Determination of binding affinity of poly-γ-glutamate to Shiga toxin

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Short title: Binding affinity of poly-γ-glutamate to Shiga toxin

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ABSTRACT

We examined poly-γ-glutamate from natto, a Japanese fermented food, in the ability to adsorb Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). The polymer was immobilized by direct coupling to EAH-Sepharose™. The poly-γ-glutamate-Sepharose (about 10 mg of ligand/mL of gel) adsorbed Stx2, but not Stx1: its dissociation constant (*K*d) against Stx2 was calculated to be 14.0 μM. To analyze the binding site of poly-γ-glutamate against Stx2, we similarly immobilized glutamate and glutarate. Glutamate- and glutarate-Sepharoses (each 7 μmol of ligand/mL of gel) similarly adsorbed Stx2, but not Stx1; *K*d values against Stx2 were

calculated to be 14.0 and 30.0, respectively, μM. The common structures of PGA-, glutamate-, and glutarate-Sepharoses were considered to be glutaryl groups. When we added the mixture of Stx2 and poly-γ-glutamate-Sepharose to Caco-2 cells (a human colon epithelial cell line), poly-γ-glutamate-Sepharose was found to reduce the cytotoxicity of Stx2.

PRACTICAL APPLICATION

Poly-γ-glutamate is a component of a traditional Japanese food, and is believed to be absolutely safe. Present study revealed that immobilized poly-γ-glutamate adsorbed Stx2 produced by enterohemorrhagic *Escherichia coli*. To our knowledge, this is the first report on adsorbents specific to Stx2. Our results are probably useful for development of new functional foods with the ability to adsorb Stx2.

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

INTRODUCTION

Poly-γ-glutamate (PGA) is a component of a traditional Japanese food, natto, prepared from steamed soybeans by the biological action of *Bacillus subtilis*. PGA is an anionic polypeptide in which glutamate is polymerized via γ-amide linkages. Therefore, PGA is indigestible, and is believed to be absolutely safe. Potential applications of PGA have been of interest in a broad range of industrial fields such as medicine, food, and cosmetics (Shih & Van 2001; Bajaj & Singhal 2011).

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. The toxins also traverse the epithelium, invade the blood circulation, and cause neurological damage and hemolytic-uremic syndrome. 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.

EHEC O157:H7 produces two types of Stx, Stx1 and Stx2. The amino acid sequence identity between Stx1 and Stx2 is about 56%, and they are immunologically distinct from each other (Bergan *et al*. 2012). Several variants of both Stx1 and Stx2 have also been identified (Bergan *et al*. 2012; Melton-Celsa 2014). Stx1 has been linked to human illness, but Stx2 are more frequently associated with the development of hemorrhagic colitis and hemolytic uremic syndrome (Melton-Celsa 2014). Louise & Obrig (1995) reported that Stx2 was 1000 times more cytotoxic than Stx1 toward human renal microvascular endothelial cells, the putative target of Stx. Both Stx1 and Stx2 belong to the AB5 family of protein toxins, with 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). Although Stx1 and Stx2 showed a similar affinity to soluble Gb3 (Kitova *et al*. 2007), lipid environment around Gb3 is reported to influence the binding affinity of Stx1 and Stx2 to Gb3 (Bergan *et al*. 2012; Karve & Weiss 2014). Cholesterol and phosphatidylcholine were reported to neutralize the cytotoxicity of Stx1, but not Stx2 (Gallegos *et al*. 2012). The binding ability of Stx1 to Gb3 under the lipid environment may be lower than that of Stx2. Interaction between the toxins and Gb3 is reported to be multivalent; three binding sites on each B subunit monomer for Gb3 (Fraser *et al*. 2004; Ling *et al*. 1998). 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; Watanabe *et al*. 2004), there are few reports on foods with an ability to adsorb Stx. Previously, we reported that Stx bound to PGA precipitated beyond the saturated density, but not to soluble PGA (Goto *et al*. 2016). If chemically insolubilized PGA adsorbs Stx, the toxin in gut lumen may

be excreted with the PGA into feces. To obtain an insoluble PGA as a functional food with ability to adsorb Stx, we prepared and analyzed PGA-Sepharose and related resins.

MATERIALS AND METHODS

Materials

PGA was purified from natto as described previously (Goto *et al*. 2016). EAH-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. Dowex Mac 3 (3.8 μmol of carboxyl groups/mL of resin), an anion-exchange resin, was obtained from Dow Chemical Japan, Tokyo, Japan. An Stx (VT) detection kit including Stx1 and Stx2 as references (variants: Stx1a and Stx2a) was obtained from Denka Seiken (Tokyo, Japan). 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). Millex-GV (pore size 0.22 μm) was also obtained from Merck Millipore (Billerica, Mass. USA). The 96-well microplates (V-bottom) used for a reversed passive latex agglutination (RPLA) assay of Stx was supplied from Greiner Japan (Tokyo, Japan). Caco-2 (a human colon epithelial cell line) was obtained from DS Pharma Biomedical Co. Ltd., Tokyo, Japan. A minimum essential medium (MEM), fetal bovine serum (FBS), and non-essential amino acids (NEAA) were obtained from Thermo Fisher Scientific (Yokohama, Japan). Cell counting kit-8 was purchased from Dojindo (Kumamoto, Japan).

Preparation of immobilized PGA

PGA was immobilized by direct coupling to EAH-Sepharose™ 4B with EDC. 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 EDC (228 mg, final conc. 0.1 M), and incubated at

25ºC for 17 h. During the initial 1 h of the reaction, the pH value of mixture was kept at 5.0 according to the manufacture's protocol. After the reaction, the resin was washed three times with 10 mL of distilled water and kept at 4˚C until use. About 10 mg of PGA was found to be immobilized on 1 mL of gel, as judged from increase in weight of dried resin before and after the immobilized reaction. Glutamate-Sepharose and glutarate-Sepharose were similarly prepared: about 1.5 mg ligands were immobilized on mL of gel.

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 references Stx1 and Stx2 provided in the kit. The 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 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.

Determination of *K*d values

We analyzed PGA-, glutamate-, and glutarate-Sepharoses in the ability to adsorb Stx by an equilibrated dialysis method with Float-A-Lyzer G2 as described previously (Badr *et al*. 2017). The references 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 (BSA) to avoid non-specific adsorption. A constant amount of each resin (0.2 mL) and varying concentrations of Stx solutions (0.2 mL) were mixed, put in the dialysis bag of the Float-A-Lyzer G2, and dialyzed against 50 mL of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% BSA 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 the resins in the dialysis bag was dissociated by addition of NaOH (final pH 10), and free Stx was separated from the resins by the centrifugal ultrafiltration with a filter unit (Amicon Ultra-4, MWCO: 100 kDa). After the ultrafiltration at 4,000 *g* for 30 min, filtrates containing free Stx were neutralized with HCl and analyzed by the RPLA assay. The ratio (ν) of [bound Stx] to [the Stx-adsorbent] was plotted against ν /[free Stx]. The *K*d values were obtained by calculating the reciprocal of slopes of lines, as described previously (Badr *et al*. 2017).

Preparation of crude Stx

Stx1 localized in the periplasm fraction of bacterial cells and Stx2 secreted into culture media (Shimizu *et al*. 2007) were separately prepared. EHEC O157:H7 Sakai was aerobically cultivated at 37°C for 24 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 10,000 *g* for 15 min, the supernatant solution obtained was sterilized with a filter unit (Millex-GV, pore size 0.22 μm) and used as extracellular Stx preparation. The precipitated cells were suspended in 2 mL of 10 mM phosphate buffer (pH 7.4) and disrupted by vigorous mixing with 1 g of glass beads (average diameter of 0.1 mm). After centrifugation of the cell extract at 10,000 *g* for 15 min, the supernatant solution obtained was sterilized with the filter unit and used as an intracellular Stx preparation. The extracellular and intracellular Stx preparations were mixed, dialyzed against 10 mM phosphate buffer (pH 7.4) and used as a crude Stx (a mixture of Stx1 and Stx2).

Specificity of PGA-Sepharose against Stx

The crude Stx was put on a PGA-Sepharose column (ϕ 0.5 x 5 cm) equilibrated with 10 mM phosphate buffer (pH 7.4). After the column was washed with the same buffer, elution was made by a linear gradient of 0-20 mM NaOH at a flow rate of 1.0 mL/min. After neutralization of eluate with HCl, the amounts of Stx1 and Stx2 in fractions were determined by the RPLA assay.

Protection of Caco-2 cells by PGA-Sepharose from Stx2

We prepared the Stx2 bound to PGA-Sepharose, and examined the cytotoxicity of the complex as follows. Mixtures of PGA-Sepharose (40 μL of 10% suspension) and several concentrations of Stx2 solutions (40 μL) were incubated at 37°C for 1 h with shaking. After the incubation, the mixture was directly used for the cell based assay with Caco-2 cells. Caco-2 cells were inoculated in 96 well microplates (about 250 cells/well) with 45 μL MEM containing 20% FBS and 0.1mM NEAA. After incubation at 37°C for 2 h in a humidified atmosphere with 5% CO_2, 5 μL of the mixture was added into each well. The cells were cultured at 37°C for 4 days in a humidified atmosphere with 5% CO_2. Living cells in each well were measured with Cell counting kit-8 according to the manufacture's protocol.

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

Binding of Stx to PGA-Sepharose

We examined the binding of Stx to PGA-Sepharose by the equilibrated dialysis method. Fig. 1A showed the binding of Stx2 to PGA-Sepharose. The amounts of Stx2 bound to PGA-Sepharose increased with increase of those of Stx2 used. The *K*d value of PGA-Sepharose against Stx2 was obtained by calculating the reciprocal of slope of the line in Fig. 1B, and shown in Table 1. However, Stx1 did not bind to PGA-Sepharose (data not shown).

Similarly, we examined the binding of Stx against glutamate-Sepharose, glutarate-Sepharose and Dowex Mac 3. Figure 2A showed the adsorption of Stx2 to glutamate-Sepharose. The *K*d value of glutamate-Sepharose to Stx2 was similarly calculated based

upon the results of Fig. 2B and was identical to that of PGA-Sepharose (Table 1). Figure 3A showed the binding of Stx2 to glutarate-Sepharose. Stx1 did not bind to glutamate- and glutarate-Sepharoses (data not shown). We also calculated the *K*d value of glutarate-Sepharose against Stx2 based upon the results of Fig. 3B. The glutarate-Sepharose showed a similar *K*d value to PGA- and glutamate-Sepharoses (Table 1). While, neither Stx1 nor Stx2 bound to Dowex Mac 3 (data not shown).

Specificity of PGA-Sepharose against Stx

As described above, PGA-Sepharose bound Stx2, but not Stx1. We examined the specificity of PGA-Sepharose against Stx2 by the column chromatography. The crude Stx containing each 100 ng of Stx1 and Stx2 (1 mL) was applied to the column. As shown in Fig. 4, Stx1 was passed through the column, but Stx2 was adsorbed to the column and eluted with about 10 mM NaOH. The yields of Stx1 and Stx2 were both about 100%. When more than 100 ng of Stx2 were put on the column, a part of Stx2 passed through the column.

Protection of Caco-2 cells by PGA Sepharose from Stx2

Figure 5 shows the effect of PGA-Sepharose on protection of Caco-2 cells from Stx2. We confirmed in advance that PGA-Sepharose induced neither increase nor decrease of the living cells number of Caco-2 cells. The number of living cells decreased with increase of the concentration of Stx2. However, the mixtures of Stx2 and PGA-Sepharose seemed to have lower cytotoxicity than only Stx2. Thus, Caco-2 cells were partially protected by PGA-Sepharose from cytotoxicity of Stx2.

DISCUSSION

PGA-Sepharose bound Stx2, but not Stx1. Isoelectric points of Stx1 and Stx2 were reported to be 7.0 and 4.1, respectively (Dickie *et al*. 1989; Oku *et al*. 1989). Thus, Stx2 is an acidic protein. The binding of Stx2 to PGA-Sepharose was considered not to be ionic

interaction. Since glutamate-Sepharose also showed a similar affinity against Stx2 to PGA-Sepharose, the binding between PGA-Sepharose and Stx2 seemed not to depend on the conformation of PGA (Shih & Van 2001). In addition, amino groups of glutamate-Sepharose seemed not to influence on the binding of Stx2. While, Dowex Mac 3 adsorbed neither Stx1 nor Stx2. The structure of polyacrylic acid seemed to be unsuitable for adsorbing Stx.

We used EDC for the direct coupling of PGA, glutamic acid, and glutaric acid to EAH-Sepharose™ 4B. 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 & Gergely 1990). Therefore, there is no modification in the PGA-Sepharose and other related resins except for cross-linking formed by the coupling reaction with EDC. Since the *K*d values of PGA-, glutamate-, and glutarate–Sepharoses to Stx2 were similar to each other, the common binding site of Stx2 against these resins was considered to be glutaryl groups.

There are several reports on Gb3 as an Stx-adsorbent for clinical practice. Since interaction between Stx and Gb3 was reported to be multivalent (Fraser *et al*. 2004; Ling *et al*. 1998), clustered Gb3 was required for strong binding (Nishikawa 2011). Free Gb3 showed a low affinity to Stx (*K*d value: about 1 mM) (St Hilaire *et al*. 1994), but a conjugate of Gb3-chitosan 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). Thus, the *K*d value of PGA-Sepharose as a functional food was higher than that of the Gb3-chitosan conjugate as a medicine. However, our results are probably useful for development of functional foods with ability to prevent diseases caused by EHEC and to mitigate symptoms of the infectious disease, because functional foods can be taken in larger quantities than medicine. 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. Karve & Weiss (2014) also reported that the binding of Stx variants increased in the presence of phosphatidylcholine and cholesterol. Therefore, we examined the protective effect of PGA-Sepharose on the cytotoxicity of Stx2 with Caco-2 cells. The cells

are widely used for investigating Stx-mediated intestinal cytotoxicity (Kouzel et al. 2017). We found that Caco-2 cells were partly protected by PGA-Sepharose from Stx2 under the conditions we used. Because of the deference in affinities to Stx2 between Caco-2 cells and PGA-Sepharose, Stx2 bound to PGA-Sepharose may partly dissociate and exhibit the cytotoxicity to Caco-2 cells. However, our results suggested that PGA-Sepharose had the ability to bind Stx2 even in the presence of Caco-2 cells.

Difference in toxicity of Stx1 and Stx2 is speculated to be that in receptor binding preferences between the two toxins (Fraser *et al*. 2004; Ling *et al*. 1998; Karve & Weiss 2014). Since Stx2 has markedly higher toxicity than Stx1, removal of Stx2 from intestinal environment of patients is important for prevention and treatment of infectious diseases by EHEC. However, there was no report about Stx2-adsorbents, to the best of our knowledge. PGA-Sepharose was found to specifically adsorb Stx2 when we used the crude Stx.

Our results are probably useful for development of new functional foods with the ability to adsorb Stx2.

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CONPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The author declares that they have no conflict of interest.

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

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Figure Legends

FIG. 1. Binding of Stx2 to PGA-Sepharose by the equilibrated dialysis method. PGA-Sepharose (0.2 mL) and several concentrations of Stx solution (0.2 mL) were mixed, put in a bag of the Float-A-Lyzer G2 (MWCO = 300 K), and dialyzed against 50 mL of 10 mM phosphate buffer (pH 7.4) supplemented with 0.85% NaCl and 0.5% BSA at 4°C for 16 h. The amounts of Stx in the dialysis bag (bound Stx, open bars) and outer liquid (free Stx, closed bars) were analyzed by the RPLA assay (A). The ratios (ν) of [bound Stx] to [PGA] were plotted against ν /[free Stx] (B). The *K*d value was obtained by calculating the reciprocal of slope of a line in Fig. 1B. Three independent experiments were performed twice, and the vertical bars show the standard errors.

FIG. 2. Binding of Stx2 to glutamate-Sepharose by the equilibrated dialysis method. Experiments were performed as described in Fig. 1 except that PGA-Sepharose was replaced with glutamate-Sepharose. The amounts of Stx in the dialysis bag (bound Stx, open bars) and outer liquid (free Stx, closed bars) were analyzed by the RPLA assay (A). The ratios (ν) of [bound Stx] to [glutamate] were plotted against ν /[free Stx] (B). The *K*d value was obtained by calculating the reciprocal of slope of a line in Fig. 2B. Three independent experiments were performed twice, and the vertical bars show the standard errors.

FIG. 3. Binding of Stx2 to glutarate-Sepharose by the equilibrated dialysis method. Experiments were performed as described in Fig. 1 except that PGA-Sepharose was replaced with glutarate-Sepharose. The amounts of Stx in the dialysis bag (bound Stx, open bars) and outer liquid (free Stx, closed bars) were analyzed by the RPLA assay (A). The ratios (ν) of [bound Stx] to [glutarate] were plotted against ν /[free Stx] (B). The *K*d value was obtained by calculating the reciprocal of slope of a line in Fig. 3B. Three independent experiments were performed twice, and the vertical bars show the standard errors.

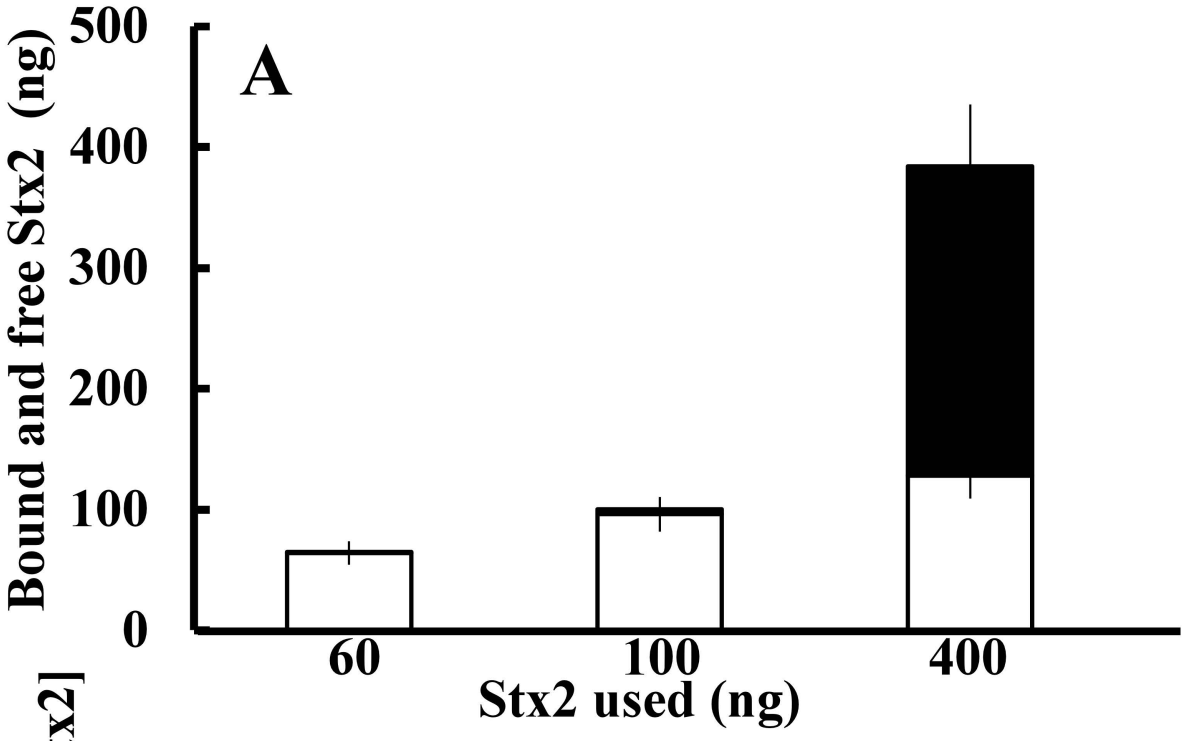
FIG. 4. PGA-Sepharose column chromatography of Stx. The crude Stx (each 100 ng of

Stx1 and Stx2, 1 mL) was put on a PGA-Sepharose column (ϕ 0.5 cm x 5 cm, about 1 mL) equilibrated with 10 mM phosphate buffer (pH 7.4) at a flow rate of 1.0 mL/min. After washing the column with the same buffer, elution was made by a linear gradient of 0-20 mM NaOH at a flow rate of 1.0 mL/min. After neutralization of eluate with HCl, the amount of Stx in each fraction was determined by the RPLA assay. Three independent experiments were performed, and the representative result was shown. Black bar: Stx1, white bar: Stx2.

FIG. 5. Protection of Caco-2 cells by PGA Sepharose from Stx2. Mixtures of PGA-Sepharose (40 μL of 10% suspension) and several concentrations of Stx2 solutions (40 μL) were incubated at 37°C for 1 h with shaking. The mixtures were directly used for the cell based assay with Caco-2 cells. Caco-2 cells were inoculated in 96 well microplates (about 250 cells/well) with 45 μL MEM containing 20% FBS and 0.1 mM non-essential amino acids. After incubation at 37°C for 2 h in a humidified atmosphere with 5% CO_2, 5 μL of the mixture was added into each well. As controls, the mixture was replaced with Stx2 solution or PGA-Sepharose. The cells were cultured at 37°C for 4 days in a humidified atmosphere with 5% CO_2. Living cells in each well were measured with the Cell counting kit-8 according to the manufacture's protocol. Six independent experiments were performed, and the vertical bars show the standard errors. Closed circles: the mixture, open circles: Stx2, and closed triangles: PGA-Sepharose.

Fig. 1

Top


A
Bound and free Stx2 (ng)
500
400
300
200
100
0
60
100
400
Stx2 used (ng)

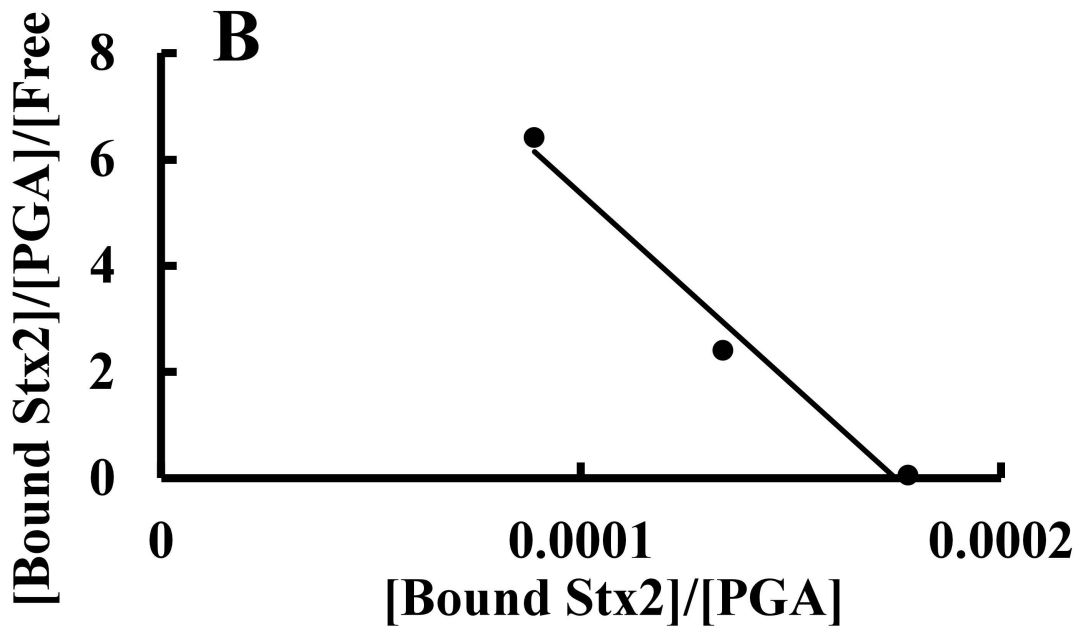

B
[Bound Stx2]/[PGA]/[Free Stx2]
8
6
4
2
0
0
0.0001
0.0002
[Bound Stx2]/[PGA]

Fig. 2
Top

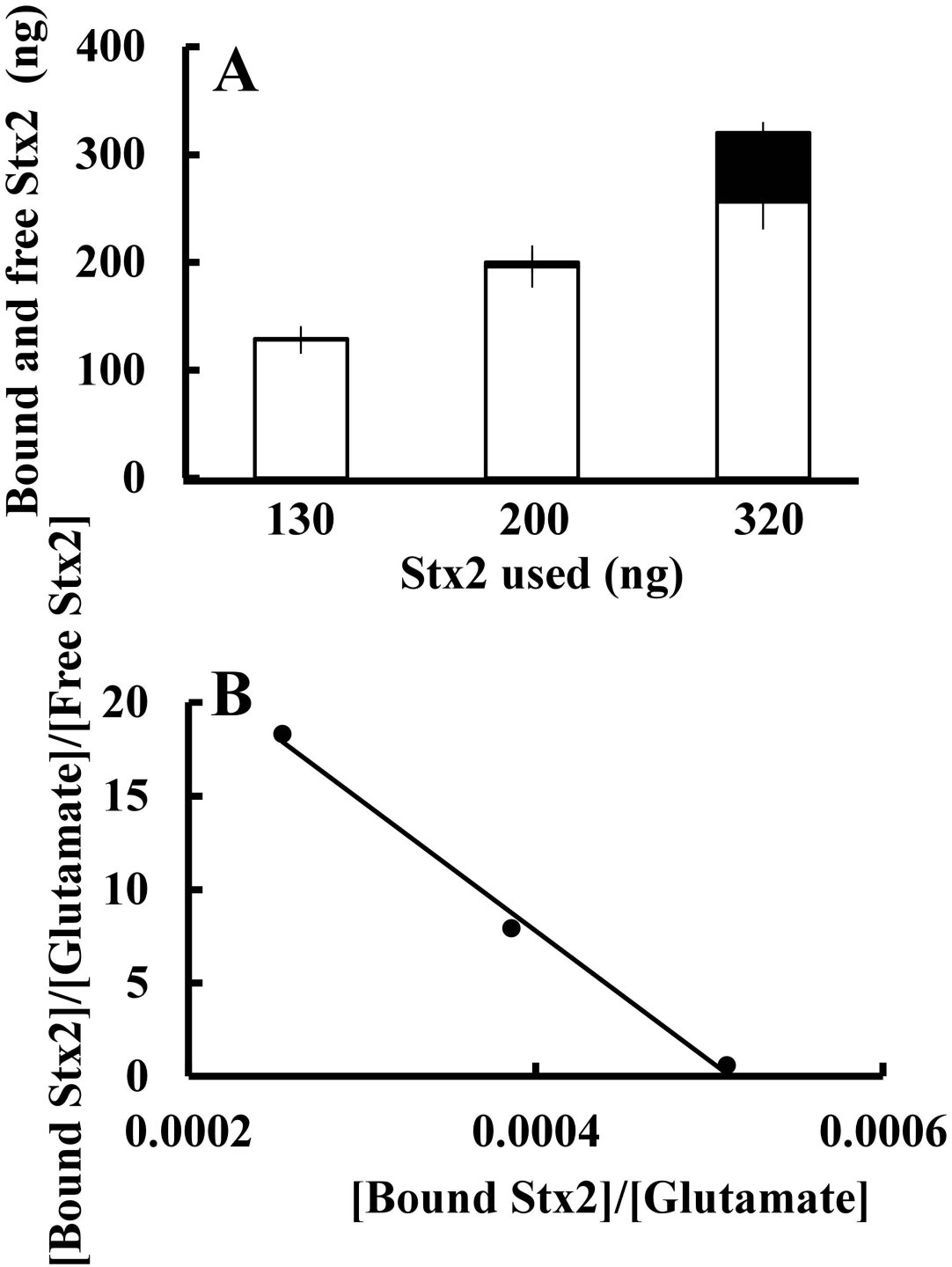

A
Bound and free Stx2 (ng)
400
300
200
100
0
130
200
320
Stx2 used (ng)
B
[Bound Stx2]/[Glutamate]/[Free Stx2]
20
15
10
5
0
0.0002
0.0004
0.0006
[Bound Stx2]/[Glutamate]

Fig. 3

Top

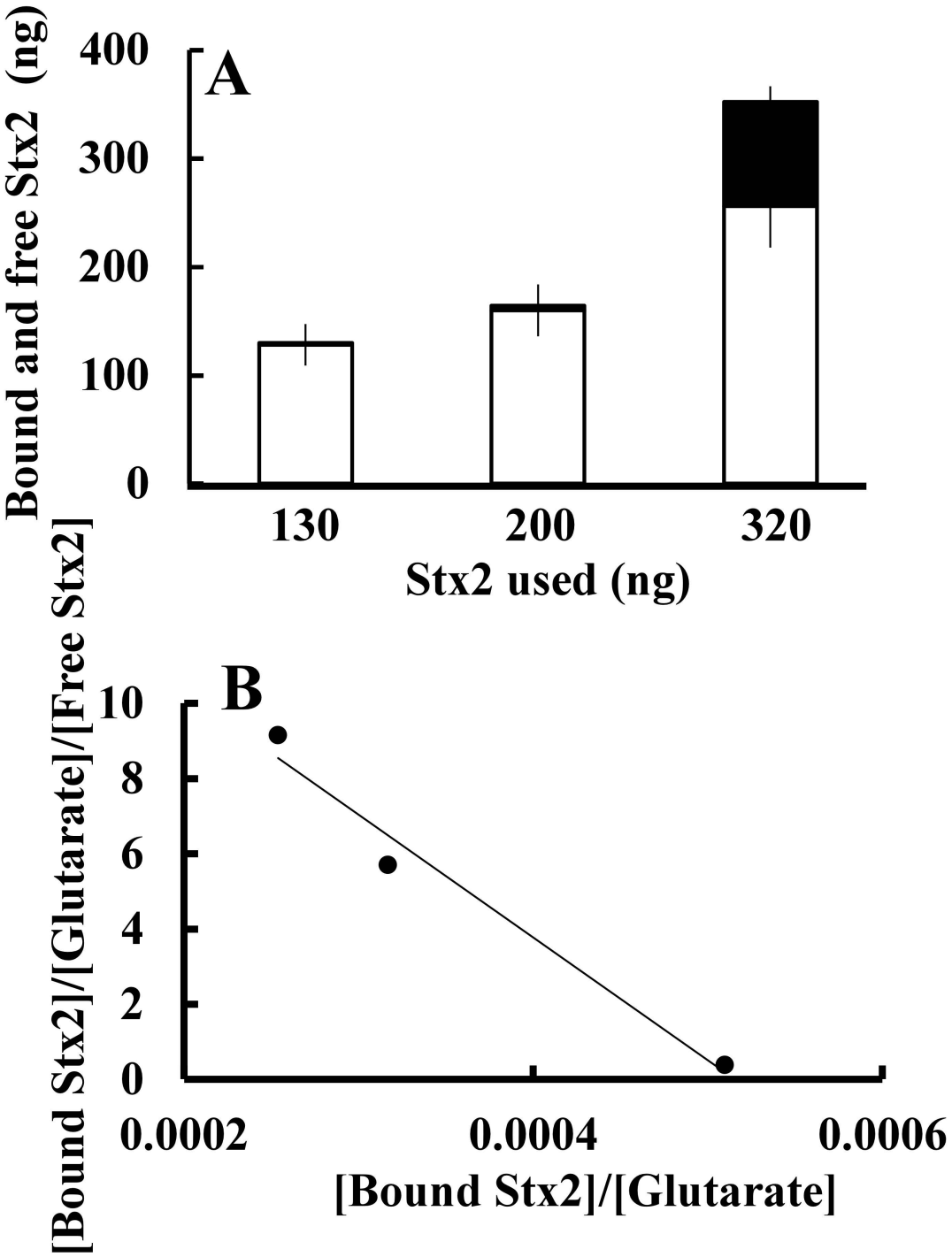

A
Bound and free Stx2 (ng)
400
300
200
100
0
130
200
320
Stx2 used (ng)
B
[Bound Stx2]/[Glutarate]/[Free Stx2]
10
8
6
4
2
0
0.0002
0.0004
0.0006
[Bound Stx2]/[Glutarate]

Fig. 4

Top

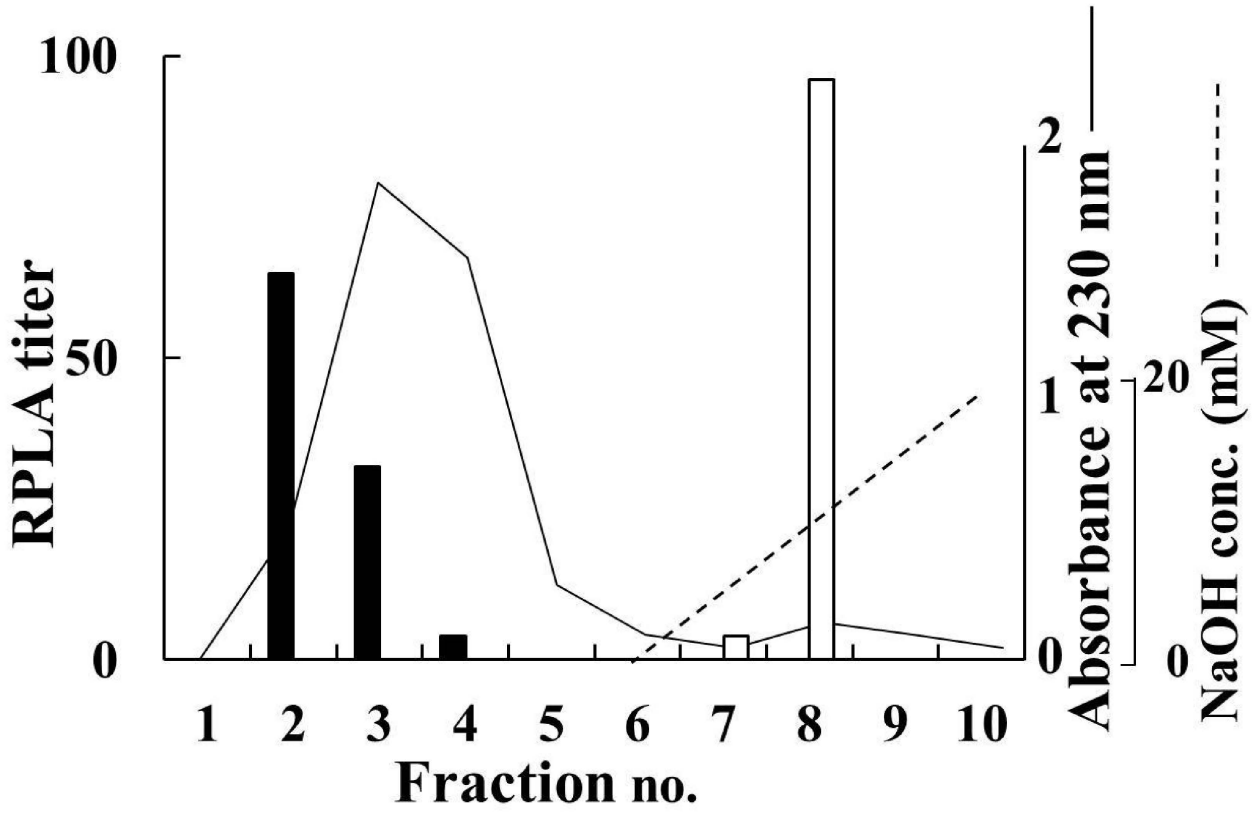

100
50
0
RPLA titer
1
2
3
4
5
6
7
8
9
10
Fraction no.
2
1
0
Absorbance at 230 nm
20
0
NaOH conc. (mM)

Fig. 5

Top

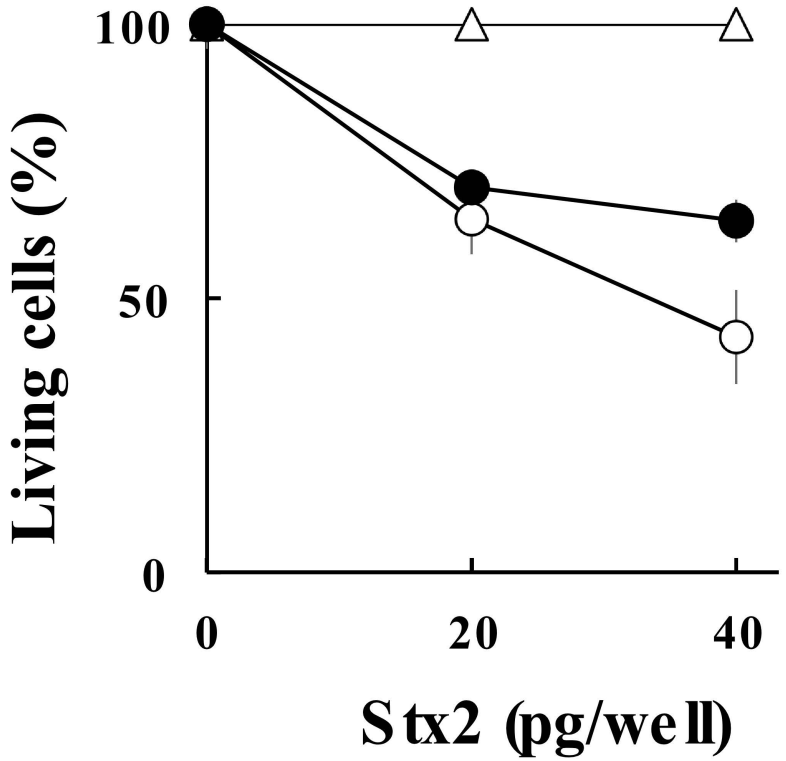


Table 1 The *K*d values of PGA-, glutamate-, and glutarate-Sepharoses to Stx2.

Resins	*K*d (μM)
PGA-Sepharose	14.0
Glutamate-Sepharose	14.0
Glutarate-Sepharose	30.0