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Sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage root mucilage with antioxidant activities in vitro

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Abstract

Sweet potato storage root mucilage was extracted and purified by SDS and heating treatments. Total antioxidant activity, DPPH (1,1-diphenyl-2-picrylhydrazyl) staining, reducing power method, metal ion-dependent hydroxyl radical, FTC (ferric thiocyanate) method, and protection of calf thymus DNA against hydroxyl radical-induced damage were studied. Half-inhibition concentrations, IC_{50}, were 0.08 mg/ml and $IC_{50} > 0.1$ mg/ml, respectively, for the crude and purified mucilage in the total antioxidant activity test. In the DPPH staining, the crude and purified mucilage appeared as white spots when they were diluted to 50 and 100 μg per application, respectively. Like total antioxidant activity, reducing power, scavenging capacity against hydroxyl radical, FTC activity and protection of calf thymus DNA against hydroxyl radical-induced damage were found in the mucilage. It is suggested that the mucilage might contribute its antioxidant activities against both hydroxyl and peroxyl radicals.

Keywords: Sweet potato; Antioxidant; Mucilage; Hydroxyl radical; Peroxyl radical; 1,1-Diphenyl-2- picryl hydrazyl (DPPH)

1. Introduction

It is commonly accepted that, in a situation of oxidative stress, reactive oxygen species, such as superoxide ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($^{\bullet}OOH$, $ROO^{\bullet}$) 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, Frei, Vita, & Keaney, 1997), neurodegenerative disorders, atherosclerosis, cataracts, and inflammation (Aruoma, 1998). Traditional medicine is widespread and plants still presents a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activity (Perry, Pickering, Wang, Houghton, & Perru, 1999; Lin & Huang, 2002; Repetto & Llesuy, 2002). In the search for sources of natural antioxidants and compounds with radical scavenging activity during recent years, some have been found, such as echinacoside in *Echinaceae* root (Hu & Kitts, 2000), anthocyanin (Espin, Soler-Rivas, Wichers, & Viguera-Garcia, 2000), phenolic compounds (Rice-Evans, Miller, & Paganga, 1997), water extracts of roasted *Cassia tora* (Yen & Chuang, 2000), whey proteins (Allen & Wrieden, 1982; Tong, Sasaki, McClements, & Decker, 2000), and thioredoxin *h* protein from sweet potato (Huang, Lin, Chen, Hou, & Lin, 2004a).

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The major components of plant mucilage are pectins. Pectins are largely acidic polysaccharides that form gels in the extracellular matrix and are present in all cell walls. The two most common pectins found in dicotyledonous plants are polygalacturonic acid (PGA) and rhamnogalacturonan I (RG I) (Brett & Waldron, 1990; Carpita & Gibeaut, 1993; Cosgrove, 1997). PGA is an unbranched chain of α-1,4-linked galacturonic acid (GalUA) residues, while RG I is a highly substituted, branched polysaccharide with a backbone of alternating α-1,4-linked GalUA and α-1,2-linked rhamnose residues (Brett & Waldron, 1990). There are reports concerning the physiological activities of pectins on the interactions between fibroblast growth factors and receptors (Liu et al., 2001), on the modulation of lung colonization of B16-F1 melanoma cell (Platt & Raz, 1992), and on the inhibition of human cancer cell growth and metastasis in nude mice (Nangia-Makker et al., 2002). Pectin diets could also reduce the incidence of colon cancer in rats (Hardman & Cameron, 1995). Yam (*Dioscorea batatas*, Dioscoreaceae) is a major tuber crop and its mucilages are mainly composed of mannan-protein macromolecules with antioxidant activities (Tsai & Tai, 1984). A G009 fraction (polysaccharide fraction) of *Ganoderma lucidum* showed inhibitory activity against iron-induced lipid peroxidation and dose-dependent hydroxyl radical scavenging activity (Lee et al., 2001). The arabinogalactan polysaccharide showed good protection against iron-mediated lipid peroxidation of rat brain homogenate, as revealed by the thiobarbituric acid-reactive substances (TBARS) and lipid hydroperoxide (LOOH) assays (Subramanian, Chintalwar, & Chattopadhyay, 2002).

No report on the antioxidant activities of mucilage of sweet potato is presently available. In this work we report (for the first time) that purified mucilage from sweet potato displayed antioxidant activities in comparison with chemicals such as butylated hydroxytoluene (BHT), reduced glutathione, or ascorbic acid in a series of in vitro tests.

2. Materials and methods

2.1. Mucilage extractions and purifications

Mucilage extractions and purifications were done according to the method of Hou, Hsu, and Lee (2002). Fresh sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage roots were purchased from a local market. After being cleaned with water, the storage roots were cut into strips for crude mucilage extractions. Sweet potato storage root was homogenized with two volumes (w/v) of 50 mM Tris–HCl buffer (pH 8.3) containing 1% vitamin C. After centrifugation at 14,000*g* for 30 min, the supernatants were mixed with isopropanol to a final concentration of 70%, and stirred quickly at 4 °C overnight. The precipitates were filtered and dehydrated with 100% isopropanol, then rinsed with acetone. After drying at 40 °C in an oven, the crude mucilage was ground and collected for further purification by both SDS and heating procedures. About 1.0 g crude mucilage powder was dissolved in 200 ml of distilled water and kept in a 50 °C water bath. Forty ml of 5% SDS solution (dissolved in 45% ethanol) were added to the crude mucilage solution. The mixture was kept with gentle stirring at 50 °C for 30 min, then at room temperature for another 2 h. After that, the mucilage solution was placed in an ice bath to quickly lower the temperature in order to precipitate the SDS-protein complex. After centrifugation at 14,000*g* at 0 °C for 30 min, the supernatants were precipitated with isopropanol and dried at 40 °C in an oven as described earlier. The semi-purified mucilage was again ground, dissolved, and then heated in boiling water for 20 min. After centrifugation at 14,000*g* at 0 °C for 30 min, the supernatants were mixed with isopropanol to a final concentration of 70%. The purified mucilage was filtered, dehydrated, rinsed with acetone, dried, and then collected for further uses.

2.2. Protein and PAS stainings on 10% SDS–PAGE 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 2-mercaptoethanol, and subjected to electrophoresis according to the method of Laemmli (1970). Coomassie brilliant blue G-250 was used for protein staining (Huang, Chen, Hou, Chen, & Lin, 2004b). Periodic acid–Schiff (PAS) staining was used for oligosaccharide staining. After electrophoresis, the gel was placed in fixative (7.5% acetic acid), and shaken gently for 60 min. To oxidize the oligosaccharides, the gel was treated with 0.2% periodic acid for 45 min at 4 °C. The gel was washed with distilled water and stained with Schiff's reagent in the dark at 4 °C (Deepak, Thippeswamy, Shivakameshwari, & Salimath, 2003).

2.3. Total antioxidant status

Total antioxidant status of the mucilage was measured using 2,2′-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) assay (Re et al., 1999). ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12–16 h) in the dark before use. The resultant intensely-coloured $ABTS^{\cdot+}$ radical cation was diluted with 0.01 M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test compound was diluted 100 × with the ABTS solution to

a total volume of 1 ml. Absorbance was measured spectrophotometrically at time intervals of 1, 2, 5, 10, and 15 min after addition of each extract in a range of four to eight concentrations. The assay was performed at least in triplicate. Controls containing 990 μl of PBS, to replace ABTS, were used to measure absorbance of the extract themselves. The assay relies on the antioxidant ability of the samples to inhibit the oxidation of ABTS to $ABTS^{\bullet+}$ radical cation.

2.4. Rapid screening of antioxidant by dot-blot and DPPH staining

Each diluted sample of the mucilage was carefully loaded onto 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 Soler-Rivas, Espin, and Wichers (2000). 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 colour depends upon the amount and nature of radical scavenger present in the sample.

2.5. Determination of antioxidant activity by reducing power measurement

The reducing powers of the mucilage and glutathione were determined according to the method of Yen and Chen (1995). The mucilage (0, 20, 40, 60, 80, 100 μg/ml)and ascorbic acid (0, 1.5, 2.5, 3.5, 5 μg/ml)were 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 period ferricyanide was reduced to ferrocyanide. Then an equal volume of 1% trichloroacetic acid was added to the mixture, which was then centrifuged at 5000*g* for 10 min. The upper layer of the solution was mixed with distilled 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.

2.6. Scavenging activity of mucilage against metal ion-dependent hydroxyl radical

The hydroxyl radical was determined by the deoxyribose method (Halliwell, Gutteridge, & Aruoma, 1987). Every 0.5 ml of sample containing different amounts (0, 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) of crude mucilage and purified mucilage was added to 1.0 ml solution of 20 mM potassium phosphate buffer (pH 7.4), 2.8 mM 2-deoxy-ribose, 104 μM EDTA, 100 μM $FeCl_3$, 100 μM ascorbate and 1 mM hydrogen peroxide. The mixtures were incubated for 1 h at 37 °C. After incubation, equal volumes of 0.5% thiobarbituric acid in 10% trichloroacetic acid were added and the mixtures were boiled at 100 °C for 15 min. Deionized water was used as a blank, and reduced glutathione (0, 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) was used as a positive control. The absorbance at wavelength 532 nm was measured. The scavenging activity of hydroxyl radical (%) was calculated by the equation: $[(A532_{blank} - A532_{sample})/A532_{blank}] \times 100\%$.

2.7. 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 distilled 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 were 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 colour principle $[Fe(SCN)_3]$, which was formed after lipid peroxide was produced and Fenton reaction had occurred, was measured every 24 h until the day when the absorbance of the control reached the maximum value. All tests were run in duplicate and analyses of all samples were run in triplicate and averaged.

2.8. Protection of hydroxyl radical-induced calf thymus DNA damage by mucilage

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno, Yamada, Mitsuta, Mizuta, and Yoshikawa (1991). Fifteen μl of reaction mixture containing the crude and purified mucilage (25, 50, 100, and 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 were added to stop the reaction. The blank test contained only calf thymus DNA and the control test contained all reaction components except mucilage. The treated DNA solutions were subjected to agarose electrophoresis and then stained with ethidium bromide and examined under UV light.

2.9. Statistical analysis

Means of triplicate analyses were calculated. The Student's *t* test was used for comparison between two

treatments. A difference was considered to be statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Extraction and purification of mucilage from sweet potato storage root

The crude mucilage was obtained by isopropanol precipitation and the yield was about 1.5%. For further purification, it was first treated by SDS, which binds to proteins, and the complexes formed were removed by centrifugation. The semi-purified mucilage was then heated in boiling water for 20 min. The total recovery of purified mucilage, after the two purification steps, was about 24% that of the crude one. After drying at 40 °C in an oven, the purified mucilage was ground and then collected for further uses (Fig. 1).

3.2. Dose effect of the mucilage on the inhibition of $ABTS^{\cdot+}$ formation

Inhibition of generation of the $ABTS^{\cdot+}$ radical cation is the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activities of solutions of pure substances, aqueous mixtures and beverages.

Total antioxidant activities of the crude and purified mucilage were measured using the $ABTS^{\cdot+}$ method (Fig. 2). Different amounts of mucilage exhibited a dose-dependent total antioxidant activity within the applied concentrations (0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml). At 0.1 mg/ml, the crude and purified mucilage displayed the highest total antioxidant activity (percent inhibition $58.4 \pm 1.00\%$ and $36.9 \pm 0.95\%$, respectively), with a half-inhibition concentration (IC_{50}) of 0.08 mg/ml and $IC_{50} > 0.1$ mg/ml, respectively. Reduced glutathione and ascorbic acid (0, 0.0015, 0.0025, 0.00625, 0.0125 and 0.025 mg/ml) were used as positive controls.

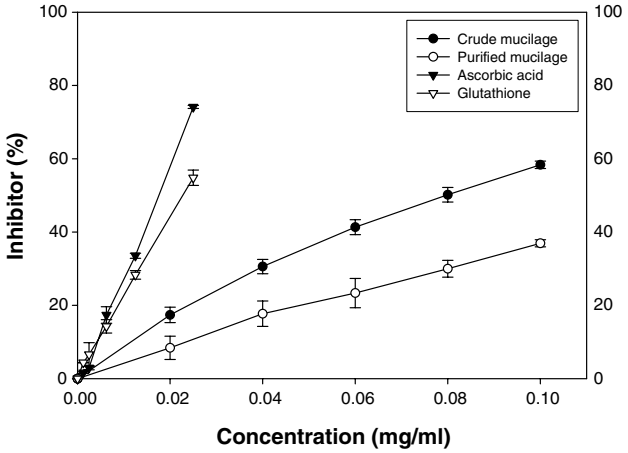

Fig. 2. The concentration effect of the crude and purified mucilage of sweet potato on the inhibition of the $ABTS^{\cdot+}$. Concentration range includes 0, 0.02, 0.04, 0.06, 0.08, and 0.01 mg/ml. Absorbance value represents average of triplicates of different samples analyzed.

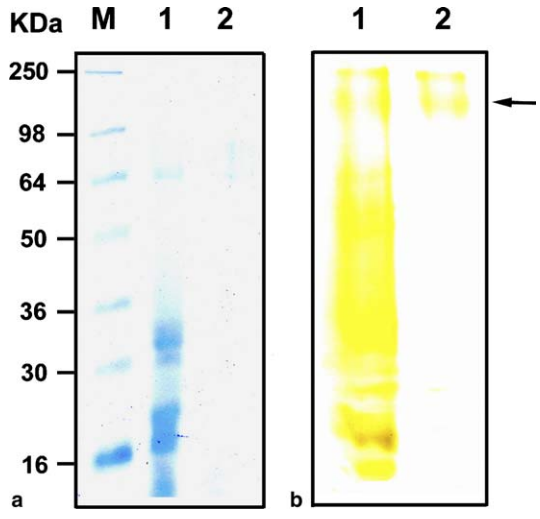

Fig. 1. The protein (a) and PAS (b) stainings of the crude (lane 1) and purified (lane 2) sweet potato storage root mucilage on SDS–PAGE gels after 2-mercaptoethanol treatment. The gel system contained 2.5 cm, 4% stacking gel and 4.5 cm, 15% separating gel. M indicates the Seeblue prestained markers of SDS–PAGE. The mucilage (15 μg) was loaded into each well. Arrow indicates the position of the mucilage.

3.3. Rapid screening of antioxidant by dot-blot and DPPH staining

Antioxidant capacity of the mucilage from sweet potato was eye-detected semi-quantitatively by a rapid DPPH staining TLC method. 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 of

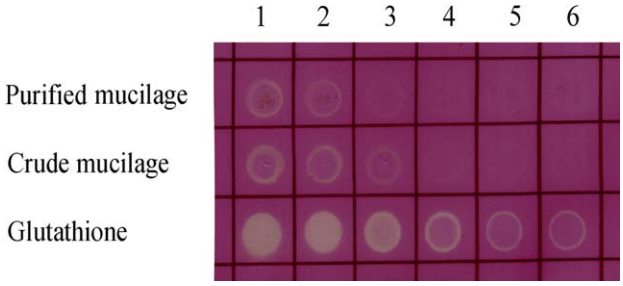

Fig. 3. Dot blot assay of the mucilage from sweet potato on a silica sheet stained with a DPPH solution in methanol. The crude and purified mucilage (200, 100, 50, 25, and 12.5 μg) were applied from left to right in sample row; glutathione (30, 15, 7.5, 3.75, 1.875, and 0.9375 μg) was applied from left to right in control row.

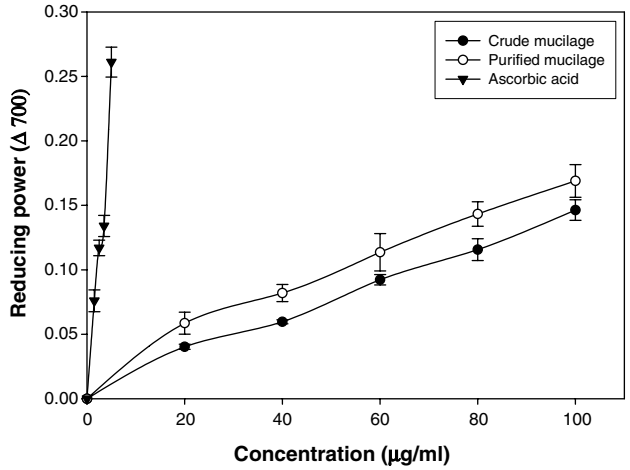

Fig. 4. Antioxidative activities of the crude and purified mucilage (0, 20, 40, 60, 80, and 100 μg/ml) from sweet potato, as measured by the reducing power method. Ascorbic acid (0, 1.5, 2.5, 3.5, and 5.0 μg/ml)was used as a positive control. Each absorbance value represents the average of triplicates of different samples analyzed.

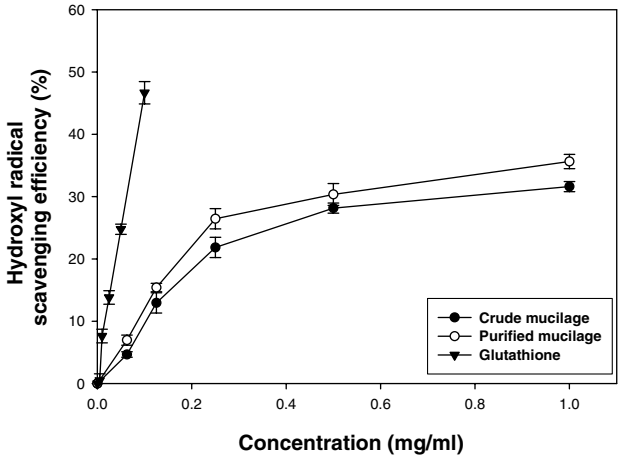

Fig. 5. The concentration effect on the scavenging activity levels of crude and purified mucilage against hydroxyl radical assayed by spectrophotometry. Concentration range includes 0, 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml. Reduced glutathione (0, 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) was used as a positive control. Each absorbance value represents the average of triplicates of different samples analyzed.

the free radicals shifts the end point. The appearance of a white colour spot on a purple background has potential value for the indirect evaluation of antioxidant capability of the mucilage in the dot blots (Chang et al., 2002). White spots with strong intensity appeared quickly up to the dilution with 50 μg of crude mucilage per application, and up to the dilution with 100 μg of the purified mucilage. The reduced glutathiones was used as a positive control.

3.4. Measurement of reducing power

We investigated the Fe^{3+}–Fe^{2+} transition to measure the mucilage's reducing capacity. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Hadas, 1995). The antioxidant activities of putative antioxidants have been attributed to various mechanisms, among which 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 the mucilage is shown in Fig. 4, with ascorbic acid as a positive control. The reducing power activity of the mucilage exhibited a dose-dependence (significant at $p < 0.05$) within the applied concentrations (0, 20, 40, 60, 80, and 100 μg/ml). The purified mucilage had a better reducing power than the crude mucilage.

3.5. The scavenging activity of mucilage on hydroxyl radical

The effects of the crude and purified mucilage on hydroxyl radical are shown in Fig. 5. Mucilage exhibited dose-dependent hydroxyl radical-scavenging activity within the applied concentrations (0, 20, 40, 60, 80, and 100 μg/ml). The purified mucilage had a higher scavenging activity than the crude one on the same weight basis. The capacity of reduced glutathione against hydroxyl radical was apparently lower than that of the mucilage. The scavenging activity levels of purified mucilage against hydroxyl radical were 6.97% and 35.6%, respectively, for 20 and 200 μg/ml samples. The reduced glutathione (0, 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) was used as a positive control. The hydroxyl radical was very reactive, and could be formed by transition of metal ion catalysts (iron, copper, in Fenton reaction) (Halliwell, 1999). Our results suggest that mucilage might scavenge hydroxyl radical formed in metal ion-dependent processes, and thus reduce cell damage caused by hydroxyl radical.

3.6. Ferric thiocyanate (FTC) method

Low-density lipoprotein (LDL) peroxidation has been reported to contribute to atherosclerosis development (Steinbrecher, 1987). Therefore, prevention or delay of LDL peroxidation is an important function of antioxidants. Fig. 6 shows the time-course for the antioxidative activity of the mucilage from sweet potato, BHT and H_2O by the FTC method. BHT was used as a positive control, and H_2O as a negative control. The results indicate that mucilage has antioxidative activity. The mucilage may act as a significant LDL peroxidation inhibitor ($p < 0.05$). The purified mucilage had a better ability to prevent LDL peroxidation than did the crude one.

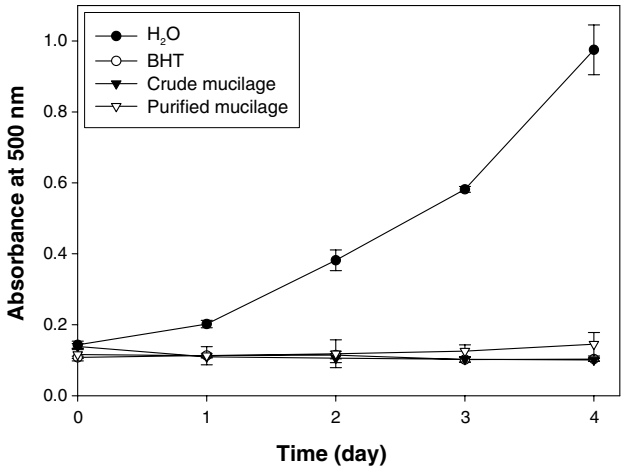

Fig. 6. Inhibition of linoleic peroxidation by the crude and purified mucilage as measured by the FTC method. Each absorbance value represents the average of triplicates of different samples analyzed.

3.7. Protection against hydroxyl radical-induced calf thymus DNA damage by mucilage

Free radicals could damage macromolecules in cells, such as DNA, protein, and membrane lipids (Halliwell, 1999). Fig. 7 shows that mucilage protected against hydroxyl radical-induced calf thymus DNA damage. The blank contained only calf thymus DNA, and the control contained all components except mucilage. Compared to the blank and control, mucilage added above 25 μg could reduce hydroxyl radical induced calf thymus DNA damage during 15-min reactions. The purified mucilage performed better than the crude one in this test.

In results mentioned above, the purified mucilage performed better than the crude one in tests of Figs. 4,5, and 7 while, in the tests of Figs. 2 and 3, the opposite was found. In Fig. 6, the antioxidant abilities of the two samples are equal. The reason why the crude mucilage performed better than the purified one in the results of Figs. 2 and 3 may be the existence of other components in the crude sample (Fig. 1) that have antioxidant activities. Other components of the extract might contain isoflavonoids, anthocyanins, phenolic compounds and some proteins. Because the mucilage was extracted with isopropanol, some pigments and proteins could dissolve in the organic solution.

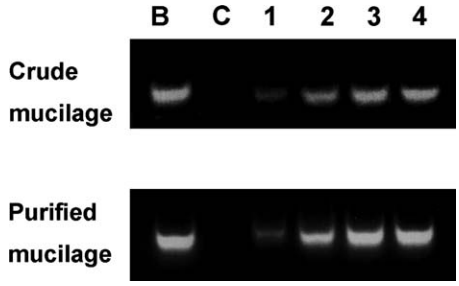

Fig. 7. Protection capacity of the mucilage against hydroxyl radical-induced calf thymus DNA damage. Sample lanes 1–4 contained 25, 50, 100, and 200 μg/ml of mucilage, respectively. Blank (B) contained calf thymus DNA only; the control (C) contained all reaction components except mucilage.

Several pectin derivatives have been reported to have pharmacological and biological activities toward cancer, cardiovascular diseases, Alzheimer's disease, and tuberculosis (Muri, Nieto, Sindelar, & Williamson, 2002). Succinimide hydroxamic acids were proved to be potent inhibitors of histone deacetylase and tumor cell proliferation (Curtin et al., 2002). Hydroxamic acid derivatives of salicylic acid are cyclooxygenase (COX-1 and COX-2) inhibitors (Dooley et al., 2003). Oxal hydroxamic acid derivatives are potent inhibitors of matrix metalloproteinases (Krumme & Tschesche, 2002). Aspartic acid, β-hydroxamate exhibited antitumor activity on L5178Y leukemia (Thomasset et al., 1991), therapeutic effect on Friend erythroleukemia (Tournaire et al., 1994), and antiproliferative activity on Friend virus-infected erythropoietic progenitor cells (Tournaire et al., 1994). We have also reported that different degrees of esterification (DE) of pectin hydroxamic acids (PHAs) exhibited semicarbazide-sensitive amine oxidase, angiotensin converting enzyme (ACE) inhibitory activities (Hou, Lee, Hsu, & Lin, 2003), antioxidant and antiradical activities (Yang, Cheng, Lin, Liu, & Hou, 2004). The resonance properties of hydroxamic acid moieties (RCONHOH) in the galacturonic acid monomer of pectin molecules might explain the differences between pectin hydroxamic acids and the corresponding esterified pectins.

4. Conclusions

In conclusion, the results from in vitro experiments, including total antioxidant activity assay (Fig. 2), DPPH staining (Fig. 3), reducing power method (Fig. 4), scavenging hydroxyl radical (Fig. 5), FTC method (Fig. 6), and prevention of hydroxyl radical-induced calf thymus DNA damage (Fig. 7), demonstrated that the mucilage of sweet potato roots may have significant antioxidant activities and behave as a potent antioxidant against both hydroxyl and peroxyl radicals when people consume sweet potato. The ex vivo or in vivo antioxidant activity of the mucilage should be studied in the near future.

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