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Water soluble feruloyl arabinoxylans from rice and ragi: Changes upon malting and their consequence on antioxidant activity

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Abstract

The objective of this study is to determine the changes brought about by germination on water soluble feruloyl arabinoxylans (feraxans), one of the major components of soluble fibre from rice and ragi and their consequence on antioxidant activity. Soluble feraxans, isolated from native and malted rice and ragi were fractionated on DEAE-cellulose. Ferulic acid content of the major [0.1 molar ammonium carbonate (AC) eluted] fraction was higher in malts (rice: 1045 μg/g; ragi: 1404 μg/g) than in native (rice: 119 μg/g; ragi: 147 μg/g) and this fraction was separated by Sephacryl S-300 chromatography into two peaks each in rice (native: 232 and 24.4 kDa; malt: 75.4 and 39.6 kDa) and ragi (native: 140 and 15.4 kDa; malt: 38.9 and 15.4 kDa). 0.1 molar AC eluted fractions showed very strong antioxidant activity in vitro as determined by β-carotene–linoleate emulsion (IC_{50}: 0.16–0.24 mg), DPPH* (IC_{50}: 4.1–11.4 mg) and Ferric reducing/antioxidant power, FRAP (EC_1: 0.76–3.1 mg) assays. Antioxidant activity of feraxans was several (4.9–1400) folds higher than the expected activity due to their bound ferulic acid content. Apart from ferulic acid, presence of sugars with >C=O (uronyl/acetyl) groups and degree/nature of glycan-polymerization were observed to influence antioxidant activity of the polysaccharides. Malting resulted in many dynamic changes in the ferulic acid content in different feraxan types and affected their antioxidant activity.

Keywords: Antioxidant; Arabinoxylan; Cereals; Dietary fibre; Ferulic acid; Non-starch polysaccharide; Ragi

1. Introduction

Cereals, the staple food for millions of people across the world, are the chief source of soluble dietary fibre (SDF) (Plaami, 1997). Arabinoxylans, along with some amount of β-D-glucans, are the major components of SDF (Rao and Muralikrishna, 2004). These water soluble non-starch polysaccharides are known to have many beneficial roles in human nutrition and health such as lowering cholesterol and fat availability, reducing the disease symptoms of constipation and reducing the risk of diabetes, atherosclerosis and colorectal cancer (Morris et al., 1977; Plaami, 1997; Willett, 1994). They are also known to influence the quality of bakery products due to their physicochemical properties like viscosity and water holding capacity (Izydorczyk and Biliaderis, 1995). Being potent natural immunomodulators and prebiotic, of late, they are considered as functional food ingredients (Charalampopoulos et al., 2002).

Ferulic acid, a major bound phenolic acid, is known to exist ester linked mainly to arabinoxylans and influence

Abbreviations: A:X, arabinose:xylose; AA, antiradical activity; AAC, antioxidant activity coefficient; AC, ammonium carbonate; BHA, butyrated hydroxy anisole; BHT, butyrated hydroxy toluene; BSA, bovine serum albumin; DEAE, diethyl amino ethyl; DPPH*, 1,1-diphenyl-2-picryl-hydrazyl; EC_1, equivalent concentration 1; FRAP, ferric reducing antioxidant power; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibition concentration; IR, infra red; kDa, kilo Dalton; M, malt; N, native; NSP, non-starch polysaccharides; P:H, pentose:hexose; SDF, soluble dietary fibre; TPTZ, 2,4,6-tri (2-pyridyl)-triazine; UV, ultra violet; w/v, weight/volume.

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their physicochemical properties (Ishii, 1997). Feruloyl polysaccharides can interlink each other and form gel in aqueous solutions in the presence of peroxidase. Ferulic acid is supposed to have a number of health benefits. It is known to decrease total cholesterol and increase vitamin-E bioavailability, increase vitality of sperms and a good protective agent against UV radiation–induced skin damage. Ferulic acid exhibits very strong antioxidant, free radical scavenging and anti-inflammatory activity (Castelluccio et al., 1995; Shahidi et al., 1992). It is known to have anti-tumor and anti-cancer effects (Mori et al., 1999).

Apart from dietary fibre, several reports have proved that ferulic acid is a potential chemo-preventive agent for colorectal cancer (Kawabata et al., 2000; Mori et al., 1999). Colorectal cancer is a major cause for concern in the developed and developing nations' health program. For example, it is the third leading cause of cancer death and malignancy in the United States. The 5-year survival rate for colon cancer is only 60%. Epidemiological studies have shown that consumption of whole grain and grain-based diet is associated with reduced risk of chronic diseases including colorectal cancer (Jacobs et al., 1995). This has been linked to the phytochemical profile and antioxidant activity of the grains (Adom and Liu, 2002; Adom et al., 2003; Charalampopoulos et al., 2002; Mori et al., 1999). Although antioxidants can prevent oxidative stress caused by amines and nitroso-compounds, delivery of enough amount of antioxidant to the colon is essential for its good health. However, being small molecules, most antioxidants, including free ferulic acid and feruloyl oligosaccharides, are absorbed in the small intestine and do not enter entero-hepatic circulation (Bourne and Rice-Evans, 1998; Zhao et al., 2003). Thus, oral or intravenous free ferulic acid administration does not reach the colon.

Recently, efforts are made to synthesize enzyme-resistant starch-ferulate to deliver enough ferulic acid to the colon and shown to release ferulic acid by microbial fermentation (Ou et al., 2001). On the other hand, cereal fibre – bound ferulic acid can get into the colon and is partly released by colon microorganisms. However, as complex dietary fibre resists complete fermentation, the concentration of released ferulic acid may be too low to act as a chemo-preventive agent. Although free ferulic acid (Subba Rao and Muralikrishna, 2002) and feruloyl oligosaccharides are known to exhibit antioxidant activity in vitro (Ohta et al., 1994, 1997), it is not shown if feruloyl polysaccharides as such exhibit any antioxidant activity. In case, they may be the better candidates as chemopreventive agents.

The aim of the present study is to observe the biochemical changes in the water soluble feraxans brought about by malting (controlled germination – a process known to be nutritionally beneficial) of cereal grains – rice and ragi, and also to determine the possible antioxidant activity of feraxans, a ferulic acid reservoir and a parent molecule to feruloyl oligosaccharides.

2. Results and discussion

2.1. Isolation, fractionation and characterization of NSP

Water soluble non-starch polysaccharides (NSP) were obtained from native (N, ungerminated) and malted (M, germinated for 96 h) rice and ragi. The yield of water soluble NSP increased by around 3 and 5 folds upon malting of rice and ragi, respectively (Table 1). A similar pattern of increase was observed in water extractable non-starch polysaccharides (WEP) (Rao and Muralikrishna, 2004). However, the yield of water soluble NSP was low compared to WEP (rice: N, 1.2%, M, 2.2%; ragi: N, 0.6%, M, 2.1%). WEP contained small amount of starch contamination (less than 5%) as degraded starch might get extracted with cold water, and was only partially soluble in water. This might be due to the altered physicochemical and hydration characteristics of polysaccharides during processing (Fig. 1). Similar to WEP, water soluble NSP has over 98% sugar and less than 1% protein. The uronic acid content of malt (rice, 4.0%; ragi, 6.1%) NSP was higher than native (rice, 2.6%; ragi, 4.8%) NSP (Table 1). This could be due to the mobilization of high uronic acid con-

Table 1
Yield, ferulic acid and uronic acid contents, and antioxidant activity (IC_{50}, as determined by emulsion assay) of water soluble non-starch polysaccharides obtained from rice and ragi

	Yield (%)	Ferulic acid (μg/g)	Uronic acid (%)	Activity, IC_{50} (mg)	Expected[a] activity, IC_{50} (mg)
Rice					
N	0.15	510.6	2.6 ± 0.1	1.14	54.8
M	0.44	492.5	4.0 ± 0.2	1.24	56.9
Ragi					
N	0.13	528.0	4.8 ± 0.2	0.92	53.0
M	0.61	503.1	6.1 ± 0.3	1.05	55.7

N, native; M, malt.
IC_{50} (mg), the concentration of polysaccharides at which 50% inhibition of β-carotene oxidation was attained.
[a] The amount of polysaccharides containing ferulic acid equivalent to IC_{50} (mg) of free ferulic acid.

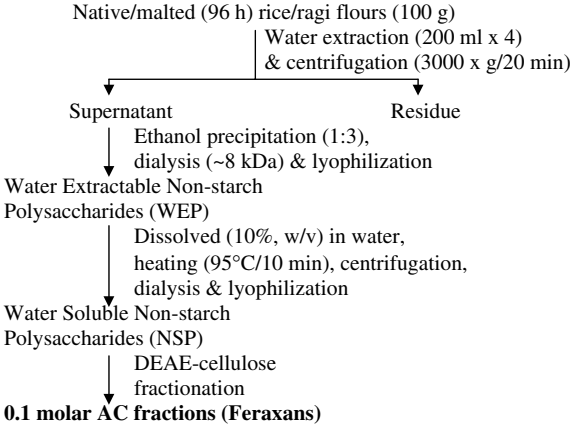

Fig. 1. Scheme for obtaining water soluble NSP/feraxans from native and malted rice and ragi.

taining arabinoxylans during malting. Neutral sugar composition indicated that over 60% of the polysaccharides were of arabinoxylan type (data not shown). It was observed that the arabinose to xylose ratio increased upon malting and was more so in case of ragi. This was probably due to the high activity of induced carbohydrate degrading enzymes during malting (Nirmala et al., 2000). In particular, xylanase activity increased by several folds (rice: N, 0.19 U, M, 0.78 U; ragi: N, 0.23 U, M, 0.98 U per gram flour) (Fig. 4). Xylanase would act on the region of xylan backbone relatively less substituted, yielding an arabinoxylan population more substituted with arabinose upon malting. Ferulic acid content of malt (rice, 510.6 μg/g; rice, 528.0 μg/g) NSP was slightly higher than native (rice, 492.5 μg/g; ragi, 503.1 μg/g) NSP (Table 1). This could be due to ~100 fold increase in the feruloyl esterase activity (rice: N, 0.001 mU, M, 0.123 mU; ragi: N, 0.0029 mU, M, 0.2633 mU) induced during malting (Fig. 4). Overall, malting (controlled germination of cereals) has resulted in increased solubility of NSP, and expression of NSP-degrading enzymes, and enhancement of nutrient quality (Nirmala et al., 2000).

Water soluble NSP was fractionated on DEAE-cellulose anion exchange column by eluting successively with water, 0.1 and 0.2 molar ammonium carbonate (AC) and 0.1 and 0.2 molar NaOH. Neutral polysaccharides (~20–25%) were eluted with water, whereas charged polysaccharides were eluted with AC (0.1 and 0.2 M) and NaOH (0.1 and 0.2 molar). 0.1 molar AC eluted fraction was in maximum yield (40–60%), whereas 0.2 molar AC, 0.1 and 0.2 molar NaOH eluted fractions accounted for 5–10%. However, high amount (10–20%) of polysaccharides was retained in the column uneluted. This was not surprising since high amount of uronic acid containing polysaccharides would require higher concentrations of alkali (>0.3 molar NaOH). However, it was not carried out since high concentrations of alkali would remove uronic acid by β-elimination.

Water eluted fractions contained no uronic acid. Minor amount (N, 3.7 μg/g; M, 14.9 μg/g) of ferulic acid observed (Table 2) in water eluted fractions from ragi might have come from small amount of neutral arabinoxylans wherein ferulic acid would be ester linked to side chain arabinose. Water eluted fractions are chiefly glucan type as indicated by GLC analysis, which showed glucose (80–90%) as the major sugar (data not shown). 0.2 molar AC eluted fractions contained high amount of uronic acid (11.2–15.9%) (Table 2) and GLC analysis showed them to be chiefly of arabinoxylan type of polysaccharides (data not shown). Being arabinoxylan type of polysaccharides, they contained ferulic acid whose content was very high in native (rice: 1182.0 μg/g; ragi: 1641.4 μg/g) than malt (rice: 83.7 μg/g; ragi: 189.5 μg/g) polysaccharides (Table 2).

Neutral sugar composition of 0.1 molar AC eluted fractions is shown in Table 3. Arabinose and xylose are the major sugars identified. Similar to that of water soluble

Table 2
Ferulic acid and uronic acid contents, and antioxidant activity (IC_{50}, as determined by emulsion assay) of water soluble NSP fractions (DEAE-cellulose fractionation) obtained from rice and ragi

		Ferulic acid (μg/g)	Uronic acid (%)	Activity, IC_{50} (mg)	Expected activity, IC_{50} (mg)
Rice					
Water eluted fraction	N	nd	nd	5.6	–
	M	nd	nd	6.3	–
0.2 molar AC eluted fraction	N	1182.0	11.2 ± 0.3	0.47	23.7
	M	83.7	12.8 ± 0.3	1.8	334.5
Ragi					
Water eluted fraction	N	3.7	nd	4.7	–
	M	14.9	nd	4.8	–
0.2 molar AC eluted fraction	N	1641.4	14.3 ± 0.4	0.35	17.1
	M	189.5	15.9 ± 0.4	1.5	147.8

nd, not detected.

Table 3
Ferulic acid and uronic acid contents, and neutral sugar composition (%) of 0.1 molar ammonium carbonate eluted fractions obtained from rice and ragi

	Ferulic acid (μg/g)	Uronic acid (%)	Rha	Ara	Xyl	Man	Gal	Glc	A/X
Rice									
N	119.3	8.0 ± 0.2	1.7	40.2	49.4	0.0	8.7	0.0	0.81
M	1404.3	8.9 ± 0.2	2.0	44.3	46.7	0.0	7.0	0.0	0.95
Ragi									
N	146.6	12.1 ± 0.1	3.1	39.5	45.3	0.9	6.5	4.7	0.87
M	1044.6	13.7 ± 0.1	2.3	43.9	40.3	0.0	8.0	5.5	1.09

Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; A/X, arabinose/xylose.

NSP, malting resulted in an increase in arabinose content. 0.1 molar AC eluted fractions contained high amount of uronic acid (rice: N, 8.0%, M, 8.9%; ragi: N, 12.1%, M, 13.7%) and ferulic acid (rice: N, 119.3 μg/g, M, 1404.3 μg/g: ragi: N, 146.6 μg/g, M, 1044.6 μg/g) (Table 3). Interestingly, ferulic acid content of malt feraxans was 11.8 and 7.1 folds higher compared to native rice and ragi, respectively. Contrary to this, ferulic acid content of 0.2 molar AC eluted fractions was 14.1 and 8.7 folds higher in native compared to malted rice and ragi, respectively (Table 2). This could be due to the mobilization of feruloyl arabinoxylans (Rao and Muralikrishna, 2004) by induced xylanolytic enzymes during malting. These data indicated that malting results in dynamic changes in the feraxans.

Due to their very low yield, 0.1 and 0.2 molar NaOH eluted fractions were not studied in detail.

Molecular weight of 0.1 molar AC eluted fractions was determined on Sephacryl S-300 using standard T-dextran markers. Two peaks each are observed for native and malt feraxans (Fig. 3). In case of rice native feraxans, average molecular weights are 231.5 kDa (peak 1; yield, ~65%), and 24.4 kDa (peak 2; yield, ~35%). Up on malting, average molecular weight of peak 1 decreased to 75.4 kDa (yield, ~50%) and that of peak 2 has slightly increased to 39.6 kDa (yield, ~50%). Similarly, in ragi, native feraxans have an average molecular weight of 139.9 kDa (peak 1; yield, ~65%) and 15.4 kDa (peak 2; yield, ~35%). Up on malting, average molecular weight of peak 1 decreased to 38.9 kDa (yield, ~35%) and that of peak 2 remained unchanged. However, its yield has increased (~65%). These results showed that malting causes many molecular changes in feraxans (0.1 molar AC eluted fractions) due to the induction of several non-starch polysaccharidases (Nirmala et al., 2000). In particular, xylanase (~4 fold) (Fig. 4) induced during malting would act on large molecular weight feraxans, leading to decreased molecular weight (Fig. 3) and increased solubility (Table 1).

UV absorption spectra of native and malt feraxans from rice and ragi are shown in Fig. 2. Supporting the HPLC

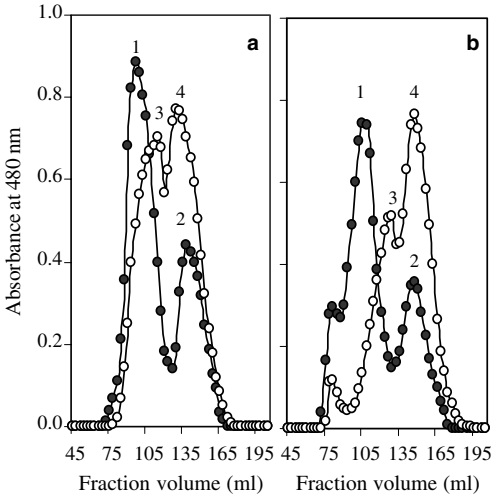

Fig. 3. Sephacryl S-300 gel filtration profile of water soluble feraxans obtained from rice (a) and ragi (b): native feraxans (●) and malt feraxans (○). Average molecular weight of peaks – rice: native peak (NP) 1, 231.5 kDa (1), NP2, 24.4 kDa (2), malt peak (MP) 1, 75.4 kDa (3) and MP2, 39.6 kDa (4); ragi: NP1, 139.9 kDa (1), NP2, 15.4 kDa (2), MP1, 38.9 kDa (3) and MP2, 15.4 kDa (4).

data, malt feraxans showed very strong absorption at ~320 nm, spectra much similar to the free ferulic acid (obtained from Sigma). Native feraxans too showed absorption (less prominent) at ~320 nm, with a slightly higher absorption at ~280 nm, perhaps due to the presence of a small amount of associated proteins.

Purified feraxans obtained on Sephacryl S-300 are further characterized by other spectroscopic methods. IR spectra (not shown) of all the individual peaks are much similar, and signals typical to arabinoxylans are observed (Subba Rao and Muralikrishna, 2004). In particular, signals at around 1730, 1635 and 1415 cm^{-1} indicated the presence of >C=O group probably from ferulic/uronic acid. Optical rotation values (rice: −5.1 to −7.4; ragi:

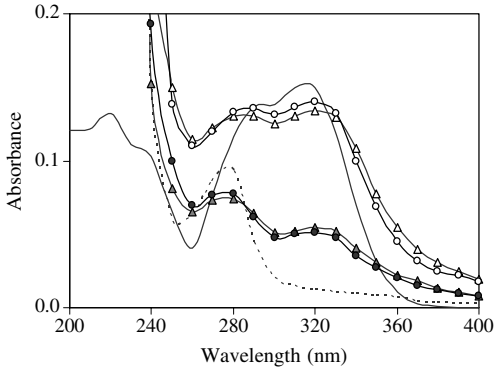

Fig. 2. UV absorption spectra of water soluble feraxans obtained from rice (circle) and ragi (triangle): native feraxans (filled), malt feraxans (open), ferulic acid (solid line) and BSA (dotted line).

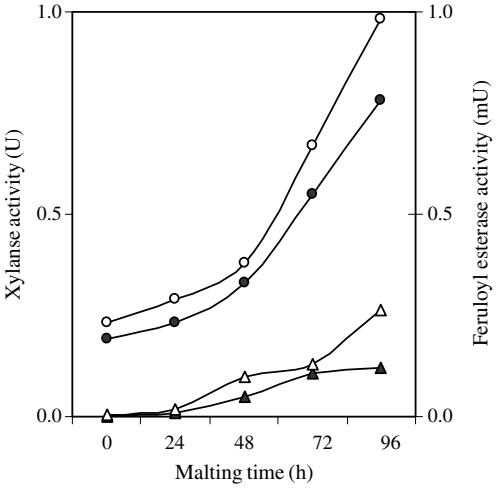

Fig. 4. Xylanase (circle) and feruloyl acid esterase (triangle) activity per gram of rice (filled) and ragi (open) flours.

−0.3 to −2.3) indicated them to be chiefly β-linked polymers. ^{1}H and ^{13}C NMR spectra (data not shown) were much similar to the spectra reported for arabinoxylans (Subba Rao and Muralikrishna, 2004).

Presence of high uronic acid, ferulic acid, galactose contents and arabinose substitution and relatively small molecular weight made water soluble feraxans from rice and ragi unique from other arabinoxylans reported (Dervilly-Pinel et al., 2001). To our knowledge, this is the first report on biochemical changes in water soluble feraxans brought about by germination.

2.2. Determination of antioxidant activity

Antioxidant activity of water soluble NSP from rice and ragi was determined by well established emulsion assay (Subba Rao and Muralikrishna, 2002). Antioxidant activity, which was expressed in IC_{50}, of water soluble NSP is given in Table 1. Synthetic antioxidants, BHA (IC_{50}, 26.4 μg) and BHT (IC_{50}, 26.2 μg) showed very strong activity. Ferulic acid too is shown to be a strong antioxidant (IC_{50}, 28.0 μg). By the virtue of their bound ferulic acid, NSP showed high antioxidant activity (Table 1). Activity pattern could roughly be correlated with the bound ferulic acid content of NSP (Table 1). However, activity of polysaccharides is roughly 48 to 58 folds (ratio of IC_{50} of ferulic acid to ferulic acid equivalent of polysaccharides) higher than the expected activity (Table 1) due to their bound ferulic acid content. Some of the possible reasons for this abnormal behavior are discussed later.

Similar to water soluble NSP, NSP fractions (fractionated on DEAE-cellulose) from rice and ragi showed high antioxidant activity with emulsion assay. Activity of water and 0.2 molar AC eluted fractions is given in Table 2. Water eluted fractions, which contained neither uronic acid nor ferulic acid (small amount of ferulic acid is detected in water eluted fractions of ragi), showed very low activity. Activity of water eluted fractions from rice might be due to the presence of very small amount of undetected ferulic acid. Contrary to water eluted fraction, with their high ferulic acid content, 0.2 molar AC eluted fractions from native rice and ragi gave very high antioxidant activity. As expected, with low amount of bound ferulic acid, 0.2 molar AC eluted fractions from malts showed lower activity. Relative activity of different NSP fractions could very well be compared with their bound ferulic acid content. However, similar to the water soluble NSP, antioxidant activity of fractions is several folds (49 to 186, for 0.2 molar AC eluted fractions) higher than the expected activity (Table 2) due to their bound ferulic acid content.

2.3. Antioxidant activity of water soluble feraxans

The antioxidant activity of fairly well characterized water soluble feraxans (0.1 molar AC eluted fractions) from rice and ragi is determined in vitro by 3 different assays namely, emulsion, DPPH* and FRAP. The IC_{50} values of soluble feraxans in emulsion and DPPH* assays and EC_1 values in FRAP assay are given in Table 4. Soluble feraxans are found to be very strong antioxidants, which could very well be explained on the basis of their molecular characteristics. Rice malt exhibited higher activity followed by ragi malt, rice native and ragi native; the order could roughly be correlated with the amount of bound ferulic acid they contain. Having less ferulic acid, rice native exhibited stronger activity than ragi native feraxans. In case of emulsion assay, activity of rice native is even higher than the ragi malt feraxans.

Both in DPPH* and FRAP assays, feraxans exhibited several folds higher activity (Table 4) than the expected activity due to their bound ferulic acid content. Moreover, activity fold increase is higher in rice native (20 to 31 folds) followed by ragi native (13 to 18 fold) feraxans. Malt feraxans showed almost equal activity fold increase (5 to 6 folds). However, while same pattern could be observed in emulsion assay, fold increase is almost 50 (for native feraxans) and 25 (for malt feraxans) times higher compared to

Table 4
Antioxidant activity of water soluble feraxans (0.1 molar AC eluted fractions) obtained from rice and ragi

	Antioxidant activity					
	Emulsion		DPPH*		FRAP	
	IC_{50} (mg)	Fold increase	IC_{50} (mg)	Fold increase	EC_1 (mg)	Fold increase
Ferulic acid	0.028		0.031		0.0059	
Rice						
N	0.163 (0.02)[a]	1400.0	8.3 (0.99)	31.1	2.4 (0.29)	20.3
M	0.156 (0.219)	127.9	4.1 (5.76)	5.4	0.76 (1.07)	5.5
Ragi						
N	0.236 (0.035)	800.0	11.4 (1.67)	18.4	3.1 (0.46)	12.8
M	0.186 (0.194)	144.3	6.0 (6.27)	4.9	0.92 (0.96)	6.2
Glucuronic acid	5.0		14.8		27.5	
Galacturonic acid	2.4		6.5		7.9	
Polygalacturonic acid	1.8		3.1		1.2	

Fold increase is the ratio of IC_{50} or EC_1 of ferulic acid to ferulic acid equivalent of polysaccharides.
[a] Values in parentheses – ferulic acid equivalent of polysaccharides in μg.

other two assays. This anomalous result could not be explained here.

2.4. Antioxidant activity of feraxans – role of saccharides

Although ferulic acid is known to be a strong antioxidant (Shahidi et al., 1992; Kikuzaki et al., 2002; Nenadis et al., 2003) and free and bound (up on alkaline hydrolysis) ferulic acid extracted from cereals is shown to have antioxidant activity (Adom and Liu, 2002; Adom et al., 2003; Subba Rao and Muralikrishna, 2002), there are no reports on the antioxidant activity of feruloyl arabinoxylans, the major ferulic acid reservoir/parent molecules in plants. However, corn bran hemicellulose fragments are shown to possess antioxidant activity, which is even higher than free ferulic acid (Ohta et al., 1994, 1997). While antioxidant activity of phenolic acids could be related to structural features such as position of hydroxyl groups and other side groups (Shahidi et al., 1992; Subba Rao and Muralikrishna, 2002; Nenadis et al., 2003; Cuvelier et al., 1992), it is believed that esterification of ferulic acid resulted in increasing activity and it could be influenced by the chain length of alcohol moiety (Kikuzaki et al., 2002). In case of feruloyl arabinoxylo-oligosaccharides, the activity is much stronger than the free ferulic acid and the activity increased with the increasing number of sugar moieties (Ishii, 1997; Ohta et al., 1994, 1997). While presence of ferulic acid is important for the activity, glycosyl group by itself showed no activity.

Our study showed that the feruloyl arabinoxylans exhibit antioxidant activity several fold higher than the activity expected due to their bound ferulic acid content and this could be explained on the basis of their molecular characteristics (Table 4) (Xue et al., 1998, 2001). While the increase in the activity might be small in low molecular weight esters (Ohta et al., 1994, 1997; Kikuzaki et al., 2002), it could be very high (several folds) in case of feraxans having very high molecular weight. For example, among the feraxans tested, although rice native contained less ferulic acid than ragi native, its higher molecular weight (NP1, 231.5 kDa and NP2, 24.4 kDa) gave stronger activity compared to ragi native (in all three assays) and stronger still, compared to ragi malt (in emulsion assay) (Table 4). In general, higher activity fold increase of native feraxans (especially rice) compared to malt is due to their higher molecular weight.

Among water soluble NSP, the high antioxidant activity-pattern that could not be correlated well with their bound ferulic acid content, might be due to the different average molecular weight of feruloyl arabinoxylans. Moreover, it is found that different antioxidants can have synergistic effects and this might be particularly true with water soluble NSP, where feruloyl arabinoxylans of different molecular nature can have a combined effect.

Further, we believe that the nature of polysaccharides such as sugar composition (Xue et al., 2001), type (α , β) of linkage, amount and nature of branching, monosaccharides' arrangements can all influence the activity. However, study is warranted to test this hypothesis.

2.5. Antioxidant activity of feraxans – role of uronic acid

Feruloyl arabinoxylans are negatively charged molecules with particularly high amount of uronic acid. This made us speculate the role of uronic acid in antioxidant activity of feraxans. We have evaluated the antioxidant activity of glucuronic, galacturonic and polygalacturonic acid by all three above-mentioned methods. Results (Table 4) showed that uronic acid by itself exhibits very strong antioxidant activity in vitro. Moreover, galacturonic acid, with different –OH group orientation, exhibits stronger activity than glucuronic acid. And consistent with our earlier explanation, being a polymer of galacturonic acid, polygalacturonic acid was a much stronger antioxidant. Therefore, the presence of uronic acid by itself (Xue et al., 1998, 2001) might impart antioxidant property to a polymer like arabinoxylan. The nature of uronic acid such as glucuronic/galacturonic/4-*O*-methyl uronic acid can further influence this property. Thus we believe that higher activity exhibited by feraxans, in part, might be due to the presence of high amount of uronic acid.

The antioxidant activity of sulfated polysaccharides reported earlier (Rupérez et al., 2002; Xue et al., 1998) was related to the presence of sulfate content and other anionic groups. Here, we showed that feruloyl polysaccharides can exhibit very strong antioxidant activity (FRAP assay: 347.7 to 1311.4 μmol $Fe^{(II)}$/mg polysaccharides), which could be 1300 to 5000 folds higher than the activity exhibited by sulfated polysaccharides (FRAP assay: 0.11 to 0.26 μmol $Fe^{(II)}$/mg polysaccharides at 37 °C) (Rupérez et al., 2002), despite the lower (~25 °C) assay temperature.

The antioxidant activity of glucose and other polysaccharides are screened by emulsion assay (Fig. 5). While

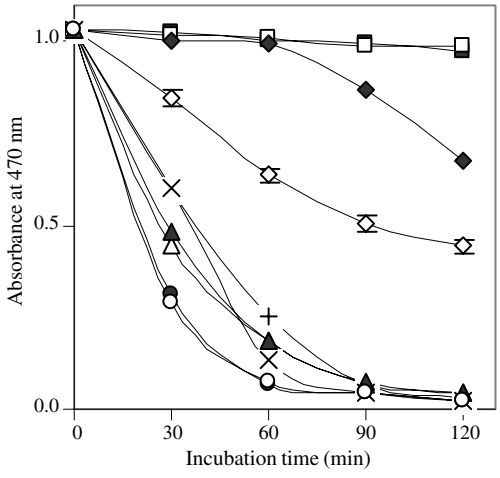

Fig. 5. Antioxidant activity (as determined by emulsion assay) of known antioxidants and neutral sugar/polysaccharides: BHA (■), BHT (□), gallic acid (◆), ethyl ferulate (◇), glucose (+), soluble starch (×), laminarin (△), xylan (▲), methanol (●) and water (○).

ethyl ferulate, gallic acid and synthetic antioxidants like BHA and BHT could exhibit strong activity, neither glucose nor soluble starch, even at very high concentrations (2 mg), showed any activity. Similarly, having no uronic acid, laminarin, a 1,3 β-D-glucan showed no activity (at 2 mg level). However, larch wood xylan (made suspension in water, as such it is insoluble) exhibited some activity [IC_{50}, 45.5 mg), perhaps due to its uronic acid content (~6.8%). Further, a number of compounds having –COOH group such as formic, acetic, propionic, butyric, succinic and citric acids are screened by all the three above-mentioned assays to see any activity that might be exerted by them. These compounds gave very low and inconsistent activity (data not shown) with all the three above-mentioned assays, indicating that the presence of >C=O group in open chain (like acetic or propionic acid) exerts no activity. However, as in phenolic acid or uronic acid, >C=O group attached to ring molecule (like phenolic or glycosyl/glucuronyl ring) can exhibit activity. Therefore, we presume that the antioxidant activity of water soluble NSP from cereals is due to the presence of feruloyl arabinoxylans and negatively charged (uronyl) moieties in arabinoxylans and not due to β-D-glucans, which contained neither ferulic acid nor uronic acid.

3. Conclusions

In summary, malting of cereal grains is shown to bring about dynamic changes in the physicochemical/structural features of water soluble feraxans. For the first time, we have shown that a widely consumed non-starch polysaccharide, i.e., water soluble feraxans from cereals can exhibit very strong antioxidant activity, which could be 5000 times higher than the activity exerted by sulfated polysaccharides. Further, we believe, apart from phenolic acids, presence of sugars with >C=O (uronyl/acetyl) groups and degree/nature of polymerization impart strong antioxidant activity to the polysaccharides. In contrary to the earlier reports (Adom and Liu, 2002; Adom et al., 2003), we have shown that the ferulic acid, a major phytochemical in cereals, can exhibit strong antioxidant activity in its bound form and thus it need not get digested and be released in the colon through the action of microflora to exert its activity (Ohta et al., 1994, 1997). Presence of good amount of antioxidants like feraxans, in colon, may be essential for scavenging cancer causing amines and nitroso-compounds formed due to protein fermentation. Moreover, as synthetic antioxidants like BHA and BHT were suspected carcinogens, ferulic acid and feraxans could be used as natural antioxidants by the food industry. Consumption of naturally occurring charged polysaccharides like water soluble feraxans might be beneficial in place of neutral (such as resistant starch and β-D-glucans) and synthetic (starch ferulate) (Ou et al., 2001) polysaccharides for maintaining good colorectal health and combating chronic diseases.

4. Experimental

4.1. Materials

Rice (*Oryza sativa* var. Jaya) and finger millet – ragi (*Eleusine coracana* var. Indaf-15) were procured from V. C. Farm of the University of Agricultural Sciences, located at Mandya, Karnataka. Fine chemicals were purchased from Sigma Chemical Company, USA. HPLC (C_{18}) and GLC (OV-225) columns were obtained from Shimadzu Corporation, Tokyo, Japan and Pierce Chemical Company, Rockford, USA, respectively. All the solvents and other chemicals used were of analytical grade.

4.2. Malting

Rice and ragi seeds were cleaned, steeped in double distilled water for 16 h at 25 °C and germinated under controlled conditions at 25 °C for 96 h in a B.O.D. incubator, as reported earlier (Nirmala et al., 2000). After germination, seeds were kilned at 50 °C for 24 h and powdered to obtain malted flour. Ungerminated seeds were powdered to obtain native flour.

4.3. Isolation, fractionation and characterization of NSP

Water soluble NSP was obtained from native and malted rice and ragi flours as described earlier (Rao and Muralikrishna, 2004). In brief (Fig. 1), flour was extracted with water (200 ml × 4 at 25 °C) and the supernatant obtained after centrifugation (3000*g* for 20 min) was precipitated with 3 volumes of ethanol. Precipitate was separated out, dialyzed (~8 kDa cutoff) and lyophilized. This water extractable NSP was further dissolved (10%, w/v) in water and insoluble portion was separated out by centrifugation. Soluble portion was heated (95 °C for 10 min) to denature enzymes and precipitate proteins. It was further centrifuged and supernatant thus obtained was dialyzed and lyophilized to obtain water soluble NSP.

Water soluble NSP was fractionated on DEAE-cellulose anion exchange column by eluting successively with water, 0.1 and 0.2 molar ammonium carbonate (AC) and 0.1 and 0.2 molar NaOH. Major (0.1 molar AC eluted) fraction was subjected to Sephacryl S-300 chromatography to determine its molecular mass. T-Dextran (T-10, T-40, T-70, T-150, T-500 and T-2000 kDa) standards were used for obtaining molecular weight calibration curve. Upon elution, fractions were dialyzed and lyophilized.

Polysaccharides were acid hydrolyzed and derivatized to determine the neutral sugar composition by GLC and ferulic acid was quantified by HPLC upon alkaline hydrolysis (Rao and Muralikrishna, 2004). Uronic acid content was quantified by Carbazole method (Dische, 1947). UV–absorption spectra of water soluble feraxans were recorded between 200 and 400 nm using a UV–Vis spectrophotometer (Shimadzu, Tokyo, Japan). IR spectra of feraxans in KBr pellets were recorded between 4000 and 400 cm^{-1}

(4 cm^{-1} resolution) using a Perkin–Elmer 2000 GC-IR spectrometer (Norwalk, USA).

4.4. Enzyme assays

Xylanase was extracted from the flour (2.5 g) with 10 ml of 0.1 molar sodium acetate buffer (pH 4.8) at 4 °C for 2 h. After centrifugation (3000*g*), supernatant was dialyzed (~8 kDa cutoff) overnight against the same buffer and assayed for enzyme activity (Nirmala et al., 2000). Enzyme extract (0.1 ml) was incubated with 1 ml (0.5%) of larch wood xylan in 0.1 molar sodium acetate buffer for 30 min at 50 °C. Reaction was stopped and the reducing sugar was quantified by dinitrosalycilic acid method (Miller, 1959). One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of xylose/min under assay conditions.

Ferulic acid esterase was extracted same as above, but using 0.05 molar tris buffer (pH 7.5) (Humberstone and Briggs, 2000). Enzyme extract (0.3 ml) was incubated with 0.1 ml (8 mmol) of ethyl ferulate in 0.05 molar tris buffer for 1 h at 37 °C. Reaction was stopped by adding 3 volumes of methanol and the ferulic acid released was quantified by HPLC (Sancho et al., 1999). One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of ferulic acid/min under assay conditions.

4.5. Determination of antioxidant activity

4.5.1. β-Carotene linoleate emulsion assay

Antioxidant activity of the samples was determined by monitoring the inhibition of coupled oxidation of β-carotene and linoleic acid (henceforth called emulsion assay) (Miller, 1971; Subba Rao and Muralikrishna, 2002). In brief, 0.1 ml of sample (aqueous solution of polysaccharides) was mixed with 2.4 ml of freshly prepared emulsion (400 μg of β-carotene in 2 ml chloroform plus 40 μl linoleic acid and 400 mg of Tween-40 were mixed well. Chloroform was evaporated by nitrogen flush. 100 ml of oxygenated (O_2) water was added to the mixture and shaken well in dark) and incubated at ~50 °C. Absorbance was read against an emulsion/sample blank (without β-carotene) at 470 nm over a 2 h period at 30 min intervals. Antioxidant activity coefficient (AAC) (Cruz et al., 1999) of the sample is expressed as the percentage inhibition of β-carotene oxidation/loss:

$$\mathrm{AAC}\ (\%) = \frac{A_{\text{sample } 120'} - A_{\text{control } 120'}}{A_{\text{control } 0'} - A_{\text{control } 120'}} \times 100,$$

where A_{control} is the absorbance of the emulsion (with out sample). Concentration providing 50% inhibition (IC_{50}) (Güllüce et al., 2003) is calculated from the graph – plotted AAC (%) against concentration.

4.6. DPPH* assay

Antiradical activity of soluble feraxans was estimated using a slight modification to the procedure reported elsewhere (Guérard and Sumaya-Martinez, 2003; Güllüce et al., 2003; Kikuzaki et al., 2002; Ohta et al., 1994). In brief, an aliquot of sample (1 ml) was added to 1 ml of a solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH*), prepared fresh, at a concentration of 80 mg/l in ethanol. After the incubation period (up to 30 min) at room temperature (~25 °C), absorbance was read against a suitable blank at 517 nm. Antiradical activity (AA) (Guérard and Sumaya-Martinez, 2003; Kikuzaki et al., 2002) of the sample is expressed as the percentage disappearance of DPPH*:

$$\mathrm{AA}\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where A_{control} is the absorbance of the DPPH* solution (1:1 dilution). IC_{50} is calculated as above.

4.7. Ferric reducing/antioxidant power (FRAP) assay

Reducing power of soluble feraxans was determined according to the existing method (Benzie and Strain, 1999; Rupérez et al., 2002). Briefly, 0.1 ml of sample was mixed with 0.9 ml of freshly prepared FRAP reagent (contained 2.5 ml of 10 mmol/l TPTZ [2,4,6-tri (2-pyridyl)-triazine] in 40 mmol/l HCl plus 2.5 ml of 20 mmol/l $FeCl_3 \cdot 6H_2O$ and 25 ml of 300 mmol/l acetate buffer, pH 6.3). After the incubation period (up to 30 min) at room temperature (~25 °C), absorbance was read against a suitable blank at 595 nm. Aqueous solution of known $Fe^{(II)}$ concentrations in the range of 100–1000 μmol/l ($FeSO_4 \cdot 7H_2O$) was used for calibration. Equivalent concentration 1 (EC_1) (Benzie and Strain, 1999) is defined as the concentration of sample having a reducing ability equivalent to that of 1 mmol/l $FeSO_4 \cdot 7H_2O$. EC_1 is calculated from the graph – plotted absorbance against concentration.

All assays were performed in triplicate and mean value was obtained. Standard deviation is given wherever appropriate.

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References

Adom, K.K., Liu, R.H., 2002. Antioxidant activity of grains. J. Agric. Food Chem. 50, 6182–6187.

Adom, K.K., Sorrells, M.E., Liu, R.H., 2003. Phytochemical profiles and antioxidant activity of wheat varieties. J. Agric. Food Chem. 51, 7825–7834.

Benzie, I.F.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27.

Bourne, L.C., Rice-Evans, C., 1998. Bioavailability of ferulic acid. Biochem. Biophys. Res. Commun. 253, 222–227.

Castelluccio, C., Paganga, G., Melikian, N., Bolwett, G.P., Pridham, J., Sampson, J., Rice-Evans, C., 1995. Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. FEBS Lett. 368, 188–192.

Charalampopoulos, D., Wang, R., Pandiella, S.S., Webb, C., 2002. Application of cereals and cereal components in functional foods: a review. Int. J. Food Microbiol. 79, 131–141.

Cruz, J.M., Domínguez, J.M., Domínguez, H., Parajó, J.C., 1999. Solvent extraction of hemicellulosic wood hydrolysates: a procedure useful for obtaining both detoxified fermentation media and polyphenols with antioxidant activity. Food Chem. 67, 147–153.

Cuvelier, M.-E., Richard, H., Berset, C., 1992. Comparison of the antioxidative activity of some acid-phenols: structure–activity relationship. Biosci. Biotechnol. Biochem. 56, 324–325.

Dervilly-Pinel, G., Thibault, J.-F., Saulnier, L., 2001. Experimental evidence for a semi-flexible conformation for arabinoxylans. Carbohydr. Res. 330, 365–372.

Dische, Z., 1947. A new specific color reaction of hexuronic acids. J. Biol. Chem. 67, 189.

Guérard, F., Sumaya-Martinez, M.-T., 2003. Antioxidant effects of protein hydrolysates in the reaction with glucose. J. Am. Oil Chem. Soc. 80, 467–470.

Güllüce, M., Sökmen, M., Daferera, D., Ağar, G., Özkan, H., Kartal, N., Polissiou, M., Sökmen, A., Şahin, F., 2003. In vitro antibacterial, antifungal and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of *Satureja hortensis* L. J. Agric. Food Chem. 51, 3958–3965.

Humberstone, F.J., Briggs, D.E., 2000. Extraction and assay of ferulic acid esterase from malted barley. J. Inst. Brew. 106, 21–29.

Ishii, T., 1997. Structure and functions of feruloylated polysaccharides. Plant Sci. 127, 111–127.

Izydorczyk, M.S., Biliaderis, C.G., 1995. Cereal arabinoxylans: advances in structure and physicochemical properties. Carbohydr. Polym. 28, 33–48.

Jacobs, D.R., Slavin, J., Marquart, L., 1995. Whole grain intake and cancer: a review of literature. Nutr. Cancer 22, 221–229.

Kawabata, K., Yamamoto, T., Hara, A., Shimizu, M., Yamada, Y., Matsunaga, K., Tanaka, T., Mori, H., 2000. Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. Cancer Lett. 157, 15–21.

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., Taniguchi, H., 2002. Antioxidant properties of ferulic acid and its related compounds. J. Agric. Food Chem. 50, 2161–2168.

Miller, G.L., 1959. Use of dinitrosalycilic acid reagent for the determination of reducing sugar. Anal. Chem. 31, 426–428.

Miller, H.E.A., 1971. A simplified method for the evaluation of antioxidants. J. Am. Oil Chem. Soc. 48, 91.

Mori, H., Kawabata, K., Yoshimi, N., 1999. Chemopreventive effects of ferulic acid and rice germ on large bowel carcinogenesis. Anticancer Res. 19, 3775–3779.

Morris, J.N., Marr, J.W., Clayton, D.G., 1977. Diet and heart: a postscript. Br. Med. J. 2, 1307–1314.

Nenadis, N., Zhang, H.-Y., Tsimidou, M.Z., 2003. Structure–antioxidant activity relationship of ferulic acid derivatives: effect of carbon side chain characteristic groups. J. Agric. Food Chem. 51, 1874–1879.

Nirmala, M., Subba Rao, M.V.S.S.T., Muralikrishna, G., 2000. Carbohydrates and their degrading enzymes from native and malted finger millet (Ragi, *Eleusine coracana*, Indaf-15). Food Chem. 69, 175–180.

Ohta, T., Semboku, N., Kuchii, A., Egashira, Y., Sanada, H., 1997. Antioxidant activity of corn bran cell-wall fragments in the LDL oxidation system. J. Agric. Food Chem. 45, 1644–1648.

Ohta, T., Yamasaki, S., Egashira, Y., Sanada, H., 1994. Antioxidant activity of corn bran hemicellulose fragments. J. Agric. Food Chem. 42, 653–656.

Ou, S., Li, A., Yang, A.A, 2001. A study on synthesis of starch ferulate and its biological properties. Food Chem. 74, 91–95.

Plaami, S.P., 1997. Content of dietary fibre in foods and its physiological effects. Food Rev. Int. 13, 29–76.

Rao, R.S.P., Muralikrishna, G., 2004. Non-starch polysaccharide–phenolic acid complexes from native and germinated cereals and millet. Food Chem. 84, 527–531.

Rupérez, P., Ahrazem, O., Leal, J.A., 2002. Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. J. Agric. Food Chem. 50, 840–845.

Sancho, A.I., Faulds, C.B., Bartolome, B., Williamson, G., 1999. Characterization of feruloyl esterase activity in barley. J. Sci. Food Agric. 79, 447–449.

Shahidi, F., Janitha, P.K., Wanasundara, P.D., 1992. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr. 32, 67–103.

Subba Rao, M.V.S.S.T., Muralikrishna, G., 2002. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Eleusine coracana* Indaf-15). J. Agric. Food Chem. 50, 889–892.

Subba Rao, M.V.S.S.T., Muralikrishna, G., 2004. Structural analysis of arabinoxylans isolated from native and malted finger millet (*Eleusine coracana*, ragi). Carbohydr. Res. 339, 2457–2463.

Willett, W.C., 1994. Diet and health: what should we eat? Science 264, 532–537.

Xue, C., Fang, Y., Lin, H., Chen, L., Li, Z., Deng, D., Lu, C., 2001. Chemical characters and antioxidative properties of sulfated polysaccharides from *Laminaria japonica*. J. Appl. Phycol. 13, 67–70.

Xue, C., Yu, G., Hirata, T., Terao, J., Lin, H., 1998. Antioxidative activities of several marine polysaccharides evaluated in a phosphatidylcholine–liposomal suspension and organic solvents. Biosci. Biotechnol. Biochem. 62, 206–209.

Zhao, Z., Egashira, Y., Sanada, H., 2003. Digestion and absorption of ferulic acid sugar esters in rat gastrointestinal tract. J. Agric. Food Chem. 51, 5534–5539.