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Protection mechanism of Se-containing protein hydrolysates from Se-enriched rice on Pb^{2+}-induced apoptosis in PC12 and RAW264.7 cells

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ABSTRACT

This study aimed to investigate the protection mechanism of Se-containing protein hydrolysates (SPH) from Se-enriched rice on Pb^{2+}-induced apoptosis in PC12 and RAW264.7 cells. Results showed that SPHs could alleviate Pb^{2+}-induced morphological changes of apoptosis and the loss of mitochondrial transmembrane potential in both cell types. Besides this, SPHs could significantly reduce the activation of caspase-3, -8, -9 induced by Pb^{2+}, reverse the Pb^{2+}-induced upregulation of Bax and release of cytochrome C, and downregulate Bcl-2 in cells. HPLC-ICP-MS and SEC-HPLC assays showed that SPHs were low molecular weight peptides (229.4–534.9 Da), and the major Se species found in SPHs was SeMet. Taken together, these findings suggested that SPHs could possibly protect the cells against Pb^{2+}-induced apoptosis *via* a caspase-dependent mitochondrial pathway, and the primary effective constituents in SPHs were SeMet and Se-containing peptides, suggesting that SPHs might be a novel potential candidate to improve the health of people with Se deficiency or in Pb-contaminated areas.

1. Introduction

Lead (Pb) is a heavy metal element that has recently attracted much attention because of pollution and adverse events (Toscano & Guilarte, 2005). Although great efforts have been made to reduce the utilization and distribution of Pb in the environment, it still remains a significant health hazard and has become even more common in daily life following rapid development through modernization and urbanization (Palmer et al., 2015). Generally, Pb could enter and accumulate in the body through the food chain, water, soil, and air, subsequently causing long-term or potential harm for humans. The central nervous system is the primary target of Pb-induced toxicity, in which neurotoxic effects, particularly in infants and children, have been highly documented (Luo, Ruan, Yan, Yin, & Chen, 2012). The PC12 cell is a frequently used neural cell line as an ideal model to study the neurotoxic mechanism of Pb (Lassiter, MacKillop, Ryde, Seidler, & Slotkin, 2009). In addition, Pb is an immunotoxicant that is potentially toxic to the immune function and consequently to human health (Paul, Chakraborty, & Sengupta, 2014). Some studies have proved that treatment of macrophages with Pb resulted in the dysregulation of cell-mediated immune responses, suggesting that Pb might cause impairment of the immune system (Krocova, Macela, Kroca, & Hernychova, 2000). Therefore, as a type of macrophage that can be easily obtained and cultured, the RAW264.7 cell has been commonly used to study cellular immunity (Sun et al., 2015).

Selenium (Se) is an essential trace element for both humans and animals. Currently, the biochemical functions of Se have been widely investigated. The antagonism and toxicity prevention of heavy metals have been assessed in several different biological systems (Lin et al., 2012). The antagonistic and synergistic effect of Pb and Se has been well studied. Wang et al. (2013) suggested that low-level organic Se could prevent and reverse Pb-induced alterations in the expression of neural cell adhesion molecules. Deng et al. (2015) proved that Se could attenuate the alterations in Bcl-2 family proteins induced by Pb exposure in cells.

In nature, Se exists in both organic and inorganic forms. The inorganic forms primarily include selenate and selenite, whereas the organic forms primarily include selenoamino acid, selenopeptide and selenoprotein. However, different Se forms possess different bioavailability and toxicity. In general, the biological effect of organic Se is significantly superior to that of inorganic Se in an

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appropriate concentration range (McKelvey, Horgan, & Murphy, 2015). Therefore, the dose, and also the bioavailability, of Se should be taken into account for Se ingestion; the latter depends on the available species of Se. Available data showed that the primary form of organic Se existing in rice was selenoamino acid and its derivatives, such as selenomethionine (SeMet), selenocysteine ($SeCys_2$), methylselenocysteine (MeSeCys), selenopeptide and selenoprotein (Liu, Cao, Bai, Wen, & Gu, 2009).

It is currently believed that most of the protein in the human body is absorbed in the form of peptide, and the absorption rates of dipeptide and tripeptide are faster than those of amino acid with the same composition (Dei Piu et al., 2014). As a result of lower molecular weight and very high biological activity, peptides have a special role in an organism and could achieve several physiological functions in the human body (Renukuntla, Vadlapudi, Patel, Boddu, & Mitra, 2013). Interestingly, the peptides obtained from rice protein not only surpass the advantages of protein but also have unique strengths and functions, such as hypoallergenic, digestive, and absorption roles (Zhang et al., 2010). Therefore, integration of the excellent characteristics of Se and rice peptides has important academic value and widespread application prospects. Therefore, the Se-containing peptides with high biological activities extracted from Se-enriched rice are of particular interest.

In our previous study, we found that addition of Se-enriched rice significantly inhibited the incidence of cyclophosphamide-induced micronuclei and mitomycin C-induced chromosomal aberration in mice. Moreover, Se-enriched rice could significantly increase the activity of glutathione peroxidase in the liver and the Se concentration in blood compared with regular rice (Hu, Xu, & Chen, 2005). Subsequently our results showed that low molecular weight peptides and SeMet were the major Se-containing moieties found in the Se-containing rice protein, following an *in vitro* gastrointestinal digestion (Fang et al., 2010). Our recent study showed that pretreatment of cells with SPHs could reduce the levels of ROS, LDH, NO and MDA in a dose-dependent manner. Instead, cell viability, SOD activity and GSH content increased as a result of this treatment, suggesting that SPHs from Se-enriched rice could indeed prevent cytotoxicity induced by Pb^{2+} (Xu et al., 2016). However, the protection mechanism of the action is still not clear.

In order to better understand the protection mechanism and biological function of SPHs, PC12 and RAW264.7 cell models were established and cellular apoptosis, mitochondrial membrane potential (MMP) and caspase activity were investigated in order to reveal the protective mechanism of SPHs on Pb^{2+}-induced cells. In addition, molecular weight distribution and Se species of SPHs were determined using SEC-HPLC and HPLC-ICP-MS to further understand their effective constituent.

2. Materials and methods

2.1. Materials and reagents

Se-enriched rice and regular rice were supplied by Nanjing Yuanwang Rich Selenium Agricultural Products Company (Nanjing, China). The preparation method was described by Fang et al. (2010).

Se standard solution with a concentration of 1000 μg of Se ml^{-1} was purchased from Aladdin Industrial Corporation (Shanghai, China). SeMet (99%), $SeCys_2$ (98%), sodium selenite (Se^{IV}, 98%), sodium selenate (Se^{VI}, 98%), MeSeCys (95%), α-amylase (from porcine pancreas), glucoamylase (from *Aspergillus niger*), trypsin (from bovine pancreas), HPLC peptide standard mixture, and Rhodamine-123 were acquired from Sigma (St Louis, MO, USA). Hoechst staining kit, caspase 3 activity assay kit, caspase 8 activity assay kit, caspase 9 activity assay kit, SDS-PAGE gel preparation kit, and SDS-PAGE sample loading buffer (5×) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Total protein quantitative assay kit and Rainbow pre-stained protein Marker (10–170 kDa) were purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). Rabbit anti-Bax, rabbit anti-Bcl-2, rabbit anti-cytochrome C, rabbit anti-GAPDH, and HRP-goat anti-rabbit IgG were obtained from Boster Biological Technology Corporation Limited (Hubei, China). Polyvinylidene fluoride (PVDF) membrane was acquired from Beijing Solarbio Science & Technology Corporation Limited. (Beijing, China). Enhanced chemiluminescence (ECL) western blotting substrate was purchased from Thermo Fisher (Massachusetts, USA).

2.2. Preparation and isolation of SPHs

The preparation of SPHs was performed according to Yan, Huang, Sun, Jiang, and Wu (2015). Briefly, defatted rice flour (10 g) was dispersed in 90 ml deionized water and incubated with 150 U α-amylase and 0.02 g $CaCl_2$ at a pH of 6.2–6.3 at 90 °C for 1 h. Then, the pH of the reaction mixture was adjusted to 4.5 and further incubated at 55 °C with 250 U glucoamylase with constant stirring for an additional 2 h. The pH was adjusted to 5.0 by adding 0.1 mol/l HCl. The final mixture was centrifuged at 4000*g* for 20 min and the supernatant was discarded. The resultant precipitate was washed with deionized water three times before freeze-drying. The freeze-dried product (10 g) was dissolved in 170 ml deionized water, and the pH was adjusted to 8.5 using 0.1 mol/l NaOH. To this solution, 3 g of trypsin was added and incubated for 3 h at 50 °C at static pH 8.0. The reaction was stopped by heating the dispersion to 90 °C to inactivate the enzyme. Supernatants were collected after centrifugation at 4000*g* for 20 min, and they were finally freeze-dried and stored at −20 °C. Subsequently, the products were ultrafiltered through 1-kDa ultracentrifugation membranes to give the fraction with molecular weight below 1 kDa (SPHs). A similar method was used to prepare regular rice protein hydrolysates (RPHs) with molecular weights below 1 kDa.

2.3. Cell culture

Mouse macrophages from RAW264.7 cell line (RAW264.7) and rat pheochromocytoma-derived cell line (PC12) obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin sodium, and 100 mg/ml streptomycin in a humidified incubator at 37 °C with 5% CO_2. Logarithmic growth phase cells were used in subsequent experiments.

2.4. Experimental groups and sample treatments

The PC12 and RAW264.7 cells were subjected to the following treatments: control group (serum-free medium); Pb^{2+}-only group (0.3 mmol/l Pb^{2+}); Pb^{2+} plus SPHs group (0.3 mmol/l Pb^{2+} and 100 μg/ml SPHs); and Pb^{2+} plus RPH group (0.3 mmol/l Pb^{2+} and 100 μg/ml RPHs). Pb^{2+}, SPHs, and RPHs were dissolved in serum-free medium. The cells were treated with SPHs for 12 h after incubation in serum-free medium for 24 h. The culture medium was removed and the cells were washed twice with phosphate-buffered saline (PBS). Then, the cells were exposed to Pb^{2+} for 4 h. Cells were harvested and rinsed with PBS three times. The cell pellets were stored at −20 °C for subsequent experiments.