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Evaluation of the Antioxidant Properties of Free and Bound Phenolic Acids from Native and Malted Finger Millet (Ragi, *Eleusine coracana* Indaf-15)

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Free and bound phenolic acids were isolated from native and malted finger millet (ragi, *Eleusine coracana* Indaf-15), and their antioxidant properties were evaluated. Protocatechuic, gallic, and caffeic acids were found to be the major free phenolic acids. A 3-fold decrease was observed in protocatechuic acid content, whereas the decrease was marginal in the case of caffeic acid upon 96 h of malting. However, the contents of other free phenolic acids such as gallic, vanillic, coumaric, and ferulic acids increased. Ferulic, caffeic, and coumaric acids were found to be the major bound phenolic acids, and a 2-fold decrease was observed in their contents upon 96 h of malting. The antioxidant activity of a free phenolic acid mixture was found to be higher compared to that of a bound phenolic acid mixture. An increase in antioxidant activity coefficient was observed in the case of free phenolic acids from 770.0 ± 7.8 to 1686.0 ± 16.0, whereas the same was decreased from 570.0 ± 6.0 to 448.0 ± 4.5 in bound phenolic acids upon 96 h of malting. Therefore, the antioxidant capacity of phenolic acids changes during the malting of ragi.

KEYWORDS: Antioxidants; finger millet; malting; phenolic acids

INTRODUCTION

Phenolic acids and their derivatives are widely distributed in plants (*1*). A number of phenolic acids are linked to various cell wall components such as arabinoxylans and proteins (*2*, *3*). Among various bound phenolic acids, ferulic acid is the major one found in Graminacious plants (*4*, *5*). Phenolic acids are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because of their stable radical intermediates, which prevent the oxidation of various food ingredients, particularly fatty acids and oils (*6*, *7*). Fortification of diets with food materials rich in phenolic acids was shown to impart antimutagenic, antiglycemic, and antioxidative properties, and this can be exploited in developing health foods (*8*). In addition, phenolic acids such as caffeic, coumaric, ferulic, and protocatechuic acids are shown to exert an antifungal effect (*9*).

Finger millet, also known as "ragi" in India, is an important staple food for people belonging to low-income groups. It is rich in phenolic compounds compared to other continental cereals such as barley, rice, maize, and wheat. The majority of the phenolic compounds present in ragi was reported to exist in the form of glycosides (*10*). Malting is known to improve the quality of cereals (*11*), and malted ragi flour or extracts derived from it are extensively used in the preparation of weaning and infant foods, beverages, and pharmaceutical preparations (*12*, *13*). Studies were carried out with respect to the contents of phenolic acids and tannins in different varieties of ragi (*14*, *15*). Changes in the contents of nutrients and antinutrients (*16*), nonstarch polysaccharides, and bound phenolic acids (*17*) during malting of ragi were also reported. Similar studies were also carried out on sorghum (*18*), barley (*7*, *19*), and different varieties of rice (*20*). However, information with particular reference to changes in free and bound phenolic acids and their antioxidant properties during malting of ragi is not reported and hence the present study.

MATERIALS AND METHODS

Materials. Finger millet (Indaf-15) seeds were procured from the V. C. farm of the University of Agricultural Sciences, located at Mandya, Karnataka, India, and used for all of the studies. Phenolic acid standards, such as caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic, and vanillic acids, β-carotene, and synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. HPLC column (Shimpak C_{18}) was obtained from Shimadzu Corp., Tokyo, Japan. All of the solvents used were of HPLC grade, and other chemicals were of analytical grade.

Methods. *Malting.* Malting was carried out according to the method of Nirmala et al. (*21*). Ragi seeds (100 g) were cleaned, steeped for 24 h, and germinated under controlled conditions on a moist cloth at 25° C in a B.O.D. incubator for 96 h. Germinated seeds were taken out at 24 h intervals and dried at 50 °C for 12 h in a hot air oven. Growth portions were removed, and devegitated seeds were weighed, powdered, and used for the experiments, along with ungerminated ragi flour, which served as control.

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Table 1. Changes in Free and Bound Phenolic Acid Contents during Malting of Ragi

	phenolic acid contents (mg/100 g of flour)											
malting time (h)	gallic acid[a]	protocatechuic acid[a]	vanillic acid[a]	caffeic acid		coumaric acid		ferulic acid		total	
				free	bound	free	bound	free	bound	free	bound
0	4.50	45.0	1.00	2.60	1.64	0.10	1.20	0.20	18.60	53.40	21.44
24	3.40	33.5	0.20	1.65	2.20	0.02	1.40	0.40	17.20	39.17	20.80
48	6.40	24.0	1.20	1.00	1.50	0.20	1.00	2.30	14.30	35.10	16.80
72	6.60	20.0	1.10	0.70	1.60	0.20	1.00	2.25	13.20	30.85	15.80
96	19.0	16.0	1.35	1.60	0.90	0.25	0.70	2.00	09.60	40.20	11.20

[a] Gallic, protocatechuic, and vanillic acids were not detected in bound phenolic acids.

Isolation and Characterization of Free Phenolic Acids. Free phenolic acids were isolated according to the method of Ayumi et al. (*20*). Two grams of native and malted flours was extracted with 70% ethanol (4 × 50 mL, 1 h each); the supernatants were obtained by centrifugation and concentrated, and the pH was adjusted in the range of 2−3 with 4 M HCl. Phenolic acids were separated by ethyl acetate phase separation (5 × 50 mL), and the pooled fractions were treated with anhydrous disodium sulfate to remove moisture, filtered, and evaporated to dryness. Phenolic acids taken in methanol were estimated colorimetrically by using Folin−Cioclateu method with gallic acid as the reference standard (*22*), as well as by HPLC (model LC-10A, Shimadzu), on a reversed phase Shimpak C_{18} column (4.6 × 250 mm), using a diode array detector (operating at 280 nm). A solvent system consisting of water/acetic acid/methanol (isocratic, 80:5:15) was used as mobile phase at a flow rate of 1 mL/min (*23*). Standards such as caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic, and vanillic acids were used for identification and quantification of phenolic acids present in the native and malted samples. Quantification of phenolic acids present in the sample was carried out by measuring the area under respective peaks and plotting against a standard graph prepared (2−10 μg) for each individual phenolic acid using the above-mentioned standards.

Isolation and Characterization of Bound Phenolic Acids. Bound phenolic acids were extracted according to the method of Eric-Nordkvist et al. (*24*). Native and malted flours (2 g each) were extracted with 70% ethanol (4 × 50 mL) and hexane (4 × 50 mL) to remove free phenolic acids and fat, respectively. The dried samples were extracted with 1 M sodium hydroxide (2 × 100 mL, 2 h each) containing 0.5% sodium borohydride under nitrogen atmosphere, and the clear supernatants were collected followed by centrifugation. The combined supernatants were acidified with 4 M HCl to pH 1.5, and the phenolic acids were processed and analyzed by colorimetry as well as by HPLC as mentioned in the case of free phenolic acids.

Assay of Antioxidant Activity. Antioxidant activity was measured by monitoring the coupled autoxidation of β-carotene and linoleic acid emulsion (*25*, *26*). β-Carotene (2 mg) was dissolved in 20 mL of chloroform, and 3 mL of this solution was added to 40 mg of linoleic acid and 400 mg of Tween-20 mixtures. The chloroform was removed under a stream of nitrogen gas. Oxygenated, deionized water (100 mL) was added to the above mixture, and the solution was kept in the dark after thorough mixing. Phenolic acid extract (50 μg/50 μL) was mixed well with β-carotene−linoleic acid emulsion and incubated at 50° C. Oxidation of the emulsion was monitored spectrophotometrically by measuring the absorbance at 470 nm at regular intervals of 30 min for a total period of 120 min. Control was prepared by adding 50 μL of methanol in place of the extract. Because the decrease in antioxidant activity coefficient (AAC) was almost linear, the results were expressed as percent inhibition relative to the control after a 120 min incubation period. AACs of samples were also expressed for gram of flour, and the values obtained were the average of triplicates.

$$\mathrm{AAC} = \frac{S_{\mathrm{abs}} \text{ at } 120 \text{ min} - C_{\mathrm{abs}} \text{ at } 120 \text{ min}}{C_{\mathrm{abs}} \text{ at } 0 \text{ min} - C_{\mathrm{abs}} \text{ at } 120 \text{ min}} \times 100$$

S_{abs} is the absorbance of the sample and C_{abs} is the absrobance of the control.

RESULTS AND DISCUSSION

Changes in Phenolic Acids. *Free Phenolic Acids.* Protocatechuic acid was found to be the major free phenolic acid in the native ragi flour along with small amounts of gallic, caffeic, vanillic, ferulic, and coumaric acids. A 3-fold decrease was observed in protocatechuic acid content (from 45 to 16 mg) upon 96 h of malting. In the case of caffeic acid a decrease of ~4-fold (from 2.6 to 0.7 mg) was observed at 72 h of malting; however, the content increased to 1.6 mg at 96 h of malting, and this may be due to the interconversion among cinnamic acid derivatives. However, the contents of other free phenolic acids such as coumaric, gallic, and ferulic acids was increased by 2-, 4-, and 10-fold upon 96 h of malting. Most of the phenolic acid contents decreased slightly in the 24 h malted sample, and it could be due to initial loss during steeping for 24 h (**Table 1**).

Bound Phenolic Acids. Ferulic (18.6 mg/100 g of flour), caffeic (1.64 mg/100 g of flour), and coumaric (1.20 mg/100 g of flour) acids were found to be the major bound phenolic acids along with traces of protocatechuic and syringic acids in the native ragi flour. Their contents were decreased by 2-fold upon 96 h of malting (**Table 1**). Ferulic, coumaric, caffeic, and gallic acids are known to be ester linked to various cell wall polymers in cereals such as rice (*20*), barley (*19*), sorghum (*22*), and millets such as ragi (*17*). However, in the present study bound gallic acid was not found.

The changes observed in the contents of free and bound phenolic acids upon malting can be explained either by the possible action of induced esterases on phenolic acid−polysaccharide and/phenolic acid−protein complexes, which might have resulted in the liberation of phenolic acids as reported in barley (*7*), or by de novo synthesis of phenolic acids, followed by their storage in seeds instead of their migrating to shoots during malting as reported in some of the sorghum varieties (*27*).

Changes in AACs. *AACs of Some Standard Phenolic Acids.* Various naturally occurring phenolic acids such as caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic, and vanillic acids along with synthetic antioxidants such as BHA and BHT were tested for their antioxidant activity, and the results are shown in **Figure 1**. AACs of BHA (171/50 μg) and BHT (137/50 μg) were found to be greater than those of other naturally occurring phenolic acids. This could be due to the emulsion system used in the present study. BHA and BHT were known to have higher AACs than other phenolic acids in an emulsion system (*28*) compared to in a lipophilic solvent system. Cinnamic acid derivatives, such as ferulic, caffeic, and coumaric acids, have higher AACs than their respective benzoic acid derivatives vanillic, protocatechuic, and *p*-hydroxybenzoic acids. This could be due to the resonance of the C=C bond, which facilitates stabilization of the radicals. Among cinnamic acid derivatives caffeic and ferulic acids were found to be stronger

Table 2. AAC of Free and Bound Phenolic Acids Derived from Native and Malted Ragi Flours[a]

malting time (h)	AAC/g of flour (colorimetric)		AAC/g of flour (HPLC)[a]	
	free	bound	free	found
0	770.00 ± 7.8 (11.00 ± 1.0)	570.0 ± 6.0 (9.5 ± 0.9)	762.60 ± 9.0 (71.40 ± 0.8)	370.20 ± 3.5 (86.30 ± 0.8)
24	584.00 ± 6.0 (10.00 ± 1.0)	577.0 ± 5.8 (8.5 ± 0.8)	562.40 ± 6.5 (72.00 ± 0.6)	358.20 ± 4.0 (86.00 ± 0.9)
48	908.00 ± 9.0 (12.50 ± 1.3)	512.0 ± 5.5 (7.0 ± 0.8)	526.00 ± 6.0 (75.00 ± 0.8)	289.00 ± 3.5 (86.25 ± 0.8)
72	1398.00 ± 14.0 (13.00 ± 1.2)	489.0 ± 5.0 (6.0 ± 0.6)	468.50 ± 5.0 (76.00 ± 0.7)	272.50 ± 2.4 (86.20 ± 0.8)
96	1686.00 ± 16.0 (15.50 ± 1.4)	448.0 ± 4.5 (5.5 ± 0.5)	676.00 ± 8.6 (84.00 ± 1.0)	193.00 ± 1.8 (86.15 ± 0.8)

[a] AAC were calculated by multiplying the amount of constituent phenolic acid as analyzed by HPLC with their respective antioxidant activity coefficients derived from individual standard phenolic acids. Values in parentheses indicate the AAC/50 μg of phenolic acid.

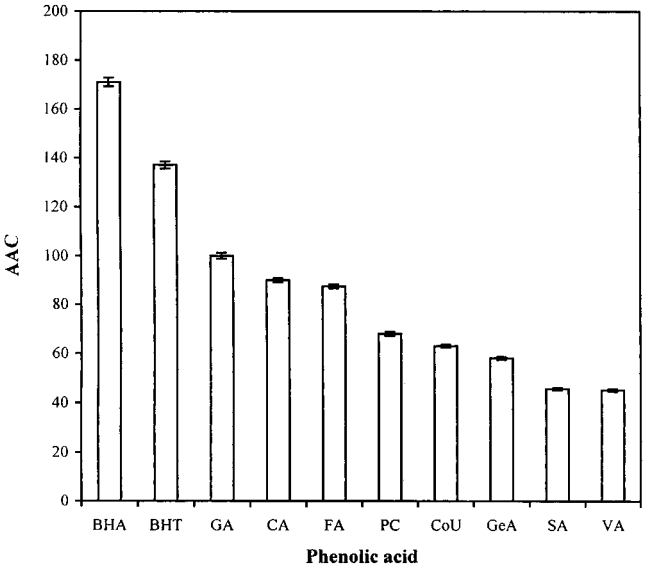

Figure 1. Antioxidant activity coefficients of different standard phenolic acids: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; GA, gallic acid; CA, caffeic acid; FA, ferulic acid; PC, protocatechuic acid; CoU, coumaric acid; GeA, gentisic acid; SA, syringic acid; VA, vanillic acid.

than coumaric acid. Gallic acid, which has three −OH groups, was stronger than protocatechuic, gentisic, and syringic acids with respect to their antioxidant property.

Antioxidant Activities of Free and Bound Phenolic Acids. The antioxidant activity of free and bound phenolic acid mixture was found to be significantly lower than that of synthetic antioxidants such as BHA and BHT (**Figure 1** and **Table 2**). Similar observations were also made in the case of barley, for which the antioxidant activity of methanolic extracts was found to be lower than that of BHT and gallic acid (*7*). The antioxidant activity of the free phenolic acid mixture, in the present study, was found to be higher compared to that of the bound phenolic acid mixture (**Table 2**). This is in contrast to the results reported for some barley varieties, for which higher antioxidant activity was reported in bound phenolic acids (*19*). However, no correlation was made between the phenolic acid content and antioxidant activity in some varieties of barley (*7*). High amounts of gallic and protocatecheuic acids present in ethanolic extracts, along with other constituents, might have resulted in the high AACs, due to their cumulative effect, as observed in the present study. Gallic acid is known to be a potent antioxidant compared to other phenolic acids such as protocatechuic, gentisic, and syringic acids and some of the cinnamic acid derivatives such as coumaric acid (*6*) (**Figure 1**). Correlations were made between the AACs calculated by HPLC and by colorimetric methods (**Table 2**). The AAC of free phenolic acids decreased from 762.6 ± 9.0 (71.4 ± 0.8) to 468.5 ± 5.0 (76.0 ± 0.7) at 72 h of malting, and the same has increased to 676.0 ± 8.6 (84.0 ± 1.0) in 96 h malted flour as measured by HPLC (**Table 2**). The same can be attributed to the decrease in the phenolic acid content (from 53.4 to 30.85), particularly free protocatechuic acid, in 72 h malt. However, the AAC increased in 96 h malt, and this could be due to the increase in the gallic and ferulic acids (**Table 2**). In the case of bound phenolic acids the AAC decreased from 370.2 ± 3.5 to 193.0 ±1.8 upon 96 h of malting, which could be due to a 2-fold decrease in the contents of bound phenolic acids, particularly ferulic acid (**Tables 1** and **2**). The AAC calculated via the colorimetric method indicated an increase in free phenolic acid mixture from 770.0 ± 7.8 (11.0 ± 1.0) to 1686.0 ± 16.0 (15.5 ± 1.4), and the same decreased from 570.0 ± 6.0 (9.5 ± 0.9) to 448.0 ± 4.5 (5.5 ± 0.5) in the case of bound phenolic acid mixture upon 96 h of malting (**Table 2**). Even though the total amount of free phenolic acid content obtained by HPLC analysis decreased from 53.4 to 40.2 upon 96 h of malting (**Table 1**), the AAC determined by colorimetry increased (**Table 2**), indicating the participation of other phenolic compounds in ethanolic extract, which were not identified by HPLC.

Conclusions. The above results clearly indicate changes in the contents of both free and bound phenolic acids upon malting. Protocatechuic acid, the major free phenolic acid, decreased by 3-fold, whereas a marginal decrease was observed with respect to free caffeic acid. However, the contents of other free phenolic acids increased upon 96 h of malting. A decrease of ∼2-fold was observed in all of the bound phenolic acids. The changes in the phenolic acid contents reflected their antioxidant properties, and this may have a bearing on the keeping quality of the products prepared from ragi.

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