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 angiotensinconverting 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 μ g/ml, 80 μ g/ml and 50 μ g/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 TensguardTM, a protein hydrolysate derived from cow's milk and have been tested for their toxicological effects and it was reported that casein and TensguardTM 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 37°C, 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 twofold 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 5×10^4 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 37°C. After incubation, the neutral red solutions were added into the cells suspension and were subjected to ten minutes incubation at 37°C. 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 EC₅₀ 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 37°C 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 $\mu g/ml,$ 25 $\mu g/ml$ and 50 $\mu g/ml$ 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 μ g/ml, 300 μ g/ml, 890 μ g/ml, 2670 μ g/ml and 8000 μ g/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.

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)	1			
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

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

* 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 EC₅₀ of the test hydrolysate samples was determined by plotting the percentage of cell viability versus concentration of hydrolysate samples. The EC₅₀ 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 EC₅₀ 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

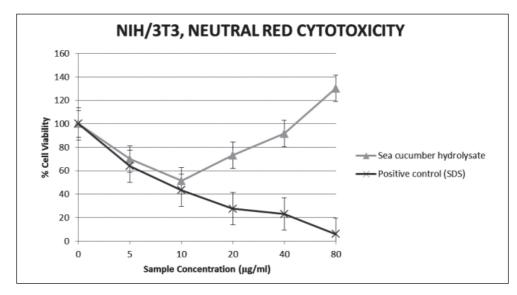


Fig. 1. The cytotoxic effect of *S. horrens* protein hydrolysates on NIH/3T3 mouse fibroblast cells.

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

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

* 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*.

ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Science, Technology and Innovation (MOSTI) to finance the project under the MOSTI fund (100-RMI/MOSTI 16/6/2 (1/2012)).

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