Analysis of cereal extracts as conditioning solutes to suppress the initial attachment of *Escherichia coli* to abiotic surfaces

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Abstract We examined the initial attachment of *E. coli* to abiotic surfaces conditioned with cereal extracts. The extracts were water-soluble fractions prepared from flours of barley, quinoa, rice and wheat. Strains used were *E. coli* ATCC 8739, *E. coli* NBRC 3301, *E. coli* NBRC 3302, *E. coli* NBRC 13168, *E. coli* NBRC 13891, and *E. coli* O157:H7 sakai. When surfaces of glass and stainless steel were conditioned at 25°C for 30 min with 0.5% cereal extracts, significantly lower numbers of *E. coli* cells attached to the conditioned surfaces than unconditioned ones, irrespective of strains used. The highest activity in reduction of the number of *E. coli* cells attached to the abiotic surfaces was found in the wheat extract. The suppressive activity was stable after treatments of the extract by autoclave and enzymatic digestion with α-amylase and Proteinase K. We purified the active compound by ammonium sulfate fractionation and gel filtration with HiPrep 16/60 Sephacryl S-200 HR after the enzymatic treatments. The purified compound showed an average molecular mass of about 300 kDa by light-scattering measurements. Analyses of its components indicated that the active compound was arabinoxylan; the molar ratios were 1.0 (arabinose) to 2.46 (xylose). Commercially available arabinoxylan (average molecular mass: 370 kDa) also showed the similar

activity. To our knowledge, this is the first report on a dietary fiber from cereals which suppresses the initial attachment of *E. coli* to abiotic surfaces.

Keywords Cereals, *Escherichia coli*, Attachment, Abiotic materials

Introduction

Cooking utensils and equipment of food factory are often contaminated with microbes. Although these surfaces are carefully washed and sterilized with disinfectants, bacteria attached to the surfaces sometimes survive on the surfaces to cause the secondary contamination of foods. *Escherichia coli* is an important species for judging hygienic status and contains pathogenic strains. Several reports have been published on attachment of *E. coli* to stainless steel surfaces [1, 2], probably because this is the most common material used in food processing environments. Some reports have been published on attachment of *E. coli* to polystyrene, glass, and Teflon [3-5]. Attachment of bacteria to abiotic surfaces is reported to start with the surface conditioning with surrounding molecules, since these molecules will diffuse faster than bacterial cells due to their reduced size [6]. Whitehead and Verran [7] also reported that bacteria would become attached, adhered and then retained on abiotic surfaces after conditioning of the surfaces. Thus, bacteria probably attach to the conditioned surfaces of cooking utensils and equipment of food factory.

There are several reports on conditioning materials that affect the attachment of bacteria. Initial adhesion of *Burkholderia cepacia* onto glass surfaces was reported to increase by alginate and other organic materials, but not by bovine serum albumin [8]. Ribeiro et al. [9] observed higher adhesion of *Bacillus cereus* spores to a stainless steel surface conditioned with whole milk than the unconditioned one. Aqueous cod muscle extract was reported to suppress the adhesion of *Pseudomonas fluorescens* to a stainless steel [10]. Adsorption of alginate and albumin onto aluminum coatings was reported to inhibit adhesion of *Escherichia coli* [11]. We also reported that beef tallow inhibited the attachment of *E. coli* O157 cells to surfaces of stainless steel, titanium, glass, aluminum, and several plastics [12]. However, there is no report on cereal components that influence the attachment of *E. coli* to abiotic surfaces as conditioning materials, to the best of our knowledge.

Since cereals are staple foods and widely used in food industry and home cooking, many cooking utensils and equipment of food factory are exposed to cereal components as conditioning materials. We here describe the effects of cereal extracts on the initial attachment of *E. coli* to abiotic surfaces.

Materials and Methods

Materials

Cereal grains of barley (Codex code: GC 0640), quinoa (GC 0648), rice (GC 0649), and wheat (GC 2086) were purchased from a food store in Tokushima, Japan. Millser-620DG was obtained from Iwatani Co. (Tokyo, Japan). α-Amylase from *Baccillus licheniformis* (type XII-A, 25.1 mg protein/ml, 932 units/mg protein, Sigma-Aldrich) and Proteinase K (30 mAnson-U/mg, Merck KGaA, Damstadt, Germany) were obtained from Nakalai Tesque, Kyoto, Japan. Arabinoxylan (wheat flour; high viscosity, average molecular mass: 370 kDa) was obtained from Megazyme Ltd., Ireland). Arabinogalactan was obtained from Tokyo chemical industry Co., Ltd, Tokyo, Japan. Pectin, xylan, inulin, amylose, and mannan were obtained from Nakalai Tesque, Kyoto, Japan. Amicon Ultra-15 (MWCO: 10 kDa) was obtained from Merck Millipore (Billerica, Mass., USA). Millex GV filter (0.22 μm pore size) was obtained from Millipore Japan Co., Tokyo, Japan. HiPrep 16/60 Sephacryl S-200 HR column was obtained from GE Healthcare (Buckinghamshire, UK). A trimethylsilylating reagent, *N*,*O*-bis(trimethylsilyl) acetamide (BSA) + trimethylchlorosilane (TMCS) + *N*-trimethylsilyimidazole (TMSI), 3:2:3, was obtained from Supelco (Bellefonte, PA., USA). Trace Gold TG-1MS GC column (30 m × 0.25 mm × 0.25 μm) was obtained from Thermo Fisher Scientific (Waltham, MA., USA).

Strains and culture conditions

We used six strains of *E. coli* [*E. coli* ATCC 8739 (Japanese pharmacopeia control), *E. coli* K12 NBRC 3301, *E. coli* W-1485 NBRC 3302, *E. coli* B NBRC 13168, *E. coli* NBRC 13891, and *E. coli* O157:H7 sakai], and five strains of other genera (*Aeromonas hydrophila* subsp. *hydrophila* NBRC 13286, *Brevundimonas diminuta* NBRC 14213, *Pseudomonas aeruginosa* NBRC 13275,

Pseudomonas fluorescens NBRC 14160, and *Staphylococcus aureus* subsp. *aureus* NBRC 12732).

E. coli strains and *S. aureus* subsp. *aureus* NBRC 12732 were aerobically cultivated at 37°C to the stationary phase with Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1 mM NaOH). Other strains were aerobically cultivated at 30°C with LB medium. The growth was analyzed by measuring the absorbance at 660 nm or using the standard plate count method with LB agar medium.

Preparation of cereal extracts

Cereal grains of barley, quinoa, rice, and wheat were milled at 20,000 rpm for 1 min with Millser-620DG. The cereal flours obtained were suspended in distilled water (6 g flour/30 ml), and vigorously shaken at 25°C for 30 min. Each supernatant solution obtained by centrifugation at 10,000*g* for 15 min was filtered through a Millex GV filter, and used as 20% cereal extract.

Abiotic materials

Petri dishes made of glass and stainless steel (type: SUS304, no. 4 finish) were obtained from AGC Techno Glass (Shizuoka, Japan) and AsOne Co. (Osaka, Japan), respectively. All undersurfaces of these petri dishes were washed with a neutral detergent, rinsed with distilled water, and sterilized by autoclave.

Attachment assay of *E. coli* to abiotic surfaces

Attachment assay was performed as described previously [12]. Undersurfaces of petri dishes (ϕ 90 mm) were conditioned with 0.5% cereal extracts (15 ml), unless otherwise indicated, at 25˚C for 30 min, and washed five-times with sterilized water. *E. coli* cells grown to the stationary phase (OD660 = about 2.8) were harvested by centrifugation and suspended in 0.85% NaCl at the cell density of about 1000 cells/ml. The cell suspension (15 ml) was poured onto the conditioned and unconditioned undersurfaces, and incubated at 25˚C for 30 min with swirling at 5 min interval according to the method of Rivas and others [13]. After removal of the cell suspensions from petri dishes, the undersurfaces were gently washed five-times with 0.85% NaCl. EMB (eosin-methylene blue) agar

medium (20 ml) incubated at 48˚C was poured on the undersurfaces, and solidified at room temperature. After cultivation at 37˚C for 48 h, colonies formed between the undersurfaces and agar medium were enumerated. When we used strains of other genera, the cells grown to the stationary phase (OD660 = 2.5 to 2.8) were similarly used.

Purification of an active compound from the wheat flour extract

α-Amylase (1 mg) was added to 100 ml of 20% wheat flour extract, and incubated at 80˚C for 8 h. Next, Proteinase K (5 mg) was added to the extract, and incubated at 37˚C for 5 h. After these enzymatic treatments, the solution was dialyzed at 4˚C for 24 h against 10 L distilled water. The inner solution was subjected to ammonium sulfate fractionation according to the method of Izydorczyk and Biliaderis [14]. Precipitates formed at 65%, 65-75%, and 75-100% saturation of ammonium sulfate were collected by centrifugation, dissolved in 20 ml distilled water, and dialyzed at 4˚C for 24 h against 2 L distilled water. After the dialysis, each inner solution was freeze-dried. A freeze-dried sample was dissolved in 10 ml H_2O, and subjected to gel filtration with a HiPrep 16/60 Sephacryl S-200 HR column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. Fractions that showed the suppressive activity against attachment of *E. coli* to glass surfaces were combined, dialyzed against distilled water, and freeze-dried.

Components of a suppressive compound against attachment of *E. coli* to abiotic surfaces

Sugar composition was determined as follows. An active compound was hydrolyzed with 2M trifluoroacetic acid (TFA) at 100˚C for 4 h. After removal of TFA under N_2 gas, the hydrolysate was dried up under reduced pressure. The dried sample (1 mg) was incubated at 70˚C for 1 h with 0.1 ml of (BSA + TMCS + TMSI, 3:2:3) and then analyzed with GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) and Trace Gold TG-1MS GC column. Identification and quantification of sugars were made with NIST 14 Mass Spectral Library ver. 1.0 and GCMSsolution ver. 4.30 (Shimadzu, Kyoto, Japan). L-Arabinose, glucose and D-xylose were used as references.

Analytical methods

Molecular mass of a compound purified from cereal extracts was determined by dynamic light scattering analysis with Zetasizer Nano ZS (Spectris Com., Ltd., Malvern, UK). Average molecular mass was calculated based on the light scattering/viscometry method [15] using the d*n*/d*c* value of 0.146 mL/g for samples in the water-based eluent [16] and 0.064 mL/g for the DMSO-based eluent [17]. Sugar content was determined by phenol-sulfuric acid method with xylose as the standard sugar [18].

Statistical analysis

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

Results

Analysis of cereal extracts in the ability to suppress attachment of *E. coli* to abiotic surfaces

Figure 1 shows the effects of 0.5% cereal extracts on attachment of *E. coli* ATCC 8739 cells to the abiotic surfaces. Lower numbers of *E. coli* cells attached to the glass and stainless steel surfaces conditioned with cereal extracts than those unconditioned. The wheat extract among the extracts surveyed showed the highest suppression against the cell attachment to both surfaces of glass and stainless steel. When we examined the effect of concentration of wheat extract on the attachment of *E. coli* ATCC 8739 to both surfaces, the numbers of attached cells were decreased with increase of the concentration wheat extract (Fig.2). Similar results were obtained when we used other five strains of *E. coli* (data not shown). Also, cereal extracts prepared at 100˚C showed similar activities to those prepared at 25˚C.

Purification and properties of an active compound

The activity of wheat extract was stable after autoclave for 15 min and the enzymatic treatments with α-amylase and Proteinase K. After these enzymatic treatments of wheat extract, the solution was

subjected to ammonium sulfate fractionation. Three fractions precipitated with 65%, 65-75% and 75-100% saturations of ammonium sulfate were dialyzed against H_2O, freeze-dried, and designated as F65, F75 and F100, respectively. We obtained about 840, 520, and 27 mg of the freeze-dried F65, F75, and F100, respectively, from 100 g of wheat flour. F65 among the three fractions showed the highest suppression against the attachment of *E. coli* to glass surfaces (Fig. 3). F65 (100 mg) was dissolved in 10 ml of H_2O, and further subjected to the gel filtration with a HiPrep 16/60 Sephacryl S-200 HR column. As shown in Fig 4, a broad peek was found in the elution profile. The suppressive activity against the attachment of *E. coli* to glass surfaces was found in the fractions from 22 to 30 in Fig. 4. Therefore, the active fractions were combined, dialyzed against deionized water, and freeze-dried. We obtained about 60 mg of the freeze-dried sample from 100 mg of F65. When the purified substance was subjected to polyacrylamide gel electrophoresis, we could not detect any band stained with both Coomassie Brilliant Blue R-250 and methylene blue (data not shown). The active substance purified from F65 showed an average molecular mass of about 300 kDa by light-scattering measurements; 278 kDa in H_2O and 336 kDa in DMSO. The active substance treated with 2 N trifluoroacetic acid did not show any anti-attachment activity.

Analysis of anti-attachment compound

The sugar composition was analyzed by GCMS (Fig. 5). Monosaccharides including their anomers were clearly separated from each other under the conditions. Peaks I and II in Fig. 5 were identical with those of arabinose in the retention times and mass spectra. Similarly, peaks III and IV were identical with those of xylose, and peaks V and VI were identical with those of glucose. The molar ratios of neutral sugars were 1.0 (arabinose), 2.46 (xylose), and 0.0053 (glucose). The trace amount of glucose seemed to be derived from remaining starch. Although amino acids and lipids can also be detected by the GCMS analysis, we could not detect them as components of the purified substance. These results indicated that the purified substance was an arabinoxylan.

Effect of purified arabinoxylan on attachment of several strains of *E. coli* and other genera to abiotic surface

We also examined the effect of the purified arabinoxylan on attachment of several strains of *E. coli* and other genera to glass surfaces. As shown in Fig. 6, attachment of these bacterial cells to glass surface was suppressed by the surfaces conditioned with arabinoxylan solution (0.02 mg/ml), irrespective of strains. Similar results were also obtained with stainless steel surfaces (data not shown). When the glass and stainless steel surfaces conditioned with the arabinoxylan were washed with a neutral detergent, the effect of arabinoxylan was completely eliminated.

Effects of commercially available arabinoxylan and other polysaccharides on attachment of *E. coli*

As shown in Fig. 7, commercially available arabinoxylan significantly suppressed the attachment of *E. coli* O157 cells to glass and stainless steel surfaces. Similar results were also obtained with other strains of *E. coli* (data not shown). Other polysaccharides used did not suppressed the attachment.

Discussion

Since cereals are widely used in food industry and home cooking, we here chose several cereal extracts to examine the ability as conditioning materials to suppress the attachment of *E. coli* to abiotic surfaces of cooking utensils and equipment of food factory. We also chose glass and stainless steel as abiotic surfaces to examine the ability of cereals, because preliminary experiments indicated that *E. coli* cells attached to stainless steel and glass surfaces, irrespective of strains used.

Generally, cereal flours are rapidly processed after mixing with H_2O in food industry and home cooking. We chose 30 min for conditioning of abiotic surfaces with cereal extracts. In addition, the number of bacterial cells attached to abiotic surfaces depends on their contact time of bacterial cells and abiotic surfaces [19]. Therefore, we chose a short contact time of 30 min according to the method of Rivas et al. [13].

Cereal extracts used reduced the numbers of *E. coli* cells attached to glass and stainless steel surfaces. Since the living cell numbers of *E. coli* were not reduced during the contact time at 25°C for 30 min (data not shown), the attachment of cells seemed to be suppressed by the conditioning.

Moreira et al. also reported that planktonic bacterial cells attached to solid surfaces conditioned with various solutes [20]. Our results suggested that water-soluble components in the cereal extracts adsorbed on the abiotic surfaces, modified the surface properties, and reduced the number of the cells attached to the surfaces. In comparison with glass and stainless steel surfaces unconditioned, larger numbers of *E. coli* cells attached to the surface of stainless steel than that of glass. This is similar to a previous result with *E. coli* O157 [12].

Since the wheat extract among the extracts used showed the highest activity in reduction of the number of cells attached to the abiotic surfaces, we examined the properties of active compounds in the wheat extract. The activity was stable after treatments of the wheat extract by autoclave and enzymatic digestions with Proteinase K and α-amylase. In addition, the active compound was found to have a molecular mass of more than 10 kDa by ultrafiltration with Amicon Ultra-15. Therefore, the active compound seemed to be a non-proteinaceous macromolecule.

The active compound purified from F65 was found to be arabinoxylan by GC-MS analysis. Wheat endosperm arabinoxylans are reported to have structural heterogeneity in their molecular mass and xylose content [14]. The arabinoxylan purified in this study was similar in the average molecular mass and the molar ratio of neutral sugars to a high molecular mass of arabinoxylan reported (the average molecular mass of 320 kDa; the molar ratio of arabinose to xylose was 1.0 to 1.96) [21]. Since arabinoxylan is a hemicellulose found in cereals, all cereal extracts used probably contain arabinoxylan. Difference of these cereal extracts in their anti-attachment activity may be due to the arabinoxylan content and molecular mass. The arabinoxylan purified in this study was confirmed to be suppressive against not only six strains of *E. coli*, but also five strains of other genera, *Aeromonas*, *Brevundimonas*, *Staphylococcus* and *Pseudomonas*, in their initial attachment to stainless steel and glass surfaces. Commercially available arabinoxylan also showed the similar activity. Arabinoxylan may be useful as an anti-bacterial reagent for cooking utensils and equipment of food factory.

Conclusions

We analyzed water soluble fractions extracted from flours of barley, quinoa, rice and wheat in the ability to suppress the initial attachment of *E. coli* to surfaces of glass and stainless steel. Significantly lower numbers of *E. coli* cells attached to the surfaces conditioned with wheat extract than those unconditioned. An active compound was purified from the wheat extract and analyzed. The purified compound showed an average molecular mass of about 300 kDa by light-scattering measurements. Analyses of its components indicated that the active compound was arabinoxylan; the molar ratios were 1.0 (arabinose) to 2.46 (xylose). Commercially available arabinoxylan also suppressed the attachment of *E. coli* cells to glass and stainless steel surfaces. To our knowledge, this is the first report on a dietary fiber from cereals which suppresses the initial attachment of *E. coli* to abiotic surfaces.

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Compliance with ethical standards

Conflict of interest The authors declare 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 captions

Fig. 1 Attachment of *E. coli* cells to abiotic surfaces conditioned with cereal extracts. *E. coli* ATCC 8739 cells grown at 37˚C to the stationary phase (OD660 = about 2.8) were used for the attachment assay with glass and stainless steel surfaces conditioned with cereal extracts. Control indicated unconditioned surfaces. The numbers of attached cells were measured by enumerating colonies formed between the undersurfaces and EMB agar medium after cultivation at 37˚C for 48 h. Three independent experiments were performed in triplicate, and the vertical bars show the

standard deviations. Black bars; stainless steel, white bars; glass.

Fig. 2 Attachment of *E. coli* cells to glass surfaces conditioned with several concentrations of wheat extract. *E. coli* ATCC 8739 cells grown at 37˚C to the stationary phase (OD660 = about 2.8) were used for the attachment assay with the glass surface conditioned with 0-2% wheat extracts. The numbers of attached cells were measured as described in the caption of Fig. 1. Three independent experiments were performed in triplicate, and the vertical bars show the standard deviations. Black bars; stainless steel, white bars; glass.

Fig. 3 Attachment of *E. coli* cells to glass surfaces conditioned with F65, F75 and F100. *E. coli* ATCC 8739 cells grown at 37˚C to the stationary phase (OD660 = about 2.8) were used for the attachment assay with the glass surfaces conditioned with some concentrations of F65, F75 and F100. The numbers of attached cells were measured as described in the caption of Fig. 1. Three independent experiments were performed in triplicate, and the vertical bars show the standard deviations.

Fig. 4 Elution profile of F65 on gel filtration with a HiPrep 16/60 Sephacryl S-200 HR column. The F65 solution (100 mg in 10 ml) was applied to the column (1.6 x 60 cm) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. Elution was made with the same buffer. Each fraction (5 ml) was analyzed in their sugar contents and suppressive activity for attachment of *E. coli* ATCC8739 cells to glass surfaces.

Fig. 5 GCMS analysis of a purified chemical component. A chemical component was hydrolyzed with 2M TFA at 100˚C for 4 h. After removal of TFA under N_2 gas, the hydrolysate was dried up under reduced pressure. The dried sample (1 mg) was trimethylsilylated at 70˚C for 1 h with 0.1 ml of (BSA + TMCS + TMSI, 3:2:3), and then analyzed with GCMS-QP2010 Ultra and Trace Gold TG-1MS GC column. Injection temperature was 250˚C; oven temperature was from 80˚C to 320˚C with a increasing rate of 15˚C/min; split ratio was 10:1; and injected sample was 1 μl.

Fig. 6 Effect of arabinoxylan on attachment of six strains of *E. coli* and five strains of other genera to glass surfaces. *E. coli* strains used were ATCC 8739, NBRC 3301, NBRC 3302, NBRC 13168, NBRC 13891, and O157:H7 sakai. Strains of other genera were *A. hydrophila* subsp. *hydrophila* NBRC 13286, *B. diminuta* NBRC 14213, *P. aeruginosa* NBRC 13275, *P. fluorescens* NBRC 14160, *S. aureus* subsp. *aureus* NBRC 12732. These cells grown to the stationary phase were used for the attachment assay with the glass surfaces conditioned with and without arabinoxylan solution (0.02 mg/ml). Three independent experiments were performed in triplicate, and the vertical bars show the standard deviations. White bars: unconditioned, and black bars: conditioned with arabinoxylan.

Fig. 7 Effects of commercially available polysaccharides on attachment of *E. coli* O157:H7 sakai. Polysaccharides used were pectin, xylan, inulin, amylose, mannan, arabinogalactan and arabinoxylan. The cells grown to the stationary phase were used for the attachment assay with the glass and stainless steel surfaces conditioned with and without each polysaccharide solution (0.02 mg/ml). Three independent experiments were performed in triplicate, and the vertical bars show the standard deviations. Black bars; stainless steel, white bars; glass.

Fig.1

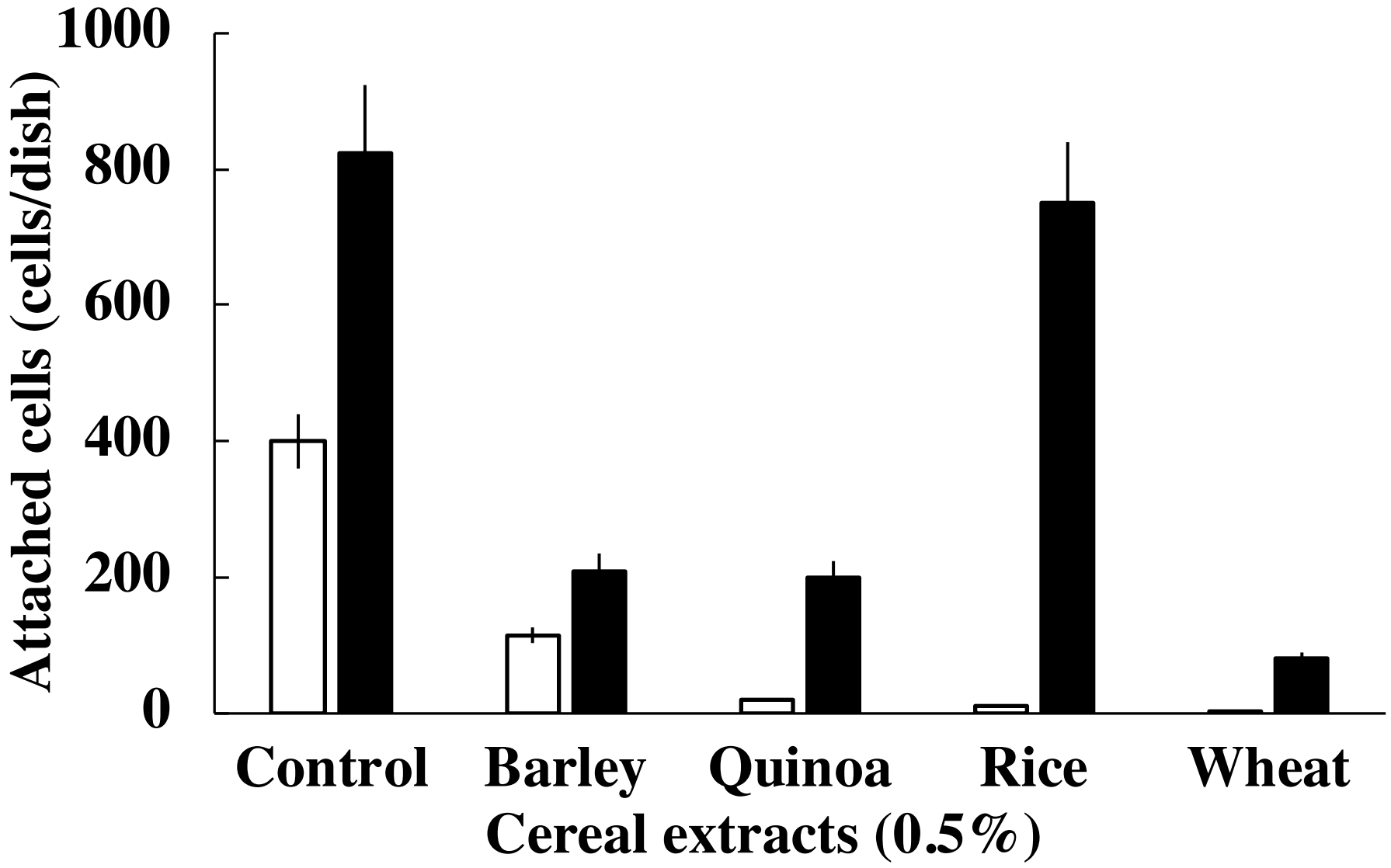

1000
800
600
400
200
0
Attached cells (cells/dish)
Control
Barley
Quinoa
Rice
Wheat
Cereal extracts (0.5%)

Fig.2

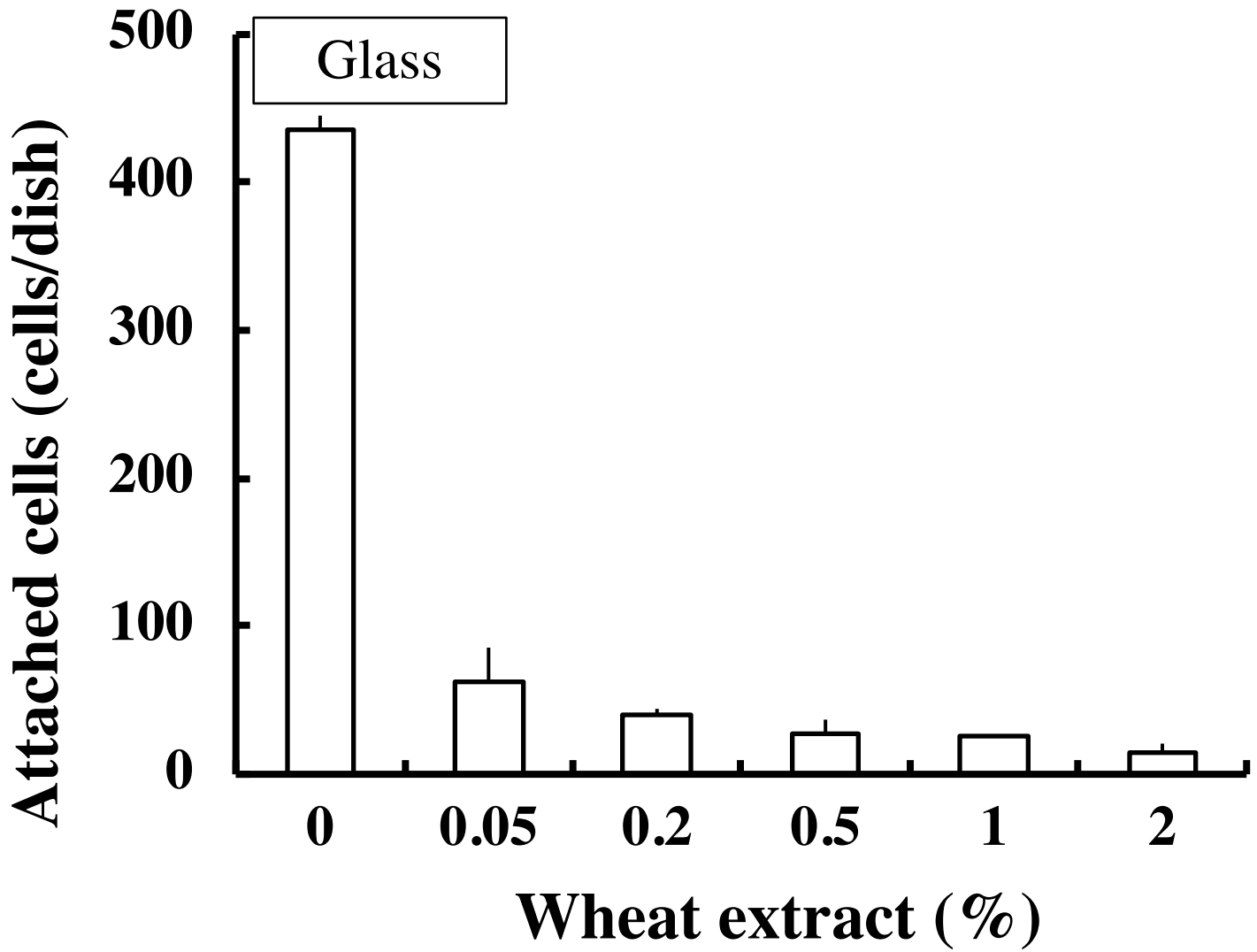

Glass
500
400
300
200
100
0
Attached cells (cells/dish)
0
0.05
0.2
0.5
1
2
Wheat extract (%)

Fig.3

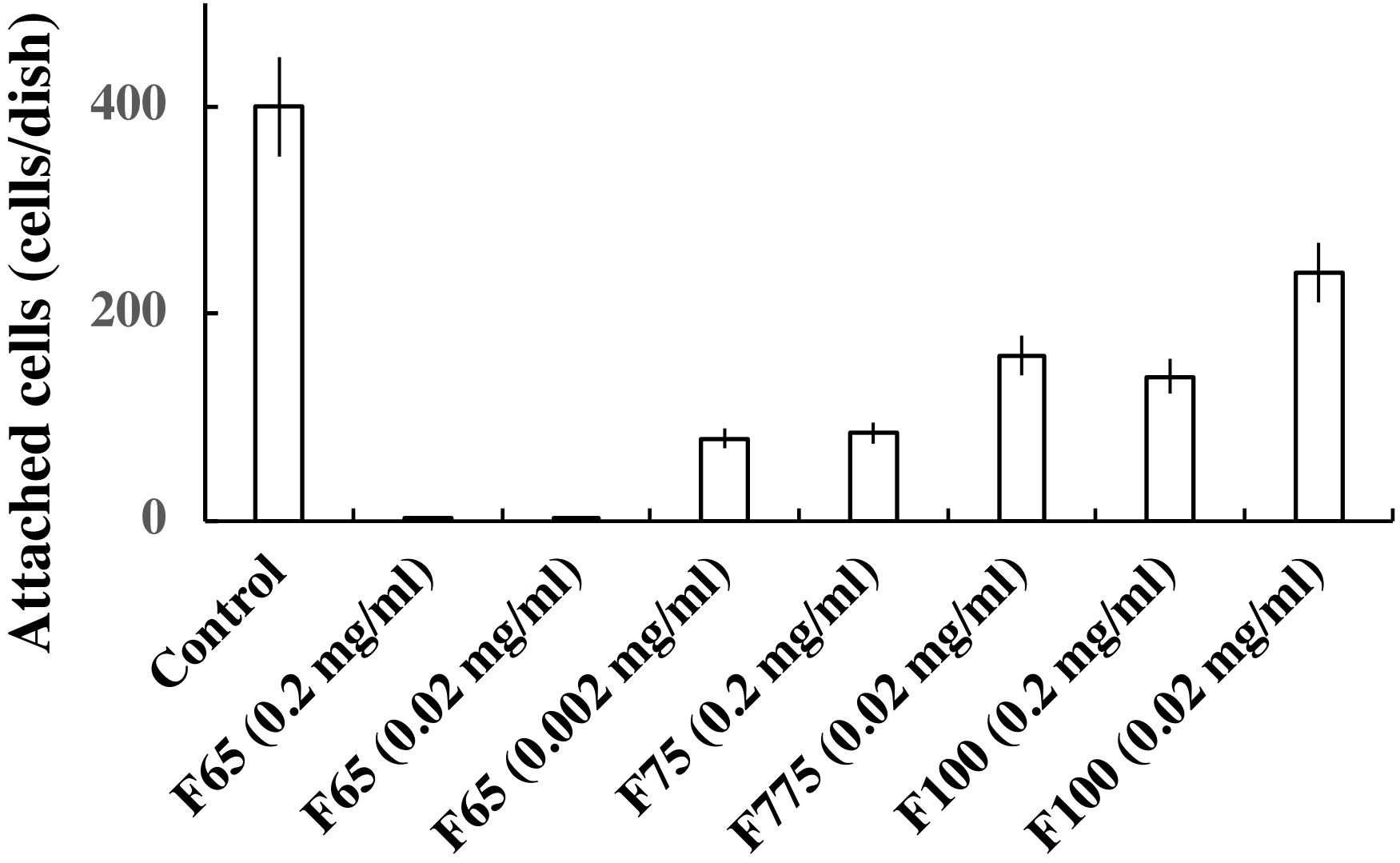

Attached cells (cells/dish)
400
200
0
Control
F65 (0.2 mg/ml)
F65 (0.02 mg/ml)
F65 (0.002 mg/ml)
F75 (0.2 mg/ml)
F775 (0.02 mg/ml)
F100 (0.2 mg/ml)
F100 (0.02 mg/ml)

Fig. 4

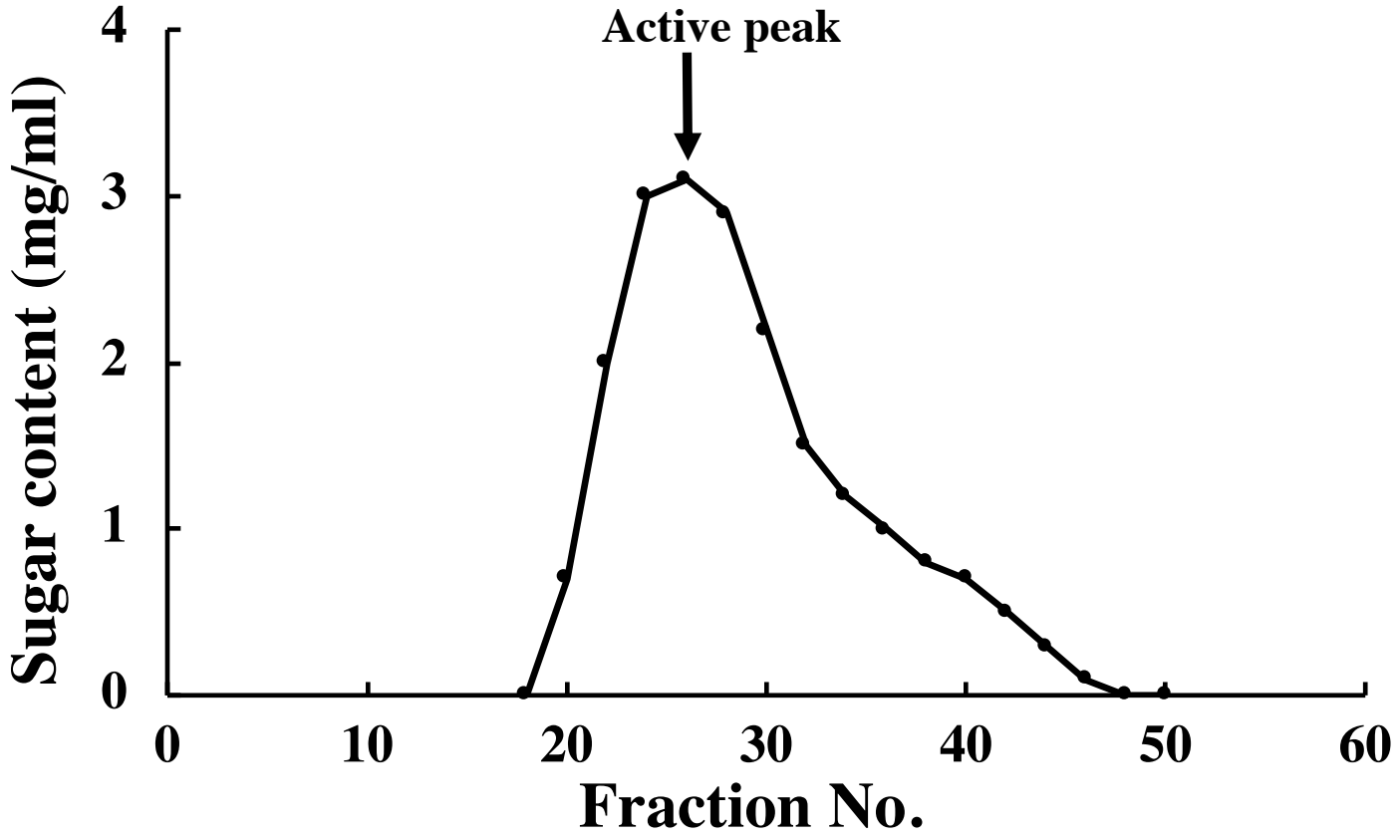

Active peak
Sugar content (mg/ml)
4
3
2
1
0
0
10
20
30
40
50
60
Fraction No.

Fig.5

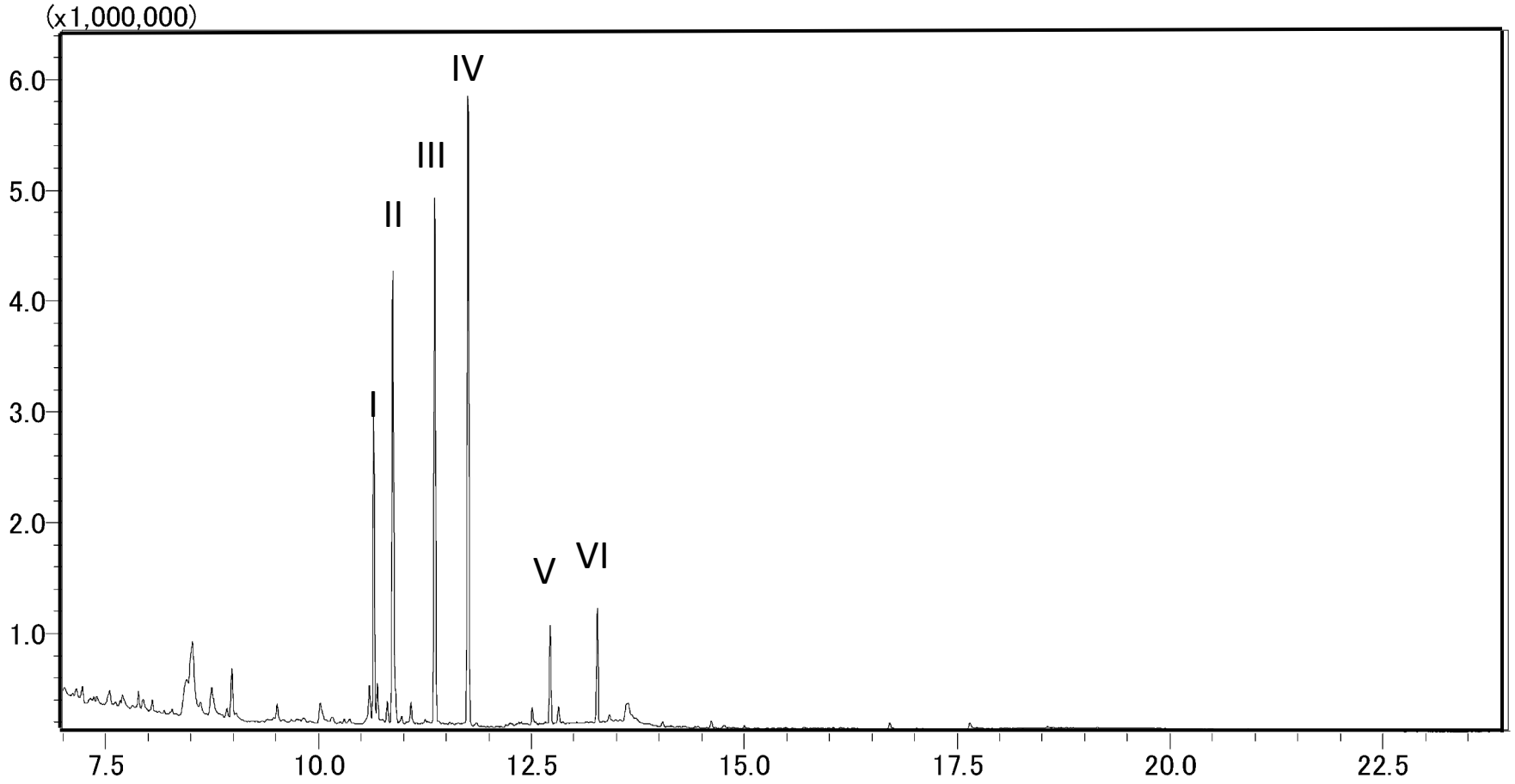

(x1,000,000)
6.0
5.0
4.0
3.0
2.0
1.0
I
II
III
IV
V
VI
7.5
10.0
12.5
15.0
17.5
20.0
22.5

Fig. 6

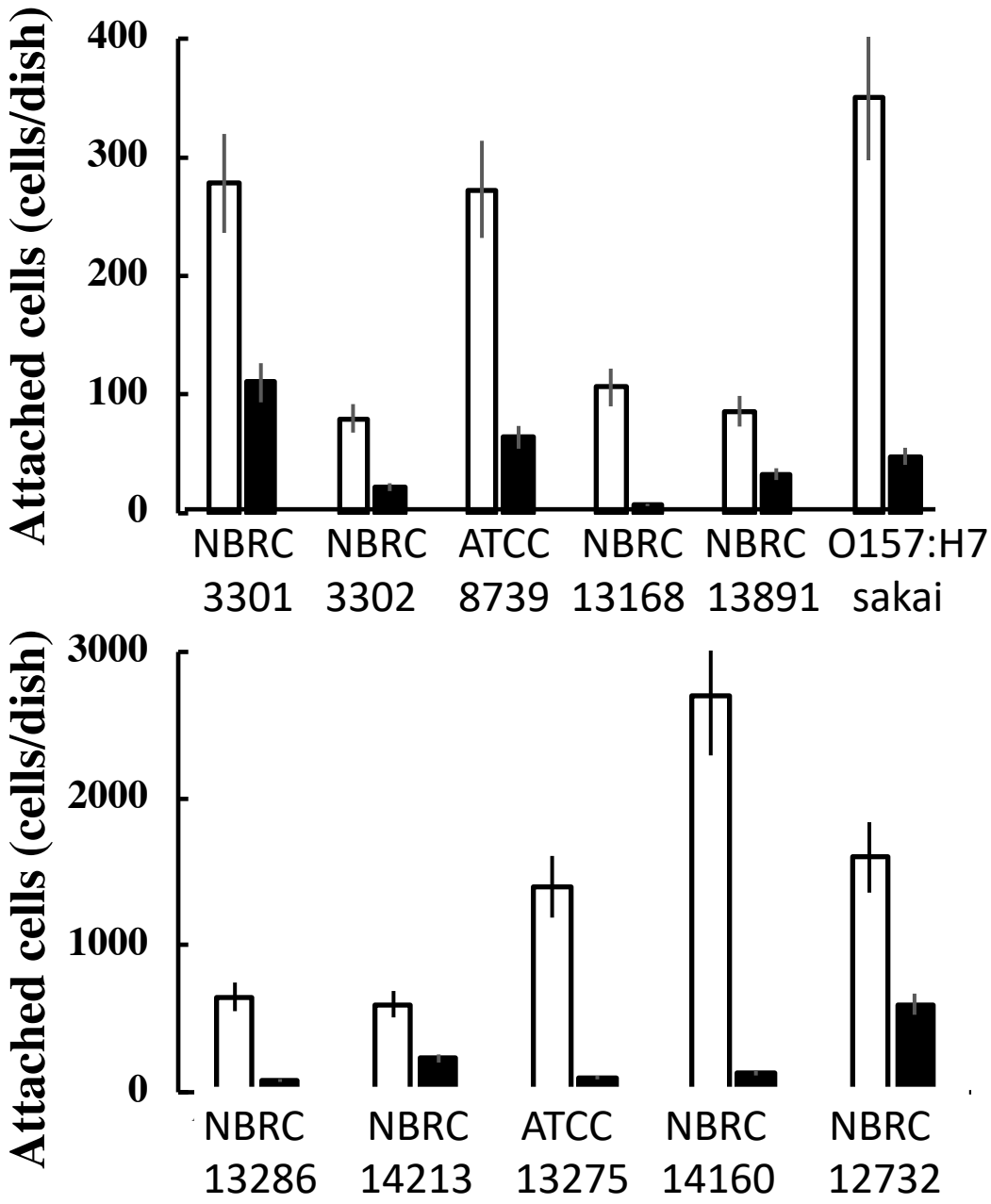
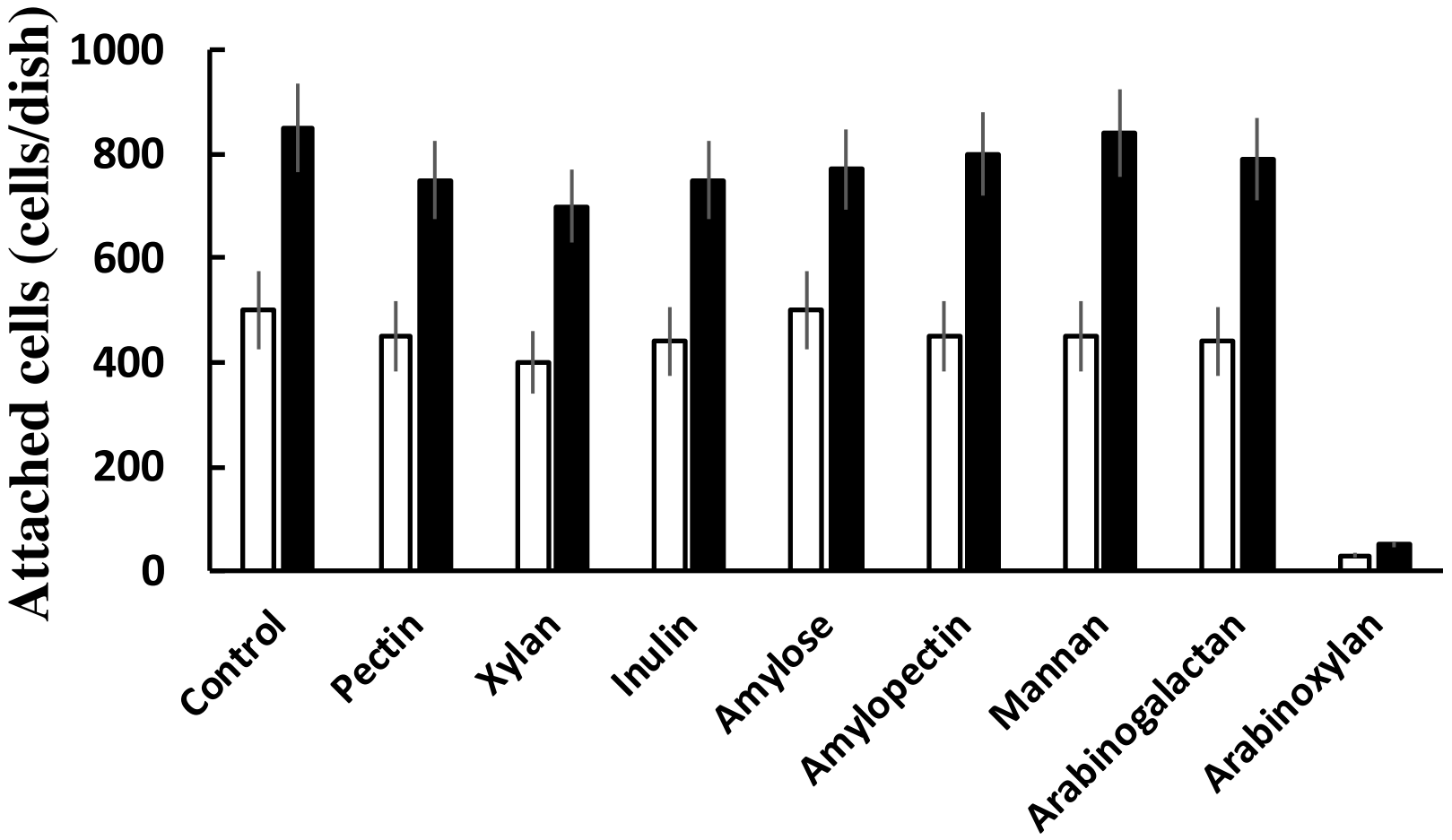

Attached cells (cells/dish)
400
300
200
100
0
NBRC 3301
NBRC 3302
ATCC 8739
NBRC 13168
NBRC 13891
O157:H7 sakai
Attached cells (cells/dish)
3000
2000
1000
0
NBRC 13286
NBRC 14213
ATCC 13275
NBRC 14160
NBRC 12732

Fig. 7


Attached cells (cells/dish)
1000
800
600
400
200
0
Control
Pectin
Xylan
Inulin
Amylose
Amylopectin
Mannan
Arabinogalactan
Arabinoxylan