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Cytotoxicity effect of oil palm (*Elaeis guineensis*) kernel protein hydrolysates

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Abstract

This study was conducted to ascertain the cytotoxicity effect of oil palm (*Elaeis guineensis*) kernel protein hydrolysates (OPKHs) produced from its protein isolate. A modified microplate titer WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] assay was used to investigate the cytotoxicity of hydrolysates produced from protease and pepsin-pancreatin hydrolysis at various concentrations (0.1, 1, 10, 100 µg/ml and 1 mg/ml) using HepG2 cell model. Additionally, peptide stimulation test using OPKHs at 1 mg/ml was carried out to investigate whether OPKHs could serve as growth factor for HepG2 cells other than affecting its viability. As a result, oleic acid appeared to normalize the WST-1 readings of HepG2 cells treated with both hydrolysates at 1 mg/ml. The presence of amino acids in OPKHs could stimulate the growth and prolongs the viability of HepG2 cells. Both OPKHs were non-cytotoxic to HepG2 cells at all tested concentrations even at high concentrations. This study indicated that pepsin-pancreatin and protease hydrolysates produced from oil palm kernel protein were non-cytotoxic on HepG2 cells.

Introduction

The area of nutritional sciences has projected towards 'nutrition for optimal health' in order to contribute for disease prevention (Sedef Nehir and Sebnem, 2012). Hence, natural foods are essential sources of energy and basic components for the maintenance and growth of human body as well as a source of beneficial bioactive compounds in humans (Sedef Nehir and Sebnem, 2012). Food proteins are one of the major nutritional components. Food proteins could exert their beneficial effects through biologically active amino acids or peptides (Appel, 2003) that possess various physiological bioactivities.

Malaysia is one of the world's largest producers and exporters of palm oil and its products (Ofori-Boateng and Lee, 2013). After the extraction of palm kernel oil, the remaining palm kernel cake has been used as an important resource to feed animals like ruminants, swine, horses and poultry (Ofori-Boateng and Lee, 2013). Malaysia produced 1.64 million tons of PKC in 2000 from about 40 crushing plants nationwide. This shows that the resource of PKC is huge and will cause environmental disposal problem in Malaysia due to the ineffective utilization (Yusoff, 2006). Our results showed that the defatted oil palm kernel meal and its protein isolate contain about 50-55% and 75% crude protein respectively (Chang *et al*., unpublished results). This indicates that the kernel of oil palm could be utilized as a functional food ingredient rather than being thrown away (Megías *et al*., 2004). The oil palm kernel protein isolate and protein hydrolysates were shown to have acceptable amino acid profiles that consists of high amounts of cysteine (Cys), methionine (Met), lysine (Lys), aromatic amino acids, glutamic and aspartic acids (Glu and Asp) (Chang *et al*., unpublished results). The utilization of oil palm kernel protein hydrolysates and the raw materials which are the protein isolates and the defatted meal have great potential as a source of peptides with health-promoting bioactivities (Megías *et al*., 2004). These properties should be examined to identify potential value-added products for human

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nutrition besides minimizing the cost of waste management and disposal problem by Malaysian palm oil industry related to legal restrictions (Harnedy and Fitz Gerald, 2012).

Cytotoxicity assays were commonly used in *in vitro* toxicology studies to study the potential cytotoxic effect of the compounds being studied (Freshney, 2005). Compounds must be shown to be nontoxic to human first if they were to be used as pharmaceuticals or nutraceuticals in the food industry (Freshney, 2005; Navarro *et al*., 2013). Our liver functions to metabolize most of the potentially toxic 'foreign materials' consumed into our body (Liu and Zeng, 2009). Thus, hepatic toxicity tests should be able to determine whether the liver could metabolize the test sample either to a less or more toxic substances (Liu and Zeng, 2009; Navarro *et al.*, 2013). LDH (lactate dehydrogenase) leakage assay, the neutral red assay, MTT [3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide] assay, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] and XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] are most commonly used to assay for cytotoxicity or cell viability of any sample tested (Fotakis, 2009).

To the best of our knowledge, this is the first study to evaluate the cytotoxicity of the oil palm kernel protein hydrolysate (OPKH) systematically. WST-1 assay were performed using HepG2 cells as they are the most commonly used human derived cells that retain many of the biochemical functions of human liver cells (Dixon and Ginsberg, 1993; Yanagita *et al*., 2008). Since OPKH contains mixture of peptides/amino acids that might stimulate the growth of cells, the term 'peptide stimulation' test is used to represent the addition of OPKH into the HepG2 cell model in this study.

Materials and Methods

Materials

Bovine serum albumin (BSA; fatty acid free), sodium hydroxide (NaOH), pepsin, pancreatin, protease (from *Bacillus licheniformis*) and Sodium Oleate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, trypsin-EDTA and non-essential amino acids were purchased from GIBCO (Santa Clara, CA, USA). Penicilin (1×10^5 units/l) and streptomycin (100 mg/l) were bought from Meiji Confectionery Co., Ltd. (Tokyo, Japan). WST-1 reagent was purchased from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of the highest grade commercially available.

Preparation of the raw material (oil palm kernel meal)

The oil palm kernels used in this study were collected from an oil palm plantation from Bagan Datok, Perak, Malaysia. Only the yellowish-red coloured but not the overripe fruits were chosen. The peeled kernels were homogenized using a commercial food blender after removing the testae and rediculae. The ground kernel meal was defatted six times with n-hexane (1:10, meal to hexane ratio) at room temperature and then subjected to overnight air-drying. The defatted meal was stored at -20°C until further analysis.

Preparation of protein isolate

The protein isolate of oil palm kernel was produced from the defatted oil palm kernel flour according to the method described by Aluko and Monu (2003).

Protein hydrolysis

Hydrolysis of oil palm kernel protein isolate using protease from *Bacillus licheniformis* was carried out according to the method of Aluko and Monu (2003). Following that, hydrolysis was done using enzyme pepsin and pancreatin according to a methodology described by Megías *et al.* (2004). The OPKHs obtained contain mixture of short or medium chain peptides with various essential and non-essential amino acids.

HepG2 cell culture

HepG2 cell culture was prepared following a modified method of Liu and Zeng (2009). The HepG2 cells were purchased from the American Type Culture Collection (ATCC, USA). Cells within three passages which were stored in liquid nitrogen were used for further experiments. HepG2 cells from one vial (about 10^6 cells) were thawed immediately by immersing in a 37°C water bath. The cells were transferred to a 15 ml centrifuge tube containing 10 ml Dulbeccos's Modified Eagle's Medium (DMEM) and re-suspended by gentle aspiration with a pipette. After centrifugation (model 2410, KUBOTA, Tokyo, Japan) for 5 min at 1000 × *g*, the supernatant was removed. Then, HepG2 cells were cultured in DMEM containing 100 units/ml penicillin and 100 µg/ml streptomycin together with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. The medium was renewed at an interval of 2 to 3 days. At approximately 70-80% confluence, they were trypsinized (0.25% trypsin – 0.04% EDTA) and passaged. The medium was pre-incubated with 1%

bovine serum albumin (BSA)-DMEM with 1 mM oleic acid for at least 24 hr (Yanagita *et al.*, 2008) before subsequent cell viability assay. The detailed experimental procedures was outlined in the part of cell viability assay and illustrated in Figure 1.

Preparation of oleic acid-BSA complex

The oleic acid-BSA complex was prepared according to the method described by Van Harken *et al.* (1969). Concentrations of oleic acid and BSA were 1 mM and 1% respectively.

Cell viability assay

The Cell Proliferation Assay (Takara Bio Inc., Shiga, Japan) was performed according to the manufacturer's instructions. Briefly, a 100 µl of HepG2 cells preincubated in DMEM containing 1% BSA-1 mM Oleic acid was transferred onto a 96-well microtiter plates (Nunclon TM, VWR International Inc., USA) and was incubated for 72 hr in a 95% air and 5% CO_2 incubator at 37°C. Then, the medium was replaced with 100 µl of experimental medium [control: 1% BSA; protein sample: 1% BSA containing 1 mM oleic acid with both OPKHs (0.1, 1, 10 or 100 µg/ml)] for 24 hr as illustrated in Figure 1. Additionally, peptide stimulation test was carried out as illustrated in Figure 2 where OPKHs of 1 mg/ml was added. Then, it was washed once with PBS followed by the addition of 90 µl of DMEM without phenol red solution. 10 µl working solution containing 10% WST-1 was then added and incubated for another 90 min. Finally, the conversion of WST-1 reagent into chromogenic formazan was measured at 450 nm by using a Microplate Reader (model 550, Bio-Rad Co. Ltd., Tokyo, Japan) at 24, 48 and 72 hr intervals. Analyses were done in triplicate. The normalized WST-1 readings indicating the cell viability was calculated by using the following formula: result of each well/blank well.

Statistical analysis

Statistical analyses were conducted using Statistical Package for Social Sciences (SPSS) software (version 20; Chicago, Illinois, USA). All independent analyses were done in triplicate, and data were reported as means ± standard deviation. Data were analyzed for significance using one-way ANOVA followed by Bonferroni's test as a post hoc test. Means were considered significantly different at $p < 0.05$.

Results and Discussion

It is a known fact that liver is the major location for xenobiotic metabolism. Xenobiotic is a foreign

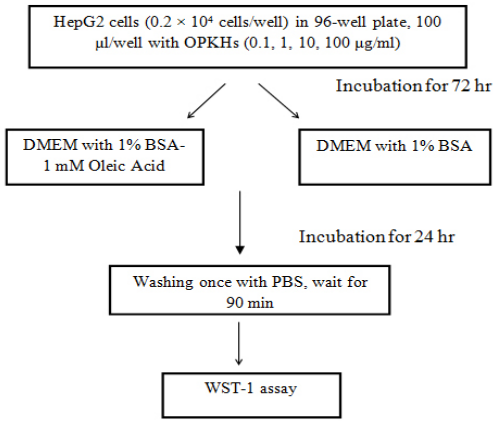

Figure 1. Schematic representation of the experimental design to investigate the viability of HepG2 cells after the addition of pepsin-pancreatin and protease hydrolysates.

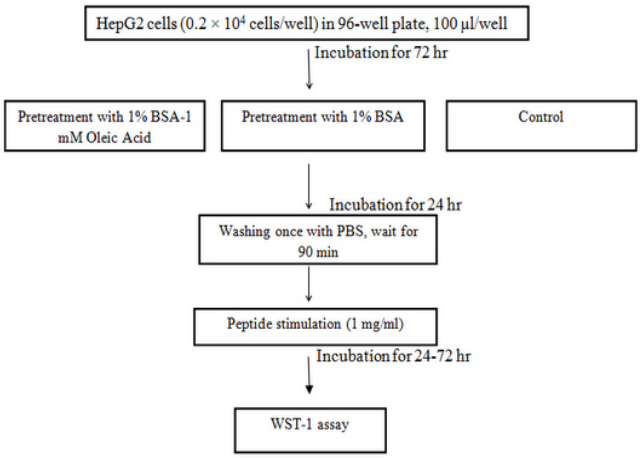

Figure 2. Schematic representation of the experimental design to investigate the effect of peptide stimulation using both OPKHs.

chemical substance found within an organism that is not naturally produced or expected to be present within that organism. Hence, research on xenobiotics should be focused first using hepatocytes. HepG2 cells are a transformed cell line, which was well differentiated and characterized (Navarro *et al.*, 2013). Some studies have used HepG2 cell model to determine the toxicity effect of peptides from various natural sources (Yanagita *et al.*, 2008; Inoue *et al.*, 2011).

Figure 3 demonstrates the effect of protease and pepsin-pancreatin hydrolysates on WST-1 readings in HepG2 cells with the presence of 1% BSA-1 mM oleic acid. WST-1 readings increased dose-dependently as both OPKHs were cultured from 24 to 72 hr. However, WST-1 readings of the control (2% DMSO) increased together with the wells 0.1-100 µg/ml of both OPKHs after culturing for 24, 48 and 72 hr respectively (Figures 3a and 3b). Moreover, there was no significant difference ($p > 0.05$) between the WST-1 readings of control and both OPKHs. This indicated that protease and pepsin-pancreatin hydrolysates were non-toxic to

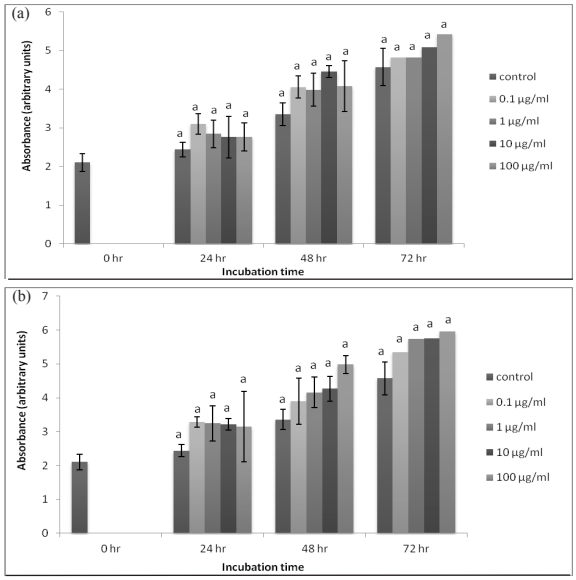

Figure 3. Effect of (a) protease hydrolysate and (b) pepsin-pancreatin hydrolysate on WST-1 readings in HepG2 cells. Absorbance was recorded at 24 hr intervals during the incubation time until 72 hr. Values are expressed as mean ± SD of triplicate independent analyses. Coefficient of Variance (CV) of the experiment was 1%. Columns marked with lower case letter (a) denote no significant difference ($p > 0.05$) with respect to control (2% DMSO).

HepG2 cells and it was negligible from 0.1-100 µg/ml. Figure 4 shows the effect of pretreatment with the presence or absence of 1 mM oleic acid and peptide stimulation on WST-1 readings in HepG2 cells. WST-1 readings were also not significantly different ($p > 0.05$) compared to control (2% DMSO) for both OPKHs with the absence of 1 mM oleic acid during peptide stimulation (Figures 4a and 4b). However, Figure 4c shows that WST-1 readings of both OPKHs were slightly higher than the control (2% DMSO) at 48 and 72 hr. The findings indicate that protease and pepsin-pancreatin hydrolysates were not toxic at 1 mg/ml.

Higher WST-1 readings shown by both OPKHs compared to control (Figure 4c) in the peptide stimulation test as well as the increase of WST-1 readings after incubating for 72 hr (Figure 3) could be due to the presence of various essential and non-essential amino acids in OPKHs (Chang *et al.*, unpublished results) that stimulates growth and prolongs the viability of HepG2 cells in culture. Both OPKHs contained essential growth factors for the growth of HepG2 cells. In fact, there should be no changes on the viability of the control in figure 4c compared to the samples tested as shown in figures 4a and 4b. However, the WST-1 readings of both OPKHs increased throughout the incubation period. From that, it could also be postulated that the presence of oleic acid at 1 mM normalized WST-1 readings

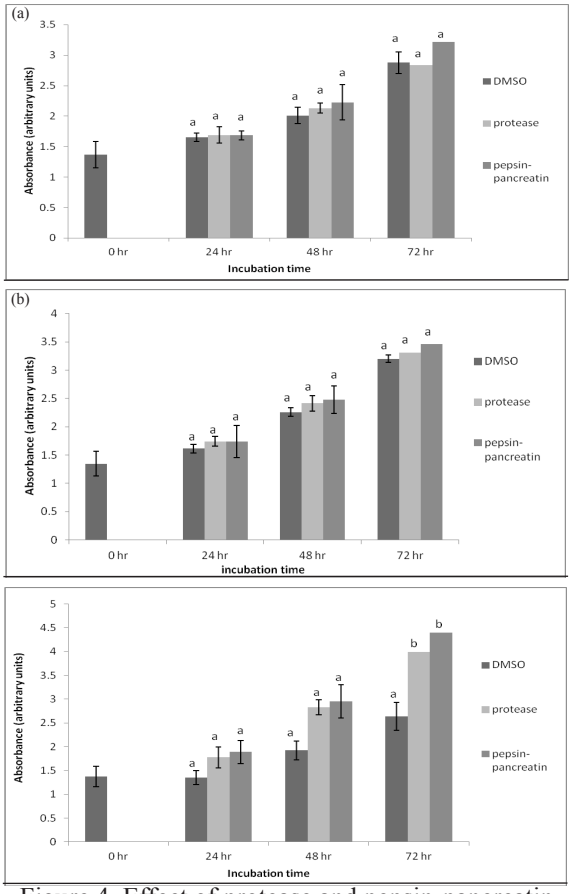

Figure 4. Effect of protease and pepsin-pancreatin hydrolysates on WST-1 readings in HepG2 cells whereby the cells were subjected to pretreatment with a) 1% BSA-1 mM oleic acid, b) 1% BSA and c) control, 24 hr prior to the experiment. Subsequently, the cells were then stimulated with peptide samples (protease and pepsin hydrolysates at 1 mg/ml). The results are means of triplicate independent analyses. Columns marked with different lower case letters (a-b) denote significantly different ($p < 0.05$) with respect to control (2% DMSO).

in HepG2 cells (demonstrated in Figures 4a and 4b) compared to Figure 4c. As shown in Figure 3, Figures 4a and 4b, reduction of cell viability determined as the relative density of the adherent cells was not observed. Cell viability remained constant even at high concentrations of both OPKHs (100 µg/ml and 1 mg/ml), which represents a high dose of the natural substances.

Initially, oleic acid (1 mM) was added as a stimulant for the HepG2 cells so that the cells are not at the resting state. HepG2 cells need to be stimulated before subsequent test is being carried out. Since the physiological concentration of oleic acid was 0.1-0.8 mM (Dixon *et al*., 1991), hence the oleic acid added into the HepG2 cells in this experiment (1 mM) was considered equal to the actual concentration in the human body. However, it didn't damage HepG2 cells because as a mono-unsaturated fatty acid (MUFA), oleic acid would be metabolized into more complex metabolites such as arachidonic acids

that could promote cell growth. From the results above, we hypothesized that oleic acid could serve as a modulator in normalizing the cell viability of OPKHs in WST-1 assay. Future studies using more sophisticated methodology with suitable biomarkers should be carried out to prove this hypothesis.

By observing the increasing trend of WST-1 readings from 0 to 72 hr (Figures 3 and 4), we could partially suggest that this was the mitotic effect (cell division) of HepG2 cells because the presence of growth factors such as essential and non-essential amino acids. Since the concentration of serum added into the culture was only at 1% BSA, hence the effect of serum on HepG2 cell growth was considered negligible. An expansion in the number of viable cells resulted in an increase in the overall activity of mitochondrial dehydrogenases in the HepG2 cells (Ganner *et al*., 2010). This leads to an increase in the amount of WST-1 formed, which directly correlates to the number of metabolically active cells in the culture. WST-1 produced by the metabolically active HepG2 cells was then quantitated by a scanning multi-well spectrophotometer (ELISA plate reader) (Ganner *et al*., 2010). In conjunction with that, incubations of the HepG2 cells with OPKHs at various concentrations at several time points (24, 48 and 72 hr) were carried out (Figure 2) to be able to distinguish or validate between effects on specific organelles or cytotoxicity (Fotakis and Timbrell, 2006).

HepG2 cell was used as a cellular model to determine the safety of OPKHs in this study. Cells were pretreated with a range of OPKH concentrations (0.1-100 µg/ml and 1 mg/ml) and it was found that OPKHs did not have cytotoxic effect. WST-1 assay is based on the cleavage of WST-1 salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] into chromogenic formazan by reductases and mitochondrial dehydrogenases (Weir *et al.*, 2011). WST-1 reagent was reduced extracellularly which was different compared to MTT. Being a negatively charged molecule, WST-1 possesses two sulfonate groups and hence preventing it from entering cells (Weir *et al*., 2011). Being positively charged, MTT penetrates the plasma membrane easily and was reduced by microsomal enzymes in endoplasmic reticulum and mitochondria intracellularly (Weir *et al.*, 2011). Due to these reasons, we did not perform a comparison study by comparing the cytotoxicity of both OPKHs using MTT assay.

The good solubility of reduced WST-1 salt makes this WST-1 assay the most convenient and commonly used tetrazolium salt technique (Weir *et al*., 2011). WST-1 has a wider linear range and shows accelerated colour development compared to MTT. Besides, WST-1 assay also showed very low background absorbance and high sensitivity, enabling it to be conveniently adapted to microplate format without compromising sensitivity (Tan and Berridge, 2000). Moreover, WST-1 formazan end-product is stable, which is very suitable for sophisticated screening of cytotoxicity assay (Carlson, 2006). However, one disadvantage of WST-1 assay is that it is not a hard measure and hence it only yields relative quantitation (Carlson, 2006). Hence, the results above should be interpreted carefully. On the other hand, cytotoxicity assays are unable to differentiate between a reduction in metabolic or proliferative activity per cell and a reduced number of cells. Hence, any novel observation has to be confirmed by clonogenic survival assays (Freshney, 2005) or *in vivo* studies (Navarro *et al.*, 2013).

Conclusion

The protease and pepsin-pancreatin hydrolysates from oil palm kernel protein were non cytotoxic on HepG2 cells even at high exposure levels as determined using WST-1 assay. Oleic acid appeared to normalize the WST-1 readings of HepG2 cells treated with both hydrolysates at 1 mg/ml. Although *in vivo* studies are warranted in further studies, the results suggest that both hydrolysates can safely been used, in terms of cell toxicity, as potential functional foods.

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