Characterization of a Natural Antioxidant from Shrimp Shell Waste

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The chemical structure of an antioxidant purified from shrimp shell was analyzed. Positive reactions with both Folin−Ciocalteu's phenol reagent and ferric chloride−potassium ferricyanide reagent indicated the antioxidant was a phenolic compound. The presence of a primary amino group was indicated by reaction with ninhydrin. The base peak of the CI mass spectrum, at 165, and the presence of a peak in the EI spectrum at 164 suggested a MW of 164 for the compound. A base peak of 94 in the EI spectrum suggested the presence of a phenolic ring. The proton NMR spectrum was consistent with an ortho-disubstituted benzene. FT-IR indicated hydrogen bonded O−H (3400 cm^{-1}) and N−H (3000 cm^{-1}) stretching bands, and conjugated C=C stretching or N−H bending (1650 cm^{-1}). UV absorption maxima were at 268 and 323 nm with ϵ_{max} of 10 492 and 1246, respectively. The antioxidant was proposed to be 1,2-diamino-1-(*o*-hydroxyphenyl)propene.

Keywords: *Antioxidant; shrimp shell; shell waste; o-phenolic; lipid oxidation*

INTRODUCTION

Natural antioxidants have received considerable interest from the food industry because of the concern over the safety of synthetic antioxidants (Braner, 1975; Colbert and Decker, 1991; Haschek and Witschi, 1979; Ito et al., 1986). Tocopherols are the most widely used natural antioxidants found in plant tissue as a blend of α, β, δ, and γ homologues (Becker, 1993). Although tocopherols have been widely used in food as safe antioxidants, the disadvantages of tocopherols are high manufacturing costs and low effectiveness in some food products such as cereals and citrus oils (Osawa and Namiki, 1981, 1985; Becker, 1993). Thus, these situations led to the search for new natural antioxidants from various sources.

Many spices have been extensively studied for their natural antioxidant characteristics. Chang et al. (1977) described a patented procedure to extract antioxidants from rosemary and sage. A number of antioxidant components from *Rosemarinus officianalis* L. have been isolated, such as carnosol, rosmanol, rosmariquinone, and rosmaridiphenol (Inatani et al., 1982; Wu et al., 1982; Houlihan et al., 1985). Natural rosemary antioxidant is currently being marketed (Kanner et al., 1994). Several antioxidants from sage (*Salvia officinalis*) have also been identified (Cuvelier et al., 1994). Various antioxidants have also been found in other sources such as young green barley leaves, tanshen (*Salvia miltiorrhiza* Bung), *Polygonum hydropiper* leaves, oregano (*Origanum vulgare* L.), *Eucalyptus* leaf waxes, and red turnip (*Brassica campestris* L.). The majority of these natural antioxidants possessed phenolic rings (Osawa and Namiki, 1985; Zhang et al., 1990; Ishikawa et al., 1991; Haraguchi et al., 1992; Osawa et al., 1992; Kikuzaki and Nakatani, 1993).

The disposal or utilization of seafood processing wastes is a critical issue for the seafood industry. Shrimp discard represents more than 30 million pounds of waste material per year on the Pacific coast of the United States. The bulk of waste materials is underutilized and represents potential resources for bioactive materials. The isolation and characterization of a natural antioxidant from shrimp shell could represent a viable alternative to current shrimp waste utilization on the west coast. Pasquel and Babbitt (1991) showed that an ethanol extract from shrimp meat had considerable antioxidant activity. We have previously reported that a polar compound was responsible for antioxidant activity in shrimp shell (Li et al., 1994). The objective of this study was to characterize the natural antioxidant in shrimp shell.

MATERIALS AND METHODS

Materials. Shrimp shell, from the species *Pandalus jordani*, was obtained from an Oregon seafood processor during the shrimp fishing season (April through October). Samples were kept on ice and transported to the Oregon State University Seafood Laboratory (Astoria, OR). Two hundred gram samples were vacuum packed, blast frozen, and stored at −20 °C. Frozen samples to be analyzed were thawed at refrigerated temperatures. Crystalline β-carotene, linoleic acid, salicylamide, and Folin−Ciocalteu's phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO).

Purification of the Antioxidant. One kilogram of shrimp shell waste was extracted with 2 L of 95% ethanol. Mixtures were blended until homogeneous using a Waring blender. The slurry was filtered through a Büchner funnel using Whatman No. 1 filter paper. The filtrate was evaporated by a rotary evaporator and redissolved into 50 mL of ethanol solution. The crude extract was applied to preparative TLC plates (Kiesel 60 F_{254}, 1000 μm thickness, EM Science, Gibbstown, NJ) developed by a mobile phase of benzene−methanol−acetic acid (45:8:4). Phenolic compounds were visualized by spraying plates with reagents used to detect phenolic compounds: (1) Folin−Ciocalteu's phenol reagent followed by an overspray of 14% sodium carbonate and (2) ferric chloride−potassium ferricyanide reagent (1:1 v/v). Antioxidant activity on plates was detected by inhibition of yellow color formation after spraying with an aqueous emulsion of β-carotene−linoleic acid (see below for method of preparation) and exposing to normal room light. Plates were sprayed with 0.1% ninhydrin in 2-propanol reagent to detect the presence of amino groups. A band at R_f 0.05 that tested positive for both phenol and amino groups and showed antioxidant activity was scraped off the

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TLC plates and further purified by high-performance liquid chromatography (HPLC).

HPLC was carried out on a Bio-Rad 2700 gradient pump system equipped with a Bio-Rad Model 1706 UV detector (Bio-Rad, Hercules, CA). A preparative column packed with R Sil C_{18} HL (250 × 10 mm i.d., 5 μm particle size) was used. Samples of TLC fractions (R_f 0.05) were injected onto HPLC. The eluants used were water and acetonitrile with a gradient of 0−20% acetonitrile for 15 min at a flow rate of 2.50 mL/min. Absorbance at 280 nm was monitored. Peak fractions were collected and measured for antioxidant activity. Antioxidant activity of fractions was evaluated in a β-carotene−linoleic acid emulsion as described in a later section. The most active fractions (retention time of 8.4 min) were collected. Fractions from several runs were pooled together and freeze-dried for subsequent structural analysis. The fraction was also applied to analytical TLC plates (Kiesel 60F_{254}, 250 μm thickness, EM Science) developed with benzene−methanol−acetic acid (45:8:4 v/v). When plates were sprayed for detection of phenol groups, a single band at R_f 0.05 was visible. Purity of the sample was also confirmed by TLC developed with butanol−water−acetic acid (12:5:3 v/v) as described previously (Li et al., 1994).

Measurement of Antioxidant Activity. Antioxidant activity was determined according to the method of Marco (1968). Crystalline β-carotene was dissolved in 10 mL of chloroform. One milliliter of this solution was then pipetted into a round-bottom flask that contained 20 mg of purified linoleic acid and 200 mg of Tween 40. After evaporation of chloroform on a rotary evaporator, 50 mL of ddH_2O was added to the flask with vigorous stirring. A 5 mL aliquot of the aqueous emulsion was then pipetted into a series of spectrophotometer tubes that contained 0.2 mL aliquots of various antioxidant fractions. After an initial reading was taken at 470 nm, the tubes were stoppered and placed in a water bath at 50 °C. Tubes were read at 470 nm at regular intervals. The ability of the antioxidant to prevent the oxidation of the β-carotene−linoleic acid emulsion was expressed as the decrease in absorbance at 470 nm.

Mass Spectrometry. Electron impact (EI) and positive chemical ionization (CI) were operated with an HP 5985 mass spectrometer. Samples were introduced into the instruments via direct insertion. The source was maintained at 200 °C, and the electron energy was 70 eV. Positive CI spectra were taken with methane as the reagent gas. The source temperature was 150 °C, and the source pressure was 2×10^{-4} Torr.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR spectra was recorded with a Bruker AM 400-MHz spectrometer. Samples were dissolved in D_2O into a semimicrotube with a cylindrical cavity. Water peak (δ 4.8 ppm) was used as reference.

Infrared Spectroscopy (IR). An IR spectrum was obtained using an IR Nicolet 510P apparatus with Fourier transform (FT-IR). Dry samples were mixed with heavy mineral oil (Nujol), and the resulting mull was examined as a film between flat salt plates.

UV Spectrophotometry. Purified antioxidant in acetonitrile−water (10:90) was scanned in the UV range (200−380 nm) using a Beckman Model DU 640 spectrophotometer (Beckman, Fullerton, CA).

Quantitative Measurement of the Antioxidant. The concentrations of purified antioxidant samples from HPLC were estimated by using a structurally similar compound, salicylamide, as a standard. The phenolic content was determined spectrophotometrically with Folin−Ciocalteu's phenol reagent. Samples (1 mL) were mixed with 1 mL of phenol reagent and 3 mL of 14% Na_2CO_3 solution. Absorbance was determined at 660 nm after 15 min with a Beckman DU-600 spectrophotometer.

RESULTS AND DISCUSSION

Purification of the active antioxidant fraction was accomplished with TLC and HPLC. Positive reactions, as shown by blue color formation with Folin−Ciocalteu's phenol reagent and deep blue color formation with ferric chloride−potassium ferricyanide reagent, indicated a phenolic compound was responsible for antioxidant activity in shrimp shell. The antioxidant also showed a purple color after spraying with ninhydrin, indicating the presence of an amino group. The yield of the antioxidant was estimated to be 0.18 mg/100 g of shrimp shell, by Folin−Ciocalteu's phenol reagent using salicylamide as a standard.

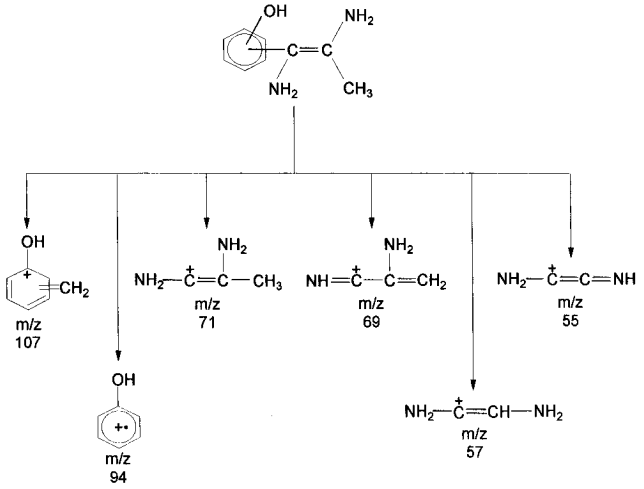

Figure 1. Proposed mass fragmentation pattern of the antioxidant compound isolated from shrimp shell waste.

Table 1. Mass Spectrometric Data of the Antioxidant Compound Isolated from Shrimp Waste

MS method	*m/z* (rel abundance)
EI	164 (M^+, 21), 107 (18), 96 (15), 95 (55), 94 (100), 83 (14), 80 (27), 79 (19), 71 (16), 69 (31), 57 (50), 56 (22), 55 (48)
CI	165 (M^+ + H, 100), 108 (17), 104 (17), 94 (21), 84 (39), 80 (19), 69 (22), 61 (70), 58 (88), 55 (36)

The molecular weight of the purified antioxidant was determined from electron impact (EI) and chemical ionization (CI) mass spectra. In the EI mass spectra of the purified antioxidant, a peak at *m/e* 164 was detected with a relative abundance of 20.9% (Table 1), which is possibly the molecular ion. The location of the base peak of the positive CI spectra at 165 further supports a molecular weight of 164 (Table 1). Since the compound is ninhydrin-positive and the MW is an even number, it will contain an even number of nitrogens, in accordance with the nitrogen rule. However, it is unlikely that a phenolic compound of molecular mass 164 would contain 4 or more nitrogens or more than 10 carbons. The most probable molecular formulas are $C_9H_{12}N_2O$ and $C_8H_8N_2O_2$.

The major peaks of both EI and CI spectra are listed in Table 1. The fragment ion, *m/e* 94, is a strong base peak supporting the presence of a phenolic compound from loss of side chain with rearrangement. The fragment ion at *m/e* 107 could be generated from cleavage of the side chain at the bond beta to the ring. The fragment ions of *m/e* 71 and 69 are possibly side chain fragments with formulas $C_3H_7N_2$ and $C_3H_5N_2$, respectively. We also speculate that fragment ions *m/e* 57 and 55 were formed by loss of $-CH_2$ from the side chain. Proposed fragment ion structures are shown in Figure 1.

Strong and moderate infrared absorptions were observed. A broad peak at 3500−2900 cm^{-1} probably is the result of superimposed OH, CH, and NH stretching bands. Broad peaks centered at 3400 and 3050 cm^{-1}

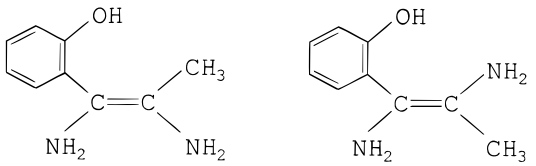

Figure 2. Proposed structures of the antioxidant compound from shrimp shell waste.

are consistent with the presence of hydrogen-bonded phenolic hydroxyl and amino groups, respectively. A strong, sharp absorption peak at 1650 cm^{-1} could indicate C=C stretching of a conjugated system, although N−H bending is another possibility. An absorption band at 1370−1380 cm^{-1} probably indicates CH_3 bending.

In the ^1H NMR spectrum, four single proton peaks in the far downfield range, at δ 8.75 (1H, d, $J = 4.6$ cps), 8.58 (1H, t, $J = 6.3$ cps), 8.10 (1H, t, $J = 6.7$ cps), and 7.95 (1H, d, $J = 4.1$ cps), are strong evidence for a disubstituted benzene. Furthermore, the pattern of peak splitting (two doublets, two triplets) indicates ortho substitution. A three-proton absorption peak at δ 4.4 appears to be an isolated $-CH_3$, although the chemical shift is more downfield than expected. Any NH_2 and OH absorption would be eliminated due to solvent exchange with D_2O. A peak at δ 3.3 is believed to be a methanol solvent peak. Since seven protons were detected by ^1H NMR, and the compound was ninhydrin-positive, the molecular formula must contain at least nine protons if a primary amine is present, thus ruling out the formula $C_8H_8N_2O_2$. The formula $C_9H_{12}N_2O$ fits well with these data, with five extra protons being accounted for by the presence of two NH_2 groups and one OH group.

The UV spectrum of the antioxidant showed a major absorption peak at 268 nm and a minor peak at 323 nm. Molar extinction coefficients were estimated to be 10 492 and 1246, respectively. The intense K-band at 268 nm is indicative of a chromophore conjugated with the ring. B-band absorption at 323 nm also suggests a chromophore conjugated with an aromatic ring.

On the basis of analysis of the data, a reasonable molecular formula and structure are proposed. The data are most consistent with a molecular formula of $C_9H_{12}N_2O$. The data were not sufficient to assign the position of the amino groups unambiguously. The structure of the compound is therefore proposed to be either *cis*- or *trans*-1,2-diamino-1-(*o*-hydroxyphenyl)-propene (Figure 2).

Pasquel and Babbitt (1991) reported that a natural antioxidant was present in shrimp meat. Although they were not able to purify and identify the compound, they postulated the active compound was a polyhydroxylated derivative of an aromatic amino acid. The compound we have isolated appears to possess chromatographic characteristics similar to those of the antioxidant from shrimp meat, but differs in being monohydroxylated.

Most synthetic and natural antioxidants have phenolic hydroxyl groups in their structures (Osawa and Namiki, 1981; Houlihan et al., 1985; Zhang et al., 1990; Wanasundara et al., 1994). It has been reported that antioxidant activity of monophenols is increased substantially by alkyl or methoxy substitution at the ortho position relative to the hydroxyl because ortho substitution with an electron donor alkyl or methoxy group increases the stability of the aryloxyl radical (Pokorny, 1987; Cuvelier et al., 1992). Cuvelier et al. (1992) illustrated that a double bond in the side chain of a phenolic compound, such as caffeic acid, participates in stabilizing the radical by delocalizing resonance. These theories may explain the increased antioxidant efficiency of the active compound. The mechanism of a primary antioxidant is to act as a free radical scavenger by donating hydrogen or electrons, while synergists, such as EDTA, act as metal chelators. Thus, the phenolic antioxidant in shrimp shell could play a role in providing hydrogen or electrons to a free radical.

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