Adsorption of Shiga Toxin to Poly-γ-Glutamate

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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 was first recognized as a food-borne pathogen in 1982 (Riley and others 1983). EHEC O157:H7 is a member of a large group of Shiga toxin (Stx)-producing *E. coli*. General symptoms of the diseases caused by EHEC are bloody diarrhea and hemorrhagic colitis 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 O157 infection were reported to activate toxin genes and induce the release of accumulated intracellular toxin (Walterspiel and others 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 and others 1991). The B subunit of Stx binds to globotriaosylceramide (Gb3) on the cell surface of renal endothelial cells, and ferries the A subunit into the cells (Lingwood and others 1987). The A subunit activated by a membrane-anchored protease furin impairs renal function by inhibiting eukaryotic protein synthesis (Garred and others 1995; Lea and others 1999). Stx is also reported to bind to the P1 blood group antigen that is present in human erythrocyte glycolipid extracts (Jacewicz and others 1986), and avian ovomucoid from pigeon egg white with the antigen is reported to adsorb Stx1

(Miyake and others 2000). Although several polymers including Gb3 have been reported to adsorb Stx (Nishikawa and others 2002; Watanabe and others 2004; Miyagawa and others 2006;Li and others 2012), there is no report on foods with an ability to adsorb Stx.

Many foods contain indigestible ingredients such as dietary fibers. If indigestible ingredients adsorb Stx, the toxin may be excreted with the ingredients into feces. In chapter 1, the author explored and analyzed several foods in the ability to adsorb Stx. Stx appeared to be adsorbed by poly-γ-glutamate (PGA), the main component of mucilage of natto (fermented soybeans, a traditional Japanese food).

PGA is an anionic polypeptide in which glutamate is polymerized via γ-amide linkages. PGA is tasteless, odorless, biodegradable and edible (Shih and Van 2001). In addition, PGA has various characteristics such as a highly water-absorbing ability, a metal-absorbing ability and antifreeze-activity (Shih and Van 2001, Mitsuki and others 1998). Therefore, potential applications of PGA have been of interest in a broad range of industrial fields such as medicine, food, and cosmetics. The author reported that Stx adsorbed to precipitated poly-γ-glutamic acid (PGA), but not to soluble PGA (Goto and others 2016). Since the interaction between Stx and cell surface receptor (Gb3) is reported to be multivalent (Ling and others 1998, Fraser and others 2004), clustered PGA may be required for strong binding of Stx. In chapter 2, the author describes the adsorption of Stx against chemically insolubilized PGA as clustered PGA.

Chapter 1

Adsorption of Shiga toxin to poly-γ-glutamate precipitated

I screened foods containing indigestible ingredients in the ability to adsorb Shiga toxin (Stx). When 5 mg of foods and dietary fibers such as dry vegetables and inulin were mixed and incubated with 0.5 ml of Stx solution (100 ng/ml) containing 0.5% bovine serum albumin, both Stx1 and Stx2 seemed to be adsorbed by only a fermented food, natto (a traditional Japanese food prepared from steamed soybeans by the biological action of *Bacillus subtilis*). I purified the Stx-adsorbing substance from natto by extraction with H_2O, acid treatment, Proteinase K treatment, and an ion exchange chromatography. The purified substance showed an average molecular mass of about 600 kDa. I identified it as poly-γ-glutamate (PGA) by amino acid analysis of its hydrolysate and peptide analysis after its treatment with Proteinase K. Purified PGA (MW: molecular weight = about 600 kDa) was considered to adsorb both Stx1 and Stx2 when I separated adsorbed and unadsorbed Stxs (MW= about 72 kDa) by an ultrafiltration method with a centrifugal filter unit (MWCO: molecular weight cut-off =100 kDa). However, PGA with the ability to adsorb Stx was an insoluble form precipitated in the filter unit during centrifugation. PGA precipitated beyond the saturated density was also confirmed to well adsorb both

Stx1 and Stx2 by an equilibrated dialysis method. To the best of our knowledge, this is the first report on food adsorbing Stx.

Materials and Methods

Materials

An Stx (VT) detection kit including standard Stx1 and Stx2 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).

Foods were purchased from a food store in Tokushima, Japan. They are two types of cereals, soybean and rice flours; three types of dry vegetables, carrot, eggplant, and onion; and one fermented food, natto. Dietary fibers (chitin, chitosan, pectin, and inulin) were obtained from Nakarai tesque Co., Kyoto, Japan. A commercial PGA preparation (Na salt) with the average molecular mass of 1000 kDa from *Bacillus subtilis* (chungkookjang) was provided by Bioleaders (Osaka, Japan). Float-A-Lyzer G2 (MWCO=300 kDa) was obtained from Spectrum Labs. Com. (Rancho Dominguez, Calif., U.S.A.).

Preparation of food samples

Dry vegetables (5 g) were milled at 20,000 rpm for 1 min with Millser-620DG (Iwatani Co., Tokyo, Japan), and used. Natto (5 g) was freeze-dried, and then similarly milled.

Adsorption of Stx to food samples

Standard Stx1 and Stx2 in the Stx detection kit were dissolved in 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% bovine serum albumin (BSA) to give a final concentration of 100 ng/ml. Each food and dietary fiber (5 mg) was suspended in the Stx solution (0.5 ml), and incubated at 25^{o}C. After incubation for 30 min, the suspensions were centrifuged at 10,000x*g* for 10 min. Supernatant solutions obtained were subjected to the RPLA assay of unadsorbed Stx as described below. Stx adsorbed to insoluble ingredients of food was dissociated by addition of NaOH (final pH 10), and free Stx was recovered by an ultrafiltration method with a centrifugal filter unit (pore size: 100 kDa, Amicon Ultra-4, Merck Millipore: Darmstadt, Germany). After neutralizing the filtrate with HCl, Stx in the solution was subjected to the RPLA assay of Stx as described below.

Adsorption of Stx to soluble PGA

I examined the adsorption of Stx to low concentrations of PGA using the centrifugal ultrafiltration to separate unadsorbed Stx from Stx adsorbed to PGA. The filtrate obtained as the unadsorbed Stx was subjected to the RPLA assay of Stx as below. The adsorbed Stx was dissociated by addition of NaOH (final pH 10), and free Stx was recovered by the ultrafiltration. After neutralizing the filtrate with HCl, Stx in the solution was subjected to the RPLA assay of Stx as described below.

I also analyzed the ability of PGA to adsorb Stx by an equilibrated dialysis method with Float-A-Lyzer G2 (MWCO=300 kDa). Stx solution (1 ml, 100

ng/ml) containing 3 mg PGA was put in a bag of Float-A-Lyzer G2 (MWCO=300 kDa), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% BSA at 4^{o}C for 16 h. Stx concentrations in the dialysis bag and outer buffer were analyzed.

Adsorption of Stx to PGA precipitated

I examined the Stx-adsorbing ability of PGA precipitated beyond the saturated density. Several amounts of PGA (10, 20, and 30 mg) were suspended in Stx solution (1 ml, 100 ng/ml), and the suspension was incubated 25^{o}C for 30 min with shaking. After centrifugation of the suspension at 10,000xg for 10 min, supernatant solution and precipitate obtained were subjected to the measurement of unbound and bound Stx, respectively. Stx bound to the precipitate (insoluble PGA) was dissociated by addition of NaOH (final pH 10), and free Stx was separated from PGA by the ultrafiltration method. After neutralizing the filtrate with HCl, Stx in the solution was subjected to the RPLA assay as described below.

I also examined the Stx-adsorbing ability of precipitated PGA by the equilibrated dialysis method as described above. The precipitated PGA (5, 10, and 15 mg as dry weight) and Stx solution (1 ml, 100 ng/ml of 10 mM phosphate buffer, pH 7.4, supplemented with 0.85% NaCl, 0.5% BSA, and 1.8 % PGA at the saturated density) were mixed, put into the dialysis bag, and dialyzed against the saturated PGA solution. Stx concentrations in the dialysis bag and outer solution were analyzed.

RPLA assay of Stx

The amounts of Stx1 and Stx2 were determined by an RPLA assay with 96-well microplates (V-bottom) and a Stx detection kit as previously reported (Takemasa and others 2009). The lower detection limit of 1 ng/ml of Stx was confirmed with the standard Stx1 and Stx2 provided in the kit. The Stx solutions 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-Stx1 or anti-Stx2 antibody in 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 the RPLA titers of Stx1 and Stx2 in the original samples.

Purification of Stx-adsorbent from natto

Natto (10 g) was suspended in 20 ml of distilled water and gently stirred at 25°C for 30 min. After centrifugation at 10,000×*g* for 15 min, pH of the supernatant solution was adjusted to 3.0 with H_2SO_4 to hydrolyze polysaccharides as reported by Ashiuchi and others (1999) and then incubated at 4°C for 16 h. After the incubation, the solution was mixed with three volumes of ethyl alcohol, and centrifuged at 4°C for 20 min (10,000×*g*). The precipitate obtained was dissolved in 10 mM Tris-HCl (pH 8.0) supplemented with 1mM $MgCl_2$, DNase (20 μg/ml), and RNase (20 μg/ml), and incubate at 37°C for 2 h. After the incubation, I added Proteinase K (0.1 mg/ml) to the solution, and further incubated at 37°C for 5 h. After treatment with these enzymes, the

solution was dialyzed against 10 mM Tris-HCl (pH 8.0) with a cellulose membrane (pore size 12 kDa), and put on a Q Sepharose Fast Flow column (GE Healthcare, Japan) equilibrated with the same buffer. After the column was washed with the same buffer, the Stx-adsorbent was eluted with a linear gradient from 0 to 1 M NaCl in the same buffer at a flow rate of 1.0 ml/min. The Stx-adsorbing fractions were combined, concentrated with Amicon ultra-4 (pore size: 100 kDa), dialyzed against distilled water, and freeze-dried.

Analytical methods

The molecular mass of Stx-adsorbent was measured by polyacrylamide gel electrophoresis (PAGE) with NuPAGE 4 to 12% Bis-Tris Gel (Invitrogen) and HMW-Native Marker Kit (GE Healthcare Japan). The gel was stained by the method of Ito and others (1996) as described below. First, the gel was stained for proteins with Coomassie Brilliant Blue, and then destained in 7% acetic acid and 10% methanol. Next, it was stained for PGA with 0.5% methylene blue in 3% acetic acid and destained in water. For amino acid analysis, the Stx-adsorbent was hydrolyzed with 6 *N* HCl at 105^{o}C for 8 h *in vacuo*. The hydrolysate was lyophilized, dissolved in distilled water, and analyzed with a CHIRALPAK MA (+) column (Daicel, Tokyo, Japan) according to the method by Ashiuchi and others (1998). PGA precipitated beyond its saturated density was visualized using scanning electron microscopy (SEM). PGA precipitated was washed twice with ethyl alcohol, dried under reduced pressure, and coated with Au (15 nm thick) using a Hitachi E-1020 ion sputter (Hitachi Ltd., Tokyo, Japan). The sample was visualized using a Hitachi TM 3030 Miniscope

(Hitachi Ltd.).

Statistical analysis

Three independent experiments were performed twice, and the results (n = 6) were analyzed by analysis of variance (ANOVA) using a software, StatView (SAS Institute, Inc., Cary, NC, USA).

Results

Adsorption assay of Stx to foods containing indigestible ingredients

I screened foods containing indigestible ingredients in the ability to adsorb Stx. Vegetables, cereals, natto, and dietary fibers were chosen for this screening. The standard Stx1 and Stx2 were dissolved in the buffer supplemented with 0.5% bovine serum albumin to avoid non-specific adsorption. As shown in Fig. 1, most foods and dietary fibers did not show the ability to adsorb Stx as judged from the amounts of unadsorbed Stx. However, unadsorbed Stx was slightly decreased by natto. When I analyzed the amounts of Stx adsorbed to natto by the ultrafiltration method after the alkaline treatment as described in "Materials and Methods," about 4 ng of Stx1 or 8 ng of the Stx2 were adsorbed by 5 mg of the freeze-dried natto sample. Since Stx was not adsorbed by steamed soybeans, *B. subtilis* seemed to produce something to adsorb Stx.

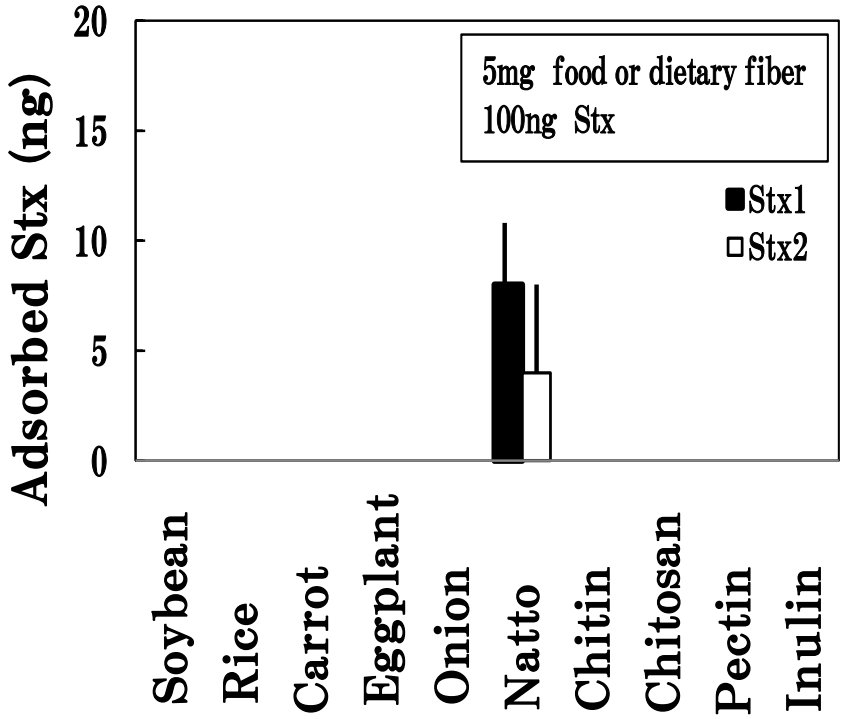

Figure 1-Adsorption of Stx to food and dietary fibers.

Each dry food and dietary fiber (5 mg) was suspended in the Stx solution (0.5 ml), and incubated at 25°C. After incubation for 30 min, the suspensions were centrifuged at 10,000×g for 10 min. Stx adsorbed to the precipitate was dissociated by addition of NaOH, and free Stx was separated by ultrafiltration with a centrifugal filter (pore size: 100 kDa). After neutralizing the filtrate with HCl, Stx in the solution was subjected to the RPLA assay. Black bars, adsorbed Stx1; and white bars, adsorbed Stx2.

Purification and properties of Stx-adsorbing substance from natto

I preliminarily examined some properties of the Stx-adsorbing substance in natto before its purification. The active substance was found to be a macromolecule, judging from the result of an ultrafiltration with a membrane of the molecular weight cutoff, 100 kDa. The activity was stable even after a thermal treatment at 121^{o}C for 15 min and an enzymatic treatment at 37^{o}C for 5 h with 0.1 mg/ml of Proteinase K. Based upon these results, I chose several purification steps as shown in Table 1. I also showed the elution profile of Q-Sepharose column chromatography (Fig. 2A). An active substance was eluted as a single peak (Fra. 19). The fraction appeared to contain the active compound with an average molecular mass of about 600 kDa (Fig. 2B). Dry weight of the substance purified from 10 g natto was about 40 mg. Hydrolysate of the purified substance with 6 *N* HCl was found to contain only glutamic acid, and did not show the Stx-adsorbing activity. In addition, I could not detect any peptides after Proteinase K-treatment of the purified substance. Therefore, the purified substance was considered to be a polymer of glutamic acid with γ-peptide linkage. In the assay of Stx-adsorbing ability of PGA at low concentrations by the centrifugal ultrafiltration method, I found PGA precipitated beyond its saturated density in the centrifugal filter unit. Therefore, I further examined both dissolved and precipitated PGA in the ability to adsorb Stx.

Table 1-Purification of Stx-adsorbent from natto.

Steps	Vol. (ml)	Stx2 adsorption (RPLA titer)
Natto (10 g)	—	
Natto extract	20	1800
Acid treatment with sulfuric acid (pH 3)	22	1600
Proteinase K treatment	22	1460
Q Sepharose Fast Flow	30	1200

RPLA titer : adsorbed Stx (ng)

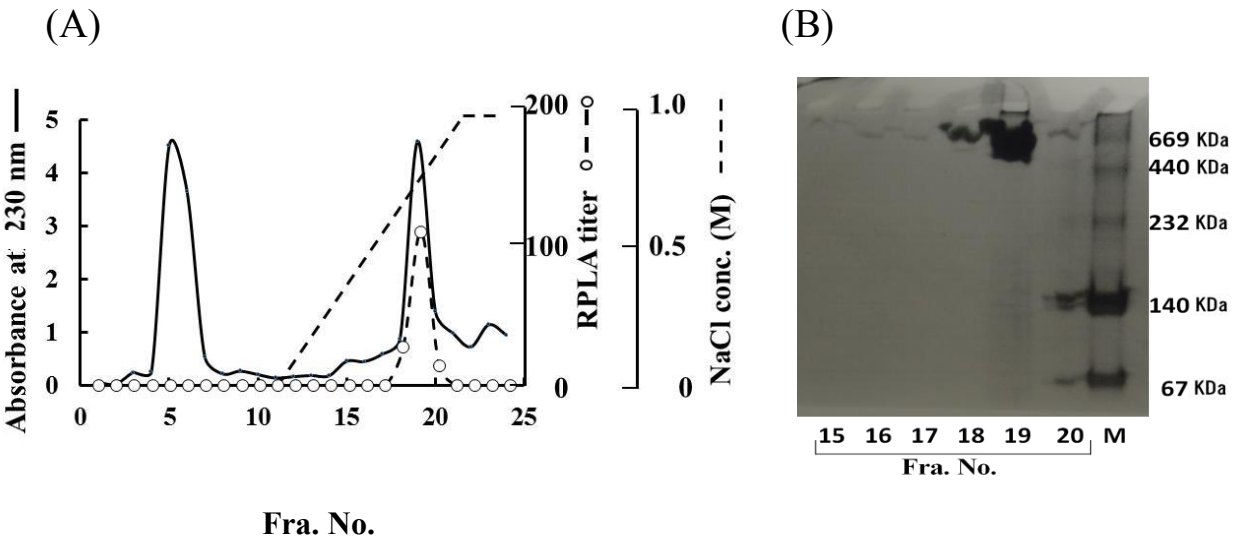

Figure 2-Elution profile of natto extract after treatment with Proteinase K on a Q Sepharose Fast Flow column (1.6 x 10 cm) (A), and PAGE of fractions 15-20 (B).

(B) Fractions 15 to 20 in Fig. 2A were subjected to polyacrylamide gel electrophoresis. First, the gel was stained for protein with Coomassie Brilliant Blue R-250 and destained in 7% acetic acid and 10% methyl alcohol. Next, it was stained for acidic polymers with 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).

Stx-adsorbing activity of PGA

The PGA purified was analyzed in the ability to adsorb Stx by the ultrafiltration method as described in "Materials and Methods." As shown in Fig. 3A, the PGA at low concentrations showed the tendency to adsorb Stx. However, I could not observe the Stx-adsorbing activity of PGA at the low concentrations by the equilibrated dialysis method with Float-A-Lyzer G2 (MWCO=300 kDa); Stx concentration in the dialysis bag was almost similar to that in the outer buffer (data not shown). Next, I examined the ability of PGA suspension beyond its saturated density to adsorb Stx. As shown in Fig. 3B, PGA precipitated in the suspension (20 and 30 mg/ml) adsorbed both Stx1 and Stx2.

I also examined the ability of the precipitated PGA to adsorb Stx by the equilibrated dialysis method. As shown in Fig. 4, the Stx concentration in the dialysis bag was clearly higher than that in the outer solution. These results suggested that PGA with an ability to adsorb Stx was its precipitated form. When I used a commercial PGA Na salt with an average molecular mass of 1000 kDa, similar results were obtained.

In order to examine the surface structure of precipitated PGA, the PGA was visualized using SEM (Fig. 5). The morphology seemed to be chain-like spheres composed of small pieces, and the surface looked rough and crumbly.

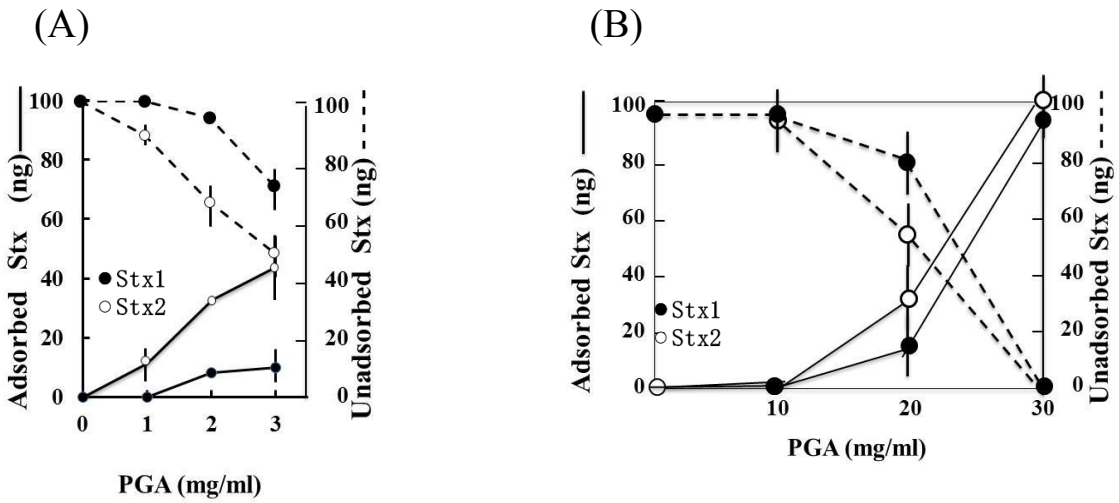

Figure 3-Adsorption of Stx to the soluble PGA using an ultrafiltration method to separate adsorbed and unadsorbed Stx (A), and to the PGA precipitated beyond its saturated density (B).

Purified PGA was added to 1 ml of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% bovine serum albumin, and incubated at 25°C for 3 h to fully saturate with water. The PGA solution or suspension was added to the standard Stx (lyophilized preparation, 100 ng). (A) After incubation at 25°C for 30 min, the solution was subjected to the ultrafiltration method with a filter unit (pore size: 100 kDa) to separate unadsorbed Stx from Stx adsorbed to PGA. (B) After incubation at 25°C for 30 min, the suspension was centrifuged at 10,000×g for 10 min. Supernatant solution and precipitate obtained were analyzed as unadsorbed and adsorbed Stx, respectively, to the precipitated PGA. Three independent experiments were performed twice (n = 6), and the vertical bars show the standard deviations. Solid lines, bound Stx; broken lines, free Stx; closed circles, Stx1; and open circles, Stx2.

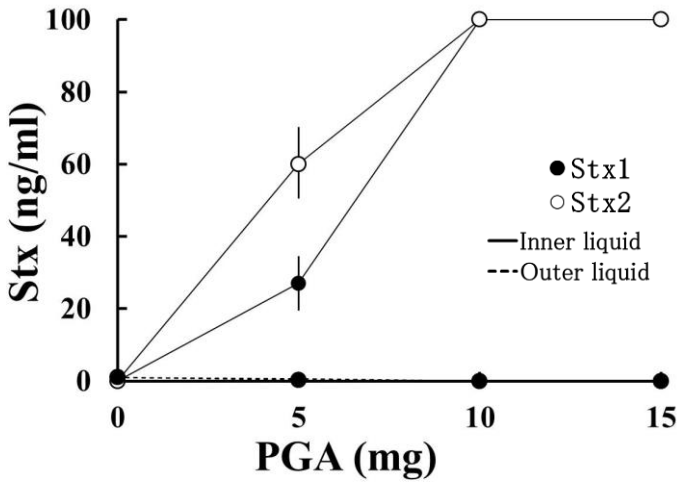

Figure 4-Adsorption of Stx to the PGA precipitated beyond its saturated density using the equilibrated dialysis method.

PGA precipitated (5, 10, 15 mg as dry wt.) and Stx solution (1 ml, 100 ng/ml of 10 mM phosphate buffer, pH 7.4, supplemented with 0.85% NaCl, 0.5% BSA, and 1.8% PGA) were mixed, put into the dialysis bag, and dialyzed against the saturated PGA solution (1.8%). Stx concentrations in the dialysis bag and outer solution were analyzed. Three independent experiments were performed twice (n = 6), and the vertical bars show the standard deviations. Solid lines, Stx in the dialysis bag; broken lines, Stx in the outer solution; closed circles, Stx1; and open circles, Stx2.

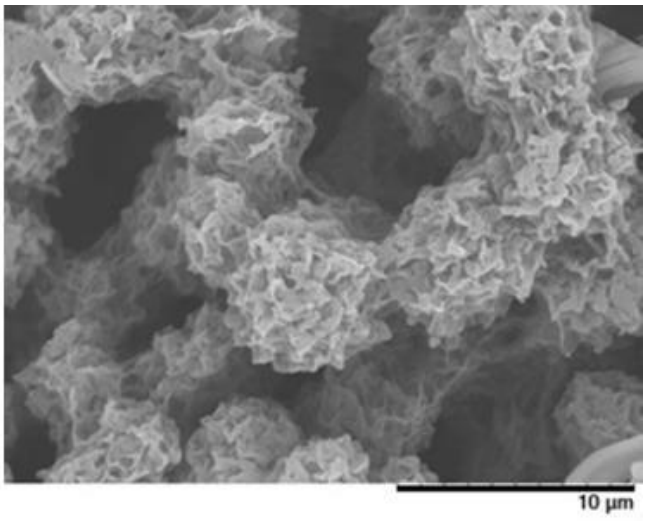

Figure 5-Scanning electron micrograph of PGA precipitated.

PGA precipitated beyond its saturated density was washed twice with ethyl alcohol, and dried under reduced pressure. After coating the dry powder with Au (15 nm thick), the sample was visualized using SEM.

Chapter 2

Binding of Shiga toxin to chemically insolubilized PGA

In chapter 1, the author described that Shiga toxin (Stx) adsorbed to precipitated PGA, but not to soluble PGA. To clarify the mechanism, I prepared and analyzed two types of chemically insolubilized PGA preparations. One is intermolecularly cross-linked PGA and another is immobilized PGA on Sepharose 4B. PGA preparations with the average molecular mass of about 50, 1000, 6000, and 8000 kDa were intermolecularly cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as a condensation reagent. The PGA preparations were also immobilized by direct coupling to EAH-Sepharose^{TM} 4B with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; PGA-Sepharose prepared contained about 10 mg of PGA in 1 ml of the swelling gel. The intermolecularly cross-linked PGA did not adsorb Stx1 and Stx2, irrespective of the average molecular mass of PGA used. The PGA-Sepharose adsorbed Stx2, but not Stx1: the dissociation constant (*K*d) of PGA-Sepharose against Stx2 was 0.25 mM. To analyze the mechanism of the adsorption, I similarly prepared glutamic acid-Sepharose and glutaric acid-Sepharose (each 7 μmol of ligand/ml of gel), and analyzed their ability to adsorb Stx. Both resins adsorbed Stx2, but not Stx1; *K*d values of Stx2 against glutamic acid-Sepharose and glutaric acid-Sepharose were almost identical to each other (0.12 mM).

Dowex Mac 3, polyacrylic acid, with carboxyl groups (3.8 μmol/ml of gel) did not adsorb both Stx1 and Stx2. Stx2, an acidic protein with pI of 4.1, seemed not to interact only with carboxylic groups of PGA.

Materials and Methods

Materials

PGA Na salt with the average molecular mass of 50,1000, 6000, 8000 kDa from *Bacillus subtilis* (chungkookjang) were provided by Bioleaders (Osaka, Japan). EAH-Sepharose^{TM} 4B, which is formed by covalent linkage of 1,6-diamonohexane to Sepharose 4B, was obtained from GE healthcare, Uppsala, Sweden. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), glutamic acid and glutaric acid were from Nakarai tesque Co., Kyoto, Japan. Dequalinium chloride was obtained from Sigma Aldrich (St. Louis, MO, USA). Dowex Mac 3, a cation-exchange resin, was obtained from Dow Chemical Japan, Tokyo, Japan (3.8 μmol of carboxyl groups/ml of resin). *Escherichia coli* O157:H7 sakai was used for preparation of crude Stx1 and Stx2. An Stx (VT) detection kit including standard Stx1 and Stx2 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).

Preparation of crude Stx1 and Stx2

Escherichia coli O157:H7 was aerobically cultivated at 37◦C for 18 h in 20

mL of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1 mM NaOH). After centrifugation of the culture at 10,000xg for 15 min, the supernatant solution obtained was sterilized with a filter unit (Millex-GV, pore size 0.22 μm) and used as crude Stx2 preparations. The precipitated cells were suspended in 2 ml of 10 mM phosphate buffer (pH 7.0) and disrupted by vigorous mixing with 1 g of glass beads (average diameter of 0.1 mm). After centrifugation of the cell extract at 10,000xg for 15 min, the supernatant solution obtained was sterilized with a filter unit (Millex-GV, pore size 0.22 μm) and used as crude Stx1 preparations.

Preparation of intermolecularly cross-linked PGA

Each PGA preparation with the average molecular mass of about 50, 1000, 6000, or 8000 kDa was dissolved in distilled H_2O (10 mg/ml), and the pH was adjusted to 5.0 with NaOH. Several amounts of solid EDC were added to the PGA solution, and pH value was kept at 5.0 at 25◦C during the initial 1 h after addition of EDC. Then, the cross-linking reaction was further continued for 16 h. After the reaction, precipitate formed was collected by centrifugation at 10,000xg for 15 min, washed twice with distilled H_2O, and dried up under reduced pressure.

Preparation of immobilized-PGA, -glutamic acid and -glutaric acid

The amount of amino group in EAH-Sepharose^{TM} 4B was in advance determined by colorimetric method with 2,4-dinitrobenzenesulfonic acid (Fields 1971). PGA preparations with the average molecular mass of about 50, 1000,

6000, and 8000 kDa were immobilized by direct coupling to EAH-Sepharose™ 4B with a condensation reagent. Six ml of EAH-Sepharose™ 4B (10 μmole amino groups/ml of gel) was mixed with 6 ml of PGA solution (10 mg/ml, pH 5.0) and solid 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (228 mg, final conc. 0.1 M), and incubated at 25°C for 17 h according to the manufacture's protocol. During the initial 1 h, pH value was kept at 5.0. About 10 mg of PGA was immobilized on 1 ml of gel. Glutamic acid-Sepharose and glutaric acid-Sepharose were similarly prepared: 1.47 mg ligand were immobilized on ml of gel.

Determination of *K*d values

I analyzed PGA-, glutamic acid-, and glutaric acid-Sepharoses in the ability to adsorb Stx by an equilibrated dialysis method with Float-A-Lyzer G2 (MWCO=300 kDa). Stx solution (0.5 ml, 64-512 ng/ml), 0.25 ml of each gel, and 0.25 ml 10 mM phosphate buffer (pH 7.0) was put in a bag of Float-A-Lyzer G2 (MWCO=300 kDa), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.0) supplemented with 0.85% NaCl and 0.5% BSA at 4°C for 16 h. Stx concentrations in the dialysis bag and outer buffer were analyzed. *K*d values of PGA-, glutamic acid-, and glutaric acid-Sepharoses against Stx were calculated using Scatchard plot analysis.

Binding of Stx to PGA-, glutamic acid- and glutaric acid-Sepharoses

Several concentrations of Stx solution were put on a PGA-Sepharose, glutamic acid-Sepharose and glutaric acid-Sepharose columns (0.5 x 10 cm)

equilibrated with 10 mM phosphate buffer (pH 7.0). After the column was washed with the same buffer, Stx adsorbed was eluted with 0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min. The amount of Stx in each fraction was determined by RPLA assay.

RPLA assay of Stx

The amounts of Stx1 and Stx2 were determined by a reversed passive latex agglutination (RPLA) assay with 96-well microplates (V-bottom) and a Stx detection kit as described in chapter 1.

Results

Preparation and properties of intermolecularly cross-linked PGA

All PGA preparations used were insolubilized by addition of EDC at the concentration of more than 0.1 M. EDC is known to specifically react with carboxylic groups to yield *o*-acylisourea active esters those specifically react with primary amino groups. The active esters are unstable in the absence of amino groups, and easily release isourea as a by product to yield original carboxylic groups (Grabarek and Gergely 1990). Therefore, a carboxylic group of PGA molecule seemed to be condensed with an α-amino group of other PGA molecule. When I examined the intermolecular cross-linked PGA preparations in the ability to adsorb Stx, both Stx1 and Stx2 were not adsorbed to the preparations (Fig. 6).

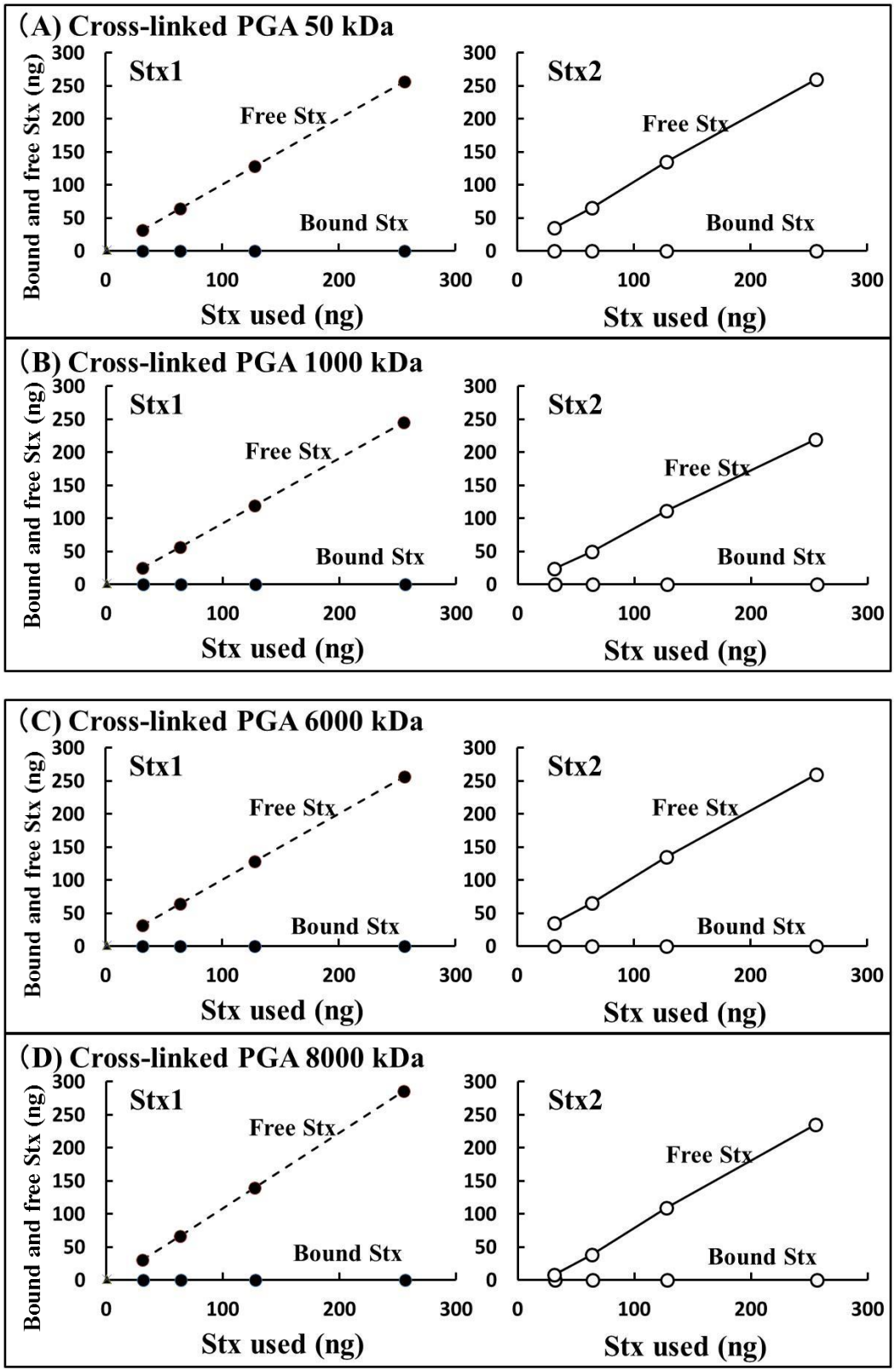
(A) Cross-linked PGA 50 kDa
Stx1
Stx2
Free Stx
Bound Stx
Bound and free Stx (ng)
Stx used (ng)
(B) Cross-linked PGA 1000 kDa
(C) Cross-linked PGA 6000 kDa
(D) Cross-linked PGA 8000 kDa
0
50
100
150
200
250
300

Figure 6-Adsorption of Stx to the cross-linked PGA.

The cross-linked PGA Preparations (10 mg as dry wt.) and Stx solution (1 ml, 30-300 ng/ml of 10 mM phosphate buffer, pH 7.4, supplemented with 0.85% NaCl, and 0.5% BSA) were mixed, put into the dialysis bag, and dialyzed against the same buffer. Stx concentrations in the dialysis bag and outer solution were analyzed. (A) cross-linked PGA 50 kDa, (B) cross-linked PGA 1000 kDa, (C) cross-linked PGA 6000 kDa, (D) cross-linked PGA 8000 kDa.

Adsorption of Stx to PGA-Sepharose

I examined the adsorption of Stx to PGA-Sepharose. As shown in Fig. 7, Stx2 adsorbed to PGA-Sepharose, but Stx1 did not adsorb to the resin. The author calculated the *K*d values of PGA-Sepharose.

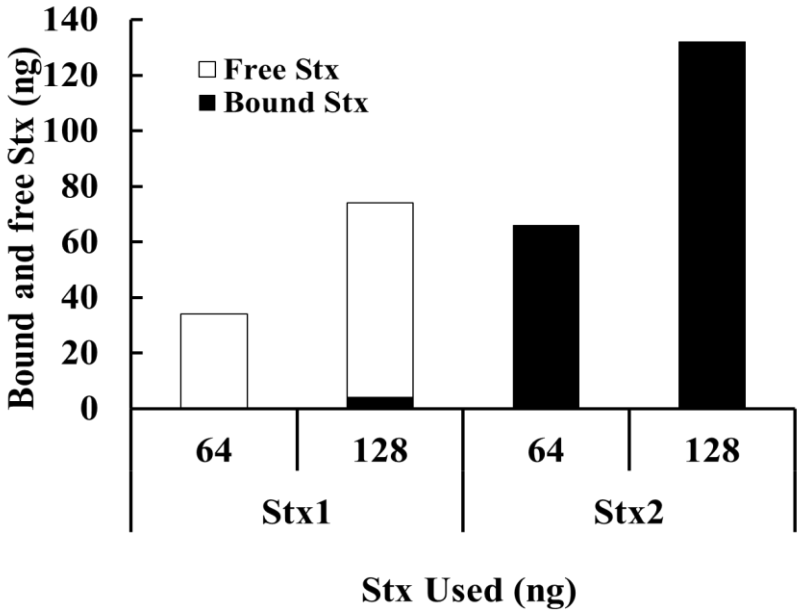

Figure 7-Binding of Stx to PGA-Sepharose.

Stx solution (0.5 ml, 64-256 ng/ml), 0.25 ml of PGA-Sepharose, and 0.25 ml of 10 mM phosphate buffer (pH 7.0) was put in a bag of Float-A-Lyzer G2 (MWCO=300 kDa), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.0) supplemented with 0.85% NaCl and 0.5% BSA at 4^{o}C for 16 h. Stx concentrations in the dialysis bag and outer buffer were analyzed.

The *K*d value of PGA-Sepharose against Stx2 was determined by Scatchard plot analysis (Fig. 8), and calculated to be 0.25 mM (Fig. 8B).

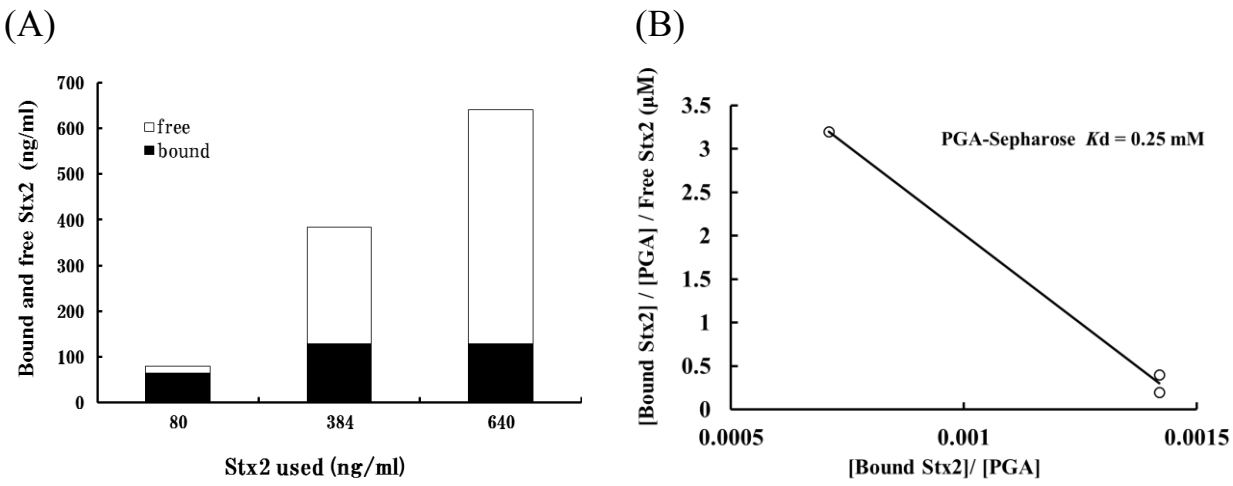

Figure 8-Binding analysis of Stx2 to PGA-Sepharose (A) and Scatchard plot analysis (B).

Stx solution (0.5 ml, 160-1280 ng/ml), 0.25 ml of PGA-Sepharose, and 0.25 ml of 10 mM phosphate buffer (pH 7.0) was put in a bag of Float-A-Lyzer G2 (MWCO=300 kDa), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.0) supplemented with 0.85% NaCl and 0.5% BSA at 4^{o}C for 16 h. Stx concentrations in the dialysis bag and outer buffer were analyzed. *K*d value of PGA-Sepharoses against Stx2 was calculated using Scatchard plot analysis (B).

The author also examined three types of resins that contained carboxylic groups; glutamic acid-Sepharose, glutaric acid-Sepharose, and Dowex Mac 3. As shown in Fig. 9, glutamic acid-Sepharose was found to adsorb Stx2. However, Stx1 was not adsorbed.

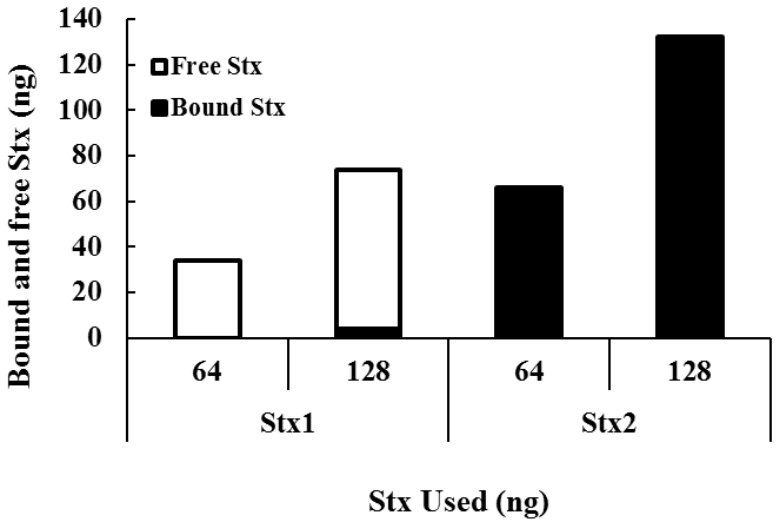

Figure 9-Binding of Stx to glutamic acid-Sepharose.

Stx solution (0.5 ml, 128-256 ng/ml), 0.25 ml of glutamic acid-Sepharose, and 0.25 ml of 10 mM phosphate buffer (pH 7.0) was put in a bag of Float-A-Lyzer G2 (MWCO=300 kDa), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.0) supplemented with 0.85% NaCl and 0.5% BSA at 4^{o}C for 16 h. Stx concentrations in the dialysis bag and outer buffer were analyzed.

Glutaric acid-Sepharose showed almost identical results with those of glutamic acid-Sepharose (data not shown). Dowex Mac 3 did not adsorb Stx1 and Stx2 (data not shown). As a whole, glutamic acid- and glutaric acid-Sepharoses adsorbed slightly larger amounts Stx2 than PGA-Sepharose. The author also calculated the *K*d value of glutamic acid-Sepharose against Stx2 (Fig. 10). Glutamic acid-Sepharose showed smaller *K*d value (0.12 mM) than PGA-Sepharose (0.25 mM). Glutaric acid-Sepharose showed an almost identical *K*d value (0.12 mM) with that of glutamic acid-Sepharose.

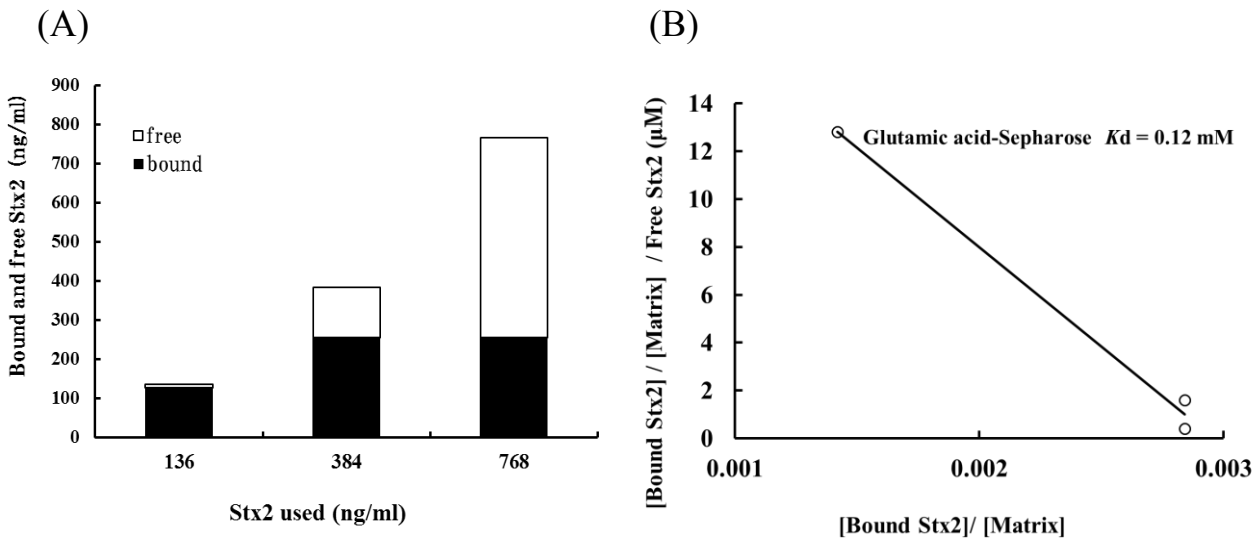

Figure 10-Binding analysis of Stx2 to glutamic acid-Sepharose (A) and Scatchard plot analysis (B).

Stx solution (0.5 ml, 272-1536 ng/ml), 0.25 ml of glutamic acid-Sepharose, and 0.25 ml of 10 mM phosphate buffer (pH 7.0) was put in a bag of Float-A-Lyzer G2 (MWCO=300 kDa), and dialyzed against 100 ml of 10 mM phosphate buffer (pH 7.0) supplemented with 0.85% NaCl and 0.5% BSA at 4^{o}C for 16 h. Stx concentrations in the dialysis bag and outer buffer were analyzed. *K*d value of glutamic acid -Sepharoses against Stx2 was calculated using Scatchard plot analysis (B).

Separation of Stx1 and Stx2 by PGA-Sepharose column chromatography

As described above, PGA-Sepharose adsorbed Stx2, but not Stx1. The author examined specific separation of Stx1 and Stx2 by PGA-Sepharose column chromatography. Crude Stx1 and Stx2 mixture was applied to the column. As shown in Fig. 11, Stx1 was passed through the column, but Stx2 was adsorbed to the column and eluted by a buffer with 0.5 M NaCl. Yields of Stx1 and Stx2 were 81% and 103%, respectively.

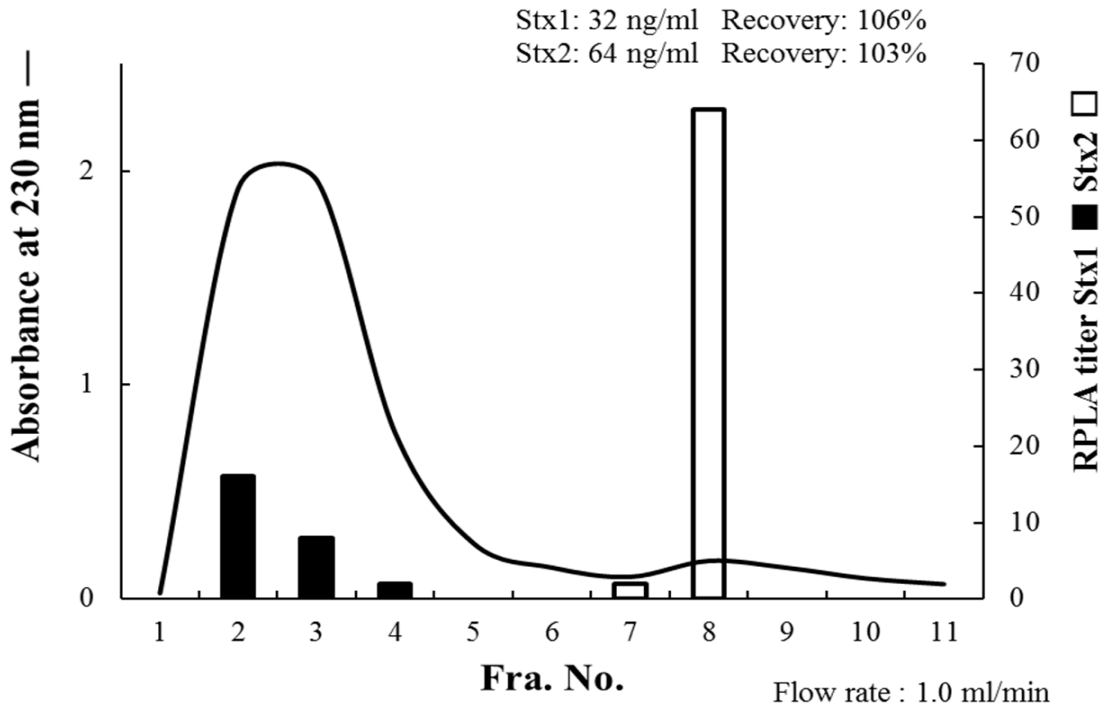

Figure 11-PGA-Sepharose column chromatography.

Crude Stx1 and Stx2 mixture (32 ng Stx1 and 64 ng Stx2 in 1 ml) was applied to the column (0.5 x 10 cm) equilibrated with 10 mM phosphate buffer (pH 7.0). After the column was washed with the same buffer, Stx adsorbed was eluted with 0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min. The amount of Stx in each fraction was determined by RPLA assay. The experiments was performed three times, and the representative result was shown. Black bar: Stx1, white bar:Stx2.

Discussion

I screened and analyzed several foods containing indigestible ingredients and some dietary fibers in their ability to adsorb Stx. Although most food samples surveyed did not adsorb Stx, natto appeared to adsorb Stx. The active substance purified from natto was considered to be poly-γ-glutamate by analysis of its constituents after hydrolysis with 6 *N* HCl, molecular mass, and products after Proteinase K treatment.

Purified PGA showed a single but broad band in a polyacrylamide gel. The broad band was not stained with Coomassie Brilliant Blue R-250, but stained with methylene blue. Thus, the purified PGA seemed to be not contaminated with proteins. PGA was considered to adsorb both Stx1 and Stx2 when I separated adsorbed and unadsorbed Stxs by the ultrafiltration method. However, I could not observe the adsorption of Stx to PGA by the equilibrated dialysis method. When I used PGA suspension beyond its saturated density, Stx was well adsorbed to the precipitated PGA. PGA precipitated was also confirmed to adsorb both Stx1 and Stx2 by the equilibrated dialysis method. These results suggested that PGA with an ability to adsorb Stx was its precipitated form. The ultrafiltration method was considered to yield PGA precipitated in the concentrated sample.

PGA has various characteristics such as high water-absorbing ability, metal-absorbing ability and antifreeze activity (Shih and Van 2001, Mitsuki and others 1998). Therefore, the potential applications of PGA have been of interest in a broad range of industrial fields such as medicine, food, and

cosmetics (Bajaj and Singhal 2011). Yokoigawa and others reported that PGA suppressed the decrease in leavening ability during prolonged fermentation time, probably because PGA adsorbed the inhibitory metabolites that accumulated in the dough (Yokoigawa and others 2006). They also reported that PGA showed high anti-mutagenic activity by adsorbing chemical mutagens (Sato and others 2008). Here, I found that PGA precipitated beyond its saturated density well adsorbed Stx.

Interaction between Stx and cell surface receptor (Gb3) is reported to be multivalent (Ling and others 1998, Fraser and others 2004), and clustered trisaccharides of receptors are required for strong binding (Nishikawa 2011). Surface of insoluble PGA may be required to its multivalent interaction with Stx.

PGA is reported to have different conformations depending on its concentration, pH, and ionic strength (Shih and Van 2001). It adopts a helical conformation at low pH, but has β-sheet structure at neutral pH. In addition, the structure is helical at low ionic strength and low PGA concentration (0.1%), but changes to β-sheet structure at high ionic strength or high PGA concentration. Therefore, PGA is considered to have β-sheet structure at high concentrations and neutral pH. The morphology of PGA precipitated beyond its saturated density seemed to be chain-like spheres composed of small pieces as judged from the scanning electron micrograph. Although PGA was reported to be nylonlike fibers (Ashiuchi 2013), the morphology of PGA precipitated was different from that of fibers. The surface structure may provide the multivalent binding sites for Stx. In the case of soluble and immobilized Gb3, the *K*d value

of Stx for soluble Gb3 was reported to be about 1 mM (St. Hilaire and others 1994), whereas that of the immobilized Gb3 was below 1 μM (Li and others 2012). Binding sites of Gb3 and PGA against Stx may be clustered by immobilization and precipitation, respectively, although further experiments are required.

In chapter 1, I described that precipitated PGA well adsorbed two types of Stx. PGA is easy to take in large quantities from natto; one pack of natto (about 50 g) as a side dish contains about 200 mg PGA. Since PGA is indigestible, tasteless and odorless (Shih and Van 2001), oral administration of PGA may be useful for removal of intestinal Stx. Although further experiments are required, precipitated PGA seemed to have a potential for treatment of the disease by Stx-producing pathogen. Preparation and analysis of PGA insolubilized by chemical modification are described in chapter 2. To the best of our knowledge, this is the first report on a food ingredient adsorbing Stx.

The author described that Shiga toxin (Stx) adsorbed to precipitated PGA, but not to soluble PGA in chaper 1. To clarify the mechanism, I prepared and analyzed two types of chemically insolubilized PGA preparations. Insolublilized PGA was prepared by direct coupling between PGA molecules with EDC. Insoluble PGA, intermolecularly cross-linked PGA, was easily formed by addition of relatively high concentratons of EDC (more than 0.1 M). Since a molecule of PGA contains only one amino group at N-terminal, the amino group seemed to be condenced with carboxylic groups of other PGA molecules by EDC. The intermolecularly cross-linked PGA prepared did not

adsorb both Stx1 and Stx2, irrespective of the average molecular mass of PGA tested. The surface structure of intermolecularly cross-linked PGA was considered to be different from that of PGA precipitated beyond its saturated concentration. One possible explanation for the difference is the pH value under which PGA became insoluble. Intermolecular cross-linked PGA was prepared at pH 5.0 from the standpoint of specificity of EDC. While, the author prepared the PGA precipitated beyond the saturated density at pH 7.0, because solubilty of PGA is low at acidic pH. PGA has helix-conformation at acidic pH, but sheet-conformation at neutral pH. This difference may be related to different surface structures of insolubilized PGA.

The author also prepared and analyze PGA-Sepharose in the ability to adsorb Stx. PGA-Sepharose prepared adsorbed Stx2, but not Stx1: the dissociation constant (*K*d) of PGA-Sepharose against Stx2 was 0.25 mM. Since Stx2 is reported to be acidic protein (pI 4.1), the binding of Stx2 with PGA-Sepharose is considered not to be ionic interaction. When the author examined glutamic acid-Sepharose and glutaric acid-Sepharose (each 7 μmol of ligand/ml of gel), both resins adsorbed Stx2, but not Stx1; *K*d values of glutamic acid-Sepharose and glutaric acid-Sepharose were almost identical to each other (0.12 mM). These results are similar to those of PGA-Sepharose. Carboxylic groups seemed to play a role in the interaction of Stx2 and PGA. While, Dowex Mac 3 (polyacrylic acid) with carboxyl groups did not adsorb both Stx1 and Stx2. The low density of carboxylic groups in Dowex Mac 3 (3.8 μmol/ml of gel) and structure of polyacrylic acid may be unsuitable for adsorbing Stx.

PGA-Sepharose was found to adsorb only Stx2. Glutamic acid-Sepharose

and glutaric acid-Sepharose also showed similar affinity. Stx2 has markedly higher toxicity than Stx1. Removal of Stx2 from blood stream of patient by EHEC-infection is an important treatment. Up to now, there is no report about Stx2-adsorbent. This is the first report as adsorbents to adsorb Stx2, although avian ovomucoid from pigeon egg white is reported to adsorb only Stx1 (Miyake and others 2000).

Conclusions

I found that Stx1 and Stx2 were adsorbed by natto, fermented soybeans. The Stx-adsorbing substance was purified from natto and identified as PGA. Purified PGA was considered to adsorb both Stx1 and Stx2 when I separated adsorbed and unadsorbed Stxs by the ultrafiltration method. However, PGA with an ability to adsorb both Stx1 and Stx2 was found not to be a soluble form by an equilibrated dialysis method, but an insoluble one by analysis with PGA precipitated beyond its saturated density.

To clarify the mechanism of Stx-adsorption by precipitated PGA, the author prepared and analyzed chemically insolubilized PGA. Intermolecularly cross-linked PGA did not adsorb Stx1 and Stx2. The surface structure of intermolecularly cross-linked PGA was considered to be different from that of PGA precipitated beyond its saturated concentration. PGA-Sepharose was found to adsorb only Stx2. Glutamic acid-Sepharose and glutaric acid-Sepharose also showed similar affinity. Carboxylic groups seemed to play a role in the interaction of Stx2 and PGA. Stx2 has markedly higher toxicity than Stx1. Up to now, there is no report about Stx2-adsorbent. This is the first report as adsorbents to adsorb Stx2. Insolubilized PGA may have a therapeutic potential in the treatment of disease by Stx-producing bacteria.

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Publications

Main publications

1. Tsukie Goto, Makiko Tsuji, Kaori Kanemaru, and Kumio Yokoigawa. 2016. Adsorption of Shiga Toxin to Poly-γ –Glutamate Precipitated. Journal of Food Science 81: 2977-2981

2. Tsukie Goto, Makiko Tsuji, Kaori Kanemaru, and Kumio Yokoigawa. Binding of Shiga toxin to chemically insolubilized PGA. in preparation

Sub publications

1. Tsukie Goto, Junko Matsushita, Kaori Kanemaru, Chizuru Endo, Kumiko Nagao, Naoko Ariuchi, Keiko Takahashi. 2013. A survey of foods for Rites of passage in Tokushima Prefecture. Journal of Cookery Science of Japan 46:389-394

2. Junko Matsushita, Tsukie Goto, Kaori Kanemaru, Chizuru Endo, Kumiko Nagao, Naoko Ariuchi, Keiko Takahashi. 2014. Survey of Annual Events and Their Special Foods in Tokushima Prefecture. Journal of Cookery Science of Japan 47:42-48