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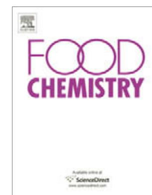

Partial hydrolysis enhances the inhibitory effects of konjac glucomannan from *Amorphophallus konjac* C. Koch on DNA damage induced by fecal water in Caco-2 cells

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ABSTRACT

This study determined effects of diets containing 5% (w/w) unhydrolysed konjac glucomannan (KGM), and its acid-hydrolysate, fractions 1 and 2 (F1, F2), with degrees of polymerisation 8 and 4, respectively, on the cytotoxicity and DNA damage of fecal water-treated Caco-2 cells. Oligofructose was used as a positive control. In addition, the possible mechanisms for these effects were investigated. Results indicated that KGM, F1, F2 and oligofructose diets similarly increased the survival rate of fecal water-treated Caco-2 cells compared with the FF diet. F2 exerted the greatest protective effects, amongst KGM-based fibres, on fecal water-induced DNA damage. The prebiotic effects of F1 and F2, were also better than that of KGM. However, the fecal water from the group fed KGM, rather than partially-hydrolysed KGMs, exerted the greatest ferrous ion-chelating ability. In conclusion, partially-hydrolysed KGMs exerted greater protective effects than did the unhydrolysed KGM on fecal water-induced DNA damage, mainly by their prebiotic effects.

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1. Introduction

Konjac glucomannan (KGM) is a well-known highly viscous dietary fibre extracted from the tuber of *Amorphophallus konjac* C. Koch of the botanical family Araceae (Tye, 1991). It is mainly composed of a β-(1 → 4)-linked D-glucosyl and D-mannosyl polymer with a mean molecular weight of $9.0 \pm 1.0 \times 10^5$ g mol^{-1} (Ratcliffe, Williams, Viebke, & Meadows, 2005). Recent studies have shown that addition of KGM to a low fibre diet could improve bowel habit in children (Loening-Baucke, Miele, & Staiano, 2004) and adults (Chen, Cheng, Liu, Liu, & Wu, 2006). In addition, KGM is also a prebiotic fibre in animals (Chen, Fan, Chen, & Chan, 2005) and humans (Chen et al., 2006). We have previously shown that BALB/c mice fed a 5% KGM diet, as compared to a fibre-free diet, could reduce the toxicity of water-soluble fecal material in Caco-2 cells (Yeh, Lin, & Chen, 2007).

Although KGM exerts many beneficial physiological functions, its high viscosity limits its application in food. Downsizing, by hydrolysing the glucomannan polymer or by pulverising of natural particles, increases the solubility of KGM and may expand its application. The change of particle size (Li, Xia, Wang, & Xie, 2005; Onishi et al., 2007) or degree of polymerisation (DP) (Chen et al., 2005) of KGM may modulate its physiological function. For example, pulverisation of native KGM (grain size 657.3 mm) to a small-sized KGM (grain size 23.7 mm) increases the swelling rate, which may promote the anti-obesity effect (Li et al., 2005). Similarly, small-sized KGM rather than native KGM suppresses the development of allergic rhinitis-like symptoms and IgE response in mice (Onishi et al., 2007). Furthermore, we have previously demonstrated that a raw KGM hydrolysate (average DP of 12) exerts a greater prebiotic effect than does the native KGM in BALB/c mice (Chen et al., 2005). Therefore, partial hydrolysis of KGM may enhance its beneficial physiological effects; however, the optimal DP of this fibre, for various functions, remains unclear.

Anti-carcinogenic effects of prebiotic dietary fibres are associated with probiotics, such as bifidobacteria and lactobacilli (Pool-Zobel et al., 1996); those have been found to decrease fecal water-induced DNA damage in the colonic mucosa (Burns & Rowland, 2004). In agreement with that, we recently proposed that the prebiotic

Abbreviations: FF, fibre-free; KGM, konjac glucomannan; DP, degree of polymerisation; F1 and F2, fractions 1 and 2; FPG, formamidopyrimidine DNA glycosylase; *C. perfringens*, *Clostridium perfringens*.

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characteristic is one of the possible mechanisms whereby KGM decreases the toxicity of faeces (Yeh et al., 2007). In addition, our previous study also suggests that the anti-toxic effect of KGM on colonic cells is partly due to its ferrous ion-chelating ability (Yeh et al., 2007). However, the optimal DP of KGM hydrolysate, for decreasing the toxicity of fecal water toward intestinal cells and for the mechanisms mediating the anti-toxic effect, has not been investigated.

The main goal of the present study was therefore to compare effects of native KGM and its partial hydrolysate, fractions 1 and 2 (F1 and F2) (average DPs 8 and 4, respectively), on the toxicity of fecal water obtained from BALB/c mice toward Caco-2 cells, derived from a human colon adenocarcinoma (Sambuy et al., 2005). We also compared the effect of DP on fecal microflora in BALB/c mice and on ferrous ion-chelating ability of fecal water. Oligofructose, a well-known prebiotic, was used as a positive control in this study.

2. Materials and methods

2.1. Dietary fibres

The konjac powder (purity 80.0%, Fukar Co., Taipei, Taiwan) was boiled in 0.2 N HCl (25 g/l) for 40 min in a round-bottom flask with a reflux condenser and then condensed in vacuum to 1/10 of its original volume. After this, raw KGM hydrolysate was composed to make a 75% ethanol solution (v/v); it was centrifuged at 10,000*g* for 30 min at 4 °C. The pellet was denoted as F1. F2 was precipitated from the same condensed solution with raw KGM hydrolysate by adjusting its ethanol concentration to 90% (v/v). F1 and F2 were successively washed with ethanol to remove the residual acid, lyophilised, and stored in desiccators until used. The sugar composition of each fraction was reported in our previous study (Wang, Lai, Chen, & Chen, 2008), and the mean DPs of F1 and F2, calculated as the ratios of total sugar content (Dubois, Gilles, Hamilton, Robers, & Smith, 1956) and reducing sugar content (Aued, Carvalho, Tavares, Zanelatto, & Bacetti, 1990), were 7.8 ± 0.2, and 3.9 ± 0.1, respectively. The purities of F1 and F2 were 94.0% and 97.7%, respectively. Oligofructose syrup contained 67.0% of oligofructose (Taiwan Sugar Co., Taipei, Taiwan).

2.2. Animals and experimental design

Seven-week-old male BALB/c mice (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were housed in solid-bottomed plastic cages with wood shavings for bedding in a room maintained on a 12 h light–dark cycle (0800–2000) at 24 ± 1 °C and 50% humidity. All animals were allowed free access to water and food in the study. Animal care followed the guidelines of the National Research Council (National Institutes of Health, 1985) and was approved by the Institutional Animal Care and Use Committee at the Chung Shan Medical University.

After a 10 days adaptation period, the mice were randomly divided into five groups ($n = 8$/group) and fed a fibre-free AIN-93 diet (FF) or an AIN-93 modified diet containing KGM, F1, F2, or oligofructose (Taiwan Sugar Co., Taipei, Taiwan). The composition of the diets was as follows (g/kg): casein, 200.0; corn starch, 529.5; sucrose, 100.0; corn oil, 70.0; AIN-93G mineral mix, 35; AIN-93 vitamin mix, 1.0; L-cystine, 3.0; choline bitartrate, 2.5; butylated hydroxytoluene, 0.014; and dietary fibre (corrected for its purity), 50. The powdered diet was mixed with an equal weight of distilled water and made into pellets.

Food intake was weighed every day, and body weight was measured twice weekly. Faeces voided were collected in ice-bathed tubes, weighed, lyophilised, and stored at −20 °C during days 18–21. The faeces voided from 0900 to 0930 on day 21 were placed directly into the anaerobic solution for analysis of fecal microflora.

2.3. Fecal water preparation

In an adaptation of the method described previously (Rieger, Parlesak, Pool-Zobel, Rechkemmer, & Bode, 1999), lyophilised fecal composites were rehydrated to 3-fold their original fecal weight. The samples were centrifuged at 36,000*g* for 2 h. The supernatant fluid, i.e., fecal water, was collected and used immediately for incubation with Caco-2 cells.

2.4. Cell culture

Caco-2 cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and were cultured in Dulbecco's modified Eagle's medium (DMEM, containing 10% fetal bovine serum, 4 mM L-glutamine, 1.5 g/l of $NaHCO_3$, 4.5 g/l of glucose, 0.01 g/l of human transferrin, and 1 mM sodium pyruvate (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified incubator under 5% CO_2 and 95% air according to the method described previously (Yeh et al., 2007). The cells were harvested at approximately 90% confluence (approximately 10^6 cells/10 cm dish). For use in the assay of fecal water toxicity, cells were detached with trypsin–EDTA, centrifuged for 5 min at 200*g*, and resuspended in Hank's balanced salt solution (HBSS; 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.4 mM $MgSO_4{\cdot}7H_2O$, 137 mM NaCl, 4.2 mM $NaHCO_3$, and 0.3 mM $NaH_2PO_4{\cdot}H_2O$) at a concentration of 2×10^6 cells/ml. The cell suspension (900 µl) was incubated with 100 µl of fecal water or HBSS buffer (as control) at 37 °C in a gently shaking water bath for 3 h (Rieger et al., 1999). An aliquot (400 µl) was taken to assess cell viability by trypan blue exclusion staining (Freshney, 2005), and the rest of the mixture was centrifuged (600*g*, 10 min) to collect the cells used for the comet assay.

2.5. Comet assay

The comet assay (Pool-Zobel, Bub, Muller, Wollowski, & Rechkemmer, 1997) was used to determine DNA damage. According to the method described previously (Yeh et al., 2007), the treated cells were suspended in low-melting-point agarose in phosphate-buffered saline (PBS) at 37 °C and placed onto a frosted glass microscope slide precoated with a layer of 1% normal-melting-point agarose. After application of a third layer of 1% normal-melting-point agarose, the slides were immersed in cold-lysing solution (10 mM Tris, 2.5 M NaCl, 100 mM Na_2EDTA, 1% sodium *N*-laurylsarcosine, 1% Triton X-100, and 10% dimethylsulphoxide) for 1 h at 4 °C. Afterwards, the slides were divided into two subgroups. One of these was treated with formamidopyrimidine glycosylase (FPG, 50 µl, 1 mg/ml) at 37 °C for 30 min for quantification of the oxidised nucleic acids in Caco-2 cells. FPG is a specific enzyme for nicking the DNA at sites of oxidised purine (Collins, Ma, & Duthie, 1995). All of the slides were placed in an electrophoresis tank, and the DNA was allowed to unwind for 15 min in the alkaline solution, followed by electrophoresis. Each value presented was calculated from three batches of experiments, each of which included two slides with at least 50 comets (Collins et al., 1995). The image was analysed (by computer) by using the Interactive Image Analysis Comet Assay III (Perceptive Instrument, Haverhill, Suffolk, UK), and DNA strand breaks were described as tail moment, where

$$\text{Tail moment} = \%\text{DNA in tail} \times \text{tail length}.$$

2.6. Enumeration of intestinal microflora

According to the method described previously (Yeh et al., 2007), Brucella blood agar (Pankuch & Appelbaum, 1986), Lactobacilli MRS agar, bifidobacteria iodoacetate medium-25 (Munoa & Pares, 1988), and modified differential clostridial agar supplemented

with polymyxin B sulphate (8.5 mg/l) (Gibb & Freame, 1965) were used to selectively identify anaerobes, lactobacilli, bifidobacteria and *Clostridium perfringens*, respectively, in an anaerobic chamber ($H_2/CO_2/N_2$, 10:10:80). Plates were inoculated in triplicate and incubated at 37 °C for 3 days. The polymerase chain reactions were performed to confirm the identity of the colonies by using specific primers, as described by Wang, Cao, and Cerniglia (1996).

2.7. Ferrous ion-chelating ability of fecal water

According to the method described previously (Dinis, Maderia, & Almeida, 1994), an aliquot of fecal water was first diluted with 5 vol of deionised water, and then 5 μl of the diluted sample were mixed with 555 μl of methanol, 15 μl of 2 mM $FeCl_2$, and 30 μl of 5 mM ferrozine solution. After reaction for 10 min, absorbance was measured at 562 nm. In addition, we determined the absorbance of each diluted fecal water sample alone, at 562 nm, as a sample control. The percentage of ferrous ion-chelating ability was calculated as: [1 – (sample absorbance – sample control absorbance)/blank absorbance] × 100%.

2.8. Statistical analysis

Values are expressed as means ± SD. Differences amongst groups were analysed by one-way ANOVA, followed by Duncan's test. A P value < 0.05 was considered statistically significant.

3. Results

The Caco-2 cell viability was lowest in the FF group, approximately suppressed by 23% as compared with the HBSS buffer-treated control group (Fig. 1). KGM, F1, F2, and oligofructose similarly and significantly suppressed the fecal water-induced cell death by 13–19% as compared with the FF diet ($P < 0.05$). In addition, the DNA damage of Caco-2 cells, induced by the fecal water, was significantly suppressed by oligofructose (62%) and by KGM-based fibres, in descending order: F2 (53%), F1 (48%), and KGM (37%) (Table 1). All KGM-based and oligofructose diets also alleviated the additional DNA damage induced by FPG. These protective effects of F1 and F2 on FPG-lesion were superior to those of KGM and oligofructose, respectively.

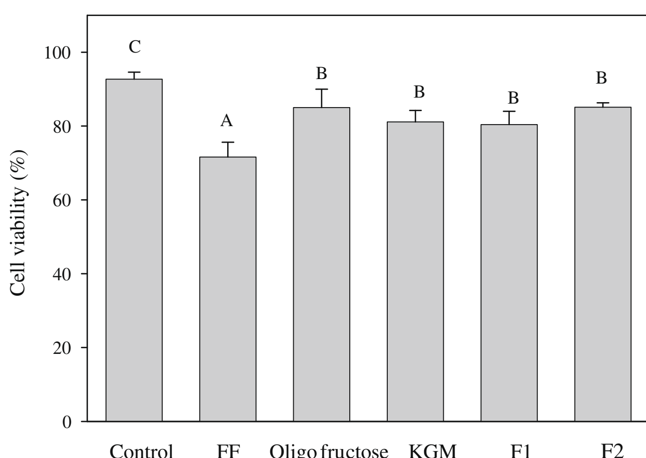

Fig. 1. The effect of fecal water from mice fed different diets on the viability of Caco-2 cells. The cells were cultured in DMEM medium and harvested at 90% confluence (viability refers to 100%), followed by washing with PBS before incubation with HBSS solution (control) or the fecal water at 37 °C for 3 h in a shaking water bath. Bars (means ± SD, $n = 8$) not sharing a common superscript are significantly different ($P < 0.05$). FF = fibre-free; KGM = konjac glucomannan; F1, 2 = hydrolysed KGM with DP = 8 and 4, respectively.

Table 1
DNA damage (tail moment) induced by different fecal waters, alone or in combination with FPG in Caco-2 cells.[a,b]

Treatments[c]	Fecal water only	Fecal water + FPG	FPG-lesion
Control	3.62 ± 0.18[A]	20.8 ± 0.58	17.2 ± 0.50[A]
FF	13.5 ± 0.42[F]	38.4 ± 0.58	25.0 ± 0.50[D]
Oligofructose	5.10 ± 0.24[B]	27.7 ± 0.78	22.6 ± 0.49[C]
KGM	8.42 ± 0.44[E]	30.9 ± 0.40	22.4 ± 0.37[C]
F1	7.05 ± 0.15[D]	25.3 ± 0.54	18.2 ± 0.48[B]
F2	6.39 ± 0.36[C]	24.9 ± 0.60	18.5 ± 0.52[B]

[a] The cells were preincubated in HBSS solution (control) or with fecal water at 37 °C for 3 h in a shaking water bath. Afterwards, half of the slides were treated with formamidopyrimidine glycosylase (FPG, 50 μl, 1 mg/ml) as described in the method. DNA damage of FPG-lesion = DNA damage of (fecal water + FPG) – DNA damage of fecal water only.
[b] Data (means ± SD, $n = 8$) in the same column not sharing a common superscript are significantly different ($P < 0.05$).
[c] FF, fibre-free; KGM, konjac glucomannan; F1 and F2, hydrolysed KGM with mean; and DP = 8 and 4, respectively.

Table 2
The effects of different dietary fibres on fecal weight and moisture in BALB/c mice.[a]

Treatments[b]	Fecal weight (g/d)	Fecal moisture (%)
FF	0.76 ± 0.25[A]	57.2 ± 8.3[A]
Oligofructose	1.12 ± 0.25[B]	67.9 ± 6.1[B]
KGM	1.06 ± 0.34[B]	62.7 ± 8.9[AB]
F1	1.15 ± 0.26[B]	65.2 ± 4.8[B]
F2	1.13 ± 0.24[B]	63.6 ± 4.7[AB]

[a] Data (means ± SD, $n = 8$) in the same column not sharing a common superscript are significantly different ($P < 0.05$).
[b] FF, fibre-free; KGM, konjac glucomannan; F1 and F2, hydrolysed KGM with mean; and DP = 8 and 4, respectively.

All KGM-based diets and the oligofructose diet significantly increased the fecal weight in a similar manner, as compared with the FF diet (Table 2). In addition, only the F1 diet, like the oligofructose diet, significantly increased the fecal moisture, as compared with the FF diet.

Oligofructose, KGM, F1 and F2 significantly increased the concentrations of fecal lactobacilli and bifidobacteria without significant effects on total anaerobes and *C. perfringens*, as compared with the FF diet (Table 3). Furthermore, the enhancing effects of F1 and F2 were similar to those of oligofructose on fecal lactobacilli concentration and on bifidobacteria concentration. Effects of KGM on fecal probiotics were inferior to those of its hydrolysed fractions, and oligofructose.

In addition, the ferrous ion-chelating abilities of the fecal waters obtained from the oligofructose, KGM, F1 and F2 groups were 47.0 ± 1.0%, 53.5 ± 0.5%, 40.5 ± 1.0%, and 45.5 ± 1.5%, respectively, which were all significantly greater than that of fecal water

Table 3
The effects of different dietary fibres on the fecal microflora concentration in BALB/c mice.[a]

Treatment[b]	Cell counts (log_{10} CFU/g faeces)			
	TA	*Lactobacilli*	*Bifidobacterium*	*C. perfringens*
FF	10.0 ± 0.3[A]	8.7 ± 0.1[A]	8.5 ± 0.1[A]	8.9 ± 0.1[AB]
Oligofructose	10.4 ± 0.2[AB]	9.3 ± 0.2[C]	9.3 ± 0.1[C]	9.0 ± 0.2[B]
KGM	10.3 ± 0.3[AB]	9.0 ± 0.1[B]	9.0 ± 0.1[B]	8.7 ± 0.2[A]
F1	10.4 ± 0.3[AB]	9.3 ± 0.1[C]	9.4 ± 0.1[C]	8.8 ± 0.2[AB]
F2	10.5 ± 0.1[B]	9.3 ± 0.1[C]	9.5 ± 0.1[C]	9.1 ± 0.1[B]

[a] Data (means ± SD, $n = 8$) in the same column not sharing a common superscript are significantly different ($P < 0.05$).
[b] FF, fibre-free; KGM, konjac glucomannan; F1 and F2, hydrolysed KGM with mean; and DP = 8 and 4, respectively.

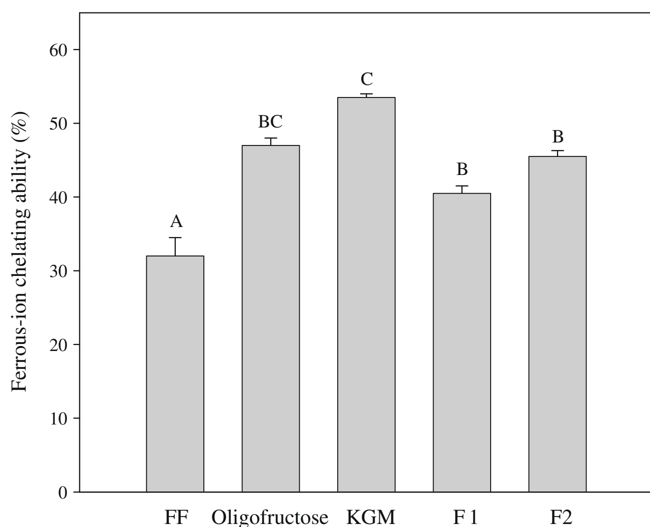

Fig. 2. The ferrous ion-chelating abilities of fecal water obtained from various groups. Bars (means ± SD, $n = 8$) not sharing a common superscript are significantly different ($P < 0.05$). FF = fibre-free; KGM = konjac glucomannan; F1, 2 = hydrolysed KGM with mean DP = 8 and 4, respectively.

obtained from the FF group (32.0 ± 2.5%) (Fig. 2). The fecal water obtained from the KGM group exerted greater ferrous ion-chelating ability than did those from the F1, F2, and oligofructose groups.

4. Discussion

There are several innovative findings in this study. First, we showed that the unhydrolysed KGM and the partially-hydrolysed F1 and F2, could similarly reduce the cytotoxicity of fecal water from mice, using Caco-2 cells as an assessment tool. We further used comet assay to assess the toxicity of fecal water on DNA integrity of Caco-2 cells and found differential effects amongst native and partially-hydrolysed KGM. These results confirm that the comet assay is a better tool for assessing the toxicity of fecal water obtained from animals fed with different dietary fibre sources (Yeh et al., 2007). Second, this study demonstrated that partially-hydrolysed KGMs significantly enhanced the protective effects of native KGM against DNA damage of Caco-2 cells induced by fecal water whilst F2, with DP = 4, exerted a better protective effect than did F1, with DP = 8. Third, this study demonstrated that KGM oligomers also enhanced the protective effect of KGM against FPG-induced DNA damage. Furthermore, F1 and F2 were more potent than was oligofructose in reducing FPG-induced DNA damage.

The prebiotic effects of inulin-type fibres and KGM with various DP have been compared in several studies (Chen et al., 2005; van de Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007; van Loo, Coussement, de Leenheer, Hoebregs, & Smits, 1995), and most of these studies have demonstrated the benefit of a lower DP (Chen et al., 2005; van Loo et al., 1995). The present study compared the prebiotic effects of KGM hydrolysates with different DP, and also showed the benefit of lower DP on fecal bifidobacteria. We again demonstrated that the prebiotic effect of KGM was not as potent as was oligofructose, a well-known superior prebiotic (Yeh et al., 2007). However, the prebiotic effects of both F1 and F2, on fecal lactobacilli concentration, were as good as those of oligofructose and tended to be better than those of oligofructose on bifidobacterial concentration, in this study.

The previous study suggests that the protective effect of dietary fibre on fecal water-induced DNA damage in Caco-2 cells may be partly mediated by its prebiotic effect (Yeh et al., 2007). In agreement with that, the present study indicated that the stimulatory effects of KGM-based fibres on fecal bifidobacteria counts correlated with their inhibitory effects on fecal water-induced DNA damage. In addition, since the FPG-lesion is an index of oxidised nucleic acids, this study confirmed that fecal water caused oxidative damage in intestinal epithelial cells, and suggested that prebiotic dietary fibres could reduce this fecal water-induced oxidative damage (Venturi, Hambly, Glinghammar, Rafter, & Rowland, 1997). Furthermore, the present study showed that the trend of inhibitory effects in KGM-based fibres, on the FPG-lesion, was approximately correlated with the trend of their prebiotic effects. Therefore, our study suggests that the prebiotic effects of oligofructose and KGM-based fibres play a vital role in protection against formation of oxidised DNA bases. This observation was supported by studies that demonstrate the antioxidative capacity of probiotics (Kaizu, Sasaki, Nakajima, & Suzuki, 1993; Virtanen, Pihlanto, Akkanen, & Korhonen, 2007).

The metal ions in faeces may lead to DNA damage and even cell death by their pro-oxidative activity (Halliwell & Gutteridge, 2007). The dietary fibre, either from mixed sources of vegetables and whole meal products (Rieger et al., 1999), purified soluble dietary fibres, such as native KGM, oligofructose and inulin (Yeh et al., 2007) or from KGM hydrolysates (in this study), increased iron-chelating ability of fecal water. In addition, our present study, in agreement with the previous study (Yeh et al., 2007), indicated that the KGM polysaccharide caused greater fecal water ferrous ion-chelating ability than did oligosaccharides. However, although KGM possessed the greatest ferrous ion-chelating ability, it exerted the lowest protective effect on DNA damage (as compared to either inulin-type oligosaccharide (Yeh et al., 2007) or any KGM hydrolysate fractions). Therefore, the ferrous ion-chelating ability of fecal water might not be a good indicator of the protective effect of soluble dietary fibre on DNA damage of colonic epithelial cells, particularly when the fibre was incorporated in a normal, non-iron-enriched diet.

The fecal weight and fecal moisture did not significantly differ amongst KGM and hydrolysate groups, suggesting that these two factors did not significantly contribute to the protective effects of these fibres on fecal water-induced DNA damage and oxidised DNA base formation.

The present study indicates that the KGM hydrolysates of DP ranging from 4 to 8 enhanced effects of native KGM on reducing fecal water-induced DNA damage in Caco-2 cells. A similar trend in the effect of KGM-based fibres on probiotics growth was also shown. The fecal probiotics may be the major mechanism for the beneficial effects of KGM and its hydrolysates in protecting against fecal water-induced DNA damage in Caco-2 cells.

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