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Glycopeptide Derived from Hen Egg Ovomucin Has the Ability To Bind Enterohemorrhagic *Escherichia coli* O157:H7

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Ovomucin glycopeptide (OGP) was prepared by size exclusion chromatography after Pronase digestion of hen egg ovomucin, and the binding of OGP to foodborne pathogens (*Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enteritidis*, *Salmonella typhimurium*, and *Staphylococcus aureus*) was investigaed. Binding assays with biotinylated bacteria as probes in microtiter plates showed that OGP bound to only *E. coli* O157:H7 among these foodborne pathogens. Periodate treatment markedly reduced the binding ability, indicating that *E. coli* O157:H7 bound to carbohydrate moieties of OGP. Lectin blot analysis with *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA), which are specific for oligosaccharides containing sialic acid, revealed their binding sites in OGP were similar to the *E. coli* O157:H7 binding sites that were probed with biotinylated *E. coli* O157:H7 after Western blotting of OGP. Sialydase treatment of OGP abolished its ability to bind *E. coli* O157:H7, demonstrating that sialic acid played an important role in the binding. These results suggest that OGP has *E. coli* O157:H7-specific binding sites that consist of sialic acid. On the basis of these properties, OGP has the potential to be an ingredient with a protective effect against *E. coli* O157:H7 infection and to be a novel probe for the detection of *E. coli* O157:H7 in the food hygiene field.

KEYWORDS: Ovomucin; glycopeptide; foodborne illness; *Escherichia coli* O157:H7; sialic acid; bacterial adhesion

INTRODUCTION

In foodborne illnesses, the adhesion of bacteria to host tissues is the first step in the infection process. In many cases, the adhesion is mediated by interaction between lectins on the surface of bacteria and carbohydrates (glycoproteins or glycolipids) on the mucosal surface of the host (*1*). It is considered that oligosaccharides and glycoconjugates such as the analogues of carbohydrates on the mucosal surface would competitively inhibit the bacteria−carbohydrate adhesion on intestinal cells. Studies have shown that the carbohydrate components in food can have protective effects against foodborne pathogens. For instance, as for carbohydrate components in bovine and human milk, sialylated oligosaccharides suppressed the adhesion of *Helicobacter pyroli* (*2*) to gastrointestinal epithelial cells. Fucosyloligosaccharides blocked the adhesion of enteropathogenic *Escherichia coli* (EPEC) (*3*, *4*) or *Campyrobacter jejuni* (*4*) to Hep-2 cells. Mannosylated glycopeptides have been shown to inhibit the binding of enterohemorrhagic *Escherichia coli* (EHEC) to intestinal cells (*5*). As for the carbohydrate components in hen eggs, Sugita-Konishi et al. have reported that sialylglycopeptides and their derivatives from egg yolk strongly inhibited the binding of *Salmonella enteritidis* to Caco-2 cells and that oral administration of the components in mice prevented *Salmonella* infection (*6*). Thus, it is expected that carbohydrate components such as oligosaccharides and glycoconjugates in food would protect against foodborne illness by inhibiting the adhesion of pathogens to host cells. The carbohydrate components of food would not induce undesired phenomena such as the emergence of drug resistance bacteria due to the overuse of antibiotics. In addition, carbohydrate components would make

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a novel probe for the detection of pathogens if they show exclusive binding to specific bacteria.

Our aim was to find a new carbohydrate component in food with specific ability to bind foodborne pathogens and apply it as a protective agent against foodborne illness and a novel probe for the detection of foodborne pathogens. In this study, we selected hen egg ovomucin as the carbohydrate component for the following reasons: (1) Ovomucin is a highly glycosylated glycoprotein. It is gelatinous and accounts for ~1.5−3.5% of egg white proteins (*7, 8*). Ovomucin consists of a carbohydrate-poor subunit (α-ovomucin) that has ~11−15% carbohydrate and a carbohydrate-rich subunit (*β*-ovomucin) that has ~50−57% carbohydrate (*8*−*10*). (2) Ovomucin has various carbohydrates, including mannose (Man), galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetylneuraminic acid (NeuAc), fucose (Fuc) (*7*), and sulfated saccharides (*8*, *11*−*13*), and several carbohydrate structures in OGP have been reported (*11*−*14*). Hence, it was speculated that ovomucin would have multivalent ligands to pathogens and that the diversity of the carbohydrate would bring about an ability to bind various pathogens. (3) Although it has been reported that ovomucin and its derivatives had virus-binding ability (*15*−*17*), antitumor effects (*18*), and macrophage-stimulating activity (*19*), the ability of ovomucin to bind foodborne pathogens has not been examined. Finding a novel function of ovomucin would lead to effective utilization of the glycoprotein.

Ovomucin is usually prepared from egg white by dilution in distilled water and by acidification followed by washing with 2% KCl, but the ovomucin obtained using this method is insoluble in aqueous buffer (*20*). Because the ovomucin needed to be solubilized, we prepared ovomucin glycopeptide (OGP) by size exclusion chromatography after digesting ovomucin with Pronase. After that, we examined the ability of OGP to bind foodborne pathogens using an assay with biotinylated pathogens as probes in microtiter plates. After identifying the pathogen that binds to OGP, we characterized the binding sites. Our data show that OGP had the ability to bind *E. coli* O157:H7 and that sialic acid in OGP was involved in the binding.

MATERIALS AND METHODS

Preparation of Ovomucin. Ovomucin was prepared by referring to the method of Kato et al. (*20*) and Tsuge et al. (*15*). In brief, thick egg white was separated from whole egg white with a sieve. The thick egg white was homogenized for 5 min and diluted with distilled water (3-fold volume). After 1 h of stirring at 25 °C, the mixture was adjusted to pH 6.0 with 1 M HCl and then incubated at 4 °C overnight. The crude gel-like ovomucin recovered by centrifugation (12000 rpm, 4 °C, 20 min) was thoroughly washed with a 2% (w/v) KCl solution until contaminating proteins were removed in the washing solution. Confirmation that no contaminants were present was obtained by monitoring the absorbance of the washing solution at 280 nm. The ovomucin obtained was dialyzed against distilled water at 4 °C and then lyophilized.

Preparation of Ovomucin Glycopeptide. Ovomucin (1 g) dispersed into 200 mL of 0.1 M Tris-HCl buffer (pH 8.0) was incubated at 37 °C for 10 min, and then 40 mg of Pronase (Kaken Pharmaceuticals Co., Ltd., Tokyo, Japan) was added to the suspension. After 24 h of incubation at 37 °C, the mixture was boiled at 100 °C for 5 min to stop the enzymatic reaction and then centrifuged at 12000 rpm for 20 min at 4 °C to recover the supernatant. After the supernatant was dialyzed against distilled water at 4 °C and lyophilized, ovomucin hydrolysate was obtained. The ovomucin hydrolysate dissolved in distilled water was applied to a column of Sephadex G-50 (2.4 i.d. × 110 cm; Amersham Biosciences, Piscataway, NJ) equilibrated with distilled water and then eluted with distilled water at a flow rate of 1 mL/min. To detect protein and carbohydrate, absorbance was monitored at 280 and 490 nm after coloring using the phenol−sulfuric acid method (*21*), respectively. The fraction with the highest carbohydrate content and the lowest protein content was collected. After lyophilization of the fraction, OGP was obtained.

Sodium Dodecyl Sulfate−Polyacrylamide Electrophoresis (SDS-PAGE). Samples were boiled in SDS-PAGE loading buffer containing 2-mercaptoethanol (2-ME) for 5 min and then applied to a 12.5% SDS−polyacrylamide gel. SDS-PAGE was performed according to the method of Laemmli et al. (*22*). Protein and carbohydrate bands in the gel were stained with Coomassie Brilliant Blue (CBB) and Schiff reagents, respectively.

Periodate and Sialydase Treatments of Ovomucin Glycopeptide. Periodate treatment of OGP was performed by referring to the method of de Repentighy et al. (*23*). OGP at 5 mg/mL in 0.1 M acetate buffer containing 50 mM sodium periodate (pH 5.5) was incubated for 20 min at 25 °C at dark. After dialysis using distilled water at 4 °C and lyophilization, periodate-treated OGP was obtained.

In the case of sialydase treatment, OGP (10 mg) and sialydase (0.2 unit as a final concentration) from *Arthrobacter urefaclens* (Nacalai Tesque, Inc., Kyoto, Japan) were added to 1 mL of 0.1 M acetate buffer (pH 5.5). The solution was incubated at 37 °C for 48 h before being heated (100 °C, 10 min). After dialysis using distilled water at 4 °C and lyophilization, desialylated OGP (asiao-OGP) was obtained. The desialylation of OGP was confirmed using the periodate−resorcinol method (*24*), and its degree was ~100%.

Biotinylation of Bacteria. *Bacillus cereus* strain EKN 5196, *Clostridium perfringens* strain EKN 2955, *Lactobacillus casei* strain EKN 5700, *Listeria monocytogenes* strain EKN 4042, *Salmonella enteritidis* strain EKN 5785, *Salmonella typhimurium* strain EKN 5784, and *Staphylococcus aureus* strain EKN 5723 were provided by Eiken Kagaku Co., Ltd. (Tokyo, Japan). *E. coli* O157:H7 strain 212 was supplied by the National Institute of Infectious Diseases (Tokyo, Japan). All bacteria were subject to heat treatment and then suspended in phosphate-buffered saline (PBS). The suspension was adjusted to 1×10^{10} cells/10 mL (PBS) or to 1.0 (as absorbance at 660 nm) with PBS. After the addition of 10 mg of EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL), the suspension was incubated for 2 h on ice. The biotinylated bacterial suspension was washed three times with PBS and then resuspended in PBS (10 mL) or PBS (10 mL) containing 0.5 or 1.0 M NaCl. The biotinylated bacteria were stored at −80 °C prior to use.

Binding Assay with Biotinylated Bacteria. One milligram of OGP, periodate-treated OGP, asiao-OGP, or gelatin hydrolysate (as a negative control; Nippi, Inc., Tokyo, Japan) was dissolved in 1 mL of PBS, and each solution was serially diluted with PBS. The dilution (100 μL) was added to wells of a polystyrene microtitration plate (Maxisorp; Nunc, Roskilde, Denmark), and the plate was incubated at 4 °C overnight or at 25 °C for 2 h. After removal of the solution, each well was washed three times with PBS (200 μL/well). Next, 200 μL of 1% bovine serum albumin (BSA)/PBS was added to the wells, and the plate was incubated at 25 °C for 2 h before being washed. Biotinylated bacteria/PBS (100 μL) or biotinylated bacteria/PBS containing 0.5 or 1.0 M NaCl (100 μL) was added to each well, and the plate was incubated at 25 °C for 1 h. After further washing, alkaline phosphatase-labeled streptavidin (Zymed Laboratories, Inc., South San Francisco, CA) diluted 1000-fold with the blocking reagent was added to each well (100 μL). The plate was incubated at 25 °C for 1 h and then washed. Next, 100 μL of a 0.1% (w/v) sodium *p*-nitrophenyl phosphate disodium/diethanolamine hydrochloride buffer (pH 9.8) solution was added to each well, and the plate was incubated at 25 °C. A 5 M sodium hydroxide solution (20 μL) was added to each well to stop the reaction, and the absorbance at 405 nm was measured with a microplate reader (MPR-A4i; Tosoh Co., Tokyo, Japan).

Chemical Compositions of Ovomucin Glycopeptide. The monosaccharide components (Fuc, Gal, GalNAc, GlcNAc, Man, and NeuAc) of OGP were measured with an ABEE labeling kit plus S (Honen Co., Tokyo, Japan) and a Honenpak C18 column (Honen, Co., Tokyo, Japan) according to the manufacturer's directions. The amount of protein in OGP was measured with Micro BCA protein assay reagent (Pierce,

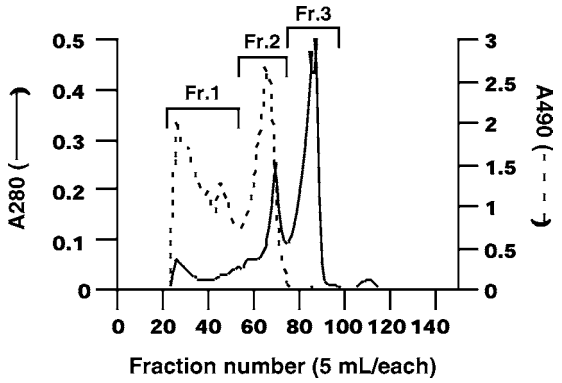

Figure 1. Size exclusion chromatography of Pronase-digested ovomucin. Conditions are as follows: column, Sephadex G-50 (2.4 i.d. × 110 cm); flow rate, 1 mL/min; eluent, distilled water. To detect the protein and carbohydrate, the absorbance was monitored at 280 nm (solid line) and 490 nm (dashed line) after coloring using the phenol–sulfuric acid method, respectively.

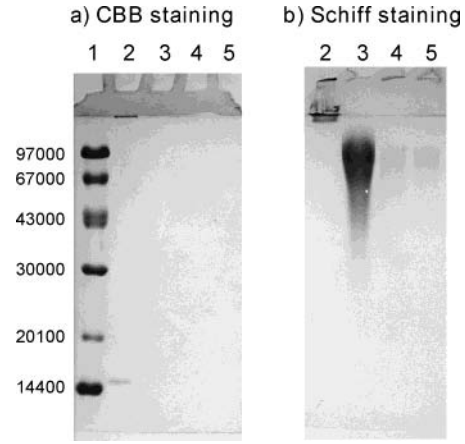

Figure 2. SDS-PAGE of each fraction after size exclusion chromatography of Pronase-digested ovomucin. The gels were stained with CBB (**a**) and Schiff reagents (**b**). Lanes 1, 2, 3, 4, and 5 are the molecular weight marker, ovomucin, fraction 1, fraction 2, and fraction 3, respectively.

Rockford, IL), in which BSA was used for preparing a standard curve, according to the manufacturer's instructions.

Lectin Blot Analysis and Probing of *E. coli* O157:H7 Binding Sites of Ovomucin Glycopeptide. Biotinylated *Ricinus communis* agglutinin (RCA) and peanut agglutinin (PNA) were purchased from Vector Laboratories, Inc. (Burlingame, CA). Biotinylated *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) were purchased from EY Laboratories, Inc. (San Mateo, CA). The binding sites in OGP were probed with biotinylated *E. coli* O157:H7 by referring to the method of de Repentighy et al. (*23*). OGP boiled in SDS-PAGE loading buffer containing 2-ME for 5 min was subjected to SDS-PAGE and then transferred (100 μg) onto a membrane (Immobilon-P; Millipore Billerica, MA). The membrane was incubated at 25 °C for 1 h with a blocking reagent and then washed three times with a washing buffer. The blocking reagents used for lectin blot analysis and the probing of *E. coli* O157:H7 binding sites were 1% (w/v) BSA/PBS and Blocking reagent for ELISA (Roche Molecular Biochemicals, Basel, Switzerland), respectively. The washing buffers used for the lectin blot analysis and the probing of *E. coli* O157:H7 binding sites were PBS containing 0.05% Tween 20 (PBST) and PBS, respectively. In the lection blot analysis, the membrane was incubated at 25 °C for 1 h with a biotinylated lectin/PBST solution (5−20 μg/mL). In the probing of *E. coli* O157:H7 binding sites, the membrane was incubated at 4 °C overnight with a biotinylated *E. coli* O157:H7/PBS suspension (1×10^9 cells/mL) without shaking. After being washed and then incubated at 25 °C for 1 h with alkaline phosphatase-labeled streptavidin (Zymed Laboratories, Inc.) diluted 1000-fold with each blocking reagent, the membrane was washed three times and subsequently once with Milli-Q water. To visualize the lectin binding sites and *E. coli* O157:H7 binding sites in OGP, the membrane was incubated with Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI).

RESULTS AND DISCUSSION

Ovomucin prepared from hen egg white was digested with Pronase and subjected to size exclusion chromatography. **Figure 1** shows the chromatographic pattern of the Pronase-digested ovomucin. Three peaks were observed. Fractions 1 and 2 had both protein and carbohydrate components, whereas fraction 3 was mainly composed of protein. The yields of fractions 1, 2, and 3 were 21.2, 12.8, and 2.4%, respectively. The three fractions and ovomucin were subjected to SDS-PAGE, and then the gel was stained with CBB and Schiff reagents (**Figure 2**). In the case of CBB staining (**Figure 2a**), the band between the stacking gel and the separating gel and a band of ∼14.4 kDa in ovomucin were stained (**Figure 2a**, lane 2). The band of 14.4 kDa was considered to be a contaminant, lysozyme, as described by Itoh et al. (*10*). Although ovomucin was thoroughly washed with 2% KCl, a small amount of lysozyme remained. However, we used this preparation because lysozyme is not a glycoprotein. On the other hand, the three fractions of Pronase-digested ovomucin were not stained with CBB (**Figure 2a**, lanes 3−5). Using the Schiff reagent (**Figure 2b**, lane 2), the band in the stacking gel was broad (**Figure 2b**). As for the three fractions, fraction 1 was stained from ∼40 to ∼100 kDa, whereas fractions 2 and 3 were weakly stained (**Figure 2b**, lanes 3−5). The glycopeptide content of fraction 1 was considered to be greater than that of fraction 2 or 3. Hence, fraction 1 was the ovomucin glycopeptide (OGP) and used in subsequent experiments.

Next, to investigate the ability of OGP to bind various foodborne pathogens, we established a binding assay with biotinylated pathogens as probes using microtiter plates. In this assay, OGP immobilized on the solid phase of microtiter plates reacts with biotinylated foodborne pathogens and then the binding is visualized with enzyme-labeled streptavidin. The assay is simple and able to detect the binding of OGP to several pathogens at a time. The binding of OGP to the solid phase of the microtiter plates was confirmed by using the method of Steinitz and Baraz (*25*) (data not shown). As representative foodborne pathogens, we used *B. cereus*, *C. perfringens*, *L. monocytogenes*, *S. typhimurium*, *S. enteritidis*, *St. aureus*, and *E. coli* O157:H7. It was reported that dead and live bacteria adhered equally to human intestinal cells and that the dead bacteria not only competed with the adhesion but also interfered with the invasion of live bacteria (*26*). In addition, it was shown that when dead EPEC and *Citrobacter rodentium* were administered to mice, the dead bacteria recapitulated the immunopathology (elicitation of a strong Th1 response, mucosal thickening, and crypt cell hyperplasia) observed in the live infection (*27*). Therefore, we used dead bacteria in this study. **Figure 3** shows the binding of OGP to foodborne pathogens. *B. cereus* (**Figure 3a**), *C. perfringens* (**Figure 3b**), *L. monocytogenes* (**Figure 3c**), *S. typhimurium* (**Figure 3d**), *S. enteritidis* (**Figure 3e**), and *St. aureus* (**Figure 3f**) did not bind to OGP or gelatin hydrolysate (as a negative control) up to a concentration of 10^2 μg/well. In addition, *L. casei*, representative of the useful bacteria in the human intestinal tract, also showed no binding to OGP (**Figure 3g**). By contrast, in *E. coli* O157:H7 (**Figure 3h**), the signal at 405 nm increased as the concentration of OGP, but not gelatin hydrolysate, increased. This increase in the signal was not due to nonspecific binding between OGP on the solid phase of the plates and the alkalinephosphatase-labeled strepta-

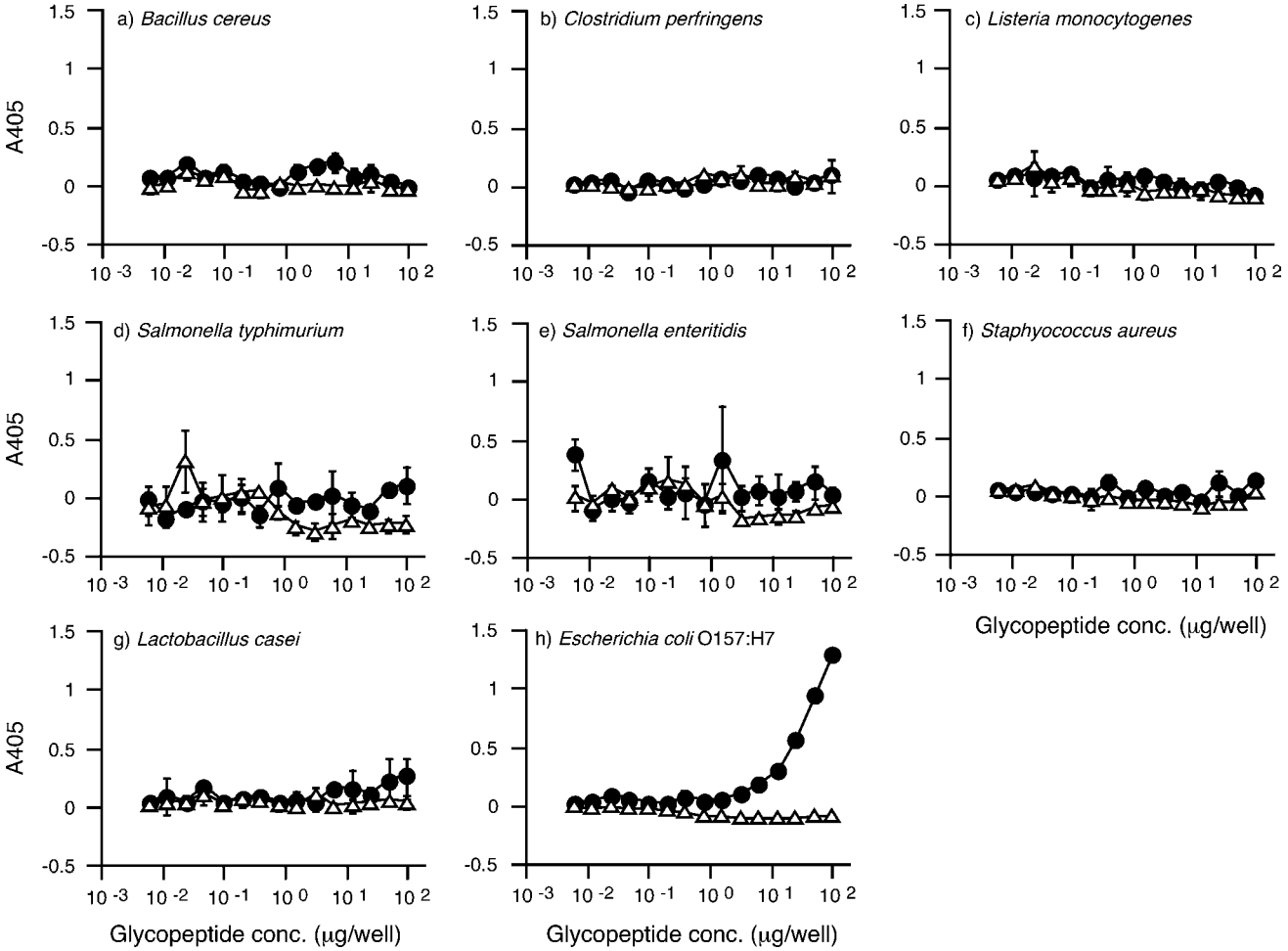

Figure 3. Binding of ovomucin glycopeptide to foodborne pathogens. The binding assay was performed as described under Materials and Methods. Bacteria used are *B. cereus* (**a**), *C. perfringens* (**b**), *L. monocytogenes* (**c**), *S. typhimurium* (**d**), *S. enteritidis* (**e**), *St. aureus* (**f**), *L. casei* (**g**), and *E. coli* O157:H7 (**h**). Each value against gelatin hydrolysate (△) (as a negative control) and OGP (●) is expressed as the mean absorbance at 405 nm and standard deviation of triplicate wells after subtraction of the background values.

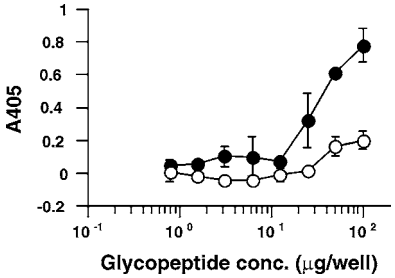

Figure 4. Effect of periodate treatment on the binding of ovomucin glycopeptide to *E. coli* O157:H7. The binding assay with native OGP (●) and periodate-treated OGP (○) was performed as described under Materials and Methods.

vidin, as was confirmed in a binding assay with PBS instead of biotinylated *E. coli* O157:H7 (data not shown). These results indicate that OGP bound exclusively to *E. coli* O157:H7 among the foodborne pathogens tested.

To clarify whether the carbohydrate moieties in OGP are involved in the binding to *E. coli* O157:H7 or not, a binding assay with periodate-treated OGP, which has a disrupted carbohydrate structure, was performed. As shown in **Figure 4**, the periodate-treated OGP had a markedly reduced ability to bind *E. coli* O157:H7 as compared with the native OGP, which indicates that the carbohydrate moieties play an important role in the binding to *E. coli* O157:H7.

Table 1. Chemical Composition of Ovomucin Glycopeptide[a]

Gal	GalNAc	GlcNAc	Man	NeuAc	Fuc	protein
23.6	11.2	17.4	0.9	33.8	0	9.8

[a] All values are percent weight.

Because the carbohydrate moieties in OGP were important for the binding of OGP to *E. coli* O157:H7, the carbohydrate composition of OGP was measured (**Table 1**). In terms of monosaccharides, OGP contained 23.6% Gal, 11.2% GalNAc, 17.4% GlcNAc, 0.9% Man, 33.8% NeuAc, and 0% Fuc as percent weight. The protein content of OGP was 9.8%. The carbohydrate and protein contents were similar to those of the main fraction after size exclusion chromatography of Pronase-digested ovomucin reported by Tsuge et al. (*15*): that is, 35.3, 11.4, 18.5%, 0, 28.4, and 6.4% for Gal, GalNAc, GlcNAc, Man, NeuAc, and protein, respectively. In the experiments on the binding of OGP to various foodborne pathogens (**Figure 3**), *S. typhimurium*, *S. enteritidis*, and *L. casei* did not bind to OGP. The small amount of mannose in OGP would indicate the inability to bind *S. enteritidis* and *S. typhimurium*, which recognize mannose in adhesion events (*6*, *28*). As for *L. casei*, because OGP was rich in sialic acid, the inability to bind may be explained by the finding that *L. casei* did not bind to GM1, a glycolipid containing sialic acid, but to desialylated GM1 (*29*).

Next, to identify the carbohydrates in OGP recognized by *E.*

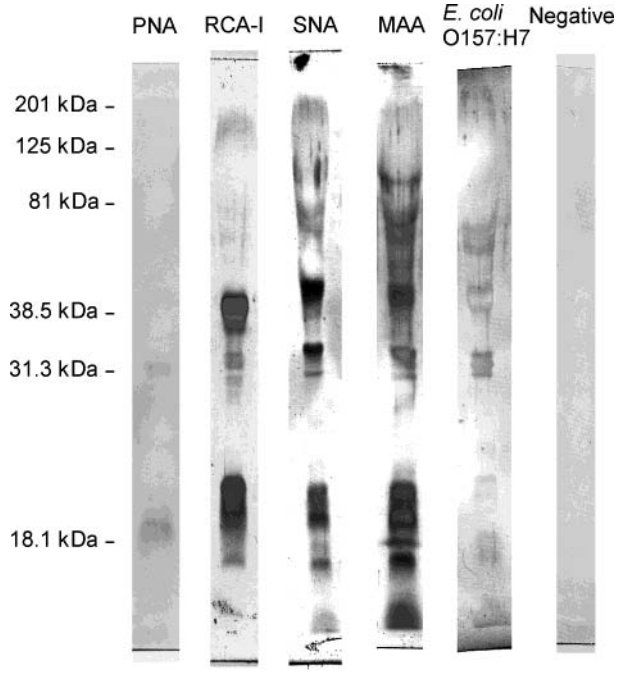

Figure 5. Lectin blot analysis and *E. coli* O157:H7 binding sites of ovomucin glycopeptide. After SDS-PAGE, OGP (100 μg) was transferred onto an Immobilon-P membrane, and lectin staining of OGP and probing of *E. coli* O157:H7 binding sites in OGP were performed with biotinylated lectins and biotinylated *E. coli* O157:H7, respectively, as described under Materials and Methods. The carbohydrate recognized by the lectins used is as follows: PNA, Galβ1–3GalNAc; RCA-I, Galβ1–4GlcNAc; MAA, NeuAcα2–3Gal; SNA, NeuAcα2–6Gal/GalNAc. The negative control indicates the result of an assay with PBS instead of adding biotinylated lectins and biotinylated *E. coli* O157:H7.

coli O157:H7, the lectin staining sites and *E. coli* O157:H7 binding sites of OGP were investigated. Because OGP had only a small amount of mannose (**Table 1**), it was considered to have mainly O-linked carbohydrates. The carbohydrate structures in ovomucin have been reported to be Galβ1−4GlcNAc-β1−6-[Galβ1−3]-GalNAc, NeuAcα2−3Galβ1−3GalNAc, NeuAcα2−3Galβ1−3GalNAc-6-SO_3H, Galβ1−4GlcNAc-6-SO_3H-β1−6-[Galβ1−3]-GalNAc, NeuAcα2−3Galβ1−3-[Galβ1−4GlcNAc-6-SO_3H-β1−6]-GalNAc, NeuAcα2−3Galβ1−3-[GlcNAcβ1−6]-GalNAc, and Galβ1−3-[NeuAcα2−6]-GalNAc (*11*−*14*). Therefore, OGP was reacted with lectins specific for these carbohydrates in O-linked glycan (**Figure 5**). In the lectin blot analysis, unlike the Schiff staining in SDS-PAGE (**Figure 2b**), several bands were observed below ∼40 kDa. These bands were not stained with CBB and Schiff reagents. On the other hand, the broad band beyond 40 kDa that was stained with Schiff reagent was faint after the lectin staining. This was because OGP was not completely immobilized onto the membrane owing to its high carbohydrate content. Thus, it was considered that the bands below ∼40 kDa were strongly stained in comparison. PNA that recognizes Galβ1−3GalNAc reacted very weakly with the bands below 40 kDa in OGP. On the other hand, RCA-I, MAA, and SNA, which are specific for Galβ1−4GlcNAc, NeuAcα2−3Gal, and NeuAcα2−6Gal/GalNAc, respectively, recognized bands above as well as below 40 kDa. Next, the binding sites in OGP were probed with biotinylated *E. coli* O157:H7. The *E. coli* O157:H7 binding sites were similar to the sites recognized by SNA and MAA. Hence, it was suggested that *E. coli* O157:H7 bound to NeuAcα2−3Gal and NeuAcα2−6Gal/GalNAc in OGP.

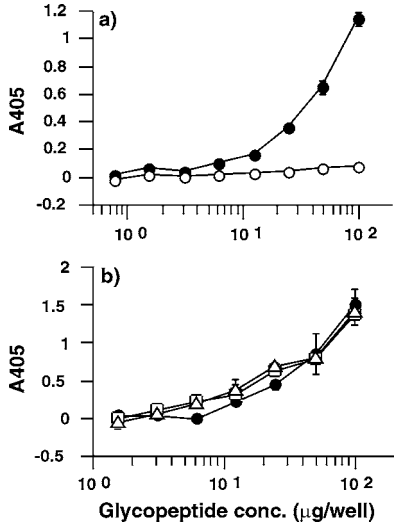

Figure 6. Effect of sialic acid on the binding of ovomucin glycopeptide to *E. coli* O157:H7: (**a**) the binding assay with native OGP (●) and sialydase-treated OGP (○) was performed as described under Materials and Methods: (**b**) the binding assay with biotinylated *E. coli* O157:H7 suspended in normal PBS (●), PBS containing 0.5 M NaCl (□), and PBS containing 1.0 M NaCl (△) was performed as described under Materials and Methods.

On the basis of the results of the lectin blot analysis, we investigated the influence of sialic acid in OGP on the binding to *E. coli* O157:H7 (**Figure 6**). The binding assay was performed with sialydase-treated OGP (asiao-OGP) and biotinylated *E. coli* O157:H7 (**Figure 6a**). Sialydase treatment abolished the ability to bind *E. coli* O157:H7. This result indicates that sialic acid plays an important role in the binding between OGP and *E. coli* O157:H7. In an assay with *E. coli* O157:H7 and group O erythrocytes, sialic acid as well as mannose was reported to have an inhibitory effect on the hemagglutinating activity of *E. coli* O157:H7 (*30*). In contrast, *E. coli* O157:H7 was shown to bind Gb4, which is the desialylated form of GM1, in a binding assay with radiolabeled *E. coli* O157:H7 strain CL56 (*31*). The difference between this result and ours may be due to the strain of *E. coli* O157:H7 because the inhibitory effect of carbohydrate on the binding to intestinal cells was not consistent among O157:H7 strains (*32*). As a putative adhesin of EHEC, fimbriae and outer membrane proteins have been reported, but most studies have focused on intimin, which is an outer membrane protein (*33*). EHEC and EPEC, which cause attaching and effacing (A/E) lesions, translocate the intimin receptor, Tir, into the membrane of mammalian cells using the type III secretion system, and then the bacteria−cell adhesion is mediated by the intimin−Tir interaction (*34*). Luo et al. (*34*) have shown that the tip of the intimin of EPEC was positively charged, on the basis of a crystal structural analysis, and suggested that the intimin binds to an anionic sialylated cellular mask. To investigate this possibility, the binding of OGP to *E. coli* O157:H7 was measured in PBS containing high concentrations of NaCl (**Figure 6b**). However, the binding in PBS containing 0.5 or 1.0 M NaCl was similar to that in normal PBS (∼0.14 M NaCl), which indicates that the interaction of *E. coli* O157:H7 to OGP was not electrostatic. Indeed, if electrostatic interaction was involved in the binding, asiao-OGP would retain some binding ability because ovomucin has sulfated saccharides (*8*, *11*−*13*), which have a negative charge. Hence, it is suggested that *E. coli* O157:H7 recognizes sialic acid in OGP via a lectin-like interaction. The site in intimin involved in the intimin−Tir interaction has a C-type lectin-like domain (*34*). Whether

this domain recognizes carbohydrates on host cells or not has been debated (*34*−*36*), but the possibility cannot be ruled out. There is also possibility that fimbriae of *E. coli* O157:H7 are involved in the binding to OGP. It was reported that LP fimbriae, which resemble the *E. coli* type 1 fimbriae, were distributed peritrichously on *E. coli* O157:H7, suggesting that the LP fimbriae participate in the adherence of *E. coli* O157:H7 to eukaryotic cells and play a role in microcolony formation (*37*). Such fimbriae may recognize the sialic acid in OGP in a lectin-like manner similar to the type 1 fimbriae and S fimbriae of *E. coli*, which recognize mannose and NeuAcα2−3Galβ1−3GalNac, respectively (*1*, *38*).

In conclusion, we have demonstrated that OGP obtained through size exclusion chromatography of Pronase-digested hen egg ovomucin bound exclusively to *E. coli* O157:H7 among foodborne pathogens tested and that the sialic acid in OGP played an important role in the binding. The results of the present study show that the glycopeptide derived from hen egg ovomucin has a novel function, the binding of *E. coli* O157:H7. In addition, the identification of the *E. coli* O157:H7 binding sites in OGP is important for the practical use of OGP in the future. OGP has the potential to provide protection against *E. coli* O157:H7 infection and to be a novel probe for the detection of *E. coli* O157:H7 in the food hygiene field. Further studies focusing on such aspects are in progress.

ABBREVIATIONS USED

BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; ELISA, enzyme-linked immunosorbent assay; EPEC, enteropathogenic *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; 2-ME, 2-mercaptoethanol; OGP, ovomucin glycopeptide; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide electrophoresis; Fuc, fucose; Gal, galactose; Man, mannose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; NeuAc, *N*-acetylneuraminic acid; MAA, *Maackia amurensis;* PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin; SNA, *Sambucus nigra*.

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