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Recombinant sporamin and its synthesized peptides with antioxidant activities *in vitro*

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ABSTRACT. Recombinant sporamin B overproduced in *E. coli* (M15) was purified by Ni^{2+}-chelated affinity chromatography. The molecular mass of sporamin B is ca. 26 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total antioxidant status, 1,1-dipheny-2-picrylhydrazyl (DPPH) staining, reducing power method, Fe^{2+}-chelating ability, ferric thiocyanate (FTC) method, and protecting calf thymus DNA against hydroxyl radical-induced damage were studied. The sporamin B protein with a concentration of 100 μg/mL exhibited highest activity (expressed as 4.21 ± 0.0078 mM Trolox equivalent antioxidative value, TEAC) in total antioxidant status test. In the DPPH staining sporamin B appeared as a white spot when the concentration was diluted to 25 mg sporamin B/mL (with an absolute amount of 75 μg). Like total antioxidant status, the reducing power, Fe^{2+}-chelating ability, FTC activity and protection calf thymus DNA against hydroxyl radical-induced damage all showed that sporamin B polypeptides have significant antioxidant activities. It was found that antioxidant activities of sporamin B increased from 19% (0 h) to about 29% (24 h) after 24 h hydrolysis by pepsin. Smaller peptides increased with hydrolytic times. Eight peptides for testing antioxidative activity were synthesized according to peptic hydrolysis simulation. The obtained SNIP, VRL, SYCQ, GTEKC, RF, VKAGE, AH, KIEL showed IC_{50} values of 8.36, 4.23, 0.206, 0.0884, 9.72, 14.9, 13.8 and 24.9 mM, respectively, when scavenging activity of DPPH radicals (%) was measured. These findings mean that cysteine residue is most important in antiradical activities. It was suggested that sporamin B might contribute to its antioxidant activities against hydroxyl and peroxyl radicals.

Keywords: Antioxidant activity; Recombinant protein; Gene expression; Sporamin; Sweet potato.

INTRODUCTION

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$) and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) radicals are generated. The reactive oxygen species play an important role related to the degenerative or pathological processes of various serious diseases, such as aging (Burns et al., 2001), cancer, coronary heart disease, Alzheimer's disease (Ames, 1983; Gey, 1990; Smith et al., 1996; Diaz et al., 1997), neurodegenerative disorders, atherosclerosis, cataracts, and inflammation (Aruoma, 1998). The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several antiinflammatory, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or antiradical scavenging mechanism as part of their activity (Perry et al., 1999; Repetto and Llesuy, 2002). In the search for sources of natural antioxidants and compounds with radical scavenging activity during the last few years, some have been found, such as whey proteins (Allen and Wrieden, 1982; Tong et al., 2000), phenolic compounds (Rice-Evans et al., 1997), anthocyanin (Espin et al., 2000), echinacoside in *Echinaceae* root (Hu and Kitts, 2000), water extract of roasted *Cassia tora* (Yen and Chuang, 2000), both thioredoxin *h* protein (Huang et al., 2004a) and mucilage (Huang et al., 2006a, b) from sweet potato root.

Sporamin was the major storage protein in sweet potato tuberous roots, first described by Maeshima et al. (Maeshima et al., 1985). It accounted for 60% to 80% of the total soluble protein in the sweet potato tuber. Expression of

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sporamin was shown to be mainly associated with tubers (Hattori et al., 1990). Sporamin was found to strongly inhibit trypsin activity, and insect-defense capabilities were confirmed in insect bioassays with transgenic tobacco (Yeh et al., 1997). These showed that sporamin functions not only as a storage protein for nutrient supply but also as a factor against herbivore attacks. S*poramin* genes belonged to a large multigene family that was divided into subfamilies A and B. There was over 90% nucleotide homology among intrasubfamily genes, and about 80% nucleotide homology among inter-subfamily genes (Hattori et al., 1989). The objectives of this work were to investigate the antioxidant property of sporamin B from sweet potato in comparison with chemical compounds such as butylated hydroxytoluene (BHT), reduced glutathione or ascorbate in a series of in vitro tests.

MATERIALS AND METHODS

Expression of sporamin B in *E. coli*

Sporamin B (Gene Bank accession number: M16883) was expressed in *E. coli*. The coding sequence was amplified from sporamin B cDNA using an oligonucleotide (5′-GGATC CACAT GAAAG CCT TC G-3′), with a *Bam*HI site (underlined) at the putative initial Met residue, and an oligonucleotide (5′-TCAGA AGCTT TGATC ACA-3′), with a *Hind*III site at the 3′ end. The PCR fragment was subcloned in pGEM T-easy vector. And the plasmid was then digested with *Bam*HI and *Hind*III and subcloned in pQE-30 expression vector (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-sporamin B, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-nitrilotriacetic acid (NTA) columns (Qiagen), according to the manufacturer's instructions.

Protein staining on 15% SDS-PAGE gels

Sporamin B was detected on 15% SDS-PAGE gels. Samples treated with sample buffer and β-mercaptoethanol (2-ME) with a final concentration of 14.4 mM were heated at 100°C for 5 min before 15% SDS-PAGE.

Measurement of total antioxidant status

Total antioxidant status of the sporamin B 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 sporamin B protein decreases the absorbance at 600 nm in proportion to its concentration. The final antioxidant capacity of sporamin B protein was calculated by the following formula: Trolox equivalent value (mmol/L)= [factor × (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 sporamin B was carefully loaded on a 20 cm × 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. (2005). 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 radical scavenger present in the sample.

Scavenging activity against DPPH radical

The effect of sporamin B on the DPPH radical was estimated according to the method of Huang et al. (2004b). An aliquot of sporamin B (30 μL) was mixed with 100 mM Tris-HCl buffer (120 μL, pH 7.4) and then 150 μL of the DPPH in ethanol with a final concentration of 250 μM was added. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance at 517 nm of the reaction solution was measured spectrophotometrically. The percentage of DPPH decolourization of the sample was calculated according to the equation: % decolourization= $[1- Abs_{sample} / Abs_{control}] \times 100$.

Determination of antioxidant activity by reducing power measurement

The reducing powers of the sporamin B and glutathione were determined according to the method of Yen and Chen (1995). Sporamin B (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) or glutathione was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, during which time ferricyanide was reduced to ferrocyanide. Then an equal volume of 1% trichloroacetic acid was added to the mixture, which was then centrifuged at 6,000 *g* for 10 min. The upper layer of the solution was mixed with deionized water and 0.1% $FeCl_3$ at a radio of 1:1:2, and the absorbance at 700 nm was measured to determine the amount of ferric ferrocyanide (Prussian Blue) formed. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

Determination of antioxidant activity by Fe^{2+}-chelating ability

The Fe^{2+}-chelating ability was determined according to the method of Decker and Welch (1990). The Fe^{2+} was

monitored by measuring the formation of ferrous iron-ferrozine complex at 562 nm. Sporamin B (0, 0.4, 0.8, 1.2, 1.6 and 2 mg/mL) was mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 10:1:2. The mixture was shaken and left to stand at room temperature for 10 min. The absorbance of the resulting solution at 562 nm was measured. The lower the absorbance of the reaction mixture the higher the Fe^{2+}-chelating ability. The capability of the sample to chelate the ferrous iron was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [1 - Abs_{sample} / Abs_{control}] \times 100$$

Determination of antioxidant activity by the ferric thiocyanate (FTC) method

The FTC method was adapted from the method of Osawa and Namiki (1981). Twenty mg/mL of samples dissolved in 4 ml of 99.5% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 ml), 0.05 M phosphate buffer pH 7.0 (8 ml) and deionized water (3.9 ml) and kept in a screw-cap container at 40°C in the dark. Then, to 0.1 ml of this solution was added 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red color [Fe $(SCN)^{2+}$, Fe^{3+} was formed after linoleic acid peroxide was produced and Fenton reaction occurred.] was measured every 24 h until the day when the absorbance of the control reached the maximum value. The inhibition of linoleic acid peroxidation was calculated as (%) inhibition = 100 - [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate and analyses of all samples were run in triplicate and averaged.

Protection of sporamin against hydroxyl radical-induced calf thymus DNA damage

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (1991). The 15 μL reaction mixture containing sporamin B (0, 50, 100, or 200 μg), 5 μL of calf thymus DNA (1 mg/mL), 18 mM $FeSO_4$, and 60 mM hydrogen peroxide were incubated at room temperature for 15 min. Then 2 μL of 1 mM EDTA was added to stop the reaction. Blank test contained only calf thymus DNA and the control test contained all reaction components except sporamin B. The treated DNA solutions were subjected to agarose electrophoresis and then stained with ethidium bromide and examined under UV light.

Determination of the antioxidative activity of sporamin B peptic hydrolysates

Six mg of sporamin B was dissolved in 1 mL of 0.1 M KCl buffer (pH 2.0). Then 0.1 mL (12 mg) of pepsin was added at 37°C for 0 and 24 h. After hydrolysis, 0.5 mL of 0.5 M Tris-HCl buffer (pH 8.3) was added, and the solution was heated at 100°C for 5 min to stop enzyme reaction. The pepsin was heated before sporamin B hydrolysis for the 0 h reaction. Each of the 60 μL sporamin B hydrolysates was used for determinations of the DPPH antioxidative activities by spectrophotometry.

Chromatograms of peptic hydrolysates of trypsin inhibitor on a Sephadex G-50 column

The unhydrolyzed sporamin B and peptic sporamin B hydrolysates at 24 h were separated by Sephadex G-50 column chromatography (1 × 60 cm). The column was eluted with 20 mM Tris-HCl buffer (pH 7.9). The flow rate was 30 mL/h, and each fraction contained 2 mL of which the absorbance at 280 nm was then determined.

Statistical analysis

Means of triplicates were calculated. Student's *t* test was used for comparison between two treatments. All data (expressed as percent of control value) were means ± SE. A difference was considered to be statistically significant when $p < 0.05$, $p < 0.01$ or $p < 0.001$.

RESULTS and DISCUSSION

Purification of expressed sporamin B

Sporamin B cDNA clones from sweet potato storage roots were isolated. *Sporamin B* was subcloned in a pQE-30 expression vector in *E. coli* and sporamin B was produced with a 6x His-tag at the N-terminus. SDS-PAGE analysis of crude extracts from transformed *E. coli* (M15) showed a high level of a polypeptide with the expected molecular mass (ca. 26 kDa). This polypeptide was found as a soluble protein in the supernatant (Figure 1, lane 2), and was absent in protein extracts obtained from *E. coli* transformed with pQE-30 vector (Figure 1, lane 1). The expressed protein was purified from crude extracts by Ni^{2+} -chelate affinity chromatography, which yielded highly purified His-tagged sporamin B (Figure 1, lane 3).

Measurement of total antioxidant status

This was measured using the total antioxidant status assay kit (Figure 2). Sporamin B protein exhibited a dose-dependent total antioxidant activity within the applied concentrations (0, 5, 10, 20, 40, 60, 80, and 100 μg/mL), the highest at 100 μg/mL (expressed as 4.21 ± 0.008 mM Trolox equivalent antioxidative value, TEAC). At 5 μg/mL, sporamin B displayed the lowest total antioxidant status (1.95 ± 0.002 mM TEAC).

Rapid screening of antioxidant by dot-blot and DPPH staining

Antioxidant capacity of expressed sweet potato sporamin B was eye-detected semi-quantitatively by a rapid DPPH staining method in TLC. Each diluted sample was applied as a dot on a TLC layer that was then stained with DPPH solution (Figure 3). This method is typically based on the inhibition of the accumulation of oxidized

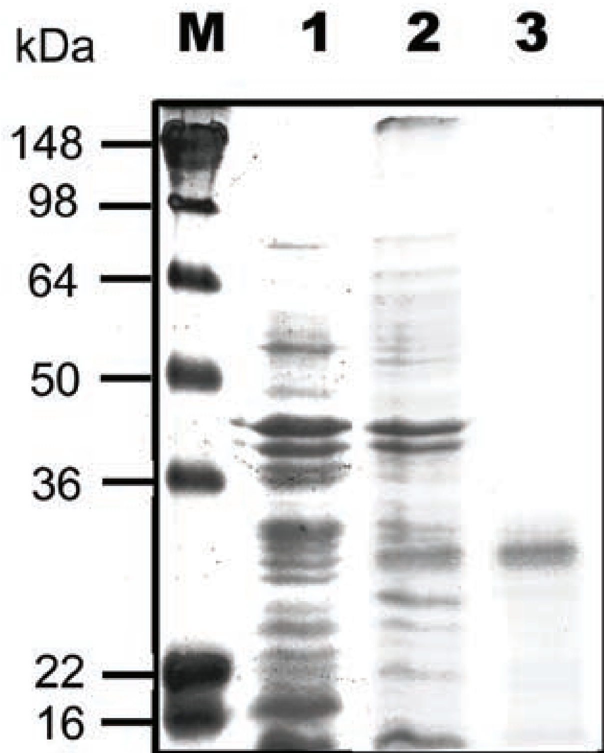

Figure 1. SDS-PAGE analysis of purified recombinant sweet potato sporamin B. Crude extracts from *E. coli* (M15) transformed with pQE30 (lane 1) or with pQE30-spormin B1 (lane 2) were analyzed by 15% (w/v) SDS/PAGE with 10 μg protein applied on each lane, and then the gel was stained with Coomassie blue G-250. Molecular masses of standard proteins are indicated at the left of the figure. His-tagged sporamin B was purified by Ni^{2+}-chelated affinity chromatography (lane 3). The experiments were done twice and a representative one is shown.

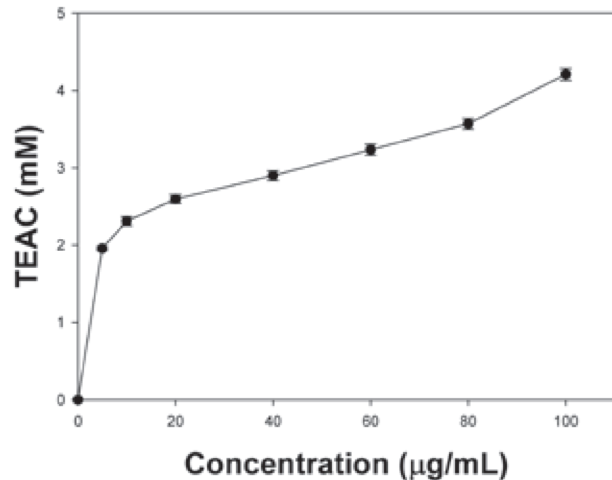

Figure 2. Total antioxidant activity of recombinant sporamin B from sweet potato, as measured by the total antioxidant status assay. Absorbance value represents average of triplicates of different samples analysed.

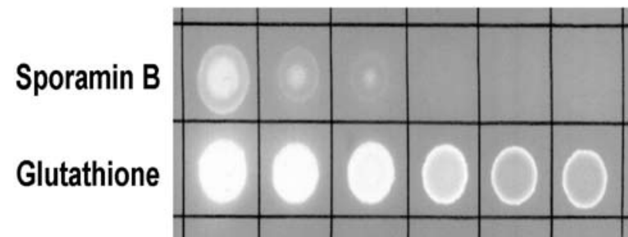

Figure 3. Dot blot assay of recombinant sporamin B from sweet potato on a silica sheet stained with a DPPH solution in methanol. Each 3 μl of sporamin B (100, 50, 25, 12.5, 6.25, and 3.125 mg/mL) was applied from left to right in sample row; while each 3 μl glutathione (10, 5, 2.5, 12.5, and 6.25 mg/mL) was applied from left to right in control row.

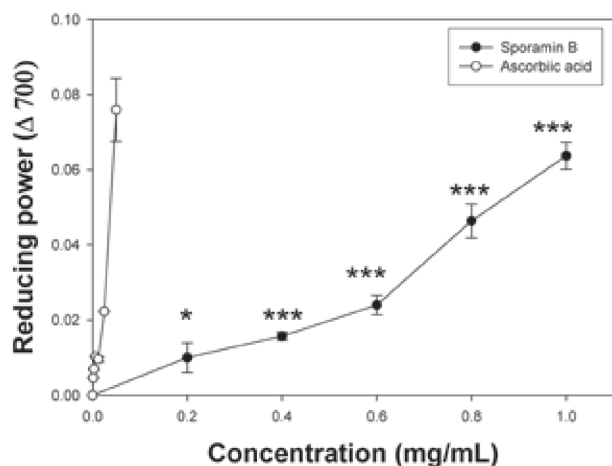

Figure 4. Antioxidative activities of recombinant sporamin B from sweet potato, as measured by the reducing power method. Each absorbance value represents average of triplicates of different samples analysed. Results represent the means ± SE from at least 3 separate experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (unpaired t test) compared to sporamin unsupplemented samples.

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 expressed sporamin B in the dot blots (Soler-Rivas et al., 2000). Fast-reacted and strong intensites of white spots appeared up to the dilutions of 25 mg sporamin B/mL (with an absolute amount of 75 μg). The reduced glutathione was used as a positive control.

Measurement of reducing power

We investigated the Fe^{3+}-Fe^{2+} transformation in the presence of the samples of sporamin B to measure its reducing capacity. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among them are 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 sporamin B is shown in Figure 4 with ascorbic acid served as a positive control. The reducing power activity of sporamin B exhibited a dose-dependence (significant at $p<0.05$) within the applied concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL).

Measure of Fe^{2+}-chelating ability

The metal chelating capacity of sporamin B and standard antioxidants was determined by assessing their ability to compete with ferrozine for the ferrous ions. The Fe^{2+}-chelating ability of the sporamin B is shown in Figure 5. EDTA was used as a positive control. The Fe^{2+}-chelating ability of sporamin B was lower than that of EDTA and this difference was statistically significant ($P<0.05$). Sporamin B at doses of 0.4, 0.8, 1.2, 1.6 and 2 mg/mL exhibited 65.26, 70.15, 72.41, 74.61 and 76.03% iron binding capacity, respectively. On the other hand, EDTA at doses of 0.02, 0.1, 0.2, 0.3 and 0.4 mg/mL had 33.74, 78.16, 95.68, 96.68 and 96.69% chelating activity of iron, respectively.

Ferric thiocyanate (FTC) method

Low-density lipoprotein (LDL) peroxidation has been reported to contribute to atherosclerosis development (Steinbrecher, 1987). Therefore, delay or prevention of LDL peroxidation is an important function of antioxidants. Figure 6 shows the time-course curve for the antioxidative activity of the sporamin B from sweet potato, BHT and H_2O by the FTC method. The BHT was used as a positive control, and H_2O as a negative control. The results indicate that sporamin B has antioxidative activity. Sporamin B may act as a significant LDL peroxidation inhibitor ($P<0.05$).

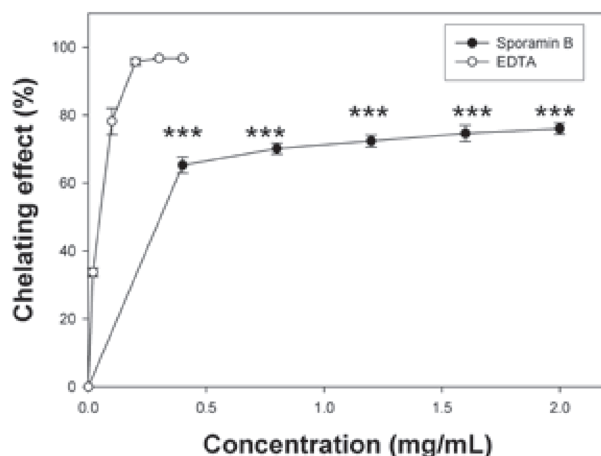

Figure 5. Antioxidative activities of recombinant sporamin B from sweet potato, as measured by the Fe^{2+}-chelating ability method. Each absorbance value represents average of triplicates of different samples analysed. Results represent the means ± SE from at least 3 separate experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 (unpaired *t* test) compared to sporamin unsupplemented samples.

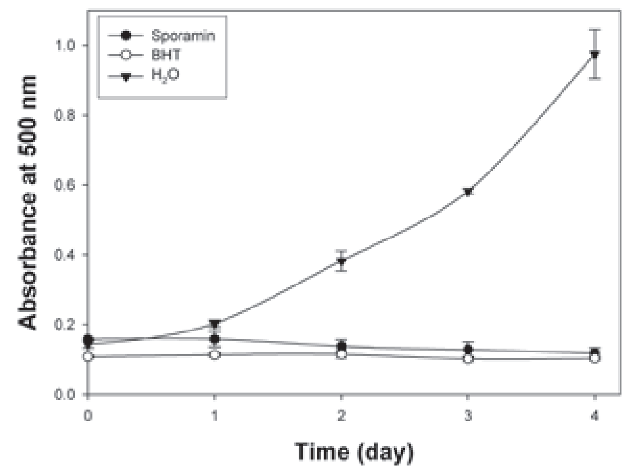

Figure 6. Inhibition of linoleic acid peroxidation by recombinant sporamin B from sweet potato, as measured by the FTC method. Each absorbance value represents average of triplicates of different samples analysed.

Protection against hydroxyl radical-induced calf thymus DNA damage by sporamin B

Free radicals could damage macromolecules in cells, such as DNA, protein, and lipids in membranes (Kohno et al., 1991). Figure 7 shows that sporamin B protected against hydroxyl radical-induced calf thymus DNA damages. The blank contained calf thymus DNA only, and the control contained all components except sporamin B. Compared to the blank and control, it was found that 100 μg sporamin B could protect against hydroxyl radical induced calf thymus DNA damages during 15-min reactions.

Determination of the antioxidative activity of peptic sporamin B hydrolysates and their peptide distributions

We used pepsin to hydrolyze sporamin B to mimic the hydrolysis course during digestion in human's (or animal's) stomach. Figure 8 shows the antioxidative activity of peptic sporamin B hydrolysates and the antioxidative activity (scavenging activity of DPPH radicals, percent) of peptic sporamin B hydrolysates collected at different pepsin hydrolysis times. From the results, it was found that the antioxidative activity increased from 19% (0 h) to about 29% (24 h). It was found that smaller peptides increased with pepsin hydrolytic time. The purifications of potential peptides of antioxidative activity need further investigations. We used synthetic peptides to measure antioxidative activity. Synthetic peptides were designed by simulating pepsin cutting sites of sporamin A and B gene products from sweet potato (pH>2, http://expasy.nhri.org.tw/tools/peptidecutter/). New peptides (Table 1) for antioxidative activity, that is, SNIP, VRL, SYCQ, GTEKC, RF, VKAGE, AH, KIEL were synthesized according to simulation. IC_{50} values of individual peptides were 8.36, 4.23, 0.206, 0.0884, 9.72, 14.9, 13.8 and 24.9 mM,

Figure 7. Protection against hydroxyl radical-induced calf thymus DNA damage by recombinant sporamin B. Sample lanes 1-3 contained 2.5, 5, and 10 mg/mL sporamin B, respectively. Blank (B) contained calf thymus DNA only; while the control (C) contained all reaction components except sporamin B.

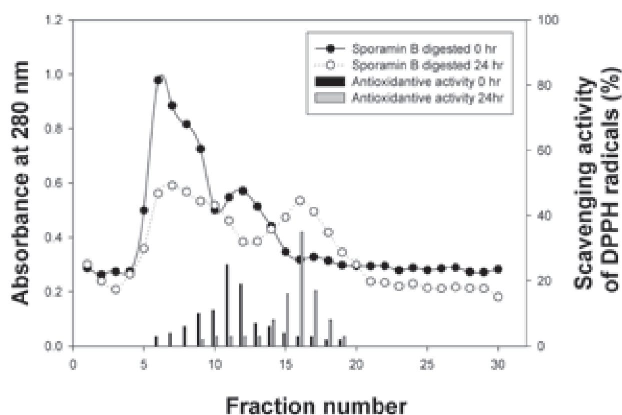

Figure 8. Antioxidative activity of recombinant sporamin B peptic hydrolysates. The plot shows the antioxidative activity (%) of sporamin B hydrolysates at different pepsin hydrolysis time (0 h and 24 h). The proteins and the scavenging activity of DPPH radicals (%) were showed. The scavenging effect (%) was calculated according to the equation [1-(Abs 517 nm of sample ÷ Abs 517 nm of control)] × 100%.

Table 1. Sporamin B peptides with antioxidant activity.

Peptide	Scavenging activity of DPPH radicals (%), IC_{50} (mM)
GSH (control)	0.0748
SNIP	8.36
VRL	4.23
SYCQ	0.206
GTEKC	0.0884
RF	9.72
VKAGE	14.9
AH	13.8
KIEL	24.9

respectively, when scavenging activity of DPPH radicals (%) was measured. Cysteine residues with free-SH in whey proteins (Allen and Wrieden, 1982; Tong et al., 2000) were reported to have antioxidant activities. Our results further indicate that cysteine residues (GTEKC or SYCQ) in sweet potato sporamin B contribute to the antiradical activities. The synthetic peptide, GTEKC, has the highest antioxidant activity (IC_{50} is 0.0884 mM) as good as reduced glutathione (IC_{50} is 0.0748 mM). Another synthetic peptide, SYCQ, also has a good antioxidant activity (IC_{50} is 0.206 mM). These results demonstrated that simulated synthetic peptides from peptic sporamin B hydrolysates exhibited antioxidative activity.

In conclusion, the results from in vitro experiments, including total antioxidant status assay (Figure 2), DPPH staining (Figure 3), reducing power method (Figure 4), Fe^{2+}-chelating ability (Figure 5), FTC method (Figure 6), and hydroxyl radical-induced calf thymus DNA damage (Figure 7), demonstrated that sporamin B in sweet potato may have significant antioxidant activities. Sporamin B may contribute significantly to change the redox states and as a potent antioxidant against both hydroxyl and peroxyl radicals when people consume sweet potato. The *ex vivo* or *in vivo* antioxidant activity of sporamin B should be performed in near further.

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重組蛋白質 sporamin 及其合成之胜肽含有抗氧化的活性

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在大腸桿菌 (M15) 中大量表現重組蛋白質 sporamin B，然後利用鎳離子螯合之親和性管柱純化。Sporamin B 經 SDS-PAGE 分析其分子量約為 26 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、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。Sporamin B 在總抗氧化能力分析上在100 μg/mL 時可達最高的抗氧化活性(以 4.21 ± 0.0078 mM Trolox equivalent antioxidative value, TEAC, 表示)。在 DPPH 染色法中，25 mg sporamin B/mL (實際使用量為 75 μg) 開始具有抗氧化活性。在所有分析項目中，重組之 sporamin B 蛋白質都具有顯著的的抗氧化活性。利用胃蛋白酶水解 sporamin B 時，小分子的胜肽會隨著水解時間增加。24 小時後抗氧化活性(對 DPPH 之清除能力)可以從 19% (0 h) 增加到 29% (24 h)。利用電腦模擬胃蛋白酶水解 sporamin B 蛋白質的結果，八種人工合成具有抗氧化活性胜肽：SNIP, VRL, SYCQ, GTEKC, RF, VKAGE, AH, KIEL，利用 DPPH 自由基清除率測定其 IC_{50} 為 8.36, 4.23, 0.206, 0.0884, 9.72, 14.9, 13.8 和 24.9 mM。結果發現胜肽上具有半胱胺酸基者具有很好的抗自由基活性。

關鍵詞：抗氧化活性；基因表現；重組蛋白質；sporamin；甘藷。