STUDIES ON WATER EXTRACTABLE FERULOYL POLYSACCHARIDES FROM NATIVE AND GERMINATED RICE (*Oryza sativa*) AND RAGI (*Eleusine coracana*)

A thesis submitted to the
UNIVERSITY OF MYSORE

For the award of the degree of
DOCTOR OF PHILOSOPHY

In
BIOCHEMISTRY

By
R. SHYAMA PRASAD RAO, *M.Sc.*

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
MYSORE – 570 020, INDIA

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DECLARATION

I declare that the thesis entitled **"STUDIES ON WATER EXTRACTABLE FERULOYL POLYSACCHARIDES FROM NATIVE AND GERMINATED RICE (***Oryza sativa***) AND RAGI (***Eleusine coracana***)"** submitted to the **UNIVERSITY OF MYSORE** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** is the result of the work carried out by me under the guidance of **Dr. G. MURALIKRISHNA**, scientist E-II, department of Biochemistry and Nutrition during the period of November 2000 – September 2005. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

(R. SHYAMA PRASAD RAO)

Date:

Place: Mysore

Dr. G. MURALIKRISHNA **Date:**
Scientist E-II
Department of Biochemistry and Nutrition

CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON WATER EXTRACTABLE FERULOYL POLYSACCHARIDES FROM NATIVE AND GERMINATED RICE (***Oryza sativa***) AND RAGI (***Eleusine coracana***)"** submitted by **R. SHYAMA PRASAD RAO** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** to the **UNIVERSITY OF MYSORE** is the result of the research work carried out by him in the department of Biochemistry and Nutrition, under my guidance during the period of November 2000 – September 2005.

(Dr. G. MURALIKRISHNA)
GUIDE

to

my esteemed teachers

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(R. SHYAMA PRASAD RAO)

CONTENTS

ABBREVIATIONS

Abbreviations	Expansions
α	Alpha
β	Beta
δ	Delta
μ	Micro
°C	Degree centigrade
η_r	Relative viscosity
4-O-Me	4-O-Methyl
Ara	Arabinose
AA	antiradical activity
AAC	Antioxidant activity coefficient
AACC	American Association of Cereal Chemists
Ac	Acetyl
AC	Ammonium carbonate
BCP	Bromo cresol purple
BHA	Butyrated hydroxy anisole
BHT	Butyrated hydroxy toluene
BOD	Biological oxygen demand
BSA	Bovine serum albumin
BU	Brabender unit
BV	Breakdown viscosity
cm	Centimeter
CA	Coumaric acid
CPV	Cold paste viscosity
D_2O	Deuterium oxide
DDT	Dough development time
DEAE	Diethyl amino ethyl
DF	Dietary fibre
DMSO	Dimethyl sulphoxide
DP	Degree of polymerization
DPPH*	1,1-diphenyl-2-picrylhydrazyl
EC_1	Equivalent concentration 1
EDTA	Ethylene diamino tetra acetic acid
Em	Maximum extensibility
EV	Electron volts
f	Furanose
FA	Ferulic acid
FRAP	ferric reducing antioxidant power
FU	Farinograph unit
Fuc	Fucose
fxn	Feraxan
g	Grams
Gal	Galactose

GalA	Galacturonic acid
GLC	Gas liquid chromatography
GlcA	Glucuronic acid
Glc	Glucose
GPC	Gel permeation chromatography
GT	Gelatinization temperature
h	Hour
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography
HPV	Hot paste viscosity
IC_{50}	50 % inhibition concentration
IR	Infra red
kDa	kilo Dalton
L	Litre
M	Malt
MALDI-TOF	Matrix assisted laser desorption ionization – time of flight
Man	Mannose
Me	Methyl
Min	Minute
mm	Millimeter
MS	Mass spectroscopy
N	Native
$NaBD_4/NaBH_4$	Sodium borodeuteride/borohydride
nd	Not detected
ND	Not determined
nm	Nanometer
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
OD	Optical density
p	Para
p	Pyranose
PMAA	Permethylated alditol acetate
PMR	Proton magnetic resonance
ppm	Parts per million
Rha	Rhamnose
R_m	Maximum resistance
SCFA	Short chain fatty acid
SDF	Soluble dietary fibre
Sec	Second
SV	Set back viscosity
TCA	Trichloro acetic acid
TFA	Trifluoro acetic acid
TI	Tolerance index
TPTZ	2,4,6-tri (2-pyridyl)-triazine

U	Unit (s)
UV	Ultra violet
v/v	Volume/volume
w/v	Weight/volume
WEP	Water extractable non-starch polysaccharides
WUP	Water unextractable non-starch polysaccharides
$_{x}$ g	Gravity
Xyl	Xylose

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Synopsis of the thesis submitted for the award of Ph. D. degree (Biochemistry) of the University of Mysore, Mysore, India

Title of the thesis 'Studies on water extractable feruloyl polysaccharides from native and germinated rice (*Oryza sativa*) and ragi (*Eleusine coracana*)'

Candidate R. Shyama Prasad Rao

Carbohydrates are the principal components/macronutrients of food, and apart from providing bulk of the caloric intake they play a variety of functions in human food and nutrition. In particular, arabinoxylans have attracted the attention of many researchers because of the complexity of their structure-function relationships. They are the major non-starch polysaccharides in cereals and represent bulk of the soluble and insoluble dietary fiber (unavailable/un-digestible carbohydrates) intake in human food. Nutritionally, dietary fibers/non-starch polysaccharides are known to exert many physiological/metabolic effects in reducing the risks of diseases (known as diseases of lifestyle/civilization) such as diabetes, obesity, atherosclerosis, hypertension, constipation, diverticulosis, colorectal cancer and so on. Due to their various physicochemical properties, water soluble non-starch polysaccharides (NSP), mainly arabinoxylans, are also known to have many functional roles in human food.

Although general structure of arabinoxylans is known from many cereals, detailed investigations pertaining to the water soluble arabinoxylans/feruloyl arabinoxylans (feraxans) are sparse. Moreover, large variations in the fine structure of arabinoxylans isolated from various cereals are observed and they in turn may have influence on their physicochemical/functional roles. Thus for better understanding of fine structure of arabinoxylans and for their utilization in human food and nutrition with precise functional effects, arabinoxylans are characterized from diverse sources and conditions.

The present study is taken up as there was no detailed investigation on the water soluble feraxans from rice and ragi, the major cereal and millet respectively. Structural and functional characterizations of water soluble feraxans from these two cereal grains are investigated with the following objectives:

(a) Isolation and preliminary characterization of water extractable feruloyl polysaccharides from native and germinated rice and ragi,

(b) Fractionation and purification of water extractable feruloyl polysaccharides,

(c) Structural characterization of purified polysaccharides using methylation, GLC-MS analysis, Smith degradation and ^{13}C-NMR and

(d) Functional properties of water soluble feruloyl polysaccharides i.e., viscosity, gelling and foam stabilization and effect on dough characteristics, determination of their antioxidant and prebiotic activity *in vitro*.

The research work carried out towards achieving these objectives forms the subject matter of the thesis. The thesis is divided into 5 chapters:

Chapter 1: Introduction

This chapter begins with the general account on carbohydrates, their classification, digestibility and importance in human nutrition. A brief account of the various methods employed in the structural characterization of carbohydrates/polysaccharides is given. Cereal feruloyl arabinoxylans, their biosynthesis and degradation, fine structures and physicochemical/functional roles in food and nutrition are discussed. Finally, with a brief account on rice and ragi, the chapter highlights the aims and scopes of the present study.

Chapter 2: Material and Methods

This chapter starts with the information on the general procedures, and various chemicals and instruments used in the present study. Various

colorimetric estimation methods employed for the analysis of feraxans and enzyme assay methods are discussed. Isolation, fractionation and purification procedures employed for water soluble feraxans are described. Structural characterization procedures included chemical methods such as methylation analysis, periodate oxidation and Smith degradation, and spectroscopic methods such as GLC-MS, NMR, IR and UV studies.

Methods employed for the functional characterization of feraxans included various antioxidant assays; viscosity, gelation and foam stabilization experimental protocols; and farinograph, extensograph, amylograph and bread making procedures. A list of bacteria used for *in vitro* fermentation experiments, their media and growth conditions, and *in vitro* fermentation procedures are also described.

The following three chapters present the findings of the investigation in the form of results and discussion.

Chapter 3: Isolation, fractionation and purification of water soluble feraxans

Results on the characterization of water soluble non-starch polysaccharides (NSP), phenolic acids, variations in feraxanases during malting and fractionation and purification results are presented.

Water extractable non-starch polysaccharides (WEP) represent a small proportion (0.6 – 2.2%) of the total flour and their content increased by 2 to 3 folds upon malting (96 h controlled germination). Their water un-extractable counterparts (WUP) are present in higher proportions (7.5 – 20.3%). The WEP and WUP have high amount (2.8 – 11.0%) of uronic acid, which is slightly higher in malts, probably due to the faster degradation of mixed glucans than arabinoxylans as indicated by pentose to hexose ratio.

Ferulic acid is the major bound phenolic acid ester-linked both in WEP and WUP, and over 90% of the total ferulic acid are bound to the latter. Malting resulted decrease in the bound ferulic acid content, due to

the action of induced ferulic acid esterase. p-Coumaric acid is also found as bound phenolic acid mainly in WUP, in addition to ferulic acid.

Protocatachuic acid is the major free phenolic acid with small amounts of gallic, caffeic and ferulic acids and their overall contents decreased upon malting. Presence of very low amount of free ferulic acid suggested that the bound ferulic acid hydrolyzed during malting would be quickly degraded in the system.

All the major feraxanases were detected in both rice and ragi flours with many folds higher activity in malts indicating their induction during malting. In specific, xylanase activity increased by 2 to 3 folds and ferulic acid esterase activity increased by 50 to 100 folds upon malting. Arabinofuranosidase and xylopyranosidase, two key enzymes in the feraxanase system also induced during malting. These xylanolytic enzymes, acting together, are responsible for the loosening/degradation of cell wall matrix during germination and in turn increasing the content of WEP.

WEP is sparingly soluble in water and its content (water soluble non-starch polysaccharides – NSP) increased by 3 to 5 folds up on malting. The major portion of water soluble NSP is arabinoxylan type of polysaccharide as indicated by sugar composition and it contained high amount of uronic (2.6 – 6.1%) and ferulic (492.5 – 528.0 μg/g) acids.

Water soluble NSP was fractionated on DEAE-cellulose into 5 fractions by eluting with water, 0.1 and 0.2 molar ammonium carbonate (AC) and 0.1 and 0.2 molar NaOH. The major (0.1 molar AC eluted) fraction is arabinoxylan type of polysaccharide with high amount of ester-linked ferulic acid as indicated by its strong UV absorption and HPLC analysis, and thus was designated as water soluble feruloyl arabinoxylans (feraxans). Interestingly, ferulic acid content of malt feraxans is around 12 and 7 folds higher than native (un-germinated) feraxans for rice and ragi respectively. On the contrary, ferulic acid content of 0.2 molar AC eluted fractions was higher in native polysaccharides compared to malts. This indicated possible

mobilization of feruloyl arabinoxylans during malting due to the action of xylanolytic enzymes.

Sephacryl S-300 gel permeation chromatography yielded two peaks each for native and malted rice and ragi water soluble feraxans. They were further purified on Sephacryl S-300 and their homogeneity was ascertained by HPSEC, capillary and cellulose-acetate paper electrophoresis.

Chapter 4: Structural characterization of water soluble feraxans

This chapter highlights the results regarding structural features of water soluble feraxans from rice and ragi.

The molecular weight of purified feraxans ranged between 15,400 to 2,31,500. Molecular weight of feraxans decreased upon malting and the yield of high molecular weight peaks also decreased. This is due to the action of xylanolytic enzymes, in turn leading to the better extractability/solubility of degraded polysaccharides in water.

Purified feraxans have high arabinose to xylose (Ara/Xyl) ratio and are rich in uronic (8.0 – 13.4%) and ferulic (54.0 – 1471.6 μg/g) acids, which are higher in malt feraxans. The presence of high amount of galactose seems to be the characteristic feature of rice and ragi water soluble feraxans.

Methylation analysis of the carboxyl reduced feraxans showed very high amount of 2,3,5-Me_3-arabinose indicating that majority of arabinose residues are terminally linked. Detection of di-methylated arabinose residues indicated the presence of branching site provision for arabinose and ester-linked ferulic acid. Presence of terminally linked galactose and glucuronic acid (4-O-Me) are confirmed by their tetra methyl derivatives. Di and mono-methylated xylose residues are in almost equal amounts and un-methylated xylose is found in good amount indicating high branching.

Periodate oxidation and Smith degradation studies showed that about 60% of sugar residues have adjacent free hydroxyl groups, which is in close agreement with the methylation and PMR data.

The low negative optical rotation values (-0.3 to -7.4) indicated the polymer primarily to be β-linked. Signals corresponding to α-L-arabinofuranoside (δ ~110 ppm) and β-D-xylopyranoside (δ ~104 ppm) are detected in the ^{13}C-NMR spectra of water soluble feraxans. Glucuronic acid is found to be in 4-O-Me form as indicated by ^{13}C-NMR spectral signals at ~178 ppm (for >C=O), ~98.8 and ~72.1 ppm (for C-1 and C-3 of α-D-glucuronic acid) and ~59.5 and ~18.0 ppm (for -O-CH_3). It is also confirmed by GLC-MS analysis.

Proton magnetic resonance (PMR) spectra of feraxans showed almost equal distribution of di, mono (2/3) and un-substituted xylose residues as quantified by the integration of the anomeric signals arising from the arabinose residues. Interestingly, the amount of di-substituted xylose increased in malt feraxans with concomitant decrease in the content of mono-substituted residues. On the other hand, amount of un-substituted residues remained almost equal in both native and malt feraxans. Similar trend is observed both in rice and ragi feraxans.

With their higher Ara/Xyl ratio and lower molecular weight, malt feraxans have higher di-substituted xylose residues. The substitution pattern of xylose residues is correlated with Ara/Xyl ratio and molecular weight of feraxans. There is a trend in the xylose substitution pattern. As the Ara/Xyl ratio increases and/or molecular weight decreases, content of di-substituted xylose residues increases while the un-substituted residues remain overall same. A trend of decrease in the Ara/Xyl ratio with increasing molecular weight is also observed.

The PMR spectra showed the signals corresponding to ferulic acid bound to the water soluble feraxans. Infrared spectra of feraxans showed signals typical to arabinoxylans with uronic/ferulic acid >C=O signal at ~1730 cm^{-1}.

With this information in hand, a structural model has been proposed for rice and ragi water soluble feraxans. They have a β-linked xylose backbone with α-linked arabinose residues as side branches, similar to

other cereal arabinoxylans. However, they differed in many other respects. They are of small molecular weight and have high Ara/Xyl ratio and hence highly branched, with almost equal amount of di, mono and un-substituted xylose residues. They are particularly rich in O-2 substituted xylose residues unlike many other cereal arabinoxylans especially from wheat. Presence of high amounts of galactose, glucuronic (4-O-Me) and ferulic acids are the characteristic features of water soluble feraxans.

In spite of their positions in the widely separated clades, water soluble feraxans from rice and ragi are essentially similar, and structurally resembled highly branched regions of rye and maize arabinoxylans than to wheat arabinoxylans. Water soluble feraxans from malts are of low molecular weight with higher Ara/Xyl ratio and higher content of ferulic acid. This is probably due to the action of xylanolytic enzymes induced during malting which preferentially acted upon the less substituted region of large molecular (native) feraxans.

Chapter 5: Functional characterization of water soluble feraxans

This chapter presents the findings of functional characterization of water soluble feraxans.

Water soluble NSP/feraxans showed many functional characteristics. With their high amount of bound ferulic acid, water soluble NSP/feraxans exhibited very high antioxidant activity. The activity pattern observed for different fractions could well be correlated with their bound ferulic acid content. However, antioxidant activity of feraxans is several folds higher than the expected activity due to their bound ferulic acid content. This is, in part, related to the molecular weight/chain length of the polysaccharides. Possible antioxidant effect of negatively charged sugar residues is also shown.

Water soluble NSP/feraxans exhibited very low viscosity except for ragi malt NSP. This property may make them ideal to be incorporated in fibre/antioxidant depleted/deprived foods/drinks requiring low viscosity.

Changes in the viscosity in relation to concentration, temperature and pH are also shown. Interestingly, due to the bound ferulic acid, feraxans showed different trends in viscosity with respect to pH in different buffers. The presence of NaOH in the alkaline pH hydrolyses hydrophobic bound ferulic acid and increases the viscosity of feraxans due to freed –OH groups and increased hydrophilic interactions.

Despite considerable amount of bound ferulic acid, water soluble NSP/feraxans showed no gelling ability. However, they showed good foam stabilization property. Water soluble NSP has higher foam stabilization effects compared to purified feraxans possibly due to the cumulative effect of several polysaccharide populations in NSP.

Incorporation of water soluble NSP into wheat dough resulted in overall positive effects. Farinograph values indicated higher water absorption and lower dough development time with slightly lower dough stability. Both extensibility and resistance to extension are increased upon the addition of water soluble NSP, the effect is similar to that of dough improvers. Amylograph studies showed increased viscosity of wheat dough upon the addition of NSP.

Test baking indicated improved bread characteristics with the addition of water soluble NSP. Weight, loaf volume and specific volume are increased, while firmness of bread decreased. Thus addition of water soluble NSP/feraxans has overall positive functional effects on dough compared to the negative effect exerted by their insoluble counterparts.

The *in vitro* fermentation characteristics/prebiotic activity of water soluble NSP/feraxans are studied with probiotic cultures of lactic acid bacteria. In general, feraxans are only partly fermented by few lactic acid bacteria, which are able to utilize arabinose or xylose. Feraxan non-fermenters could not utilize constituent sugars – especially xylose. Degradation/fermentation of feraxans is constrained by the xylanolytic enzymes especially lack of xylanase in the probiotic bacteria.

Utilization of feraxans by lactic acid bacteria resulted in increased OD, dry cell mass and viable cell counts, and concomitant decrease in the pH, which is related to the production of SCFA. Acetate is the chief SCFA produced. Arabinofuranosidase, the key enzyme in the feraxans' degradation is shown to be induced in cells by the presence of pentose sugars/feraxans in the culture medium. Rat cecal/faecal mixed cultures completely degraded feraxans, which is related to their high xylanase activity. Pre-hydrolysis of feraxans with xylanase facilitated their fermentation by lactic acid bacteria. Pure cultures of lactic acid bacteria, thus have limited ability to ferment feraxans and their complete fermentation might require consortium of bacteria like in mixed cultures.

Although many food borne pathogenic bacteria are able to ferment constituent sugars, they are unable to utilize feraxans. The culture broth of lactic acid bacteria grown on feraxans showed antimicrobial/bacterio-static activity towards these pathogenic bacteria. The water soluble feraxans with their ability to support the growth of probiotic lactic acid bacteria are shown to have prebiotic activity. The malt feraxans showed slightly better functionality compared to the native ones.

Overall, a comparative investigation is made on the structural and functional characteristics of water soluble feraxans from rice and ragi, and their changes upon germination.

A summary and conclusions are given at the end of results and discussion section.

The thesis ends with a list of references arranged in alphabetical order.

(Dr. G. MURALIKRISHNA) (R. SHYAMA PRASAD RAO)
Guide Applicant

'... for certainly, all beings here are indeed born from food; having been born, they remain alive by food; and on departing, they enter in to food ...'

Taittiriyopanishad III, 2 (~ 600 BC)

'Let food be your medicine', advised Hippocrates (460 – 377 BC), the father of medicine, centuries ago. Living in the industrialized world, we seem to forget this advice each time we reach the latest pharmaceutical wonder. Nutritious food is the basic requirement of body and in fact body is transformed food. Eating highly refined foods that lack essential nutrients has resulted in the most technologically advanced and wealthy country in the world suffering from all forms of malnutrition and degenerative diseases. Former surgeon general, C. Everett Koop said the following concerning the American diet, "your choice of diet can influence your long-term health prospects more than any other action you might take".

Food is not just for energy, but contains biologically active components which offer the potential of enhanced health or reduced risk of diseases. It can be both preventive and curative. While there are a number of nutrients (individual food components), scientists are only now discovering the healing power of each nutrient. Food is also linked with changing mood and mind functions. Hippocrates's words are being realized in today's new, emerging type of foods – functional foods.

Carbohydrates are the most important energy provider among the macronutrients, accounting between 40 and 80 percent of the total energy intake. The role of dietary carbohydrates in human nutrition has been less extensively studied than those of protein and fat, till date. The main reason for this has been the absence of sound and rapid methodologies regarding carbohydrate analysis. Of late, it is recognized that apart from providing energy, carbohydrates play more subtle functions in the human nutrition.

1.1. Carbohydrates

The name 'sugar' is often used as a synonym for carbohydrates in general (Lindhorst, 2003). Carbohydrates, the most abundant bio-molecules on earth, are defined as 'polyhydroxy aldehydes or ketones, and their derivatives'. They can be classified as mono/di/oligo/poly-saccharides based on the number of sugar residues they possess. Plants synthesize sugars by an endothermic process called 'photosynthesis', using the light energy from sun and inorganic carbon. Individual sugar units (monomers) are then linked together to yield a vast and diverse array of oligo/polysaccharide types.

Carbohydrates, oligo/polysaccharides in general, are extremely difficult to synthesize in laboratory and has kept researchers in dark from knowing their exact function. Sugars in polysaccharides have numerous points of attachment and link together in myriad complex three-dimensional shapes, unlike the building blocks of nucleic acids and peptides/proteins that bind in linear chains. A single glucose unit, for example, has four hydroxyl groups that can bind to other sugars. Each bond that forms between separate units can take one of two different shapes. As a result, just four sugars can be strung together in more than 5 million possible arrangements.

Far from being inert, carbohydrates are now known to play very important roles in every aspect of living things from recognizing pathogens, to blood clotting, to enabling sperm to penetrate an ovum. The list of things they are already known to do includes regulating the half-life of hormones in the blood, directing embryonic development, and acting as 'address code' for directing traffic of various cells and proteins throughout the body. They are also involved in cell-cell adhesion, immune response, and parasitic infections and vaccines against bacteria and cancer based on carbohydrate antigens have spurred substantial interest in recent years. Polysaccharides in particular, are now known to have several important functions (table 1) (Pigman and Horton, 1970).

They are of much interest in food as they influence the food texture, consistency, water binding and other characteristics (Fincher and Stone, 1986.

Table 1. Some of the functions of polysaccharides/glycoconjugates.

Polysaccharides/glycoconjugates	Function
Starch and glycogen	Food reserve
Cellulose, hemicelluloses and chitin	Structural molecules
Gums and mucilages	Defensive, prevent tissue desiccation
Seaweed polysaccharides	Cementing materials for the cell walls
Bacterial polysaccharides	Antigenic
Lipopolysaccharides	Recognition markers and protect the surface of microorganisms
Hyluronic acid, condroitin sulphate and keratin	Lubricants and thickeners in connective tissues
Mucopolysaccharides	Calcification process in animal wounds
Heparin	Anticoagulant
Glycolipids	Membrane components and receptors for toxins
Glycoproteins	Enzymes, recognition molecules, membrane components and hormones

1.2. Carbohydrate classification

The FAO/WHO (1998) report provides a classification for the main categories of food carbohydrates based on their degree of polymerization and chemical nature. Monosaccharides are the individual sugar units and are the building blocks of higher order structures of carbohydrates. Most common types of sugars are hexoses (6 carbon chain) and pentoses (5 carbon chain) and based on their functional group, they may be aldoses, ketoses or polyols (reduced form). They may exist either in open or closed chain forms in aqueous solution, and later form may be either in pyranosidic (6 member ring) or in furanosidic (5 member ring)

structure. Disaccharides contain two and oligosaccharides contain 3 – 9 sugar units linked by glycosidic bonds. The glycosidic bond is formed between the hemiacetal/hemiketal hydroxyl group of one monosaccharide (glycosyl donor) and a hydroxyl group of the succeeding monosaccharide (glycosyl acceptor or aglycone) with the elimination of a water molecule. Polysaccharides are naturally occurring condensation polymers of monosaccharides with a degree of polymerization of 10 or more and in many times it may run into millions. Polysaccharides may be homo or hetero-polymers based on the type of constituent monosaccharide units. Glycoconjugates – glycolipids, glycoproteins and proteoglycans are included under the broad definition of carbohydrates.

1.3. Dietary fibers, functional foods and their health benefits

The nature of carbohydrates in food is growing field of interest within the food industry because of the potential of some types of carbohydrates to help prevent diseases of lifestyle. Non-glycemic carbohydrates, i.e., those carbohydrates (or their components) that are not absorbed in the small intestine and, therefore, transit down to become fermented in the colon, have drawn lot of attention. In fact, food carbohydrates can be broadly classified on the basis of their *in vivo* digestibility into digestible and non-digestible carbohydrates (table 2) (Asp, 1996; Englyst et al., 1992). Non-digestible carbohydrates have been collectively referred to as 'dietary fibre' (Hipsley, 1953). Some of these carbohydrates are of particular interest to the food industry for the purpose of developing 'functional foods', i.e., foods that are able to exert positive health effects. Non-digestible oligo/polysaccharides are considered as prebiotics, which stimulate the growth of bifidobacteria in the colon.

Table 2. Classification of carbohydrates based on their *in vivo* digestibility.

	Subgroup	Components
Digestible	Monosaccharides	Glucose, galactose, mannose, fructose (ketose), Arabinose, xylose Sorbitol, mannitol
	Disaccharides	Sucrose, maltose, lactose,
	Oligosaccharides	malto-oligosaccharides
	Polysaccharides	Starch – amylose and amylopectin
Non-digestible	Disaccharides	Trehalose
	Oligosaccharides	Raffinose, stachyose, verbascose, fructo- and xylo-oligosacchairdes
	Polysaccharides	Starch - modified and resistant, Non-starch – cellulose, ligno-cellulose, arabino-xylans, mixed glucans, mannans, pectins

A distinction was established between insoluble DF and soluble DF. The effects of insoluble DF are of limited interest because of their low functionality and fermentability (Hsu and Penner, 1989). By contrast, soluble DF in general has a wide functionality due to its ability to interact with water, and is almost fully fermented by the large intestine micro flora, bringing about much desired physiological/metabolic effects (Lopez et al., 1999). Cereals, the staple food for millions of people across the world, are the chief source of both soluble and insoluble DF (Plaami, 1997). Arabinoxylans, along with some amount of β-D-glucans, are the major components of soluble DF (Rao and Muralikrishna, 2004).

1.4. Diseases of lifestyle/civilization – role of dietary fibre

Interest in carbohydrates/polysaccharides is increasing due to the recent worldwide concern about the continuously increasing rates of many common diseases, known as diseases of lifestyle/civilization. Some of these common diseases in western countries are linked to the deficiency of complex carbohydrates/dietary fibre in food. The list includes obesity, diabetes, atherosclerosis and chronic heart problems,

increased cholesterol in the body, hypertension, constipation and diverticulosis, colorectal cancer and many more. Obesity in particular, is raising in adults and now in children as well (FAO/WHO, 1998). Although obesity as a significant phenomenon has usually been associated with developed countries, it is now also on the rise in the developing countries.

Cereals form the quantitatively most important source of DF. Consuming cereals and cereal based products are known to have beneficial roles in human nutrition and health and have been linked to their phytochemical profiles (Adom and Liu, 2002; Adom et al., 2003; Charalampopoulos et al., 2002; Mori et al., 1999). After more than 30 years of research into many and varied claims for its benefits, it is now clear that fibre has uniquely significant physical/physiological effects. Accumulating evidence favors the view that increased intake of DF can have positive health effects against chronic diseases, such as cardiovascular diseases, diverticulosis, diabetes and colon cancer. Prevention of constipation and regulation of transit time are mainly caused by the bulking effect of DF. It is also partly fermented in large intestine by a mixed flora of anaerobic bacteria and most of the physiological effects of DF are thought to be based on this property (Scheeman, 1998).

A daily intake of approximately 30 g is encouraged to promote health benefits associated with fibre. Because of the increased nutritional awareness, the food industry is facing the challenge of developing new food products with special health enhancing characteristics (Charalampopoulos et al., 2002). To meet this challenge, it must identify new sources of neutraceuticals and other natural and nutritional materials with the desirable functional characteristics (Izydorczyk et al., 2001). In view of the therapeutic potential of DF, more fibre incorporated food products are being developed all over the world. However, consumer acceptability of these functional foods depends not only on the

nutritional property, but also on the functional and sensory quality. These factors are considered while developing functional foods.

1.5. Characterization of polysaccharides

Simple monosaccharides can be built into giant molecules called complex polysaccharides that rival DNA and proteins in size and complexity. It's a testament to the importance of sugars that scientists have granted them an 'ome' of their own. Just as the 'genome' and 'proteome', the 'glycome' of an organism or cell encompasses all the sugars it makes. Still in its infancy, glycomics is slowly revealing its huge cast of sugar-related characters – structure and their myriad roles. The glycome – study of carbohydrates, of a single cell type or creature is probably many thousands of times more complex than the genome (Schmidt, 2002). It is the polysaccharides – their structure or bonding that makes the characterization very difficult. Even chemically synthesizing oligosaccharides by capping sugar molecules with 'protecting' groups at all but one branch point leaves compounds with a mixture of bonds formed in different orientations, requiring extensive purification procedures after each new sugar building block is added.

A polysaccharide may contain between ten and a million sugar residues. Polysaccharides are rarely homogeneous and usually have a very wide molecular weight distribution; often they are regarded as group of very closely related molecular species varying in both molecular architecture and size ('polydisperse'). Structural characterization of polysaccharides usually requires extensive purification procedures.

1.5.1. Isolation, fractionation and purification

Characterization of polysaccharides first requires them to be isolated from biological samples. They may be extracted with various extractants such as water (for water soluble arabinoxylans and mixed glucans), polar non-aqueous solvents (for starch and glycogen) (Leach

and Schoch, 1962), chelating agents (for pectins) (Selvendran, 1985), N-methyl morpholine-N-oxide (MMNO, for cellulose) (Chanzy et al., 1979) and alkali (for hemicellulose A and B) (Wilkie, 1979). Water extraction at different temperatures can be carried out to obtain gums and mucilages.

Polysaccharides thus isolated from the biological samples are rarely homogeneous and require extensive fractionation and purification steps before proceeding further with structural characterization. Polysaccharides differ in their molecular size, shape and charge, and can be fractionated using various methods such as fractional precipitation with solvents (ethanol, acetone), salts (ammonium sulphate) or methods such as ion exchange/affinity/gel permeation chromatographies.

1.5.2. Homogeneity criteria

Polysaccharides are highly complex and diverse, and unlike proteins, they are heterogeneous in their chemical characteristics like molecular weight and composition which in turn affects their physical properties. The heterogeneity arises because their biosynthesis, which is controlled indirectly by glycosyltransferase genes, unlike direct templates in case of DNA and proteins. Glycosyltransferases, the enzymes with individual specificities, are responsible for the transfer of sugar residues from particular glycosyl donor to the growing polysaccharide chain. Variations in polysaccharide structures may result from (a) departure from absolute specificity of the transferases, (b) incomplete formation of segments/side chains and (c) post polymerization changes. If these variations are continuous with respect to parameters such as molecular size, proportions of sugar constituents and linkage type, separation into discrete molecular species will be impossible and the polysaccharide sample would be called ‘polydisperse’. If the heterogeneity lies in their molecular size, but not in their chemical composition, they are called ‘polymolecular’ (Aspinall, 1983).

It is difficult to establish the purity of any polysaccharide sample unambiguously. Showing the absence of heterogeneity by as many independent criteria as possible is considered to be sufficient to go ahead with the structural characterization.

There are number of methods to show the absence of overall heterogeneity of a polysaccharide sample. Some of them are (a) consistency in chemical composition and physical properties such as optical rotation and viscosity, (b) chromatographic methods such as ion exchange, affinity and gel filtration, (c) ultra centrifugation pattern, (d) electrophoretic methods such as cellulose acetate and capillary electrophoresis and (e) spectroscopic methods such as IR and NMR. Purified sample is then subjected to the structural characterization (Aspinall, 1982).

1.5.3. Structural characterization

Sugar residues forming the polysaccharide chain may either be in linear or branched arrangements. They may all be of the same type (homoglycan) or of different types (heteroglycan). The length of a polymer chain, called degree of polymerization, is specified by the number of structural units it contains. The structural units may either in pyranose or in furanose ring form.

The structure of a polysaccharide can be organized into four different levels similar to that of proteins; they are (a) primary, (b) secondary, (c) tertiary and (d) quaternary.

The covalent sequence of monomeric units along with the respective glycosidic linkages is known as ‘primary structure’. Depending on the primary structures, polysaccharide chains may also adopt characteristic shapes such as ribbons, extended helices and hollow helices, which are known as ‘secondary structures’. Energetically favored interactions between chains of well defined secondary structures result in ordered organizations, which are known as ‘tertiary structures’.

Further associations between well-defined entities result in higher levels of organizations, known as 'quaternary structures' (Perez and Kouwijzer, 1999).

The major problems in the determination of the molecular structure of complex carbohydrates/polysaccharides are to establish (a) the molecular weight and nature of constituent sugar residues including their ring size, (b) the position and anomeric configurations of the inter-glycosidic linkages, (c) the sequence of residues/linkages and (d) overall arrangement of polymeric chains.

Structural elucidation of plant polysaccharides is a very tough task due to their non-periodic repeating units unlike microbial polysaccharides. However, several methods are available for the determination of the polysaccharide structure and are broadly categorized into three main classes: (a) chemical, (b) enzymatic and (c) spectroscopic methods (Aspinall, 1982).

1.5.3.1. Chemical methods

1.5.3.1.1. Molecular size

As the polysaccharides are 'polydisperse' in nature, their molecular size is represented as average of either weight (M_w) or number (M_n). The determination of both weight and number average gives an indication of the molecular size distribution, greater the difference between M_w and M_n, greater the polydispersity of the sample. Number average can be obtained by membrane osmometry (M_n > 20 kDa) and vapor pressure osmometry (van Dam and Prins, 1965) and weight average can be obtained by light scattering (Manley, 1963). Similarly, weight average can also be obtained by ultracentrifugation (sedimentation equilibrium or approach to equilibrium). These methods, however, are based mainly on the theoretically calculated average values.

Gel filtration chromatography, on the other hand, is a simple and widely used method to obtain the average molecular weight of the

polysaccharide sample. The column needs to be pre-calibrated with known molecular weight markers to determine the molecular weight of the unknown samples.

1.5.3.1.2. Sugar composition

Determination of the sugar composition of the polysaccharides involves the identification and quantification of sugar constituents. De-polymerization of the polysaccharide is a prerequisite, for which various methods have been developed and complete acid hydrolysis is the most common and widely used one. Aldose containing polysaccharides can be completely hydrolyzed with minimum loss of constituent sugars either by 0.5 or 1.0 molar sulfuric acid at 100°C for about 6 h (Selvendran et al., 1979) or by 1.0 molar trifluoro acetic acid at 120°C for 1 h (Albersheim et al., 1967). However, ketose containing sugars are very unstable under these conditions and thus mild acid hydrolysis either by 0.1 molar oxalic acid at 70°C for 1 h (Aspinall et al., 1953) or by 0.05 molar sulfuric acid at 80°C for 1 h (Codington et al., 1976) is followed for their de-polymerization. Incomplete hydrolysis takes place when a polysaccharide contains either amino sugars or uronic acid residues. Amino sugar containing polysaccharides require stronger acid and they can be completely hydrolyzed by 4 molar HCl at 100°C for about 6 h (Spiro, 1972). Uronic acid containing polysaccharides undergo decomposition (liberate carbon dioxide) upon acid hydrolysis by 12% HCl (Whyte and Englar, 1974). This can be circumvented by reducing the carboxyl group with water soluble carbodiimide/sodium borohydride mixture followed by acid hydrolysis. Determining the difference in the sugar composition before and after carboxyl reduction gives the amount of uronyl residues (Lindberg et al., 1972).

Monosaccharides released upon acid hydrolysis can easily be identified and quantified either by HPLC of GLC method. HPLC method is non-destructive, requires no derivatization and sample can be recovered

after the analysis. Separation of individual sugar residues is based either on cation/anion exchange (water) or on partition (acetonitrile: water) chromatography. Detection of sugars is done using refractive index (RI) detector (McGinnis and Fang, 1980). However, low sensitivity of RI detector requires large amount of sample (in micrograms) for analysis.

GLC is a destructive method and requires sample derivatization. However, it is much more sensitive than HPLC and requires very less amount of sample (in nanograms) for analysis. Trimethylsilyl (TMS) ethers, trifluoroacetyl (TFA) esters and alditol acetates are the most commonly prepared derivatives. Constituent monosaccharides are reduced with sodium boro-hydride or deuteride to obtain acyclic form and then acetylated using either acetic anhydride and pyridine (1:1) (Sawardekar et al., 1965) or acetylated aldonitrilation (hydroxylamine/pyridine and acetic anhydride, PAAN derivatives) (Dmitriev et al., 1971). Unlike TMS derivatization, acetylation eliminates the formation of multiple derivatives when different rings are formed (pyranose or furanose) or different anomeric forms are generated from reducing sugars or from equilibrium mixture of methyl glycosides. The acyclic derivatives can be identified by their retention time and if necessary by mass.

The enantiomeric forms of the constituent sugars can not be distinguised by the above mentioned methods. Although majority of the sugars are in D form, sugars such as rhamnose (in pectins) and arabinose (in arabinoxylans) are in L-form. These enantiomeric forms can be distinguished by converting into equilibrium mixtures of glycosides of chiral alcohols (+/- 2-butanol or +/- 2-octanol) followed by GC analysis using capillary columns (Leontein et al., 1978).

1.5.3.1.3. Linkage analysis

Methylation (conversion of all the free hydroxyl groups into methoxyl groups) is the most versatile and widely used technique for the determination of linkages in polysaccharides. It gives information on linkage positions, ring size (pyranose or furanose), non-reducing end groups, and kind and extent of branching (Hirst and Percival, 1965). The method involves complete etherification of free/un-substituted hydroxyl groups; i.e., those not involved in ring formation, inter sugar glycosidic linkages, or carrying substitutions stable at conditions used for methylation of the polysaccharides and for subsequent hydrolysis of the methylated derivatives (Lindberg, 1972).

In particular, Hakomori methylation is the one which is most reliable and extensively used method for the complete methylation of the polysaccharides (Hakomori, 1964). In this method, polysaccharide is dispersed in dimethyl sulfoxide (DMSO), treated with sodium methyl sulfinyl methanide (sodium dimsyl) and then reacted with methyl iodide. Hakomori's method is very effective compared to other methods wherein etherification may not be achieved in a single step or complete alkoxide formation may not take place. Haworth (dimethyl sulfate as alkylating agent and aqueous 30% sodium hydroxide as base), Purdie (methyl iodide as both solvent and alkylating agent and silver oxide as base) and Kuhn (N,N-dimethyl formamide/DMSO as dipolar aprotic solvent, methyl iodide/dimethyl sulfate as alkylating agent and silver/barium oxide as base) methylation are some other methods, which have restricted use and can be employed when the sample is partially methylated. The completeness of methylation can be ascertained either by methoxyl group determination or by the absence of O-H stretching vibrations in the IR spectrum.

The characterization of per-methylated polysaccharides requires identification and quantification of all the sugar derivatives formed upon de-polymerization and is performed by GLC-MS (Dutton, 1973). Per-

methylated polysaccharides are hydrolyzed and reduced to form acyclic derivatives. Since per-methylated polysaccharides are less soluble in aqueous solvents, initial partial hydrolysis is done with organic solvents such as formic acid and then complete hydrolysis with dilute aqueous acids is performed. Acyclic derivatives obtained after reduction are acetylated to obtain alditol acetates, which is the most widely used derivatization method for the characterization of per-methylated sugars. The mass spectra of per-methylated alditol acetates are generally simple to interpret, with fragmentation patterns characteristic of constituent sugars and their substitution pattern. However, methylation analysis does not give information on stereo-chemical nature (α/β) of the constituent sugars (Lonngren and Svensson, 1974).

Primary fragment ions from per-methylated alditol acetates arise by α-cleavage with preferred formation of (a) ions with lower molecular weight, (b) ions from cleavage between two methoxyl bearing carbon atoms, (c) ions from cleavage of a methoxyl bearing and an acetoxyl bearing carbon atom with marked preference for the methoxyl bearing species to carry the positive charge and (d) ions from very low abundance of cleavage between two acetoxyl bearing carbon atoms. Primary fragment ions undergo a series of subsequent elimination reactions to give secondary fragment ions which include losses by (a) β-elimination of acetic acid (m/e 60) or methanol (m/e 32), (b) α-elimination of acetic acid but not methanol and (c) via cyclic transition states of formaldehyde, methoxy-methyl acetate or acetoxy-methyl acetate (Jansson et al., 1976).

1.5.3.1.4. Oxidation

Characterization of the products obtained from oxidative cleavage of polysaccharides can give details about the mode of linkage, substitution pattern and configuration of sugar residues/linkages (α/β). Two important methods of oxidative cleavage are chromium trioxide and periodate oxidations.

CrO_3 oxidation: The configuration of glycosidic linkages in polysaccharides can be determined by chromium trioxide oxidation, which is shown to preferentially oxidize β-linked polysaccharides over α-linked ones. The difference is attributed to the easy formation of a keto-ester by cleavage at the bridge oxygen of β-anomeric compounds (Lindberg et al., 1975).

Periodate oxidation: It is the widely used method for the determination of linkages in polysaccharides. Glycol cleavage via oxidation by sodium metaperiodate gives formic acid (usually from triol cleavage in pyranose) or formaldehyde (from exocyclic diol, CHOH-CH_2OH groups) and the oxidant is reduced to iodate. The liberated products can be estimated by various methods such as titrimetry and spectrophotometry for the oxidant reduced, acid-base titration for the formic acid liberated or colorimetry for the formaldehyde formed (Hay et al., 1965).

1.5.3.1.5. Smith degradation

The aldehydes liberated upon periodate oxidation, and the sugar residues in the polysaccharide, which are resistance to oxidation are reduced with sodium borohydride and hydrolyzed to obtain monosaccharides along with residual stubs of oxidized units; either glycerol (from pentitol) or erythritol and threitol (from 1→4 and 1→6 linked hexitols, respectively) (Goldstein et al., 1965). Smith degradation products are identified and quantified by GLC-MS.

1.5.3.1.6. Oligosaccharide analysis

Polysaccharides can be partially fragmented/de-polymerized and the analysis of the oligosaccharides thus obtained can give information regarding the distribution of side chains (random/uniform or non-random) in turn providing complete structural information of the parent polysaccharides. Oligosaccharides can be obtained by several chemical

methods such as partial acid hydrolysis, acetolysis, trifluoroacetolysis, mercaptolysis and methanolysis. They can be fractionated/purified and characterized by following similar methods employed for polysaccharides. In particular, MALDI-TOF-MS and FAB-MS are useful for oligosaccharide characterization, providing molecular mass and sequence of the constituent sugar residues, respectively (York et al., 1990).

1.5.3.2. Enzymatic method

Oligosaccharides can be obtained by fragmentation/de-polymerization of polysaccharides with the use of specific polysaccharide degrading enzymes. Enzymes cleave the polysaccharides with the varying degree of polymerization. By characterizing the oligosaccharides released and also the leftover polysaccharides, one can get information regarding the structure of parent polysaccharides. Based on the mode of action, polysaccharide degrading enzymes – glycosidases are classified into two groups: exo- and endo-glycosidases. Exo-glycosidases act on polysaccharides and release mono/disaccharide units from the non-reducing terminal, whereas endo-glycosidases cleave the polysaccharides randomly (at the un-branched regions of both main and side chains), resulting in the release of oligosaccharides with varying degree of polymerization. Enzymatic method of obtaining oligosaccharides has several advantages over chemical method, viz. (a) their specificity, both to linkage type and substitution pattern, (b) lack of by-products, (c) high reaction rates and (d) control over the reaction. Various cell wall polysaccharides such as arabinoxylans (Hoffmann et al., 1992; Subba Rao and Muralikrishna, 2004) and xyloglucans (Lerouxel et al., 2002) have been characterized by analyzing the oligosaccharides obtained on enzymatic method.

1.5.3.3. Spectroscopic methods

Spectroscopic methods are much easier to perform compared to chemical and enzymatic methods of oligo/polysaccharide analysis and they complement the data obtained from other two methods. Some of the important spectroscopic methods are: nuclear magnetic resonance (NMR), infra red (IR), mass spectrometry (MS), optical rotatory dispersion (ORD), circular dichroism (CD) and X-ray diffraction.

1.5.3.3.1. NMR spectrometry

NMR spectrometry is the rapid and non-destructive method to study the structure of polysaccharides, requiring no modification or degradation of the sample. ^{13}C and ^{1}H NMR together can give the details on molecular complexity and fine structure of the polysaccharides. ^{13}C NMR can give details about the composition, linkage and conformation of polysaccharides (Jennings and Smith, 1978) and can also ascertain the purity of the polysaccharide sample. However, it can not differentiate the enantiomeric configuration of sugars. Various plant polysaccharides like arabinoxylans (Hoffmann et al., 1991; Izydorczyk and Biliaderis, 1993; Subba Rao and Muralikrishna, 2004), mixed glucans (Uzochukwu et al., 2002) and pectins (Ryden et al., 1989) have been characterized using ^{13}C NMR spectroscopy.

1.5.3.3.2. IR spectroscopy

Infrared waves are absorbed by the vibrating chemical bonds in the polysaccharides giving characteristic IR spectra (vibrational) in the frequency range of 4000 to 400 cm^{-1}. IR spectroscopy can be used for the detection of functional groups, configuration of sugar residues and to know the substitution pattern. It is used to characterize arabinoxylans and their oligosaccharides (Kacurakova et al., 1998).

1.5.3.3.3. Mass spectrometry

Mass spectrometry is the indispensable technique in the characterization of oligo/polysaccharides. In the conventional mass spectrometry, polysaccharide sample can not be analyzed directly and hence it is separated into small molecules/constituent sugar residues and derivatized with acetylation/alkylation in order to make them volatile. Mass spectrometry is based on the principle that ions of different mass: charge ratio (m/e) are separated due to their differential diffraction in the combined electric and magnetic fields. Chemical ionization and electron ionization are the two important methods by which ionization can be achieved. In chemical ionization, molecular ions remain intact and spectra obtained are simple to interpret. On the other hand, electron ionization may result in complicated spectra because ions entering the analyzer may get fragmented by the high energy transferred from the bombarding electrons.

The advent of recent mass spectrometric techniques such as matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and fast atom bombardment-mass spectrometry (FAB-MS) have revolutionized the oligo/polysaccharide analysis. These techniques do not need laborious sample derivatization steps, but provide valuable information on the molecular mass and sequence of constituent residues. Many plant oligosaccharides including arabinoxylans have been characterized using these techniques (Jacobs et al., 2003; Lerouxel et al., 2002; Subba Rao and Muralikrishna, 2004).

1.6. Cereal Polysaccharides

The major constituent (60 – 80%) of cereals is starch, a storage polysaccharide, which is made up of two constituents: a linear α 1→4 linked amylose and branched α 1→4 linked amylopectin. Apart from starch, cereals also contain other polysaccharides known as non-starch polysaccharides, which include cellulose, hemicelluloses, arabinoxylans,

1-3/1-4 β-D-glucans, glucomannans, pectins and arabinogalactans (Fincher, & Stone, 1986; Izydorczyk, & Biliaderis, 1995). These non-starch polysaccharides mainly occur in the cell walls, where they play both structural and growth-regulating role and are divided into two types: 'fibrillar' and 'matrix' polysaccharides. Cellulose, a β 1→4 linked polymer of glucose forms the micro-fibrils in the cell wall. All other non-starch polysaccharides belong to the 'matrix' polysaccharide group, are very heterogeneous in structure. They form complexes with each other and with other cell-wall components such as cellulose, proteins (extensins, rich in hydroxy proline residues), lignin (polymer of cinnamyl alcohol) and other phenolic constituents.

Together, alkali extractable matrix polysaccharides have been termed 'hemicelluloses' as they were considered to be chemically and structurally related to cellulose.

The rigidity and strength of the cell wall is related to the integrity of cellulose/hemicellulose network. During cell growth, however, wall expansion has been found to be dependent on the enzymatic modification of the hemicellulosic component (Pauly et al., 2001).

1.7. Arabinoxylans/feruloyl arabinoxylans

In 1927, non-starchy, gummy polysaccharides were isolated from bread wheat flours and shown to consist predominantly of pentoses, arabinose and xylose (Freeman and Gortner, 1932; Hoffman and Gortner, 1927). Similar polysaccharides were also found in durum wheat, rye and barley, and were initially referred to as pentosans and later as arabinoxylans. Pentosans, in general represent a heterogeneous group of polysaccharides which, in addition to pentose sugars, may also contain hexoses, hexuronic acids and some proteins, and hence current nomenclature is more structure descriptive, identifying several polymeric components such as arabinoxylans or arabinogalactan peptides, depending on the molecular constitution of the polysaccharides.

Arabinoxylans have been identified in a variety of tissues of the main cereals of commerce: wheat, rye, barley, oat, rice and sorghum (Fincher and Stone, 1986) as well as in some other plants: rye grass (Hartley and Jones, 1976), pangola grass (Ford, 1989) and bamboo shoots (Ishii, 1991). Although arabinoxylans are minor components (but some times up to 10% as in barley grain) of entire cereal grains, they play important structural and functional role in plant cell.

Arabinoxylan consists of a linear backbone of β-(1→4)-D-xylopyranosyl residues, partly substituted with single α-L-arabinofuranosyl residues at O-2, and O-3, or at both O-2 and O-3 positions of the xylose residues (McNeil et al., 1975; Vietor et al., 1994). The presence of arabinosyl substituents and their distribution over the xylan backbone affect such arabinoxylan properties as solubility and interaction with other polymeric cell wall components (Andrewartha et al., 1979; McNeil et al., 1975) as well as restrict the enzymic degradation by endoxylanase (Vietor et al., 1994). Some arabinose residues are covalently linked through ester linkages to ferulic acid (Smith and Hartley, 1983). General structure of feruloyl arabinoxylan is depicted in the figure 1.

In type II walls, which are present in grasses, arabinoxylans are the major non-cellulosic polysaccharides in the primary walls. The arabinoxylans in type II walls have abundant arabinosyl side chains. They are also substituted with galactose and high amounts of glucuronic acid. In general, arabinoxylans were divided into water extractable and water un-extractable, based on the extractability, and this difference largely arises from their degree and pattern of substitution, feruloylation and non-covalent interactions with other wall components.

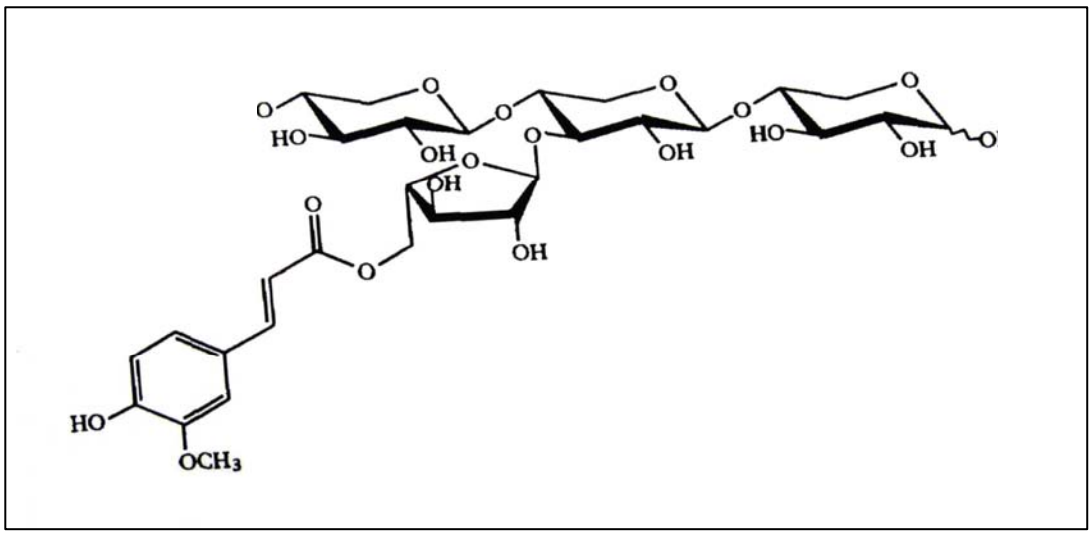

Figure 1. General structure of feruloyl arabinoxylan.

Ferulic acid, a hydroxycinnamic acid is the major bound phenolic acid in cereals arabinoxylans, and is synthesized by phenylpropanoid pathway. It is concentrated mainly in the aleurone layer (~ 75%) of the grain and comprises about 0.5% in wheat and 0.14% in barley grains.

Ferulic acid is a strong antioxidant and known to protect cells from UV radiation. Ferulic acid groups present in the arabinoxylans, on oxidative coupling yield diferulates/dimers which link adjacent polymers together, tightening the structure of the cell wall and thus restricting cell expansion. Ferulic acid protects the polysaccharides against enzymic hydrolysis. It also protects the plants from microbial/pathogen invasion.

1.7.1. Biosynthesis of arabinoxylan

The cell wall component-polysaccharide biosynthesis takes place in different sub-cellular compartments. Cellulose and callose are made at the plasma membrane, whereas pectin and hemicelluloses (arabinoxylans) are believed to be synthesized in the Golgi apparatus (Carpita and Gibeaut, 1993).

Xylans are common polysaccharides in plant cell walls, particularly in secondary cell walls where they are deposited as the major

non-cellulosic polysaccharides. The xylans in type II walls, which are present in grasses and some related plants, have abundant α-L-arabinofuranosyl side chains attached through (1→3) and (1→2) linkages apart from a small amount of glucuronosyl and other side chains (Aspinall, 1980; McNeil et al., 1984). This type of xylan, a heteropolysaccharide is known as arabinoxylan.

Heteropolysaccharide biosynthesis can be divided into four steps: chain or backbone initiation, elongation, side chain addition, and termination and extracellular deposition (Iiyama et al., 1993; Waldron and Brett, 1985). Our understanding of these different steps in biosynthesis is still very incomplete. The main enzymes responsible for heteropolysaccharide biosynthesis are glycosyltransferases, but only very few genes for these have been identified, and the enzymes responsible for synthesizing the backbone of xylans are only partially characterized (Porchia et al., 2002). The backbone-synthesizing enzymes may belong to the cellulose synthase-like proteins, but this assumption may be false as it is now known that callose synthase does not resemble cellulose synthase (Hong et al., 2001).

The biosynthesis of (1→4) linked β-xylosyl backbones in xylans is catalyzed by β-1,4-xylosyltransferase. This enzyme has been investigated in many plants, including wheat seedlings wherein the activity was characterized from microsomal membranes (Porchia and Scheller, 2000). An UDP-D-glucuronate decarboxylase (E.C. 4.1.1.35) was shown to catalyze the synthesis of UDP-D-xylose from UDP-D-glucuronate in an essentially irreversible reaction that is believed to commit glycosyl residues to heteroxylan synthesis (Zhang et al., 2005).

The addition of side chains to xylans has been less investigated and little is known about the way in which the different glycosyltransferases interact to form the complete polysaccharide. A study of glucuronosyltransferase has shown an interaction with xylosyltransferase (Baydoun et al., 1989). Although arabinose is a

common monosaccharide in plant polysaccharides and glycoproteins, there are few reports on the arabinosyltransferases involved in polysaccharide synthesis. Recently, Porchia et al. (2002) reported the presence of arabinoxylan arabinosyltransferase (AX-AraT) in microsomal and Golgi membranes isolated from wheat seedlings and showed that AX-AraT is dependent on the synthesis of unsubstituted xylan acting as acceptor. They have also demonstrated the formation of a single arabinosylated protein and its possible role in arabinoxylan biosynthesis.

1.7.2. Biosynthesis of ferulic acid

Being a secondary metabolite, biosynthesis of ferulic acid is fairly well understood. Ferulic acid, a hydroxy-cinnamic acid derivative, is synthesized in plants via shikimate/phenylpropanoid pathway from phenylalanine or L-tyrosine. Shikimate/arogenate pathway leads, through phenylalanine, to the majority of plant phenolics, the phenylpropane (C_6-C_3) derivatives (phenylpropanoids). p-Coumaric acid is formed as an intermediate in the ferulic acid biosynthesis.

1.7.3. Feruloylation of arabinoxylans

One of the characteristic features of arabinoxylans is their high content of bound ferulic acid (and small amount of p-coumaric acid), chiefly ester linked to α-L-arabinofuranose usually at O-5 position. The feruloylation and p-coumaroylation occur on highly specific hydroxyl groups of polysaccharides. However, there is no complete agreement on to the site of feruloylation of wall polysaccharides or the nature of the feruloyl donor. Fry and Miller (1989) administered (^{3}H) arabinose into spinach cultured cells and traced its incorporation into arabinose units of the major wall polysaccharides. The authors showed that arabinosylation and feruloylation occurred co-synthetically and intracellularly. Similarly, Obel et al. (2003) showed the intracellular feruloylation of arabinoxylans in wheat suspension-cultured cells. On the

other hand, Yamamoto et al. (1989) suggested that feruloylation site is located within the matrix of barley coleoptile cell walls.

Meyer et al. (1991) showed that feruloyl-CoA is a donor for feruloylation. A microsomal preparation from suspension cultured parsely (*Petroselinum crispum*) cells was able to transfer ferulic acid from feruloyl-CoA to uncharacterized endogenous wall polysaccharides. An alternative feruloyl donor may be the glycosidic ester of ferulic acid (1-O-feruloyl-β-D-glucose). Mock and Strack (1993) demonstrated that 1-O-sinapoyl-β-D-glucose is formed by UDP-glucose: hydroxycinnamate D-glucosyltransferase (E.C. 2.4.1.120).

1.7.4. Oxidative gelation *in vivo*

Feruloyl arabinoxylans are known to undergo oxidative phenolic coupling (dimerization) (figure 2) reactions in walls; the coupling reactions themselves *in vivo* would be remarkably specific. To permit a coupling reaction, feruloyl groups on the same or different polysaccharide chains must be juxtaposed. Matrix polysaccharides could be imagined in gelatinous form and they would have enough mobility to place feruloyl residues in close proximity. But at present there is no definite proof for this theory. Peroxidases are candidates for the catalysis of the dehydrogenative dimerization of feruloyl residues in the cell wall. The peroxidases not only generate free radical intermediates of ester-linked feruloyl residues, but may also generate the hydrogen peroxide needed to achieve this from various hydrogen donors. Several mechanisms have been proposed for hydrogen donor generation. Ogawa et al. (1996) showed that one of the physiological functions of the cytosolic CuZn-superoxide dismutase is supplying hydrogen peroxide for lignification.

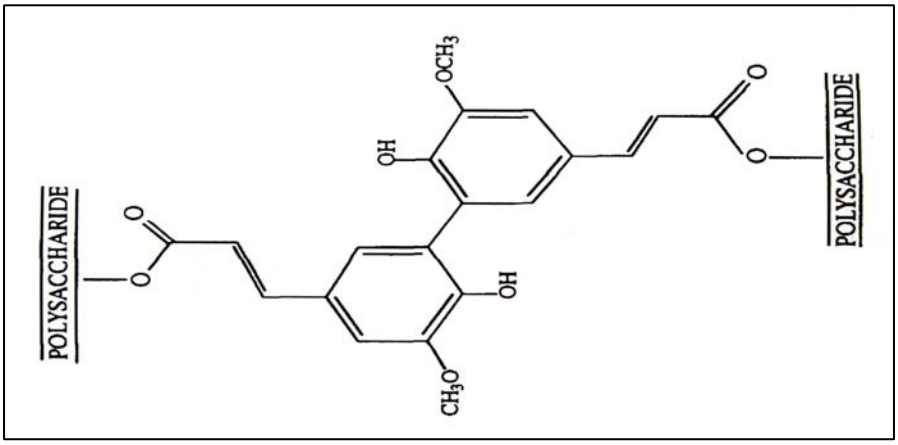

Figure 2. Covalent diferulate cross-link between arabinoxylan molecules.

Obel et al. (2003) have observed the intracellular formation of ferulic acid dimer, which is limited to 8,5'-diferlulic acid, while other dimers appeared to be formed extracellularly in wheat suspension-cultured cells. Similarly, Fry et al. (2000) reported the intraprotoplasmic and wall-localized formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. It is argued that feruloyl arabinoxylans that are cross-linked before and after secretion are likely to loosen and tighten the cell wall, respectively and have control on cell expansion.

1.7.5. Functions of arabinoxylans and feruloyl arabinoxylans *in vivo*

Feruloyl arabinoxylans (feraxans) are the major polysaccharides in the type II walls, which are present in grasses. With the very complex and diverse structure, arabinoxylans may have roles in the cross-linking of cellulose microfibrils and may thereby regulate cell development, expansion and strengthen the wall by mechanical resistance (Carpita, 1996). These polysaccharides, by means of oxidative coupling, also become polymerized into the lignin macromolecules. Such polymerizations decrease wall extensibility and may ultimately be involved in the control of cell growth. They also limit

biodegradation/digestibility of polysaccharides, thus forming an effective barrier against microbial invasion. Feruloyl oligosaccharides are known as signal molecules between plants and microorganisms (Darvill et al., 1992).

1.7.6. Degradation of arabinoxylans and feruloyl arabinoxylans *in vivo*

Feruloyl arabinoxylans (feraxans) (Nishitani and Nevins, 1989) are highly complex and diverse in structure and therefore require an array of hydrolytic enzymes for their degradation (figure 3). Collectively these enzymes are referred to as feraxanases (Nishitani and Nevins, 1989). Xylanase and feruloyl esterase are perhaps the key enzymes involved in the biodegradation of feraxans and they need to act synergistically. Xylanase would break the long-chain xylans into feruloyl-arabino-xylo-oligosaccharides, which in turn would be easily accessed by feruloyl esterase for the de-esterification of ferulic acid. On the other hand ferulic acid esterase may act upon feraxans to cleave the feruloyl moieties, thus facilitating their degradation by xylanase. Arabinofuranosidase, xylopyranosidase, glucuronidase, galactosidase and acetyl esterase are some of the other enzymes in the feraxanase group which are required for the complete biodegradation of feraxans.

Feraxan biodegradation is supposed to be a constant/continuous process in the cellular maintenance. However, during seed germination/malting, their biodegradation is hastened in the endosperm and aleurone cell wall by the induced feraxanases/xylanolytic enzymes. There are some reports on the *in vivo* biodegradation of feraxans during malting/germination of cereals such as wheat, barley, rye and ragi (Autio et al., 2001; Obel et al., 2002; Rao and Muralikrishna, 2004; Subba Rao and Muralikrishna, 2004).

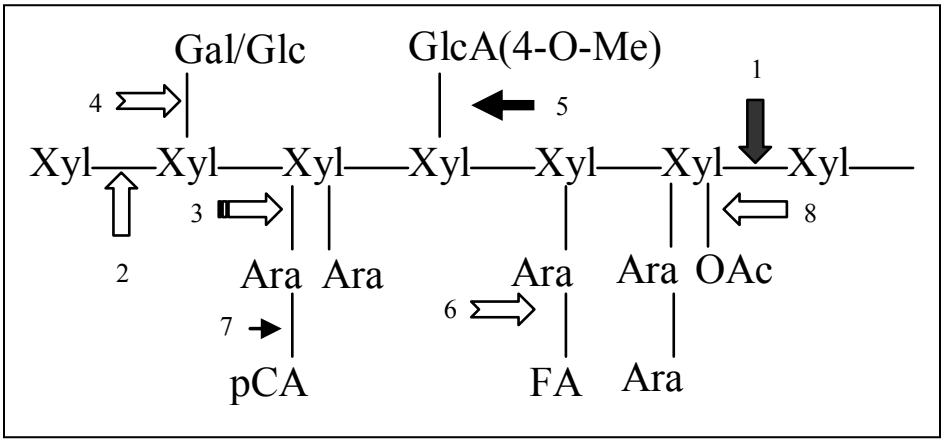

Figure 3. Feraxan – feraxanases system. Each arrow represents a different enzyme: xylanase (1), xylo-pyranosidase (2), arabino-furanosidase (3), galacto/gluco-pyranosidases (4), glucuronidase (5), feruloyl esterase (6), p-coumaroyl esterase (7) and O-acetyl esterase (8).

The ferulic acid degradation is not well understood, however, it may take place by chain shortening via β-oxidation process (figure 4) (Gasson et al., 1998) directly analogous to the well known β-oxidation pathway of fatty acids. Vanillin, a highly valued flavor compound, is the main degradation product of ferulic acid.

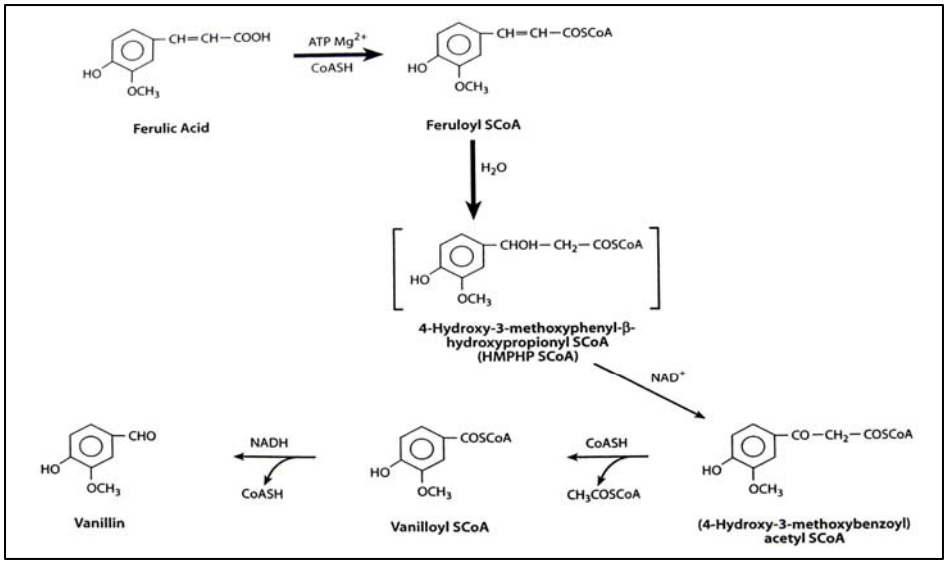

Figure 4. One of the (partial) biodegradation pathways for ferulic acid leading to vanillin via β-oxidation.

1.8. Fine structure of arabinoxylans

Although arabinoxylans have been of interest to cereal chemists and technologists for many years, structural studies initiated in 1951 by Perlin were taken up only in the 1990s when a number of workers focused on the detailed structural characteristics of these polysaccharides. General structure of arabinoxylans is now well known. However, these polymers are highly heterogeneous in chemical structure and molecular weight. They vary not only from source to source, but also in different parts and fractionation and purification methods employed. This prompts arabinoxylans to be studied from different cereal sources both from structural and functional viewpoint.

In general, arabinoxylans from various cereals and/or other plants share the same basic chemical structure. However, they differ in the manner of substitution of the xylan backbone. The main differences are found in the ratio of arabinose to xylose, in the relative proportions and sequence of the various linkages between these two sugars, and in the presence of other substituents.

The ratio of Ara/Xyl in arabinoxylans from wheat endosperm may vary from 0.50 to 0.71 (Rattan et al., 1994) but it is usually lower than that found in bran (Shiiba et al., 1993) (figures 5A and 5B). Similarly rye endosperm arabinoxylans are less substituted (0.48 – 0.55) (Bengtsson and Aman, 1990) than their bran counterparts (0.78) (Ebringerova et al., 1990). In general rice (Shibuya and Iwasaki, 1985) and sorghum (Vietor et al., 1994) seem to consist of more highly branched xylan backbones than those from wheat, rye and barley (figures 5E and 5F), and they may contain galactose and glucuronic acid substituents, in addition to the pentose sugars.

With a relatively low degree of branching, arabinoxylans from wheat, rye and barley contain a rather high amount of un-substituted Xyl*p* residues and a relatively low amount of mono-substituted Xyl*p* residues, compared to the more highly branched arabinoxylans from rice

and sorghum. The proportion of doubly substituted residues seems not to be related to the arabinose to xylose ratio and varies substantially among various arabinoxylans; highest amount has been reported for wheat bran arabinoxylans. The presence of O-2 mono-substituted xylose residues has been verified in all cereal arabinoxylans except those of rye endosperm. This type of xylose substitution appears to be a structural feature characteristic especially of barley arabinoxylans; a close to one ratio of O-3 to O-2 mono-substituted Xyl*p* residues suggest almost equal distribution of both linkages in the polysaccharide (Vietor et al., 1992).

Cereal arabinoxylans exhibit a high degree of endogenous micro-heterogeneity. It is, therefore, not possible to assign a single structure to arabinoxylans. In order to get better insight into the structural characteristics of individual homogeneous arabinoxylans, several investigators extensively fractionated arabinoxylans using ethanol or ammonium sulphate graded precipitation techniques (Gruppen et al., 1992a; Gruppen et al, 1992b; Izydorczyk and Biliaderis, 1992; Vietor et al., 1992). Increased concentration of ethanol/ammonium sulphate resulted in arabinoxylan fractions in continuously increasing Ara/Xyl ratios. The higher degree of branching was also accomplished by variations in the relative proportions of un-, mono- and di-substituted Xyl*p* residues. Highly substituted arabinoxylan fractions contained less un-substitued Xyl*p* residues.

The distribution of arabinosyl substituents along the xylan backbone is probably of greater importance than the degree of substitution itself, since it affects the conformation (Andrewartha et al., 1979) and the capacity of arabinoxylans to interact with each other and/or with other polysaccharides. According to the early work by Perlin and co-workers (Ewald and Perlin, 1959; Goldschmid and Perlin, 1963), wheat endosperm arabinoxylans consist branched regions where O-3 or O-2,3 substituted xylose residues are separated by single un-substituted xylose residues. At lengths of approximately 20 – 25 xylose units,

relatively smooth domains of at least two to five (and possibly more) un-substituted xylosyl residues may be present.

Based on ammonium sulphate fractionation and oligosaccharide analysis upon xylanase hydrolysis, wheat (endosperm) water-soluble arabinoxylans are reported to have three structural domains. Region I is highly substituted (more of O-2,3), and periodate oxidation/Smith degradation studies demonstrated that substituted xylose residues are present either isolated, in pairs or even as three contiguous residues, which may in large be limited by steric hindrance. Region II is similarly substitution, but contains more of O-3 xylose residues. Region III, which separates highly substituted domains, contains sequence of 2 – 6 or more un-substituted xylose residues. Different fractions differ in the proportion/ratio of these regions.

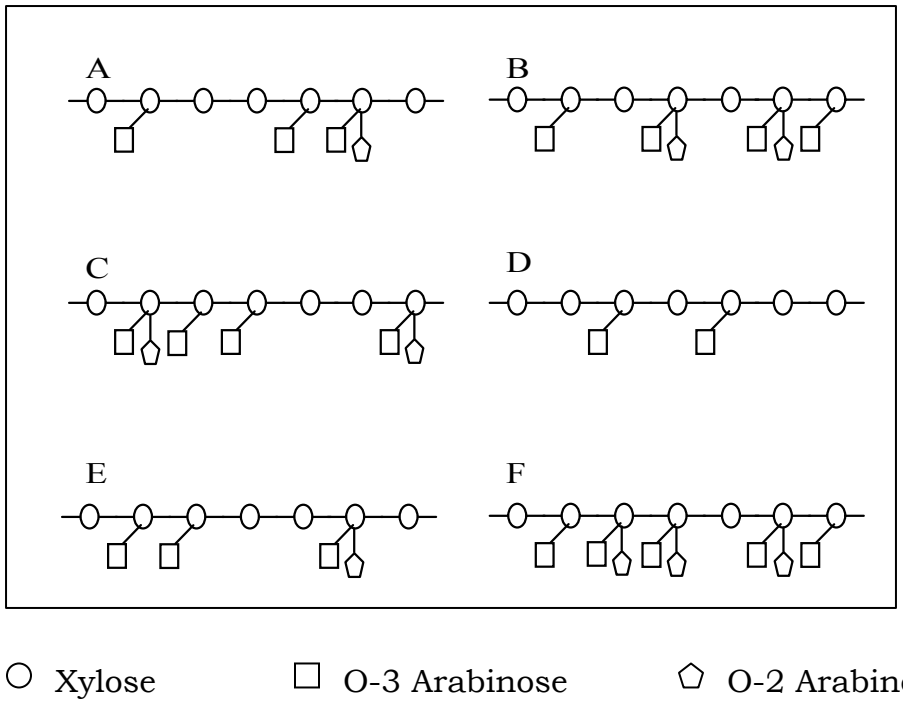

Figure 5. Structural models for cereal arabinoxylans. Less branched endosperm/insoluble (A) and more branched bran/soluble (B) arabinoxylans. Highly branched (*region A*) (C) and less branched (*region B*) (D) arabinoxylans. Less branched wheat (E) and more branched rice (F) arabinoxylans.

Wheat alkali-extractable arabinoxylans differ in their fine structure from water-soluble arabinoxylans and presumed to have two regions (A and B) (figure 5C and 5D). The highly branched region A composed mostly of repeating tetrameric units of un- and di-substituted xylose residues. This region also contains some O-2 substituted xylose residues. The less dense region B, which alternates with region A, includes at least seven contiguous un-substituted xylose residues.

The structure of arabinoxylans from barley endosperm (Vietor et al, 1992) was shown to be more regular than that from wheat. The major region, mono- (enriched with O-2) and di-substituted xylose residues are separated by un-branched xylose residue, and the clusters are separated by regular un-branched region of at least four xylose units.

Rye arabinoxylans have a different structure; the major polymer structure (arabinoxylan I) has xylose chain substituted exclusively at O-3, and minor polymer (arabinoxylan II) contains di-substituted O-2,3 xylose residues.

Rice and sorghum arabinoxylans are highly substituted and overall they resemble branched regions of other cereal arabinoxylans.

1.9. Physicochemical/functional roles of arabinoxylans in relation to food and nutrition

In the past few decades, arabinoxylans have stimulated research interest since they have been proven to have significant influence on the water balance (Jelaca and Hlynka, 1971) and rheological properties of dough (Meuser and Suckow, 1986; Michniewicz et al., 1991), retrogradation of starch (Biliaderis and Izydorczyk, 1992; Gudmundsson et al., 1991) and bread quality (Delcour et al., 1991; McCleary, 1986).

The chemical nature, including the subtle difference in the structure of the polysaccharides, is important in knowing their exact functional roles. Further, the multitude of free hydroxyl groups occurring in any polysaccharide allow for an infinite amount of hydrogen bonding

(intra and inter-bonding), which again influence the physical behavior of the polysaccharides. The distribution of arabinosyl substituents along the xylan backbone is known to affect the conformation of arabinoxylans (Andrewartha et al., 1979) and the intermolecular associations, which in turn have a direct bearing on certain physical and functional properties of these macromolecules.

Cereal arabinoxylans widely vary in their molecular weight and different methods of determination of molecular weight may give different values for the same arabinoxylan population (Fincher and Stone, 1986). Very high molecular weight of up to 5,000,000 has been reported for barley endosperm arabinoxylans (MacGregor and Fincher, 1993). The conformation of arabinoxylans, which can be determined by X-ray diffraction analysis, is dependent on substitution patterns. Arabinoxylans are shown to have a 3-fold, left handed helix and in the solid state they appear as an extended, twisted ribbon when xylan backbone is un-substituted (Fincher and Stone, 1986). This conformation is relatively flexible, supported by one H-bond between adjacent xylose residues and forms aggregates into insoluble complexes, stabilized by intermolecular H-bonding. Presence of arabinosyl substitution stiffens the molecule by maintaining the xylan backbone more extended and thus prevents its aggregation. Flexibility of xylan backbone is limited by the steric hindrance/interaction of arabinose side groups (Yui et al., 1995).

As a result of their rather stiff conformation, arabinoxylans exhibit very high viscosity in aqueous solutions, compared to the intrinsic viscosity of other polysaccharides such as dextran and gum arabica (Fincher ad Stone, 1986). In general, increased arabinose substitution was associated with increased asymmetry of arabinoxylan molecules and thus with higher hydrodynamic volume/viscosity. However, other factors such as xylan chain length, presence of ferulic acid and specific

arrangement of arabinose residues along the xylan backbone influence this property.

In the presence of free radical-generating agents (e.g. hydrogen peroxide/peroxidase, ammonium persulphate, ferric chloride, linoleic acid/lipoxygenase), arabinoxylans are capable of forming three-dimensional networks (gels or viscous solutions). This unique property, now known as 'oxidative gelation' of water extracts of wheat flour was first described by Durham (1925). A number of factors such as molecular weight and substitution of the arabinoxylans influence the gelling property. However, presence of ferulic acid is prerequisite for the gelling ability of the polysaccharide and numerous hypotheses concerning the mechanism of this reaction have been developed. Detection of diferulic acid in oxidized arabinoxylan systems indicates that cross linking occurs through the coupling of two adjacent ferulic acid residues (Geissmann and Neukom, 1973).

Arabinoxylans are known to influence the quality of bakery products due to their physicochemical properties like viscosity and water holding capacity (Izydorczyk and Biliaderis, 1995). They absorb high amounts of water (6 – 8 times their weight) and when added to wheat flour, they compete with other constituents of dough for water. Studies showed significant increase in the farinograph water absorption, dough development time and loaf volume when arabinoxylans are added to the bread dough (Biliaderis et al., 1995; Vanhamel et al., 1993). However, at very high concentrations, due to the increase in viscosity, arabinoxylan addition adversely affected the bread quality (Biliaderis et al., 1995). Arabinoxylans are shown to protect protein foams against thermal disruption and retain gas in the dough (Hoseney, 1984). Viscosity of arabinoxylans adds to the strength and elasticity of gluten-starch films surrounding the gas bubbles and slows down the rate of CO_2 diffusion from dough during baking, affecting firmness and homogeneity of crumb texture.

Arabinoxylans are now considered to be prebiotics and used especially as arabinoxylo-oligosaccharides in functional foods for actively managing the colonic micro-flora with the aim of improving host health. A prebiotic is defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid, 1995).

They are also emerging as natural antioxidants, immuno-modulators and components of edible films.

1.9.1. Uses of ferulic acid

There have been studies showing potential health benefits of ferulic acid, such as anti-carcinogenic and anti-inflammatory properties. Ferulic acid is a strong UV absorber and constitutes the active ingredient in many skin lotions and sunscreens. It is also part of the gel matrix of wound healing, in a chemical form similar to the diferulated cross-links between arabinoxylan polymers in the cell walls. In the food industry, it is extracted from agro-industrial waste and bio-converted using fungi to vanillin, a much valued flavor compound. Its ability to inhibit peroxidation of fatty acids finds as natural food preservative and antioxidant.

1.10. Introducing the present investigation

Arabinoxylans are the chief non-starch polysaccharides (NSP) in cereals, the staple food for millions. NSP, arabinoxylans in particular, are shown to have much functionality and health benefits and for this reason structure and functions of cereal arabinoxylans were studies over the past few decades. However, study is largely confined to wheat, maize, oat, sorghum and others, and only to a limited extent to rice and ragi. It is believed that water soluble arabinoxylans are the one which exert much functionality, but study is sparse on these polysaccharides.

Moreover, structure and functions of water soluble feruloyl polysaccharides, especially from rice and ragi are scarce and for this reason these two cereal grains are chosen for investigation.

1.10.1. Rice and ragi – the grasses

The grass family includes all the major cereals, such as rice, wheat, maize, and oats, and most of the minor grains as well, such as rye, common millet, finger millet, teff, and many others that are less familiar (figure 6) (Kellogg, 1998).

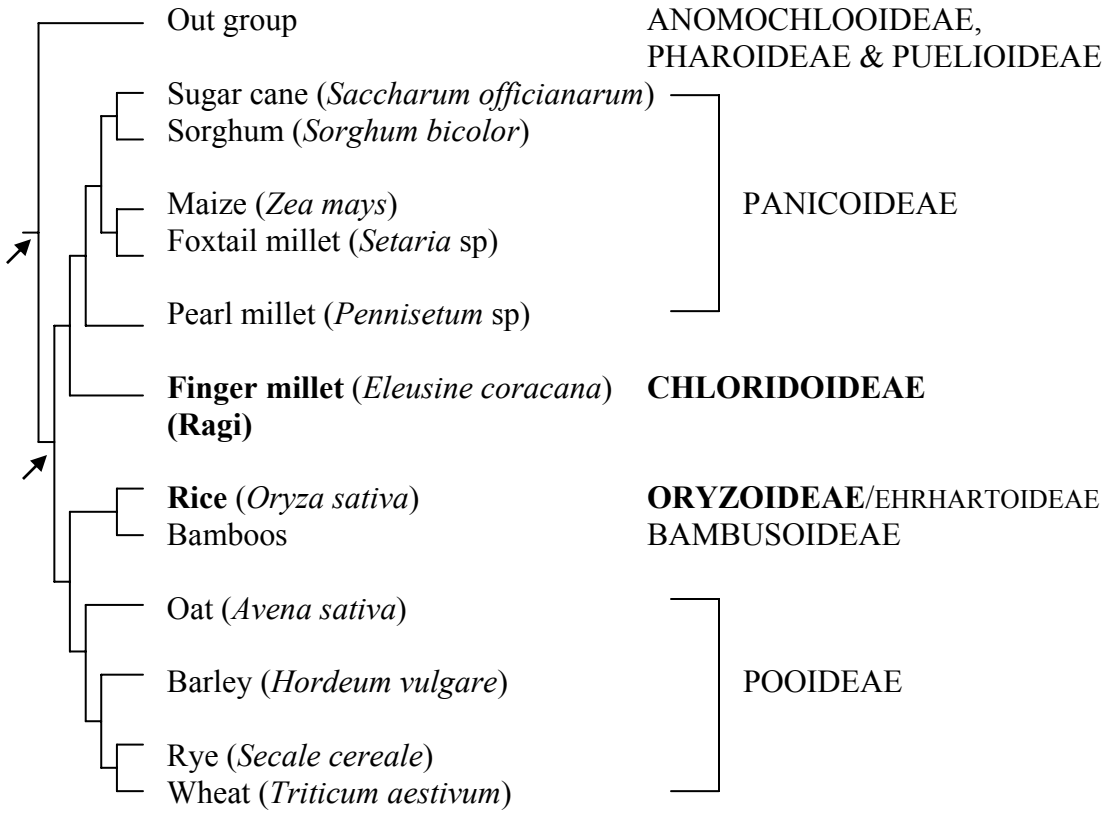

Figure 6. Phylogeny of the grass family (originated about 70 million years ago, upper arrow) based on the combination of morphological/anatomical and biochemical/molecular data shows that rice and ragi belong to different clades, widely separated in the evolutionary process of divergence dating back to over 66 million years (lower arrow) (Kellogg, 1998; Kellogg, 2001).

Since their domestication 10,000 years ago, the grasses have been of paramount importance to agriculture and human sustenance. This fact alone has been sufficient to make them the traditional focus of intensive scientific research. They also emerged in recent years as a collective model genetic system that stands beside and complements *Arabidopsis*.

Rice and ragi (figures 7A and 7B) belong to different clades in the grass family (figure 6), as they diverged over 66 million years ago. It would be interesting to see and compare any biochemical/molecular similarities/differences in these two distinct groups. Therefore, rice and ragi, a cereal and a millet respectively, were selected for the study of structural and functional aspects of water soluble feraxans, chief soluble fibers in cereals, and changes upon malting is investigated and a comparison is made between two.

Figure 7. The study subjects – rice (paddy – lower right side) (*Oryza sativa* var. Jaya) (A) and ragi (*Eleusine coracana* var. Indaf-15) (B) grains.

Rice (*Oryza sativa*) is the staple food grain and provides 25 to 80 percent of the calories in the daily diet of over 3 billion people or half the world's population (White, 1994). Probably native to the deltas of the great Asian rivers, rice is known to exist in over 1,20,000 varieties, and

now is emerging as a model monocotyledonous plant and key subject of intensive plant research.

Apart from staple diet, rice is gaining importance in food and pharmaceutical products. Oryzanol, a by product of rice mill has emerged as major neutraceutical. Over 10% of the rice grown in the U.S. each year goes into beer as rice gives lighter color and refreshing taste. Soaking/malting of rice is shown to induce GABA, a well-known blood pressure lowering compound and it has been proposed that it can be used as health food for hypertension sufferers (Saikusa et al., 1994).

Ragi (*Eleusine coracana*), also known as finger millet, is an important staple food in parts of India and Africa for people in low income groups. Nutritionally its importance is well recognized because of its high content of calcium (0.38%) and dietary fibre (18%), compared to the continental cereals such as rice, maize, wheat and barley (Kamath and Belavady, 1980; Ravindran, 1991). Ragi contains less protein (6 – 12%) and fat (1.0 – 1.4%), but contains high amount (3%) of an essential amino acid – methionine, an exceptional figure for a cereal grain (National Research Council, 1996). It is consumed as whole flour, thereby retaining the fibre, phenolics, minerals and vitamins present in the outer layer of the grain, which is nutritionally beneficial. Ragi is usually malted and the malt flour is used in the preparation of weaning food, beverages, ready-to-eat food items and other pharmaceutical products.

Ragi is supposed to be originated in Uganda, Africa (National Research Council, 1996). It is a C_4 plant, drought tolerant and quite resistance to diseases and pests and hence has the potential to be the leading/future food crop.

1.10.2. Malting

Malting (controlled germination) is the important process for the quality enhancement of cereal grains especially barley, for brewing

purposes. Hydrolases induced during malting act upon cell wall polysaccharides and bring many desirable changes including partial degradation and increased solubility of complex polysaccharides. High proportion of ragi is malted to prepare weaning and geriatric food with increased nutritional quality. Malting involves mainly three steps: steeping (soaking), germination and kilning (drying). As the seed germination (or malting) is an important biochemical process, a comparative malting study is undertaken between rice and ragi.

1.11. Scope of the present investigation

Cereals are the predominant staple food for millions of people across the world and are chief sources of non-starch polysaccharides/dietary fibre (Bunzel et al., 2001), whose consumption is linked with health benefits. Being major NSP, arabinoxylans stimulated considerable interest due to their functional properties such as water absorption, viscosity enhancing, and gelling quality and their impact on the rheological behavior of dough as well as the loaf volume and texture of bakery products (Meuser and Suckow, 1986). Functional properties, at least in part, are now related to the structural features of NSP. Despite the large amount of information available on the structural, nutritional and physiological properties of fibre, very little information is available on the functional effects of various fibre types (Özboy and Köksel, 1997). A great deal of uncertainty, however, remains as to the exact functional role and contribution of NSP from different sources to overall product characteristics; several research reports in this area are contradictory (Cawley, 1964; Courtin and Delcour, 2002; Jelaca and Hlynka, 1972; Kim and D'Appolonia, 1977). It is believed that much of the functionalities of these polysaccharides are due to their water soluble nature. They are almost completely fermented in large intestine by a mixed flora of anaerobic bacteria and most of the physiological effects are thought to be based on this property (Scheeman, 1998). Water insoluble

pentosans are shown to have an overall negative impact on product characteristics (Abdul-Hamid and Luan, 2000; Kulp and Bechtel, 1963), whereas their soluble counterparts have a beneficial impact (Delcour et al., 1991; Meuser and Suckow, 1986).

There are a number of individual reports on the overall structure and function of cereal water insoluble-hemicelluloses/arabinoxylans, which are mainly obtained by alkali extraction (Izydorczyk and Biliaderis, 1995; Subba Rao and Muralikrishna, 2004). However, information regarding water extractable non-starch polysaccharides is largely confined to mixed glucans with limited information on water extractable arabinoxylans.

Rice and ragi, a major cereal and millet respectively, are widely used as food and are the major sources of non-starch polysaccharides, water soluble (feruloyl) arabinoxylans in particular. However, there are no detailed studies on soluble feruloyl polysaccharides/arabinoxylans. In particular, information on detailed structural characteristics, their functional role in relation to structure and changes brought about by germination are lacking.

An attempt is therefore made in the present investigation to isolate and characterize these water extractable/soluble feruloyl polysaccharides from native and malted rice and ragi with the following objectives:

(a) Isolation and preliminary characterization of water extractable feruloyl polysaccharides and changes in feraxanases during malting,

(b) Fractionation and purification of water extractable feruloyl polysaccharides,

(c) Structural characterization of purified feruloyl polysaccharides using methylation, GLC-MS analysis, Smith degradation and ^{13}C-NMR, and

(d) Functional properties of water extractable feruloyl polysaccharides i.e., viscosity, gelling and foam stabilization and effect on dough characteristics, determination of their antioxidant and prebiotic activity *in vitro*.

In the present investigation detailed structural characteristics of water soluble feruloyl arabinoxylans are undertaken. Their functionalities, effect on dough properties and baking quality and fermentation properties are also studied. Functional characteristics, in part, are related to the structure of these polysaccharides. Knowledge on the structure and functionality of these soluble polysaccharides/fibre components may lead to an increased use in cereal-based products and functional foods for health benefits.

2.1. General

- All the results are average values of minimum of three experiments.
- Extractions and reagents were done using double glass-distilled water.
- Room temperature was ~ 25°C.
- Boiling water bath temperature was ~ 95°C, unless otherwise mentioned.
- Dialysis, against double distilled water or buffer, was carried out at ~ 4°C by using dialysis bags with a cutoff of ~ 8 kDa.
- Concentration/evaporation of samples was carried out by using Buchi Rotavapor (RE 111) with a water bath temperature ranging from 30 to 40°C.
- Colorimetric and spectrophotometric readings of test solutions with appropriate blanks were taken by using Shimadzu double beam Spectrophotometer (UV – 160A).
- Lyophilization was carried out using Virtis Freeze Mobile (12 SL).
- Centrifugation was carried out either in Sigma (202 C), Hermle (Z 320 K) or Remi (RC 8) centrifuges.
- Gel permeation fractions were collected, by using LKB Bromma 2211 fraction collector.
- Autoclaving was done at ~ 121°C, ~ 15 lbs for ~ 20 min.

2.2. Chemicals

2.2.1. Sigma Chemical Company, St. Louis, USA:

Enzymes: Glucoamylase (E.C. 3.2.1.3) from *Aspergillus niger*, Termamyl (E.C. 3.2.1.1) from *Bacillus licheniformis*, glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger*, peroxidase (E.C. 1.11.1.7) from horse radish, driselase (E.C. 3.2.1.8) from *Basidiomycetes* sp. and xylanase (E.C. 3.2.1.8) from *Thermomyces lanuginosus*.

Substrates: Larch wood xylan, 1,3 β-D-glucan (laminarin) from *Laminaria digitata*, p-nitrophenyl acetate, p-nitrophenyl glycosides of xylopyranose, arabinofuranose, and α and β galactopyranose, and ethyl ferulate.

Phenolic acids: Ferulic, coumaric, caffeic, gentisic, protocatechuic, vanillic, syringic, gallic and cinnamic acids.

Other chemicals: Blue dextran, coomassie brilliant blue G–250, carbodiimide (1-cyclo-hexyl-2 (4-methylmorpholino)–ethyl p-toluene sulfonate), carbazole, dinitrosalicylic acid, D_2O, O-diansidine, iodomethane (methyl iodide), ruthenium red, sodium azide, sodium borohydride, sodium borodeuteride, tris (hydroxy methyl) methyl amine (2-amino-2-(hydroxy methyl) propane-1,3-diol), DPPH* (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-tri (2-pyridyl)-triazine) and β-carotene.

Gel matrices and ion exchange resins: DEAE-cellulose (0.99 meq/g), Amberlite IR 120-P (8% cross linked, 16 – 50 mesh).

2.2.2. Pharmacia fine chemicals, Uppsala, Sweden:

Sephacryl S-300 (fractionation range for dextrans: 10 to 400 kDa) and T series dextran standards (T-10, T-20, T-40, T-70, T-150, T-500, T-2000).

2.2.3. Other sources:

Sugar standards (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and inositol) were from ICN Pharmaceuticals Inc., Life Sciences group, Cleveland, USA.

Cellulose acetate membranes were from Beckman Instruments International, S. A., Geneva, Switzerland.

Sodium hydride (99%) was from Aldrich Chemical Company, Milwaukee, USA.

Sep-Pak C_{18} cartridges were from Waters Associates, Milford, USA.

Dimethyl sulphoxide (UV spectroscopic grade), Folin phenol reagent (2 normal), bovine serum albumin (BSA) and hydrogen peroxide were from Sisco Research Laboratories, Mumbai, India.

Vials, Crimper and decapitator for methylation were from Pierce Chemical Company, Rockford, Illinois, USA.

Microbiological culture media and media ingredients were obtained from HiMedia (Mumbai, India). Microbiological filters (0.2 micron) were from Millipore (Bangalore, India).

All other chemicals/solvents used were of analytical grade and were obtained either from E-Merck, SRL or BDH (India).

2.3. Instruments

Following instruments were used for various experiments:

Beckman microzone cellulose acetate electrophoresis unit (model R 101) from Beckman Instruments International, S. A., Geneva, Switzerland.

B.O.D. incubator from Industrial and Laboratory Tools Corporation, Chennai, India.

Brabender Farinograph, Extensograph and Micro-Visco-Amylograph from Duisburg, Germany.

GC-15A, equipped with FID, from Shimadzu, Japan

GC-17A QP-5000 Mass Spectrometer, Shimadzu, Japan

GC-FT-IR – Perkin Elmer SPECTRUM system GC-IR 2000 Spectrometer, equipped with Windows 2.1 version, from Norwalk, USA.

HPLC – LC 10A, equipped with RI and UV – photo diode array detectors, from Shimadzu, Japan.

NMR – Bruker AMX 400 MHz Spectrometer from Bruker BioSpin, Germany.

Perkin Elmer (Model 243) polarimeter from Norwalk, USA.

Prince CE 560 capillary electrophoresis unit from Netherlands.

Texture analyzer (model Tehdi) from Stable Microsystems, Surrey, UK.

UV-visible Spectrophotometer (UVH 60A) from Shimadzu, Japan.

2.4. Columns

2.4.1. GLC Analysis

OV-225 (ϕ 1/8″ x 8 ft), 3% on Chromosorb W (80 – 100 mesh) and PEG-20M (ϕ 1/8″ x 8 ft) were obtained from Pierce Chemical Company, Rockford, Illinois, USA

SP-2330 (ϕ 0.32 mm x 30 M) was obtained from Supelco, Tokyo, Japan.

SC-30 (ϕ 0.32 mm x 30 M, 0.25 μm film thickness) was obtained from J & W Scientifics, USA.

2.4.2. HPLC Analysis

Shimpack C_{18} column (ϕ 4.6 mm x 250 mm) was obtained from Shimadzu Corporation, Tokyo, Japan.

E-linear (ϕ 7.8 mm x 300 mm) and E-1000 (ϕ 3.9 mm x 300 mm) gel permeation columns were obtained from Waters Associates, USA.

Silica capillary column (ϕ 75 μm x 1 M) was obtained from Netherlands.

2.5. 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.

Maida, refined wheat flour devoid of most of the NSP, was obtained from the local market.

2.6. Methods

2.6.1. Distillation/purification of solvents

Phenol, acetic anhydride, pyridine, diethyl ether and DMSO were purified by distilling in all glass distillation apparatus following standard distillation protocols.

2.6.2. Malting

Rice and ragi seeds (200 g each) 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 (Malleshi and Deshikachar, 1986; Nirmala et al., 2000). After germination, seeds were kilned at 50°C for 24 h and milled to obtain malted flour. Ungerminated seeds were milled to obtain native flour. Malting loss was calculated by using the following formula:

$$\text{Malting loss} = \frac{W_1 - W_2}{W_1} \times 100\ \%$$

Where, W_1 = initial weight of the seeds and W_2 = final weight of the seeds after malting and removing growth portions.

2.6.3. Colorimetric estimations

2.6.3.1. Total sugar

To the sample (0.5 ml) in a test tube (ϕ 12 x 150 mm), phenol (0.3 ml, 0.5%) and concentrated H_2SO_4 (1.8 ml, specific gravity: 1.84) were added successively and mixed thoroughly. After incubation at room temperature for 20 min, absorbance was read at 480 nm (McKelvy and Lee, 1969). Sugar content was determined by referring to the standard graph, prepared by using either D-glucose or D-xylose (5 – 50 µg/0.5 ml).

2.6.3.2. Reducing sugar

Preparation of DNS reagent: Dinitro salicylic acid (1g) was dissolved in a solution containing sodium potassium tartrate (30 g) and 2.0 molar NaOH (20 ml) and the content was made up to 100 ml with water. The reagent was filtered though a Whatman No. 1 filter paper.

To the sample (1 ml) in a test tube, DNS reagent (1 ml) was added and incubated in boiling water bath for 10 min. Content was then cooled and diluted with equal volume (2 ml) of distilled water. Absorbance was read at 550 nm (Miller, 1959). Reducing sugar content was determined

by referring to the standard graph prepared by using D-glucose (0.1 – 1.0 mg/ml).

2.6.3.3. Uronic acid

To the sample (0.5 ml) in a test tube (ϕ 12 x 150 mm), kept in an ice-cold water bath concentrated H_2SO_4 (3 ml) was added and mixed well. After incubation in boiling water bath for 20 min, carbazole* solution (0.1 ml, 0.1% in ethanol) was added and kept in dark for 2 h. Absorbance was read at 530 nm (Knutson and Jeanes, 1968). Uronic acid content was determined by referring to the standard graph prepared by using either D-glucuronic or D-galacturonic acid (10 – 50 µg/0.5 ml).

* Carbazole was re-crystallized in benzene to remove contaminants.

2.6.3.4. Protein

a. **Lowry method**

Reagent A: Na_2CO_3 (2%) in NaOH (0.1 molar)

Reagent B: $CuSO_4.5H_20$ (0.5%) in sodium potassium tartrate (1%)

Reagent C: Reagent A (50 ml) and reagent B (1 ml) were mixed together prior to estimation.

Reagent D: Folin-phenol reagent was diluted to 1 normal (based on the titration with NaOH to phenolphthalein end-point).

To the sample (1 ml) in a test tube reagent C (5 ml) was added and allowed to stand for 10 min at room temperature. Reagent D (0.5 ml) was added to it and again allowed to stand for 30 min. Absorbance was read at 700 nm (Lowry et al., 1951). Protein content was determined by referring to the standard graph prepared by using BSA (20 – 100 µg/ml).

b. **Bradford method**

Preparation of Bradford reagent: Coomassie brilliant blue G-250 (10 mg) was dissolved in ethanol (5 ml, 95%) and phosphoric acid (10 ml, 85%)

was added. The solution was made up to 100 ml with water and filtered through Whatman No. 1 filter paper.

To the sample (0.2 ml) Bradford reagent (0.8 ml) was added and mixed well. Absorbance was read at 595 nm (Bradford, 1976). Protein content was determined by referring to the standard graph prepared by using BSA (2 – 10 µg/0.2 ml).

2.6.3.5. Glucose oxidase method

Preparation of glucose oxidase reagent: Glucose oxidase (125 mg, ~6250 units) was dissolved in tris buffer (0.1 molar, pH 7.0) and peroxidase [0.5 ml (10 mg/ml in water), ~1250 units], O-dianisidine (0.5 ml, 10 mg/ml in 95% ethanol) and triton X-100 (1 ml, 10 ml detergent in 40 ml ethanol) were added. The solution was made up to 100 ml with tris buffer.

To the sample (0.5 ml) glucose oxidase reagent (3 ml) was added and incubated at 37°C for 1 h. Absorbance was read at 420 nm (Dahlqvist, 1964). Glucose content was determined by referring to the standard graph prepared by using glucose (10 – 50 µg/0.5 ml).

2.6.4. Glucoamylase digestion

Sample (10 g) was dissolved in sodium acetate buffer (100 ml, 0.1 molar, pH 4.8) and incubated with termamyl (1 ml, ~1000 units) at 95°C for 1 h. After cooling glucoamylase (100 mg, ~7000 units) was added and incubated at 37°C for 48 h. Glucose released from the starch hydrolysis was quantified by glucose oxidase method.

2.6.5. Enzyme assays

Native and malt fours (2.5 g) were extracted with different buffers (10 ml each) for obtaining different enzymes; i.e., sodium phosphate buffer (0.05 molar, pH 6.0) for obtaining amylase and 1,3 β-D-glucanase (Nirmala et al., 2000), sodium acetate buffer (0.1 molar, pH 4.8) for obtaining xylanase, sodium phosphate buffer (0.1 molar, pH 5.7) for

obtaining arabinofuranosidase, xylopyranosidase, α-D-galactopyranosidase and β-D-galactopyranosidase, sodium potassium phosphate buffer (0.2 molar, pH 6.5) for obtaining acetyl esterase and tris buffer (0.05 molar, pH 7.5) for obtaining ferulic acid esterase (Humberstone and Briggs, 2002). Extractions were performed at 4°C for 2 h. After centrifugation (3000 x g for 20 min), supernatant was dialyzed overnight against the same buffer and assayed for the presence of various enzyme activities using appropriate substrates and conditions.

For assaying the enzymes in the microbial culture broths, broth (24 h old) was directly used as the sample and incubated with the substrates. For assaying enzymes in the rat cecal and faecal samples, weighed samples were suspended in normal saline (0.85%).

2.6.5.1. Amylase

Gelatinized soluble starch (1 ml, 1% in sodium phosphate buffer) was incubated with sample/enzyme extract (0.1 ml) for 30 min at 45°C (Bernfeld, 1955). Reaction was stopped by adding DNS reagent (1 ml) 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 glucose/min under assay conditions.

2.6.5.2. 1,3 β-D-Glucanase

Laminarin (1 ml, 0.2% in sodium phosphate buffer) was incubated with sample (0.1 ml) for 4 h at 37°C (Fink et al., 1988). Reducing sugar liberated was quantified as above.

2.6.5.3. Xylanase

Larch wood xylan (1 ml, 0.5 % in sodium acetate buffer) was incubated with sample (0.1 ml) for 30 min at 50°C (Cleemput et al., 1997). Reducing sugar liberated was quantified as above. One unit of

activity is defined as the amount of enzyme required to liberate 1 μmol of xylose/min under assay conditions.

2.6.5.4. β-D-Xylopyranosidase

The substrate, p-nitrophenyl β-D-xylopyranoside (glycoside) (0.5 ml, 2 mmol in sodium phosphate buffer) was incubated with sample (0.1 ml) for 1 h at 37°C (Beldman et al., 1996). Reaction was stopped by adding saturated solution of sodium tetraborate (0.5 ml). Absorbance was read at 400 nm. One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of p-nitrophenol/min under assay conditions.

p-Nitrophenol (2 – 10 μg/0.5 ml in sodium phosphate buffer) was mixed with equal volume of saturated solution of sodium tetraborate (0.5 ml). Absorbance was read at 400 nm, and a standard curve was prepared.

2.6.5.5. α-L-Arabinofuranosidase

p-Nitrophenyl α-L-arabinofuranoside was taken as the substrate (Beldman et al., 1996) and the assay was performed as mentioned above.

2.6.5.6. α-D-galactopyranosidase

p-Nitrophenyl α-D-galactopyranoside was taken as the substrate and the assay was performed as mentioned above.

2.6.5.7. β- D-galactopyranosidase

p-Nitrophenyl β-D-galactopyranoside was taken as the substrate and the assay was performed as mentioned above.

2.6.5.8. Acetyl esterase

The substrate, p-nitrophenyl acetate (1 ml, saturated solution in sodium potassium phosphate buffer) was incubated with sample (0.1 ml) at 25°C and absorbance was read at 400 nm for up to 3 min (time scan) (Chung et al., 2002). One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of p-nitrophenol/min under assay conditions.

p-nitrophenol (2 – 10 μg/ml in sodium potassium phosphate buffer) absorbance was read at 400 nm and a standard curve was prepared.

2.6.5.9. Feruloyl esterase

Ethyl ferulate (0.1 ml, 8 mmol in tris buffer) (Humberstone and Briggs, 2000) was incubated with sample (0.3 ml) for 1 h at 37°C (Blum et al., 2000). Reaction was stopped by adding 3 volumes of methanol and the ferulic acid released was quantified by HPLC (Rao and Muralikrishna, 2004). One unit of activity is defined as the amount of enzyme required to liberate 1μmol of ferulic acid/min under assay conditions.

2.6.6. Isolation of WEP and WUP

Flour (100 g) was extracted with water (200 ml x 4 at 25°C) for 2 h each and the supernatant obtained after centrifugation (3000 x g for 20 min) was precipitated with 3 volumes of ethanol. Precipitate was separated out, dialyzed and lyophilized to obtain water extractable polysaccharides (WEP). The residue obtained after centrifugation was digested with glucoamylase to remove the starch and the undigested material was dried by solvent exchange and designated as water unextractable polysaccharides (WUP) (Rao and Muralikrishna, 2004).

2.6.7. Isolation and characterization/quantification of free and bound phenolic acids

a. **Free phenolic acids**

Flour (1 g) was extracted with ethanol (70%, 50 ml x 4, at room temperature) for 1 h each and the supernatant was obtained upon centrifugation (3000 x g for 20 min) (Ayumi et al., 1999). Its pH was adjusted to 2 – 3 with HCl (4 molar) and phenolic acids were phase separated into ethyl acetate (50 ml x 4). Combined solvent was treated with anhydrous sodium sulphate, filtered and evaporated to dryness. Phenolic acids present were taken in methanol (1 ml) and analyzed by HPLC using C_{18} column (4.6 x 250 mm) and UV diode array detector (280 and 320 nm) with a solvent system of water: acetic acid: methanol (isocratic, 80:5:15 v/v/v, flow 0.6 ml/min). Caffeic, cinnamic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids were used as standards (2 – 10 μg/10 μl) (figure 8). Phenolic acids in the sample are quantified by using peak area standard curve.

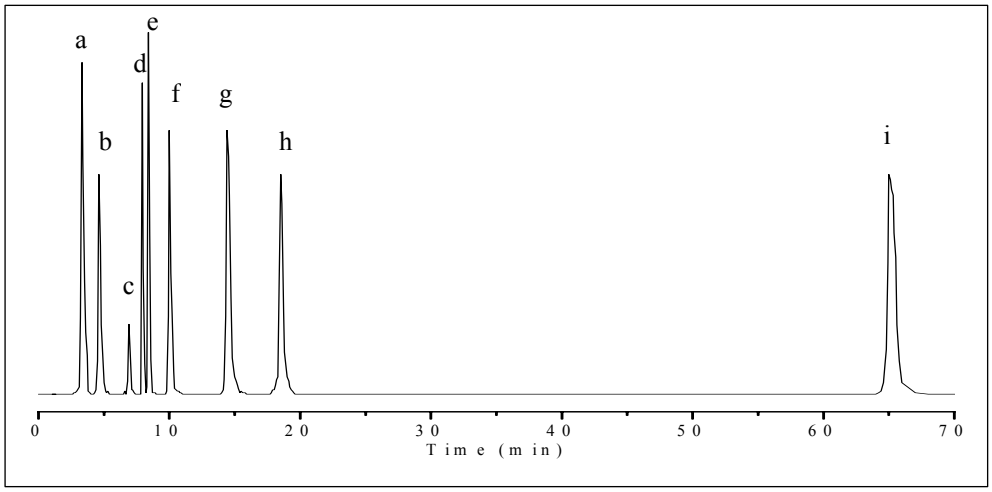

Figure 8. HPLC profile of standard phenolic acids on C_{18} column (detection was performed at 320 nm): gallic acid (3.2 min)(a), protocatechuic acid (4.6 min) (b), gentisic acid (6.9 min) (c), vanillic acid (7.9 min) (d), caffeic acid (8.4 min) (e), syringic acid (9.6 min) (f), coumaric acid (14.4 min) (g), ferulic acid (18.5 min) (h) and cinnamic acid (65.0 min) (i).

b. **Bound phenolic acids**

WEP and WUP (1 g each) were extracted with NaOH (1 molar, 100 ml x 2, at room temperature) containing sodium borohydride (0.5%) under nitrogen atmosphere for 2 h each and the supernatant was collected upon centrifugation (3000 x g, for 20 min), acidified (pH 1.5) with HCl (4 molar) (Nordkvist et al., 1984) and processed further as described for free phenolic acids.

2.6.8. Fractionation of NSP on DEAE-cellulose column

a. **DEAE-cellulose regeneration**: It was washed with water and then treated successively with five volumes of HCl (0.5 molar) and NaOH (0.5 molar) for 30 min each. After each treatment it was thoroughly washed with water to bring the pH to neutrality. The regenerated anion exchanger was suspended in five volumes of ammonium carbonate solution (0.5 molar, pH 9.3), packed in to a column and (ϕ 3 x 25 cm) and excess carbonate was washed off with water.

b. **Preparation of NSP sample**: WEP was dissolved in water (1:10, w/v) and insoluble portion was separated out by centrifugation (3000 x g, for 20 min). Soluble portion was heated (95°C for 10 min) to denature enzymes and precipitate proteins. It was further centrifuged and the supernatant thus obtained was dialyzed and lyophilized to obtain water soluble non-starch polysaccharides (NSP).

c. **Fractionation of NSP**: water soluble NSP (1 g) was dissolved in water (1:10, w/v) and loaded to the pre-equilibrated DEAE-cellulose column and the elution was carried out with water, followed by ammonium carbonate (0.1 and 0.2 molar) and NaOH (0.1 and 0.2 molar) solutions (Neukom and Kuendig, 1965). The flow rate was maintained at 60 ml/h and fractions (5 ml each) collected were assayed for total sugar. Peak fractions were pooled, dialyzed and lyophilized.

2.6.9. Homogeneity criteria

2.6.9.1. Molecular sieving

Gel permeation chromatography (Anderson and Stoddart, 1966; Izydorczyk and Biliaderis, 1993) was performed on Sephacryl S-300 gel matrix. Sample (0.1 molar AC eluted fraction, 10 mg/ml) was loaded to the column (dimensions: ϕ 1.6 x 90 cm). The elution was carried out by using NaCl (0.05 molar) containing sodium azide (0.05%) at a constant flow rate of 18 ml/h. Fractions (3 ml) were collected and assayed for total sugar. Peak fractions were pooled, dialyzed and lyophilized. The column was pre-calibrated with T-series dextran standards (T-10, T-20, T-40, T-70, T-150, T-500 and T-2000 kDa), glucose (to determine bed volume) and blue dextran (to determine void volume). Calibration curve is prepared by plotting V_e/V_o versus log molecular weight (where V_e = elution volume, V_o = void volume) and molecular weight of the sample is determined.

2.6.9.2. High Performance Size Exclusion Chromatography (HPSEC)

HPSEC (Gruppen et al., 1992a; Gruppen et al., 1992b) was carried out using E-linear (ϕ 7.8 x 300 mm) and E-1000 (ϕ 3.9 x 300 mm) columns connected in series. HPLC was equipped with a refractive index (RI) detector and a CR 4A recorder. Samples (10 mg/ml) and T-series dextran standards were injected (10 μl) and eluted with water at a flow rate of 0.6 ml/min.

2.6.9.3. Electrophoresis of acidic polysaccharides

a. **Cellulose acetate paper electrophoresis**: Paper electrophoresis was performed on cellulose acetate membrane using Beckman Microzone Electrophoretic Cell (model: R 101). Ammonium carbonate – NaCl (0.05 molar, pH 9.3) was used as the running buffer. Membrane was immersed with running buffer prior to run and excess buffer was removed by using

blotters. Sample (10 mg/ml) was applied on the membrane by using an applicator and run at 180 volts. The rate of migration was monitored by using prucion red marker dye. After run, membrane was immersed in ruthenium red dye (0.5% in water) to stain the acidic polysaccharides and the excess dye was washed with water.

b. **Capillary electrophoresis**: Capillary electrophoresis was performed over a silica capillary column (ϕ 75 μm x 100 cm) (Soga and Serwe, 2000) using Prince CE 560 capillary electrophoresis unit. Prior to run column was thoroughly washed with NaOH (0.1 molar) and equilibrated with borate buffer (0.5 molar, pH 8.3). Sample was injected and run at a column pressure of 100 mbar and voltage maintained at 20 k-volts. Elution was monitored at 253 nm.

2.6.10. Structural methods

2.6.10.1. Determination of neutral sugar composition by GLC

a. **Hydrolysis of polysaccharide with sulfuric acid:** Polysaccharide sample (10 mg) was dissolved in water (0.2 ml) and ice-cold sulfuric acid (0.6 ml) and water (6.4 ml) were added to obtain a final acid concentration of 8%, and hydrolyzed by keeping in boiling water bath for 12 h (Selvendran and O'Neil, 1987). Content was neutralized with barium carbonate, filtered, de-ionized with Amberlite IR 120-H^+ resin and concentrated.

b. **Preparation of alditol acetate derivatives:** To the polysaccharide hydrolysate inositol (1 mg) was added as an internal standard and sodium carbonate (0.1 ml, 2 molar) to saponify any glucuronolactones formed. Reduction was done by adding either sodium borohydride (10 mg) or sodium borodeuteride (sample was taken in D_20, in case of methylated sugars) and kept at room temperature for 6 h. Excess borohydride was destroyed by adding acetic acid (2 normal) drop wise till

the hydrogen-effervescence stopped. Boric acid formed was removed by co-distillation with methanol (2 ml x 5). To the resultant glycitol, acetic anhydride (1 ml) and pyridine (1 ml) were added and kept in boiling water bath for 2 h (Sawardekar et al., 1965). After acetylation excess reagents were removed by co-distillation with water (2 ml x 3) and toluene (2 ml x 3). Alditol acetates were extracted with chloroform, filtered though glass wool and dried using a nitrogen stream. They were further dissolved in a known amount of chloroform and analyzed by GLC and MS.

c. **GLC analysis**: Alditol acetates (figure 9) and per-methylated sugars were analyzed on GLC fitted with an OV-225 (3%) stainless steel column (ϕ 1/8″ x 8 ft) using flame ionization detector (FID). Column, injector and detector port temperature were maintained at 200°C (185°C in case of per-methylated sugars), 250°C and 250°C respectively. Nitrogen was used as the carrier gas (40 ml/min).

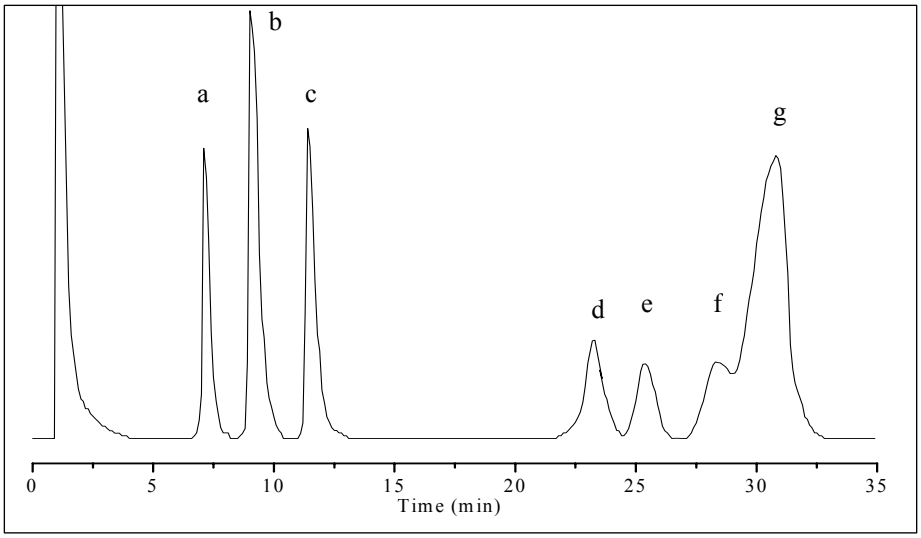

Figure 9. GLC profile of standard sugars on OV-225 column: rhamnose/fucose (7.1 min) (a), arabinose (9.0 min) (b), xylose (11.4 min) (c), mannose (23.1 min) (d), galactose (25.3 min) (e), glucose (28.5 min) (f) and inositol (31.0 min) (g).

2.6.10.2. GLC-MS Analysis

GLC-MS analysis (Jansson et al., 1976) was performed on a Shimadzu GC 17A QP-5000 system using SP 2330 capillary column (ϕ 0.31 mm x 30 m) operating at an ionization potential of 70 electron volts with a temperature program (180 - 200°C, 4°C raise per min, 200°C for 50 min). Mass range between 40 – 400 amu (m/e) was taken for analysis. Helium was used as the carrier gas.

2.6.10.3. Methylation analysis

a. P**reparation of methyl sulphinyl carbanion (MSC)**: Sodium hydride (99%, 500 mg washed trice with dry ether and flushed with nitrogen) was treated with DMSO (5 ml) in a reaction vial (25 ml capacity) at 60°C for 4 h. Hydrogen liberated was intermittently removed by using a needle. The resulting greenish yellow MSC solution (anion) was tested for its characteristic blood red color with triphenyl methane (Rauvala, 1979). The vial containing anion was covered with parafilm and stored at -20°C till use.

b. **Methylation of polysaccharides**: Polysaccharide sample (5 – 10 mg) was dissolved in DMSO (1 ml) in a reaction vial with stirring and ultrasonication. MSC (1 ml) was added drop wise to the solution with the help of a syringe, stirred at room temperature for 4 h and presence of excess anion was tested by using triphenyl methane. Methyl iodide (1 ml) was added to the reaction mixture at ice-cold temperature with the help of a syringe and stirred for 2 h (Hakomori, 1964).

c. **Purification of methylated polysaccharides:** Permethylated polysaccharide was purified on Sep-Pak C_{18} cartridge, which was activated by flushing ethanol (40 ml) followed by acetonitrile (2 ml) and water (4 ml). The reaction mixture was diluted with equal volume of water (3 ml) and passed through the cartridge (Waeghe et al., 1983). More polar contaminants were eluted with water (2 ml x 4) followed by successive elution with acetonitrile:water (3:17, 2 ml x 4),

acetonitrile:water (1:4, 2 ml x 4), acetonitrile (100%, 2 ml x 4), methanol (100%, 2 ml x 4) and ethanol (95%, 2 ml x 4). Elution was carried out at a flow rate of 1 – 2 drops/sec. Individual fractions were tested for the presence of sugar by spotting a drop on silica gel TLC strip and charring with sulfuric acid (5%, in methanol) at 120°C for 1 h. Fractions (eluted with 100% acetonitrile and 100% methanol) giving positive test were pooled, concentration and hydrolyzed.

d. **Hydrolysis of methylated polysaccharides**: Permethylated polysaccharides were hydrolyzed by using formic acid (90%, 2 ml) at 100°C for 2 h. Excess acid was removed by flash evaporation and the dried sample was further hydrolyzed with sulfuric acid (2 ml, 0.5 normal) at 100°C for 12 h. Acid was neutralized with barium carbonate and hydrolysate was filtered and evaporated to dryness. It was exchanged with D_20 (2 ml x 2), reduced with sodium borodeuteride and derivatized as mentioned earlier.

2.6.10.4. Reduction of polysaccharide carboxyl groups

To the polysaccharide solution (100 mg/20 ml) 1-cyclohexyl-2(4-methylmorpholino)-ethylcarbo-diimide-p-toluene sulphonate (1 g) was added in small portions over a period of 2 h. the pH of the solution during reaction was maintained at 4.8 by the addition of HCl (0.1 molar). Reduction was carried out by the addition of sodium borohydride (10 ml, 2 molar) over a period of 4 h and during which the pH was maintained at around 7.0 by the addition of HCl (4 molar) (Taylor and Conrad, 1972). The reaction mixture was dialyzed and lyophilized. The above sequence was repeated 2 to 3 times for the quantitative conversion of carboxyl groups into primary alcohol (-COOH to $–CH_2OH$).

2.6.10.5. Periodate oxidation

To the polysaccharide solution (10 mg/5 ml) sodium meta periodate (5.0 ml, 20 mmol) was added, mixed well and kept at 4°C in the dark. Aliquots (0.5 ml) were withdrawn from the sample at regular intervals (4 h) and the amount of periodate remaining was determined by TPTZ method (Avigad, 1969).

TPTZ reagent preparation: TPTZ (75 mg, 0.24 mmol) was dissolved in acetic acid (46 ml) and sodium acetate buffer (210 ml, 1 molar, pH 4.0), mixed with freshly prepared solution of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (31.4 mg, 0.08 mmol/100 ml) and the volume was made up to 1000 ml with water.

Appropriately diluted sample (0.5 ml) was mixed with TPTZ violet reagent (4.5 ml) and the residual color was read at 593 nm. A standard graph was prepared by taking known amount (5 – 200 nmol/0.5 ml) of periodate.

2.6.10.5.1. Formic acid liberation

An aliquot (1 ml) was withdrawn from the above reaction mixture after the periodate consumption became constant. Ethylene glycol (1 ml) and methyl red indicator (2 drops, 0.02% in ethanol) were added to it and titrated against sodium hydroxide (0.01 normal). Change in the color of the solution from pink to yellow indicated the endpoint. A reagent blank was prepared in the same way with ethylene glycol and the difference in the acidity between the blank and sample represented the formic acid liberation from the polysaccharide sample (Brown et al., 1948).

2.6.10.6. Smith degradation

To the polysaccharide solution (10 mg/5 ml) sodium meta periodate (5 ml, 20 mmol) was added and kept at 4°C for 48 h. Reaction was stopped by the addition of ethylene glycol (0.1 ml) and the oxidized polysaccharide sample was reduced with sodium borohydride (100 mg) at room temperature for 16 h. Excess borohydride was destroyed by using

acetic acid (2 normal) and the solution was dialyzed. The polyalcohol was hydrolyzed with sulfuric acid (2 ml, 0.5 normal) at room temperature for 48 h, derivatized and analyzed by GLC (Abdel-Akher et al., 1952).

2.6.10.7. Optical rotation

Optical rotation of the polysaccharide solution (1%, in water) was determined at 20°C (Saavendra et al., 1988) by using Perkin Elmer (model 243) polarimeter and was calculated by using the following formula:

$$\text{Optical rotation } [\alpha]_D = \frac{100\ \theta}{lc}$$

Where, θ is the angle of rotation of plane polarized light, l is the path length (1 cm) and c is the concentration (%) of the polysaccharide solution.

2.6.10.8. Ultra violet (UV) spectroscopy

UV absorption spectra of polysaccharide solution taken in quartz cuvette were recorded between 200 – 400 nm using a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

2.6.10.9. Infra red (IR) spectroscopy

Polysaccharide sample (1 mg) was blended with KBr (150 mg) and prepared the pellet by using a palletizer (Kacurakova et al., 1994). IR spectra were recorded between 4000 – 400 cm^{-1} (4 cm^{-1} resolution) using a Perkin-Elmer 2000 spectrometer (Norwalk, USA).

2.6.10.10. ^{13}C and ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy

Polysaccharide solution (100 mg/ml in D_2O) was taken in sample probe (ϕ 5 mm x 15 cm) and resonance spectrum was recorded in a

Bruker AMX 400 MHz NMR spectrometer operating at 60°C for 4 h with a spectral width of 22,272 Hz and about 3000 scans. Deuterium resonance was used as a field frequency lock and the shifts were recorded with reference to external TMS (Hoffmann et al., 1991; Hoffmann et al., 1992).

2.6.11. Functional characterization

2.6.11.1. Determination of antioxidant activity

2.6.11.1.1. 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 (Miller, 1971; Subba Rao and Muralikrishna, 2004). Sample (0.1 ml, aqueous solution of polysaccharide) 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:

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

Where, A_{control} is the absorbance of the emulsion (without sample). Concentration providing 50 % inhibition (IC_{50}) (Güllüce et al., 2003) was calculated from the graph – plotted AAC (%) against concentration.

2.6.11.1.2. DPPH* assay

Antiradical activity of soluble feraxans was estimated according to the slight modification of the procedure reported elsewhere (Guérard and Sumaya-Martinez, 2003; Güllüce et al., 2003; Kikuzaki et al., 2002; Ohta

et al., 1994). 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 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*:

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

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

2.6.11.1.3. 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). Sample (0.1 ml) 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 $FeCl_3.6H_2O$ and 25 ml of 300 mmol/L acetate buffer, pH 6.3). After the incubation period 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 ($FeSO_4.7H_2O$) 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 $FeSO_4.7H_2O$. EC_1 was calculated from the graph – plotted absorbance against concentration.

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

2.6.11.2. Relative viscosity

Relative viscosity of water soluble NSP, with respect to water, was determined as described by Muralikrishna et al. (1987). Water soluble NSP was dissolved in water/buffer and the viscosity was determined in an Ostwald viscometer, with respect to concentration (0.2 – 1.0 %), temperature (20 – 80°C) and pH (2.0 – 10.0).

2.6.11.3. Oxidative gelation

Oxidative gelation of water soluble NSP was carried out according to Vinkx et al. (1991). To an aqueous solution of NSP (0.2 – 1.0 %, 20 ml) 0.1 ml of H_2O_2 (0.39 g/l) and 0.1 ml (1 Sigma purpurogallin unit) of horse radish peroxidase (E.C. 1.11.1.7, 200 – 300 units/mg solid) were added and incubated at 30°C. Relative viscosity, with respect to water, was determined at regular intervals (0, 20, 40 and 60 min) as mentioned above. Appropriate controls were taken to determine the oxidative gelation capacity of water soluble NSP.

2.6.11.4. Foam stabilization

Foam stabilization effect of water soluble NSP was determined according to Izydorczyk et al. (1991), and Susheelamma and Rao (1979). In brief, 1 ml of 2 % BSA containing 0.2 to 1.0 % additive was mixed (for 20 sec) with 0.25 ml of 5 % $NaHCO_3$ in a graduated tube and the initial foam volume was noted. For the above mixture 0.25 ml of 5 % citric acid was added and foam volume was noted after mixing for 30 sec and 10 min holding. The solution was then heated at 90°C for 3 min and the final foam volume was noted. Suitable blanks/controls were taken to obtain an indication of the foam stabilization effect of the water soluble NSP.

2.6.11.5. Farinograph characteristics

Effect of addition of water soluble NSP on mixing properties of wheat dough was determined by Brabender Farinograph, using a 50 g bowl, as approved by AACC (2000). 50 g of maida, on 14 % moisture basis, was kept in the bowl with or with out additive (0.25 and 0.50 %) and during mixing, water was added from the burette to give a dough consistency of 500 BU. Following parameters were derived from the resulting Farinogram: (a) water absorption (%), (b) dough development time (min), (c) dough stability (min) and (d) mixing tolerance index (FU).

2.6.11.6. Extensograph characteristics

Effect of addition of water soluble NSP on extensibility of wheat flour dough was determined by Brabender Extensograph, as approved by AACC (2000). Dough containing 2 % NaCl (on flour basis), with or without additive (0.25 and 0.50 %) and having a water content resulting in a Farinograph consistency of 500 BU, was prepared in a Farinograph. Dough (150 g) was rounded in to a ball, shaped into a cylinder and clamped horizontally in a cradle in the Brabender Extensograph. After the resting period (45, 90 and 135 min) it was subjected to Extensograph studies. Maximum extensibility (mm) and maximum resistance to extension (BU) were obtained. The results are the average of duplicate measurements.

2.6.11.7. Micro-Visco-Amylograph studies

Effect of addition of water soluble NSP on pasting characteristics of wheat starch was determined by Brabender Micro-Visco-Amylograph. 15 g wheat flour containing 0.25 and 0.50 % of additive was mixed with 100 ml of water. The resultant slurry was transferred to an Amylograph bowl and heated at a rate of 7.5°C/min from 30°C to 92°C. After 3 min holding, the paste was cooled at a rate of 7.5°C/min to 50°C and held at same temperature for 2 min. Following parameters were noted from the

resulting Amylogram: (a) gelatinization temperature (GT, in °C), (b) peak viscosity (PV), (c) hot paste viscosity (HPV) and (d) cold paste viscosity (CPV).

2.6.11.8. Bread quality studies

Bread quality studies were carried out by adding 0.25 and 0.50 % of water soluble NSP to bread formulation (AACC, 2000; Abdul-Hamid and Luan, 2000). Maida (100 g) with yeast (2 %), fat (1 %), sugar (2.5 %) and salt (0.5 %) were mixed with water, fermented at 30°C, molded and baked at 220°C for 25 min.

Loaf volume (ml) was determined by volume displacement method using mustard seeds. Firmness or texture of 1 day old bread was determined by Texture analyzer (model Tehdi). Bread slice was compressed (25%) with the compression plunger and the resulting peak force was measured in gram. Significance of difference between tests was evaluated using Duncan's multiple range test at 5 % level.

2.6.11.9. Microbiological methods

2.6.11.9.1. Organisms and culture conditions

Bifidobacteria and lactic acid bacteria: This included the cultures of *Bifidobacterium adolesentis* NDRI 236, *Lactobacillus acidophilus, Lb. acidophilus* B 4495, *Lb. amylovorus* B 4437, *Lb. brevis* NDRI 253, *Lb. bulgaricus* CFR 2028, *Lb. casei, Lb. casei* B 1922, *Lb. casei* DSM 20011, *Lb. casei* NCIM 2586, *Lb. casei* Sweden, *Lb. helveticus* B 4526, *Lb. plantarum* NCIM 2084, *Lb. plantarum* B 4496, *Lb. plantarum* CFR 2164, *Lb. rhamnosus* NDRI 018, *Lb. salivarius* CFR 2158 and *Pediococcus pentosaceus* NDRI 035, which were available in the institute culture collection. The cultures of lactic acid bacteria were individually maintained at 6°C in lactobacillus MRS broth and that of bifidobacteria

in MRS broth supplemented with cysteine HCl (0.05%) and sub-cultured at regular intervals of 30 days.

Yeast: This included cultures of *Brettanomyces claussenii* CFR 501, *Candida bacarum* CFR 502, *C. fragariorum* CFR 503, *C. fragariorum* CFR 504, *C. versatilis* CFR 505, *Saccharomyces cerevisiae* Boulderi, *S. cerevisiae* CC 127, *S. cerevisiae* CFR 101, *S. carlsbergensis* NCIM 3224 and *Schizosaccharomyces pombe* CFR 506. The yeast cultures were individually maintained at 6°C in potato dextrose broth and sub-cultured at regular intervals of 60 days.

Food borne pathogenic bacteria: This included cultures of *Bacillus cereus* F 4810, *Escherichia coli* D 21, *Staphylococcus aureus* FRI 722 and *Yersinia enterocolitica* MTCC 859. The cultures were individually maintained at 6°C in brain heart infusion (BHI) broth and sub-cultured at regular intervals of 60 days.

Cecal and faecal contents: Cecal and faecal contents collected from the healthy control laboratory rats were suspended in 0.85% normal saline and used as the source of mixed flora.

2.6.11.9.2. Inoculum

Individual cultures were grown (24 h) in respective culture media and subjected to centrifugation (at 3000 x g for 20 min, 20°C) and resultant cells were suspended in 0.85% normal saline. Serial dilutions were prepared to get the requisite cell population. Cecal and faecal contents were suspended in normal saline, serially diluted and used as inoculum.

2.6.11.9.3. Filter sterilization

Sugars were prepared in 10x concentration and filter sterilized (0.22 mμ, Millipore) and stored in refrigerator till use. Ferulic acid and enzyme solutions were also filter sterilized.

2.6.11.9.4. Media composition and preparation

2.6.11.9.4.1. Lactobacillus MRS Broth

Ingredients (g/L)	
Proteose peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00
Polysorbate 80	1.00
Ammonium sulphate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00

Final pH (at 25°C) 6.5 ± 0.2

The broth was prepared (55.15 g/L) and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. 10 ml of this broth base was inoculated with inoculum (0.1%, v/v - 100 µl) and incubated at 37°C for 48 h.

The MRS broth for fermentation experiments was formulated by excluding beef extract, yeast extract and dextrose and replacing protease peptone with tryptone. Sodium acetate was added as per the requirement. 2 ml of broth containing required sugar (1%) was inoculated with 20 µl of inoculum and incubated at 37°C for 48 h.

2.6.11.9.4.2. Tryptone (a pancreatic hydrolysate of casein)

Total nitrogen	15.0%
Amino nitrogen	3.5%
Sodium chloride	5.0%
pH (1% solution)	6.5

2.6.11.9.4.3. Lactobacillus MRS Agar

For the ready made or formulated broth base containing required sugar (1%), agar (15.0 g/L) was added and heated till the agar liquefied. During pour plating of lactobacilli, medium was melted (~ 45°C) and added to the sterile plates containing 1 ml of culture broth. Plates were allowed to set, inverted and kept for incubation at 37°C for 48 h.

2.6.11.9.4.4. Bromo Cresol Purple (BCP) Broth Base

Ingredients (g/L)	
Peptic digest of animal tissue	10.00
Beef extract	3.00
Sodium chloride	5.00
Bromo cresol purple	0.04
Final pH (at 25°C) 7.0 ± 0.2	

The broth base was prepared (18.0 g/L) and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. 2 ml of this broth base containing required sugar (1%) was inoculated with 20 µl of inoculum and incubated at 37°C for up to 5 days. A change in the broth color from purple to deep yellow was considered as positive test.

2.6.11.9.4.5. BCP Agar

For the above broth base containing required sugar (1%), agar (15.0 g/L) was added and heated till the agar liquefied. During pour plating of lactobacilli, medium was melted (~ 45°C) and added to the sterile plates containing 1 ml of culture broth. Plates were allowed to set, inverted and kept for incubation at 37°C for 48 h.

2.6.11.9.4.6. Yeast Nitrogen Base (without amino acids and ammonium sulfate)

Ingredients (g/L)	
Biotin	0.000002
Calcium pantothenate	0.0004
Folic acid	0.000002
Inositol	0.002
Niacin	0.0004
P Aminobenzoic acid (PABA)	0.0002
Pyridoxine hydrochloride	0.0004
Riboflavin (vitamin B2)	0.0002
Thiamine hydrochloride	0.0004
Boric acid	0.0005
Copper sulphate	0.00004
Potassium iodide	0.0001
Ferric chloride	0.0002
Manganese sulphate	0.0004
Sodium molybdate	0.0002
Zinc sulphate	0.0004
Monopotassium phosphate	1.0
Magnesium sulphate	0.5
Sodium chloride	0.1
Calcium chloride	0.1

Final pH (at 25°C) 4.5 ± 0.2

The broth base was prepared in 10x concentration (1.7 g in 100 ml of distilled water) and 5 g ammonium sulphate, 10 mg L-histidine hydrochloride, 20 mg DL-methionine and 20 mg DL-tryptophan were added. The solution was filter sterilized and stored in refrigerator till use. Final medium was prepared by aseptically pipetting 0.5 ml of the 10x sterile medium in 4.5 ml sterile distilled water containing required sugar (5 mg/ml).

Media tubes were inoculated with very light inoculum and incubated at 25°C for up to 7 days. Lines (with India ink) were drawn on a paper and culture tubes were held in front of them. If lines were not seen or appear diffused through the culture, the test was considered positive and if lines were distinguishable, test was negative (Wickerham, 1951).

2.6.11.9.4.7. Nutrient Broth

Ingredients (g/L)

Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0

Final pH (at 25°C) 7.4 ± 0.2

The broth was prepared (13.0 g/L) and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. 10 ml of this broth was inoculated with 100 µl of inoculum and incubated at 37°C for 48.

2.6.11.9.4.8. Nutrient Agar

For the above broth, agar (15.0 g/L) was added and heated till the agar liquefied. Plates were allowed to set, inverted and kept for incubation at 37°C for 48 h.

2.6.11.9.4.9. Potato Dextrose Broth

Ingredients (g/L)

Potatoes, infusion from	200.0
Dextrose	20.0

Final pH (at 25°C) 5.6 ± 0.2

Broth was prepared and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2.6.11.9.5. Characterization of rat cecal/fecal culture isolates

a. **Isolation**: Cecal/fecal samples were obtained from rat, suspended in normal saline (0.85%) and xylose utilizing cultures were isolated from the pour-plate of MRS-BCP agar. Isolates were sub-cultured in MRS broth containing xylose.

b. **Catalase assay**: A loop full of 24 h old test culture was placed on a drop of H_2O_2 (3%). The effervescence observed due to the release of nascent oxygen was considered as an indication of catalase activity and considered positive.

c. **Grams staining**: A loop full of 24 h old test culture was smeared over a glass slide and heat fixed. It was stained with crystal violet stain preparation (mixture of crystal violet (10%) in 20 ml ethanol and ammonium oxalate (0.8%) in 80 ml water) for 1 min and excess stain was washed off with water. It was then treated with Gram's iodine solution (mixture of iodine (1 g) and potassium iodide (2 g) in 300 ml water) for 1 min. The smear was washed with water, decolorized with ethanol (70%) rewashed with water and counter stained with Saffranin (Saffranin-O (200 mg) in ethanol (10 ml, 95%) was diluted to 100 ml with water) for 30 sec. Excess stained was washed with water and the stained smear was air dried.

d. **Microscopy**: Stained smear was observed under an oil immersion of a compound microscope. Staining characteristics and morphology were noted.

2.6.11.9.6. *In vitro* fermentation experiments

Membrane filter (0.22 mμ, Millipore) sterilized individual sugars (arabinose, galactose, glucose, lactose and xylose) and feraxans in 10x concentration were incorporated at 1% level into BCP broth medium (2

ml) and inoculated with 20 µl aliquots of culture suspension giving a cell number of $5x10^3$ CFU and incubated at 37°C (25°C for yeast) for up to 48 h. A change in the broth color from purple to deep yellow was considered as positive test.

Lactic acid bacteria were grown (at 37°C) in MRS broth (formulated without beef extract, yeast extract, sodium acetate and dextrose and replaced protease peptone to tryptone) with individual sugars and feraxans at 1% level. Growth and utilization was monitored by measuring pH and absorbance of culture broth at regular time intervals. Turbidity was monitored spectrophotometrically with an UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) at 625 nm.

After 48 h of incubation, cultures were centrifuged (at 3000 x g for 20 min) and cells were oven dried (for constant weight, at 120°C) to determine the dry cell mass. Resultant, supernatant was analyzed for SCFA. Besides, viable cell population was enumerated by pour plating using BCP agar containing glucose and incubating at 37°C for 48 h.

Yeast cultures were screened for the utilization of individual sugars and feraxans incorporated into yeast nitrogen broth base medium and incubating at 25°C for up to 5 days. A visual turbidity of the culture broth indicated the utilization of substrate.

2.6.11.9.7. Polysaccharide hydrolysis with feraxanases

Driselase and ragi malt extract (filter sterilized) were used as the enzyme sources. MRS broth (2 ml) containing feraxans (1%) was incubated with either driselase (1 mg/0.1 ml in sodium phosphate buffer, 0.1 molar, pH 6.5) or ragi malt extract (equivalent to 25 mg flour per 0.1 ml buffer) at 37°C for 24 h. To this broth required culture was inoculated, incubated further for 48 h and growth was monitored.

2.6.11.9.8. SCFA analysis

Culture supernatant was acidified with sulfuric acid (50%) and extracted with diethyl ether (Karppinen et al., 2000) and analyzed for SCFA by GLC on PEG-20M with column, injector and detector temperatures of 120°C, 220°C and 230°C respectively, using N_2 (40 ml/min) as the carrier gas (Silvi et al., 1999). Acetate, propionate and butyrate (all 10 μmol/ml in diethyl ether) were used as standards. Quantity of individual SCFA in the sample was estimated by using peak area standard curve.

2.6.11.9.9. Antimicrobial assay

This was carried out using agar well diffusion assay as described by Brantner et al. (1994). Aliquots of 0.1 ml supernatant of 48 h old culture broth of specific lactic acid bacteria were added to individual agar well made in the nutrient agar plates pre-inoculated with individual pathogenic cultures and incubated for 24 h at 37°C. The zone of inhibition formed around the well was measured and recorded.

3.1. Introduction

Non-starch polysaccharides from cereals and millets form the quantitatively most important source of both soluble and insoluble dietary fibers (Bunzel et al., 2001). Arabinoxylans and β D-glucans, the chief cereal non-starch polysaccharides, are partially water-soluble and thus impart functionality. They are also known to reduce the risk of diseases such as diabetes, atherosclerosis and colon cancer (Karppinen et al., 2000; Plaami, 1997). Phenolic acids such as coumaric and ferulic acids, mainly bound to arabinoxylans, further influence these properties, in addition to their strong antioxidant properties. (Dervilly et al., 2000; Dervilly-Pinel et al., 2001b; Subba Rao and Muralikrishna, 2002). There are a number of individual reports on the overall sugar composition of cereal fibers (Cyran et al., 2002; Dervilly et al., 2000; Shibuya, 1984) and the amount of bound phenolic acids (Durkee and Thivierge, 1977; Hahn et al., 1983; Harukaze et al., 1999; Huang et al., 1986; Nordkvist et al., 1984). However, comparative information on different non-starch polysaccharides and bound phenolic acids and their changes brought about by malting (controlled germination) of cereals is very much limited (Dervilly-Pinel et al., 2001a; Glennie, 1983; Salomonsson et al., 1978; Subba Rao and Muralikrishna, 2001; Voragen et al., 1987).

Initial studies on native and malted ragi indicated 96 h malting resulted in maximum changes in non-starch polysaccharide composition and bound phenolic acid contents (Subba Rao and Muralikrishna, 2001). This is perhaps due to the induction of cell wall degrading enzymes, i. e., xylanase, arabinase and 1-3/1-4 β-D-glucanase, whose activities were higher in 96 h malts (Nirmala et al., 2000). Hence this condition was chosen to isolate water extractable (WEP) and water un-extractable (WUP) non-starch polysaccharide – phenolic acid complexes from native and germinated (malted, for 96 h) rice and ragi. A comparative study

pertaining to the changes (brought about by malting) in the water soluble non-starch polysaccharides/feraxans of rice and ragi was undertaken.

3.2. Malting loss and yields of WEP and WUP

Rice and ragi were malted for 96 h and changes in the non-starch polysaccharides and their degrading enzymes were monitored. The malting loss at 96 h was found to be lower for rice (17.1%) than ragi (24.7%), which was due to the low and high vegetative growth rates respectively.

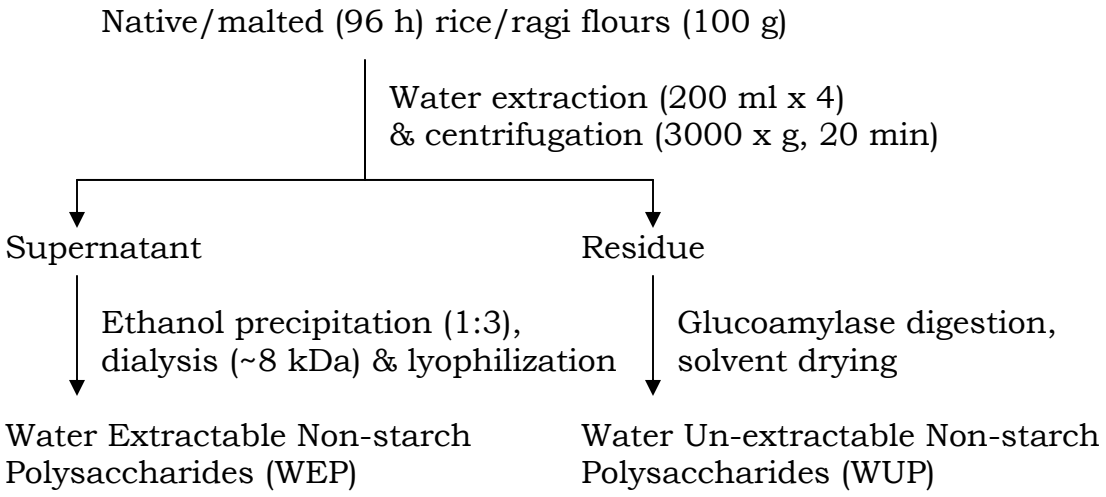

Figure 10. Scheme for obtaining WEP and WUP from native and malted rice and ragi flours.

WEP and WUP were obtained from native and malted flours as shown in figure 10. WEP content increased upon malting (table 3), which might be perhaps due to the preferential degradation of high amount of water-soluble mannan type of polysaccharides during malting as indicated by its sugar composition (table 5). In general, WEP content might have been increased due to the loosening of cell walls during malting, which may facilitate improved solubility of various non-starch polysaccharides (Palmer and Duffus, 1986). Results emanated from previous study indicated an apparent increase in non-starch

polysaccharide contents upon malting of ragi (Subba Rao and Muralikrishna, 2001). The yields of WUP did not change much upon malting. Few studies have been carried out earlier, regarding changes in the extraction/solubility of non-starch polysaccharides upon malting (Malleshi et al., 1986).

Table 3. Changes in WEP and WUP contents during malting of rice and ragi.

	Malting time (h)	Malting loss (%)	WEP yield (%)	WUP yield (%)
Rice	0 (N)	-	1.2	8.2
	24	3.8	1.5	ND
	48	6.6	1.6	ND
	72	8.3	1.9	ND
	96 (M)	17.1	2.2	7.5
Ragi	0 (N)	-	0.6	20.3
	24	4.7	0.8	ND
	48	10.2	1.2	ND
	72	16.2	1.7	ND
	96 (M)	24.7	2.1	20.3

N – Native; M – Malt/Malted; ND – not determined

3.3. Characterization of WEP and WUP

WEP from both rice and ragi contained small amount of starch (Rice: N – 3.8%, M – 5.0%; Ragi: N – 2.6%, M – 4.7%). Small amount of starch may be soluble in cold water and also degraded starch might have been extracted with cold water. Probably for this reason WEP from malts had higher percentage of glucose, which might have originated from starch as contaminant. WEP also contained small amounts of proteins (table 4), whose contents have increased upon malting because of the expression of several hydrolytic enzymes (Nirmala et al., 2000) and these proteins might get co-extracted with cold water. Amylase, a chief enzyme in the cereal grains, is induced during malting and known to be extractable with water or dilute buffers (Nirmala et al., 2000). WEP

contained high amylase activity, several folds higher in malts (Rice: N – 28.6, M – 1841.7; Ragi: N – 20.2, M – 491.9 U per gram WEP). Both WEP and WUP contained about 90% sugar and small amounts of uronic acid (table 4).

Table 4. Total sugar (%), uronic acid (%) and protein (%) contents of WEP and WUP from rice and ragi.

		WEP			WUP		
		Total Sugar*	Uronic acid	Protein	Total sugar	Uronic acid	Protein
Rice	N	93.0	2.8	3.5	90.3	8.5	ND
	M	93.4	3.5	4.6	90.8	9.2	ND
Ragi	N	92.0	4.3	3.1	88.4	10.7	ND
	M	91.2	5.2	4.9	90.0	11.0	ND

* uronic acid gives partial positive answer for total sugar.

3.3.1. Neutral sugar composition of WEP and WUP

WEP from all, native and malted rice and ragi, mainly consisted of arabinose, xylose and glucose in different proportions (table 5), which is in agreement with the one reported earlier on barley (Voragen et al., 1987). In general, glucose is the most predominant sugar in WEP and hexoses are in higher amount compared to the pentoses. Upon malting, change in the ratio of pentose to hexose (P/H) is observed, which increased both in rice and ragi indicating higher rate of degradation of hexoses like mannose and galactose. It might be due to the induction of hydrolytic enzymes such as mannosidase and galactosidase. The change in the content of rhamnose is not much in WEP of rice. However, it has increased by ~2.5 folds in ragi WEP. Malting resulted increase in glucose, arabinose and xylose both in rice and ragi. The ratio of arabinose to xylose (Ara/Xyl) has increased up on malting of rice, but has decreased slightly in case of ragi WEP, which is in accordance with the earlier study (Subba Rao and Muralikrishna, 2001). Mannose present in native WEP has disappeared up on malting. This might be due to the degradation of

mannan/glucomannan type of polysaccharides, which are present in small amounts in cereals (Fincher, 1975; Voragen et al., 1987). Disappearance of mannose in WEP might be taken as an index of malting.

Table 5. Neutral sugar composition (%) of WEP from native and malted rice and ragi.

		Rha	Ara	Xyl	Man	Gal	Glc	Ara/Xyl	P/H
Rice	N	6.1	12.5	15.2	8.6	1.3	56.3	0.82	0.42
	M	5.9	22.1	9.3	0.0	1.8	61.0	2.38	0.50
Ragi	N	5.4	23.7	11.9	3.6	12.0	43.4	1.99	0.60
	M	13.0	21.8	14.6	0.0	0.0	50.6	1.49	0.72

Rha – Rhamnose; Ara – Arabinose; Xyl – Xylose; Man – Mannose; Gal – Galactose; Glc – Glucose; Ara:Xyl - Arabinose:Xylose; P:H - Pentose:Hexose

WUP of rice and ragi consisted mainly of arabinose, xylose and glucose with small amounts of other sugars (table 6). The pentose content is more in all WUP except in native rice. The P/H ratio of rice WUP has increased upon malting in favor of pentoses. The P/H ratio of ragi WUP has decreased upon malting in favor of hexoses, which indicated the pentosan degradation as evident in barley and ragi (Okokon, 1992; Subba Rao and Muralikrishna, 2001). The xylose content of rice WUP has increased upon malting but is slightly decreased in case of ragi. The mannose content of WUP has disappeared upon malting, similar to the one observed with respect to WEP.

Table 6. Neutral sugar composition (%) of WUP from native and malted rice and ragi.

		Rha	Ara	Xyl	Man	Gal	Glc	Ara/Xyl	P/H
Rice	N	0.0	28.8	15.4	0.0	3.2	52.6	1.87	0.79
	M	5.1	33.5	43.6	0.0	0.0	17.9	0.77	4.31
Ragi	N	0.0	34.1	22.0	1.5	3.6	38.9	1.55	1.28
	M	4.2	29.7	17.8	0.0	0.0	48.3	1.67	0.98

3.4. Bound phenolic acids from WEP and WUP

Ferulic acid is the major bound phenolic acid identified in WEP (table 7), which is in accordance with earlier reports on cereals (Durkee and Thivierge, 1977; Hahn et al., 1983; Harukaze et al., 1999; Salomonsson et al., 1978; Shibuya, 1984; Subba Rao and Muralikrishna, 2001). Ferulic acid content is higher in native ragi WEP. Both ferulic and coumaric acids have undergone several fold degradation upon malting, which is in accordance with the earlier report on ragi (Subba Rao and Muralikrishna, 2001). This might be due to the induction of phenolic acid esterases during germination (Humberstone and Briggs, 2000; Maillard et al., 1996; Sancho et al., 2001).

Table 7. Bound phenolic acids (μg/g) of WEP and WUP from rice and ragi.

		WEP		WUP	
		Coumaric acid	Ferulic acid	Coumaric acid	Ferulic acid
Rice	N	9.4	104.4	387.5	1426.0
	M	3.0	68.0	360.0	915.0
Ragi	N	5.9	209.1	77.5	1519.0
	M	0.9	86.8	75.6	891.0

Similar to WEP, ferulic and coumaric acids are the main bound phenolic acids identified in WUP (table 7). However, the ratio of ferulic: coumaric acid is less in WUP, especially in rice, which has good amount of coumaric acid, in agreement with the earlier reports (Harukaze et al., 1999; Shibuya, 1984). Even in WUP, the phenolic acid content decreased by several folds upon malting. Ferulic acid degradation is higher in ragi compared to rice. However, coumaric acid did not undergo considerable degradation upon malting. About 90% of the phenolic acids are bound to WUP of both rice and ragi.

As per literature, major amount of phenolic acids are present in bran portion, whereas the cell walls of endosperm contain very less amount (Nordkvist et al., 1984). However, there is no report on the distribution of phenolic acids based on the solubility of non-starch polysaccharides. Comparative studies in the present investigation clearly showed that the major amount of phenolic acids is bound to WUP rather than to WEP (Rao and Muralikrishna, 2004). This finding can also be supported by the fact that diferulates are 8 - 39 times higher in cereal insoluble dietary fibre compared to soluble dietary fibre (Bunzel et al., 2001).

3.5. Free phenolic acids

Protocatechuic acid is the major free phenolic acid both in rice and ragi (table 8). Similar observation has been made in the earlier study (Subba Rao and Muralikrishna, 2002). Gallic acid and caffeic acids are the other minor phenolic acids seen. Although ferulic acid is the major bound phenolic acid, its amount is too less in the free form. Other phenolic acids, like p-coumaric acid, may be present, but in very minute quantities and hence were undetected/non-quantifiable. Upon malting, there is a decrease in the amount of free phenolic acids, both in rice and ragi. Malting lead to the overall decrease in the ferulic acid, the main bound phenolic acid, possibly due to the action of ferulic acid esterase, and it might be expected that amount of free ferulic acid should be high upon malting. However, interestingly, the free ferulic acid upon malting has decreased. The possible explanation is that ferulic acid might have got degraded or decarboxylated to other flavor compounds.

Table 8. Free phenolic acids (μg/g) of rice and ragi flours.

		Gallic acid	Protocatechuic acid	Caffeic acid	Ferulic acid
Rice	N	19.6	496.5	17.0	6.4
	M	22.4	320.1	1.8	5.1
Ragi	N	29.2	503.7	10.8	12.1
	M	15.9	243.4	9.4	1.2

3.6. Cell wall degrading enzyme activities

Arabinoxylans, β-D-glucans and cellulose are the major non-starch polysaccharides in the cereals and are the key components of the cell wall (Fincher and Stone, 1986). During malting/germination, which is largely a degradative process with reference to polysaccharides in the grains, several carbohydrases are induced. Carbohydrases are classified into cytolytic (cell wall degrading) and amylolytic (starch degrading) enzymes (Ballance and Manners, 1975).

In general, amylase is the major enzyme in cereal grains, both in resting stage as well as during malting and ragi (96 h malt) was shown to be a good source of amylase (Nirmala et al., 2000). In the present study, amylase is the main carbohydrase, induced to the high extent both in rice (N, 3.9 U; M, 162.0 U per gram flour) and ragi (N, 1.5 U; M, 97.0 U per gram flour) during malting.

Among the cell wall/non-starch polysaccharide degrading enzymes, basal activity can be detected in resting grains. However, during malting all these enzyme activities increase by several folds (Nirmala et al., 2000), which may be essential for the degradation of the cell wall polysaccharides.

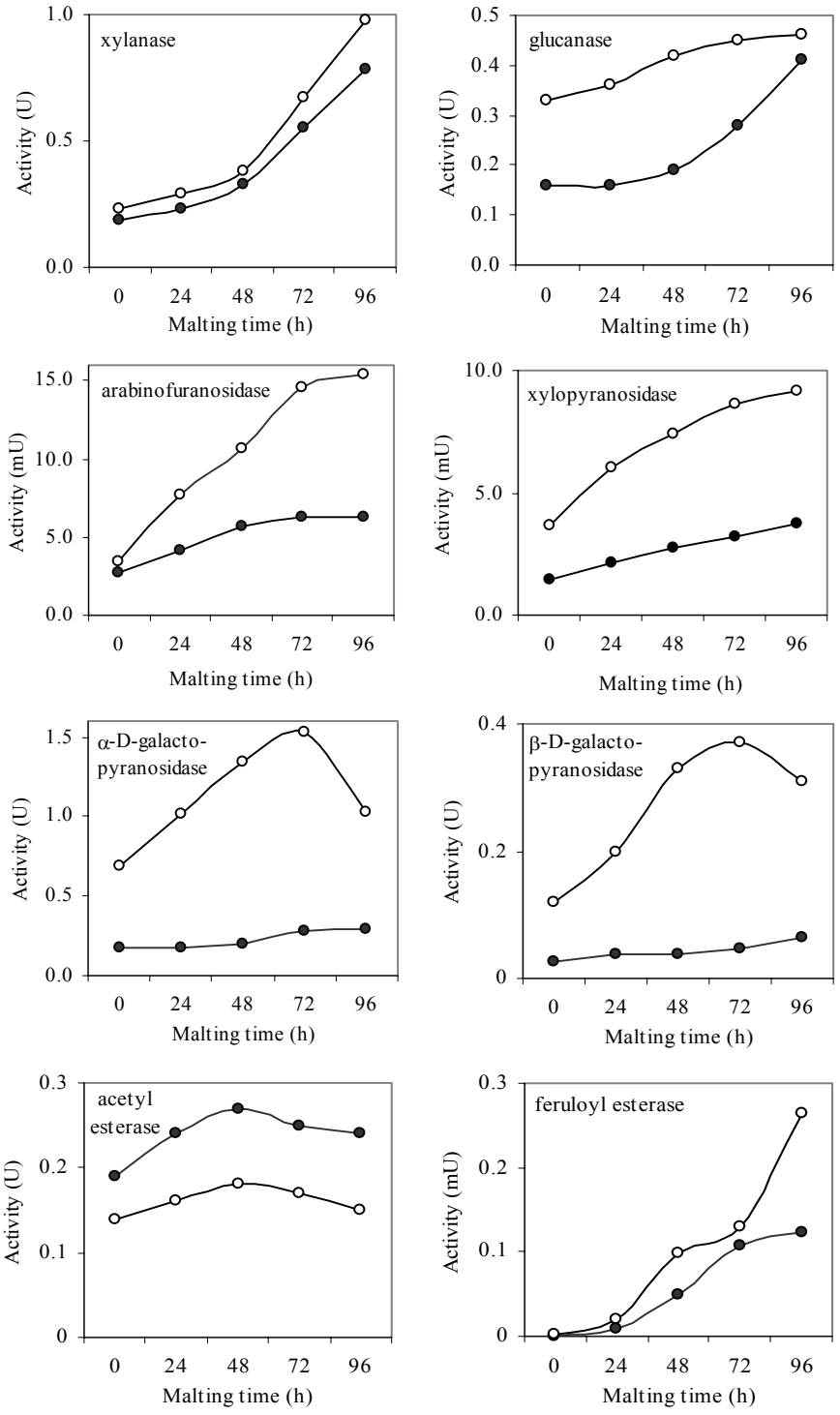

Figure 11. Variations in the NSP degrading enzyme activities (per gram flour) during malting of rice (●) and ragi (○).

In particular, xylanase is the chief non-starch polysaccharide degrading enzyme and its activity increased by 2 – 3 folds upon malting (figure 11). Similar high activity/induction during malting of xylanase was observed in earlier studies with ragi (Nirmala et al., 2000), wheat (Corder and Henry, 1989) and rye (Rasmussen et al., 2001). 1,3 β-D-glucanase activity is less than xylanase, but showed similar increase during malting. In contrary to this, however, Autio et al. (2001) reported high β-D-glucanase activity (compared to xylanase) in barley and this has been linked with its high β-D-glucan content. The other xylanolytic enzymes, namely, arabinofuranosidase and xylopyranosidase are also present in resting grains and their activity increase upon malting. High α-D-galactopyranosidase and β-D-galactopyranosidase activities are detected both in rice and ragi. These enzymes are essential for the degradation of arabinogalactans found in the cereal cell wall and hydrolysis of small amounts of galactose residues present in the heteroxylans/arabinoxylans.

Xylans are known to be partially acetylated (Biely et al., 1985; Chung et al., 2002; Humberstone and Briggs, 2002) and its hydrolysis requires the acetyl (xylan) esterase activity. High amount of acetyl esterase activity is detected both in rice and ragi, and there is only a slight increase in their activity during malting. On the contrary, ferulic acid esterase, the enzyme essential for the hydrolysis of the high amount of bound ferulic (phenolic) acid is increased by several folds upon malting. This might be the reason for the lower amount of over all bound ferulic acid in WEP and WUP of malted rice and ragi.

Cytolytic enzymes act on/degrade various non-starch polysaccharides leading to their better extractability in water. This is the reason for the increased yield of WEP both from rice and ragi during malting. In general, ragi showed slightly higher enzyme activities compared to rice.

3.7. Fractionation and purification

Water extractable NSP(s) comprise a group of heterogeneous polysaccharides, of which feruloyl-arabinoxylans and β-D-glucans are the predominant ones. For the structural characterization of feruloyl arabinoxylans, they must be in pure form, devoid of glucans and contaminant proteins. Further, as there are several sub-populations (polydisperse) in the arabinoxylans, extensive fractionations steps are often required to obtain purified polysaccharides.

3.7.1. Characterization of water soluble NSP

Although WEP can be obtained in high yield from rice and ragi, they are only sparingly soluble in water. Solubility of polysaccharides is known to be affected by many factors (Izydorczyk and Biliaderis, 1995) and extraction process/drying may also bring changes in this property. Thus, WEP was dissolved in water to separate insoluble portions (figure 12) and soluble portion was further fractionated and characterized.

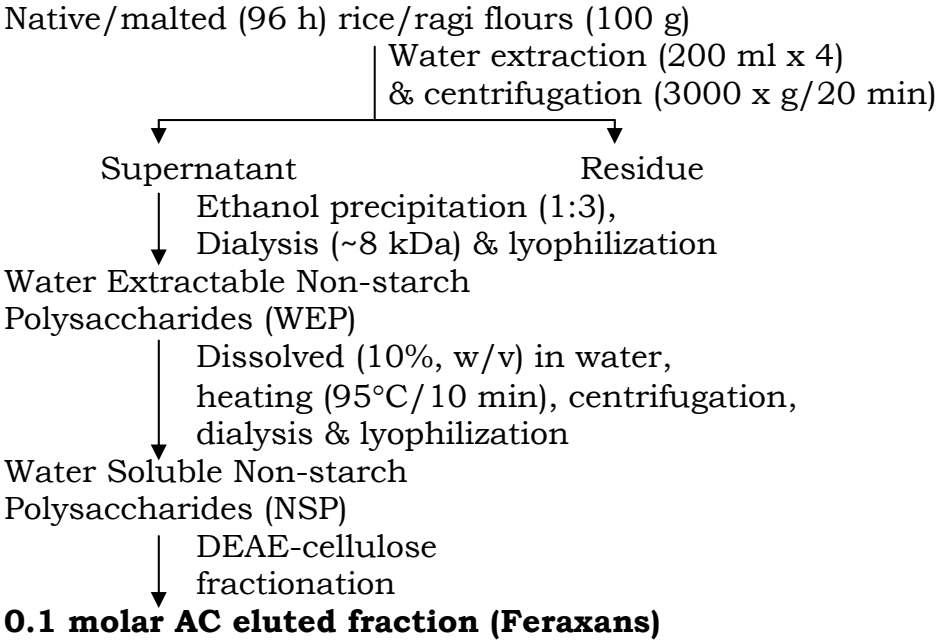

Figure 12. Scheme for obtaining water soluble NSP/feraxans from native and malted rice and ragi.

Yield and uronic/ferulic acid contents of water soluble NSP obtained from rice and ragi are presented in table 9. The yields of water soluble NSP increased by around 3 and 5 folds upon malting of rice and ragi respectively. A similar pattern was observed in water extractable non-starch polysaccharides (WEP) (Rao and Muralikrishna, 2004). However, the yield of water soluble NSP is low compared to WEP (rice: N, 1.2%, M, 2.2%; ragi: N, 0.6%, M, 2.1%). Water soluble NSP has over 96% sugar and less than 1 % protein (table 9). The uronic acid content ranged between 2.6 – 6.1%, which is slightly higher in malts. This could be due to the mobilization of uronic acid containing arabinoxylans during malting. Ferulic acid content ranged from 492.5 – 528.0 μg/g, slightly less in malts. This could be due to ~ 100 fold increase in the ferulic acid esterase activity (rice: N, 0.001 mU, M, 0.123 mU; ragi: N, 0.0029 mU, M, 0.2633 mU), which is induced during malting (figure 11). The relative viscosity of water soluble NSP is low, except for the one isolated from ragi malt, which is over three times higher than the native ones.

Table 9. Yield, ferulic acid and uronic acid contents of water soluble NSP from native and malted rice and ragi.

		Yield (%)	Total sugar (%)	Uronic acid (%)	Protein (%)	Ferulic acid (μg/g)	Relative viscosity (1%, 25°C)
Rice	N	0.15	97.7	2.6	0.8	510.6	1.13
	M	0.44	97.0	4.0	1.1	492.5	1.19
Ragi	N	0.13	97.1	4.8	0.6	528.0	1.15
	M	0.61	96.3	6.1	0.9	503.1	3.71

Neutral sugar composition indicated that over 60% of the polysaccharides are of arabinoxylan type (table 10). Arabinose to xylose ratio increased upon malting and more so in the case of ragi. This is probably due to the high activity of induced carbohydrate degrading enzymes during malting (Nirmala et al., 2000). In particular, xylanase

activity increased by many folds (rice: N, 0.19 U, M, 0.78 U; ragi: N, 0.23 U, M, 0.98 U per gram flour) (figure 11). Xylanase would act on the relatively less substituted xylan backbone, yielding an arabinoxylan population more substituted with arabinose upon mating.

Table 10: Neutral sugar composition (%) of water soluble NSP from native and malted rice and ragi.

		Rha	Ara	Xyl	Man	Gal	Glc	Ara/Xyl	P/H
Rice	N	1.0	28.3	37.0	0.4	3.5	29.8	0.77	1.94
	M	1.2	29.4	32.8	0.0	1.6	35.0	0.90	1.70
Ragi	N	2.2	27.3	32.5	1.0	9.3	27.7	0.84	1.57
	M	1.7	33.1	29.6	0.6	6.5	28.5	1.12	1.76

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).

3.7.1.1. Fractionation of water soluble NSP

Water soluble NSP were fractionated (figure 12) on DEAE-cellulose (CO_3^{2-} form) 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 (figure 13). Neutral polysaccharides (~ 10 – 25 %) were eluted with water, whereas charged polysaccharides were eluted with AC (0.1 and 0.2 molar) and NaOH (0.1 and 0.2 molar). 0.1 molar AC eluted fraction is in maximum yield (50 – 60 %) (table 13), whereas 0.2 molar AC, and 0.1 and 0.2 molar NaOH eluted fractions accounted for 5 to 15 % (table 11). However, high amount (10 – 20 %) of polysaccharides was retained in the column uneluted. This is not surprising since high amount of uronic acid containing polysaccharides require higher concentrations of alkali (> 0.3 M NaOH). However, it was not carried out since high concentrations of alkali removes uronic acid by β-elimination. DEAE-cellulose fractionation

was routinely employed for the study of arabinoxylans (Nilsson et al., 1999; Subba Rao and Muralikrishna, 2004; Woolard et al., 1976) and similar fractionation profiles/results were obtained (Subba Rao and Muralikrishna, 2004).

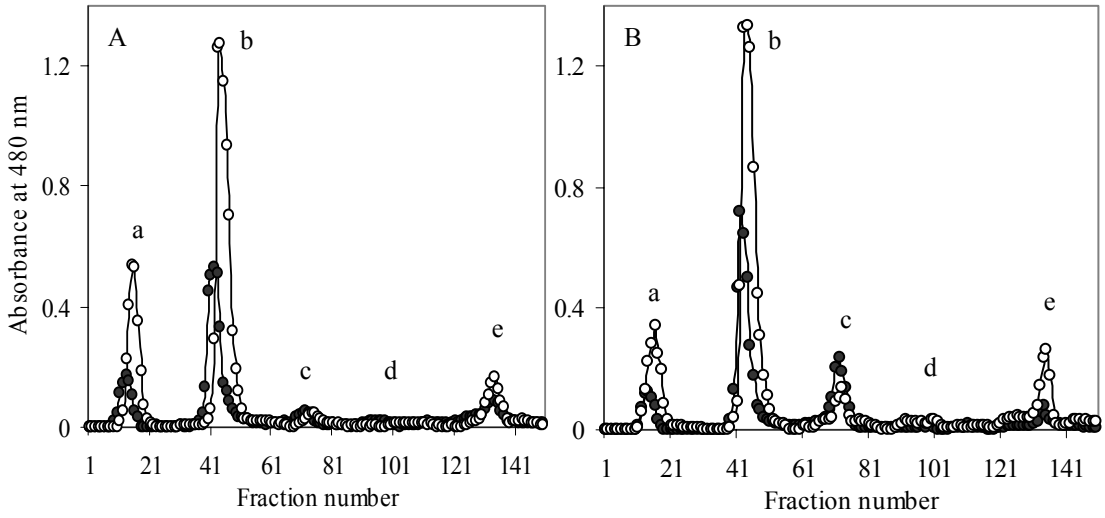

Figure 13. Fractionation profile on DEAE-cellulose of water soluble NSP from native (●) and malted (○) rice (A) and ragi (B): water eluted fraction (a), 0.1 molar ammonium carbonate eluted fraction (b), 0.2 molar ammonium carbonate eluted fraction (c), 0.1 molar NaOH eluted fraction (d) and 0.2 molar NaOH eluted fraction (e) (fraction size, 5 ml).

Water eluted fractions contained no uronic acid (table 11). Minor amount (3.7 – 14.9 µg/g) of ferulic acid observed (table 11) in water eluted fractions from ragi might have come from small amount of neutral arabinoxylans wherein they are ester linked to side chain arabinose. All other fractions have high amount of uronic acid. However, uronic acid content of NaOH eluted fractions are less than ammonium carbonate eluted fractions. This might be due to the partial elimination of uronic acid in the alkaline condition. 0.2 molar ammonium carbonate eluted

fractions contained high amount of ferulic acid. Interestingly upon malting, their content decreased by about ten fold. Ferulic acid content of NaOH eluted fractions were not determined as they are likely to be de-esterified in the alkaline condition. All fractions contained about 95% sugar and less than 1% protein (table 11).

Table 11. Yield, ferulic acid and uronic acid contents of water soluble NSP fractions (DEAE-cellulose fractionation) from native and malted rice and ragi.

		Yield (%)*	Total sugar (%)	Uronic acid (%)	Protein (%)	Ferulic acid (µg/g)
Water eluted fraction						
Rice	N	23.4	97.0	nd	1.0	nd
	M	21.0	97.0	nd	1.0	nd
Ragi	N	11.2	98.0	nd	0.6	3.7
	M	13.1	98.0	nd	0.7	14.9
0.2 molar AC eluted fraction						
Rice	N	3.8	93.0	11.2	0.7	1182.0
	M	3.1	92.5	12.8	0.6	83.7
Ragi	N	9.7	92.0	14.3	0.4	1641.4
	M	5.9	92.5	15.9	0.5	189.5
0.1 molar NaOH eluted fraction						
Rice	N	1.1	93.5	10.0	0.8	ND
	M	0.9	93.0	10.7	0.8	ND
Ragi	N	1.3	92.5	12.1	0.4	ND
	M	1.0	93.0	11.9	0.4	ND
0.2 molar NaOH eluted fraction						
Rice	N	7.5	94.0	7.6	0.5	ND
	M	7.8	93.5	8.3	0.7	ND
Ragi	N	3.4	94.0	9.0	0.6	ND
	M	8.9	93.0	10.0	0.6	ND

* Percent of water soluble NSP loaded to the column; nd – not detected; ND – not determined.

Water eluted fractions are chiefly glucan type as indicated by GLC analysis, which showed glucose (75 – 95 %) as the major sugar (table 12).

0.2 molar ammonium carbonate and 0.1 and 0.2 molar NaOH eluted fractions are arabinoxylan type of polysaccharides (table 12).

Table 12. Neutral sugar composition (%) of water soluble NSP fractions (DEAE-cellulose fractionation) from native and malted rice and ragi.

		Rha	Ara	Xyl	Man	Gal	Glc	Ara/Xyl	P/H
Water eluted fraction									
Rice	N	0.0	3.7	0.7	0.0	0.0	95.6	5.29	0.05
	M	0.0	3.0	1.1	0.0	0.0	95.9	2.73	0.04
Ragi	N	0.4	10.6	1.9	4.1	6.5	76.5	5.58	0.14
	M	0.0	8.5	7.3	0.0	0.0	84.2	1.16	0.19
0.2 molar AC eluted fraction									
Rice	N	1.0	37.6	47.7	3.0	6.7	4.0	0.79	6.23
	M	0.0	43.9	46.2	0.0	8.0	1.9	0.95	9.10
Ragi	N	0.0	41.7	43.6	2.5	9.1	3.1	0.96	5.80
	M	0.0	35.0	49.9	3.0	7.6	4.5	0.70	5.62
0.1 molar NaOH eluted fraction									
Rice	N	0.2	40.3	48.5	1.0	8.0	2.0	0.83	8.07
	M	1.3	46.7	42.8	0.0	9.2	0.0	1.09	9.73
Ragi	N	0.3	37.8	39.8	4.8	8.2	9.1	0.95	3.51
	M	0.0	40.1	39.3	5.1	9.5	6.0	1.02	3.85
0.2 molar NaOH eluted fraction									
Rice	N	0.0	45.5	47.2	0.5	6.0	3.8	0.96	9.00
	M	0.9	48.1	41.7	0.0	9.3	0.0	1.15	9.66
Ragi	N	0.7	42.7	37.4	3.2	10.5	5.5	1.14	4.17
	M	0.5	39.0	37.2	4.0	12.0	7.3	1.05	3.27

3.7.2. Characterization of feraxans

As the 0.1 molar ammonium carbonated eluted fraction was obtained in maximum yield (table 13), it was selected for subsequent studies. Neutral sugar composition (table 14) of this major fraction indicated it to be arabinoxylan type of polysaccharide. Interestingly, the fraction showed strong absorbance at UV range (figure 14) indicating the presence of ferulic acid and thus it is designated as feruloyl arabinoxylan (feraxan). This fraction is taken for functional studies and purified further for the structural elucidation.

The UV absorption of the native feraxans is less compared to the malts, indicating lower amount of bound ferulic acid in the native feraxans. Both rice and ragi feraxans showed similar UV absorption spectra.

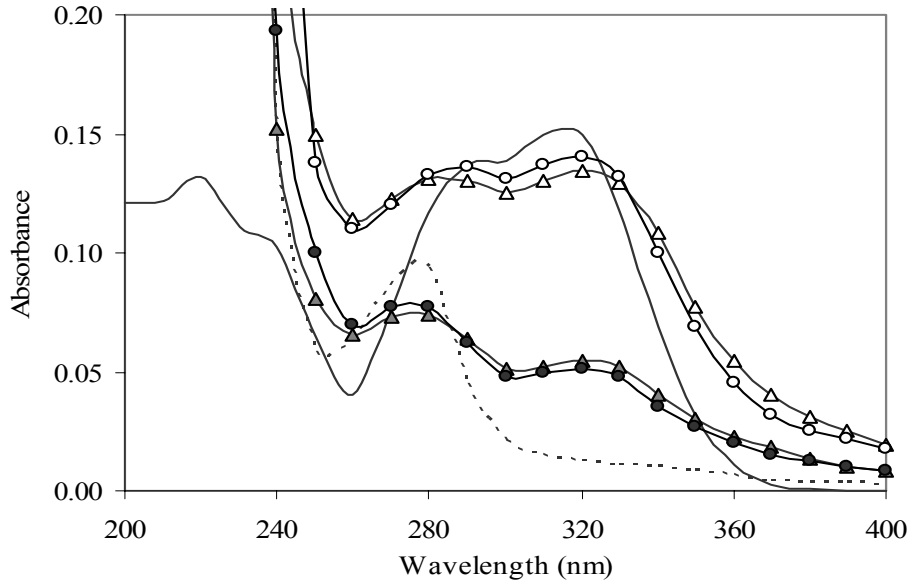

Figure 14. UV – absorption spectra of water soluble feraxans from native (solid) and malted (open) rice (circle) and ragi (triangle). Ferulic acid (solid line) and BSA (dotted line) spectra are shown.

Apart from arabinose and xylose, the main sugars, some amount of galactose/glucose is also identified in the feraxans (table 14). Arabinoxylans from many cereals such as rye, sorghum and maize are known to contain small amounts of galactose/glucose as side groups (Cyran et al., 2002, Cyran et al., 2003; Dervilly et al., 2000; Izydorczyk and Biliaderis, 1995; Saulnier et al., 1995a, Saulnier et al., 1995b). Similar to that of water soluble NSP, malting resulted in slight increase in the arabinose content of feraxans.

Table 13. Yield, ferulic acid and uronic acid contents of feraxans from native and malted rice and ragi.

		Yield (%)*	Total sugar (%)	Uronic acid (%)	Protein (%)	Ferulic acid (µg/g)
Rice	N	50.3	96.0	8.0	0.7	119.3
	M	54.1	94.5	8.9	0.5	1404.3
Ragi	N	59.6	94.8	12.1	0.2	146.6
	M	55.1	94.5	13.7	0.3	1044.6

* Percent of water soluble NSP loaded to the column

Feraxans contained high amount of uronic acid (8.0 – 13.7 %) and ferulic acid (119.3 – 1404.3 µg/g) (table 13). Interestingly, ferulic acid content of malt feraxans is 11.8 and 7.1 folds higher compared to native in rice and ragi respectively. Contrary to this, ferulic acid content of 0.2 molar AC eluted fractions is 14.1 and 8.7 folds higher in native compared to malt in rice and ragi respectively (table 11). This could be due to the mobilization of feruloyl arabinoxylans by induced xylanolytic enzymes during malting. These results indicated that malting brought in dynamic changes in the feraxans.

Table 14. Neutral sugar composition (%) of feraxans from native and malted rice and ragi.

		Rha	Ara	Xyl	Man	Gal	Glc	Ara/Xyl	P/H
Rice	N	1.7	40.2	49.4	0.0	8.7	0.0	0.81	10.3
	M	2.0	44.3	46.7	0.0	7.0	0.0	0.95	13.0
Ragi	N	3.1	39.5	45.3	0.9	6.5	4.7	0.87	7.0
	M	2.3	43.9	40.3	0.0	8.0	5.5	1.09	6.2

3.7.2.1. Fractionation/purification of feraxans

Feraxans were found to contain at least two sub-populations of arabinoxylans as analyzed by HPSEC and cellulose acetate paper electrophoresis and hence were fractionated on Sephacryl S-300 gel permeation chromatography. All the four feraxans yielded two major sub-

populations of polysaccharides (figure 15). Molecular weight of individual polysaccharides is determined by using a standard calibration curve (figure 16). Each sub-population of feraxans was re-loaded separately to the Sephacryl S-300 column and was eluted as single peak (figure 17).

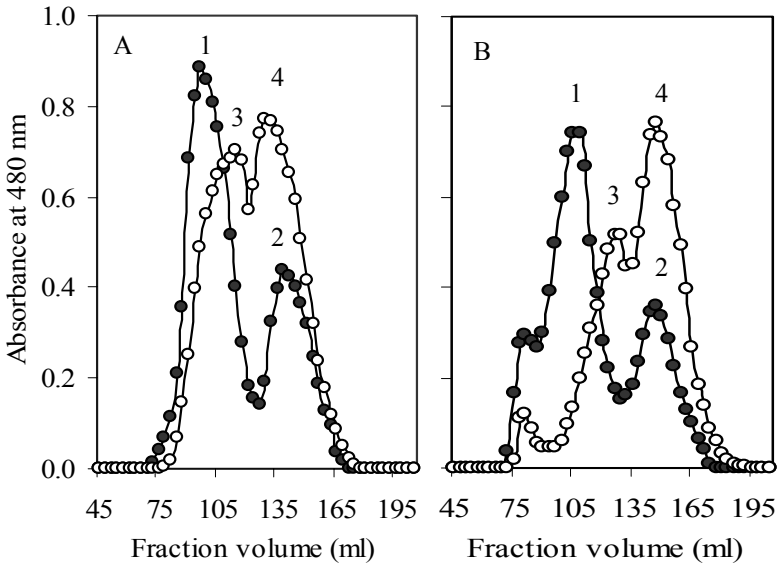

Figure 15. Sephacryl S-300 gel filtration profile of feraxans from native (●) and malted (○) rice (A) and ragi (B). Average molecular weight (in kDa) of peaks – rice: Native Peak (NP) 1, 231.5 (1), NP2, 24.4 (2), Malt Peak (MP) 1, 75.4 (3) and MP2, 39.6 (4); ragi: NP1, 139.9 (1), NP2, 15.4 (2), MP1, 38.9 (3) and MP2, 15.4 (4).

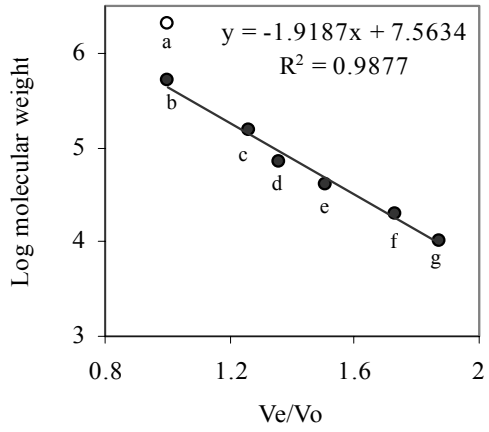

Figure 16. Calibration curve for Sephacryl S-300 (Dextran standards – T-2000 (a), T-500 (b), T-150 (c), T-70 (d), T-40 (e), T-20 (f) and T-10 (g).

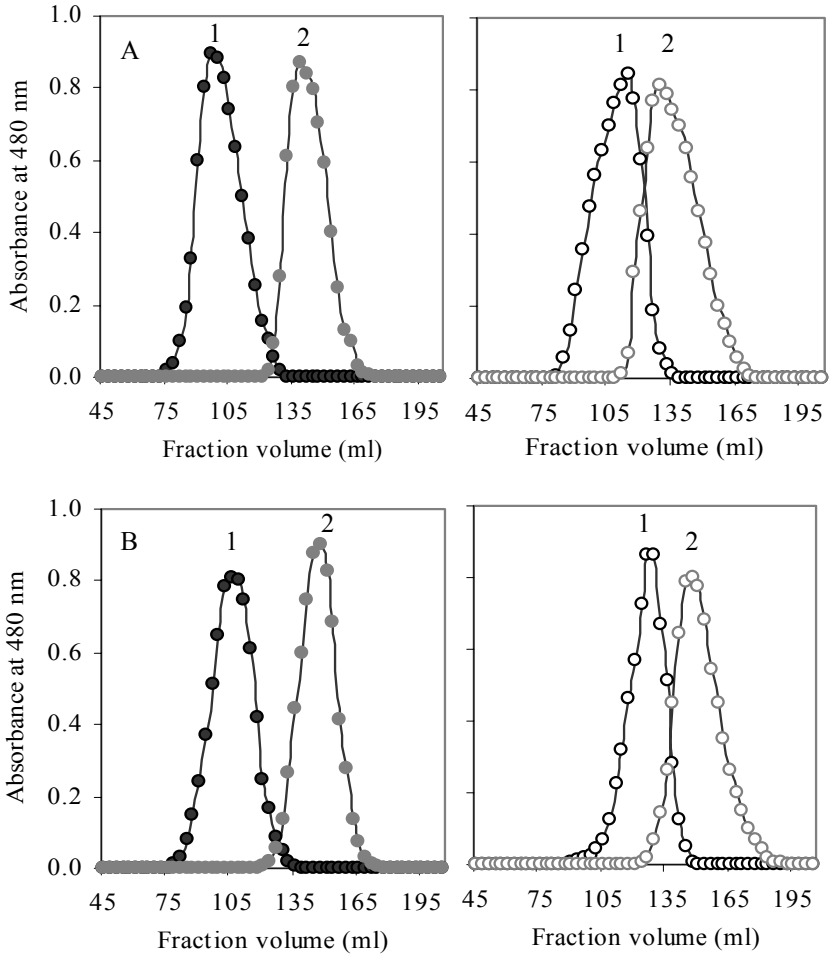

Figure 17. Gel filtration profile on Sephacryl S-300 of individual feraxans from native (●) and malted (○) rice (A) and ragi (B). Peaks – 1 and 2.

The purity of water soluble feraxans was ascertained by HPSEC, capillary and cellulose acetate paper electrophoreses.

All the eight purified feraxans are eluted as single peak in HPSEC, indicating their purity (figure 18). However, peaks are not very sharp, similar to the profile observed for other purified arabinoxylans obtained from wheat (Dervilly et al., 2000; Dervilly-Pinel et al., 2001a; Maes and Delcour, 2002; Roels et al., 1999), barley (Cyran et al., 2002; Trogh et al., 2004) and maize (Saulnier et al., 1995a).

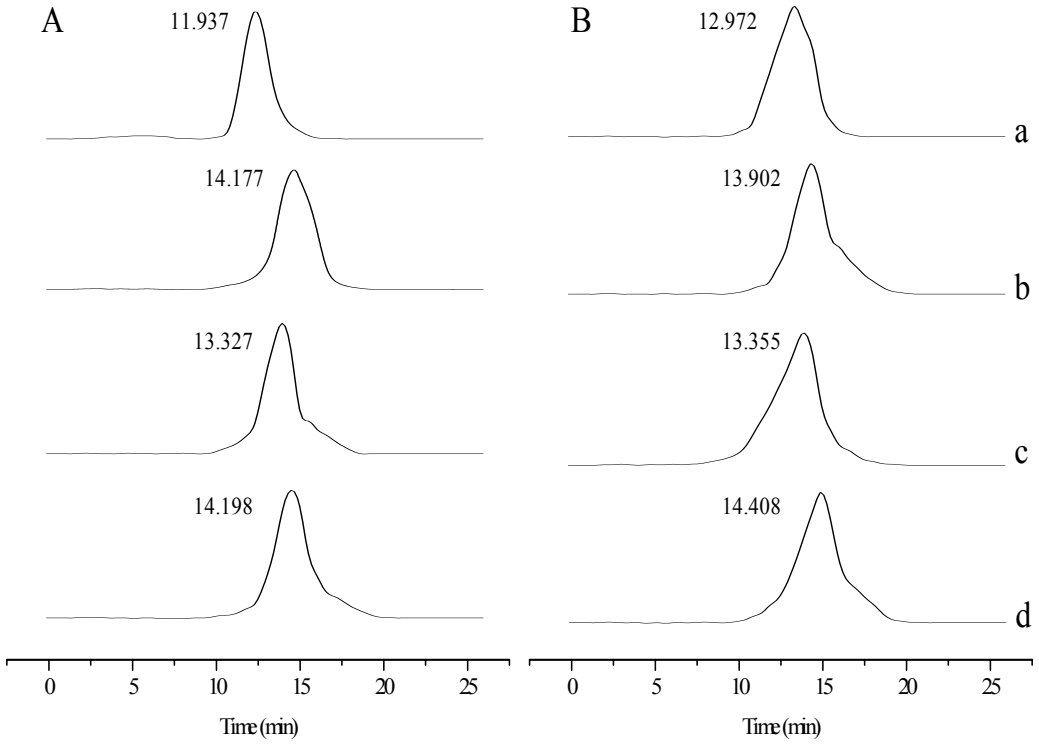

Figure 18. HPSEC profile of rice (A) and ragi (B) feraxans: native peak (NP) 1 (a), NP2 (b), malt peak (MP) 1 (c) and MP2 (d).

Purity of arabinoxylans is commonly ascertained by HPSEC using various columns like TSK columns in case of wheat water unextractable arabinoxylans (Gruppen et al., 1992a; Gruppen et al., 1992b), Waters Hydrogel 1000 column in case of wheat bran arabinoxylans (Shiiba et al., 1993), Shodex OH pack columns in case of maize bran heteroxylans (Chanliaud et al., 1995) and E-linear and E-1000 columns in case of ragi arabinoxylans (Subba Rao and Muralikrishna, 2004).

Capillary electrophoresis of purified feraxans, similar to HPSEC, showed single peak (figure 19). However, retention times could not be correlated to the overall molecular weight of the feraxans. This might be

due to their differences in the degree of substitution, branching pattern, and uronic acid content. Capillary electrophoresis is widely used for the study of proteins and being increasingly utilized in the carbohydrates' analyses (Soga and Serwe, 2000; Subba Rao and Muralikrishna, 2004).

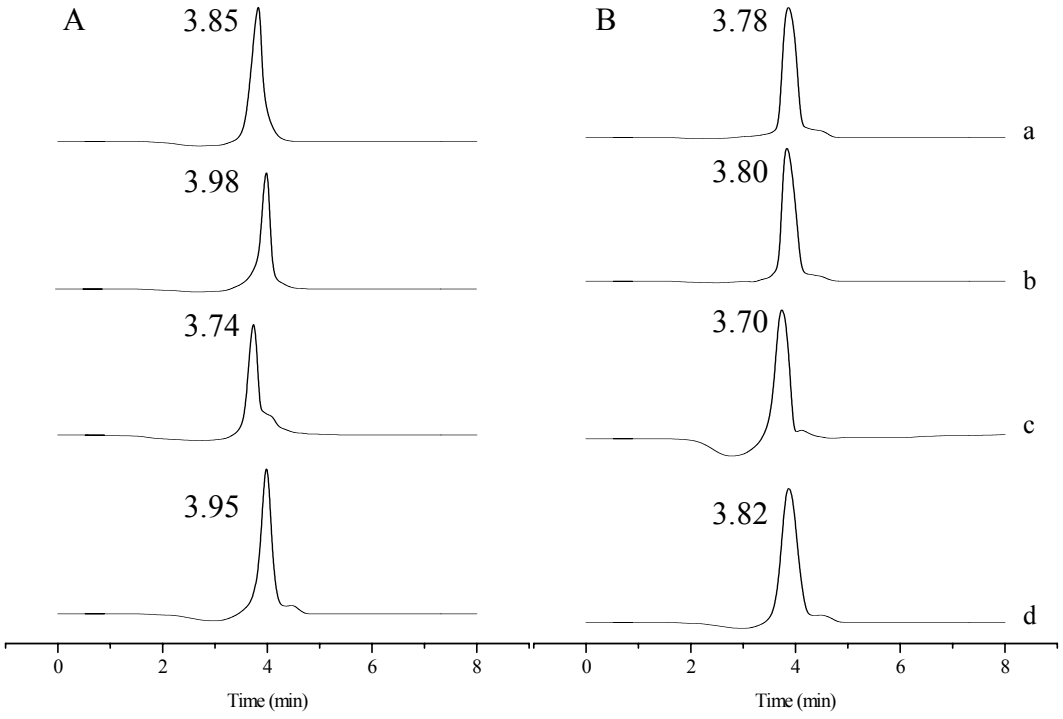

Figure 19. Capillary electrophoresis profile of rice (A) and ragi (B) feraxans: NP1 (a), NP2 (b), MP1 (c) and MP2 (d).

Purified feraxans were also subjected to cellulose acetate paper electrophoresis. All feraxans showed single band (figure 20) and differences in the electrophoretic mobility were attributed to the uronic acid content.

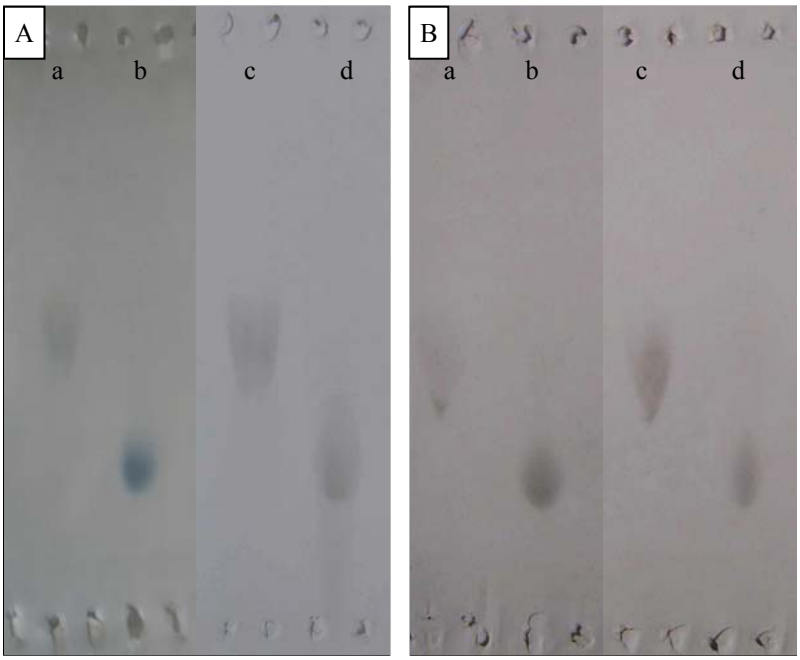

Figure 20. Cellulose acetate electrophoresis of rice (A) and ragi (B) feraxans. NP1 (a), NP2 (b), MP1 (c) and MP2 (d).

Presence of high uronic acid, ferulic acid, arabinose substitution and galactose content and relatively small molecular weight made water soluble feraxans from rice and ragi unique from other arabinoxylans reported (Dervilly-Pinel et al., 2001b).

In conclusion, malting of cereal grains has brought about dynamic changes in the water soluble feraxans.

4.1. Introduction

Although general structure of arabinoxylans from rice (Shibuya and Iwasaki, 1985; Shibuya et al., 1983) and ragi (Subba Rao and Muralikrishna, 2004) is known, detailed structural characterization of water soluble feruloyl arabinoxylans from these cereals is lacking. Further, comparative analysis of feraxans in these two grasses and the changes brought about by germination have not been looked at, employing similar study condition. Thus, for a detailed structural characterization, water soluble feruloyl arabinoxylans were obtained from native and malted rice and ragi following isolation, fractionation and purification of water extractable non-starch polysaccharides (WEP) (figure 21).

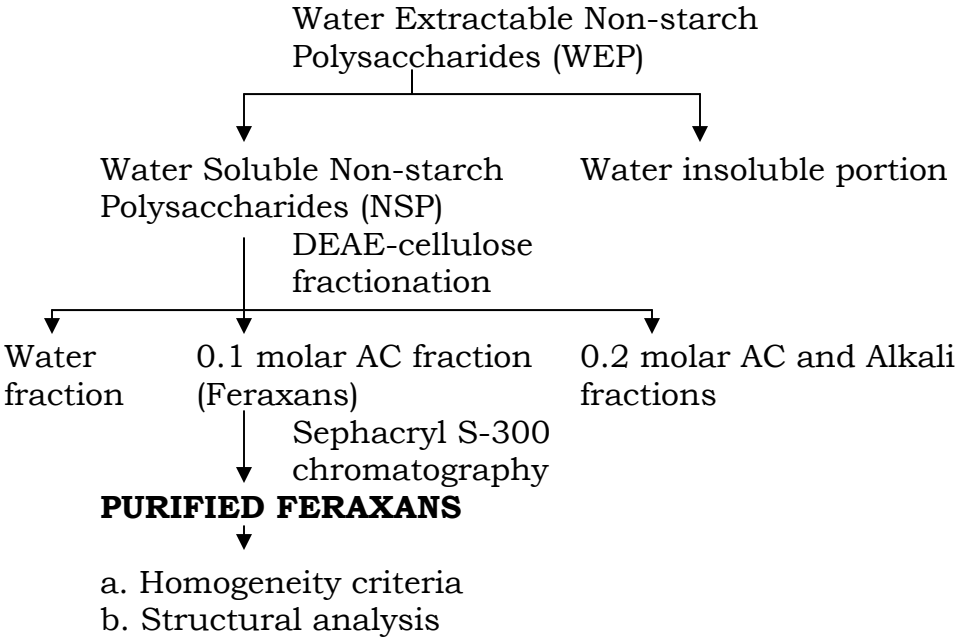

Figure 21. Scheme for obtaining purified (water soluble) feraxans from native and malted rice and ragi for structural analysis.

Homogeneity of these purified water soluble feraxans was tested by four different methods, namely reloading the each fraction obtained on

Sephacryl S-300 on the same column, HPSEC, capillary electrophoresis and cellulose acetate paper electrophoresis. Individual fractions were found to be pure/monodisperse and thus taken up for structural characterization.

4.2. Characterization of purified water soluble feraxans

Purified water soluble feraxans, similar to the 0.1 molar AC eluted fractions (feraxans), showed strong absorbance at UV range with maximum absorption at around 320 nm (figure 22). The spectra are similar to that of ferulic acid, indicating the presence of ferulic acid in the polysaccharide.

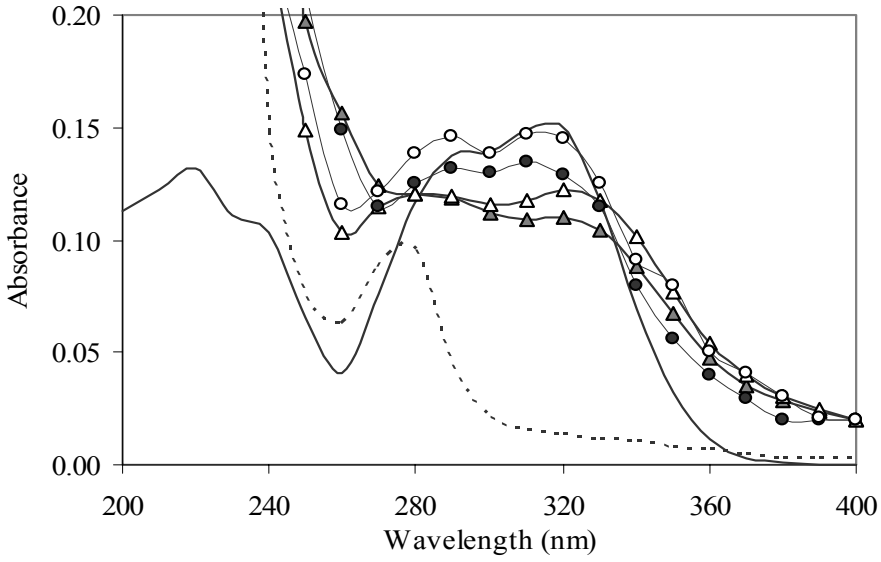

Figure 22. UV – absorption spectra of purified feraxans from malted (Peak 1 – filled and Peak 2 – open symbols) rice (circle) and ragi (triangle). Ferulic acid (solid line) and BSA (dotted line) spectra are shown

The molecular weight of purified feraxans (table 15) was determined on the Sephacryl S-300 column using standard dextran markers (figure 16). In case of rice native, 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 is slightly increased to 39.6 kDa (yield: ~ 50 %). Similarly, in ragi, native feraxans has 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 caused 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) (figure 11) induced during malting would act on large molecular weight feraxans, bring down their molecular weight (figures 15 & 17) and increase solubility/yield (table 3 & 9). Water soluble feraxans from both native and malted rice and ragi are found to be relatively small molecules compared to other arabinoxylans reported (Dervilly-Pinel et al., 2001a).

Table 15. Yield, molecular weight and ferulic acid contents, and specific rotations of purified feraxans (Sephacryl S-300) from native and malted rice and ragi.

			Yield (%)*	Molecular weight (kDa)	Ferulic acid (µg/g)	Total sugar (%)	Uronic acid (%)	Protein (%)	Optical rotation $[\alpha]_D$
Rice	N	P1	65.0	231.5	130.9	94.9	8.0	0.2	- 5.1
		P2	35.0	24.4	78.5	94.5	8.3	0.5	- 7.4
	M	P1	50.0	75.4	1388.2	94.5	9.5	0.5	- 5.9
		P2	50.0	39.6	1471.6	95.0	8.7	0.4	- 5.9
Ragi	N	P0	4.8	> 400.0	-	-	-	-	-
		P1	60.5	139.9	161.4	95.0	9.0	0.3	- 1.6
		P2	34.7	15.4	54.0	94.0	13.4	0.2	- 0.3
	M	P0	1.9	> 400.0	-	-	-	-	-
		P1	33.5	38.9	843.8	94.5	8.2	0.3	- 2.3
		P2	64.6	15.4	949.9	94.2	12.9	0.3	- 0.8

* Percentage of sample loaded, P - Peak

Feraxans contained around 95 percent sugar (uronic acid also gives partial positive answer for total sugar test) and less than one percent protein. The presence of high amount of uronic acid seems to be the characteristic of water soluble feraxans, especially ragi (Subba Rao and Muralikrishna, 2001). Supporting the UV absorption profiles, feraxans contained high amount of bound ferulic acid, which was determined by HPLC after alkaline hydrolysis. Ferulic acid identification was confirmed by its characteristic fragmentation pattern in the MS (figure 23). Interestingly, ferulic acid content of malt feraxans is much higher compared to the native ones.

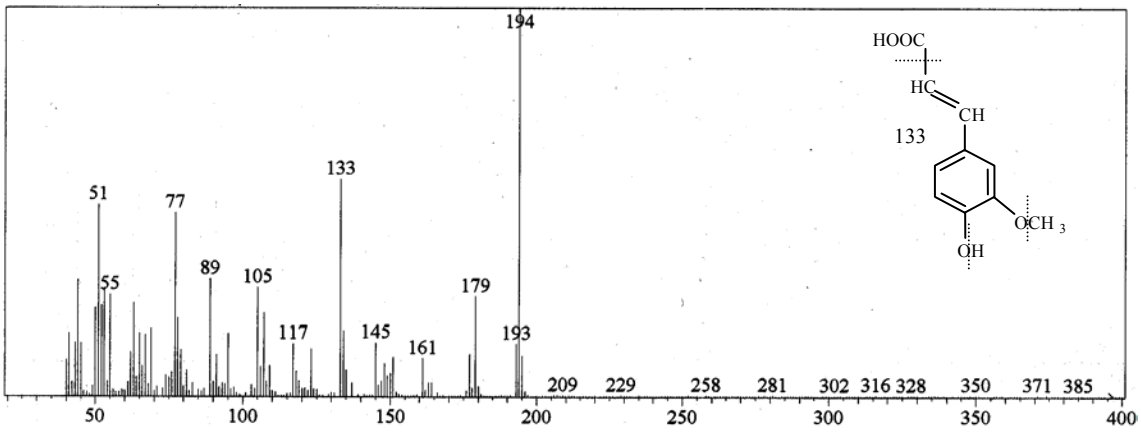

Figure 23. Fragmentation pattern of ferulic acid.

The neutral sugar composition analysis showed chiefly arabinose and xylose (table 16). However, considerable amount of galactose is also observed, which might be the part of arabinoxylans (or might arise from the contaminant arabinogalactoproteins) as arabinoxylans are shown to contain galactose (Cyran et al., 2002; Dervilly et al, 2000; Izydorczyk and Biliaderis, 1995; Saulnier et al., 1995a; Saulnier et al., 1995b). Feraxans contained very less protein, contrary to many other purified arabinoxylans which contained high amounts (about 5 percent) of proteins (Dervilly-Pinel et al., 2001a; Roels et al, 1999; Saulnier et al.,

1995a). Although small amount of glucose is the inherent feature of arabinoxylans, feraxans, especially from rice contained little glucose. This also suggested the absence of any contaminant glucans.

Table 16. Neutral sugar composition (%) of purified feraxans (Sephacryl S-300) from native and malted rice and ragi.

			Rha	Ara	Xyl	Man	Gal	Glc	Ara/Xyl	P/H
Rice	N	P1	0.4	42.3	48.5	0.0	8.8	0.0	0.87	10.3
		P2	0.0	40.5	50.3	1.3	7.9	0.0	0.81	9.9
	M	P1	0.5	45.4	47.6	0.0	6.5	0.0	0.95	14.3
		P2	0.5	43.4	48.1	0.0	8.0	0.0	0.90	11.4
Ragi	N	P1	0.0	39.7	49.3	1.8	9.2	0.0	0.81	8.1
		P2	1.3	40.1	45.6	1.5	7.0	4.5	0.88	6.6
	M	P1	0.7	46.6	44.2	0.0	8.5	0.0	1.05	10.7
		P2	1.0	43.5	43.7	0.0	8.2	3.6	1.00	7.4

4.3. Structural characterization

4.3.1. Chemical methods

4.3.1.1. Carboxyl reduction

As these purified water soluble feraxans are found to contain high amount of uronic acid (around 8 – 13 %), carboxyl reduction seemed to be an essential step prior to methylation. Thus they were reduced with carbodiimide. Reduced feraxans contained about 2 – 3 % uronic acid (rice 1.8, 1.5, 2.2 and 2.3; ragi 2.0, 2.9, 2.5 and 2.8 % for peak 1 and 2 from native and malt feraxans respectively). Arabinoxylans from cereals are shown to contain very high amount of uronic acid (Saulnier et al., 1995b; Shibuya et al., 1983; Subba Rao and Muralikrishna, 2004). Many cereals polysaccharides such as rice bran arabinoxylans (Shibuya and Iwasaki, 1985), sorghum endosperm cell walls (Verbruggen et al., 1995) and ragi arabinoxylans (Subba Rao and Muralikrishna, 2004) were reduced prior to methylation study for the quantitative conversion of uronyl group to primary alcohol.

4.3.1.2. Methylation

Feraxans were methylated by the method of Hakomori (1964) in order to know the linkage type, substitution and ring size. Per-methylated sugars were identified based on their retention time with respect to 2,3,4,6-Me_4-Glc. Representative GLC profile of per-methylated sugars obtained for feraxans is shown below (figure 24).

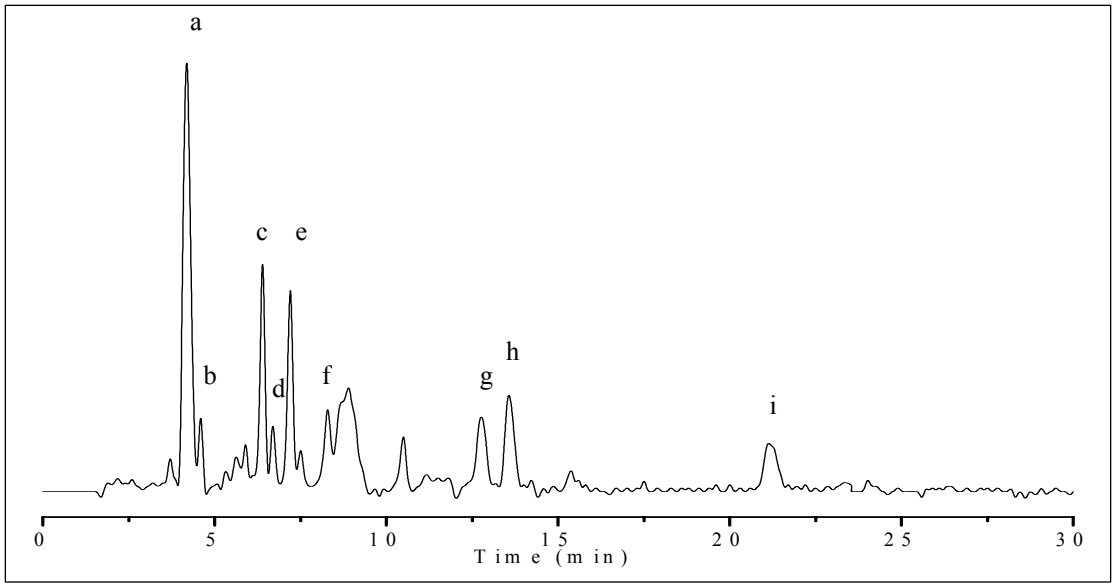

Figure 24. Representative GLC profile of per-methylated alditol acetates of water soluble feraxan from ragi (NP1): 2,3,5-Me_3-Ara (a), 2,3,4-Me_3-Xyl (b), 2,3,4,6-Me_4-Glc/2,3,4,6-Me_4-Gal (c), 2,3-Me_2-Ara (d), 2,3-Me_2-Xyl (e), 2-Me-Ara (f), 3-Me-Xyl (g), 2-Me-Xyl (h) and Xyl (i).

Permethylated sugars can not be identified based on retention time alone and hence identification is always assisted by their characteristic mass spectra. Mass spectral profile with diagnostic fragments of major per-methylated sugars obtained for feraxans are shown below (figures 25 to 30). In the present study major O-Me sugars obtained are 2,3,5-Me_3-Ara, 2,3,4-Me_3-Xyl, 2,3,4,6-Me_4-Glc, 2,3,4,6-Me_4-Gal, 2,3-Me_2-Ara, 2,3-Me_2-Xyl, 2-Me-Ara, 2/3-Me-Xyl and Xyl.

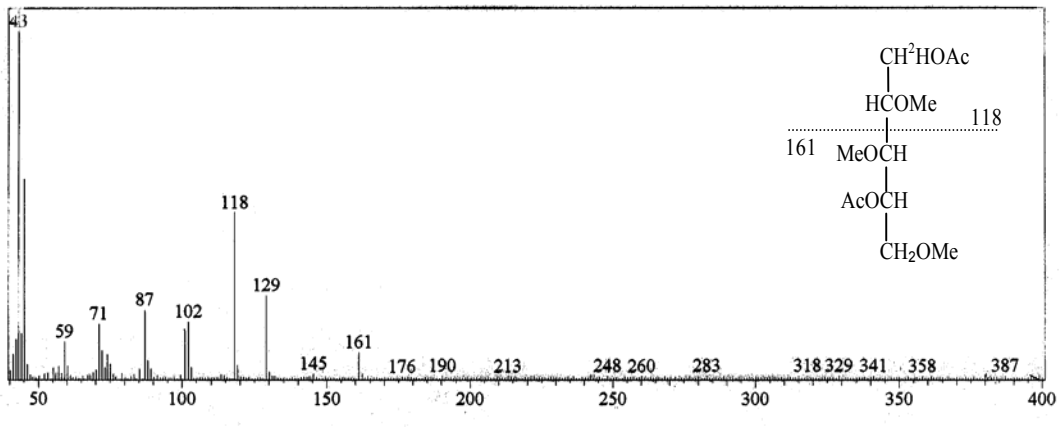

Figure 25. Fragmentation profile of 2,3,5-Me_3-Arabinose.

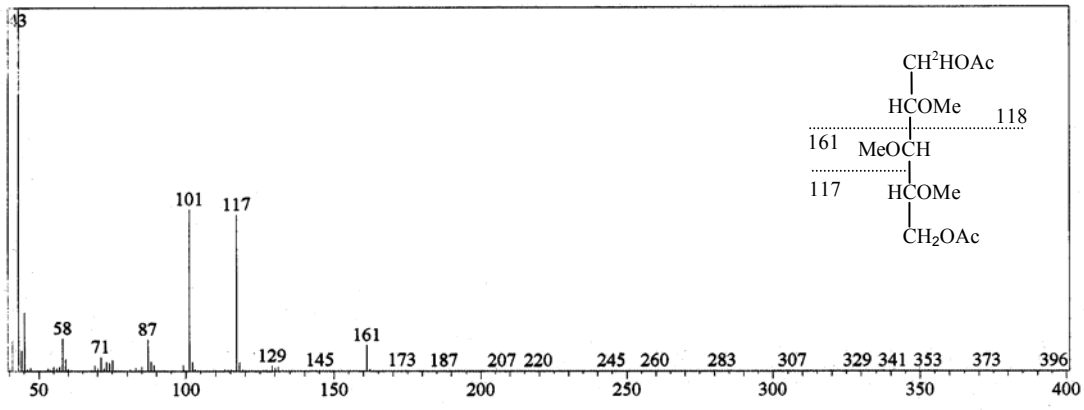

Figure 26. Fragmentation profile of 2,3,4-Me_3-Xylose.

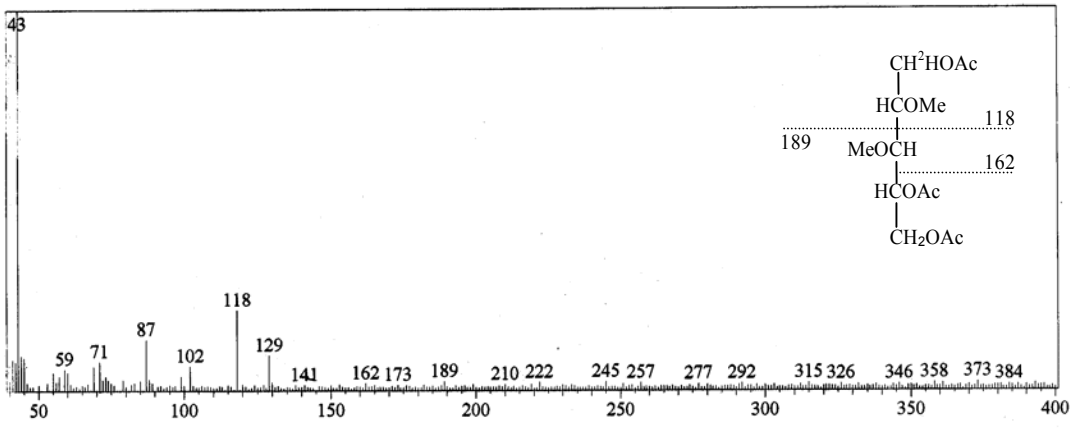

Figure 27. Fragmentation profile of 2,3-Me_2-Arabinose/Xylose.

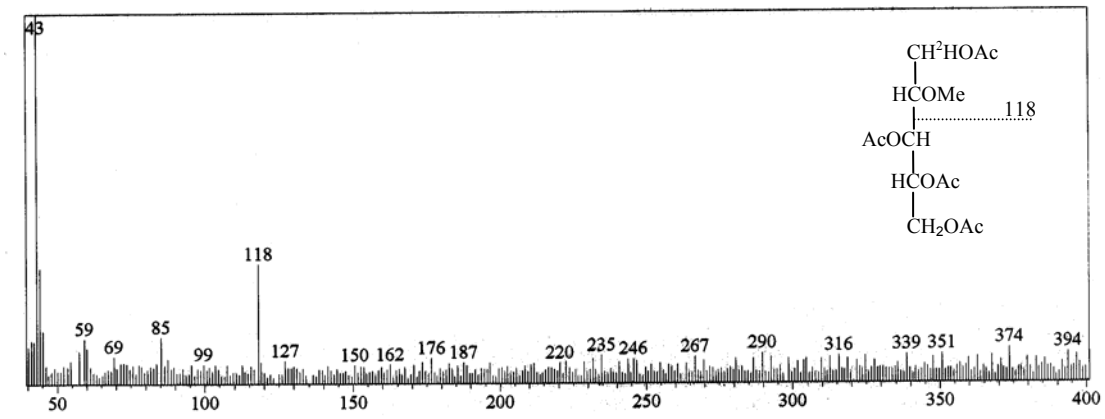

Figure 28. Fragmentation profile of 2-Me-Arabinose/Xylose.

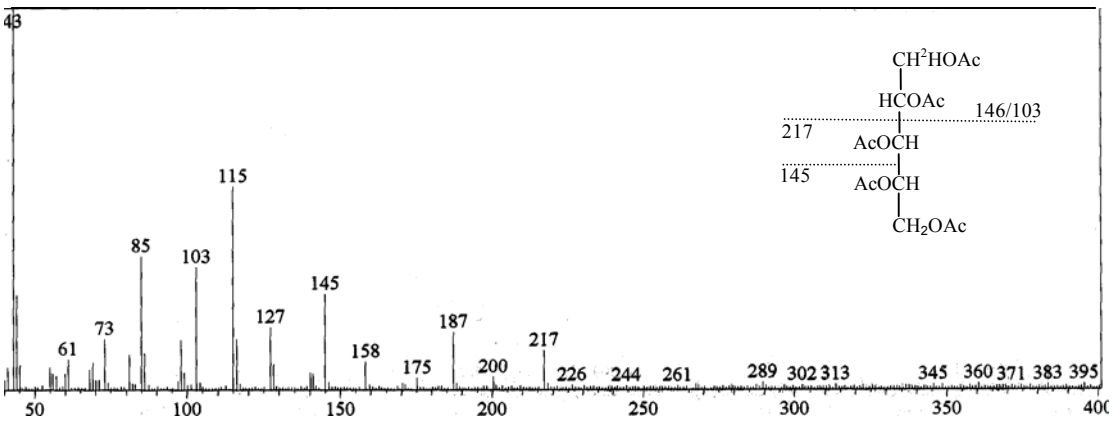

Figure 29. Fragmentation profile of Arabinose/Xylose.

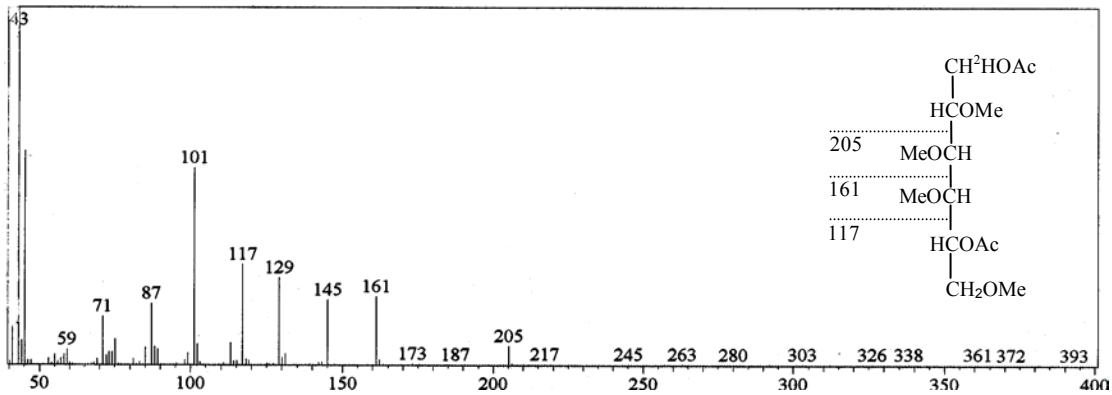

Figure 30. Fragmentation profile of 2,3,4,6-Me_4-Galactose/Glucose.

The relative amount of each per-methylated sugar is quantified (table 17) based on the peak area and neutral sugar composition.

Table 17. Methylation analysis (peak area, mol %) of feraxans from native and malted rice and ragi.

O-Methyl ether	Linkage	Rice				Ragi			
		NP1	NP2	MP1	MP2	NP1	NP2	MP1	MP2
2,3,5-Ara	Terminal	25.9	27.5	29.0	28.9	25.4	29.1	31.5	29.2
2,3-Ara	1,5	4.9	4.2	6.1	4.8	5.6	4.3	6.7	7.1
2-Ara	1,3,5	8.5	5.7	6.4	6.4	7.2	5.5	5.2	2.1
2,3,4-Xyl	Terminal	1.1	1.3	1.4	1.3	1.8	1.9	1.4	1.3
2,3-Xyl	1,4	13.5	18.7	18.4	18.0	18.9	15.5	13.5	13.2
2/3-Xyl	1,3/2,4	22.8	17.6	9.2	12.5	15.6	16.3	11.7	12.2
Xyl	1,2,3,4	7.5	9.3	14.6	12.8	9.8	9.5	14.1	12.6
2,3,4,6-Gal	Terminal	7.0	6.3	5.5	6.5	7.1	5.4	6.9	6.0
2,3,4,6-Glc	Terminal	5.0	5.4	5.9	5.1	5.3	8.2	4.6	9.8
2,3-Xyl/[2(3)-Xyl + Xyl]		0.45	0.70	0.77	0.71	0.74	0.60	0.52	0.53
Xyl/2(3)-Xyl		0.33	0.53	1.59	1.02	0.63	0.58	1.21	1.03

It is clear from the methylation analysis that the 2,3,5-Ara is the major product, which indicated it to be terminally linked to xylose residue. 2,3-Me_2-Ara and 2-Me-Ara are detected in good yield (over 30% of free arabinose). These arabinose residues might be present in short side-chains on the xylose backbone. They also provided a site for the covalent linkage of ferulic acid, the major bound phenolic acid in cereal arabinoxylans. Arabinose, however, is not detected in appreciable amounts.

On the other hand, 2,3-Me_2-Xyl, 2/3-Me-Xyl and Xyl are detected almost in equal quantity. The backbone of the polysaccharides is clearly shown to be made up of 1→4 linked D-Xylose residues. It is evident that around one third of xylose residues were un-substituted, another one third is mono-substituted and remaining xylose residues are di-substituted. Small amount of 2,3,4-Me_3-Xyl, which might be originating from terminally linked xylose or from end residue, is also seen. It is clear

from the ^{1}H NMR results (figure 37 and table 20) that 2/3-Me-Xyl contained good amount of 2-Me-Xyl.

Arabinose residues might have been linked to xylose at O-3 or O-2 or both at O-2 and O-3 as indicated by equal amount of mono and di-substituted xylose residues.

Galactose is mostly terminally linked as indicated by the presence of 2,3,4,6-Me_4-Gal. However, traces of 2,3,4/2,3,6-Me_3-Gal could also be seen. Similarly uronic acid is also linked terminally to xylose as indicated by the presence (around 5%) of 2,3,4,6-Me_4-Glc, which might be originated from the carboxyl reduced glucuronic acid (Bergmans et al., 1996). Increase in the 2,3,4,6-Me_4-glucose was seen for the carboxyl reduced arabinoxylans from rice bran (Shibuya and Iwasaki, 1985).

Hakomori methylation is widely used for the structural characterization of polysaccharides, in particular for the linkage study of arabinoxylans from wheat (Shiiba et al., 1993), barley (Han, 2000), rice (Shibuya and Iwasaki, 1985), sorghum (Woolard et al., 1976), maize (Saulnier et al., 1995a; Saulnier et al., 1995b) and ragi (Subba Rao and Muralikrishna, 2004).

Methylation results of cereal arabinoxylans showed, in general, that the amount of 2,3,5-Me_3-Ara and 2-Me-Xyl are more compared to the other O-Me ether derivatives obtained for maize (Saulnier et al., 1995), sorghum (Woolard et al., 1976) rye (Vinkx et al., 1995) and ragi arabinoxylans (Subba Rao and Muralikrishna, 2004). However, in the present study both mono and di-substituted xylose residues are almost in equal proportions indicating high amount of di-substitution especially in malt feraxans. A heteroxylan isolated from the pericarp of wheat kernel was shown to be highly substituted glucuronoarabinoxylan in which 80% of the β-D-xylosyl residues carry one or two substitutions (Brillouet and Joseleau, 1987) These results are in contrary to the one reported for wheat (Cleemput et al., 1995; Izydorczyk and Biliaderis, 1994; Shiiba et al., 1993) and barley arabinoxylans (Han, 2000), wherein

high amount of 2,3-Me_2-Xyl was detected indicating less branching or low substitution of xylose.

4.3.1.3. Evidence for the presence of 4-O-Me-glucuronic acid

Carboxyl reduced feraxans were hydrolyzed and acetylated in order to find the nature of uronic acid. GLC analysis (figure 31) showed the presence of 4-O-Me-glucose, which is further authenticated with mass spectra (figure 32) by the presence of diagnostic fragments (129, 189 and 217).

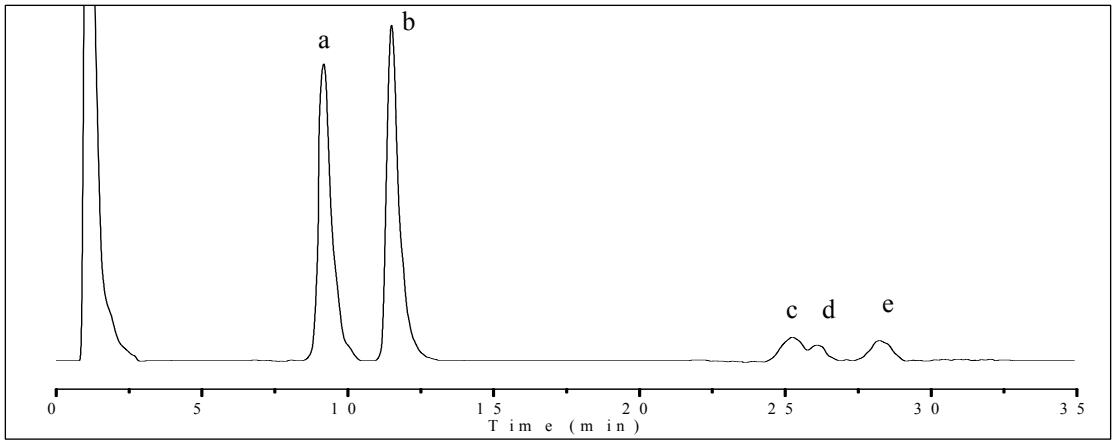

Figure 31. Representative GLC profile of carboxyl reduced water soluble feraxan from ragi (NP2): arabinose (a), xylose (b), galactose (c), 4-O-Me-glucose (d) and glucose (e).

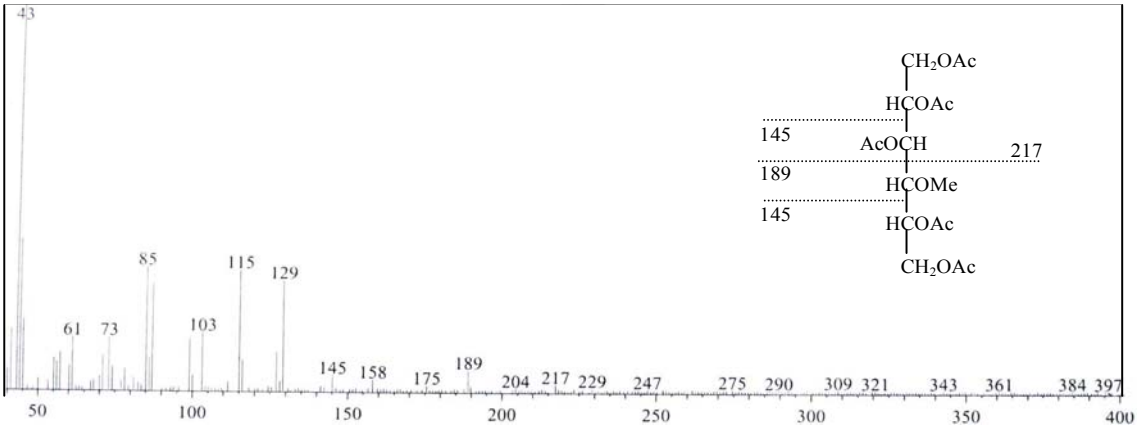

Figure 32. Fragmentation profile of 4-O-Me-Glc.

Cereal arabinoxylans are shown to contain very high amount of uronic acid (Saulnier et al., 1995a; Shibuya et al., 1983) and are generally presence in the form of 4-O-Me-glucuronic acid. They are also shown to occur in high amount in ragi arabinoxylans (Subba Rao and Muralikrishna, 2004).

4.3.1.4. Periodate oxidation

The consumption of periodate during oxidation of feraxans are measured to know the degree of substitution and the kinetics of periodate oxidation are shown in figure 33. Eight purified feraxans consumed between 4.02 to 4.30 μmol of periodate per mg of arabinoxylans (AX) indicating that about 60 to 65 percent sugars have adjacent free hydroxyl groups.

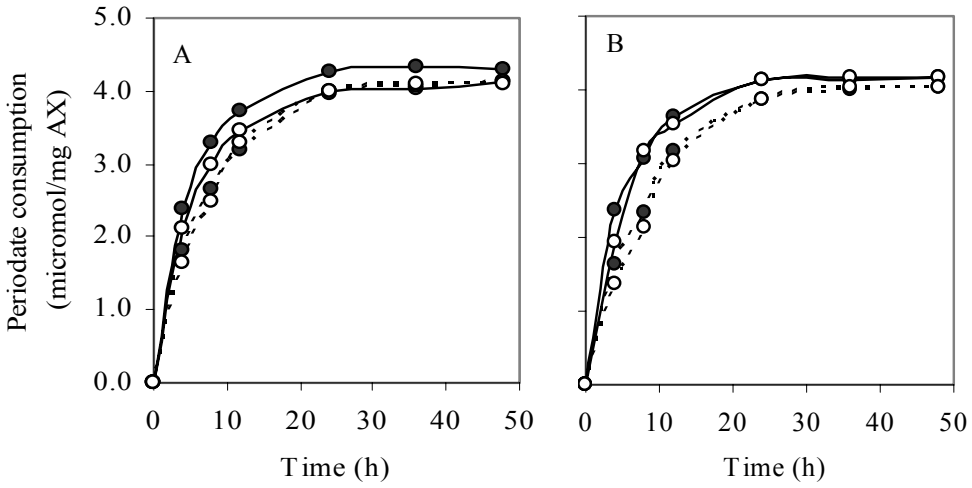

Figure 33. Kinetics of periodate oxidation of feraxans from native (solid line) and malted (dotted line) rice (A) and ragi (B). Peak 1 – solid symbol and Peak 2 – open symbol.

Periodate consumption is maximum initially and reached plateau after 24 h. Periodate consumption by native arabinoxylan fractions is

slightly higher compared to malts, which may be an indication of slightly higher branched nature of malt arabinoxylans. In general, arabinose content of malt arabinoxylans is slightly higher compared to native ones. Overall, periodate oxidation study showed high degree of branching in feraxans. Similar to this, highly branched glucuronoarabinoxylans obtained from sorghum husk were found to consume about 0.64 moles of periodate over 27 h of oxidation (Woolard et al., 1976). In a recent study, Dervilly-Pinel et al. (2004) showed almost equal consumption (4.27 and 4.11 μmol/mg AX) of periodate by two arabinoxylan populations with different levels of substitution (Ara/Xyl = 0.38 and 0.82). The periodate consumption rate was maximum during the first 5 h and reached plateau after 24 h.

4.3.1.4.1. Formic acid liberation

There was no detectable level of formic acid in the reaction mixture. This indicated the absence/low amount of 3 consecutive hydroxyl groups in the sugars. It also suggested that the high amount (about 10 percent) of uronic acid present in the arabinoxylans are chiefly in 4-O-methyl form. This was further substantiated by the GLC analysis of carboxyl reduced feraxans wherein the presence of 4-O-methyl glucose was observed. Small amounts of galactose/glucose may be present in short side chains, thus reducing the further oxidation. Methylation analysis showed trace amount of 2,3,6-Me_3- galactose/glucose, indicating the absence of 3 contiguous –OH groups.

4.3.1.5. Smith degradation

Periodate consumption was halted after 48 h and analysis of Smith degradation products showed high amount of glycerol and xylose (table 18). Glycerol might chiefly have been originated from side chain arabinose. Similar to this study, Smith degradation analysis of the

glucuronoarabinoxylans from sorghum husk showed high amount of glycerol and mild acid hydrolysis yielded many oligosaccharides with different xylose values (Woolard et al., 1976). Based on periodate oxidation and Smith degradation study of wheat water unextractable arabinoxylans, Gruppen et al. (1993) reported that most of the branched residues are present as isolated units of blocks of two contiguous substituted xylose residues.

Table 18. Analysis of Smith degradation products (%) obtained form feraxans.

			Glycerol	Ara	Xyl	Ara/Xyl
Rice	N	P 1	63.1	10.5	26.4	0.40
		P 2	59.1	3.7	37.2	0.10
	M	P 1	58.9	2.5	38.6	0.07
		P 2	61.5	2.1	36.4	0.06
Ragi	N	P 1	55.6	7.6	36.8	0.21
		P 2	54.5	7.4	38.1	0.19
	M	P 1	48.1	2.1	49.8	0.04
		P 2	50.0	3.7	46.3	0.08

P - Peak

Aspinall and Ross (1963) obtained glycerol xylosides with one to three xylopyranosyl residues in the molar ratio of 7.5:2.2:1 upon periodate oxidation, followed by mild acid hydrolysis of rye flour arabinoxylans. They concluded that arabinofuranosyl side chains are attached to isolated and, less frequently, to two and three, but not more, continuous xylopyranosyl residues. However, in a much recent study on rye arabinoxylans, Aman and Bengtsson (1991) concluded that the distribution of units of small blocks of two branched residues is isolated and not random as previously reported.

4.3.2. Spectroscopic methods

4.3.2.1. ^{13}C Nuclear magnetic resonance

The ^{13}C nuclear magnetic resonance spectra obtained for purified feraxans are shown in figure 34. Chemical shifts (δ) are expressed in ppm downstream from external Me_4Si.

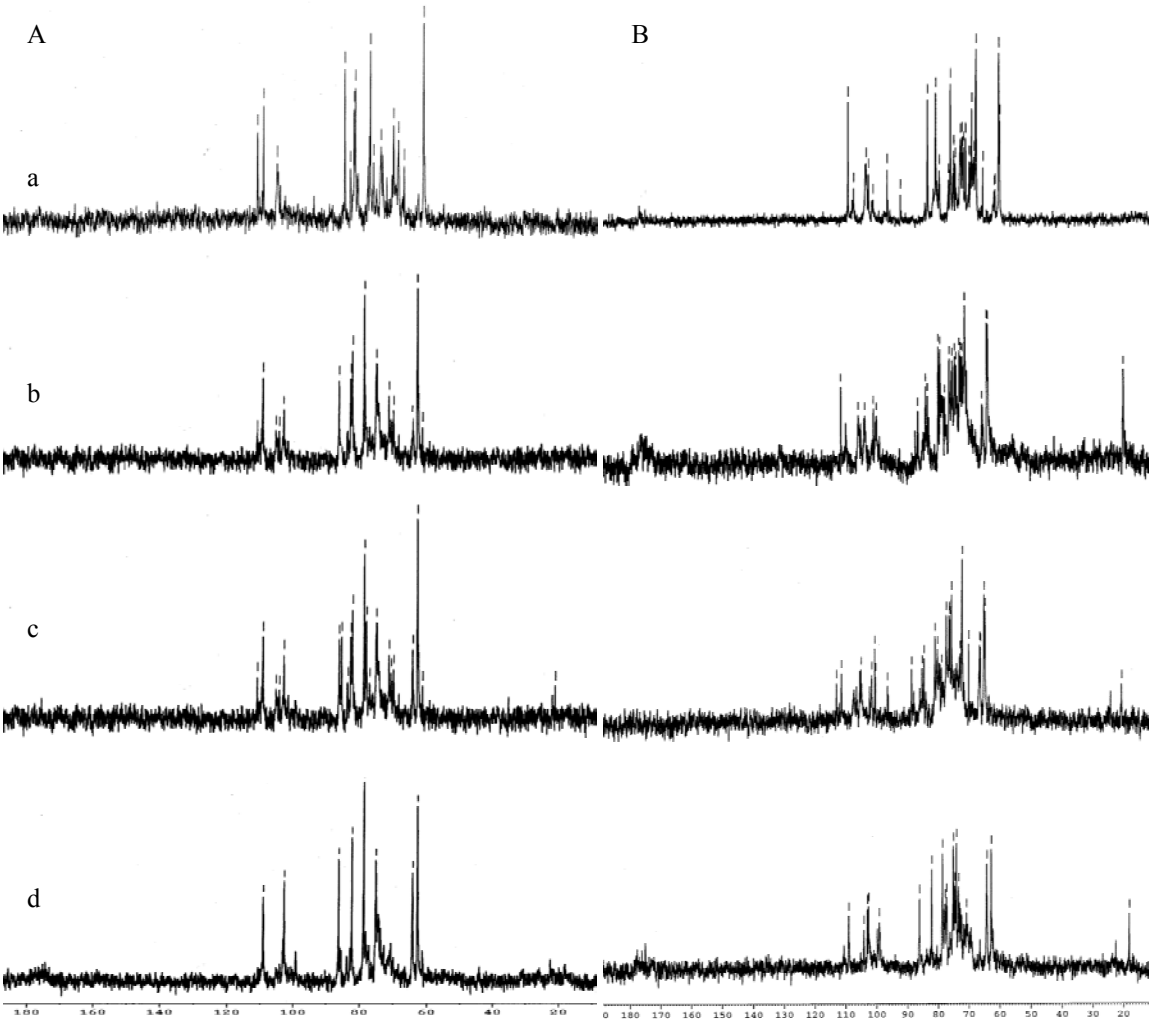

Figure 34. ^{13}C NMR spectra of water soluble feraxans obtained from native and malted rice (A) and ragi (B) feraxans: NP1 (a), NP2 (b), MP1 (c) and MP2 (d).

The ^{13}C NMR spectra obtained for eight purified feraxans are very much similar and data are compiled in table 19. The ^{13}C NMR spectra of

feraxans, similar to the other cereal arabinoxylans showed distinguishable clusters of signals (Hoffmann et al., 1991). The chemical shift values of the signals for anomeric carbon of Ara_f (δ = 108.8 – 110.7 ppm) and Xyl_p (δ = 102.6 – 104.7 ppm) indicated that Ara_f has α and Xyl_p has β configuration (Bock and Pedersen, 1983; Hoffmann et al., 1991; Joseleau et al., 1977). C-1 signals for mono (element B) and di-substituted (element A) xylose residues might be observed at around 104.7 and 102.6 ppm respectively.

Table 19. Assignments* of ^{13}C NMR signals (chemical shifts, ppm) obtained for feraxans from rice and ragi.

Residue	Chemical shifts (ppm)				
	C-1	C-2	C-3	C-4	C-5
β-D-Xyl_p	104.7	73.2	74.8	77.0	64.1
β-D-Xyl_p-(adj)	104.0				64.1
Element A					
β-D-Xyl_p	102.6	73.1			63.8
α-L-Ara_f-(1→2)	110.5	82.7		85.2	62.3
α-L-Ara_f-(1→3)	108.8	81.6		85.2	62.3
Element B					
β-D-Xyl_p	104.7	73.8	78.6	74.3	63.8
α-L-Ara_f-(1→3)	108.8	81.6	78.6	85.2	62.7

* Assignments are based on Hoffmann et al., (1991) and references thereof.
Element A = →4)[α-L-Ara_f-(1→2)][α-L-Ara_f-(1→3)]-β-D-Xyl_p(1→
Element B = →4)[α-L-Ara_f-(1→3)]-β-D-Xyl_p(1→
β-D-Xyl_p = →4)-β-D-Xyl_p(1→
β-D-Xyl_p-(adj) = →4)-β-D-Xyl_p(1→ adjoining element A and element B at the non-reducing end.

Partial structure of the arabinoxylan with differently linked sugar residues responsible for the observed ^{13}C NMR signals is shown in the figure 35. Signals are seen for Ara_f C-2, C-4 and C-5 (ring carbon atoms) at around 82.6, 85.2 and 62.3 ppm respectively. However, signal

intensities could not clearly be assigned to the relative abundance of the two elements (A and B) as all the feraxans are found to be heavily branched (from methylation and ^{1}H NMR results). Otherwise, signal intensities of these 'structural-reporter-group' regions could give information regarding the relative abundance of mono and di-substituted xylose (Hoffmann et al., 1991).

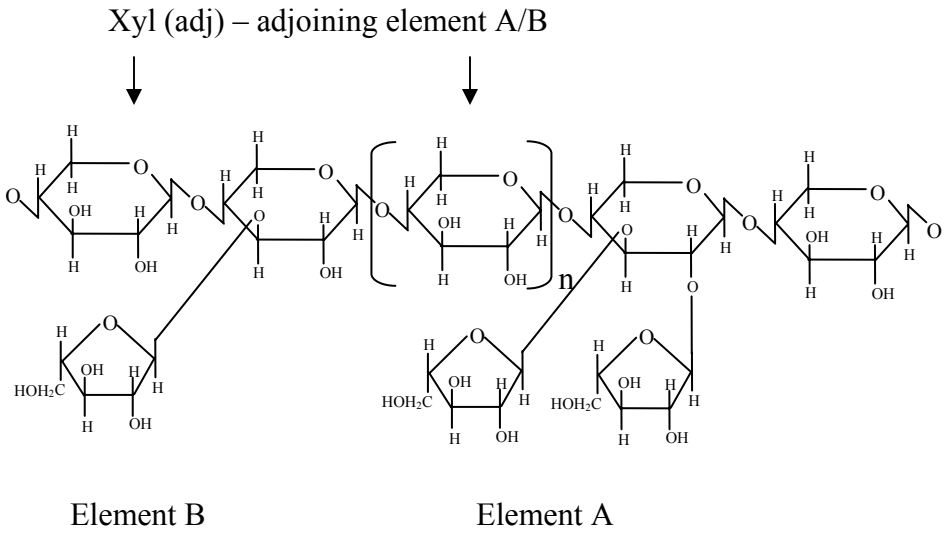

Figure 35. Tentative/probable partial structure of rice/ragi arabinoxylan.

Signals observed at around 98.8 and 72.1 ppm could be assigned to the C-1 and C-3 of α-D-glucuronic (or 4-O-Me) acid respectively. Low intensity signals at around 59.5 and 18.0 ppm might be arising from -O-CH_3 of 4-O-Me-α-D-glucuronic acid (Brillouet and Joseleau, 1987). Low intensity signal for >C=O (C-6 carbonyl group) of 4-O-Me-α-D-glucuronic acid are detected at around 178.0 ppm. Similar observations are made with the ^{13}C NMR spectra obtained for ragi arabinoxylans (Subba Rao and Muralikrishna, 2004). Carbonyl signals of -$NHCOCH_3$ group are also

observed at around 176.0 in the ^{13}C NMR spectrum of antigenic polysaccharides isolated from *Neisseria meningitides* serogroup A (Jennings and Smith, 1978). Some of these signals might also be arising from acetyl groups as arabinoxylans are known to contain acetyl groups (Saavendra et al., 1988).

As the arabinoxylans contained ferulic acid side groups, one would expect the signals pertaining to ferulic acid (figure 36) in the ^{13}C NMR spectra of arabinoxylans. However, ^{13}C NMR spectra of feraxans did not show prominent signals that could be assigned to ferulic acid. This might not be surprising as ferulic acid in feraxans is present in low amounts. On the other hand signals seen at around 178.0 and 59.5 ppm may partly be originated from bound ferulic acid. However, signals corresponding to ferulic acid were very well seen in case of feruloyl oligosaccharides obtained from arabinoxylans (Kato and Nevins, 1985; Colquhoun et al., 1994).

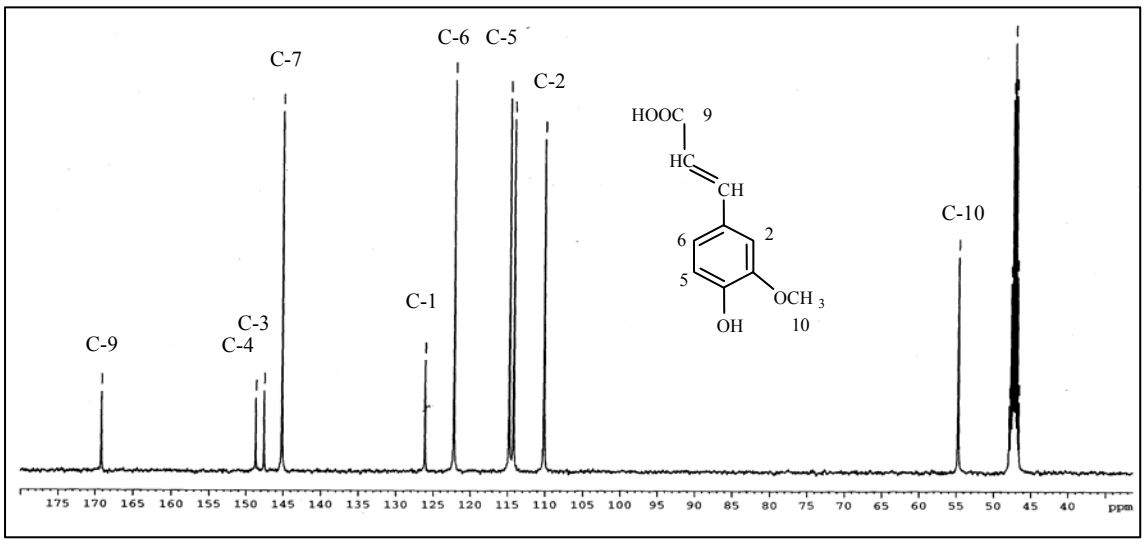

Figure 36. ^{13}C NMR spectrum of ferulic acid.

In general ^{13}C NMR data obtained for water soluble feraxans from native and malted rice and ragi are similar to the data obtained for other

cereal arabinoxylans (Hoffmann et al., 1991; Izydorczyk and Biliaderis, 1995; Subba Rao and Muralikrishna, 2004).

4.3.2.2. ^{1}H Nuclear magnetic resonance

The ^{1}H (proton) nuclear magnetic resonance (PMR) spectra with expanded anomeric regions of arabinose proton obtained for purified feraxans are shown in figure 37.

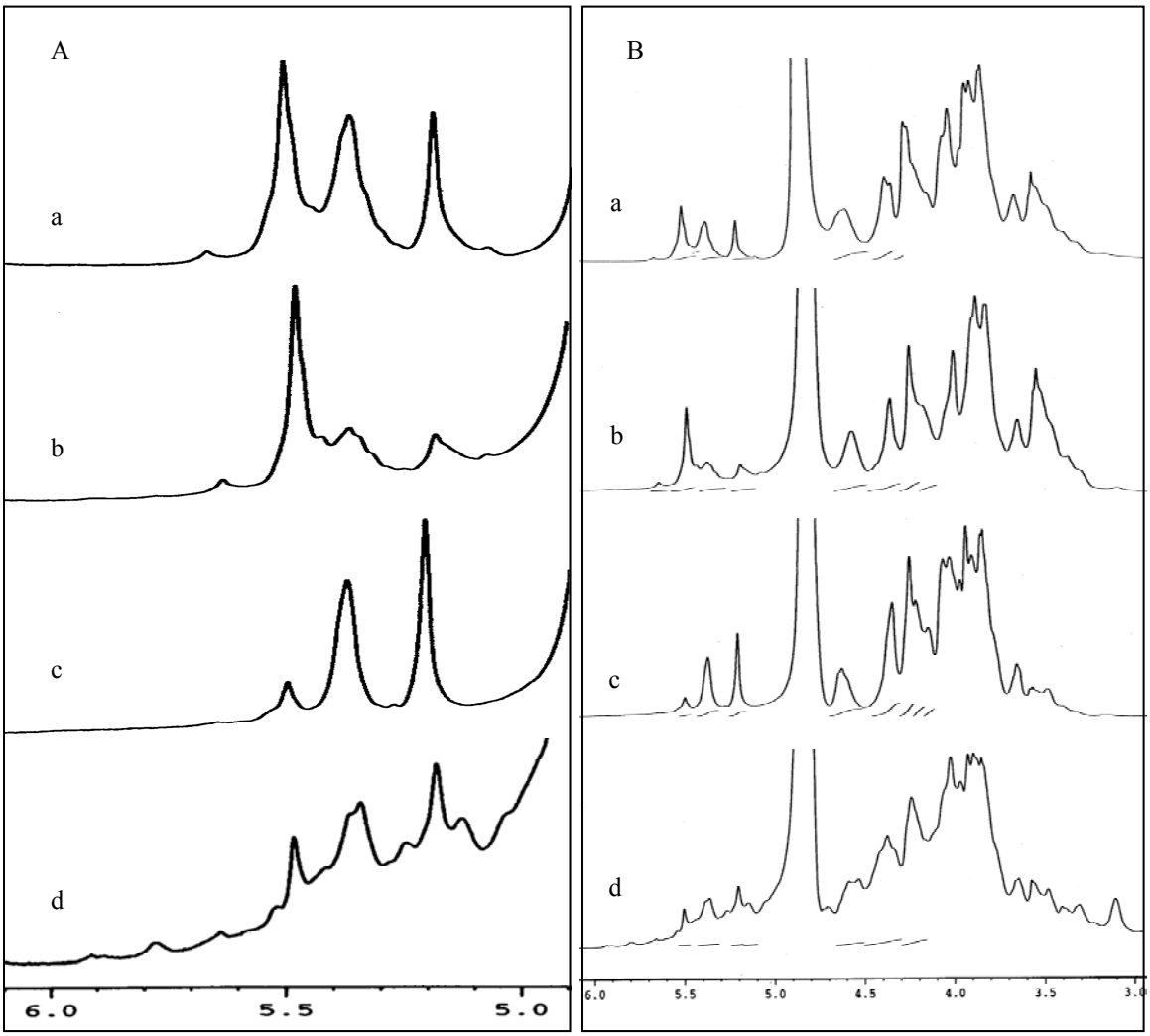

Figure 37. ^{1}H NMR spectra (B) of water soluble feraxans obtained from native and malted rice. Anomeric signals of arabinose are expanded at the left (A): NP1 (a), NP2 (b), MP1 (c) and MP2 (d).

The peak at around δ 5.47 ppm represented the anomeric protons of arabinose linked to the O-3 position of xylose residues, while the two peaks at around δ 5.34 and δ 5.18 ppm are from anomeric protons of arabinose residues linked to O-2 and O-3 of the same xylose residue. The unresolved signals or shoulders downstream from the peaks at δ 5.34 and δ 5.18 ppm resulted from two neighboring di-substituted xylose residues in the arabinoxylan chain (Hoffmann et al., 1992; Vinkx et al., 1993) which indicated that the feraxans contained both isolated and paired di-substituted xylose residues similar to other arabinoxylans from wheat (Cleemput et al., 1995) and barley (Trogh et al., 2004). The presence of unresolved signal or shoulder downstream from the peak at around δ 5.47 ppm represented the presence of O-3 mono-substituted xylose next to di-substituted xylose (Hoffmann et al., 1992). The O-2 mono-substitution of xylose cannot be detected directly by ^{1}H NMR spectroscopy because its signal (at around δ 5.34 ppm) overlaps with that of di-substituted xylose (Vinkx et al., 1995). Theoretically signals at around δ 5.34 and δ 5.18 ppm should have equal intensity as they represent the anomeric protons of arabinose linked at O-2 and O-3 position of the same xylose residues. However, as the O-2 mono-substituted arabinose protons give the signal at around δ 5.34 ppm, the combined signal at around δ 5.34 is higher than the signal at around δ 5.18 ppm. Therefore, the content of O-2 mono-substituted xylose is estimated as the difference between the integrals of the two peaks of arabinose residues linked to di-substituted xylose (Oscarsson et al., 1996).

The proportions of un, mono (O-2 and O-3) and di-substituted xylose in the purified feraxans from native and malted rice and ragi are given in table 20.

Table 20. Substitution pattern of xylose in feraxans.

			un-xyl	2-xyl	3-xyl	2,3-xyl	Di/mono	Un/substituted
Rice	N	P 1	30.6	13.9	37.9	17.6	0.34	0.44
		P 2	40.4	3.7	34.5	21.4	0.56	0.68
	M	P 1	41.8	7.0	14.5	36.7	1.71	0.72
		P 2	41.1	11.6	16.2	31.1	1.12	0.70
Ragi	N	P 1	42.2	11.5	23.1	23.2	0.67	0.73
		P 2	36.7	5.3	33.3	24.7	0.64	0.67
	M	P 1	33.1	10.3	18.4	38.2	1.33	0.50
		P 2	34.2	5.3	26.3	34.2	1.08	0.52

On average, the levels of un, O-2 mono, O-3 mono and di-substituted xylose are around 35, 10, 25 and 30% respectively. Cereal arabinoxylans, especially from ragi are shown to contain high amount of substitution with very low amount of un-substituted xylose residues (Subba Rao and Muralikrishna, 2004). More specifically, the levels of un-substituted xylose residues varied at 30.6 – 42.2%. The content of O-2 substituted xylose is low and varied at 3.7 – 13.9%, comparable with other arabinoxylans like one from barley (Dervilly et al., 2002; Oscarsson et al., 1996; Trogh et al., 2004). The content of O-3 substituted xylose varied at 14.5 – 37.9%, which is higher compared to barley (~ 20%) (Oscarsson et al., 1996) and wheat (~ 20%) (Cleemput et al., 1995), but lower compared to other cereal arabinoxylans (Saulnier et al., 1995; Subba Rao and Muralikrishna, 2004). The amount of di-substituted xylose is quite high and ranged at 17.6 – 38.2%. This value is comparably higher than the di-substitution level in other arabinoxylans especially from barley (~ 24%) (Dervilly et al., 2002; Oscarsson et al., 1996; Trogh et al., 2004).

While the un-substituted xylose residues remained overall same, feraxans from native samples contained higher amount of O-3 substituted xylose residues compared to malt feraxans. On the contrary, levels of di-substituted xylose residues are comparably higher for malt

feraxans both from rice and ragi. This is evident in the ratio of di/mono-substitution, which is very high for malts indicating higher amount of di-substitution. However, the ratio of un/substituted xylose residues ranged at 0.44 – 0.73, with only a slight increase in the substitution level for malt feraxans.

The four structural elements in the xylan backbone, i.e., un, mono (O-2), mono (O-3) and di-substituted xylose are correlated with the Ara/Xyl ratio and results are shown in figure 38.

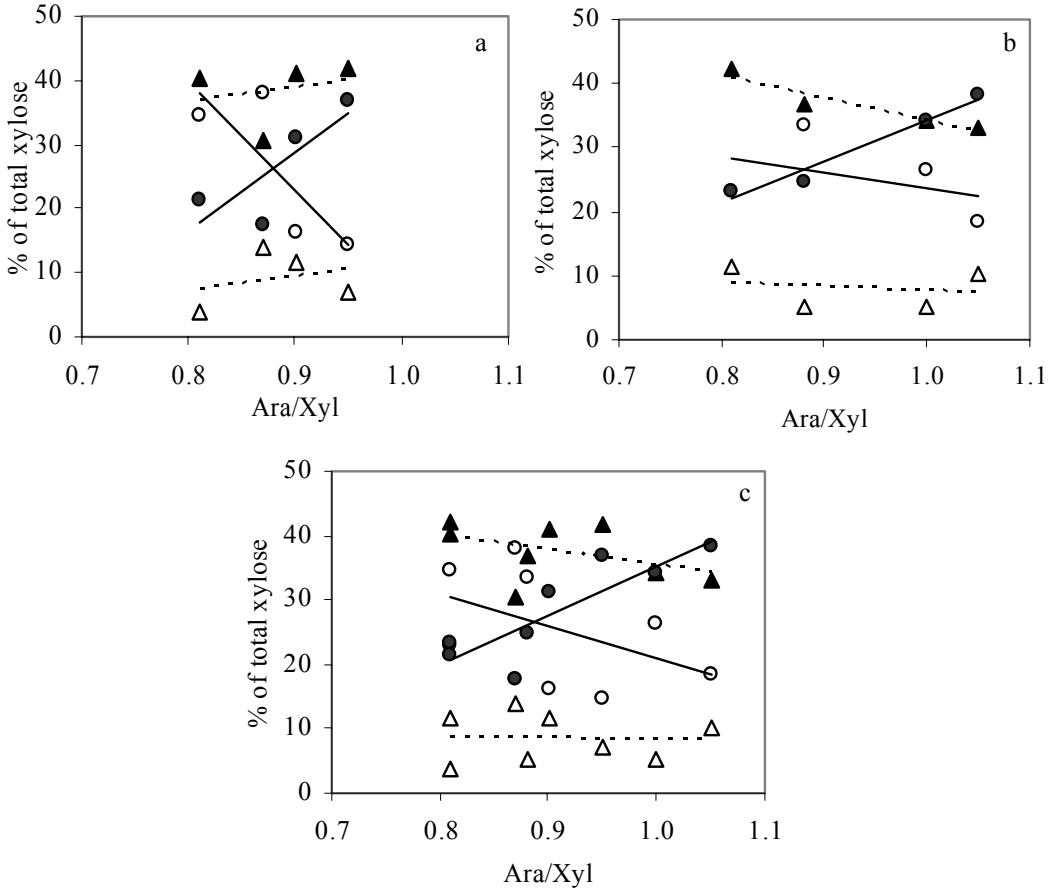

Figure 38. Relationship between the relative proportion of differently linked xylose residues (unsubstituted – ▲, O-2 – ∆, O-3 – ○ and O-2,3 – ●) and the ratio of Ara/Xyl of feraxans from native and malted rice (a) and ragi (b). a and b combined (c).

It is observed that overall levels of un and mono (O-2) substituted xylose residues remained constant with increasing Ara/Xyl ratio. However, the level of mono (O-3) substituted xylose residues decreased and di-substitution increased with the increase in Ara/Xyl ratio. Similar relationships are reported previously for wheat and rye water extractable arabinoxylans (Cyran et al., 2003; Dervilly et al., 2000; Vinkx, 1995).

Similar trend is observed in rice and ragi feraxans individually as well as when both data are combined. Since malt feraxans have higher Ara/Xyl ratio, their di-substitution level is higher to accommodate the extra arabinose without much change in the level of un-substituted xylose.

Arabinoxylan fractions obtained with increased concentrations of ethanol/ammonium sulphate were observed to have higher Ara/Xyl ratio and lower molecular weight (Izydorczyk and Biliaderis, 1992; Mares and Stone, 1973). In other words, Ara/Xyl ratio decreased with increasing molecular weight of the arabinoxylans. This is substantiated in the present study wherein malt feraxans with lower molecular weight have higher Ara/Xyl ratio. The relationships: Ara/Xyl ratio, un/substituted xylose and di/mono-substituted xylose with that of molecular weight of feraxans are plotted and the results are shown in figure 39.

From the figure 39a, it is clear that Ara/Xyl ratio and di-substitution decreased with increasing molecular weight of the feraxans, whereas un-substitution level remained still or slightly increased. However, this relationship might disappear in arabinoxylans with relatively narrow molecular weight range (figure 39b). To validate this relationship, data for barley and wheat arabinoxylans are taken from the literature and plotted individually and combined, and the results are shown in figure 40.

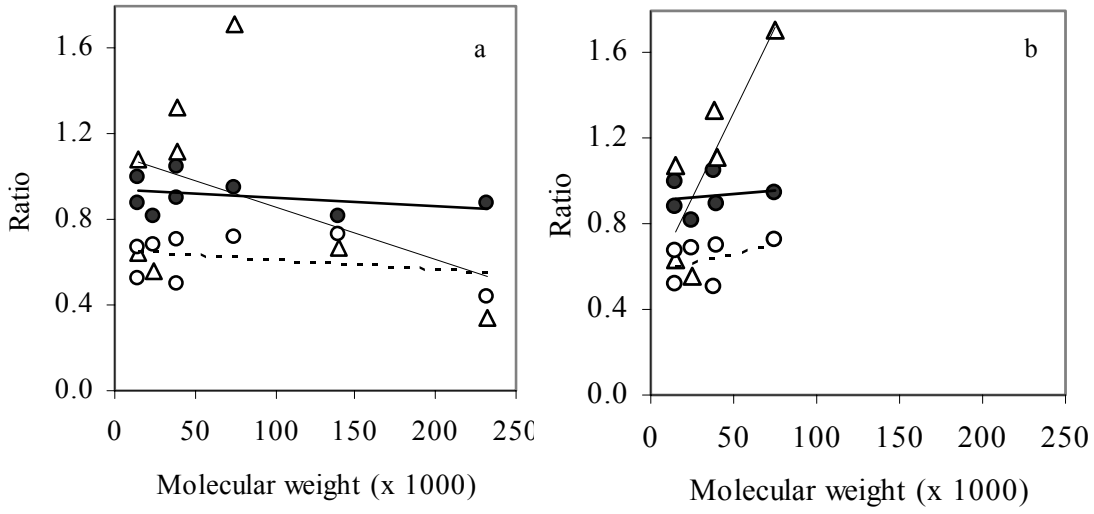

Figure 39. Relationships of molecular weight with ratios of Ara/Xyl (solid circle), un-substituted/substituted xylose (open circle) and di/mono-substituted xylose (open triangle) in all feraxans (a) and low molecular weight feraxans (b) from native and malted rice and ragi.

It is observed both in barley and wheat arabinoxylan that arabinose and di-substitution decreased with increasing molecular weight whereas the un-substitution increased. Same relationship is observed even when data from rice, ragi, barley and wheat are combined. It may be noted that graded precipitation of arabinoxylans takes place by the virtue of hydrophobic interactions, which in turn is governed by the molecular weight and Ara/Xyl ratio. Arabinoxylan precipitates when either molecular weight is higher or Ara/Xyl ratio is lower than the general pool. This results in the overall increase in the Ara/Xyl ratio with decreasing molecular weight.

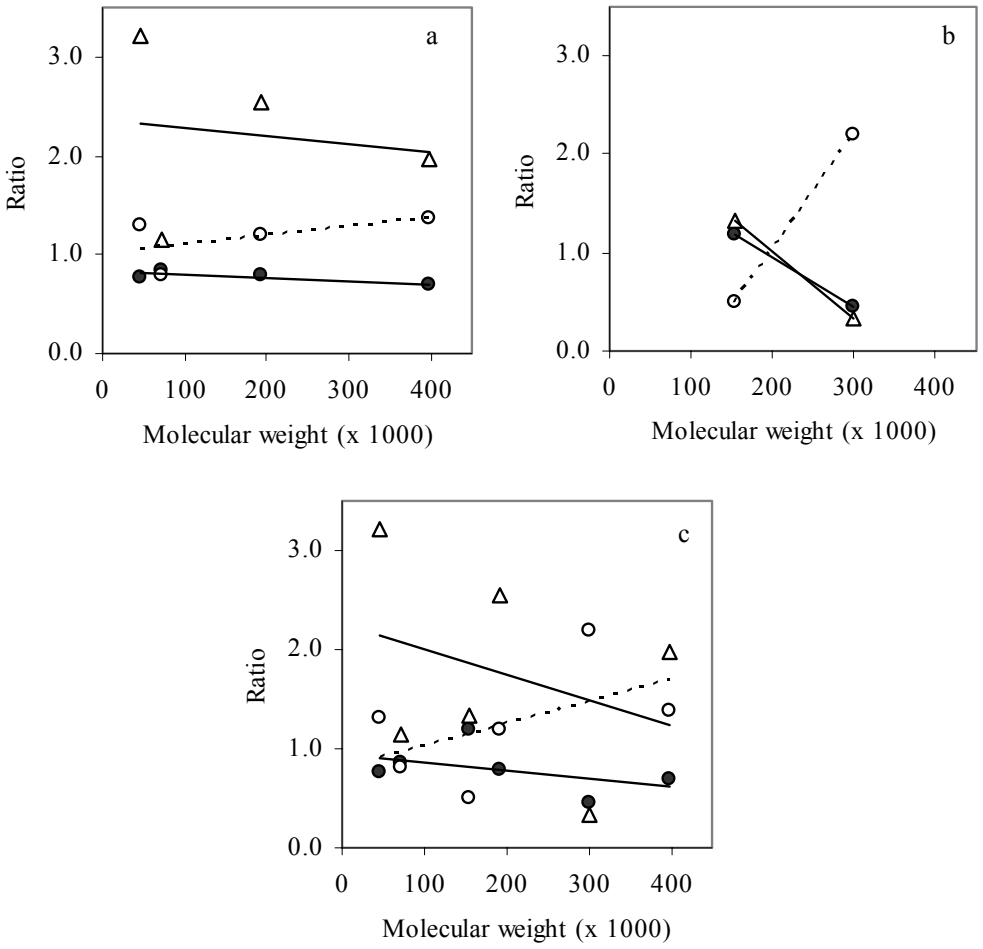

Figure 40. Relationships between molecular weight and ratios of Ara/Xyl (solid circle), un-substituted/substituted xylose (open circle) and di/mono-substituted xylose (open triangle) in barley malt arabinoxylans (raw data from Cyran et al., 2002) (a), wheat water-soluble arabinoxylans (raw data from Dervilly-Pinel et al., 2004) (b) and both (a) and (b) combined (c).

As the feraxans contained bound ferulic acid, PMR spectrum of rice feraxan (MP2) (figure 42) showed signals at around δ 6 – 8 ppm, which might be assigned to ferulic acid (figure 41) (Cyran et al., 2003; Ralph et al., 1994; Saulnier et al., 1999).

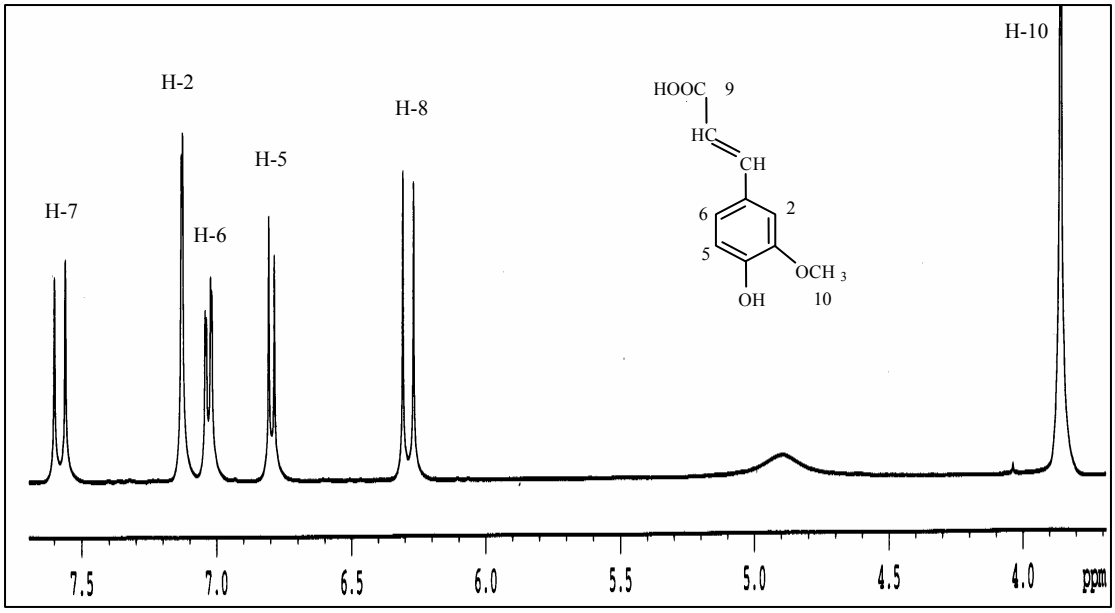

Figure 41. ^{1}H NMR spectrum of ferulic acid.

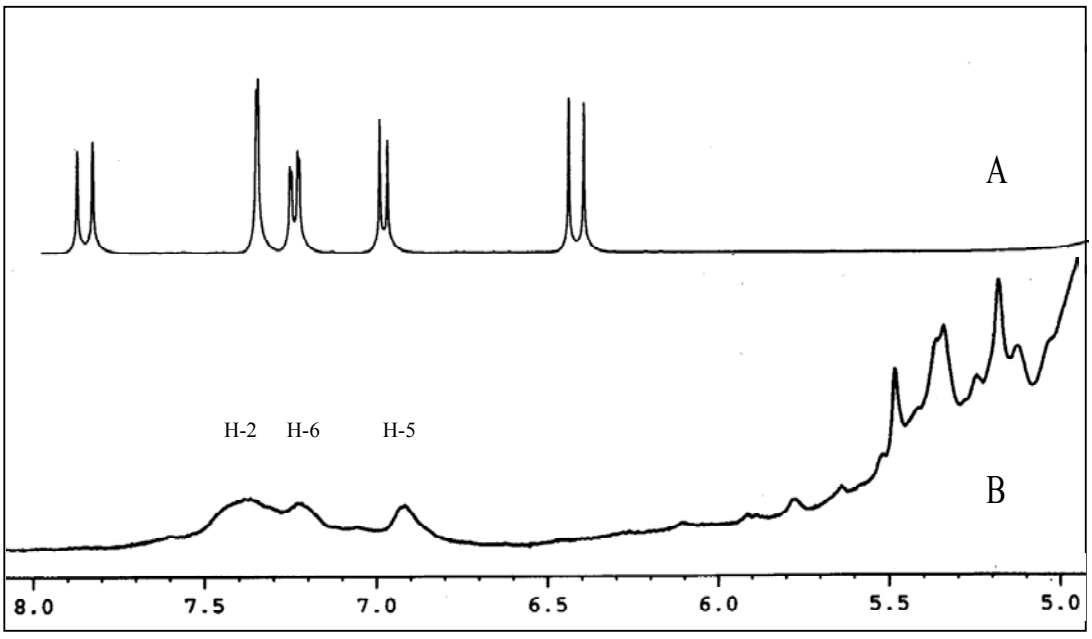

Figure 42. ^{1}H NMR spectrum of water soluble feraxan (rice MP2) (B) showing signals corresponding to ferulic acid (aligned with ferulic acid) (A) along with the anomeric signals of arabinose.

4.3.2.3. Infra red spectroscopy

The IR spectra obtained for feraxans from native and malted rice and ragi are shown in figure 43. IR spectra of carboxyl reduced feraxans were similar to the spectra obtained for unreduced feraxans, except that former spectra showed lower peak intensity at around 1728.6 cm^{-1} corresponding to the signal of >C=O group of uronic acid residue.

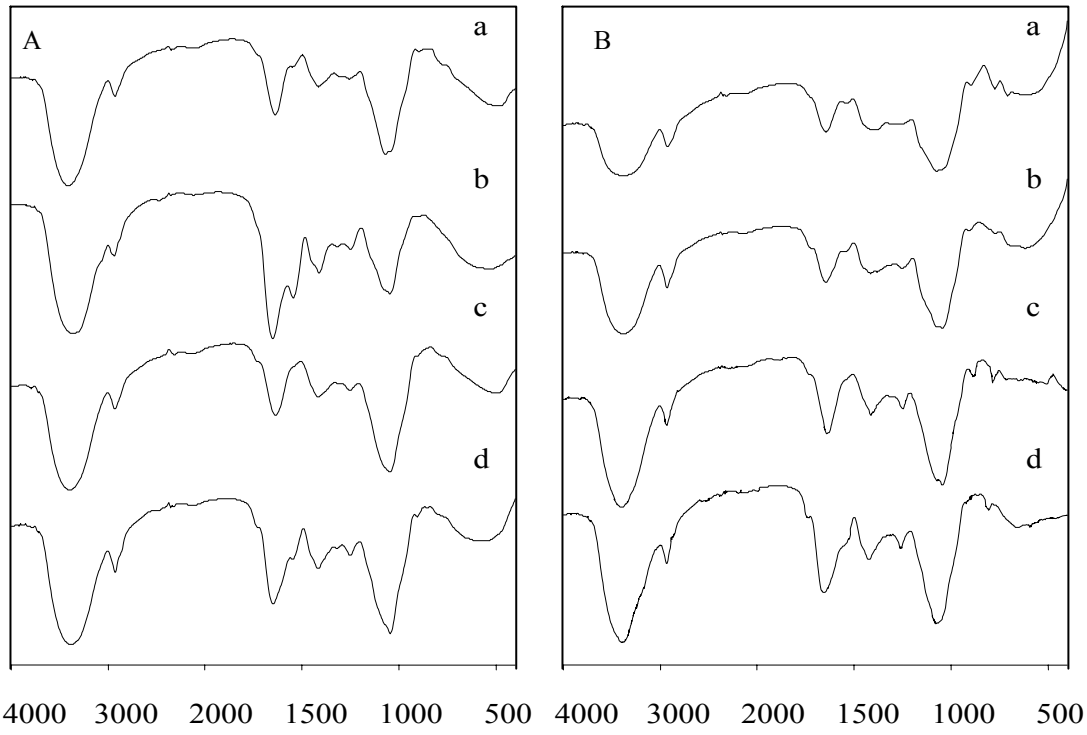

Figure 43. Infra red spectra of water soluble feraxans from native and malted rice (A) and ragi (B). NP1 (a), NP2 (b), MP1 (c) and MP2 (d).

The signals observed at around 1417.0 and 2930.0 cm^{-1} are due to $-CH_2$ and –CH stretching vibrations respectively, and the signal observed at around 3365.0 cm^{-1} is due to –OH stretching vibrations of polysaccharide, and water involved in hydrogen bonding (Fringant et al., 1995). The signal at around 1415.0 cm^{-1} is due to C-C, C-O and C-O-H bending vibrations. Signals at this region are known to show variations

(in the spectra) depending on the amount of substitution at O-2 and O-3 positions. The intensity of signals at this region decreases (coupled with the loss of peak multiplicity) with the increased substitution (Kacurakova et al., 1994).

Only a few reports are available on the IR spectral study of cereal arabinoxylans (Kacurakova et al., 1994; Kacurakova et al., 1998; Subba Rao and Muralikrishna, 2004). However, IR has been used to study supramolecular structure of xylans obtained from algae (Aspinall, 1983), gum exudates (Lelliott et al., 1978) and hard woods (Kalutskaya, 1988).

4.3.2.4. Ultra violet spectroscopy

The UV spectrum of purified feraxans showed characteristic pattern with maximum absorption at around 320 nm (figure 22). The spectra are very similar to the spectrum obtained for trans ferulic acid. Interestingly malt feraxans showed higher UV absorption compared to native (figure 14), indicating the higher amount of bound ferulic acid in malt feraxans. The spectra are very much similar to the spectra obtained for feruloyl oligosaccharides from wheat (Ralet et al., 1994).

4.3.2.5. Optical rotation

Optical rotation values of purified feraxans obtained from native and malted rice and ragi ranged at – 0.3 to – 7.4 (table 15). The negative value indicates that the polymer is primarily β linked. However, this value is low compared to the high negative values of other arabinoxylans (Saavendra et al., 1988; Subba Rao and Muralikrishna, 2004). This may be partly because the feraxans contain higher α linkages due to their high arabinose, galactose and uronic acid contents. On the contrary, primarily α linked polymers are shown to have high positive optical rotation values (Saavendra et al., 1988).

4.4. Possible structural models for water soluble feraxans

Based on the data obtained from various chemical and spectroscopic studies, a model is being put forward to depict the structural characteristics of water soluble arabinoxylans from native and malted rice and ragi (figure 44). Although the general structure of cereal arabinoxylans is known (Izydorczyk and Biliaderis, 1995), elucidation of fine structure of arabinoxylans still remains as a matter of interest and importance.

It is clear from the data that water soluble feraxans from rice and ragi are of low molecular weight compared to many other cereal arabinoxylans (Izydorczyk and Biliaderis, 1995) and have higher arabinose content (nearly equal to xylose). These two factors made them particularly water soluble. Feraxans also contained high amount of galactose and uronic acid, whose content is slightly higher in malts. They also contained high amount of bound ferulic acid, which is several folds higher in native compared to malt feraxans.

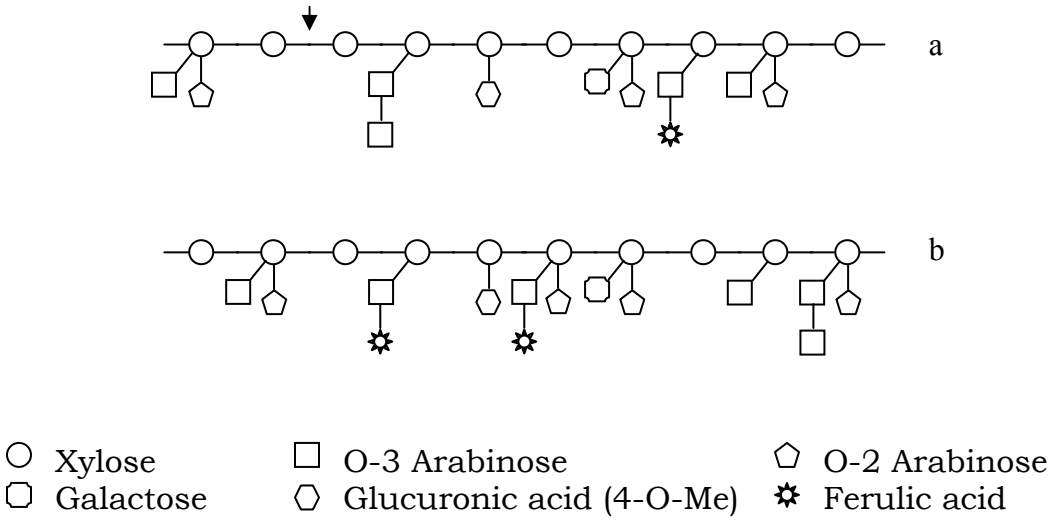

Figure 44. Possible structural models for feraxans obtained from native (a) and malted (b) rice and ragi. Native feraxan is less branched and has easy access point for xylanase (arrow).

The backbone of the feraxans is made up of 1→4 linked β-D-xylose residues to which α-L-arabinose residues are linked at O-2 and/or O-3 position. The amount of un-substituted xylose residues (30 – 40%) is nearly equal in both or slightly more in malt feraxans. However, xylose residues in native feraxans are more O-3 substituted (~ 40%) and less di-substituted (~ 20 %). Malt feraxans have higher di-substitution compared to mono-substitution. The arabinose residues (~ 20%) are also present in short side branches (either O-3 or O-5 linked to arabinose). Overall, around 40% of sugar residues have substitution as indicated by periodate oxidation data. Due to their lower branching and di-substitution, native feraxans might contain 2 or more contiguous un-substituted xylose residues. This served as the easy access point for the cleavage of native feraxans by xylanase. On the other hand, although malt feraxans are of low molecular weight, their further degradation might require synergistic action of xylanolytic enzymes.

It is interesting to note that malt feraxans contained very high amount of ferulic acid. On the contrary, ferulic acid content of 0.2 molar ammonium carbonate eluted fractions for both native rice and ragi contained very high amount of ferulic acid, whereas their malt counterparts had low ferulic acid. It might be speculated from the above observations that during malting there is a degradation of highly feruloylated high molecular weight arabinoxylans (which might be otherwise insoluble and water un-extractable due to molecular complexity). This lead to the formation of water soluble, highly feruloylated small molecular weight arabinoxylans (feraxans) during malting (figure 45).

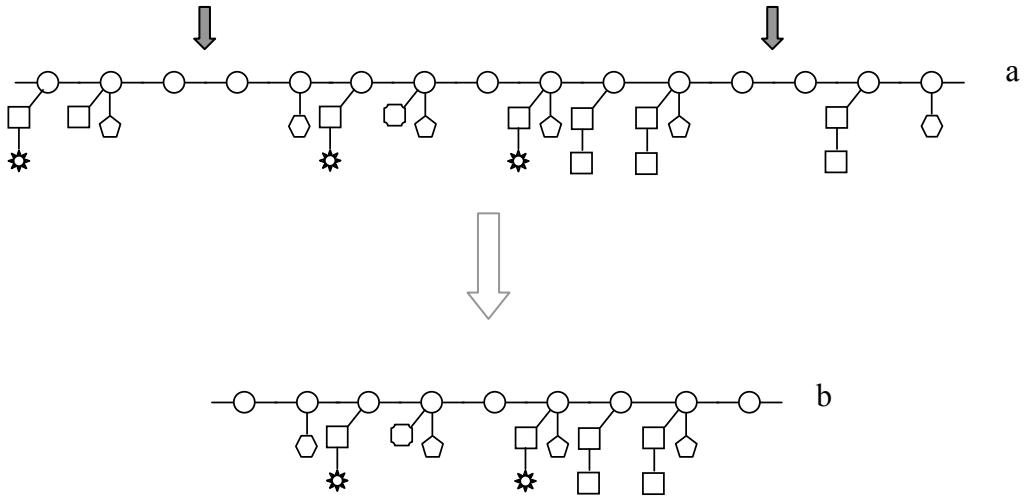

Figure 45. Partial biodegradation of high molecular weight feruloyl arabinoxylan [having easy access points for xylanase (small arrows)] leading to highly feruloylated low molecular weight arabinoxylan with higher Ara/Xyl ratio.

It may be noted that as the xylanase preferentially cleaved the high molecular weight feraxans in the un-substituted or mono/low substituted regions, the resultant small molecular feraxans contained higher arabinose content. It also leads to overall increase in the level of substitution and/or di-substitution. With their high substitution levels, rice and ragi water soluble feraxans structurally resembled rye (Bengtsson et al., 1992) and maize (Saulnier et al., 1995) arabinoxylans than wheat arabinoxylans (Izydorczyk and Biliaderis, 1995).

Although rice and ragi belong to two separate clades, which diversified about 66 million years ago, the structural characteristics of water soluble feraxans from the grains of these two grasses are relatively similar. Also, similar changes upon germination/malting are observed to have occurred in the water soluble feraxans of these two grains.

5.1. Introduction

Of late non-starch polysaccharides, feraxans in particular, are considered essential in food and nutrition as they are observed to have considerable functionality. The water soluble non-starch polysaccharides are known to have many beneficial roles as they 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, they are considered as functional food ingredients (Charalampopoulos et al., 2002). Although functional properties arise due to their distinct physical/structural features, the relation between structure and function is only partly understood. A distinction can be made between water soluble and water insoluble feraxans. While water insoluble feraxans are of limited interest, water soluble feraxans are given lot of attention as they exert considerable functional effects (Lopez et al., 1999). Moreover, functionalities of different polysaccharides/fractions isolated from different sources are studied to obtain the best results and relate them to the physicochemical/structural features of the polysaccharides. There is no report on the functionalities of water soluble feraxans from rice and ragi. Hence their functional characteristics and possible implications are investigated, and related in part to their structural features. Differences that arise in them from the malting are also considered.

5.2. Antioxidant activity

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 sperm and a good protective agent against UV radiation – induced skin damage. Ferulic acid exhibits very strong antioxidant, free radical scavenging and anti-inflammatory activities (Castelluccio et al., 1995; Shahidi et al., 1992). It is known to have anti-tumorogenic and anti-cancerogenic effect, and also considered

as a potential chemo-preventive agent for colorectal cancer (Kawabata et al., 2000; Mori et al., 1999).

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 amounts of antioxidants 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 might be too low to act as a chemo-preventive agent. Although free ferulic acid (Subba Rao and Muralikrishna, 2002) and feruloyl oligosaccharides (Ohta et al., 1994; Ohta et al., 1997) are known to exhibit antioxidant activity *in vitro*, it is not shown if feruloyl polysaccharides as such exhibit any antioxidant activity. In case, they may be the better candidates as chemopreventive agents.

In the present study possible antioxidant activity of feraxans, a ferulic acid reservoir and a parent molecule to feruloyl oligosaccharides is investigated.

5.2.1. Antioxidant activity of NSP

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 is expressed in IC_{50}, of water soluble NSP is given in table 21. 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 21). Activity pattern could roughly be correlated with the bound ferulic acid content of NSP (table 9). 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 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 22. 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 the high ferulic acid content, 0.2 molar AC eluted fractions from native rice and ragi showed 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 due to their bound ferulic acid content.

Table 21. Antioxidant activity (IC_{50}, as determined by emulsion assay) of water soluble NSP from rice and ragi.

		Activity, IC_{50} (mg)	Expected* activity, IC_{50} (mg)
Rice	N	1.14	54.8
	M	1.24	56.9
Ragi	N	0.92	53.0
	M	1.05	55.7

IC_{50} (mg), the concentration of polysaccharides at which 50% inhibition of β-carotene oxidation is attained.
* the amount of polysaccharides containing ferulic acid equivalent to IC_{50} (mg) of free ferulic acid.

Table 22. Antioxidant activity (IC_{50}, as determined by emulsion assay) of water soluble NSP fractions (DEAE-cellulose fractionation) from rice and ragi.

			Activity, IC_{50} (mg)	Expected activity, IC_{50} (mg)
Rice	Water eluted fraction	N	5.6	-
		M	6.3	-
	0.2 molar AC eluted fraction	N	0.47	23.7
		M	1.8	334.5
Ragi	Water eluted fraction	N	4.7	-
		M	4.8	-
	0.2 molar AC eluted fraction	N	0.35	17.1
		M	1.5	147.8

5.2.2. 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 are 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 23. Soluble feraxans are found to be very strong antioxidants, which could very well be explained on the basis of their molecular characteristics. Rice malt feraxans exhibited higher activity followed by ragi malt, rice native and ragi native

feraxans, the order could roughly be correlated with the amount of bound ferulic acid they contain. Having less ferulic acid, rice native feraxans exhibited stronger activity than ragi native feraxans. In case of emulsion assay, activity of rice native feraxans is even higher than the ragi malt feraxans.

Both in DPPH* and FRAP assays, feraxans exhibited several folds higher activity (table 23) 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) feraxan. Malt feraxans showed almost equal activity fold increase (5 to 6 folds). However, while same pattern could be observed in emulsion assay, fold increase was almost 50 (for native feraxans) and 25 (for malt feraxans) times higher compared to other two assays.

Table 23. Antioxidant activity of water soluble feraxans (0.1 molar AC eluted fractions) 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)[+]	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	

[+] values in parentheses – ferulic acid equivalent of polysaccharides in µg
Fold increase is the ration of IC_{50} or EC_1 of ferulic acid to ferulic acid equivalent of polysaccharides.

5.2.3. Antioxidant activity of feraxans – role of saccharides

Although ferulic acid is known to be a strong antioxidant (Kikuzaki, 2002; Nenadis et al., 2003; Shahidi et al., 1992) and free and bound (up on alkaline hydrolysis) ferulic acid extracted from cereals are 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 the free ferulic acid (Ohta et al., 1997). While antioxidant activity of phenolic acids can be related to structural features such as position of hydroxyl groups and other side groups (Cuvelier et al., 1992; Nenadis et al., 2003; Shahidi et al., 1992; Subba Rao and Muralikrishna, 2002), it is believed that esterification of ferulic acid results in increasing activity and it can 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; Ohta et al., 1997). While presence of ferulic acid is important for the activity, glycosyl group by itself showed no activity.

The present study showed that the feruloyl arabinoxylans exhibited 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 23) (Xue et al., 1998; Xue et al., 2001). While the increase in the activity might be small in low molecular weight esters (Kikuzaki et al., 2002; Ohta et al., 1994; Ohta et al., 1997), it can be very high (several folds) in case of feraxans having very high molecular weight. For example, among the feraxans tested, although rice native feraxans contained less ferulic acid than ragi native feraxans, its higher molecular weight (NP1, 231.5 kDa and NP2, 24.5 kDa) gave stronger activity compared to ragi native (in all

three assays) and stronger still, compared to ragi malt (in emulsion assay) (table 23). In general, higher activity fold increase of native feraxans (especially rice) compared to malt is due to their larger molecular weight.

Among water soluble NSP, the high antioxidant activity-pattern that could not be correlated well with the bound ferulic acid content, might be due to the different average molecular weight of feruloyl arabinoxylans. Moreover, it is presumed 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, 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, this hypothesis requires further validation.

5.2.4. Antioxidant activity of feraxans – role of uronic acid

Feruloyl arabinoxylans are negatively charged molecules with particularly high amount of uronic acid. This prompted to speculate the role of uronic acid in antioxidant activity of feraxans. The antioxidant activity of glucuronic, galacturonic and polygalacturonic acid is evaluated by all three above-mentioned methods. Results (table 23) showed that uronic acid by itself exhibits very strong antioxidant activity *in vitro*. Moreover, galacturonic acid, with different –OH group orientation, exhibited stronger activity than glucuronic acid. And consistent with the earlier explanation, being a polymer of galacturonic acid, polygalacturonic acid is a much stronger antioxidant. Therefore, the presence of uronic acid by itself (Xue et al., 1998; Xue et al., 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 it is supposed that the 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, it is shown 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 – 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 (figure 46). While 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 any 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 %).

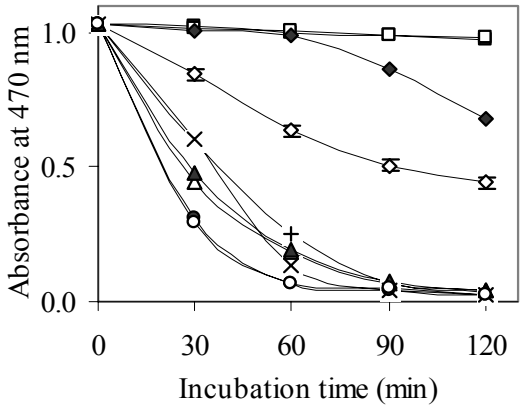

Figure 46. Antioxidant activity (as determined by emulsion assay) of known antioxidants and neutral sugar/polysaccharides. BHA (■), BHT (□), gallic acid (♦), ethyl ferulate (◊), glucose (+), soluble starch (×), laminarin (Δ), xylan (▲), methanol (●), water (○).

Further, a number of compounds having –COOH group such as formic, acetic, propionic, butyric, succinic and citric acids are screened by all three above-mentioned assays to see any activity exerted by them. These compounds gave very low and inconsistent activity with all three above-mentioned assays, indicating that the presence of >C=O group in open chain (like acetic and 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, it is presumed 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 contain neither ferulic acid nor uronic acid.

In summary, it is shown that a widely consumed non-starch polysaccharide, i. e., water soluble feraxans from cereals can exhibit very strong antioxidant activity, which can be 5000 times higher than the activity exerted by sulfated polysaccharides. Further, apart from phenolic acids, presence of sugars with >C=O (uronyl) 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), it is 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; Ohta et al., 1997). Presence of good amount of antioxidants like feraxans, in colon, might be essential for scavenging cancer causing amines and nitroso-compounds formed due to protein fermentation. Moreover, as synthetic antioxidants like BHA and BHT are suspected carcinogens, ferulic acid and feraxans can be used as natural antioxidants by the food industry. Consumption of naturally occurring charged polysaccharides like water soluble feraxans may 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.

5.3. Rheological properties

Many common diseases in western countries are thought to be due to a deficiency in DF, like water soluble feraxans. A daily intake of approximately 30 g is encouraged to promote health benefits associated with fibre. Because of the increased nutritional awareness, the food industry is facing the challenge of developing new food products with special health enhancing characteristics (Charalampopoulos et al., 2002). To meet this challenge, it must identify new sources of neutraceuticals and other natural and nutritional materials with the desirable functional characteristics (Izydorczyk et al., 2001). In view of the therapeutic potential of DF, more fibre incorporated food products are being developed. However, consumer acceptability of these functional foods depends not only on the nutrition, but also on the functional and sensory quality.

Being major NSP, water soluble feraxans stimulated considerable interest due to their water absorption, viscosity enhancing, and gelling properties and their impact on the rheological behavior of dough as well as the loaf volume and texture of bakery products (Meuser and Suckow, 1986). Despite the large amount of information available on the structural, nutritional and physiological properties of fibre, very little information is available on the functional effects of various fibre types (Özboy and Köksel, 1997). Incorporation of NSP is shown to have an impact on dough rheology and on bread quality parameters such as loaf volume, crumb texture and staling characteristics of the bread (Biliaderis et al., 1995). Water insoluble pentosans are shown to have an overall negative impact on product characteristics (Abdul-Hamid and Luan, 2000; Kulp and Bechtel, 1963), whereas their soluble counterparts have

a beneficial impact (Delcour et al., 1991; Meuser and Suckow, 1986). Functional properties, at least in part, are now related to the structural features of NSP. A great deal of uncertainty, however, remains as to the exact functional role and contribution of NSP from different sources to overall product characteristics; several research reports in this area are contradictory (Cawley, 1964; Courtin and Delcour, 2002; Jelaca and Hlynka, 1972; Kim and D'Appolonia, 1977).

Rice and ragi, a major cereal and millet respectively, are widely used as staple food. However, functional properties of their NSP have not been explored. Better knowledge on the functionality of these fibre components might lead to an increased use in cereal-based products. Thus functional characteristics of water soluble NSP/feraxans from rice and ragi with respect to dough properties and baking quality is studied. Consequence of malting, which is largely considered to be nutritionally beneficial, has also been addressed in view of NSP functionality.

5.3.1. Viscosity

The water soluble NSP showed low viscosity except for ragi malt (figure 47). The viscosity increased upon malting, despite the partial degradation of long chain NSP. This might be due to the increase in arabinose content, especially in ragi malt. Increased arabinose substitution is known to stiffen the xylan backbone due to a rigid rod like conformation of the polymer, thus increasing the viscosity (Andrawartha et al., 1979). Malting is shown to bring about changes in the viscosity of several types of NSP (Subba Rao and Muralikrishna, 2004).

The viscosity of water soluble NSP evidently increased with increase in concentration (0.2 to 1.0%) and decreased with temperature (20 to 80°C). This is due to the greater chain interactions and increased thermal mobility of polysaccharide molecules respectively (Whistler, 1973).

The viscosity is maximum at pH 6 to 7, which is perhaps due to the repulsive effects of the negatively charged uronyl group of arabinoxylans, increasing its water binding capacity, typical of acidic polysaccharides, gums and mucilages (Muralikrishna et al., 1987). Viscosity decreased with decreasing pH, however, at basic pH, it showed different pattern at different conditions (figure 47c). In carbonate buffer (0.05 molar) it decreased, but in glycine NaOH buffer (0.05 molar) viscosity remained equal to the neutral pH. This is probably due to the presence of hydrophobic feruloyl moiety whose hydrolysis by alkali increased the polysaccharide – water interaction.

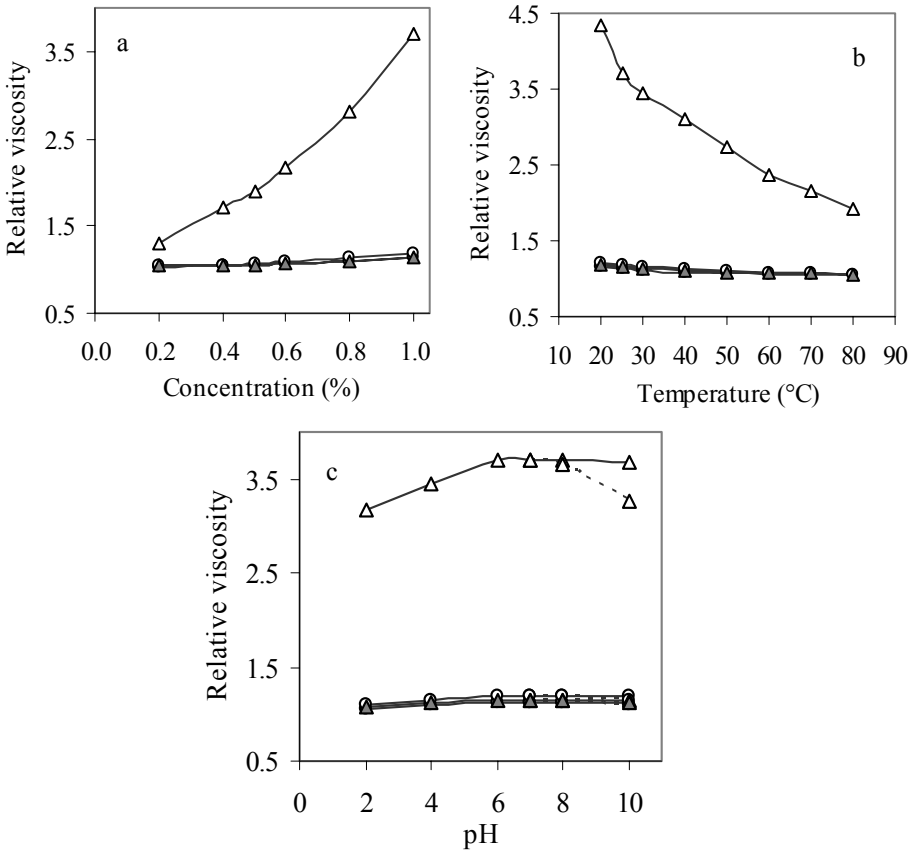

Figure 47. Effect of concentration (a), temperature (b) and pH (dotted line – carbonate buffer) (c) on viscosity (η_r) of water soluble NSP from native (solid symbol) and malted (open symbol) rice (circle) and ragi (triangle).

It is known that pentosans impart high viscosity in the aqueous solution, which is governed not only by their structure-type, but also by conformation of the chain and specific arrangement of substituent residues along the backbone (Izydorczyk and Biliaderis, 1995).

Water soluble feraxans too showed low viscosity and rheological properties (figure 48), similar to the one observed for water soluble NSP (figure 47).

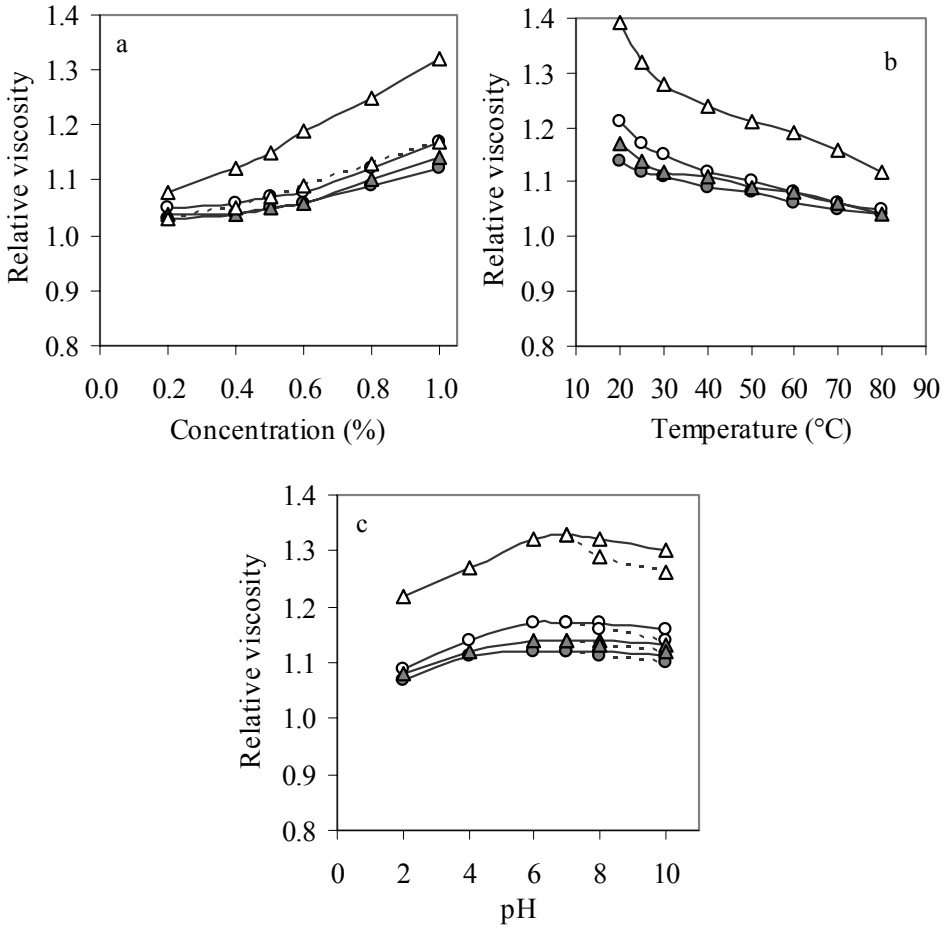

Figure 48. Effect of concentration (a) (dotted line – water fraction), temperature (b) and pH (dotted line – carbonate buffer) (c) on viscosity (η_r) of water soluble feraxans from native (solid symbol) and malted (open symbol) rice (circle) and ragi (triangle).

Contrary to the ragi malt NSP, ragi malt feraxan has low viscosity. Moreover, ragi malt water fraction too exhibited very low viscosity (figure 48a). The higher viscosity of ragi malt NSP is probably imparted by 0.1 and 0.2 molar NaOH fractions as these two fractions are observed to have very high viscosity. Due to their very low yield they are not studied further.

As the water soluble NSP/feraxans from rice and ragi showed low viscosity, they might be ideally used in fibre deprived health drinks.

5.3.2. Oxidative gelation

Hydrogen peroxide/peroxidase mediated cross linking of WEP, especially arabinoxylans, has been investigated for over 30 years and this cross linking ability of polysaccharides is attributed to the associated ferulic acid moiety (Schooneveld-Bergman et al., 1999). Although water soluble NSP from rice and ragi contained substantial amount of ferulic acid (Rice: N – 510.6 μg/g, M – 492.5 μg/g; Ragi: N – 528.0 μg/g, M – 503.1 μg/g) they showed little gelling ability even at 1% concentration (figure 49a). Water soluble feraxans too showed no gelling ability (figure 49b), despite having good amount of ferulic acid (Rice: N – 119.3 μg/g, M – 1404.3 μg/g; Ragi: N – 146.6 μg/g, M – 1044.6 μg/g). Similar observations were made by others (Subba Rao et al., 2004; Vinkx et al., 1991). This is probably due to the relatively low molecular weight of rice and ragi water soluble NSP compared to rye, which was shown to possess gelling ability. Similar observations were made, wherein inability of wheat NSP having much higher ferulic acid content to gel compared to rye NSP, was related to their low molecular weight (Vinkx et al., 1991).

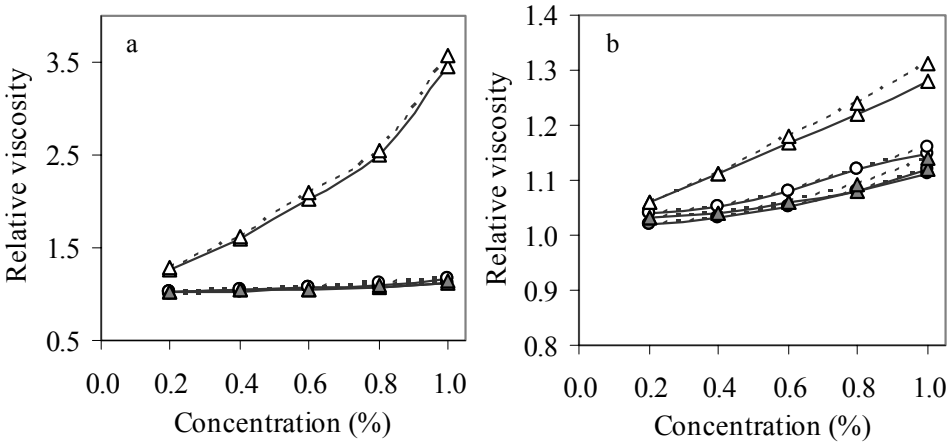

Figure 49. Gelling ability (dotted line – gelled) of water soluble NSP (a) and feraxans (b) from native (solid symbol) and malted (open symbol) rice (circle) and ragi (triangle).

5.3.3. Foam stabilization

Many polysaccharide solutions are known to stabilize the protein foams against thermal disruption by virtue of their high viscosity and ability to interact with the proteins absorbed to the foam cells (Sarker et al., 1998; Susheelamma and Rao, 1979). Water soluble NSP from rice and ragi showed good foam stabilization activity, comparable with highly viscous gums (table 24). Although it is not strictly linear, activity increased with concentration (0.2 to 1.0%). This is due to the increase in the viscosity. Due to their higher viscosity, NSP from malts, especially ragi showed higher foam stabilization activity. Similar results are reported for other polysaccharides (Izydorczyk and Biliaderis, 1992; Muralikrishna et al., 1987; Subba Rao et al., 2004). Since the formation of foam is usually impeded by increasing viscosity of the liquid medium, initial foam volume decreases with increased concentration/viscosity (Izydorczyk and Biliaderis, 1992). However, activity is proven during the

thermal treatment, wherein added NSP prevented the disruption of gas cells during thermal expansion of CO_2 (Izydorczyk et al., 1991).

Table 24. Effect of water soluble NSP obtained from native/malted rice and ragi on protein foam.

		Additive concen. (%)	Foam volume (ml)			
			$NaHCO_3$	Citric acid	10′ at 25°C	3′ at 90°C
Control		-	4.4 ± 0.6	5.6 ± 0.6	4.6 ± 0.4	1.2 ± 0.4
Acacia gum		0.2	1.8 ± 0.4	4.4 ± 0.4	3.8 ± 0.4	5.6 ± 0.6
Guar gum		0.2	1.0 ± 0.4	3.6 ± 0.6	3.2 ± 0.2	6.8 ± 0.4
Xanthan gum		0.2	2.2 ± 0.4	4.0 ± 0.4	3.8 ± 0.4	6.6 ± 0.4
Rice NSP	N	0.2	2.6 ± 0.4	4.8 ± 0.2	3.0 ± 0.4	2.6 ± 0.4
		0.4	2.6 ± 0.2	4.8 ± 0.2	3.2 ± 0.4	2.8 ± 0.4
		0.6	2.4 ± 0.2	4.4 ± 0.4	3.0 ± 0.4	3.0 ± 0.2
		0.8	2.2 ± 0.2	4.6 ± 0.2	3.0 ± 0.2	3.0 ± 0.2
		1.0	2.0 ± 0.2	4.2 ± 0.2	3.0 ± 0.4	3.0 ± 0.2
	M	0.2	2.4 ± 0.2	4.6 ± 0.2	3.0 ± 0.4	3.0 ± 0.4
		0.4	2.2 ± 0.4	4.2 ± 0.4	3.0 ± 0.2	3.2 ± 0.4
		0.6	2.4 ± 0.2	4.4 ± 0.2	3.0 ± 0.2	3.4 ± 0.4
		0.8	2.0 ± 0.2	3.8 ± 0.4	2.8 ± 0.2	3.4 ± 0.2
		1.0	1.8 ± 0.2	3.8 ± 0.2	3.0 ± 0.2	3.6 ± 0.2
Ragi NSP	N	0.2	2.4 ± 0.2	4.6 ± 0.4	3.0 ± 0.2	2.6 ± 0.4
		0.4	2.2 ± 0.4	4.6 ± 0.2	2.8 ± 0.4	3.0 ± 0.2
		0.6	2.2 ± 0.2	4.2 ± 0.2	2.6 ± 0.4	3.0 ± 0.2
		0.8	2.0 ± 0.4	4.0 ± 0.4	2.8 ± 0.4	3.2 ± 0.2
		1.0	1.8 ± 0.2	4.0 ± 0.2	2.8 ± 0.2	3.2 ± 0.2
	M	0.2	2.2 ± 0.4	4.0 ± 0.4	3.2 ± 0.2	3.8 ± 0.4
		0.4	2.2 ± 0.4	3.8 ± 0.2	3.0 ± 0.2	4.0 ± 0.2
		0.6	2.0 ± 0.2	3.4 ± 0.4	2.6 ± 0.2	4.2 ± 0.2
		0.8	1.8 ± 0.2	3.4 ± 0.4	2.6 ± 0.4	4.2 ± 0.4
		1.0	1.6 ± 0.2	3.2 ± 0.4	2.6 ± 0.4	4.6 ± 0.2

Similar to the water soluble NSP, water soluble feraxans too exhibited foam stabilization activity. However, as ragi malt feraxan has a lower viscosity compared to ragi malt NSP, it showed lower foam stabilization property (table 25).

Table 25. Effect of feraxans obtained from native/malted rice and ragi on protein foam.

		Additive concen. (%)	Foam volume (ml)			
			$NaHCO_3$	Citric acid	10′ at 25°C	3′ at 90°C
Control		-	4.4 ± 0.6	5.6 ± 0.6	4.6 ± 0.4	1.2 ± 0.4
Acacia gum		0.2	1.8 ± 0.4	4.4 ± 0.4	3.8 ± 0.4	5.6 ± 0.6
Guar gum		0.2	1.0 ± 0.4	3.6 ± 0.6	3.2 ± 0.2	6.8 ± 0.4
Xanthan gum		0.2	2.2 ± 0.4	4.0 ± 0.4	3.8 ± 0.4	6.6 ± 0.4
Rice feraxans	N	0.2	3.0 ± 0.2	4.8 ± 0.4	3.2 ± 0.4	2.4 ± 0.2
		0.4	2.6 ± 0.4	4.6 ± 0.4	3.2 ± 0.4	2.8 ± 0.2
		0.6	2.6 ± 0.4	4.6 ± 0.4	3.2 ± 0.2	2.6 ± 0.4
		0.8	2.2 ± 0.2	4.2 ± 0.4	3.0 ± 0.2	2.8 ± 0.2
		1.0	2.2 ± 0.2	4.4 ± 0.4	3.2 ± 0.2	2.8 ± 0.4
	M	0.2	2.4 ± 0.4	4.6 ± 0.4	3.4 ± 0.2	2.6 ± 0.2
		0.4	2.4 ± 0.2	4.4 ± 0.2	3.2 ± 0.2	2.6 ± 0.2
		0.6	2.2 ± 0.2	4.4 ± 0.4	3.2 ± 0.2	2.8 ± 0.2
		0.8	2.2 ± 0.2	4.2 ± 0.2	3.2 ± 0.4	2.8 ± 0.2
		1.0	2.0 ± 0.2	4.0 ± 0.2	3.0 ± 0.4	3.2 ± 0.4
Ragi feraxans	N	0.2	2.8 ± 0.2	4.6 ± 0.4	3.2 ± 0.4	2.6 ± 0.2
		0.4	2.4 ± 0.4	4.4 ± 0.4	3.2 ± 0.2	2.8 ± 0.4
		0.6	2.4 ± 0.2	4.2 ± 0.4	3.0 ± 0.4	2.6 ± 0.4
		0.8	2.2 ± 0.4	4.2 ± 0.2	3.2 ± 0.2	2.8 ± 0.2
		1.0	2.0 ± 0.2	4.2 ± 0.2	3.0 ± 0.4	3.0 ± 0.2
	M	0.2	2.4 ± 0.2	4.4 ± 0.2	3.2 ± 0.4	3.0 ± 0.2
		0.4	2.4 ± 0.2	4.4 ± 0.2	3.0 ± 0.2	3.2 ± 0.4
		0.6	2.4 ± 0.2	4.0 ± 0.4	3.0 ± 0.2	3.0 ± 0.4
		0.8	2.0 ± 0.2	3.8 ± 0.2	3.0 ± 0.4	3.4 ± 0.2
		1.0	1.8 ± 0.4	3.6 ± 0.2	2.8 ± 0.4	3.8 ± 0.2

5.3.4. Effect on dough characteristics and baking quality

5.3.4.1. Farinograph characteristics

The changes in the water absorption of the wheat flour (maida, refined wheat flour) and dough characteristics upon the addition (0.25 and 0.50%) of rice and ragi water soluble NSP are assessed using Brabender farinograph and the results are shown in table 26. The water absorption values slightly increased (0.4 to 1.3%) and the higher water absorption of the malt NSP might be due to their higher arabinose

content. In general, pentosans are known to absorb high amount of water and similar results are reported for other cereal NSP (Biliaderis et al., 1995; Jelaca and Hlynka, 1971; Subba Rao et al., 2004). However, these values are lower than those reported for pentosans obtained from wheat endosperm (Kulp, 1968). Water absorption capacity of NSP is shown to depend on the source of pentosans (Jelaca and Hlynka, 1971), which might be due to their fine physicochemical differences.

Dough development time (DDT) decreased by 0.2 to 1.1 min upon the addition of water soluble NSP and the effect is higher with malt samples, especially from rice. Addition of soluble pentosans is shown to marginally increase or decrease DDT depending upon their chemical nature (Biliaderis et al., 1995; Kim and D'Appolonia, 1977; Kulp and Bechtel, 1963; Pence et al., 1951; Subba Rao et al., 2004).

Table 26. Effect of water soluble NSP obtained from native/malted rice and ragi on farinograph characteristics.

		Additive concentration (%)	Water absorption (%)	Dough development time (min)	Dough stability (min)	Mixing tolerance index (FU)
Control		-	57.4	4.4	5.9	40
Rice	N	0.25	57.8	4.0	4.5	45
		0.50	58.0	3.8	4.7	47
	M	0.25	57.9	3.7	4.0	52
		0.50	58.2	3.3	3.7	60
Ragi	N	0.25	57.9	4.0	4.5	45
		0.50	58.1	4.2	4.6	47
	M	0.25	57.9	4.2	4.6	44
		0.50	58.7	4.0	4.4	57

FU – Farinograph Unit

Addition of water soluble NSP decreased the stability of dough and the effect is more pronounced with rice malt. Pentosan additions brought about changes in the dough stability (Jelaca and Hlynka, 1971; Kulp and Bechtel, 1963). The starch – gluten network is one of the major factors determining the stability of the dough and added pentosans are known to

interact with this system (Delcour et al., 1991). The pattern of increase in the mixing tolerance index (TI) is roughly similar to that of increase in water absorption.

Although prolonged mixing of the wheat flour with water soluble NSP from rice and ragi increased the TI of the dough, the changes may marginal and no adverse effect is observed.

5.3.4.2. Extensograph characteristics

Addition of water soluble NSP from rice and ragi is observed to have strengthened the wheat flour dough (table 27). The effect is better with native NSP. Although native NSP is less viscous, its higher molecular weight might allow a better interaction with starch – gluten complex. Addition of water insoluble NSP is shown to decrease dough extensibility (Kulp and Bechtel, 1963). However, addition of water soluble NSP increased both resistance to extension and extensibility, one similar to the effect observed with the dough improvers (Bloksma, 1971). Addition of WEP is known to interfere indirectly by competing for water and directly by cross-linking to bring about increased resistance of gluten against extension (Wang et al., 2002).

Table 27. Effect of water soluble NSP obtained from native/malted rice and ragi on extensograph characteristics.

		Additive concentration (%)	Energy (cm^2)	Maximum resistance to extension (BU)	Maximum extensibility (mm)	Maximum ratio number (BU/mm)
Control		-	70	361	138	2.6
Rice	N	0.25	99	520	146	3.6
		0.50	112	573	159	3.6
	M	0.25	88	446	150	3.0
		0.50	92	484	154	3.1
Ragi	N	0.25	93	481	142	3.4
		0.50	103	531	163	3.3
	M	0.25	87	476	139	3.4
		0.50	88	484	145	3.3

5.3.4.3. Micro-Visco-Amylograph studies

The results obtained by amylograph studies indicated a marginal decrease (0.1 to 1.2°C) in the gelatinization temperature (table 28). Other parameters, such as peak viscosity, hot and cold paste viscosity are increased and concomitant increase in break down and set back viscosity is observed with the addition of water soluble NSP. These results showed that the addition of water soluble NSP has a positive bearing in contrast to the water insoluble NSP, wherein decrease in these values was reported (Kulp and Bechtel, 1963).

Table 28. Effect of water soluble NSP obtained from native/malted rice and ragi on starch pasting characteristics by Brabender micro-visco-amylograph.

		Additive concentration (%)	Gelatinization temperature (°C)	Peak viscosity (BU)	Hot paste viscosity (BU)	Cold paste viscosity (BU)	Break down PV-HPV	Setback total CPV-HPV
Control		-	64.0	950	665	1199	285	534
Rice	N	0.25	62.8	1019	693	1276	326	573
		0.50	62.8	1039	703	1324	336	612
	M	0.25	62.8	1027	699	1301	328	591
		0.50	62.9	1160	728	1446	432	697
Ragi	N	0.25	63.7	974	690	1270	284	568
		0.50	63.9	995	704	1295	291	574
	M	0.25	63.7	988	701	1266	287	550
		0.50	63.8	1019	700	1333	319	617

5.3.4.4. Bread quality studies

As the addition of water soluble NSP showed an overall positive bearing on dough properties, it was incorporated in similar concentration to see its effect on baking quality. Test baking showed that the weight of loaf is slightly increased (0.8 to 3.0 g) due to the higher moisture content – as a result of water retention property of added polysaccharides (Biliaderis et al., 1995). A significant increase in loaf volume and specific loaf volume is observed upon the addition of NSP (table 29). Similar

observations are made in the earlier study with the addition of low concentrations of arabinoxylans (Biliaderis et al., 1995). The expansion of dough and consequently bread volume upon addition of arabinoxylans is probably due to increased strength and elasticity of the gluten-starch composite network. However, ferulic acid residues are shown not to involve in the interactions leading to larger loaf volume as removal of these moieties from water soluble polysaccharides or the addition of an excess of ferulic acid in the baking formulation did not impair the starch-gluten system (Delcour et al., 1991). Similarly, crumb firmness value decreased significantly with the addition of NSP indicating improvement in the texture of breads. The improving effect is more pronounced with the malt samples, at 0.50% concentration. Water soluble arabinoxylans, due to their viscosity and interfacial activity, may add to the elasticity and strength of the films surrounding the gas cells, thereby resulting in an even bread crumb texture (Izydorczyk et al., 1991).

Table 29. Effect of water soluble NSP obtained from native/malted rice and ragi on bread characteristics.

		Additive concentration (%)	Weight (g)	Loaf volume (ml)*	Specific loaf volume (ml/g)*	Firmness (peak load, g)*
Control		-	136.2	480.0^{e}	3.52^{f}	843.8^{a}
Rice	N	0.25	137.9	487.5^{d}	3.54^{ef}	798.8^{b}
		0.50	139.6	497.5^{c}	3.56^{de}	742.5^{d}
	M	0.25	137.4	487.5^{d}	3.55^{ef}	778.8^{c}
		0.50	138.7	497.5^{c}	3.59^{cd}	722.5^{e}
Ragi	N	0.25	137.0	492.5^{cd}	3.59^{cd}	802.5^{b}
		0.50	139.2	510.0^{b}	3.66^{b}	711.3^{f}
	M	0.25	136.9	492.5^{cd}	3.60^{c}	807.5^{b}
		0.50	138.8	525.0^{a}	3.78^{a}	631.3^{g}

* means within a column, values bearing the same letter are not significantly different at 5 % level, as determined by the Duncan's multiple range test.

There were no noticeable changes in the sensory parameters such as crust and crumb color and crumb grain size, compared to the control.

In contrary to the water insoluble NSP, which are shown to have a negative effect on baking quality (Abdul-Hamid and Luan, 2000), addition of water soluble NSP form rice and ragi has a positive effect.

In summery, the results obtained in the present study indicated overall positive functional attributes of water soluble NSP from rice and ragi. They has a relatively lower viscosity but showed a good foam stabilization activity. Addition of water soluble NSP also imparted positive effect on properties of wheat dough similar to the studies reported earlier (Subba Rao et al., 2004), which is in contrary to the effect exerted by water insoluble NSP (Abdul-Hamid and Luan, 2000).

The positive effect on dough characteristics is reflected in the baking studies; wherein significant increase in the bread quality is attained with the addition of water soluble NSP from rice and ragi. Taken together, water soluble NSP from rice and ragi has a functional bearing and can be incorporated (as soluble dietary fibre) in various fibre deprived health foods and bakery products.

5.4. Prebiotic activity

The gastrointestinal tract of human adults contains a vast and complex consortium of more than 500 different species of bacteria that play a major role in colonic function and affect host homoeostasis (Guarner and Malagelada, 2003). They might even confer health benefits by helping to digest dietary complex carbohydrates and by maintaining the appropriate balance among the different types of gut bacteria (Kraehenbuhl and Corbett, 2004). They also produce vitamins, short-chain fatty acids (SCFA) and other nutrients for their hosts, providing up to 15% of total caloric intake.

The gut microflora is affected by many factors such as age, drug therapy, diet, host physiology, peristalsis, local immunity and *in situ* bacterial metabolism, of which diet is probably the most significant factor

determining the gut flora since foodstuffs provide the main nutrient sources for colonic bacteria (Berg, 1996).

There is currently much interest in the concept of actively managing the colonic microflora with the aim of improving host health. This is attempted with the consumption of probiotics (live microbial food supplements) and recently with prebiotics. A prebiotic is defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). The usual target species for such a dietary intervention are bifidobacteria and lactobacilli. However, the prebiotic substrate may also be fermentable to a lesser degree by potential pathogenic bacteria. It is, therefore, desirable that the prebiotic is fermented by beneficial bacteria with a very high degree of selectivity.

Many studies have now confirmed that the prebiotics are a valid approach to the dietary manipulation of the colonic microflora (Bouhnik et al., 1997; Gibson et al., 1995; Kleesen et al., 1997). In addition to the desirable effect of increased bifidobacteria and lactobacilli by prebiotic substrates, SCFA produced as the end product of fermentation can be nutrients as well as growth signals for the intestinal epithelium, an example being butyrate with its pro-differentiation, anti-proliferation and anti-angiogenic effects on colonocytes (Mai and Morris, 2003).

Dietary fibre (DF) is a general term for different types of carbohydrates derived from plant cell walls that are not hydrolyzed by human digestive enzymes. Many of the health effects are believed to be related to the microbial fermentation of DF in the large intestine. The extent of fermentation depends on the nature of substrate. A distinction is made between insoluble and soluble DF. The metabolic effects of insoluble DF such as cellulose and a part of hemicellulose are of limited interest because of their low digestibility in most mono-gastric species. By contrast, soluble DF is generally broken-down by the large intestine

microflora, and leads to the much desirable physiological effects (Lopez et al., 1999).

Feruloyl arabinoxylans (feraxans) are the chief soluble DF components and are consumed with the cereal based food products. While ferulic acid is a potent antioxidant and prevents LDL oxidation, inhibits tumor promotion and protects against chronic diseases such as coronary heart disease and cancer (Bravo, 1998), prebiotic potential of feruloyl polysaccharides is not known. Only few reports mention the fermentability of crude cereal non-starch polysaccharides and oligosaccharides (Cotta, 1993; Jaskari et al., 1998; Karppinen et al., 2000; Kontula et al., 1998; Korakli et al., 2002; Rycroft et al., 2001). Monitoring fermentation *in vivo* is very difficult. The digestion of DF can be measured from faeces, but SCFA are readily absorbed in the colon and the amount found in faeces does not describe the true situation. Knowledge of the extent of fermentation of DF and of the SCFA production *in vitro* is therefore of great importance.

The present study is to examine the *in vitro* fermentability pattern of well characterized water soluble feraxans from native and malted rice and ragi by bifidobacteria and lactic acid bacteria and to establish the prebiotic property of these polysaccharides.

5.4.1. Fermentation of Individual sugars

Individual sugars were added at 1% level to the bromocresol purple (BCP) broth medium and tested for its fermentation. Of the 18 lactic acid bacterial cultures tested, only 3 (*Bifidobacterium adolesentis* NDRI 236, *Lactobacillus brevis* NDRI 253 and *Pediococcus pentosaceus* NDRI 035) are able to utilize both arabinose and xylose, constituent sugars of feraxans. Additional 3 strains (*Lb. plantarum* NCIM 2084, *Lb. plantarum* CFR 2164 and *Lb. salivarius* CRF 2158) utilized arabinose. Rests of the cultures are unable to utilize arabinose and xylose. Lactic acid bacteria are known to have limited ability to utilize pentoses, especially xylose

(Chaillou et al., 1998; Erlandson et al., 2001). However, all 18 cultures utilized galactose and especially lactose, which indicated the presence of disaccharidases in these cultures. The arabinose/xylose utilizing lactic acid bacterial cultures are grown in MRS broth medium supplemented with individual sugars. All cultures utilized arabinose and grew to near maximum OD compared to the ones grown in glucose (table 30). However, xylose utilizing cultures grew slowly and reached only intermediate OD. Bifidobacterial species are shown to grow better on xylooligosaccharides than on xylose, suggesting a lack of specific transport system for the monomer (Palframan et al., 2003). Similarly, *Ped. pentosaceus* is shown to utilize xylose, but growth occurred only at a very slow rate (Dobrogosz and DeMoss, 1963). Culture broth pH decreased to near 4 after 48 h fermentation in glucose and arabinose, whereas it remained near 5 in case of xylose, indicating its slower utilization (table 30). There is an increase in dry cell mass compared to blank (0.2 – 0.3 mg/ml culture broth) and the pattern is similar to their absorbance profile, higher the absorbance more the dry cell mass. Xylose utilizing cultures reached just over double the dry cell mass compared to control.

Table 30. Growth characteristics of lactic acid bacteria on different carbon sources.

	Glc			Ara			Xyl		
	OD	pH	Cell mass*	OD	pH	Cell mass	OD	pH	Cell mass
B. adolesentis NDRI 236	1.35	4.3	1.4	0.85	4.6	0.8	0.89	4.8	0.8
Lb. brevis NDRI 253	1.86	4.1	1.9	1.13	4.6	1.0	0.57	5.5	0.6
Lb. plantarum NCIM 2084	2.21	3.9	2.7	2.00	4.2	1.7	0.46	6.0	0.3
Lb. plantarum CFR 2164	2.14	4.0	2.4	1.97	4.1	2.0	0.28	6.1	0.3
Lb. salivarius CFR 2158	2.17	4.2	2.3	1.61	4.2	1.5	0.26	6.2	0.3
Ped. pentosaceus NDRI 035	1.91	3.3	2.0	1.42	3.8	1.3	0.89	5.0	0.7
Cecal mixed flora	1.95	3.6	1.8	1.87	4.1	1.6	1.51	5.0	1.5
Fecal mixed flora	1.90	3.7	1.8	1.83	4.2	1.5	1.4	4.7	1.3

* Cell mass in mg/ml culture broth.

The amount of total SCFA produced varied for individual strains and sugars, however, arabinose is observed to yield higher total SCFA (table 31). Acetate is the chief SCFA produced and its amount account for over 90%.

Table 31. SCFA production (acetate/propionate/butyrate - μmol/ml culture broth) by lactic acid bacteria.

	Glc	Ara	Xyl
B. adolesentis NDRI 236	3.75/0.05/0.02	8.19/0.05/0.02	5.80/0.10/0.03
Lb. plantarum NCIM 2084	2.41/0.02/0.00	2.95/0.07/0.00	nd
Lb. plantarum CFR 2164	2.13/0.00/0.07	1.83/0.05/0.00	nd
Lb. salivarius CFR 2158	2.91/0.07/0.03	4.14/0.00/0.00	nd
Ped. pentosaceus NDRI 035	8.55/0.00/0.00	10.26/0.03/0.02	3.18/0.25/0.02
Cecal mixed flora	3.87/0.04/0.02	11.36/0.12/0.00	2.47/0.28/0.07
Fecal mixed flora	5.08/0.09/0.01	7.46/0.44/0.05	3.50/0.62/0.11

nd – not detected; SCFA from *Lb. brevis* NDRI 253 was not determined as it did not grow in the acetate free culture broth.

Rat cecal and faecal mixed flora (contains gram positive rods similar to lactobacilli/bifidobacteria) utilized both arabinose and xylose with high OD, dry cell mass and total SCFA. Acetate is the chief SCFA produced, similar to the one reported for human faecal bacteria (Karppinen et al., 2000). In the absence of carbon source, mixed flora readily utilized proteins, leading to a blank OD of near 0.8 and similar increase in dry cell mass (0.6 mg/ml) is observed.

5.4.2. Fermentation of feraxans

Xylose utilizing cultures namely, *B. adolesentis* NDRI 236, *Lb. brevis* NDRI 253 and *Ped. pentosaceus* NDRI 035 turned the BCP broth color into deep yellow indicating feraxan fermentation. However, arabinose utilizing cultures are unable to ferment feraxans. Similar profile is observed when these cultures are grown in MRS broth medium supplemented with feraxans (table 32). This is probably due to the lack

of feraxan degrading enzymes (feraxanases) in these cultures. Enzyme assays did not show any detectable feraxanases' activity. Although these cultures could utilize arabinose, its hydrolysis/mobilization from the feraxans required arabinofuranosidase activity. Such enzyme constrain is reported in earlier study (Cotta, 1993). Due to their complex nature, arabinoxylan fraction of NSP is shown to be resistance to fermentation by many lactic acid bacteria (Korakli et al., 2002).

On the contrary, all the three xylose utilizing cultures are able to degrade feraxans and grew to intermediate OD (table 32) compared to the ones grown in arabinose/glucose, pattern much similar to the one observed with xylose (table 30). Of the 3 cultures, *B. adolesentis* NDRI 236 grew to the maximum OD of 0.89 followed by *Ped. pentosaceus* NDRI 035 (0.66) and *Lb. brevis* NDRI 253 (0.61). Similar increase in the viable cell count is also observed.

Table 32. Growth characteristics of lactic acid bacteria on native and driselase/ragi malt extract hydrolyzed feraxans.

	Native			Driselase			Ragi malt extract		
	OD	pH	Cell mass*	OD	pH	Cell mass	OD	pH	Cell mass
B. adolesentis NDRI 236	0.89	5.4	0.8	1.28	5.0	1.2	1.25	5.0	1.2
Lb. brevis NDRI 253	0.61	5.5	0.7	1.15	5.3	1.3	1.25	5.2	1.4
Lb. plantarum NCIM 2084	0.25	6.2	0.3	1.36	5.1	1.5	1.48	4.9	1.7
Lb. plantarum CFR 2164	0.28	6.3	0.3	1.13	5.1	1.3	1.31	5.0	1.5
Lb. salivarius CFR 2158	0.32	6.3	0.3	1.40	5.0	1.6	1.51	5.1	1.6
Ped. pentosaceus NDRI 035	0.66	5.3	0.7	1.38	5.2	1.5	1.25	5.1	1.3
Cecal mixed flora	2.26	5.7	1.9	-	-	-	-	-	-
Fecal mixed flora	2.27	5.7	1.8	-	-	-	-	-	-

* Cell mass in mg/ml culture broth

Interestingly, 24 h old culture broths of these cultures showed feraxanases activities (table 33). The culture of *Ped. pentosaceus* NDRI 035 showed highest activity for both arabinofuranosidase (323.2 μU/ml) and xylopyranosidase (256.0 μU/ml) followed by *B. adolesentis* NDRI 2136 and *Lb. brevis* NDRI 253.

Table 33. Enzyme activities (μU/ml) in lactic acid bacterial culture broth.

	Xylanase	Xylosidase	Arabinosidase	α galactosidase	β galactosidase	Acetyl esterase
B. adolesentis NDRI 236	nd	85.0	266.2	104.0	28.0	1532.0
Lb. brevis NDRI 253	nd	66.0	85.0	76.0	9.0	nd
Ped. pentosaceus NDRI 035	nd	256.0	323.2	180.0	142.0	nd
Cecal culture	43.5	294.1	836.0	3100.0	2700.0	4800.0
Fecal culture	27.4	203.6	798.0	3400.0	2600.0	3300.0

nd – not detected

Arabinofuranosidase has been characterized and a large number of genes/proteins appeared to be specialized for catabolism of a variety of plant oligosaccharides are reported in bifidobacteria (Schell et al., 2002; Shin et al., 2003). However, these cultures showed no detectable xylanase activity. This may be the probable reason why these cultures slowly utilized feraxans. In fact arabinofuranosidase and xylopyranosidase cannot degrade long chain xylan and they act best on arabino-xylo-oligosaccharides. Lactic acid bacteria are shown to poorly utilize arabinoxylan, but readily utilized arabinoxylan hydrolysates (Jaskari et al., 1998; Korakli et al., 2002).

This is reflected in the sugar composition of the polysaccharides (table 34). GLC analysis of the feraxans precipitated from 48 h culture broths showed that arabinose content decreased upon fermentation and the profile is similar to the arabinofuranosidase activity. The culture of *Ped. pentosaceus* NDRI 035 removed much of the arabinose residues present as side groups in the xylan backbone. However, the enzyme activities, xylopyranosidase in particular, are limited by the long chain xylan backbone. There is a slight decrease in the arabinose content of feraxans from other culture broths especially *Lb. salivarius* CFR 2158 (table 34). This may either be due to the low, undetected arabinofuranosidase activity or may due to the non-specific activity of

other hydrolases. In fact, *Lb. salivarius* CFR 2158 showed slightly higher growth in feraxans compared to the blank. Growth may also be due to the galactose, made available by galactosidase activity, which would hydrolyze small amount of galactose residues present as side chains in the feraxans. A similar profile of decrease in pH, increase in dry cell mass and production of SCFA are observed.

Table 34. Neutral sugar composition (%) of feraxans after 48 h fermentation by lactic acid bacteria.

	Ara	Xyl	Ara/Xyl
Blank	44.3	55.7	0.80
B. adolesentis NDRI 236	28.7	71.3	0.40
Lb. brevis NDRI 253	37.0	63.0	0.59
Lb. plantarum NCIM 2084	44.2	55.8	0.79
Lb. plantarum CFR 2164	43.9	56.1	0.78
Lb. salivarius CFR 2158	41.3	58.7	0.70
Ped. pentosaceus NDRI 035	24.4	75.6	0.32

Table 35. SCFA production (acetate/propionate/butyrate - μmol/ml culture broth) by lactic acid bacteria.

	Native	Driselase	Ragi malt extract
B. adolesentis NDRI 236	2.07/0.20/0.00	2.36/0.03/0.00	2.75/0.05/0.01
Lb. plantarum NCIM 2084	0.05/0.00/0.00	1.79/0.03/0.00	2.04/0.09/0.00
Lb. plantarum CFR 2164	0.07/0.00/0.00	1.96/0.00/0.00	1.87/0.03/0.03
Lb. salivarius CFR 2158	0.06/0.01/0.00	2.24/0.03/0.00	2.64/0.01/0.00
Ped. pentosaceus NDRI 035	3.71/0.02/0.02	4.45/0.18/0.04	4.78/0.09/0.02
Cecal mixed flora	3.41/6.80/1.45	-	-
Fecal mixed flora	1.28/3.73/1.70	-	-

SCFA from *Lb. brevis* NDRI 253 was not determined as it did not grow in the acetate free culture broth.

It is observed that feraxans from malts supported a slightly better growth of these bacteria, indicated by slight increase in OD (0.1 – 0.2). This might be due to the lower molecular weight of malt feraxans which could better be accessed by arabinofuranosidase and xylopyranosidase.

Malting of rice and ragi caused degradation of large molecular feraxans due to the induction of xylanolytic enzymes. In particular, high activity of xylanase (rice 0.78 U and ragi 0.98 U per gram flour) is detected in malt flour. Along with the induction of several hydrolytic enzymes, malting would be beneficial as it results in the partial degradation of feraxans leading to a better substrate for prebiotic bacteria.

As there is no drastic difference in the fermentability of native and malt feraxans and both rice and ragi feraxans showed similar characteristics, further fermentation studies are carried out with ragi (native) feraxans.

Interestingly, rat cecal and faecal mixed flora readily utilized feraxans and grew to the highest OD. This is evident by their high feraxanases activity. In particular, xylanase activity (27.4 – 43.5 μU/ml) could be detected in these cultures. Xylanase acts on xylan backbone leading to xylooligosaccharides which in turn could be easily degraded by arabinofuranosidase and xylopyranosidase. Very high amounts of α and β galactosidases and acetyl esterase activity is also detected.

Concomitant decrease in the pH and increase in the dry cell mass is observed. They also produced high amounts of SCFA. Propionate is the chief SCFA produced (55.6 – 58.3%) by mixed flora and butyrate is present in good amounts (12.4 – 25.3%). Fibers, especially arabinoxylans are known to be butyrogenic and lead to the high amount of propionate and butyrate in contrary to acetate, especially by mixed flora. Butyrate producing bacteria in mixed flora are said to utilize acetate leading to the production of high amounts of butyrate (Duncan et al., 2004).

It is worthwhile to note that mixed flora first utilized proteins present in the culture broth and hence its pH increased to above 7 at around 24 h incubation. However, later cells utilized feraxans, bringing down the pH with the production of SCFA. This might be due to the non-preference to feraxans or initial constrains due to the lack of enzymes or slower hydrolysis/degradation. Complete degradation of feraxans may

require a consortium of different microbial species, as in mixed flora, acting synergistically.

It is observed that arabinofuranosidase is induced by the presence of arabinose, xylose or feraxans (figure 50). Glucose being a readily utilizable carbon source did not induce this enzyme. Earlier study showed the induction of xylopyranosidase and arabinofuranosidase in selected ruminal bacteria only when grown in xylose and not on arabinose and glucose and the activity is observed to be intracellular (Cotta, 1993).

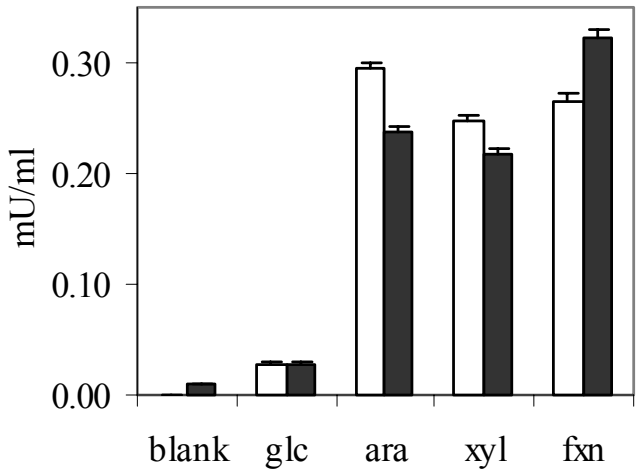

Figure 50. Arabinofuranosidase induction in *Bifidobacterium* (□) and *Pediococcus* (■) grown in different carbon sources. fxn – feraxan.

A clear induction of arabinofuranosidase in both arabinose and xylose and their polymer – feraxan has been observed in the present study. However, no distinction is made as to the site of enzyme activity – intracellular or extracellular. In part, this activity might be intracellular as the cells in the broth sample may take up the substrate, releasing out the p-nitrophenol after intracellular hydrolysis. During starvation there is a need to hydrolyze large molecular weight xylans outside the cells. For

this reason, it is supposed that the xylanolytic enzymes/activities, xylanase in particular to be largely extracellular in nature.

5.4.3. Fermentation with the aid of feraxanases

Feraxans in the MRS broth medium was pre-hydrolyzed with feraxanases (driselase or ragi malt extract in phosphate buffer). Ragi malt extract is rich in xylanolytic enzymes (Nirmala et al., 2000). Driselase, a source of xylanase (~ 0.28 U/mg protein) is also found to contain high amount of arabinofuranosidase activity (~ 1.64 mU/mg protein). Pre-hydrolysis of feraxans facilitated the growth of lactic acid bacteria, indicating the enzyme constrain, specifically xylanase. After 48 h incubation, OD of the culture broth is doubled compared to the one with native feraxans (table 32). A similar increase in the dry cell mass is also observed. Interestingly, cultures which are unable to grow on native feraxans fermented pre-hydrolyzed feraxans and concomitant change in pH, dry cell mass (table 32) and SCFA (table 35) are also observed. The growth is mainly due to the utilization of arabinose. The culture of *Lb. plantarum* is known to utilize only arabinose from the xylooligosaccharide mixture (Kontula et al., 1998). Unlike mixed flora, which produce high amounts of propionate and butyrate, pure cultures of lactic acid bacteria produced acetate as the chief SCFA on feraxan fermentation.

5.4.4. Fermentation of feraxans by yeast and pathogens

Of the 10 yeast cultures tested, none were able to grow on either feraxan or its individual sugars – arabinose and xylose. They grew luxuriantly in glucose, however, grew weakly on galactose but not on lactose. Yeasts in general, are not known to ferment pentose sugars especially xylose.

On the contrary, except *B. cereus* F 4810, other pathogenic cultures tested utilized either arabinose or xylose, but not both (table

36). Some of them also utilized galactose and lactose. However, they are unable to grow on feraxans. This may particularly be due to the lack of feraxanases in these strains. Many intestinal (pathogenic) bacteria are reported to be unable to utilize xylooligosaccharides and xylans (Van Laere et al., 2000). Being complex molecules, feraxans may act as a better prebiotics, able to be degraded only by probiotic strains.

Table 36. Sugar fermentation (in BCP broth) by pathogenic bacteria.

	Glc	Gal	Lac	Ara	Xyl	Feraxans
B. cereus F 4810	+	-	-	-	-	-
E. coli D 21	+	+	+	+	-	-
S. aureus FRI 722	+	+	+	-	+	-
Y. enterocolitica MTCC 859	+	-	-	+	-	-

5.4.5. Antimicrobial activity

Prebiotics are known to affect the growth of probiotic bacteria, bringing the much desired effects such as lowered pH, production of SCFA and vitamins and immune activation (Schley and Field, 2002). It is also been considered that prebiotics/probiotics can modulate growth/activity of pathogenic bacteria by the virtue of their antimicrobial activity. Culture broths (48 h old) of lactic acid bacteria grown in native feraxans showed a mild bacteriostatic activity towards *B. cereus* F 4810 (table 37, figure 51) and *E. coli* D 21 and there was no apparent effect on *S. aureus* FRI 722 and *Y. enterocolitica* MTCC 859. The activity might either be due to the lactic acid or any bacteriocin produced. The milder activity may due to the low amount of antimicrobial compounds produced as the native feraxans supported only a mild growth of lactic acid bacteria.

Table 37. Antimicrobial activity of lactic acid bacterial culture broth against pathogens.

	Blank	*Ped. pentosaceus* NDRI 035	*B. adolesentis* NDRI 236	*Lb. plantarum* NCIM 2084
B. cereus F 4810	-	+	+	-
E. coli D 21	-	+	+	
S. aureus FRI 722	-	-	-	-
Y. enterocolitica MTCC 859	-	-	-	-

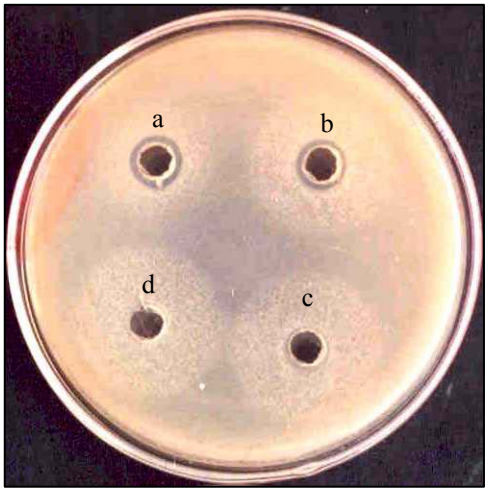

Figure 51. Antimicrobial activity of lactic acid bacterial culture broth on *B. cereus* F 4810. Culture broths from *Ped. petosaceus* NDRI 035 (a), *B. adolesentis* NDRI 236 (b), *Lb. plantarum* NCIM 2084 (c) and MRS/feraxans blank (d).

5.4.6. Role of ferulic acid

Feraxans are the parent molecules to which ferulic acid is ester linked and cereals are a rich source of this antioxidant compound. Ferulic acid is shown to have a number of health benefits (Bravo, 1998) and there was also an attempt to synthesize enzyme resistance starch ferulate to deliver enough antioxidant in to the colon (Ou et al., 2001). Here an attempt is made to see the role of ferulic acid in prebiotics. Supplementing ferulic acid with arabinose (to which ferulic acid is esterified in the parent molecule) in the lactic acid bacteria culture broth did not show any growth difference compared to the control. Leaving

apart any growth enhancing activity, administered ferulic acid concentration (0.01 – 0.05 μmol/10 mg, equivalent to the concentration found in feraxans) might be too low even to impart a growth inhibitory activity. Similarly, removal of ferulate moieties by alkali hydrolysis did not show any effect on the growth of lactic acid bacteria. However, earlier study reported a growth inhibitory effect of phenolics esterified to arabinoxylans that are used as substrates for ruminal bacteria (Akin et al., 1993). A recent study showed growth promoting activity of feruloyl oligosaccharides towards *B. bifidum* (Yuan et al., 2005). Growth is due to the utilization of sugar moieties and is not suppressed by the ferulic acid moiety. In the present study, ferulic acid (in the similar concentrations found with feraxans) showed no inhibitory/antimicrobial activity towards pathogenic cultures either.

In summary, lactic acid bacteria utilized water soluble feraxans from native and malted rice and ragi as carbon source and produced SCFA, chiefly acetate, and reduced the pH of culture broth. Due to the enzyme limitation, however, feraxans are only partly degraded by individual cultures and may require a consortium of (probiotic) bacteria to degrade it fully. Xylanase is not detected and arabinofuranosidase is shown to be induced by pentose sugars and their polymers. Common pathogenic cultures are unable to ferment feraxans. Despite the enzyme constrain, lactic acid bacteria partly utilized feraxans, justifying their prebiotic nature.

In conclusion, water soluble arabinoxylans from native and malted rice and ragi are shown to have considerable functionality. In fact, antioxidant activity of feraxans might be a novel property, and they are also shown to be prebiotic.

The results of the present investigation on the water soluble feraxans from native and malted rice and ragi have been summarized and concluded as follows:

- Water extractable non-starch polysaccharides (WEP) represent a small proportion (0.6 – 2.2%) of the total flour and their content increased by 2 to 3 folds upon malting (96 h controlled germination). Their water un-extractable counterparts (WUP) are present in higher proportions (7.5 – 20.3%). The WEP and WUP contained high amount (2.8 – 11.0%) of uronic acid, which is slightly higher in malts, probably due to the faster degradation of mixed glucans than arabinoxylans as indicated by P/H ratio **(page 77, chapter 3)**.
- Ferulic acid is the major bound phenolic acid ester-linked both in WEP and WUP and over 90% of the total ferulic acid are bound to the later. Malting resulted decrease in the bound ferulic acid content, due to the action of induced ferulic acid esterase. p-Coumaric acid is also found as bound phenolic acid mainly in WUP **(page 78, chapter 3)**.
- Protocatachuic acid is the major free phenolic acid with small amounts of gallic, caffeic and ferulic acids and their overall contents decreased upon malting. Presence of very low amount of free ferulic acid suggested that the bound ferulic acid hydrolyzed during malting would be quickly degraded in the system **(page 80, chapter 3)**.
- All the major feraxanases were detected in both rice and ragi flours with many folds higher activity in malts indicating their induction during malting. In specific, xylanase activity increased by 2 to 3 folds and ferulic acid esterase activity increased by 50 to 100 folds upon malting. Arabinofuranosidase and xylopyranosidase, two key enzymes in the feraxanase system also induced during malting. These xylanolytic enzymes, acting together, are responsible for the

loosening/degradation of cell wall matrix during germination and increasing the content of WEP **(page 81, chapter 3)**.

- WEP is sparingly soluble in water and its content (water soluble non-starch polysaccharides – NSP) increased by 3 to 5 folds up on malting. The major portion of water soluble NSP is arabinoxylan type of polysaccharide as indicated by sugar composition and it contained high amount of uronic (2.6 – 6.1%) and ferulic (492.5 – 528.0 μg/g) acids **(page 84, chapter 3)**.
- Water soluble NSP was fractionated on DEAE-cellulose into 5 fractions with water, 0.1 and 0.2 molar ammonium carbonate (AC) and 0.1 and 0.2 molar NaOH elution. The major (0.1 molar AC eluted) fraction is arabinoxylan type of polysaccharide with high amount of ester-linked ferulic acid as indicated by its strong UV absorption and HPLC analysis, and thus was designated as water soluble feruloyl arabinoxylans (feraxans). Interestingly, ferulic acid content of malt feraxans is around 12 and 7 folds higher than native (un-germinated) rice and ragi respectively. On the contrary, ferulic acid content of 0.2 molar AC eluted fractions was higher in native compared to malts. This indicated possible mobilization of feruloyl arabinoxylans during malting due to the action of xylanolytic enzymes **(pages 86, 89 and 90, chapter 3)**.
- Sephacryl S-300 gel permeation chromatography yielded two peaks each for native and malted rice and ragi water soluble feraxans. They were further purified on Sephacryl S-300 and their homogeneity was ascertained by HPSEC, capillary and cellulose-acetate paper electrophoresis. The molecular weight of purified feraxans ranged between 15,400 to 2,31,500. Molecular weight of feraxans decreased upon malting and the yield of high molecular weight peaks also decreased. This is due to the action of xylanolytic enzymes, in turn

leading to the better extractability/solubility of degraded polysaccharides in water **(pages 91 and 92, chapter 3)**.

- Purified feraxans have high Ara/Xyl ratio and are rich in uronic (8.0 – 13.4%) and ferulic (54.0 – 1471.6 μg/g) acids, which were higher in malts. The presence of high amount of galactose seems to be the characteristic of rice and ragi water soluble feraxans **(pages 98 and 100, chapter 4)**.
- Methylation analysis of the carboxyl reduced feraxans showed very high amount of 2,3,5-Me_3-arabinose indicating that majority of arabinose residues are terminally linked. Detection of di-methylated arabinose residues indicated the presence of branching site provision for arabinose and ester-linked ferulic acid. Presence of terminally linked galactose and glucuronic acid (4-O-Me) are confirmed by their tetra methyl derivatives. Di and mono-methylated xylose residues are in almost equal amounts and un-methylated xylose is found in good amount indicating high branching **(page 104, chapter 4)**.
- Periodate oxidation and Smith degradation studies showed that about 60% of sugar residues have adjacent free hydroxyl groups, which is in close agreement with the methylation and PMR data **(pages 107 and 109, chapter 4)**.
- The low negative optical rotation values (-0.3 to -7.4) indicated the polymer primarily to be β-linked. Signals corresponding to α-L-arabinofuranoside (δ ~110 ppm) and β-D-xylopyranoside (δ ~104 ppm) are detected in the ^{13}C-NMR spectra of water soluble feraxans. Glucuronic acid is found to be in 4-O-Me form as indicate by ^{13}C-NMR spectral signals at ~178 ppm (for >C=O), ~98.8 and ~72.1 ppm (for C-1 and C-3 of α-D-glucuronic acid and ~59.5 and ~18.0 ppm (for -O-CH_3). It is also confirmed by GLC-MS analysis **(pages 110 and 111, chapter 4)**.

- Proton magnetic resonance (PMR) spectra of feraxans showed almost equal distribution of di, mono (2/3) and un-substituted xylose residues as quantified by the integration of the anomeric signals arising from the arabinose residues. Interestingly, the amount of di-substituted xylose increased in malt feraxans with concomitant decrease in the content of mono-substituted residues. On the other hand, amount of un-substituted residues remained almost equal in both native and malt feraxans. Similar trend is observed both in rice and ragi feraxans **(pages 114 and 116, chapter 4)**.
- With their higher Ara/Xyl ratio and lower molecular weight, malt feraxans have higher di-substituted xylose residues. The substitution pattern of xylose residues is correlated with Ara/Xyl ratio and molecular weight of feraxans. There is a trend in the xylose substitution pattern. As the Ara/Xyl ratio increases and/or molecular weight decreases, content of di-substituted xylose residues increases while the un-substituted residues remain overall same. A trend of decrease in the Ara/Xyl ratio with increasing molecular weight is also observed **(pages 117, 119 and 120, chapter 4)**.
- The PMR spectra showed the signals corresponding to ferulic acid bound to the water soluble feraxans. Infrared spectra of feraxans showed signals typical to arabinoxylans with uronic/ferulic acid >C=O signal at ~1730 cm^{-1} **(pages 121 and 122, chapter 4)**.
- With this information in hand, a structural model has been proposed for rice and ragi water soluble feraxans. They have a β-linked xylose backbone with α-linked arabinose residues as side branches, similar to other cereal arabinoxylans. However, they differed in many other respects. They are of small molecular weight and have high Ara/Xyl ratio and hence highly branched, with almost equal amount of di, mono and un-substituted xylose residues. They are particularly rich in O-2 substituted xylose residues unlike many other cereal

arabinoxylans especially from wheat. Presence of high amounts of galactose, glucuronic (4-O-Me) and ferulic acids are the characteristic features of water soluble feraxans **(page 124, chapter 4)**.

- In spite of their positions in the widely separated clades, water soluble feraxans from rice and ragi are essentially similar, and structurally resembled highly branched regions of rye and maize arabinoxylans than to wheat arabinoxylans. Water soluble feraxans from malts are low molecular with higher Ara/Xyl ratio and higher content of ferulic acid. This is probably due to the action of xylanolytic enzymes induced during malting which preferentially acted upon the less substituted region of large molecular (native) feraxans **(page 126, chapter 4)**.
- Water soluble NSP/feraxans showed many functional characteristics. With their high amount of bound ferulic acid, water soluble NSP/feraxans exhibited very high antioxidant activity. The activity pattern observed for different fractions could well be correlated with their bound ferulic acid content. However, antioxidant activity of feraxans is several folds higher than the expected activity due to their bound ferulic acid content. This is, in part, related to the molecular weight/chain length of the polysaccharides. Possible antioxidant effect of negatively charged sugar residues is also shown **(page 131, chapter 5)**.
- Water soluble NSP/feraxans exhibited very low viscosity except for ragi malt NSP. This property may make them ideal to be incorporated in fibre/antioxidant depleted/deprived foods/drinks requiring low viscosity. Changes in the viscosity in relation to concentration, temperature and pH are also shown. Interestingly, due to the bound ferulic acid, feraxans showed different trends in viscosity with respect to pH in different buffers. The presence of NaOH in the alkaline pH hydrolyses hydrophobic bound ferulic acids and increases viscosity of

feraxans due to freed –OH groups and increased hydrophilic interactions **(pages 138 and 139, chapter 5)**.

- Despite considerable amount of bound ferulic acid, water soluble NSP/feraxans showed no gelling ability. However, they showed good foam stabilization property. Water soluble NSP has slightly better effects compared to feraxans possibly due to the cumulative effect of several polysaccharide populations in NSP **(pages 141 to 143, chapter 5)**.
- Incorporation of water soluble NSP into wheat dough resulted in overall positive effects. Farinograph values indicated higher water absorption and lower dough development time with slightly lower dough stability. Both extensibility and resistance to extension are increased upon the addition of water soluble NSP, the effect is similar to that of dough improvers. Amylograph studies showed increased viscosity of wheat dough upon the addition of NSP **(pages 144 to 146, chapter 5)**.
- Test baking indicated improved bread characteristics with the addition of water soluble NSP. Weight, loaf volume and specific volume are increased, while firmness of bread decreased. Thus addition of water soluble NSP/feraxans has overall positive functional effects on dough compared to the negative effect exerted by their insoluble counterparts **(page 147, chapter 5)**.
- The *in vitro* fermentation characteristics/prebiotic activity of water soluble NSP/feraxans are studied with probiotic cultures of lactic acid bacteria. In general feraxans are only partly fermented by few lactic acid bacteria, which are able to utilize arabinose or xylose. Feraxan non-fermenters could not utilize constituent sugars – especially xylose. Degradation/fermentation of feraxans is constrained by the xylanolytic enzymes especially lack of xylanase in the probiotic bacteria **(pages 153 and 154, chapter 5)**.

- Utilization of feraxans by lactic acid bacteria resulted in increased OD, dry cell mass and viable cell counts, and concomitant decrease in the pH, which is related to the production of SCFA. Acetate is the chief SCFA produced. Arabinofuranosidase, the key enzyme in the feraxans' degradation is shown to be induced in cells by the presence of pentose sugars/feraxans in the culture medium. Rat cecal/faecal mixed cultures completely degraded feraxans, which is related to their high xylanase activity. Pre-hydrolysis of feraxans with xylanase facilitated their fermentation by lactic acid bacteria. Pure cultures of lactic acid bacteria, thus have limited ability to ferment feraxans and their complete fermentation may require consortium of bacteria like in mixed cultures **(pages 153 and 154, chapter 5)**.
- Although many food borne pathogenic bacteria are able to ferment constituent sugars, they are unable to utilize feraxans. The culture broth of lactic acid bacteria grown on feraxans showed antimicrobial/bacterio-static activity towards these pathogenic bacteria. The water soluble feraxans with their ability to support the growth of probiotic lactic acid bacteria are shown to have prebiotic activity. The malt feraxans showed slightly better functionality compared to the native ones **(page 160, chapter 5)**.

Overall, a comparative investigation is made on the structural and functional characteristics of water soluble feraxans from rice and ragi, and their changes upon germination.

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LIST OF PUBLICATIONS AND PATENTS

PUBLICATIONS

1. **R. Shyama Prasad Rao** and G. Muralikrishna, 2004. Non-starch polysaccharides – phenolic acid complexes from native and germinated cereals and millet. *Food Chemistry*, 84, 527 – 531.
2. **R. Shyama Prasad Rao** and G. Muralikrishna, 2005. Water soluble feruloyl arabinoxylans from rice and ragi: changes upon malting and their consequence on antioxidant activity. Communicated to *Phytochemistry*.
3. **R. Shyama Prasad Rao**, R. Sai Manohar and G. Muralikrishna, 2005. Functional properties of water soluble non-starch polysaccharides from rice and ragi: effect on dough characteristics and baking quality. Communicated to *Journal of Cereal Science*.
4. **R. Shyama Prasad Rao**, M. C. Varadaraj and G. Muralikrishna, 2005. *In vitro* fermentation of water soluble feruloyl arabinoxylans from rice and ragi by lactic acid bacteria: enzyme constrain and prebiotic activity. Communicated to *Applied and Environmental Microbiology*.
5. **R. Shyama Prasad Rao** and G. Muralikrishna, 2005. Structural characteristics of water soluble feruloyl arabinoxylans from rice and ragi: variations upon malting. To be communicated to *Carbohydrate Polymers*.

PATENTS

1. G. Muralikrishna and **R. Shyama Prasad Rao**, 2002. A process for obtaining phenolic acid rich dietary fibre from cereal malts. US Patent, 0415/DEL/2002.
2. G. Muralikrishna and **R. Shyama Prasad Rao**, 2003. A process for obtaining ferulic acid esterase from cereal malts. Patent submitted to CSIR.
3. **R. Shyama Prasad Rao** and G. Muralikrishna, 2004. A process for obtaining xylooligosaccharides form cereals and their malts. Patent submitted to CSIR.