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*Original Article*

# Neuroprotective Activities of Enzymatically Hydrolyzed Peptides from Porcine Hide Gelatin

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**Abstract:** Central nervous system disorders, including cerebrovascular disease, neurodegenerative diseases and head trauma are the most common cause of severe disability in adults and share a number of pathophysiological features. The therapeutic strategy of neuroprotection has been well accepted as one of the promising approaches in treating such brain disorders, and searching for the effective neuroprotective agents is still an open-ended task for neurologists and neuro-pharmacologists. In this study, we report for the first time that the enzymatic hydrolysates from type-B porcine hide gelatin has potent neuroprotective activity against $H_2O_2$- or serum deprivation-induced injuries of cultured SH-SY5Y cells. The peptides used in this study were prepared from type-B porcine hide gelatin digested with pepsin and papain. The neuroprotective activity of the porcine hide gelatin hydrolysate (PHH) was evaluated using MTT reduction assay. From the pre-screening of PHH, we found that the whole porcine hide gelatin hydrolysate obtained from papain digestion (PHH-I) showed significant neuroprotective activities ($P<0.05$). After further separation of PPH-I through SP-Sephadex C-50 and Sephadex G-25, only the fraction with smaller molecular weight from Sephadex G-25 (PHH-Ic) demonstrated potent neuroprotective activities ($P<0.01$). The active fraction showed a molecular mass between 1,000-3,000Da in SDS-polyacrylamide gel electrophoresis, and was rich in Glycine, Proline and Hydroxyproline in amino acid composition, indicating that peptides with a spectrum of molecular sizes and certain amino acids are critical for the neuroprotective activities of gelatin peptides. The viability of cultured cells treated with gelatin peptides was significantly improved in a dose-dependent manner. Further studies are necessary to establish the neuroprotective activity of hydrolyzed peptides for the neurons *in vivo*.

**Key Words:** Gelatin, hydrolysate, antioxidative, neuroprotective, neurotrophic

## Introduction

The likelihood of brain disease at least once in a person's life has skyrocketed. Central nervous system disorders, including cerebrovascular diseases such as stroke, neurodegenerative diseases like Alzheimer's and Parkinson's disease, increase in their frequency with the increasing life expectancy of the population. They represent the most common cause of severe disability in adults. In addition, early onset of brain disorders including head trauma, multiple sclerosis, and schizophrenia also take a remarkable socioeconomic significance. To date, the etiology and pathogenesis of these diseases are not well addressed. Causal therapies or even prophylactic measures are available in only few and selected conditions. Despite the variability of predominant symptoms, age, cause and prognosis, the above-mentioned brain diseases share a number of pathophysiological features that display a uniform pattern of acute or delayed tissue destruction. It has been well accepted that therapeutic approaches should aim at harm reduction and brain function protection [1-5]. The strategies can be described with the term "neuroprotection". Neuroprotection as a means to prevent or delay pathological neuronal loss in central nervous system disease of various pathophysiological origins represents a novel therapeutic approach. They target nonspecifically mechanisms of the "final

common pathway" and/or the intrinsic balance between degeneration and regeneration.

The research on "neuroprotection" has been one of popular topics due to its potential significance. When compared with a large number of research on compounds such as xanthate [6] and vitamine E [7], the reports related to hydrolyzed peptides with neuroprotective activity from natural sources are very small.

Gelatin is a heterogeneous mixture of high molecular weight, water-soluble protein derived from collagen hydrolysis, and it contains relatively high amounts of amino acids such as glycine, proline, and alanine. Gelatin is normally classified as two categories, type A and type B. Type A gelatin is obtained from collagen material by acid treatment, while type B gelatin is referred to those obtained by alkali treatment. It is a translucent, colorless (or a light brown), brittle, and nearly tasteless solid substance. Gelatin is extensively used as a food additive to increase the texture, water-holding capacity, and the stability of several food products [8]. It is also commonly used in the photography and cosmetic industries [8]. Up to now, gelatin has been modified into biologically active peptides by protease treatments and hydrolyzed peptides with potentials to act as inhibitors of angiotensin I converting enzyme [9] and as antioxidants against peroxidation of linoleic acid [10]. A study also showed that the antioxidative peptides in the gelatin hydrolysate of bovine skin significantly enhanced the cell viability of cultured liver cells [11]. However, it is lacking in reported literatures regarding neuroprotective activity of gelatin peptides.

The objective of this study was to investigate the neuroprotective activity of type-B porcine hide gelatin hydrolysate (PHH). Specifically, we investigated the effect of enzymatically hydrolyzed gelatin on cultured SH-SY5Y cells. The results demonstrated that gelatin peptides, with smaller molecular weight and rich in Glycine, Proline and Hydroxyproline in the amino acid composition, could improve the viability of SH-SY5Y cells treated by $H_2O_2$ or cultured in serum-free media.

## Materials and Methods

### *Materials*

The type-B porcine hide gelatin was obtained from the Xinwulong Gelatin Co. (Xiamen, China). Pepsin and papain were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). SP-Sephadex C-50 and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the Amersham Biosciences (Sweden), respectively. Standard proteins for molecular mass determination were purchased from Gibco-BRL (Life Technologies, Gaithersburg, MD, USA). SH-SY5Y neuroblastoma cells were purchased from the American Type Culture Collection (ATCC, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM), F12 medium and fetal bovine serum were from Gibco (Grand Island, NY, USA). 3-[4,5-dimethylthiazol-2-yl]- 2, 5-diphenyltetrazolium bromide (MTT), penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, IL). All other reagents used were of the highest grade available commercially.

### *Sample Preparation*

*Enzymic Digestion*. According to the preliminary experiment results, the type-B porcine hide gelatin was hydrolyzed for 2 h separately with two different enzymes, pepsin and papain, under the conditions of pH 7.0 (phosphate buffer, 20 mM), 37°C, and at an enzyme-to-gelatin weight ratio of 1:10.

*Ultrafiltration*. The hydrolysates obtained from papain and pepsin were pooled, dialyzed against MilliQ water (pH 7.0) at 4°C for 24 hours with several changes, then initially fractionated through an ultrafiltration membrane system (Millipore Co., Bedford, MA) separately with molecular weight cutoffs (MWCO) 8,000 Da, and subsequently lyophilized and stored at -80°C until use. The prepared hydrolysates obtained from papain and pepsin were designated as porcine hide gelatin PHH-I and PHH-II, respectively.

### *Fractionation of gelatin hydrolysate*

*Cation-exchange chromatography*. The hydrolysed peptides of PHH-I demonstrating neuroprotective activities during preliminary scanning test below 8000 Da was dissolved in 50 mM sodium acetate buffer (pH 4.0) and applied onto a SP-Sephadex C-50 cation

exchange column (2.5 cm × 58 cm ) previously equilibrated with the starting buffer (50 mM sodium acetate buffer, pH 4.0). After elution of unadsorbed materials, bound peptides were eluted at a flow rate of 1 mL/min using a linear gradient of 0–1.0 M NaCl in the same buffer and the optical density was monitored at 225 nm. 5 mL fractions come under a single elution peak were pooled. Resulted fractions were lyophilized and tested for its neuroprotective activity.

*Gel filtration*. The adsorbed fraction on SP-Sephadex C-50 column demonstrating neuroprotective activity was pooled, dialyzed against 10 mM Tris – HCl buffer (pH 7. 2) at 4°C for 24 hours with several buffer changes and concentrated, then applied to the gel filtration chromatography on a Sephadex G-25 column (2.0 cm × 78 cm) previously equilibrated with the starting buffer. The flow rate was 0.30 mL/min, 10 min/tube, and the elution was monitored at 225 nm. The protective activity to cultured cells of each fraction was also determined.

*SDS-PAGE*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20% T, 4% C) was performed according to the method of Laemmii and Favre [12]. Gels were stained in 0.1% (w/v) Coomassie blue-30% (v/v) methanol-10% (v/v) acetic acid in water. The destaining solution was 30% (v/v) methanol-10% (v/v) acetic acid in water.

*Amino Acid Composition*

For the analysis of amino acid composition, 20 mg lyophilized type-B porcine hide gelatin PHH-Ic showing potent neuroprotective activity was hydrolyzed in 6 N HCl containing 0.1% thioglycolic acid under a vacuum at 110°C for 24 h. Amino acids generated with phenylisothio cyanate cleavage were identified and quantified using an automatic amino acid analyzer (HP1050, Hewlett-Packard Biotech, U.S.A).

*Cell Culture and Treatments*

SH-SY5Y cells were maintained in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. Cells were seeded into cell culture plates or dishes in DMEM/F12 (1:1) medium, supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Experiments were carried out 24–48 h after the cells were seeded. All components were dissolved in PBS and diluted freshly before adding to the cultures. The cyto-protective activity of compounds was estimated by two approaches, serum deprivation and $H_2O_2$-induced cell damage. In order to produce oxidative stress, $H_2O_2$ was freshly prepared from 30% stock solution prior to each experiment. Pre-incubation with gelatin hydrolysates at concentration of 5 ~ 150 mg/L was conducted 2 h before $H_2O_2$ addition. After 30 min exposure of 100 or 200 μM $H_2O_2$, the cultures were replaced by fresh medium. Cells were cultured continuously for another 24 hours before cell viability determination. For serum deprivation injury, SH-SY5Y cells were incubated in serum-free high glucose DMEM/F12 medium for 1-4 days with/without hydrolyzed peptides. All samples of gelatin peptides were dissolved and diluted in PBS and added into the cultures 2 h before serum deprivation. Assays for cell viability and morphological observation were performed at different time after $H_2O_2$ exposure and serum starvation.

*Cell Survival Determination*

Cell survival was evaluated by the ability to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), an indication of metabolic activity. MTT stock solution in PBS was added to each well with a final concentration of 0.5 mg/mL, and the incubation was continued for another 4 h at 37°C. Finally, an equal volume of a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (SDS, pH 4.8) was added. The mixtures were kept overnight at room temperature, and then the amount of MTT formazan was quantified by determining the absorbance at 570 nm and 630 nm using a Universal Microplate Reader (Bio-Tek).

*Morphological Observation*

Cell morphology was routinely assessed by phase contrast microscopic observation. Cells were observed and photographed via a CCD camera attached to the microscope.

*Statistical Analysis*

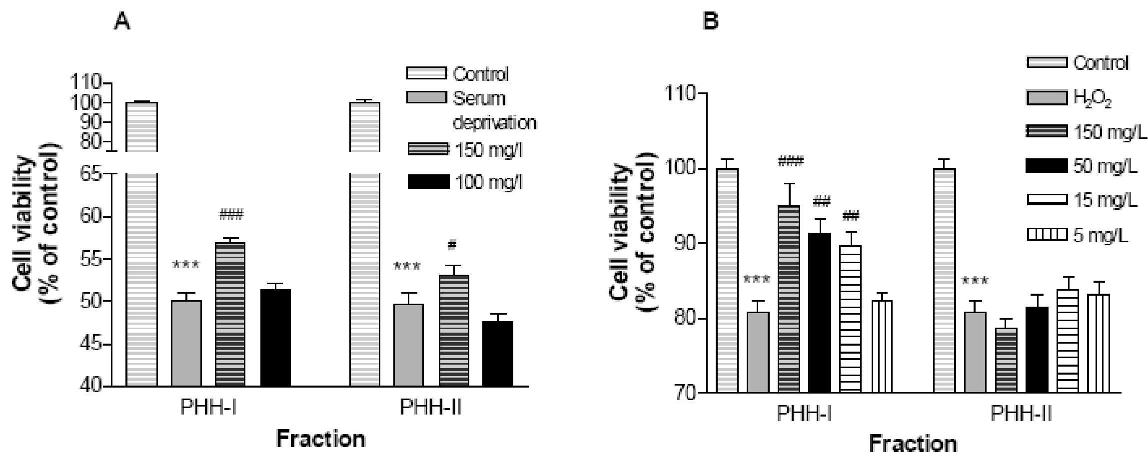


**Figure 1**. Effect of PHH-I and PHH-II on cell survival (MTT reduction) in human neuroblastoma SH-SY5Y cells stressed by serum deprivation (A) and $H_2O_2$ (B) injury. (A): Neurons were serum starving for 3 days. PHH-I and PHH-II at concentrations of 100 and 150 mg/L were added to the culture 2 h in advance. (B): Pre-incubation with PHH-I and PHH-II was conducted 2 h before $H_2O_2$ addition. After 30 min exposure of 100 μM $H_2O_2$, the medium was replaced by fresh medium. Cells were cultured continuously for another 24 hours before cell viability determination. Data expressed as means ± S.E.M. indicated by vertical bar. *** P < 0.001 *vs.* control; #P<0.05, ##P<0.01, ###P<0.001 *vs.* serum deprivation or $H_2O_2$ treated group. At least three independent experiments were carried out, n = 6 in each group.

At least three independent experiments were performed in double triplicate. The results were expressed as means ± S.E.M. Data were evaluated for significance with one-way ANOVA followed by Duncan's multiple range testing.

## Results

### *Hydrolysis of gelatin from porcine hide*

The type-B porcine hide gelatin was hydrolyzed for 2 h separately with two different enzymes, pepsin and papain, the total hydrolysates were pooled, dialyzed and initially fractionated through an ultrafiltration membrane system separately with MWCO 8,000 Da. The prepared PHH-I and PHH-II subsequently lyophilized for preliminary scanning test on neuroprotective activities.

### *Preliminary screening of PHH on neuroprotective activities*

In order to evaluate whether PHH possesses neuroprotective property, we first examined the effects of crude components of PHH, PHH-I and PHH-II, on serum deprivation and $H_2O_2$-induced neuronal injury. Active mitochondrial dehydrogenase of living cells can cleave MTT to produce formazan, the amount of which directly correlates with the number of metabolically active cells. As determined by MTT reduction, the survival of neurons was markedly decreased by about 50 % after 3 days serum deprivation. Treatments with both PHH fraction I and II at concentrations of 100-150 mg/L were able to attenuate cell injury and death, with statistical significance in 150 mg/L group (P < 0.05) (**Figure 1A**). Similar to the effect on serum starving injury, PHH-I also showed obvious protective activity against $H_2O_2$-induced cell death. The neuroprotective activities of PHH-I were stronger than that of PHH-II in both models (**Figure 1B**). Soy bean protein total hydrolysate was used as control for excluding the non-specific effect of hydrolyzing protein. Not surprisingly, soy bean protein total hydrolysate did not show significant activity on the above-mentioned injuries (data not shown).

### *Fractionation of gelatin hydrolysate*

According to the results of preliminary screening, the active component, PHH-I, was selected to further studies. PHH-I was collected and applied to an open column of a SP-Sephadex C-50 cation exchanger, and the adsorbed fraction exhibiting cell activity (data not shown) was desorbed from the column

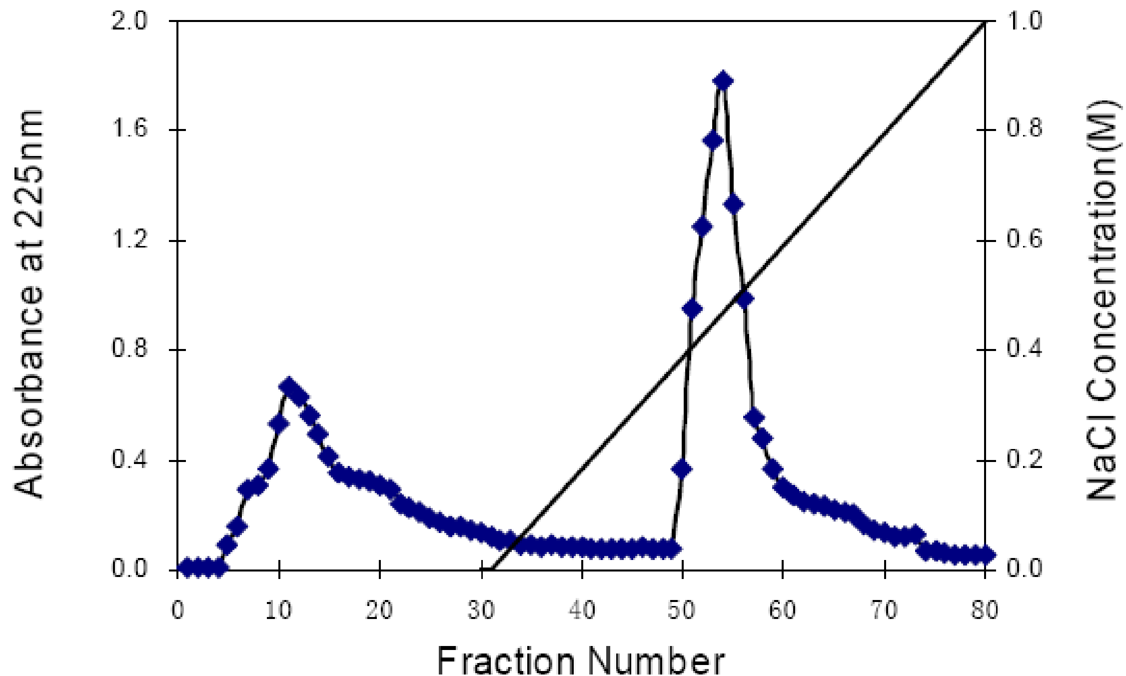


**Figure 2**. Elution profile of fractions from the SP-Sephadex C-50 column equilibrated with the binding buffer (50 mM sodium acetate buffer, pH 4.0). Unbound peptides were then washed through the column with binding buffer. Adsorbed peptides were eluted with a linear gradient of NaCl from 0 to 1.0 M in the same buffer.

with a linear NaCl concentration gradient (**Figure 2**). Subsequently, the adsorbed peak was pooled, concentrated, and further purified by chromatography on a Sephadex G-25 column (**Figure 3**). Three peaks, designated as PHH-Ia, PHH-Ib and PHH-Ic, appeared in the eluted fractions. Among them, the fraction PHH-Ic with smaller molecular weight, showed the strongest cytoprotective activity ($P < 0.05$-$0.001$) as shown in **Figure 5**. Its SDS-PAGE pattern was shown in **Figure 4**. The peptides exhibited a molecular mass among approximately 1,000-3,000 Daltons in SDS-PAGE analysis.

*Amino acid composition*

The approximate amino acid composition of PHH-Ic was listed as follows: glycine 23%, proline 15%, hydroxyproline 13%, glutamic acid 9%, alanine 8%, arginine 7%, aspartic acid 5%, lysine 4%, serine 4%, leucine 3%, valine 2%, phenylalanine 2%, threonine 2%, isoleucine 1%, hydroxylysine 1%, methionine, histidine, and tyrosine 1% (**Table 1**). These values varied, depending on the source of the raw material and the specific composition of hydrolysate fraction.

*Effect of PHH-I components on cell survival under serum deprivation*

To further assess the protective effect of PHH-I components on serum starving injury, we observed the cell survival curve with/without FBS. As showed in **Figure 5**, cell viability in the control with serum increased continuously and reached at peak on day 4-5. While in serum deprivation group, cell viability did not change obviously on day 2 and then reduced after day 3, indicated that the cells stop growing and then died gradually. Addition of PHH-I in the concentration of 25-150 mg/L into the serum starving cultures significantly reduced and postponed the cell death with no significant difference among four doses. The viabilities were averagely increased about 10% when compared to serum deprivation control (**Figure 5A** and **B**).

To find the active components of PHH-I, we evaluated the effects of PHH-Ia, PHH-Ib and

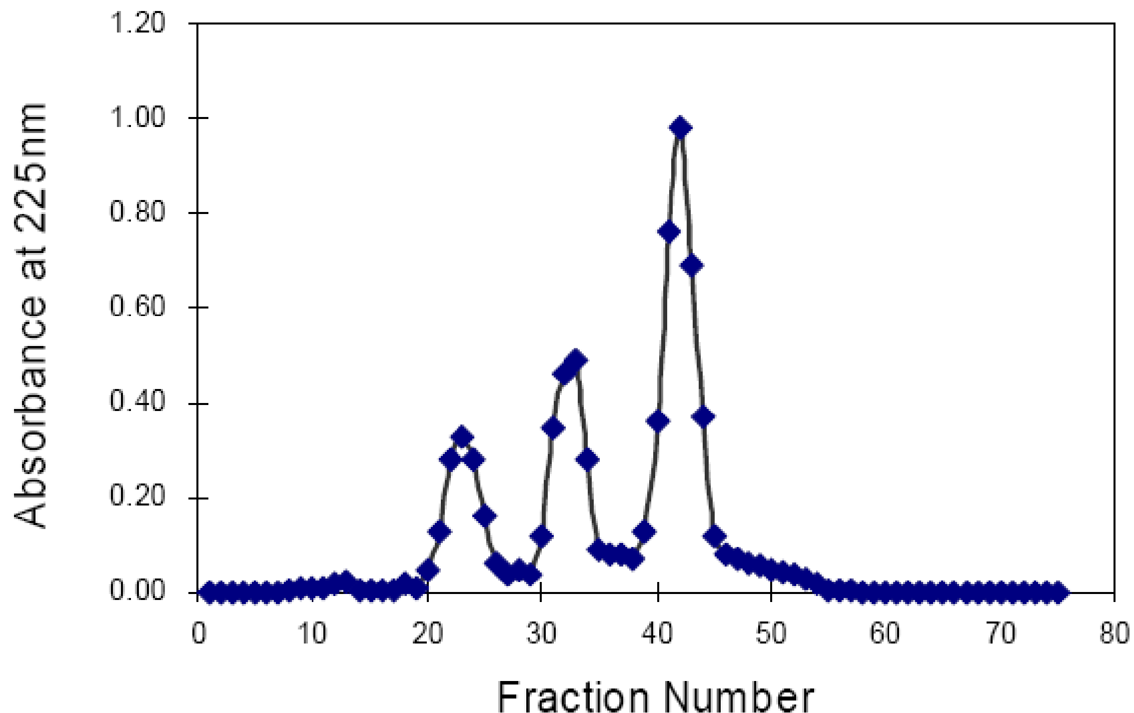


**Figure 3.** The adsorbed fraction with antioxidant activity from SP-Sephadex C-50 column chromatography was pooled, concentrated and applied to a Sephadex G-25 column. Peptides were eluted with 10 mM Tris-HCl buffer, pH 7.2.

PHH-Ic, derived from chromatography separation through SP-Sephadex C-50 and Sephadex G-25, on serum deprivation-induced cell death. As showed in **Figure 5**, PHH-I components, PHH-Ia, PHH-Ib had no cell protective activity. However, PHH-Ic showed dramatic protective effect, even stronger than the total component of PHH-I. PHH-Ic also increased cell viability significantly from day 2 through day 4 compared to serum deprivation treatment ($P < 0.05$-$0.001$), which indicated the PHH-Ic was the activating component of PHH-I.

*Evaluation of PHH-I and its components on protecting neurons against $H_2O_2$-induced cell death*

To further confirm the cell protective effect of PHH-I and its components (PHH-Ia, Ib and Ic), the similar experiments were performed in $H_2O_2$-treated SH-SY5Y cells. Cell viability as determined by MTT reduction was markedly decreased after temporarily exposure to 100 or 200 μM $H_2O_2$ for 30 min (**Figure 6**). In morphological observation, as shown in **Figure 7**, there was a significant decrease in cell number. Most cells lost neurites and exhibited round shape or replaced by debris following

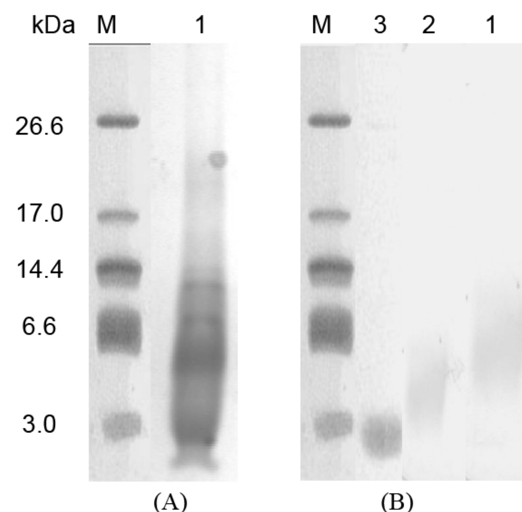


**Figure 4.** SDS-PAGE of PHH-I and its components (PHH-Ia,b,c) (A) Lane M: molecular mass standards; lane 1: 12 μg PHH-I; (B) Lane M: molecular mass standards; Lane 1: 12 μg PHH-Ia; Lane 2: 12 μg PHH-Ib; Lane 3: 10 μg PHH-Ic.

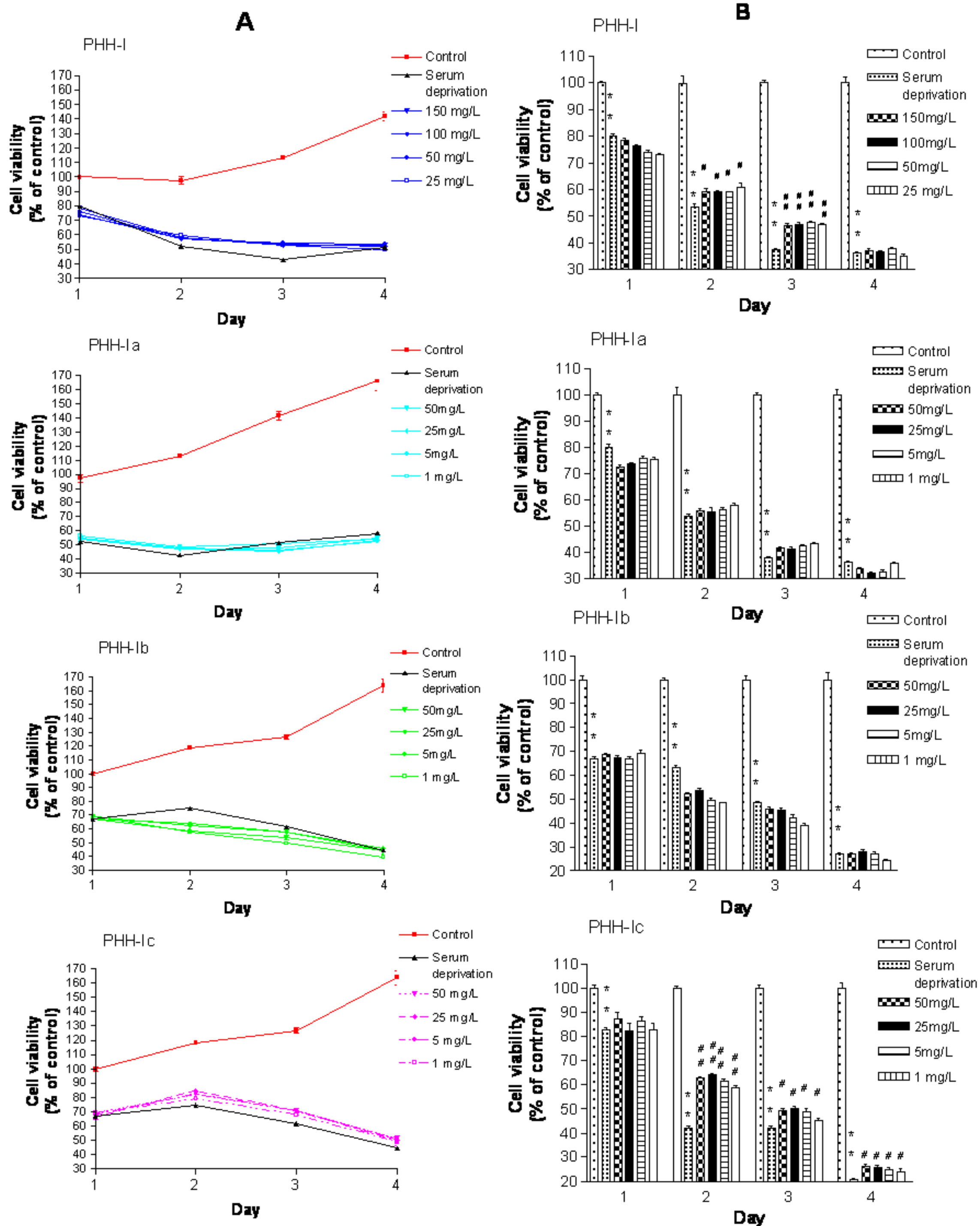


Figure 5. Effect of PHH-I and its components (PHH-Ia,b,c) on cell survival (MTT reduction) in human neuroblastoma SH-SY5Y cells suffering serum deprivation. Neurons were serum starving for 1-4 days. PHH-I (25, 50, 100 and 150 mg/L) and its components (PHH-1a, Ib and Ic at 1, 5, 25, and 50 mg/L) were added to the culture 2 h in advance. MTT reduction was detected in day 1 through day 4, respectively. A, cell growth curve, OD value of control at day 1 as 100%. B, indicated by vertical bar, OD values of control of same days as 100%. Data expressed as means ± S.E.M. ** P<0.01 *vs.* control; # P<0.05, ## P<0.01 *vs.* serum deprivation treated group. At least three independent experiments were carried out, n = 6 in each group.

Table 1. Amino acid compositions of porcine gelatin hydrolysate PHH-Ic

| Amino acids | Gelatin hydrolysate (%) |
|---|---|
| glycine | 23 |
| proline | 15 |
| hydroxyproline | 13 |
| glutamic acid | 9 |
| alanine | 8 |
| arginine | 7 |
| aspartic acid | 5 |
| lysine | 4 |
| serine | 4 |
| leucine | 3 |
| valine | 2 |
| phenylalanine | 2 |
| threonine | 2 |
| isoleucine | 1 |
| hydroxylysine | 1 |
| methionine, histidine, and tyrosine | 1 |

temporary exposure of $H_2O_2$. It suggested that SH-SY5Y cells were very sensitive to $H_2O_2$. Pretreatment with PHH-I and PHH-Ic at different concentrations showed to a different degree of protection against $H_2O_2$-induced decrease in cell viability determined by MTT reduction assay. In accordance with serum deprivation model, PHH-Ia and Ib did not show protective activity on $H_2O_2$ injury model (data not shown). Only PHH-Ic possessed the cytoprotective activity. However, inconsistent with their activities in serum deprivation model, the total component, PHH-I was more effective against $H_2O_2$-induced injury than PHH-Ic demonstrated by MTT reduction assay and morphological changes (Figure 6 and 7).

## Discussion

As a means to prevent or delay pathological neuronal loss in central nervous system diseases of various pathophysiological origins, such as stroke, neurotrauma, neuroinflammatory and neurodegenerative diseases, neuroprotection represents a novel therapeutic approach that gains support from a large number of studies in cultured cells and animal models demonstrating beneficial effects of growth factors on neuronal survival and functional recovery [3, 5, 22]. Neuroprotection can be defined as an approach toward the best possible maintenance/restoration of cellular interactions in the brain resulting in a maximum protection of neural function [13]. As one of the pathophysiological features, oxidative damage by radical oxygen species has been well documented to play a pivotal role in many brain diseases [14-17]. Treatments that decrease oxidative stress can protect neurons following serum withdrawal or β-amyloid insult [18, 19, 20], further indicating that oxidative stress is one of the key players in these pathological processes. Therefore, therapeutics addressing these fundamental mechanisms may not only slow down neuronal degeneration, but also restore their function. Thus the therapeutic strategy aimed at alleviating oxidative damage might be beneficial in above-mentioned brain diseases. $H_2O_2$- and serum deprivation-induced cell injury models have been widely used in evaluating neuroprotectors [4, 21].

In preliminary screening of PHH on neuroprotective activities, PHH-I showed stronger activity than that of PHH-II. PHH-I showed robust protective activity against $H_2O_2$- and serum deprivation-induced cell death. The reason why PHH-I showed cell protective activity while PHH-II did not is probably because both the sequences for amino acids and length of peptide bonds are somewhat different when using different enzyme in digestion. In the mean time, total hydrolysate of soy bean protein did not show significant protective effects on the injuries. It suggested that the bioactivity of protein hydrolysate depends on its original source, and the specific amino acid components and/or sequences of the hydrolysed peptides are critical for their bioactivity. Papain used in the research is consisted of a single polypeptide chain with three disulfide bridges and a sulhydryl group necessary for activity of the enzyme. It has broad specificity and cleaves peptide bonds of some amino acids such as glycine, leucine and arginine. Therefore, the neuroprotective activities such as antioxidative activities [11], was supposed to be attributable to an amino acid residue, Glysine, at the terminus of active peptide.

The 3rd fraction with smaller molecular mass (PHH-Ic) from Sephadex G-25 column showed stronger cell protective activity when compared to other two fractions with larger molecular mass (PHH-Ia and PHH-Ib). It suggests that the neuroprotective peptides of

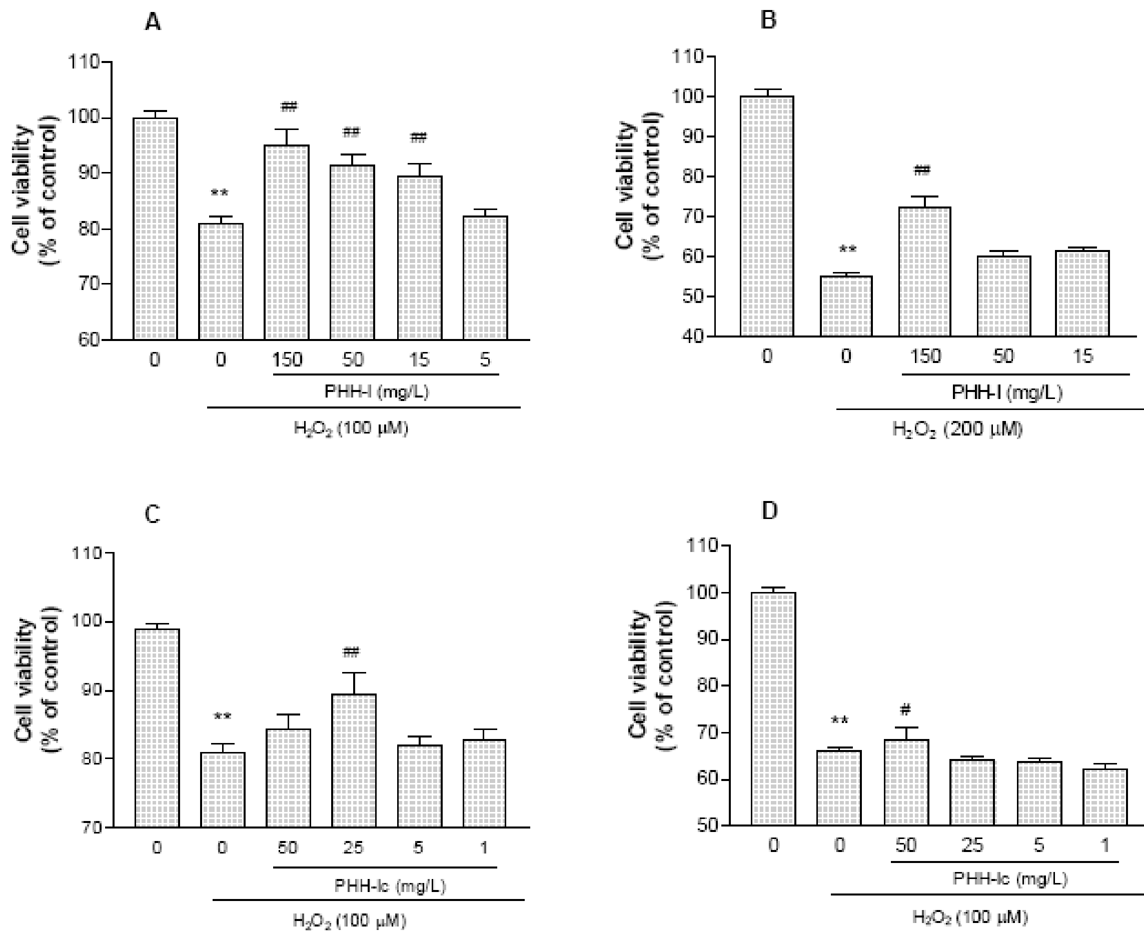


Figure 6. Attenuation of $H_2O_2$-induced SH-SY5Y cell damage by PHH-I and PHH-Ic. Cells were incubated with 100 (A, C) or 200 (B, D) μM $H_2O_2$ for 30 min and cultures continued another 6 h. PHH-I (A, B) and Ic (C, D) were added to the culture 2 h prior to $H_2O_2$ addition. Cell viability was assessed by measuring the MTT reduction. Three independent experiments were carried out (n = 6 in each treatment). The data were means ± S.E.M. expressed as percent of control value. ** $P < 0.01$ *vs*. control; # $P<0.05$, ## $P<0.01$ *vs*. $H_2O_2$ group, respectively.

PHH are largely from peptides smaller than 3,000 Daltons.

Amino acid composition showed that PHH-Ic had higher percentage of proline and hydroxyproline (Table 1) compared to its counterparts from Alaska Pollack skin gelatin and bovine skin gelatin hydrolysate [9, 10]. The amino acid components and residue-composition should play important roles in the neuroprotective activities. It has been reported that proline and hydroxyproline served as reducing agents in the antioxidative activity [10, 11], therefore its cell protective activity might be partially due to the reducing property of amino acids. Our results suggest that the antioxidative activity might contribute to the neuroprotective effects of PHH and its components.

Except for oxidative injury, withdrawal of neurotrophic factors is also the key factor in serum deprivation-induced cell death. Serum withdrawal from neurons causes apoptosis and constitutes a useful model to study mechanisms underlying the neuroprotection afforded by target-derived neurotrophic support [23, 24, 25]. In present study, PHH-I and its component PHH-Ic dramatically increased the cell survival on serum

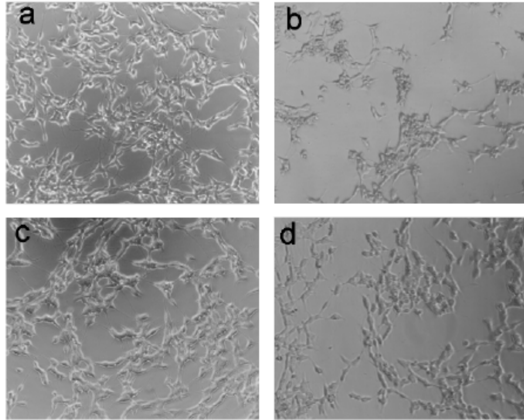


**Figure 7.** Attenuation of $H_2O_2$-induced neuronal injury by PHH-I and PHH-Ic. After pretreatment with 150 mg/L of PHH-I and 25 mg/L PHH-Ic for 2 hr followed by temporary exposure to $H_2O_2$ (200 μM), cells were cultured continuously for another 24 hours before morphology observation. a, control cells; b, $H_2O_2$ (200 μM) treatment; c, co-treated with PHH-I; d, co-treated with PHH-Ic. Morphology was observed by phase-contrast microscopy (200×). Numerous cells lost neurites and demonstrated a round shape following exposure to $H_2O_2$. In contrast, many cells appeared normal in cultures exposed to the same amount of $H_2O_2$ in the presence of PHH-I and PHH-Ic.

withdrawal cell model, which might imply the potential of PHH components with neurotrophic activity.

Protease hydrolysis of gelatin has been shown to produce biologically active peptides that inhibited angiotensin I converting enzyme and linoleic acid peroxidation [9]. However, there are few reports about the neuroprotective activity of its hydrolyzed peptides so far. The present study is herein the first report that the gelatin hydrolysate of type-B porcine hide, designated PHH-I, exhibited potent neuroprotective activity on SH-SY5Y neuroblastoma cells. It also implied that the smaller peptides of gelatin hydrolysates are more potent in protecting neuronal cells.

As antioxidant, vitamin E and flavonoid have been widely used in the treatment or the prevention of oxidative stress-involved diseases. However, due to their hydrophobic properties, the assimilation of vitamin E and flavonoid need the aid of fat, which is not the favorite way for some group of people such as hyperlipoidemia and obesity. On the other hand, the cumulative toxicity and some side-effects also limit their usages. Compared to the above-mentioned disadvantages of vitamin E and flavonoid, PHH-Ic provides a food-sourced and safer alternative of antioxidant. These characteristics make PHH-Ic a desirable component for potential application in food therapy and medicine.

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