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Description	

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Peptides binding a Gb3 mimic selected from a phage library

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Abstract

Peptides binding to a Gb3 mimic were selected from 12mer peptide library. The self-assembled monolayer (SAM) of a Gb3 mimic was formed on the gold surface, and biopanning was carried out with the phage display peptide library. After 3 rounds of biopanning, four individual sequences were obtained from 10 phage clones, and the selected peptides having the specific 7mer sequence (FHENWPS) showed affinities to the Gb3 mimic as strong as to RCA_{120}. Molecular dynamics calculations suggested that the peptides bound to the Gb3 mimic by hydrophobic interaction and hydrogen bonding formation, and the cooperative interactions played an important role in the recognition. The Stx-1 binding was inhibited by the peptides.

Key Words

phage display peptide library, protein-oligosaccharide interaction, Gb3, Shiga toxin, quartz crystal microbalance,

Abbreviations: ABTS, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BOP, benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; BSA, albumin from bovine; DMF, *N, N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; FAB, fast atom bombardment; F-MOC, fluorenylmethoxycarbonyl; FTIR, fourier transform infrared spectrophotometer; Gal, galactose; Gb3, globotriaosylceramide Gal(α1–4)Gal(β1–4)GlcβCer; Glc, glucose; GM1,

Gal(β1-3)GalNAc(β1-4)NeuAc(α2-3)Gal(β1-4)GlcβCer; Lac, lactose; LacNAc, Gal(β1-4)GlcNAc; HOBt, 1-hydroxybenzotriazole hydrate; MALDI, matrix assisted laser desorption ionization; ODS, octadecyl silane; QCM, quartz crystal microbalance; RAS, reflection-absorption spectroscopy; RCA_{120}, *Ricinus communis* agglutinin 120; SAM, self-assembled monolayer; SPR, surface plasmon resonance; Stx, Shiga toxin; TBS, tris buffered saline; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TOF, time-of flight

1. Introduction

Protein-oligosaccharide interactions play key roles in the control of various biological processes [1,2]. The importance has driven better understanding of the saccharide binding specificity of lectins [3]. Progress has been made using X-ray crystallography [4, 5], isothermal titration microcalorimetry [6] and site-directed mutagenesis [7]. However, it is difficult to obtain detailed information on thermodynamic and kinetic parameters resulting from the analysis of a large lectin-oligosaccharide complex because the binding of saccharides to protein involves a variety of recognition processes and the protein themselves exhibit considerable structural diversity.

Phage display has been a powerful tool for identifying peptides that bind to a particular target, such as an antigen, epitope or receptor [8]. It allows rapid screening of random peptide libraries that can be used for diagnostic and therapeutic researches. This methodology has been applied to glyco-science. Peptides, which bind to carbohydrate-binding molecules such as lectins and antigens, were identified, and showed the mimic function of carbohydrates, including inhibitor of enzymes and tumor metastasis [9-16]. Peptides binding to the specific carbohydrates were also isolated [17,18].

In this report, we carried out affinity selection, so-called “biopanning”, with a globotriaosylceramide (Gb3) mimic (Figure 1). Gb3 is reported to be the ligand of Shiga toxins (Stxs) (verotoxins) of *Escherichia coli* O-157: H7 which causes the serious clinical diseases [19]. Binding to Gb3 is the primary event for the internalization of Stxs to cell and plays important roles [20, 21]. Though the interaction of Stxs with Gb3 or Gb3 mimics have been studied [22-27], the detailed interaction and the infection mechanism are still unclear. We have reported that the Gb3 mimics bound and

neutralized Stxs [29, 30]. The simple oligopeptides selection via a biopanning is useful for analysis of the interaction between Stxs and the Gb3 mimic, and could contribute to develop inhibitors of toxin infection [17,18].

The interaction of an isolated saccharide to a specific protein is so weak that glyco-cluster formation is needed to achieve efficient molecular recognition. In addition, the biopanning of a ganglioside was reported to be dependent on the formation of an ordered glycolipid assembly [17]. Liposomes in solution, Langmuir monolayers at air-water interfaces, and self-assembled monolayers (SAMs) on solids are known to be well-ordered glycolipid assemblies [31, 32]. We have recently reported the quantitative estimation of affinity of Stxs for SAM of a Gb3 mimic [30]. Densely packed SAM of the Gb3 mimic is suitable for the selection of peptides that recognize glycolipids presented in biomembranes. We report the fast analysis of the interaction between the Gb3 mimic (Figure 2) and Stx-1 by phage-display and the fabrication of a small peptide recognizing the Gb3 mimic.

2. Materials and Methods

^{1}H NMR spectra were recorded at 25 °C in D_2O with a Varian Inova 500 spectrometer equipped with Sun Workstation (Varian, Inc. Palo Alto, CA). For the peptide conformation analysis, 5 mg of peptide was dissolved in 600 μl of D_2O. FAB-MS and MALDI-TOF-MS were taken with a JMS-700 (JEOL Ltd., Tokyo) and a Voyager (Applied Biosystems, Foster City, CA), respectively. Surface plasmon resonance (SPR) measurements were performed at 25 °C at a flow rate of 5 ml/min in aqueous solution with an SPR 670 (Nippon Laser & Electronics Labs, Nagoya). The quartz crystal microbalance (QCM) in this study was commercially available 9 MHz AT-cut

quartz deposited with Au electrodes on both sides, purchased from Sogo Pharmaceutical Co. Ltd. (Kanagawa). The frequency changes of QCM were followed by a universal frequency counter (53131A, Hewlett-Packard Co., Ltd., Pala Alto, CA). CD spectrum of peptides in aqueous solution at 25 °C was collected on a JASCO J-725 using optical cell of 0.1-cm path length. Fluorescence experiments were performed in a JASCO FP-777 using 1.0 cm path length cell. The sample peptide solution (4.0×10^{-4} mol/l) in aqueous solution was excited at the 295 nm, and the emission was scanned from 310 to 450 nm.

2.1. Glycolipid Synthesis

The compounds used in this article are shown in Figure 2. The synthesis of Gb3C10 disulfide was carried out according to our previous method [29, 30].

p-*N*-Undecanoylamidophenyl *O*-[α-D-galactopyranosyl-(1–4)-β-D-galactopyranosyl] -(1–4)-β-D-glucopyranoside (Gb3C10')

Undecanoic acid (Kishida, Osaka) (3.5 mg, 19 μmol), *p*-aminophenyl [α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl]-(1→4)-β-D-glucopyranoside (pAP-Gb3) (12 mg, 19 μmol) (21), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)(Tokyo Kasei Kogyo Co., Ltd, Tokyo) (7.3 mg, 38 μmol), and triethylamine (7.8 μl, 57 μmol) were dissolved in DMF (2 ml) in a round-bottomed flask, and the reaction mixture was stirred for 26 h. The reaction was followed by TLC (silica gel 60 and reversed phase RP-18, Merck, Darmstadt, Germany). The solvent was removed under vacuum, and the crude product was purified by gel permeation chromatography of LH20 (methanol) and freeze-dried to yield a white solid (7.7 mg, 40%).

TLC: R_f (reversed phase: MeOHe) = 0.82, R_f (silica-gel: MeOH) = 0.0. FAB-MS (HR-MS) for C35H57NO17: m/z 764.0 $[M+H]^+$ (calculated $[M+H]^+$ 764.4). ^{1}H-NMR(D_2O,

δ, ppm) : 0.93(t, 3H, J = 7.0Hz C*H*$_3$), 1.04 (m, 2H, C*H*$_2$CH$_3$), 1.12 (m, 12H, NHCOCH$_2$(C*H*$_2$)$_6$), 1.73 (m, 2H, NHCOCH$_2$C*H*$_2$), 1.86 (t, 2H, NHCOC*H*$_2$), 3.42-3.90 (overlapped m, 17H, C*H*(OH) and C*H*$_2$OH GalH2"-4",6" GalH2'-6' and GlcH2-6), 4.02 (br, 1H, Gal-H5"), 4.34 (d, 1H, J = 8.0Hz GalH-1"), 4.77 (d, 1H,J = 3.5 Hz, GalH-1'), 5.03 (d, 1H, GlcH-1), 7.04 (dd, 2H, Ar*H* meta to amide), 7.16 (dd, 4H, Ar*H* ortho to amide).

2.2. Preparation of Self-Assembled Monolayers

The gold substrate was treated with a freshly prepared pirania solution (H_2SO_4 (96 %): H_2O_2 (31 %)=3:1 in volume) twice for 10 min, washed thoroughly with deionized water and methanol, and then incubated in a DMSO solution of Gb3C10 (0.01 mM) for 16 h. The substrate was rinsed thoroughly with DMSO and deionized water, and dried in a stream of dry nitrogen gas.

2.3. Screening of Peptides for Gb3C10 SAM.

Biopanning was carried out according to the procedures [17] using a phage display library kit (New England Biolabs Inc., Cambridge, MA), which is based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (pIII) of the filamentous coliphage M13. The phage library (4×10^{10} transducing units (TU)) was added to the SAM substrate. After incubation for 60 min at room temperature, the SAM substrate was washed three times with Tris buffered saline (TBS). The phages bound to the Gb3C10 SAM were eluted with 0.1 N Gly-HCl buffer (pH 2.2). The eluate was neutralized with 1 M Tris-HCl buffer. The phage clones obtained were amplified by infecting them into host bacterial cells (*E. coli* ER2738).

2.4. Peptide Synthesis

Peptides were synthesized by the solid phase method using standard Fmoc chemistry on Wang resin. Coupling of amino acids was carried out using 1-hydroxybenzotriazole

hydrate (HOBt) and benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Kokusan Chemical Co., Ltd, Tokyo) in DMF. Fmoc group was removed in 20 % piperidine/DMF. The peptides were purified by reversed phase HPLC on an HPLC column (JASCO, Tokyo) with a gradient of actonitrile/0.1 % TFA. The peptide purities were estimated by HPLC (purity >97 %). MALDI-TOF-MS; peptide **1** $[M+H]^+$ 1482.9 (calculated $[M+H]^+$ 1484.7), peptide **2** $[M+H]^+$ 1432.4 (calculated $[M+H]^+$ 1432.7), peptide **3** $[M+H]^+$ 1539.1 (calculated $[M+H]^+$ 1538.6), peptide **4** $[M+Na]^+$ 1267.0 (calculated $[M+Na]^+$ 1267.3), motif $[M+H]^+$ 917.0 (calculated $[M+H]^+$ 917.0).

2.5. Assay for Interaction between Gb3C10 SAM and Peptides.

Interaction between Gb3C10 SAM and peptides were analyzed by the quartz crystal microbalance (QCM), enzyme-linked immunosorbent assay (ELISA), and surface plasmon resonance (SPR).

The QCM plates modified with Gb3C10 SAM were immersed in the aqueous phage solution for 10 min. The frequency change of QCM in aqueous solution correlates the mass increase by the empirical formula based on the calibration of Sauerbrey equation.

$$\Delta F(\mathrm{Hz}) = (-2.10 \pm 0.10)\Delta m(\mathrm{ng}) \qquad (1)$$

ΔF and Δm represent the frequency change (Hz) and the mass change (ng), respectively.

A 96-well plate was coated with Gb3C10' prior to the ELISA measurements. The relative affinities were estimated by normalizing the phage titers of each mutant, serially diluting the phage 1:2, and binding to the 96-well plate coated with glycolipids. After a washing step, the assay was visualized by adding H_2O_2 and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) followed by quenching with sodium citrate for 60 min. The absorbance at 405 nm was measured as a function of

the diluted phage using a microplate reader.

SPR measurements were performed with Gb3C10 SAM and peptide at 25°C, at a flow rate of 5 μl/min in aqueous solution. After the measurement, the surface of Gb3C10 SAM was regenerated with 100 mM HCl and 100 mM NaOH. The association and dissociation rate constants as well as the dissociation equilibrium constants were calculated based on the SPR evaluation software.

2.6. Assay for Binding Inhibition of Stx-1

The QCM plate with Gb3C10 SAM was immersed into the synthetic peptide solution (1 x 10^{-6}). After the frequency change was saturated, the Stx-1 was injected to the solution and the amount of Stx-1 adsorbed on the SAM was determined [20].

2.7. Molecular Dynamics Calculation

The interactions between peptides and Gb3C10' were visualized using a biopolymer module of the Insight II software (ver. 97, MSI, San Diego, CA). The CVFF force field was used during minimization of the interaction by the Discover software (ver. 3.0, MSI, San Diego, CA). Water was appended to the compounds following the force field command.

3. Results

3.1. Selection of Gb3 Binding Peptides from a Phage-Displayed Peptide Library

The Gb3C10 SAM (Figure 2) was used to select phage clones. The binding of the phages to the SAM was monitored by the frequency changes of QCM (Figure 3). The frequency change was increased after each biopanning procedure. The amino acid sequences after 2nd and 3rd round selections were determined from DNA sequences. No identical sequence was found after the 2nd round selection, and only 4 different

sequences were obtained from 10 phage clones after the 3rd round selection (Table 1). The repetition of biopanning was effective on the successful library selection. Interestingly, the consensus sequence of the 7mer peptide, FHEWPS (Phe-His-Glu-Asn-Trp-Pro-Ser), was observed in phages **3** and **4**.

3.2. Affinities of the Selected Peptides

The affinities of the four clones were estimated by ELISA (Figure 4). The selected phage clones showed much stronger affinity to Gb3C10 SAM than random control peptides. Phages **3** and **4** showed stronger binding than phages **1** and **2**, which suggests that the consensus sequence (motif: FHENWPS) induced the binding to the Gb3 mimic.

The peptides of phages **1-4** and the consensus motif were chemically synthesized and their binding affinities to Gb3C10 SAM were analyzed by SPR (Figure 5, Table 2). The association constants of the peptide **3** and **4** to Gb3 C10 SAM were higher than those of peptide **1** and **2**, almost of the same order as that of RCA_{120} (lectin) to Gb3C10 SAM (K_a, 1.7×10^5 M^{-1}), and comparable to that of Stxs to Gb3C10 SAM [30]. Considering the weak affinities of sugars for lectins [2, 3] and the inert properties of sugar layer for protein adsorption [33], these results show that the biopanning of phage display library was successful and that this method is a promising approach for analyzing sugar protein interaction.

The adsorption of Stx-1 to Gb3C10 SAM was measured in the absence and in the presence of the excess amount of peptides **3**, **4** and the 7mer motif (1×10^{-6} M) (Figure 6). The amount of adsorbed Stx-1 was reduced to ca. 70 % by the 7mer motif peptide and ca. 20 % by the peptides **3** and **4.** These inhibition data indicated that the peptides are regarded as a possible inhibitor of Stx-1.

4. Discussion

Several amino acids were reported to be specific to the interactions of lectins with carbohydrates [3, 4, 34, 35]. Hydrophilic Ser (S), His (H), Glu (E), Asn (N), Tyr (Y) and Arg (R) are considered to bind to the hydroxyl groups via hydrogen bonding and coordination with cations. For example, Ser in peanut agglutinin forms hydrogen bonding with lactose and LacNAc [35], His in galectin interacts with lactose by hydrogen bonding [36], and Glu and Asn in E-selectin bind via ion-pairing with Ca^{2+}. Strong hydrophobic residues (Trp (W) and Phe (F)) in galectins, ricins, and enterotoxins were reported to interact with hydrophobic α-anomeric surface of galctose. The amino group of small hydrophobic amino acids (Leu (L), Gly (G) and Ala (A)) was reported to interact with carbohydrates via hydrogen bonding. Pro (P) was found to contribute to the peptide conformation suitable for binding to the Gb3 mimic [37]. Pro tends to occur abundantly in phage peptide library based on the amino acid sequence bias of M13 phages [38].

The interactions between the selected peptides and Gb3 mimic (Gb3C10') were analyzed by molecular dynamics calculations. The Gb3 mimic was found to bind the motif moiety of the peptide **3** and **4** (Figure 8 (a) and (c)). The expanded view (Figure 8(b) and (d)) demonstrated the interaction between Trp and the anomeric surface of Gal and the hydrogen bonding between Glu and Gal. The molecular dynamics (Figure 9) of the 7 mer motif and the Gb3C10' suggested densely packed interactions between Phe and the aromatic moiety of Gb3C10' aglycon in addition to those between Trp and Gal and between Glu and Gal. These computer simulations suggest that both of the hydrophobic interaction and hydrogen bond formation contribute to Gb3 recognition

and that the cooperation of these two interactions in the peptide is a key to strong recognition of Gb3.

The very strong binding between Stxs and Gb3Cer was contributable to the hydrophobic interactions [23, 25, 27]. The interaction of Phe30, Trp34, and Phe63 with the hydrophobic surface of Gb3 was essential to the Stx-1 binding, as suggested by the crystal structure analysis [23] and point mutation [28]. Though the amino acid sequences in the selected phages were not found in Stx-1, Phe and Trp are present with high frequencies in all of the selected phages and the consensus motif, which indicated that they are related to the Gb3 mimic recognition.

Interestingly, the sequence of the motif and the Stx-1 B subunit have a similarity in the hydrophobic resides. That is, Phe and Trp residues in the motif are separated by 4 amino acids, which is similar to Phe30 and Trp34 in the Stx-1 B subunit. The conformation of the motif peptide was analyzed on the basis of the CD, fluorescence and ^{1}H NMR spectra of peptide **4**. The negative band at 200 nm in the CD spectrum of peptide **4** in water was indicative of random-coil structure (Figure 10), which is in contrast to the β-sheet structure of Stx-1 in the crystal. Fluorescence emission spectrum of peptide **4** presented the maximum at 356 nm, indicative of the exposure of Trp to hydrophilic environment, but Stx-1 presented the maximum at 347 nm [39]. To attain strong binding of this motif peptide like Stx-1, the formation of specific conformation for the Gb3 mimic binding is required. The H C^{α} protons of the motif region in peptide **4** were up-field shifted from the random coil chemical shifts (Figure 11). The conformation of peptide **4** for the Gb3 binding is future investigation.

Peptides **3** and **4** showed strong interaction to Gb3C10 SAM, though the interaction of the 7mer motif itself was weak in SPR analysis. The observation is similar to the

results of point mutation of Stx-1 [28], in which the mutation of one or two residues decreases the affinities. Not only the motif moiety but also other residues in peptides **3** and **4** might contribute to the Gb3 recognition, and the cooperativity of motif and other residues is supposed to induce the strong bindings.

5. Conclusion

The biopanning with the self-assembled monolayer of the Gb3 mimic was successful in selecting the Gb3 mimic-binding peptides. The densely packed Gb3C10 SAM was suitable for the biopanning. Three rounds of biopanning selected two peptides (**3** and **4**) containing the consensus sequence FHENWPS with 4/10 frequencies. The selected peptides inhibited Stx-1 binding to the Gb3C10 SAM. Molecular dynamics calculation suggested that the cooperative action in hydrophobic and hydrogen bonds plays an important role in the binding between the peptides and the saccharide moieties of Gb3.

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Figure legends

Figure 1 Schematic illustration of *in vitro* selection for a Gb3 mimic.

Figure 2 Chemical formula and abbreviation of the glycolipids.

Figure 3 Frequency change (ΔF) and mass increase (Δm) of QCM by binding of selected phages onto the Gb3C10 SAM.

Figure 4 Binding assay of selected phage clones to the Gb3C10' by ELISA.

Figure 5 SPR analysis of Gb3C10 SAM with varying concentration of peptides in an aqueous solution at 25 ° C.

Figure 6 The amount of Stx-1 binding to Gb3C10 SAM in the absence and in the presence of peptides.

Figure 7 Frequency of amino acids in the selected phage clones (**1**-**4**).

Figure 8 Computer simulated binding mode of peptide **3** and **4** with Gb3C10' in water by molecular dynamics calculations. ((a) view of peptide **3** with Gb3C10', (b) expanded view of (a), (c) view of peptide **4** with Gb3C10', and (d) expanded view of (c)).

Figure 9 Computer simulated binding mode of the 7mer with a Gb3C10' in water by molecular dynamics calculations.

Figure 10 CD spectrum of peptide **4** in PBS buffer (pH 7.4) at 4.0×10^{-4} mol/l.

Figure 11 The deviation of $H^{\alpha}C$ proton NMR chemical shift of peptide 4 from the random coil values in D2O at 298 K.

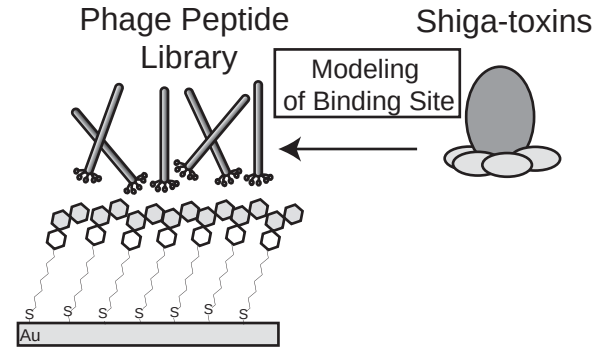
Tabular material

Table 1. Gb3 mimic-binding peptides after the 3rd round selection from the phage display library.

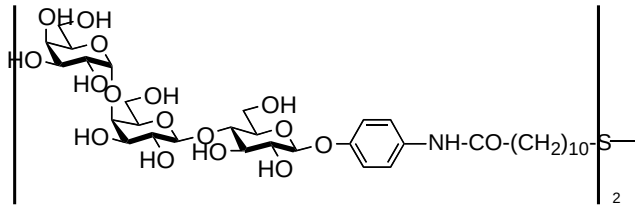
Name	Sequence	Frequency
phage **1**	WAPPLFRSSLFY	4/10
phage **2**	NFMESLPRLGMH	2/10
phage **3**	FYSHS**FHENWPS**	2/10
phage **4**	**FHENWPS**GGGSA	2/10
motif	**FHENWPS**	----

Table 2. Affinity constants and dissociation constants of the peptides with Gb3C10 SAM.

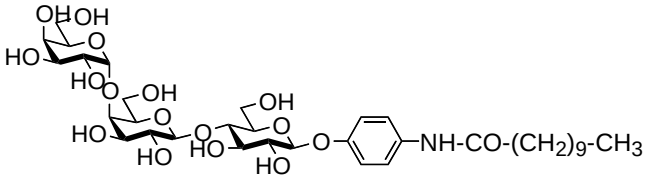
Name	$K_a(M^{-1})$	$K_D(M)$	$k_a(M^{-1}s^{-1})$	$k_d(s^{-1})$
peptide **1**	3.1×10^{4}	3.2×10^{-5}	1.2×10^{3}	3.9×10^{-2}
peptide **2**	5.3×10^{4}	1.9×10^{-5}	2.3×10^{2}	4.4×10^{-3}
peptide **3**	1.0×10^{5}	9.6×10^{-6}	7.8×10	7.5×10^{-4}
peptide **4**	3.0×10^{5}	3.4×10^{-6}	2.4×10^{2}	8.2×10^{-4}
motif	1.2×10^{4}	8.3×10^{-5}	3.7×10	3.1×10^{-3}



Self-Assembled Monolayer
of Glycolipid

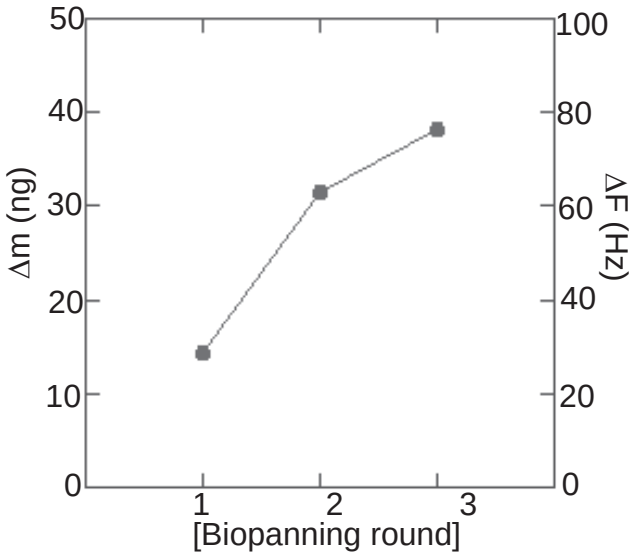


Gb3C10 disulfide



Gb3C10'

Figure2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30
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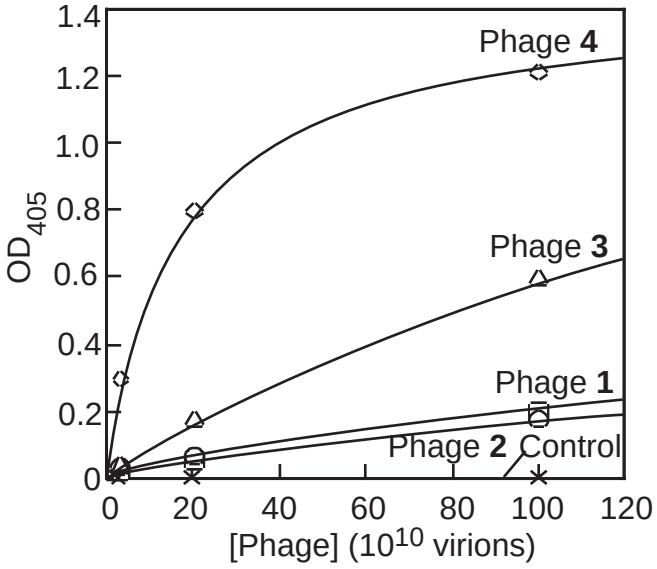
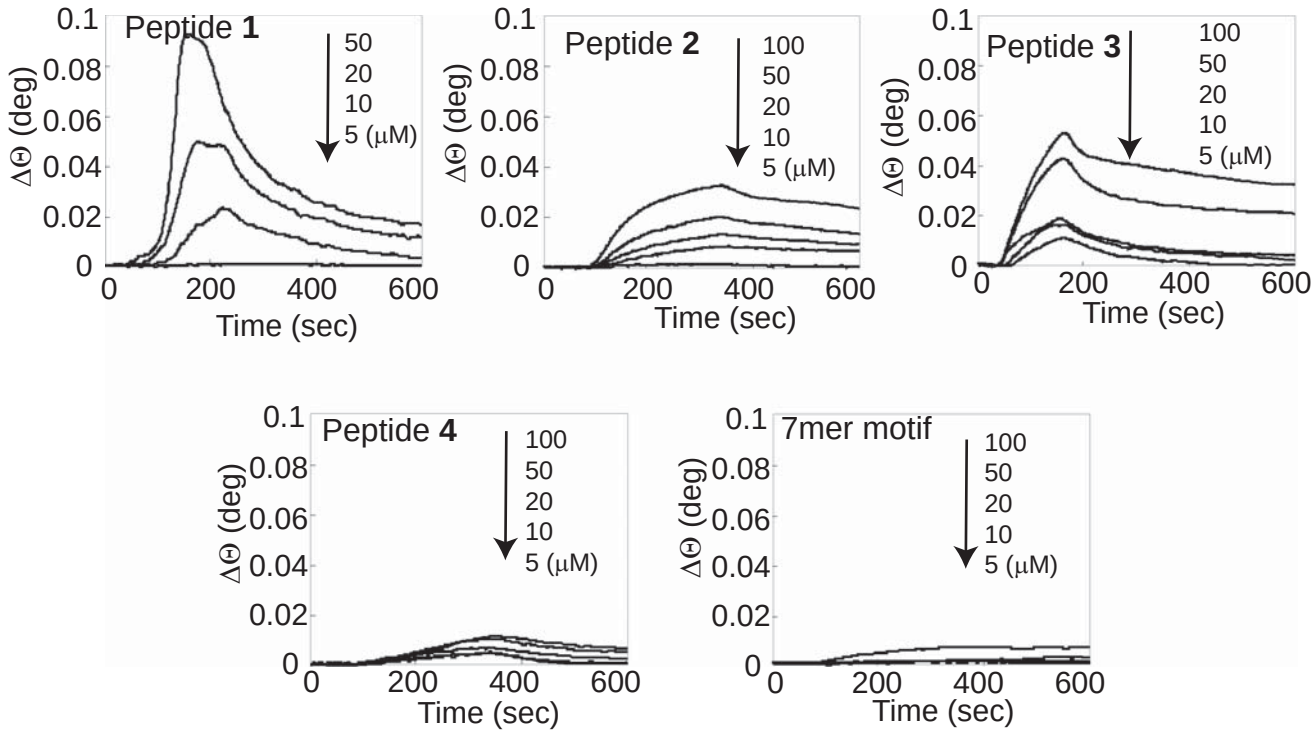
80
60
40
20
0
ΔF (Hz)
1
2
3
[Biopanning round]


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD405
Phage 4
Phage 3
Phage 1
Phage 2 Control
0
20
40
60
80
100
120
[Phage] (10^10 virions)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10
5 (μM)
Peptide 2
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50
20
10
5 (μM)
Peptide 3
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50
20
10
5 (μM)
Peptide 4
100
50
20
10
5 (μM)
7mer motif
100
50
20
10
5 (μM)
ΔΘ (deg)
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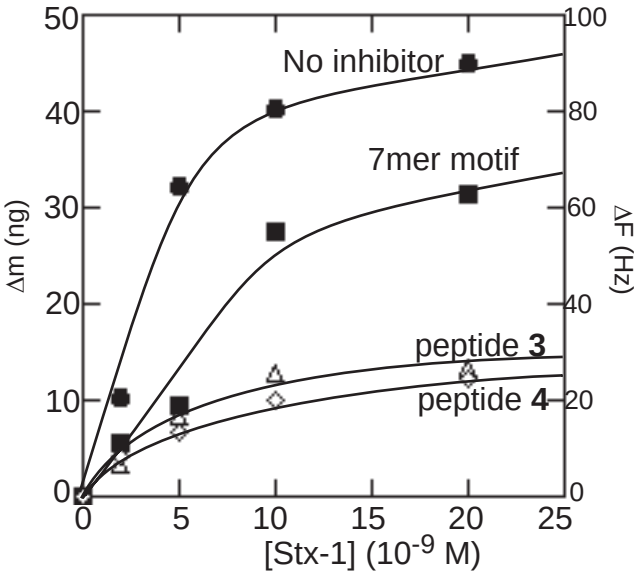
400
600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40
30
20
10
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Δm (ng)
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80
60
40
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ΔF (Hz)
No inhibitor
7mer motif
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peptide 4
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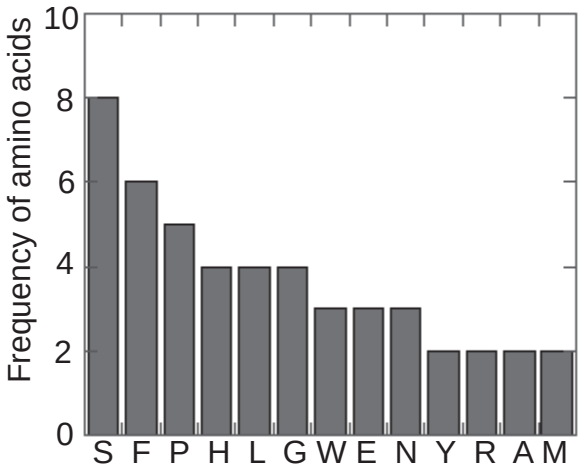
5
10
15
20
25
[Stx-1] (10-9 M)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6
4
2
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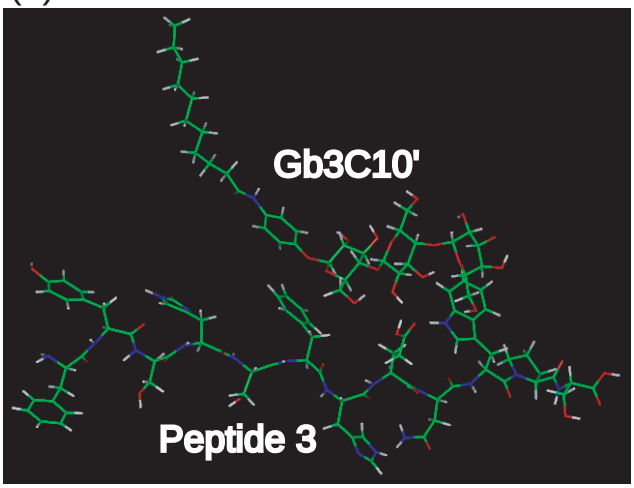
S F P H L G W E N Y R A M

(a)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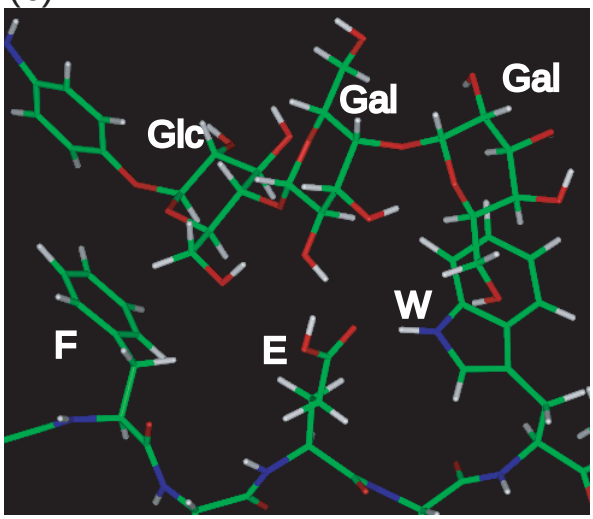
Peptide 3

(b)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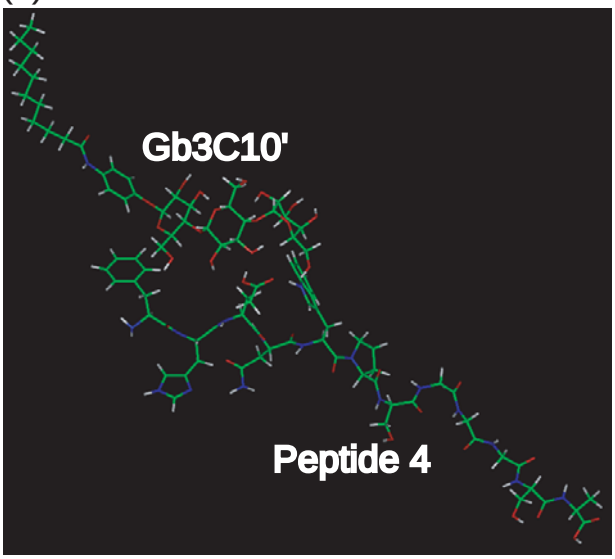
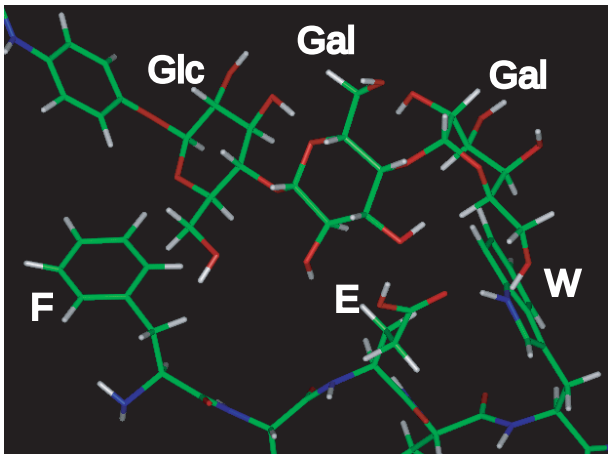
W
F
E

(c)


Gb3C10'
Peptide 4

(d)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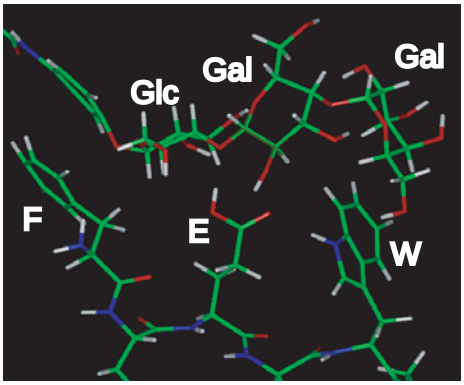
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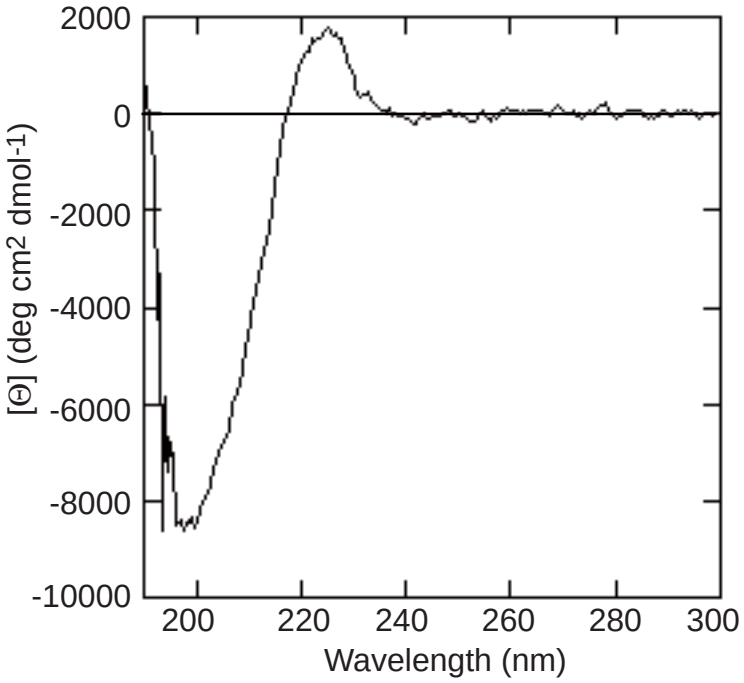
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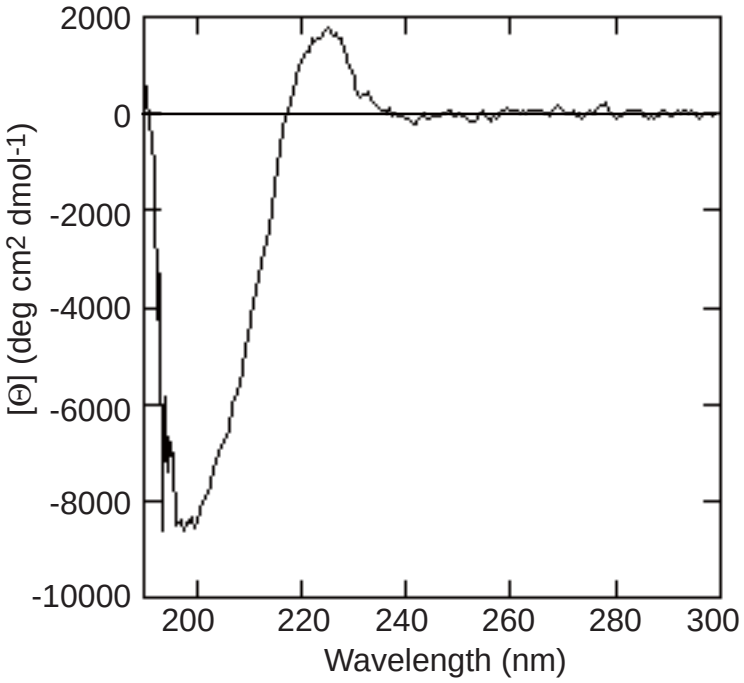
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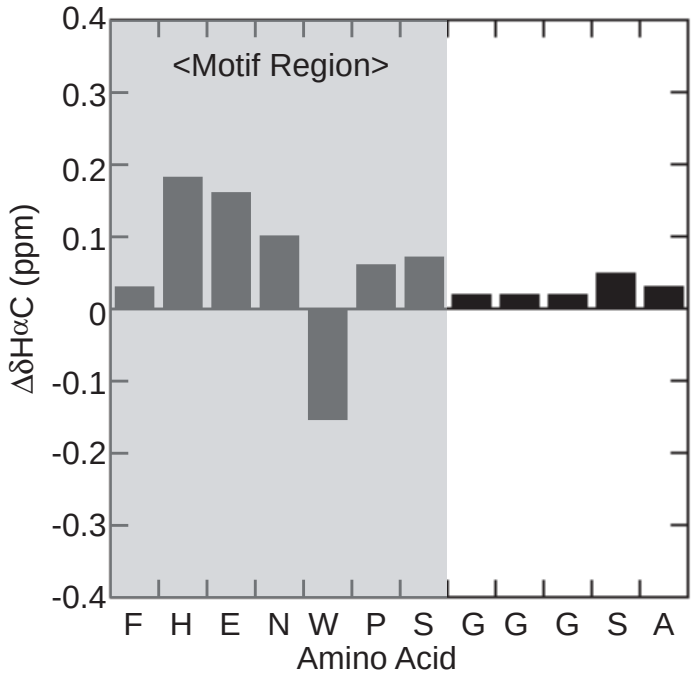
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Amino Acid