Running title: redox status of Bowman-Birk inhibitor

Title of article: Redox Status of Bowman-Birk Inhibitor from soybean Influence its *in vitro* Antioxidant Activities

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Abstract

Soybean (*Glycine max*) is a major protein source for animal and human nutrition. The Bowman–Birk protease inhibitor (BBI), ranking 3rd among soybean seed storage proteins, is a major antinutritional factor. BBI was incubated with 1 mM DTT at 37ºC for 2 h and loaded directly onto a Sephadex G-25 gel column for purification. The molecular mass of the reduced form of BBI is ca. 0.8 kDa determined by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis). The methodology we used includes total antioxidant status, (1,1-dipheny-2-picrylhydrazyl) DPPH staining, DPPH radical scavenging activity, reducing power method, Fe^{2+} -chelating ability, FTC (ferric thiocyanate) method, and protection calf thymus DNA

against hydroxyl radical- induced damage. The oxidized and reduced form of BBI with a concentration of 200 μg/mL exhibited the highest activity (expressed as 4.74± 0.36 and 7.20 ± 0.20 mM Trolox equivalent antioxidative value, TEAC) in total antioxidant status test. In the DPPH staining the reduced form of BBI appeared as white spots when it was diluted to 12.5 μg/mL (a final amount of 0.6 μg). Like total antioxidant status, the reducing power, Fe^{2+}-chelating ability, FTC activity and protection against hydroxyl radical-induced calf thymus DNA damage all showed that the reduced BBI exhibited higher antioxidative activities than the oxidized BBI. The results suggested that the reduced BBI exhibited higher antioxidative activities than the oxidized BBI in a series of *in vitro* tests. These findings provide one of the molecular bases for BBI applications to treat various serious diseases.

INTRODUCTION

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species such as superoxide ($O_2^{\cdot -}$, $HOO^{\cdot -}$), hydroxyl ($OH^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals are generated. The reactive oxygen species play an important role in the degenerative or pathological processes of various serious diseases, such as cancer, coronary heart disease, Alzheimer's disease (Ames, 1983), neurodegenerative disorders, atherosclerosis, cataracts, inflammation (Aruoma, 1998) and aging (Burns

et al., 2001). The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several antiinflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as part of their activity (Lin and Huang, 2002). In the search for sources of natural antioxidants, substances such as phenolic compounds (Rice-Evans et al., 1997), anthocyanin (Espin et al., 2000), echinacoside in *Echinaceae* root (Hu and Kitts, 2000), whey proteins (Tong wt al., 2000) and water extracts of roasted *Cassia tora* (Yen and Chuang, 2000), have been extensively studied for their antioxidant activity and radical-scavenging activity.

Soybean (*Glycine max*) is an ancient legume traditionally used in the preparation of fermented and a staple dietary component among Asian populations (Sarkar et al.,1998). Substantial epidemiological evidence suggests that Asian populations consuming a high amount of soybean foods have a lower risk of certain chronic diseases such as cardiovascular disease and cancer (Becker-Ritt et al., 2004). Soybean contains several biologically active compounds such as isoflavones, saponins, peptides, and proteins. Genistein is the isoflavone present in high concentration in soybean (Bau et al., 1997) and is proposed to be the most biologically active (Cohen et al., 2002). The biological properties of isoflavones are associated with its capability

to prevent osteoporosis, cancer, and cardiovascular disease (Isanga and Zhang, 2008). Soybean also contains peptides and proteins that possess certain biological activities such as Bowman−Birk inhibitor (BBI), Kunitz inhibitor, and lunasin. BBI is a small serine protease inhibitor. BBI from soybean consists of 71 amino acid residues and has 7 disulfides bonds. BBI can withstand boiling water temperature for 10 min, resistant to the pH range and proteolytic enzymes of the gastrointestinal tract, is bioavailable, and is not allergenic. BBI inhibits the proteolytic activities of trypsin, chymotrypsin and elastase. Several studies have demonstrated the efficacy of BBI against tumor cells *ex vitro*, in animal models, and in human phase IIa clinical trials (Vaughn, et al., 2008).

The objectives of this work were to investigate antioxidant properties of the oxidized and reduced forms of BBI from soybean in comparison with reduced glutathione in a series of *in vitro* tests.

MATERIALS AND METHODS

Materials. 1,1-dipheny-2-picrylhydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA), sodium bicarbonate, Tris(hydroxylmethyl) aminomethane and Bowman-Birk inhibitor (BBI, 90% purified) were purchased from Sigma Chemical Co. (St. Louis, MO USA). The total antioxidant status assay kit was purchased from

Calbiochem-Novabiochem Corporation (La Jolla, CA, USA).

Protein staining and thiol-label staining of BBI on 15% denaturing polyacrylamide gels. Samples were mixed with sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with or without 2-mercaptoethanol. Coomassie brilliant blue G-250 was used for protein staining (Huang et al., 2009). The method of thiol-label staining on an SDS-PAGE gel basically followed the report of Huang et al. (Huang et al., 2004a) using the mBBr (monobromobimane) reagent as a probe.

Purification of reduced Bowman-Birk inhibitor. BBI was incubated with 1 mM DTT at 37ºC for 2 h and then the sample was loaded directly onto a Sephadex G-25 gel column (Amersham PD-10 desalting column). The BBI was eluted with 100 mM Tris-HCl buffer (pH 7.5).

Measurement of Total Antioxidant Status. Total antioxidant status of the BBI protein was measured using the total antioxidant status assay kit (Calbiochem Corp) according to the manufacturer's instructions. The assay relies on the antioxidant ability of the protein to inhibit oxidation of 2, 2' azino-bis-[3-ethylbenz-thiazoline-

6-sulfonic acid] (ABTS) to $ABTS^+$ by metmyoglobin. The amount of $ABTS^+$ produced is monitored by reading the absorbance at 600 nm. Under these reaction conditions, the antioxidant ability of BBI protein suppresses the absorbance at 600 nm in proportion to its concentration. The final antioxidant capacity of BBI protein was calculated by the following formula: concentration of $ABTS^+$ being cleared (mM) = [factor x (absorbance of blank-absorbance of sample)]; factor= [concentration of standard/(absorbance of blank-absorbance of standard)].

Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining. An aliquot (3 µL) of each diluted sample of the BBI was carefully loaded on a 20 cm x 20 cm TLC layer (silica gel 60 F254; Merck) and allowed to dry (3 min). Drops of each sample were loaded in order of decreasing concentration along the row. The staining of the silica plate was based on the procedure of Huang et al. (Huang et al., 2004b). The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM DPPH solution. Then the excess of solution was removed with a tissue paper and the layer was dried with a hair-dryer blowing cold air. Stained silica layer revealed a purple background with white spots at the location where radical scavenger capacity presented. The intensity of the white color depends upon the amount and nature of

138 radical scavenger present in the sample.

139

140 **Determination of Antioxidant Activity by Reducing Power Measurement.** The
141 reducing powers of the BBI and glutathione were determined according to the method
142 of Huang et al. (Huang et al., 2005). The BBI (0, 12.5, 25, 50, 100, and 200 μg/mL) or
143 glutathione was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1
144 % potassium ferricyanide. The mixture was incubated at 50ºC for 20 min, during
145 which time ferricyanide was reduced to ferrocyanide. Then an equal volume of 1%
146 trichloroacetic acid was added to the mixture, which was then centrifuged at 3,500 *g*
147 for 10 min. The upper layer of the solution was mixed with distilled water and 0.1 %
148 $FeCl_3$ at a radio of 1 : 1 : 2, and the absorbance at 700 nm was measured to determine
149 the amount of ferric ferrocyanide (Prussian Blue) fromed. Increased absorbance of
150 the reaction mixture indicated increased reducing power of the sample.

151

152 **Determination of Antioxidant Activity by Fe^{2+}-Chelating Ability.** The Fe^{2+}
153 -chelating ability was determined according to the method of Huang et al. (Huang et
154 al., 2007). The Fe^{2+} was monitored by measuring the formation of ferrous
155 iron-ferrozine complex at 562 nm. The BBI (0, 12.5, 25, 50, 100, and 200 μg/mL) was
156 mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 10 : 1 : 2. The mixture was

157 shaken and left to stand at room temperature for 10 min. The absorbance of the
158 resulting solution at 562 nm was measured. The lower the absorbance of the reaction
159 mixture the higher the Fe^{2+}-chelating ability. The capability of the sample to chelate
160 the ferrous iron was calculated using the following equation:
161 Scavenging effect (%) = 【 1- ABS_{sample} / $ABS_{control}$ 】×100.
162
163 **Protection of Bowman-Birk inhibitor against Hydroxyl Radical-Induced Calf**
164 **Thymus DNA Damage.** The hydroxyl radical was generated by Fenton reaction
165 according to the method of Huang et al. (Huang et al., 2007). The 15 μL reaction
166 mixture containing BBI (0, 2.5, 5, 10, or 20 mg/mL), 5 μL of calf thymus DNA (1
167 mg/mL), 18 mM $FeSO_4$, and 60 mM hydrogen peroxide were incubated at room
168 temperature for 15 min. Then 2 μL of 1 mM EDTA was added to stop the reaction.
169 Blank test contained only calf thymus DNA and the control test contained all
170 components except BBI. After agarose electrophoresis, the treated DNA solutions
171 were stained with ethidium bromide and examined under UV light.
172
173 **Statistical Analysis.** Averages of triplicates were calculated. Student's *t* test was used
174 for comparison between two treatments. A difference was considered to be
175 statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Purification of the reduced Bowman-Birk inhibitor. BBI was incubated with 1 mM DTT at 37ºC for 2 h and then the sample was loaded directly onto a Sephadex G-25 gel column (Amersham PD-10 desalting column). BBI was eluted with 100 mM Tris-HCl buffer (pH 7.5). The PD-10 desalting columns contain Sephadex G-25 for gel filtration (size exclusion) of biomolecules. Proteins with molecular mass >5,000 Da pass quickly through the column via outside space of gel particles and salts such as DTT with molecular mass <1,000 Da pass through the column slowly via inside space of gel particles. Thus, we can purify the reduced form of BBI without DTT (Fig. 1).

Measurement of Total Antioxidant Status. Total antioxidant status of the BBI protein was measured using the total antioxidant status assay kit (Fig. 2). Both the oxidized and reduced forms of BBI show a dose-dependent total antioxidant activity within the applied concentrations (0, 12.5, 25, 50, 100, and 200 μg/mL). At 200 μg/mL, both the oxidized and reduced forms of BBI displayed the highest total antioxidant status (4.74 ± 0.36 and 7.20 ± 0.20 mM ABTS* radical cation being cleared). The reduced BBI had higher total antioxidant status than the oxidized one.

Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining and Scavenging Activity against DPPH Radical. Antioxidant capacity of the oxidized and reduced forms of the BBI was eye-detected semi-quantitatively by a rapid DPPH staining method using TLC. Each diluted sample was applied as a dot on a TLC layer that was then stained with DPPH solution (Fig. 3). This method is typically based on the inhibition of the accumulation of oxidized products, since the generation of free radicals is inhibited by the addition of antioxidants and scavenging the free radicals shifts the end point. The appearance of white color spot vs a purple background has a potential value for the indirect evaluation of antioxidant capability of the oxidized and reduced forms of BBI in the dot blots (Chang, et al., 2007a). Fast-reacted and strong intensities of white spots appeared up to the dilution of 200 μg/BBI/mL (with an absolute amount of 0.6 μg). The oxidized form of BBI had lower antioxidant activity than the reduced form of BBI at 200 μg/mL.

The DPPH radical was widely used in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins or crude mixtures (Huang et al., 2008a). DPPH radical is scavenged by antioxidants through the donation of a hydrogen forming the reduced DPPH-H. The color changed from purple to yellow after reduction, which could be quantified by its decrease of absorbance at wavelength 517 nm. Figure 4 shows the dose-response

curve for the radical-scavenging activity of the different concentrations of BBI and glutathione using the DPPH coloring method. It was found that both the oxidized form and reduced form of BBI had the highest radical-scavenging activity (44.0 ± 0.35 and 60.0 ± 1.50 %, respectively) at 200 μg/mL. Free cysteine residues in whey proteins and trypsin inhibitor from sweet potato were reported to have antioxidant activities (Allen and Wrieden, 1982; Huang et al., 2008b). These findings suggest that cysteine residues in soybean BBI might also participate in antiradical activity.

Measurement of Reducing Power. We measure BBI's reducing capacity using Fe^{3+}-Fe^{2+} transformation process. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Chang et al., 2007b). The antioxidant activity of putative antioxidants have been attributed to various mechanisms including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Diplock, 1997). The reducing power of BBI is shown in Fig. 5 with reduced glutathione as a positive control. The reducing activity of reduced form of BBI exhibited a dose-dependence within applied concentrations (0, 12.5, 25, 50, 100, and 200 μg/mL)**.** The oxidized form of BBI had no reducing capacity.

Measurement of Fe^{2+}-Chelating Ability. The metal chelating capacity of BBI and standard antioxidants were determined by assessing their ability to compete with ferrozine for the ferrous ion. The Fe^{2+}-chelating ability of BBI with a concentration dependent mode is shown in Fig. 6. EDTA was used as a positive control. The Fe^{2+} -chelating ability of the BBI was lower than that of EDTA. The dose of 200 μg/mL of oxidized and reduced form of BBI exhibited 45.6 ± 1.29 and 68.0 ± 2.93 % iron binding capacity, respectively. The action of the BBI, as a peroxidation protector, may be mainly due to its iron-binding capacity. The reduced form of the BBI protein had better iron binding capacity than the oxidized form of BBI.

Protection against Hydroxyl Radical-Induced Calf Thymus DNA Damage by BBI. Free radicals could damage macromolecules in cells, such as DNA, proteins, and lipids in membranes (Halliwell, 1999). Figure 7 show that BBI protected calf thymus DNA against hydroxyl radical-induced damages. Compared to the blank test and control test, it was found that the reduced form of the BBI added above 5 mg/mL (the final absolute amount of 25 μg) could protect calf thymus DNA against hydroxyl radical-induced damages during 15-min reactions. While the oxidized form of the BBI added above 10 mg/mL (the final absolute amount of 50 μg) could protect calf thymus DNA.

BBI and other anticarcinogenic protease inhibitors can prevent radicals from being produced in cells and thereby decrease the amount of oxidative damage (Kennedy, 1999). A strong correlation exists between the ability of a protease inhibitor to prevent the release of oxygen free radicals from cells and its ability to inhibit carcinogenesis, with inhibitors with chymotrypsin inhibitor activity—such as BBI—having the greatest potency (Kennedy, 1999). The ability to prevent the release of oxygen free radicals is also assumed to be related to the potent anti-inflammatory activity of BBI,

In conclusion, the results from *in vitro* experiments, including total antioxidant status assay (Fig. 2), DPPH staining (Fig. 3), scavenging activity against DPPH radical (Fig. 4), reducing power method (Fig. 5), Fe^{2+}-chelating ability (Fig. 6), and hydroxyl radical-induced calf thymus DNA damage (Fig. 7), demonstrated that BBI of soybean has various antioxidant activities and the reduced form of BBI had higher antioxidantive activities than the oxidized one. Because BBI can serve as an electron donor in a variety of cellular redox reactions or during removal of hydrogen peroxide and free cysteine residues in reduced form of BBI might also participate in antiradical activity. Hence, BBI may contribute significantly to change the redox states and as a potent antioxidant against hydroxyl and peroxyl radicals when people consume soybean. The *ex vivo* or in *vivo* antioxidant activity of BBI should be performed in near further.

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大豆 Bowman – Birk 蛋白酶抑制劑之氧化還原狀態影響其體外之抗氧化活性

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大豆（*Glycine max*）是動物和人類營養之一個重要的蛋白質來源。Bowman-Birk蛋白酶抑制劑（BBI）為大豆種子中排名第三之貯藏蛋白，是一個重要的抗營養因子。將BBI 在 37ºC 下 1 mM DTT 溶液中培育2 小時，然後直接加入Sephadex G-25 凝膠管柱中純化。還原態之 BBI 經SDS-PAGE 測定其分子量約 0.8 kDa。本研究分析的項目有：總抗氧化能力、DPPH (1,1-dipheny-2-picrylhydrazyl)染色法、DPPH自由基清除活性、還原力、亞鐵離子螯合能力、抑制過氧化物形成能力和保護DNA免於氫氧自由基傷害。氧化態和還原態BBI 在總抗氧化能力分析上在200 μg/mL 時可達最高的抗氧化活性(以 4.74± 0.36 和 7.20 ± 0.20 mM Trolox equivalent antioxidative value, TEAC, 分別表示)。在DPPH 染色法中，12.5 μg/mL（實際使用量為 0.6 μg）開始具有抗氧化

384 活性。像在總抗氧化能力、還原力、亞鐵離子螯合能力、抑制過氧化物形成能力
385 和保護DNA免於氫氧自由基傷害分析還原態BBI比氧化態BBI具有較高的抗氧化能
386 力。由實驗結果得知在一系列的體外分析試驗中還原態BBI比氧化態BBI具有較高
387 的抗氧化能力。這些發現可提供 BBI 應用在治療其他各種疾病的一個分子基
388 礎。
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390 **關鍵詞：**大豆；Bowman-Birk 蛋白酶抑制劑; 抗氧化; 氧化還原狀態