Genotoxicity and Cytotoxicity Evaluation of Winged Bean (*Psophocarpus Tetragonolobus*) Protein Hydrolysate

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Abstract: *Winged bean (Psophocarpus tetragonolobus) protein hydrolysates were known as potential functional food sources that have angiotensin-I-converting enzyme (ACE) inhibitory and antioxidative properties. The genotoxicity properties of winged bean protein hydrolysates have been investigated through two different in vitro tests: Ames test and in vitro micronucleus test while the cytotoxicity properties of winged bean 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 winged bean 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, winged bean protein hydrolysate did not induced clastogenicity in V79 cell while in neutral red test, winged bean protein hydrolysate did not shows any cytotoxic effects on NIH/3T3 mouse fibroblast cell. In conclusion, winged bean protein is safe in term of genotoxic and cytotoxic and hence has the potential to be used in pharmaceutical and food industries as functional ingredient.*

Keywords: *genotoxicity, cytotoxicity, winged bean, protein hydrolysate.*

1. Introduction

Bioactive-peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. Traditionally, peptides preparations are frequently use as a source of protein substances in different kind of dietetic, nutritional and medicinal supplement. It has been shown that such peptides, which can be specifically produced through biotechnological means, have functional activities such as antithrombotic, antioxidative [6], immune defense [15] and antihypertensive [17] apart from improving the nutritional values of food depending.

The development of functional foods is likely to entail the increased use of different protein sources. These protein sources may be natural constituents of plant or animal origins or genetically modified. Bioactive peptides have been produced from a wide range of food materials including egg, milk, fish, soy products, wheat and mushroom [3]. The introduction into the diet of functional foods supplemented with these compounds may raise the issue of toxicity. There are, however, a number of scientific and technological issues to be resolved before these substances can be optimally exploited for human nutrition and health. Basic research on the effect of bioactive proteins and their potential side-effects, e.g. allergenicity and toxicity, is an important future research needs related to bioactive proteins [7].

Dietary proteins, beside for their ability to supply calories and amino acids, the proteins also been known to offer health benefits *in vivo* and *in vitro* in the intact form or as hydrolysates form. Food protein hydrolysates that may induce beneficial biological functionalities are called bioactive peptides. Protein hydrolysates with both antimicrobial and cancer cell cytotoxic effects have been reported in several studies [4] [2].

Recently, the study on winged bean *(Psophocarpus tetragonolobus)* protein hydrolysate bioactivites has been conducted [16]. The results of the study shows that winged bean protein hydrolysate demonstrated angiotensin-I-converting enzyme (ACE) inhibitory and antioxidative activities hence has the potential to be a bifunctional food ingredient [16].

To date, several bioactive peptides have been characterized functionally and toxicologically. Tensguard™ is a protein hydrolysate derived from the hydrolysis of the GlycoMacroPeptide fraction of cow's milk and marketed as a food supplement. Tensguard contains high amount of a peptide Isoleucine-Proline-Proline (IPP) and is reported to reduce blood pressure in hypertensive rats [13]. The safety profile of Tensguard™ was evaluated using a panel of genotoxicity tests – the Ames bacterial reverse mutation test (OECD number 471), the (OECD number 476) and the human lymphocyte chromosomal aberration test. Results indicated that Tensguard is not mutagenic or clastogenic [13]. A 90-day subchronic animal toxicology test further showed Tensguard is not toxic to rats even under excessive amounts of consumption [13]. There was, however, an indication of cytotoxicity when the lymphoma cell cultures were continuously exposed at a high concentration of 5 mg/ml.

Valyltron is a protein hydrolysate derived from the enzymatic hydrolysis of sardines. Valyltron contains high amount of the dipeptide valyl-tyrosine (VY) and has also been reported to lower the blood pressure of mildly-hypertensive human subjects [11]. The safety profile of Valyltron was evaluated using genotoxicity and animal toxicology tests. The Ames test and an *in vivo* micronucleus assay indicate that Valyltron is not mutagenic or clastogenic. At high dose levels, however, the dipeptide appears to have a potential effect on erythropoiesis. Acute and subchronic feeding test in rats did not reveal any toxic effects of the intermediate product alpha-5000 (the precursor to Valyltron). Based on these observations, it was concluded that the dipeptide has no systemic toxic effects [11].

Considering the above fact, the safety of winged bean (*Psophocarpus tetragonolobus*) protein hydrolysates were assessed *in vitro* in this study. Ames test was performed based on OECD guidelines number 471 to determine the ability of the hydrolysate sample to induce mutagenic effects on the indicator microorganisms. *In vitro* micronucleus test were performed based on OECD guidelines number 487 to evaluate the effects of the hydrolysate sample on the genetic system by analyzing the formation of micronuclei in the V79 chinese hamster lung cell. The cytotoxic effects of the hydrolysates were evaluated trough neutral red test by analyzing the viability percentage of NIH/3T3 mouse fibroblast cell after treated with winged bean protein hydrolysate.

2. Materials and Methods

2.1. Chemicals

Dulbecco's modified eagle medium (DMEM), sodium pyruvate, sodium bicarbonate ($NaHCO^3$), glucose-6-phosphate, dimethyl sulfoxide (DMSO), nicotinamide adenine dinucleotide phosphate (NADP) and Foetal bovine serum (FBS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The metabolic activation system Arochlor™ 1254-induced S9 fraction (Sprague Dawley rats), mutant *Salmonella typhimurium* strains (TA98, TA100, TA1535 and TA1537), Sodium azide, ICR 191 acridine, daunomycin, 2-aminoanthracene, mitomycin C were purchased from Moltox. Trypsin-EDTA was purchased from Gibco Life Technologies (Grand Island, NY). All other chemicals and solvents used in this study were of the highest analytical grade available.

2.2. Protein Hydrolysate Sample

The winged bean protein hydrolysate was supplied in crude powder form by Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

2.3. Bacterial Strains and Cell Lines

The Ames test was performed with Salmonella typhimurium histidine auxotrophs, TA98, TA100, TA1535 and TA1537. Freshly thawed frozen strains of 10 µl were inoculated in 10 ml of growth medium and the cultures were grown overnight at 37 ºC in an environmental shaker at 180 rpm. The Chinese hamster lung cell V79 and mouse fibroblast cell NIH/3T3 cell was purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 10

mM sodium pyruvate in a 37 ºC incubator maintained at 5% CO2. Subcultures were carried out every 2 days using 0.25% Trypsin-EDTA solution.

2.4. Ames Test

The bacterial reverse mutation test was performed in compliance with OECD guideline number 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^{TM} 1254-induced rats for metabolic activation (S9-mix). The final concentration of liver homogenate fraction was 10%. Five winged bean hydrolysates concentrations were used ranging from 100 µg/ml to 8000 µg/ml. Negative controls (DMSO) 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 approximately 72 hours. 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.

2.5. Neutral red test

Cytotoxicity assays was done to investigate the toxicity effects of the winged bean 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 winged bean protein hydrolysate samples for up to 24 hours. Then, the cell culture 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. Then, the absorbance of mixtures of the resulting solutions was measured using the microplate spectrophotometer system (Spectra max190-Molecular Devices) with wavelength at 540 nm. The results will be analyzed with the Soft max pro software. Triplicate test will be 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 (Swanson and Pezzuto, 1990).

2.6. *In vitro* micronucleus test

In vitro micronucleus test was conducted for genotoxicity evaluation of the winged bean 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 winged bean 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 agents mitomycin C as positive control. The cells were observed under fluorescence microscope after stained with acridine orange dye for the formation of micronuclei.

3. Results

3.1. Ames Test

The mean numbers of histidine revertants at all the tested concentrations,i.e., 100 µg/ml, 300 µg/ml, 890 µg/ml, 2670 µg/ml and 8000 µg/ml of winged bean protein hydrolysates and positive controls in the absence and presence of S9 fraction in *S. typhimurium* mutant strains TA98, TA100, TA1535 and TA1537 was studied. The positive controls demonstrated a high increase in the number of revertant colonies both in the absence and

presence of metabolic activation. On the other hand, no significant increase in the number of histidine revertants observed at any concentration level of winged bean protein hydrolysates in five strains of the *S. typhimurium* (TABLE 1).

TABLE I: Response of winged bean hydrolysates in the *Salmonella typhimurium* reverse mutation assay. Results represent mean number of revertant colonies/3 replicate plates ± standard deviation.

Concentration (µg/ml)	TA98	TA100	TA1535	TA1537
Without metabolic activation				
Negative control	22±3	240±21	23±2	28±11
100	28±5	239±26	15±3	36±6
300	30±6	213±20	18±6	36±12
890	31±8	192±39	12±4	37±11
2670	25±10	233±16	17±2	34±15
8000	29±8	251±42	17±6	25±8
Positive control	1475±240	1003±131	817±52	5112±645
With metabolic activation (10% S9-mix)				
Negative control	46±11	303±56	16±2	26±11
100	40±11	202±20	14±4	33±6
300	41±7	260±9	19±4	36±10
890	47±12	266±36	16±5	33±6
2670	51±3	278±17	17±1	14±2
8000	60±6	256±21	21±3	37±3
Positive control	6700±717	6061±613	152±51	594±38

3.2. Neutral Red Test

The cytotoxic assay (neutral red) results obtained with the protein hydrolysates of winged bean against NIH/3T3 mouse fibroblast cell lines are summarized in Fig. 1.

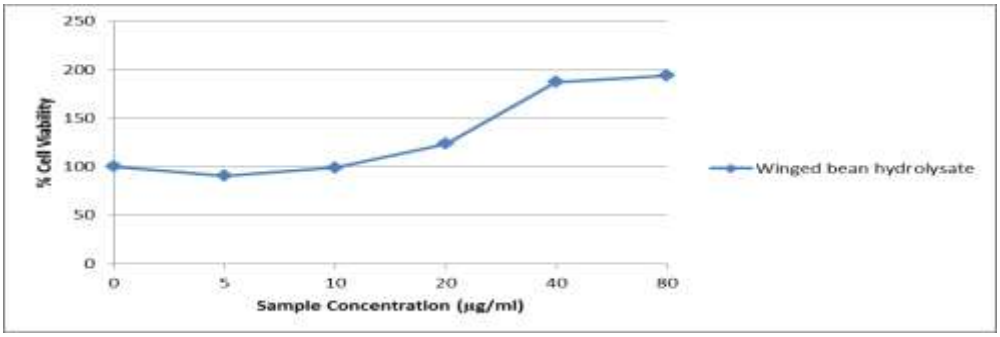

Fig. 1: The cytotoxic effect of winged bean protein hydrolysates on NIH/3T3 mouse fibroblast cells.

3.3. *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 winged bean protein hydrolysates with and without metabolic activation. Percentage of micronucleated cells was presented in TABLE 4. Winged bean protein hydrolysates had no significant effect on the number of micronuclei induced at all the concentrations tested in the study when compared to the negative control. Both the positive clastogens, mitomycin C, significantly enhanced the number of micronuclei.

TABLE IV: Effect of the protein hydrolysates on micronucleus induction in V79 cells.

Treatment	**Micronucleated cells (%)[a]**
Without metabolic activation	
Negative control	1.8
Winged bean (12.5 µg/ml)	0.4
Winged bean (25 µg/ml)	0.8
Winged bean (50 µg/ml)	1.7
Positive control	6.1

[a] Percentage of micronucleated cells per 1000 cells per treatment.

4. Discussion

The exploitation of biopeptides from winged bean as functional foods was a great achievement for the food technology industries. However, we believe that there are no previous *in vitro* studies conducted for genotoxicological evaluation of winged bean protein hydrolysates.

By taking into account the safety of the hydrolysates, a series of *in vitro* toxicological studies were performed to evaluate the genotoxic and cytotoxic profile of winged bean protein hydrolysates. The three *in vitro* models employed for screening the genotoxic and cytotoxic potential of winged bean hydrolysates were Ames test, cytotoxicity test and *in vitro* micronucleus assays both in the presence and absence of metabolic activation.

The significance of the Ames test has been clearly verified as a suitable primary test for the indicator of potential mutagens and carcinogens, and since mid-1970s this assay has been routinely used as a screening assay to detect carcinogens [8]. The mutagenicity of winged bean protein hydrolysates was determined using *Salmonella* reverse mutation assay both in the presence and absence of metabolic activation in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 at all the tested concentrations, i.e.,100 µg/ml, 300 µg/ml, 890 µg/ml, 2670 µg/ml and 8000 µg/ml. The number of histidine revertants in negative and positive controls was in accordance with the acceptable mean revertant values.

The results from the Ames test shows that in the absence of metabolic activation, winged bean protein hydrolysates did not exhibit any mutagenicity towards *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 up to a concentration of 8000 µg/ml. This implies that winged bean protein hydrolysates neither produced frame shift mutations in TA98 and TA1535 nor base pair mutations in TA100 and TA1537 strains of *S. typhimurium*. The addition of the hydrolysates at concentrations ranging from 300 µg/ml to 8000 µg/ml did not lead to a reproducible increase in the number of revertant colonies at one or more concentrations. The fold inductions in revertant histidine colonies over the solvent control were found to be less than two in the four mutant strains. However, the treatment with the positive controls sodium azide (TA100 and TA1535), ICR 191 Acridine (TA1537) and Daunomycin (TA98) exert their mutagenic effects on *Salmonella* tester strains in the absence of metabolic activation significantly amplified the frequency of histidine revertants.

Some of chemical substances are not directly genotoxic *in vitro* due to the absence of specific metabolic enzymes that are required to trigger the metabolic activation system, which will convert the pro drugs into their metabolically active forms or secondary metabolites [5]. As a result in this study, S9 mix solutions from Arochlor treated rats have been utilized as a source of metabolic activation system to reveal the possible risk of the test substances after metabolic activation.

In the presence of metabolic activation (S9), winged bean protein hydrolysates up to a concentration of 8000 µg/ml did not display any significant increase in histidine revertants when compared to negative control. S9 dependent mutagen, 2-aminoanthracene exhibited significant amplified number of revertant colonies in both the *S. typhimurium* mutant strains confirming its mutagenicity. 2-aminoanthracene is a heterocyclic amine and is reported to act as promutagens or procarcinogens requiring metabolic activation system for DNA adduct formation. Many studies have identified cytochrome P 450/A2 as the catalytic enzyme essential for bioactivation of this heterocyclic amine [1][14].

In order to evaluate the cytotoxic effect of winged bean protein hydrolysates, the neutral red cytotoxicity assay was performed. 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 [12]. In this study, the cells treated with winged protein hydrolysates do not exert EC_{50} since none of them causing 50% cell death.

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

5. Conclusion

In conclusion, the results in all the three *in vitro* tests demonstrated that winged bean protein hydrolysates were free of significant genotoxic and cytotoxic effect under our experimental conditions.

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