Adsorption of Shiga toxin to poly-γ-glutamate precipitated

Tsukie Goto[1,2], Makiko Tsuji[3], Kaori Kanemaru[1], and Kumio Yokoigawa[1]

[1]Graduate School of Integrated Arts and Sciences, Tokushima University, 1-1 Minamijosanjima-cho, Tokushima 770-8502, Japan.

[2]Department of Science for Human Health, Junior College, Shikoku University, 123-1 Ebisuno, Furukawa, Ojin-cho, Tokushima 771-1192, Japan.

[3]Kobe Women's Junior college, Chuo-ku, Kobe 650-0046, Japan.

Contact information for Corresponding Author

Kumio Yokoigawa, Graduate School of Integrated Arts and Sciences, University of Tokushima, 1-1 Minamijosanjima-cho, Tokushima 770-8502, Japan. Tel/Fax: +81-88-656-7267; E-mail: yokoigawa@tokushima-u.ac.jp

Short version of title: Adsorption of Stx to PGA

Choice of journal section: Food Microbiology and Safety

ABSTRACT: We 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*). We 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. We 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= about 600 kDa) was considered to adsorb both Stx1 and Stx2 when we separated adsorbed and unadsorbed Stxs (MW= about 72 kDa) by an ultrafiltration method with a centrifugal filter unit (MWCO=100 K). 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.

Keywords: *Escherichia coli* O157:H7, Shiga toxin, poly-γ-glutamate

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 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 (Li and others 2012; Nishikawa and others 2002; Watanabe and others 2004; Miyagawa and others 2006), 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. We here describe the adsorption of Stx to food containing indigestible ingredients. Stx appeared to be adsorbed by poly-γ-glutamate (PGA), the main component of mucilage of natto (fermented soybeans, a traditional Japanese food).

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=300K) was obtained from Spectrum Labs. Com. (CA, USA).

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 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 10,000 x*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). 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 low concentrations of PGA

We 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.

We also analyzed the ability of PGA to adsorb Stx by an equilibrated dialysis method

with Float-A-Lyzer G2 (MWCO=300K). Stx solution (1 ml, 100 ng/ml) containing 3 mg PGA was put in a bag of Float-A-Lyzer G2 (MWCO=300K), 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

We 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 10,000 xg 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.

We also examined the Stx-adsorbing ability of precipitated PGA by the equilibrated dialysis method as described above. The precipitated PGA (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 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 a reversed passive latex agglutination (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^{o}C for 30 min. After centrifugation at 10,000 x*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^{o}C for 16 h. After the incubation, the solution was mixed with three volumes of ethyl alcohol, and centrifuged at 4^{o}C for 20 min (10,000 x*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^{o}C for 2 h. After the incubation, we added Proteinase K (0.1 mg/ml) to the solution, and further incubated at 37^{o}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 4-12% 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., Tokyo, Japan).

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

We 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 we 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.

Purification and properties of Stx-adsorbing subsatance from natto

We 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, we chose several purification steps as shown in Table 1. We 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, we 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, we found PGA precipitated beyond its saturated density in the centrifugal filter unit. Therefore, we further examined both dissolved and precipitated PGA in the ability to adsorb Stx.

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, we could not observe the Stx-adsorbing activity of PGA at the low concentrations by the equilibrated dialysis method with Float-A-Lyzer G2 (MWCO=300K); Stx concentration in the dialysis bag was almost similar to that in the outer buffer (data not shown). Next, we 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.

We 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 we 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.

Discussion

We 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 we separated adsorbed and unadsorbed Stxs by the ultrafiltration method. However, we could not observe the adsorption of Stx to PGA by the equilibrated dialysis method. When we 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). Previously, we 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). We also reported that PGA showed high anti-mutagenic activity by adsorbing chemical mutagens (Sato and others 2008). Here, we 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 nylon–like 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 free and immobilized Gb3, the *K*d value of free Gb3 for Stx was reported to be about 1 mM (St Hilaire and others 1994), whereas that of the immobilized Gb3 for Stx 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 this paper, we 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 is under investigation. To the best of our knowledge, this is the first report on a food ingredient adsorbing Stx.

Conclusion

We 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 we 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 a precipitated one by analysis with PGA precipitated beyond its saturated density. Insolubilized PGA may have a therapeutic potential in the treatment of disease by Stx-producing bacteria.

Acknowledgments

We thank Prof. Sachio Nishio, Shikoku University, for his kind supports on the scanning electron microscopy of PGA precipitated.

References

Ashiuchi A. 2013. Microbial production and chemical transformation of poly-γ-glutamate. Microb Biotechnol 6: 664–74.

Ashiuchi M, Tani K, Soda K, Misono H. 1998. Properties of glutamate racemase from *Bacillus subtilis* IFO 3336 producing poly-gamma-glutamate. J Biochem 123:1156-63.

Ashiuchi M, Soda K, Misono H. 1999. A poly-gamma-glutamate synthetic system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly-gamma-glutamate produced by *Escherichia coli* clone cells. Biochem Biophys Res Commun 263:6-12.

Bajaj I, Singhal R. 2011. Poly (glutamic acid) - an emerging biopolymer of commercial interest. Bioresour Technol 102:5551-61.

Donohue-Rolfe A, Acheson DW, Keusch GT. 1991. Shiga toxin: purification, structure, and function. Rev Infect Dis 13:S293-7.

Fraser ME, Fujinaga M, Cherney MM, Melton-Celsa AR, Twiddy EM, O'Brien AD, James MN. 2004. Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. J Biol Chem 279:27511-7.

Garred O, van Deurs B, Sandvig K. 1995. Furin-induced cleavage and activation of Shiga toxin. J Biol Chem 270:10817-21.

Ito Y, Tanaka T, Ohmachi T, Asada Y. 1996. Glutamic acid dependent production of poly(γ-glutamic acid) by *Bacillus subtilis* TAM-4. Biosci Biotech Biochem 60:1239-1242.

Jacewicz M, Clausen H, Nudelman E, Donohue-Rolfe A, Keusch GT. 1986. Pathogenesis of Shigella diarrhea. XI. Isolation of a Shigella toxin-binding glycolipid from rabbit jejunum

and HeLa cells and its identification as globotriaosylceramide. J Exp Med 163;1391–1404.

Lea N, Lord JM, Roberts LM. 1999. Proteolytic cleavage of the A subunit is essential for maximal cytotoxicity of *Escherichia coli* O157:H7 Shiga-like toxin-1. Microbiology 145 (Pt 5):999-1004.

Li X, Wu P, Cheng S, Lv X. 2012. Synthesis and assessment of globotriose-chitosan conjugate, a novel inhibitor of Shiga toxins produced by *Escherichia coli*. J Med Chem 55:2702-10.

Ling H, Boodhoo A, Hazes B, Cummings MD, Armstrong GD, Brunton JL, Read RJ. 1998. Structure of the shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb3. Biochemistry 37:1777-88.

Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S, Karmali M. 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *in vitro*. J Biol Chem 262:8834-39.

Miyake M, Utsuno E, Noda M. 2000. Binding of avian ovomucoid to Shiga-like toxin type 1 and its utilization for receptor analog affinity chromatography. Anal Biochem 281: 202-8.

Mitsuki, M., Mizuno, A., Tanimoto, H., and Motoki, M. 1998. Relationship between the antifreeze activities and the chemical structures of oligo- and poly(glutamic acid)s. J. Agric Food Chem 46: 891-5.

Miyagawa A, Watanabe M, Igai K, Kasuya MC, Natori Y, Nishikawa K, Hatanaka K. 2006. Development of dialyzer with immobilized glycoconjugate polymers for removal of Shiga-toxin. Biomaterials 27:3304-11.

Nishikawa K. 2011. Recent progress of Shiga toxin neutralizer for treatment of infections by Shiga toxin-producing *Escherichia coli*. Arch Immunol Ther Exp 59:239-47.

Nishikawa K, Matsuoka K, Kita E, Okabe N, Mizuguchi M, Hino K, Miyazawa S, Yamasaki C, Aoki J, Takashima S, Yamakawa Y, Nishijima M, Terunuma D, Kuzuhara H, Natori Y. 2002. A therapeutic agent with oriented carbohydrates for treatment of infections by

Shiga toxin-producing *Escherichia coli* O157:H7. Proc Natl Acad Sci U S A. 99:7669-74.

Riley LW, Remis RS, Mcgee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hagrett NT, Blake PA, Cohen ML. 1983. Hemorrhagic colitis associated with a rare type *Escherichia coli* serotype. N Engl J Med 308:681-5.

Sato M, Kanie K, Soda K, Yokoigawa K. (2008). Suppressive effect of poly-γ-glutamate on SOS response of *Salmonella typhimurium* induced by chemical mutagens. J Biosci Bioeng 105:690-3.

Shih, I.-L. and Van, Y.-T. 2001. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresour Technol 79: 207-25.

St Hilaire PM, Boyd MK, Toone EJ. 1994. Interaction of the Shiga-like toxin type 1 B-subunit with its carbohydrate receptor. Biochemistry 33: 14452-63.

Takemasa N, Ohnishi S, Tsuji M, Shikata T, Yokoigawa K. 2009. Screening and analysis of spices with ability to suppress verocytotoxin production by *Escherichia coli* O157. J

Food Sci 74: 461-6.

Walterspiel JN, Ashkenazi S, Morrow AL, Cleary TG. 1992. Effect of subinhibitory concentrations of antibiotics on extracellular Shiga-like toxin I. Infection 20:25-9.

Watanabe M, Matsuoka K, Kita E, Igai K, Higashi N, Miyagawa A, Watanabe T, Yanoshita R, Samejima Y, Terunuma D, Natori Y, Nishikawa K. 2004. Oral therapeutic agents with highly clustered globotriose for treatment of Shiga toxigenic *Escherichia coli* infections. J Infect Dis 189:360-8.

Yokoigawa K, Sato M, Soda K. 2006. Simple improvement in freeze-tolerance of bakers' yeast with poly-γ-glutamate. J Biosci Bioeng 102:215-9.

Table 1-Purification of Stx-adsorbent from natto

Step	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

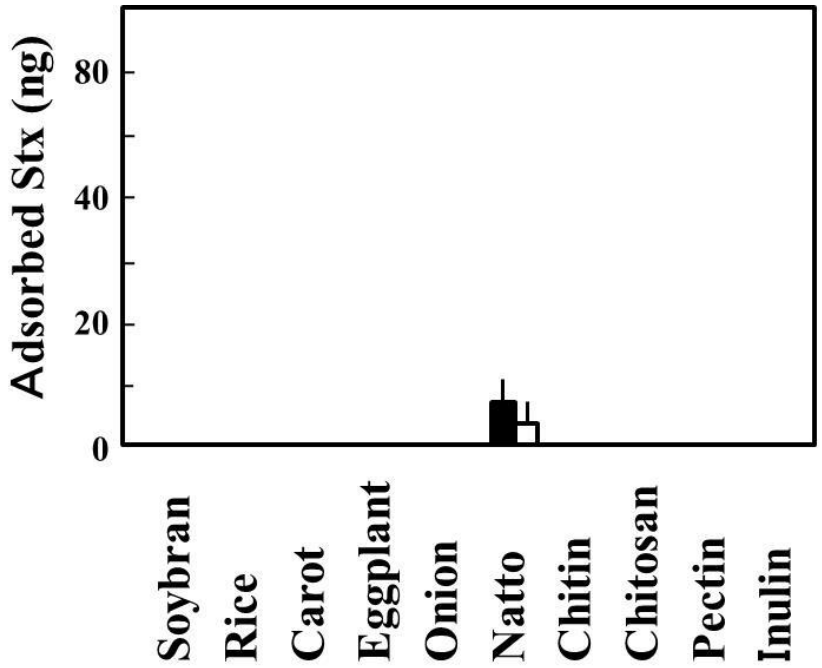

Figure 1-Adsorption of Stx to food and dietary fibers. Each dry food and dietary fiber (5 mg) was suspended in the Stx solution (0.5 ml), and incubated at 25°C. After incubation for 30 min, the suspensions were centrifuged at 10,000 x*g* for 10 min. Stx adsorbed to the precipitate was dissociated by 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.

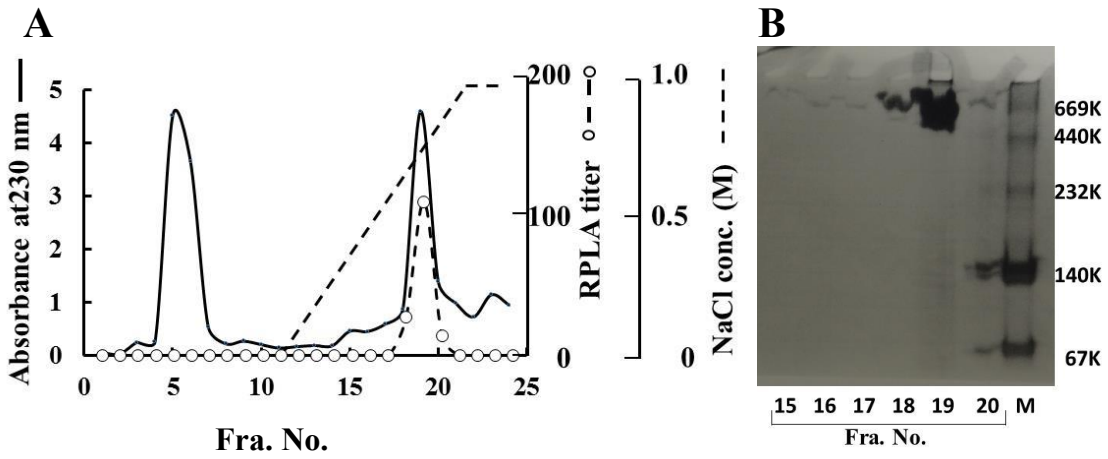

Figure 2-Elution profile of natto extract after treatment with Proteinase K on a Q Sepharose Fast Flow column (1.6 x 10 cm) (A), and PAGE of fractions 15-20 (B).

(B) Fractions 15-20 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).

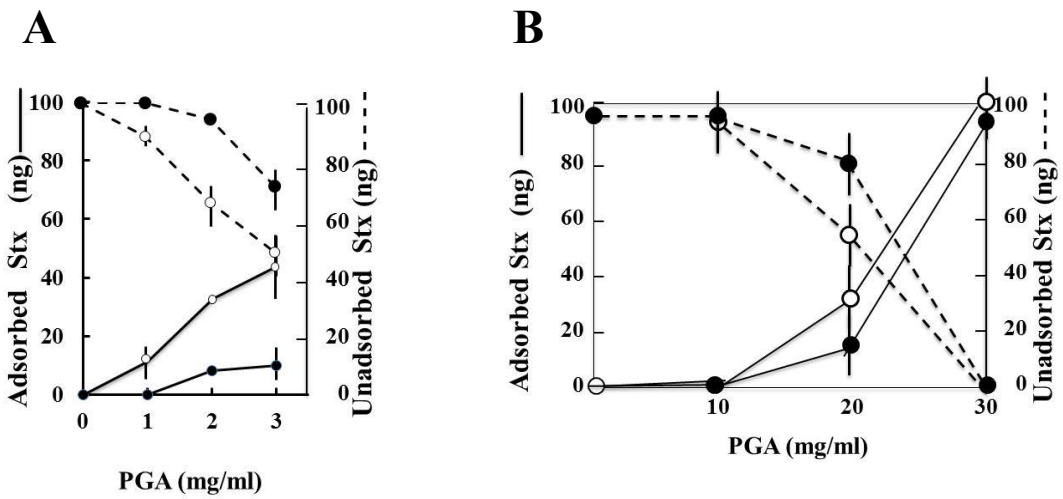

Figure 3-Adsorption of Stx to the soluble PGA using an ultrafiltration method to separate adsorbed and unadsorbed Stx (A), and to the PGA precipitated beyond its saturated density (B). Purified PGA was added to 1 ml of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% bovine serum 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.

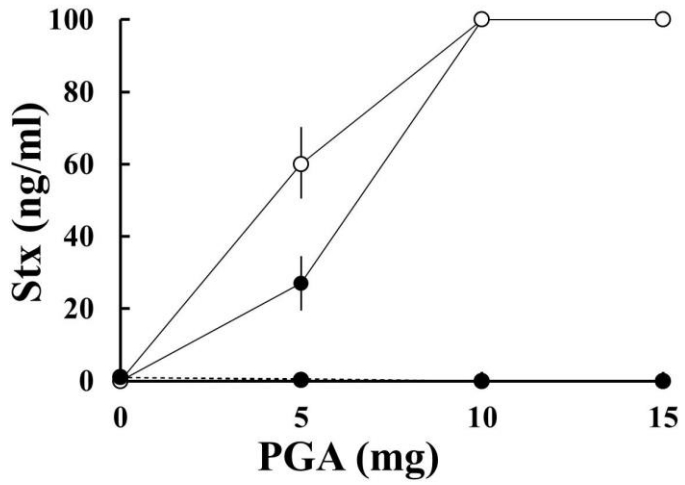

Figure 4-Adsorption of Stx to the PGA precipitated beyond its saturated density using the equilibrated dialysis method. PGA precipitated (5, 10, 15 mg as dry wt.) and Stx solution (1 ml, 100 ng/ml of 10 mM phosphate buffer, pH 7.4, supplemented with 0.85% NaCl, 0.5% BSA, and 1.8% PGA) were mixed, put 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.

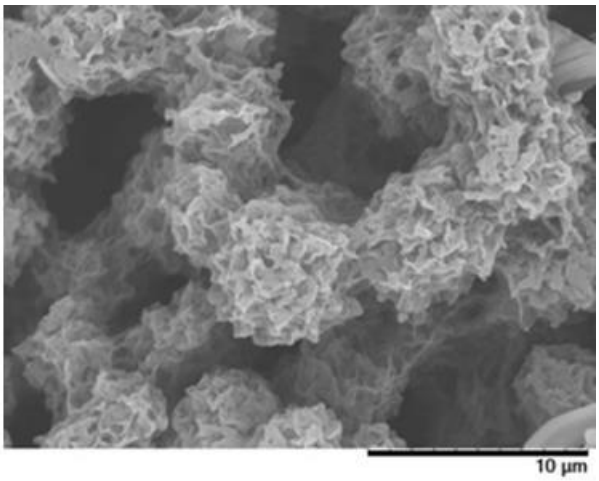

Figure 5-Scanning electron micrograph of PGA precipitated. PGA precipitated beyond its saturated density was washed twice with ethyl alcohol, and dried under reduced pressure. After coating the dry powder with Au (15 nm thick), the sample was visualized using SEM.