Studies on edible seaweeds in the ability to adsorb Shiga toxin and mutagens

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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 was first recognized as a food-borne pathogen in 1982 (Riley et al., 1983). 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 (Walterspiel et al., 1992), 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) (Donohue-Rolfe et al., 1991). The B subunit pentamer binds to globotriaosylceramide (Gb3) on the cell surface of renal endothelial cells, and ferries the A subunit into the cells (Lingwood et al., 1987). The A subunit activated by a membrane-anchored protease furin impairs renal function by inhibiting eukaryotic protein synthesis (Garred et al., 1995 and Lea et al., 1999). Stx is also reported to bind to the P1 blood group antigen that is present in human erythrocyte glycolipid extracts (Jacewicz et al., 1986), and ovomucoid from pigeon egg white with the antigen is reported to adsorb Stx1 (Miyake et al., 2000). Although several polymers including the globotriose moiety of Gb3 have been reported to adsorb Stx (Miyake et al., 2000; Li et al., 2012; Miyagawa et al., 2006; Nishikawa et al., 2002 and Watanabe

et al., 2004), 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 (Al-Amoudi et al., 2009), anticoagulant, anti-inflammatory (Ananthi et al., 2010), antitumor (Synytsya et al., 2010), contraceptive, and antiviral activities, for the treatment of several diseases (Wang et al., 2010). Especially, a sulfated polysaccharide, fucoidan, is extensively explored for its medicinal properties and being isolated from various species of seaweed (Wijesinghe and Jeon 2012). 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. In chapter 1, I explored and analyzed polysaccharides from some edible seaweeds in the ability to adsorb Stx.

DNA damage is crucial in variety of diseases and degenerative processes. In recent years there has been great interest in investigating natural compounds with ability to protect cells from carcinogens and mutagens. These compounds could act against the initiation, promotion or progression stages of carcinogenesis (Edenharder *et al.*, 1993) and destroy or block the DNA-damaging mutagens from outside of cells (Ruan, 1989). Seaweeds have been known to have multiple therapeutic properties such as suppression against some types of cancer (Carper, 1987). Synytsya *et al*. (2010) studied antitumor activity of fucoidan isolated from brown seaweed *Undaria pinnatifida*. Also, Yamamoto and Maruyama (1985) and Yamamoto *et al*. (1987) reported that oral intake of seaweed powder or its extract caused a decrease in the incidence rate of *invivo* chemically induced carcinogenesis. Studies on antimutagenic compounds in edible seaweeds are interesting and promising for the chemoprevention against carcinogenesis (Okai *et al*., 1994). Some investigators reported antimutagenic activity of some seaweeds (Okai *et al*., 1993) Hot water extracts of edible brown seaweeds *Laminaria japonica* and *Undaria pinnatifida*

were reported to have antimutagenic activity against typical genotoxic substances by the *umu* gene expression system with *Salmonella typhimurium* (AT 1535/pSK 1002). The significant antimutagenic activity in the extracts was found in polysaccharides and non-polysaccharide substances. A significant antimutagenic activity was also found in the hot water extract of an edible brown alga, *Hijikia fusiforme* (Okai and Higashi-Okai, 1994). Antimutagenic activity of methanol extracts of eight edible seaweeds in Japan has been reported. Among them *Porphyra tenera* showed the highest antimutagenic activity (Okai et al., 1994). Seaweeds from the Maxican Pacific ocean have been evaluated as a source of chemoprotectants and *Rhizoclonium riparium* seaweed has been reported to show highest mutagenesis inhibition (Osuna-Ruiz et al., 2016). In chapter 2, I examined antimutagenic activity of hot water extracts of various edible seaweed through the suppressive effect on the SOS response of *Salmonella typhimurium* induced by several chemical mutagens. β-Galactosidase activity of the bacterium with a fusion gene umuC'-'lacZ was measured to estimate the antimutagenic activity of the extracts of seaweeds.

Chapter 1

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

I screened edible seaweeds in the ability to adsorb Shiga toxin (Stx) by an equilibrated dialysis method. Although water insoluble fractions of fourteen edible 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. I 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. Analysis 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 my knowledge, this is the first report on Stx-adsorbing dietary fibers.

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 (30m×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, *Ulva Linnaeus*, 1753 (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), *Chondracanthus tenellus* (Harvey) Hommersand(suginori),*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 (Takemasa et al., 2009). 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 polysaccharidesby 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 I 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 (Dubois et al., 1956). Uronic acid content of Stx-adsorbent was determined by *m*-hydroxydiphenylmethod (Blumenkrantz and Asboe-Hansen, 1973). Sulfate content was determined by rhodizonate method (Silvestri et al., 1982). 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 from80^{o}C to 320^{o}C. Identification and quantification of sugars were made with NIST 14 Mass Spectral Library ver. 1.0 and GCMS solution 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 (Ito et al., 1996) 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

I screened 14 edible seaweeds in the ability to adsorb Stx. Although I 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, *Ulva Linnaeus* 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, I chose the extract for further experiments.

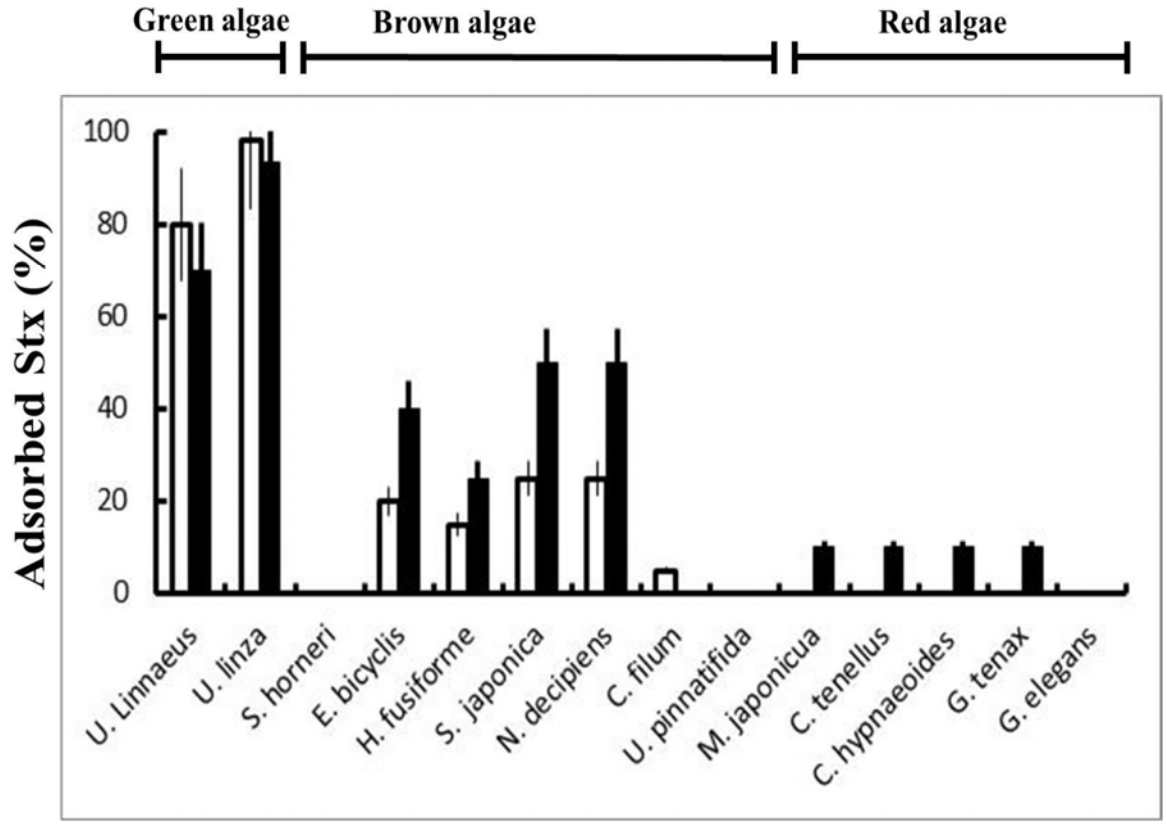

Figure 1 Adsorption of Stx to the extracted chemical components of seaweeds.

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.

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°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.

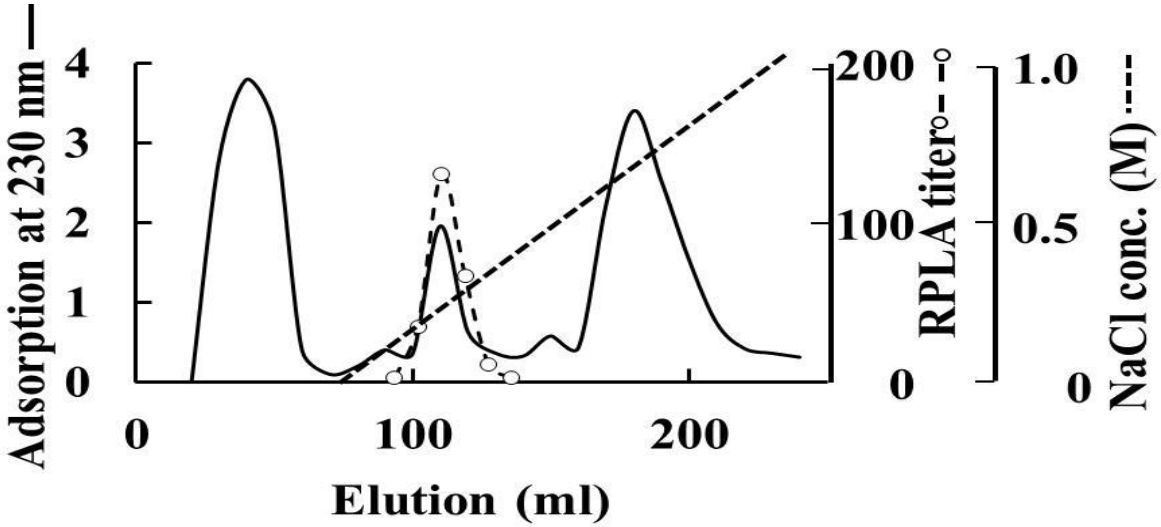

Figure 2 Elution profile of the *U. linza* seaweed extract on DEAE-Toyopearl column chromatography.

The seaweed extract (5%, 100 ml) was subjected to the column (2.0 × 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.

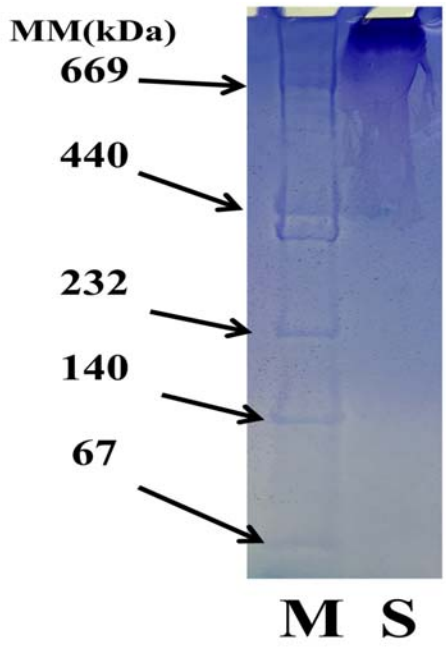

Figure 3 Polyacrylamide gel electrophoresis of Stx-adsorbent.

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).

Analysis of Stx-adsorbent

When I 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). Mono saccharides 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, I could not detect them as components of the purified substance. These results indicated that the purified substance was a rhamnan sulfate.

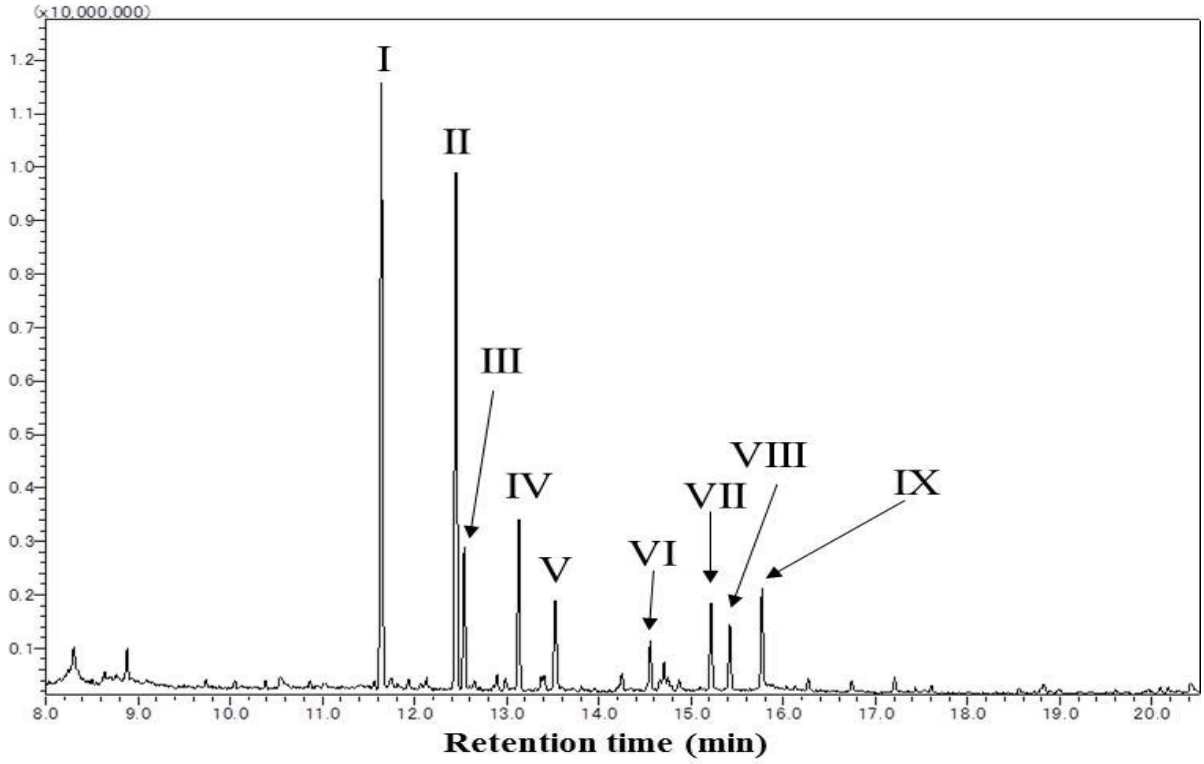

Figure 4 GCMS analysis of purified Stx-adsorbent.

Stx-adsorbent (50 mg) was hydrolyzed with 2 M TFA at 100 °C for 4 h. After removal of TFA under N2 gas, the hydrolysate was dried up under reduced pressure. The dried sample (1 mg) was trimethylsilylated at 70 °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 °C; oven temperature was from 80 to 320 °C with an increasing rate of 15 °C/min; split ratio was 10:1; and injected sample was 1 μl.

Stx-adsorbing activity of the rhamnan sulfate

I 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).

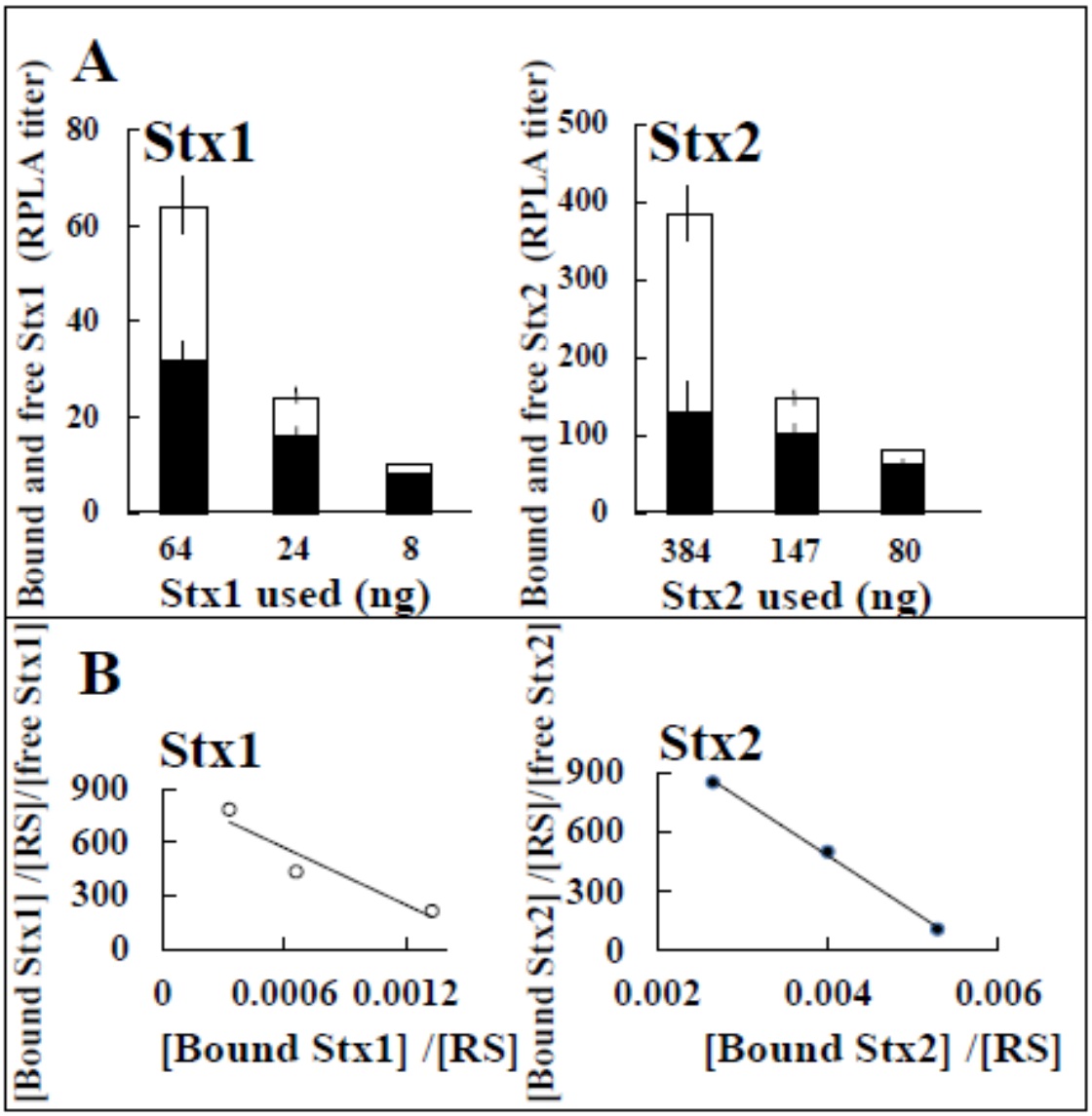

Figure 5 Binding of Stx to the rhamnan sulfate by the equilibrated dialysis method.

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 °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.

Chapter 2

Suppressive effect of some edible seaweeds on SOS response of *Salmonella typhimurium* induced by chemical mutagens

I examined antimutagenic activity of hot water extracts of twelve edible seaweeds by analyzing the suppressive effect on the SOS response of *Salmonella typhimurium* induced by direct [furylframide, AF-2 and 4-nitroquinoline 1-oxide, 4NQO] and indirect [3-amino-1-methyl-5H-pyrido-(4,3-b) indole, Trp-P-2 and 2-amino-3-methylimidazo (4,5-f) quinoline, IQ] mutagens.Dry seaweeds were milled, suspended in water, and autoclaved. Supernatant solutions obtained by centrifugation of the autoclaved suspensions were used as hot water extracts. Antimutagenic activities of the seaweed extracts were different form each other against each mutagen used. The extract of a brown alga *E.bicyclis* showed a strong activity against AF-2 and that of *C.filum had* moderate activity. The extracts of *E.bicyclis, C.filum* and *H.fusiforme* seaweeds exhibited strong activities against 4NQO. Against an indirect mutagen Trp-P-2, the extracts of two brown algae (*E.bicyclis and N.decipiens*) and those of three red algae (*M.japonicua, G.tenax and C.tenellus*) showed strong activities. The extracts of six brown seaweeds (*H.fusiforme, S.japonica, E.bicyclis, N.decipiens, S.horneri,* and *C.filum*) and the green seaweed (*U. linza*) exhibited strong antimutagenic activities. Thus, the extract of *E.bicyclis* among the extracts used was found to have the highest activity irrespective of the types of mutagens. The brown seaweed *E.bicyclis* could prove useful as an antimutagenic seaweed.

Materials and Methods

S. typhimurium TA1535/pSK1002 was provided by Dr. Yoshimitsu Oda, Osaka Prefectural Institute of Public Health, Osaka.The direct mutagens used were furylframide (AF-2) and 4-nitroquinoline-1-oxide (4NQO). The indirect mutagens used, which were activated by the S9 mix (cofactor A set for Ames test, Oriental Yeast Industries, Osaka), were 3-amino-1-methyl-5H-pyrido (4,3-b) indole (Trp-P-2)and 2-amino-3-methylimidazo (4,5-f) quinoline (IQ) . These chemical mutagens were obtained from Wako Pure Chemical Industries (Osaka).

Modified *umu* test

The antimutagenicity of seaweed extracts was analyzed by a slightly modified *umu* test (Oda et al., 1985), which is based on the ability of DNA-damaging agents to induce the *umu* operon expression and the ability of antimutagenic substances to suppress the expression. A plasmid (pSK 1002) carrying a fusion gene *umu*C'-'*lacZ* was introduced into *Salmonella typhimurium* TA1535. The expression and suppression of the *umu* operon were analyzed by measuring the β-galactosidase activity of the cells. Strain TA1535 with pSK1002 was pre-cultured at 37℃ for 16 h in TGA medium (1% bactotrypton, 0.5% NaCl, 0.2% glucose and 0.002% ampicillin). The culture was diluted 50-fold with TGA medium and further incubated to an appropriate bacterial cell density (absorbance of 0.2 - 0.3 at 600 nm). The standard assay mixture for analyzing the suppressive effect on the SOS response contained 20 μl of each direct mutagen (final concentration: AF-2, 0.03 μg/ml; 4NQO, 0.3 μg/ml), 1 and 3% hot water-soluble extracts of seaweeds, and 2.0 ml of the cell suspension described above. For the assay using indirect mutagens, 1.7 ml of the cell suspension supplemented with 0.3 ml of the S9 mix was mixed with

PGA and each indirect mutagen (IQ, 0.3 μg/ml; Trp-P-2, 3 μg/ml). The concentration of each mutagen at which the β-galactosidase activity was induced without the inhibition of bacterial growth was selected. After incubation at 37°C for 2 h, the mixture was diluted with 0.85% NaCl, and the cells were harvested by centrifugation at 3000 × g for 15 min. The precipitate was resuspended in 0.85% NaCl, and then the β-galactosidase activity of the cell extract was assayed (A). The standard assay mixture without seaweed extract was used for the test as the control (B). The percentage suppression of the SOS response was calculated using the equation

$$(1 - A/B) \times 100 \quad (1)$$

The cell extract used for measuring the β-galactosidase activity was prepared as follows: 200 μl of the cell suspension in 0.85% NaCl was vigorously mixed with 1.8 ml of Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05 M β-mercaptoethanol), 50 μl of 0.1% sodium dodecyl sulfate and 10 μl of chloroform. The enzyme reaction was initiated by adding 0.2 ml of 2-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 0.1 M phosphate buffer, pH 7.2) to the cell extract, followed by incubation at 28°C for 20 min; the reaction was stopped with 1.0 ml of 1 M Na_2CO_3. β-Galactosidase activity was calculated as follows (12).

$$\beta\text{-galactosidase unit} = 1000(C - 1.75 \times D)/0.2 \times E \quad (2)$$

Here, C and D represent the absorbance at 420 and 550 nm, respectively, of the enzyme reaction mixture, and E shows the absorbance at 600 nm of the bacterial suspension grown in the *umu* test.

Seaweeds

Dry edible seaweeds were purchased from a food store in Tokushima, Japan. They are twelve seaweeds, *Ulva Linnaeus*, 1753 (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), *Chondracanthus tenellus* (Harvey) Hommersand (suginori), *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 were milled at 20,000 rpm for 1 min with Millser-620DG, and hot water-soluble extracts of seaweeds (1% and 3%) were prepared as described in chapter 1.

Results

Conditions for assay of antimutagenic activity

I examined the concentration of seaweeds extracts used for determination of antimutagenic activity by the *umu* C gene expression system. All extracts showed very low activities below 0.1% concentration. Therefore, I used the extracts at 1% and 3% in the system. Neither inhibition nor activation against β-galactosidase activity was observed when I used all extracts at 1% and 3%.

Antimutagenic activity against direct mutagens

As shown in Fig. 1, antimutagenic activities of the seaweed extracts were different form each other against each mutagen used. The 3% extract of a brown alga *E.bicyclis* showed a strong activity against AF-2 according to the classification proposed by Ikken et al. (1999) and that of *C.filum* had moderate activity (Fig. 1A). Five extracts of *M.japonicua, U.linnaeus, N.decipiens and G.tena* showed very weak activity at 3% concentration, and the other investigated seaweeds did not show any activity. The extracts of *E.bicyclis, C.filum* and *H.fusiforme* seaweeds exhibited strong activities against 4NQO, while other extracts showed weak activities or no activity (Fig. 1B). Thus, the extract of *E.bicyclis* among the 12 extracts used was considered to have the highest activity against the direct mutagens.

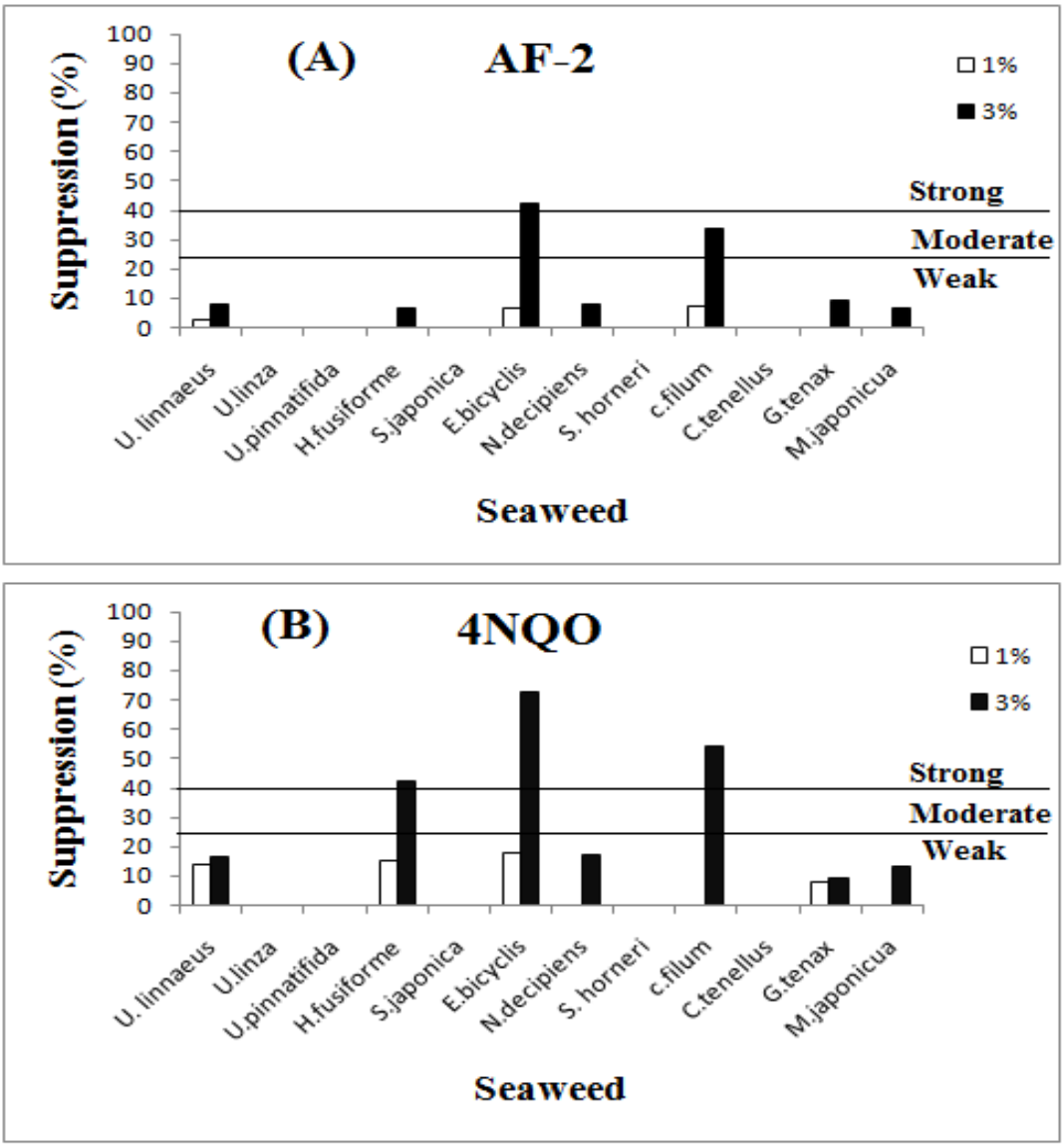

Figure 1 Effects of seaweed extracts on SOS response induced by direct mutagens

(A): (AF-2 ,0.03 mg/ml) and (B): (4NQO 0.3 mg/ml).Seaweed extracts (final conc.: 1% and 3%) were added to the standard assay mixture of the *umu C* gene expression system. Each result is expressed as the mean value of triplicate experiments, White bars: suppression at 1% seaweed extracts; and black bars: suppression at 3% seaweed extracts. Activity reference values (% of suppression: >40= strong, 25-40= moderate and < 25= weak antimutagenic activity) were used according to Ikken et al., (1999).

Antimutagenic activity against indirect mutagens

Against an indirect mutagen IQ, most seaweed extracts showed high antimutagenic activities (Fig. 2A). The 3% extracts of six brown seaweeds (*H.fusiforme, S.japonica, E.bicyclis, N.decipiens, S.horneri,* and *C.filum*) and the green seaweed (*U. linza*) exhibited strong antimutagenic activities. The extracts of red alga *M.japonicua* and green alga *U.linnaeus* showed moderate activity, while the extract of red alga G.tenax showed very low activity. Only two extracts showed no activity against IQ mutagen.

Against another indirect mutagen Trp-P-2, most of the extracts showed antimutagenic activities (Fig. 2B). The 3% extracts of two brown algae (*E.bicyclis and N.decipiensand*) and those of three red algae (*M.japonicua, G.tenax and C.tenellus*) showed strong activities. The extract of *E.bicyclis* among the extracts used was found to have the highest activity irrespective of the types of mutagens. The brown seaweed *E.bicyclis* could prove useful as an antimutagenic seaweed.

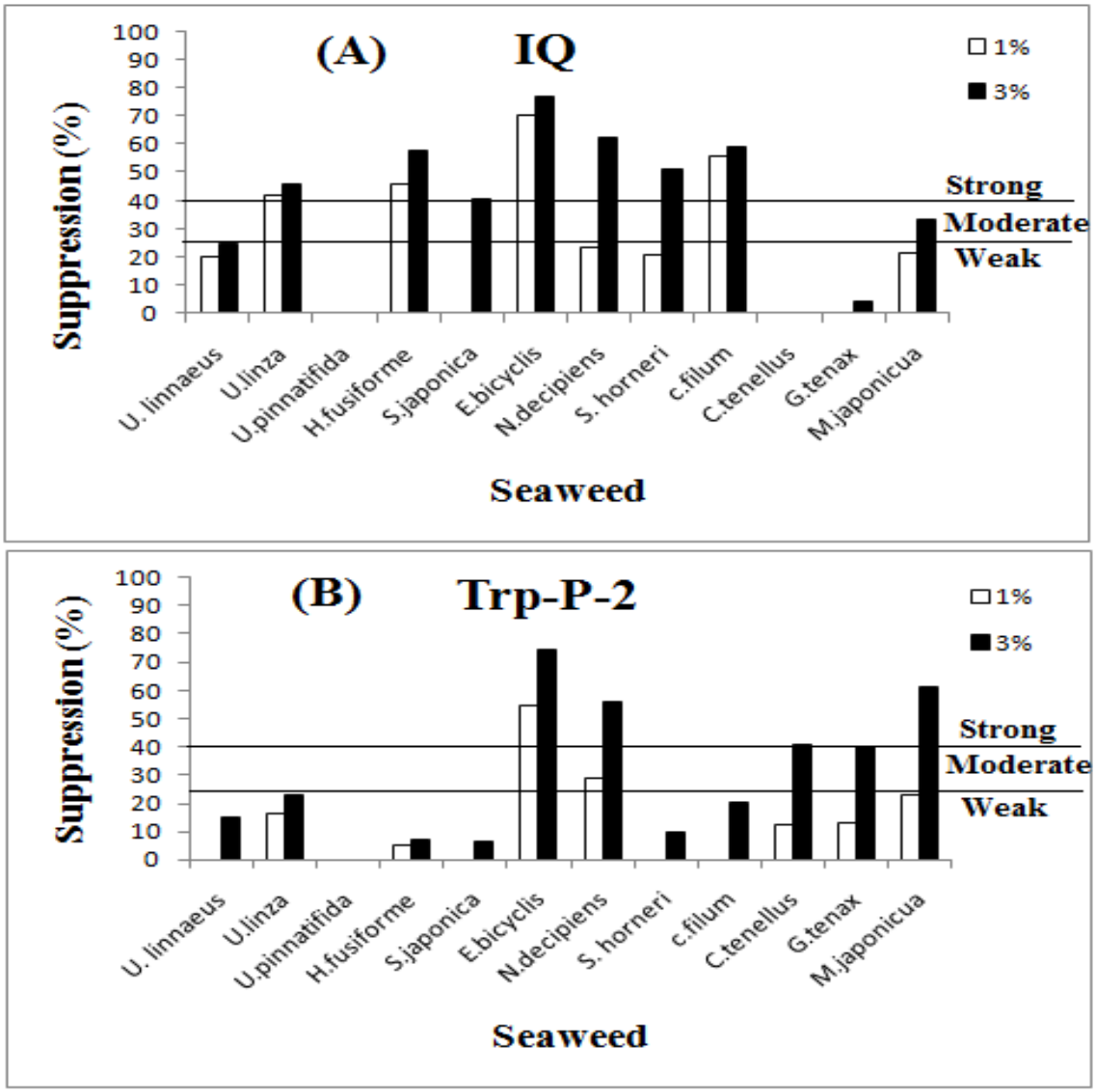

Figure 2 Effects of seaweed extracts on SOS response induced by indirect mutagens

(A): (IQ , 0.3mg/ml) and (B): (Trp-P-2, 3 mg/ml).Seaweed extracts (final conc.: 1% and 3%) were added to the standard assay mixture of the *umu C* gene expression system. Each result is expressed as the mean valueof triplicate experiments. White bars: suppression at 1% seaweed extract; and black bars: suppression at 3% seaweed extracts. Activity reference values (% of suppression: >40= strong, 25-40= moderate and < 25= weak antimutagenic activity) were used according to Ikken et al., (1999).

Discussion

In chapter 1, I 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) (Wanget al., 2013), 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) (St Hilaire et al., 1994). Interaction between Stx and cell surface receptor (Gb3) is reported to be multivalent; three binding sites on each B subunit monomer for Gb3 (Fraser et al.,2004 and Ling et al., 1998). Therefore, clustered Gb3is required for strong binding (Nishikawa, 2011). 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 (Li et al., 2012). 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 (Lahaye and Robic, 2007). 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. (Gallegos et al., 2012) reported that binding of Stx2 to Gb3 was influenced by

residues in the ceramide portion of Gb3 and the lipid environment. (Karveand Weiss, 2014) 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 (*Ulva Linnaeus*) was also found to adsorb both Stx1 and Stx2. Lee et al. (2010) reported that rhamnan sulfate from *Ulva Linnaeus* 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 sulfate from *U. linza* was different from that of the *Ulva Linnaeus* 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 (Dickieet al., 1989 and Okuet al., 1989). Since the rhamnan sulfates from *U. linza* and *Ulva Linnaeus* 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 = 25.5μM). 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.

In chapter 2, I examined antimutagenic activity of hot water extracts of twelve edible seaweeds by analyzing the suppressive effect on the SOS response of *S. typhimurium* induced by direct and indirect mutagens. The results indicated that some of the investigated hot water extracts of twelve edible seaweeds had a potent protective activity against mutagenesis induced by directly acting mutagens and indirect mutagens. As a whole, the activity was higher against indirect mutagens than direct mutagens. Among the investigated seaweed extracts, an extract from the brown seaweed *E.bicyclis* showed the strongest activity against all the investigated mutagens. Some investigators studied antimutagenic activity of the hot water extracts from edible brown seaweeds, *Laminaria japonica* and *Undaria pinnatifida* against mutagenesis using direct mutagens (MNNG & AF-2) and indirect mutagens (2-AAF & Trp-P-1). They reported that these seaweed extracts contain heterogenous antimutagenic activities against genotoxic substances, and that the major activity was detected in a non-polysaccharide fraction. The fraction exhibited a relatively strong antimutagenic activity against indirect mutagens (2-AAF or Trp-P-1) but a weak activity against direct mutagens (MNNG or AF-2). Minor activity was found in the polysaccharide fraction of the extracts. My results in this study indicated that most seaweeds extracts exhibited strong activity against indirect mutagens (Trp-P-2 or IQ) but a weak activity against direct mutagens (AF-2 or 4NQO), but that any antimutagenic activity was not detected in the extract of *U. pinnatifia*. The difference may be due to those in algal species, habitat, and time of collection (Khotimchenko, 2010). Okai and Higashi-Okai (1994) also found a significant antimutagenic activity in the hot water extract of the brown alga, *Hijikia fusiforme*. However, they reported that the major activity was detected in the polysaccharide fraction, and

that the minor activity was found in the non-polysaccharide fraction of the extracts. Depending on the species of algae, the active components with antimutagenic activity may be different from each other, although further study is required. Antimutagenic activity of methanol-soluble extracts of eight varieties of edible seaweeds against Trp-P-1 mutagen was also studied by Okai et al, 1994. Their results indicated that *Enteromorpha prolifera* and *Porphyra tenera* showed strongest suppressive activities compared with the other investigated seaweeds, and that these seaweeds contained considerable amounts of β-carotene. Okai et al. (1996) analyzed active components with antimutagenic activity in methanol extract of the red seaweed *Porphyra tenera*. They found that the antimutagenic activity of the seaweed extract was strongly associated with the functions of β-carotene, chlorophyll a and lutein. Nantes *et al*. (2014) reported that carrageenan had significant chemopreventive potential that is mediated by both demutagenic and bio-antimutagenic activities. They also reported that the polysaccharide could adsorb agents that are toxic to DNA and inactivate them. Although red algae contain carrageenan, the extracts of red seaweeds I used showed relatively low antimutagenic activities. Probably because their experimental conditions were different from my conditions; they used an *Allium cepa* assay in which the cells were incubate for 24-48 h with carrageenan.

The results in this study indicated that 3% concentration of seaweed extracts showed higher antimutagenic activities than 1% concentrations. This was similar to that of Osuna-Ruiz *et al*.(2016). They studied antimutagenic activity of acetone extracts of *C. sertularioides, R. riparium* and *S. filamentosa* at different concentrations (0.003-3.0 mg/plate) on *S. typhimurium* TA98 and TA100 tester strains, and found that inhibition of aflatoxin B_1 (AFB_1) mutagenicity on both tester strains decreased with decrease of the seaweed extract concentration.

This study indicated that the extract of brown seaweed *E.bicyclis* exhibited the strongest

activity against all the investigated mutagens among the seaweed extracts used. The brown seaweed *E.bicyclis* seemed to be used as an alternative source of biological active substances for the mutagenesis.

Conclusions

I 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 *K*d values of the rhamnan sulfate to Stx1 and Stx2 were 1.9 and 3.5 μM, respectively. This is the first report on Stx-adsorbing dietary fibers. In addition, most seaweeds extracts showed antimutagenic activities. Especially, the extract of brown seaweed *E.bicyclis* showed the highest antimutagenic activity among the 12 edible seaweed extracts, irrespective of the types of mutagens. Edible seaweeds could prove useful as adsorbents of chemical mutagens.

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Publications

Main publications

1. Hoida Ali Badr, Keiko Takahashi, Ryushi Kawakami, Yasuo Oyama, Kumio Yokoigawa, and Kaori Kanemaru.(2017): Screening and analysis of edible seaweeds in the ability to adsorb Shiga toxin. European Food Research and Technology, 243:2147-2153

Sub publications

1. Hoida Ali Badr Badr,Kaori Kanemaru, Kumio Yokoigawa.(2017) Rhamnan sulfate attenuates methylmercury cytotoxicity in rat thymic lymphocytes. Natural Science Research 30: 5-8.

2. Kaori Kanemaru, Tsukie Goto, Hoida Ali Badr and Kumio Yokoigawa.Determination of binding affinity of poly-g-glutamate from a fermented food to Shiga toxin. Submitted to J Food Biochemistry.