Screening and analysis of edible seaweeds in the ability to adsorb Shiga toxin

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ABSTRACT We screened edible seaweeds in the ability to adsorb Shiga toxin (Stx) by an equilibrated dialysis method. Although water insoluble fractions of fourteen dry seaweeds did not adsorb Stx, most water soluble fractions were found to adsorb it to one degree or another. Among the seaweed tested, the extract of the *Ulva linza* Linnaeus [*Enteromorpha linza* (Linnaeus) J. Agardh] was found to well adsorb both Stx1 and Stx2. We purified the Stx-adsorbing substance from the *U. linza* extract by DEAE-Toyopearl column chromatography and gel filtration with HiPrep 16/60 Sephacryl S-300 HR column. The purified substance showed an average molecular mass of about 800 kDa by polyacrylamide gel electrophoresis. Analyses of its components indicated that the substance was a highly rhamnose–containing polysaccharide with sulfate esters of 18%. Apparent dissociation constants (*K*d) of the polysaccharide to Stx1 and Stx2 were calculated to be 1.9 and 3.5 μM, respectively. To our knowledge, this is the first report on Stx-adsorbing dietary fibers.

Keywords Shiga toxin, seaweed, *Ulva linza* Linnaeus, rhamnan sulfate

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 was first recognized as a food-borne pathogen in 1982 [1]. EHEC O157:H7 is a member of a large group of Shiga toxin (Stx)-producing *E. coli*. A general symptom of the diseases caused by EHEC is hemorrhagic diarrhea in human, and Stx produced in the gut lumen is closely related to the intestinal diseases. Stx also traverses the epithelium, invades the blood circulation, and causes neurological damage and hemolytic-uremic syndrome (HUS). The pathogen produces two immunologically distinct Stx (i.e., Stx1 and Stx2). Stx1 and Stx2 are referred to as verocytotoxin 1 (VT1) and verocytotoxin 2 (VT2), respectively. Since some of the antibiotics used for the treatment of EHEC infection were reported to activate toxin genes and induce the release of accumulated intracellular toxin [2], new types of therapeutic agents are required to this pathogen. Stx is composed of one toxic subunit (A subunit) and five sugar recognizing subunits (B subunit) [3]. The B subunit pentamer binds to globotriaosylceramide (Gb3) on the cell surface of renal endothelial cells, and ferries the A subunit into the cells [4]. The A subunit activated by a membrane-anchored protease furin impairs renal function by inhibiting eukaryotic protein synthesis [5, 6]. Stx is also reported to bind to the P1 blood group antigen that is present in human erythrocyte glycolipid extracts [7], and ovomucoid from pigeon egg white with the antigen is reported to adsorb Stx1 [8]. Although several polymers including the globotriose moiety of Gb3 have been reported to adsorb Stx [8-12], there are few reports on foods with an ability to adsorb Stx.

Polysaccharides from seaweeds have been reported to have various characteristics such as antioxidant [13], anticoagulant, anti-inflammatory [14], antitumor [15], contraceptive, and antiviral activities, for the treatment of several diseases [16]. Especially, a sulfated polysaccharide, fucoidan, is extensively explored for its medicinal properties and being isolated from various species of seaweed [17]. Up to now, there is no report on the Stx-adsorbing ability of polysaccharides from seaweeds. If the indigestible polysaccharides

adsorb Stx, the toxin may be excreted with the polysaccharides into feces. We here describe the adsorption of Stx to polysaccharides from edible seaweed.

Materials and Methods

Materials

An Stx (VT) detection kit including Stx1 and Stx2 as references (variants: Stx1a and Stx2a) was obtained from Denka Seiken (Tokyo, Japan). The 96-well microplates (V-bottom) used for a reversed passive latex agglutination (RPLA) assay of Stx was supplied from Greiner Japan (Tokyo, Japan). A trimethylsilylating reagent, *N*,*O*-bis(trimethylsilyl) acetamide (BSA) + trimethylchlorosilane (TMCS) + *N*-trimethylsilyimidazole (TMSI), 3:2:3, was obtained from Supelco (Bellefonte, PA, USA). Float-A-Lyzer G2 (MWCO=300K) was obtained from Spectrum Labs Com (Rancho Dominquez, CA, USA). Amicon Ultra-4 and Amicon Ultra-15 (MWCO: 10 kDa and 100 kDa) were obtained from Merck Millipore (Billerica, Mass. USA). Trace Gold TG-1MS GC column (30 m × 0.25 mm × 0.25 μm) and NuPAGE 4-12% Bis-Tris Gel were obtained from Thermo Fisher Scientific (Waltham, MA USA). DEAE-Toyopearl was obtained from Toso Co. (Tokyo Japan). HiPrep 16/60 Sephacryl S-300 HR column and HMW-Native Marker Kit was obtained from GE Healthcare (Buckinghamshire, UK). Millser-620DG was obtained from Iwatani Co. (Tokyo, Japan). Dry edible seaweeds were purchased from a food store in Tokushima, Japan. They are fourteen seaweeds, *Monostroma nitidum* Wittrock (Aosa in Japanese), *Ulva linza* Linnaeus [*Enteromorpha linza* (Linnaeus) J. Agardh] (Usuba-aonori), *Eisenia bicyclis* (Kjellman) Setchell (Arame), *Nemacystus decipiens* (Suringar) Kuckuck (Mozuku), *Sargassum horneri* (Turner) C. Agardh (Akamoku), *Chorda filum* (Linnaeus) Stackhouse (Turumo), *Gloiopeltis tenax* (Turner) Decaisne (Funori), *Mazzaella japonicua* (Mikami) Hommersand (Akaba-ginnansou), *Gelidium elegans* Kützing (Tengusa), *Campylaephora hypnaeoides* J. Agardh (Egosou), *Ulva prolifera* Mueller (Akanori), *Undaria pinnatifida* (Harvey) Suringar (Wakame), *Saccharina japonica* (Areschoug) Lane, Mayes, Druehl et Saunders (Kombu), and

Hizikia fusiforme (Harvey) Setchell (Hijiki).

Preparation of seaweed extracts

Dry seaweeds (5 g) were milled at 20,000 rpm for 1 min with Millser-620DG, and suspended in 100 ml H_2O. The suspensions were autoclaved at 121°C for 15 min, and centrifuged at 10,000*g* for 15 min. The supernatant solutions and precipitates were used as 5% seaweed extracts and insoluble residues, respectively.

Reversed passive latex agglutination (RPLA) assay of Stx

The amounts of Stx1 and Stx2 were determined by RPLA assay with 96-well microplates (V-bottom) and the Stx detection kit as previously reported [18]. The lower detection limit of 1 ng/ml of Stx was confirmed with the Stx1 and Stx2 provided in the kit. Sample solutions containing Stx were subjected to twofold serial dilution, and each diluted sample (25 μl) was mixed with the suspension (25 μl) of latex beads coated with anti-Stx1a or anti-Stx2a antibody in each well of 96-well microplates. After incubating the microplates at 30°C overnight, the agglutination of latex beads in each well was examined with the naked eye. The reciprocal of the maximal dilution rate showing agglutination was expressed as RPLA titers of Stx1 and Stx2 in the original samples.

Adsorption of Stx to chemical components of seaweed extracts

The Stx1 and Stx2 with a molecular weight of about 72 kDa were dissolved in 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% bovine serum albumin to avoid non-specific adsorption. Each seaweed extract (5%, 0.1 ml) or insoluble residue (0.1 g) was mixed with 0.4 ml of Stx solution to give the final Stx conc. of 100 ng/ml. The mixture was put in a bag of the Float-A-Lyzer G2 (MWCO: 300 kDa), and dialyzed against 50 ml of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% bovine serum albumin at 4°C for 16 h. After the dialysis, the outer liquid was concentrated with a

filter unit (Amicon Ultra-15, MWCO: 10 kDa), and subjected to the RPLA assay. The Stx bound to polysaccharides of extracts or also of insoluble residues in the dialysis bag was dissociated from them by addition of NaOH (final pH 10), and free Stx was separated from the polysaccharides by the centrifugal ultrafiltration with a filter unit (Amicon Ultra-4, MWCO: 100 kDa). After the ultrafiltration at 4,000*g* for 30 min, filtrates containing free Stx were neutralized with HCl and analyzed by the RPLA assay.

When we determined apparent dissociation constants (*K*d) of a chemical component of seaweeds against Stx, an equilibrated dialysis method was used. A constant amount of the component and varying concentrations of Stx solutions were mixed, put in the dialysis bags, and dialyzed as described above, until the concentrations of free and bound Stx were in a state of equilibration. The ratio (ν) of [bound Stx] to [the Stx-adsorbent] was plotted against ν /[free Stx] in the equilibrated dialyses with varying concentrations of Stx. The *K*d values were obtained by calculating the reciprocal of slopes of lines.

Purification of Stx-adsorbent from the *U. linza* extract

The 5% extract (100 ml) of the *U. linza* seaweed was put on a DEAE-Toyopearl column (2.0 x 20 cm) equilibrated with 20 mM Tris-HCl (pH 7.5). After the column was washed with the same buffer, bound chemical components were eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions that showed the Stx-adsorbing ability were combined, concentrated with Amicon ultra-15 (MWCO: 10 kDa), and subjected to the gel filtration with a HiPrep 16/60 Sephacryl S-300 HR column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. Fractions that showed the Stx-adsorbing ability were combined, dialyzed against distilled water and freeze-dried.

Components of Stx-adsorbent

Total sugar content was determined by phenol-sulfuric acid method [19]. Uronic acid content of Stx-adsorbent was determined by *m*-hydroxydiphenyl method [20]. Sulfate

content was determined by rhodizonate method [21]. Sugar composition was determined as follows. The Stx-adsorbent (50 mg) was hydrolyzed with 2M trifluoroacetic acid (TFA) at 100^{o}C for 4 h. After removal of TFA under N_2 gas, the hydrolysate was dried up under reduced pressure. The dried sample (1 mg) was incubated at 70^{o}C for 1 h with 0.1 ml of (BSA + TMCS + TMSI, 3:2:3) and then analyzed with GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) and Trace Gold TG-1MS GC column with the oven temperature from 80^{o}C to 320^{o}C. Identification and quantification of sugars were made with NIST 14 Mass Spectral Library ver. 1.0 and GCMSsolution ver.4.30 (Shimadzu, Ktoto, Japan). L-Rhamnose, D-xylose, D-glucose and D-glucuronic acid were used as references.

Analytical methods

The molecular masses of chemical components purified from seaweeds by gel filtration were measured by polyacrylamide gel electrophoresis with NuPAGE 4-12% Bis-Tris Gel and HMW-Native Marker Kit. The gel was stained with two types of dyes [22] as described below. First, the gel was stained for proteins with 0.25% Coomassie Brilliant Blue R-250, and then destained in 7% acetic acids/10% methyl alcohol. Next, it was stained for acidic polysaccharides with 0.5% methylene blue in 3% acetic acid and destained in water.

Statistical analysis

Three independent experiments were performed twice, and statistical analysis was performed by analysis of variance (ANOVA) using a software, StatView (SAS Institute, Inc., Cary, NC, USA).

Results

Adsorption assay of Stx to chemical components of seaweed extracts

We screened 14 edible seaweeds in the ability to adsorb Stx. Although we could not detect the Stx-adsorbing ability in the insoluble residues of these seaweeds (data not shown), most

seaweed extracts showed the ability to adsorb Stx (Fig. 1). The extracted chemical components of two green algae, *M. nitidum* and *U. linza*, adsorbed higher amounts of Stx1 and Stx2 than those of other algae. Four brown algae, *E. bicyclis*, *H. fusiforme*, *S. japonica,* and *N. decipiens,* partially adsorbed both Stx1 and Stx2. However, *S. horneri* and *U. pinnatifida* did not adsorbed Stx, and *C. filum* adsorbed small amounts of Stx1. Almost red algae, *C. hypnaeoides, M. japonicua, G. tenax* and *U. prolifera,* adsorbed small amounts of Stx2, and *G. elegans* did not adsorb Stx. Since the *U. linza* extract well adsorbed both Stx1 and Stx2, we chose the extract for further experiments.

Purification and properties of Stx-adsorbent

The Stx-adsorbing substance in the *U. linza* seaweed extract was bound to DEAE-Topearl and eluted by about 0.25 M NaCl (Fig. 2). The active fractions were concentrated and then subjected to the gel filtration with HiPrep 16/60 Sephacryl S-300 HR column (data not shown). Active fractions of the gel filtration were harvested and dialyzed against deionized water and freeze-dried. We obtained about 50 mg of the freeze-dried sample from 5 g of the dry seaweed. The purified substance was subjected to polyacrylamide gel electrophoresis. Although we could not detect any band stained with Coomassie Brilliant Blue R-250, a broad band was observed after staining with methylene blue (Fig. 3). Average molecular mass of the purified substance was about 800 kDa. The activity was stable even after an enzymatic treatment at 37^{o}C for 5 h with 0.1 mg/ml of Proteinase K. Hydrolysate of the purified substance with 2 N TFA did not show the Stx-adsorbing activity.

Analysis of Stx-adsorbent

When we examined the sulfate content of the purified substance, it contained about 18% (w/w) of sulfate. Total sugar and uronic acid of the purified substance were about 60% (w/w) and 20% (w/w), respectively. The sugar composition was analyzed by GCMS (Fig. 4). Monosaccharides including their anomers were clearly separated from each other under the

conditions. Peaks I and II in Fig. 4 were identical with those of rhamnose in the retention times and mass spectra. Similarly, peaks III and IV were identical with those of xylose, peaks VI and VIII were identical with those of glucose, and peaks V, VII and IX were identical with those of glucuronic acid. The content of glucuronic acid in this polysaccharide was calculated to be 17.0% (w/w). Major uronic acid in this polysaccharide was considered to be glucuronic acid. The molar ratio of neutral sugars were 3.87 (rhamnose), 1.0 (xylose), and 0.12 (glucose). Although amino acids and lipids can also be detected by the GCMS analysis, we could not detect them as components of the purified substance. These results indicated that the purified substance was a rhamnan sulfate.

Stx-adsorbing activity of the rhamnan sulfate

We analyzed the ability of the purified rhamnan sulfate to adsorb Stx by the equilibrated dialysis method with Float-A-Lyzer G2 (MWCO=300 kDa). Figure 5A showed the amounts of Stx in the inner and outer liquid of equilibrated dialysis. The *K*d values of the rhamnan sulfate against Stx1 and Stx2 were calculated to be 1.9 and 3.5 μM, respectively (Fig. 5B).

Discussion

We previously reported that Stx adsorbed to poly-γ-glutamic acid (PGA) precipitated beyond its saturated density, but that the toxin did not adsorb to the soluble form of PGA (23). In order to explore foods with the ability to adsorb Stx under intestinal environments, we screened and analyzed fourteen edible seaweeds containing indigestible polysaccharides. Among the seaweed extract tested, the *U. linza* extract was found to adsorb both Stx1 and Stx2. Stx-adsorbing substance purified from the *U. linza* extract showed a single but broad band by polyacrylamide gel electrophoresis with average molecular mass of about 800 kDa. Analysis of the components of the purified substance indicated that the active substance was a rhamnan sulfate. This rhamnan sulfate was different in the average molecular mass from that of *Enteromorpha linza* (about 108 kDa) [24], probably because of differences in species

and variety.

The *K*d values of the rhamnan sulfate to Stx1 (1.9 μM) and Stx2 (3.5 μM) were obviously lower than that of free Gb3 (about 1 mM) [25]. Interaction between Stx and cell surface receptor (Gb3) is reported to be multivalent; three binding sites on each B subunit monomer for Gb3 [26, 27]. Therefore, clustered Gb3 is required for strong binding [28]. A Gb3-chitosan conjugate was reported to have lower *K*d values (0.05 μM for Stx1B, 0.43 μM for Stx2B) to Stx than free Gb3 [9]. From this standpoint, clustered rhamnan sulfate may strongly adsorb Stx, although further study is required. In the case of the rhamnan sulfate, its binding site to Stx is probably different from that of Gb3, because their sugar components are different from each other. Structure of rhamnan sulfates from green algae shows great complexity and variability as evidenced by the numerous oligosaccharide repeating structural units (29). The binding site of the rhamnan sulfate to Stx may be one of the repeating structural units, and clusters of the structural unit may be required for strong adsorption of Stx, although further study is required. In addition, Gallegos et al. [30] reported that binding of Stx2 to Gb3 was influenced by residues in the ceramide portion of Gb3 and the lipid environment. Karve at al. [31] also reported that the binding of Stx variants increased in the presence of phosphatidylcholine and cholesterol. Therefore, further study on *in vivo* affinity of rhamnan sulfate to Stx is required from these standpoints.

The *U. linza* seaweed is absolutely safe, and it is easy to take the rhamnan sulfate in relatively large quantities from the seaweed; 5 g of the *U. linza* seaweed contains about 50 mg of the rhamnan sulfate as described above. The seaweed seemed to be a potential resource for removal of free Stx from the gut of patients.

Another green alga extract (*M. nitidum*) was also found to adsorb both Stx1 and Stx2. Lee et al. [32] reported that rhamnan suflfate from *M. nitidum* was a high rhamnose–containing polysaccharide with 31.7% sulfate esters, 4.6% uronic acid, and trace amounts of D-glucose and D-xylose. Although the rhamnan suflfate from *U. linza* was different from that of the *M. nitidum* in the ratio of components, these rhamnan sulfates may

have similar binding sites for Stx. Isoelectric points of Stx1 and Stx2 were reported to be 7.0 and 4.1, respectively [33, 34]. Since the rhamnan sulfates from *U. linza* and *M. nitidum* showed an affinity to acidic Stx2, the mechanism of adsorption seemed not to be an ionic interaction.

Almost brown and red algae partially adsorbed Stx. These algae are known to contain sulfated polysaccharides such as fucoidan and sulfated galactans. When we analyzed commercial fucoidan from *Fucus vesiculosus* (Sigma-Aldrich) in the ability to adsorb Stx, it showed a relatively low affinity to Stx1 (Kd = 1.8 mM) and Stx2 (Kd = 25.5 μM). The difference in the ability to adsorb Stx between brown algae tested may be due to that in the content of fucoidan. Red algae with sulfated galactans were considered to have low ability to adsorb Stx. Thus, green algae containing rhamnan sulfate showed a higher ability to adsorb Stx than brown and red algae.

Conclusions

We screened and analyzed 14 edible seaweeds in the ability to adsorb Stx. Although the insoluble fractions of 14 seaweeds surveyed did not adsorb Stx, most seaweed extracts showed the ability to adsorb Stx. The *U. linza* extract among them well adsorbed both Stx1 and Stx2. The Stx-adsorbing substance was purified and analyzed. The average molecular mass of the purified substance was about 800 kDa. The content of sulfate, total sugar, and uronic acid were about 18%, 60%, and 20%, respectively. The mole ratios of neutral sugars were calculated to be 3.87 (rhamnose), 1.0 (xylose), and 0.12 (glucose). Thus, the purified substance was regarded as a rhamnan sulfate. The Kd values of the rhamnan sulfate to Stx1 and Stx2 were 1.9 and 3.5 μM, respectively. To our knowledge, this is the first report on Stx-adsorbing dietary fibers.

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Conpliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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Figure Captions

Fig. 1 Adsorption of Stx to the extracted chemical components of seaweeds. The 5% seaweed extract (0.1 ml) and the Stx solution (0.4 ml) were mixed to give the final Stx conc. of 100 ng/ml, and subjected to the equilibrated dialysis. After the dialysis, the amounts of Stx1 and Stx2 in the inner and outer liquids were analyzed by the RPLA assay. Three independent experiments were performed twice, and the vertical bars show the standard errors. White bars = adsorbed Stx1; black bars = adsorbed Stx2.

Figure 2-Elution profile of the *U. linza* seaweed extract on DEAE-Toyopearl column chromatography. The seaweed extract (5%, 100 ml) was subjected to the column (2.0 x 20 cm) equilibrated with 20 mM Tris-HCl (pH 7.5). After washing the column with the same buffer, elution was made with a linear gradient from 0 to 1.0 M NaCl in the same buffer.

Fig. 3 Polyacrylamide gel electrophoresis of Stx-adsorbent. The Stx-adsorbent purified (7.5 μg) was subjected to polyacrylamide gel electrophoresis (lane S). First, the gel was stained for protein with 0.25% Coomassie Brilliant Blue R-250 and destained in 7% acetic acid and 10% methyl alcohol. Next, it was stained for acidic polymers with 0.5% methylene blue in 3% acetic acid, and destained in water. Lane M contained standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

Fig. 4 GCMS analysis of purified Stx-adsorbent. Stx-adsorbent (50 mg) was hydrolyzed with 2M TFA at 100^{o}C for 4 h. After removal of TFA under N_2 gas, the hydrolysate was dried up under reduced pressure. The dried sample (1 mg) was trimethylsilylated at 70^{o}C for 1 h with 0.1 ml of (BSA + TMCS + TMSI, 3:2:3), and then analyzed with GCMS-QP2010 Ultra and Trace Gold TG-1MS GC column (30 m × 0.25 mm × 0.25 μm). Injection temperature was 250^{o}C; oven temperature was from 80^{o}C to 320^{o}C with a increasing rate of 15^{o}C/min; split ratio was 10:1; and injected sample was 1 μl.

Fig. 5 Binding of Stx to the rhamnan sulfate by the equilibrated dialysis method. Purified rhamnan sulfate (0.25 ml, 1.88 μM) and several concentrations of Stx solution (0.25 ml) were mixed, put in a bag of the Float-A-Lyzer G2 (MWCO = 300 K), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% bovine serum albumin at 4^{o}C for 16 h. The amounts of Stx in the dialysis bag (bound Stx, closed bars) and outer liquid (free Stx, open bars) were analyzed by the RPLA assay (A). The ratios (ν) of [bound Stx] to [the rhamnan sulfate (RS)] were plotted against ν /[free Stx] (B). The *K*d values were obtained by calculating the reciprocal of slopes of lines in Fig. 5B. Three independent experiments were performed twice, and the vertical bars show the standard errors.

Fig.1

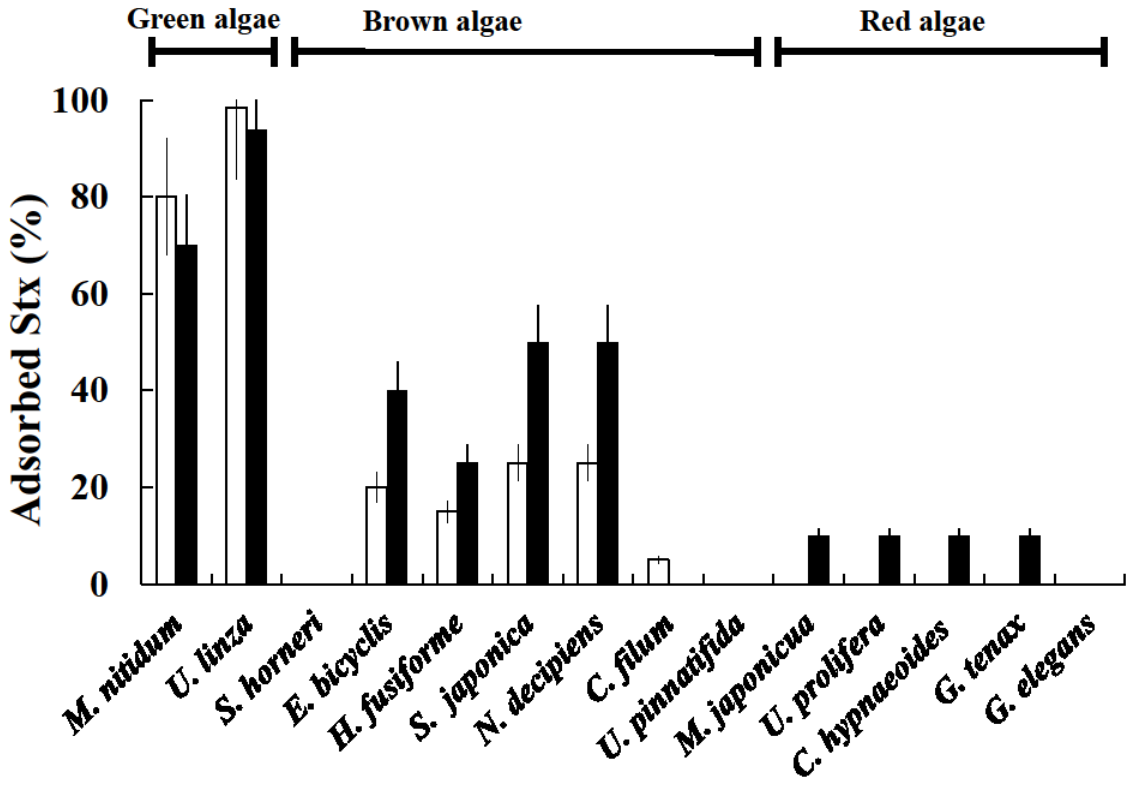

Green algae
Brown algae
Red algae
Adsorbed Stx (%)
100
80
60
40
20
0
M. nitidum
U. linza
S. horneri
E. bicyclis
H. fusiforme
S. japonica
N. decipiens
C. filum
U. pinnatifida
M. japonicua
U. prolifera
C. hypnaeoides
G. tenax
G. elegans

Fig. 2

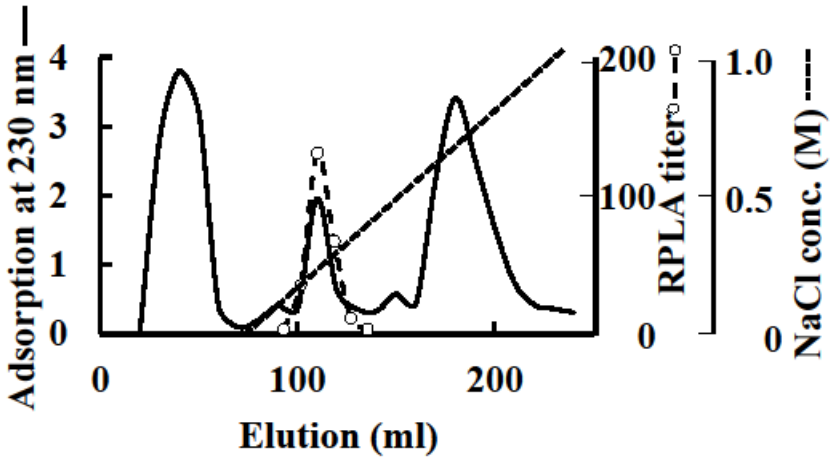

Adsorption at 230 nm
4
3
2
1
0
0
100
200
Elution (ml)
200
100
0
RPLA titer
1.0
0.5
0
NaCl conc. (M)

Fig. 3

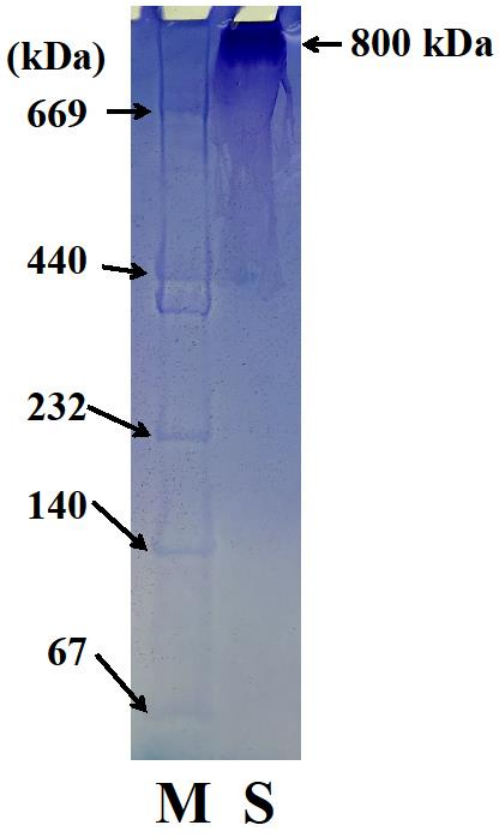

(kDa)
800 kDa
669
440
232
140
67
M S

Fig.4

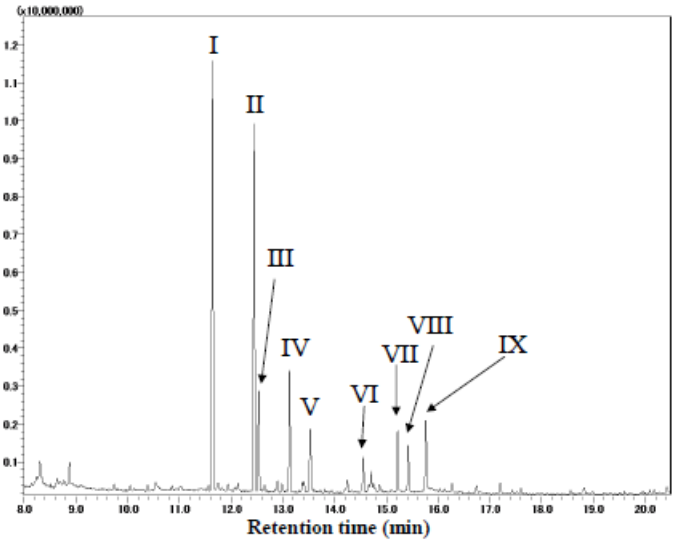
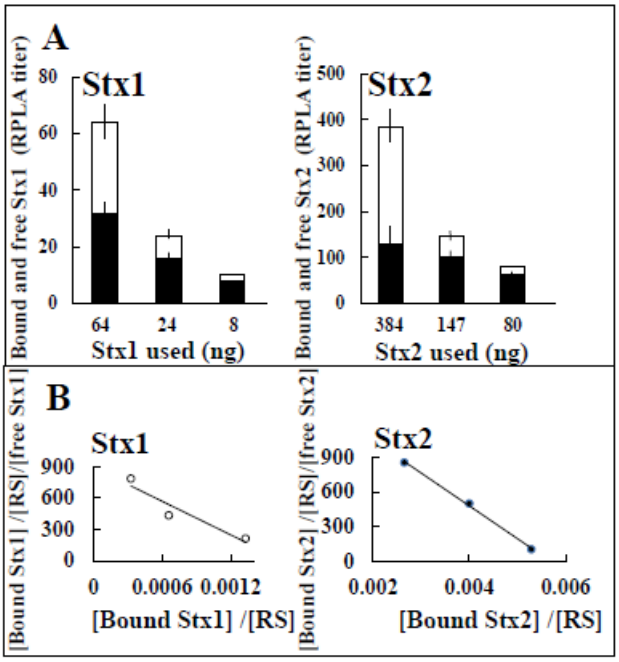

(x10,000,000)
I
II
III
IV
V
VI
VII
VIII
IX
Retention time (min)

Fig. 5


A
Stx1
Bound and free Stx1 (RPLA titer)
80
60
40
20
0
64
24
8
Stx1 used (ng)
Stx2
Bound and free Stx2 (RPLA titer)
500
400
300
200
100
0
384
147
80
Stx2 used (ng)
B
Stx1
[Bound Stx1] /[RS]/[free Stx1]
900
600
300
0
0
0.0006
0.0012
[Bound Stx1] /[RS]
Stx2
[Bound Stx2] /[RS]/[free Stx2]
900
600
300
0
0.002
0.004
0.006
[Bound Stx2] /[RS]