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Oat avenanthramides exhibit antioxidant activities in vitro☆

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Abstract

Avenanthramides are a group of alkaloids that consist of an anthranilic acid derivative linked to a hydroxycinnamic acid derivative by a pseudo peptide bond, which are constituents of oat (*Avena sativa* L.) grain. The three most abundant avenanthramides, *N*-(4′-hydroxy-3′-methoxycinnamoyl)-5-hydroxyanthranilic acid (Bf), *N*-(4′-hydroxycinnamoyl)-5-hydroxyanthranilic acid (Bp), and *N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid (Bc), were synthesized and purified. These were tested for antioxidant activity using two in vitro systems: inhibition of β-carotene bleaching and reaction with the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Each avenanthramide displayed antioxidant activity in both systems. Bc had greater activity than Bp and Bf. Bc was nearly as active as the standard synthetic antioxidant, butylated hydroxytoluene (BHT) in the β-carotene system. In the DPPH system, Bc and Bf were more active than 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®). The relative activities of the avenanthramides corresponded to those determined for their component hydroxycinnamic acid moieties using an aqueous DPPH system or in a lipophilic system that measured the autoxidation of methyl linoleate.
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Keywords: Oat; Avenanthramide; Antioxidant

1. Introduction

Oat (*Avena sativa* L.) has long been recognized as a healthful and nutritious food, containing high concentrations of well-balanced protein and soluble fiber, energy in the form of carbohydrate and oil, and several vitamins and minerals (Peterson, 1992; Welch, 1995). In addition, oat is a source of antioxidants, such as tocols (Peterson & Qureshi, 1993) and various phenolic compounds (Daniels, King, & Martin, 1963; Daniels & Martin, 1967, 1968; Durkee & Thivierge, 1977; Sosulski, Krygier, & Hogge, 1982; Xing & White, 1997). Collins (1989) identified and characterized a group of novel alkaloids that contained phenolic groups in oat groats and hulls, which were given the trivial name 'avenanthramides'. These avenanthramides are substituted hydroxycinnamic acid conjugates, and more than 25 distinct entities were separated by TLC from methanol extracts of groats. Dimberg, Theander, and Lingnert (1993) determined that avenanthramides had antioxidant activity, and that the concentrations of Bf exceeded 100 mg/kg in the groats of some cultivars. Bp, Bf and Bc (previously called A, B and C, respectively, Fig. 1) occur in higher concentrations than all other avenanthramides (Emmons & Peterson, 1999). Their concentrations are affected by genotype and growing environment, and at one location, the combined concentration of Bp, Bf and Bc exceeded 300 mg/kg (Emmons & Peterson, 2001).

Avenanthramides can be synthesized by condensing the acyl chloride derivative of the protected aromatic acid with the appropriate free anthranilic acid in the presence of pyridine (Bain & Smalley, 1968). Following these general procedures, Collins (1989) synthesized avenanthramide Bp (A) and Bf (B). Bc (C) was synthesized by condensing caffeic acid with 5-hydroxyanthranilic acid (Ishihara, Miyagawa, Matsukawa, Ueno, Mayama, &

☆Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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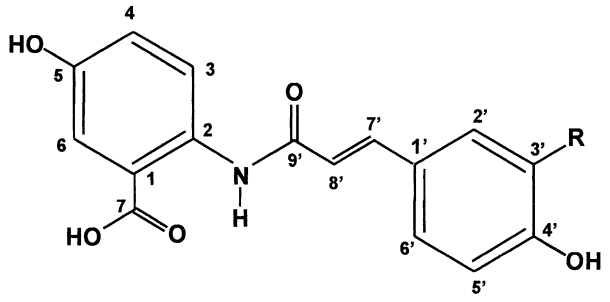

Avenanthramide	R
Bp	H
Bf	OCH_3
Bc	OH

Fig. 1. The structures of avenanthramides Bc, Bp and Bf.

Iwamura, 1998). The synthesized avenanthramides were purified by chromatography, and their structures were verified by NMR spectroscopy.

Antioxidants are believed to play an important role in preventing or alleviating chronic diseases by reducing the oxidative damage to cellular components caused by reactive oxidant species. Some plant phenols act as antioxidants due to their chemical structures (Hollman, 2001).

As a first step in determining the potential for avenanthramides to affect human health, we synthesized the three predominant ones and assessed their antioxidant capacities using two different in vitro tests. Prior studies have shown that oat extracts contain antioxidant activity (Emmons, Peterson, & Paul, 1999; Handelman et al., 1999), but this activity could not be attributed to any specific components in the extracts. The only information on avenanthramide antioxidant activities was published by Dimberg et al. (1993) using a linoleic acid oxidation test system. They found that the activities of Bf, called avenanthramide 1, and another minor avenanthramide were higher than those of several simple phenolics (caffeic acid, ferulic acid, vanillin), but lower than that of α-tocopherol.

Although there is yet no direct evidence that avenanthramides are absorbed and metabolized, the inclusion of avenanthramide Bc in rat diets selectively affected oxidant–antioxidant balance in various tissues (Ji, Lay, Chung, Fu, Parkin, & Peterson, 2002). An analogous molecule, tranilast [*N*-(3,4-dimethoxycinnamoyl)-anthranilic acid], has been studied extensively as a novel antiproliferative drug (Isaji, Miyata, & Ajisawa, 1998). Because avenanthramides have similar chemical structures, their bioavailability is inferred.

Results can vary among in vitro antioxidant assays, because of the different chemistries and reaction environments (aqueous or lipophilic) involved in these assays (Baderschneider, Luthria, Waterhouse, & Winterhalter, 1999; Peterson, 2001). Our objectives were to synthesize the three major avenanthramides and test their antioxidant activities using two different in vitro analysis systems. The purpose of the study was to identify which of these oat avenanthramides should be tested in model animal systems. Should such tests prove successful, strategies for increasing their concentrations in oat grain through plant breeding or metabolic engineering should be considered.

2. Materials and methods

2.1. Materials

Thionyl chloride was obtained from Aldrich (Milwaukee, WI, USA), 4-acetoxycinnamic acid, 4-acetoxy-3-methoxycinnamic acid and 5-hydroxyanthranilic acid from Alfa-Aesar (Ward Hill, MA, USA), caffeic acid, acetic anhydride, DPPH, β-carotene, linoleic acid, BHT and Folin and Ciocalteau's phenol reagent from Sigma (St. Louis, MO, USA), and pyridine and Trolox® from Fluka (Milwaukee, WI, USA). BHT is a widely used synthetic antioxidant, and Trolox is a water-soluble α-tocopherol derivative that is used as a comparative standard antioxidant.

2.2. Methods

2.2.1. Synthesis of avenanthramides

Bp and Bf were synthesized following the methods of Collins (1989). For Bp, 212 mg (1 mmol) of 4-acetoxycinnamic acid was refluxed with 1 ml thionyl chloride in 4 ml of $CHCl_3$ for 30 min. The solution was rotary-evaporated, and the residue was repeatedly dissolved in acetone and rotary evaporated to remove all thionyl chloride, yielding 4-acetoxycinnamoyl chloride. This product was dissolved in 10 ml of acetone and combined with 157 mg (1 mmol) of 5-hydroxyanthranilic acid dissolved in 10 ml of pyridine. The solution was warmed for 10 min with stirring on a sand bath at 100 °C and cooled to room temperature. The solvents were removed by repeated rotary evaporation and washing with acetone/H_2O (80/20, v/v). The residue was dissolved in acetone/acetic acid/H_2O (80/10/10, v/v/v) and allowed to stir overnight at room temperature, after which the solvents were removed by rotary evaporation. The residue was refluxed in methanol/H_2O/NH_4OH (50/40/10, v/v/v) for 60 min to remove the acetyl protecting group, cooled, and rotary evaporated to dryness. The products were chromatographed on a 2.5×18 cm Sephadex LH-20 column, using acetone/H_2O/acetic acid (30/65/5) to elute the column. Fractions that eluted after a blue-purple band, visible with long wavelength UV light, were collected and analyzed for the presence of Bp by HPLC. The Bp-containing fractions were combined

and refrigerated overnight. Crystals of the E isomer of Bp, and a small amount of the Z isomer, were formed. The E isomer was recrystallized from hot acetone/H_2O.

For Bf, the starting material was 241 mg (1 mmol) 4-acetoxy-3-methoxycinnamic acid. The synthesis and purification followed the same steps as described for Bp.

To synthesize Bc, 1 g of caffeic acid was reacted overnight with 20 ml of acetic anhydride in the presence of 2 ml of pyridine at room temperature. Fifty milliliters of ice cold H_2O was added and the solution was refrigerated overnight. The resulting precipitate was collected on a Hirsch funnel, washed several times with ice cold H_2O, and oven dried (40 °C). The residue was refluxed with 10 ml of thionyl chloride for 3 h, and then the procedures described for Bp were followed.

The purities of the avenanthramides were verified by HPLC, and their structures confirmed to be authentic by HPLC retention times, UV spectra, and NMR spectroscopy. The ^1H NMR spectra corresponded closely with those reported by Collins (1989).

2.2.2. High-performance-liquid chromatography (HPLC)

The quantitative analyses of the synthesized avenanthramides and determinations of their purities were made by HPLC on a C18 column with an acetonitrile gradient as previously described (Emmons et al., 1999; Emmons & Peterson, 2001).

2.2.3. β-Carotene bleaching assay

The synthesized avenanthramides were tested for their abilities to inhibit the autoxidation of linoleic acid and β-carotene as previously described (Emmons & Peterson, 1999). A range of concentrations of each avenanthramide was tested, and the percent inhibition of absorbance loss, as compared to a blank, was plotted against concentration. Triplicate analyses were run at each concentration. BHT was used as a control. The concentration of each avenanthramide that inhibited the rate of β-carotene bleaching by 50% is defined as its EC_{50}.

2.2.4. DPPH assay

Aliquots of the synthesized avenanthranudes were added to a DPPH solution and incubated for 3 h, after which the absorbance change at 515 nm was used to calculate the amount of DPPH reduced, as previously described (Peterson, Emmons, & Hibbs, 2001). Single analyses at each concentration were run, except for two concentrations each of Bf and Bc, where duplicates were run. The number of μmoles of DPPH reduced was plotted against the number of μmoles of avenanthramide, and an EC_{50} value was calculated (Brand-Williams, Cuvelier, & Berset, 1995). This is defined as the amount of antioxidant necessary to reduce the concentration of DPPH by 50%. Trolox was used as a control.

2.2.5. Total phenolic content

The colour yields of the avenanthramides with the Folin–Ciocalteau reagent were measured using a gallic acid standard (Ragazzi & Veronese, 1973). Folin–Ciocalteau reagent (125 μl) was added to 250 μl of avenanthramide (100 μg/ml) or gallic acid (50 or 100 μg/ml) solutions (in methanol), followed by 750 μl of 200 mg/ml Na_2CO_3. After 15 min, 2.5 ml of deionized H_2O were added, the mixture was centrifuged, and the absorbance of the supernatant was read at 725 nm.

3. Results

All three avenanthramides inhibited β-carotene bleaching in the in vitro test system, and the degree of inhibition depended on the concentration of avenanthramides in the test solutions (Fig. 2). With each avenanthramide, the plot of percent inhibition vs. μmoles of avenanthramide was asymptotic. A comparison of the concentrations that caused a 50% inhibition

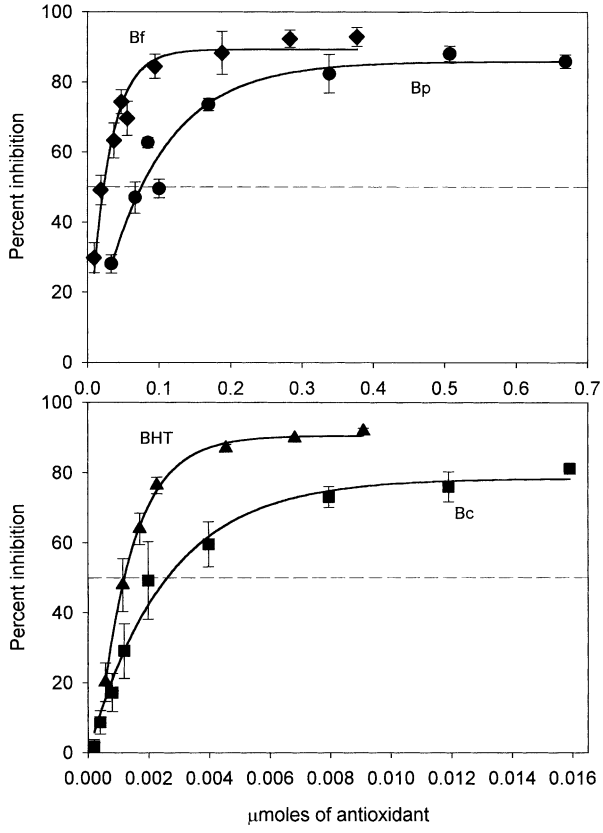

Fig. 2. Antioxidant activities, as indicated by the inhibition of β-carotene bleaching, by various concentrations of avenanthramides Bc, Bp and Bf as compared to BHT. Data are means of three analyses, and error bars indicate the standard deviations. The dashed horizontal line indicates 50% inhibition.

revealed that the order of effectiveness was BHT > Bc > Bf > Bp. Compared to BHT, the EC_{50} concentration of Bc was 2.4-fold higher, whereas that of Bf was 15-fold higher and Bp was 62-fold higher (Table 1). Neither the avenanthramides nor BHT completely inhibited β-carotene bleaching, even at the highest concentrations tested (Fig. 2). When the inhibition percentages were plotted against antioxidant concentrations expressed on a log scale, parallel lines with similar slopes were obtained for each antioxidant (Fig. 3).

In the DPPH test, the amounts of DPPH reduced were linearly proportional to the amounts of the antioxidants over the ranges tested (Fig. 4). The relative effectiveness of the antioxidants at the 50% reduction level was identical to that observed in the β-carotene test. In the case of this test, the range in concentrations of the antioxidants required to achieve the 50% reduction in DPPH was much smaller than had been found for the β-carotene test (Table 1). Both Bc and Bf reduced 50% of the DPPH at lower concentrations than that of Trolox. The slopes of the lines were different, with that of Bc > Bf > Trolox > Bp. Thus, the relative effectiveness is not the same at different levels of DPPH reduction.

Table 1
Antioxidant activities of avenanthramides relative to Trolox of BHT, as determined by reaction with DPPH and inhibition of β-carotene bleaching

Compound	EC_{50} (DPPH assay)[a] μmoles	EC_{50} (β-carotene assay)[b] μmoles	Phenolic content (gallic acid equiv.) mol/mol
Bp	0.198	0.074	0.93
Bf	0.105	0.018	1.09
Bc	0.074	0.0029	1.89
Trolox	0.160		
BHT		0.0012	

[a] Quantity required to reduce half of the DPPH.
[b] Quantity required to inhibit β-carotene oxidation rate by 50%.

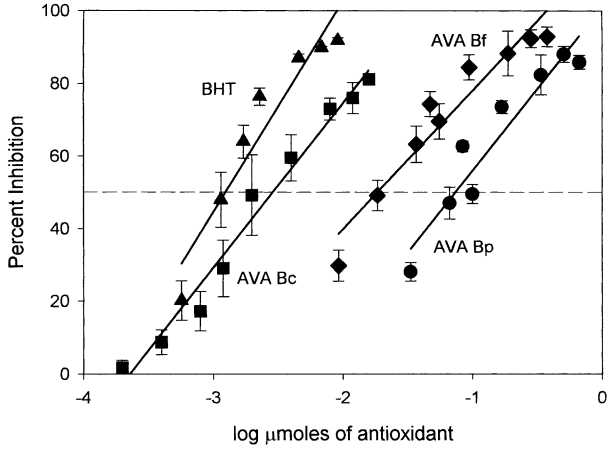

Fig. 3. The inhibition of β-carotene bleaching by Bc, Bp and Bf as compared to BHT. The results are graphed as the log concentrations of antioxidants. The dashed horizontal line indicates 50% inhibition.

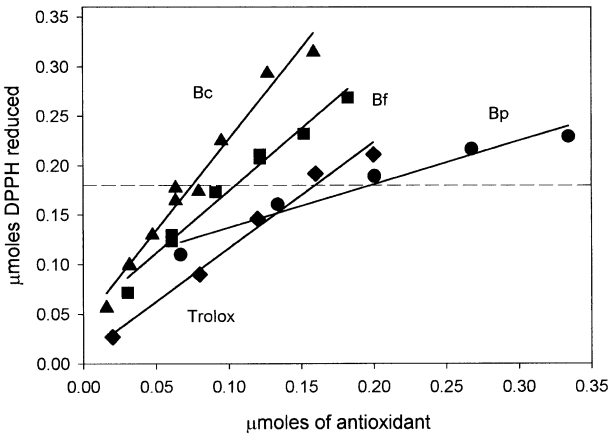

Fig. 4. Antioxidant activities of Bc, Bp and Bf as compared to Trolox as indicated by the reduction of DPPH. The dashed line represents the reducation of 50% of the DPPH in the assay tube.

4. Discussion

Although there are reportedly as many as 25 different avenanthramides from oat groats (Collins, 1989), three of them consistently appear in higher concentrations than the others (Dimberg, Molteberg, Solheim, & Frølich, 1996; Dimberg, Sunnerheim, Sundberg, & Walsh, 2001; Emmons & Peterson, 1999, 2001; Peterson et al., 2001). These three, Bp, Bf, and Bc, were synthesized and their antioxidant activities were analyzed in vitro. This paper is the first report on the in vitro antioxidant activities of Bp and Bc. Previously, Dimberg et al. (1993) reported that purified Bf was 18% as active as α-tocopherol in inhibiting the oxidation of linoleic acid, whereas it was three times as active as caffeic acid.

The β-carotene bleaching test (Marco, 1968; Miller, 1971) has been used by many workers to measure the antioxidant activities of plant extracts (Al-Saikhan, Howard, & Miller, 1995; Auerbach & Gray, 1999; Lee, Howard, & Villalon, 1995). In this test system, the components are maintained as an emulsion with Tween 40, so the lipid-soluble antioxidants are measured more efficiently than in an aqueous system. For the avenanthramides, which are soluble in mixtures of water and organic solvents but nearly insoluble in water, this test was much more sensitive than the aqueous DPPH test. Reaction rates were linear over 60 min (not shown), and the curves of percent inhibition versus concentration showed similar kinetics for each avenanthramide (Figs. 2 and 3). The concentration of Bc required to achieve 50% inhibition was lower than that of Bp or Bf by more than an order of magnitude. However, complete inhibition was not achieved, even at the highest concentrations tested.

The DPPH assay measures the loss of absorbance that occurs when the DPPH radical is reduced by an antioxidant. The method is popular because of its simplicity. We measured the absorbance of the reaction mix after 3 h of incubation, when the reaction was complete or nearly so (data not shown). Other workers have measured DPPH concentration at earlier time points (Bryngelsson, Mannerstedt-Fogelfors, Kamal-Eldin, Andersson, & Dimberg, 2002; Chen, Wang, Rosen, & Ho, 1999) , which would give an indication of the initial rate of reaction, but not the total capacity. By comparing the number of μmoles of avenanthramide required to reduce 50% of the DPPH, our assay method gave the same order of activity as did the β-carotene bleaching assay, Bc > Bf > Bp.

Three different kinetic types of the reaction between antioxidants and DPPH have been described (Brand-Williams et al., 1995), and the avenanthramides seem to be among the slow reaction types, which take 1–6 h to reach steady state (data not shown). The plots of μmoles of DPPH reduced versus μmoles of avenanthramide produced straight lines for each antioxidant, but the slopes were different (Fig. 4). The slope of the Trolox line was slightly greater than the slopes of the Bc and Bf lines, which were similar. However, the slope for the Bp line was lower, and it intersected the other lines at low concentrations. These data could result from the stoichiometry of the functional groups on the reactants. Bp has two hydroxyls whereas Bc has three. Therefore, Bc reacts with more DPPH per unit increase in concentration than Bp. Bf, with a methoxy and two hydroxy groups, was intermediate in slope.

The relative reactivities of the three avenanthramides reflected the antioxidant activities of their constituent hydroxycinnamic acid moieties. Using the DPPH test, the order of reactivity was caffeic > ferulic > *p*-coumaric (Brand-Williams et al., 1995; Chen & Ho, 1997). In a lipophilic system that measures the autoxidation of methyl linoleate, the order of reactivity was the same (Cuvelier, Richard, & Berset, 1992). The antioxidative efficiency of a *para* hydroxy phenol was enhanced by the addition of an adjacent methoxy group, and an adjacent hydroxy group enhanced reactivity even more (Cuvelier et al., 1992). These results are consistent with the relative calculated and measured bond dissociation enthalpies for phenol and its ortho methoxy and hydroxy monosubstituted derivatives (Wright, Johnson, & DiLabio, 2001). In contrast, the addition of a hydroxy (caffeic acid) or methoxy (ferulic acid) group to *p*-hydroxy cinnamic acid (*p*-coumaric acid) weakened the reactivity in an ABTS [2,2′-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid)] radical scavenging reaction (Rice-Evans, Miller, & Paganga, 1996). The Folin–Ciocalteau reaction, which measures the quantity of oxidizable groups, such as phenolic groups, indicated that there were approximately 1.9 gallic acid equivalents for Bc, 1.1 for Bf and 0.9 for Bp (Table 1). The higher value for Bf than for Bp may indicate that the methoxy group increases the potential of the adjacent hydroxy to be oxidized.

The concentrations of avenanthramides in oat groats are affected by both the genotype and the growing environment (Emmons & Peterson, 2001). The average concentrations for three cultivars that were grown in seven locations ranged from 9 to 52 mg/kg for Bp, 13 to 78 mg/kg for Bf, and 25 to 145 mg/kg for Bc. Considering the in vitro antioxidant activities determined in this study and the naturally occurring concentrations, it is apparent that Bc contributes substantially more to the total antioxidant activity measured in extracts of oat groats than the other avenanthramides. Thus, Bc appears to have the most potential for animal studies on in vivo effects. Furthermore, oat may be bred, or grown in particular environments, to enhance antioxidant potential.

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