

Effect of selected cereal grains on *in vitro* bioaccessibility of isoflavones in soymilk

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Abstract

Soymilk, which can be called as plant based “milk” is a favourite traditional beverage in Asian with various beneficial health protective effects due to the presence of isoflavones. Mixing cereal grains into soymilk to make a wholesome beverage for breakfast becomes popular. However, dietary factors may affect the bioaccessibility of isoflavones. The aim of this current work was to evaluate changes in the bioaccessibility of isoflavones in soymilk after mixing with selected cereal grains following *in vitro* gastrointestinal digestion. The samples were subjected to *in vitro* gastrointestinal digestion with its isoflavones content determined before and after simulated gastric digestion and intestinal digestion with dialysis membrane. Soymilk contained 1.80 ± 0.03 mg daidzein and 2.12 ± 0.08 mg genistein in 100 ml, while bioaccessibility of soymilk daidzein and genistein were $11.24 \pm 0.46\%$ and $5.09 \pm 0.25\%$ respectively. The addition of cereal grains except barley in soymilk showed significant reduction ($p < 0.05$) in bioaccessibility of isoflavones. Dietary fiber content, especially the insoluble fiber of cereal grains was related to the reduction of bioaccessibility of isoflavones by its entrapping affinity of isoflavones and viscosity effect in the gut. The higher the dietary fiber added into the soymilk, the lower the bioaccessibility of isoflavones in soymilk.

Keywords

In vitro gastrointestinal digestion
Bioaccessibility
Daidzein
Genistein
Soymilk
Cereal grains

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Introduction

Soymilk is an aqueous extract of whole soybeans. It is produced by soaking soybeans, then finely grinding soybeans with water. Recently, as consumers become more health conscious, soymilk is becoming more popular due to the association of soybean consumption with variety health protective effects. Growing evidence showed that soy based foods may contribute to lower occurrences of non-communicable disease, such as cardiovascular disease (Siow and Mann, 2010), type 2 diabetes (Nakamoto *et al.*, 2014), and decreased risk of certain types of cancers such as breast cancer (Hilakivi-Clarke *et al.*, 2010; D’Adamo and Sahin, 2014; Wu *et al.*, 2015) and prostate cancers (Lakshman *et al.*, 2008; Xu *et al.*, 2009) as well as bone health (Ma *et al.*, 2008) and relief menopausal symptoms (Goodman *et al.*, 2011; Taku *et al.*, 2012; Chen *et al.*, 2015). These health protective effects from soy are due to the presence of soy proteins with excellent source of isoflavones. Isoflavones are flavonoids, have a structural similarity to estrogens and therefore also named as phytoestrogen. It confers pseudo-hormonal properties on isoflavones, including the ability to

bind estrogen receptors (Manach *et al.*, 2004).

Apart of soymilk, cereal grains, which are seeds from the grass family Gramineae have gained reputation in contributing to healthy eating behavior due to the presence of antioxidant properties (Fardet *et al.*, 2008). Several studies showed that high intake of dietary fiber, particular grain is associated with lower mortality (Chuang *et al.*, 2012; Johnsen *et al.*, 2015). Thus, there is a trend of increase in consumption of breakfast cereals as consumers nowadays are more knowledgeable and health conscious. Breakfast cereals are commonly mixed into beverage, such as cow milk or soymilk and generally taken during breakfast.

However, when studying the benefits of isoflavones on human health, the bioaccessibility of bioactive compounds is crucial. Before become accessible for absorption, isoflavones must be released from food matrix and modified by the gastrointestinal enzymes. Thus, analyzing whether the digestion process affects the bioactive compounds and their stability is utmost important before concluding on any potential health effect. Soy isoflavones bioaccessibility in humans appears to depend on a variety of factors, which are isoflavone compounds,

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sources, dose, gender, age, gut transit time, diet and food matrix, and lastly effect of processing and storage (Nielsen and Williamson, 2007). For food matrix and diet, polyphenol bioaccessibility can be influenced by macro-constituents, which are dietary fiber, dietary lipids, proteins and digestible carbohydrates, as well as minor-constituents, such as minerals and trace elements (Bohn, 2014).

To date, little data exists regarding the bioaccessibility of isoflavones in soymilk with added cereal grains. Due to current consumption pattern, the aim of this current study was to examine the effect of four different types of cereal grains (oats, barley, wheat, corn) and multigrain powder on *in vitro* bioaccessibility of isoflavones in soymilk.

Materials and Methods

Materials

Fresh home-made soymilk was purchased at a soymilk stall in Seremban, Negeri Sembilan, Malaysia. Oat, barley, corn cereals, and wheat cereals were categorized and randomly selected from a supermarket in Seri Kembangan, Selangor, Malaysia. For multigrain powder, it was randomly purchased at an organic shop in Seremban, Negeri Sembilan, Malaysia.

Oat, barley, corn cereals, wheat cereals and multigrain powder was measured according to the amount of half serving size as recommended on pack, then it was grinded and homogenized. Two hundreds milliliters of soy milk which served as one serving size was added with oat, barley, corn cereals, wheat cereals, and multigrain powder. After that, all the samples were freeze dried using free dryer (Virtis Benchtop K, Germany), then grinded into powder form and homogenized.

Pepsin (P-7000, from porcine stomach mucosa), pancreatin (P-1750, from porcine pancreas), bile extract (B-8631, from porcine), cellulose dialysis membrane (molecular weight cutoff of 12 000 Da; flat width 33mm), genistein analytical standards and daidzein analytical standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States). Hydrochloric acid, HPLC-grade methanol, HPLC-grade acetonitrile and sodium bicarbonate were purchased from RandM Chemical (United Kingdom). Filters (0.2 μ m) were purchased from Millipore Iberica S.A. (Madrid, Spain). Ethanol was obtained from Fisher Scientific (Leicestershire, England).

In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion model

adapted from Gil-Izquierdo *et al.* (2002), with some modification was used to stimulate the gastrointestinal digestion. This digestion model was carried out in two phases, namely gastric and intestinal digestion with dialysis membrane. It was performed in triplicate for each sample.

For gastric digestion stage, 3 g of each sample was mixed with 20 ml distilled water and 1ml pepsin solution. The pH was adjusted to 2 with addition of 5 M hydrochloric acid and incubated at 37°C in an incubator shaker (Heidolph Unimax 1000, Germany) at 90 rpm for 2h.

Titrate acidity was determined on a 20 ml homogenous aliquot of the pepsin digest added with 5ml of pancreatin-bile extract mixture. It was defined as the amount of 0.5N sodium bicarbonate required to titrate the pepsin digest pancreatic bile extract mixture to pH 7.5.

A segment of dialysis membrane with 18 cm of length was filled with 25 ml water and the amount of sodium bicarbonate (0.5N) equivalent to the titratable acidity determined as above was added. For intestinal digestion, 20 ml of gastric digest was placed into polyethylene tube and then the dialysis membrane containing water-sodium bicarbonate mixture was completely immersed into the gastric digest. It was incubated in a 37°C incubator shaker for 30 minutes or longer until the pH of the digest reached 5.0. Then, a 5 ml of pancreatin-bile mixture was added to the tube, and the incubation continued for another 2 hours at 90 rpm and 37°C. After 2 hour, the dialysis membrane was removed and rinsed with distilled water. Two fractions were obtained after intestinal digestion, which were duodenal and dialysed fraction. Duodenal fraction was referred as portion of bioactive compounds that remained outside the dialysis membrane and were also considered as unabsorbed compounds, whereas the bioactive compounds available for absorption were inside the dialysis membrane. Only the dialysed fractions were collected for isoflavones content analyze.

After each digestive phase, the aliquots were taken and immediately placed in cold water bath for 10 minutes to stop digestion. Subsequently, the aliquots were frozen to -40°C until the isoflavone contents were analyzed by HPLC.

Determination of isoflavones

Isoflavones (Daidzein and Genistein) were analysed using the method adapted from Rodríguez-Roque *et al.* (2013), with some modifications. Aliquots of non-digested and dialysed fraction of soymilk and 5 types of soymilk mixed with cereal grains (soymilk with wheat cereal; soymilk with

corn cereal; soymilk with barley; soymilk with oats; soymilk with multigrain) were analyzed for its isoflavones contents.

For the extraction and hydrolysis of isoflavones, 4ml of the aliquots were mixed with 4 ml of 80% ethanol acidified to 1M with HCl into a set of 20 ml screw-top centrifuge tubes. Then, the tubes were capped and this mixture was incubated in water bath (Memmert, Schwabach, Germany) for 1 hour at 80°C. After 1 hour, samples were cooled and shaken vigorously for 2 minutes. Cooled samples were centrifuged at 9000 rpm for 10 minutes at 4°C using Allegra 64R centrifuge (Beckman Coulter, Mervue Galway, Ireland). Supernatant was removed and transferred into a 10 ml polyethylene tube. For the residue, it was re-extracted with 2 ml of 80% ethanol. Samples were re-centrifuged at 9000rpm for another 10 minutes at 4°C. Both supernatant were combined into polyethylene tube, and then was filled to 10 ml with 80% ethanol. The samples were filtered throughout with 0.2 µm millipore filter and kept at -40°C until HPLC analysis.

A HPLC analysis was carried out using HPLC system equipped with two precision pump with a diode array detector set at 248 nm and 260 nm. The chromatographic separation was performed using a C18 SunFire (5 µm) stainless steel column (3 mm x 150 mm) connected to a guard column C28 SunFire (5 µm). A sample of 50 µl was injected to the HPLC system, with a flow rate of 1ml/min and operating temperature of 35°C. The chromatographic separation was carried out in a gradient profile as shown in Table 1. The total run time was 55 minutes. A gradient elution (refer Table 1) was employed with a mixture of two solvents: (A) water/methanol (80:20 v/v) and (B) water/methanol/acetonitrile (40:40:20 v/v/v).

The identification of isoflavone (Daidzein and Genistein) was done by comparing the retention time with the respective reference standards. Quantification of isoflavones was carried out by integration of peak areas. Data were compared to calibration curves of each isoflavones and results were expressed as milligrams of isoflavones per 100ml of soymilk.

Bioaccessibility calculations

Bioaccessibility was considered as the concentration of bioactive compounds which were released from the food matrix after in-vitro gastrointestinal digestion and which is available for absorption. The bioaccessibility calculation as below:

$$\text{Bioaccessibility (\%)} = \left(\frac{BC_{\text{dialysed}}}{BC_{\text{(non-digested)}}} \right) \times 100$$

Table 1. Mobile Phase Gradient for Determination of Isoflavones by HPLC

Time (min)	Flow (ml/min)	A (%)	B (%)
0	1.0	80	20
15	1.0	80	20
25	1.0	0	100
50	1.0	0	100
52	1.0	80	20
55	1.0	80	20

Where BC_{dialysed} and $BC_{\text{(non-digested)}}$ corresponded to the bioactive compound concentration (mg/100 ml) in dialysed fraction and non-digested soymilk respectively.

Statistical analysis

IBM SPSS version 20.0 was used in the data analysis. The amount and percentage of bioaccessibility of isoflavones were reported as mean and standard deviation for all three replicate measurements. Besides that, One-way analysis of variance (ANOVA) was used in order to determine the significant differences percentages of bioaccessibility of isoflavones in soymilk with selected types of grains added followed by Turkey HSD post hoc test. The limit of probability of significance was fixed at $p < 0.05$.

Results and Discussion

Isoflavones content

In this study, the soymilk isoflavones profiles were 1.80 ± 0.03 mg/100 ml daidzein and 2.12 ± 0.08 mg/100ml genistein. As can be seen in Table 2, it was the lowest as compared with the USDA database as well as previous study. However in term of daidzein : genistein ratio in soymilk, the current study had quite similar ratio with the USDA database, which were 1:1.2 and 1:1.3, while other studies found different ratio of 1:2.1 (Rodríguez-Roque *et al.*, 2013) and 3:1 to 13:1 (Hasnah *et al.*, 2009). Variations of daidzein and genistein contents in soymilk could be due to different variety of soybeans used and processing techniques in producing soymilk (Jackson *et al.*, 2002; Huang *et al.*, 2006; Hasnah *et al.*, 2009). Besides that, some of the isoflavones may also be lost in the water used to soak raw soybeans, and by-products such as okara (Jackson *et al.*, 2002).

Thermal treatment, which is a key process of production of soymilk significantly influenced content and profile of isoflavones (Huang *et al.*,

Table 2. Isoflavones Profile in Soymilk Compared to USDA Database and Other Studies

Isoflavones	Current study* (n=3)	Nutrient Contents (mg/100ml)		
		USDA Database (Bhagwat <i>et al.</i> , 2008)*	Malaysia (Hasnah <i>et al.</i> , 2009)	Spain (Rodríguez- Roque <i>et al.</i> , 2013)*
Daidzein	1.80 ± 0.03	4.84 ± 1.71	19-24	3.40 ± 0.12
Genistein	2.12 ± 0.08	6.07 ± 0.47	1-4	7.13 ± 0.05

* Values were expressed as mean ± standard deviation.

Table 3. Mean Percentage of Daidzein and Genistein Bioaccessibility of Different Type of Soymilk-Based Samples after Undergone *In Vitro* Gastrointestinal Digestion

Samples	Bioaccessibility (%)*	
	Daidzein	Genistein
Soymilk	11.24 ± 0.46 ^a	5.09 ± 0.25 ^a
Soymilk with Wheat Cereal	8.90 ± 0.43 ^b	4.01 ± 0.20 ^{be}
Soymilk with Corn Cereal	9.83 ± 0.14 ^{bc}	4.54 ± 0.05 ^{cde}
Soymilk with Barley	10.18 ± 0.30 ^{acd}	4.81 ± 0.27 ^{ac}
Soymilk with Oats	7.64 ± 0.80 ^e	3.77 ± 0.09 ^b
Soymilk with Multigrain	9.89 ± 0.20 ^{bd}	4.20 ± 0.23 ^{bd}

* Values were expressed as mean ± standard deviation of three replicates (n=3). Different letters in the same column indicate significant differences (p<0.05).

2006; Xu and Chang, 2009). From study by Huang *et al.* (2006), thermal treatment significantly influenced the contents of daidzein and genistein in soymilk. In raw soymilk, this study showed that the ratio between daidzein and genistein was found to be present in the ratio of roughly 10:1. After the thermal treatment of soymilk, the daidzein content was about half that genistein. Studies showed that genistein was rather heat stable compound compared with daidzein, thus causing different extent of loss of daidzein and genistein in soymilk during thermal processing (Huang *et al.*, 2006; Stintzing *et al.*, 2006). This result is consistent with our finding showing that genistein content in ready-to-drink soymilk is higher than daidzein as shown in Table 2.

Bioaccessibility of daidzein and genistein

The bioaccessibility of isoflavones was expressed as a percentage. The dialysed fraction of soymilk was obtained after gastric and intestinal digestion through *in vitro* assay with controlled pH, temperature, enzyme and chemical conditions. Overall, this study showed that daidzein bioaccessibility (11.24 ± 0.46%) was almost two times higher than genistein bioaccessibility (5.09 ± 0.25%) as shown in Table 3.

In other words, daidzein was more accessible for body absorption compared with genistein. Studies done by Walsh *et al.* (2003) and Ma *et al.* (2014) also revealed similar result. Bioaccessibility could be defined as dietary compound release from its parent matrix into aqueous fraction to be uptake by absorptive epithelial cells (Walsh *et al.*, 2003). A recent study by Simmons *et al.* (2012) presented that the efficiency of partitioning in aqueous fraction was greater by daidzein than genistein. Therefore, this statement further support our current result that daidzein was more accessible for potential uptake by absorptive epithelial cells due to the greater hydrophilicity of daidzein.

In term of comparing between samples, the bioaccessibility of daidzein and genistein showed statistically significant difference (p<0.05) between soymilk and soymilk mixed with different types of cereal grains, except soymilk mixed with barley (Table 3). Thus, adding cereal grains showed significant decreased bioaccessibility of daidzein and genistein in soymilk except barley.

Influence of macro-constituents, such as fats, protein, and dietary fiber on the bioaccessibility of isoflavones have been widely investigated (Tew *et*

al., 1996; Walsh *et al.*, 2003; Motoi *et al.*, 2009). In a review later, dietary fiber is mentioned as the key influence on polyphenol bioaccessibility (Bohn, 2014). Chitindingu *et al.* (2015) observed that higher dietary fiber content indicated lower bioaccessibility levels of polyphenol in cereal grains. A randomized controlled trial study showed that the addition of 40g dietary fiber to a meal containing 15 g wheat fiber diet decreased plasma appearance of isoflavonoid genistein by 55% in humans (Tew *et al.*, 1996). In an animal model, the addition of dietary fiber by 5% hemicellulose to a 5% cellulose diet was demonstrated to reduce plasma daidzein concentration, showing decrease availability of daidzein (Motoi *et al.*, 2009). All these statements showed that addition dietary fiber into soymilk was expected to impact the bioaccessibility of daidzein and genistein.

In current study, adding cereal grains into soymilk except barley showed significant decreased bioaccessibility of isoflavones. It could be due to the general binding effect of dietary fiber from cereal grains by physically trapping the isoflavones during digestion (Palafox-Carlos *et al.*, 2011; Bohn, 2014). Besides that, the added dietary fiber enhances viscosity of gastric fluids restricting the peristaltic mixing process that promotes transport of enzymes to their substrates (Palafox-Carlos *et al.*, 2011). Previous studies showed that increase aglycone contents, which are more accessible in intestinal environment during the gastric phase was due to acid hydrolysis of ester and glycosidic bonds of glucosides (Simmons *et al.*, 2012; Ma *et al.*, 2014). Thus, the viscosity effect of dietary fiber from cereal grains would affect the enzymatic process during gastric stage and reduce the hydrolysis process to break down glucosides into aglycone form of isoflavones in soymilk.

Soymilk mixed with barley had slightly lower daidzein and genistein bioaccessibility compared with soymilk, which were $10.18 \pm 0.30\%$ and $4.81 \pm 0.27\%$ respectively and the value showed no significant difference ($p \geq 0.05$) with soymilk. Barley used in the current study was pearled barley which undergoes de-hulling and pearling. Dehulling mainly removes the hull and only a small portions of bran, germ and endosperm, while pearling barley grains further removes the remaining hull, bran, germ and also part of the endosperm (Baik and Ullrich, 2008). Pearling and dehulling reduce the contents of insoluble fibre, protein, ash and free lipids due to removing hull (palea and lemma), bran (pericarp, testa) and germ (embryo) (Baik and Ullrich, 2008). In brief, added pearled barley has less impact on bioaccessibility of isoflavones in soymilk due to low dietary fiber content.

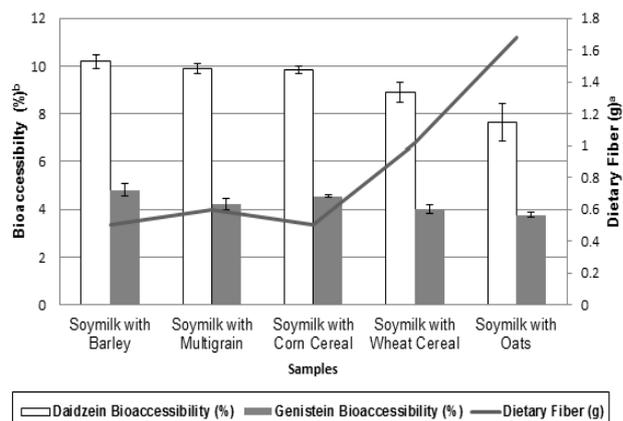


Figure 1. Comparison between Dietary Fiber Content with Daidzein and Genistein Bioaccessibility

^a Values of dietary fiber are according to the nutrition labeling of the cereal grains.

^b Values were expressed as mean \pm standard deviation of three replicates (n=3)

Adding oats into soymilk beverage showed the lowest bioaccessibility of daidzein and genistein with $7.64 \pm 0.80\%$ and $3.77 \pm 0.09\%$ respectively. The bioaccessibility of daidzein in soymilk mixed with oats was significantly different ($p < 0.05$) as compared to others. However, genistein bioaccessibility was only significantly different with soymilk, soymilk mixed with corn cereal and barley. This observation is supported as oats had high dietary fiber content among the cereal grains used in this study (Figure 1). Previous study reported that oats was excellent binders of 17β -estradiol, whereas corn had a lower binding capacity with a relatively low affinity (Arts *et al.*, 1991). Thus, oats may affect the bioavailability of estrogens as well as phytoestrogen which have similar chemical structure, such as daidzein and genistein. The binding of estrogens has been shown to be related to the presence of high insoluble fiber, known as lignin (Arts *et al.*, 1991). Oats showed highest percentage of lignin content, which was more than 3%, while other grains, such as barley, wheat and corn showed less than 1.2% of lignin content (Arts *et al.*, 1991). Thus, the presence of lignin in cereal grains would affect the isoflavones bioaccessibility in soymilk.

Insoluble fiber may be more effective in binding and excreting estrogen in stool consequential with the decrease in serum estrone and estradiol (Park *et al.*, 2009). Insoluble fibers, such as lignin, cellulose and some hemicellulose are not water soluble, could not form gel and limited fermentation ability (Lattimer and Haub, 2010). A study on composition in oats revealed higher insoluble fiber content (6.0-7.1%) compared with soluble fiber (4.1-4.9%) (Manthey *et al.*, 1999). All these evidences further supported that

insoluble fibers, especially lignin which is present in huge amount in oats may contribute to the binding effect of phytoestrogen in soymilk and consequently lowering the bioaccessibility of daidzein and genistein. In short, the higher the dietary fiber added, the lower the bioaccessibility of daidzein and genistein in soymilk.

Conclusion

Soymilk contained 1.80 ± 0.03 mg daidzein and 2.12 ± 0.08 mg genistein in 100ml. Daidzein bioaccessibility ($11.24 \pm 0.46\%$) was two-fold higher than genistein bioaccessibility ($5.09 \pm 0.25\%$), showing daidzein more accessible for absorption in gastrointestinal environment. Soymilk was found to have the highest isoflavones bioaccessibility and statistical test showing significant difference with samples which mixed with cereal grains except soymilk with barley. Dietary fiber has been known as the key influence on the isoflavones bioaccessibility. The added cereal grains in soymilk may increase the amount of dietary fiber, thus entrapment of isoflavones from added dietary fiber from cereal grains occurred during digestion in gastrointestinal environment. In short, the higher the dietary fiber added expressed the lower the bioaccessibility of daidzein and genistein in soymilk. Further research is needed to gain further insight into the fiber composition of cereal grains with the interactions of isoflavones on the bioaccessibility of isoflavones in the gastrointestinal tract.

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