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Enzymatic digestion optimization of dietary fiber from cassava pulp and their effect on mercury bioaccessibility and intestinal uptake from fish using an *in vitro* digestion/Caco-2 model

[1]Kachenpukdee, N., [2]Santerre, C.R., [3]Ferruzzi, M.G.. and [1*]Oonsivilai, R.

[1]School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand
[2]Department of Nutrition Science, Purdue University, West Lafayette, United State of America
[3]Department of Food Science, Purdue University, West Lafayette, United State of America

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Abstract

The objectives of this study were to determine the optimal extraction condition of cassava pulp and their effects on mercury bioaccessibility and bioavailability. The extraction process requires the starch be separated from the fiber by enzyme application. The enzyme reaction conditions for the solubilization were optimized via a response surface methodology (RSM). The selected dependent variable was percentage of neutral detergent fiber (NDF). The highest NDF (79.68%) of crude dietary fiber could be gotten from enzymatic digestion condition of 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v) and 1% of neutrase (v/v). In addition, NDF affecting the mercury bioavailability was estimated by using *in vitro* digestion and Caco-2 human intestinal cell model system. *In vitro* digestion (bioaccessibility) showed that the fiber could reduce mercury bioaccessibility to 2-57% compared with the control (0-1000 mg of NDF 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 NDF was added in digestion model test showed that the NDF did assist with reduction of mercury amount in fish tissue from 39% to 21% compared with control (the control lacks NDF). 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 6.54-4.63% in the media containing 500 mg NDF. In conclusion, this study suggests that NDF prepared from cassava pulp could decrease mercury bioavailability by inhibiting the mercury transfer to the aqueous fraction and could be applied in functional food and dietary supplement products.

Introduction

Mercury (Hg) is a highly toxic element effecting the nervous system, heart, kidneys, lung, and immune system. The toxicity varies according to the level of exposure (Sharma *et al.*, 2014). Humans can be exposed to Hg though various pathways. Eating fish contaminated with methylmercury (MeHg) is the major source of human exposure (Bose-O'Reilly *et al*., 2010). Chelating agents are commonly used for the removal of heavy metal. However, their side effects have been reported (Tandon and Singh, 2000). Recently, many researchers have been reported applying dietary fibers as adsorbents of heavy metal because of their nontoxic property (Idouraine *et al*., 1996; Sembries *et al.*, 2004; Nawirska, 2005; Shim *et al.*, 2009; Hu *et al*., 2010; Zhang *et al.*, 2011). Two possible mechanisms of binding are chemisorption and physical sorption. Chemisorption is the main one; free carboxyl group from uronic acids/phenolic groups from lignin form a coordination complex with di/trivalent metal anions or the positive charge of trivalent cations is connected with carboxyl anion (Zhang *et al*., 2011). Physical adsorption is used to characterize the surface and pore features of fiber. So, Dietary fiber can be absorbed/bind and carries them through the digestive tract because it is resistant to digestion by human elimentary enzyme (Kay, 1982). Cassava pulp is a by-product of cassava starch factory processing which accounts for approximately 10–30% by weight (wet) of the original tubers (Kosugi *et al*., 2009). It contains approximately 60% starch and 30% fiber (Apiwatanapiwat *et al*., 2011). Therefore, the concept in the extraction process of dietary fiber is treating with starch and proteins with enzymes for hydrolysis. The result is Neutral Detergent Fiber (NDF), which is the most abundant structural component in plant cells (lignin, hemicellulose and cellulose). This is because cassava pulp mainly contain insoluble fiber (Dhingra *et al*., 2012).

*Corresponding author.
Email: *roonsivi@sut.ac.th*
Tel: 66914197991; Fax: +66-44224387

The couples *in vitro* digestion/Caco-2 cell model are useful to assess chemical risk to humans (Ferruzzi *et al.*, 2002). *In vitro* digestion is conducted to simulate the human digestive system for measuring bioaccessibility (Etcheverry *et al*., 2011). Caco-2 cell can be used to evaluate intestinal cell uptake and how reliable it is to the *in vivo* situation in estimating bioavailability (Au and Reddy, 2000). In addition, *in vitro* method is less expensive, faster and it is easier to control the experimental factors than in human or animal studies (Sandberg, 2005). However, *in vitro* studies cannot be substituted for *in vivo* studies. Confirmations should be done via *in vivo* studies (Donhowe *et al*., 2014).

Materials and Methods

Preparation of dried cassava pulp

Dried cassava pulp were obtained from Sanguan Wongse Starch Co., Ltd. After drying at 60°C for 8-12 hours leaves were ground with a grinder (High speed grinder, 3500 w, Simon, Inc., Foodmachine, China) until they were a fine powder. The cassava pulp powder was then kept in a sealed container until further treatment.

Enzymatic digestion process

The aim of this study was to find optimal conditions to obtained NDF. Response Surface Methodology was used to find the optimum points. Independent variables such as amount of X1: α-amylase (EC 3.2.1.1, Merk, Darmstadt, Germany), X2: neutrase (EC 3.4.24.28, Novozymes Co., Bagsvaerd, Denmark),) and X3: amyloglucosidase (EC 3.2.1.3, Bray, Co. Wicklow, Ireland) were optimized using 3-factors with 3-levels. Box-Behnken design requires fewer than 15 runs in 3-factor experimental design. Dried cassava pulp was treated with enzyme for starch and protein hydrolysis. The cassava pulp solution was prepared by mixing 2 g of dried cassava pulp with 50 ml of phosphate buffer (pH 6). The cassava pulp solution was treated with α-amylase (0.1%, 0.2% and 0.3% w/v) for 30 min, at pH 6, 95°C. The pH was adjusted to 7.5 with sodium hydroxide solution (NaOH) and treated with neutrase (0.5%. 1.0% and 1.5% v/v) for 30 min at 60°C. Using hydrochloric acid solution, the pH was adjusted to 6 and treated with amyloglucosidase (0.1%, 0.3% and 0.5% v/v) for 30 min at 95°C. The resulting hydrolysate is separated by centrifugation at 10000xg for 10 minutes. The sediment was washed with doubly distilled water, re-centrifuged for 10 min at 10000xg and dried at 50°C in hot air oven. The selected dependent variables were cumulative percentage of NDF (Y1). Each experimental treatment was performed in triplicate. After extraction, the fiber is refered to as crude dietary fiber (CDF).

Preparation of fish tissue

Swordfish was obtained from Gulf of Mexico and shipped frozen the Department of Nutrition Science, Purdue University, West Lafayette, Indiana, USA. From sample mercury analysis, it contains total Hg of 1.17 ppm. Fish tissue was homogenized in a blender. Fish homogenized tissue were weighed (0, 1, 2 and 4 g) 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 cell disruptor at 20 kHz at 150-500 Watts for 30 s and mixed with CDF (0, 50, 100, 500 and 1000 mg).

In vitro digestion

The 2 stages in *in vitro* digestion model (Garrett *et al*., 1999; Ferruzzi *et al.*, 2002). The gastric phase was initiated with the addition of porcine pepsin (3 mg/ml, Sigma Chemical Co., St. Louis, MO) and adjustes to a pH of 2 with 0.1 M HCl (Analytical grade, Sigma Chemical Co.) Samples were vortexed and the top of the tube was flushed with Nitrogen gas, N_2 (99.99%, Air Gas, Indianapolis, IN) and incubated at 37ºC for 1 h in shaking water bath at 150 rpm (VWR, Cornelius, OR). The intestinal phase was initiated by adjusting the 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 pH adjusted 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 vortexed and the top of the tube flushed with N_2 and incubated at temperature 37ºC for 1 h in shaking water bath at a speed of 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 (the slurry of sample after digestion), aqueous fraction (the supernatant after centrifugation) and residual pellets were collected and stored at -80 ºC prior to analysis. Each experimental treatment was performed in triplicates.

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

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

Caco-2 human intestinal cell culture

The procedure for cellular uptake was described by Ferruzzi *et al.* (2002) with slight modifications applied. Hg 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 4.5 g/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/CO_2 (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 of 1:3. Cells were incubated at 37°C for 6 h. Then medium was removed by aspiration and cells were 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 triplicates.

$$\text{Uptake efficiency (\%)} = \frac{\text{Accumulation of Hg in cell (ng/well)}}{\text{Hg content in test media (ng/well)}} \times 100$$

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 by adding MTT solution (5 µL MTT solution/300 µL DMEM with no phenol red) into each well in 24 well plates (The test media was added into the monolayer and incubated at 37ºC for 6 h and washed twice with DPBS) and incubated at a temperature of 37ºC for 2 h. The purple product was dissolved with 300 µL of dimethylsulfoxide (DMSO). The purple solution (50 µL) was loaded in 96-well plates and into it was added 50 µL of DMSO. The absorbance was read at 570-630 nm with 96-well plate reader (Bio-Tek Instruments. Inc. Tustin, CA). Each experiment was performed in duplicates.

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).

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 cell was also determined. Total Hg data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

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 $p < 0.05$.

Results and Discussion

Enzymatic digestion optimization

Dried cassava pulp contains 59.39 ± 0.02% of starch and 2.60 ± 0.06% of protein (Table 1). Therefore, the process was started by enzymatic starch and protein hydrolysis instead of the fiber extraction process. The first step was to digest starch by using α-amylase and amyloglucosidase because there are two types of linkages for starch molecules. α-Amylase are enzymes that hydrolyze alpha-1, 4-glycosidic linkages polysaccharides to yield dextrins, oligosaccharides, maltose and D-glucose (Sundarram and Murthy, 2014). Amyloglucosidase was used for the decomposition of starch into glucose by tearing-off glucose units from the non-reduced end of the polysaccharide chain. Neutrase was used for hydrolysis of internal peptide bonds (Apiwatanapiwat *et al*., 2011). Finally, all of the digested materials were

Table 1. Physicochemical properties of dried cassava pulp

Component	% Content (dried basic)	
	Dried cassava pulp	Crude dietary fiber
Crude protein	2.60±0.06	1.06±0.61
Ash	2.10±0.03	3.92±0.74
Moisture	4.11±0.23	5.41±0.87
Fat	0.13±0.03	0.33±0.63
Starch	59.39±0.02	9.6±0.51
Neutral detergent fiber (NDF)	31.67±1.78	79.68±0.97
Acid detergent fiber (ADF)	28.21±0.10	78.26±0.64
Acid detergent lignin (ADL)	2.44±0.27	4.09±0.95
Cellulose[a]	25.78± 0.16	74.16± 0.78
Hemicellulose[b]	3.46± 0.67	1.42± 0.76

[a] ADF -ADL, b NDF-ADF

solubilized in water and separated by centrifugation. The percentage of NDF increased when the starch and protein were removed (Table 1). The highest NDF is 79.68 ± 0.97% (cellulose 74.16%, hemicellulose 1.42 and lignin 4.09 ± 0.95%) could be gotten from enzymatic digestion conditions at 0.1% of α-amylase (w/v), 0.1% of amyloglucosidase (v/v) and 1% of neutrase (v/v) (Figure 1). The result had shown high content of cellulose because the major components of plant cell walls are cellulose. The proportions of these components in a fiber depend on age, source, and extraction process (Malik and Grohmann, 2011). In Table 1, starch are still remained in dried cassava pulp, in comparison with the CDF, suggesting that the enzymatic digestion process was successful enough to have removed starch from fiber structure.

Bioaccessibility and absolute bioaccessibility of MeHg

Total mercury that accumulates in fish tissue is MeHg because it is assumed that the majority (95-99%) of the Hg has already been converted to the methyl form (Bloom, 1992; Wiener and Spry, 1996). *In vitro* digestion model can be used to evaluate the amount of MeHg released from fish tissue to the aqueous fraction (absolute bioaccessibility) that is available for absorption by intestinal cells, the percentage of MeHg in digesta from fish tissue (bioaccessibility), interactions between MeHg and CDF and the effect of luminal factors. The first step involved studying the effect of CDF amount on Hg bioaccessibility and absolute bioaccessibility from 1 g of fish tissue. The result shows that CDF significantly reduced Hg bioaccessibility and absolute bioaccessibility in amount of fiber from 0-1000 mg ($p < 0.05$) and appears to be linearly correlated to the amount of dietary fiber (Figure 3). The optimal amount of CDF to be used in next step is 500 mg, even though 1000 mg shows the highest reducing Hg bioaccessibilyty due to the sticky nature of the experiment.

The effect of fish tissue amount (0-4 g) with 500 g of CDF on Hg bioaccessibility and absolute bioaccessibility was studied. The bioaccessibility of control (the control lacks NDF) from different fish tissues (0, 0.5, 1, 2 and 4 g) increased between 0.5-1 g of fish tissue that ranged from 75.00 ± 3.57% to 87.50 ± 2.78%. After 1 g of fish tissue, the bioaccessibility of Hg becomes lower and quite stable, ranging from 38.21 ± 2.13% to 27.45 ± 2.64%. The results of absolulute bioaccessibility are the same as shown in Figure 4. These results were similar to those previously reported by Shim *et al.* (2009), who reported that the bioaccessibility of Hg becomes lower when increasing fish tissue. This would suggest that increasing fish tissue is not necessarily increasing bioaccessibility. In contrast, the Hg released from fish tissue became 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 tract. Hence, the Hg bound to them would not be soluble in aqueous fraction (Calatayud *et al*., 2012). The concentration of Hg depends on the fish type analyzed and there are reports that Hg is higher in swordfish than tuna and sardine; ages, size, sex, metabolism and feeding habits also counts (Cabañero *et al.*, 2005). However, the bioaccessibilites could be different depending on factors such as the composition of food matrix,

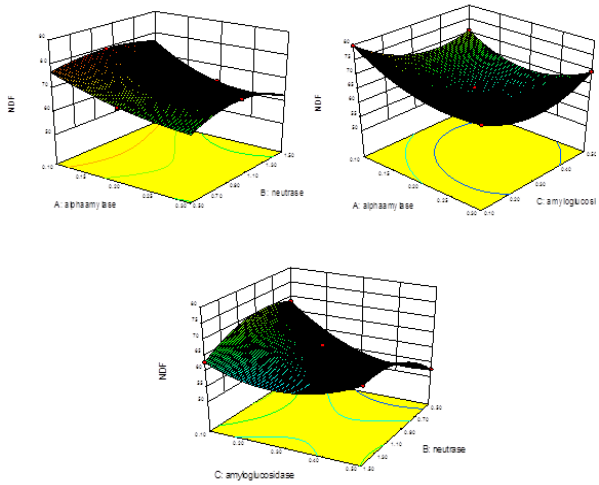

Figure 1. Contour plot showing the yield of NDF (%). The interaction effect between (a) α-amylase and neutrase (b) α-amylase and amyloglucosidase (c) neutrase and amyloglucosidase

pH, shaking time and enzyme conditions (Laird, 2010). This result implies that total Hg in food is not necessarily bioavailable.

The result of samples with 500 mg of CDF shows that crude dietary fiber significantly reduced Hg bioaccessibility and absolute bioaccessibility in amount of fish tissue from 0-4 g ($p<0.05$) (Figure 4). The absolute bioaccessibility decreased approximately 50% for 0.5 g of fish tissue when compared with control and increased to 67% for 1 g and becomes lower at level of 31% for 2 g and 21% for 4 g of fish tissue. These results suggest that CDF decreased Hg bioavailability by inhibition of Hg transfer to the aqueous fraction. Adsorption or binding of MeHg is dependent on the ratio of fiber and fish tissue, composition and physiological function of the fiber and luminal factor. The most important component of CDF is cellulose (74.16%). So, the main mechanism of MeHg inhibition is the positive charge of Hg which is not fully neutralized by carboxyl anion. Thus, free valences would be available to bind external anions.

MeHg uptake by Caco-2 cells

Caco-2 cell nutrient uptake from *in vitro* digests offered a physiological assessment of nutrient as well as reliable predictors of nutrient absorption in humans (bioavailability) (Wood and Tamura, 2001). The combination of them has presented the fastest approach to determine availability of contaminants (Shi *et al*., 2005).

Figure 5 shows uptake efficiency and accumulation of Hg intestinal uptake by using TC7 clone of the Caco-2 cell with media containing aqueous fraction from *in vitro* digestion. Sample from previous studies were diluted 1:3 with basal DMEM

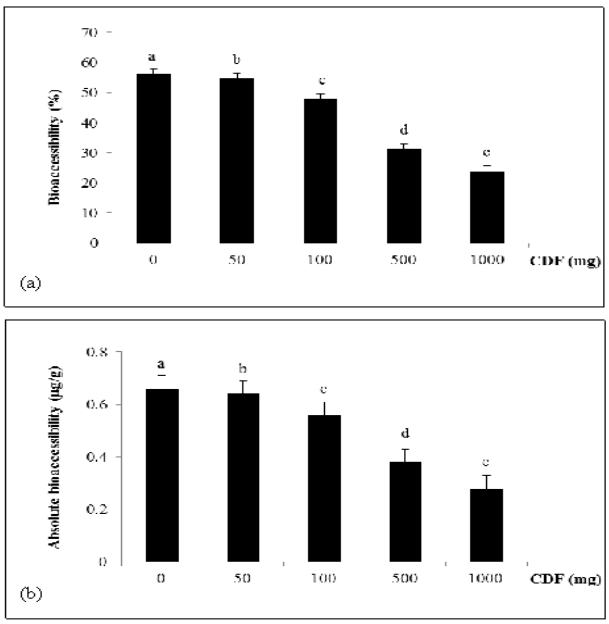

Figure 2. Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 1 g fish tissue in the presence of increasing amount of dietary fiber. Data represent mean ± SEM from n=3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant difference between treatments as determined by a Tukey's post hoc test ($p<0.05$)

(test media) and incubated at temperature of 37°C for 6 h with different amounts of fish tissue (0-4 g) and CDF (500 mg). This system could be used to evaluate the level of cellular Hg uptake and approximation to in vivo situation (Calatayud *et al*., 2012).

Total Hg in test media of control ranged from 5.7 ± 0.3 to 21.8 ± 0.3 ng and when transferred to the intracellular; it was approximated to range from 0.44±1.4 to 1.42 ± 1.6 ng of Hg/mg protein. These levels represent uptake efficiency of approximately 9.07-5.97%. Increasing amounts of fish tissue showed decreasing patterns of cellular Hg uptake. Mercury uptake with 500 mg of CDF was decreased by 40% for 0.5 g of fish tissue compared to the control, 55% for 1 g, 47% for 2 g and 30% for 4 g. The difference of uptake efficiency depends on the Hg concentration in the test media (Calatayud *et al.*, 2012). With high concentration (21.8 ng) of Hg, 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%). Therefore, these results suggest that eating food which is rich in fiber may be efficient for long term chronic MeHg exposure in fish eating populations by reducing Hg bioavailability. These data are similar to previous studies that reported that the fiber from wheat, soy protein, and phytochemical such as catechins (green

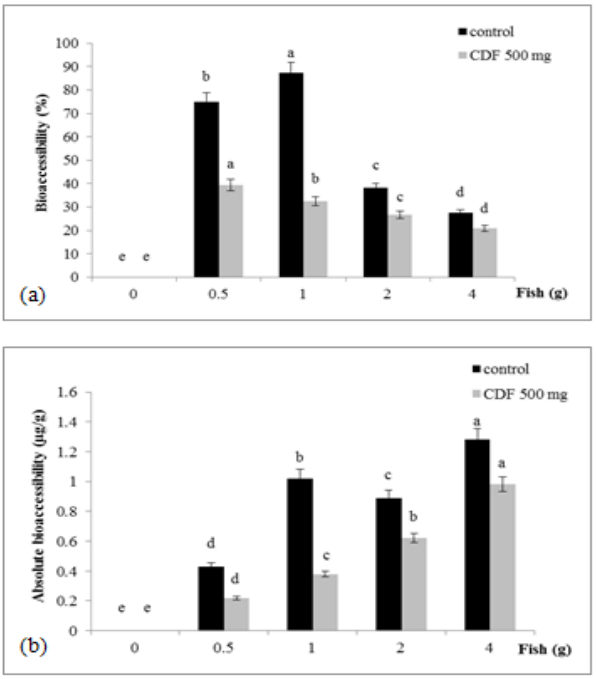

Figure 3. Bioaccessibility (a) and absolute bioaccessibility (b) of MeHg following *in vitro* digestion of 0-4 g fish tissue with 500 mg of dietary fiber in the presence of increasing amount of dietary fiber. Data represent mean ± SEM from n=3 digestion experiment. Bioaccessibility represents the percentage of MeHg transferred from fish tissue into aqueous fraction. Presence of different letters indicate significant difference between treatments as determined by a Tukey's post hoc test ($p<0.05$)

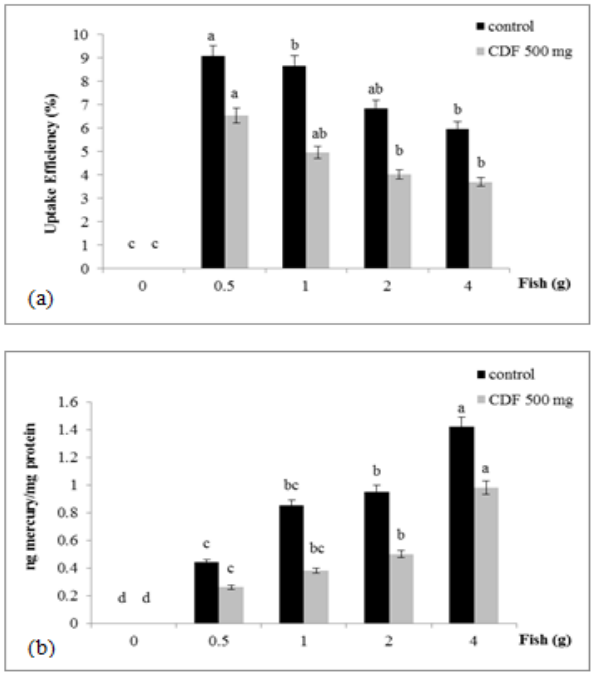

Figure 4. Uptake efficiency (a) and accumulation (b) of MeHg by Caco-2 human intestinal cells from test media containing MeHg from aqueous fraction of in vitro digestion. Data represent mean ± SEM from n=3 independent Caco-2 uptake experiment. Presence of different letters indicate significant difference between treatments as determined by a Tukey's post hoc test ($p<0.05$).

tea) and theaflavins (black tea) could reduce the bioavailability of Hg which might be more efficient than synthetic chelating agents (e.g., DMPS) (Shim *et al*., 2009). This result implies that total Hg might be bioaccesssible but not necessarily bioavailable (Wang *et al*., 2012).

Conclusion

These results suggest that dietary fiber might have acted as a chelating agent for reducing Hg bioavailability. It also shows the potential of dietary fiber prepared from cassava pulp to decreased Hg bioavailability. However, confirmations with in vivo systems should be done.

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