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The identification of antioxidants in dark soy sauce

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Abstract

Soy sauce is a traditional fermented seasoning in Asian countries, that has high antioxidant activity *in vitro* and some antioxidant activity *in vivo*. We attempted to identify the major antioxidants present, using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay as a guide. 3-Hydroxy-2-methyl-4*H*-pyran-4-one (maltol) was one of several active compounds found in an ethyl acetate extract of dark soy sauce (DSS) and was present at millimolar concentrations in DSS. However, most of the antioxidant activity was present in colored fractions, two of which (CP1 and CP2) were obtained by gel filtration chromatography. Their structural characteristics based on nuclear magnetic resonance (NMR) and electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) analysis suggest that carbohydrate-containing pigments such as melanoidins are the major contributors to the high antioxidant capacity of DSS.

Keywords: *Soy sauce, maltol, melanoidin, antioxidant, ABTS*

Introduction

Imbalance between formation of reactive oxygen species and levels of antioxidants *in vivo* has been suggested to play a role in the development of various diseases, such as atherosclerosis, diabetes, rheumatoid arthritis, cancer and neurodegenerative diseases [1,2]. Some (but not all) studies show that nutritional antioxidants can decrease oxidative damage in the human body and may have beneficial effects on disease prevention [2–5]. This has led to a growing interest in antioxidants from natural products [2–7].

Soy sauce is a traditional fermented seasoning of East Asian countries and is currently used in cooking worldwide [8]. It is traditionally prepared from soy bean and wheat flour [9]. Several papers have alluded to the presence of antioxidants in soy sauce [10–14], and we found earlier that dark soy sauce (DSS) had extremely high total antioxidant activity (TAA) *in vitro* [14] as judged by the ability to scavenge the nitrogen-centred $ABTS^{\cdot+}$ radical, an assay that is frequently used to assess the antioxidant activity of beverages, food extracts and body fluids [15]. DSS also decreased lipid peroxidation *in vivo* in human volunteers [16]. In the present study, we attempted to identify the major components that contribute to the high antioxidant activity of DSS.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich, Singapore unless otherwise stated. Ethyl acetate, formic acid, hydrochloric acid fuming 37% [Guaranteed Reagent (GR) for analysis], sodium hydroxide

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(GR for analysis) were from Merck, Germany; methanol (HPLC grade) was from Labscan Analytical Science, Thailand.

DSS (Tiger brand, Chuen Cheong Food Industries, Singapore) from a single commercial batch was purchased from a local supermarket.

ABTS assay

This was carried out as described in Ref. [14,15]. 2,2′-Azino-bis[3-ethylbenzothiazoline-6-sulfonate] (ABTS) in water (7 mM final concentration) was oxidized using potassium persulfate (2.45 mM final concentration) for at least 12 h in the dark. The $ABTS^{\cdot+}$ solution was diluted to an absorbance of 0.7 ± 0.02 at 734 nm (Beckman UV–VIS spectrophotometer, Model DU640B, UK) with phosphate-buffered saline (PBS 10 mM, pH 7.4). Extracts of DSS (10 μl) or trolox standard (10 μl) were added to 1 ml of $ABTS^{\cdot+}$ solution. Absorbance was measured 1 min after initial mixing. Antioxidant properties of fractions of DSS extracts were expressed as Trolox equivalent antioxidant capacity (TEAC), calculated from at least three different concentrations of extract tested in the assay and giving a linear response.

Isolation of low molecular mass components from ethyl acetate extract

DSS (6.4 L) was extracted three times with a four-fold volume of methanol. The methanol extracts were combined, filtered by filter paper and evaporated to dryness under vacuum at 40°C (MeOH-extract, yield 2.6 kg). No significant retention of antioxidant components from soy sauce by the filter paper was detected.

The residue after methanol extraction (MeOH-R) yielded 1.4 kg. The MeOH-extract was suspended in water and partitioned with ethyl acetate three times. The ethyl acetate fractions were combined and evaporated to dryness under vacuum at 30°C (EtOAc-extract, yield 11.6 g). The remaining aqueous fraction (EtOAc-R) yielded 2.6 kg.

As the EtOAc-extract exhibited strong ABTS radical scavenging activity, it was subjected to flash chromatographic separation with a silica gel RP18 (particle size 40–63 μm, Merck KGaA, Darmsadt, Germany) packed column (6 × 42 cm) eluting with methanol and water. Fractions eluted with 10% methanol had the highest $ABTS^{\cdot+}$ radical scavenging activity and were further purified by a prep-HPLC (Agilent 1100 Series, equipped with a fraction collector) using a ZORBAX SB-C18 PreP HT column (21.2 × 250 mm, 7 μm) (Agilent, USA) at 20 ml/min with methanol—0.1% formic acid in MilliQ water (10:90, v/v) as mobile phase.Ten fractions showing antioxidant activity in the ABTS assay, Fr.1 − 10, were obtained. Fraction 9 was found to have the most ABTS scavenging activity and yielded Compound **1** (41 mg).

Fractionation of colored components

Approximately, 1 gm of ethyl acetate extract residue (EtOAc-R) was re-suspended in 25 ml distilled water and dialyzed against distilled water for seven days [17], using a cellulose dialysis tubing (Pierce, Rockford, USA; molecular weight cutoff 3500). Initially, we investigated the influence of dialysis times and temperatures, room temperature (~25°C) and cold-room temperature (~4°C), on the antioxidant capacity of the colored products as measured by the ABTS assay and found no significant effect (data not shown). For convenience, the dialysis experiments were carried out at room temperature. The nondialyzable fraction was freeze-dried. Approximately, 84.5 mg of the freeze-dried product was dissolved in 5 ml of water and loaded onto a fine Sephadex G-75 gel filtration chromatography column (2 × 100 cm). The colored fractions (absorbance at 470 nm), 4 ml per tube, were collected. The fractions from No. 26 to 34 possessed significantly higher TEAC values and consequently were combined as Colored Product 1 (CP1) (yield 61 mg).

The MeOH-R fraction (approximately 2 g) was dialyzed against distilled water. The nondialyzable fraction (85 mg) was fractionated with gel chromatography in the same way as EtOAc-R. The fractions from No. 3 to 9 were combined as Colored Product 2 (CP2) (yield 47 mg).

HPLC determination of maltol

DSS (10 ml) was extracted with 40 ml methanol on an orbital shaker (SLOS-20, Seoulin Bioscience, Seoul, Korea) at a speed of 150 rpm for 24 h, and then centrifuged at 3000*g* for 30 min. This procedure was repeated three times. The supernatants were pooled and dried using a rotary evaporator under vacuum at 40°C. The residue was dissolved in 20 ml water and extracted three times with 20 ml ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness at 30°C. Prior to HPLC analysis, the dried samples were dissolved in 10 ml methanol-0.1% formic acid (9:1) and then filtered through 0.45 μm disposable nylon filters (Agilent Technology, USA).

Analysis was performed using an Agilent 1100 HPLC with an Agilent ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm), maintained at 35°C. The mobile phase was formic acid in MilliQ water (0.1%, v/v) (A) and methanol (B) with a gradient program as follows: 10% of B for 15 min, 10–90% of B in 6 min, 90% of B for another 5 min, with flow rate at 1 ml/min. The injection volume for all samples was 10 μl and absorbance was monitored at 270 nm. Spectra were recorded from 190 to 400 nm.

Mass spectrometry

An Agilent XCT Plus ion trap mass spectrometer (ITMS) (Agilent Technology, US) was used to analyze the fractions, Fr. 1–10, obtained from ethyl acetate extracts. Atmospheric pressure chemical ionization (APCI) MS was performed in the positive mode. The dry gas and vaporiser temperatures were 350 and 400°C, respectively.

For the analysis of CP1, Electrospray-Ionization Mass Spectrometry (ESI-MS) was performed using a Waters Micromass Q-Tof micro mass spectrometer (Waters, USA). Sample was directly infused at a speed of 10 μl/min for acquiring mass spectra. The capillary and sample cone voltages were maintained at 3.0 kV and 50 V, respectively. The source and desolvation temperatures were 80 and 250°C, respectively. The mass spectra were acquired from m/z 100 to 5000 in the positive ion mode.

Nuclear magnetic resonance spectrometry (NMR)

The NMR spectra of compound **1**, CP1 and CP2 were recorded on a Bruker Advance AMX500 NMR spectrometer (Rheinstetten, Germany) at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C), respectively. Tetramethylsilane (TMS) was used as an internal standard. Compound **1** was dissolved in methanol-*d*4; CP1 and CP2 were dissolved in D_2O.

Statistical analysis

The mean values were calculated from data taken from at least three separate experiments. Where significance testing was performed, a Student's *t*-test was used; *P*-values of <0.05 were considered to be statistically significant.

Results

No significant differences in TEAC value or HPLC profile were detected between different bottles of the same commercial batch. The antioxidant components in DSS were found to be stable during 1–2 months storage, based on the TEAC value and their HPLC profile.

Separation and characterization of low molecular mass components

DSS was extracted with methanol, and the methanol extract was further partitioned with ethyl acetate as described in Material and Methods. The three fractions of DSS, MeOH-R, EtOAc-extract and EtOAc-R were subjected to the ABTS assay. The MeOH-R fraction contributed about 60% of the TAA of DSS. However, the EtOAc-extract showed the strongest antioxidant activity, approximately 12-fold higher than that of the DSS (Figure 1(a)).

After pretreatment with a RP18 column (mainly to remove benzoate, which has no antioxidant activity in the ABTS assay), the EtOAc-extract was separated with preparative HPLC resulting in 10 fractions

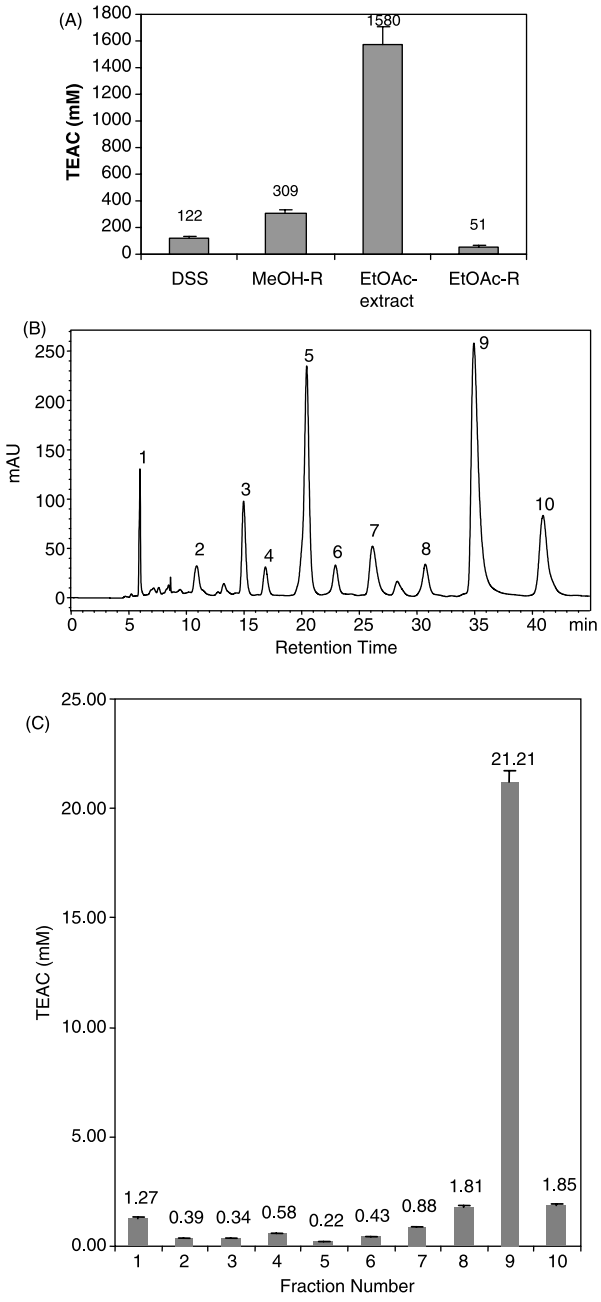


Figure 1. (a) TEAC values per g/ml of DSS and three fractions: MeOH-R, EtOAc-extract and EtOAc-R. The DSS and three fractions were dried using a rotary evaporator, and then dissolved in water. One ml of $ABTS^{\cdot+}$ ($A_{734nm} = 0.7 \pm 0.02$) was added to 10 μl of DSS or several different amounts of its fractions at at least three different dilutions as described under Materials and Methods. Absorbance was read 1 min after initial mixing. Values mean ± SD, $n = 3$; (b) typical HPLC chromatogram of EtOAc-extract after RP-18 column pretreatment. The absorbance was monitored at 270 nm; (c) TEAC values of 10 fractions of EtOAc-extract per μg/ml.

(Figure 1(b)). Among them, the ABTS assay (Figure 1(c)) showed that Fr.9 has by far the strongest activity. Fr.9 was further purified to yield compound **1**.

The positive APCI-ITMS spectrum of Compound **1** showed a protonated molecular ion peak at *m/z* 127 $[M + H]^+$. So its molecular weight was determined to be 126. The ^{1}H-NMR spectrum (Table I) of **1** (500 MHz, methanol-*d*4) showed two *cis*-olefinic proton signals at δ_H 7.94 (1H, d, $J = 5.5$ Hz) and 6.39 (1H, d, $J = 5.5$ Hz). In addition, the signal of one methyl group directly attached to an olefinic carbon at δ_H 2.35 (3H, s) was observed. The ^{13}C-NMR spectrum (Table I) showed one carbonyl carbon signal at δ_C175.3, and four olefinic carbon signals at δ_c 156.3, 152.2, 144.6 and 114.4, indicating the presence of a pyranone ring. The signal at δ_c 14.2 corresponded to the carbon of the methyl group. Compound **1** was also analyzed by HPLC-DAD. Its retention time and UV spectra (absorption maxima at 210 and 275 nm) agreed very closely with those obtained from authentic maltol. So the MS spectrum, NMR data, the retention time and UV spectrum support the identity of compound **1** as 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol) (Figure 2).

Fractions 1–8 and 10 were also analyzed by APCI-ITMS, suggesting these fractions contain molecules with molecular weight ranging from 124 to 214 (data not shown).

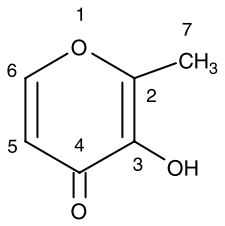


Figure 2. Structure of 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol).

Content of maltol and its contribution to the TAA of dark soy sauce

A considerable part of the TAA of the DSS was contained in MeOH-R and EtOAc-R fractions and in fractions 1–8 and 10 (Figure 1). Although maltol was identified as the active fraction of Fr. 9, we needed to determine its overall contribution to TAA. Thus an HPLC method was developed for determination of maltol in DSS.

Maltol standard was dissolved in methanol-0.1% formic acid (10:90, v/v) yielding concentrations of 0.25, 0.5, 1.0, 1.5, 2.0 mM. Three different sets of standard solutions were prepared and analyzed each day and in three continuous days. Calibration curves for the quantification of maltol were obtained by plotting concentration (mM) against peak area. The calibration curve was linear within the investigated concentration range with the following regression equation:

$$A = 4869.4C - 43.5 (R^2 = 0.9999, n = 9)$$

where A is the peak area (mAU·S), and C is the concentration of maltol solution (mM).

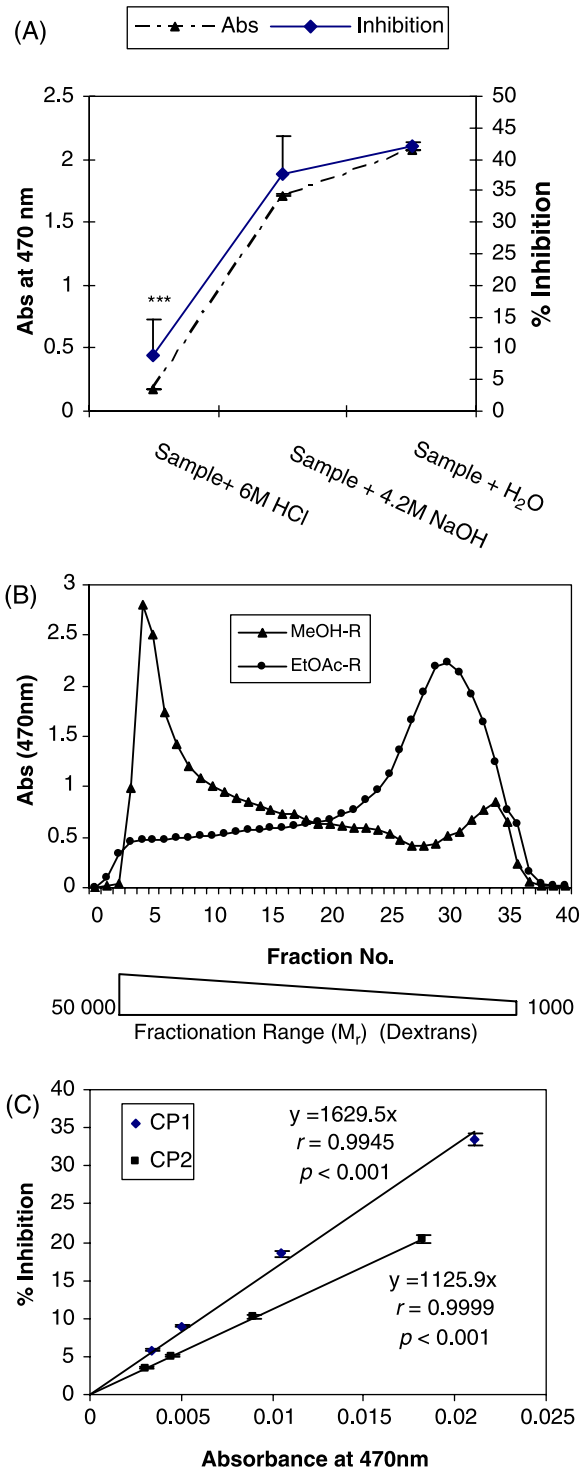
The within- and between-assay precision were established by injecting three sets of samples at three spike concentration levels (0.25, 1.0 and 2.0 mM) within one and three days, respectively. The within- and between-assay was in the range 0.3–1.8% and 1.4–3.4%, respectively (Table II). The limit of detection (LOD) was determined as the amount that resulted in a peak with a height two or three times that of the baseline noise. The limit of quantification (LOQ) was determined as the amount that resulted in a peak with a height ten times that of the baseline noise. The LOD and LOQ were 8 and 25μM, respectively. The accuracy was evaluated through recovery studies by adding known amounts of maltol at three different levels to the sample. The unspiked samples and each of the spiked samples were analyzed in triplicate. The recoveries at the different concentration levels were in the range 89.8–94.2% (Table II). Using this validated method, the average concentration of maltol in DSS was determined to 1.15 ± 0.04 mM ($n = 5$). The antioxidant activity of maltol (TEAC value) was 2.67 ± 0.05 mM ($n = 5$). This concentration of maltol was calculated to contribute around 2.5% of the TAA of DSS. HPLC analysis of pure maltol illustrated that this aromatic

Table I. ^{1}H and ^{13}C NMR data of Compound **1**.

C/H number	δ_C (ppm)	δ_H (ppm)
2	152.2	
3	144.6	
4	175.3	
5	114.4	6.39 (1H, d, $J = 5.5$ Hz)
6	156.3	7.94 (1H, d, $J = 5.5$ Hz)
7	14.2	2.35 (3H, s)

Table II. Within- and between-assay precision and recoveries of the assay used to measure maltol.

Concentration level/mM	Within-assay precision (% RSD, $n = 3$)	Between-assay precision (% RSD, $n = 3$)	Recovery (%)
0.25	1.8	3.4	89.8
0.5			89.9
1.0	0.4	2.4	92.9
1.5			94.2
2.0	0.3	1.4	93.9



compound was stable for at least one week in water at room temperature.

Fractionation and characterization of the colored components

When the MeOH-R fractions of DSS were incubated in 6 M HCl *in vacuo* at 110°C for 18 h, the colored components became insoluble (the solvent became transparent, and colored precipitate was present at the tube bottom), while in 4.2 M NaOH they were stable. Removal of colored components dramatically decreased the antioxidant activity of the hydrolysate (Figure 3(a)), suggesting that the colored components could greatly contribute to the TAA of DSS.

To further investigate the colored components, we used gel filtration chromatography to fractionate the DSS. The MeOH-R and EtOAc-R fractions were separately dialyzed against distilled water and further fractionated with gel filtration chromatography as described in Material and Methods. Figure 3(b) shows the gel filtration chromatograms of these two fractions. We obtained a high-molecular-mass fraction (CP2) from the MeOH-R, and a lower-molecular-mass fraction (CP1) from the EtOAc-R. Indeed, the absorbance at 470 nm and $ABTS^{\cdot+}$ scavenging activity of CP1 and CP2 were highly correlated, $r = 0.9945$ for CP1 and 0.9999 for CP2 (Figure 3(c)). However, the ratios of scavenging activity to absorbance for the two fractions were not equal, 1629.5 for CP1 and 1125.9 for CP2, suggesting that different compositions are present.

The ^{1}H-NMR and ^{13}C-NMR spectra of CP1 (Figure 4(a),(c)) are very complex. Despite this, it was possible to observe the resonance signals related to carbonyl groups (δ 166.8). The spectra also indicated the presence of carbohydrates: the proton signals at δ 5.36–5.33 and carbon signals at δ 99.9–98.5 were due to the anomeric hydrogens and carbons, and a complex of multiplet between δ 3.93 and δ 3.37 and the carbon signals at the range of δ 77.6–δ 60.5 corresponded to other sugar backbone protons and carbons, respectively.

The ^{1}H-NMR spectrum of CP2 (Figure 4(b)) showed similar signals to those of CP1 (Figure 4(a)). However, the ratios of integral of the signal peaks at δ 8.25 to that at δ 5.36–5.33 are different for these two fractions; around 1:2 for CP1 and 1:1 for CP2. This difference suggested that their compositions are somewhat different.

Figure 3. (a) The absorbance and $ABTS^{\cdot+}$ scavenging activity of MeOH-R fraction under acidic and basic conditions. The MeOH-R samples were incubated in 6 M HCl or 4.2 M NaOH *in vacuo* at 110°C for 18 h. The colored components became insoluble in acidic condition, while in alkaline condition they were stable. Removal of colored components dramatically decreased the antioxidant activity of the acidic hydrolysate, indicating that the colored components could greatly contribute to the TAA of DSS. Value[illegible] $n = 3$. ***Comparision between the $ABTS^{\cdot+}$ scav[illegible] sample + 6 M HCl and that of sample + H_2O (***$p < 0.001$); (b)

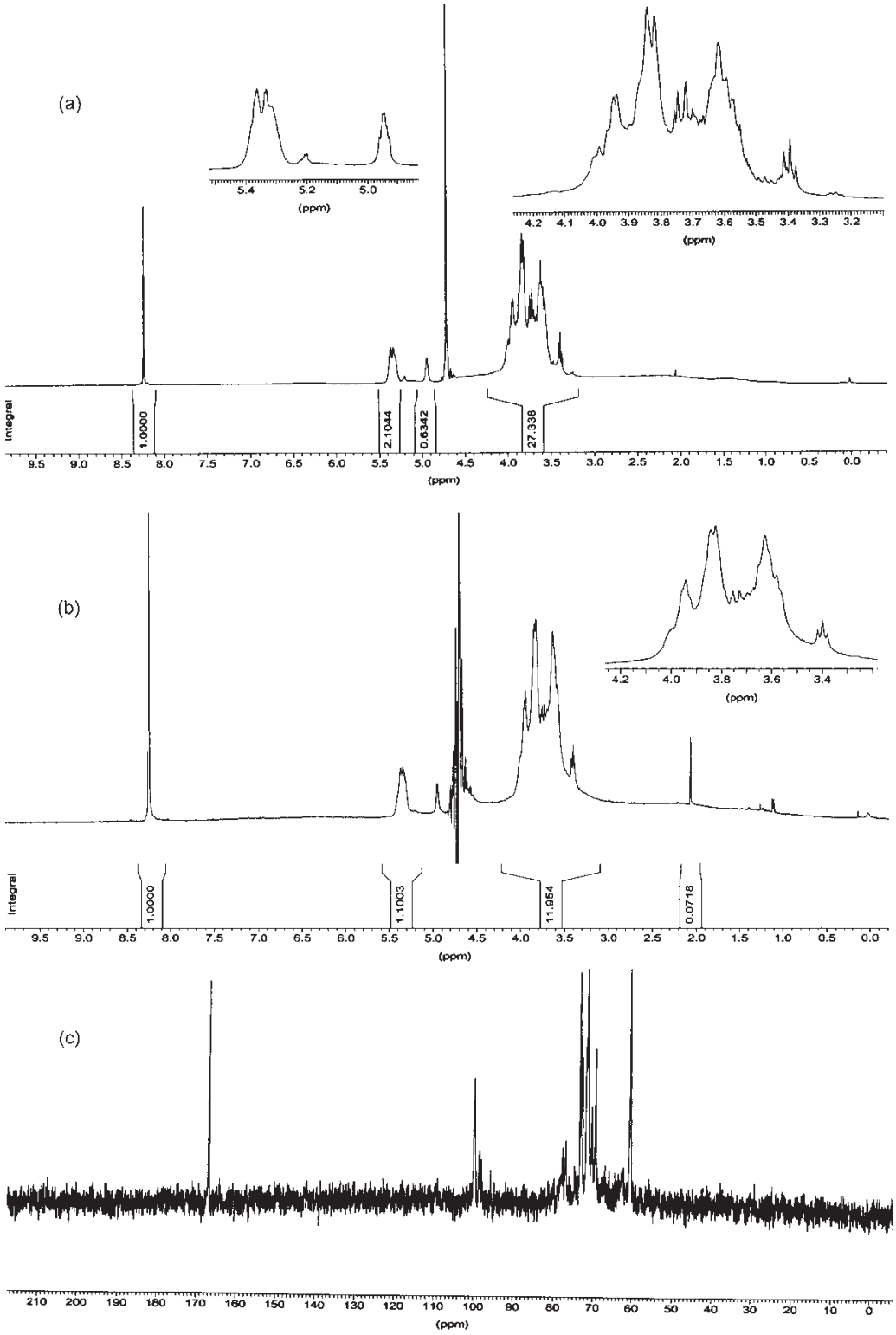


Figure 4. ^{1}H NMR spectra of CP1 (a) and CP2 (b), and ^{13}C NMR spectrum of CP1 (c).

The ESI-TOF-MS spectrum of CP1 (Figure 5) showed that most of the observed ions were doubly-charged (as shown in Figure 5, the signal was observed at 1001.3 with the peaks of the isotopic pattern at 0.5 Dalton distance), and the *m*/*z* range was from 200 to 2200, centered at 1000, indicating that the average molecular weight was about 2000. As shown in Table III, the observed ions can be assigned to several series. Interestingly, within each series, *m*/*z*s of the doubly-charged or the singly-charged ions consist-

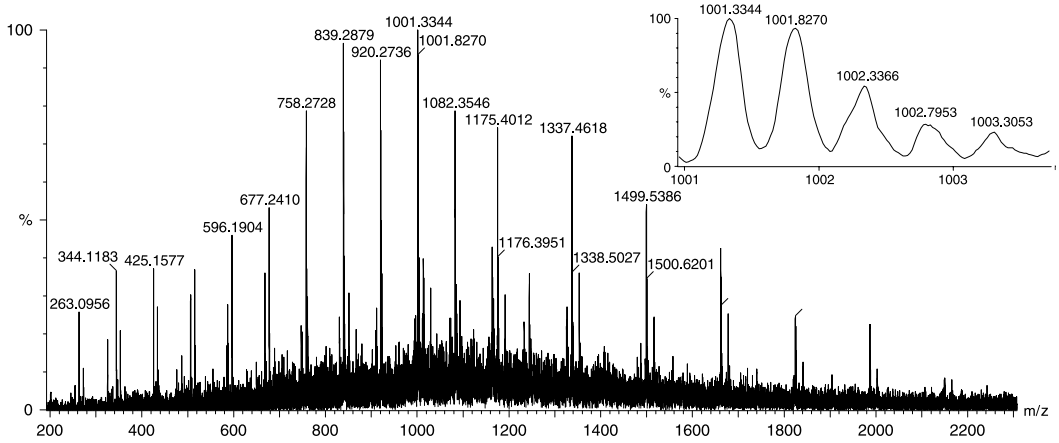


Figure 5. ESI-TOF-MS spectrum of CP1. The insert shows a typical doubly charged ion. The peaks observed at around 1001.3 show the isotopic pattern at 0.5 Dalton distance.

ently increase by 81 or 162, respectively. This moiety with a mass of 162 could be a hexose losing one molecule of water (180−18). These results were consistent with our NMR data, suggesting that CP1 is a sugar-containing fraction.

Using ESI-TOF-MS, we also investigated the effect of adding the $ABTS^{\cdot+}$ free radical on the structure of the colored components. We compared the mass spectra of the CP1 solution spiked with $ABTS^{\cdot+}$ stock solution with those without spiking. Interestingly, we found the intensities of doubly-charged ions, especially those in Series 2 (Table III), were markedly decreased, while the singly-charged ions have no or little change. These findings suggest that the sugars contribute little to the antioxidant activity, while possibly chromophore units are present, contributing to both the color and the antioxidant activity.

Discussion

Our data show that the ethyl acetate extract of DSS has the strongest antioxidant activity based on the ABTS assay, which detects a wide range of antioxidants [14,15]. Fractionation of that extract produced several components with antioxidant activity, but the most active was indentified as maltol. We also developed an HPLC method to determine the maltol level in DSS. Although maltol has been identified in many soy sauce products [18−20], to the authors' knowledge, this is the first study to

Table III. The observed ions in TOF-MS spectrum of CP1. Three series were observed. Within each series, *m/z*s of the doubly-charged or the singly-charged ions consistently increase by 81 or 162, respectively.

Series 1			Series 2*			Series 3		
Ions	Charge states	M.W. (Da)	Ions	Charge states	M.W. (Da)	Ions	Charge states	M.W (Da)
263.0956	2	528.2	353.1367	2	708.3	1191.4094	1	1192.4
344.1483	2	690.3	434.1462	2	870.3	1353.4515	1	1354.5
425.1577	2	852.3	515.1589	2	1032.3	1515.6155	1	1516.6
506.1745	2	1014.3	596.1904	2	1194.4	1677.6451	1	1678.6
587.2086	2	1176.4†	677.2410	2	1356.5	1839.7515	1	1840.8
						15		
668.2075	2	1338.4†	758.2728	2	1518.6			
749.2477	2	1500.5†	839.2879	2	1680.6			
1175.4012	1	1176.4†	920.2736	2	1842.5			
1337.4618	1	1338.5†	1001.3334	2	2004.7			
1499.5386	1	1500.5†	1082.3546	2	2166.7			
1661.6138	1	1662.6	1163.4160	2	2328.8			
1823.7094	1	1824.7	1244.4283	2	2490.9			
1985.7924	1	1986.8	1325.4880	2	2653.0			
24								
			1406.5306	2	2815.1			
			1487.5840	2	2977.2			

* Intensities of peaks which were markedly decreased after reaction of CP1 with $ABTS^{\cdot+}$; † Both singly-charged and doubly-charged ions were observed.

determine its concentration in soy sauce and its contribution to the TAA of DSS. Maltol can be formed from reducing sugars during food processing and has been identified in a wide variety of other heated materials such as bread crusts, coffee, cocoa beans, cereals, dried whey and chicory [21]. Therefore, the amount of daily maltol intake can be considerable. Maltol has even been suggested to be capable of exerting neuroprotective activity by inhibiting oxidative damage [22–24], and its iron chelate has been used to treat anaemia in humans [25]. At present the pharmacokinetics of maltol after oral dosing have only been investigated in dogs and indicate rapid and extensive absorbtion, followed by conjugation and rapid excretion [26]. However, the oral bioavailability of maltol in humans has not yet been examined.

Flavonoids, e.g. daidzein and genistein, have been identified in some soy sauce products and postulated to have antioxidant activities [27], but in our study, we found the levels in the DSS to be undetectable (data not shown). This is not perhaps surprising, since soy sauce is generally regarded as a poor source of isoflavones [28].

However, the major antioxidant activity of DSS appears to reside in high molecular weight colored components, some soluble in methanol and others not, containing carbohydrate residues. Indeed, depletion of coloured components by acidic hydrolysis from the colored fraction dramatically decreased antioxidant activity. These brown polymers are most probably melanoidins, formed by Maillard reactions between reducing sugars and compounds possessing a free amino group, such as free amino acids and the amino groups of peptides [29].

Although soy sauce is a traditional cooking ingredient throughout Asia, the actual process and the composition of the starting ingredients for making the soy sauce is known to differ between countries. It is highly likely that differences in the raw materials, fermentation time and heating processes used during the manufacture of soy sauce may affect the composition and antioxidant activity of the final products. Indeed, we have previously demonstrated a variation in the antioxidant capacity of different soy sauce products [14]. Conventional soy sauce making involves a solid-state aerobic fermentation process by *Aspergillus* species on a soybean and wheat mixture. *Aspergillus* produces extracellular enzymes, in particular proteases and amylases, which hydrolyze the proteins and polysaccharides of soybean and wheat. Subsequently, the sugars and amino acids produced are further digested during a brine fermentation process and, depending on the extent of fermentation process that takes place, the amount of Maillard reaction products can vary. Full details of commercial soy sauce manufacture tend to be carefully guarded by Asian food companies and the exact details of manufacture for the brand examined in this study is not known.

The antioxidant characteristics of Maillard related products from fermented soy sauce have been studied [30] and melanoidins have previously been isolated from soy sauce [31]. Melanoidins have also been characterized in many other foods, such as beer [32], bread [33] and roasted coffee [34,35]. Many studies have attempted to elucidate the structural characteristics of melanoidins [29,36], using a variety of starting materials. Ando et al. [10] investigated different soy sauce products, and found that the color of soy sauce was not proportional to its antioxidant capacity. In our study, even for one soy sauce product, the ratios of free radical scavenging ability to color density of two different fractions, CP1 and CP2, were different. This could be due to the variety of chemical structures of the colored components. In this study, NMR and MS were used to eludicate the structural characteristics of melanoidins. NMR and MS data of CP1 indicate the presence of carbohydrate residues, which agrees with the finding that sugar moieties could be part of the melanoidin backbone [37]. The carbonyl resonance and non-saturated proton signals of CP1 suggest that amino acids may be involved in the formation of the chromophore unit. Based on our ESI-TOF-MS data, the molecular weights of CP1 are in the range of 400–4400, which is comparable to soy sauce pigments fractionated by Motai et al. [38].

Maillard reaction products add the flavor and brown color to soy sauce [9]. One of these antioxidant flavor components, 4-hydroxy-2(or5)-ethyl-5(or2)-methyl-3(2H)-furanone (HEMF) was reported to inhibit benzo[a]pyrene-induced forestomach neoplasia in mice and decrease the hydrogen peroxide concentration in human polymorphonuclear leucocytes [12]. The colored components of soy sauce have also been linked to the antioxidant capacity of soy sauce [10,13]. For example, soy sauce melanoidin strongly inhibited $NO^{\cdot}$-induced DNA damage in a dose dependent manner, as determined by the comet assay [39]. In an *in vitro* study, melanoidin has been demonstrated to affect the growth of human colonic bacteria [40]. Melanoidins extracted from soy sauce have been suggested to inhibit colon cancer cell growth [41]. Our unpublished data also indicate that desalted DSS exibits some inhibitory effect on growth of the HT29 colon cancer cell line. We have found that DSS has a rapid antioxidant effect against lipid peroxidation *in vivo* [16]. As has been suggested for other nutritional antioxidants [2,42,43], melanoidins may play a role in the antioxidant defence of the gastrointestinal tract, since they are unlikely to be absorbed as such. Maltol may well be absorbed [25], however, and could conceivably exert systemic antioxidant effects.

Acknowledgements

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