purpose of investigating binding of the DCA group to the peptides, we studied the interaction between the peptides and DCA derivatives using surface plasmon resonance (SPR). SPR allows monitoring of biomolecular interactions in real time and enables determination of binding kinetics and affinities without the need for fluorescent-labeled ligands. Thus, if the SPR chip bearing the peptides produces a sufficient signal upon binding of the herbicides, the chip can be directly used as a biosensor. In order to immobilize

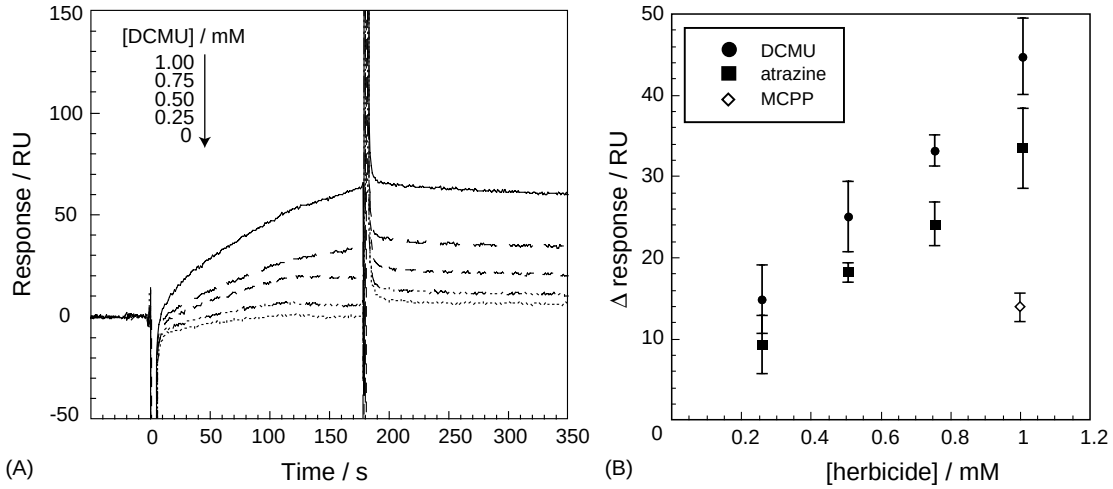


Fig. 2. (A) Sensorgrams obtained from injections of DCMU at various concentrations over the D1C peptide surface. (B) Sensorgram responses after 180 s that were plotted against the herbicide concentrations.

the peptides a Cys residue was attached to the C-terminus of the peptides. The Cys-containing peptides D1C–D5C were synthesized using standard Fmoc-SPPS and purified with RP-HPLC. The peptides were immobilized on the CM5 sensor chip using a standard ligand–thiol coupling procedure.

We tested the ability of peptide D1C to bind to the DCMU molecule by injecting different concentrations of DCMU (from 0 to 1 mM) over the D1C sensor chip surface. A reference surface without immobilized peptide was used as a control. Fig. 2A shows the sensorgrams obtained after data preparation by subtraction of the reference cell data. The binding responses proved to be reproducible and concentration-dependent. Fig. 2B displays plots of the binding responses between the D1C sensor chip and various herbicides. The increase in response was greater with DCMU than either atrazine or MCPP. In particular, the response with MCPP was very small, suggesting that the peptides bound molecules containing a chlorinated aniline ring, such as DCMU or atrazine. These results imply that the screening process carried out in this study was successful since the selected peptide D1C was able to recognize the chemical structure of the target molecule.

From the SPR measurements we were able to analyze the kinetic parameters for binding DCA to the peptides. Fig. 3 shows plots of the kinetic parameters for the peptides with the herbicides. We were however unable to obtain parameters for D3C since its response deviated significantly from the average values. According to the association constant K_a, the peptides D1C, D2C and D4C showed selectivity for DCMU which posses a chemical structure of DCA. The ratios of K_a (DCMU)/K_a (atrazine) were calculated to compare the specificity of the peptides to DCMU. The ratios for

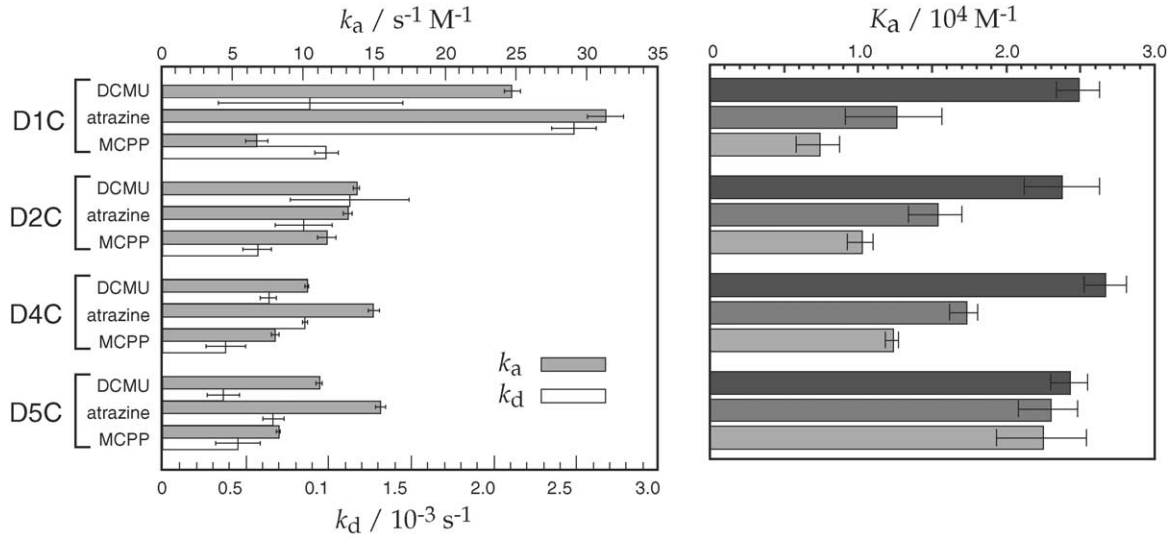


Fig. 3. Plots of the kinetic parameters for peptides (D1C, D2C, D4C and D5C)/herbicide interactions. k_a, association rate constant; k_d, dissociation rate constant; K_a, association constant.

D1C, D2C, D4C and D5C were 1.98, 1.55, 1.54 and 1.06, respectively, indicating that D1C was the most specific binder for DCMU. Although the association rate of atrazine for D1C was larger than that of DCMU, the dissociation rate of atrazine was also large, resulting in the association constant of DCMU with D1C being the largest. The low affinities of D1C, D2C and D4C for MCPP were due to the large k_d values, which contributed to the specificity of the peptides for DCMU. Among the peptides, only D5C showed less specific binding for DCMU. Although D5C bound DCMU with an association constant that was similar to the others, it also bound atrazine and MCPP at the same level. Using only the data from this present study we are unable to predict the structure of the peptides, but we can speculate that specificity for the ligands is possibly due to amino acids that restrict the conformation of the main-chain, such as aromatic residues (Tyr and Phe) and β-branched residues (Val and Ile). The conformation of D5C may be relatively flexible due to amino acid side-chains (D, Q and L) and would explain why the specificity of D5C was lower than that of the other sequences. Thus, only the screened peptides D1C, D2C and D4C bound specifically to DCMU and of these peptides D1C was found to be the best binder.

In order to examine the importance of an Asp residue at the N-terminus, modified peptides E1C (ETYYC) and N1C (NTYYC) were designed and synthesized. Fig. 4 shows the response of the peptides with various concentrations of DCMU. E1C showed an increase in response depending on the concentration of DCMU, whilst N1C showed little increase in response. These results strongly suggest that an N-terminal amino acid bearing a carboxylate group is crucial for binding of DCMU. The carboxylate group may play a role in controlling peptide conformation by electrostatic interactions with the N-terminal amine or by forming hydrogen bonds with the ligand or peptide chain. The specificity of E1C for DCMU was different to that of D1C. The association constant of E1C for atrazine was similar to that for DCMU, but was significantly smaller for MCPP. The mutation from Asp to Glu modulated the specificity for the herbicides despite a comparable binding ability to DCMU.

Thus, the question remains as to why peptide sequences such as E-X-X-X do not survive. We believe the reason may be due to the fact that these peptide beads were not efficiently stained with DCA–NBD. The response signal of DCA–NBD over the E1C surface (4.8 RU) was smaller than over the D1C surface (12.6 RU). The first and second rounds of screening after staining were the stages in which the majority of the beads were excluded. Therefore, sequences such as E-X-X-X could not survive during the last stage of the competitive-screening. Although structural information is necessary to explain the difference between D-X-X-X and E-X-X-X, the interaction between the peptide and bait affected the survival in the present screening process.

The association constants, K_a, of the peptides for DCMU were smaller than that of the natural protein for the ligand. In particular, the association rate constants, k_a, of the peptides ($10–10^2\ s^{-1}\ M^{-1}$) were much lower than of the natural proteins. For example, the association rate constant of an estrogen receptor with an agonist ligand is about $10^6\ s^{-1}\ M^{-1}$ [10]. We believe the tetrapeptides might not provide an effective surface for the small ligands, resulting in the small association rate constant. The extension of an effective surface for the ligand is likely to improve the binding ability by applying scaffolds that can accommodate small ligands. Surface of ligand–peptide interaction can be extended by using multivalent functional surface with peptide dendrimers or repeating sequence of peptide. Moreover, predetermined secondary structure of polypeptide such like α-helices β-turn might be candidates for the scaffolds that provide an interaction surface to the target ligand.

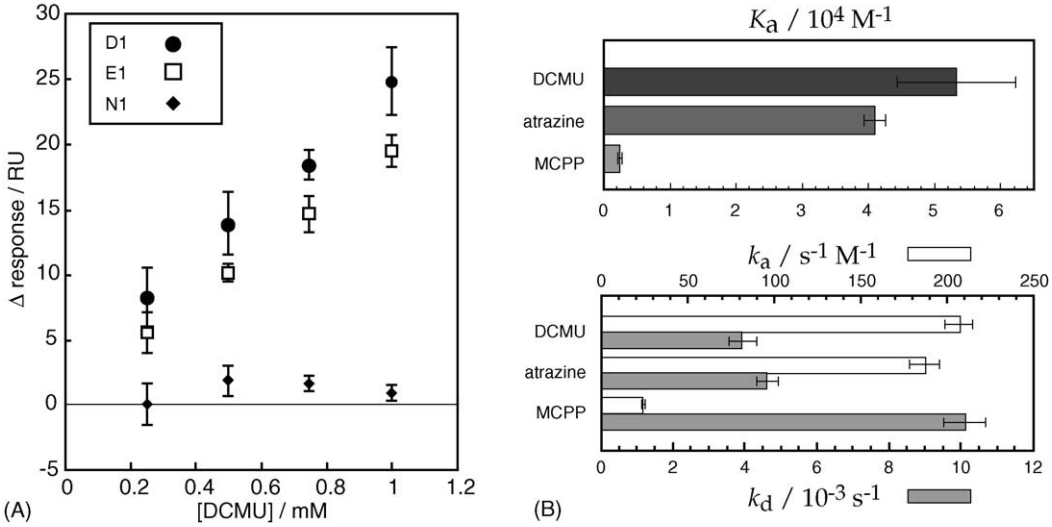


Fig. 4. (A) Sensorgram responses after 180 s obtained from injection of DCMU at various concentrations over the D1C, E1C and N1C peptide surfaces. (B) Plots of kinetic parameters for peptide (E1C)/herbicide interactions.

4. Conclusions

We have successfully obtained peptides (D1–D5) that bind to the herbicides DCMU and atrazine from a tetrapeptide library. The peptide sequences consist of hydrophobic amino acids at the C-terminus and an Asp residue at the N-terminus. SPR analyses revealed binding of the peptides to the herbicides. The peptides bound DCMU selectively and atrazine moderately. These results correspond to the chemical structure of the herbicides. The carboxylic acid group on the side-chain of Asp is crucial for binding ability as demonstrated by the results of the E1 peptide. Our findings suggest that this screening approach is applicable to other small molecules.

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