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Black Sesame Pigment: DPPH Assay-Guided Purification, Antioxidant/Antinitrosating Properties, and Identification of a Degradative Structural Marker

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Supporting Information

ABSTRACT: An improved purification procedure leading to black sesame (*Sesamum Indicum* L.) pigment was developed involving fat removal by treatment of ground black sesame seeds with dichloromethane followed by an optimized hydrolytic protocol with 6 M HCl, at 100 °C, overnight. The black pigment thus obtained displayed good antioxidant efficiency by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay (82% reduction at 0.5 mg/mL), good ferric ion-reducing capacity (61 μM Trolox equivalent concentration at 0.5 mg/mL), and potent antinitrosating properties (74% inhibition of 2,3-diaminonaphthalene (DAN) nitrosation at gastric pH at 2.5 mg/mL). A synthetic pigment obtained by oxidative polymerization of coniferyl alcohol (polyconiferyl alcohol, PCA), the putative biosynthetic precursor to the sesame pigment, was characterized as a reference standard. FT IR spectra of the purified sesame pigment and PCA supported the structural similarity. HPLC analysis of degradation products by alkaline hydrogen peroxide of purified black sesame pigment showed the formation of vanillic acid (VA) as the main isolable fragment. Similar yields of VA were obtained by degradation of PCA. A positive correlation between VA yields and DPPH activity was determined in samples of different purities. It is suggested that VA is a structural marker of black sesame pigment, confirming the biosynthetic origin from coniferyl alcohol and pointing to the *o*-methoxyphenol motif as the key factor accounting for the potent antioxidant properties of the pigment.

KEYWORDS: *black sesame, antioxidant, antinitrosating, vanillic acid, coniferyl alcohol*

INTRODUCTION

Black sesame seed (*Sesamum indicum* L.), traditionally used in Chinese folk medicine and as food for humans in China and other East Asian countries, has attracted interest because of its potent antioxidant activity, superior to that of white sesame seed.[1] This activity is commonly attributed to a number of lipid-soluble lignan antioxidants, including primarily sesamin and sesamolin[2] but also sesaminol, sesamol, and pinoresinol,[3,4] which are found in sesame seeds, partly as glycosylated derivatives,[5,6] and have been shown to display health beneficial effects. For example, mouse lipid peroxidation was effectively decreased by black sesame seeds.[7] Ethanol extracts of black-coated sesame seed induced suppression of growth of cultured malignant cells.[8] Sesamol and sesaminol, in particular, have been identified as the main antioxidants believed to protect sesame seed oil from peroxidative deterioration.[4,9] Pinoresinol, sesaminol, sesamol, and sesamolin exhibited powerful inhibitory effects on lipid peroxidation of liposomes in rat liver and kidney.[10] Sesamol was shown to exert strong antimutagenic activity in the Ames tester strains TA100 and TA102.[11] Sesamin was found to decrease cholesterol levels in blood.[12] Dietary use of sesame seeds or sesame oil in healthy individuals was found to cause a significant increase in plasma levels of γ-tocopherol.[13,14] Sesame lignans were also found to possess neuroprotective, antihypertensive, and antiinflammatory properties.[15]

In contrast to liposoluble lignans, little attention has been directed so far to the structure and antioxidant properties of the insoluble pigment of black sesame seeds. Pursuit of this goal is yet prompted by the current burst of interest in the black components of various foods or drinks of plant origin, including black rice, tea, sunflower, black beans, and grapes. For most of these pigments structural formulations have been put forward; these were characterized as anthocyanins in the case of black rice, black beans, sunflower, and grapes, whereas catechin polymers were identified as the main components of black tea pigments.[16−19]

Several literature papers deal with purification protocols of black sesame pigment (BSP). They are based on the use of various solvents under different experimental conditions, such as aqueous NaOH followed by potassium borohydride;[20] 80% ethanol at 70 °C for 30 min;[21] 75% ethanol at room temperature for 24 h;[22] and supercritical carbon dioxide.[23] However, the structural characteristics of the purified pigment have remained largely unknown. Most of the current

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knowledge is based on a supposed biogenetic origin from coniferyl alcohol (Figure 1),[24] but how this phenolic precursor

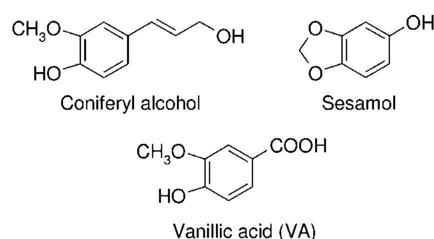

Figure 1. Chemical structures of coniferyl alcohol, sesamol, and vanillic acid (VA).

is converted to produce a black insoluble pigment is unknown. On this basis, the present study was designed with the dual scope of gaining for the first time a structural insight into BSP, following optimization of purification protocol, and of assessing the antioxidant properties of pigment as a function of the purification protocol and degree of purity. More specifically, the aims of the study include developing a convenient isolation and purification protocol of BSP allowing for efficient removal of fats and other impurities; setting up procedures for the preparation of reduced pigment samples (hereafter referred to as reduced black sesame pigment or rBSP) and of a synthetic reference polymer by oxidative polymerization of coniferyl alcohol (polyconiferyl alcohol or PCA); determining the antioxidant efficiency as well as the antinitrosating properties of purified BSP and rBSP by different assays; and performing chemical degradation experiments for the identification of structural markers.

■ MATERIALS AND METHODS

General Experimental Methods. Black sesame seeds (*S. indicum* L.) were provided by Belan Ziviltechniker GmbH (Wels, Austria). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, iron(III) chloride, 2,4,6-tris(2-pirydyl)-*s*-triazine, 2,3-diaminonaphthalene (DAN), horseradish peroxidase (EC 1.11.1.7), hydrogen peroxide (30% v/v), sodium metabisulfite, 4-hydroxy-3-methoxybenzoic acid (vanillic acid, VA), 3,4-dihydroxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), 3,4-methylenedioxybenozic acid (piperonylic acid), *p*-methoxycinnamic acid, and 3,4-methylenedioxyphenol (sesamol) were from Sigma-Aldrich (Milan, Italy). 4-Hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol) was from Acros Organics (Geel, Belgium).

UV−vis spectra were performed using a Beckman (Milan, Italy) DU 640 spectrophotometer. FT IR spectra were run on a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using the Smart ATR accessory on ZnSe crystals. HPLC analysis was performed with an Agilent (Santa Clara, CA, USA) instrument equipped with a UV−vis detector (G1314A). The chromatographic separation was performed on a Phenomenex (Castel Maggiore, Italy) Sphereclone ODS column (250 × 4.60 mm, 5 μm), at a flow rate of 0.8 mL/min. The mobile phase was a 0.2% formic acid (solvent A)/methanol (solvent B) gradient as follows: 2% B, 0−15 min; from 2 to 80% B, 15−45 min. The detection wavelength was 254 nm. LC-MS analysis was performed with an LC/MSD 1100 VL system (Agilent) equipped with a UV−vis detector (G1314A) and a quadrupole mass spectrometer with electrospray ionization (ESI) source (G1956A) operating in positive ionization mode in the following conditions: nebulizer pressure, 50 psi; drying gas (nitrogen), 10 L/min, 350 °C; capillary voltage, 4000 V; fragmentor voltage, 50 V. The chromatographic separation was performed on an Eclipse XDB-C18 (150 × 4.60 mm, 5 μm, Agilent) column, at a flow rate of 0.4 mL/min, using the same eluant system as above.

Removal of Fats from Crude Black Sesame Seeds. Sesame seeds (15 g) were finely ground in a mortar and stirred in 1 L of dichloromethane overnight, at room temperature. The solvent was removed by settling and the solid residue treated twice as before. The final residue was collected by filtration (5 g).

Harsh Acid Hydrolysis Purification Protocol. Defatted sesame seeds (500 mg) were treated with 35 mL of 6 M HCl under stirring, at 100 °C, overnight. After cooling at room temperature, the mixture was centrifuged (3000 rpm, 1248*g*, 15 min), and the precipitate was washed three times with water and then freeze-dried to give a black powder. In other experiments the reaction was run by varying (i) HCl concentration (0.5 or 3 M), (ii) temperature (25 or 60 °C), and (iii) reaction time (1 or 5 h). Yields of pigment are reported in Table S1 in the Supporting Information.

Ten milligrams of the pigment obtained by treatment with 6 M HCl, at 100 °C, overnight, was acetylated with 500 μL of acetic anhydride and 10 μL of pyridine.

Mild Acid Hydrolysis Purification Protocol. Defatted sesame seeds (3.5 g) were stirred in 200 mL of dioxane/1 M HCl solution (9:1 v/v) at 100 °C for 2 h. After cooling at room temperature, the mixture was filtered to remove small amounts of insoluble material. The solution was concentrated in a rotary evaporator until most of the organic solvent was removed and taken at 4 °C. After ca. 3 h, when a dark yellow precipitate was observed, the mixture was centrifuged (3000 rpm, 1248*g*, 15 min, 4 °C). The precipitate was washed with 0.01 M HCl and collected by freeze-drying to give a dark orange powder (40 mg).

Reductive Treatment. The pigment obtained by the harsh acid hydrolysis protocol (100 mg) was treated overnight under stirring with $NaBH_4$ (1 mg) in 7 mL of 0.1 M phosphate buffer (pH 7.4) at room temperature. The mixture was then acidified to pH 3 with 3 M HCl and centrifuged (3000 rpm, 1248*g*, 15 min). The precipitate was washed three times with water and freeze-dried to give a gray-brown powder (75 mg). In other experiments the same treatment was performed on the defatted sesame seeds.

Preparation of Reference Synthetic Pigments. A solution of coniferyl alcohol (300 mg) dissolved in the minimum quantity of methanol was added to 0.1 M phosphate buffer (pH 6.8) (400 mL) (4.2 mM final concentration of coniferyl alcohol). Horseradish peroxidase (10.8 units/mL final concentration) and hydrogen peroxide (9.8 mM final concentration) were then added in two portions at 1 h intervals, and the mixture was allowed to stand at room temperature under vigorous stirring. After 24 h, the mixture was acidified to pH 3 with 3 M HCl and washed with ethyl acetate (3 × 100 mL). The aqueous layer was centrifuged (7500 rpm, 7798*g*, 30 min) to give a light brown precipitate, which was washed with water and freeze-dried (146 mg). In other experiments the reaction was run as above starting from 100 mg of sesamol.

Ten milligrams of pigment from coniferyl alcohol was acetylated with 500 μL of acetic anhydride and 10 μL of pyridine.

DPPH Assay.[25] One milligram of pigment was added to 2 mL of a freshly prepared 0.2 mM solution of DPPH in methanol, and the mixture was vigorously stirred at room temperature. After 10 min, the absorbance at 515 nm was measured. Results were expressed as percentage of reduction of the initial DPPH radical absorption by the pigments. Experiments were run in triplicate.

Ferric Reducing/Antioxidant Power (FRAP) Assay.[26] One milligram of pigment was added to 2 mL of a solution of 1.7 mM $FeCl_3$ and 0.83 mM 2,4,6-tris(2-pirydyl)-*s*-triazine in 0.3 M acetate buffer (pH 3.6). The reaction mixture was vigorously stirred at room temperature. The reduction of Fe^{3+} to Fe^{2+} was monitored by measuring the absorbance at 593 nm after 10 min. Results were expressed as Trolox equivalents. Experiments were run in triplicate.

DAN Assay.[27] One milligram of pigment was added to 400 μL of a solution of DAN (0.2 mM) in 50 mM sodium acetate buffer (pH 4.0), followed by sodium nitrite (20 mM). After 30 min, 50 mM potassium phosphate buffer (pH 7.4, 3.6 mL) was added to stop the reaction, and the mixture was filtered on Chromafil PVDF (0.20 μm). Naphtho[2,3-*d*]triazole was quantified by measuring the fluorescence of each sample using an excitation wavelength of 375 nm and an emission wavelength

of 450 nm. Results were expressed as percentage of inhibition of DAN nitrosation by the pigments. Experiments were run in triplicate.

Chemical Degradation. The reaction was run as described.[28] Briefly, 5 mg of pigment was suspended in 1 M NaOH (1 mL) and treated with 1.5% H_2O_2 at room temperature and under vigorous stirring. After 24 h, the mixture was treated with 5% $Na_2S_2O_5$ (200 μL), taken to pH 3 with 6 M HCl, filtered on Chromafil PVDF (0.20 μm), and analyzed by HPLC.

RESULTS AND DISCUSSION

BSP Purification, Preparation of rBSP and PCA, and Evaluation of Antioxidant Activity. After several trials changing solvents and various experimental parameters, two hydrolytic procedures were eventually selected and optimized for the purification of BSP. All procedures involved an initial treatment of crude ground seeds with CH_2Cl_2 to remove fat components.

The first purification protocol (referred to as harsh acid hydrolysis protocol) involved hydrolysis of defatted pigment with 6 M HCl at 100 °C for 18 h to produce a black powder (20% yield w/w). The variations in pigment yields with changing HCl hydrolysis conditions are reported in Table S1 in the Supporting Information. Data indicated that varying the acid concentration (0.5−6 M) or time of hydrolysis (1−18 h) did not cause significant variations in the pigment yield, whereas temperature was the most influential parameter in this respect. In particular, a decrease in the yield from 34 to 20% was observed when the temperature was raised from 25 to 100 °C, suggesting more effective removal of colorless impurities. Interestingly, however, increasing acid concentration from 0.5 to 6 M resulted in an apparent darkening of pigment color, from gray to black. Close inspection of the material during acid hydrolysis even at low temperatures revealed that the overall blackening effect was actually due to removal of the light gray material with an associated increase of the proportions of the dark pigment rather than to turning of the light-colored material to a heavily pigmented one. On this basis it seems likely that the color changes are not the result of a browning of other components, for example, polysaccharides due to thermal treatments.

To assess the effects of purification on the antioxidant capacity, in subsequent experiments the variations of the properties of BSP with the purification conditions were determined using the DPPH assay. This assay evaluates the ability of the substance under examination to donate H atoms and quench the visible absorption at 515 nm of the nitrogen radical of DPPH.[25]

The data in Table 1 reveal marked differences in the hydrogen donor properties of the pigments purified by the various hydrolytic protocols examined.

Table 1. DPPH Reducing Properties of BSP Preparations (0.5 mg/mL) Obtained by Different Hydrolysis Protocols (n = 3, SD < 3%)

sample	[HCl] (M)	T (°C)	time (h)	reduced DPPH (%)
1	0.5	100	18	39
2	3	100	18	51
3	6	100	18	82
4	6	25	18	27
5	6	60	18	68
6	6	100	1	72
7	6	100	5	74

The highest activity (82%) was exhibited by sample 3 obtained under the harshest conditions (hereafter referred to as hBSP), whereas the pigment obtained in higher yields by HCl hydrolysis (sample 4) (see Table S1 in the Supporting Information) was far less active. Closer inspection of these data showed that acid concentration and temperature are most critical for high DPPH quenching activity (see Figure S1 in the Supporting Information). This would indicate that the increase of the DPPH reducing activity follows from either removal of impurites or liberation of redox active functionalities by hydrolytic processes.

The second protocol examined involved mild acid hydrolysis of defatted sesame seeds with an organic solvent. In particular, a mixture made of dioxane/1 M HCl (9:1 v/v) at 100 °C for 2 h[29] resulted in a significant percent mass loss, leading to a dark orange powder in about 1% yield w/w (sample referred to as mBSP).

A protocol for the preparation of rBSP was also developed, involving treatment of hBSP with $NaBH_4$ at pH 7.4 under stirring followed by acidification to give eventually a dark gray material.

Finally, a coniferyl alcohol polymer (PCA) serving as a reference synthetic pigment because of its expected biogenetic relationship with BSP and lignin-like polymers was prepared. To this aim, coniferyl alcohol was oxidized with horseradish peroxidase and hydrogen peroxide in 0.1 M phosphate buffer (pH 6.8) to give eventually a light brown precipitate.

Table 2 shows a comparison of DPPH reducing properties of BSP samples representative of the various purification and reduction protocols against the crude defatted pigment and PCA.

Table 2. DPPH Reducing Properties of BSP Samples (0.5 mg/mL) (n = 3, SD < 3%)

sample	reduced DPPH (%)
hBSP	82
rBSP	100
mBSP	34
defatted sesame seeds	0
reduced defatted sesame seeds	7
PCA	69

The hBSP sample showed better hydrogen donor properties than mBSP and higher than those of PCA. Complete DPPH reduction was observed with the rBSP sample, indicating a potentiating effect of the reductive treatment. Direct treatment of defatted sesame seeds with $NaBH_4$, as for preparation of rBSP, led to a material with very low hydrogen donor capacity (7% DPPH reduction), indicating that acid hydrolysis is critical to generate an antioxidant pigment. No reduction was observed in the case of the simply defatted sample, confirming that the observed antioxidant activity is due to the pigment and not to seed matrix constituents. Moreover, an acetylated sample of hBSP showed a very low DPPH reducing activity (13%), pointing to phenolic groups as major determinants of the antioxidant activity of the pigment. In line with this hypothesis, the hydrogen donor capacity of PCA was completely abated following the acetylation treatment.

In a related set of experiments the antioxidant capacity of the various pigment preparations was determined using the FRAP assay. This assay is based on the reaction of a Fe^{3+}−tripyridyltriazine complex with the antioxidant under acidic

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conditions to generate Fe^{2+}, giving an intense absorption maximum at 593 nm. The antioxidant effect (reducing ability) can thus be evaluated spectrophotometrically by monitoring the formation of the Fe^{2+}−tripyridyltriazine complex[26] and is reported as Trolox equivalent concentration.

The results in Table 3 are consistent with those of the DPPH assay, highlighting the good antioxidant (ferric reducing) properties of the hBSP sample. Also in this case, a significant improvement was observed after the reductive treatment.

Table 3. Fe^{3+} Reducing Properties of BSP Samples (0.5 mg/mL) (n = 3, SD < 3%)

sample	Trolox equivalent concentration (μM)
hBSP	61
rBSP	91
mBSP	9
defatted sesame seeds	14
reduced defatted sesame seeds	12
PCA	19

Antinitrosating Efficiency. Protection of the stomach against mutagenic and carcinogenic nitrosation processes and nitrosamine formation from ingested nitrite[30] is an important step in the prevention of tumors of the gastrointestinal tract. Accordingly, we were prompted to test the efficacy of BSP preparations in a model test of antinitrosating properties. The test is based on the inhibition of nitrosation of DAN and determination of the relative fluorescence at 450 nm of the naphtho[2,3-*d*]triazole formed upon reaction with nitrous acid, in the presence and absence of the inhibitor.[27]

Again, the best antinitrosating activity was associated with hBSP but, at variance with previous data, no effect of reduction in rBSP was observed (Table 4).

Table 4. DAN Nitrosation Inhibiting Activity of BSP Samples (2.5 mg/mL) (n = 3, SD < 3%)

sample	inhibition (%)
hBSP	74
rBSP	75
mBSP	33
defatted sesame seeds	18
reduced defatted sesame seeds	17
PCA	47

Structural Studies. In a subsequent series of experiments, we sought insight into the main structural properties of BSP and the key functionalities underlying the antioxidant and antinitrosating properties. Due to the difficulty of applying common spectral techniques to the sesame pigment because of its unfavorable solubility properties, preliminary FT IR analysis was carried out on the solid pigments in the ATR mode in comparison with reference PCA. The FT IR spectra showed very similar features (Supporting Information, Figure S2) indicating strict analogy of functional groups: in addition to a broad peak at around 3400 cm^{-1}, due likely to OH groups, intense signals were present in the 1700−1500 cm^{-1} range due to the aromatic moieties and at around 1300−1100 cm^{-1}, indicating C−O bonds. To get additional structural information we resorted to investigating the potential of an oxidative degradation approach commonly used for the qualitative and quantitative determination of melanin pigments. This approach was motivated by the close analogy of BSP and eumelanin pigments, sharing insolubility in all solvents, high chemical heterogeneity, and the presence of impurities from the biological matrix affecting interpretation of data. Accordingly, BSP suspensions were treated with alkaline hydrogen peroxide, and the degradation products were analyzed by HPLC.[28]

Figure 2 shows the elutograms of the mixtures obtained by degradation of the BSP preparations.

All samples gave similar well-defined HPLC profiles, although with variable peak distributions. LC-MS analysis with ESI+ detection indicated for product I eluted under the peak at ca. 32 min a pseudomolecular ion peak at m/z 169. The product was thus identified as 4-hydroxy-3-methoxybenzoic acid (vanillic acid, VA) also on the basis of comparison with an authentic sample. It is interesting to note that VA had been previously identified in methanolic extracts of defatted sesame meal,[31] indicating that this product may be a precursor to sesame pigment or may result from oxidative fission of units taking part in the pigment scaffold enhanced by the far-infrared irradiation treatment. As to the other components of the degradation mixture, it was not possible on the basis of only LC-MS analysis to make a structural formulation, nor was it secured that these components derived from degradation of the polyphenolic components of sesame as these prevailed over VA under milder HCl hydrolysis conditions.

The formation yields of VA from various pigment samples are reported in Table 5.

A plot of formation yields of VA against DPPH reducing properties of the various BSP samples indicated a positive correlation (Figure 3), suggesting that VA may be a useful index of the relative antioxidant capacity of BSP preparations, likely reflecting the amount of phenolic groups responsible of the antioxidant activity in the different BSP samples. A significantly high correlation coefficient of 0.81 is calculated for this plot.

No detectable VA was found in methanol extracts of black sesame seeds, thus ruling out its occurrence among low molecular weight constituents of black sesame seeds and supporting its origin by degradation of specific structural units of BSP.

The identification of VA as the main detectable degradation product of BSP provided additional support for the proposed biosynthesis of the pigment from coniferyl alcohol. To corroborate this conclusion, the synthetic polymer PCA was subjected to oxidative degradation under the same conditions as BSP and gave, as expected, VA as main detectable product (330 μg/mg of pigment) (Figure 4).

On the basis of this result, it could be argued that coniferyl alcohol-derived units account for some 40% of hBSP. Because VA could be obtained also by direct oxidative degradation of coniferyl alcohol (754 μg/mg) under argon atmosphere, it is not possible at present to draw any conclusion regarding the precise nature of the VA-forming units or the degree of polymerization of coniferyl alcohol in BSP.

Because sesame seeds are rich in sesamol and related lignans, in subsequent experiments a synthetic polymer was prepared by oxidation of sesamol under the same conditions adopted for PCA synthesis. The dark precipitate thus obtained (9% yield) was degraded to give an elution profile (see Figure S3 in Supporting Information) profoundly different from that of hBSP. It can thus be concluded that sesamol is not a main biogenetic precursor to BSP. As expected, no VA was formed.

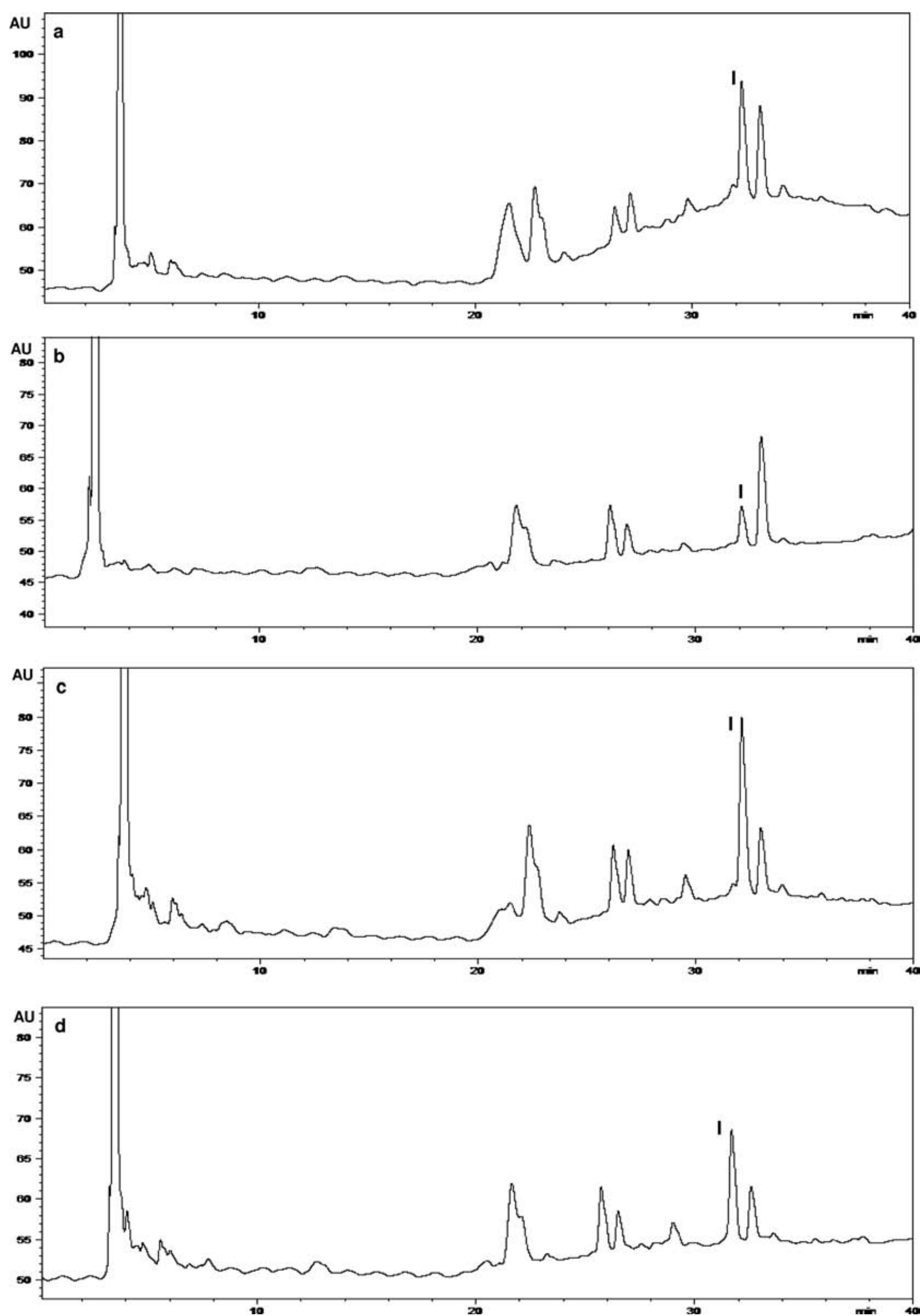

Figure 2. HPLC elution profiles of the mixtures produced by oxidative degradation of BSP samples purified as in Table 1: (a) sample 3 (6 M HCl, 100 °C, 18 h); (b) sample 1 (0.5 M HCl, 100 °C, 18 h); (c) sample 6 (6 M HCl, 100 °C, 1 h); (d) sample 4 (6 M HCl, 25 °C, 18 h).

Inspection of the hBSP degradation profiles for the presence of isovanillic acid, piperonylic acid, 3,4-dihydroxybenzoic acid, and 4-methoxycinnamic acid using authentic standards allowed the presence of aromatic units bearing 3-hydroxy-4-methoxy, methylenedioxy, dihydroxy, or *p*-methoxy functionalities to be ruled out.

In conclusion, the results described herein provide for the first time an insight into some key structural characteristics of the insoluble pigment of black sesame seeds. The main outcomes can be summarized as follows.

(1) An efficient isolation and purification protocol for BSP has been set out, allowing for removal of fats and other impurities. The reported hydrolytic procedure is experimentally

Table 5. Formation Yields of VA by Alkaline Degradation of BSP Preparations as a Function of Hydrolysis Conditions as in Table 1

sample	VA (μg/mg of pigment)
1	30
2	104
3	126
4	66
5	162
6	130
7	144

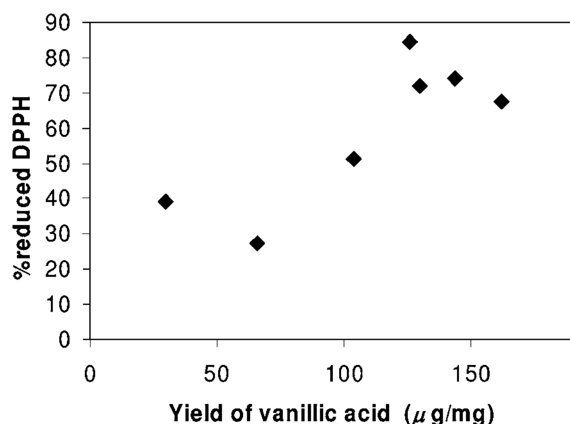

Figure 3. Plot of VA formation yields against DPPH reduction (%) for BSP samples 1−6 as in Tables 1 and 5 (r = 0.81).

simple and leads to a pigment (hBSP) with a low impurity content (based on pigment yield) and superior antioxidant and antinitrosating properties.

(2) A reduced pigment sample (rBSP) and a synthetic reference polymer (PCA) have been prepared. The reductive procedure is simple, cheap, and leads to a pigment with a significantly improved antioxidant behavior. Development of a reference synthetic model of the sesame pigment from a natural substrate under biomimetic conditions may also be useful to guide the design of bioinspired polymers for potential application as antioxidants. The structural similarity of PCA to the black sesame pigment was also supported by FT IR analysis.

(3) The antioxidant efficiency of purified BSP was determined by two different and complementary assays, the DPPH and FRAP tests. hBSP exhibited highly efficient antioxidant activity at 0.5 mg/mL in both tests. Most interestingly, evidence was obtained for the first time that hBSP is also an effective inhibitor of nitrosation reactions under gastric-like conditions, a property that may prompt evaluation of the pigment as a supplement in chemopreventive dietary regimens for nitrite- and nitrosamine-induced carcinogenesis in the gastrointestinal tract.

(4) Key structural moieties of BSP have been identified as 4-hydroxy-3-methoxyphenyl motifs, and their levels in different pigment preparations have been found to positively correlate with antioxidant properties. Chemical degradation experiments indicated that such moieties can derive biogenetically from coniferyl alcohol units and may account for almost half of the pigment at the maximum degree of purity examined. Besides the coniferyl alcohol derived units, other structural components may help to determine the overall antioxidant properties of BSP. This conclusion is based on the observation that the antioxidant and antinitrosating capacities of the model pigment PCA are lower than those of hBSP.

Overall, these findings provide novel methodological guidelines and practical analytical tools to orient the development of BSP purification protocols for biological evaluation purposes and may stimulate further research aimed at assessing the health beneficial effects of black sesame seeds.

■ ASSOCIATED CONTENT

Supporting Information

Yields of BSP by different acid hydrolysis treatments on defatted seeds; correlation between DDPH reducing activity of BSP samples and hydrolysis conditions; FT IR spectra of PCA and hBSP; HPLC elution profile of the mixture obtained by oxidative degradation of polymer from sesamol. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

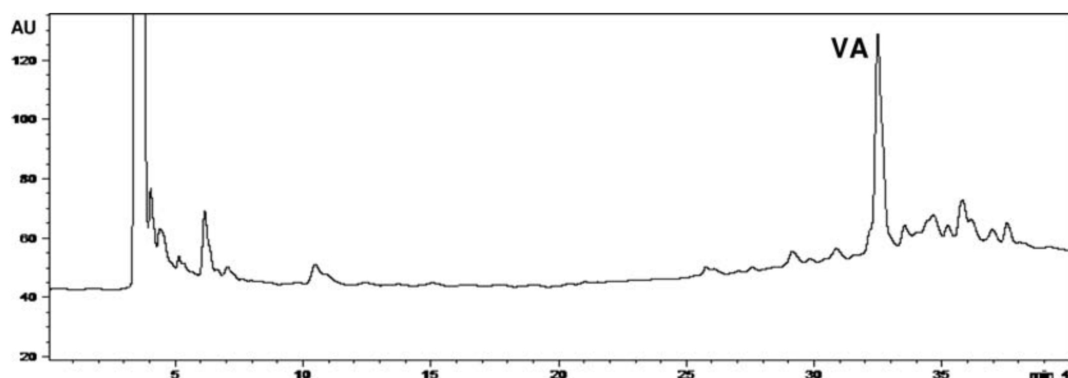

Figure 4. HPLC elution profile of the mixture obtained by oxidative degradation of PCA.

■ ABBREVIATIONS USED

DPPH, 2,2-diphenyl-1-picrylhydrazyl; DAN, 2,3-diaminonaphthalene; PCA, polyconiferyl alcohol; VA, vanillic acid; BSP, black sesame pigment; rBSP, reduced black sesame pigment; ESI, electrospray ionization; FRAP, ferric reducing/antioxidant power; hBSP, black sesame pigment obtained by harsh hydrolysis; mBSP, black sesame pigment obtained by mild hydrolysis.

■ REFERENCES

(1) Shahidi, F.; Liyana-Pathirana, C. M. Antioxidant activity of sesame fractions. In *Phenolic Compounds in Foods and Natural Health Products*; Shahidi, F., Ho, C. T., Eds.; ACS Symposium Series 909; American Chemical Society: Washington, DC, 2005; Chapter 4, pp 33−45.

(2) Namiki, M. The chemistry and physiological functions of sesame. *Food Rev. Int.* **1985**, *11*, 281−329.

(3) Suja, K. P.; Jayalekshmy, A.; Arumughan, C. Free radical scavenging behavior of antioxidant compounds of sesame (*Sesame indicum* L.) in DPPH$^{\bullet}$ system. *J. Agric. Food Chem.* **2004**, *52*, 912−915.

(4) Kuo, P. C.; Lin, M. C.; Chen, G. F.; Yiu, T. J.; Tzen, J. T. C. Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans. *J. Agric. Food Chem.* **2011**, *59*, 3214−3219.

(5) Katsuzaki, H.; Kawakishi, S.; Osawa, T. Sesaminol glucosides in sesame seeds. *Phytochemistry* **1994**, *35*, 773−776.

(6) Ryu, S. N.; Ho, C. T.; Osawa, T. High performance liquid chromatographic determination of antioxidant lignan glycosides in some varieties of sesame. *J. Food Lipids* **1998**, *5*, 17−28.

(7) Ma, Y. H.; Wang, Y.; Wang, H. H. Effects of sesame and oat on blood-lipid and lipid peroxidation in growing mice. *J. Chin. School Health* **1998**, *19*, 164−166.

(8) Kamei, H.; Koide, T.; Kojima, T.; Hasegawa, M.; Umeda, T. Suppression of growth of cultured malignant cells by allomelanins, plant-produced melanins. *Cancer Biother. Radiopharm.* **1997**, *12*, 47−49.

(9) Shyu, Y. S.; Hwang, L. S. Antioxidative activity of the crude extract of lignan glycosides from unroasted Burma black sesame meal. *Food Res. Int.* **2002**, *35*, 357−365.

(10) Uchida, M.; Nakajin, S.; Toyoshima, S.; Shinoda, M. Antioxidant effect of sesamol and related compounds on lipid peroxidation. *Biol. Pharmacol. Bull.* **1996**, *19*, 623−626.

(11) Kaur, P. I.; Saini, A. Sesamol exhibits antimutagenic activity against oxygen species mediated mutagenicity. *Mutat. Res.* **2000**, *470*, 71−76.

(12) Hirata, F.; Fujita, K.; Ishikura, Y.; Hosoda, K.; Ishikawa, T.; Nakamura, H. Hypocholesterolemic effect of sesame lignan in humans. *Atherosclerosis* **1996**, *122*, 135−136.

(13) Lemcke-Norojärvi, M.; Kamal-Eldin, A.; Appelqvist, L.-Å.; Dimberg, L. H.; Öhrvall, M.; Vessby, B. Corn and sesame oils increase serum gamma-tocopherol concentrations in healthy Swedish women. *J. Nutr.* **2001**, *131*, 1195−1201.

(14) Cooney, R. V.; Custer, L. J.; Okinaka, L.; Franke, A. A. Effects of dietary sesame seeds on plasma tocopherol levels. *Nutr. Cancer* **2001**, *39*, 66−71.

(15) Park, S. H.; Ryu, S. N.; Bu, Y.; Kim, H.; Simon, J. E.; Kim, K. S. Antioxidant components as potential neuroprotective agents in sesame (*Sesamum indicum* L.). *Food Rev. Int.* **2010**, *26*, 103−121.

(16) Kim, M. K.; Kim, H.; Koh, K.; Kim, H. S.; Lee, Y. S.; Kim, Y. H. Identification and quantification of anthocyanin pigments in colored rice. *Nutr. Res. Pract.* **2008**, *2*, 46−49.

(17) Kuhnert, N. Unraveling the structure of the black tea thearubigins. *Arch. Biochem. Biophys.* **2010**, *501*, 37−51.

(18) Tsuda, T.; Osawa, T.; Ohshima, K.; Kawakishi, S. Antioxidative pigments isolated from the seeds of *Phaseolus vulgaris* L. *J. Agric. Food Chem.* **1994**, *42*, 248−251.

(19) He, F.; Liang, N. N.; Mu, L.; Pan, Q. H.; Wang, J.; Reeves, M. J.; Duan, C. Q. Anthocyanins and their variation in red wines II. Anthocyanin derived pigments and their color evolution. *Molecules* **2012**, *17*, 1483−1519.

(20) Eidenberger, T. Sesame seed derived pigments. PCT Int. Appl. WO 2010012751, 2010.

(21) Shahidi, F.; Liyana-Pathirana, C. M.; Wall, D. S. Antioxidant activity of white and black sesame seeds and their hull fractions. *Food Chem.* **2006**, *99*, 478−483.

(22) Xu, J.; Chen, S.; Hu, Q. Antioxidant activity of brown pigment and extracts from black sesame seed (*Sesamum indicum* L.). *Food Chem.* **2005**, *91*, 79−83.

(23) Hu, Q.; Xu, J.; Chen, S.; Yang, F. Antioxidant activity of extracts of black sesame seed (*Sesamum indicum* L.) by supercritical carbon dioxide extraction. *J. Agric. Food Chem.* **2004**, *52*, 943−947.

(24) Kato, M. J.; Chu, A.; Davin, L. B.; Lewis, N. G. Biosynthesis of antioxidant lignans in *Sesamum indicum* seeds. *Phytochemistry* **1998**, *47*, 583−591.

(25) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.

(26) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(27) Kono, Y.; Shibata, H.; Kodama, Y.; Sawa, Y. The suppression of the N-nitrosating reaction by chlorogenic acid. *Biochem. J.* **1995**, *3*, 947−953.

(28) Panzella, L.; Manini, P.; Monfrecola, G.; d'Ischia, M.; Napolitano, A. An easy-to-run method for routine analysis of eumelanin and pheomelanin in pigmented tissues. *Pigment Cell Res.* **2007**, *20*, 128−133.

(29) Salanti, A.; Zoia, M.; Orlandi, L.; Zanini, F.; Elegir, G. Structural characterization and antioxidant activity evaluation of lignins from rice husk. *J. Agric. Food Chem.* **2010**, *58*, 10049−10055.

(30) d'Ischia, M.; Napolitano, A.; Manini, P.; Panzella, L. Secondary targets of nitrite-derived reactive nitrogen species: nitrosation/nitration pathways, antioxidant defense mechanisms and toxicological implications. *Chem. Res. Toxicol.* **2011**, *24*, 2071−2092.

(31) Lee, S. C.; Jeong, S. M.; Kim, S. Y.; Nam, K. C.; Ahn, D. U. Effect of far-infrared irradiation on the antioxidant activity of defatted sesame meal extracts. *J. Agric. Food Chem.* **2005**, *53*, 1495−1498.