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Norovirus-binding proteins recovered from activated sludge microorganisms with an affinity to a noroviral capsid peptide

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Running headline

NOROVIRUS-BINDING PROTEINS

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ABSTRACT

Aims: Transmission routes of noroviruses, leading etiological agents of acute gastroenteritis, are rarely verified when outbreaks occur. Since the destination of norovirus particles being firmly captured by microorganisms could be totally different from that of those particles moving freely, microorganisms with natural affinity ligands such as virus-binding proteins would affect the fate of viruses in environment, if such microbial affinity ligands exist. The aim of this study is to identify norovirus-binding proteins (NoVBPs) that are presumably working as natural ligands for norovirus particles in water environments.

Methods and Results: NoVBPs were recovered from activated sludge microorganisms by an affinity chromatography technique in which a capsid peptide of norovirus genogroup II (GII) was immobilized. The recovered NoVBPs bind to Norovirus-like particles (NoVLPs) of norovirus GII, and this adsorption was stronger than that to NoVLPs of norovirus genogroup I. The profile of two-dimensional electrophoresis of NoVBPs showed that the recovered NoVBPs included at least 7 spots of protein. The determination of N-terminal amino acid sequences of these NoVBPs revealed that hydrophobic interactions could contribute to the adsorption between NoVBPs and norovirus particles.

Conclusions: NoVBPs conferring a high affinity to norovirus GII were successfully isolated from activated sludge microorganisms.

Significance and Impact of Study: NoVBPs could be natural viral ligands and play an important role in the NoV transmission.

Keywords: norovirus, virus-binding protein, activated sludge microorganisms, virus-like particle, affinity chromatography, hydrophobic interaction

INTRODUCTION

Noroviruses are important etiological agents for acute gastroenteritis all over the world (Widdowson et al., 2005). Since a large amount of norovirus is included in feces of gastroenteritis patients (Chan et al., 2006), it is very crucial to remove or inactivate noroviruses in wastewater treatment processes to prevent water environments from contamination. However, there have been difficulties in sufficiently reducing viruses in wastewater with conventional wastewater treatment processes (Lodder and de Roda Husman, 2005; Ueki et al., 2005), which has resulted in the frequent detection of noroviruses from water environments (Rutjes et al., 2005; Haramoto et al., 2005). Noroviruses discharged to water environments can reach coastal areas and are accumulated by bivalves such as oysters (Nishida et al., 2003), and the food poisoning caused by contaminated bivalves has been established as one of the main transmission routes of noroviruses (Cheng et al., 2005). However, the transmission routes of noroviruses are rarely identified once outbreaks occur, and the fate of noroviruses in human society has not been fully elucidated.

In our previous study, bacterial proteins that have an ability to bind human virus-binding proteins (VBPs) were discovered in a bacterial culture of activated sludge (Sano et al., 2004). These VBPs are located on the surface of bacterial cells and exhibit a

virus-binding ability under the conditions of water environments. Since viruses adsorbed to suspended solids can survive for a longer period than viruses suspended freely (Foster et al., 1980), bacteria harboring VBPs for noroviruses (NoVBPs) could prolong norovirus lives in an aquatic environment, if such microbial affinity ligands exist.

In this study, NoVBPs were newly recovered from activated sludge microorganisms using an affinity chromatography technique. The C-terminal amino acid sequence of norovirus genogroup II genotype 4 (GII.4) was used as a ligand. The norovirus-binding ability of the recovered proteins was evaluated by ELISA using norovirus-like particles (NoVLPs). Two-dimensional (2D) electrophoresis was employed to show molecular weight and isoelectric point (pI) of NoVBPs, and N-terminal amino acid sequences were determined in order to identify NoVBPs.

MATERIALS AND METHODS

Affinity isolation of NoVBPs.

Sludge from a municipal wastewater treatment plant (100 mL) was centrifuged (1,000 x *g*, 10 min, 4 ˚C), and activated sludge microorganisms in the supernatant liquid

were cultivated in 2 L of a nonselective nutrient broth (Kyokuto, Tokyo, Japan) under aerobic conditions. The culture in the stationary phase was centrifuged (3,000 x *g*, 10 min, 4 ˚C), and crude proteins were extracted from the pellet as described previously (Sano et al., 2004). Extracted crude proteins were filtered with 0.45-µm-pore-size membrane for low protein binding (Millex-HV, PVDF, SLHV 025 LS, Millopore) and applied to the affinity column (HiTrap NHS-activated, Amersham Bioscience Corp., NJ, USA), in which a custom-made polypeptide of noroviral capsid protein was immobilized as a ligand. The peptide ligand corresponds to a C-terminal region of the noroviral capsid protein, which faces the hollows on the surface of the noroviral particle (Prasad et al., 1999; Parker et al., 2005) and can interact with a monoclonal antibody (Shiota et al., 2007). The sequence of the immobilized peptide was H_2N-WVNQFYTLAPMG-COOH, which was produced by the Peptide Institute, Inc. (Osaka, Japan). The amino acid sequence of this region is well conserved among genotypes, thereby indicating the identity between 75 and 92% in norovirus GII (Fig. 1). Affinity chromatography was performed using the AKTA FPLC system (Amersham Bioscience Corp., NJ, USA) at room temperature (the room temperature is controlled at 23˚ C). Start and elution buffers of the affinity chromatography were 2mM Tris-HCl (pH: 7.2) and 6M urea, 0.5M NaCl in 20mM Tris-HCl (pH: 3.5), respectively. The flow rate was set at 1 ml/min, and 1 ml each of affinity chromatographic

fraction was collected by a fraction collector (Frac-900, Amersham Bioscience Corp., NJ, USA). Affinity chromatographic fractions were then desalted by dialysis against 10 mM NH_4HCO_3 (pH: 8.0) for at least 12 hours, and proteins in these fractions were concentrated with a vacuum and centrifugal dehydrator (CVE-100, EYELA, TOKYO RIKAKIKAI CO. LTD., Tokyo, Japan). Concentrated proteins in the pellet were suspended in 100 µl of double autoclaved MilliQ water. The water was evaporated with the dehydrator for washing. This washing step was repeated twice, and recovered proteins in the pellet were preserved at –20˚ C until further analysis.

Evaluation of virus-binding ability with ELISA

The recovered proteins from 10 ml of the affinity chromatographic fraction were dissolved in 350 µl of 50 mM sodium carbonate buffer (pH: 9.0). Then, a 50 µl portion of the dissolved proteins was added to each well of a microtiter and left for 2 hours to coat the well. Triplicate wells were used for each sample. Then, the wells were washed twice with phosphate-buffered saline (PBS; Nissui Pharmaceutical Corporation Limited, Tokyo, Japan) and blocked with 5% bovine serum albumin (BSA) in PBS. After incubation at 4˚ C overnight, the wells were washed twice with PBS, and NoVLPs in 100 µl of PBS containing 5% BSA were applied to the wells. Plates were incubated at room temperature

for 1 hour and washed twice with PBS, and then mouse anti-NoVLP IgG in 100 µl of PBS containing 5% BSA was inoculated to each well. NoVLPs and anti-NoVLP IgG were kindly supplied by Dr. Takeda, National Institute of Infectious Diseases, Japan. The genotypes of NoVLPs used in this study were norovirus GI.1 (r124), GI.3 (r645) and GII.4 (r104). After incubation at room temperature for 1 hour, the wells were washed twice with PBS. Rabbit anti-mouse antibody modified by horseradish peroxydase (Funakoshi, Tokyo, Japan) was diluted in PBS containing 5% BSA, and 50 µl of the diluted antibody was added to each well. After incubation at room temperature for 1 hour and washing three times with PBS, secondary antibody bound was measured by coloring with o-phenylenediamine (P-7288, SIGMA CHEMICAL CO., ST. Louis, MO., USA) and H_2O_2 in citrate-phosphate buffer for 30 min. The coloring reaction was stopped with 2 M H_2SO_4. The absorbance at 492 nm was determined with a plate reader (Multiskan MS, Labsystems, Finland).

Determination of N-terminal amino acid sequences

Prior to the analysis of amino acid sequences, the isolated proteins were processed for 2D electrophoresis. First, proteins were purified with 2D Clean-Up Kit (GE Healthcare) and dissolved in a swelling buffer (8M urea, 2% CHAPS, 0.002% Bromophenol blue). Dissolved proteins were applied to isoelectric electrophoresis with the

Immobilite Drystrip pH 4-7 (GE Healthcare), and then denatured by a buffer containing sodium dodecyl sulfate (SDS). Denatured proteins were processed for polyacrylamide gel (12.5%) electrophoresis. After the 2D electrophoresis, proteins were transferred to a polyvinylidine difluoride filter with a protein blotting device and stained by Coomassie Brilliant Blue (CBB). CBB stained proteins were cut out from the filter, and N-terminal amino acid sequences of these proteins were analyzed with a peptide sequencer (Applied Biosystem). The protein-protein BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used for the homology search of the determined amino acid sequences against all protein sequences in the NCBI database.

RESULTS

Isolation of NoVBPs from activated sludge microorganisms

Fig. 2 shows the affinity chromatographic profile of crude proteins extracted from activated sludge microorganisms. The large absorbance peak between buffer volumes of 0 and 3 indicates that the vast majority of bacterial proteins were immediately washed out because they do not have the binding ability to the viral capsid peptide immobilized in the

affinity column. However, a small absorbance peak was obtained just after the injection of the elution buffer, which implies that a fraction of proteins was separated from the extracted bacterial proteins with an affinity to the noroviral capsid peptide. This peak was reproducibly obtained in all trials at buffer volumes between 10 and 12 mL, although the peak height was slightly decreasing, probably due to the deterioration of peptide ligand when the affinity column was used more than 20 times. These outcomes for the recovery of NoVBPs are similar to those for poliovirus-binding proteins (PoVBPs), in which one small peak was reproducibly obtained after the injection of the elution buffer (Sano et al., 2004).

The affinity chromatographic fraction, including the small peak after the injection of the elution buffer was collected, and the binding ability of the isolated proteins to noroviral particles were investigated with ELISA using NoVLPs. Fig. 3 shows that the signal/noise (S/N) ratio in the protein-immobilized well with NoVLP of norovirus GII.4 reached 8.55 (±1.38), indicating that NoVLP of norovirus GII.4 significantly adsorbed to the proteins isolated by the affinity chromatography. This result means that the recovered proteins include NoVBPs for norovirus GII.4. Meanwhile, S/N ratios in the adsorption of NoVBPs to NoVLPs of norovirus GI.1 and GI.3 were 3.18 (±0.27) and 3.03 (±0.15), respectively. These S/N ratios imply that NoVBPs bound to NoVLPs of norovirus GI

strains as well as that of GII.4. However, the statistically significant larger S/N value for GII.4 NoVLP compared to that for GI NoVLPs means that the binding affinity of recovered NoVBPs to norovirus GII.4 was stronger than to the norovirus GI strains. In the affinity ligand region, 5 or 6 out of 12 amino acid residues are different between norovirus GII.4 and GI strains (Fig. 1). It could be speculated that the conserved amino acid residues in this region have a significant role in the binding between NoVBPs and NoVLPs.

Determination of N-terminal amino acid sequences

The profile of 2D electrophoresis shows that the isolated proteins included a variety of proteins (Fig. 4). In order to identify proteins, 7 spots were randomly selected. The analysis of N-terminal amino acid sequences revealed that NoVBPs in spots 1 to 3 and 7 originated from chaperon protein GroEL (Table 1). It is well known that the chaperon protein GroEL captures denatured hydrophobic proteins (Bergonzelli et al., 2006). Since the C-terminal peptide of norovirus capsid protein immobilized in the affinity column has a relatively high content of hydrophobic amino acid (6 out of 12 residues), isolated proteins related to GroEL could be recovered by the hydrophobic interaction with norovirus capsid peptide.

The determined amino acid sequence of NoVBP in spot 1 was identical to that

spot 3. Furthermore, the N-terminal amino acid sequence of NoVBP in spot 2 was identical between 35th and 40th amino acid residues of NoVBP in spot 1. The relative molecular weights of these NoVBPs estimated by 2D electrophoresis (Fig. 4) are different from each other, which implies that NoVBPs in spots 1-3 were modified proteins translated from an identical gene related to GroEL.

NoVBPs in spots 4 and 5 were predicted and hypothetical proteins, respectively (Table 1). NoVBP in spot 6 riginated from riboflavin synthase, beta chain (ribH). The reason two predicted and hypothetical proteins and riboflavin synthase were recovered in the affinity chromatography has remained unclear. However, a hydrophobic interaction also could be the main factor for the recovery of riboflavin synthase, because it has been reported that the hydrophobic interaction has an important role in the formation of a homotrimer (Liao et al., 2001).

DISCUSSION

The peptide immobilized in the affinity column originated from Lordsdale virus belonging to norovirus GII.4, which is the dominant genotype of norovirus and easily

detected from fecal samples of non-bacterial gastroenteritis patients, domestic sewage and environmental water (Lee et al., 2007; Siebanga et al., 2007; Sano et al., 2006), and even from stool samples of asymptomatic persons (Ozawa et al., 2007). S/N ratios indicated in Fig. 3 clearly show that NoVBPs recovered by affinity chromatography have a strong affinity to NoVLP of norovirus GII.4. Our previous study suggests that NoVBPs obtained in this study have a binding affinity to the other strains of norovirus GII. In the previous study, PoVBPs isolated by the affinity to Poliovirus 1 mahoney exhibited the binding ability to a capsid peptide from Poliovirus 1 sabin, even though 2 out of 12 amino acid residues of the affinity ligand region are different from each other (Sano et al., 2004). Only one or two amino acid residues in the affinity ligand region were replaced between norovirus GII genotypes except Amsterdam strain (Fig. 1), which could allow NoVBPs recovered in this study to have a wide binding spectrum to norovirus GII strains. It would be of importance to analyze the binding specificity spectrum of NoVBPs with norovirus GII strains in a future study.

The chaperon protein GroEL was retrieved by the blast search for N-terminal amino acid sequences of proteins in spots 1 to 3 and 7 (Table 1). GroEL is known as a heat shock protein 60 (Hsp60) of *E. coli*, which is involved in the folding of denatured proteins and de novo proteins just after translation (Lesk, 2004). GroEL can interact with denatured

proteins that are exposing hydrophobic amino acid residues (Ewalt et al., 1997). GroEL has a relatively broad spectrum of binding, and thousands of proteins that have different amino acid sequences and conformations can be captured by GroEL (Kawata et al., 1994). Since the ligand region for the affinity chromatography (C-terminal region of norovirus capsid protein exposed to outside of noroviral particle) has 6 hydrophobic residues out of 12 amino acids, GroEL is likely to be recovered by the hydrophobic interaction with the ligand in the affinity column. Besides, this region of the norovirus capsid protein is being exposed to outside of norovirus particles as described above; therefore, the interaction of NoVLPs and NoVBPs shown in Fig. 3 could be partially explained by the contribution of GroEL proteins.

There are no conclusive results showing that proteins recovered in this study are involved in the movement of noroviruses in water environments. Since GroEL and riboflabin synthase are generally celluar proteins, it is impossible for these proteins to commit the adsorption of noroviruses to activated sludge microorganisms in their native state. However, these cellular proteins could be released from dead bacterial cells and could adsorb to noroviral particles. The physicochemical properties of the conjugate of noroviral

particles and these cellular proteins are totally different from that of the noroviral particle alone, which results in a different destination of noroviruses in water environments. Furthermore, extracellular GroEL conferring adhesive traits to bacteria has been reported (Frisk et al., 1998). Bergonzelli et al. reported that the GroEL protein of a gram-positive probiotic bacterium, *Lactobacillus johnsonni* La1, is surface-associated and is involved in the adsorption of this bacterium to host cells or the activities of immune regulation (Bergonzelli et al., 2006). Pantzar et al. reported that GroEL of a human pathogen, *Haemophilus ducreyi*, is surface-located and is responsible for the attachment of this bacterium to host cells (Pantzar et al., 2007). Extracellular GroEL may affect the movement of human pathogenic viruses, and it would be worth evaluating the commitment of these proteins to the norovirus transportation in water environments.

In this study, the peptide sequence originated from the capsid protein of norovirus GII was used as a ligand for the affinity isolation of NoVBPs, but there is a possibility that the three-dimensional morphology of the peptide immobilized in the affinity column is different from that of the norovirus particle, which could alter the composition of recovered proteins. The recovery of NoVBPs with the affinity to noroviurs particle (such as NoVLPs)

might give a new spectrum of recovered proteins, which should be the next challenge in a future study.

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Figure captions

Fig. 1. Multiple alignments of amino acid sequences from the carboxy-terminal region of the norovirus capsid protein. The sequence surrounded by the rectangle is the region that was used as the ligand in the affinity chromatography. Residues indicated by a gray box are amino acids different from that of the Lordsdale virus (norovirus genogroup II genotype 4).

Fig. 2. Affinity chromatographic profile of crude proteins extracted from activated sludge microorganisms. The carboxy-terminal region of the norovirus capsid protein was used as the ligand in the affinity chromatography. Arrow indicates the point at which the injection of the elution buffer was started.

Fig. 3. ELISA of the norovirus-binding ability of the recovered proteins by the affinity chromatography. Values of signal/noise ratio are the arithmetic mean of triplicate trials, and bars represent standard deviations.

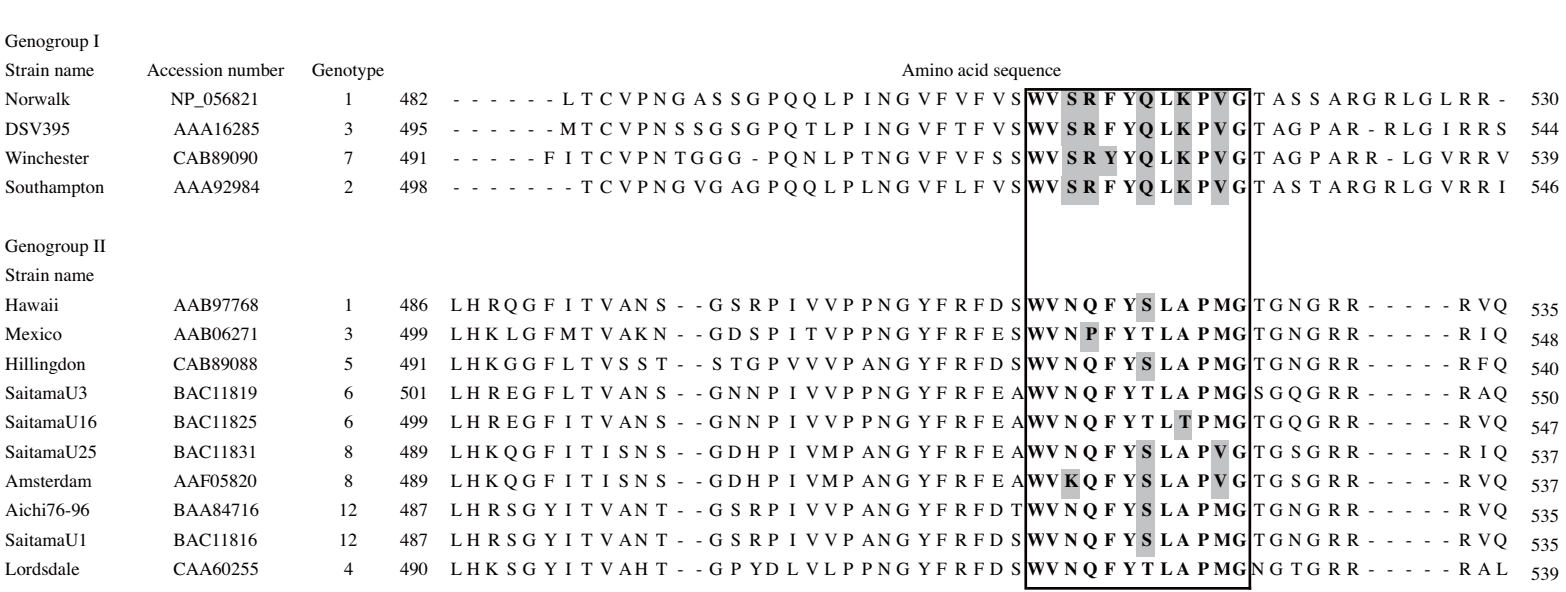
Fig. 4. Two-dimensional electrophoretic profile of the recovered proteins by affinity chromatography. The gel was stained by silver. N-terminal amino acid sequences of

proteins in spots indicated by circles were analyzed.

Table 1. N-terminal amino acid sequences of the isolated norovirus-binding proteins

Spot number	Sequence	Protein with the highest identity (accession number, identity (%))
Spot 1	AAKEVKFGNEARIKM LEGVNILADAVKVTL GPKGRNVVLD	chaperonin GroEL (YP_001143150.1, 100)
Spot 2	RNVVLDKSFGAPTITK DGVS	chaperone GroEL (ZP_06637554.1, 100)
Spot 3	AAKEVKFGNEARIKM LEGVNILADAVKVTLG PKGRNVVLD	chaperonin GroEL (YP_001143150.1, 100)
Spot 4	SMQFDLLDL	predicted protein (XP_002296287/1, 88)
Spot 5	QDAFSYAKGSIT	hypothetical protein ASA_3233 (YP_001142972.1, 83)
Spot 6	MKIIEGNIAAPEARVAI VVARFN	riboflavin synthase, beta chain (ribH) (YP_001140861.1, 95)
Spot 7	RAEAMDKVGRDDGV VIV	heat shock protein 60 family chaperone GroEL (ZP_06156329.1, 81)

Spot number corresponds to that in Fig. 3.


Genogroup I
Strain name
Accession number
Genotype
Amino acid sequence
Norwalk NP_056821 1 482 - - - - - - L T C V P N G A S S G P Q Q L P I N G V F V F V S W V S R F Y Q L K P V G T A S S A R G R L G L R R - 530
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Winchester CAB89090 7 491 - - - - - F I T C V P N T G G G - P Q N L P T N G V F V F S S W V S R Y Y Q L K P V G T A G P A R R - L G V R R V 539
Southampton AAA92984 2 498 - - - - - - - T C V P N G V G A G P Q Q L P L N G V F L F V S W V S R F Y Q L K P V G T A S T A R G R L G V R R I 546
Genogroup II
Strain name
Hawaii AAB97768 1 486 L H R Q G F I T V A N S - - G S R P I V V P P N G Y F R F D S W V N Q F Y S L A P M G T G N G R R - - - - - R V Q 535
Mexico AAB06271 3 499 L H K L G F M T V A K N - - G D S P I T V P P N G Y F R F E S W V N P F Y T L A P M G T G N G R R - - - - - R I Q 548
Hillingdon CAB89088 5 491 L H K G G F L T V S S T - - S T G P V V V P A N G Y F R F D S W V N Q F Y S L A P M G T G N G R R - - - - - R F Q 540
SaitamaU3 BAC11819 6 501 L H R E G F L T V A N S - - G N N P I V V P P N G Y F R F E A W V N Q F Y T L A P M G S G Q G R R - - - - - R A Q 550
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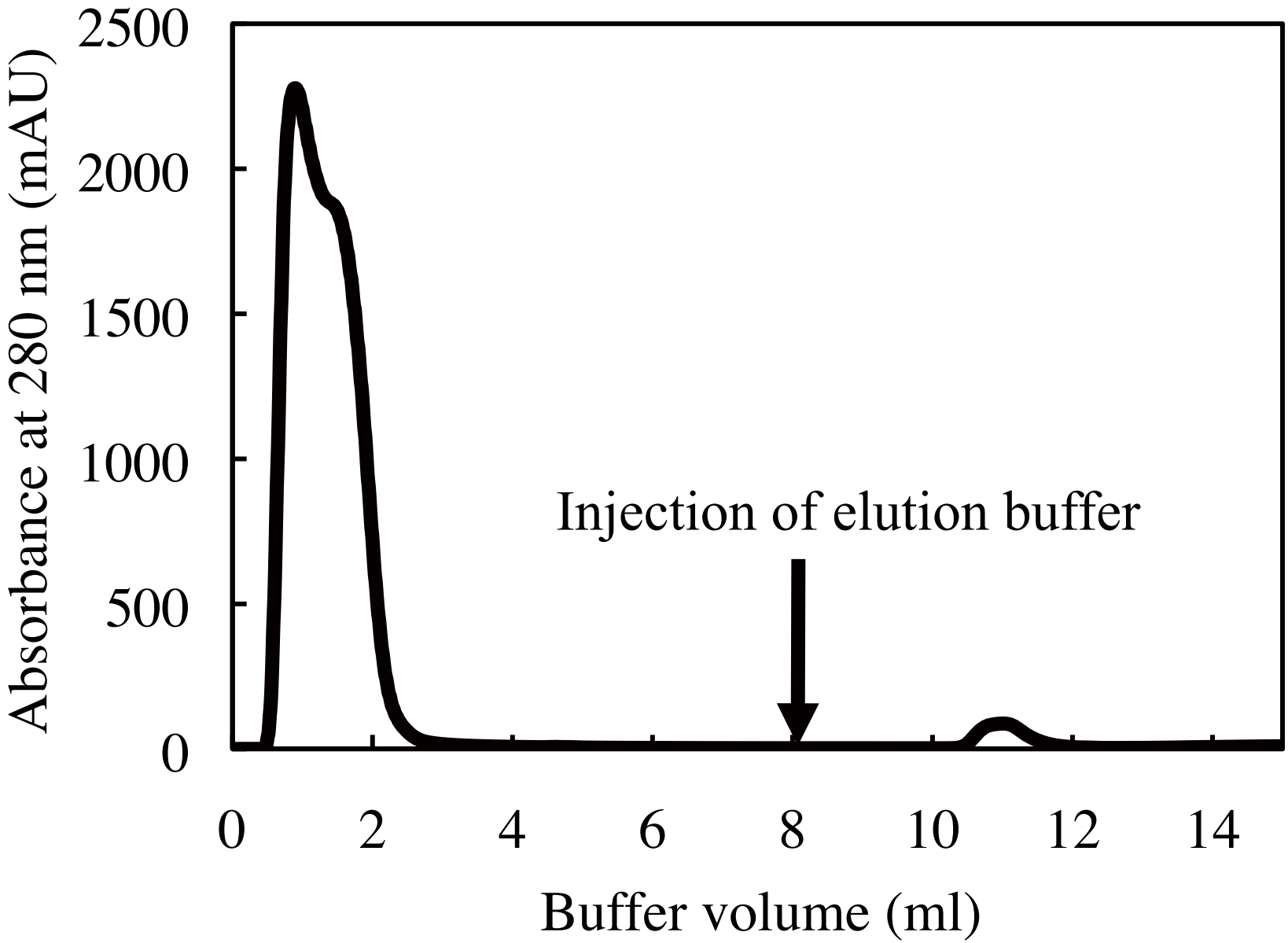
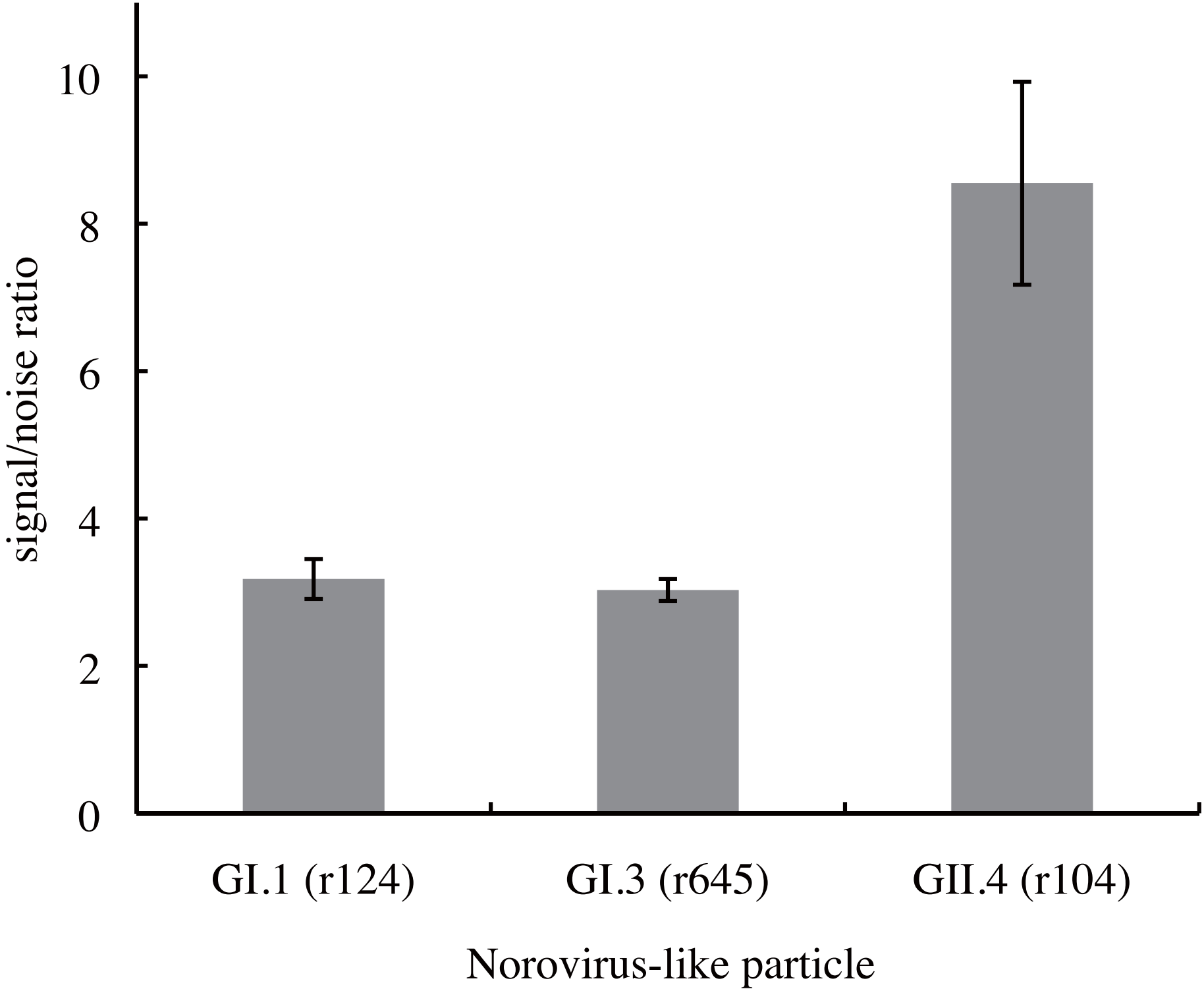
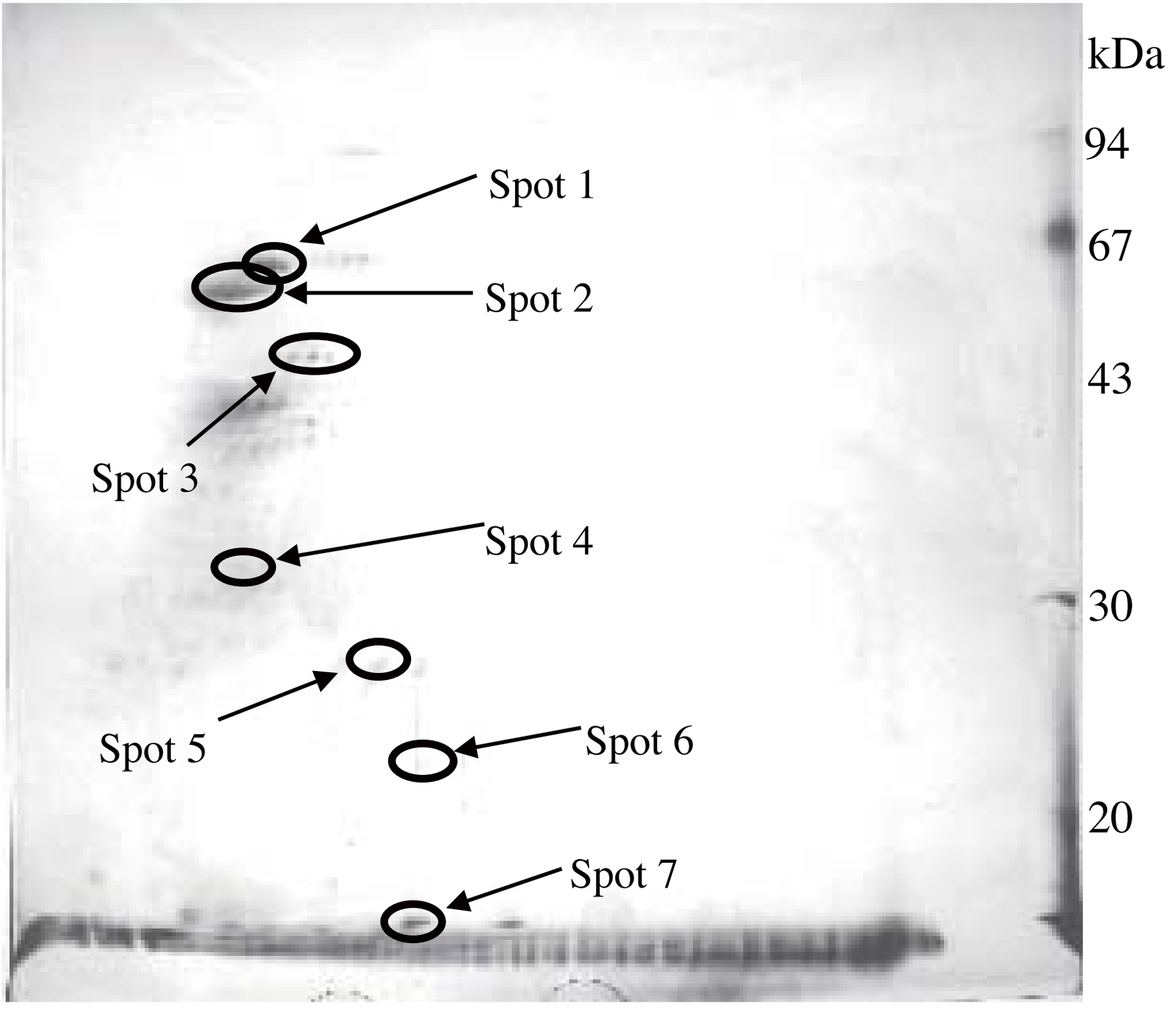

Absorbance at 280 nm (mAU)
2500
2000
1500
1000
500
0
Injection of elution buffer
0
2
4
6
8
10
12
14
Buffer volume (ml)


10
8
6
4
2
0
signal/noise ratio
GI.1 (r124)
GI.3 (r645)
GII.4 (r104)
Norovirus-like particle


kDa
94
67
43
30
20
Spot 1
Spot 2
Spot 3
Spot 4
Spot 5
Spot 6
Spot 7
3
4
5
6
7
pI