Full Length Research Paper

Crude proteins from pigeon pea (*Cajanus cajan* (L.) Millsp) possess potent SOD-like activity and genoprotective effect against H\textsubscript{2}O\textsubscript{2} in TK6 cells

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Accepted 31 October, 2011

In the present study, we reported new investigations on antioxidant potential of biological proteins from pigeon pea (*Cajanus cajan* (L.) Millsp) seeds. The crude pigeon pea proteins (PPP) were obtained by extraction with PBS, NaCl and water. The PPP extracts were precipitated by ammonium sulfate [(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}] in various percentage. All fractions were investigated in their potential antioxidant activity of both enzymatic antioxidant-like activity and anti-oxidative cellular damage in TK6 human lymphoblasts. The protective effect of membrane damage was investigated by employing mitochondrial dehydrogenase (MDH) and lactate dehydrogenase (LDH) activity assays and the anti-oxidative DNA damage was assed using comet assay. Results revealed a potent superoxide dismutase (SOD) like activity in three protein fractions including P-II, N-II, and W-III. Their molecular weight distributions were located mainly in a range of 6 to 66 kDa. The highest inhibitory effect on mitochondrial and cell membrane damage was found in N-II fraction. Interestingly, the results from comet assay demonstrated the anti-oxidative DNA damage in H\textsubscript{2}O\textsubscript{2}-induced TK6 cells. This activity was indicated by a significant reduction in tail length (TL) damage parameter. According to our results obtained in this study, we conclude that proteins isolated from pigeon pea seeds possess anti-oxidative effects on H\textsubscript{2}O\textsubscript{2}-induced cellular damage.

Key words: Pigeon pea, antioxidant-like activity, anti-oxidative activity, H\textsubscript{2}O\textsubscript{2}, comet assay.

INTRODUCTION

Reactive oxygen species (ROS) are continuously generated as part of our normal process of cellular metabolism and also by environmental factors, including smoking, pollution, radiation, toxins and toxic metals. An imbalance between ROS production and body’s antioxidant defense system leads to an accumulation of ROS. The ROS induces cell damage by modifying molecules, including proteins, lipids, and DNA (Kehrer, 1993) and leads to oxidative stress that is associated with tissue damage, accelerated aging, and degenerative diseases. Oxidative degradation of membrane lipids can result in loss of membrane integrity, defective membrane and transport mechanisms. This contributes to cancer development through increasing cell proliferation and inducing DNA damage that lead to genetic lesions (Storz, 2005). The mechanisms of mammalian cells to scavenge and/or neutralize ROS are mainly due to antioxidant enzymes. Among these enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are considered to be the most important (Baker and Baker, 1992). SOD is the first antioxidant mobilized by our cells for defense.

It converts superoxide anion (O\textsubscript{2}\textsuperscript{-}) to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (Fridovich, 1997). CAT is mostly located in peroxisomes and converts H\textsubscript{2}O\textsubscript{2} to water and oxygen...
GPx enzyme has even been called the master antioxidant. It is a tripeptide found inside every single cell in body and is responsible for removal of \( \text{H}_2\text{O}_2 \) and other organic hydroperoxides (Lawrence and Burk, 1978). Recently, the use of natural protein extracts or purified proteins as antioxidants has attracted particular interest. This has led to new investigations into assessing the antioxidant potential of biologically active proteins or peptides from natural sources. For examples, soy proteins, wheat proteins, albumin, fish proteins, maize zein, egg albumin proteins and egg yolk phosvitin, potato patatin and yam Dioscorin, mushroom proteins were reported to have antioxidant activity (Chen et al., 1996; 2000; Hu et al., 2003; Liu et al., 2003; Zhao et al., 2004).

Pigeon pea *Cajanus cajan* (L.) Millsp. known as red gram is a famous and multi-use grain legume crop. It is commonly found and consumed in semi-tropical and tropical developing countries. Young pods are used as an indigenous vegetable in northern Thailand. It is an ideal source of protein and vitamin B in human diet, especially in the vegetarian population (Duke, 1981; Salunkhe et al., 1986). Pigeon pea has many uses as traditional folk medicinal plants. It has been used for many years for treating diabetes, expelling bladder stones, stabilizing the menstrual period, applying to sores, skin irritations, hepatitis, measles, jaundice, dysentery and many other illnesses (Grover et al., 2002; Sharma et al., 2006; Odeny, 2007). Pigeon pea seeds contain proteinaceous inhibitors of trypsin, chymotrypsin and amylases (Emmanuel, 1987; Akand et al., 2010) as well as phytolectins, and secondary metabolites (Bressani and Elias, 1979; Grant et al., 1983), which constitute the defense machinery. Biochemical characterization of pigeon pea proteinase inhibitors has revealed that these are Kunitz type proteinase inhibitors having inhibitory activity against trypsin and chymotrypsin (Godbole et al., 1994). Previously, seven isoforms of trypsin/chymotrypsin inhibitors (TCIs) and two isoforms of trypsin inhibitors (TIs) have been reported from pigeon pea seeds (Pichare and Kachole, 1996).

However, there is little information concerning the antioxidant properties of pigeon pea. This study therefore investigates the antioxidant enzyme properties of protein isolated from pigeon pea seeds and protective effects against \( \text{H}_2\text{O}_2 \)-induced oxidative stress in TK6 human lymphoblast cells.

**MATERIALS AND METHODS**

Seeds of pigeon pea (*C. cajan* (L.) Millsp.) were cultivated and harvested from Khon Kaen Field Crop Research Center (KFCRC), Khonkaen, Thailand. Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) Merck (Hohenbrunn, Germany). RPMI medium and horse serum, Gibco Invitrogen (Grand Island, N.Y. USA). Normal melting point (NMP) and low melting point (LMP) agarose gel, Promega (Madison, WI, USA).

**Cell culture**

TK6 human lymphoblast cell line (CRL-8015) American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were routinely maintained as suspension in 75 cm\(^2\) polystyrene flasks in commercial growth media RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% inactivated horse serum in presence of penicillin-streptomycin (50 units/ml) and maintained at 37°C in humidified atmosphere containing 5% \( \text{CO}_2 \). Subculturing cell was performed every 48 h.

**Isolation of crude protein from pigeon pea**

Crude protein isolated from pigeon pea was conducted following the method described by Benjakul et al. (2000), with a slight modification. Dried pigeon pea pods were ground to a powder in an electric mill. The powder was mixed (at 1:5, w/v) with three different extraction solvents including distilled water, 0.15 M NaCl and 0.1 M PBS for 3 h at 4°C and in a presence of 1% protease inhibitor. The mixtures were filtered through clean gauze cloth to remove debris. The filtrates were subsequently recovered by a refrigerated centrifugation (4°C) at 5,000 rpm for 30 min and followed by filtration using No. 42 filter paper. The supernatants were brought to 0 to 30, 30 to 60 and 60 to 90% saturation with ammonium sulfate (\( \text{NH}_4\text{SO}_4 \)), respectively. The precipitates were collected by centrifugation at 10,000 rpm for 15 min and resuspended in above mentioned extraction solutions. The solutions were desalted to remove (\( \text{NH}_4\text{SO}_4 \)) using HiTrap™ Desalting Column (Sephadex™ G-25 Superfine, 1.6×2.5 cm), (GE Healthcare, Uppsala, Sweden). Crude protein fractions were obtained with distilled water at flow rate of 5 ml/min. All crude protein fractions from PBS (P), NaCl (N) and water (W) were lyophilized and stored at -20°C.

**Determination of molecular weight distribution**

The protein fractions those possessed active anti-oxidative activity in TK6 cells were further analyzed for molecular weight distribution. This was performed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in accordance with the method of Laemmli (1970), on 15% separating and 4% stacking gel. A wide range of molecular mass protein marker (Bio-rad, USA) was run on the gel to determine the molecular weight (MW) of protein fractions.

**Determination of antioxidant enzyme-like activity**

**Superoxide dismutase (SOD) activity**

SOD activity was measured using SOD assay kit-WST (Sigma Aldrich, Switzerland). The assay is base on the reduction rate of \( \text{O}_2^- \) by SOD. The \( \text{O}_2^- \) were generated by xanthine oxidase (XO) activity. The water-soluble tetrazolium salt (WST-1) produced a water-soluble formazan dye up on reduction with a superoxide anion. The absorbance of the WST-1 formazan in assay kit was measured by spectrophotometer at 440 nm.

**Glutathione peroxidase (GPx) activity**

GPx activity was determined using cellular activity assay kit CGP-1 (Sigma Aldrich, USA). This kit used an indirect method based upon the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which was then coupled with recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The
Determination of intracellular anti-oxidative activity

TK6 cells at 2 x 10^5 cells/ml were incubated with pigeon pea protein fractions (0.01 to 2 mg/ml) in 12-well culture plates (Corning Costar, USA) for 24 h. Subsequently, cells were exposed to 1 mM H2O2 for 30 min to allow an induction of intracellular oxidative stress. Results were analyzed by mitochondrial dehydrogenase (MDH) and lactate dehydrogenase (LDH) assays.

Determination of cytotoxicity

The possible cytotoxic effect of protein fractions and H2O2 was investigated in TK6 cells using the trypan blue dye exclusion (TBE) method (Subhashini et al., 2005). TK6 were exposed to various concentrations of protein fractions for 24 h and H2O2 (0.01 to 1.00 mM) for 30 min. By the end of exposure, culture medium was removed by centrifugation. Cells were washed twice and resuspended in PBS. Approximately 20 μl of cell suspension was stained with trypan blue dye where dead cells were stained blue. The stained (dead) cells and the total cells per square of a cell chamber were counted on a Neubauer hemocytometer (Boeco, Germany) under the light microscope (Olympus CX21, Tokyo, Japan).

Determination of mitochondrial dehydrogenase (MDH) activity

The protective effect of protein fractions to mitochondrial membrane against H2O2-induced oxidative stress was evaluated employing MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) assay (Cilla et al., 2008) which based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenase. The culture medium was removed by centrifugation at 5,000 rpm for 3 min. Cells were treated with 0.5 mg/ml MTT for 2 h at room temperature and then 500 μl DMSO was added to solubilize the intracellular formazan crystal. Results were analyzed by spectrophotometer at 570 nm.

Determination of lactate dehydrogenase (LDH) activity

Cell membrane damage by H2O2 was determined by LDH leakage from injured cells. After oxidative stress induction in TK6 cells by 1 mM H2O2, culture medium from each well was collected by centrifugation at 5,000 rpm for 3 min. Release of LDH into supernatant was determined using the In Vitro Toxicology (TOX-7) assay kit (Sigma-Aldrich, USA). The reduction of NAD to NADH by LDH in assay kit was examined by spectrophotometer at 490 nm.

Determination of anti-oxidative DNA damage by comet assay

The comet assay or single cell gel electrophoresis (SCGE) was performed following method described by Singh et al. (1988). After incubation with protein fractions for 24 h, TK6 cells (2 x 10^5 cells/mL) were suspended in 1 ml lypendorf tubes and exposed to 0.5 mM H2O2 for ROS generation. The H2O2 treatment was performed at 4°C for 5 min to avoid DNA repair process. Following H2O2 exposure, cells were washed twice with ice-cold PBS and resuspended in 200 μl PBS. A mixture of 20 μl cell suspension and 75 μl of 0.5% low melting point (LMP) agarose were immediately layered onto a glass microscope slide pre-coated with 0.75% normal melting point (NMP) agarose. The slides were covered with the coverslips and placed on a cold flat to allow the agarose to solidify. The coverslips were gently removed and 80 μl of 0.5% LMP agarose was spread on glass slides, recovered with the coverslips, and left for agarose to completely solidify. Slides were immersed in pre-chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) and kept at 4°C for 2 h.

Slides were incubated in fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C to allow unwinding of DNA. The slides were then transferred into an electrophoresis unit with the same buffer and subjected to an electrophoretic field at 300 mA and 25 V at 4°C for 20 min in dark condition. After electrophoresis, slides were fixed in neutralizing buffer (0.4 M Tris, pH 7.5) for 10 min and then gently rinsed in ice chilled distilled water. The slides were stained with 30 μl of 0.2% ethidium bromide (EBR) and cells were analyzed using a fluorescence microscope (Olympus BX51, Tokyo, Japan). Images of one hundred randomly selected cells per experimental point were scored and analyzed of damage using Comet III analysis software (Perceptive Instruments, Halstead, UK). Two major parameters including tail length (TL= distance of DNA migration) and tail moment (TM= Distance between the centre of gravity of the head to the centre of gravity of the tail) X (Tail DNA Intensity / Total Comet DNA intensity)) were taken for result analysis.

Statistical analysis

The parameter values were all expressed as the mean±SD from triplicate measurements. Significant differences among the groups were determined by Turkey’s HSD test using SPSS 11.0 software package program (SPSs Inc., USA). The results were considered significant if the value is p≤0.05.

RESULT AND DISCUSSION

Pigeon pea protein (PPP) isolations

Eight fractions of crude PPP from different extraction solutions including PBS (P-I, P-II, P-III), NaCl (N-I, N-II), and water (W-I, W-II, W-III) were obtained as demonstrated in Table 1. It was shown that precipitation at 30 to 60% (NH4)2SO4 saturation produced greater protein amount than other precipitation ratios. The highest protein concentration (18.07 μg/ml) was found with PBS extraction (P-II) and precipitated at 30 to 60% (NH4)2SO4 whereas the lowest (2.20 μg/ml) was obtained from NaCl extraction and of 30-60% saturated (NH4)2SO4 precipitate. The protein amount of 60 to 90% saturated (NH4)2SO4 precipitate of NaCl extraction (N-III) was too low and undetectable. Therefore, the N-III fraction was excluded from further studies.

Effect of crude PPP fractions on SOD and GPx –like activities

The anti-oxidant SOD and GPx like activity was shown in
Table 1. Yields of crude protein fractions isolated from pigeon pea employing (NH₄)₂SO₄ precipitation method.

<table>
<thead>
<tr>
<th>Extraction solution</th>
<th>Yield of protein precipitation (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (0-30%)</td>
</tr>
<tr>
<td>PBS (P)</td>
<td>6.78</td>
</tr>
<tr>
<td>NaCl (N)</td>
<td>2.20</td>
</tr>
<tr>
<td>Water (W)</td>
<td>3.47</td>
</tr>
</tbody>
</table>

*Protein precipitate in this fraction is undetectable.

Table 2. Antioxidant enzyme-like activities of isolated pigeon pea protein.

<table>
<thead>
<tr>
<th>Fractions (1.25 µg protein)</th>
<th>SOD activity (% Inhibition)</th>
<th>GPx activity (µmole/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-I</td>
<td>58.21±0.77</td>
<td>5.95±1.03</td>
</tr>
<tr>
<td>P-II</td>
<td>83.24±1.70</td>
<td>6.14±1.30</td>
</tr>
<tr>
<td>P-III</td>
<td>36.79±0.89</td>
<td>6.15±0.85</td>
</tr>
<tr>
<td>N-I</td>
<td>33.96±0.25</td>
<td>6.30±0.80</td>
</tr>
<tr>
<td>N-II</td>
<td>90.91±2.58</td>
<td>5.50±0.49</td>
</tr>
<tr>
<td>W-I</td>
<td>58.06±1.66</td>
<td>4.13±1.70</td>
</tr>
<tr>
<td>W-II</td>
<td>82.84±3.33</td>
<td>4.64±0.44</td>
</tr>
<tr>
<td>W-III</td>
<td>93.63±0.22</td>
<td>4.45±1.97</td>
</tr>
</tbody>
</table>

Results are mean values of triplicate ± SD. * indicate significantly different (p≤0.05) from each extraction solution.

Table 2. All PPP fractions revealed considerable inhibition of O₂⁻ indicated by SOD activity. The results showed that SOD activity of 30 to 60% (NH₄)₂SO₄ precipitated of PBS (P-II), NaCl (N-II) and 60 to 90% of water (W-III) were significantly (p≤0.05) greater than that of other PPP fractions. The O₂⁻ inhibition rates of P-II, N-II and W-III were 83.24±1.70, 90.91±2.58 and 93.63±0.22%, respectively. All the protein fractions isolated from pigeon pea possessed SOD antioxidant activity in non-cellular system by scavenging of O₂⁻ generated by xantine oxidase system. It was noticed that the P-II, N-II and W-III fractions exhibited enzymatic antioxidant-like activity significantly greater than other fractions. These findings were similar to results of protein hydrolysed from chickpea (Li et al., 2008).

GPx is considered to be important enzyme in decomposition of H₂O₂. The GPx activity of PPP fractions was expressed in µmole/min/ml and represented an inhibitory effect on H₂O₂. However, there was no significant difference of GPx activity in all PPP fractions. In contrast with SOD, all of the protein fractions did not show any significant differences in GPx activity in non-cellular system. It was similar to the results from Subhashinee et al. (2005), when used H₂O₂ at low concentration (<100 µM). H₂O₂ is present in aerobic cells as a metabolite in low concentrations, generated by non-enzymatic and superoxide dismutase-catalyzed dismutation reactions (Kanner, 1992). The protective effect of SOD upon GPx activity against inactivation by O₂⁻ by protecting cellular function had been reported (Gebhardt et al., 1999). Based on these results, three active PPP fractions from SOD-like activity (P-II, N-II and W-III) were selected to determine future anti-oxidative cellular damage.

Effect of PPP fractions and H₂O₂ on cell viability

Results from TBE assay suggested that exposure of TK6 cells to PPP fractions at concentration ranging 0.01 to 2.00 mg/ml and H₂O₂ at 0.01 to 1.00 mM for 24 h resulted in no significant effect on cell viability (data not shown). Therefore, H₂O₂ at concentration of 1 mM was selected for oxidative mitochondrial and cell membrane damage activity studies.

Effect of PPP fractions on mitochondrial enzyme activity

The cytoprotective effect of PPP fractions was demonstrated in term of activity of mitochondrial dehydrogenases (MDH). The amount of formazan production was proportional to the number of TK6 viable cells, since the reduction of MTT to formazan in viable cells by mitochondrial dehydrogenase. The effects of
H$_2$O$_2$-induced oxidative stress on mitochondrial dehydrogenase activity in TK6 cells preincubated with three PPP fractions including P-II, N-II and W-III were shown in Figure 1. Oxidative induction by H$_2$O$_2$, produced a statistically significant reduction in mitochondrial dehydrogenase activity with respect to the control. Preincubation of TK6 cells with P-II, N-II and W-III fractions (1 mg/mL) exhibited MDH activity at greater levels than that of H$_2$O$_2$ exposure alone. The maximum MDH activity found in TK6 cells treated with N-II (59.48%) and W-III (58.08%) fractions. The biologically significant reaction of H$_2$O$_2$ is its spontaneous conversion, catalyzed by Fe$^{2+}$ (Fenton reaction), to the highly reactive HO$^-$ that react instantaneously with any biological molecule from which it can abstract a hydrogen atom (Storz, 2005). Mitochondrion is considered to be the major cellular source for generation of reactive oxygen species (ROS) through one-electron carriers in the respiratory chain. Two to five percent of oxygen used in the mitochondrial oxidative phosphorylation forms ROS.

Mitochondrion is also very susceptible to oxidative stress as evidenced by massive information on lipid peroxidation, protein oxidation, and mitochondrial DNA (mtDNA) mutations (Lenaz, 1998). Among several harmful ROS, H$_2$O$_2$ is considerable to be the most toxic due to its stability. Stimulation with H$_2$O$_2$ at concentration of 1 mM, could produce a significant reduction in mitochondrial enzyme activity with respect to the control. Similar observations was described by previous studies in HepG2 and Caco-2 cells (Lee et al., 2005), which suggested that this effect was due to cell proliferation inhibition caused by the intracellular accumulation of H$_2$O$_2$.

Pretreatment TK6 cells with pigeon pea protein fractions significantly increased mitochondrial dehydrogenase (MDH) activity after exposure to H$_2$O$_2$. In the same way, results demonstrated by Cilla et al. (2008), of culture preincubate with bioaccessible fraction of samples treated with milk revealed higher percentages of MTT conversion to formazan by mitochondrial dehydrogenases than the control. These latter authors reported for the MTT conversion detected after exposure to the samples with milk accords with the more preserved mitochondrial integrity observed in their work. In addition, the results suggest that pigeon pea protein isolated (N-II and W-III) could exert a certain cytoprotective effect, or at least a potential modulating effect upon mitochondrial metabolic enzymes which could contribute to the...
increased adaptive response observed against the oxidative stress caused.

**Effect of PPP fractions on LDH activity**

The release of LDH from injured cells to culture medium following H\textsubscript{2}O\textsubscript{2}-induced oxidative membrane damage and LDH activity were used as an indicator of membrane integrity in TK6 cells. The increasing of LDH activity represents the higher degree of cell membrane damage. Results on LDH activity of injured TK6 cells following H\textsubscript{2}O\textsubscript{2} induction and inhibitory effect of cell membrane damage by PPP fractions (P-II, N-II and W-III) were shown in Figure 2. Treatment of H\textsubscript{2}O\textsubscript{2} at 1 mM exhibited a markedly increase in LDH activity as compared to control cells. LDH activity was significantly decreasing in TK6 cells preincubated with 1 mg/ml P-II, W-III and 1 mM GSH (positive control) in respect to H\textsubscript{2}O\textsubscript{2} exposure. The inhibitory effect on H\textsubscript{2}O\textsubscript{2} – induced cell membrane damage of P-II, W-III and GSH was about 30, 29 and 28 %, respectively. Leakage of cellular LDH was measured as a common marker of cell membrane integrity to characterize the stress-induced membrane damage (Gebhardt et al., 1999). H\textsubscript{2}O\textsubscript{2} rapidly permeates the cell membrane and reacts with Fe\textsuperscript{2+} or Cu\textsuperscript{+} ions forming toxic HO\textsuperscript{•} that lead to propagation of oxidative damage to the cell membrane (Halliwell and Gutteridge, 1989). Marked cellular damage in TK6 cells was observed in response to H\textsubscript{2}O\textsubscript{2} concentration of 1mM compared to the control cells as detected by increased LDH leakage, which indicates a change in membrane permeability or cell necrosis. This observation was similar to results reported by Subhashinee et al. (2005), that H\textsubscript{2}O\textsubscript{2} at higher concentrations (>100 µM) cause cell membrane damage to Caco-2 cells.

A change in membrane permeability disturbs the structural integrity. It could lead to the increased entry of toxic chemicals to cells and hence eventually results in cell death at a later stage. According to our study, LDH leakage was suppressed when cells were pre-incubated with pigeon pea protein fractions (P-II and W-III) and GSH. These suggestions with the effects of these proteins to inhibit the membrane disruption caused by H\textsubscript{2}O\textsubscript{2} as revealed from the less LDH level outside the cells. The results agree with previous study in hepatocytes (Sarkar et al., 2001) cells, which reported the effect of pretreatment of protein isolated from
Phyllanthus niruri inhibited the membrane damage against tertiary butyl hydroperoxide.

Effect of PPP fractions on H$_2$O$_2$-induced DNA damage

The more DNA (single- and double-strand) breaks produced, the greater the fluorescent intensity of the comet tail relative to the head. In present study, the TL and TM were employed as major damage parameters in result analysis. Images of comet cells evaluated for the different treatment groups were demonstrated in Figure 3. Cells of the control group (Figure 3A) illustrated baseline data on TL at 14.52 ± 0.17 (Figure 4) and TM at 0.271 ± 0.004 μm (Figure 5). Exposure of TK6 cells to 50 μM H$_2$O$_2$ for 5 min produced a marked increase in both TL (65.07 ± 2.28) and TM (10.53 ± 0.57) values. The results from 3 independent experiments exhibited significant (p≤0.05) decreases in both TM and TL values in preincubated cells with P-II (TM=4.16±1.06; TL=52.94±0.51), N-II (TM=3.02±0.16; TL=43.73±1.04) W-III (TM=3.38 ± 0.76; TL=42.67±1.49) and GSH (TM=2.83±0.70, TL=53.47±2.14) respect to H$_2$O$_2$ (TM=10.53±0.56; TL=65.07±2.28). These results indicate protective effect of P-II, N-II and W-III protein fractions against H$_2$O$_2$-inducible DNA damage in TK6 cells. However, there was no significant difference of TM in anti-oxidative DNA damage among the three protein fractions.

It has been well accepted that oxidative DNA damage has been implicated in various degenerative diseases (Halliwell and Gutteridge, 1981). A huge number of studies exhibited anti-oxidative damage activity of natural compounds found in plants. Here, we demonstrated the
protective effect of pigeon pea protein on H$_2$O$_2$-induced oxidative damage in TK6 cells analyzed by comet assay. By this assay, the DNA fragments (measured in term of tail moment, TM) are generated by DNA double-strand breaks (DSB), single-strand break (SSB) induced by alkali-labile sites (Ross et al., 1995; Collins et al., 1997). Results of present study revealed the protective effect of pigeon pea protein fractions particularly P-II, N-II and W-III against H$_2$O$_2$-induced DNA damage. Similar studies had been reported that 35 kDa protein isolated from curry leaves could exert a protective from ferrous sulphate: ascorbate (Fenton reactant) induced DNA damage (Ningappa and Srinivas, 2008).

Moreover, Sivapriya et al. (2007), previously reported that the novel proteins from the water extract of Sundakai seed showed the protective effect on H$_2$O$_2$-meated DNA damage. The protective effect of against H$_2$O$_2$-induced oxidative damage in TK6 cells could be concern with SOD activity because of the respiratory chain in mitochondrial was a powerful source of ROS, primarily the O$_2^-$ and consequently H$_2$O$_2$, either as a product of SOD (Fridovich, 1986). In this study, proteins isolated from pigeon pea were exhibit SOD activity could be suppress the superoxide radical which produced at mitochondrial. It agrees with study of Cemeli et al. (2009), the SOD concentrations at 50 and 150 IU/mL had been proven to reduce the levels of DNA damage in some oxidative system. Similarly with the results reported by Birnboim et al. (1985), demonstrated that the majority of DNA damage remaining was inhabitable by SOD with the presence of catalase. In the other way, the antioxidant action could probably be counteracting/ quenching of ROS in cellular system, thereby reducing the potential of H$_2$O$_2$ to attack cellular components.

Protein molecular weight distribution

Considering among 8 PPP fractions, P-II, N-II and W-III were found to possess the highest SOD-like activity. Therefore, analysis on molecular weight distribution was performed only on these three fractions. The band pattern of protein fractions on 15% SDS-PAGE exhibited molecular weight distribution mainly in range of 6 to 66 kDa (Figure 6).

A number of studies had already shown that the antioxidant activity of protein is dependent on their molecular weight distribution (Pena-Ramos et al., 2004;...
Moure et al., 2006; Wang et al., 2007). In this study, results revealed that protein fraction with molecular weight ranging from 6.5 to 60 kDa. This latter was probably associated with higher antioxidant activity. Therefore, the mechanism underlying the protective effect of protein isolated from pigeon pea act as antioxidant needs to be further explored.

Conclusion

The results obtained in present study demonstrated SOD and GPx enzymatic antioxidant activity of crude protein fractions from pigeon pea. At 1.25 µg/ml of pigeon pea precipitate protein exhibited significantly different of fractions (P-II, N-II and W-III) in SOD activity but did not show in GPx activity. The pigeon pea protein fractions possessing the highest SOD activity were proven to be effective on multiple anti-oxidative activities in TK6 cells including protective effect on mitochondrial membrane, cell membrane and DNA damage induced by H2O2. The molecular weight distribution of these 3 active fractions including P-II, N-II and W-III revealed protein bands mainly located at 6.5 to 60 kDa. Further studies at peptide level may be required in order to identify active molecules responsible of pharmacological activity as well as the mechanisms of their anti-oxidative property.

ACKNOWLEDGEMENTS

This project was financially supported by the Ministry of Science and Technology (2009 to 2011) to the Thailand Institute of Scientific and Technological Research (TISTR) and the study was carried under the collaboration between TISTR and the Faculty of Pharmacy, Mahidol University, Bangkok Thailand. Pigeon pea seeds used in this study was kindly supplied by Dr.Chalaem Martwanna from the Field Crops Research Institute, Khon Kaen Field Crops Research Center (KFCRC), Khon Kaen, Thailand. The authors would like to thank Mr.Srisak Trangvacharakul, Director of Food Technology Department, TISTR for his encouragement. Thanks to all members of Department of Pharmaceuticals and Natural Products (PNPD) of TISTR and colleagues at Department Biochemistry, Faculty of Pharmacy, Mahidol University for their kind support.

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