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Modified Dietary Fiber from Cassava Pulp and Assessment of Mercury Bioaccessibility and Intestinal Uptake Using an In vitro Digestion/Caco-2 Model

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Abstract

The objectives of this study were to determine the effects of modified dietary fiber (MDF) on mercury bioaccessibility and bioavailability. The preparation modified dietary fiber from cassava pulp was start by separation starch and protein from fiber by enzyme application to prepare crude dietary fiber and modified them with etherification method. The MDF affecting the mercury bioavailability was estimated by using couple in vitro digestion and Caco-2 human intestinal cell model system. In vitro digestion (bioaccessibility) showed that the MDF could reduce mercury bioaccessibility to 35-85% compared with control (0-1000 mg of MDF in 1 g of fish tissue) in a dose dependent manner. The effect of fish tissue amount (0 - 4 g) on mercury quantification when 500 mg of MDF was added in digestion model test showed that the MDF did assist with reduction of mercury amount in fish tissue from 70-84% compared with control that MDF was not added. Furthermore, the Caco-2 cell was utilized for evaluation of intestinal cell accumulation and supporting reliable estimating bioavailability. The results showed that the mercury transfer to intracellular range from 9.07-5.97% for control and 5.09-6.68% in the media containing 500 mg MDF. In conclusion, this study suggests that MDF prepared from cassava pulp could decrease mercury bioavailability by inhibition the mercury transfer to the aqueous fraction and could be applied in functional food and dietary supplement products.

Keywords: *Cassava pulp, Dietary Fiber, Modified Dietary Fiber, In vitro Digestion, Caco-2 cell line*

1. Introduction

Mercury is naturally occurring elements which is in the earth's crust and released in to the environment when coal is burned that are the largest source of mercury emission to the air (US EPA (US Environmental Protection Agency), 2013). It found in various form: elemental or metallic mercury, inorganic mercury compounds, and organic mercury compounds (WHO, 2007). Methyl mercury is a form of mercury, which is form in water when other forms of mercury in the water react with certain bacteria. Human could be exposed mercury in methyl mercury form by various ways. Eating fish or shellfish that contaminated with mercury are the main cause of methyl mercury exposure. (WHO, 2013; ATSDR, 1999). For example, bass (striped), bluefish, chilean sea bass, golden snapper, walley fish, king mackerel, tile fish, swordfish, shark and tuna have the highest levels of mercury (Gochfeld 2003).

Mercury is highly toxic to human health effecting on nervous system, heart, kidneys, lung, immune system and eventually death (US FDA, 2013). The nervous system is high sensitive to all forms of mercury. Methyl mercury and metallic mercury vapors give more damage to central nervous system than other forms. Exposure to high level of metallic mercury vapors in short term cause lung damage, nausea, vomiting, diarrhea, increase in blood pressure or heart rate, skin rashes and eye irritation (ATSDR, 1999). Life-stage other than the embryo and fetus stage may be less sensitive to methyl mercury (JECFA, 2006). Children have more sensitive than adults. For adults (up to about 17 years) tolerable intake of mercury about twice per week would not any risk of neurotoxicity (WHO, 2007).

Ethylenediaminetetraacetic acid (EDTA), Diethylenetriaminepentaacetic acid (DTPA) 2,3-mercaptopropanol (BAL), D-Penicillamine (D-β, β-dimethylcysteine), Deferoxamine dimercaptosuccinic acid (DMSA), and penicillamine (Tandon et al. 2001) are commonly used for metal toxicity removal but reported for their side effects (Karlsson et al. 2010). Recently, many researchers have been reported applying dietary fibers from various food products to be heavy metal adsorbents because of their nontoxic and biodegradable nature (Nawirska 2005, Hu et al. 2010).

Cassava pulp is a by-product of cassava starch factory processing. Cassava (Manihot esculenta Crantz.) is the main crop of Thailand especially in the northeast of Thailand. Total cassava root crops of Thailand in 2011 are about 21,060,903 tones (Thailand Tapioca starch, 2011). Cassava pulp, accounts for approximately 10–30% by weight (wet) of the original tubers (Kosugi et al., 2009). It's contains approximately 50% starch, and 15% fiber (Sriroth et al., 2000). Most of cassava pulps are used for feed. So, there is a potential cassava pulp application for heavy metals binding to prevent their toxicities.

Bioaccessibility is a concept related to bioavailability. Bioaccessibility is the fraction of a chemical that could be dissolved or released from food by the digestive fluids and available for absorption in the gastrointestinal tract (Tokalıoğlu et al. 2014). Bioavailability is the fraction of

chemical that is released from food through desorption processes under physiological condition (Donhowe et al. 2014)

2. Materials and methods

2.1 Sample preparation

Dried Cassava Pulp leaves were used as a source of fiber in this study will be obtained from Sanguan Wongse Starch Co.,Ltd. Drying at 60 °C for 8-12 hours. Grinding with a grinder until a fine power will be ground until they were fine powder. The cassava pulp powder was then kept in a sealed container until further treatment.

Preparing the crude dietary fiber, start with dried cassava pulp 5 g in phosphate buffer 50 ml. Adjust pH to 6 and add 0.1% of α-amylase (w/v) for 30 min at 95°C. Then, adjust pH to 7.5 with sodium hydroxide solution and add 1% of neutrase (v/v) for 30 min at 60°C. Adjust pH to 6 with hydrochloric acid solution and add 0.1% of amyloglucosidase (v/v) for 30 min at 60°C. The resulting hydrolysate is separate by centrifugation 10 min at 30000xg. The sediment is wash with doubly distilled water, re-centrifuge for 10 min at 30000xg and drying at 60°C in hot air oven.

The modified dietary fiber prepared by using crude dietary fiber with CS2 and NaOH (17.5%w/v) in a ratio of 1:3:6, respectively. Samples were placed in a reaction bottle of 100 mL at 30°C for 1 h; the bottle was shaken for another 1 h at room temperature. Homogenously with AN in the ratio of 1:3 (crude fiber: AN (1:3) at 30°C for 2 h with occasional shaking. Dilute HCl, as precipitating agent, precipitates which is then filtered and washed with distilled water until neutrality, dried at 60°C for 24 h under vacuum (Kamel et al., 2005). Sample is treated either by an aqueous solution of hydroxylamine at a known concentration or by an aqueous solution of hydroxylamine hydrochloride, whose pH is adjusted to between 9 and 10 by adding sodium After treatment at a constant temperature (from 60 to 80°C) for a given time (from 30 min to 3 h), the modified fiber is filtered, washed with deionized water, and dried under a vacuum (Saliba et al., 1999). After modification, the fiber calls modified dietary fiber (MDF)

Sword fish was obtained from the Department of Food and Nutrition, Purdue University, West Lafayette, Indiana, USA. From sample mercury analysis, it contains total mercury of 1.17 ppm. Fish tissue was homogenized in a blender. Replicate samples (1 g of fish homogenized tissue) were weighed into 50 ml polypropylene centrifuge tube with screw cap. 1 ml of saline (0.9% NaCl, Sigma-Aldrich) was added in to test tube and homogenized twice by a cell disruptor at 20 kHz at 150-500 Watts for 30 s and mixed with dietary fiber.

2.2 Analytical methods

The physicochemical properties including crude protein, moisture, ash, fat,carbohydrate, acid detergent fiber (ADF) and acid detergent lignin (ADL) was performed according to AOAC method (2005). Neutral detergent fiber (NDF) (Van Soest et al., 1991).

The functional properties such as water holding capacity (WHC) (Jasberg et al., 1989), oil holding capacity (OHC) (Caprez, et al., 1986), solubility (AACC, 2000) method of No. 44-19, swelling (Robertson et al., 1999) and COOH content (USP, 1990)

2.3 In vitro digestion

The 2 stages in vitro digestion model was described by (Garrett, Failla and Sarama 1999). The gastric phase was initiated with addition porcine pepsin (3 mg/ml, Sigma Chemical Co., St. Louis, MO) and adjusts of the pH to 2 with 0.1 M HCl (Analytical grade, Sigma Chemical Co.) Samples were vortex and flushed the top of the tube with nitrogen gas (99.99%, Air Gas, Indianapolis, IN) and incubation at 37ºC for 1 h in shaking water bath at 150 rpm (VWR, Cornelius, OR). For the intestinal phase was initiated by adjustment pH to 5.3 with 100 mM sodium bicarbonate solution (Sigma Chemical Co.) and addition of 9 ml of bile extract/pancreatin/lipase mixture: pancreatin (0.4 mg/ml, Sigma Chemical Co., St. Louis, MO), lipase (0.2 mg/ml, Sigma Chemical Co.) and porcine bile extract (2.4 mg/ml, Sigma Chemical Co.) and adjusted pH to 7.0 ± 0.5 with 0.1 M NaOH (Analytical grade, Sigma Chemical Co.), made up to 30 ml with 0.9% saline (pH 7) Samples were vortex and flushed the top of the tube with nitrogen gas and incubation at temperature 37ºC for 1 h in shaking water bath at speed 150 rpm. One sample tube was separated for digesta and other 3 sample tubes were centrifuged at 167,000 g for 35 min (Beckman L8-70M, Beckman Coulter, San Antonio, TX). Aliquots of raw materials, digesta, aqueous phases, and residual pellets were collected and stored at -80 ºC prior to analysis.

Data Analysis

Relative bioaccessibility (%) = $\frac{\mu g/kg \text{ of Hg in aqueous}}{\mu g/kg \text{ of Hg in digesta}} \times 100$

Absolute bioaccessibility (µg/g) = $\frac{\%\ \text{bioaccessibility} \times \text{starting material}}{100}$

2.4 Caco –2 human intestinal cell culture

The procedure for cellular uptake described by (Ferruzzi, Failla and Schwartz 2002) with slight modification was applied. A TC7 clone of Caco-2 cell was kindly provided by Dr. Jim Fleet, Department of Foods and Nutrition at Purdue University (West Lafayette, IN). Mercury intestinal accumulate investigation was done using the Caco-2 human intestinal cell culture model (TC7 clone) between passages 85-94. Cells were seeded in 6-well plastic dishes (35 mm X 10 mm, Costar Coming, New York, NY) Cells were maintained in Dulbecco Modified Eagle's Medium (DMEM, BioWhittaker, Lonza) with 4.5g/L Glucose and L-glutamine. The medium was supplemented with 1% v/v of autoclaved HEPES (10 mM, Sigma-Aldrich, St. Louis, MO), 1% v/v of non-essential amino acids (0.1 mM, BioWhittaker, Lonza), 1% v/v of P/S (penicillin/streptomycin, 100 U/L/100 U/L, BioWhittaker, Lonza), and 0.1% v/v of gentamicin (50 ug/L, Sigma-Aldrich) and 10% v/v of fetal bovine serum (FBS, Atlanta Biologicals) and incubated in humidified atmosphere of air/CO2 (95%/5%) at 37 °C. Uptake experiments were performed as monolayer was 11-14 day post-confluent. Culture medium was changed every 2 day.

Monolayer was washed twice with 1 ml of Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS, BioWhittaker, Lonza) before adding 2 ml of test media. Test media was prepared by diluting filtered aqueous fraction from in vitro digestion and basal DMEM at a ratio 1:3. Cells were incubated at 37 °C for 6 h. Then medium was removed by aspiration and cells was washed twice with 1 ml of DPBS. Cells were collected by scraping into 0.75 mL of ice-cold PBS and stored at -80°C until analysis. Each experimental treatment was performed in triplicate.

Data Analysis

$$\text{Uptake efficiency (\%)} = \frac{\mu g/kg \text{ of Hg in cell}}{\mu g/kg \text{ of Hg in test media}} \times 100$$

2.5 Assessment of toxicity and cellular viability

Cellular viabilities in all treatments were generally between 90 and 95 %. Cellular viability was determined using a methylthiazoletetrazolium-(MTT, [3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], Sigma Chemical Co., St. Louis MO). The method was initiated with adding MTT solution (5 µL MTT solution/300 µL DMEM with no phenol red) into each well in 24 well plate (The monolayer was added test media and incubated at 37 ºC for 6 h. Washing twice with DPBS) and incubated at temperature 37 ºC for 2 h. The purple product was dissolved with 300 µL of dimethylsulfoxide (DMSO). The purple solution (50 µL) was loaded in 96-well plate and added 50 µL of DMSO. Read the absorbance at 570-630 nm with 96-well plate reader (Bio-Tek Instruments. Inc. Tustin, CA). Each experiment was performed in duplicate.

2.6 Protein assay

Cells were homogenized by sonic disruption, and cell protein was measured by using a BC A (Bicinchonic Acid) protein assay kit according to manufacturer's protocols (Bio-Rad Laboratories, Rockford, IL).

2.7 Determination of mercury

Cells were centrifuged at 200 rpm for 10 min at room temperature (20-25 °C) (Eppendorf Centrifuge 5415 D, Hamburg, Germany) and the supernatant was discarded. An aliquot of cells was analyzed for total mercury using a Thermal Decomposition (Gold) Amalgamation Atomic Absorption Spectrophotometer (TDA/AAS) Mercury Analyzer (DMA-80, Milestone Inc., Pittsburgh, PA) as described by Shim et al (2009). Total mercury in the aqueous fraction and pellet also was determined. Total mercury data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

2.8 Statistical analysis

Results are presented as representative data from at least two sets of experiments. Data are expressed as the mean ± standard error. For cellular uptake studies, a sample size of n=3 was

used. Statistical analysis for each parameter assessed was performed by using analysis of variance (ANOVA) followed by Tukey's post hoc test (SAS, Gary, NC). Differences among means were considered statistically significant at $p < 0.05$

3. Results

3.1 Physicochemical and functional properties of modified dietary fiber

Table 1 shows physicochemical properties of modified dietary fiber (MDF). the physicochemical properties of MDF percentage of crude protein, ash, moisture, fat, starch, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicelluloses were 0.87, 6.76, 3.54, 0.12, 1.13, 87.58, 80.21, 5.67, 74.54 and 7.37 respectively (table 3). The functional properties of modified dietary fiber shows high water holding capacity, oil binding capacity, water solubility index, swelling capacity and COOH content (table 2).

Table 1 Physicochemical properties of modified dietary fiber

Component	Modified
Crude protein	0.87±0.23
Ash	6.76±0.57
Moisture	3.54±0.45
Fat	0.12±0.08
Starch	1.13±0.32
Neutral detergent fiber (NDF)	87.58±1.32
Acid detergent fiber (ADF)	80.21±2.34
Acid detergent lignin (ADL)	5.67±1.08
Cellulose[a]	74.54
Hemicellulose[b]	7.37

[a] ADF -ADL, [b] NDF-ADF

Table 2 Functional properties of modified dietary fiber

Functional properties	Modified dietary fiber
Water holding capacity (WHC)	7.12
Oil binding capacity (OBC) (g/g sample)	5.87
Water solubility index (WSI) (%)	6.13
Swelling capacity (SC) (mL/g DM)	10.4
COOH content (%)	6.86

3.2 Bioaccessible concentration and bioaccessibility of Hg

In this study was assumed the mercury in fish tissue to be in the methyl mercury form. The first step was study bioaccessibility of fish tissue (no MDF added) by in vitro digestion method. The slurry of sample after digestion called digesta, after centrifuged to separate pellet called aqueous fraction. Bioaccessibility is amount of Hg release from fish tissue to aqueous fraction. Table 3 shows the effect of modified crude dietary fiber amount on mercury bioaccessibility from fish tissue using an in vitro digestion model including defined as the fraction of mercury available for absorption by human intestinal cells. MDF significantly reduced mercury bioaccessibility in amount of fiber from 0-1000 mg ($p<0.05$) and appears to be linearly correlation related to amount of dietary fiber. Mercury bioaccessibility decreased to 35-85% compared to the control.

Table 3 Modofied dietary fiber on amount 0-1000 mg with 1 g of fish tissue. Total mercury in each phase (μg/g) and relative bioaccessibility following in vitro digestion. Data represent the absolute bioaccessibility

Fiber (mg)	Total mercury (μg)		Relative Bioaccessibility (%)	Absolute bioaccessibility (ug/g)
	Digesta	Aqueous		
0	0.80 ± 0.08^{d}	0.46 ± 0.03^{c}	57.50 ± 1.21^{a}	0.67 ± 0.13^{a}
50	0.78 ± 0.10^{e}	0.29 ± 0.10^{b}	37.17 ± 1.02^{b}	0.44 ± 0.08^{b}
100	0.87 ± 0.09^{c}	0.21 ± 0.04^{ab}	24.14 ± 0.87^{c}	0.28 ± 0.04^{c}
500	0.90 ± 0.04^{b}	0.14 ± 0.03^{a}	15.55 ± 0.68^{d}	0.18 ± 0.03^{d}
1000	0.93 ± 0.04^{a}	0.08 ± 0.01^{a}	8.60 ± 0.56^{e}	0.10 ± 0.02^{e}

* Data represent mean +/- SEM from n=3 independent in vitro digestion experiment
* Presence of different letters indicate significant difference between treatments as determined by a Tukey's post hoc test ($p<0.05$)

Figure 1 shows the effect of MDF (500 mg) on difference amount of fish tissue (0-4 g). MDF significantly reduced mercury bioaccessibility in amount of fish tissue from 0-4 g ($p<0.05$) when compared with control (not dietary fiber added). MDF with 0.5 g of fish tissue, the mercury bioaccessibility decreased approximately 79% when compared with control and increased to 86% for 1 g and decreased to 71% in 2-4 g of fish tissue. The resulting shows, the bioaccessibility of Hg becomes lower and quite stable after 1 g of fish tissue. For control, the amount of mercury in the aqueous fraction from different of fish tissue (0, 0.5, 1, 2 and 4 g) range from 0.24± 0.01 to 0.81± 0.01 μg/g and the bioaccessibility of Hg increased between 0.5-1 g of fish

tissue that range from 75.00 ± 3.57% to 87.50 ± 2.78% of total mercury in digesta. After 1 g of fish tissue, the bioaccessibility of Hg becomes lower and quite stable (range from 38.21 ± 2.13 to 27.45 ± 2.64).

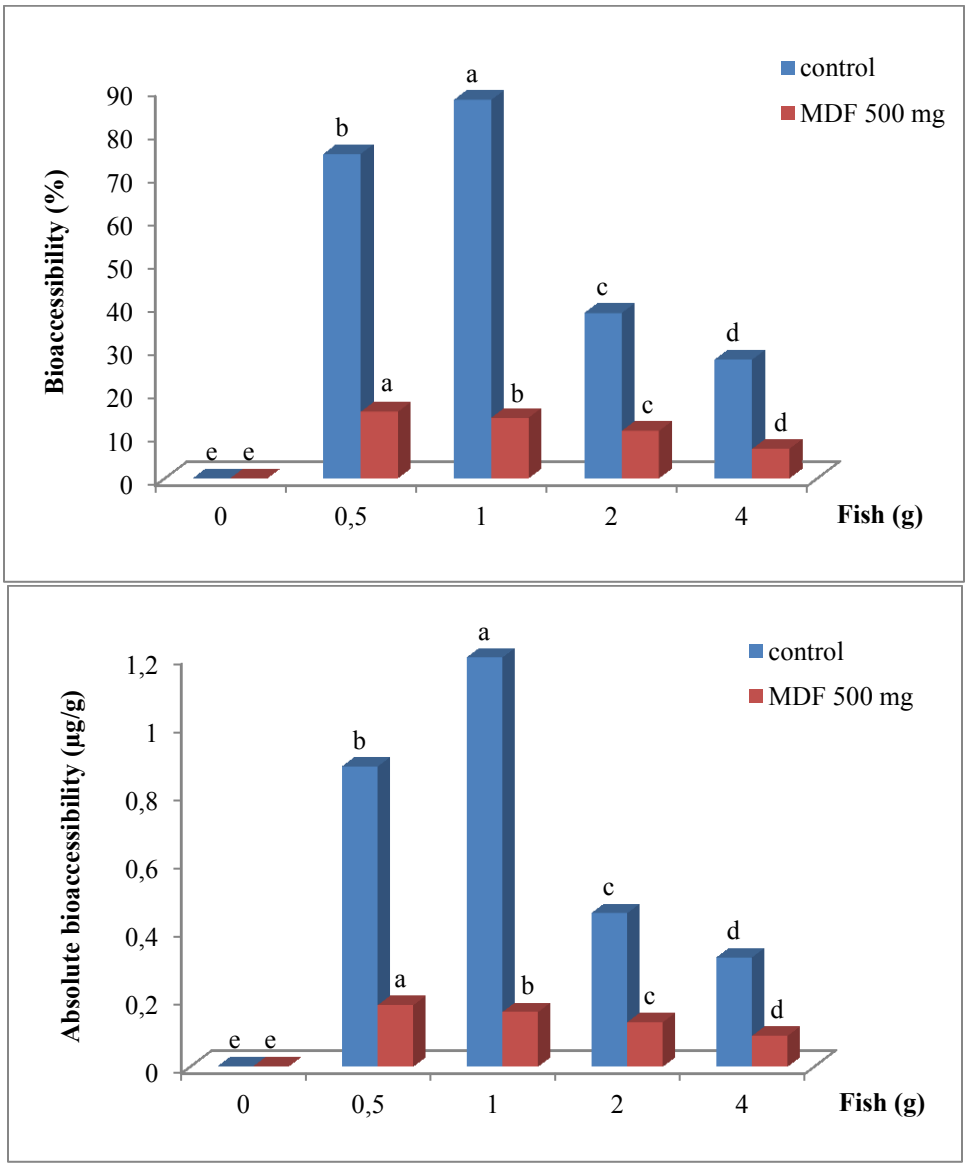

Fig. 1 Bioaccessibility and absolute bioaccessibility of mercury on different amount of fish tissue (0-4 g) with 500 mg of MDF

3.2 Hg uptake by Caco-2 cells

Determination of bioaccessibility is the first step to measuring bioavailability, bioaccessibility is amount of ingested mercury in aqueous that is available for absorption in to intestinal mucosa. The study could be used Caco-2 cell to evaluate intestinal cell accumulation and support reliable estimating bioavailability (Torres-Escribano et al. 2011, He and Wang 2013, Laird and Chan 2013, Hu et al. 2011, Das, Jean and Kar 2013). Figure 1 shows accumulation of mercury intestinal uptake by using TC7 clone of the Caco-2 cell with media containing aqueous fraction from in vitro digestion. Sample from previous studied were diluted 1:3 with basal DMEM (test media) and incubated at temperature 37°C for 6 h with different amounts of fish tissue (0-4 g) and different types of dietary fiber 500 mg. This system could be used to evaluate level of cellular mercury uptake and approximation to in vivo situation (Moreda-Piñeiro et al. 2011). Total mercury in test media range from 1.12-7.01 and 5.78-21.84 ng for no fiber added (control). The amount of Hg transfer to the intracellular was decrease when increasing of fish tissue by range from 9.07-5.97% of the mercury in the test media for control and 5.09-6.68% in the media containing 500 mg MDF. The difference of uptake efficiency depend on mercury concentration in the test media (Calatayud et al. 2012). The high concentration (21.8 ng) of Hg in control, efficiency of the uptake was low (5.97%), whereas with low concentration (5.7 ng) of Hg, efficiency of the uptake was increased (9.07%) but present non significant in sample with modified dietary fiber. These data are similar to previous studies that reported the fiber from wheat, soy protein, and phytochemical such as catechins (green tea) and the aflavins (black tea) could reduce the bioavailability of Hg and which might be more efficient than synthetic chelating agents (e.g., DMPS) (Shim et al. 2009).

Table 4 Accumulation of mercury by TC7 clone of Caco-2 human intestinal cells incubated for 6 h at 37ºC with different amounts of fish tissue

Fish (g)	No fiber			Modified dietary fiber (MDF)		
	Test media (ng)	ng mercury/ mg protein	Uptake Efficiency (%)	Test media (ng)	ng mercury/ mg protein	Uptake Efficiency (%)
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0
0.5	$5.78 \pm 0.3^{b,3}$	$0.44 \pm 0.1^{b,3}$	9.07 ± 0.57^{c}	$1.12 \pm 0.2^{b,1}$	$0.06 \pm 0.1^{b,1}$	5.09 ± 1.18^{ns}
1	$11.44 \pm 0.9^{c,3}$	$0.85 \pm 0.1^{bc,3}$	8.66 ± 1.43^{c}	$2.96 \pm 0.1^{c,1}$	$0.18 \pm 0.1^{bc,1}$	6.58 ± 1.77^{ns}
2	$14.72 \pm 1.6^{d,3}$	$0.95 \pm 1.2^{c,2}$	6.85 ± 0.77^{bc}	$5.32 \pm 0.2^{d,1}$	$0.37 \pm 0.5^{c,1}$	7.13 ± 2.06^{ns}
4	$21.84 \pm 0.3^{e,3}$	$1.42 \pm 1.6^{d,2}$	5.97 ± 0.54^{b}	$7.01 \pm 0.3^{e,1}$	$0.48 \pm 1.1^{d,1}$	6.68 ± 1.30^{ns}

* Data represent mean +/- SEM from n=3 independent Caco-2 uptake experiment

* Presence of different letters indicate significant difference between treatments as determined by a Tukey's post hoc test ($p<0.05$)

* Presence of different letters indicate significant difference between treatments as determined by a Tukey's post hoc test ($p<0.05$) a, b, c…. is fiber dose effect and 1, 2, 3….is fiber type effect

4. Discussion

4.1 Bioaccessibility of Hg

After 1 g of fish tissue, the bioaccessibility of Hg becomes lower and quite stable These results was similar to previous reported by Shim et al., (2009) that the bioaccessibility of Hg becomes lower when increasing fish tissue. This would suggest that increasing fish tissue is not necessarily increasing bioaccessibility. In opposite, the mercury released from fish tissue become lower. Because, most of the inorganic; Hg and MeHg, in seafood are bound to sulphydryl groups of proteins, the proteins were not completely hydrolyzed in gastrointestinal. So that the Hg bound to them would not be soluble in aqueous fraction (Calatayud et al. 2012). The concentration of Hg depend on the fish type analyzed and there are reported that Hg is higher in swordfish than tuna and sardine, also its age, size, sex, metabolism and feeding habits

(Cabañero, Madrid and Cámara 2004). However, the bioaccessibilites could be different depend on the factors such as the composition of food matrix, pH, shaking time and enzyme conditions (Laird and Chan 2013). This result implies that total mercury in food is not necessarily bioavailable (Ouédraogo and Amyot 2011).

Modified dietary fiber significantly reduced mercury bioaccessibility in amount of fiber from 0-1000 mg and appears to be linearly correlation related to amount of dietary fiber. These results suggest that crude dietary fiber decreased mercury bioavailability by inhibition of mercury transfer to the aqueous fraction. The mechanisms of dietary fiber binding with heavy metal are chemisorption and physical sorption. Physical sorption is absorption heavy metal in the fiber matrix and chemisorption, which is connected between fiber matrix of phenolic groups from lignin and carboxyl groups from uronic acid (Zhang, Huang and Ou 2011).

4.2 Hg uptake by Caco-2 cells

There are differences in the uptake efficiency, depending on the Hg concentration added to the cells. With high exposures the uptake was low, whereas with lower levels of exposure the uptake increases, the high uptake indicate greater bioavailability if the intracellular content is finally transported to the body (Xing et al. 2008). Also, the high retention of Hg could indicate that the intestinal epithelium acts as a barrier for absorption of this contaminant, although the toxicological effects on the epithelium should be evaluated (Laird and Chan 2013).

5. Conclusion

These results suggest that dietary fiber might be acted as a chelating agent for reducing mercury bioavailability also show the potential of dietary fiber prepared from cassava pulp to decreased Hg bioavailability but should be aware to confirm with in vivo system in the future.

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