Investigation of Micro-Particles Produced from Wheat Bran and Sugarcane Bagasse Fermentation by Human Faecal Flora and the Binding Capacities of Fermentation Residues

Shi Yi Ou*1,2, Jie Zheng1,2, Yanfang Xu1, Jing Zhang1 and Baoru Yang1,2

1Department of Food Science and Engineering, Jinan University, Guangzhou 510632, People’s Republic of China
2Department of Biochemistry and Food Chemistry, University of Turku, Turku FI-20014, Finland

Abstract
Wheat bran has been consistently reported to protect against colon cancer. To investigate the mechanism of the unique characteristics of wheat bran, the morphology and zeta potential of micro-particles released from wheat bran and sugarcane bagasse fermentation by human faecal flora were studied by atomic force microscopy and laser light scattering technique, respectively. Micro-particles released from wheat bran fermentation differed significantly from those produced from sugarcane bagasse fermentation in shape, size (237 VS 454 nm), and zeta potential (-20.01 VS -3.96 mV). Scanning electron microscopic observations showed that the structure of fermented residue of wheat bran was more loose and porous than that of sugarcane bagasse, and had a larger surface area. In vitro studies revealed higher binding capacities of fermented wheat bran for bile acid, cholesterol, and peanut oil compared with fermented sugarcane bagasse.

Keywords: Wheat bran; Sugarcane bagasse; Colon fermentation; Micro-particles; Binding capacity

Introduction
Dietary fibres may prevent or promote colon cancer [1]. Their preventive effects have been ascribed to the production of short-chain fatty acids (SCFAs) during fermentation in the colon [2]. Some soluble dietary fibres, such as carrageenan and citrus pectin, which produce large amounts of SCFAs in the colon, have been found to promote colon carcinogenesis [1,3,4], whilst alkaline-modified citrus pectin seems to prevent it [5]. Molecular biological research indicates that carbohydrates (dietary fibres) play a significant role in the recognition processes involved in the adhesion between cells and the extracellular matrix [5]. The charge density, molecular weight, and structure of carbohydrates are important factors that influence these processes [6].

Among water-insoluble dietary fibres, wheat bran has been consistently reported to protect against colon cancer [1,7], even when it is consumed with a high-fat/low-calcium diet [8]. However, the accurate mechanisms for its preventive effect on colon cancer are poorly understood. Some of the possible mechanisms proposed include: carcinogen binding [1]; reduction of the exposure of colon epithelia to carcinogens by increasing stool bulk and decreasing intestinal transit time [9]; and antitumour effects from phytic acid, antioxidants, and lignans in wheat bran [10-13]. Aside from wheat bran, oat bran, rice bran, maize bran, and sugarcane bagasse have all shown binding capacity for carcinogens, and also contain a series of antioxidants [14,15]. Although rice bran contains more phytic acid than wheat bran, the latter seems to be more active in preventing against colon cancer [18], and many colon carcinogens are lipophilic substances [19]. Thus, the binding capacity of fermented wheat bran and sugarcane bagasse for cholesterol, bile acid, and peanut oil were investigated in vitro to evaluate their capacity for adsorbing harmful lipophilic substances in the colon.

Materials and Methods
Reagents
Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1 from Bacillus licheniformis, 120 KNU/g), protease Alcalase 2.4 L (EC 3.4.21.62, from B. licheniformis, 2.4 AU/g), and amyloglucosidase AMG 300 L (EC 3.2.1.3, from Aspergillus niger, 300 AGU/g) were purchased from Novo Nordisk ( Bagsvaerd, Denmark). Cholic acid, cholesterol, and other chemical reagents were purchased from Sigma Company (St. Louis, MO), and peanut oil and eggs were purchased from the local market.

Wheat bran and sugarcane bagasse
Wheat bran was obtained from Guangdong Nanfang Flour Group (Guangzhou, Guangdong Province, China), and sugarcane bagasse was obtained from Overseas Chinese Sugar Factory (Taishan, Guangdong Province, China). The wheat bran was milled and passed through a 0.5 mm sieve, followed by the removal of starch with α-amylase, and amyloglucosidase and protein with proteinase, according to our previous work [20]. The residue was air-dried and then placed in an oven at 105°C for 4 h. The sugarcane bagasse was milled, passed through a 0.5 mm sieve, and then suspended in ten volumes of water.
(50°C) with constant stirring to remove the remaining sucrose. The starch and protein was removed from the solid materials using the method described above.

**Preparation of micro-particles**

An *in vitro* fermentation system was designed according to our previous work [20]. Fermentation was performed in buffer flasks containing a 100 mL mixture of the following: 5.0 mL 1% human faeces suspension, 0.1 mol/L NaH2PO4-Na2HPO4 buffer (pH=6.5), 0.63 mmol/L cysteine-HCl, and 2.0 g of destarched and deproteinised wheat bran or sugarcane bagasse. Faeces were collected from healthy male volunteer students in our university, who mainly ate in the university dining hall and did not take antibiotics for at least 6 months prior to the test. Flasks were placed in an anaerobic cabinet at 37°C for 24 h. The fermented materials were collected and filtered through a 200-mesh nylon cloth. The materials collected on the cloth were dried at 105°C for 6 h and ground using a mortar for scanning electron microscopy (SEM). The filtrates were centrifuged at 8000×g for 20 min, and then the supernatant was diluted tenfold to determine the shape and size of the distribution of the micro-particles by laser light scattering and atomic force microscope (AFM). The electrokinetic properties of the particles were determined by measuring their zeta potential.

**Observation of micro-particles by atomic force microscope**

Approximately 5 μL of fermentation solution was placed on a piece of mica and dried at room temperature. AFM imaging was performed using an Autoprobe CP Research atomic force microscope (Thermo Microscope Ltd., USA). AFM images were collected using silicon tips with a spring constant of 3.2 N/m in light tapping mode.

**Determination of size distribution and zeta potential of micro-particles**

Particle sizes and size distributions were measured by laser light scattering, with a 90 Plus Particle Size Analyser system (Brookhaven Instruments, Huntsville, NY, USA). The electrokinetic properties of the particles were determined by measuring the zeta potential on a Zeta Plus apparatus (Brookhaven Instruments, Huntsville, NY, USA).

**Observation of wheat bran, sugarcane, and fermentation residues by scanning electron microscopy**

Dried and ground samples of destarched and deproteinised wheat bran, sugarcane bagasse, and fermentation residues were spread on a double-sided conducting adhesive tape pasted onto a metallic stub, sputter-coated with gold, and observed using a Philips XL-30 scanning electron microscope connected to a digital camera. The images were collected on a computer.

**Bile acid binding capacity of wheat bran, sugarcane, and fermentation residues**

*In vitro* bile acid binding tests were carried out using the method of Kahlon et al. [21] with modifications. Two grams of each material were incubated with 10 mmol/L cholic acid in 100 mL of phosphate buffer (pH 6.3). The slurry was shaken (120 t/min) in acid-washed flasks at 37°C for 3 h and then centrifuged at 4000×g for 20min. The concentration of cholic acid in the supernatant was determined by an Agilent 1100 high performance liquid chromatograph (HPLC, Waldbronn, Germany) with a C18 column (YMC-Pack ODS-A, 5 μm, 4.6 × 150 mm). The mobile phase was acetonitrile: water: phosphoric acid (35:65:0.1). The flow rate was 1.1 mL/min, and cholic acid was detected at 192 nm.
The binding capacity of wheat bran, sugarcane, and fermentation residues for peanut oil. The binding capacity of fermented residues for oil was determined according to Sangnark and Noomhorm [22]. Three grams of the dried sample were mixed with peanut oil in a centrifugal tube and left for 1 h at room temperature (25°C). The mixture was then centrifuged at 1500 × g for 10 min, the supernatant was decanted, and the pellet was recovered by filtration through a nylon mesh. The binding capacity (BOC) was calculated as follows:

$$\text{BOC} = (W_2 - W_1)/W_1$$

Where $W_1$ and $W_2$ are the weights of the samples before and after adsorbing oil, respectively.

The binding capacity of wheat bran, sugarcane, and fermentation residues for cholesterol in egg yolk. Because cholesterol is difficult to dissolve in water even if emulsifiers are added, egg yolk was used in this research. Fresh egg yolk was homogenised with 9 volumes of de-ionised water. Mixtures of 2.0 g dried samples with 50 mL of the diluted yolk at pH 7.0 were placed in a rotator at 80 rpm, 37°C for 2 h, and then centrifuged at 3000 × g for 15 min. One millilitre of supernatant was diluted 5 times with 90% acetic acid, and its colour was developed by adding 0.1 mL of o-phthalaldehyde reagent and 2 mL concentrated H$_2$SO$_4$, according to Park [23]. Absorbance was read at 550 nm against a reagent blank 20 min after the addition of H$_2$SO$_4$, and the cholesterol concentration of the samples was determined against the standard curve generated by the cholesterol standard solution.

The binding capacity was calculated according to the cholesterol concentration before and after adsorption by fermented wheat bran and sugarcane bagasse.

Results and Discussion

Shape, size distribution, and zeta potential of the particles released from wheat bran and sugarcane bagasse fermentation by human faecal flora. AFM is capable of imaging nano-particles and living cells in their native environment with considerably higher resolution than what
can be achieved with light microscopy [24,25]. Zeta potential is electric potential in the interfacial double layer at the location of the slipping plane versus a point in the bulk fluid away from the interface [26]. Today, Zeta potential has been used to probe the interaction between cells and biomolecules, to study cell biological activation, cell agglutination and cell adhesion which are related to cell surface charge properties; it was considered as a critical parameter for cellular interaction [27]. Thus, AFM and zeta potential measurement are quite suitable for investigation of micro-particles released from wheat bran and sugarcane bagasse fermentation by human faecal flora.

The AFM results showed that the particles from the raw materials of wheat bran and sugarcane bagasse were all in irregular shape, fermentation changed their shape and size, and made them different from each other (Figure 1 and 2). The particles from wheat bran fermentation were spherical in shape (Figure 1), whereas the residues from sugarcane bagasse fermentation were more irregular and crystal-like (Figure 2). The sizes of the particles of wheat bran fermentation residues were distributed over a wider range than the residues from sugarcane bagasse fermentation. Furthermore, the particles from sugarcane bagasse fermentation conglutinated tightly together, possibly due to the low-density negative charge on the surface of the particles (Figure 3).

Laser light scattering and Zeta Plus tests showed that the micro-particles released from wheat bran and sugarcane bagasse fermentation differed in size distribution and charge density. The size of micro-particles from sugarcane bagasse fermentation focused at approximately 454 nm; however, the size distribution of particles from wheat bran fermentation was quite different from that of sugarcane bagasse (Figure 4). The particles from wheat bran fermentation were distributed over three ranges. The first was at 1-4 nm. Particles within this range were designated as nanoparticles (size below 100 nm). The second was at approximately 237 nm, whilst the third one was over 10,000 nm. Moreover, the negative charge density (zeta potential, Figure 3) of the particles from wheat bran fermentation (~20.01mv) was 5 times higher than that from sugarcane bagasse fermentation (~3.96mv).

The highly negatively charged micro particles, especially the nanoparticles may influence the growth of tumour cells and colonic mucosal proliferation. Tumor cells often bear higher negative charges than normal cells [28,29], and substances that change the density of surface charge of tumor cells may inhibit tumor growth. Moreover, Zhang et al. [27] used zeta potential measurement to study the interaction between the negative nano-particles, iron oxide nano-particles and cowpea mosaic virus with normal and cancer breast epithelial cells, and found that the zeta potential value for two kinds of cells changed in an opposite way: the zeta potential value of the normal cells changed to a slight positive, while the cancer cell increased by 40% to more negative. Since micro-particles from wheat bran fermentation had higher density negative charge than that of sugarcane bagasse, the highly negatively charged micro-particles may influence the growth of colon cancer cell by charge repulsion, which may partially explain the protective effects of wheat bran against colon cancer shown in previous investigations.

Morphologies of wheat bran and sugarcane bagasse before and after fermentation

SEM showed that the structure of unfermented wheat bran is
Highly negatively charged micro-particles were released from wheat bran after fermentation, which may be part of the mechanism involved in the preventive effect of wheat bran on colon cancer. The fermentation residue from wheat bran had large inner and outer surface areas, and showed high adsorption capacity for heavy metals, bile acid, and lipids, which may decrease the concentration of carcinogens and other harmful compounds present in the colon. Nano-particles produced by the fermentation process may be absorbed by colon epithelia and consequently have some physiological effects. Future work will focus on analysing the composition of the particles, and investigating their effects on the growth of epithelia and colon cancer cells and their behaviour after absorption by mucosal cells.

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**Table 1:** Binding capacity (means ± SD, n=3) of wheat bran and sugarcane bagasse before and after fermentation for peanut oil and cholesterol.

|                          | Wheat bran | Sugarcane bagasse |
|--------------------------|------------|-------------------|
|                          | Cholic acid (µmol/g) | Oil (g/g) | Cholesterol (mg/g) | Cholic acid (µmol/g) | Oil (g/g) | Cholesterol (mg/g) |
| Before fermentation      | 22.65 ± 1.43 | 4.93 ± 0.14 | 24.9 ± 1.4        | 24.34 ± 1.78 | 3.65 ± 0.12 | 18.6 ± 1.2        |
| After fermentation       | 38.65 ± 1.89 | 6.36 ± 0.08 | 36.2 ± 0.1        | 29.36 ± 1.54 | 4.23 ± 0.16 | 25.3 ± 1.6        |

*P < 0.05

Figure 6: SEM images of wheat bran (a) and sugarcane bagasse (b) residue after fermentation.
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