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Fingerprint-Detection of Sugar-Binding Proteins Generated by Labeled Structured Glycopeptides Arrays

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Based on a novel biochip-concept involving labeled structured peptides and the "protein-fingerprint" method, the construction of *O*-glycopeptide-array on a novel chip material and their use for biodetection are described.

Since high-throughput protein-detection and characterization systems are urgently required in the post-genomic era, we have been developing a practical biochip system using synthetic structured peptide libraries.[1–3] Recently several peptide arrays were reported for screening of epitope or discovery of ligands of proteins,[4,5] the novel concept of our system involves protein–protein interactions that are mimicked by labeled peptide–protein interactions. Visualization of interactions is not in a "one to one" manner, but as *bar-code* like patterns with fluorescent intensities generating "protein fingerprints." The structured peptide array is a sensing element for protein-structure discrimination. The use of a peptide array as a "protein-chip" affords significant advantages for industrial production and applications for practical manufacturing, storage, and delivery, compared to arrays with antibodies or recombinant proteins.[6] It is known that several toxic proteins, such as *Ricinus communis* agglutinin (RCA), cholera toxin, *staphylococcal* enterotoxin B, and *Pseudomonas aeruginosa* lectin (PA-I) recognize cell-surface carbohydrates of the host cells.[7,8] Therefore, the sensitive detection system of these carbohydrate-binding species in addition to protein-structure recognition will be an effective tool for rapid detection and may contribute to protection against various toxin-related diseases. Hence, our peptide libraries consisting of several hundreds of α-helix, β-sheet, and β-loop peptides have been expanded to incorporate glycosides.

Glycopeptides are more difficult to synthesize and purify than the parent peptides, although as mimetics glycopeptides are better than glycosides alone, which has been reported previously.[9] Two strategies have been employed to generate glycopeptides, the post-modification method and the building block strategy.[10] In the former method, mannosylamine or glucuronic acid have been coupled to the ε-amino group of Lys of an α-helical TAMRA (5,6-carboxytetramethylrhodamine)-labeled peptides, which gave non-natural type mannose- or glucose-attached α-helix peptides. In the latter method, a galactosylated Fmoc–threonine or a mannosylated Fmoc–threonine was introduced to the α-helical model peptides during the peptide assembly to produce *O*-glycoside peptides, respectively. The HPLC profiles of synthetic products showed that the latter method afforded a more favorable yield with better profiles of the desired products than the former method (Supporting Information). Thus, we have decided to employ the building block strategy for the construction of glycopeptide libraries. Their diversity arises from the use of four different glycosides (glucoside, galactoside, mannoside, and lactoside), their position and number (one or two) in addition to the backbone amino acid sequence. Four building blocks of *O*-glycosylated Fmoc–Thr (glucoside, mannoside, galactoside, and lactoside) were successfully prepared in good yields using BF_3OEt_2-mediated glycosylation of per-acetylated sugar (Supporting Information). These building blocks were then efficiently incorporated into peptides by using the Fmoc-solid phase syntheses to give glycopeptides (Figure 1). All glyco-

α-helix (1-27)

1:TAMRA-AT^{G}KAAKAAKKAAKA-GC
2:TAMRA-AKKAAT^{G}AAKKAAKA-GC
3:TAMRA-AKKAAKAAT^{G}KAAKA-GC
4:TAMRA-AT^{M}KAAKAAKKAAKA-GC
5:TAMRA-AKKAAT^{M}AAKKAAKA-GC
6:TAMRA-AKKAAKAAT^{M}KAAKA-GC
7:TAMRA-AKKAAKAAKKAAT^{M}A-GC
8:TAMRA-AT^{Ga}KAAKAAKKAAKA-GC
9:TAMRA-AKKAAT^{Ga}AAKKAAKA-GC
10:TAMRA-AKKAAKAAT^{Ga}KAAKA-GC
11:TAMRA-AKKAAKAAKKAAT^{Ga}A-GC
12:TAMRA-AT^{L}KAAKAAKKAAKA-GC
13:TAMRA-AKKAAT^{L}AAKKAAKA-GC
14:TAMRA-AKKAAKAAT^{L}KAAKA-GC
15:TAMRA-AKKAAKAAKKAAT^{L}A-GC
16:TAMRA-LKKLLT^{G}LLKKLLKL-GC
17:TAMRA-LKKLLKLLT^{G}KLLKL-GC
18:TAMRA-LKKLLKLLKKLLT^{G}L-GC
19:TAMRA-LKKLLKLLT^{M}KLLKL-GC
20:TAMRA-LKKLLKLLKKLLT^{M}L-GC
21:TAMRA-LKKLLT^{Ga}LLKKLLKL-GC
22:TAMRA-LKKLLKLLT^{Ga}KLLKL-GC
23:TAMRA-LKKLLKLLKKLLT^{Ga}L-GC
24:TAMRA-LKKLLKLLT^{L}KLLKL-GC
25:TAMRA-LKKLLKLLKKLLT^{L}L-GC
26:TAMRA-AKKAAKAAKKAAKAGC
27:TAMRA-LKKLLKLLKKLLKL-GC

β-sheet (28-54)

28:TAMRA-G-KAKAKAT^{G}A-GC
29:TAMRA-G-KAKAT^{G}AKA-GC
30:TAMRA-G-KAKAKAT^{M}A-GC
31:TAMRA-G-KAKAT^{M}AKA-GC
32:TAMRA-G-KAT^{M}AKAKA-GC
33:TAMRA-G-T^{M}AKAKAKA-GC
34:TAMRA-G-KAKAKAT^{Ga}A-GC
35:TAMRA-G-KAKAT^{Ga}AKA-GC
36:TAMRA-G-KAT^{Ga}AKAKA-GC
37:TAMRA-G-KLKLKAT^{G}A-GC
38:TAMRA-G-KLKAT^{G}AKL-GC
39:TAMRA-G-KAT^{G}AKLKL-GC
40:TAMRA-G-T^{G}AKLKLKL-GC
41:TAMRA-G-KLKLKAT^{M}A-GC
42:TAMRA-G-KLKAT^{M}AKL-GC
43:TAMRA-G-KAT^{M}AKLKL-GC
44:TAMRA-G-T^{M}AKLKLKL-GC
45:TAMRA-G-KLKLKAT^{Ga}A-GC
46:TAMRA-G-KLKAT^{Ga}AKL-GC
47:TAMRA-G-KAT^{Ga}AKLKL-GC
48:TAMRA-G-T^{Ga}AKLKLKL-GC
49:TAMRA-G-KLKLKAT^{L}A-GC
50:TAMRA-G-KLKAT^{L}AKL-GC
51:TAMRA-G-KAT^{L}AKLKL-GC
52:TAMRA-G-T^{L}AKLKLKL-GC
53:TAMRA-G-KAKAKAKA-GC
54:TAMRA-G-KLKLKLKL-GC

β-loop (55-80)

55:TAMRA-KKITV-DT^{G}ER-KTYTE-GC
56:TAMRA-KKITV-DT^{M}ER-KTYTE-GC
57:TAMRA-KKITV-DT^{Ga}ER-KTYTE-GC
58:TAMRA-KKITV-ET^{G}DR-KTYTE-GC
59:TAMRA-KKITV-ET^{Ga}DR-KTYTE-GC
60:TAMRA-KKITV-ET^{G}SD-KTYTE-GC
61:TAMRA-KKITV-ET^{M}SD-KTYTE-GC
62:TAMRA-KKITV-ET^{Ga}SD-KTYTE-GC
63:TAMRA-KKITV-ET^{L}SD-KTYTE-GC
64:TAMRA-KKITV-DT^{G}SE-KTYTE-GC
65:TAMRA-KKITV-DT^{M}SE-KTYTE-GC
66:TAMRA-KKITV-DT^{Ga}SE-KTYTE-GC
67:TAMRA-KKITV-DT^{L}SE-KTYTE-GC
68:TAMRA-KKITV-DT^{M}ET^{M}-KTYTE-GC
69:TAMRA-KKITV-DT^{Ga}ET^{Ga}-KTYTE-GC
70:TAMRA-KKITV-DT^{L}ET^{L}-KTYTE-GC
71:TAMRA-KKITV-ET^{M}DT^{M}-KTYTE-GC
72:TAMRA-KKITV-ET^{Ga}DT^{Ga}-KTYTE-GC
73:TAMRA-KKITV-ET^{L}DT^{L}-KTYTE-GC
74:TAMRA-KKITV-ET^{Ga}ST^{Ga}-KTYTE-GC
75:TAMRA-KKITV-DT^{G}ST^{G}-KTYTE-GC
76:TAMRA-KKITV-DT^{M}ST^{M}-KTYTE-GC
77:TAMRA-KKITV-DT^{Ga}ST^{Ga}-KTYTE-GC
78:TAMRA-KKITV-DT^{L}ST^{L}-KTYTE-GC
79:TAMRA-KKITV-DPER-KTYTE-GC
80:TAMRA-KKITV-EPSD-KTYTE-GC

Figure 1. List of glycopeptides (26, 27, 53, 54, 79, and 80 are non glycolsylated peptides as controls) synthesized in the present study; 1–27: α-helix; 28–54: β-sheet; 55–80: β-loop. T^{G}: glucosylated Thr; T^{M}: mannosylated Thr; T^{Ga}: galactosylated Thr; T^{L}: lactosylated Thr. C-Termini of peptides are amide.

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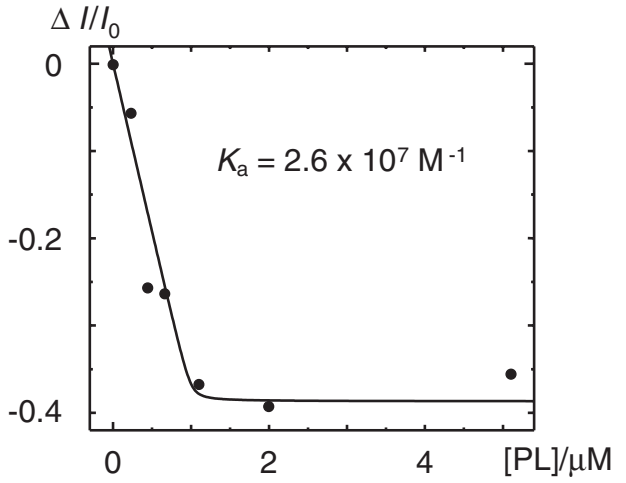

Figure 2. Binding of peanuts lectin (PL) to the glycopeptide (No. 8) in the solution assay.

peptides in the present study were purified by preparative HPLC and characterized by LCMS. Their secondary structures have been elucidated by CD (circular dichroism) spectra and indicated that the sugar moieties did not significantly influence their secondary structures (Supporting Information).

In preliminary experiments the binding ability of glycopeptides were tested with a lectin using the 96-well plate (Supporting Information). The fluorescent intensities (I_0) of a glycopeptide (No. 8) were measured at 1.0 μM, and the various concentrations of the lectin from peanuts (PL) were mixed in each well. The changes of fluorescent intensities ($\Delta I/I_0$) were measured. As shown in Figure 2 the glycopeptide was recognized by the lectin with strong affinity. As previously reported[9] the result indicates that the synthesized glycopeptides could be useful for detection of sugar-binding proteins.

The solution (ca. 350 pL) of peptides in Figure 1 were then immobilized using Piezorray™ (Perkin-Elmer, Japan) through the sulfhydryl group of the C-terminal Cys-residue using EMCA (*N*-ε-maleimidocaproic acid), on the recently developed novel chip material (based on an amorphous carbon) designated PepTenChip®, which has superior characteristics compared to those on conventional glass plates.[11] The acetamidomethyl (Acm) group of each peptide was removed by silver trifluoromethanesulfonate (5 mM) in a mixture of trifluoroacetic acid (TFA)/anisole before immobilization (Supporting Information). The resulting glycopeptides were precipitated with ether and freeze-dried for immobilization. Two isomers caused by 5- and 6-TAMRA were mixed and used for array experiments, since there were no significant differences in recognition between both isomers.

The resulting glycopeptides arrays were visualized using a fluorescence scanner (Figure 3), which showed that the averaged amount of peptides was ca. 2.1 femto mole. The toxic protein solutions (RCA from Vector lab. and PA-I from Sigma) were added on to this array and the fluorescent responses were measured. The experiments were performed twice and the peptides that showed reproducible responses were picked up for the fingerprint imaging.

As indicated in Figure 4 significant differences have been observed in the responses against RCA and PA-I, especially

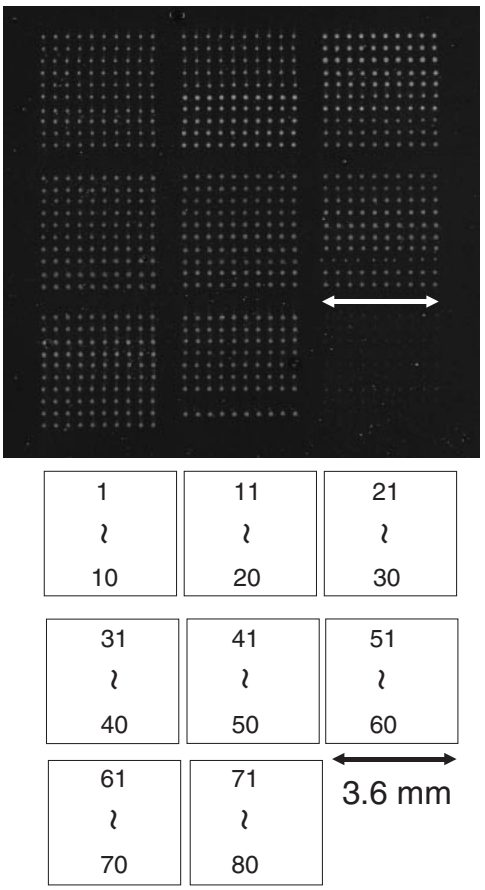

Figure 3. Fluorescent scanner image of the labeled glycopeptide array in the present study. A total of 80 peptides were spotted on the chip based on amorphous carbon, in which 10 spots of each peptide were arrayed. Their location is indicated in the lower panel.

peptides 19, 21, 22, 23, 30, 41, 42, and 52. The fluorescent changes ($\Delta I/I_0$) in α-helical peptides (19, 21, 22, and 23) decreased (−) against RCA upon increasing the concentration, in contrast they increased (+) against PA-I. The β-sheet peptides (30, 41, 42, and 52) responded more strongly against RCA than against PA-I. The results reflect differential recognition between RCA and PA-I, due to different sugar-side chains and the secondary structures of the backbone peptide sequences. As is well known RCA and PA-I have affinities for galactose (peptides: 21, 22, and 23) and lactose (peptide 52), those proteins should bind to these carbohydrates in the glycopeptides. In contrast, peptides (19, 30, 41, and 42) have mannosyl residues, which should be recognized by lectins. Thus, protein fingerprints were generated as individual peptides showed different responses to each toxic protein. It is emphasized that bio-recognition is performed both carbohydrate and their surrounding peptide structures.

These assays have also been performed in milk solution to simulate practical conditions found in food analyses. Although the range of fluorescence changes ($\Delta I/I_0$) in milk solution was weaker (within −0.3–+0.3), the pattern of responses could be converted to a *bar-code* (Supporting Information). A carbohydrate-micro array has been recently reported, in which several bacterial toxins could be detected by a micro-flow method,[8]

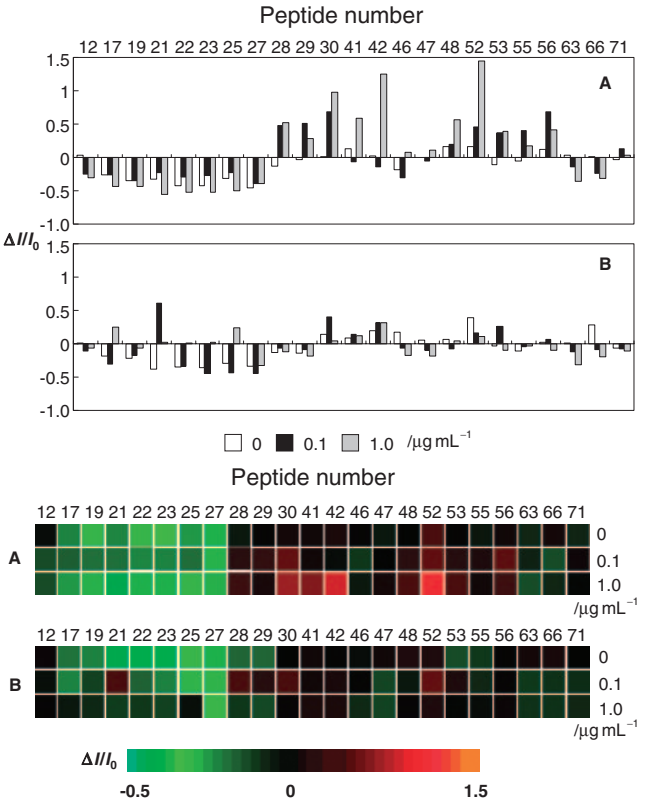

Figure 4. The fluorescent responses and their fingerprints of toxic proteins using selected glycopeptides (**A**: RCA and **B**: PA-I). Each protein in PBS was added to the array at three different concentrations (0, 0.1, and 1.0 μg mL^{-1}). Green color indicates a decrease in fluorescence, and red color shows an increase in fluorescence upon addition of proteins as analytes.

which requires comparatively large amounts of analyte (nearly one mL). Although a glycan-array generated from plant-cell wall was also developed for screening of antibody-binding specificity by another group,[9] the detection method using a secondary antibody was a time-consuming process and costly. Our detection system requires only 2 μL of protein-containing analyte and therefore is more practical compared to methods employing carbohydrate microarrays. The present glycopeptide array can detect carbohydrate-binding proteins even in a protein mixture such as milk. The present system will make possible nutrition analyses and/or eventually have anti-bioterrorism applications in addition to in vitro diagnosis.

In summary, we have developed glycopeptide-arrays by high-throughput syntheses using an improved chip material designated as PepTenChip®, which will contribute to applications in proteomic research and clinical medicine for diagnostic agents as well as a toxin detection tool.

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Supporting Information

Syntheses and characterization of glycosylated threonine building blocks and labeled glycopeptides are described. CD spectra, binding experiments, and arraying with detection methods of labeled glycopeptides are indicated. Additionally detection of analytes containing milk is also shown. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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