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GENOTOXICITY AND CYTOTOXICITY EVALUATION OF SEA CUCUMBER (*Stichopus horrens*) PROTEIN HYDROLYSATES

OBREN JAMES KAWANDING*, NORIHAM ABDULLAH, ZAINON MOHD NOOR, NOORAAIN HASHIM, NAZAMID SAARI, MOHD FAKHARUL ZAMAN RAJA YAHYA and MOHD FAIZ FOONG ABDULLAH

School of Biological Sciences, Faculty of Applied Sciences,
Universiti Teknologi MARA (UiTM), Shah Alam, 40450 Selangor Darul Ehsan
**Email: obren_123@yahoo.com*

ABSTRACT

Sea cucumber (*Stichopus horrens*) protein hydrolysates were known as a potential functional food source with angiotensin-converting enzyme (ACE) inhibitory. The genotoxicity properties of *S. horrens* protein hydrolysates have been investigated through two different *in vitro* tests: Ames test and *in vitro* micronucleus test while the cytotoxicity properties of *S. horrens* protein hydrolysate were assessed using neutral red test. The study was conducted at a concentration up to 8000 µg/ml, 80 µg/ml and 50 µg/ml for Ames test, *in vitro* micronucleus test and neutral red test respectively with and without metabolic activation. There were no increments in the number of revertant colonies observed at any concentrations of *S. horrens* protein hydrolysates with and without metabolic activation in all four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) compared to the solvent control. In *in vitro* micronucleus test, *S. horrens* protein hydrolysate did not induce clastogenicity in V79 cell while in neutral red test, *S. horrens* protein hydrolysate did not show any cytotoxic effects on NIH/3T3 mouse fibroblast cell. In conclusion, *S. horrens* protein hydrolysates are safe in terms of genotoxic and cytotoxic hence have the potential to be used in pharmaceutical and food industries as functional ingredient.

Key words: Sea cucumber (*Stichopus horrens*), protein hydrolysates, genotoxicity, cytotoxicity

INTRODUCTION

Sea cucumber (*Stichopus horrens*) protein hydrolysate was studied as a potential source of peptide containing angiotensin-converting enzyme (ACE) inhibitory (Forghani *et al.*, 2012). Angiotensin-converting enzyme (ACE) which is responsible for the elevation of blood pressure acts as an exopeptidase that converts an inactive form of decapeptide (angiotensin-I) to a potent vasoconstrictor, an octapeptide (angiotensin-II), and inactivates the catalytic function of bradykinin, exhibiting depressor action (Ondetti *et al.*, 1977; Raia *et al.*, 1990). Peptides with specific amino acid pattern at N- and C-terminal are able to inhibit ACE thus, reducing blood pressure. Such peptides which are intact within the original protein can only exhibit their inhibition upon releasing by proteolytic enzymes (Forghani *et al.*, 2012).

The high potential of *S. horrens* hydrolysate towards the development of functional foods and pharmaceutical industries development has been recognized (Aydin *et al.*, 2011; Bruckner *et al.*, 2003; Conand, 2001; Conand and Byrne, 1993; Lawrence *et al.*, 2010). Moreover, the possibility of the *S. horrens* hydrolysates that will be used as a functional ingredient to become more reactive than the native protein is high due to its low molecular weight (Choi *et al.*, 2012). Therefore, the safety of these *S. horrens* hydrolysates should be verified and evaluated. These *S. horrens* hydrolysate should be tested for the absence of cytotoxicity and genotoxicity in order to protect consumers from the potential side effects of these products that may be harmful to the human body and to ensure that these protein hydrolysates are safe to be exploited for human nutrition and health. Several protein hydrolysates such as casein was derived from powdered fermented milk and Tensguard™, a protein hydrolysate derived from cow's milk and have been tested for their toxicological effects and it was reported that casein and Tensguard™ did not show any mutagenic or claustogenic activity (Maeno *et al.*, 2005; Ponstein *et al.*, 2009). The safety evaluation of protein hydrolysate is an essential step before that particular protein hydrolysate can be applied in the manufacturing of functional foods.

* To whom correspondence should be addressed.

Concerning the safety of the protein hydrolysate derived from *S. horrens*, *in vitro* genotoxicity and cytotoxicity studies were conducted in this present paper.

MATERIALS AND METHODS

Materials

S. horrens protein hydrolysate was supplied by Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia. All other reagents used in this study were of analytical grade.

Ames test

The bacterial reverse mutation assay (Ames test) was performed in compliance with OECD guideline no. 471 using the plate incorporation method with the histidine-requiring *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 in the absence and presence of a liver fraction of Arochlor 1254-induced rats for metabolic activation (S9-mix). The final concentration of liver homogenate fraction was 10%. Five *S. horrens* hydrolysates concentrations were used ranging from 100 µg/ml to 8000 µg/ml. Negative controls (dimethyl sulfoxide) and positive controls were run simultaneously. The positive control substances were sodium azide (TA100 and TA1535), ICR 191 Acridine (TA1537), Daunomycin (TA98) and 2-aminoanthracene (TA98, TA100, TA1535 and TA1537 in the presence of the S9-mix). Bacteria were exposed to the substances at 37ºC, for 30 minutes. Toxicity was defined as a reduction (at least 50%) in the number of revertant colonies and/or clearing of the background lawn of bacterial growth. The assay was considered valid if the mean colony counts of the control values of the strains were within acceptable ranges and if the results of the positive controls met the criteria for a positive response (i.e. a two-fold increase compared to the negative control). The test substance was considered to be mutagenic if the mean number of revertant colonies on the test plates was increased in a concentration-related way or if a reproducible two-fold or more increase was observed compared to that of the negative control plates.

Neutral red test

Cytotoxicity assay was done to investigate the toxicity effects of the *S. horrens* protein hydrolysates samples on cultured cells. Cytotoxicity was determined using neutral red (NR) assay based on initial protocol described by Borenfreund and Puerner in 1984. The NIH/3T3 mouse fibroblast cell lines was cultured in appropriate media and conditioned in culture flasks. Prior to the assays, the cells were trypsinised and seeded into 96 well microplates at about 5 x 10^4 cells/well. The cells were treated with different concentrations of the *S. horrens* protein hydrolysate samples and sodium dodecyl sulfate (SDS) as positive control for up to 24 hours. Then, the cell culture was incubated in a humidified incubator at 37ºC. After incubation, the neutral red solutions were added into the cells suspension and were subjected to ten minutes incubation at 37ºC. The absorbance value of solutions were measured using the microplate spectrophotometer system (Spectra max190-Molecular Devices) with wavelength at 540 nm. The results were analyzed with the Soft max pro software. Triplicate test was performed for each concentration of protein hydrolysates in order to create significant results. The EC_{50} of the test hydrolysate samples was determined by plotting the percentage of cell viability versus concentration of hydrolysate samples. The EC_{50} is the concentration of hydrolysate samples that causes 50% cell death (Pezzuto *et al.*, 1990).

In vitro micronucleus test

In vitro micronucleus test was conducted for genotoxicity evaluation of the *S. horrens* hydrolysates sample, as per OECD guidelines number 487. Chinese hamster lung fibroblast cells (V79 cells) were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated-FBS, 0.2 mg/ml L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were kept in tissue-culture flasks at 37ºC in a humidified atmosphere, containing 5% CO_2 in air, and were harvested by treatment with 0.15% trypsin–0.08% EDTA in phosphate-buffered saline solution (PBS). The cell culture then was treated with *S. horrens* hydrolysates with concentration of 12.5 µg/ml, 25 µg/ml and 50 µg/ml and incubated. DMSO was used as negative control and clastogenic agent mitomycin C as positive control. The cells were observed under fluorescence microscope after stained with acridine orange dye for the formation of micronuclei.

RESULTS AND DISCUSSION

Ames test

S. horrens hydrolysates were treated on the plates at concentrations of 100 µg/ml, 300 µg/ml, 890 µg/ml, 2670 µg/ml and 8000 µg/ml. The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant increase in the number of revertants was observed. *S. horrens* hydrolysates did not induce an increase in the number of revertant colonies in all strains (TA98, TA100, TA1535 and TA1537) tested both in the absence and presence of metabolic activation (Table 1). The positive control substances gave the expected increase in the number of revertant showing the validity of the test.

Table 1. Response of *S. horrens* hydrolysates in the Ames test

Concentration (µg/ml)	TA98*	TA100*	TA1535*	TA1537*
Without metabolic activation				
Negative control	7±3	129±11	22±13	33±27
100	12±4	203±15	23±4	32±20
300	13±6	155±13	26±12	36±26
890	10±3	171±27	30±18	27±21
2670	11±3	129±13	32±0	34±18
8000	15±3	150±29	35±21	32±19
Positive control	148±45	938±380	615±8	5003±182
With metabolic activation (10% S9-mix)				
Negative control	16±5	117±16	22±1	37±13
100	29±6	118±12	19±6	23±17
300	20±2	129±7	26±7	24±5
890	31±4	126±20	23±1	20±6
2670	16±10	135±5	26±7	23±6
8000	31±10	134±6	21±1	28±15
Positive control	1188±424	887±43	192±11	362±63

* Mean number of revertant colonies/3 replicate plates ± standard deviation.

Neutral red test

In order to evaluate the cytotoxic effect of *S. horrens* protein hydrolysates, the neutral red cytotoxicity assay was performed. The neutral red cytotoxicity assay results obtained with the protein hydrolysates of *S. horrens* against NIH/3T3 mouse fibroblast cell lines are summarized in Fig 1. The EC_{50} of the test hydrolysate samples was determined by plotting the percentage of cell viability versus concentration of hydrolysate samples. The EC_{50} is the concentration of hydrolysate samples that causes 50% cell death (Pezzuto *et al.*, 1990). In this study, the cell treated with *S. horrens* protein hydrolysates does not exert EC_{50} since none of them caused 50% cell death.

In vitro micronucleus test

The number of micronucleus per 1000 cells per treatment was determined in V79 Chinese hamster lung cells treated at various concentrations of *S. horrens* protein hydrolysates with and without metabolic activation. Percentage of micronucleated cells is presented in Table 2. *S. horrens* protein hydrolysates had no significant effect on the number of micronuclei induced at all concentrations tested in the study when compared to the negative control. The positive clastogens; mitomycin C, significantly enhanced the number of micronuclei. The study of DNA damage at the chromosomal level and micronuclei formation is a vital part of genetic toxicity screening. The *in vitro* micronucleus assay

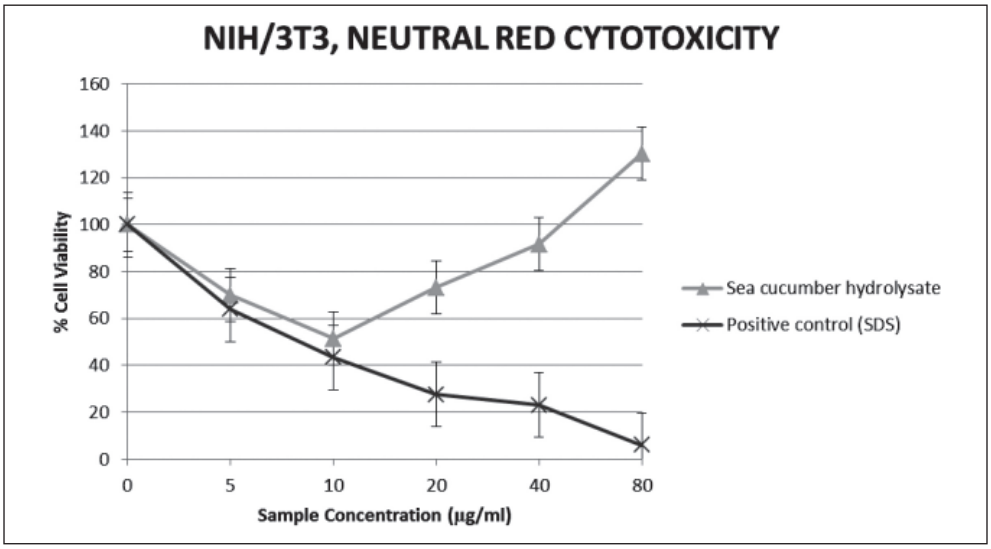

Fig. 1. The cytotoxic effect of *S. horrens* protein hydrolysates on NIH/3T3 mouse fibroblast cells.

Table 2. Effect of the protein hydrolysates on micronucleus induction in V79 cells

Treatment	Micronucleated cells (%)*
Without metabolic activation	
Negative control (DMSO)	1.1
12.5 µg/ml	0.9
25 µg/ml	0.9
50 µg/ml	0.9
Positive control (mitomycin C)	4.6
With metabolic activation (10% S9-mix)	
Negative control (DMSO)	1.0
12.5 µg/ml	0.7
25 µg/ml	0.4
50 µg/ml	0.7
Positive control (mitomycin C)	14.4

* Percentage of micronucleated cells per 1000 cells per treatment.

was carried out with *S. horrens* protein hydrolysates using V79 Chinese hamster lung cells. The hydrolysates did not induce significant micronuclei formation in V79 cells *in vitro*.

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REFERENCES

Aydin, M., Sevgili, H., Tufan, B., Emre, Y. & Köse, S. 2011. Proximate composition and fatty acid profile of three different fresh and dried commercial sea cucumbers from Turkey. *International Journal of Food Science and Technology,* **46(3)**: 500-508.

Bruckner, A.W., Johnson, K.A. & Field, J.D. 2003. Conservation strategies for sea cucumbers: Can a CITES Appendix II listing promote sustainable international trade? SPC Beche-de-mer. *Information Bulletin.,* **18**: 24-33.

Choi, J., Sabikhi, L., Hassan, A. & Anand, S. 2012. Bioactive peptides in dairy products. *International Journal of Dairy Technology,* **65(1)**: 1-12.

Conand, C. & Byrne, M. 1993. A review of recent developments in the world sea cucumber fisheries. *Marine Fisheries Review,* **55**: 1-13.

Conand, C. *Overview of Sea Cucumbers Fisheries over the Last Decade – What Possibilities for a Durable Management? The Netherlands, 2001;* pp.339-344.

Forghani, B., Ebrahimpour, A., Bakar, J., Abdul Hamid, A., Hassan, Z. & Saari, N. 2012. Enzyme Hydrolysates from Stichopus horrens as a New Source for Angiotensin-Converting Enzyme Inhibitory Peptides. *Evidence-Based Complementary and Alternative Medicine, 2012*, 9.

Lawrence, A.J., Afifi, R., Ahmed, M., Khalifa, S. & Paget, T. 2010. Bioactivity as an options value of sea cucumbers in the Egyptian Red Sea. *Conservation Biology,* **24(1)**: 217-225.

Maeno, M., Nakamura, Y., Mennear, J.H. & Bernard, B.K. 2005. Studies of the toxicological potential of tripeptides (L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline): III. Single- and/or repeated-dose toxicity of tripeptides-containing Lactobacillus helveticus-fermented milk powder and casein hydrolysate in rats. *International Journal of Toxicology,* **24 Suppl 4**: 13-23.

Ondetti, M.A., Rubin, B. & Cushman, D.W. 1977. Design of specific inhibitors of angiotensin converting enzyme: new class of orally active antihypertensive agents. *Science,* **196(4288)**: 441-444.

Pezzuto, J.M., Beattie, C.W., Lowell, K., Mar, W., Swanson, S.M., Reutrakul, V. & Farnsworth, N.R. 1990. Evaluation of the cytotoxic potential of natural products employing a battery of human cancer cell lines. *Planta Medica*, **56(6)**.

Ponstein-Simarro Doorten, A.Y., vd Wiel, J.A.G. & Jonker, D. 2009. Safety evaluation of an IPP tripeptide-containing milk protein hydrolysate. *Food and Chemical Toxicology,* **47(1)**: 55-61.

Raia, J.J., Jr. Barone, J.A., Byerly, W.G. & Lacy, C.R. 1990. Angiotensin-converting enzyme inhibitors: a comparative review. *DICP,* **24(5)**: 506-525.