NEUROPROTECTIVE EFFECTS OF THE LIPIDIC EXTRACT FROM INTEGUMENT OF SQUID DOSIDICUS GIGAS

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NEUROPROTECTIVE EFFECTS OF THE LIPIDIC EXTRACT FROM INTEGUMENT OF SQUID *DOSIDICUS GIGAS*

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Key words: *Dosidicus gigas*, integument, phospholipids, amyloid β -peptide.

ABSTRACT

The integument of the squid *Dosidicus gigas* is an industrial waste and trouble the industrial processing. In this study, we examined the lipid composition and neuroprotective effects of *D. gigas* integument lipidic extract (DGI) in SH-SY5Y neuroblastoma cells which have been exposed to H_2O_2 . The results showed that phospholipids, triacylglycerol and cholesterol accounted for 70.90 ± 4.96 (mean $\pm$ S.D.), 20.70 ± 3.54 and $8.40 \pm 0.28\%$ of DGI, respectively. And the main fatty acids were docosahexaenoic acid (30.53% of total fatty acid), palmitic acid (20.36% of total fatty acid) and eicosapentaenoic acid (11.84% of total fatty acid). DGI at the concentrations of 2, 4 and 8 mg/ml successfully protected SH-SY5Y cells against the cytotoxicity, markedly increased the cell proliferation, antioxidant enzymes of glutathione contents and catalase activities, and inhibited the secretion of amyloid β -peptide 1-42. Our results suggested that DGI may be a possible approach in the prevention of Alzheimer's disease and other neurodegenerative diseases.

I. INTRODUCTION

The first histological clinical report of Alzheimer's disease (AD) is mentioned in 1907 [1]. The observations of patients with clinically developed AD indicate that numerous psychopathological symptoms, including intellectual dysfunctions, orientation dysfunctions, memory dysfunctions, emotional disturbances, motor and psychomotor dysfunctions, etc. [3]. These psychopathological symptoms would make AD patients lose living abilities gradually and depend on families and care-givers severely, and acting like a neonate. Up to 90% of patients with dementia developed significant behavioral problems during the course of their illness [34], however the sub-

stantial proportion of patients with AD develop delusions or hallucinations sometimes over the course of their illness [32]. In other words, AD is considered simply as another form of dementia and it is common for aging individuals as well. Amyloid β -peptide ($A\beta$), an insoluble fibril deposits, is the major component of the senile plaques (SP) that characterize AD brain [15]. It has been demonstrated that $A\beta$ is toxic directly to cultured neuron [37], and $A\beta$ monomers assemble into dimers, trimers and higher aggregates, all of which are highly neurotoxic associated with AD [39]. $A\beta$ involved on various neuronal cells surface receptors to generate reactive oxidative species (ROS), and ROS also leads to the hyperphosphorylation of Tau protein that destabilizes microtubules. This process eventually forms intracellular neurofibrillary tangles, one of observable hallmark pathology of AD [45]. In brain tissue, the level of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) activity and catalase (CAT) activity are significantly decreased in AD temporal cortex when compared to age-matched controls [26]. It was observed that the sites of neurodegeneration and oxidative stress are associated with increased $A\beta$ deposits in AD brain [19]. According to these observations, it is directly responsible for oxidative damage to neuron, leading to subsequent neuronal loss in the AD brain.

The squid *Dosidicus gigas*, also known as jumbo flying squid, is a commonly used by frozen seafood processing companies. Fresh squid *D. gigas* has a sticky consistency and their tissue is chewy. Its integument is separated from the edible part when unfrozen by hot water and treated as a fishery wastes troubling industrial processing [31]. Reportedly the phospholipids is 80-85% of total lipid in squid *Ommastrephes bartrami*, and docosahexaenoic acid (DHA) is a major fatty acid of total compositional fatty acid, moreover eicosapentaenoic acid (EPA) as the second [33, 10]. Numerous studies have reported that dietary DHA is required during development when cellular differentiation [7], enhanced a maze-learning ability in aged mice [24], prevention of AD [8], and reduced fish or DHA intake increases risk for AD [25]. Besides, phospholipids is a potentially useful source of ω -3 polyunsaturated fatty acid, and it has showed that intestinal absorption of phospholipids is followed by preferential uptake of ω -3 polyunsaturated fatty acid by the brain [27]. In animal model,

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it was also proved that DHA-enriched phospholipid diets supplement could restore DHA levels and cholinergic activity and modulate age-related alterations in hippocampus [14]. However, the action of lipidic extract from squid is still unclear, even though it is rich in ω -3 fatty acid.

In this study, we are the first one to investigate the neuro-protective effect of *D. gigas* integument lipidic extract (DGI) in SH-SY5Y neuroblastoma cells which have been exposed to H_2O_2 . To determine if integument of *D. gigas* could be a new resource of ω -3 fatty acid enriched phospholipids, which could be prepared to a reasonable cost.

II. MATERIALS AND METHODS

1. Material and Reagent

The integument of *D. gigas* was kindly supplied by the Mei-er Hau Fishing Co., Ltd (Tainan, Taiwan) as refrigerated lumps. SH-SY5Y, the neuroblastoma cell line was obtained from the Culture Collection and Research Center (CCRC, Hsinchu, Taiwan), originally from the American Type Culture Collection. All other reagents used in this study were of chemical grade and commercially available.

2. Extraction of *D. gigas* Integument Lipidic Extract (DGI)

The refrigerated integument was thawed, weighed, chopped and then stirred with ten volumes of acetone for 4 hr, and filtered through a filter paper (No. 1 TOYO, Japan). After filtering, the residual integument was dried in the dryer with current air (RISEN Co., LTD, RHD-602D) at 35°C for 2 hr, and then the residual integument was extracted with ten volumes of ethanol for 12 hr and centrifuged ($2,000 \times g$, 20 min). The supernatants were combined, concentrated under reduced pressure at 45°C, and DGI was obtained, and then kept it at -20°C for the following tests.

3. Lipid and Fatty Acid Composition Analyses of DGI

Qualitative and quantitative analyses of the lipid composition present in the integument extract were performed by thin-layer chromatography (TLC) coupled to an automated flame ionization detector (FID) system (Iatroscan MK-5, Iatron Laboratories, Tokyo, Japan). Triacylglycerol (TG), cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) standards were purchased from Sigma Chemical Co. The standards and DGI were dissolved in chloroform/methanol (2:1, v/v). The samples (1.0 μ l) were spotted onto silica gel-coated Chromarods (type S-III) (Iatron Laboratories) by procedure of a 2 μ l syringe Hamilton (Bondaluz, Switzerland) coupled to an SES 3202/IS-02 semi-automatic sample spotter (Nieder-Olm, Germany). The rods were developed consecutively four times using the following mobile phase: (1) 60 ml of chloroform/methanol/water/25% NH_3 (47:20:2.5:0.28, v/v/v/v) up to 1 cm; (2) 60 ml of hexane/diethyl ether (63:7, v/v) up to 9 cm; (3) 70 ml of hexane/

benzene (35:35, v/v) up to 10 cm. After each development the rods were heated for 10-12 min at 60°C to dry the remaining solvent, and run through a flame ionization detector in the Iatroscan by using an air flow of 2.0 l/min atmospheric air, a hydrogen flow of 150 ml/min (high purity hydrogen, C50) and a scanning speed of 3.0 s/cm. A total scan was performed to identify all the lipid components. Data were processed with the Boreal software version 2.5. All data were analyzed in triplicate. The method was modified by Dominguez *et al.* [12].

DGI was prepared for fatty acid analysis according to the AOAC (FAME method) standard [16] and analyzed by capillary GC-FID (HP 5890 series II, HP-Innowax cross-linked PEG column). GC conditions were as follows: injector 250°C, detector 300°C, oven 210°C, and carrier-nitrogen 1 ml/min. The individual fatty acid peaks in the chromatograms were identified by comparison with authentic standards (Sigma Chem. Co.).

4. Cell Culture

SH-SY5Y neuroblastoma cells were grown in complete minimum essential medium (MEM) in a humidified air/5% CO_2 chamber at 37°C. Sixteen hours prior to treatment cells were washed free of fetal calf serum (FCS) containing medium and further incubated in FCS-free MEM containing SH-SY5Y cells growth supplement N_2 . All cell culture materials were purchased from Gibco Life Technologies (Rockville, MD, USA).

SH-SY5Y cells (2×10^5) were treated with 50 μ M H_2O_2 for 30 sec, and then treated with or without different concentrations of DGI (2, 4, 8 mg/ml) dissolved in 0.4% dimethyl sulfoxide (DMSO) in MEM for 24 hr. Cells treated without H_2O_2 and DGI as negative control (NC) and cells treated with H_2O_2 alone as positive control (PC). The concentration of H_2O_2 (50 μ M) was the result of previously study which demonstrated ability to induce oxidative stress cell cytotoxicity and $A\beta$ secretion [28].

5. Cell Proliferation Assay

Proliferation of cells was determined by streptomycin, 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay [38]. SH-SY5Y cells were washed with PBS three times and MTT (1 mg/ml) was added to the medium for 4 hr, and OD was read at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Wako, Tokyo) [29]. The relative cell proliferation % = $(A_1/A_0) \times 100\%$, A_0 was the absorbance of NC, and A_1 were the absorbance of DGI (0, 2, 4, 8 mg/ml) treated with H_2O_2 . The relative cell proliferation of NC was regarded as 100%.

6. Enzyme Activities Assay

For evaluating the content of glutathione (GSH) and CAT activity, SH-SY5Y cells (2×10^5) were treated with 50 μ M H_2O_2 for 30 sec, and then treated with or without different concentrations of DGI (2, 4, 8 mg/ml) dissolved in 0.4%

Table 1. Lipid composition of *D. gigas* integument lipidic extract (DGI).

Composition	Content* (%)
Triacylglycerol (TG)	20.70 ± 3.54
Cholesterol	8.40 ± 0.28
Total phospholipids	70.90 ± 4.96
Phospholipids classes	
Phosphatidylcholine (PC)	44.52 ± 5.23
Phosphatidylethanolamine (PE)	22.58 ± 0.45
Phosphatidylserine (PS)	6.80 ± 2.35
Others	26.10 ± 1.69

*Mean ± SD, n = 3

DMSO in MEM for 24 hr. Cellular GSH and CAT were determined using commercial assay kit (Cayman, Catalog No. 703002 and 707002), and OD was read at 405 and 540 nm in an ELISA reader. All assays were performed in triplicate.

7. Amyloid β -peptide (A β) assay

A β 1-42 secretion was determined by a sandwich ELISA kit (Covance, Catalog No. SIG-38942). SH-SY5Y cells (2×10^5) were treated with 50 μ M H₂O₂ for 30 sec, and then treated with or without different concentrations of DGI (2, 4, 8 mg/ml) dissolved in 0.4% DMSO in MEM for 24 hr. After incubation, cell culture medium was removed after desired time and assayed for A β . The A β ELISA assay was performed in triplicate.

8. Statistical Analysis

The data presented are mean ± S.D. of three determinations, and differences were considered to be statistically significant if $P < 0.05$.

III. RESULT AND DISCUSSION

1. Lipid Composition of DGI

The integument of *D. gigas* was tough but not fibrous. The content of DGI on dry base was 5.10%, since 550.8 mg of DGI was obtained from 10.8 g of dry *D. gigas* integument. Separation of lipid and phospholipids classes in DGI was accomplished by TLC-FID using Iatroscan technique, which was sensitive and quantitative for each lipid composition [11]. The lipid composition of DGI is given in Table 1. Phospholipids accounted for 70.90 ± 4.96% of DGI, which was lower than the phospholipids content of total lipid in integument of squid *Ommastrephes bartrami* (80-85% of total lipid) [10]. The TG and cholesterol of DGI were 20.70 ± 3.54 and 8.40 ± 0.28%, respectively, which were lower than phospholipids content (Table 1). It was also known that total lipid extracted from the mantle of female squid *Illex argentine*s had a higher percentage of phospholipids and had lower sterols [30]. But the liver lipid of squid *Watasenia scuntillans* had high level of TG (60.9-72.7%) [22].

Table 2. Fatty acid composition of total fatty acid in *D. gigas* integument lipidic extract (DGI)

Fatty acid	Area% of gas chromatography
C14:0	1.93
C16:0	20.36
C17:0	2.08
C18:0	10.69
C18:1 (ω -9)	2.83
C18:3 (ω -3)	8.05
C20:4 (ω -6)	4.66
C20:5 (ω -3)	11.84
C22:1	2.05
C22:5 (ω -3)	0.56
C22:6 (ω -3)	30.53
Others	4.42

The major component of phospholipids in DGI was PC (44.52 ± 5.23% of total phospholipids), PE (22.58 ± 0.45% of total phospholipids) and PS (6.80 ± 2.35% of total phospholipids) as follow. Our result was similar to the component of phospholipids in squid *O. bartrami* [10]. PC was found to be the main composition in the phospholipids classes of squid, such as *Sthenoteuthis oualaniensis* egg (67.50% of total phospholipids) [40], *Loligo vulgaris* (56% of total phospholipids) [23], and squid eye (30.7% of total phospholipids) [43]. Generally marine oils were vulnerable to oxidation, but in comparative study on oxidative stability indicated that PC of squid eye was oxidatively more stable than PC of soybean in liposomes [43]. Besides, in oxidative stability of lipid containing phospholipids did not always decrease with increasing phospholipids contents, and stability was strongly influenced by phospholipids composition [6]. So, it was suggested that DGI may be an oxidatively stable lipidic extract of *D. gigas* integument.

2. Fatty Acid Compositions of DGI

The composition in terms of the important fatty acid of total fatty acid in DGI was shown in Table 2. The main fatty acids in DGI were DHA (C22:6 ω -3) (30.53% of total fatty acid), palmitic acid (C16:0) (20.36% of total fatty acid) and EPA (C20:5 ω -3) (11.84% of total fatty acid). Comprising fatty acid composition of integument of *O. bartrami*, the main fatty acids were also DHA (34.4% of total fatty acid), palmitic acid (29.8% of total fatty acid) and EPA (14.1% of total fatty acid) [10]. Since phospholipids were a major composition of DGI, and total phospholipids were rich in DHA and EPA. Results implied that DGI was could be utilized as a good source of ω -3 rich phospholipids.

3. Effects of DGI on Cell Proliferation

H₂O₂ (50 μ M), a known oxidative stress inducer, which resulted in significant induction of cell cytotoxicity shown by

Fig. 1. Effect of *D. gigas* integument lipidic extract (DGI) on the relative cell proliferation by H_2O_2 -induced SH-SY5Y cells ($n = 3$). Means with asterisk were significantly different from the positive control ($P < 0.05$).

loss of cell viability, so we then tested whether DGI protected SH-SY5Y cells against H_2O_2 -induced cell death. As determined by MTT colorimetric assay, oxidative stress by H_2O_2 resulted in a decrease of the relative cell proliferation by $52.74 \pm 2.82\%$ (Fig. 1). DGI significantly inhibited cell death induced by H_2O_2 , resulting in an increase in the relative cell proliferation of $99.57 \pm 8.29\%$, $98.95 \pm 9.67\%$ and $90.11 \pm 5.42\%$ at concentrations of 2, 4 and 8 mg/ml, respectively (Fig. 1). Calvert *et al.* [5] demonstrated that post-treatment of DHA could protect SH-SY5Y cells from glutamate-induced cell death, and DHA showed significant and dose-dependent increases in the percentage of cells with longer neurites [41, 42]. Therefore, it was suggested that DGI had inhibitory effect against oxidative stress as a result of rich in ω -3 fatty acid.

4. Effect of DGI on Antioxidant Activities

It has been known that antioxidant system protects cells from H_2O_2 , oxygen free radicals, etc. This system consisted of the enzymes GSH, CAT and SOD [13]. A decrease in the activity of any of these enzymes would most likely lead to cell death in brain [35], and AD brain is subjected to increased oxidative stress resulting from reactive oxygen species [36]. In recently clinical trials, DHA enriched phospholipids were used as neuroprotective substance [9] and ω -3 fatty acid could prevent from Alzheimer's disease in animal model [8]. In an attempt to discern where DGI acted as oxidative protecting substance, we analyzed the content of GSH and the activity of CAT in follow.

The content of GSH and the activity of CAT from SHSY5Y cells were determined using ELISA kit. The content of GSH from negative control was 3.70 ± 0.22 mol/g protein and was markedly decreased by H_2O_2 in positive control to a level of 2.05 ± 0.09 mol/g protein. When DGI was added, it improved significantly the content of GSH, resulting in an increase of

Fig. 2. Effect of *D. gigas* integument lipidic extract (DGI) on the glutathione (GSH) content by H_2O_2 -induced SH-SY5Y cells ($n = 3$). Means with asterisk were significantly different from the positive control ($P < 0.05$).

Fig. 3. Effect of *D. gigas* integument lipidic extract (DGI) on the catalase (CAT) activity by H_2O_2 -induced SH-SY5Y cells ($n = 3$). Means with asterisk were significantly different from the positive control ($P < 0.05$).

3.10 ± 0.20 , 3.85 ± 0.17 and 3.84 ± 0.15 mol/g protein at concentrations of 2, 4 and 8 mg/ml respectively, and the value was closer to negative control (Fig. 2). Similarly, cellular CAT activity was decrease by H_2O_2 from 3.5 ± 0.14 to 2.3 ± 0.08 μmol/min/mg protein (Fig. 3). After DGI was adding, the CAT activities improved in an increase of 3.24 ± 0.16 , 3.37 ± 0.09 and 3.26 ± 0.20 μmol/min/mg protein at concentration of 2, 4 and 8 mg/ml respectively (Fig. 3). It was showed that DGI protected against H_2O_2 induced GSH and CAT loss.

Recently Kaur *et al.* [21] have shown that DHA acted as a neuroprotector for methylmercury-induced neurotoxicity in primary neural cell, and DHA also successfully protected

Fig. 4. Effect of *D. gigas* integument lipidic extract (DGI) on the amyloid β -peptide ($A\beta$) 1-42 secretion by H_2O_2 -induced SH-SY5Y cells ($n = 3$). Means with asterisk were significantly different from the positive control ($P < 0.05$).

neurons against the glutamate-induced cytotoxicity, markedly increased the cell viability and the activities of glutathione peroxidase and glutathione reductase [40]. So we considered that DGI was rich in DHA, thereby DGI may be a neuroprotector for H_2O_2 -induced neurotoxicity in neural cell.

5. Effect of DGI on $A\beta$ 1-42 secretion

$A\beta$ 1-42, $A\beta$ 1-40 and $A\beta$ 25-35 were all toxic to cultured hippocampal neurons [44], subsequent study showed that $A\beta$ 1-42 implicated for neurodegeneration in AD brain [4]. In order to analyze the potential anti- $A\beta$ -induced neurotoxicity properties of DGI, we analyzed the level of cellular $A\beta$ 1-42. $A\beta$ 1-42 secretion from SH-SY5Y cells was 6.63 ± 1.07 pg/mg in negative control, and was markedly stimulated by H_2O_2 to 42.30 ± 3.65 pg/mg. When DGI was added, it decreased significantly the level of $A\beta$ 1-42, resulting in a decrease of 8.30 ± 1.55 , 9.07 ± 2.87 and 8.73 ± 3.61 pg/mg protein at concentrations of 2, 4 and 8 mg/ml respectively, and the values were significantly lower than positive control (Fig. 4). In addition, enrichment of culture media with DHA has been found to reduce the level of $A\beta$ in PC12 neuron cells [20], and DHA also could significantly decreased the level of $A\beta$ in AD rats [18]. EPA, another important ω -3 fatty acid, acted as a precursor for DHA, which could ameliorate learning deficits associated with AD [17]. But, a contrary opinion was reported that DHA and EPA may accelerate neuronal loss in the terminal stages of prion or AD [2].

In conclusion, our study demonstrated the fact that DGI at physiological concentrations was able to protect neuronal cells from H_2O_2 -induced cell cytotoxicity, oxidative stress and $A\beta$ secretion for the first time. These findings were significant demonstrated that DGI may be a possible approach in the prevention of AD and other neurodegenerative diseases.

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