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Arabinoxylan from finger millet (*Eleusine coracana*, *v.* Indaf 15) bran: Purification and characterization

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ABSTRACT

Water unextractable portion from finger millet bran was sequentially extracted with saturated barium hydroxide (BE) and 1 M potassium hydroxide (KE) solutions. They consisted preponderantly of arabinose and xylose in different ratios. Ferulic, caffeic, coumaric and vanillic acids were identified as major bound phenolic acids. BE and KE were purified on DEAE-cellulose column by eluting successively with different eluants. The major fractions (0.1 M ammonium carbonate) were resolved into one (BE) and two subfractions (KE1 and KE2) respectively on Sephacryl S-400 gel filtration chromatography and their homogeneity was ascertained by gel filtration, cellulose acetate membrane electrophoresis and capillary electrophoresis. The average molecular weight of BE, KE1 and KE2 were found to be 430, 1028 and 40 kDa respectively. The structural elucidation of the purified polysaccharides by ^{1}H and ^{13}C NMR analysis indicated the backbone to be 1,4-β-D-linked xylan with substitution mainly at O-2 or O-3 and/or both by α-L-arabinose residues.

1. Introduction

Finger millet (*Eleusine coracana*), one of the widely used millets in southern part of India has many nutritional benefits because of its high content of calcium (0.34%) and dietary fiber (18%) which are higher compared to other cereals such as wheat, maize, rice and barley (Kamat & Belavady, 1980; Ravindran, 1991). Finger millet contains less protein and fat but, it contains highest amount of methionine, among cereal grains. It is consumed as whole flour, thereby retaining the fiber, phenolics, minerals and vitamins present in the outer layer of the grain. Hence, present day research is focused in identifying newer or natural sources of nutraceuticals and nutritional materials having desirable functional properties. Dietary fiber is classified into broad category, which includes constituents such as cellulose, pectin, arabinoxylan, lignin, β-glucan, etc. (DeVries, 2004; Slavin, 2008).

Among the dietary fiber components arabinoxylan (AX), has been identified in all major cereal grains such as wheat, rye, barley, oat, rice, sorghum bajra and ragi and also in some other plants such as psyllium peels, pongal grass, bamboo shoots and rye grass (Fischer et al., 2004; Izydorczyk & Biliaderis, 1995). They are also described as pentosans and are vital in cell wall formation. Additionally, they are important in determining the end use quality of cereal-based food products (Finnie, Bettge, & Morris, 2006; Hartmann, Michael, & Peter, 2005; Revanappa, Nandini, & Salimath, 2010). They are of growing importance because of their health-beneficial effects in alleviating disease symptoms such as colon cancer, atherosclerosis, and diabetes (Biliaderis, Izydorczyk, & Rattan, 1995; Lu, Walker, Muir, & O'Dea, 2004). Being potent natural immunomodulators and prebiotics, they are also considered as functional food ingredients (Charalampopoulos, Wang, Pandiella, & Webb, 2002). Arabinoxylans are composed of a main chain of (1 → 4)-linked β-D-xylopyranosyl residues occasionally with various degrees of substitution at O-2 or O-3 or at both O-2 and O-3 by arabinose and sometimes with uronic acid residue. Usually cinnamic acid derivatives such as ferulic and coumaric acids are substituted at O-5 of arabinose residues (Fincher & Stone, 1986).

The structural features and molecular weight of polysaccharides are of fundamental importance since they influence certain physicochemical and functional properties of polymers (Izydorczyk, 2005). Hence the understanding of the structure require a high-purity arabinoxylan preparation without any contamination from other associated cell–wall components such as contaminating starch, (1 → 3/1 → 4)-β-D-glucans, cellulose, lignin and associated proteins. Most of the arabinoxylans are water unextractable and can only be extracted with alkali. An efficient method that facilitates the fractionation and characterization of cereal AX was introduced in early nineties (Gruppen, Hamer, & Voragen, 1992) which involves saturated barium hydroxide as well as potassium hydroxide solutions has become the method of choice for extraction of water unextractable AX from wheat, rye, barley and corn

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fiber (Izydorczyk, Macri, & MacGregor, 1997). However it was not used with respect to isolation of finger millet arabinoxylans.

A purified arabinoxylan was isolated from native and malted finger millet by 10% NaOH, followed by ammonium sulphate precipitation, DEAE cellulose column chromatography and gelfiltration and was structurally characterized (Subba Rao & Muralikrishna, 2006). Earlier study from finger millet has indicated several AXs varying in their molecular weight and charge. Of late pure ragi bran is being incorporated in several of the low fiber rich foods in India. Water unextractable AX the major dietary fiber component enriched mainly in ragi bran is considered as functional food. Since there is no information available on the water unextractable AXs of ragi bran, emphasis is laid in the present communication on the barium hydroxide as well as potassium hydroxide isolated AXs with respect to their purification and structural characterization.

2. Materials and methods

2.1. Materials

Finger millet (*v.* Indaf-15) seeds were procured from Vishweshwaraiah Canal (VC) farm of the University of Agricultural Sciences, Mandya, Karnataka, India. DEAE-cellulose (0.99 m equiv./g, medium flow), sugar standards: rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, galacturonic acid, and inositol were obtained from ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, OH. Dextran standards were from Hi-Media, Pvt. Ltd. Mumbai, India. All other chemicals used in this study were of analytical grade. Gas chromatography column, OV-225 (3%, 3.2 mm × 15.2 cm), was purchased from Pierce Chemical Co., Rockford, IL. Beckman Microzone cellulose acetate electrophoresis unit and cellulose acetate membranes were obtained from Beckman Instruments International, S.A., Geneva, Switzerland. A plain uncoated silica capillary column was procured from Prince Co., Emmen, Netherlands.

2.2. Isolation of alkali soluble AX

Finger millet seed was milled as described (Shobana, Harsha, Platel, Srinivasan, & Malleshi, 2010) to obtain endosperm and bran. Before extraction of alkali soluble AX from bran, contaminants such as free sugars, starch, water and 0.5% EDTA-extractable polysaccharides (pectins) were removed (Subba Rao & Muralikrishna, 2001). In brief, flours (100 g) were extracted with 70% aqueous ethanol (500 ml × 3) followed by treating the residue successively with (a) water at room temperature, (b) glucoamylase (500 mg) at 55 °C in 0.05 M acetate buffer, pH 4.5 for 48 h and (c) 0.5% EDTA at 85 °C for 2 h followed by sequential extraction with saturated barium hydroxide and 1 M potassium hydroxide as described (Gruppen, Hamer, & Voragen, 1991; Izydorczyk et al., 1997) with slight modification. In brief the water insoluble residue (10 g) was extracted with saturated aqueous barium hydroxide (1 L) containing sodium borohydride (1%, w/v) to prevent alkaline degradation. The suspension was stirred for 16 h at room temperature (RT, 27 ± 1 °C) followed by centrifugation (5000 × *g*, 10 min). The residue was re-extracted with reduced volume of saturated aqueous barium hydroxide (500 ml) for 2 h at 27 ± 1 °C and centrifuged (5000 × *g*, 10 min). Both the extracts were pooled and acidified to pH 5.0 with glacial acetic acid (50%), concentrated, and precipitated with 3 volumes of ethanol. The resulting precipitate was dissolved in water and dialyzed extensively against water and then freeze dried and was named as "BE". The insoluble residue obtained after barium hydroxide extractions was further extracted with successive treatments of 1 M KOH {(a) 400 ml for 16 h and (b) 200 ml for 2 h at RT} in the presence of 1% (w/v) sodium borohydride. Further processing of the KOH extractions was done as per the procedure followed for barium hydroxide extraction and the resultant precipitate was designated as "KE". After freeze-drying of samples, total sugar (McKelvy & Lee, 1969) protein (Bradford, 1976), uronic acid (Blumenkrantz & Asboe-Hansen, 1973) and bound phenolic acids contents were determined (Nordkvist, Salomonsson, & Aman, 1984).

2.3. Neutral sugar composition

The isolated polysaccharides were suspended in water 0.5 ml and solubilized with concentrated 0.6 ml sulfuric acid for 2 h at ice-cold temperature. The sulfuric acid concentration in the reaction mixture was brought down to 8% with the addition of water, and the hydrolysis was carried out by refluxing in a boiling water bath for 12 h. After hydrolysis, the above mixture was neutralized with solid barium carbonate and the resultant white barium sulfate precipitate was removed by filtration through Whatman no. 1 filter paper. The filtrate thus obtained was concentrated, deionized with Amberlite (IR 120-P), and used for the preparation of alditol acetates.

2.3.1. Preparation and analysis of alditol acetates

Alditol acetates were prepared as previously described (Sawardekar, Slonekar, & Jeanes, 1965). Briefly, the pH of the concentrated filtrate containing monosaccharides, obtained from the above step, was adjusted to 7.0 with 1 M sodium carbonate, and the reduction was carried out by adding 10 mg anhydrous sodium borohydride followed by incubation at room temperature for 6 h. Excess sodium borohydride was neutralized with 2 N acetic acid, and the liberated borate ions were removed by repeated co-distillation with methanol. Acetylation was carried out in a boiling water bath for 2 h by adding 1 ml each of anhydrous acetic anhydride and pyridine. Excess acetylating reagents were removed by flash evaporation. The alditol acetates obtained were dried by repeated washing with water followed by toluene. Purified alditol acetates thus obtained were extracted with 1 ml of chloroform, dried, and analyzed on a 3% OV-225 column using a Shimadzu 15-A gas–liquid chromatograph equipped with a flame ionization detector and a Spinchrome PC based data acquisition system was used to data acquisition. The analysis was performed at 250 °C column temperature and 200 °C injector and detector temperatures with N_2 (40 ml/min) as carrier gas. The column was calibrated, by injecting a sugar standard mixture containing rhamnose, arabinose, xylose, mannose, galactose, glucose, and inositol. Sugars present in the samples were identified by comparing their relative retention times with standards.

2.4. DEAE-cellulose chromatography

BE and KE were fractionated on a DEAE-cellulose column (carbonate form, 3.5 cm × 25 cm) (Neukom & Kuendig, 1965). Polysaccharides 500 mg were eluted stepwise with water, ammonium carbonate (AC 0.1 and 0.2 M), and sodium hydroxide (NaOH 0.1 and 0.2 M) at 60 ml/h. Fractions of 10 ml were collected, assayed for total sugar by phenol-sulfuric acid method. Fractions containing carbohydrates were pooled, dialyzed, and freeze-dried.

2.5. Gel permeation chromatography

Gel permeation chromatography was performed as described (Izydorczyk & Biliaderis, 1993). Polysaccharides 10 mg were dissolved in 1 ml of 0.1 M NaCl containing 0.05% sodium azide and were loaded on precalibrated Sephacryl S-400 column (1.6 cm × 92 cm) and eluted using 0.1 M NaCl containing 0.05% sodium azide. Fractions (4 ml) were collected using an LKB Bromma

Table 1
Yields, total sugar, uronic acid protein contents and sugar composition of BE and KE (CA: caffeic acid, VA: vanillic acid, COA: coumaric acid, FA: ferulic acid).

Sample	Yield %	Total sugar %	Uronic acid %	Protein %	Ara:Xyl	Phenolic acids (relative ratio)			
						CA	VA	COA	FA
BE	2.9	75.0	5.1	6.8	1.0:0.8	1.0	1.1	1.0	1.9
KE	2.1	73.0	8.6	5.7	1.0:1.2	1.5	4.9	2.2	3.6

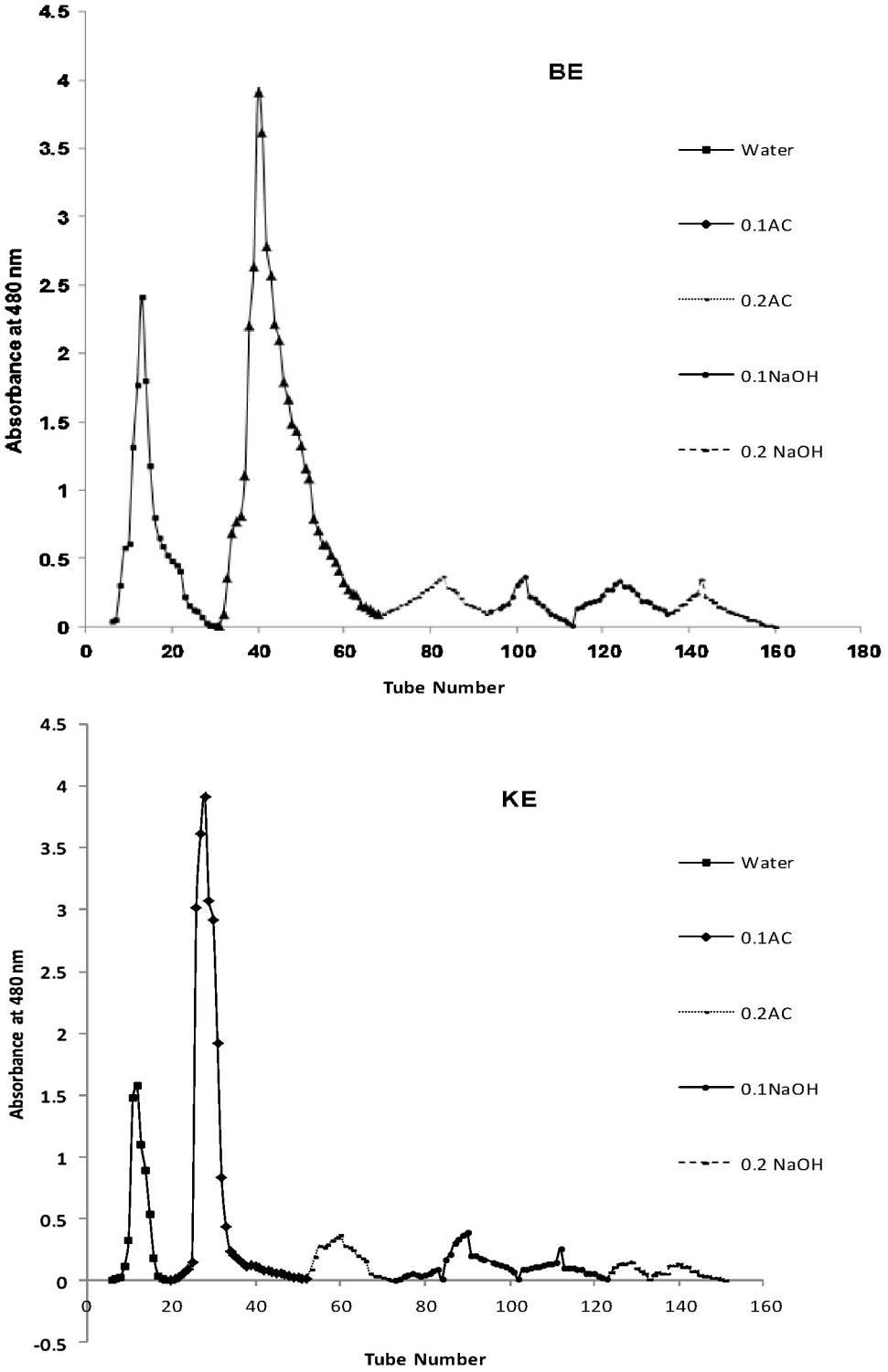

Fig. 1. Fractionation of BE and KE polysaccharide on DEAE-cellulose.

2211 fraction collector at a 16 ml/h flow rate, and aliquots were analyzed by the phenol-sulfuric acid method. Fractions containing carbohydrate were pooled, dialyzed, and freeze-dried.

2.6. Cellulose acetate electrophoresis

The electrophoretic mobility of polysaccharides was analyzed by using a Beckman Microzone electrophoretic cell (model R 101) and cellulose acetate membranes (Anderson, Hendrie, Miller, & Munro, 1971). In brief, from the purified polysaccharides (10 mg/ml), 10 μl were loaded onto a cellulose acetate membrane, prewetted with running buffer of 50 mM ammonium carbonate–NaCl buffer, pH 9.3, using an applicator and electrophoresed at constant voltage (180 V) for 30 min. The rate of migration was monitored by using porcion red dye, and the polysaccharides were visualized by staining with ruthenium red.

2.7. Capillary electrophoresis (CE)

Purified polysaccharides (0.1%, 10 μL) were dissolved in 0.2 M sodium borate buffer, pH 9.3 and were analyzed on a pure silica column (75 μm × 100 cm) maintained at 100 m bar pressure and 20 kV voltages using a Prince CE 560 model CE unit. Prior to analysis, the column was thoroughly washed with 0.1 M sodium hydroxide and equilibrated with 0.2 M sodium borate buffer, pH 9.3. Elution of the polysaccharides was monitored at 253 nm using a UV detector (model lambda 1010).

2.8. ^{13}C and ^{1}H NMR spectroscopy

^{13}C and ^{1}H NMR of purified polysaccharides were carried out by taking samples in D_2O as described (Hoffman, Geijtenbeek, Kamerling, & Vliegenthart, 1992; Hoffman, Roza, Maat, Kamerling, & Vliegenthart, 1991) (2 ml × 1 ml each, 1 ml). The deuterium resonance was used as a field frequency lock and the shifts were referenced to external TMS. The spectra were recorded in a Bruker AQS 500 MHz NMR spectrometer (5 mm BBO probe) operating at 27 °C for 4 h using spectral width of 26,455 Hz was set with offset frequency of 12,505 Hz with 10,240 scans for ^{13}C NMR whereas for ^{1}H NMR the width of 10,330 Hz was set with offset frequency of 2351.4 Hz with 64 scans.

2.9. FTIR spectroscopy

Purified polysaccharides (10 mg) was blended with dry KBr to prepare a homogeneous pellet, which was applied to the FTIR window and the spectra was recorded between 400 and 4000 cm^{-1} in Nicolet 5700 FTIR spectrometer.

3. Results and discussion

3.1. Isolation of alkali soluble AX

Alkali extractable fractions i.e. BE and KE were obtained by extraction of finger millet bran residue (left over after removal of cold water, hot water and EDTA extractable polysaccharides) sequentially with saturated barium hydroxide and 1 M potassium hydroxide respectively. Yield, total sugar, uronic acid, protein contents, sugar composition and phenolic acid are given in Table 1. The yield (2.9%) and total sugar (75%) of BE was higher compared to KE whereas uronic acid was relatively high (8.6%) in KE fractions. BE and KE prepondently consisted of arabinose and xylose, however KE in addition to the above sugars consisted minor amounts of galactose and glucose (data not shown). Glucose might have originated either from resistant starch or from mixed linked 1,3/1,4 β-D glucans. Ferulic (FA), caffeic (CA), coumaric (COA) and vanillic (VA) acids were found to be the major bound phenolic acids in both BE and KE, out of which ferulic acid was the most preponderant.

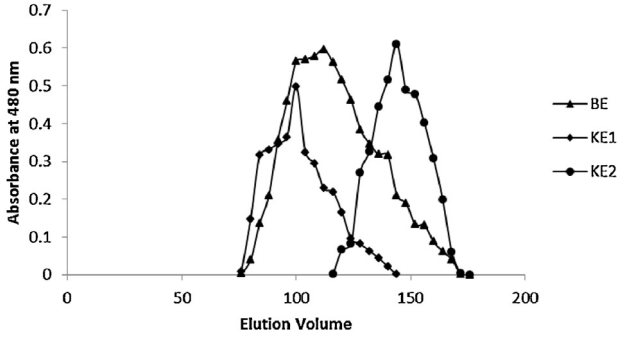

Fig. 2. Elution profile of 0.1 M AC eluted fractions of BE and KE from DEAE-cellulose on Sephacryl-S-400.

3.2. Fractionation on DEAE-cellulose

BE and KE were further fractionated on DEAE-cellulose by eluting with water, AC (0.1 and 0.2 M) and NaOH (0.1 and 0.2 N) into five major fractions (Fig. 1). Yield, total sugar, uronic acid content and sugar composition of water, AC 0.1 and 0.2 fractions are given in Table 2. Neutral sugar composition analysis of these fractions indicated arabinose, xylose, galactose and glucose in different ratios. 0.1 M AC eluted fraction from BE and KE fractions were obtained in relatively higher (42% and 39% respectively), yield. These fractions found to be completely soluble, rich in arabinose and xylose (95 and 90% respectively) and uronic acid (5.1% in BE and 8.8% in KE), which were tested for homogeneity criteria as described below. Composition of 0.1 and 0.2 N NaOH eluted fractions was not carried out because of their low yields.

3.3. Purity, molecular weight and sugar composition of 0.1 M AC eluted fractions

0.1 M AC eluted fractions of BE and KE obtained from DEAE Cellulose resolved into one (BE) and two peaks (KE1 and KE2) respectively on Sephacryl S-400 (Fig. 2). The molecular weight of BE was found to be 430 kDa, which was higher (380 kDa) compared to the one from Chinese Black-grained wheat bran (Sun, Cui, Gu, & Zhang, 2011). KE1 and KE2 have molecular mass of 1028 and 40 kDa respectively. High molecular weights were reported for purified arabinoxylans (AXs) obtained from wheat bran (Gruppen et al., 1992), barley (MacGregor & Fincher, 1993), rice, and sorghum (Verbruggen, Beldman, & Voragen, 1995). Molecular weight of 1200 and 1028 kDa of AXs were reported from native and malted finger millet with arabinose to xyloses ratio of 1.0:1.0 and 1.0:0.83 respectively (Subba Rao & Muralikrishna, 2004). Hence the AXs from ragi bran reported in the present study are of relatively low molecular weight compared to native and malted finger millet reported earlier (Subba Rao & Muralikrishna, 2004).

Table 2
Fractionation of BE and KE extract on DEAE cellulose.

Sample	Fractions	Yield %	Total sugar %	Uronic acid %	Ara:Xyl
BE	Water	6.0	46.0	1.8	2.1:1.0
	AC 0.1 M	45.0	95.0	5.2	1.0:0.7
	AC 0.2 M	10.5	40.0	2.1	1.0:0.8
KE	Water	10.0	35.0	1.1	1.0:1.0
	AC 0.1 M	40.0	90.0	8.8	1.0:1.2
	AC 0.2 M	15.0	42.0	4.1	1.0:2.5

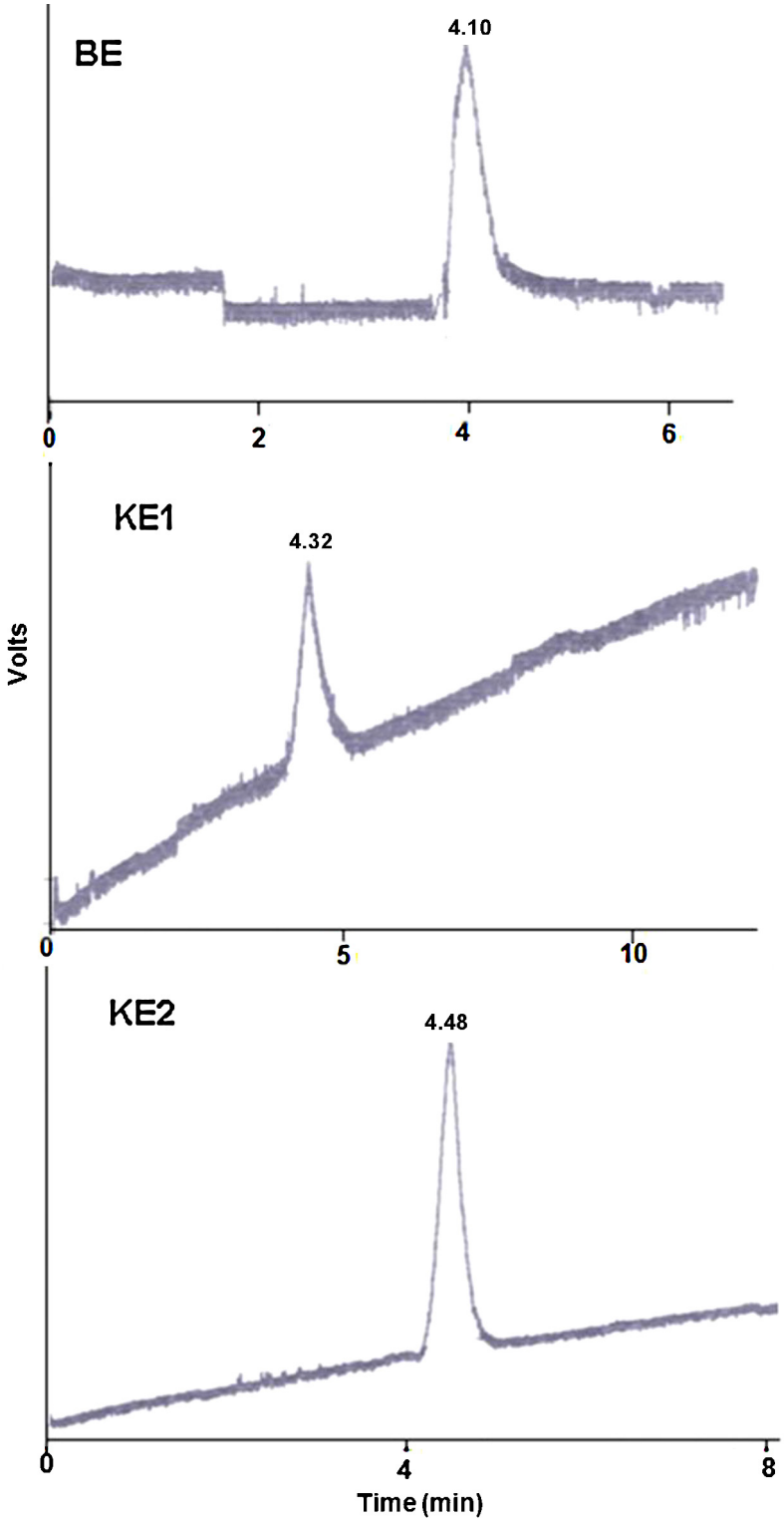

Fig. 3. Capillary electrophoresis of purified arabinoxylans (BE, KE1 and KE2).

3.4. Cellulose acetate electrophoresis

Purified AXs contain a significant amount of uronic acid (5.1, 8.8 and 5.8% for BE, KE1 & KE2 respectively,) hence the purity was also ascertained by their behavior under electric field on cellulose acetate electrophoresis. All three purified BE, KE1 and KE2 moved as single bands, indicating their homogeneity (data not shown). Cellulose acetate electrophoresis is used to determine the purity of pectic polysaccharides (Stephen, 1983).

3.5. Capillary electrophoresis

Homogeneity of pectic polysaccharides was also documented by using capillary electrophoresis (Martin et al., 2002), so the same

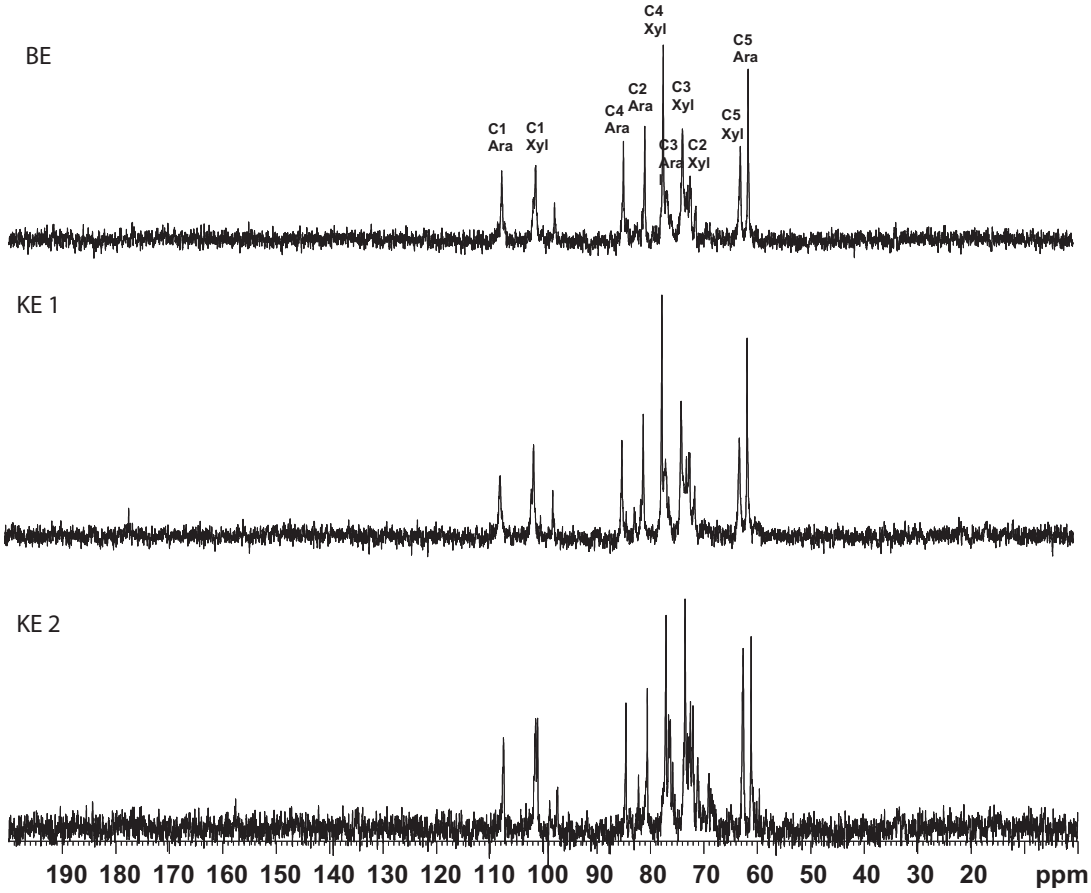

Fig. 4. ^{13}C NMR spectra of purified arabinoxylans (BE, KE1 and KE2).

method was adopted to check the homogeneity of purified arabinoxylans, since they contained significant amounts of uronic acid (Fig. 3). The BE, KE1 and KE2 were eluted as single symmetrical peaks at 4.1, 4.32 and 4.48 min respectively. The early elution of BE arabinoxylan could be due to its low uronic acid content when compared to KE1 and KE2. Capillary electrophoresis (CE) is one of the methods used routinely to establish the purity of charged components (Karger, Chu, & Foret, 1995).

3.6. ^{13}C and ^{1}H NMR spectroscopy analysis

^{13}C and ^{1}H NMR spectra of pure arabinoxylans of BE, KE1 and KE2 were analyzed to determine their primary structure. According to the characteristic signals, the spectra of the polysaccharides were completely assigned and the respected chemical shifts of signals were summarized in Table 3. These signals obtained were assigned by comparing with previously reported data (Gruppen et al., 1992; Hoffman et al., 1992; Subba Rao & Muralikrishna, 2004). The spectrum of all three purified samples showed almost similar signals with slight variation in peak intensity. In ^{13}C NMR the resonance in the region of 107.4 and 101.4 ppm was assigned to anomeric carbon atoms of α-L-arabinofuranose and β-D-xylopyranose, respectively and data also indicated the presence of xylose in mono or disubstituted forms (Fig. 4). C-2 to C-4 signals of arabinose and xylose were observed between 71 and 85 ppm and signals at 61.2 and 62.8 ppm are assigned for C-5 of arabinose and xylose, respectively. The signal for (C=O) carbonyl group of 4-O-Me-a-D-glucuronic acid is detected at around 176.8 ppm (Nandini & Salimath, 2001), however this was not prominent in BE extract due to low content of uronic acid.

^{1}H NMR spectra of all three purified arabinoxylans indicated a prominent signal at 5.4 ppm, representing the anomeric protons of arabinose substituted at O-3 of xylose residues, while the two peaks at around 5.22 and 5.29 ppm are from the terminal arabinose substituents linked to both C-2 and C-3 of the same xylose residue, respectively, indicating presence of doubly substituted xylose residues (Hoffman et al., 1992; Subba Rao & Muralikrishna, 2004). Differences in the structural features of AXs, due to the degree of branching, spatial arrangement of arabinosyl substituents along the xylan backbone and the ferulic acid content, can affect the functional properties and biological activities in particular. Even though AXs from various sources have the same basic structure, they differ in the substitution pattern of the xylan backbone that may lead to changes in their conformation, molecular weight and the capacity of AX molecules to interact with each other and with other polysaccharides.

3.7. FTIR spectroscopy analysis

FTIR spectra of BE, KE1 and KE2 are shown in Fig. 5 The bands in the region 3390 cm^{-1} indicated the hydroxyl stretching vibrations of polysaccharides and water involved in hydrogen bonding. The band in the region 2932.5 cm^{-1} is due to C—H stretching vibrations. The high arabinose substitution at C-3 of xylose residues that is low intensity shoulder at 990 and 1164 cm^{-1} and the loss of peak multiplicity in 1120–1000 cm^{-1}, is a typical characteristic of highly substituted arabinoxylans. The signal at 1744 cm^{-1} can be attributed to the carbonyl group stretching of uronic acid carboxyl and its is not prominent in BE due to low content of uronic acid. The remaining signals in the spectra at 1409.8, 1324, 1043 cm^{-1}

Table 3
Assignment of chemical shifts for the resonance of gylcosyl residues of purified BE, KE1 and KE2 in ^{13}C and ^{1}H NMR spectra.

Glycosyl residues	Chemical shifts (^{13}C), δ (ppm)	Gycosyl residues	Chemical shifts (^{1}H) δ (ppm)
α-L-Ara-f	107.4	α-L-Ara-f (0–3) mono	5.40
β-D-Xyl-p	101.4	α-L-Ara-f (0–2) di	5.21
C2,3,4-Ara & Xyl	71–85	α-L-Ara-f 0–3) di	5.29
C5-Ara	61.0	β-D-Xyl-p	4.59
C5-Xyl	62.8	H2,3,4,5-Ara & Xyl	3.66–4.9
C1 & C6 (C=O) of Uronic acid	97.4 & 176.8		

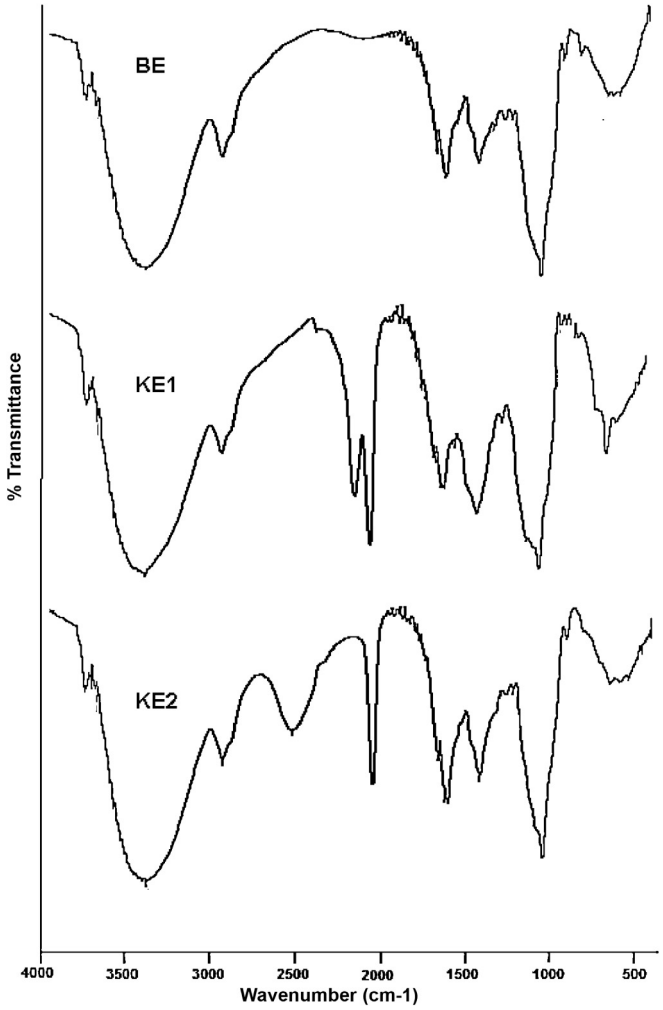

Fig. 5. FT-IR spectra of purified arabinoxylans (BE, KE1 and KE2).

are due to $-CH_2$, $-CH_2-OH$, and $-C-OH$, respectively. Absorptions in the regions 800–1450 cm^{-1} are due to C—C, C—O, C—OH and C—O—C bending vibrations (Nandini & Salimath, 2001; Subba Rao & Muralikrishna, 2004). The non-significant absorption at 2044 and 2138 cm^{-1} for KE1 and KE2 respectively are either due to C—O stretch of complex inorganic element like calcium or it might be due to residual solvent used during process.

4. Conclusions

The water and 0.5% EDTA unextractable polysaccharides from finger millet bran were isolated sequentially with saturated barium hydroxide and 1 M potassium hydroxide in 2.9 and 2.1% yields respectively and composed mainly of arabinose and xylose. The purified arabinoxylans exhibited an average molecular weight (Mw) of 460, 1028 and 40 kDa for BE, KE1 and KE2 respectively. The structural characterization of these purified arabinoxylan by ^{13}C NMR, ^{1}H NMR and FTIR indicated that they are made up of β-D-(1–4)-linked xylan backbone, which were mono and disubstituted with α-L-arabinofuranosyl groups at O-3, O-2 and both O-2 and O-3 positions along with uronic acid as well as phenolic acid residues in the side chain. The reported arabinoxylans in the present communication are relatively low molecular weight arabinoxylans compared to the ones reported earlier from finger millet and other continental cereals.

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