ESTERASES FROM FINGER MILLET MALT AND THEIR EFFECT ON THE FUNCTIONALITY OF CEREAL NON-STARCH POLYSACCHARIDES

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in

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by

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DECLARATION

I declare that the thesis entitled **"ESTERASES FROM FINGER MILLET MALT AND THEIR EFFECT ON THE FUNCTIONALITY OF CEREAL NON-STARCH POLYSACCHARIDES"** submitted to the **UNIVERSITY OF MYSORE** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** is the result of research work carried out by me under the guidance of **Dr. G. MURALIKRISHNA**, Scientist E-II, department of Biochemistry and Nutrition, during the period of March 2003 – November 2007. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date: 3-11-2007 **(G. MADHAVI LATHA)**

Place: Mysore

DR. G. MURALIKRISHNA, M.Sc., Ph.D. **Date: 3-11-2007**
Scientist E-II
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CERTIFICATE

This is to certify that the thesis entitled **"ESTERASES FROM FINGER MILLET MALT AND THEIR EFFECT ON THE FUNCTIONALITY OF CEREAL NON-STARCH POLYSACCHARIDES"** submitted **by Miss. G. MADHAVI LATHA** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** is the result of the research work carried out by her in the department of Biochemistry and Nutrition, under my guidance during the period of March 2003 – November 2007.

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DEDICATED TO THE MEMORY OF MY BELOVED FATHER

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(G. MADHAVI LATHA)

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ABBREVIATIONS

Abbreviations	Expansions
α	Alpha
β	Beta
μ	Micro
°C	Degree centigrade
η_r	Relative viscosity
Ac	Acetyl
AAE	Acetic acid esterase
APS	Ammonium persulfate
α-NA	Alpha naphthyl acetate
B.O.D	Biological oxygen demand
BSA	Bovine serum albumin
cm	Centimeter (s)
DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetra acetic acid
ESI	Electrospray ionization
FA	Ferulic acid
FAE	Ferulic acid esterase
GPC	Gel permeation chromatography
GSH	Reduced glutathione
g	Gram (s)
h	Hour
HPLC	High performance liquid chromatography
IR	Infrared
kDa	kilo Dalton
M	Molar
Me	Methyl
min	Minute

ml	Milliliter
mm	Millimeter
MS	Mass spectroscopy
µg	Microgram
MUTMAC	4- Methylumbelliferyl *p*- trimethyl ammoniocinnamate chloride
nm	Nanometer
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
OD	Optical density
p	Para
PAGE	Poly acrylamide gel electrophoresis
PNPA	Para nitrophenyl acetate
PNP	Para nitrophenol
PCMB	Para chloro mercuric benzoate
ppm	Parts per million
PVPP	Polyvinylpolypyrrolidone
SDS	Sodium dodecyl sulphate
Sec	Second
TEMED	*N, N, N*^{1}, *N*^{1}-tetramethylene diamine
U	Unit (s)
UV	Ultra violet
w/w	Weight/weight
w/v	Weight/volume
v/v	Volume/volume
WEP	Water extractable polysaccharides
WUP	Water unextractable polysaccharides
WSP	Water soluble polysaccharides

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

Title of the thesis: 'Esterases from finger millet malt and their effect on the functionality of cereal non-starch polysaccharides'

Candidate: G. Madhavi Latha

Cereal plant cell walls are complex mixture of polysaccharides consisting of arabinoxylans, (1→3)/(1→4)-β-D-glucans, arabino-galactoproteins and glucomannans, in addition to the high amounts of lignocellulosic complexes. Considerable progress was made for the past few decades on the functional aspects of cereal arabinoxylans due to their potential influence on water holding capacity and water balance, protein foam stabilization, viscosity, gelling and rheological properties. Cereal arabinoxylans are β-1, 3/1, 4 linked D-xylose polymers with a large variation on the nature and position of the substituents in the side chain. Minor substituents such as acetyl (attached at O-2/O-3 of the xylan backbone), as well as feruloyl groups (esterified to 5'-OH group of arabinosyl residues in the side chain), are also present in the side chains of arabinoxylans. The structure of these arabinoxylans varies not only from source to source, but also with in the same source. Arabinoxylans have been characterized from various sources such as wheat, barley, maize, rye and ragi with respect to their structural and functional relationship. Nutritionally, arabinoxylans are known to alleviate various disease symptoms such as diabetes, atherosclerosis and colon cancer.

Malting of cereals/millets is an important biotechnological process which helps in the induction of various hydrolytic enzymes such as α/β-amylases, α-glucosidases, pullulanases, xylanases and esterases, which in turn, controls the various physiological and biochemical processes in the grain. The information pertaining to both starch and major cell wall degrading enzymes such as xylanases and xylosidases from cereal malts is quite exhaustive. However, the same is not true with respect to the minor cell wall degrading enzymes such as O-acetyl esterases and feruloyl esterases.

Esterases are important class of hydrolytic enzymes that cleave the ester linkages i.e. acetic acid esterases (EC 3.1.1.6) cleaves the acetyl groups from the xylose residues of arabinoxylans while cinnamic acid esterases (EC 3.1.1.73) hydrolyze the cinnamic acid derivatives such as ferulic acid and coumaric acids which in turn, may modulate the functionality of cereal arabinoxylans.

Finger millet (*Eleusine coracana*), also known as ragi, is an indigenous minor millet, rich in calcium and dietary fiber. It is extensively consumed in south India, and is used in both native and processed (malted) forms. Till date, the effect of O-acetyl groups and feruloyl groups on the functionality of cereal water-soluble non-starch polysaccharides has not been addressed. Hence, the present study is undertaken as there were no reports on isolation, purification and characterization of finger millet malt esterases i.e. acetic and ferulic acid esterases with respect to their (a) kinetic properties, and (b) role in modulating the functionality of water-soluble non-starch polysaccharides from cereals with respect to their viscosity, foam stabilization and gelling characteristics, which are the most important aspects of polysaccharide functionality. Accordingly, following objectives are envisaged in the present investigation.

1. Standardization of the experimental protocols with respect to the isolation of esterases from finger millet malt, with respect to ionic strength, pH, number of extractions and temperature. Preparation of synthetic substrate(s) for ferulic acid esterases (Feruloyl glycerol).
2. Purification of esterases using fractional precipitation, ion exchange and gel filtration chromatography. Ascertaining apparent homogeneity by native PAGE/SDS-PAGE. Determination of kinetic parameters of esterases i.e. pH, temperature optima and stability.
3. Studying the functional characteristics such as viscosity and foam stabilization of water-soluble polysaccharides of cereals in the presence of enriched enzyme isolates of esterases.
4. Role of esterases with respect to the mechanism of gelling properties of polysaccharides.

An outline of the studies carried out on the above objectives is presented systematically chapter wise in this synopsis of the envisaged Ph. D. thesis.

Chapter 1. Introduction

This chapter begins with general information on the structural and functional aspects of cell wall polysaccharides of the cereals. The functional and nutritional significance of cereal arabinoxylans and its minor substitutes such as acetyl and feruloyl groups are described. The chapter also deals with the literature pertaining to the cell wall degrading enzymes essentially the esterases such as acetic and ferulic acid esterases mainly from cereals and also a few reports from microbes, i.e. definition, history, types, sources, substrates used, assay methods, isolation, fractionation, purification procedures, criteria of purity, kinetics and inhibitory studies, their active site residues, and the mechanism of their action. Few examples of recombinant esterases are also provided. This chapter also describes the various steps involved in the malting of cereals. A brief account on finger millet is also given. Finally, the chapter highlights the aim and scope of the present investigation, with defined objectives.

Chapter 2. Materials and Methods

This chapter deals with the information on the general procedures, and various chemicals and instruments used in the present investigation. Described here are malting of ragi and various calorimetric and spectrophotometric methods such as estimations of total carbohydrate, reducing sugar, protein, α-naphthol, ferulic acid and *p*-nitrophenol. Methods are described pertaining to the synthesis of ferulic acid esterase substrates such as feruloyl glycerol and *p*-nitrophenyl ferulate along with the isolation of natural substrate i.e. wheat bran polysaccharide .Enzyme assay methods for esterases such as acetic and ferulic acid esterases are described, along with a brief account on the procedure to remove starch from water-soluble polysaccharides by glucoamylase. Isolation of water-soluble polysaccharides (WSPs) and identification of their acetyl and feruloyl groups by infrared spectroscopy is also given.

Isolation of acetic and ferulic acid esterases, purification of these esterases by ammonium sulphate fractionation, anion exchange chromatography on DEAE-cellulose, gel permeation chromatography (GPC) on Sephacryl S-200/Biogel P-30 and Phenyl-Sepharose (acetic acid esterase) chromatographic methods are described in detail. The methods used to ascertain purity of these esterases by PAGE, protein/activity staining and gel permeation chromatography are described.

This chapter also deals with the various methods used for the characterization of esterases (acetic and ferulic acid esterases) such as determination of molecular weight by GPC, SDS-PAGE and electrospray ionization-mass spectroscopy (ESI-MS), pH and temperature optima's and their stability studies, and arrhenius plots. Various kinetic parameters such as determination of K_m and V_{max}, effect of metal ions, inhibitors, UV and fluorescence spectra of purified esterases (acetic and ferulic acid esterases) are mentioned. A brief account on the methods i.e. HPLC, ESI-MS and ^{1}H NMR used for the identification of the released products by deesterification (deacetylation/deferuloylation) of water-soluble polysaccharides (ferulic acid esterases) and synthetic substrates i.e. α-naphthyl acetate, *p*-nitrophenyl acetate, glucose pentaacetate (acetic acid esterase) are mentioned.

Methods employed to determine the effect of apparently purified acetic and ferulic acid esterases from ragi malt on the functional properties such as viscosity, foam stabilization and oxidative gelation of water-soluble polysaccharides isolated from wheat, ragi and other polysaccharides such as gum karaya are given. Finally, the chapter ends with the method describing the effect of acetic acid esterase from ragi malt on the synergistic gelation of blends of xanthan and locust bean gum.

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

Chapter 3. Isolation of esterases from ragi malt

This chapter for convenience sake is divided in to two sub-chapters i.e.

a. Isolation of acetic acid esterase
b. Isolation of ferulic acid esterase

Section 3a

In this sub chapter, results pertaining to the changes in the activity of acetic acid esterase during malting of ragi and optimization of the best conditions to isolate maximum activity of acetic acid esterase from ragi malt and coleoptiles are described. Activity of acetic acid esterase was maximum at 72 h of ragi malt and its coleoptiles. Different extractants, i.e. acetate, phosphate and Tris buffer at various pH, and different concentrations of polyvinylpolypyrrolidone (PVPP), reduced glutathione, Triton X-100, calcium and magnesium chlorides and different stabilizers were tried, to select the best condition for isolation of maximum acetic acid esterase activity. Accordingly, Tris buffer (pH 9.0, 75 mM) in the presence of reduced glutathione (100 mM), PVPP (0.5%), Triton X-100 (0.75%), calcium chloride (6 mM) and magnesium chloride (4 mM) was found to be the best extractant of acetic acid esterase from 72 h ragi malt. Temperature optima and thermal stability of crude extract of acetic acid esterase from 72 h malt and its coleoptiles were found to be 30°C. Several amino acids were tested for their stabilizing effect on acetic acid esterase out of which glycine alone has some stabilization effect on the enzyme at 50°C.

Section 3b

In this sub chapter results pertaining to the characterization of synthetic substrates of ferulic acid esterase i.e. feruloyl glycerol and *p*-nitrophenyl ferulate are provided. Changes in the activity of ferulic acid esterase during the malting of ragi and optimization of extraction conditions of ferulic acid esterase from 96 h ragi malt are mentioned.

Synthesis of the substrates of ferulic acid esterase i.e. feruloyl glycerol and *p*-nitrophenyl ferulate were achieved, and the identification of these compounds were done by spectrophotometry and nuclear magnetic resonance spectroscopy respectively. Activity of ferulic acid esterase was maximum at 96 h ragi malt and its coleoptiles. Different extractants, i.e. acetate, phosphate and Tris buffer at various pH in the presence of reduced glutathione (25 mM), PVPP (1%) and Triton X-100 (1%) were used. Tris buffer (pH 7.0, 50 mM) was found to be the best extractant of ferulic acid esterase from 96 h malt; however, Tris buffer (pH 9.0, 50 mM) was the best

extractant for 96 h coleoptiles. Temperature optima of crude extracts of ferulic acid esterase from 96 h malt and its coleoptiles were found to be 38°C and 40°C respectively and the enzyme was thermally stable at 30°C.

Chapter 4. Purification and characterization of esterases from ragi malt

This chapter is also divided in to 2 sub-chapters, for better presentation

a. Purification and characterization of acetic acid esterase

b. Purification and characterization of ferulic acid esterase.

Section 4a.

This sub chapter deals with the purification and characterization of acetic acid esterase from 72 h ragi malt. The Tris buffer (pH 9.0, 75 mM) extract was fractionated using ammonium sulphate fractionation in to minor (0 - 40%) and major (40 - 80%) fractions. The major fraction was separated on DEAE-cellulose column into two activity peaks representing acetic acid esterases. The major active fractions were pooled and further purified by gel permeation on Sephacryl S-200 and Phenyl-Sepharose column chromatographies. This resulted in apparently pure enzyme and its purity was ascertained by native and SDS-PAGE and also by activity staining.

This sub chapter also deals with the nature and properties of the purified acetic acid esterase. The pH and temperature optima of the enzyme were found to be 7.5 (Tris-HCl buffer, 75 mM) and 45°C respectively. The enzyme is stable in the pH range of 6.0 - 9.0 and temperature range of 30 - 40°C. The activation energy of the enzymatic reaction catalyzed by acetic acid esterase using α-naphthyl acetate was found to be 7.29 kJ mol^{-1}. The apparent K_m and V_{max} of the purified acetic acid esterase for α-naphthyl acetate were 0.04 μM and 0.175 Uml^{-1} respectively. The molecular weight of the native enzyme was found to be 79.4 kDa by GPC and ESI-MS whereas the denatured enzyme was found to be 19.7 kDa on SDS-PAGE indicating that the acetic acid esterase from 72 h ragi malt as a homotetramer.

EDTA, citric acid and metal ions such as Fe^{3+}, Cu^{2+} increased the activity while Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Al^{3+} reduced the activity. Group specific reagents such as eserine and *p*-chloromercuric benzoate at 25 mM

concentration completely inhibited while iodoacetamide showed very less inhibition. Eserine is found to be a competitive inhibitor of acetic acid esterase from ragi malt. Fluorescence spectra of purified enzyme indicated an emission maximum of 330 - 340 nm indicating the presence of aromatic amino acids in the purified acetic acid esterase from ragi malt. The products liberated from α-naphthyl acetate, *p*-nitrophenyl acetate and glucose pentaacetate by the action of purified ragi acetic acid esterase were authenticated by ESI-MS and ^{1}H NMR

Section 4b.

This sub chapter deals with the purification and characterization of ferulic acid esterase from 96 h ragi malt. Tris buffer (pH 7.0, 50 mM) extract was fractionated using ammonium sulphate fractionation in to minor (0 - 40%) and major (40 - 80%) fractions. The major fraction was separated on DEAE-cellulose column into two activity peaks, representing ferulic acid esterases. The major active fraction was pooled and further purified by gel permeation on Biogel P-30 column chromatography, which resulted in apparently pure enzyme and its purity was ascertained by native and SDS-PAGE and also by activity staining using 4-methylumbelliferyl *p*-trimethylammonio cinnamate chloride (MUTMAC).

The pH and temperature optima of the enzyme were found to be 6.0 and 45°C respectively. The enzyme is stable in the pH range of 5.5 - 9.0 and temperature of 30°C. The activation energy of the enzyme-catalyzed reaction by ferulic acid esterase for the hydrolysis of *p*-nitrophenyl ferulate was found to be 4.08 kJ mol^{-1}. The apparent K_m and V_{max} of the purified ferulic acid esterase for *p*-nitrophenyl ferulate were found to be 0.053 μM and 0.085 U ml^{-1} respectively. The molecular weight of the native as well as denatured ferulic acid esterase was found to be 16.5 kDa by GPC, ESI-MS and SDS indicating it to be a monomer.

Metal ions such as Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, oxalic and citric acids enhanced the ferulic acid esterase activity. The enzyme was completely inhibited by Fe^{3+}. Group specific reagents such as *p*-chloromercuric benzoate and iodoacetamide inhibited the enzyme indicating the possible presence of cysteine residues in the active site pocket. Phenylmethyl sulphonyl fluoride (0 - 50 mM) did not show any inhibitory effect on

the purified enzyme. Fluorescence spectrum of the purified enzyme has showed an emission maximum of 300 - 310 nm indicating the presence of aromatic amino acids. The enzyme showed substrate specificity towards small molecular weight substrates over large polymers such as water-soluble polysaccharides isolated from ragi and wheat. The products liberated from water-soluble polysaccharides of wheat and ragi by the action of purified ragi ferulic acid esterase were authenticated by ESI-MS.

Chapter 5. Role of ragi malt esterases on the functionality of cereal water-soluble non-starch polysaccharides

This chapter presents the findings on the various functional properties of water-soluble polysaccharides such as (a) viscosity, (b) foam stabilization and (c) oxidative gelation with respect to different concentrations, temperatures and the effect of enriched active fractions (DEAE-cellulose) of both esterases (acetic and ferulic acid esterases) on the functional properties.

The results indicated that the relative viscosities of the enzyme treated water-soluble polysaccharides were marginally less than the controls (untreated ones). The relative viscosities of wheat and ragi flour water-soluble polysaccharides (both enzyme treated and untreated) increased with the increase in concentration (0.2 - 1.0%), decreased with increase in temperature (30 - 70°C). Foam stabilization effect of water-soluble polysaccharides increased with increase in concentration. Enzyme treated water-soluble polysaccharides has less effect on the stabilization of the thermal disruption of protein foams compared to the untreated ones. Purified acetic acid esterase treated water-soluble polysaccharides of wheat and ragi showed increased gelation while purified ferulic acid esterase treated ones showed slight decrease in comparison with their respective controls.

Xanthan gum (XG), deacetylated by purified acetic acid esterase, showed improved gelation when blended, with locust bean gum (LBG) in the ratio of 1:1 compared to the native xanthan gum indicating the effect of acetyl groups on gelation.

The above results indicated slight variation on the functional properties of water-soluble polysaccharides isolated from wheat and ragi when de-esterified

(deacetylated and deferuloylated) with purified ragi esterases (acetic and ferulic acid esterase).

The summary and conclusions are given after the results and discussion chapter

The thesis ends with bibliography consisting of all the references cited in the present investigation with their titles arranged in alphabetical order.

CHAPTER-1
INTRODUCTION

1.1. INTRODUCTION

Plant cell walls are a composite structures of cellulose, hemicellulose, lignin and structural proteins. Cell wall construction is an important prerequisite for many central processes of plant life, such as germination, growth, fruit ripening, organ abscission, vascular differentiation and responses to pathogens. The complex nature of plant cell walls and the structure of the individual polysaccharides have been the subject of many investigations (Aspinall, 1980 & 1982; Selvendran and O'Neill, 1987). Cellulose, the major abundant and renewable organic structure in nature, is composed of D-glucopyranose residues linked by β-(1→4) glycosidic bonds and provides rigidity to the plant cell walls due to the spatial arrangement of glucose residues (De Vries and Visser, 2001). Hemicelluloses account for 20 - 30% of the dry weight of plant cell walls and are the second most abundant renewable polysaccharides in nature after cellulose (Eriksson et al., 1990; Timell, 1964).

Cereal hemicelluloses are complex mixture of polysaccharides composed of arabinoxylans, β-(1→3)/(1→4)-D-glucans, arabino-galactoproteins and glucomannans. Out of these polysaccharides, arabinoxylans received increased attention because of their importance in (a) foam stabilization (b) gelling and (c) bread making due to their high viscosity and water holding capacity. Cereal arabinoxylans are β-1, 4 linked D-xylose polymers with a large variation on the nature and position of the substituents in the side chain. The structure of arabinoxylans varies not only from different sources, but also with in the arabinoxylans isolated from the same source. These arabinoxylans constitute xylan backbone either in β-1, 3/1, 4 linkages, with very low to high substitution at O-2/O-3 of the backbone mostly by arabinose and occasionally by 4-O-methyl D-glucuronic acid and D-galactose in the side chains [(Figure 1), Biely, 1985; Subba Rao and Muralikrishna, 2004; Shyama Prasad Rao and Muralikrishna, 2007; Muralikrishna and Subba Rao, 2007].

Barley arabinoxylans are structurally uniform than wheat arabinoxylans, since the substituted residues are well separated by regions of unsubstituted xylosyl units, thereby forming a cluster (Vietor et al., 1991). Wheat bran and barley-spent grain (BSG) are the agricultural waste products rich in arabinoxylans. Wheat bran

arabinoxylans are highly heterogeneous comprising of both water extractable as well as water unextractables, whereas barley is rich in hot water extractable arabinoxylans.

Minor substituents such as cinnamic acid derivatives i.e. ferulic and coumaric acids are esterified to arabinose side chains whereas acetyl groups are found to be esterified to the free hydroxyl groups of xylose residues in the xylan backbone [(Figure 1), Harvey et al., 1986; Smith and Hartley, 1983; Timell, 1967]. Due to the presence of number of substituent groups, arabinoxylans are considered as heterogeneous group of polysaccharides which require both exo and endo hydrolases for their complete degradation.

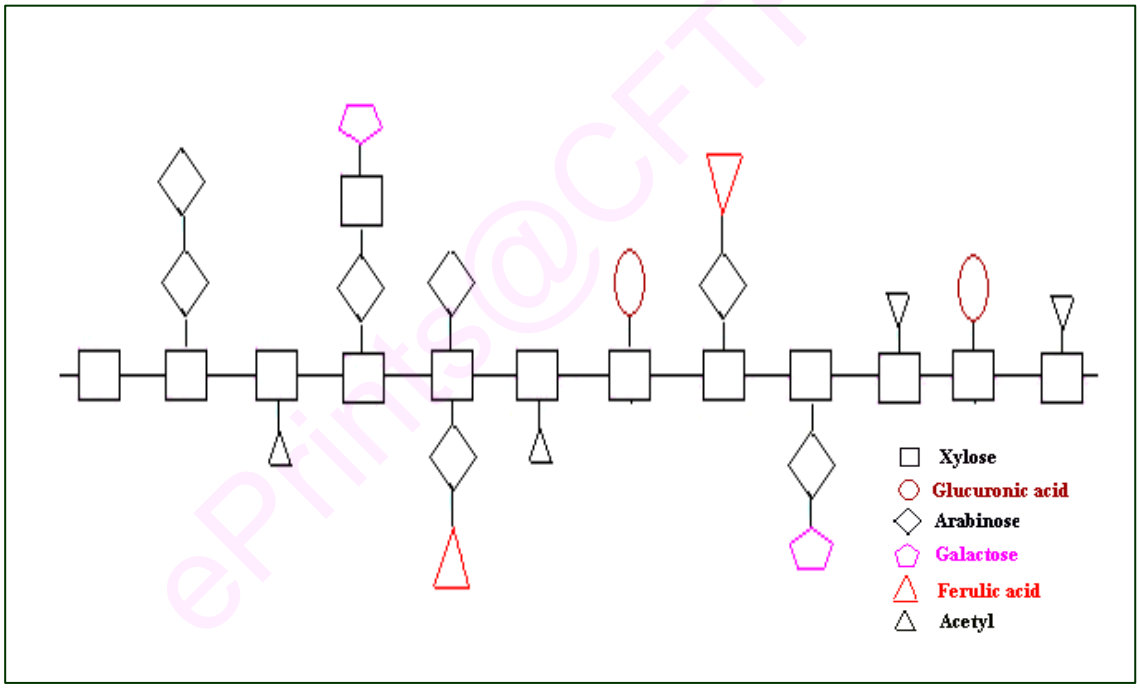


Figure 1. Schematic representation of arabinoxylan

1.2. Minor constituents of cereal arabinoxylans

1.2.1. Acetyl esters

In cereal arabinoxylans, the acetyl groups can be found at positions 2 and 3 of the xylopyranoid ring. However, in oligosaccharides, the acetyl group can be even at 4^{th} position due to spontaneous acetyl group migration in aqueous solution (Biely et al., 1996; Kabel et al., 2003). The presence of acetyl groups in the xylan backbone

contributes to its substantial solubility, while deacetylation leads to the formation of xylan aggregates (Matsuo and Mizuno, 1974). However, studies showed that extensive acetylation do contribute to the insolubility of arabinoxylans indicating the bifunctional nature of acetyl groups (Humberstone and Briggs, 2000b). Degree of acetylation has a pronounced effect on the physicochemical properties of arabinoxylans. Acetylated arabinoxylans have poor gelling properties (Morris and Bacon, 1977) and are not degraded in the rumen by microbes (Wood and McCrae, 1986; Mitchell et al., 1990). The acetylated polysaccharides are synthesized in the Golgi complex, followed by their transportation by UDP sugar complexes for incorporation into the plant cell walls (Liners et al., 1994).

1.2.2. Cinnamic acid esters

Phenolic acid esters are plant secondary metabolites which are biosynthesized through the shikimic acid pathway [(Figure 2), Dixon and Paiva, 1995]. They vary in their structure and occurrence i.e. simple phenolics such as hydroxybenzoic acids and large polymers such as condensed and hydrolysable tannins with high molecular weights. They are known to exhibit anti-platelet, anti-oxidant, anti-inflammatory, anti-tumoral, anti-glycemic and oestrogenic activities, suggesting their potential role in the prevention of coronary heart diseases and cancer (Hertog et al., 1993; Jang et al., 1997; Arai et al., 2000).

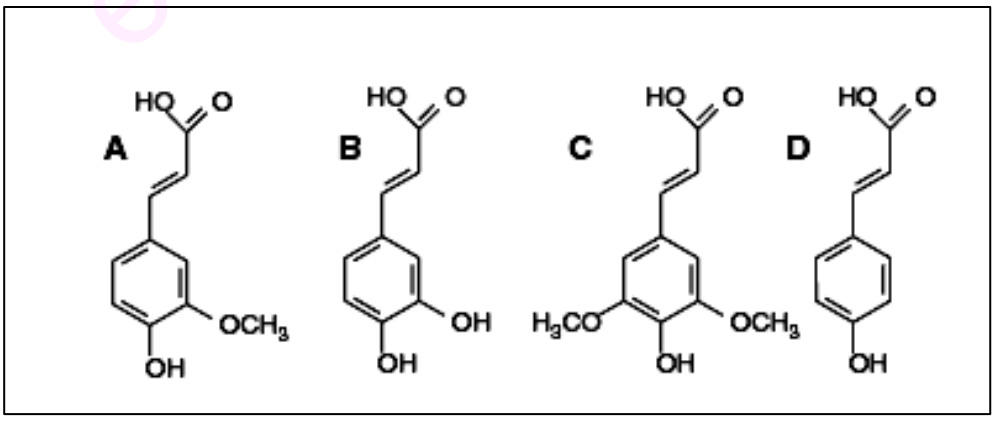


Figure 2. Structure of phenolic acids
A. Ferulic acid B. Caffeic acid C. Sinapic acid and D. *p*-coumaric acid

In addition, phenolic acids such as caffeic, coumaric, ferulic, and protocatechuic acids are shown to exert an antifungal effect (Baranowski et al., 1980). Polyphenols give an astringent taste by interacting with proteins, which provide a means of chemical defense for plants against grazing animals.

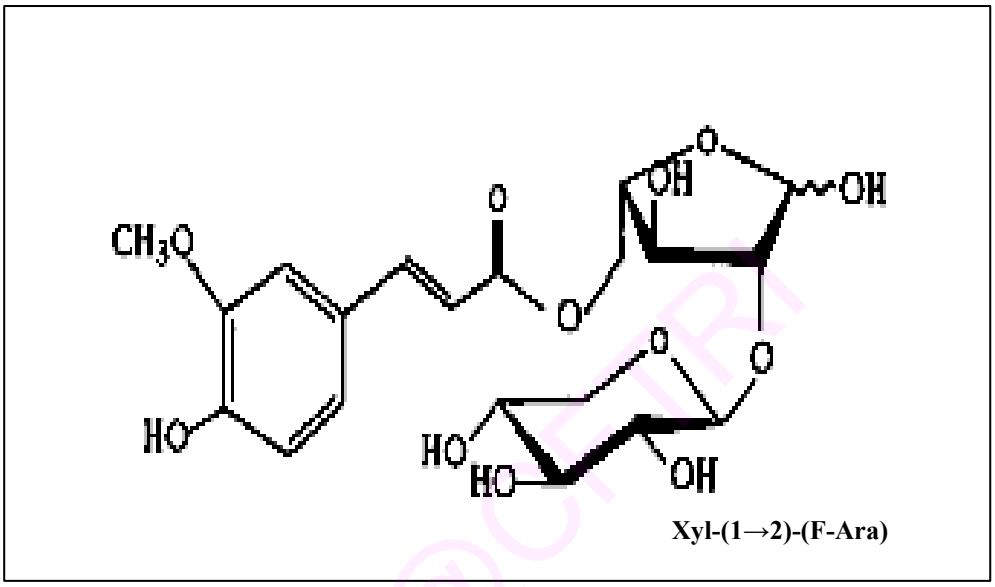
1.2.3. Distribution of ferulic acid esters

Ferulic acid esters are 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic or *trans*-4-hydroxy-3-methoxy-cinnamic acids. They are distributed throughout the primary and secondary cell walls in plants and are present relatively in high concentrations particularly in the epidermis, xylem vessels, bundle sheaths and sclerenchyma. Ferulic acid is mostly esterified to 5′-OH group of α-L-arabinofuranosyl side chains in arabinoxylans (Figure 3a), and sometimes to 2′-OH [α-L-arabinofuranosyl residues in arabinans (Figure 3b)], 6′-OH [(β-galactopyranosyl residues in pectic substances and galactans), Saulnier and Thibault, 1999] and 4′-OH [(α-D-xylopyranosyl residues in xyloglucans), Ishii et al., 1990]. Linkage of ferulic acid to polysaccharides is highly specific. The content of cinnamoyl esters in plant cell walls vary from 0.1 to 3.1%. Ferulic acid fluoresce brightly under the UV light and it constitutes about 0.14% (w/w, dry weight) in the aleurone layer of barley grains (Nordkvisk et al., 1984), 0.66% in wheat bran (Smith and Hartley, 1983), 0.8% in sugar beet pulp (Michard et al., 1994), 0.9% in rice endosperm cell wall (Shibuya, 1984) and 3.1% in maize bran (Saulnier et al., 1995).

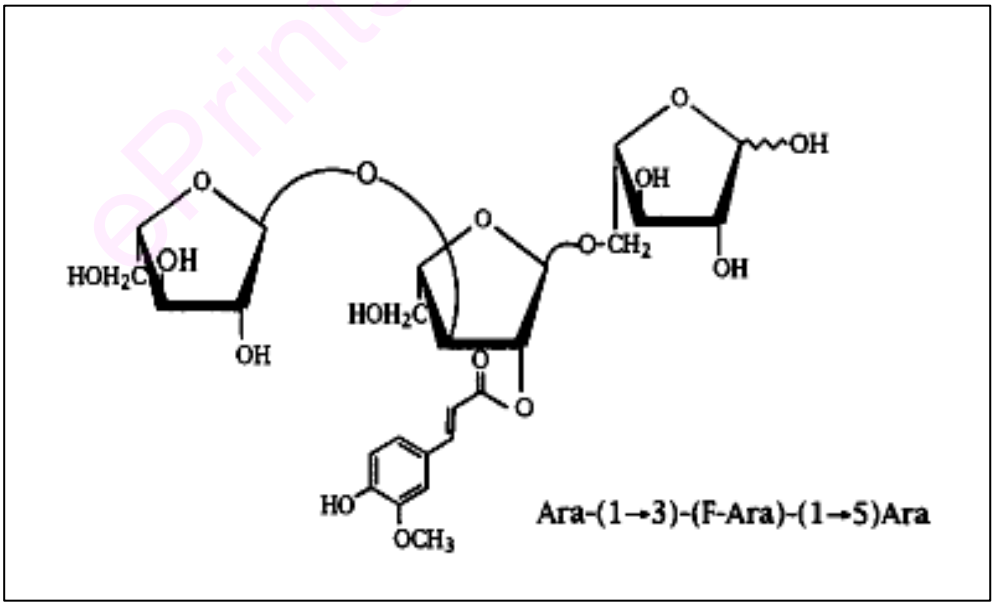
It has been estimated that every 15th arabinose unit in barley straw arabinoxylan is esterified with ferulic acid and the endosperm of barley contains 50 - 690 μg of ferulic acid per 100 mg of the cell wall material (Fincher, 1976; Ahluwalia and Fry, 1986; Kanauchi and Bamforth, 2002).

Cross linking of the cell wall polymers by ferulic acid dehydrodimers [(8-5, 8-O-, 5-5'- and 8-8'- diferulic acids), Ralph et al., 1994; Iiyama et al., 1994 (Figure 4)] is a major obstacle which limits the accessibility of main chain-degrading enzymes to the structural polysaccharides thereby reducing the cell wall digestibility and terminate the expansion phase of cell growth in grasses. Controlling the spatial

terminate the expansion phase of cell growth in grasses. Controlling the spatial arrangement of ferulic acid within the matrix could regulate diferulic acid formation, and in turn extensibility (Grabber et al., 1998a & b).



(a)



(b)

Figure 3. Structure of ferulic acid esterified to arabinose residues a) at C-5 position b) C-2 position

(Source: Plant Science 127 (1997) 111 - 127, Reprinted with permission)

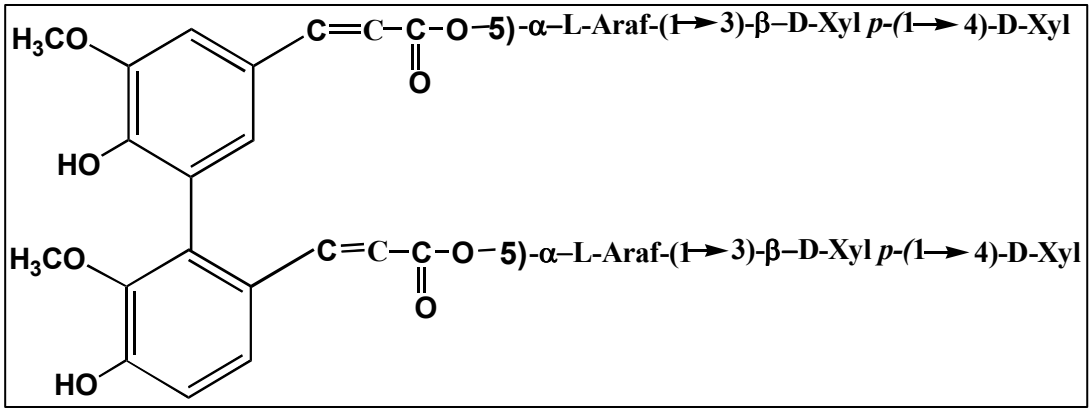


Figure 4. Structure of covalent diferulate cross link between arabinoxylans

1.2.4. Properties and uses of ferulic acid

Ferulic acid increases the hydrophobicity of the arabinoxylan molecule thereby decreasing its solubility. Ferulic acid acts as a strong antioxidant since it has the capacity to donate electrons. As the molecular mass of arabinoxylan increases, its antioxidant activity increases compared to the free ferulic acid, which may be perhaps due to the increase in both hydrophilic as well as hydrophobic regions of ester linked ferulic acid moieties (Graf, 1992).

Ferulic acid plays an important role in restricting the cell growth by the formation of cross-links between arabinoxylans and with other polysaccharides such as lignins, thereby preventing the extension of cell walls, and degradation caused by the invasion of pathogens (Hartley and Jones, 1977). It acts as a natural preservative in foods rich in fatty acids wherein it inhibits the lipid peroxidation. Because of its antioxidant nature, it has many health benefits (Bunzel et al., 2001). It has a strong absorbance of UV radiation thus can be used in sunscreen lotions and wound dressings. It acts as a precursor for various flavor compounds such as vanillin, vanillic acid and 4-vinyl-guaiacol in food industry (Lesage-Meessen et al., 1999). The removal of ferulic acid from arabinoxylans by enzymatic methods would allow the exploitation of the acid for industrial and food applications.

1.3. Cereal Non-Starch Polysaccharides-Functional and Nutritional Significance

Cereal arabinoxylans have been functionally characterized from various sources such as wheat, barley, maize, triticale, rye and ragi (Blanch Flower and Briggs, 1991; Dervilly-Pinel et al., 2001; Subba Rao et al., 2004; Shyama Prasad Rao et al., 2007). The arabinoxylans (1.5 - 2.5% in the cell walls) of wheat are classified in to water extractable (33%) and water unextractable (66%) arabinoxylans (Courtin and De1cour, 2002). The unextractable arabinoxylans in wheat are mainly due to the covalent and non-covalent interactions of arabinoxylans with the other cell wall components such as proteins, cellulose and lignin (Iiyama et al., 1994). Ferulic acid residues are mostly confined to the high molecular weight water extractable arabinoxylans in wheat. Due to less substitution, the enzymes can easily attack alkali extractable arabinoxylans than water extractable polymers (Gruppen et al., 1993).

Water extractable arabinoxylans from wheat bran contains more phenolic acid residues than water unextractable arabinoxylans from bran and water unextractable and water extractable arabinoxylans from endosperm. Furthermore, the ratio of diferulic and ferulic acid residues in the arabinoxylan are much greater in wheat bran and BSG than in endosperm, indicating a much higher level of cross-linking (Faulds et al., 2003). In solutions, the feruloylated arabinoxylans adopt more flexible conformations than non-feruloylated polymer (Izydorczyk and Biliaderis, 1995).

Acetyl groups are present at every second xylose residue in hard wood arabinoxylans. Acetylation hinders the packing of polysaccharide molecules thereby increasing their solubility in water (Bacon et al., 1975).

Considerable progress has been made for the past few decades on the functional aspects of cereal arabinoxylans because of their potential influence on water holding capacity and water balance (Jelaca and Hlynka, 1971), protein foam stabilization (Izydorczyk and Biliaderis, 1992a & b), viscosity, gelling and rheological properties of dough (Meuser and Suknow, 1986; Michniewicz et al., 1991) retrogradation of starch (Biliaderis and Izydorczyk, 1992) and bread quality (Delcour et al., 1991).

1.3.1. Viscosity

The stiff conformation of cereal arabinoxylans is due to the random arrangement of substituents, which provide high intrinsic viscosity in aqueous solutions compared to the non-cereal polysaccharides such as dextrans, arabinans, gums etc. Several factors such as overall asymmetric conformation, degree of polymerization and spatial arrangement of arabinose side chains on xylans will influence the behavior of arabinoxylans in solution. Cereal arabinoxylans with high Xyl/Ara ratio, feruloyl content, acetyl content show high η values than other non-starch polysaccharides (Izydorczyk and Biliaderis, 1993). The water extractable arabinoxylan in wheat interferes with gluten formation indirectly by cross linking ferulic acid residues there by increasing the viscosity, extensibility of dough. However, the presence of free ferulate competes with hydrogen peroxide/peroxidase in dough thereby decreasing the effect of feruloylated arabinoxylan interference (Wang et al., 2002a). Arabinoxylans in solutions behave as Newtonian fluids typical of pseudoplastic materials (Izydorczyk and Biliaderis, 1992a & b).

1.3.2. Foam stabilization

Protein solutions form a stiff viscoelastic layer at the air/water interface known as the foam. Many polysaccharide solutions are known to stabilize these protein foams against thermal disruption by interacting with them by forming electrostatic, hydrophobic and hydrogen bonds thereby maintaining the viscoelasticity of the protein foam (Susheelamma and Rao, 1979). The polysaccharide solutions increase the foam stabilization capacity of protein foam by increasing the foamability i.e. efficiency for gas encapsulation/retention with out collapsing. Surface shear or dilational viscosity shows that a polysaccharide solution forms high viscous fluids, thereby stabilizing the formed protein foam (Sarker et al., 1998).

1.3.3. Oxidative gelation

Water extractable arabinoxylans in the presence of free radical generating agents such as hydrogen peroxide/peroxidase, ferric chloride, linoleic acid/lipoxygenase, ammonium persulphate, tends to form three-dimensional networks

(gels or viscous solutions). This unique property of arabinoxylans known as oxidative gelation was first described by Durham (1925) in water extractable arabinoxylans of wheat, which mainly interferes with the gluten formation during dough preparation. Molecular weight, substitution of arabinoxylans, and especially the content of feruloyl groups influence the gelling mechanism of arabinoxylans. The cross-link formed by the coupling of ferulic acid residues i.e. the presence of diferulic cross-links in oxidized arabinoxylan is considered as the important aspect of Oxidative gelation of arabinoxylans (Geissmann and Neukom, 1973).

1.4. Arabinoxylan degrading enzymes

Enzymatic hydrolysis of cereal arabinoxylans requires the participation of several hydrolytic enzymes (Figure 5). Plant and microbial sources produce a combination of enzymes. These are classified in to two groups based on the nature of the linkages that they cleave, primarily the carbohydrases and secondly the esterases, which act synergistically in order to increase digestibility of the plant cell wall (Dekker, 1989).

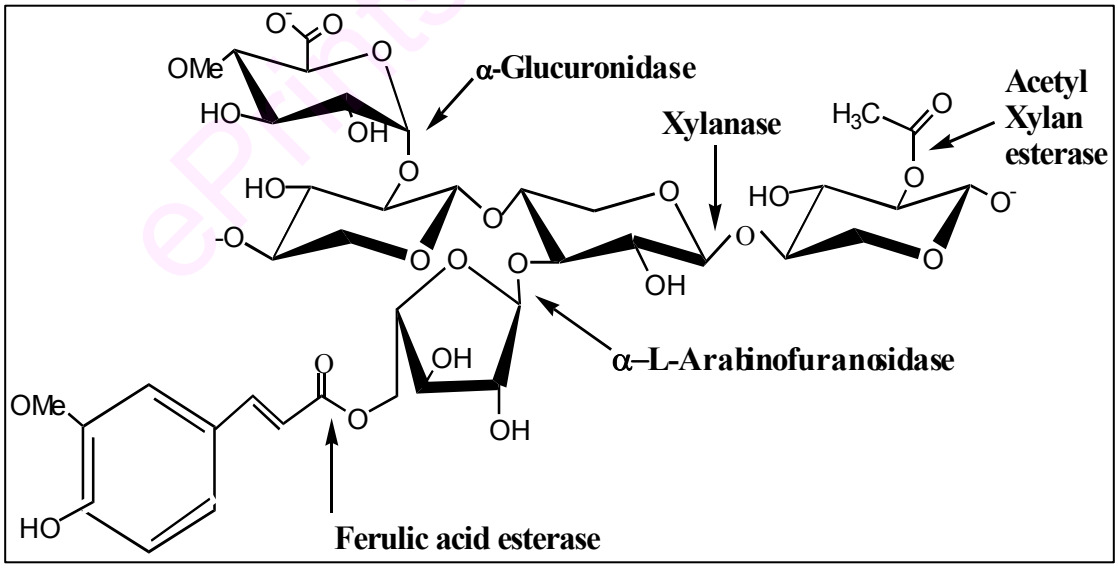


Figure 5. Structure of xylan and the different enzymes required for its complete hydrolysis

The first group of enzymes are hydrolases involved in the hydrolysis of the glycosidic bonds of xylan. These include endoxylanases (EC 3.2.1.8), which randomly degrades the xylan backbone to shorter xylooligosaccharides; β-xylosidase (EC 3.2.1.37), which cleaves the xylooligosaccharides to xylose in an exo-manner; α-L-arabinofuranosidase (EC 3.2.1.55) which removes arabinose from the xylan backbone and α-D-glucuronidase (EC 3.2.1.1) removes 4-O-methylglucuronic acid substituents from the xylan backbone.

The second group includes enzymes that cleave the ester linkages present in several biomolecules called esterases (EC 3.1.1.1) such as acetic acid esterase (AAE, EC 3.1.1.6) and cinnamic acid esterases (ferulic acid esterase, FAE, EC 3.1.1.73).

1.4.1. Esterases

Esterases belong to the class of hydrolases. Desnuelle (1951) defined esterases as the enzymes, which best hydrolyze esters of short chain fatty acids with monohydroxy alcohols (Figure 6). There are several esterases, which were purified and characterized from mammals, microbes and plant sources (few examples are shown in Table 1). Focusing the complete literature on these enzymes is beyond the scope of the present investigation. Accordingly, this introduction focuses on acetic acid esterase as well as ferulic acid esterase (AAE and FAE) mainly from cereals and to some extent from microbes.

1.4.2. Differences between esterases and lipases

Esterases and lipases catalyze the hydrolysis of an ester bond resulting in the formation of an alcohol and carboxylic acid. Both belong to the family of serine hydrolases, share structural and functional characteristics including a catalytic triad containing serine, aspartate which form hydrogen bond with histidine and show sequence homology i.e. Gly-Xaa-Ser-Xaa-Gly. Esterases differ from lipases by their substrate specificity and interfacial activation (Long, 1971). Esterases are more active towards esters of short chain fatty acids, but lipases are active towards esters of long chain fatty acids containing glycerol (Desnuelle, 1951). According to the classification of Hofstee (1960), fatty acid esterases include “esterases acting on

substrates in solution (esterase proper) and esterases acting predominantly on undissolved substrates (lipase-type esterases)".

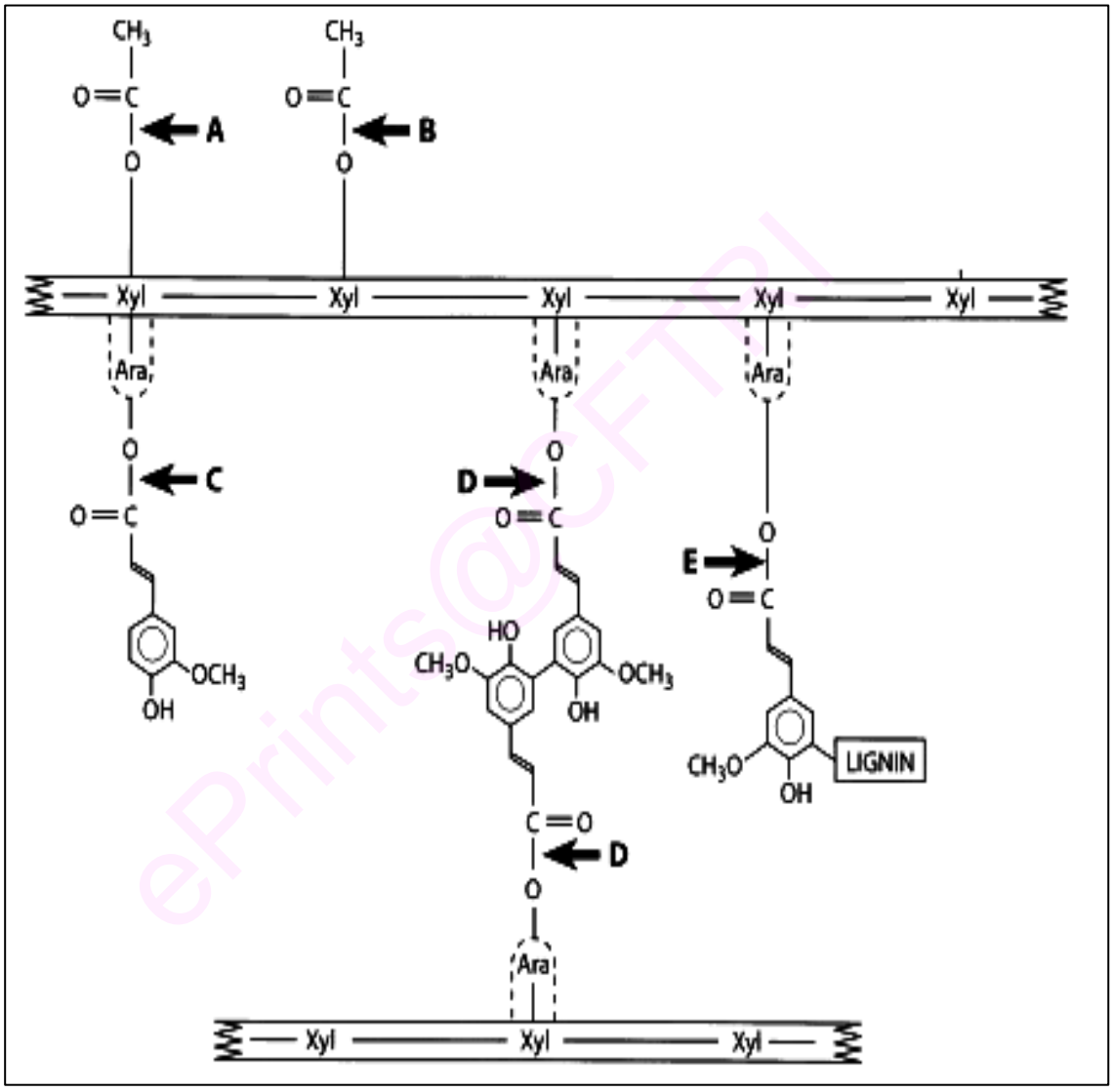


Figure 6. Mode of action of esterases
A, B-Acetyl xylan esterase; C, D, E- Ferulic acid esterase
(Source: Microbiology 144 (1998) 2011 - 2023, Reprinted with permission)

Table 1. Sources of esterases

Microbial sources	Bacteria:	*Streptomyces* (Nishimura and Inouye, 2000), *Pseudomonas* (Kim et al., 2002a & b; Horne et al., 2002), *Bacillus* (Degrassi et al., 1998), *Thermoanaerobacterium* (Shao and Wiegel, 1995), *Micrococcus* sp (Fernandez et al., 2004).
	Fungi:	*Penicillium* (Kroon et al., 2000), *Aspergillus* (Giuliani et al., 2001).
	Yeast:	*Saccharomyces* (Lomolino et al., 2003), *Candida* (Ghosh et al., 1991).
Plant sources	Barley (Sancho et al., 1999; Humberstone and Briggs, 2002a & b), Wheat (Cubadda and Quattrucci, 1974), Finger millet (Aravinda Upadhya et al., 1985), Root nodules of *Medicago sativa* (Pringle and Dickstein, 2004).	
Animal sources	Mammalian liver, Brain, Serum, Dental composites (Finer et al., 2004) etc.	

1.4.3. Applications of esterases

Some of the applications of esterases obtained from plants, microbes and animals are in the development of new drugs for Schistosomiasis, biomarkers for organo-phosphates in marine environment, assessment of poison due to pesticides and heavy metals (acetylcholine esterase); clinical medicine in human, poultry and fish (erythromycin esterase); preparation of (S)–flurbiprofen (recombinant esterase, PF1-K); transesterification reactions in organic solvents, production of isoamyl acetate and mannitol, detoxification of xenobiotics, control of physiological process of hormones, improvement of aroma and flavor, fatty acid production, resistant against inflammatory cells lysosomal enzymes, biodegradation of dental composites, metabolism of aspirin and non-narcotic analgesics, conversion of proparacetamol to paracetamol, in the resolution of α-aryl propionic acids of naproxen (anti-inflammatory agent), deblocking intermediate esters in the manufacture of β-lactam antibiotics in aqueous solutions etc (Panda and Gowrishankar, 2005).

1.4.4. Acetic acid esterase (AAE)

AAEs (EC 3.1.1.6) hydrolyse the ester linkages of the acetyl groups present in the xylan side chains. Various microbes are involved in the release of acetic acid in the presence of this enzyme (Table 2).

Very few attempts were made to purify and characterize AAE from cereals and were mostly restricted to barley (Humberstone and Briggs, 2002a). Studies on barley malt shows a range of esterases on native PAGE using α-naphthyl acetate (α-NA). The electrophoretic pattern impedes two slow moving bands which are thermo stable, cationic with a molecular weight of 47.0 kDa, and are designated as acetyl xylan esterases, while a fast moving thermo labile band was observed with an anionic charge and molecular weight of about 62.0 kDa, which is referred as ferulic acid esterase (FAE) (Sun et al., 2005). AAEs obtained from plant and animal sources differ from microbial acetyl xylan esterases (EC 3.1.1.72) with respect to their substrate specificity (Biely et al., 1996; Mitchell et al., 1990).

Structural analysis of acetyl xylan esterases from *Clostridium thermocellum* (*Ct*CE4) and *Streptomyces lividans* (*Sl*CE4) reveals that the enzyme from these sources are metal ion dependent and show chemical preference to Co^{2+} ions. *Streptomyces lividans* (*Sl*CE4) acetyl xylan esterase displays the classical His-His-Asp model while the enzyme from *Clostridium thermocellum* (*Ct*CE4) displays a different ligand coordination utilizing an aspartate, a histidine, and four water molecules (Taylor et al., 2006).

Most of the microbial acetyl xylan esterases showed sequence homology between them (Egana et al., 1996). However, the two thermo stable acetyl xylan esterases from the *Thermoanaerobacterium* sp showed differences with respect to N-terminal sequence, kinetic parameters and molecular size (Shao and Wiegel, 1995). The acetyl xylan esterase of *Bacillus pumilus* showed an N-terminal amino acid sequence of MQLFDLFLEELG with an internal sequence of ALEVIQSFPEVDEHR by Edman degradation (Degrassi et al., 1998).

The crystal structure of acetyl xylan esterase (II) from *Penicillium purpurogenum* determined at 85 k and 0.90 Å resolution has shown the presence of the catalytic triad Ser^{90}-His^{187}-Asp^{175} with a bound sulphate ion [(Figure 7a), Ghosh

et al., 2001]. Figure 7b shows the α/β helix/strands of acetyl xylan esterase (II) from *Penicillium purpurogenum* indicating the distribution of secondary structural elements of the enzyme.

Table 2. Microbial sources of AAE

Microbial source	Reference
Aspergillus aculeatus	Leeuwen et al., 1992
Aspergillus awamori	Sundberg et al., 1990
Aspergillus japonica	Khan et al., 1990
Aspergillus niger	Linden et al., 1994
Aspergillus oryzae	Tenkanen, 1998
Bacillus pumilus	Degrassi et al., 1998
Candida guilliermondii	Basaran and Hang, 2000
Cellvibrio japonicus (Pseudomonas fluorescens)	Ferreira et al., 1993
Coriolus versicolor	Tsujiyama and Nakano, 1996
Fibrobacter succinogenes	McDermid et al., 1990a
Orpinomyces sp. strain PC-2	Blum et al., 1999
Penicillium purpurogenum	Egana, et al., 1996
Ruminococcus sp	Aurilia et al., 2000
Schizophyllum commune	Halgasova et al., 1994
Streptomyces lividans	Dupont et al., 1996
Termitomyces clypeatus	Mukhopadhyay et al., 1997
Thermoanaerobacterium sp	Shao and Wiegel, 1995
Trichoderma reesei	Margoles-Clarke et al., 1996

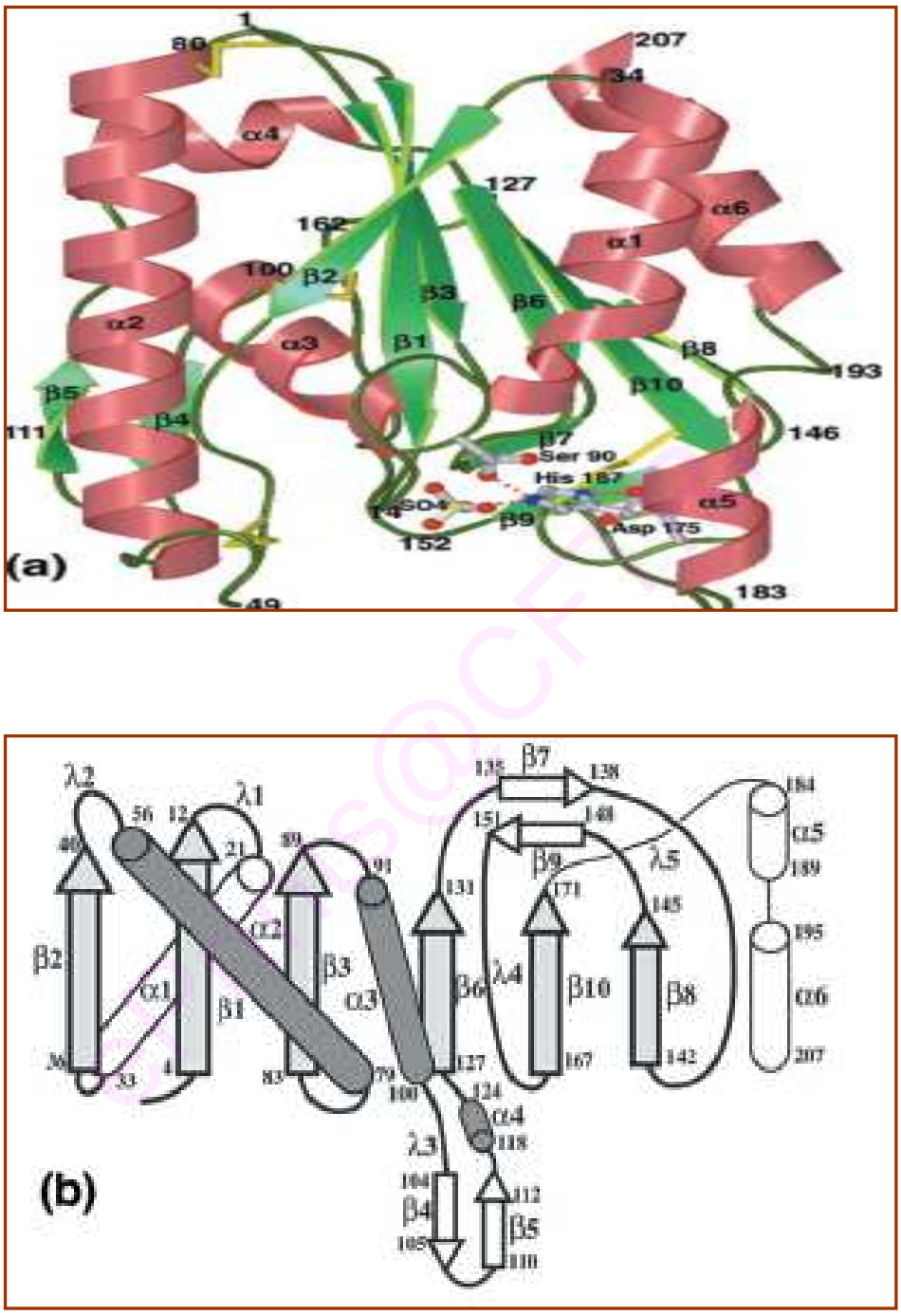


Figure 7. (a) Ribbon diagram and (b) Topology diagram of α/β hydrolase fold of acetyl xylan esterase (II) from *Penicillum purpurogenum*
(Source: JBC 276(14) (2001) 11159 – 11166, Reprinted with permission)

1.4.5. Ferulic acid esterase (FAE)

Feruloyl esterases (FAEs; also known as ferulic acid esterases, cinnamoyl esterases and cinnamic acid hydrolases; EC 3.1.1.73) are a subclass of the carboxylic acid esterases (EC 3.1.1.1) (Williamson et al., 1998). Out of several cinnamoyl esterases reported FAEs are widely studied from microbial sources. Some of the examples of microbial FAEs are shown in the **Table 3**.

Table 3. Microbial sources of FAE

Microbial source	Reference
Aspergillus awamori	McCrae et al., 1994; Koseki et al., 1998
Aspergillus niger	Kroon and Williamson, 1996
Butyrivibrio fibrisolvens	Dalrymple et al., 1996
Fibrobacter succinogenes	McDermid et al., 1990b
Fusarium sp	Topakas et al., 2003
Neocallimastix patriciarum	McSweeney et al., 1994
Schizophyllum commune	Mackenzie and Bilous, 1988
Streptomyces avermitilis CECT 3339	Ferreira et al., 1999
Streptomyces olivochromogenes	Faulds and Wiliamson, 1991

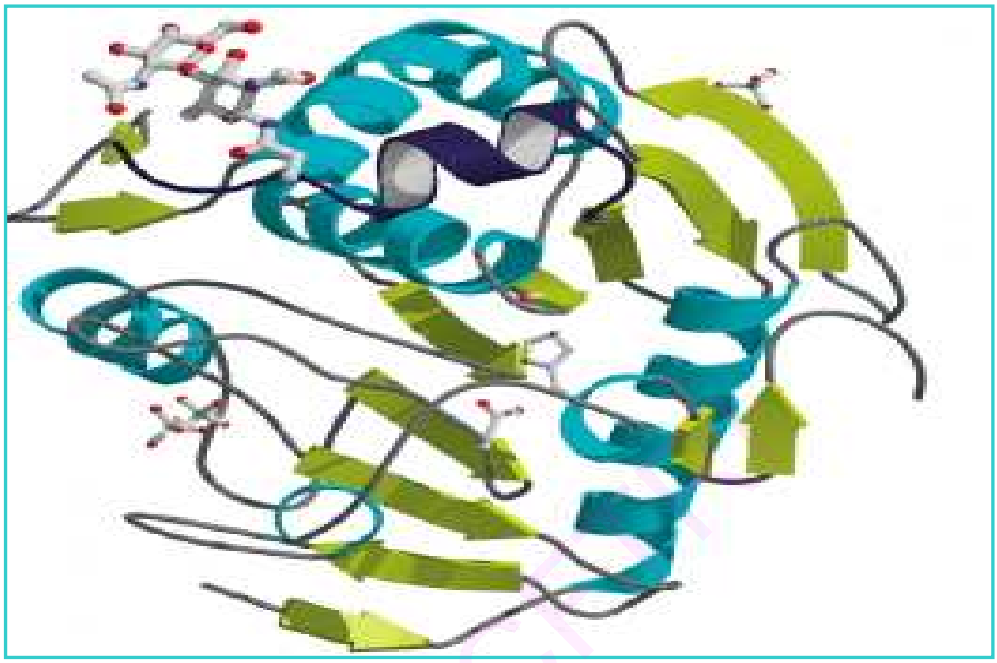
FAE from *Streptomyces olivochromogenes* was the first phenolic acid esterase to be purified to homogeneity from a bacterial source (Faulds and Williamson, 1991). FAEs have been divided into four types (A, B, C and D) depending on their (i) protein sequence (ii) specificity towards hydroxycinnamic acid methyl esters and (iii) ability to release diferulic acids from model and complex substrates. **Type A** FAEs, show specificity towards the phenolic acids such as ferulic acid and sinapic acid, which contain methoxy group at meta position. **Type B** FAEs, show specificity towards phenolic acids such as *p*-coumaric acid and caffeic acid, which contain one or two hydroxyl substitutions on phenolic moiety. **Type C** and **D** FAEs, show broad

specificity against synthetic hydroxycinnamic acids and differ in their ability to release 5, 5′ DiFerulic acids (Kroon et al., 1999).

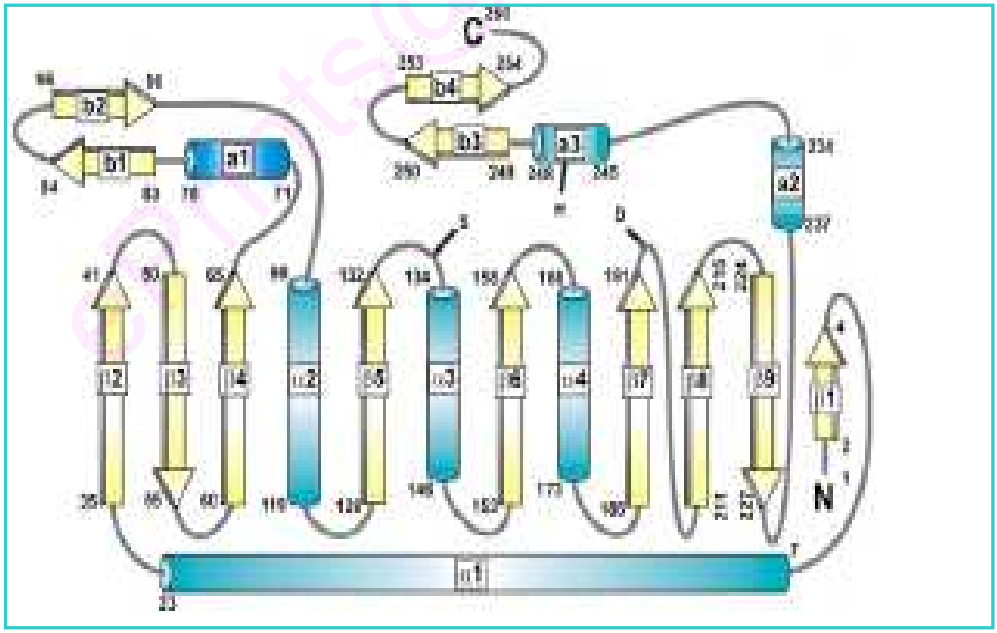
The recombinant FAE (FAEB) from *Penicillium funiculosum* showed N-terminal amino acid sequence of ASLTQVNNFGDNPGSLQMYIYVPNKLASKP which has sequence homolgy with acetyl xylan esterases from *Aspergillus* sp such as *awamori* (Koseki et al., 1997) and *niger* (De Graff et al., 1992) and also with putataive esterases from *Mycobacterium tuberculosis* (Cole et al., 1998) etc. The crystal structure and α/β hydrolase fold of FAE from *Aspergillus niger* (AnFaeA) is depicted in the figure 8a and b (Hermoso et al., 2004).

FAE can be used to remove ferulic acid from cell walls prior to treatment with polysaccharide hydrolases to assess its impact upon cell wall degradation. These enzymes show a reciprocal heterosynergy among themselves. This can be attributed by the observation that, the xylanase catalyses the breakdown of xylan into low molecular weight fragments which are appropriate substrates for esterases, whereas liberation of ferulic/acetic acids from arabinoxylan by FAE/AAE strongly enhance the accessibility of xylanases to the xylan backbone (Bartolome et al., 1995). The studies carried out by Faulds et al (2003), indicated that not only the source of xylanases but also the type of FAE present in conjunction with xylanase effect the efficient deferuloylation.

Cell wall solubilase activity of FAE was documented by the increased yields of β-glucans and pentosans isolated from the barley malt extracts (Bamforth et al., 1997). There is a considerable scope to utilize FAEs from cereals and their malts to modulate the functional properties of feruloyl arabinoxylans.



(a)



(b)

Figure 8. (a) Ribbon diagram and (b) Topology diagram of α/β hydrolase fold of FAE from *Aspergillus niger* (AnFaeA)
(Source: J. Mol. Biol. 338 (2004) 495 - 506, Reprinted with permission)

1.5. Isolation of esterases

Esterases from cereals and microbes are present either in the free or membrane bound forms. In cereals, mostly they occur as membrane bound enzymes. The free forms can be readily isolated using various buffers such as sodium acetate, sodium phosphate, Tris-HCl, MOPS buffer etc, while the membrane bound esterases were isolated using extraction media containing PVPP, reduced glutathione and Triton X-100 dissolved in buffer (Humberstone and Briggs, 2000a & b).

1.6. Purification of esterases

1.6.1. Fractional precipitation

Large volumes of crude enzyme extracts can be concentrated by fractional precipitation using salts such as ammonium sulphate and organic solvents such as ethanol and acetone. Most of the microbial esterases are precipitated and partially purified by ammonium sulphate fractionation (Egana et al., 1996; Degrassi et al., 1998). The main advantage in using such a salt is its salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Scopes, 1982). Since enzymes can be denatured more easily at high salt concentrations or in organic solvents at temperatures above 10°C, this step is carried out at low temperatures.

1.6.2. Chromatographic methods used for the purification of esterases

Several chromatographic methods i.e. both conventional and classical were used to purify esterases from cereals and microbes. The main drawbacks for the complete purification of cereal esterases are (a) the presence of multiple forms (isoenzymes) (b) broad substrate specificity and (c) high sensitivity (easy inactivation) (Ward and Bamforth, 2002).

Ion exchange is a chromatographic technique in which separation is achieved based on the charges carried by solute molecules. An ion exchanger contains insoluble matrix containing charged groups attached by covalent bonds. The fractionation of proteins/enzymes by ion exchange chromatography depends on their net charge and in turn the ionizable amino acids. Choice of buffer pH and ionic strength plays a pivotal role during ion exchange chromatography. pH of the buffer

should be one pH unit above or below the pI of the enzyme and ionic strength should be low, as the affinity of enzymes/proteins towards the ion exchanger decreases with increase in ionic strength. DEAE-cellulose anion exchange resins have been used for the purification of most of the cereal and microbial esterases (Humberstone and Briggs, 2002a & b; Dupont et al., 1996)

Size exclusion chromatography (SEC/GPC) is a column chromatographic technique, which is useful in the separation of molecules based on their size, or hydrodynamic volumes with respect to the average pore size of the gel matrix used. Sephacryl, Sephadex, Sepharose etc are the matrices used to purify esterases from cereals and microbes depending upon their molecular masses (Stellwagen, 1990).

Phenyl-Sepharose chromatography is used for the separation of molecules based on their hydrophobicity. The resin Phenyl-Sepharose CL-4B and Q-sepharose are used to purify the macromolecules especially the membrane proteins/enzymes containing hydrophobic binding sites (Steven and Konigsberg, 1981).

Chromatofocusing is a unique column chromatographic method used for the separation of proteins/enzymes based on their isoelectric points (pI). It offers high resolution due to the separation based on small difference in pI values (0.05 units) and high capacity of ion exchange technique used. The method has its own limitation due to the high cost of polybuffer used to elute proteins/enzymes (Makovee, 2000).

HPLC (High performance liquid chromatographic method)

HPLC is a non-destructive automated liquid chromatographic method with a capability for high-resolution separation of wide range of samples in a cumulative period. There is no laborious process for derivatization of samples and can be recovered after the chromatographic process. Separation is mainly by ion exchange or partition of compounds in a mobile phase. The compounds can be detected by either UV or refractive index detectors. Proteins/denatured peptides are mostly purified by preparative reverse phase HPLC using C_{18} column and are detected by UV detector both at 225 nm and at 280 nm (Mahoney and Hermodson, 1980).

1.7. Criteria of purity of esterases

There are different methods to ascertain the homogeneity of enzymes which include gel electrophoresis (native and SDS-PAGE), activity staining, charge, biological activity, reverse phase HPLC using C_{18} column, capillary electrophoresis, ESI-MS, N-terminal sequence etc (Price, 1996).

1.7.1. Gel electrophoresis

This can be carried out under native (based on the charge) and denatured (based on the mass/size) conditions to determine the purity and molecular mass of the enzyme by comparing with the standard molecular weight protein markers.

Activity staining of esterases was carried out by incubating the gel after electrophoresis in an activity staining solution. The activity staining solution for AAE (cereals/microbes) contain α/β-NA, fast blue RR/BB salt in sodium phosphate buffer (Hou and Lin, 1998), while for FAE the fluorescent substrate MUTMAC dissolved in phosphate/Tris buffer is used (Ferreira et al., 1999). The esterase activity was determined by observing a visible or fluorescent band on the gel.

1.8. Properties of esterases

1.8.1. pH optima and stability of esterases

Effect of pH on enzyme catalysis is mostly due to the ionization of amino acids at the active site regions, which in turn depicts the stability of enzyme structure and the ionization of substrate. Esterases have a pH optimum of 5.5 - 9.0 (Egana et al., 1996; Blum et al., 1999). The same esterase enzyme isolated from different sources exhibit difference in pH optima, reflects the physiological requirements of the source from which the enzyme was extracted. For the determination of pH optima of esterases (cereals/microbes) different buffers are used to maintain the maximum buffering capacity such as Glycine-HCl (2.0 - 3.0), sodium acetate (4.0 - 6.0), sodium phosphate (6.0 - 8.0), Tris-HCl (7.0 - 9.0). pH stability is one of the important criteria of an enzyme which helps to store the enzyme in pure form for longer periods. Esterases show broad pH stability especially from pH 6.0 - 9.0 retaining about 60 - 80% of the activity (Lee et al., 1987).

Ionic strength of buffer also plays an important role on enzyme activity due to the specific or non-specific effects of electrolytes. It affect the solubility of enzyme thereby the activity, because reactions require the diffusion of substrate towards the enzyme. Esterases show optimal activity at an ionic strength of 50 - 100 mM in various buffers (Humberstone and Briggs, 2000a & b).

1.8.2. Temperature optima, activation energy and thermal stability of esterases

All chemical reactions depend on temperature. It is one of the important parameter, which affect the rate of enzyme hydrolysis. The relationship between temperature and reaction rates can be described by the Arrhenius equation

$$K = Ze^{-E}a/2.3\ RT$$

where K is the rate constant, T the temperature in Kelvin, R the gas constant and Z is the frequency of favorable collisions between reactants. Arrhenius plots are used to analyze the temperature dependence of enzyme activation and inactivation. The effect of temperature on enzymatic reactions changes with pH, nature and thermal stability of substrate, ionization constants, kinetic parameters etc. The temperature optima of microbial FAEs range from 30 - 70°C (Rumbold et al., 2003; Faulds and Williamson, 1991). The thermal stability of cereal esterases range from 30 - 45°C (Sun et al., 2005), while microbial esterases show high thermal stability ranging from 30 - 90°C (Wang et al., 2004; Dupont et al., 1996).

1.8.3. Effect of metal ions

The structural studies on most of the microbial esterases indicate the possible presence of metal ions in the catalytic site, thus they can be considered as metallo enzymes (Taylor et al., 2006). Metal ions such as Cu^{2+}, Cu^{+}, Fe^{3+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Ni^{2+} etc are shown to effect the activity of esterases. The activating and inhibiting role of metal ions depends on the environmental conditions such as pH, temperature, ionic strength etc.

1.8.4. Active site analogues

The group specific compounds such as PCMB, PSMF, eserine, iodoacetamide etc are considered as active site analogues for most of the enzymes. PCMB and iodoacetamide are known to form complexes with cysteine residues in the active site region of the enzymes. Eserine, PMSF are the structural analogues of the amino acid serine, which is known to be present in the active site of several of the esterases (Kim and Lee, 2004; Cubadda and Quattrucci, 1974). Depending upon the type and source of esterases the active site analogues block the amino acids of the catalytic triad and inhibit the esterase activity.

1.9. Different techniques used to characterize the released products by the action of esterases

1.9.1. Spectroscopic methods

Spectroscopic methods give more details about the structure and they are much simpler to execute. The data obtained from these methods complement the data obtained from chemical and enzymatic methods. Some of the important spectroscopic methods are mass spectroscopy, nuclear magnetic resonance spectroscopy and infrared spectroscopy.

1.9.1.1. Mass spectroscopy

Mass spectroscopy is an analytical technique where the ions of the molecules are separated according to their m/z (mass/charge) ratio by deflection in a variable magnetic field to give a mass spectrum. A mass spectrum is a plot of relative abundance against the ratio of m/z. Mass spectrometry can be used in three principle ways

a. to measure relative molecular masses (molecular formulae).
b. to detect fragmentation pattern (presence of recognizable groups with in the molecule).
c. to identify the compound by comparing with molecular masses of known compounds.

In a mass spectrometer, organic molecules are bombarded with electrons to form high energy positively charged ions (molecular ions/parent ions) which further can break down into smaller ions (fragments/daughter ions). Mass spectrometry enables the structural information about a compound/molecule/protein. To obtain the molecular masses of a compound the three main stages involved in a mass spectrometry are a) sample preparation b) sample ionization and c) mass analysis. Sample ionization is carried out either by electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI). In both the methods, the molecules are converted into ions by the addition or loss of one or more protons. These methods do not need laborious sample derivatization steps, but provide valuable information on the molecular mass of the compounds and sequence of various biomolecules like small proteins, peptides, glycolipids, oligo and polysaccharides (Harrison and Cotter, 1990).

1.9.1.2. Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance (NMR) is a powerful non-selective analytical tool, which enables to ascertain molecular structure including relative configuration, relative and absolute concentrations, and even intermolecular interactions. ^{13}C and ^{1}H NMR together can give the molecular complexity and fine structure of compound in solution and solid state. The advent of modern pulse NMR in Fourier Transform (FT-NMR) mode shortens the time required for a scan by allowing a range of frequencies to be probed at a time, and the multi-dimensional FT-NMR provides a lot of structural information about the molecule. It can be used to ascertain the purity of biomolecules like peptides, proteins, polysaccharides etc (Lavertu et al., 1991).

1.9.1.3. Infrared spectroscopy

Infrared spectroscopy (IR) defined in a simple way, is the absorption measurement of different IR frequencies by a sample placed in the path of an IR beam. Infrared waves are absorbed by the vibrating chemical bonds in the polysaccharides giving characteristic IR spectra in the frequency range of 4000 - 400 cm^{-1}. Samples can be prepared in the form of pellets by mixing with dry KBr or in the

form of a smear by mixing with nujol (paraffin oil). In general, IR spectroscopy can be used for detection, configuration of functional groups and to know their substitution pattern. Different functional groups absorb characteristic IR frequencies. It is used for structural elucidation of arabinoxylans and their oligosaccharides (Kacurakova et al., 1998).

1.10. Substrates for esterases

1.10.1. Substrates for AAE

Various substrates both natural and synthetic were used to determine the activity of AAEs. Studies showed the use of acetylated oat spelt or birch wood or larch wood xylans (Basaran and Hang, 2000; Johnson et al., 1988b), cell wall polysaccharides of barley and wheat (Sun et al., 2005; Humberstone and Briggs, 2000b), α/β-NA (Poutanen and Sundberg, 1988), PNPA (Johnson et al., 1988a), 4-methyl umbelliferyl acetate (Shao and Wiegel, 1995).

1.10.2. Substrates for FAE

Studies on FAE revealed the use of methyl (Borneman et al., 1990b) and ethyl ferulates (McDermid et al., 1990b), feruloyl glycerol (Humberstone and Briggs, 2000a), PNPF (Mastihuba et al., 2002), 4-methyl umbelliferyl *p*-trimethyl ammonio cinnamate chloride (MacKenzie et al., 1987) as well as cell wall extracts released from cellulase-treated plant cell walls (Mackenzie and Bilous, 1988; Borneman et al., 1990a & b), feruloylated oligosaccharides obtained after enzymatic hydrolysis of sugar-beet pulp with driselase (Ralet et al., 1994) as substrates.

Studies indicate that less substituted xylans are considered the better substrates for the action of FAE (Sancho et al., 2001). Half of the commercial enzyme preparations exhibited FAE activity using feruloyl arabinose substrate (Hatfield et al., 1991). The release of ferulic acid from the feruloylated sugar esters by *Lactobacillus acidophilus* depicts that the smaller the degree of polymerization more the free ferulic acid released [Wang et al., 2004 (Figure 9)].

FAE differs from other cinnamic acid esterases in their substrate specificity. Studies on *Aspergillus niger* shows that the increase in the number of methoxy groups

and decrease in the number of hydroxyl groups on the aromatic ring increases FAE activity, while the reverse effect was observed with cinnamic acid esterases (De Vries and Visser, 2001).

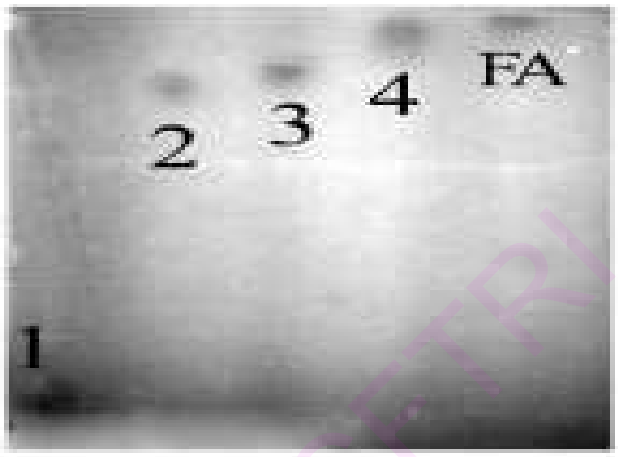


Figure 9. TLC for the release of ferulic acid from feruloylated sugars by FAE from *Lactobacillus acidophilus*
(Source: Appl. Environ. Microbiol. 70(4) (2004) 2367 - 2372, Reprinted with permission)

1.11. Assay methods of esterases

A number of analytical methods both quantitative and qualitative are available in the literature for the assay of AAEs and FAEs.

1.11.1. Assay of AAE

A spectrophotometric method described by Poutanen and Sundberg (1988) is generally used, to measure the enzyme activity by using α-NA dissolved in ethanediol as the substrate. The released product i.e. α-naphthol is monitored spectrophotometrically at 235 nm. One unit of AAE activity is defined as the amount of enzyme required to liberate one µmol of α-naphthol min^{-1}. The activity with PNPA was determined by monitoring photometrically the release of PNP at a wavelength of 400 nm making use of PNP standard graph (Chung et al., 2002).

Acetylated xylans incubated with AAE can be analyzed by HPLC for the released acetic acid using Aminex/HPX-87H ion exchange column using sulphuric acid (4 mM) as the eluent (Linden et al., 1994). The released acetate from various substrates such as glucose penta acetate, xylose tetra acetate and from acetylated polysaccharides can also be quantified using an enzymatic analysis kit from Boehringer Mannheim [(catalog no. 148261), Egana et al., 1996].

β-Xylosidase-coupled assay of acetyl xylan esterases and AAEs on various substrates like 2-*O*-acetyl 4-nitrophenyl β-D-xylopyranoside (2-Ac-NPh-Xyl), 3-*O*-acetyl 4-nitrophenyl β-D-xylopyranoside (3-Ac-NPh-Xyl), and 4-*O*-acetyl 4-nitrophenyl β-D-xylopyranoside (4-Ac-NPh-Xyl) helps in the release of PNP, xylose and acetic acid (Biely et al., 2004).

The AAE activity can also be determined on native PAGE using α/β-NA and fast blue RR salt as a greyish band (Hou and Lin, 1998).

1.11.2. Assay of FAE

A spectrophotometric method described by Mastihuba et al (2002) using PNPF dissolved in DMSO and Triton X-100 making the final volume with Tris-HCl buffer is generally the adopted procedure used for measuring the FAE activity. The released product PNP is then monitored spectrophotometrically at 400 nm. One unit of FAE activity is defined as the amount of enzyme required to liberate one μmol of PNP min^{-1}. The activity with feruloyl arabinose is determined by monitoring photometrically the release of ferulic acid at a wavelength of 340 nm as described by McCallum et al (1991).

HPLC analysis for the release of ferulic acid from ethyl ferulate, feruloyl glycerol and feruloylated oligo/polysaccharides in the presence of FAE are usually determined at both λ_{280} and at λ_{320} using C_{18} chromatographic column.

FAE activity can also be determined on native PAGE using fluorescent substrate MUTMAC where the released product methyl umbelliferone gives a green fluorescence against a blue background (Mackenzie et al., 1987). The coupled assay of FAE from *Aureobasidium pullulans* and α-L arabinofuranosidase from *Aspergillus niger* on the substrates *p*-nitrophenyl-5-feruloyl arabinofuranoside and *p*-nitrophenyl-

2-feruloyl arabinofuranoside results in the formation of ferulic acid, arabinose and PNP (Biely et al., 2002).

FAE activity is also determined by measuring the R_f values of ferulic acid and ethyl ferulate on precoated silica gel plates with UV_{254} indicator for the hydrolysis of ethyl ferulate. The solvent system containing ethyl acetate, benzene and 2-propanol (2:1:0.1) was used as mobile phase (Mastihuba et al, 2002).

1.12. Mechanism of action of AAE

The proposed mechanism of action of acetyl xylan esterase in comparison with the other carbohydrate esterases is presented in the figure 10. Aspartate anion acts as the catalytic nucleophile attacks carbon atom of ester or amide bond. Simultaneously removal of (2-amino-2-deoxy-) sugar is facilitated by a protonation of non-dissociated aspartic acid which act as the catalytic acid that remains, after the proton donation, in dissociated form.

Acetyl group is transferred to the nucleophile forming covalently linked acetyl-enzyme intermediate. The intermediate is then hydrolyzed by a water molecule whose nucleophilicity is increased by a proton removal by the catalytic aspartate anion (originating from the catalytic acid) acting as the catalytic base. Acetic acid as the second product is released and ionization states of the catalytic aspartates are restored (Puchart et al., 2006).

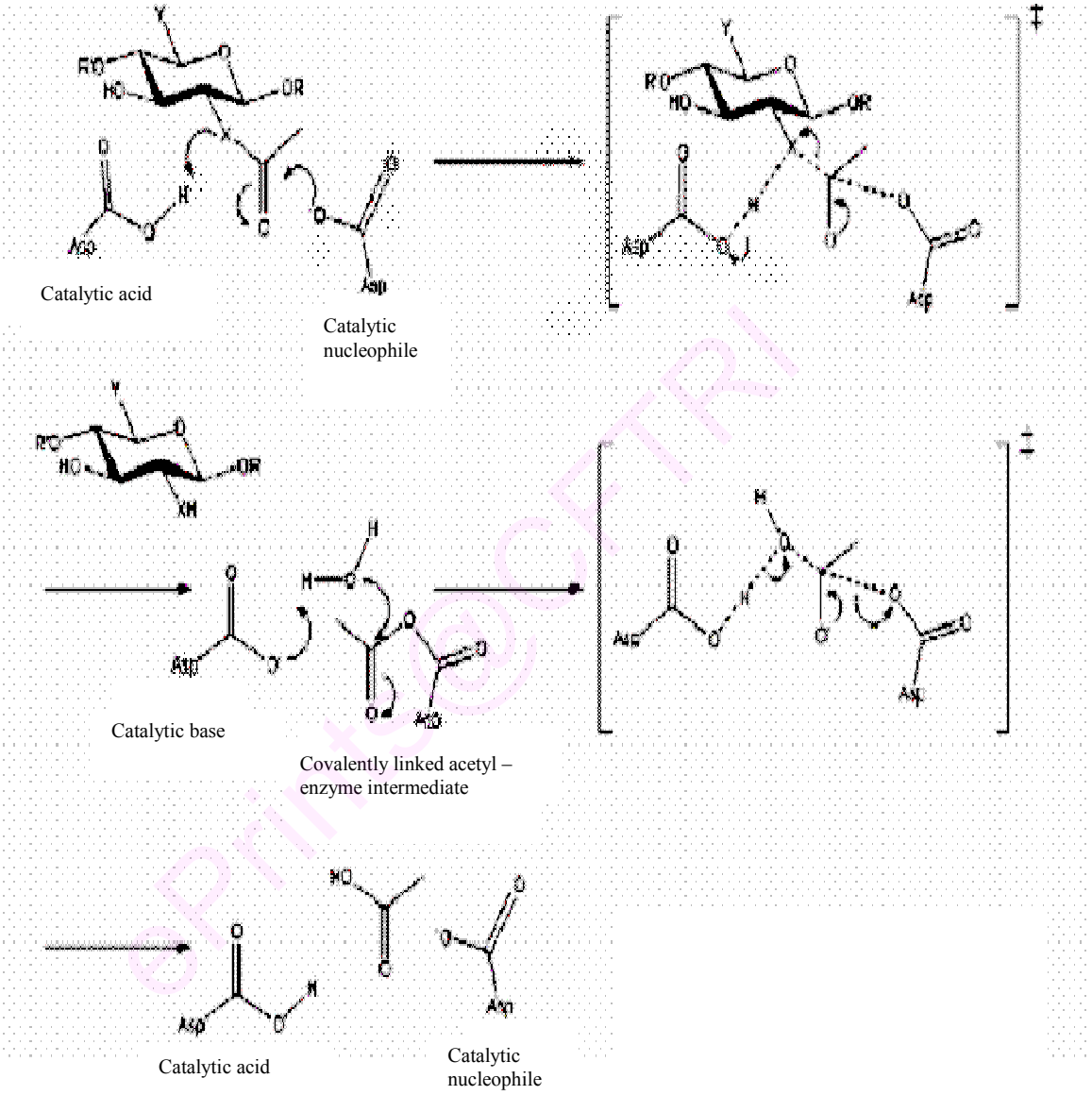


Figure 10. Mechanism of action of *Streptomyces lividans* acetyl xylan esterase
(Source: BBA 1764 (2006) 263 - 274, Reprinted with permission)

1.13. Recombinant esterases

The esterase genes of a number of microbes were cloned and expressed in *Escherichia coli* for the large-scale production of recombinant esterases. The *Bacillus* sp associated with the marine sponge *Aplysina aerophoba* shows the presence of two esterase genes (EstB1 and EstB2). Both these esterases were cloned and expressed in *Escherichia coli* under the control of T7 promoter using the vector pET-22b (+) (Karpushova et al., 2005). The cloned sequence in E. coli DNA obtained by screening the metagenomes of various soils for esterases shows ORF of 1089 base pairs of est25, which encodes a protein of 363 amino acids having conserved esterase sequence motifs and a catalytic triad of Ser, Asp and His (Kim et al., 2006). The recombinant ESTD (Figure 11) from hyperthermophilic bacterium *Thermotoga maritima* show identity with the carboxyl esterase (EST30) from *Geobacillus stearothermophilus* at the c-terminal part, thus this esterase (EST30) acts as a template for ESTD at this position.

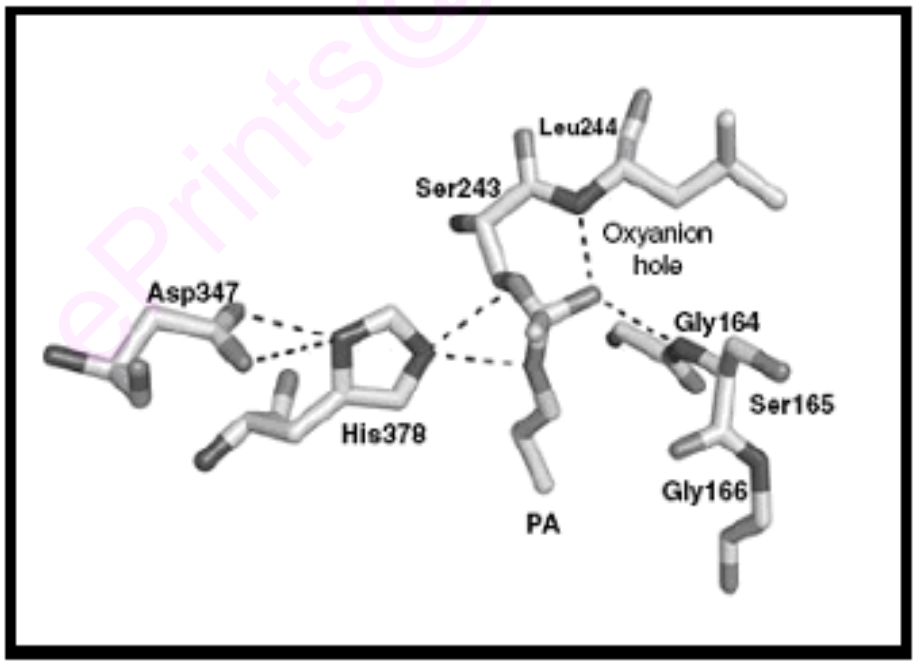


Figure 11. The active site region of ESTD with the bound ligand PA (propylacetate)
(Source: FEBS J. 274(11) (2007) 2832 - 2842, Reprinted with permission)

The recombinant esterases show identity with the non-recombinant esterases, with respect to the presence of catalytic triad containing serine, histidine and aspartate at the active site (Levisson et al., 2007; Karpushova et al., 2005).

1.13.1. Recombinant AAEs

The AAEs so far sequenced has showed a little homology between them and that of the various microbes (Dalrymple et al., 1997; Ghosh et al., 1998; Lorenz and Wiegel, 1997; Shareck et al., 1995; Tsujibo et al., 1997). In the case of *Orpinomyces* sp. strain PC-2, the purified mRNA was used as a template to construct cDNA library for the gene axeA in E. coli phage λZAPII. The gene showed an open reading frame (ORF) 939 base pairs, which encodes a protein of molecular weight 34.0 kDa (Blum, et al, 1999).

The AAE gene of hyperthermophilic archaeon *Sulfolobus solfataricus* strain MT4 was cloned and expressed in E. coli, where the complete plasmid (3.3 Kb genomic fragment) of the clone coded the complete esterase sequence of the archaeon (Morana et al., 2002). AfAXE is the gene encodes acetyl xylan esterase of *Aspergillus ficuum.* The corresponding cDNA contains two introns, one TATAA box, and two CAAT-like boxes, which was subsequently expressed in *Pichia pastoris*. The transcription initiation site was 61 bp upstream of the start codon and the amino acid sequence shows 28 amino acids (Hea-Jong et al., 2002). Acetyl xylan esterase cDNA of *Penicillium purpurogenum* was cloned with the pGEM-T vector system (Promega) (Gutiérrez et al., 1998).

1.13.2. Recombinant FAEs

Various microbial FAE genes such as *Aspergillus niger*, *Neurospora crassa*, *Cellvibrio japonicus (Pseudomonas fluorescens)*, *Butyrivibrio fibrisolvens* etc has been cloned and expressed in E. coli. The FAE (III) from *Aspergillus niger* was encoded by faeA gene. The sequence of this gene shows homology in the conserved regions with lipase from *Rhizomucor miehei*, but the FAE enzyme (FAE III) expressed from this gene could not hydrolyse di or triglycerides, the substrates of lipase from *Rhizomucor miehei* (De Vries et al., 1997).

The active site structure of the recombinant FAEs from *Neurospora crassa* (NcFae-1) (Figure 12) and *Aspergillus niger* (AnFaeA), are shown to form complexes with feruloyl oligosaccharides. Studies have showed that most of the microbial FAEs show homology and consense motifs with the catalytic triad of Ser, His, Asp.

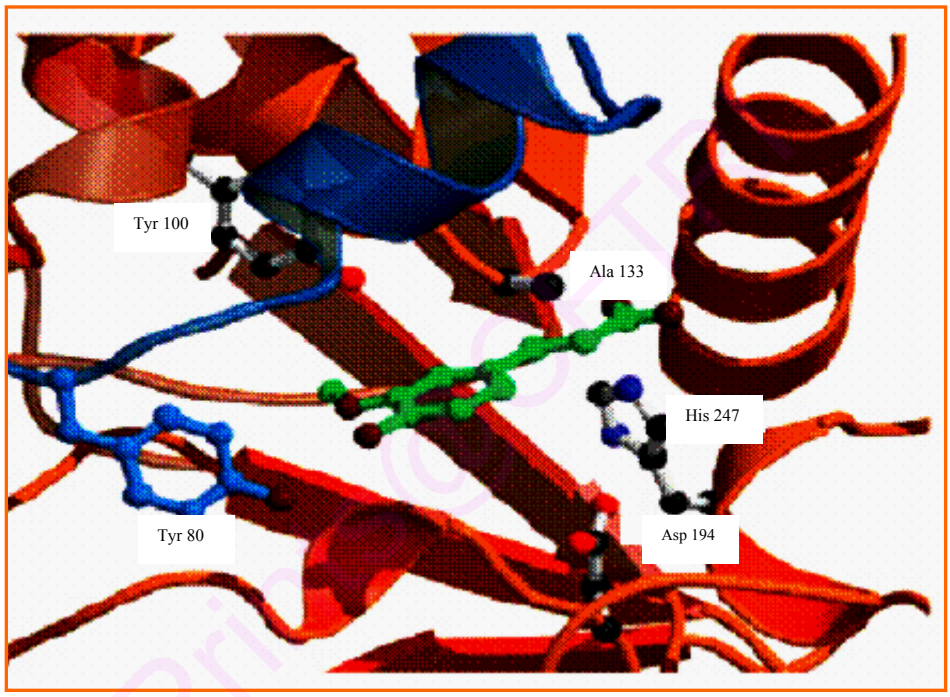


Figure 12. Active site model of *Neurospora crassa* FAE.
(Source: FEBS J. 272 (2005) 4362 - 4371, Reprinted with permission)

1.14. Malting and the biochemical changes

Malting is the controlled germination of cereals, followed by a termination of this natural process by the application of heat. Malting is carried out under three major stages i.e. steeping, germination and kilning. During steeping, the grain imbibes water until the water content of the grain reaches 42 to 48% and the moisture content increases progressively. Most of the cereal grains attain the saturation moisture content with in 24 h of steeping.

Various physiological and biochemical changes may take place during steeping like imbibition of seeds and degradation of carbohydrates by various

hydrolytic enzymes (Bacic and Stone, 1981). The key physiological events of malting, which determine the quality of the final malt, include rapid and uniform germination, the synthesis of hydrolytic enzymes in the scutellum and aleurone tissues surrounding the endosperm and finally the degradation of endosperm cell walls described as modification (Figure 13).

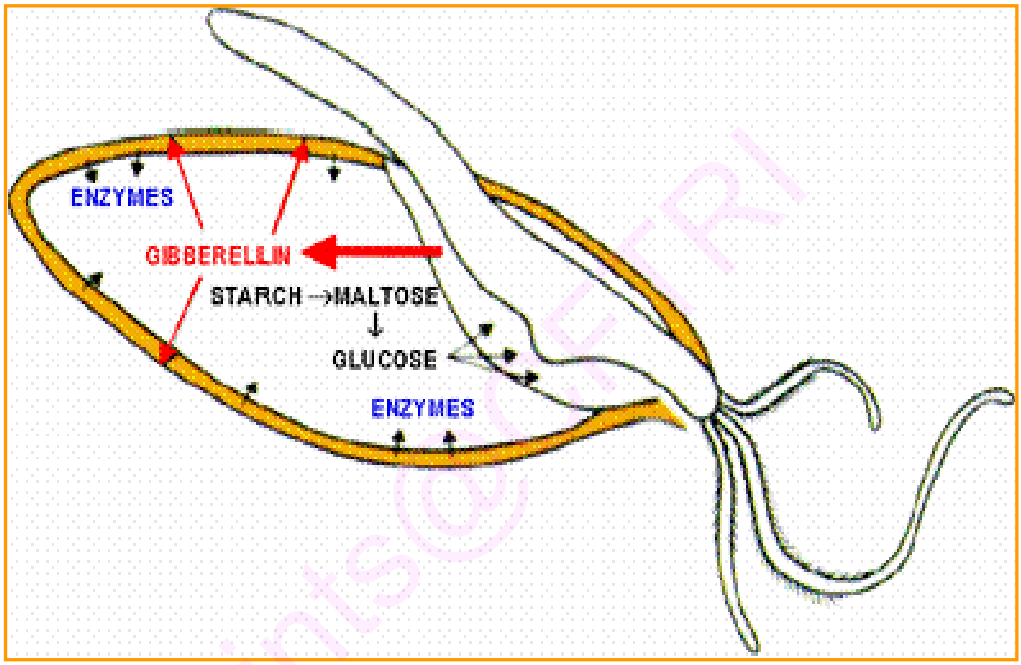


Figure 13. Hydrolase action and modification of barley grain during malting

Gibberellin, a plant hormone produced by the germinating embryo, regulates these physiological events. The enzyme activities obtained in germination can be arrested in by kilning i.e. subjecting the germinated seeds to high temperatures (>60°C). Some of the changes that occur during the process of kilning are inhibition of enzymes, maillard reactions, development of aroma and colour (Owuama and Asheno, 1994; Bathgate, 1973). Malting losses were higher for finger millet compared to rice, maize, and sorghum. Malting reduces the paste viscosity and raises the calorie density of the slurry, which is highly desirable in various food formulations (Brandtzaeg et al., 1981; Malleshi and Desikachar, 1979).

Aim and scope of the present investigation

The members (cereals/millets) of the Poaceae family (grass family) showed an enormous of agricultural importance from many decades because of their nutritional values. Millets are one of the important food crops cultivated in dry zones of Africa and in the semiarid regions of Asia. Important millets such as pearl millet, finger millet etc are usually consumed by the rural population in different forms both in the native and malted forms in India.

Finger millet

Finger millet *(Eleusine coracana)* also known as ragi (Figure 14a & b), is an indigenous minor millet, rich in calcium and dietary fiber. It is extensively consumed by south Indian rural population which is used both in the native and processed forms (Subba Rao and Muralikrishna, 2001). Content of different constituents of finger millet is shown in the **Table 4**.

(a) (b)

Figure 14. The study subject (a) Finger millet (ragi) plant (b) ragi grains

Ragi is a rich source of the amino acid methionine. Studies were reported from the department of Biochemistry and Nutrition, CFTRI, regarding (a) Isolation, purification and characterization of ragi amylases (Nirmala and Muralikrishna, 2003a; Muralikrishna and Nirmala, 2005), (b) Structure and functional relationship of alkali

soluble arabinoxylans (Subba Rao and Muralikrishna, 2004; Muralikrishna and Subba Rao, 2007) and (c) water extractable feruloyl polysaccharides and their antioxidant properties (Shyama Prasad Rao and Muralikrishna, 2006).

Table 4. Content of different constituents present in finger millet (Source: Shukla et al., 1986)

Constituents	Content present
Protein	4.6 - 5.7%
Total carbohydrate	73.7 - 83.1%
Soluble sugars	2.9 - 9.9 mg/100 g
Non protein nitrogen	0.14 - 0.34%
Calcium	325 - 474 mg/100 g
Magnesium	132 - 233 mg/100 g
Phosphorus	284 - 350 mg/100 g
Iron	6.7 - 10.2 mg/100 g
Potassium	88 - 104 mg/100 g
Zinc	2.5 - 3.5 mg/100 g
Copper	0.75 - 1.1 mg/100 g
Manganese	24 - 62 mg/100 g

Both alkali soluble arabinoxylans (hemicelluloses) and water-soluble feruloyl polysaccharides were proved to have an effect on the functionality of cereal non-starch polysaccharides with respect to (a) foam stabilization, (b) gelling and (c) viscosity (Subba Rao et al., 2004). Even though a report published in 1985, described the presence of carboxyl esterase from finger millet (Aravinda Upadhya et al., 1985) however, till date the effect of O-acetyl and feruloyl groups on the functionality of cereal water-soluble non-starch polysaccharides are not addressed.

Hence, a study was undertaken to purify and characterize AAE and FAE from

malted finger millet and explore their potential in modulating the functionality of various non-starch polysaccharides, which is one of the most important aspects in Food Science and Technology. Purification and characterization of AAEs and FAEs have not been attempted from finger millet, hence the present study with the following objectives-

1. Standardization of the experimental protocols with respect to the isolation of esterases from finger millet malt, with respect to ionic strength, pH, number of extractions and temperature. Preparation of synthetic substrate(s) for ferulic acid esterases (Feruloyl glycerol).
2. Purification of esterases using fractional precipitation, ion exchange and gel filtration chromatography. Ascertaining apparent homogeneity by native PAGE/SDS-PAGE. Determination of kinetic parameters of esterases i.e. pH, temperature optima and stability.
3. Studying the functional characteristics such as viscosity and foam stabilization of water-soluble polysaccharides of cereals in the presence of enriched enzyme isolates of esterases.
4. Role of esterases with respect to the mechanism of gelling properties of polysaccharides.

CHAPTER-2
MATERIALS AND METHODS

2.1. General

- All the results are average values of minimum of three experiments.
- Reagent preparations and extractions were carried out using double 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 range of ~10 kDa.
- Concentration/evaporation of samples was carried out by using Buchi Rota vapor (RE 111), Switzerland with a water bath temperature of 30 - 40°C.
- Colorimetric and spectrophotometric readings of test solutions with appropriate controls were taken by using Shimadzu double beam spectrophotometer (UV-160A), Japan.
- Lyophilization was carried out using Virtis Freeze Mobile (12 SL at -60°C), Germany.
- Centrifugation of samples was carried out using Sigma (202C), USA or Remi (RC 8), Bangalore or Hitachi Koki, Japan centrifuges.
- Gradient elution of ion exchange chromatography was carried out using Pharmacia gradient mixer, Uppsala, Sweden.
- Fractions eluted from ion exchange, gel permeation and hydrophobic interaction chromatographies were collected, by using Gilson FC203B fraction collector, France.

2.2. Materials

Authenticated varieties of finger millet (*Eleusine coracana*, ragi, Indaf-15) and wheat (*Triticum aestivum*, variety 225) procured from V.C. farm of the University of Agricultural Sciences, Bangalore, located at Mandya, Karnataka, India, were used for the entire studies.

2.3. Chemicals

Chemicals used in the present investigation were obtained from various agencies as described below.

2.3.1. Sigma Chemical Company, St. Louis, USA

Enzymes. Glucoamylase (EC 3.2.1.3) from *Aspergillus niger*, Peroxidase from horseradish (EC 1.11.1.7).

Substrates. α-naphthyl acetate (α-NA), *p*-nitrophenyl acetate (PNPA), ethyl ferulate, 4-methyl umbelliferyl *p*-trimethylammonio cinnamate chloride (MUTMAC), larch wood xylan.

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

Other chemicals. Ammonium persulphate, blue dextran, boron trifluoride dimethyl etherate, bovine serum albumin (BSA), coomassie brilliant blue G-250 (Bradford method of protein assay)/R-250 (Gel staining), *p*-chloromercuric benzoate (PCMB), (dimethylamino)pyridine (DMAP), ethylene diaminetetraacetic acid (EDTA), eserine, fast blue RR salt, ferulic acid, gel permeation chromatography (GPC) molecular weight markers (7.0 kDa to 210 kDa), gum karaya, locust bean gum, α-naphthol, *p*-nitrophenol, phenylmethylsulfonylfluoride (PMSF), polyvinylpolypyrrolidone (PVPP), thionyl chloride ($SoCl_2$), TEMED (N,N,N',N'-tetramethyl ethylene diamine), Triton X-100, xanthan gum.

Gel matrices and ion exchange resins. Sephacryl S-200.

2.3.2. Other sources

Genei, Bangalore, India. Protein molecular mass markers such as lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), egg albumin (43.0 kDa), BSA (66.0 kDa), phosphorylase (97.4 kDa) and myosin (205 kDa), for SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

Merck, Darmstadt, Germany. Pre-coated silica gel plates, 0.25 mm layer thickness with UV_{254} indicator.

Pharmacia fine chemicals, Uppsala, Sweden. DEAE-cellulose for anion exchange chromatography, Biogel P-30 for gel permeation chromatography, Phenyl-Sepharose CL-4B for hydrophobic interaction chromatography.

Sisco Research Laboratories, Mumbai, India. Acrylamide, bis-acrylamide, dimethyl sulphoxide (UV spectroscopic grade), glycine, hydrogen peroxide, reduced glutathione, Tris-HCl.

Qualigens fine chemicals, Bangalore, India. ammonium sulphate.

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

2.4. Instruments

Following instruments were used for various experiments-

- Magnetic stirrer from Remi, Bangalore, India.
- Analytical balance from Shimadzu, Japan
- B.O.D Incubator from Industrial and Laboratory Tools Corporation, Chennai, India.
- Incubator for enzyme assays: Julabo-Sw-20°C, Switzerland.
- Sonicator: Julabo USR 1, 220 volts, Germany
- Broviga mini slab gel electrophoresis unit from Balaji Scientific Services, Chennai, India.
- HPLC-LC 10A, equipped with RI and UV photodiode array detectors, from Shimadzu, Japan.
- GC-FT-IR-Perkin Elmer Spectrum system GC-IR 2000 Spectrophotometer, equipped with Windows 2.1 version, from Norwalk, USA.
- ESI-MS by Waters Corporation 2695 mass spectrometer (Model No. Q-Tof ultima), Manchester, United Kingdom.
- NMR by Bruker AMX 500 MHz spectrometer from Bruker Biospin, Germany.

2.5. Columns

HPLC analysis

a. Shimpak-C_{18} Column (ϕ 4.6 mm x 250 mm) from Shimadzu corporation, Tokyo, Japan.

b. Supelcosil-SAX1 ion exchange column (ϕ 4.6 mm x 250 mm) from Sigma-Aldrich Biotechnology LP and Sigma-Aldrich Co. St. Louis, USA.

2.6. Methods

2.6.1. Distillation/purification of solvents

Acetic anhydride, benzene, chloroform, dichloromethane, dimethyl sulphoxide, isopropanol, phenol, pyridine, toluene, triethylamine, were purified by distilling in all glass distillation apparatus following standard distillation procedures.

Water used in HPLC analysis was degassed millipore water. All the solvents used in the analysis were of HPLC grade. Enzyme extractions and purifications were carried out at 4°C unless and otherwise mentioned. The results obtained in the present investigation are the average values of three independent experiments.

2.6.2. Malting (Malleshi and Desikachar, 1979; Nirmala et al., 2000)

Ragi seeds (200 g each) were cleaned, surface sterilized with sodium hypochlorite (0.02%), steeped in double distilled water for 16 h (at 25°C) and germinated under controlled conditions on moist cloth in a B.O.D incubator for 120 h. Germinated seeds were withdrawn every 24 h (24, 48, 72, 96 and 120 h), dried at 50°C for 12 h and vegetative portions were removed manually by gentle brushing. Devegetated seeds and coleoptile portions were weighed, powdered and used for the experiments along with ungerminated ragi flour as control.

2.6.3. Colorimetric estimations

2.6.3.1. Total sugar (McKelvy and Lee, 1969)

To the sample (0.5 ml) in a test tube (ϕ 12 x 150 mm), phenol (0.3 ml, 0.5%) followed by concentrated H_2SO_4 (1.8 ml, specific gravity: 1.84) were added successively and mixed thoroughly. After incubation for 20 min, at room temperature

the absorbance was read at 480 nm. Total sugar content was determined from the standard graph prepared by using D-glucose as the reference standard (5.0 - 50 μg/ 0.5 ml).

2.6.3.2. Glucose estimation (Dahlqvist, 1964)

Preparation of glucose oxidase reagent. Glucose oxidase (125 mg, ~6250 units), dissolved in Tris buffer (0.1 M, pH 7.0). To this peroxidase [0.5 ml (10 mg/ml in water), ~1250 units] was added followed by O-dianisidine (0.5 ml, 10 mg/ml in 95% ethanol) and Triton X-100 (1.0 ml, 10 ml detergent in 40 ml methanol) and the solution was made up to 100 ml with Tris buffer, filtered and stored in a brown bottle.

To the sample (0.5 ml), glucose oxidase reagent (3.0 ml) was added and incubated at 37°C for 1 h. Absorbance was read at 420 nm. Glucose content was determined from the standard graph prepared by using D-glucose (10 - 50 μg/0.5 ml) as the reference standard.

2.6.3.3. Glucoamylase digestion (Gelatinization) of starch present in water extractable polysaccharides (WEPs)

WEPs (10 g) were dissolved in sodium acetate buffer (100 ml, 0.1 M, pH 4.8) and incubated with termamyl (1.0 ml, ~1000 units) at 95°C for 1 h to remove contaminating starch, cooled to room temperature. To these mixtures, glucoamylase (100 mg, ~7000 units) was added and incubated at 37°C for 48 h. Glucose released from the hydrolysis of starch of WEPs was quantified by glucose oxidase method as described above.

2.6.3.4. Protein by Bradford method (Bradford, 1976)

Preparation of Bradford reagent. Coomassie brilliant blue G-250 (10 mg) was dissolved in ethanol (5.0 ml, 95%) and to this phosphoric acid (10 ml, 85%) was added. The solution was made up to 100 ml with water, filtered through Whatman No.1 filter paper, and stored in a brown bottle at 4°C.

To the sample containing protein (0.2 ml), Bradford reagent (0.8 ml) was added and mixed well. Absorbance was read at 590 nm, after 10 min, using

appropriate reagent blank. Protein content was quantified from the standard graph prepared by using BSA (2.0 - 10 μg/0.2 ml) as the reference standard.

2.6.4. Spectrophotometric methods

2.6.4.1. Determination of protein/peptide (Peterson, 1983)

The presence of protein/peptide in column fractions was monitored by taking absorbance at 280/220 nm respectively.

2.6.4.2. Standard graph of PNP

Stock solution of PNP (5.0 mg) was prepared by dissolving in sodium/potassium phosphate buffer (0.2 M, pH 6.5). PNP solution was taken in a concentration range (2.5 - 12.5 μg) in the test tube and diluted to 1.0 ml with the above buffer. The absorbance at 400 nm was measured taking appropriate blank (buffer). The standard graph of PNP was used to quantify PNP released from PNPA/PNPF for the assay of AAE/FAE.

2.6.4.3. Standard graph of α-naphthol

α-naphthol (1.0 mg) was dissolved in ethanediol (10 ml) and the solution (1.0 - 10 μg) was taken in the test tube and diluted to 1.0 ml with Tris buffer (75 mM, pH 9.0). The absorbance at 235 nm was measured taking appropriate blank (buffer). The standard graph of α-naphthol was used to quantify α-naphthol released from α-NA (α-naphthyl acetate) for the assay of AAE.

2.6.4.4. Standard graph of ferulic acid

Ferulic acid (1.0 mg) was dissolved in minimum volume of methanol (50 μl) and made up to 10 ml with Tris-HCl buffer (50 mM, pH 7.0). Ferulic acid solution (1.0 - 10 μg) was taken in the test tube and diluted to 1.0 ml with buffer. The absorbance at 317 nm was measured taking appropriate blank (buffer). The standard graph of ferulic acid was used to quantify ferulic acid released from ethyl ferulate and WSPs for the assay of FAE.

2.6.5. Synthesis/Preparation of substrates for FAE

2.6.5.1. Feruloyl glycerol (Humberstone and Briggs, 2000a)

Ferulic acid and glycerol in a ratio of 1:20 were taken in a round bottomed flask wrapped with aluminum foil and closed by a trap containing anhydrous calcium chloride. Boron trifluoride dimethyl etherate was added (1:100 w/v) to catalyze the reaction. The mixture was stirred continuously at 50°C for 7 days or till the completion of the reaction. Shift in the absorbance spectra (200 - 400 nm) indicated the completion of reaction. Then the reaction mixture was allowed to cool and the boron trifluoride was neutralized with sodium bicarbonate (5.0 mM). The feruloyl glycerol thus formed was further purified on Amberlite XAD-4 column, where the unreacted glycerol was removed in the washing with water, and feruloyl glycerol was eluted using methanol. Anhydrous sodium sulphate was added in excess to the methanol-eluted fraction to remove moisture, filtered and concentrated, and stored at 4°C in the dark. The R_f values of feruloyl glycerol (0.52, 0.64) and ferulic acid (0.22) on a cellulose plate were detected by fluorescence under UV light.

2.6.5.2. *p*-nitrophenylferulate (PNPF) (Mastihubova et al., 2001)

PNPF was prepared according to the chemo enzymatic procedure with a slight modification. The synthesis of PNPF involved a four-step reaction.

Step I Acetylation (Hatfield et al., 1991)

In the first step, acetylation of ferulic acid (Figure 15a) was carried out to prevent side reactions of the 4-hydroxy moiety. Ferulic acid (3.0 g) was acetylated with acetic anhydride (4.8 ml) in pyridine (5.25 ml) and the reaction was kept for stirring (4 h), and monitored by TLC (using 5.0% methanol in chloroform). The reaction mixture was then quenched with 95% ethyl alcohol cooled to obtain crystals of 4-acetoxyferulic acid. The filtrate after recovery of the crystals of 4-acetoxyferulic acid was washed 3 - 4 times with toluene to remove pyridine. The resulting filtrate was re-dissolved in 95% ethyl alcohol to obtain a second crop of crystals of 4-acetoxyferulic acid. Re-crystallization of combined extracts gave pure 4-acetoxyferulic acid (3.6 g, mp 201 - 204°C, Figure 15b).

Step II Chlorination

4-Acetoxyferulate (3.2 g) obtained from Step I was dissolved in benzene (62 ml) in a round bottom flask (250 ml). Thionyl chloride (6.2 ml, 68.5 mM) was added slowly to the above solution and the mixture was refluxed in an oil bath (95°C) for 2 h. TLC was done (100% chloroform) to check the formation of 4-acetoxyferuloyl chloride. The solution was washed 2 - 3 times with toluene, and the resulting material was dissolved in boiled toluene which upon cooling, gave small white crystals of 4-acetoxyferuloyl chloride (2.8 g, mp 184 - 188°C, Figure 15c), which were stored in a vacuum desiccator over P_2O_5.

Step III Coupling reaction (Esterification) (Lu and Ralph, 1998)

Coupling of 4-acetoxyferuloyl chloride with PNP was carried out using 4-(dimethylamino) pyridine (DMAP). 4-acetoxyferuloyl chloride (2.4 g) and PNP (2.1 g, 1.5 equivalent) were dissolved in dry dichloromethane (CH_2Cl_2, 111.4 ml) to which DMAP (295 mg, 0.25 equivalent) and Triethylamine (Et_3N) (1.16 ml, 0.85 equivalent) were added. The reaction mixture was stirred until the completion for ~2 h as indicated by TLC (chloroform/ethyl acetate) analysis. The solution was diluted with CH_2Cl_2 and washed with 3.0% HCl and saturated ammonium chloride till the pH of the lower organic layer was 7.0. The organic layer was dried over anhydrous sodium sulphate and filtered. The resulting mixture obtained after coupling was purified by flash chromatography on a silica gel column by using chloroform to remove traces of unreacted PNP, ferulic acid and 4-acetoxyferuloyl chloride. The esterified 4-acetoxyferulate was eluted in the first quarter of the bed volume and the purity was checked by TLC (chloroform). The resulting *p*-nitrophenyl-4-acetoxyferulate (Figure 15d) was evaporated and preparative TLC (using chloroform as eluent) was carried out to obtain the product, which was 99% pure, as determined by NMR.

Step IV Selective deacetylation (Mastihubova et al., 2001)

In this step, the solvents CH_2Cl_2 and 2-propanol were pre-dried over molecular sieves, 3Å. The esterified 4-acetoxyferulate (100 mg) was dissolved in CH_2Cl_2 (8.0 ml) and 2-propanol (4.0 ml) and lipase PS (465 mg) from *Burkholderia*

cepacia was added to the solution. The reaction mixture was incubated on a shaker (250 rpm) for 3 days at 40°C or until the disappearance of the starting material, which was monitored by TLC at every 6 h interval. Selective deacetylation of esterified 4-acetoxyferulate gave PNPF (Figure 15e). The reaction was terminated by filtration of the enzyme and the filtrate was concentrated by evaporation of the solvents. The product of each reaction was characterized by ^{1}H and ^{13}C NMR.

^{1}H and ^{13}C NMR spectral analysis

2.0 mg of product formed in each step was dissolved in deuteriated chloroform ($CDCl_3$) and analyzed by ^{1}H NMR and ^{13}C NMR using Bruker 500 MHz spectrometer.

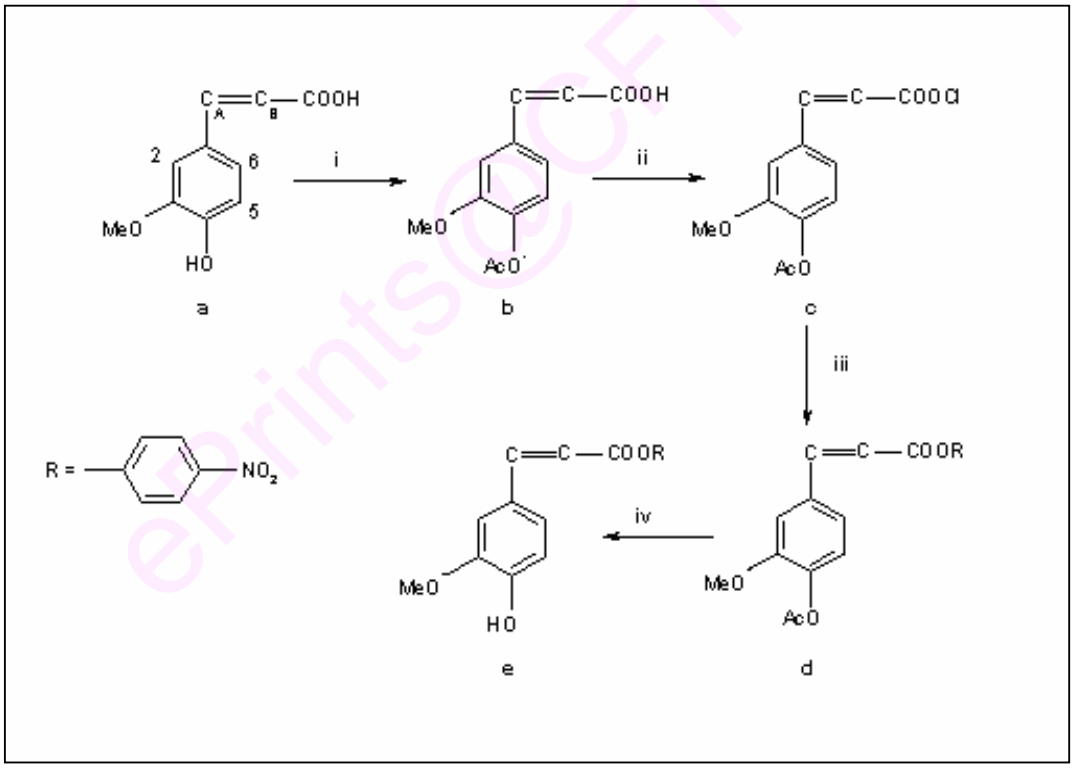


Figure 15. Scheme for the synthesis of PNPF

i) $(CH_3CO)_2O$ + Pyridine ii) $SoCl_2 + C_6H_6$ iii) DMAP + $CH_2Cl_2 + Et_3N$
iv) Lipase, 40°C, 3 days R: *p*-nitrophenol (PNP)
a) Ferulic acid b) 4-acetoxyferulate c) 4-acetoxyferuloylchloride
d) PNP-4-acetoxyferulate e) PNPF

2.6.5.3. Isolation of feruloyl polysaccharide from wheat bran (Shyama Prasad Rao and Muralikrishna, 2004)

Wheat bran (10 g) was dispersed in a beaker containing water (50 ml) and stirred for 2 h at 4°C followed by centrifugation (7000 x g, for 20 min) to separate the supernatant containing bound feruloyl polysaccharide. Supernatant was concentrated (5.0 ml) and precipitated with three volumes of ethanol. The precipitated polysaccharide was centrifuged (7000 x g, for 20 min) to separate the precipitate, which was dissolved in minimum volume of water and dialyzed against water. This substrate preparation contains covalently bound ferulic and coumaric acids.

2.6.6. Enzyme assays

Native and malted seeds (1.0 g) were extracted with different buffers for obtaining different enzymes; i.e. Tris-HCl buffer (75 mM, pH 7.5) for obtaining AAE (Humberstone and Briggs, 2000b) and Tris-HCl buffer (50 mM, pH 7.0) for obtaining FAE (Humberstone and Briggs, 2000a). Extractions were carried out at 4°C for 2 h. After centrifugation (7000 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.

2.6.6.1. AAE (Poutanen and Sundberg, 1988)

AAE activity was measured by using α-NA (1.0 mM) dissolved in ethanediol as the substrate. The reaction mixture containing AAE and α-NA (made to a final assay volume of 1.0 ml with 75 mM Tris-HCl buffer, pH 9.0) was carried out for 30 min at 30°C and the reaction was stopped by adding H_2SO_4 (100 µl of 0.33 M). One unit of AAE activity is defined as the amount of enzyme required to liberate one µmol of α-naphthol min^{-1}. The reaction was monitored spectrophotometrically at 235 nm for the release of α-naphthol.

The activity with PNPA was determined by monitoring spectrophotometrically for the release of PNP at a wavelength of 400 nm making use of PNP standard graph (2.5 - 12.5 µg) (Chung, et al., 2002).

2.6.6.2. FAE

2.6.6.2a. Spectrophotometric assay using PNPF (Standard method) (Mastihuba et al, 2002)

PNPF (2.0 mg) was dissolved in DMSO (50 µl) and Triton X-100 (50 µl), and the volume was made with Tris-HCl buffer (5.0 ml, 50 mM, and pH 7.0). The reaction mixture containing FAE and PNPF (made to a final assay volume of 1.0 ml with 50 mM Tris-HCl buffer, pH 7.0) were incubated at 37°C for 60 min. Suitable enzyme and substrate blanks were maintained, as the substrate used is highly unstable. One unit of FAE activity is defined as the amount of enzyme required to liberate one µmol of PNP h^{-1}. The reaction was monitored spectrophotometrically at 400 nm for the release of PNP from PNPF making use of PNP standard graph (2.5 - 12.5 µg).

2.6.6.2b. HPLC assay (Mastihuba et al., 2002)

One volume of ethyl ferulate (0.01 M ethyl ferulate in 0.5 ml of Tris buffer, 50 mM, pH 7.0) and three volumes of enzyme solution were incubated at 37°C for 60 min. One volume aliquot of reaction mixture was withdrawn and mixed with three volumes of methanol to stop the reaction. The reaction mixture was mixed by vortexing followed by centrifugation (7000 x g, for 5 min) to separate the supernatant from the residue. The released ferulic acid was monitored by HPLC analysis both at λ_{280} and at λ_{320} using ferulic acid (0.01 mg - 0.1 mg) as reference standard.

2.6.7. Isolation of WEP and WUP (Shyama Prasad Rao and Muralikrishna, 2004)

Flour (Ragi/Wheat, 100 g) was extracted with water (200 ml x 4 at 25°C) for 2 h each and the supernatants obtained after centrifugation (4000 x g, for 10 min) were pooled and concentrated by evaporation and precipitation with ethanol (3 volumes). Precipitate was separated by centrifugation and dissolved in minimum volume of water (10 ml), dialyzed and lyophilized to obtain water extractable polysaccharides (WEPs). The residue obtained after centrifugation was treated with glucoamylase to digest the starch present in the undigestable material followed by centrifugation to obtain the starch free residue, which was dried by solvent exchange and designated as water unextractable polysaccharides (WUPs).

2.6.7.1. Preparation of WSP

WEP was dissolved in water (1:10, w/v) by stirring for 30 min and insoluble portion if any was separated by centrifugation (4000 x g, for 10 min). Soluble portion was heated (95°C for 10 min) to denature enzymes and precipitate proteins. The supernatant obtained after centrifugation was dialyzed and lyophilized to obtain water-soluble non-starch polysaccharides (WSPs).

2.6.8. Isolation and characterization/quantification of free and bound ferulic acid

2.6.8.1. Free ferulic acid (Ayumi et al., 1999)

Ragi and wheat flours (2.0 g) were extracted with aqueous ethanol (70%, 50 ml x 4, for 1 h each, at room temperature) and the supernatants obtained upon centrifugation (4000 x g, for 10 min) were concentrated and adjusted the pH to 2 - 3 with HCl (4.0 M) and ferulic acid was separated by ethyl acetate phase separation (50 ml x 4) followed by drying with anhydrous sodium sulphate, filtered and evaporated to dryness. The dried material was taken in methanol (1.0 ml) analyzed by HPLC on C_{18} column (ϕ 4.6 x 250 mm) using photodiode array detector operating at 280 and 320 nm with a solvent system of water:acetic acid:methanol (isocratic, 75:5:20 v/v/v, flow 1.0 ml/min). Ferulic acid was used as standard (0.01 - 0.1 mg/10 µl). Ferulic acid in the sample is quantified by measuring the peak area of ferulic acid.

2.6.8.2. Bound ferulic acid (Nordkvist et al., 1984)

Ragi and wheat flours (1.0 g each) were extracted with NaOH (1.0 M, 100 ml x 2, at room temperature) containing sodium borohydride (0.5%) under nitrogen atmosphere for 2 h each and centrifuged (4000 x g, for 20 min). The supernatant was collected acidified (pH 1.5) with HCl (4.0 M) and processed further as described for free ferulic acid.

2.6.9. Isolation of esterases from ragi malt

2.6.9.1. Isolation of AAE (Humberstone and Briggs, 2000b)

AAE was extracted from 72 h malted ragi flour and powdered coleoptiles using different extractants such as Tris-HCl buffer (25 - 100 mM, pH 7.0 - 9.0),

sodium acetate buffer (50 mM, pH 4.0 - 6.0), and sodium phosphate buffer (50 mM, pH 6.0 - 8.0) in the presence of reduced glutathione (25 - 100 mM), PVPP (0.25 - 1.0%), Triton X-100 (0.25 - 1.0%), $CaCl_2$ (2 - 10 mM) and $MgCl_2$ (2 - 10 mM) for 2 h at 4°C and the supernatants were collected by centrifugation (7000 x g, 4°C for 20 min) using refrigerated centrifuge and dialyzed against the extraction buffer and used for further experiments.

2.6.9.2. Isolation of FAE (Humberstone and Briggs, 2000a)

FAE was extracted from 96 h malted ragi flour (Indaf-15) and powdered coleoptiles using different extractants such as Tris-HCl buffer (25 - 100 mM, pH 7.0 - 9.0), sodium acetate buffer (50 mM, pH 4.0 - 6.0), sodium phosphate buffer (50 mM, pH 6.0 - 8.0) in the presence of reduced glutathione (25 mM), PVPP (1.0%) and Triton X-100 (1.0%), for 2 h at 4°C and supernatants were collected by centrifugation (7000 x g,, 4°C for 20 min) using refrigerated centrifuge and dialyzed against the extraction buffer and used for further experiments.

2.6.10. Purification of esterases from ragi malt

2.6.10.1. Fractionation by ammonium sulfate (Ward and Bamforth, 2002)

AAE and FAE enzyme extracts obtained from 72 h and 96 h malted ragi flours (50 g) were extracted with 75 mM Tris-HCl buffer (1:7, pH 9.0, 350 ml) and 50 mM Tris-HCl buffer (1:7, pH 7.0, 350 ml) respectively, containing reduced glutathione, Triton X-100 (w/v) and PVPP were taken for the purification. The crude enzyme extracts obtained were subjected to ammonium sulphate precipitation and the precipitates i.e., 0 - 20, 20 - 40, 40 - 60 and 60 - 80% were removed by centrifugation (12000 x g, for 30 min), resolubilized in Tris-HCl buffer (20 mM, pH 8.5, AAE and 20 mM, pH 9.0, FAE), dialyzed, activities and protein concentration of the respective esterases were estimated

2.6.10.2. Chromatographic methods

2.6.10.2a. Ion exchange chromatography (Humberstone and Briggs, 2002a & b)

Ion exchange chromatography was carried out on DEAE-cellulose column.

Regeneration of DEAE-cellulose

The anion exchanger (100 ml) was washed 2 - 3 times with water to remove fines and treated with HCl (0.5 N) for 30 min at room temperature with occasional stirring. This was thoroughly washed with water to remove excess acid to obtain neutral pH. It was subsequently treated with NaOH (0.5 N) for 30 min and washed thoroughly with water in the similar manner to get neutral pH and stored in Tris-HCl buffer (pH 8.5/9.0, 40 mM at 4°C).

Purification of esterases on DEAE-cellulose

AAE from 40 - 80% ammonium sulphate fraction of 72 h ragi malt

Regenerated and degassed anion exchanger (30 ml) was packed in a glass column (1.5 x 18 cm), pre-equilibrated with Tris-HCl buffer (40 mM, 2 x 28 ml; 20 mM, 5 x 28 ml, pH 8.5) at a flow rate of 18 ml h^{-1}. The AAE enriched ammonium sulfate fraction (40 - 80%) was dialyzed against equilibrating buffer and loaded on to a DEAE-cellulose column. The unbound proteins were eluted with equilibrating buffer. A linear NaCl gradient (0.0 - 0.6 M) in equilibrating buffer was used to elute the bound proteins, which were collected (1.5.ml each) and monitored for protein (280 nm) as well as AAE activity.

FAE from 40 - 80% ammonium sulphate fraction of 96 h ragi malt

Regenerated and degassed anion exchanger was packed in a glass column (2.25 x 25 cm) pre-equilibrated with Tris-HCl buffer (40 mM, 2 x 56 ml; 20 mM, 5 x 56 ml, pH 9.0), at a flow rate of 24 ml h^{-1}. The FAE enriched ammonium sulfate fraction (40 - 80%) was dialyzed against equilibrating buffer and loaded on to a DEAE-cellulose column. The unbound proteins were eluted with equilibrating buffer. A linear NaCl gradient (0.0 - 0.7 M) in equilibrating buffer was used to elute the bound proteins, which were collected (4.0 ml each) and monitored for protein (280 nm) as well as FAE activity.

2.6.10.2b. Gel permeation chromatography (Humberstone and Briggs, 2002a & b)

Purification of DEAE cellulose active fractions of AAE on Sephacryl S-200

The pre-swollen gel was degassed and packed in a glass column (0.6 x 90 cm) and pre-equilibrated with Tris-HCl buffer (10 mM, pH 7.0) at a flow rate of 6.0 ml h^{-1}. The active fractions from the anion exchange chromatography were pooled, dialyzed, concentrated and loaded on Sephacryl S-200 column. Fractions (1.5 ml) were collected and monitored for protein and activity.

Purification of DEAE cellulose active fractions of FAE on Biogel P-30

The pre-swollen gel was degassed and packed in a glass column (0.7 x 100 cm) and pre-equilibrated with Tris-HCl buffer (10 mM, pH 7.0) containing NaCl (150 mM) and EDTA (1.0 mM) at a flow rate of 12 ml h^{-1}. The active fractions from the anion exchange chromatography were pooled, dialyzed, concentrated and loaded on Biogel P-30 column. Fractions (2.0 ml) were collected and monitored for protein and activity. The pooled and dialyzed active fractions obtained from Biogel P-30 were concentrated and used for further characterization of FAE.

2.6.10.2c. Hydrophobic interaction chromatography (Steven and Konigsberg, 1981)

Regeneration of Phenyl-Sepharose CL-4B

The pre-swollen gel was degassed and packed in a glass column (1.5. x 7.0 cm), pre-equilibrated with a bed volume of distilled water followed by ethanol, n-butanol and then again by ethanol and distilled water. Finally the column was equilibrated with sodium phosphate buffer (5.0 mM, pH 6.5) containing 1.0 M $(NH_4)_2SO_4$.

Purification of Sephacryl S-200 purified active fraction of AAE on Phenyl-Sepharose CL-4B

The pooled and dialyzed active fractions obtained from Sephacryl S-200 were concentrated and loaded on the column. The unbound proteins were eluted with equilibrating buffer. A linear $(NH_4)_2SO_4$ gradient (1.0 - 0.0 M) followed by 50%

ethylene glycol in sodium phosphate buffer (5.0 mM, pH 6.5) were used to elute the bound proteins (2.5 ml) each. Active fractions were monitored both for protein and activity.

2.6.11. Criteria of purity

2.6.11.1. SDS-Poly Acrylamide Gel Electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out at room temperature (Laemmli, 1970).

Reagents

a) Monomer: Acrylamide (29.2 g) and bis-acrylamide (0.8 g) were dissolved in water (50 ml) and final volume was made up to 100 ml, filtered and stored in a brown bottle at 4°C.

b) Separating gel buffer: Tris (18.15 g) was dissolved in water, pH was adjusted to 8.8 with 6.0 N HCl and made up to 100 ml and stored at 4°C.

c) Stacking gel buffer: Tris (3.0 g) was dissolved in water; pH was adjusted to 6.8 with 6.0 N HCl, volume made up to 50 ml, filtered and stored at 4^{o}C.

d) Sodium dodecyl sulfate (SDS): 10 g was dissolved in 100 ml water.

e) Ammonium persulphate: 100 mg was dissolved in 1.0 ml water.

f) Tank buffer: Tris (0.3 g) and glycine (1.44 g) and SDS (0.1 g) were dissolved in water and made up to 100 ml.

g) Cocktail buffer: Mixed 2.5 ml of solution (c), 2.0 ml solution (d), glycerol (2.0 ml), β-mercaptoethanol (1.0 ml) and bromophenol blue (0.01%, 0.5 ml) and stored at 4°C.

Running gel (12%, 0.7 mm) was prepared by mixing

2.8 ml of (a), 1.8 ml of (b) and 2.1 ml water were mixed, degassed, and added 60 µl of solution (d), 10 µl TEMED, and 60 µl solution (e). The contents were mixed well and poured between the assembled glass plates with edges sealed with 2.0% agar. Gels were allowed to polymerize at room temperature for 2 h.

Stacking gel (5.0%) was prepared by mixing solution 'a' (0.34 µl) and 'c' (0.5 ml) with water (1.1 ml) and degassed. To this mixture was added solution 'd'

(20 µl), TEMED (10 µl) and solution 'e' (20 µl) and poured above the polymerized separating gel. The polymerization was facilitated at room temperature for 2 h.

Protein samples (~5.0 µg) were prepared by dissolving in solution 'g' (50 µl). This was heated in a boiling water bath for 5 min. Cooled samples were loaded (25 µl) to the wells immersed in solution (f) and were run at a constant voltage 50 volts for 3 h or until the tracking dye was just above the lower end of the gel.

Staining reagents

a) Coomassie staining (Price, 1996)

(a.I) Staining reagent: Coomassie brilliant blue-R-250 (100 mg) was dissolved in methanol:acetic acid:water (100 ml, 12.5:7.5:30, v/v). The reagent was filtered and stored at room temperature.

(a.II) Destaining solution: methanol: acetic acid: water (12.5:7.5:30).

b) Silver staining (Wray et al., 1981)

b.I) Fixing: acetic acid (6.0 ml), methanol (25.0 ml) and formaldehyde (250 µl) were mixed and made up to 100 ml with water.

b.II) Ethanol (50%) solution.

b.III) Pretreatment solution: sodium thiosulphate (200 mg) in 100 ml water.

b.IV) Impregnating solution: silver nitrate (200 mg) dissolved in water, added formaldehyde (25 µl) and made up to 100 ml.

b.V) Developing solution: sodium carbonate (6.0 g) was dissolved in water containing sodium thiosulphate (1.0 mg), formaldehyde (75 µl) and made up to 100 ml.

b.VI) Stopping solution: acetic acid (6.0 ml) and ethanol (2.5 ml) were mixed and made up to 100 ml.

Gel staining

After electrophoresis, the gels were stained for proteins by any one the following methods.

Coomassie blue staining. After electrophoresis gels were kept in solution (a.I) for 2 h and destained by solution (a.II).

Silver staining. Gel after electrophoresis were fixed in solution (b.I) for 45 min followed with washing by solution (b.II) twice for 30 min each. Subsequently the gel was placed in solution (b.III) for 1 min with continuous shaking. After washing thoroughly with water thrice, the gel kept in solution (b.IV) for 20 min with shaking. The protein bands were visualized by treating with solution (b.V) for 10 - 15 min or till the bands appear. Staining was arrested by placing the gel in solution (b.VI) and stored in 6.0% acetic acid.

2.6.11.2. Native polyacrylamide gel electrophoresis (Walker, 1996)

PAGE under native conditions was carried out to evaluate the purity of esterases. The electrophoresis was carried out as described above (2.6.11.1) without SDS. The sample containing protein (~20 μg) was dissolved in 50 μl of sample buffer devoid of SDS and β-mercaptoethanol. 25 μl of this sample was loaded on the wells, and were run at a constant voltage of 50 volts for 3 h and the gel was stained for protein as well as for esterase activity.

2.6.11.3. Activity staining of purified esterases

2.6.11.3a. AAE activity staining (Hou and Lin, 1998)

The gel after electrophoresis was incubated in activity staining solution [50 mM sodium phosphate buffer, 100 ml, pH 7.2; α-NA (10 mg) dissolved in acetone (1.0 ml) and fast blue RR salt (50 mg)] in the dark at 37°C until dark grey or black bands appeared. The stained gel was washed with water and fixed in 3.0% acetic acid.

2.6.11.3b. Determination of the FAE activity by diffusion of enzyme in to agarose gel (Mackenzie et al., 1987)

The purified ragi FAE was inactivated by running on PAGE, and it was loaded in the agarose wells directly and checked for the activity by diffusion. For detection of FAE activity MUTMAC (at a concentration of 5.0 mM) was incorporated in Tris-HCl buffer (50 mM, pH 7.0) into agarose gel (1.5%, 1.0 mm thickness) cast on FMC gel bond agarose support film. Purified ragi FAE (~20 μg) was loaded in the wells after solidification. The gel was flooded with Tris-HCl buffer and illuminated with long

wavelength UV. FAE activity and the rate of diffusion of the enzyme were visible as light blue fluorescence against an intense green fluorescent background.

2.6.12. Estimation of molecular weight of esterases (M_r)

2.6.12.1. By SDS-PAGE (Laemmli, 1970)

The molecular weight of denatured protein was determined by SDS polyacrylamide gel electrophoresis using a 12% w/v acrylamide gel. Proteins were detected by silver staining. M_r values were estimated from a plot of log M_r versus mobility using the following protein standards such as lysozyme (14.0 kDa), soybean trypsin inhibitor (20.1 kDa, used only for AAE), carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), BSA (66.0 kDa), phosphorylase (97.4 kDa) and myosin (205 kDa used only for AAE).

2.6.12.2. By Gel permeation chromatography (Whitaker, 1963)

Native molecular weight of AAE was determined by GPC on a column of Sephacryl S-200 HR (0.6 x 90 cm). In order to reduce the interaction of eluting protein with the column matrix, NaCl (100 mM) was added to the elution buffer (Tris-HCl, 10 mM, pH 7.0). The column was calibrated by using Sigma low molecular weight standard protein markers such as papain/lysozyme (12.0 kDa), carbonic anhydrase (29.0 kDa), BSA (66.0 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). The molecular mass of purifed FAE was estimated on a column (0.7 x 100 cm) of Biogel P-30 calibrated by using standard protein markers such as aprotinin (7.0 kDa), lysozyme (12.0 kDa), and carbonic anhydrase (29.0 kDa). The molecular mass of both these enzymes were calculated from a plot of V_e/V_o against the log molecular weight, where V_o is the void volume of the gel matrix determined by blue dextran and V_e is the elution volume of the standard proteins/AAE/FAE.

2.6.12.3. By ESI-MS (Price, 1996)

Molecular mass of purified ragi AAE and FAE were also determined by ESI-MS. The purified enzymes (~5.0 µg) were taken in 50% methanolic solution and subjected (10 µl) to ESI-MS Alliance, Waters 2695 mass spectrometer using positive

mode electrospray ionization.

2.6.13. Effect of pH on purified AAE and FAE (Nirmala and Muralikrishna, 2003a)

Esterase activities were determined at various pH values using different buffers such as sodium acetate (pH 4.0 - 6.0), sodium phosphate (pH 6.0 - 8.0) and Tris-HCl (pH 7.0 - 9.0) at 0.5 unit increments, at 75 mM (AAE) and 50 mM (FAE) concentrations. The maximum activity was taken as 100% and relative activities were plotted against different pH values.

2.6.14. pH stabilities of purified AAE and FAE (Nirmala and Muralikrishna, 2003b)

Stability of purified esterases were carried out by pre-incubating these enzymes in different buffers such as Glycine-HCl (pH 2.0 - 3.0), sodium acetate (pH 4.0 - 6.0), sodium phosphate (pH 6.0 - 8.0) and Tris-HCl (pH 7.0 - 9.0), followed by determining the residual activities at different time intervals (0 - 360 min). The original activity was taken as control (100%) and relative activities were plotted against different time intervals.

2.6.15. Temperature optima of purified AAE and FAE (Nirmala and Muralikrishna, 2003a)

Freshly purified AAE enzyme (50 µl) was incubated with α-NA (1.0 mM) in Tris-HCl buffer (pH 7.5, 75 mM) in a temperature range of 30 - 80°C (with an interval of 5°C) using a thermostatically controlled incubator where as purified FAE enzyme (0.1 ml) was incubated with PNPF (1.0 mM) in sodium acetate buffer (pH 6.0, 50 mM) in a temperature range of 30 - 70°C (with an interval of 5°C). The maximum activity was taken as 100% and relative activities were plotted against different temperatures.

2.6.16. Thermal stability of purified AAE and FAE (Nirmala and Muralikrishna, 2003b)

Purified AAE and FAE were pre-incubated in a temperature range of 30 - 70/

80°C for 15 min. The residual activities were estimated taking original activity as control (100%) and relative activities were plotted against different temperatures.

2.6.17. Measurement of Activation energy

To determine the temperature dependence of purified ragi AAE and FAE activities, reaction rates/activities in temperature range of 30 - 60°C with an interval of 5°C were determined. Arrhenius plots were drawn for individual esterases taking natural log of activity on y-axis and T^{-1} in Kelvin on x-axis. The activation energies were determined from the slope of the plot using Arrhenius equation

$$\text{Slope} = -E_a / R$$

where R is universal gas constant whose value is 8.314 J mol^{-1}.

2.6.18. Spectral analysis of purified ragi esterase

a) UV absorption spectra.

The absorption spectra of purified esterases in Tris-HCl buffer (7.5, 75 mM, AAE) and sodium acetate buffer (6.0, 50 mM, FAE) respectively were recorded in a range of 200 - 400 nm using Shimadzu UV-160A spectrophotometer (Peterson, 1983).

b) Fluorescence spectra.

The excitation and emission spectra of the purified esterases was taken in an Amicon-Bowman spectrofluorimeter. The emission spectra were recorded after excitation of the sample. The relative fluorescence intensities of the enzymes were recorded.

2.6.19. Effect of substrate concentration (Lineweaver and Burk, 1934)

2.6.19.1. Effect of different concentrations of α-NA on the activity of purified AAE

Different concentrations of α-NA (2.0 - 10 µg) in Tris-HCl buffer (75 mM, pH 7.5) were incubated with purified AAE for 30 min at 45°C and activities were measured after every 5 min interval. Initial velocities (V_o) were calculated for all

substrate concentrations and the K_m and V_{max} values were calculated from the double reciprocal plot.

2.6.19.2. Effect of different concentrations of PNPF on the activity of purified FAE

Different concentrations of PNPF (2.5 - 12.5 µg) in sodium acetate buffer (pH 6.0, 50 mM) were incubated with purified FAE for 1 h at 45°C and activities were measured after every 15 min interval. Initial velocities (V_o) were calculated for all substrate concentrations and the K_m and V_{max} values were calculated from the double reciprocal plot.

2.6.20. Effect of metal ions on the activities of purified AAE and FAE (Nirmala and Muralikrishna, 2003a)

Purified ragi AAE and FAE were pre-incubated with 5.0 mM solutions of citric acid, oxalic acid, EDTA and salts of metal ions (Chlorides of Fe^{3+}, Cu^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+} etc) at 45°C for 15 min and residual activities were measured. The enzyme activities without the respective compounds were taken as control (100%) and relative activities were calculated.

2.6.21. Effect of group specific reagents (Nirmala and Muralikrishna, 2003b)

2.6.21.1. AAE

Purified AAE was incubated with PCMB, iodoacetamide and eserine (25 mM) in Tris-HCl buffer (pH 7.5) at 45°C for 15 min and the residual activities were estimated. The enzyme activity without inhibitor was taken as 100% and relative activities were plotted against inhibitor concentration (25 mM).

2.6.21.2. FAE

Purified FAE was incubated with PCMB, iodoacetamide and eserine (10 - 50 mM) in sodium acetate buffer (pH 6.0, 50 mM) at 45°C for 15 min and the residual

activities were estimated. The enzyme activity without inhibitor was taken as 100% and relative activities were plotted against inhibitors concentrations.

2.6.22. Substrate specificity of esterases

2.6.22.1. Enzymatic deacetylation of acetyl substrates using purified AAE

Water-soluble portions of larch wood xylan, gum karaya and water extractable polysaccharides (0.5%, 1.0 ml) isolated from ragi and wheat were taken in Tris-HCl buffer (75 mM, pH 7.5) and incubated with purified ragi AAE (0.1 ml). The reaction was allowed to proceed at 45°C for 2 h and was stopped by boiling for 10 min and centrifuged at 15000 x g, for 30 min to separate the supernatant from the residue. The supernatant was analyzed by HPLC method for acetic acid using Supelco-C610H ion exchange column at room temperature at 210 nm using orthophosphoric acid (0.05%) as the eluent with a flow rate of 0.5 ml min^{-1}. The retention time for acetate under these conditions was 19.8 min. The specific activities of purified ragi AAE using various water-soluble polysaccharide preparations were compared with small molecular weight synthetic substrates such as α-NA and PNPA.

2.6.22.2. Enzymatic deesterification of feruloyl substrates using purified FAE

Water-soluble portions of water extractable polysaccharides (0.5%, 1.0 ml) isolated from ragi, wheat and maize were taken in sodium acetate buffer (pH 6.0, 50 mM) and incubated with purified ragi FAE (0.1 ml). The reaction was allowed to proceed at 45°C for 1 h and was stopped by boiling for 10 min and centrifuged at 15000 x g, for 30 min to separate the supernatant from the residue. The supernatant was analyzed by HPLC method for ferulic acid using C_{18} column at room temperature at both λ_{280} and λ_{320} making use of ferulic acid (0.01 - 0.1 mg) standard graph using methanol-water-acetic acid (20:75:5) as the eluent with a flow rate of 1.0 ml min^{-1}. The retention time for ferulate under these conditions was 17.8 min. The specific activities of purified ragi FAE using various water-soluble polysaccharide preparations were compared with small molecular weight synthetic substrates such as PNPF and ethyl ferulate.

2.6.23. IR analysis of water-soluble polysaccharides for ester group (Kacurakova et al., 1998)

Freeze-dried water-soluble polysaccharides of ragi, wheat, larch wood xylan and gum karaya (2.0 mg each) were taken in KBr pellet discs (10 mg) and analyzed by infrared spectroscopy for the presence of ester group. IR spectra were recorded between 4000 - 400 cm^{-1} using a Perkin-Elmer 2000 spectrometer.

2.6.24. ESI-MS analysis for the deacetylation of substrates using purified AAE

α-NA and PNPA (1.0 mg) were dissolved in ethanediol (0.5 ml) and DMSO (0.5 ml) respectively, and incubated with purified AAE at 45°C for different time intervals (0 - 8 h). The reaction was stopped with methanol (0.3 ml) and centrifuged. The methanol layer, which consists of products, was subjected for ESI-MS Alliance, Waters 2695 mass spectrometer using negative mode electrospray ionization. Capillary voltage was 3.5 kV, core voltage 100 V, source temperature 80°C, desolvation temperature 150°C, core gas (Argon) 35 lit h^{-1} and desolvation gas (Nitrogen) 500 lit h^{-1}.

2.6.25. ESI-MS analysis for the deesterification of substrates containing feruloyl groups using purified FAE

Water-soluble portions of water extractable polysaccharides (0.5%, 1.0 ml) isolated from ragi and wheat were taken in sodium acetate buffer (pH 6.0, 50 mM) and incubated with purified ragi FAE (0.1 ml) at 45°C for different time intervals (0 - 10 h). The reaction was stopped with methanol (0.3 ml) and centrifuged. The methanol layer, which consisted of ferulic acid, was subjected for ESI-MS Alliance, Waters 2695 mass spectrometer using negative mode electrospray ionization.

2.6.26. ^{1}H NMR spectral analysis for deacetylation of substrates using purified AAE

α-NA, PNPA (2.0 mg) solutions were incubated with purified ragi AAE for 8 h at 45°C and subsequently the reaction mixture was freeze dried and dissolved in

DMSO-D_6 and the products were analyzed by ^1H NMR spectra using Bruker 500 MHz spectrometer operating at 27°C.

2.6.27. Functional properties of WSPs

2.6.27.1. Deesterification of WSPs with enriched DEAE-cellulose purified fractions of esterases

2.6.27.1a. AAE treatment

WSPs of wheat, ragi, gum karaya (GK) (0.2%) and xanthan gum (XG) (0.5%) were dissolved in buffer (Tris-HCl, pH 7.5, 75 mM) and incubated with enriched DEAE-cellulose purified fraction of ragi AAE (50 μg, for XG 200 μg), at 45˚C for 2 h. The enzyme treated and untreated (control) WSPs were taken for determining the relative viscosities, foam stabilization capacity, oxidative gelation of WSPs from wheat and ragi and synergistic gelation using XG and LBG.

2.6.27.1b. FAE treatment

WSPs of wheat and ragi were dissolved in buffer (Sodium acetate, pH 6.0, 50 mM) and incubated with enriched DEAE-cellulose purified fraction of ragi FAE (50 μg), at 45°C for 2 h. The enzyme treated and untreated (control) WSPs were taken for determining the relative viscosities, foam stabilization capacity and oxidative gelation.

2.6.27.2. Effect of enriched DEAE-cellulose purified fractions of esterases on the relative viscosities of WSPs (Muralikrishna et al., 1987)

Relative viscosities of ragi and wheat WSPs, with respect to buffer, were determined. The relative viscosities of enzyme treated (et) and untreated (substrate blanks, sb) WSPs were determined in an Ostwald viscometer, with respect to concentration (0.2 - 1.0%) and temperature (20 - 80˚C) against buffer.

2.6.27.3. Effect of enriched DEAE-cellulose purified fractions of esterases on foam stabilization capacity of WSPs (Izydorczyk et al., 1991; Shyama Prasad Rao et al., 2007)

Foam stabilization effect of WSPs (ragi, wheat, Gum karaya) on protein foam (BSA) was determined by the following procedure which in brief involves, BSA (1.0 ml, 2.0%) containing additive (0.2 - 1.0%) (polysaccharide solution treated/untreated with esterase) was mixed (for 20 - 30 sec) with $NaHCO_3$ (0.25 ml, 5.0%) in a graduated tube and the initial foam volume (V_1) was noted. To the above mixture citric acid (0.25 ml, 5.0%) was added and foam volume was noted after mixing for 30 sec and a holding period of 10 min. Then the solution was heated at 90˚C for 3 min and the final volume (V_2) was noted. Appropriate controls i.e. WSPs/BSA were maintained along with enzyme treated ones.

2.6.27.4. Effect of purified esterases on the oxidative gelation of WSPs (Vinkx et al., 1991)

To the WSP solution of ragi and wheat (treated/untreated with esterase) (0.2 - 1.0%, 20 ml), 0.1 ml of H_2O_2 (0.39 g/l), and 0.1 ml (1.0 Sigma purpurogallin unit) of horseradish peroxidase (EC 1.11.1.7, 200 - 300 units/mg solid), were added and incubated at 30˚C. Relative viscosities with respect to buffer (Tris-Hcl, pH 7.5/ Sodium acetate, pH 6.0, 50 mM) were determined at regular intervals (0, 20, 40 and 60 min) as mentioned above.

2.6.27.5. Role of enriched DEAE-cellulose purified fraction of ragi AAE on the synergistic gelation of Xanthan and Locust bean gums (XG and LBG)

XG and LBG were first dispersed in distilled water (~1:50) stirred for ~2 h and centrifuged at 5000 x g, for 10 min. The supernatant was dialyzed against distilled water and lyophilized. Water-soluble portion of XG (0.5%) was deacetylated with AAE (200 μg) for 0 - 2 h as described above. Water-soluble portion of LBG (0.5%) was dispersed in buffer and mixed with the enzyme treated XG solution (equal volume, 1:1, 0.5%) followed by stirring for 5.0 min, and the relative viscosities of

these blends along with the enzyme untreated ones were determined by ostwald viscometer for various intervals of time (0 h, 1 h and 2 h).

2.6.27.6. ESI-MS analysis of deacetylated XG

Water-soluble portion of XG (0.5%) was dispersed in Tris-HCl (50 mM, pH 7.5) and after incubation with AAE for ~2 h as described above, was precipitated with ethanol (3 volumes) and centrifuged (4000 x g, 10 min). The supernatant was evaporated completely and the sample was dissolved in minimum volume of methanol and subjected for ESI-MS analysis using negative mode electrospray ionization for the detection of released acetate.

RESULTS AND DISCUSSION

CHAPTER-3
ISOLATION OF ESTERASES FROM RAGI MALT

3.1. Introduction

Esterases from several microbial and non-microbial sources were isolated, purified and characterized. Many studies on the nature of these enzymes have been reported from microbial sources (Biely et al., 1985; Borneman et al., 1990a & b; Johnson et al., 1988a). Esterases from non-microbial sources such as porcine liver esterase type II (EC 3.1.1.1), orange peel acetyl esterase (EC 3.1.1.6), tomato pectin esterase and orange peel pectin esterase (EC 3.1.1.11) (Williamson, 1991) etc were reported. However, very few reports were present with respect to the plant sources and were restricted mainly to barley (Humberstone and Briggs, 2000a & b; 2002a & b) and wheat (Cubbada and Quatracci, 1974). Barley showed a multiplicity of esterases hydrolyzing different types of esters (Vietor et al., 1993). The present chapter mainly focuses on the malting of ragi, and different optimization conditions used for the isolation of AAEs and FAEs from ragi malt and its coleoptiles.

Malting is a process consisting of steeping, germination of the cereal grains under controlled conditions for a limited period, followed by controlled drying (kilning). The germinated ragi seeds at 24, 48, 72 and 96 h are shown in the figure 16 (a, b, c and d). The gibberellins secreted by the embryo not only stimulate the aleurone layer to induce various hydrolytic enzymes (Hough, 1985) such as amylases, xylanases, esterases (AAE and FAE), peroxidases (Jacobsen, 1973; Gubler and Ashford, 1983) etc, but also speedup the process of germination by breaking down the dormancy (Briggs, 1987).

The hydrolytic enzymes such as AAE and FAE induced during the process of germination help in the partial degradation of endosperm cell wall by cleaving the ester linkages, thereby releasing the acetyl and feruloyl groups respectively. The absence of acetyl groups in the extracts of germinating barley reveal the possible presence of specific sugar acetyl esterases (Frohwein and Leibowitz, 1961). Studies showed that FAE activity was observed during kilning and mashing of barley grains at 44 - 45°C but not during malting (Maillard and Berset, 1995).

The malting loss in Indaf-15 was found to increase with increase in the period of germination (Nirmala et al., 2000). Malting reduces the paste viscosity of cereal and millet flours such as wheat, sorghum, finger millet (Brandtzaeg et al., 1981).

Malting capacity of cereals and millets was examined due to their potential use in food industry. The treatment of GA_3 results in increase in total soluble sugars during malting. During germination, the storage protein is degraded, the amino acids are used for the synthesis of new proteins required for the growth of the seedling (Enari and Sopanan, 1986), and starch is hydrolyzed to glucose, which is used as a source of energy for the growth of seedling and synthesis of new compounds (James, 1940).

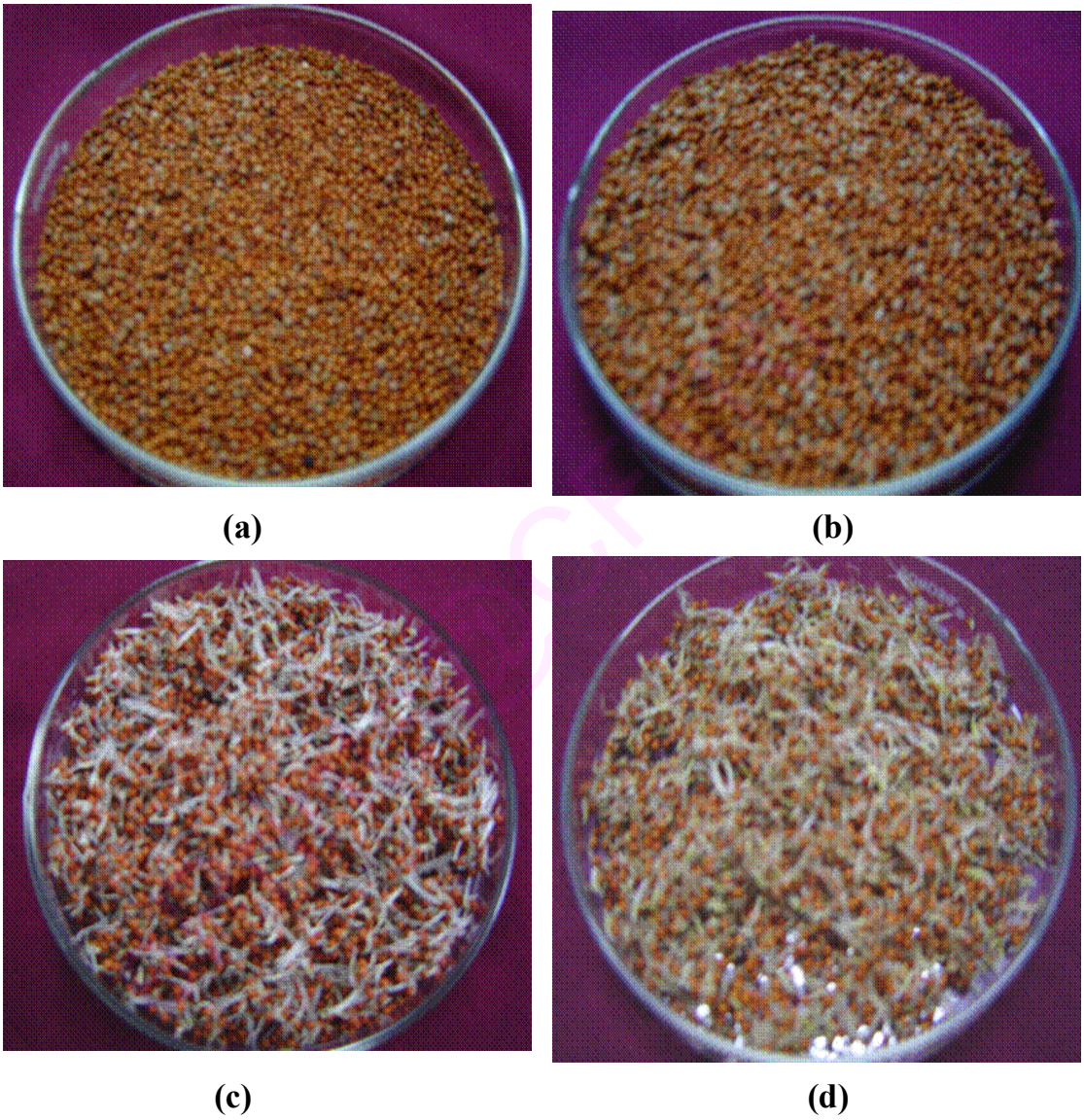


Figure 16. Germinated ragi seeds (a) 24 h (b) 48 h (c) 72 h and (d) 96 h

3a. Isolation of ragi AAE from ragi malt

3a.1. AAE levels in native and germinated ragi seeds

Malting was carried out for different periods of germination of ragi seeds i.e. 24, 48, 72, 96, and 120 h. A linear increase in the activity of AAE was observed and it was found to be maximum at 72 h malt [0.38 U gm^{-1} malt using α-NA and 0.19 U gm^{-1} of malt using PNPA as substrates, Table 5] and thereafter a decrease in the activity was noticed in the 96 and 120 h malts (Madhavi Latha and Muralikrishna, 2007). Since activity was found to be maximum in 72 h malt, the growth portions (coleoptiles) of this malt were also tested for the AAE activity, which was found to be significantly less than that of the malt (0.22 U gm^{-1} of malt).

Table 5. Changes in AAE activity during malting

Malting periods (h)	Ragi	
	α-NA	PNPA
24	0.17	0.08
48	0.25	0.08
72	0.38	0.19
96	0.16	0.10
120	0.12	0.08

Values expressed as μmoles min^{-1} gm^{-1} of malt.

AAEs during the germination process digest the endosperm cell walls by splitting the acetic acid residues from the arabinoxylans hence they are considered as the entities of 'Solubilase' complex (Kanauchi and Bramforth, 2002). The activity of AAE in barley malts increased gradually for the first 3 days (up to 72 h), followed by

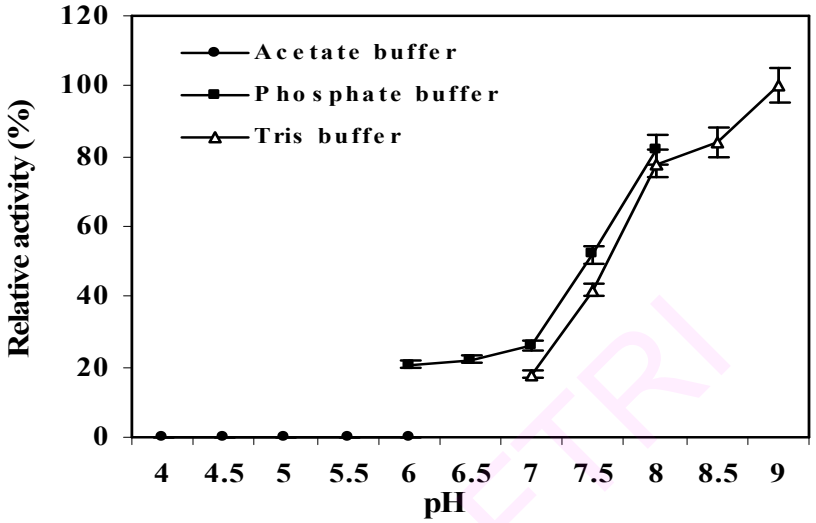
a substantial increase in the 4th and 5th days of malting (Humberstone and Briggs, 2000b).

3a.2. Isolation and optimization of the extraction conditions of AAE from 72 h ragi malt and its coleoptiles

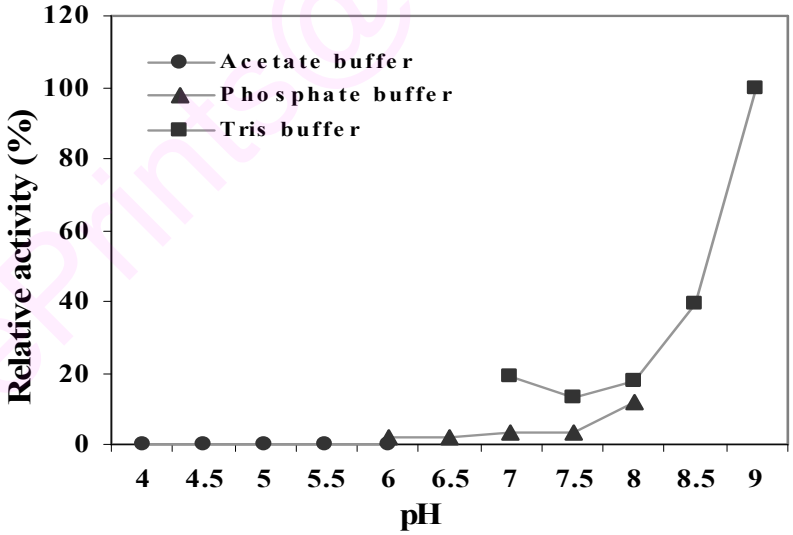
Isolation of enriched enzyme is an important aspect in an enzyme purification and characterization procedure. In the initial stages, the extraction of AAE was performed using only Tris-HCl buffer (50 mM, pH 7.5) at 4°C. More than 90% activity was obtained in the first extraction of AAE from 72 h ragi malt. Once activity was detected and the enzyme assay was developed the extraction procedure was optimized to yield maximum AAE activity from 72 h ragi malt by modifying the parameters such as pH, ionic strength, GSH (reduced glutathione), PVPP (polyvinylpolypyrrolidone), metal ions etc.

3a.2.1. Effect of pH on the extraction of crude AAE

To study the effect of pH on the isolation of enzyme, various buffers were chosen such as Tris-HCl (pH 7.0 - 9.0), acetate (pH 4.0 - 6.0) and phosphate buffers (pH 6.0 - 8.0). Tris buffer was found to be the best extractant compared to acetate and phosphate buffers for extraction of the enzyme from both 72 h ragi malt and its coleoptiles, as it yielded higher activities (Figure 17a & b). The optimum pH for the extraction of the enzyme from both malt and coleoptiles was found to be 9.0 (Tris buffer) which was higher than the one reported for barley [(7.0), Humberstone and Briggs, 2000b].



(a)



(b)

Figure 17. Effect of pH on the extraction of AAE from (a) coleoptiles (b) ragi malt

3a.2.2. Effect of ionic strength on the extraction of AAE

As Tris-HCl buffer was found be the best extractant, further experiments were carried out to test the effect of different ionic strength of Tris-HCl buffer (25 - 100 mM, pH 9.0) on the activity of AAE. 75 mM Tris-HCl buffer (pH 9.0) was considered, the best extractant for maximum extraction of the enzyme from both malt and coleoptiles (Figure 18). The ionic strength required (75 mM) for the extraction of ragi AAE is comparatively higher than the one reported (50 mM) for barley AAE (Humberstone and Briggs, 2000b). Most of the hydrolytic enzymes can be extracted in the ionic strength range of 50 - 100 mM beyond which the enzymes are likely to loose their activity (Li-Xing et al., 2005).

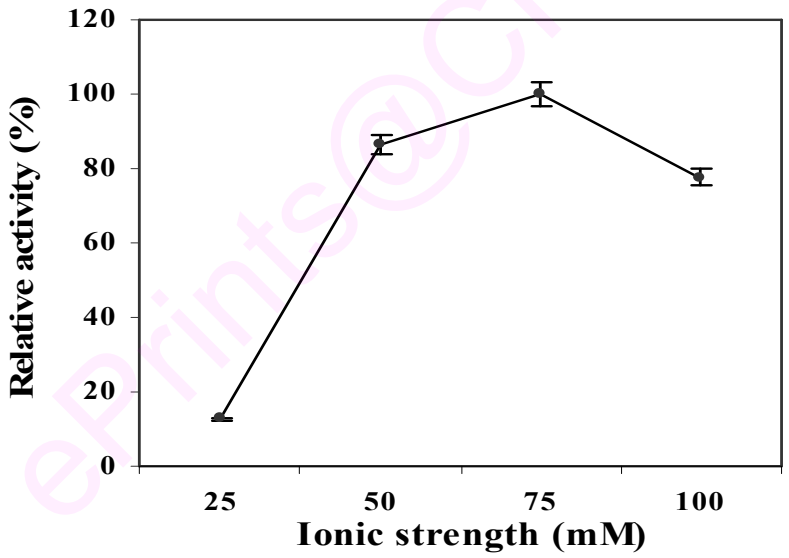


Figure 18. Effect of Ionic strength on the extraction of AAE from ragi malt

3a.2.3. Effect of GSH on the extraction of AAE

In the present study, GSH at different concentrations (25 - 100 mM) along with PVPP (1.0%) and Triton X-100 (1.0%) were used to enhance the extraction of AAE from ragi malt. The extraction of ragi AAE was maximum in 100 mM of GSH, which was found to be higher than 25, 50 and 75 mM of GSH as shown in **Table 6**.

Table 6. Effect of different concentrations of GSH on the isolation of crude AAE

Concentration of GSH	Relative activity (%)
Control	100
25 mM	100
50 mM	93
75 mM	122
100 mM	145

The activity due to the incorporation of GSH in the extraction medium did not exhibit linear increase from 25 - 100 mM, and this may be perhaps due to the presence of many AAEs showing differential behaviours at various concentrations of GSH. The amount of GSH required for the extraction of AAE from ragi was higher than the one reported for the extraction of barley malt AAE (Humberstone and Briggs, 2000b). The significant effect of GSH at higher concentrations may be due to the disruption of disulphide bonds in the enzyme and binding of the enzyme with other proteins or protection of thiol (SH) groups of the enzyme (Pompella et al., 2003).

3a.2.4. Effect of PVPP on the extraction of AAE

The extraction of AAE from ragi malt was maximum in the presence of 0.5% PVPP as shown in **Table 7**, which was found to be comparatively less than the one reported from barley malt AAE, in which 1.0% PVPP was used for the extraction (Humberstone and Briggs, 2000b).

The enzyme extract of ragi is coloured mainly due to the presence of polyphenols, which mostly form insoluble complexes by combining with enzyme during malting and are extracted along with the enzyme at alkaline pH, thus reducing the extraction and activity of the enzyme (Harris and Rickets, 1958). PVPP helps in the absorption of phenolic compounds and acts as a stabilizer of many enzymes

thereby enhances the enzyme recovery and activity (McMurrough et al., 1995). The effect of different concentrations of PVPP (0.25 - 1.0%) along with GSH (100 mM) and Triton X-100 (1.0%) on the extraction of AAE was carried in order to minimize the co-extraction of phenolic compounds present in ragi malt.

3a.2.5. Effect of Triton X-100 on the extraction of AAE

The extraction of the enzyme was maximum in the presence of 0.75% Triton X-100 (Table 7), which was found to be less than the one reported from barley malt AAE (1.0% Triton X-100, Humberstone and Briggs, 2000b).

Table 7. Effect of different concentrations of PVPP and Triton X-100 on the isolation of crude AAE

Concentration	Relative activity (%)	
	PVPP	Triton X-100
Control	100	100
0.25%	63.8	115
0.50%	122	118
0.75%	110	134
1.00%	72.5	98.5

AAE was found to be a membrane bound enzyme, hence showed maximum extraction in the presence of a nonionic detergent i.e. Triton X-100. Triton X-100 disrupts hydrophobic bonding and facilitates increased enzyme extraction from the malt (Humberstone and Briggs, 2000b).

3a.2.6. Effect of $CaCl_2$ and $MgCl_2$ on the extraction of AAE

Metal ions such as $CaCl_2$ and $MgCl_2$ in the concentration of 6 mM and 4 mM respectively increased the extraction of AAE from ragi malt (Table 8). This indicates that the enzyme requires Ca^{2+} and Mg^{2+} ions during the extraction in addition to GSH, PVPP and Triton X-100.

The activity due to the incorporation of $MgCl_2$ in the extraction medium did not exhibit linear increase from 2 - 10 mM and this may be due to the presence of many acetyl xylan esterases showing differential behaviors at various concentrations of $MgCl_2$.

Table 8. Effect of different concentrations of $CaCl_2$ and $MgCl_2$ on the isolation of crude AAE

Concentration	Relative activity (%)	
	$CaCl_2$	$MgCl_2$
Control	100	100
2 mM	95	135
4 mM	97.5	142.5
6 mM	116	122.5
8 mM	102.5	135
10 mM	72.5	122.5

3a.3. Determination of Temperature optima and stability of crude ragi AAE

Temperature is one of the important parameters that affect the rate of enzyme hydrolysis. To determine the temperature optima of crude extracts of ragi malt and coleoptiles, AAE activities were determined in the temperature range of 30 - 60°C (Figure 19). The optimum temperature was found to be 30°C and similar results were reported for barley malt AAE (Humberstone and Briggs, 2000b).

The enzyme from the malt has retained only 25% activity at 35°C and completely lost its activity above 60°C, however the enzyme isolated from 72 h coleoptiles has retained about 40% of the activity at 35 - 40°C and completely lost the activity above 45°C. This may be perhaps due to the low hydrophobic environment in the active site region of the enzyme.

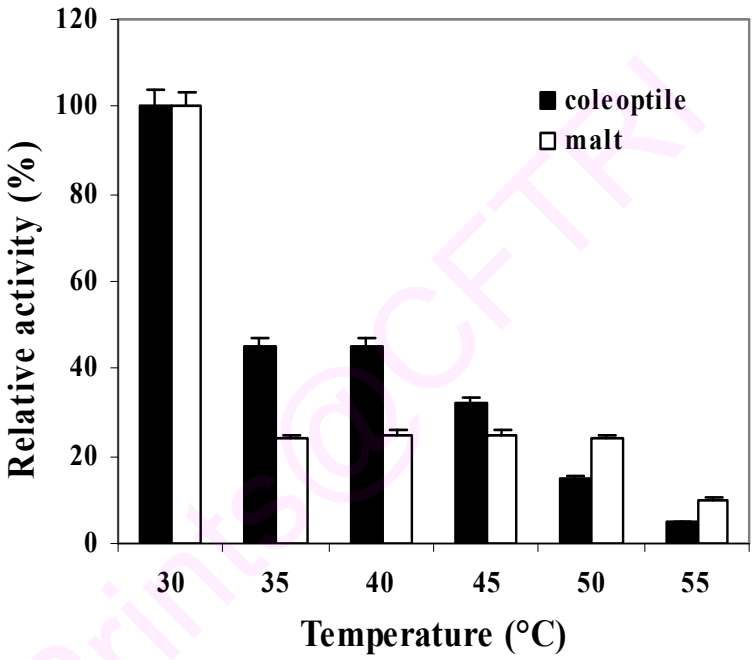


Figure 19. Temperature optima of crude AAE from ragi malt and coleoptiles

AAE from ragi was found to be thermally stable at 30°C (Figure 20), which is in tune with the published literature on hydrolytic enzymes (Humberstone and Briggs 2000b).

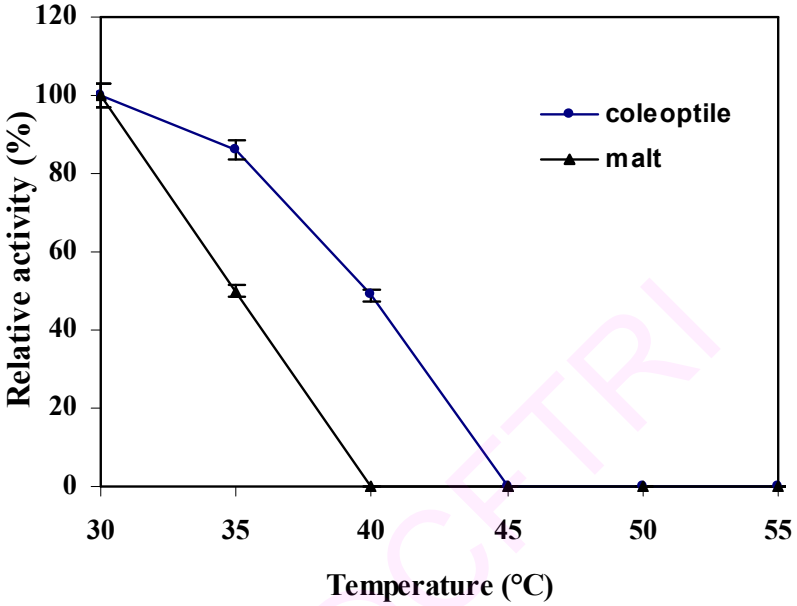


Figure 20. Thermal stability of crude AAE from ragi

3a.4. Effect of stabilizers on the activity of crude AAE

Polyhydroxy compounds such as mannitol, sorbitol, and some amino acids like glycine, asparagine, aspartic acid, glutamic acid, histidine, arginine etc are used to enhance the thermal stability of the enzyme at higher temperatures (da Costa et al., 1998). Studies showed chemical modification caused due to polyhydroxylation enhances enzyme activity. In the present study, various polyhydroxy compounds were used during the pre-incubation step at 50°C to enhance the thermal stability of AAE from ragi malt. The effect of polyhydroxy compounds on the activity of the enzyme is caused by an increase in the hydration of the soluble proteins (Arakawa and Timasheff, 1982) thereby enhancing the enzyme activity. However, in our experiments only amino acid glycine (0.1 M) could stabilize the enzyme at 50°C (Table 9), which require further, detailed experimentation.

Table 9. Effect of stabilizers on the activity of crude AAE at 50°C

Stabilizers	Relative activity (%)
Control	100
Mannitol	98
Sorbitol	100
Aspartic acid	99
Glutamic acid	100
Lysine	88
Histidine	90
Arginine	83
Glycine	115
Asparagine	93

3b. Isolation of FAE from ragi malt

3b.1. FAE levels in native and germinated seeds of ragi

Malting was carried out at different periods of germination of ragi seeds i.e. 24, 48, 72, 96 and 120 h. Maximum activity of FAE was observed in 96 h malt (0.48 μmoles $h^{-1}gm^{-1}$ malt) compared to 24, 48, 72, and 120 h malts (Table 10). The activity in coleoptiles (0.32 U gm^{-1} of malt) of 96 h germinated ragi is comparatively less than the respective malt. Studies on barley FAE impedes the rapid increase in the levels of the enzyme for 72 h and remained high for the subsequent intervals (96 and 120 h) of malting (Humberstone and Briggs, 2000a).

The decrease in the activity of FAE in 72 h malt compared to the 48 h might be due to the induction of inhibitors such as proanthocyanadins and gallic acids during this step of germination as they are known to bind to the hydrolytic enzymes irreversibly to their active sites and hinder their complete extraction from the malts. Perhaps due to this reason it is likely that the induction pattern of FAE is anomalous with respect to finger millet malt.

Table 10. Changes in FAE activity during malting

Malting periods (h)	Activity
24	0.09
48	0.25
72	0.15
96	0.48
120	0.44

Values expressed as μmoles h^{-1} gm^{-1} of malt.

3b.2. Synthesis/preparation of substrates for FAE

To determine the activity of FAE from ragi malt, substrates such as feruloyl glycerol, wheat bran polysaccharide and PNPF were synthesized/prepared.

Feruloyl glycerol was synthesized following the procedure adopted by Humberstone and Briggs (2000a). The formation of the product was identified by the change in the absorbance spectra (Figure 21) and by the relative mobility of the feruloyl glycerol on cellulose plates compared to free ferulic acid. Increase in R_f value was observed during the formation of feruloyl glycerol. This substrate was highly soluble in aqueous phase, however contain high amount of free ferulic acid, which was quantified by HPLC method and not suitable for regular spectrophotometric assay as the free ferulic acid interferes with FAE assay.

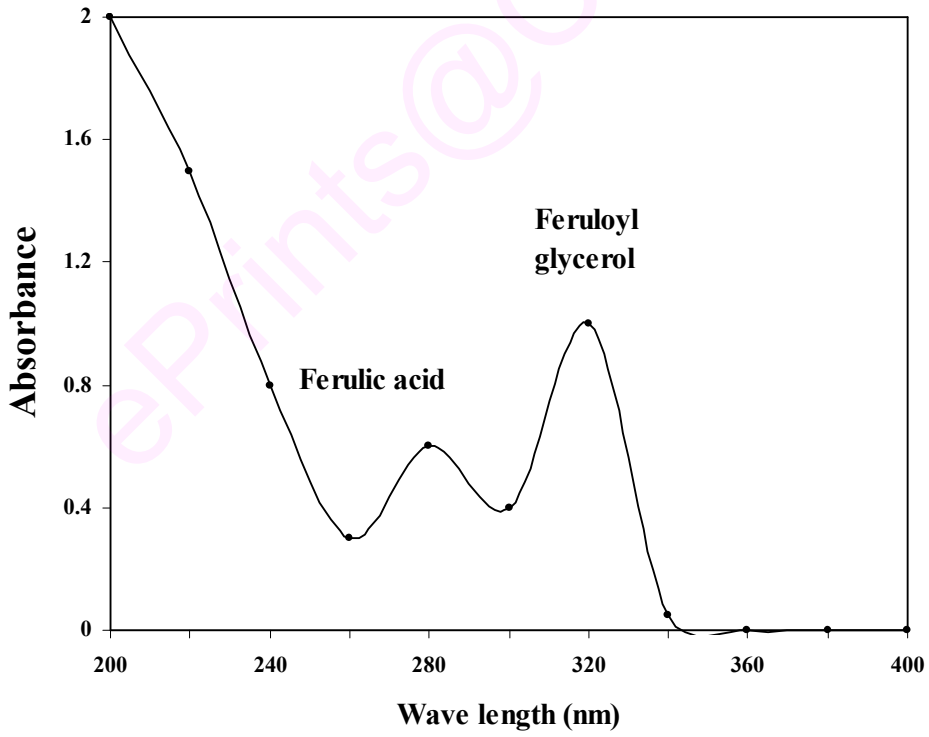


Figure 21. UV absorption spectra of ferulic acid and feruloyl glycerol

Wheat bran polysaccharide was prepared and the activity of ragi FAE was determined. The activity was comparatively less than synthetic substrates; this may be due to its large size and molecular mass of the polysaccharide and the presence of fewer number of available ferulic acid moieties for esterase action. Hence, the substrate was not used (in the present study) for the routine assay of FAE from ragi malt.

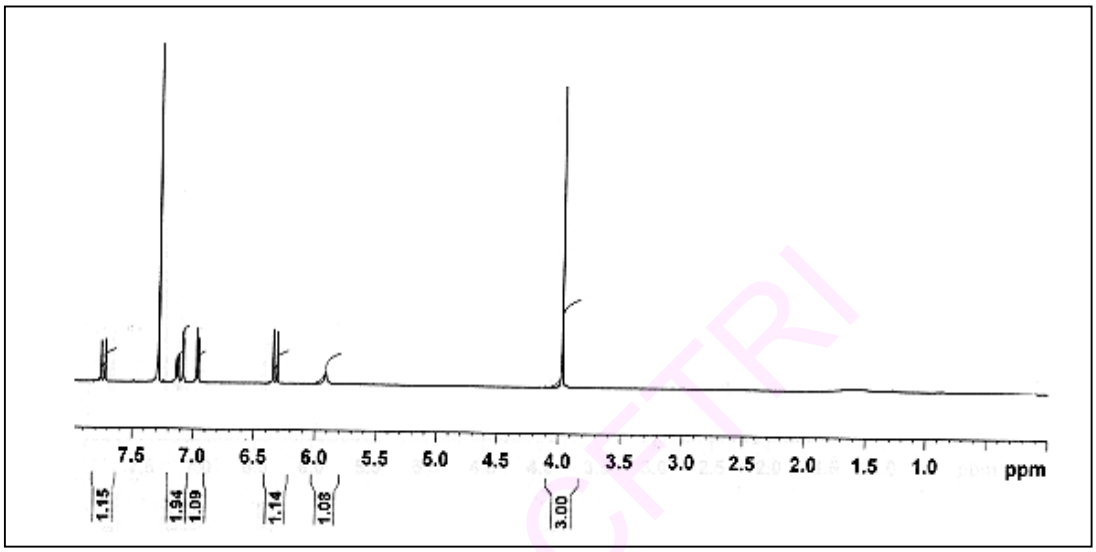
To circumvent the practical problem encountered in using the above substrates, a routine spectrophotometric assay was warranted for the isolation, purification and characterization of FAE from ragi malt. Therefore, a spectrophotometric substrate i.e. PNPF was synthesized.

The synthesis of the PNPF was carried according to the method cited by Mastihubova et al (2001). The phenolic hydroxyl moiety of ferulic acid was protected by acetylation prior to the formation of acid chloride to prevent polymerization. Esterification was carried out by coupling the acid chloride with PNP. The esterified acetoxy ferulate was subjected to selective deacetylation which was considered as a crucial step during the synthesis process, this was carried out using the commercial preparation of lipase obtained from *Burkholderia cepacia* which removes the acetyl group esterified to the hydroxyl moiety of ferulic acid with out affecting the ester bond formed between the ferulic acid and PNP, as this enzyme does not possess feruloyl esterase activity, and thus was used for effective deacetylation. This substrate models the major ferulic acid linkage to polysaccharides and provides a material with well-defined chemical properties. The complete assignments of chemical shifts are given in **Table 11**. These assignments conform well to the literature values for PNPF (Mastihubova et al., 2001).

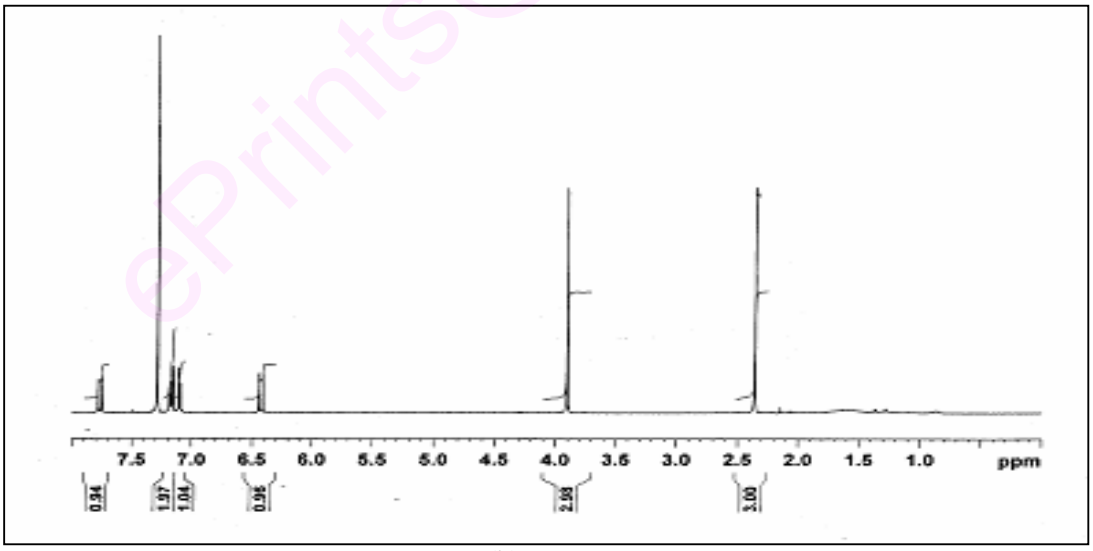
Table 11. Chemical shifts (δ) of Proton and Carbon resonances of PNPF

NMR	Chemical shifts (δ)
^{1}H NMR ($CDCl_3$)	3.96 (3H, OCH_3), 6.46 (H-A, d, J = 16 Hz), 6.97 (H-5, d, J = 8.2 Hz), 7.10 (H-2, d, J = 1.6 Hz), 7.16 (H-6, dd, J = 1.6 and 8.2 Hz), 7.38 (2H, H-2', 6', d, J = 9 Hz), 7.84 (H-B, d, J = 16 Hz), 8.32 (2H, H-3', 5', d, J = 9 Hz), 6.00 (1 H, OH)
^{13}C NMR	56.08 (OCH_3), 109.90 (C-2), 113.39 (C-A), 115.66 (C-5), 122.49 (C-2', 6'), 123.80 (C-6), 125.24 (C-3', 5'), 126.20 (C-1), 146.58 (C-4'), 147.29 (C-3), 148.23 (C-B), 148.92 (C-4), 161.81 (C-1'), 164.90 (COO)

Each step in the synthesis was characterized by NMR spectral analysis as shown in the figures 22a, b, c and d (^{1}H NMR), figure 23 (^{13}C NMR).

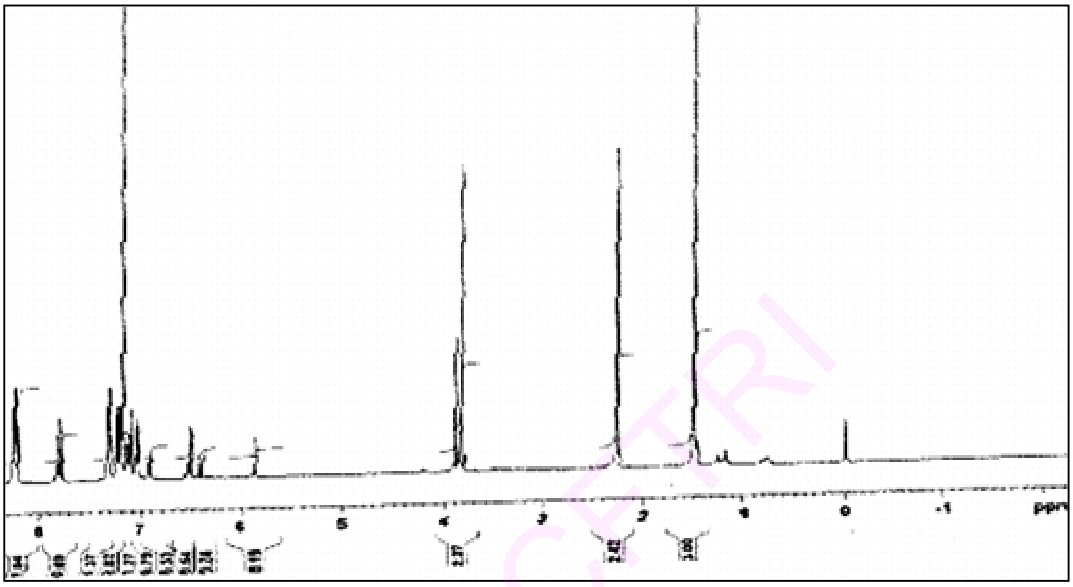


(a)

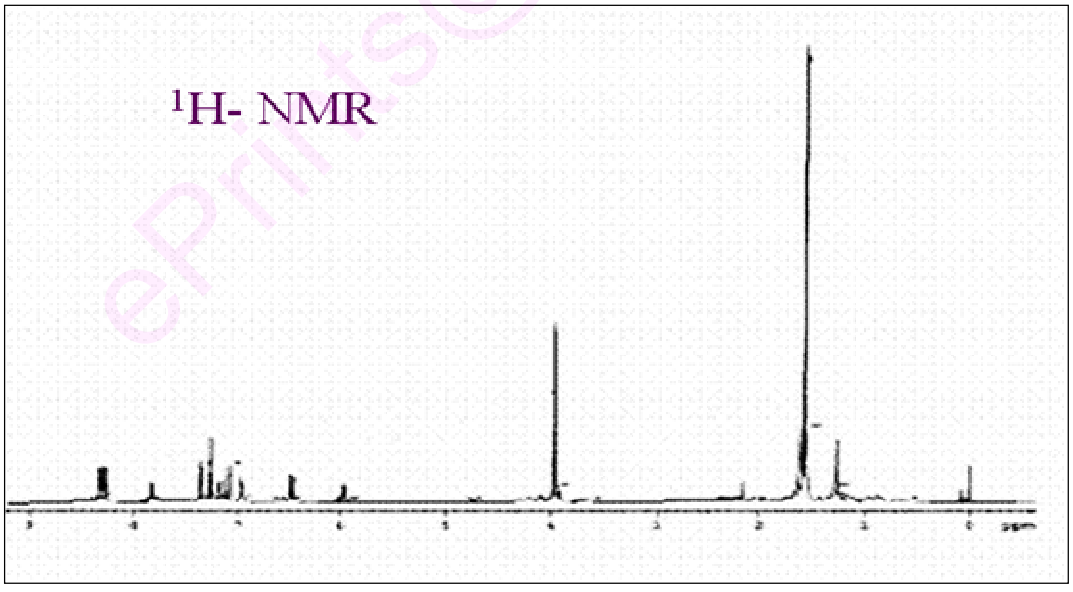


(b)

Figure 22. ^{1}H NMR spectral analysis of (a) ferulic acid and (b) 4-acetoxy ferulate



(c)



(d)

Figure 22. ^{1}H NMR spectral analysis of (c) 4-acetoxy *p*-nitrophenyl ferulate (d) PNPF

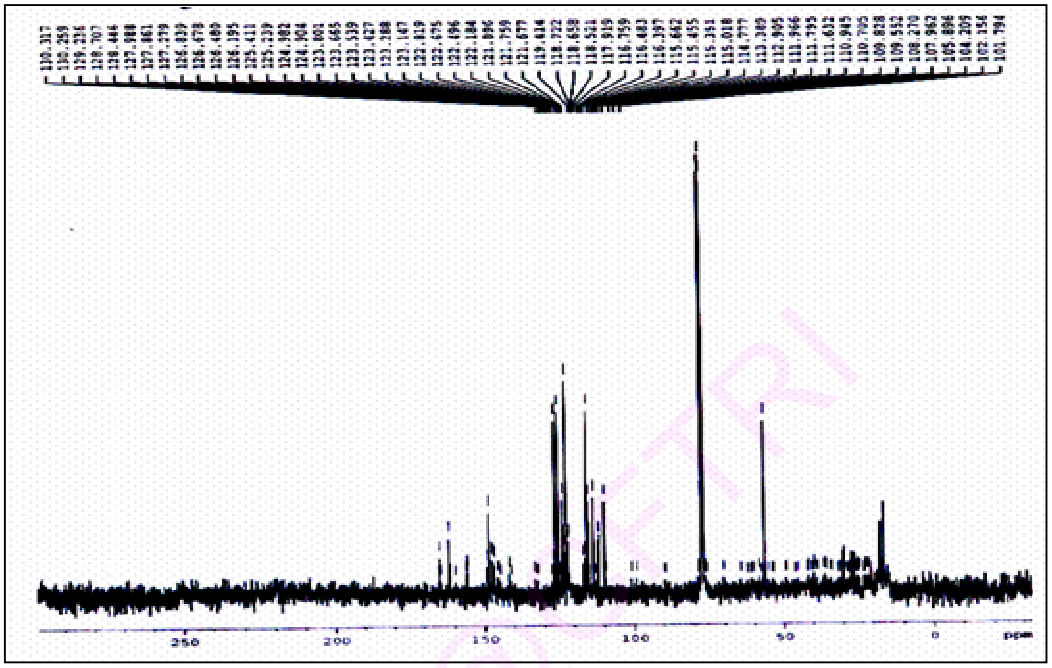
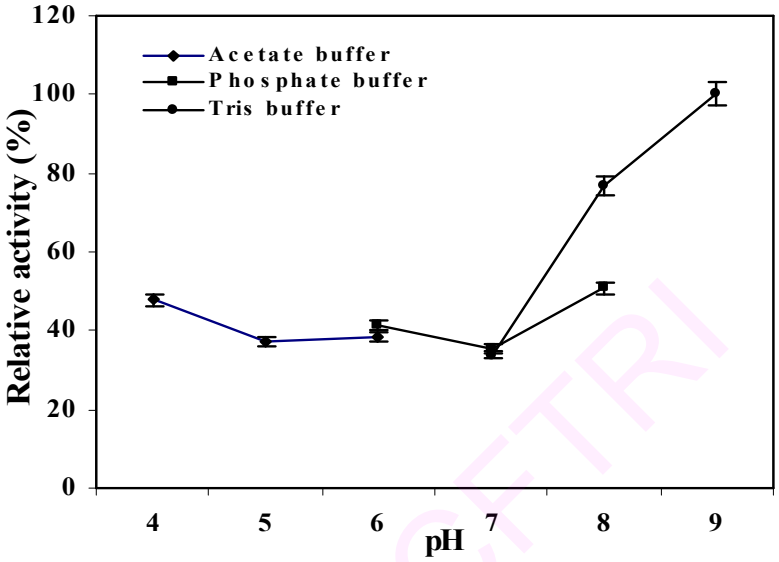


Figure 23. ^{13}C NMR spectral analysis for the synthesis of PNPF

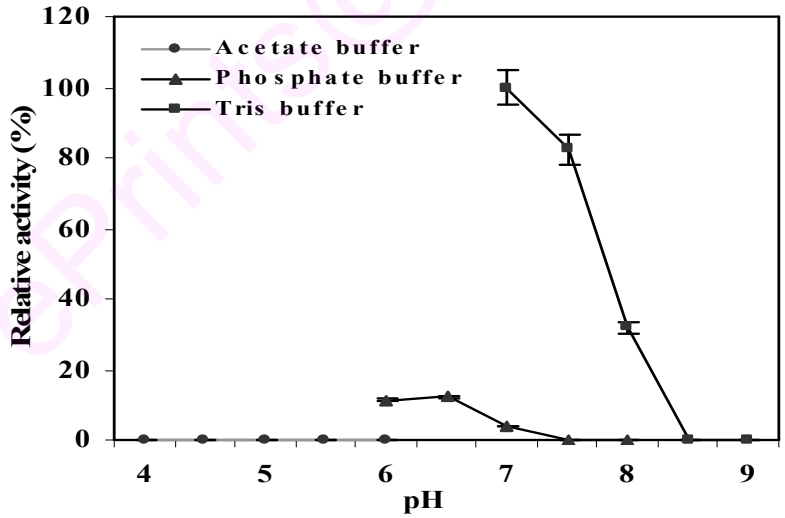
3b.3. Effect of pH on the extraction of FAE

As carried out with respect to the extraction of ragi AAE, various buffers such as Tris-HCl (pH 7.0 - 9.0), acetate (pH 4.0 - 6.0) and phosphate buffers (pH 6.0 - 8.0) were chosen to study the effect of pH on the isolation of the ragi FAE. Maximum activity was extracted in Tris buffer and it was considered as the best extractant compared to acetate and phosphate buffers for extraction of the enzyme from both malt and coleoptiles (Figure 24a & b).

The pH optimum of the enzyme from malt was found to be 7.0 (Tris-HCl buffer) which was slightly less compared to the one reported for barley enzyme (7.5, Tris-HCl, 50 mM) isolated under identical conditions (Humberstone and Briggs, 2000a), while from the coleoptiles of 96 h ragi malt the pH optima was found to be 9.0 (Tris-HCl buffer).



(a)



(b)

Figure 24. Effect of pH on the extraction of ragi FAE from (a) coleoptiles (b) malt

Extraction at pH values between 4.0 - 6.5 showed negligible FAE activity, which is in agreement with the published literature on barley malt (Humberstone and Briggs, 2000a). Phosphate buffer (6.0 - 8.0, 50 mM) did not show any effect on the extraction of ragi FAE. Enzyme activity was relatively higher in Tris-HCl buffer at pH 7.0 (0.4 µmoles h^{-1} gm^{-1} malt) compared to pH 7.5 (0.3 µmoles h^{-1} gm^{-1} malt) and pH 8.0 (0.25 µmoles h^{-1} gm^{-1} malt). Maximum activity (~88%) of FAE from 96 h ragi malt was obtained in the first extraction itself, followed by minimum activity (~12%).

3b.4. Effect of ionic strength on the extraction of FAE

In order to obtain high FAE activity Tris-HCl buffer of ionic strength 25 - 100 mM (pH 7.0) was chosen for standardization of buffer ionic strength. 50 mM Tris-HCl buffer (pH 7.0), was considered the best extractant for maximum extraction of the enzyme from the malt (Figure 25). The ionic strength required for the extraction of ragi FAE showed similarity with the one reported for barley malt (Humberstone and Briggs, 2000a).

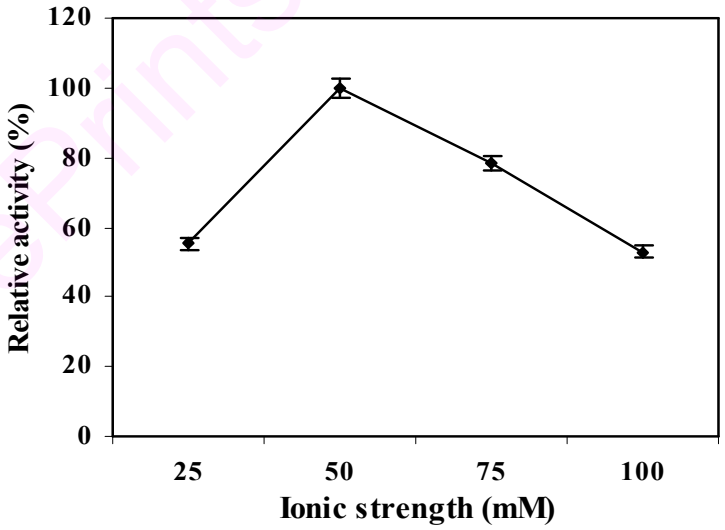
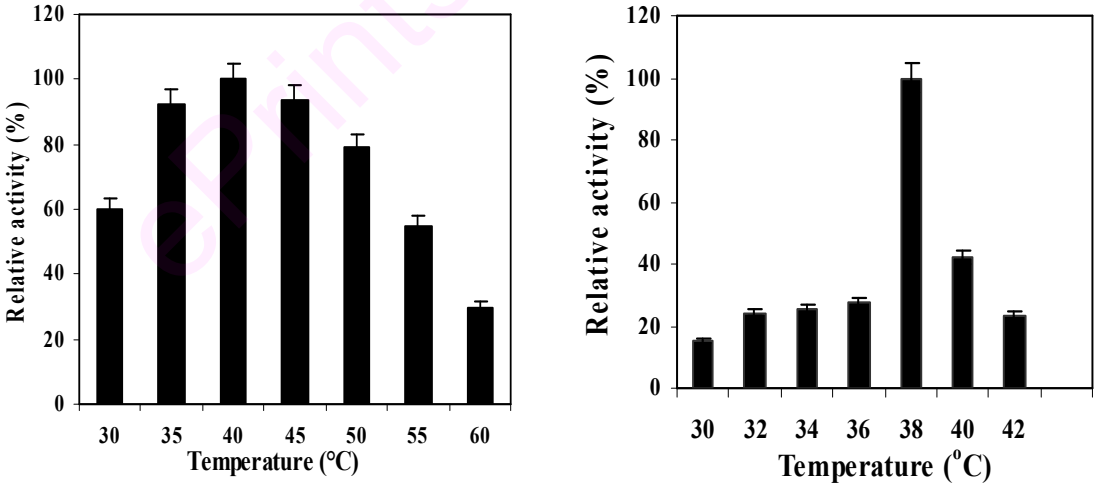


Figure 25. Effect of ionic strength on extraction of ragi FAE

Various substances such as PVPP (1.0%), reduced glutathione (25 mM) and Triton X-100 (1.0%) were added to the extracting buffer to enhance the extraction of ragi FAE as reported for the extraction of FAE and AAEs from barley malt (Humberstone and Briggs, 2000a & b) as well as for AAE from ragi malt (**Chapter 3a.2**).

3b.5.Temperature optima and stability on the activity of FAE

To determine the temperature optima of crude extracts of FAE from ragi malt and coleoptiles (Figure 26a), activities were determined in the temperature range of 30 - 60°C at every 5°C intervals. The optimum temperature was found to be 40°C for coleoptiles, and there was no significant change in the activity from the malt in this temperature range at every 5°C intervals. Hence, the activity of FAE from ragi malt was determined at every 2°C intervals of temperature, where the optimum was found to be 38°C (Figure 26b). The enzyme from the malt has retained only 22% activity at 42°C and completely lost its activity above 60°C, however from coleoptiles the enzyme has retained about 30% of the activity at 60°C.



(a) (b)

Figure 26. Temperature optima of FAE from ragi (a) coleoptiles (b) malt

Thermal stability of the crude ragi FAE was determined at a temperature range of 30 - 60°C for 15 min, the enzyme was found to be thermally stable at 30°C (Figure 27), which is in tune with the published literature on hydrolytic enzymes (Humberstone and Briggs, 2000a).

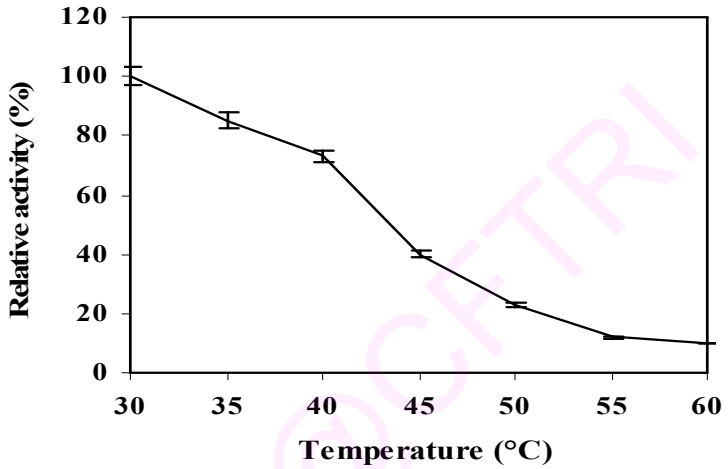


Figure 27. Thermal stability of ragi malt FAE

Conclusions

The results presented in this chapter clearly indicated that the esterases obtained from malted finger millet showed a significant increase in the activities with Tris-HCl buffer extraction in the presence of GSH, PVPP, Triton X-100, $CaCl_2$ and $MgCl_2$.

CHAPTER-4
PURIFICATION AND CHARACTERIZATION OF ESTERASES FROM RAGI MALT

4.1. Introduction

The practical use of esterases particularly from plant sources is limited due to the lack of sufficient knowledge regarding their purification and characterization with respect to their kinetic properties. This particular study is an attempt in that direction for possible exploitation of malt enzymes in modulating the functional properties of cereal non-starch polysaccharides in various food applications, using purified ragi esterases.

Several purification techniques were employed from many years to separate the individual active forms of esterases, which were characterized further biochemically, based on their kinetic parameters (Humberstone and Briggs, 2002a & b). However, most of the research findings were restricted to microbes and animals. Very few reports on plant sources i.e. from cereals are available (Cubadda and Quattrucci, 1974; Aravinda Upadhya et al., 1985; Humberstone and Briggs, 2002a & b). The present chapter focuses on the purification, characterization with respect to the kinetic studies of ragi malt esterases (AAE & FAE).

4a. Ragi malt AAE

4a.1. Purification of ragi AAE

The crude extract isolated from 72 h ragi malt with all the optimum/best conditions standardized, as indicated in the previous **chapter 3a** was purified by using a four-step purification procedure consisting of ammonium sulphate, anion exchange chromatography on DEAE-cellulose, gel filtration on Sephacryl S-200 and hydrophobic interaction chromatography on Phenyl-Sepharose were employed.

4a.1.1. Fractional precipitation of ragi AAE crude extract

Crude Tris buffer extract from 72 h ragi malt was subjected to ammonium sulfate precipitation and separated in to four fractions (0 - 20, 20 - 40, 40 - 60 and 60 - 80%). AAE activity was determined in all the ammonium sulphate fractions as shown in the **Table 12**. 0 - 20 and 20 - 40% fractions together showed a recovery of ~6.5% while, 40 - 60 and 60 - 80% fractions showed a recovery of ~93.5% respectively. As 40 - 80% ammonium sulphate fraction gave very high

recovery (~93.5%) of AAE, this fraction was taken for the further purification of this enzyme.

Table 12. Ammonium sulphate fractionation of crude AAE from 72 h ragi malt

ASP fraction (%)	Total activity (U min^{-1})	Specific activity (U min^{-1} mg^{-1})	Recovery (%)
0 - 20	0.036	0.001	2.15
20 - 40	0.074	0.002	4.40
40 - 60	0.880	0.025	52.4
60 - 80	0.690	0.015	41.0

Ammonium sulphate and acetone were used for the fractional precipitation of barley (Ward and Bamforth, 2002) and microbial AAEs (Egana et al., 1996; Degrassi et al., 1998). Ammonium sulphate fractionation (30 - 70%) of crude barley extract recovered ~80% of esterase activity (Ward and Bamforth, 2002). Microbial acetyl xylan esterase activities were present in 30 - 90% of ammonium sulphate fraction (Egana et al., 1996; Degrassi et al., 1998).

4a.1.2. Anion exchange chromatography

The enriched ammonium sulphate fraction (40 - 80%) was dialyzed against Tris-HCl buffer (pH 8.5, 20 mM), and loaded on DEAE-cellulose (anion exchange) column, which was pre-equilibrated with the Tris-HCl buffer (40 mM, 2 bed volumes; 20 mM, 5 bed volumes). The elution profile of 40 - 80% ammonium sulphate fraction on DEAE-cellulose is shown in figure 28. This step was successful in removing the coloured material, large amounts of unbound and contaminating proteins, in addition it also reduced the viscosity of this fraction. The bound proteins were eluted

with a linear gradient of NaCl (0.0 - 0.6 M). AAEs were eluted as two peaks, a major peak (P_1) at 0.32 M, and a minor (P_2) at 0.43 M NaCl concentrations, respectively. The DEAE-cellulose purified ragi AAE major fraction showed a fold purification and recovery of 16.8 and 5.2% respectively.

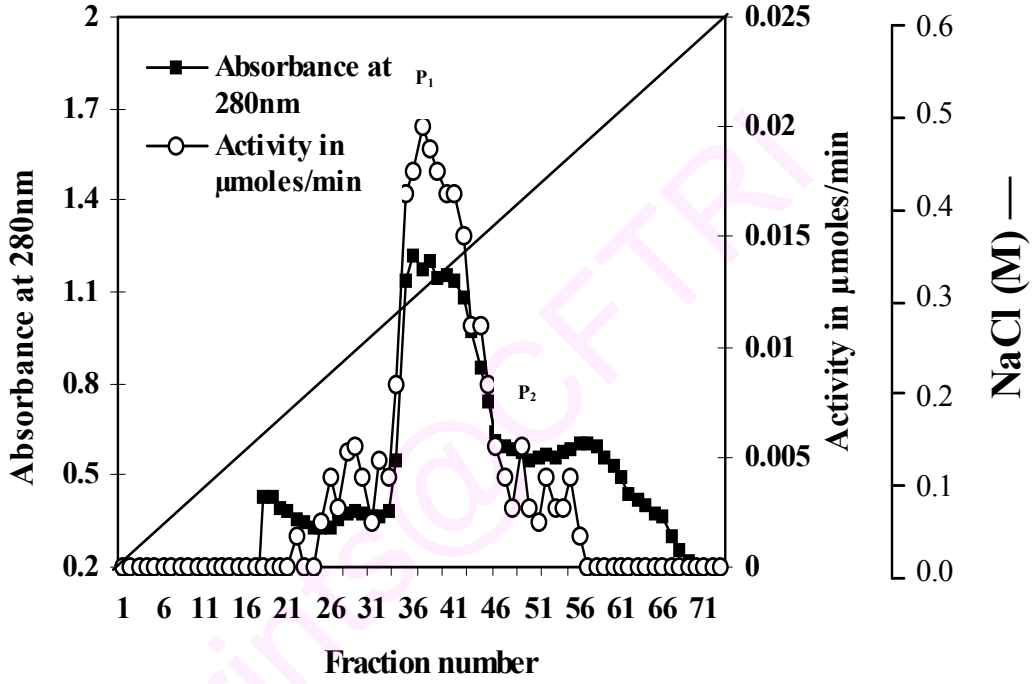


Figure 28. Elution profile of ragi AAE (40 - 80% ammonium sulfate fraction) on DEAE-cellulose column.

30 - 50% and 50 - 70% of ammonium sulphate fractions of crude barley extract showed two peaks of esterase activity with a linear gradient of NaCl (0.0 - 0.5 M) in phosphate buffer (50 mM, pH 6.5) on DE-23 cellulose ion exchange chromatographic column. The first peak of both the fractions showed activity on α-NA while the second peak of 50 - 70% fraction showed activity only towards PNPA (Ward and Bamforth, 2002).

A combination of isocratic and gradient elutions were used to separate AAE from crude barley malt extract (Humberstone and Briggs, 2002a). The recovery and fold purification after gradient elution of barley malt AAE was 21% and 15.6

respectively, which was further purified on Sephacryl S-200 column. Acetyl xylan esterases from microbes were purified by using anion (Egana et al., 1996) or cation exchangers (Dupont et al., 1996).

4a.1.3. Gel permeation chromatography

The DEAE-cellulose major peak of purified ragi AAE was further purified on Sephacryl S-200 gel permeation column. Figure 29 depicts the elution profile of ragi AAE on Sephacryl S-200 column. Ragi AAE was eluted (Tris-HCl, pH 7.0, 10 mM) as a single peak with a fold purification and recovery of 25.2 and 4.96% respectively.

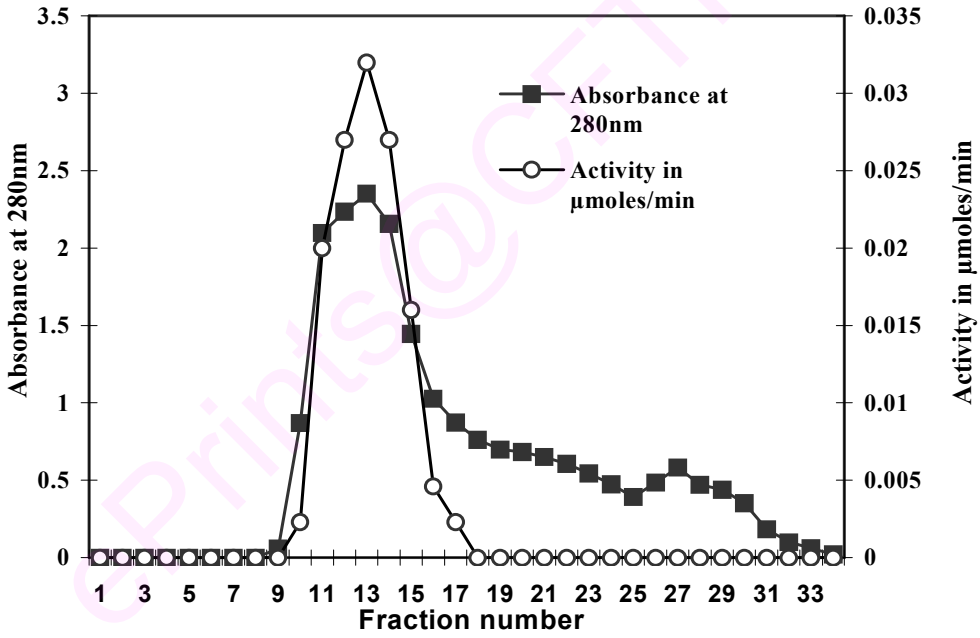


Figure 29. Elution profile of ragi AAE major fraction obtained from DEAE-cellulose column on Sephacryl S-200.

Sephacryl HR-200 gel permeation chromatography was employed in the separation of AAE from barley malt (Humberstone and Briggs, 2002a). NaCl and glucose were added to the elution buffer during the purification of barley AAE on Sephacryl HR-200 column, to increase the elution volume of the enzyme and to

reduce the ionic interactions between the solute and the gel matrix (Humberstone and Briggs, 2002a; Whitaker, 1963). The fold purification and recovery of barley AAE after purification on Sephacryl HR-200 column was 390 and 25%. The acetyl xylan esterase from *Bacillus pumilus* was eluted as a single peak on Sephacryl HR-200 column (Degrassi et al., 1998) with sodium phosphate buffer (pH 7.0, 50 mM). The fold purification and recovery of this enzyme from this microbe was 179.2 and 11.2% respectively.

4a.1.4. Hydrophobic interaction chromatography

On the native PAGE, the active Sephacryl S-200 fraction of ragi AAE showed carbohydrate streaking along with minor contaminating protein bands. Therefore, this active fraction was further subjected to hydrophobic interaction column chromatography (Phenyl-Sepharose) to remove most of the hydrophobically associated carbohydrate as well as contaminating minor proteins. The elution was performed with a decreasing linear gradient of 1.0 M - 0.0 M $(NH_4)_2SO_4$, and then with 50% ethylene glycol. AAE was eluted with 50% ethylene glycol in sodium phosphate buffer (50 mM, pH 6.5). Figure 30 shows the elution profile ragi AAE on Phenyl-Sepharose column.

The purified ragi AAE obtained by this step showed a fold purification and recovery of 34 and 0.36%, respectively. Phenyl-Sepharose hydrophobic interaction chromatography was a successful step to separate and purify various microbial acetyl xylan esterases (Degrassi et al., 1998).

Recovery of ragi AAE was significantly decreased after DEAE-cellulose column separation as well as after hydrophobic interaction chromatography, which might be due to (a) enzyme inactivation (b) removal of the carbohydrate, which might be hydrophobically associated with the AAE thereby giving stability to the enzyme. The overall scheme employed in the purification of AAE from ragi malt is summarized in **Table 13**.

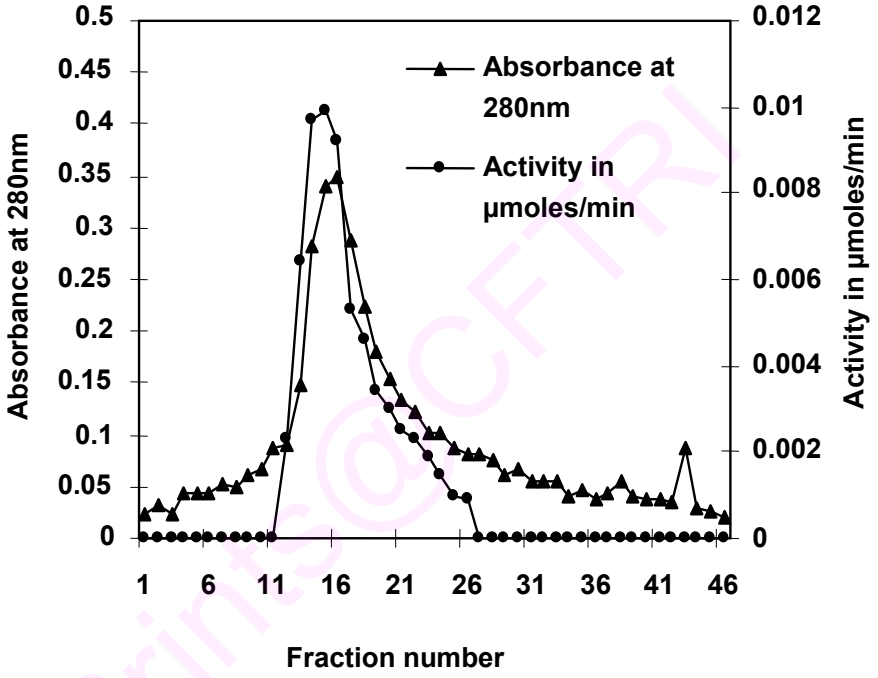


Figure 30. Elution profile of purified ragi AAE (Sephacryl S-200 active fraction) on Phenyl-Sepharose column

Table 13. Summary of the purification of AAE from 72 h ragi malt

Step	Total activity[b]	Total protein[c]	Specific activity[d]	Fold purification	Recovery (%)
Crude[a]	0.725	266	0.002	1	100
40-80% ASP fraction	0.67	16.7	0.04	14.8	92.4
DEAE-cellulose	0.04	0.83	0.045	16.8	5.24
Sephacryl S-200	0.036	0.53	0.068	25.2	4.96
Phenyl-Sepharose CL-4B	0.0016	0.028	0.093	34.3	0.36

[a] 50 g scale (Values are average of three independent experiments)
[b] One unit is equivalent to 1 μmol of α-naphthol released min^{-1}.
[c] Total protein is expressed in mg.
[d] Specific activity is expressed in 1 μmol of α-naphthol released min^{-1} mg^{-1} of protein

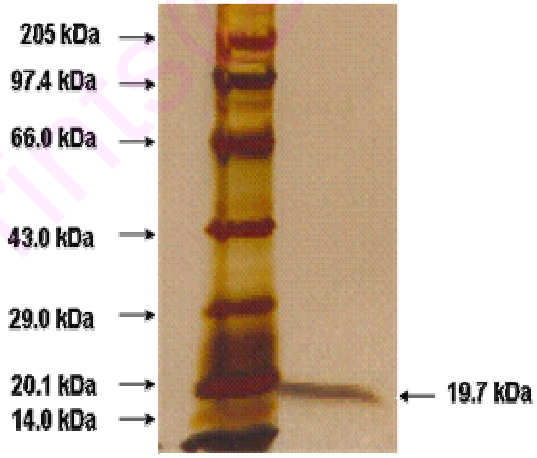
4a.2. Criteria of purity of purified ragi AAE

4a.2.1. Native/SDS-PAGE and activity staining

The purity of ragi AAE was confirmed by native and SDS-PAGE. Crude extract of ragi AAE upon four-step purification resulted in apparently homogeneous enzyme with respect to protein and activity staining. Protein and activity staining of purified ragi AAE is shown in the figure 31a & b. The activity and protein band of this purified enzyme coincides on native PAGE. By SDS-PAGE, a single subunit protein band with an estimated M_r of 19.7 kDa was observed (Figure 32).

PAGE electrophoresis is used to determine the purity of cereals such as barley (Humberstone and Briggs, 2002), wheat (Cubadda and Quattrucci, 1974) and microbial AAEs (Degrassi et al., 1998). The apparently homogeneous ragi malt AAE was taken further for kinetic studies and characterization.

Figure 31. Native PAGE of purified ragi AAE
(a) Protein staining (b) Activity staining



A B

Figure 32. SDS-PAGE of purified ragi AAE

A) Molecular weight markers: 205 kDa Myosin; 97.4 kDa Phosphorylase; 66.0 kDa Bovine serum albumin; 43.0 kDa Ovalbumin; 29.0 kDa Carbonic anhydrase; 20.1 kDa Soybean trypsin inhibitor; 14.0 kDa Lysozyme
B) 19.7 kDa purified ragi AAE

4a.3. Characterization of purified ragi AAE

4a.3.1. Determination of molecular weight of purified ragi AAE

The apparent molecular mass of purified ragi AAE determined under denaturing conditions was found to be 19.7 kDa (Figure 33) which is comparable to the ones reported from the acetyl xylan esterases isolated from *Pencillium purpurogenum* 48.0 and 23.0 kDa (Egana et al., 1996), *Bacillus pumilus* 40.0 kDa (Degrassi et al., 1998), and AAE from Barley malt ~55.0 kDa (Humberstone and Briggs, 2002a) etc. The estimated M_r of purified enzyme from ragi under non-denaturing conditions (native molecular weight) by Sephacryl S-200 (Figure 34) and ESI-MS (positive mode) (Figure 35) was found to be ~79.0 kDa indicating it to be a homotetramer, similar to the one reported from acetyl xylan esterase of *Bacillus pumilus* but it showed a molecular weight of 190 kDa (Degrassi et al., 1998). Three different esterases were identified in barley of which, one showed ~62.0 kDa, and the other two showed a molecular weight of 47.0 kDa (Ward and Bamforth, 2002).

The molecular weight of purified AAE from ragi under non-denaturing conditions is comparable to the ones reported from microbes such as *Fibrobacter succinogenes* (McDermid et al., 1990b), *Streptomyces lividans* (Dupont et al., 1996), *Candida guilliermondii* (Basaran and Hang, 2000), *Trichoderma reesei* (Poutanen and Sundberg, 1988; Sundberg and Poutanen, 1991).

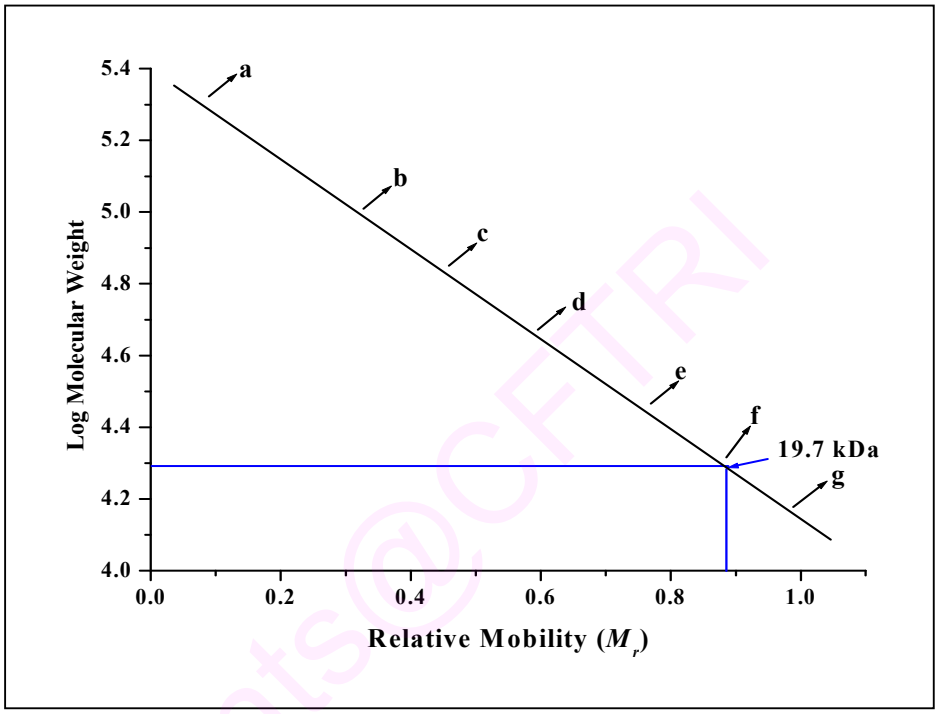


Figure 33. Calibration curve for the determination of molecular weight of purified ragi AAE by SDS-PAGE

Molecular weight markers: a) 205 kDa Myosin, b) 97.4 kDa Phosphorylase, c) 66.0 kDa, Bovine serum albumin, d) 43.0 kDa Ovalbumin, e) 29.0 kDa Carbonic anhydrase, f) 20.1 kDa Soybean trypsin inhibitor and g) 14.0 kDa Lysozyme.
19.7 kDa purified ragi AAE.

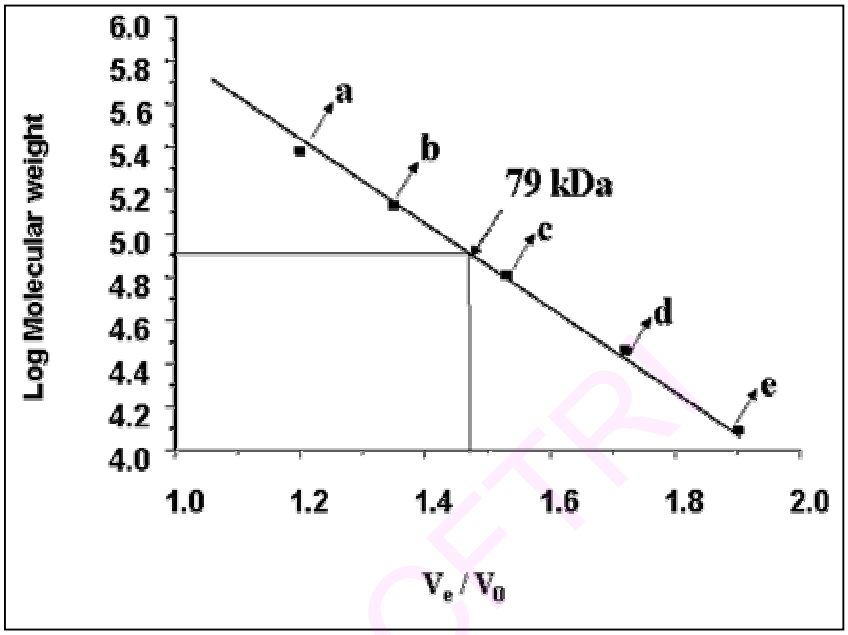


Figure 34. Calibration curve for the determination of molecular weight of purified ragi AAE by GPC on Sephacryl S-200.

a) 200 kDa β-amylase, b) 150 kDa Alcohol dehydrogenase, c) 66.0 kDa Bovine serum album , d) 29.0 kDa Carbonic anhydrase and e) 12.0 kDa Papain.
79.0 kDa purified ragi AAE.

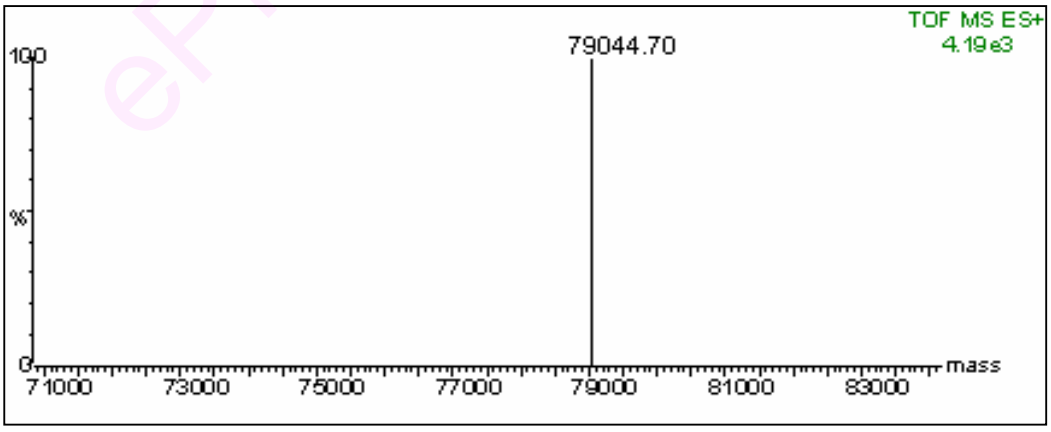


Figure 35. Determination of molecular mass of purified ragi AAE by ESI-MS (positive mode)

4a.3.2. pH optima of purified ragi AAE

To determine the pH optima of purified ragi AAE the activities were determined using different buffers such as sodium acetate (pH 4.0 - 6.0), sodium phosphate (pH 6.0 - 8.0) and Tris-HCl (pH 7.0 - 9.0) at 75 mM concentration. Purified ragi AAE was found to have pH optima of 7.5 and 80% activity was retained between pH 6.0 - 9.0 (Figure 36).

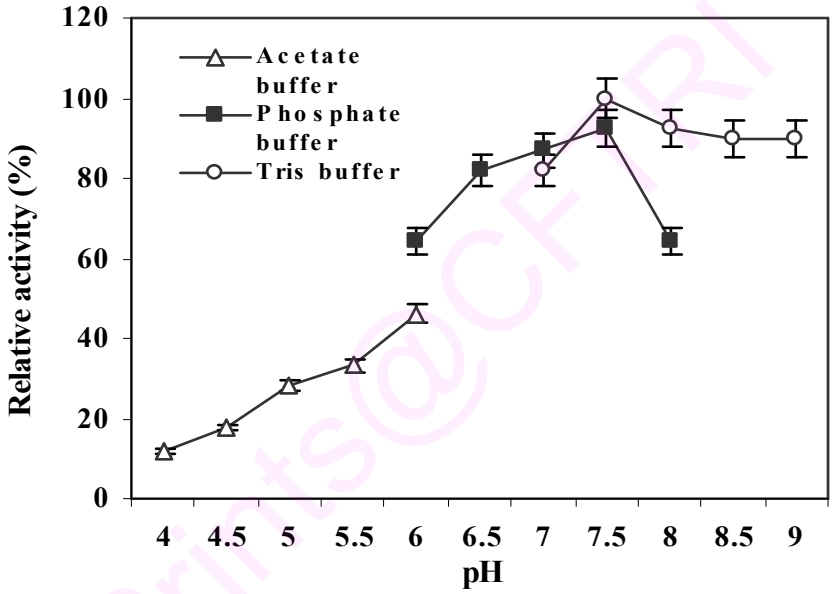


Figure 36. pH optima of purified ragi AAE

The activity of this enzyme in alkaline pH was more than the ones observed in acidic pH. The activity has decreased both in acetate and phosphate buffers at lower pH compared to Tris-HCl buffer. However, the drop in activity in acetate buffer was much more pronounced than in phosphate buffer. Most of the activity was retained in Tris-HCl buffer, indicating better stability of AAE in Tris-HCl buffer. The enzyme activity has decreased drastically in the pH range 4.0 - 5.5 in acetate buffer and 6.0 - 7.0 in phosphate buffer indicating labile nature of the enzyme in acidic as well as at neutral pH.

The optimal pH of 7.5 is similar to the pH values reported for *Bacillus pumilus* (Degrassi et al., 1998), *Schizophyllum commune* (Halgasova et al., 1994), *Streptomyces lividans* (Dupont et al., 1996) and *Candida guilliermondii* (Basaran and Hang, 2000), and comparatively less then the pH optima reported for *Rhodotorula mucilaginosa* (Lee et al., 1987) and *Orpinomyces* sp (Blum et al., 1999), and higher than the ones reported for *Caldocellum saccharolyticum* (Luthi et al., 1990) and *Penicillium purpurogenum* (Egana et al., 1996).

4a.3.3. pH stability of purified ragi AAE

pH stability of ragi AAE was determined after the pre-incubation of the enzyme at various pH values for varying intervals of time (0 - 360 min) followed by determining the residual activity of the enzyme. Buffers (75 mM) such as glycine-HCl (pH 2.0 - 3.0), sodium acetate (pH 4.0 - 6.0), sodium phosphate (7.0 - 8.0) and Tris-HCl (pH 8.0 - 9.0), were used to maintain the buffering capacity and to determine the stability of this purified enzyme at various pH. The enzyme was stable over a broad pH range from 5.5 - 9.0 retaining about 80 - 90% activity after 2 h of incubation (Figure 37).

The observation is similar to the pH stability of acetyl xylan esterase of *Rhodotorula mucilaginosa* (Lee et al., 1987), *Streptomyces lividans* (Dupont et al., 1996), *Candida guilliermondii* (Basaran and Hang, 2000). The acetyl xylan esterase (I) of *Penicillium purpurogenum* showed pH stability of 4.0 - 8.0 (Egana et al., 1996) and the AAE of *Bacillus pumilus* showed 100% stability in the pH range 8.0 - 9.5 (Degrassi et al., 1998).

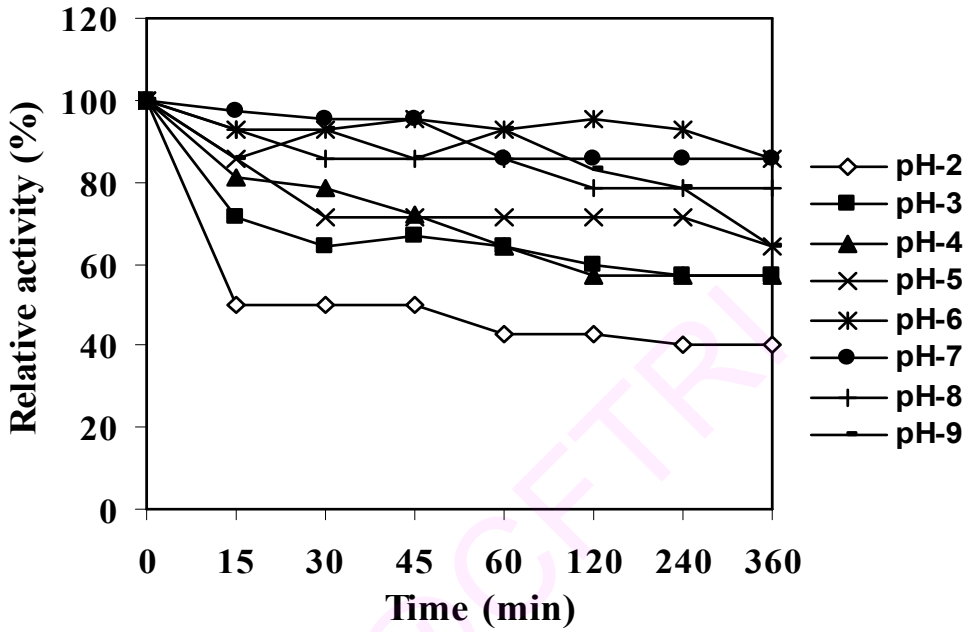


Figure 37. pH stability of purified ragi AAE

4a.3.4. Temperature optima and stability of purified ragi AAE

To determine the temperature optima of ragi AAE, activities were determined at a temperature range of 30 - 80°C (Figure 38). The optimum temperature was found to be 45°C and this was comparatively lower than the ones reported for microorganisms such as *Bacillus pumilus* (55°C), *Streptomyces lividans* (70°C), *Thermoanaerobacterium* sp (80°C) (Degrassi et al., 1998; Dupont et al., 1996; Shao and Wiegel, 1995), *Caldocellum saccharolyticum* (Luthi et al., 1990) and *Candida guilliermondii* (50 - 60°C) (Basaran and Hang, 2000) and higher than *Orpinomyces* sp. strain PC-2 (30°C) (Blum et al., 1999).

The thermal stability of purified ragi AAE was determined by pre-incubating the enzyme at different temperatures (30 - 80°C) and measuring the residual activities after 15 min. The purified enzyme was thermally active at 30°C. The stability of the enzyme decreased gradually and at 60°C, about 40% of the activity was retained. Less than 5% of the activity was retained at 80°C (Figure 38).

The acetyl xylan esterase from *Rhodotorula mucilaginosa* has showed thermal stability of ~55°C for 2 h (Lee et al., 1987), *Streptomyces lividans* ~95°C (Dupont et al., 1996), *Candida guilliermondii* ~60°C (Basaran and Hang, 2000), *Bacillus pumilus* ~50°C (Degrassi et al., 1998) while the recombinant acetyl xylan esterase of *Caldocellum saccharolyticum* expressed in E.coli showed ~80°C (Luthi et al., 1990). As the temperature increases, the three dimensional activity of the enzyme which is maintained by a number of forces mainly hydrophobic interactions, hydrogen bonds are disrupted, which result in the denaturation of the protein and lead to inactivation of the enzyme.

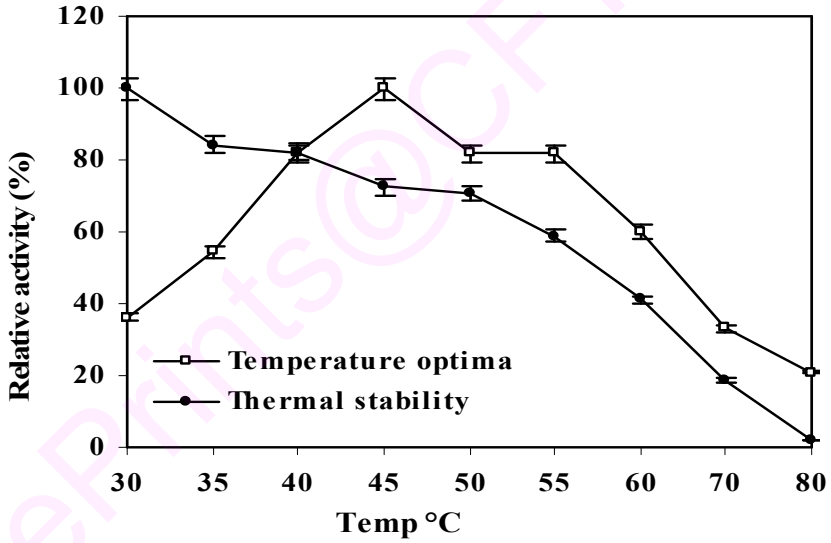


Figure 38. Temperature optima and thermal stability of purified ragi AAE

4a.3.5. Determination of energy of activation of purified ragi AAE

Activation energy, defined as the minimum energy required by the reactants in order to pass into a transition state, represents the halfway point where the bonds of substrate are distorted sufficiently so that the conversion to products becomes possible. The activation energy of the reaction was calculated at the optimum pH (7.5) of the enzyme, using α-NA as substrate. The energy of activation was calculated from the slope of the arrhenius curve obtained by plotting natural log of activity at various

temperatures on y-axis and 1/T in Kelvin on x-axis. The activation energy of purified ragi AAE was found to be 7.29 kJ mol^{-1} (Figure 39).

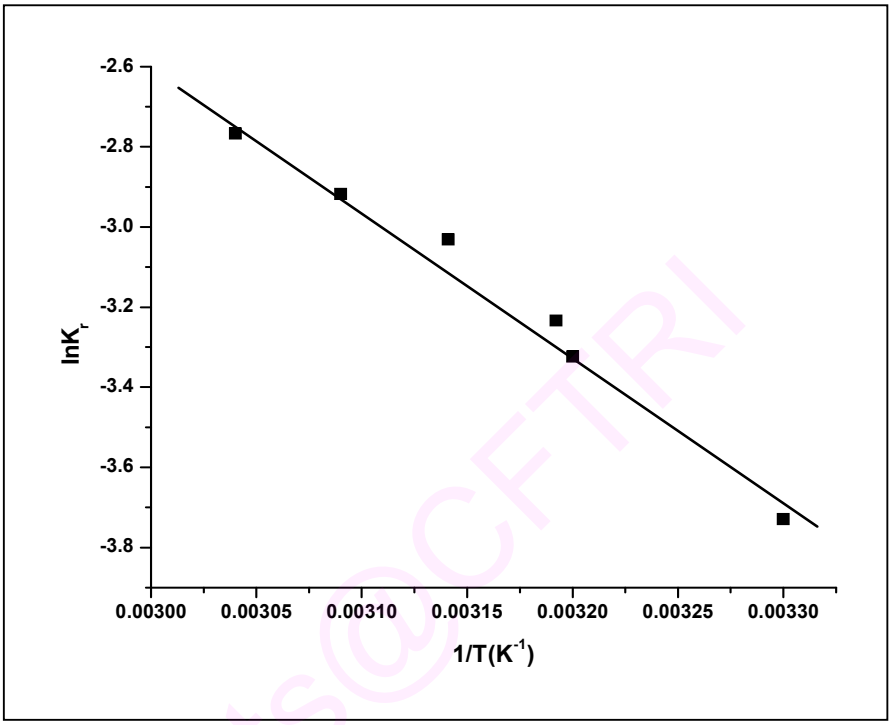


Figure 39. Arrhenius plot for the temperature dependence of AAE using α-NA as substrate.

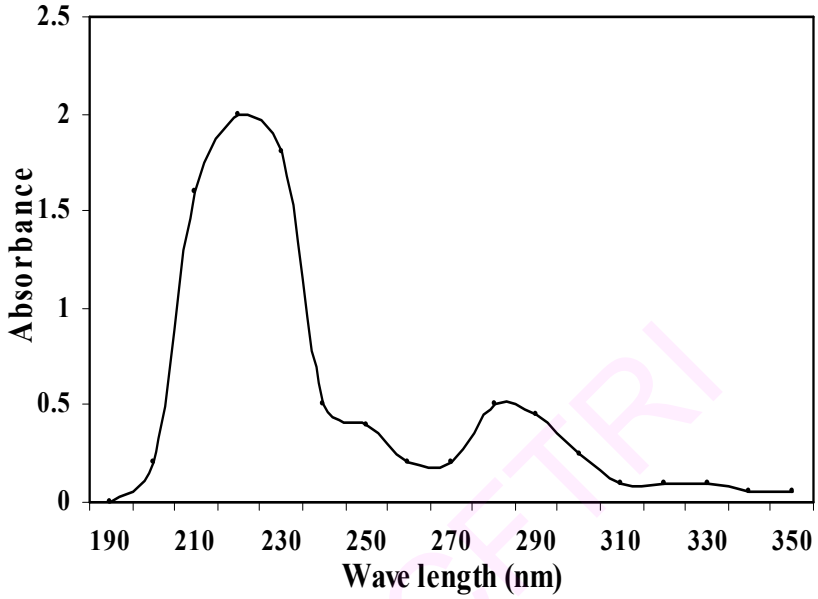
4a.3.6. Spectral analysis of purified ragi AAE

a) UV absorption spectra

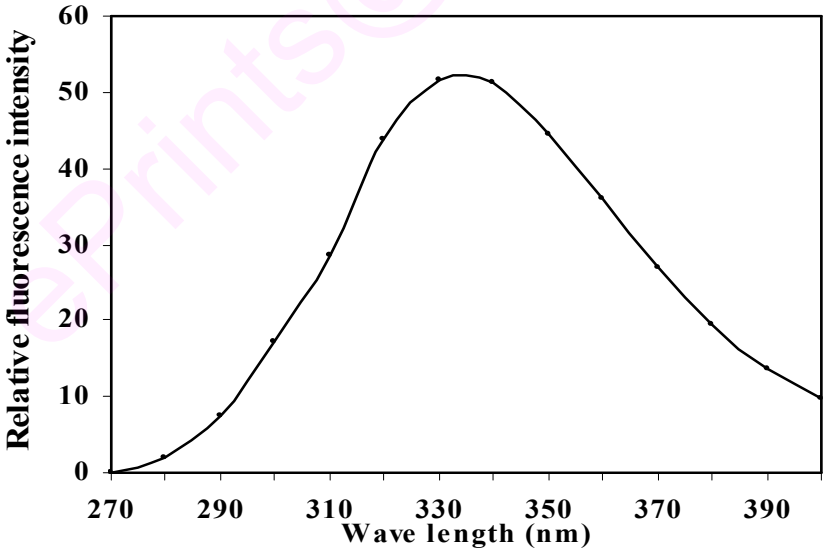
The UV absorption spectra of purified ragi AAE showed two peaks one at 225 nm (major peak) corresponding to the absorption of peptide bonds and the other at 280 nm corresponds to the absorption of aromatic amino acids (Figure 40a).

b) Fluorescence spectra

The fluorescence spectra of purified ragi AAE showed an excitation maximum of 220 nm and emission maxima range of 330 - 340 nm (Figure 40b). The fluorescence or long wave length absorption spectra of proteins is mainly due to the quenching of aromatic amino acids especially tryptophan.



(a)



(b)

Figure 40. (a) UV absorption spectra of purified ragi AAE
(b) Fluorescence spectra (emission) of purified ragi AAE

4a.3.7. Effect of substrate concentration on the activity of purified ragi AAE

Effect of different substrate concentrations on the initial velocity was calculated and the kinetic constants K_m and V_{max} were calculated from the double reciprocal plots (LB plot, Lineweaver and Burk, 1934) (Figure 41). The Michaeli constant K_m value of AAE from ragi was found to be 0.40 µM for α-NA and V_{max} was 0.175 U ml^{-1}. The K_m value of 0.40 µM determined for purified finger millet esterase is lower than the values reported for *Bacillus pumilus* (1.54 mM) (Degrassi et al., 1998), *Rhodotorula mucilaginosa* (2.6%) (Lee et al., 1987), *Candida guilliermondii* (2.63 mM) (Basaran and Hang, 2000), *Fibrobacter succinogenes* (2.7 mM) (McDermid et al., 1990b) indicating higher specificity for α-NA. K_m values for two acetyl xylan esterases from *Thermoanaerobacterium* sp were determined using 4-methyl umbelliferyl acetate as 0.45 and 0.52 mM, respectively (Shao and Wiegel, 1995).

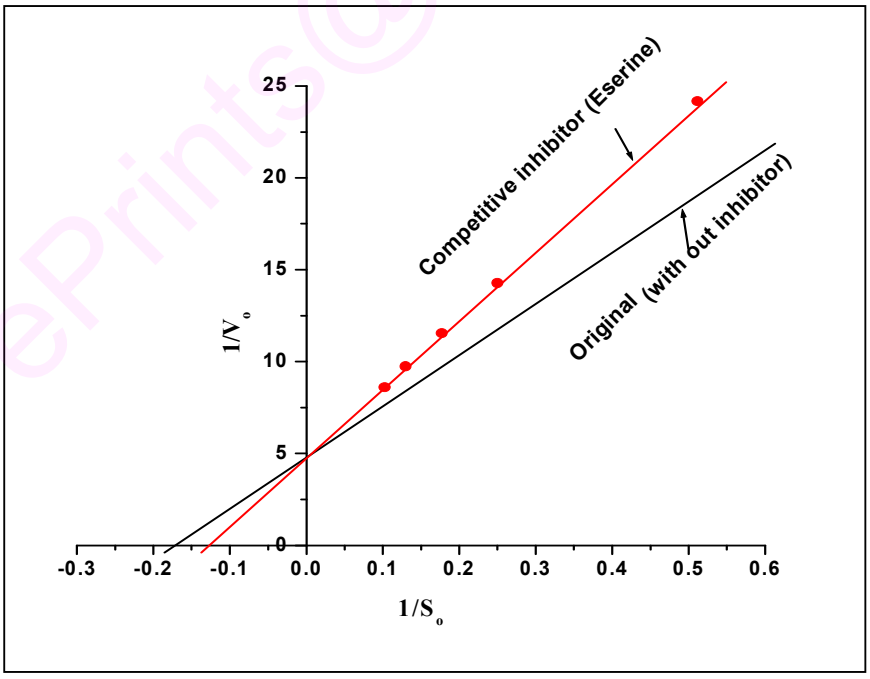


Figure 41. Determination of K_m and V_{max} of ragi AAE by Lineweaver-Burk plot with and without inhibitor (substrate used α-NA)

A K_m value of 25 mM for barley malt AAE was reported using diacetin as the substrate (Humberstone and Briggs, 2002a). K_m value of 7.94 mg ml^{-1} and V_{max} of 1977 U mg^{-1} of acetyl xylan esterase was found in *Streptomyces lividans* using acetylated birch wood xylan at concentration of 2.0 - 50 mg ml^{-1} (Dupont et al., 1996). The recombinant acetyl xylan esterase from *Orpinomyces* sp showed a K_m value of 0.9 mM and V_{max} of 785 U mg^{-1} with PNPA (Blum et al., 1999). No information is available with respect to the detailed kinetics and substrate specificity of cereal/millet AAEs.

4a.3.8. Effect of metal ions on the activity of purified ragi AAE

A range of metal ions such as Fe^{3+}, Cu^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+} at 5 mM concentration were tested for AAE activation/inhibition effect and the results are given in **Table 14.** Metal ions such as Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+} reduced the activity by 60 - 70%, while Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{+3} by 30 - 40%. Similar concentrations of Fe^{3+}, Cu^{2+}, Cu^{+} and citric acid resulted in activating the enzyme. Oxalic acid did not show any significant effect on the enzyme activity.

The inhibitory effect of Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+} and Zn^{2+} was in accordance with the results reported for AAE from *Schizophyllum commune* (Halgasova et al., 1994), *Bacillus pumilus* (Degrassi et al., 1998) and with FAE from *Aspergillus awamori* (Nakazato et al., 1999). Fe^{3+} and Cu^{2+} increased the enzyme activity which is in accordance with the 10% increase in activity of feruloyl esterase of *Pencillium expansum* by Fe^{3+} ions (Donaghy and McKay, 1997).

Cu^{2+}, Ag^{+} (10 mM, 25 - 50% inhibition) and EDTA (100 mM, 70% inhibition) inhibited the activity of acetyl xylan esterase from *Candida guilliermondii* (Basaran and Hang, 2000). The inhibitory and stimulatory effects of these ions may be important factors in the commercial exploitation of this enzyme, where enzyme stability and activity is paramount.

Table 14. Effect of metal ions, EDTA and citric acid on the activity of purified ragi AAE

Metal ions	Relative activity (%)
Control	100
Cu^{2+}	1130
Fe^{3+}	1000
Cu^{+}	728
Hg^{2+}	489
EDTA	293
Citric acid	141
Mg^{2+}	82
Zn^{2+}	79
Mn^{2+}	77
Al^{3+}	69
Co^{2+}	50
Ba^{2+}	44
Ni^{+}	36
Ca^{2+}	36

4a.3.9. Effect of group specific reagents on the activity of purified ragi AAE

Purified ragi AAE activity was determined in the presence of chemicals such as PCMB, iodoacetamide and eserine at 45°C and 25 mM concentration. The enzyme was found to be completely inactivated by PCMB as well as eserine whereas iodoacetamide had showed almost negligible effect on the activity. **Table 15** shows the effect of PCMB, eserine and iodoacetamide on the relative activity of purified ragi AAE.

Table 15. Effect of group specific reagents on the relative activity (%) of purified ragi AAE

Chemical	Relative activity (%)
Control	100
PCMB	0
Eserine	0
Iodoacetamide	95

The inhibition of PCMB and eserine suggests the possible presence of amino acids such as cysteine or serine residues at the active site pocket. The pH optima (7.5) value of the AAE also supports this statement as the ionization values of these amino acids fall in this range. PCMB is known to form complexes with cysteine residues in the active site region of the enzymes. The effect of eserine on the enzyme kinetics of purified ragi AAE showed that it is a competitive inhibitor (Figure 41). Eserine is a structural analogue of the aminoacid serine, which is known to be present in the active site of several of the esterases (Cubadda and Quattrucci, 1974).

Studies also reveal that eserine modifies histidine residues in the active site causing inhibition (Kim and Lee, 2004). Wheat esterases are completely inhibited by eserine but not sensitive to PCMB and EDTA (arylesterase inhibitors) (Cubadda and Quattrucci, 1974)

4a.3.10. Enzymatic deesterification of acetylated substrates

The amount of acetylation achieved is determined by measurement of enzymatically released acetic acid from water-soluble polysaccharides and synthetic substrates by purified ragi AAE. The specific activities of purified ragi AAE using various water-soluble polysaccharides and synthetic substrates are shown in the **Table 16.** The specific activity of this purified enzyme with respect to wheat water-soluble preparation is higher than the other polysaccharide substrates. The enzyme was active on both small molecular weight substrates as well as polysaccharides as indicated by the present study.

The anionic esterases of barley were more active towards α-NA (Ward and Bamforth, 2002). The complete deacetylation of birchwood xylan by purified acetyl xylan esterase of *Trichoderma reesei* was reported by Poutanen and Sundberg (1988). The release of acetic acid by the action of this enzyme was less compared to the theoretical amount; the reason may be due to the steric hindrance of methylglucuronosyl residues present at the vicinity of acetyl groups or due to the high specificity of the acetyl xylan esterase towards the position of acetyl groups on xylose ring (Puls et al., 1991; Poutanen and Puls, 1989).

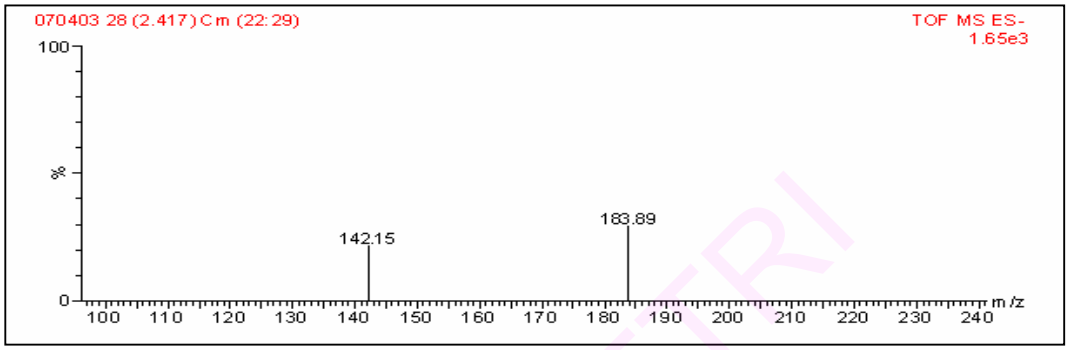
Table 16. Substrate specificity of purified ragi AAE

Substrates	Specific activity (U mg^{-1} of protein)
Ragi	0.03
Wheat	4.44
Larch wood xylan	0.06
Gum karaya	0.28
α-NA	3.38
PNPA	5.39

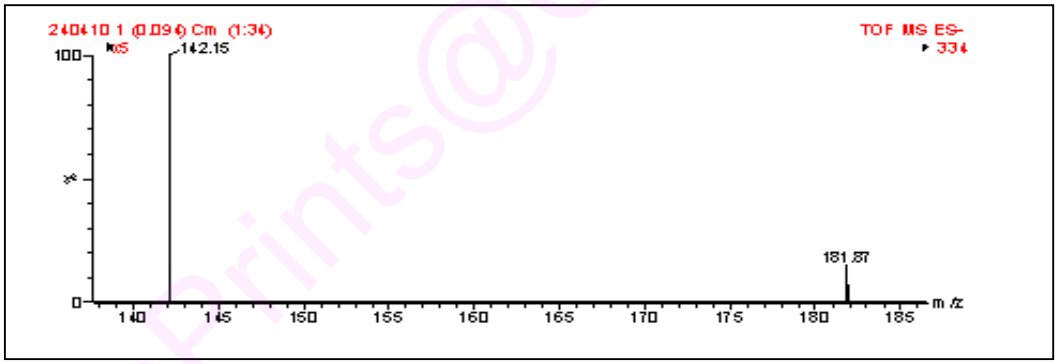
4a.3.11. Electrospray Ionization-Mass Spectrometry (ESI-MS)

Analysis by ESI-MS confirmed the gradual deacetylation of α-NA, PNPA and glucose pentaacetate to α-naphthol (Figure 42a, b & c), PNP (Figure 43a, b, c & d) and glucose triacetate (Figure 44a & b), respectively, by purified ragi AAE. The formation of PNP by the enzymatic deacetylation of PNPA using ragi AAE increased with the increase in incubation period (0 - 8 h). The ratio of signals at *m/z* for PNP and PNPA (a. 0.6:1.0, b.1.33:1.0, c. 5.0:1.0, d. 100 %) showed the complete deacetylation of PNPA to PNP after 8 h of reaction. The *m/z* values of PNPA and PNP are 193.70 and 137.15 respectively. Similar result was observed with α-NA to α-naphthol. The *m/z* value of α-naphthol is 142.70 and showed complete deacetylation of α-NA (183.89, *m/z* negative mode) to α-naphthol after 1 h of reaction

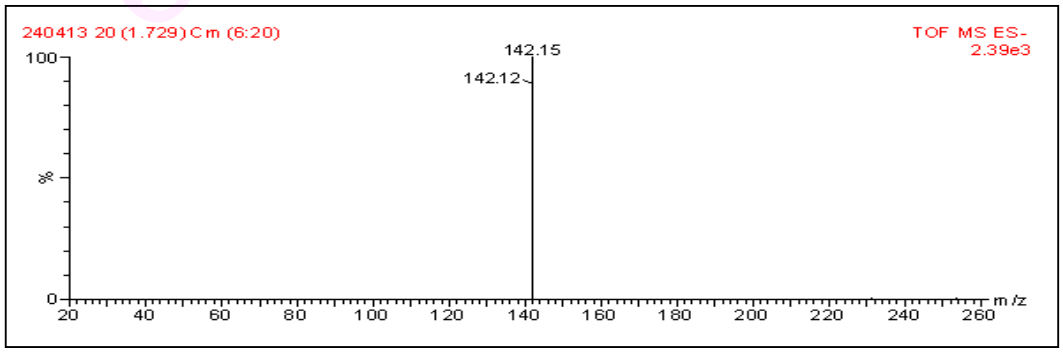
as shown in the Figure 42c. The substrate glucose pentaacetate was deacetylated to glucose triacetate after 1 h of the reaction, and did not show any change in the released product even after 8 h. The *m/z* values of glucose penta acetate and glucose triacetate are 413 and 331 respectively as shown in the Figure 44a and b.



(a)

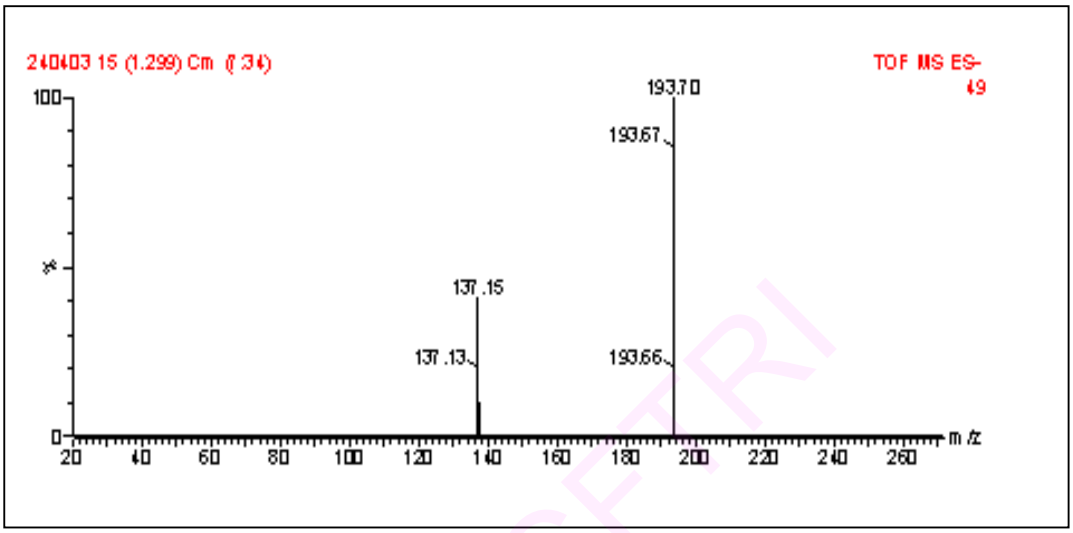


(b)

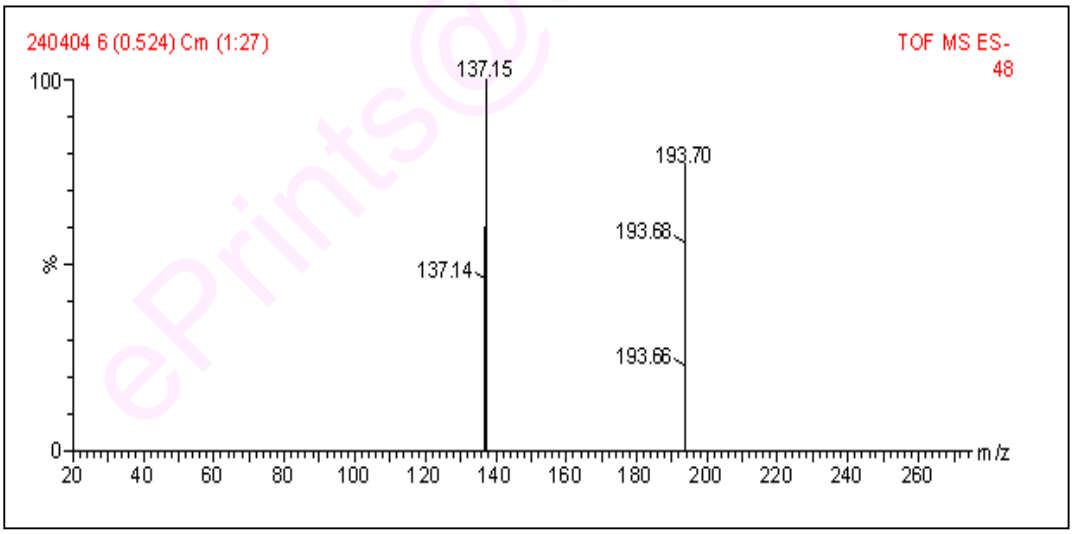


(c)

Figure 42. ESI-MS of deacetylation of α-NA to α-naphthol (0-8 h of incubation) (a) α-NA standard (b) 30 min and (c) 1 h by purified ragi AAE

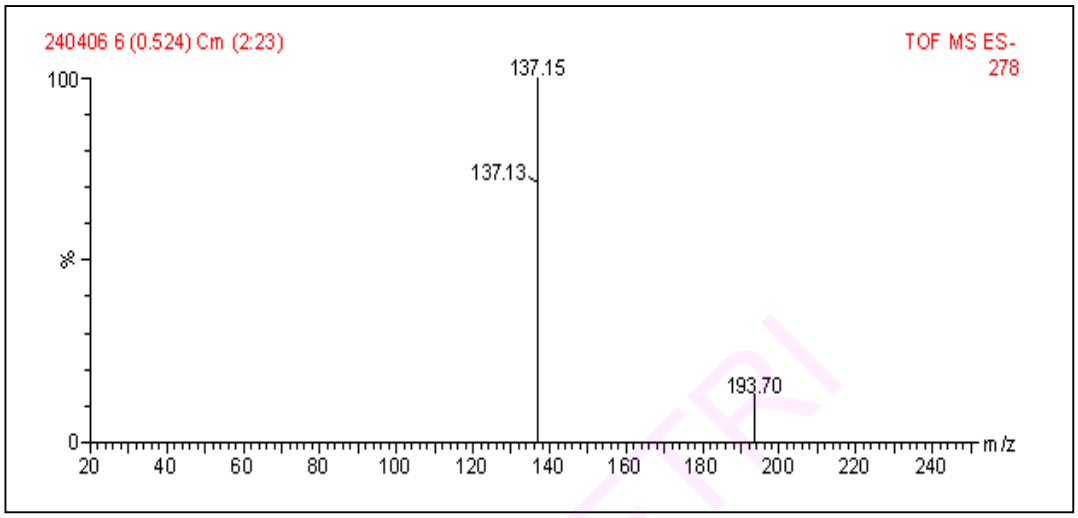


(a)

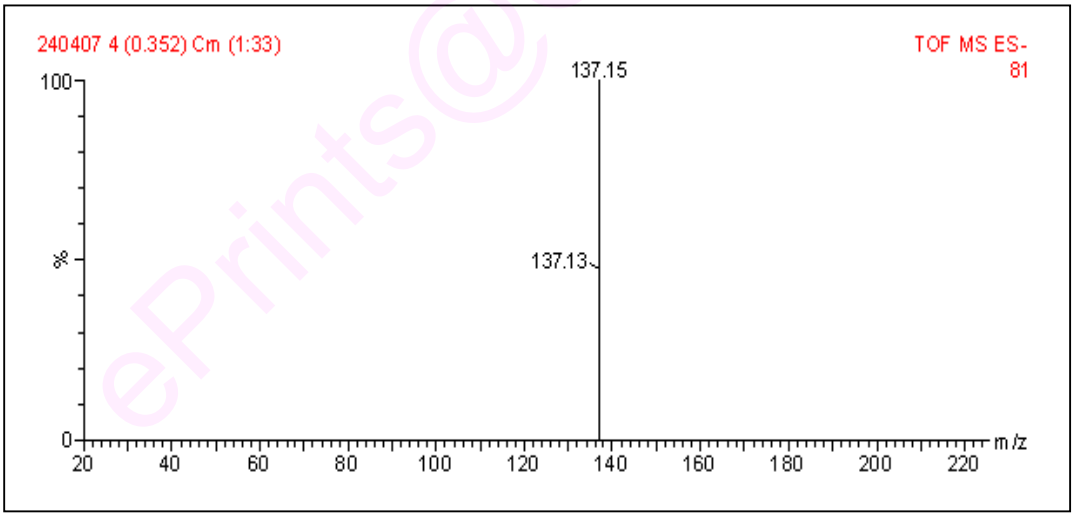


(b)

Figure 43. ESI-MS of gradual deacetylation of PNPA to PNP (1-8 h of incubation) a) 1 h and b) 2 h by purified ragi AAE

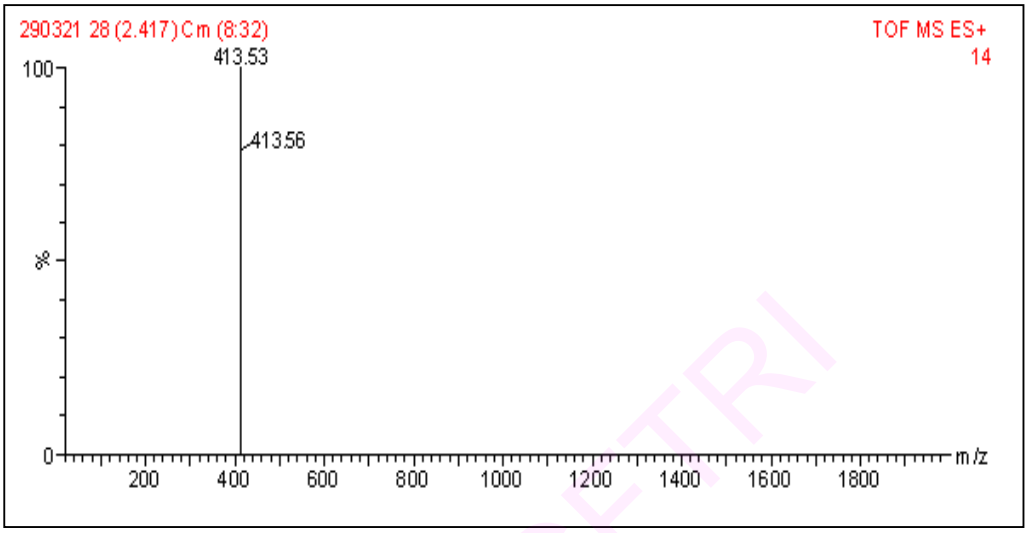


(c)

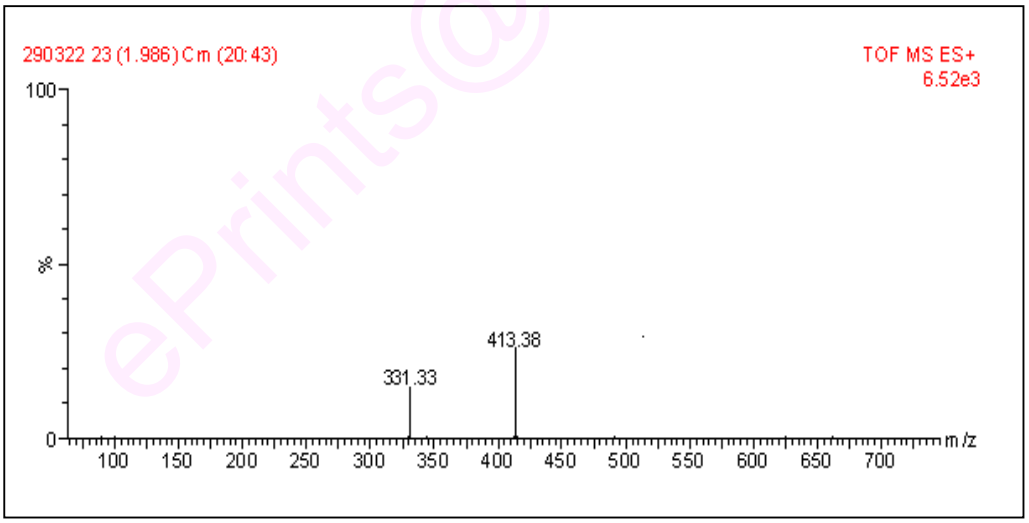


(d)

Figure 43. ESI-MS of gradual deacetylation of PNPA to PNP (1-8 h of incubation) c) 4 h d) 8 h by purified ragi AAE



(a)



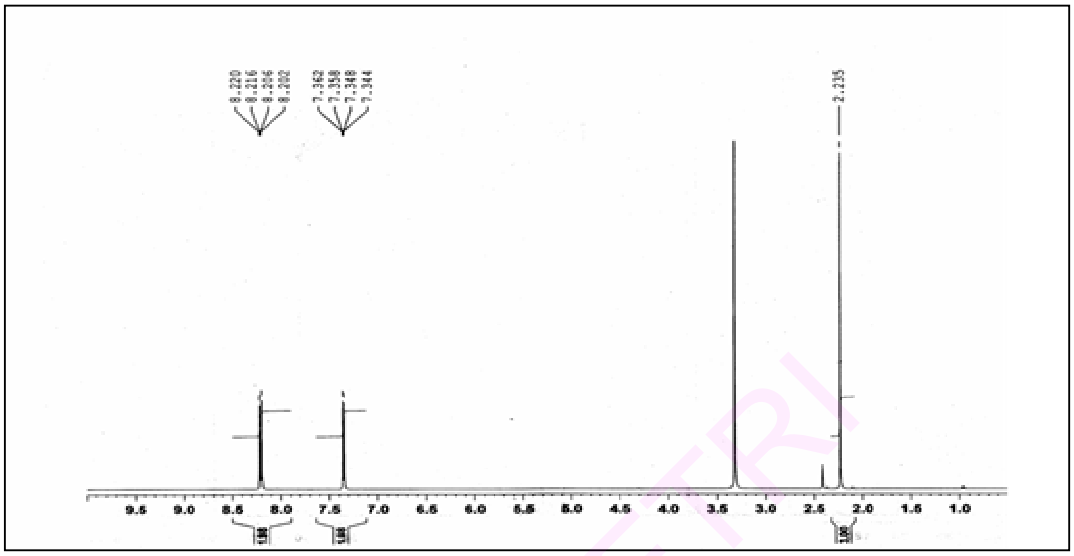
(b)

Figure 44. ESI-MS of gradual deacetylation of glucose pentaacetate to glucose triacetate (1-8 h of incubation) a) 0 h b) 1 h, by purified ragi AAE

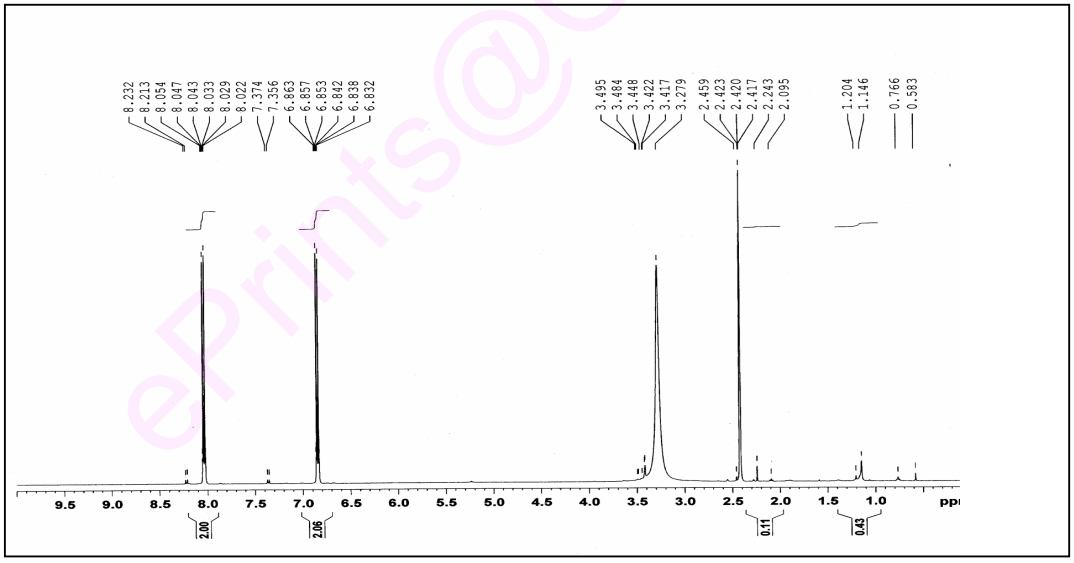
4a.3.12. ^{1}H NMR analysis of deacetylation of α-NA and PNPA by purified ragi AAE

The enzymatic deacetylation of PNPA and α-NA was confirmed by carrying out ^{1}H NMR experiments (Figure 45 & 46). The chemical shifts at 8.20 - 8.22 ppm were assigned to H-3 and H-5 protons, while 7.34 - 7.36 ppm were assigned to H-2 and H-6 protons in the aromatic ring of PNPA. The chemical shift values at 2.235 were assigned to acetyl protons of PNPA (Figure 45a).The chemical shift values for these protons were shifted after enzymatic deacetylation of PNPA to PNP (8.022 - 8053 and 6.83 - 6.86 ppm) (Figure 45b). The unreacted PNPA showed a slight chemical shift value at 7.3 and 8.2 ppm for aromatic protons and at 2.2 for acetyl protons.

In the case of α-NA the chemical shift values at 7.22 - 7.91 ppm were assigned for the aromatic protons of naphthol and 2.35 ppm assigned for the protons of the acetyl group (Lavertu et al., 1991) (Figure 46a). After enzymatic deacetylation there was a change in chemical shift values in the nucleus of naphthol ring. The complete absence of acetyl protons and presence of a carbonyl proton at 10.0 ppm indicates the complete deacetylation of α-NA to α-naphthol (Figure 46b). The complete hydrolytic cleavage of acetyl group of α-NA and PNPA by ragi AAE was found to be quite slow.

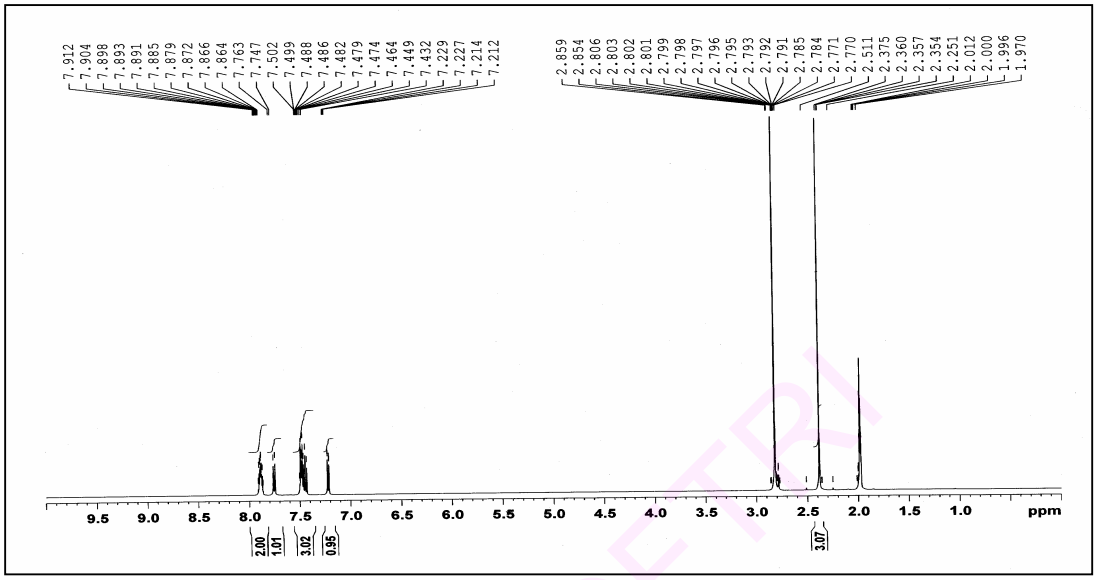


(a)

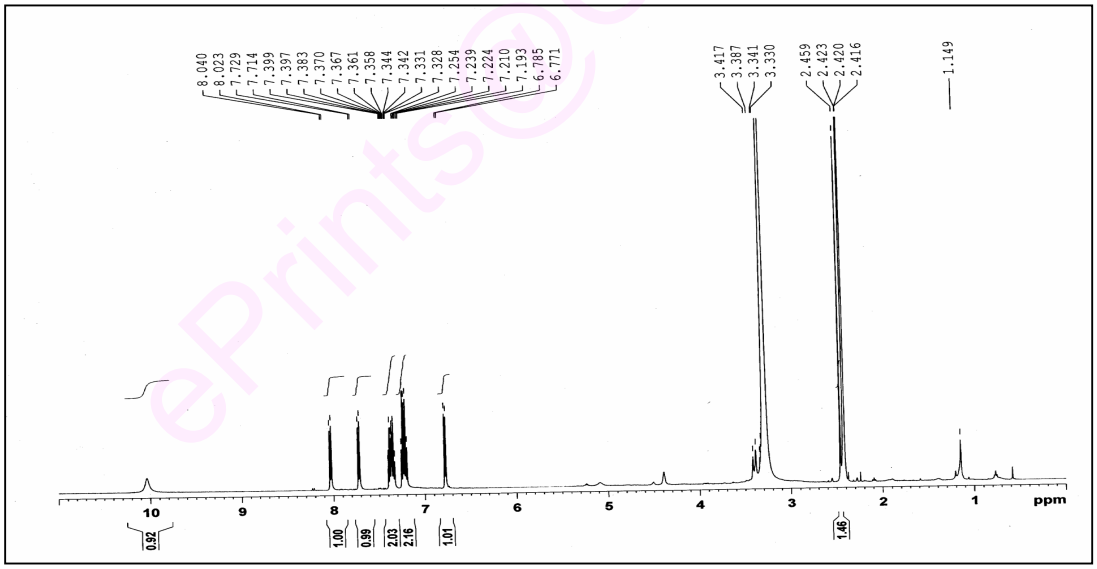


(b)

Figure 45. ^{1}H NMR spectra of a) PNPA standard b) enzymatic deacetylation of PNPA to PNP by purified ragi AAE



(a)



(b)

Figure 46. ^{1}H NMR spectra of a) α-NA standard b) Enzymatic deacetylation of α-NA to α-naphthol by ragi AAE

4b. Ragi malt FAE

4b.1. Purification of FAE

The crude extract isolated from 96 h ragi malt with all the optimum/best conditions standardized as indicated in the previous chapter (3b) was taken for purification using ammonium sulphate fractionation followed by anion exchange chromatography on DEAE-cellulose and gel filtration on Biogel P-30.

4b.1.1. Fractional precipitation

Crude Tris-HCl buffer extract (containing 1% PVPP, 1% Triton X-100 and 25 mM reduced glutathione) from 96 h ragi malt was subjected to ammonium sulfate precipitation and separated in to four fractions (0 - 20, 20 - 40, 40 - 60 and 60 - 80%). FAE activity was determined in all the ammonium sulphate fractions as shown in the **Table 17**. 0 - 20 and 20 - 40% fractions together showed a recovery of ~16.6%, while 40 - 60 and 60 - 80% showed a recovery of ~83.5%. As 40 - 80% ammonium sulphate fractions gave 83.5% recovery of FAE, which was taken for the further purification. 40 - 60% ammonium sulfate fraction of crude barley malt extract showed maximum FAE activity (Bartolome et al., 1996).

Table 17. Ammonium sulphate fractionation of crude FAE from 96 h ragi malt

ASP fraction %	Total activity (U h^{-1})	Specific activity (U h^{-1} mg^{-1})	Recovery (%)
0-20	0.02	0.004	3.0
20-40	0.09	0.02	13.6
40-60	0.30	0.07	45.5
60-80	0.25	0.05	38.0

4b.1.2. Anion exchange chromatography

The 40 - 80% ammonium sulphate fraction was dialyzed against Tris-HCl buffer (pH 9.0, 20 mM) and loaded on DEAE-cellulose (anion exchange) column, which was pre-equilibrated with the Tris-HCl buffer (40 mM, 2 bed volumes; 20 mM, 5 bed volumes). The elution profile of 40 - 80% ammonium sulphate fraction of ragi FAE is shown in figure 47. As mentioned in the purification of ragi AAE (Chapter 4a.1.2.) and barley FAE (Humberstone and Briggs, 2002b) this step was successful in removing the coloured material, large amounts of unbound and contaminating proteins and also reduced the viscosity of this fraction.

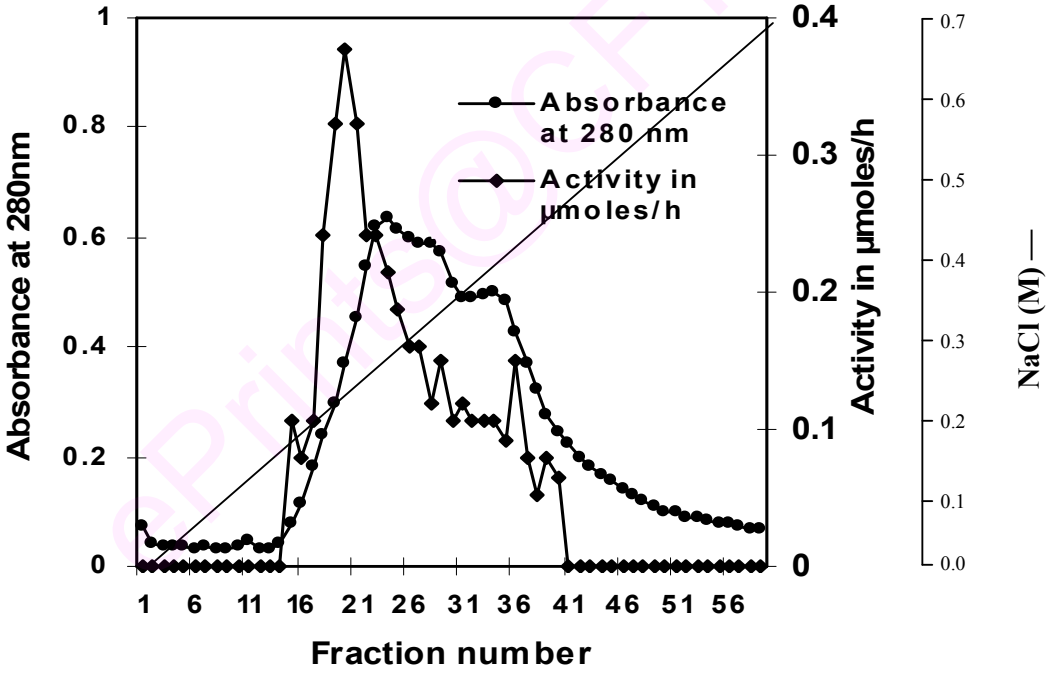


Figure 47. Elution profile of ragi FAE (40 - 80% ammonium sulfate fraction) on DEAE-cellulose column.

The bound proteins were eluted with a linear gradient of NaCl (0.0 - 0.7 M) which yielded a major peak at 0.23 M NaCl concentration. The DEAE-cellulose purified ragi FAE major fraction showed a fold purification and recovery of 15.7 and

15.4% respectively. DEAE-Biogel A and Mono Q ion exchange chromatographic methods were used for the purification of FAE from *Streptomyces olivochromogenes* (Faulds and Williamson, 1991) wherein, the active fraction of the enzyme obtained in the unbound fraction. A linear gradient of 0.0 - 0.5 M NaCl in piperazine buffer (pH 6.0, 10 mM) was used to elute bound FAE of barley malt, which gave a fold purification, and recovery of 2.88 and 5.8% respectively (Humberstone and Briggs, 2002b).

4b.1.3. Gel permeation chromatography

The DEAE-cellulose major peak of purified ragi FAE was further purified on Biogel P-30 gel permeation column. Figure 48 depicts the elution profile of ragi FAE on Biogel P-30. The enzyme was eluted (Tris-HCl pH 7.0, 10 mM) as a single peak with a fold purification and recovery of 22 and 4.75% respectively.

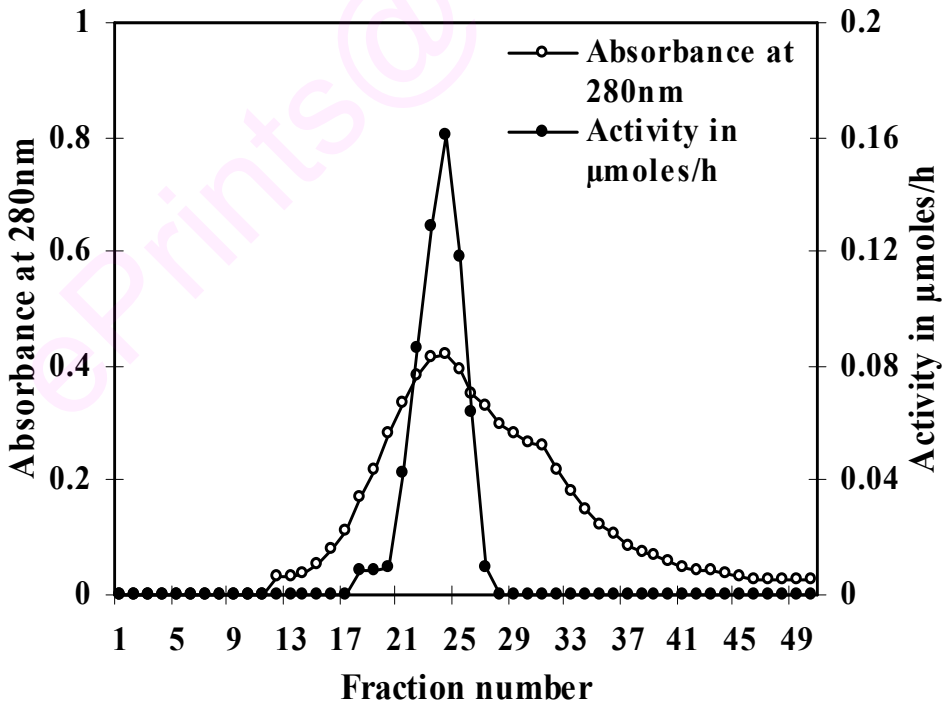


Figure 48. Elution profile of ragi FAE (DEAE-cellulose fraction) on Biogel P-30

Sephacryl HR-200 gel permeation chromatography was employed in the separation of FAE from barley malt (Humberstone and Briggs, 2002b) using NaCl (0.5 M) and glucose (2.5%) in Tris-HCl (pH 7.0, 10 mM) eluant, with a fold purification and recovery of 148 and 4.2% respectively. NaCl was included in the elution buffer to reduce ionic interactions between the solute and the gel matrix (Whitaker, 1963). Recovery of the enzyme from ragi was decreased after DEAE-cellulose chromatography, which might be due to (a) enzyme inactivation (b) removal of a synergistically acting enzyme, which might be associated with the FAE thereby stabilizing it. In our study, NaCl (10 mM) and EDTA (1.0 mM) were added to the elution buffer to retain the activity of purified ragi FAE. The overall scheme employed in the purification of FAE from ragi malt is summarized in **Table 18**.

Sephadex HR-200 gel matrix was used for the purification of the active fraction obtained from the anion exchange chromatography of FAE from *Streptomyces olivochromogenes* (Faulds and Williamson, 1991) using Tris-HCl (pH 8.0, 10 mM) eluant containing 1.0 mM each of $CaCl_2$, $MgCl_2$, EDTA, DTT (0.2 mM), sodium azide (0.02%) and aprotinin (0.05%) resulted in late elution of FAE fraction.

Table 18. Summary of the purification of FAE from 96 h ragi malt

Step	Total activity[b]	Total protein[c]	Specific activity[d]	Fold purification	Recovery %
Crude[a]	120	92	1.30	1	100
40-80% ASP fraction	100	5.2	19.2	14.8	83
DEAE-cellulose	18.5	0.9	20.5	15.7	15.4
Biogel P-30	5.7	0.2	28.5	21.9	4.75

[a] 50 g scale (Values are average of three independent experiments)
[b] One unit is equivalent to 1µmol of PNP released h^{-1}.
[c] Total protein is expressed in mg.
[d] Specific activity is expressed in 1µmol of PNP released h^{-1} mg^{-1} of protein

4b.2. Criteria of purity of purified ragi FAE

4b.2.1. Native/SDS-PAGE and activity staining

The purity of purified ragi FAE was confirmed by SDS and native PAGE. Crude ragi FAE extract upon three-step purification resulted in apparently homogeneous enzyme with respect to protein and activity staining by separation from the other contaminating proteins. Protein and activity staining of the purified ragi FAE is shown in the figure 49a & b. SDS-PAGE analysis of the purified enzyme gave a single band with an estimated molecular mass of 16.5 kDa (Figure 50).

4b.2.2. Determination of the purified ragi FAE activity by diffusion of enzyme in to agarose gel.

Activity of the purified enzyme was determined by using fluorescent substrate MUTMAC (Figure 49b). As the enzyme was inactivated immediately after running PAGE, the purified enzyme was loaded directly in the wells of agarose incorporated with substrate (MUTMAC) to check the activity. Green fluorescence was observed against blue background indicated the cleavage of MUTMAC to fluorescent methylumbelliferone (Mackenzie et al., 1987).

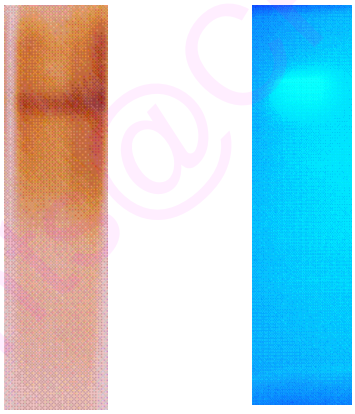


Figure 49a & b. PAGE of purified ragi FAE
(a) Protein staining and (b) Activity staining

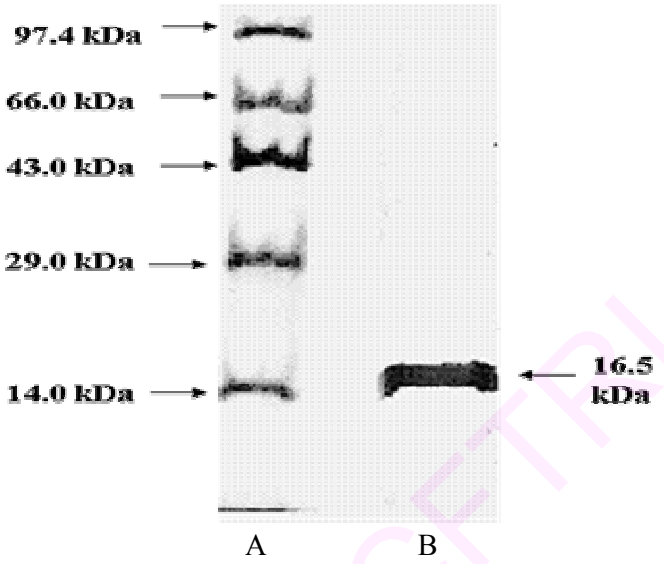


Figure 50. SDS PAGE of purified ragi FAE

A) Molecular weight markers: 97.4 kDa Phosphorylase; 66.0 kDa Bovine serum albumin;43.0 kDa Ovalbumin; 29.0 kDa Carbonic anhydrase; 14.0 kDa Lysozyme. B) 16.5 kDa purified ragi FAE.

4b.3. Characterization of purified ragi FAE

4b.3.1. Determination of molecular weight of purified ragi FAE

The molecular weight of purified ragi FAE as determined by SDS-PAGE was 16.5 kDa (Figure 51). Identical molecular mass was obtained by GPC (Figure 52) and ESI-MS (Figure 53), indicating the monomeric nature of the purified enzyme. The molecular weight of this enzyme is comparatively less than the one reported from barley malt (138 kDa, Humberstone and Briggs, 2002b).

Microbial feruloyl esterases have showed different molecular weights ranging from 24.0 kDa (FAE-II from *Neocallimastix strain* MC-2) (Borneman et al., 1991) to 132 kDa (FAE-I from *Aspergillus niger*) (Faulds and Williamson, 1993) and 210 kDa *Aureobasidium pullulans* (Rumbold et al., 2003). Three-feruloyl esterase have been characterized from *Aspergillus niger* and their estimated molecular weights are 132 kDa (FAE-I), 29.0 kDa (FAE-II) and 36.0 kDa (FAE-III) (Faulds and Williamson, 1993 & 1994).

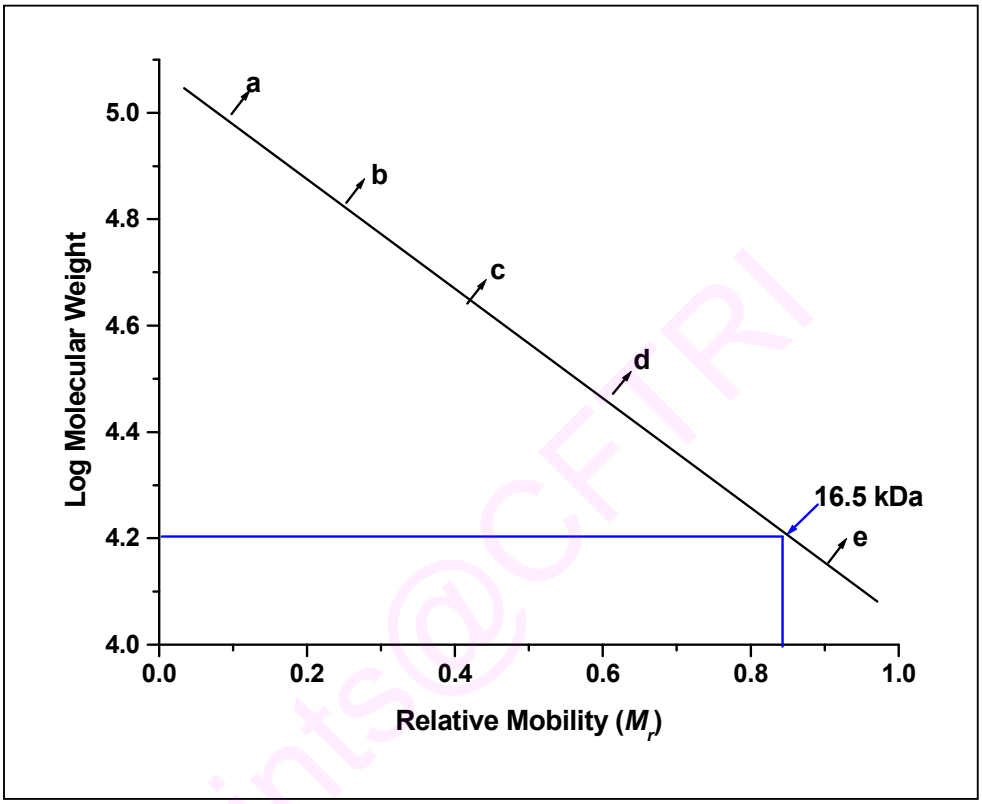


Figure 51. Calibration curve for the determination of molecular weight of purified ragi FAE by SDS-PAGE

Molecular weight markers: a) 97.4 kDa Phosphorylase, b) 66.0 kDa Bovine serum albumin, c) 43.0 kDa Ovalbumin, d) 29.0 kDa Carbonic anhydrase and e)14.0 kDa Lysozyme.
16.5 kDa purified ragi FAE.

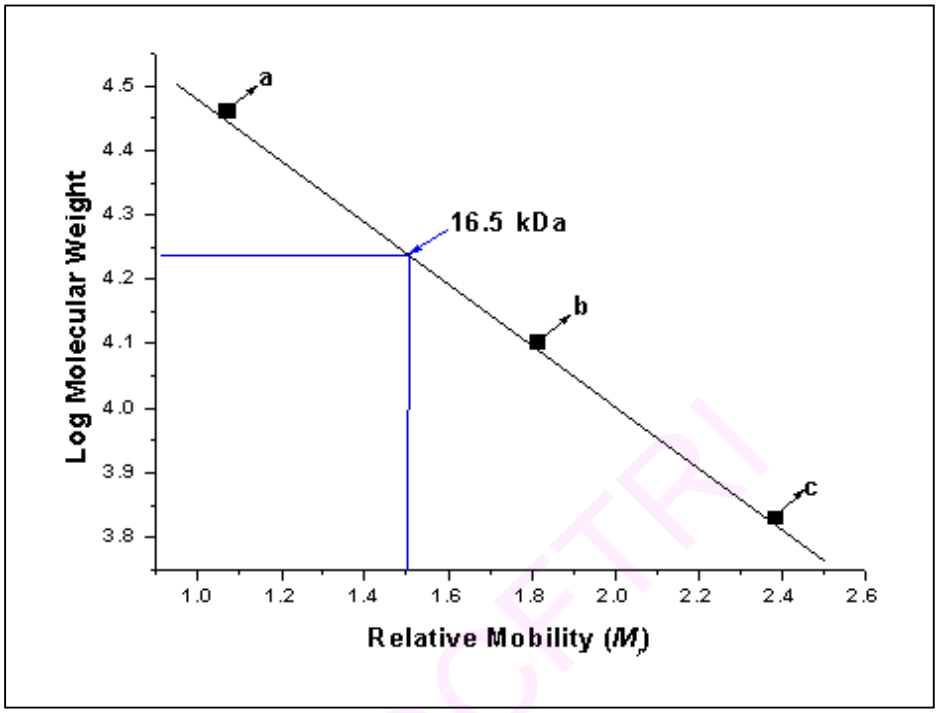


Figure 52. Calibration curve for the determination of molecular weight of purifiedragi FAE by GPC on Biogel P-30.

a) 29.0 kDa Carbonic anhydrase b) 12.0 kDa Lysozyme and c) 7.0 kDa Aprotinin.
16.5 kDa purified ragi FAE

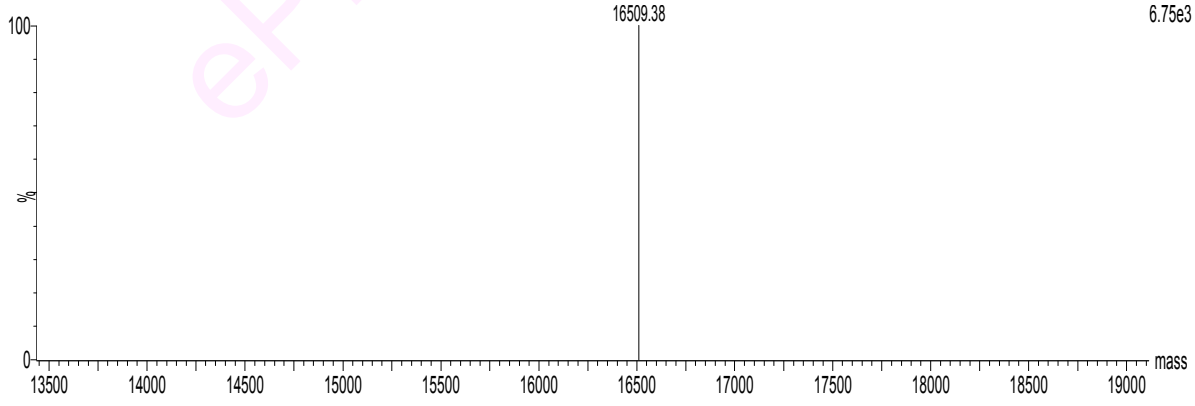


Figure 53. Determination of molecular mass of purified ragi FAE by ESI-MS positive mode)

4b.3.2. pH optima of purified ragi FAE

To determine the pH optima of purified ragi FAE the activities were determined using different buffers such as sodium acetate (pH 4.0 - 6.0), sodium phosphate (pH 6.0 - 8.0) and Tris-HCl (pH 7.0 - 9.0) at 50 mM concentration. FAE was found to have pH optima of 6.0 and retained 55 - 60% activity between pH 7.0 - 8.0 (Figure 54). Most of the activity was retained in acetate buffer, indicating better stability of the purified enzyme in this buffer. The enzyme activity has decreased drastically in the pH range 4.0 - 5.0 in acetate buffer indicating its labile nature in high acidic pH.

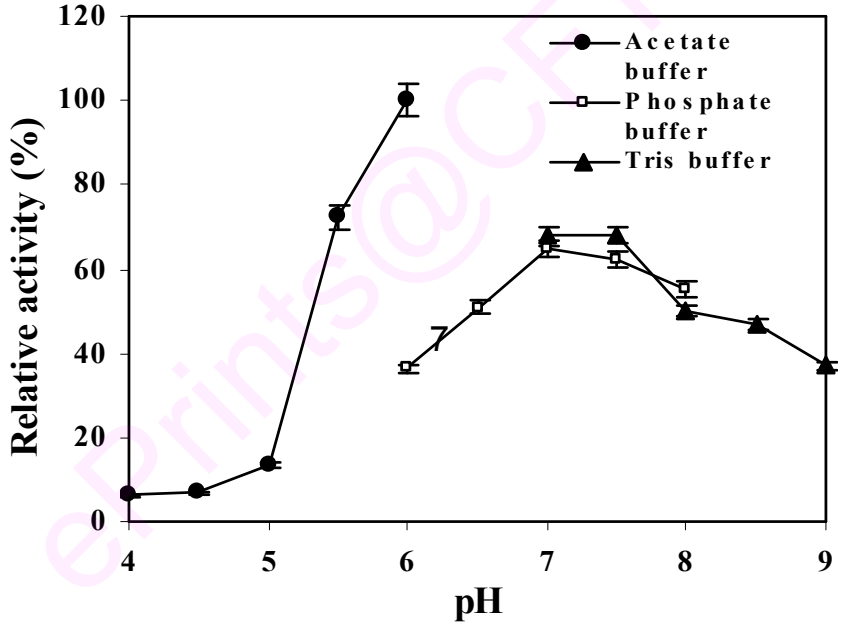


Figure 54. pH optima of purified ragi FAE

The pH optima of the enzyme (6.0) is almost similar to the pH values reported for purified FAEs from *Streptomyces avermitilis* CECT 3339 (Garcia et al., 1998), *Aureobasidium pullulans* (Rumbold et al., 2003), *Neocallimastix* strain MC-2 (Borneman et al., 1992), *Aspergillus niger* (Faulds and Williamson, 1994), *Aspergillus awamori* (McCrae et al., 1994), *Penicillium expansum* (Donaghy and Mckay, 1997), *Penicillium pinophilum* (Castanares and Wood, 1992) and

Streptomyces olivochromogenes (Faulds and Williamson, 1991) whereas it was less compared to the values reported for *Clostridium stercorarium* pH 8.0 (Donaghy et al., 2000).

4b.3.3. pH stability of purified ragi FAE

pH stability of purified ragi FAE was determined as indicated earlier for purified ragi AAE (Chapter 4a.3.3). After the pre-incubation of the enzyme using glycine-HCl (pH 2.0 - 3.0), sodium acetate (pH 4.0 - 6.0), sodium phosphate (7.0 - 8.0) and Tris-HCl (pH 8.0 - 9.0), at 50 mM for different intervals of time (0 - 360 min) the residual activity of the enzyme was determined (Figure 55).

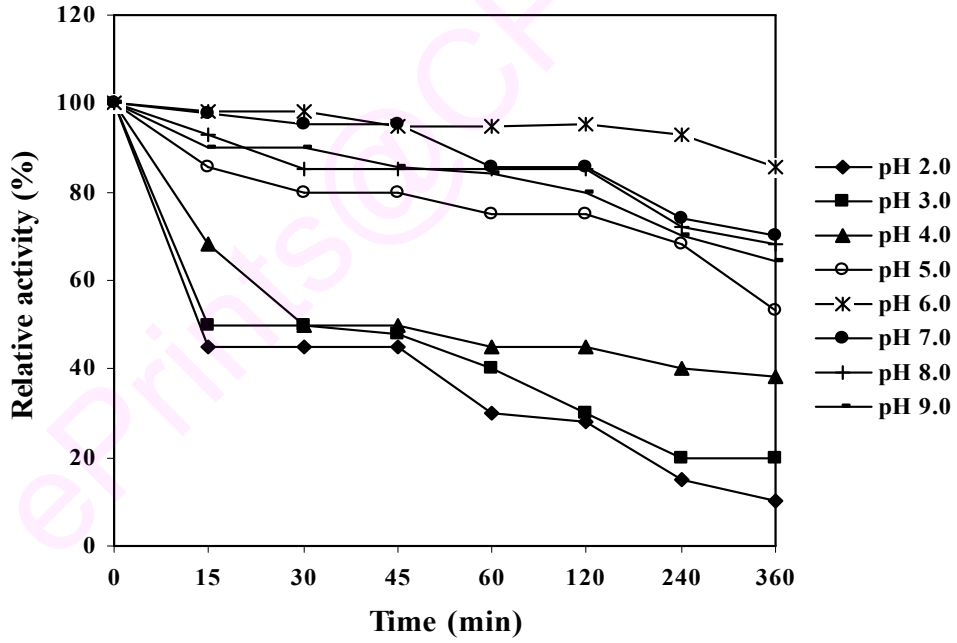


Figure 55. pH stability of purified ragi FAE

The purified ragi FAE showed 100% stability in acetate buffer (pH 6.0, 50 mM). The enzyme was stable over a broad pH range from 5.5 - 9.0 retaining about 80 - 90% activity after 2 h of incubation (Figure 55). Purified FAE of *Lactobacillus acidophilus* showed activity in the pH range 5.3 - 6.5 (Wang et al., 2004). The

recombinant FAE from *Aspergillus nidulans* showed broad pH stability from 4.0 - 9.5 (Sin and Chen, 2007).

4b.3.4. Temperature optima and stability of purified ragi FAE

To determine the temperature optima of purified ragi FAE, activities were determined at a temperature range of 30 - 70°C (Figure 56). The temperature optima of the purified enzyme was found to be 45°C similar to the one reported for *Aspergillus awamori* (McCrae et al., 1994), *Aspergillus nidulans* (Sin and Chen, 2007), and was comparatively lower than the ones reported for microorganisms such as *Aureobasidium pullulans* (60°C*)* (Rumbold et al., 2003), *Clostridium stercorarium* (65°C) (Donaghy et al., 2000), *Penicillium pinophilum* (55°C) (Castanares and Wood, 1992), *Streptomyces avermitilis CECT 3339* (50°C) (Garcia et al., 1998), *Aspergillus niger* (50°C) (Kroon et al., 1996) etc, but higher than the ones reported from microorganisms such as *Streptomyces olivochromogenes* (30°C) (Faulds and Williamson, 1991), *Lactobacillus acidophilus* (37°C) (Wang et al., 2004) and *Penicillium expansum* (37°C) (Donaghy and McKay, 1997).

The purified ragi FAE was thermally stable at 30°C and its activity gradually decreased with the increase in temperature and lost 85% of its activity at 70°C (Figure 56). Purified *Lactobacillus acidophilus* FAE was thermally stability between 37 - 75°C (Wang et al., 2004). As the temperature increases, the three dimensional structure of the enzyme which is maintained by a number of forces such as hydrophobic and hydrogen bonds will be disrupted resulting in the denaturation of the protein in turn inactivation of the enzyme.

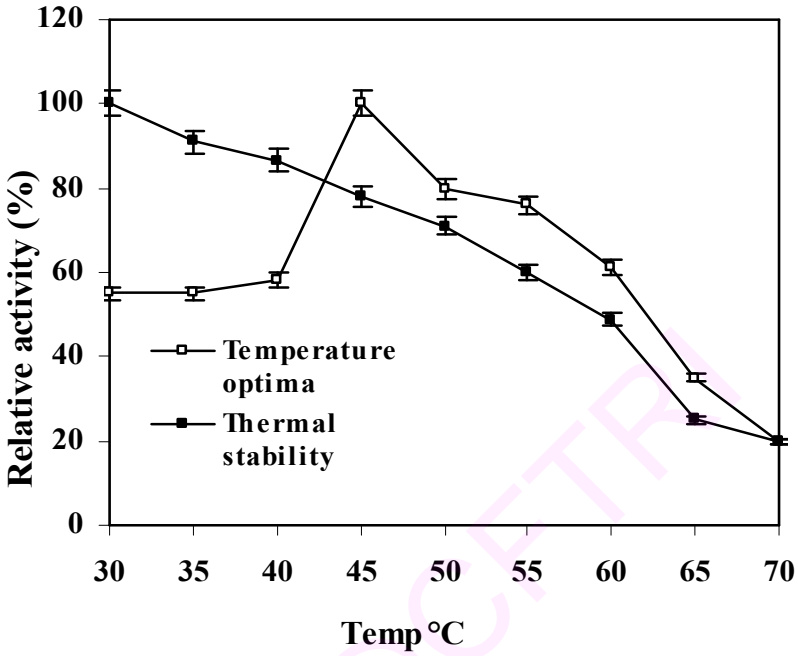


Figure 56. Temperature optima and thermal stability of purified ragi FAE

4b.3.5. Determination of energy of activation of purified ragi AAE

The activation energy of the reaction was calculated at the optimum pH of the enzyme (6.0), using PNPF as substrate The energy of activation was calculated from the slope of the arrhenius curve obtained by plotting natural log of activity at various temperatures (30 - 70°C) on y-axis and 1/T in Kelvin on x-axis. The activation energy calculated from Arrhenius plot was found to be 4.08 kJ mol^{-1} (Figure 57).

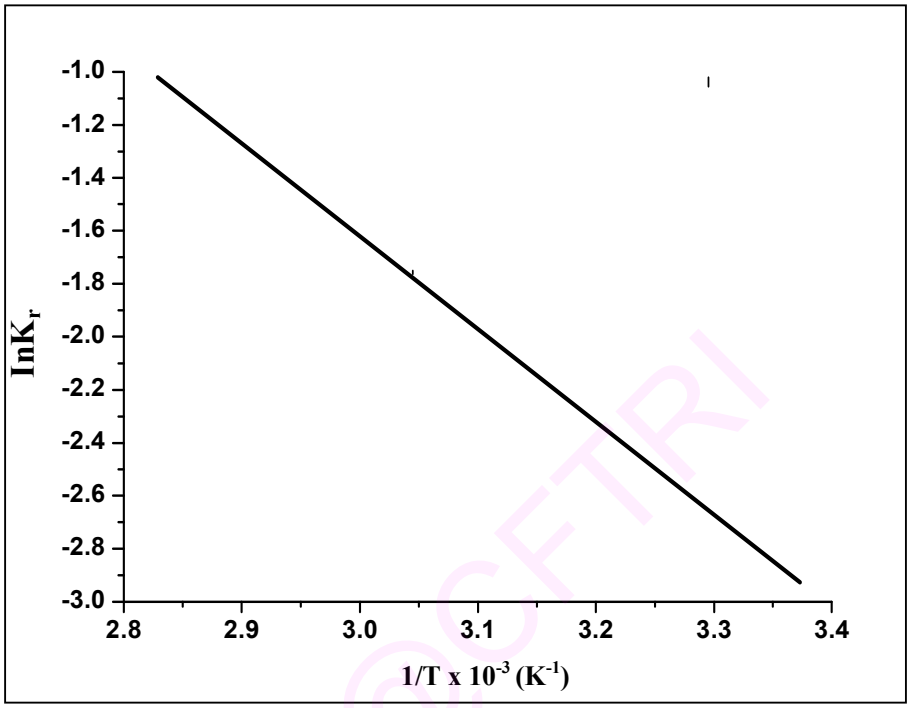


Figure 57. Arrhenius plot for the temperature dependence of purified ragi FAE using PNPF as substrate.

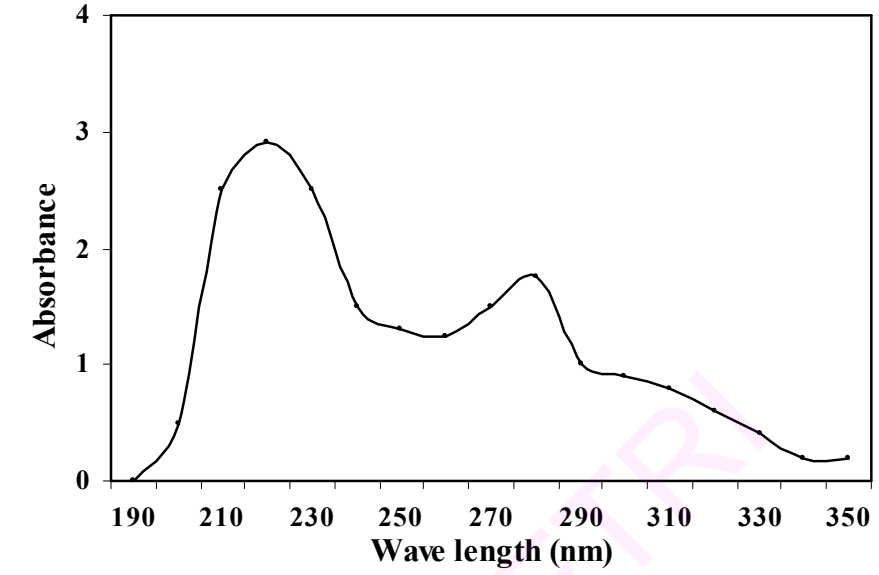
4b.3.6. Spectral analysis of purified ragi FAE

a) UV absorption spectra

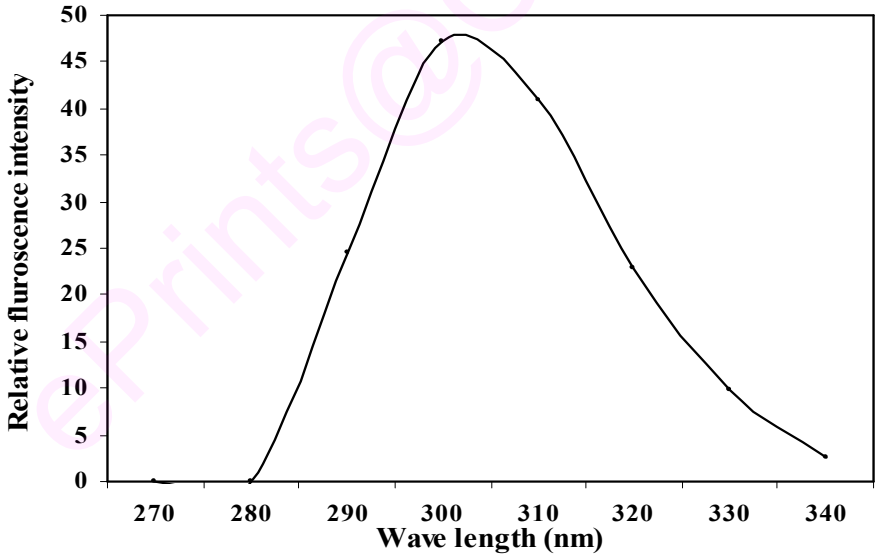
The UV absorption spectra of purified ragi FAE showed two peaks one at 225 nm (major peak) corresponding to the absorption of peptide bonds and the other at 280 nm corresponds to the absorption of aromatic amino acids (Figure 58a).

b) Fluorescence spectra

The fluorescence spectra of purified ragi FAE showed excitation maximum of 220 nm and an emission maxima range of 300 - 310 nm (Figure 58b). The fluorescence or long wave length absorption spectra of proteins is mainly due to the quenching of aromatic amino acids especially tryptophan.



(a)



(b)

Figure 58. a) UV absorption spectra and b) Fluorescence spectra (emission) of purified ragi FAE

4b.3.7. Effect of substrate concentration on the activity of purified ragi FAE

Effect of different concentration of PNPF on the initial velocity of purified ragi FAE was calculated and the kinetic constants K_m and V_{max} were calculated from the double reciprocal plots (LB plot, Lineweaver and Burk, 1934) (Figure 59). The K_m value of FAE from ragi was found to be 0.053 µM for PNPF and V_{max} was found to be 0.085 U ml^{-1}. The K_m value reported in the present study is lower than the ones reported for *Aureobasidium pullulans* (50.2 µM) (Rumbold et al., 2003), *Clostridium stercorarium* (40 µM) (Donaghy et al., 2000), *Penicillium pinophilum* (130 µM) (Castanares and Wood, 1992) using methyl ferulate as substrate.

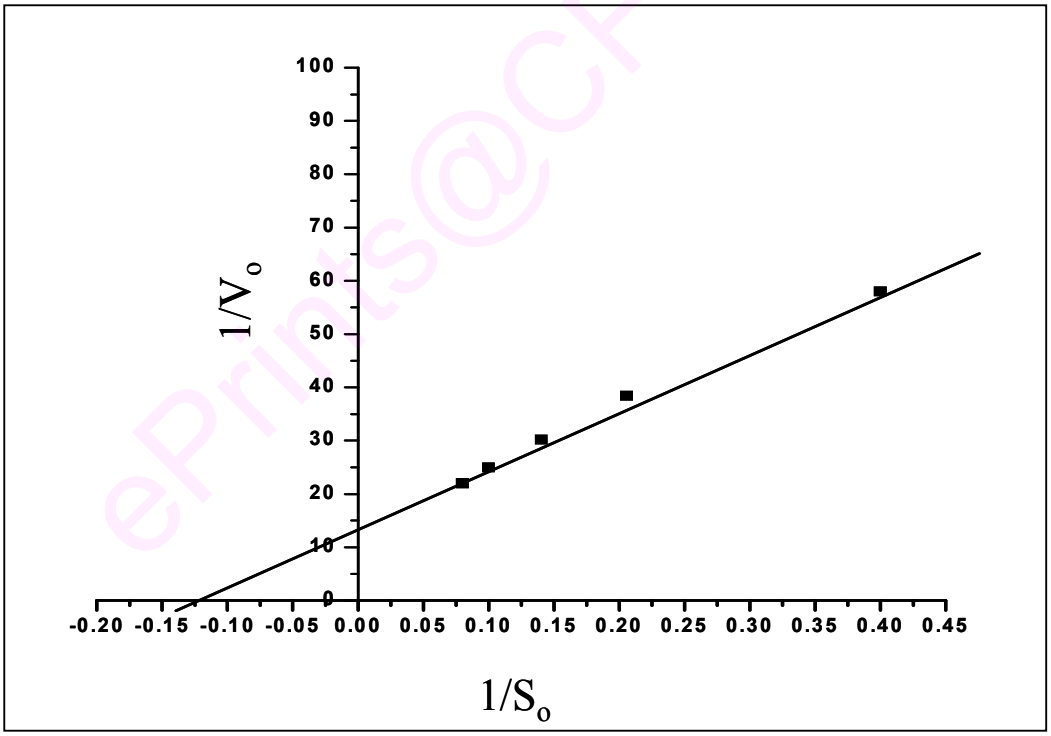


Figure 59. Determination of K_m and V_{max} of purified ragi FAE by Lineweaver-Burk plot (using the substrate PNPF)

This indicates the higher specificity of purified ragi FAE for PNPF.A K_m value of 0.46% for barley malt FAE was reported using feruloyl glycerol as the substrate (Humberstone and Briggs, 2002b). The low K_m of purified FAE from barley was due to the presence of both 1 and 2- feruloyl glycerol in the substrate solution. No information is available with respect to the detailed kinetics and substrate specificity of cereal/millet FAEs.

4b.3.8. Effect of metal ions on the activity of purified ragi FAE

A range of metal ions such as Fe^{3+}, Cu^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+} at 5 mM concentration were tested for purified ragi FAE activation / inhibition effect and the results are given in **Table 19**. Metal ions such as Mg^{2+}, Ca^{2+}, Ba^{2+} and EDTA showed no visible effect on the purified FAE. Similar concentrations of Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{+}, Cu^{2+}, oxalic and citric acid resulted in activation of the enzyme. The activity of purified ragi FAE was completely inhibited by 5 mM concentration of Fe^{3+}. The inhibitory effect of Fe^{3+} and the role of EDTA (no significant inhibition and activation) during the purification by GPC and its effect on the activity of FAE was in accordance with the results reported for feruloyl esterase from *Clostridium stercorarium* (Donaghy et al., 2000) and *Lactobacillus acidophilus* (Wang et al., 2004).

The inhibition of FAE is consistent with the phenolic acid esterases of *Neocallimastix* sp (Borneman et al., 1991 & 1992), *Aspergillus awamori* (McCrae et al., 1994), *Penicillium pinophilum* (Castanares and Wood, 1992) and *Penicillium expansum* (Donaghy and Mckay, 1997). The inhibitory and stimulatory effects of these ions may be important factors in the commercial exploitation of this enzyme wherein enzyme stability and activity are paramount.

Table 19. Effect of metal ions, EDTA and citric acid on the activity of purified ragi FAE

Metal ions	Relative activity (%)
Control	100
Cu^{2+}	217
Fe^{3+}	0
Cu^{+}	199
EDTA	100
Citric acid	119
Oxalic acid	141
Co^{2+}	140
Mg^{2+}	100
Zn^{2+}	140
Ba^{2+}	100
Ni^{+}	140
Ca^{2+}	100

4b.3.9. Effect of group specific reagents on the activity of purified ragi FAE

Purified ragi FAE activity was determined in the presence of chemicals such as PCMB, iodoacetamide and eserine and PMSF at 45°C and 10 - 50 mM concentrations (Figure 60). PCMB (50 mM) showed 67% inhibition (33% of relative activity) whereas, 45% of inhibition (55% of relative activity) was observed with iodoacetamide. Both PCMB and iodoacetamide are specific cysteine residue modifiers and result in inactivation of the enzyme by forming complexes with cysteine present in the active site region of the enzyme (Bell and Bell, 1998). The pH optima (6.0) value of the purified ragi FAE also supports this statement as the ionization values of these amino acids fall in this range.

Eserine is a structural analogue of the amino acid serine and involved in modifying histidine residues in the active site especially at pH 6.0 (Kim and Lee, 2004). In our study, eserine had showed an increase in the activity (~98% more than the control) of the enzyme instead of inhibition (Figure 60). This may be due to the phosphorylation of serine residues in the active site pocket of the enzyme, wherein the free hydroxyl group of serine might not be available to the structural analogues such as eserine for inhibition.

PMSF, a serine analogue did not show any significant activation or inhibition of purified ragi FAE, which is in accordance with the published literature on most of the microbial FAEs (Donaphy et al., 2000).

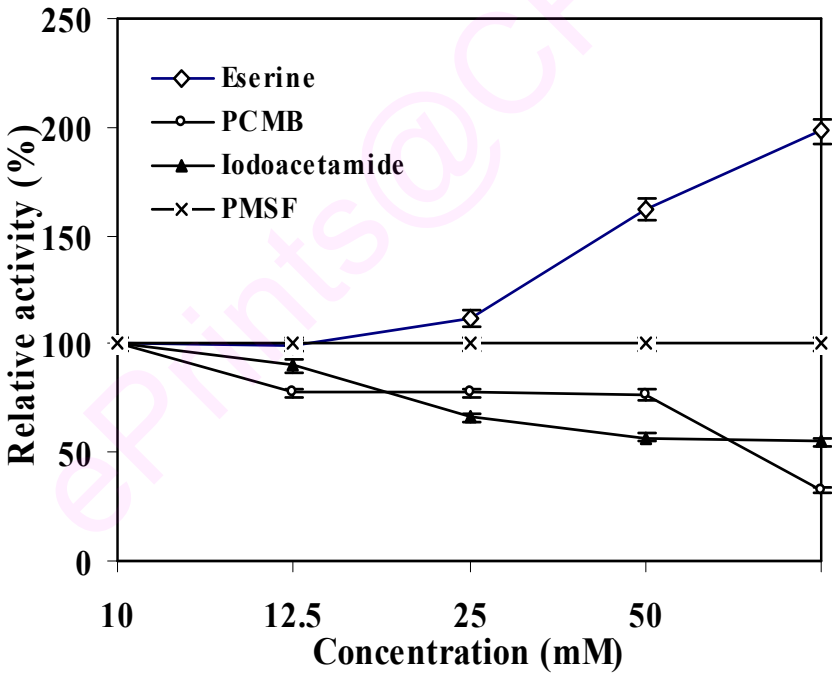


Figure 60. Effect of group specific reagents on the activity of purified ragi FAE

4b.3.10. Substrate specificity of purified ragi FAE

The substrate specificity of purified ragi FAE was achieved by measuring the enzymatically released ferulic acid from water-soluble polysaccharides and synthetic

substrates. The specific activities of purified ragi FAE using various water-soluble polysaccharides such as ragi, wheat, maize and synthetic substrates like PNPF and ethyl ferulate are shown in the **Table 20.** The specific activity of purified ragi FAE with respect to ragi water-soluble preparation is comparatively higher than the other polysaccharides. The purified enzyme was active on both small molecular weight substrates as well as polysaccharides as indicated in the present study. The maximum substrate specificity was found using synthetic substrates especially PNPF.

Table 20. Substrate specificity of purified ragi FAE

Substrates	Specific activity ($U\ mg^{-1}$ of protein)
Wheat	0.016
Ragi	0.05
Maize	0.009
Ethyl ferulate	3.0
PNPF	3.37

The result obtained for purified ragi FAE was different from the one reported on FAE activity from barley malt (Bartolome et al., 1996), which indicated the specificity of ragi FAE towards synthetic substrates than feruloyl polysaccharides. FAE isolated from various sources showed different substrate specificity, for e.g. FAE from *Cytolase* M102 showed higher activities on both PNPF and ethyl ferulate where as the enzyme from *Trichoderma reesei* showed almost negligible activity on both the substrates (Mastihuba et al., 2002).

FAE from *Aspergillus niger* showed high activity on 1% Avicel cellulose substrate compared to *Shizophyllum commune* (Mackenzie and Bilous, 1988) and *Streptomyces olivochromogenes* (Johnson et al., 1988b) and even the enzyme showed substrate selectivity for the position of ferulic acid on wheat bran and sugar beet pulp (Ralet et al., 1994).

4b.3.11. Electrospray Ionization – Mass Spectrometry (ESI-MS)

Analysis by ESI-MS confirmed the gradual deesterification of ragi and wheat water-soluble polysaccharides respectively, for the release of ferulic acid by purified ragi FAE. The amount of ferulic acid released by the enzymatic deesterification of the above polysaccharides using ragi FAE increased with increase in time (0 - 4 h) and remained same after 4 h indicating the maximum release of ferulic acid within 4 h. The *m/z* value of released ferulic acid from wheat (Figure 61) and ragi WSP (Figure 62) was 191.70 (*m/z*, ESI-negative mode). The release of ferulic acid from polysaccharides especially wheat bran was reported by feruloyl esterase reported from *Aspergillus niger* (Kim and Lee, 2004).

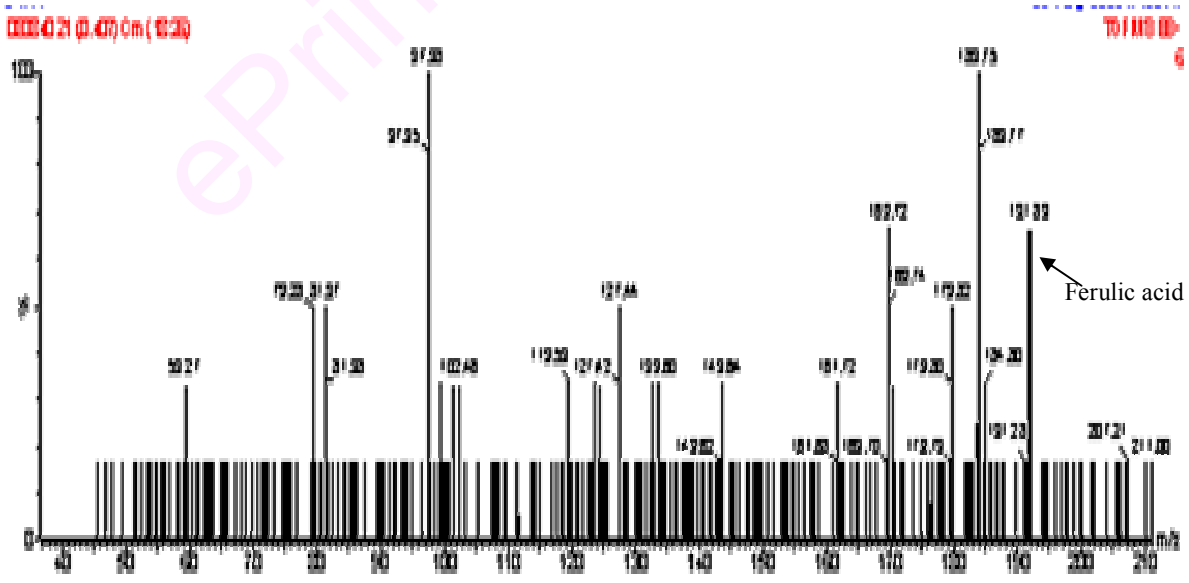
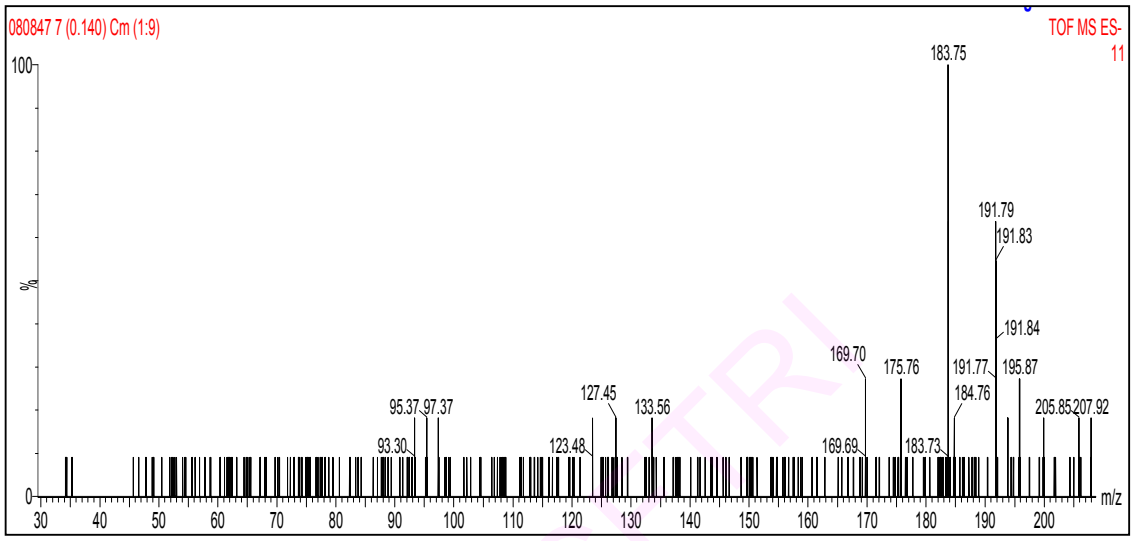
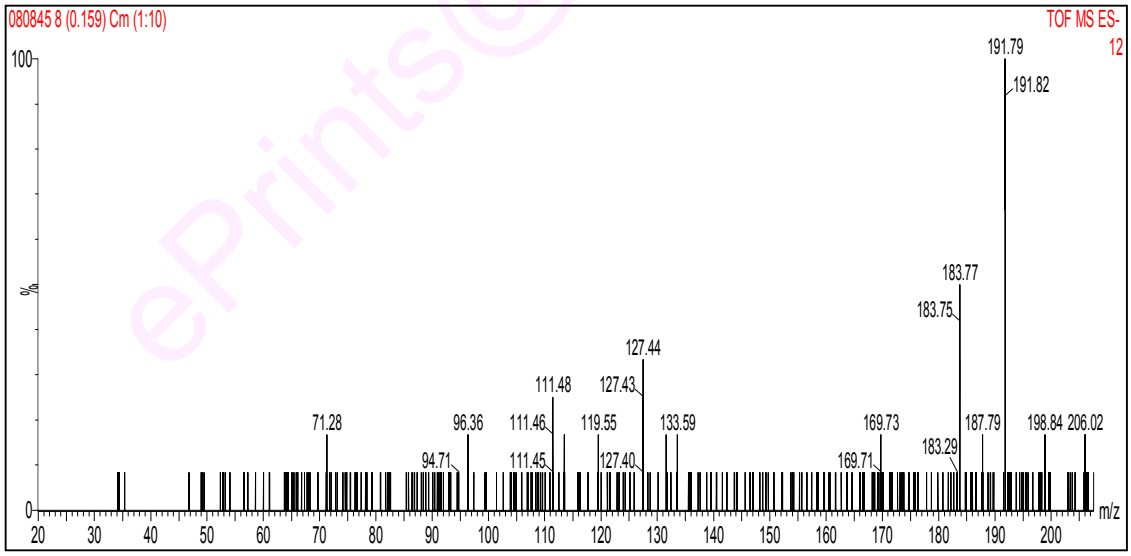


Figure 61. Enzymatic release of ferulic acid from wheat WSP by purified ragi FAE

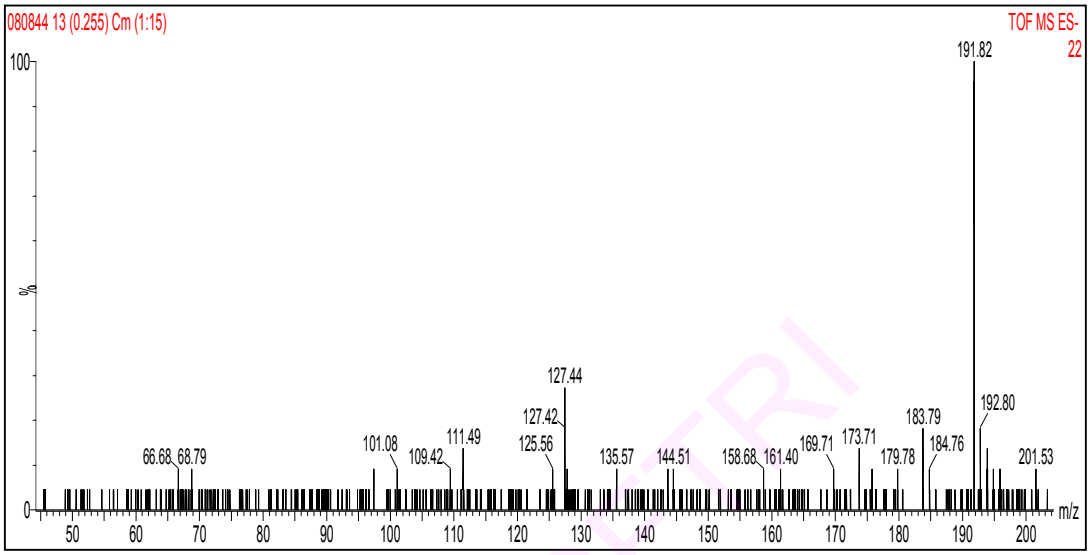


(a)



(b)

Figure 62. Enzymatic release of ferulic acid from ragi WSP (a) 1h (b) 2 h by purified ragi FAE



(c)

Figure 62. Enzymatic release of ferulic acid from ragi WSP (c) 4 h by purified ragi FAE

Conclusions

The finger millet malt esterases (AAE and FAE) were purified to apparent homogeneity. The apparently purified esterases obtained from ragi malt in the present investigation were specific for both natural polysaccharides as well as small molecular weight synthetic substrates.

CHAPTER-5
ROLE OF RAGI ESTERASES ON THE FUNCTIONALITY OF CEREAL WATER-SOLUBLE NON-STARCH POLYSACCHARIDES

5.1. Introduction

The complex and varied structure of cereal arabinoxylans, and their functional importance in food and nutritional studies made them one of the most important sought after polymers. Studies were reported on the functional characteristics of non-starch polysaccharides from native and malted finger millet (Subba Rao et al., 2004; Shyama Prasad Rao et al., 2007). However, till date, the information pertaining to the effect of O-acetyl groups and feruloyl groups on the functionality of cereal water-soluble non-starch polysaccharides (WSPs) is not available. This chapter mainly focuses on the role of ammonium sulphate and DEAE-cellulose purified finger millet malt AAE and FAEs in modulating the functionality of WSPs isolated from wheat and ragi with respect to their (a) viscosity (b) foam stabilization and (c) gelling characteristics. In addition, this chapter also addresses the effect of enriched AAE on the synergistic gelation of xanthan and locust bean gum (XG and LBG) (Dea and Rees, 1987).

5.2. Estimation of total carbohydrate

Carbohydrate contents of ragi and wheat WSPs determined by Phenol-Sulphuric acid method were found to be 100 and 90% respectively. These WSPs were taken for determination of relative viscosities, foam stabilization and oxidative gelation.

Earlier reports from our group indicated the presence of more hexoses than pentoses in native ragi WSP (Ara: Xyl 1:0.40, and pentose:hexose 0.69:1) (Subba Rao et al., 2004). Water extractable polysaccharides from native cereals such as ragi, maize, rice and wheat showed different proportions of arabinose, xylose and glucose (Shyama Prasad Rao and Muralikrishna, 2004). Native wheat WSP showed the presence of water-soluble pentosans with higher amounts of pentoses than hexoses especially xylose, arabinose and galactose (Hoseney, 1984).

5.3. IR analysis of water-soluble polysaccharides

The IR spectra of water-soluble preparations of polysaccharides such as ragi, wheat, larch wood xylan and gum karaya showed medium-intensity absorption at 1200 cm^{-1} and 1735 cm^{-1} correspond to carbonyl stretching of an ester group indicated the presence of acetyl and feruloyl groups [Johnson et al., 1988a; Madhavi Latha and Muralikrishna, 2007 (Figure 63)]. In the present study, the analysis of free and bound ferulic acid of WSPs from ragi and wheat indicated the presence of high content of bound ferulic acid than free ferulic acid (82:18). The result is in agreement with the earlier studies, which showed the high proportion of free ferulic acid in malted seeds of cereals and bound ferulic acid in native cereals (Shyama Prasad Rao and Muralikrishna, 2004).

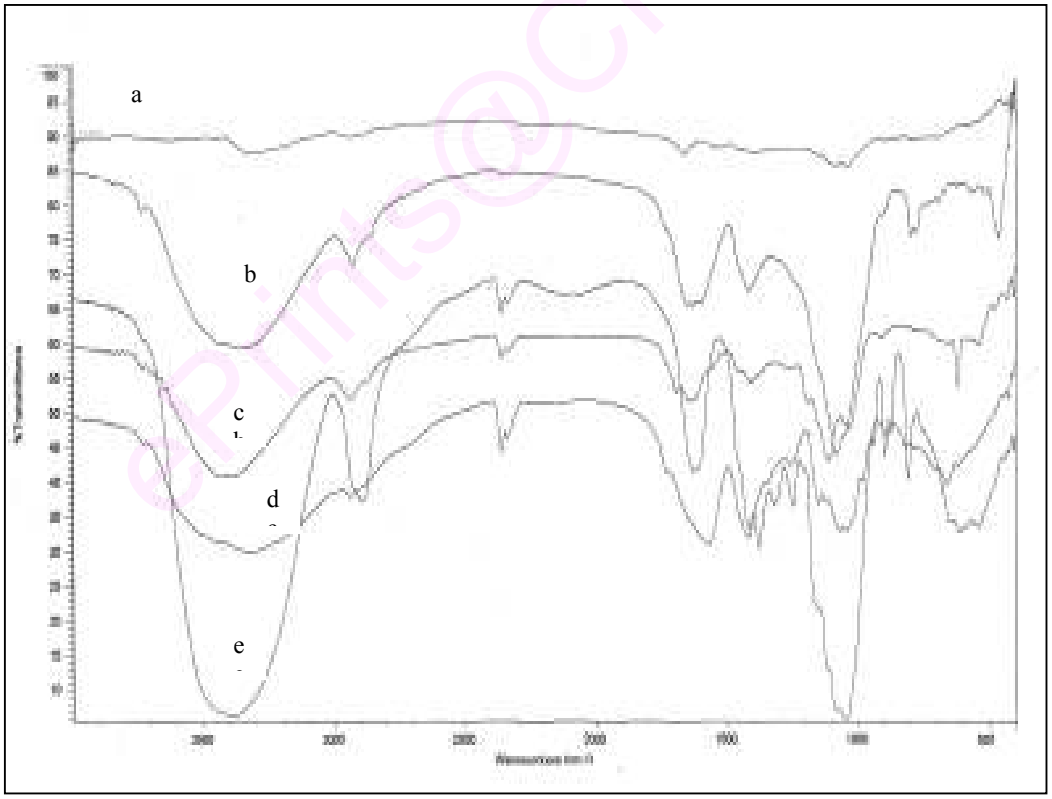


Figure 63. IR spectra of WSPs

a. Wheat
b. Ragi
c. Xanthan Gum
d. Gum karaya
e. Xylan

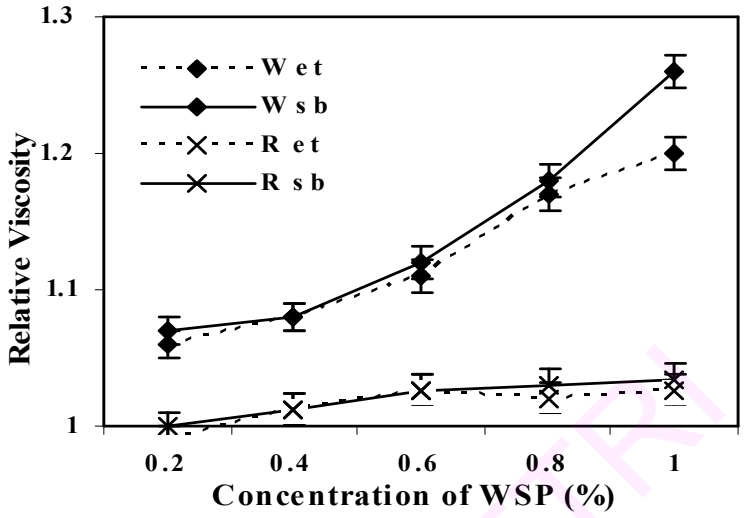
5.4. Effect of enriched DEAE-cellulose purified fractions of AAE and FAE on the relative viscosities of WSPs

The enriched DEAE-cellulose purified fractions of esterases (AAE and FAE) from ragi malt showed negligible effect on the relative viscosities of WSPs from ragi and wheat. In the present study, wheat WSP has shown high relative viscosity than ragi WSP at various concentrations and temperatures. The high relative viscosity of wheat WSP could be due to its high pentosan content and high asymmetric conformation (Hoseney, 1984). Studies show that cereal pentosans are likely to form more viscous solutions than hexosans (Fincher and Stone, 1986).

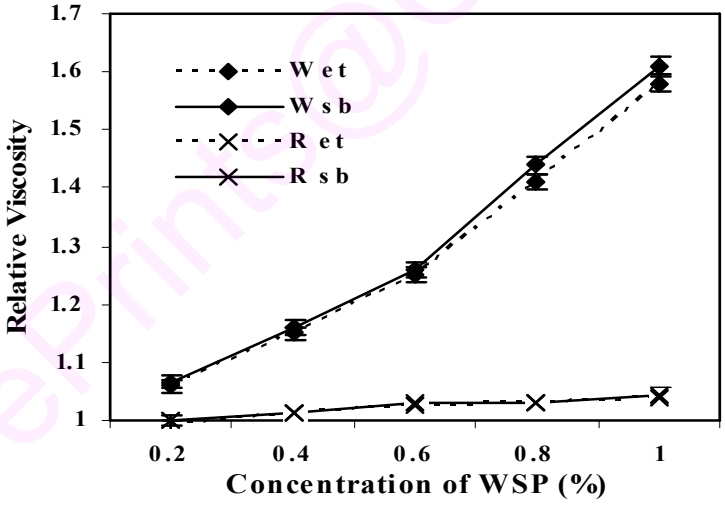
The relative viscosity of wheat WSP increased concomitantly with the increase in concentration (0.2 - 1.0%) (Figure 64a & b), while it has decreased with increase in temperature (30 - 70°C) (Figure 65 & 66). In case of ragi WSP, the change in relative viscosity with respect to increase in concentration and temperature was marginal as shown in the figures 64a & b, 65, 66. The ragi and wheat WSPs treated with enriched DEAE-cellulose purified fractions of AAE/FAE showed a marked decrease in relative viscosities when incubated at various temperatures compared to the untreated native WSPs; however, there was very little change observed with respect to increase in concentration.

The increase in viscosity of cereal arabinoxylans due to the increase in concentration is perhaps due to (a) increase in the hydrogen bonding between hydroxyl groups, (b) the movement of the liquid disturbed by hydrated solute molecules (Togrul and Arslam, 2003), and (c) decrease in the intermolecular distances between polysaccharide chains, thereby increase in their interaction (Subba Rao et al., 2004).

The decrease in viscosity with increase in temperature is due to the loss of hydration and due to the increase in the distance between the polysaccharide chains for possible interaction, thereby reducing the resistance (Whistler and Be Miller, 1973). The viscosity of polymer solution is directly related to the change in the molecular conformation, molecular weight and concentration of the polymer.



(a)



(b)

Figure 64. Effect of enriched DEAE-cellulose purified fractions of (a) AAE and (b) FAE from ragi malt on the relative viscosities of WSPs with respect to increase in concentration (R: ragi, W: wheat, sb: substrate blank, et: enzyme treated).

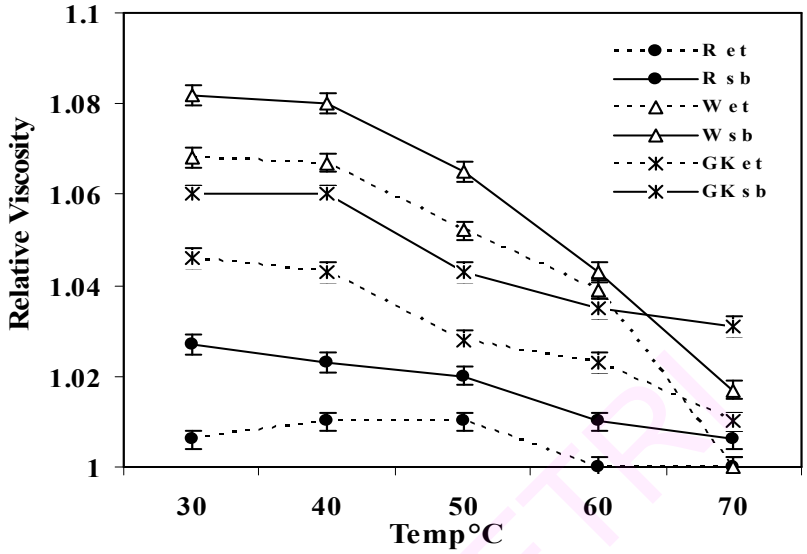


Figure 65. Effect of enriched DEAE-cellulose isolate of AAE from 72 h ragi malt on the relative viscosities of WSPs with respect to increase in temperature R: ragi, W: wheat, GK: gum karaya, sb: substrate blank, et: enzyme treated)

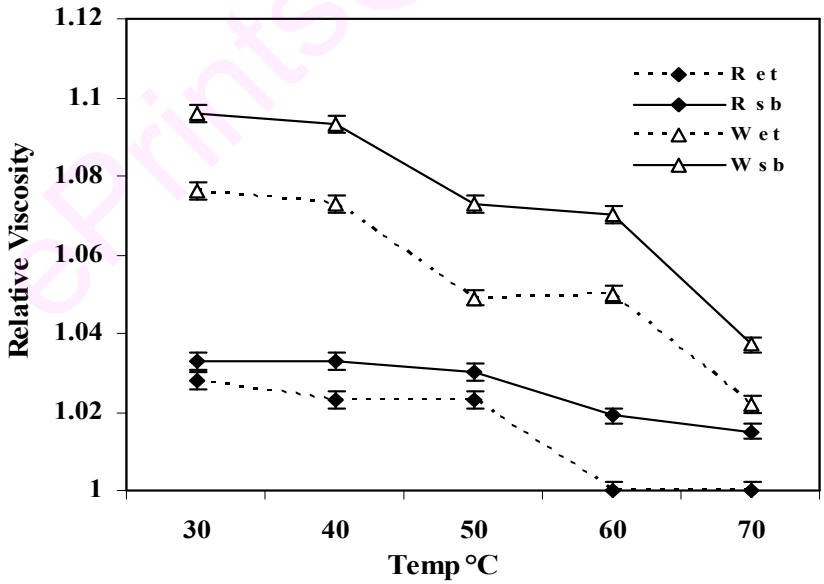


Figure 66. Effect of enriched DEAE-cellulose isolate of FAE from 96 h ragi malt on the relative viscosities of WSPs with respect to increase in temperature R: ragi, W: wheat, sb: substrate blank, et: enzyme treated)

In our study, it was observed that the relative viscosities of enzymatically treated WSPs were less than the controls, which, may be attributed due to the removal of acetyl and feruloyl groups from WSPs by enriched DEAE-purified fractions of AAE/FAE.

5.5. Effect of enriched DEAE-cellulose purified fractions of ragi AAE and FAE on the foam stabilization capacity of WSPs

The results obtained in the present study indicated that the foam stabilization effect of WSPs increased with the increase in the concentration. This may be perhaps due to the increase in the relative viscosities of WSPs (Sarker et al., 1998; Susheelamma and Rao, 1979) with increase in concentration, resulting in higher foam stabilization (Izydorczyk and Biliaderis, 1992a & b; Muralikrishna et al., 1987; Subba Rao et al., 2004: Shyama Prasad Rao et al., 2007). **Table 21, 22** and **23, 24** represents the effect of enriched DEAE-cellulose purified fractions of ragi FAE and AAE respectively on the foam stabilization property of WSPs. The removal of acetyl and feruloyl groups by the enzymatic treatment of ragi and wheat WSPs by ragi AAE and FAE respectively, decreased the foam stabilization capacity of polysaccharides, which can be attributed due to the decrease in the viscosity of the polysaccharide solution by the removal of substituent groups i.e. acetyl and feruloyl groups.

The formation of foam is usually impeded by the increase in the viscosity of the WSP solution; hence, the initial foam volume (V_1) decreased with the increase in viscosity/concentration. However, during the thermal treatment step, the added WSP prevented the disruption of gas cells during thermal expansion of CO_2 (Izydorczyk and Biliaderis, 1991). Enriched ragi AAE treatment of gum karaya resulted in substantial decrease in foam stabilization property of polysaccharide indicating the positive contribution of acetyl groups in the foam stabilization property.

Studies have showed that the pH of the protein-polysaccharide solution plays an important role in the interaction of protein with the polysaccharide, in turn the protein foam stabilization. If the pH of the solution is around the isoelectric point of the protein, the net charge on the protein is balanced, which in turn increases the interaction with the polysaccharide thereby stabilizing the foam (Phillips, 1981).

Table 21. Effect of enriched DEAE-cellulose purified fraction of FAE from 96 h ragi malt on the foam stabilization property of WSPs isolated from ragi

Sample		Additive Concentration %	Foam volume in ml			
			$NaHCO_3$	Citric acid	10' at 25°C	3' at 90°C
Control	Acetate buffer (50 mM)		5.4 ± 0.0	5.4 ± 0.0	4.3 ± 0.1	0.2 ± 0.0
Ragi WSP	Substrate blank	0.2	5.7 ± 0.3	6.5 ± 0.0	4.3 ± 0.2	0.4 ± 0.1
		0.4	6.7 ± 1.0	6.9 ± 0.1	4.7 ± 0.3	1.1 ± 0.1
		0.6	6.6 ± 0.1	8.2 ± 0.3	5.2 ± 0.3	1.4 ± 0.1
		0.8	6.5 ± 0.7	8.2 ± 0.3	5.5 ± 0.0	1.6 ± 0.1
		1	6.2 ± 0.3	8.3 ± 0.3	5.5 ± 0.1	2.1 ± 0.2
	Incubated at 45°C with FAE	0.2	4.7 ± 0.3	8.2 ± 1.0	2.6 ± 0.5	0.1 ± 0.1
		0.4	5.7 ± 1.7	7.7 ± 0.3	2.7 ± 0.3	0.2 ± 0.1
		0.6	5.5 ± 1.0	7.0 ± 1.0	2.0 ± 0.7	0.9 ± 0.1
		0.8	6.5 ± 0.0	8.2 ± 1.0	3.2 ± 0.3	1.2 ± 0.3
		1	6.5 ± 0.0	8.5 ± 1.0	3.2 ± 1.0	1.3 ± 0.3

Table 22. Effect of enriched DEAE-cellulose purified fraction of FAE from 96 h ragi malt on the foam stabilization property of WSPs isolated from wheat

Sample		Additive Concentration %	Foam volume in ml			
			$NaHCO_3$	Citric acid	10' at 25°C	3' at 90°C
Control	Acetate buffer (50 mM)		5.4 ± 0.0	5.4 ± 0.0	4.3 ± 0.1	0.2 ± 0.0
Wheat WSP	Substrate blank	0.2	4.1 ± 0.1	4.9 ± 0.1	3.3 ± 0.07	0.9 ± 0.1
		0.4	4.1 ± 0.1	4.9 ± 0.2	3.3 ± 0.4	0.9 ± 0.1
		0.6	3.5 ± 0.1	4.7 ± 0.1	3.1 ± 0.4	1.1 ± 0.1
		0.8	3.8 ± 0.0	7.6 ± 0.2	4.0 ± 0.0	1.1 ± 0.2
		1	1.9 ± 0.1	7.7 ± 0.1	3.6 ± 0.2	1.5 ± 0.1
	Incubated at 45°C with FAE	0.2	4.8 ± 0.9	4.5 ± 0.1	2.0 ± 0.1	0.6 ± 0.1
		0.4	3.2 ± 0.2	5.1 ± 0.1	2.4 ± 0.1	0.7 ± 0.1
		0.6	2.8 ± 0.0	6.8 ± 0.5	1.7 ± 0.1	0.7 ± 0.1
		0.8	4.1 ± 0.7	6.9 ± 0.7	1.9 ± 0.4	0.8 ± 0.0
		1	3.8 ± 0.2	6.6 ± 0.2	1.8 ± 0.2	1.0 ± 0.0

Table 23. Effect of enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt on the foam stabilization property of WSPs isolated from ragi

Sample		Additive concentration %	Foam volume in ml			
			$NaHCO_3$	Citric acid	10' at 25°C	3' at 90°C
Control	Tris buffer (75 mM)		5.4 ± 0.0	5.4 ± 0.0	4.3 ± 0.14	0.2 ± 0.0
Gum	Substrate	0.2	2.5 ± 0.14	5.2 ± 0.1	3.9 ± 0.14	1.6 ± 0.1
Karaya	Enz Treat	0.2	2.0 ± 0.7	2.4 ± 0.1	0.5 ± 0.0	0.2 ± 0.0
Ragi WSP	Substrate blank	0.2	4.4 ± 0.14	8.4 ± 0.14	5.15 ± 0.2	1.8 ± 0.01
		0.4	3.2 ± 0.35	6.9 ± 0.14	6.4 ± 0.1	1.8 ± 0.0
		0.6	2.0± 0.35	4.9 ± 0.1	6.5 ± 0.0	1.9 ± 0.05
		0.8	4.4 ± 0.1	9.4 ± 0.1	6.9 ± 0.1	2.1 ± 0.14
		1	4.93 ± 0.2	11.4 ± 0.14	8.5 ± 0.1	2.4 ± 0.0
	Incubated at 45°C with AAE	0.2	1.75 ± 0.35	2.5 ± 0.0	0.5 ± 0.07	0.2 ± 0.0
		0.4	1.65 ± 0.2	2.5 ± 0.0	0.4 ± 0.14	0.3 ± 0.01
		0.6	1.0 ± 0.0	2.0 ± 0.0	0.5 ± 0.1	0.2 ± 0.0
		0.8	1.4 ± 0.2	2.75 ± 0.3	0.5 ± 0.1	0.2 ± 0.01
		1	1.5 ± 0.3	2.0 ± 0.0	0.7 ± 0.1	0.2 ± 0.1

Table 24. Effect of enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt on the foam stabilization property of WSPs isolated from wheat

Sample		Additive concentration %	Foam volume in ml			
			$NaHCO_3$	Citric acid	10' at 25°C	3' at 90°C
Control	Tris buffer (75 mM)		5.4 ± 0.0	5.4 ± 0.0	4.3 ± 0.14	0.2 ± 0.0
Gum	Substrate	0.2	2.5 ± 0.14	5.2 ± 0.1	3.9 ± 0.14	1.6 ± 0.1
Karaya	Enz Treat	0.2	2.0 ± 0.7	2.4 ± 0.1	0.5 ± 0.0	0.2 ± 0.0
Wheat WSP	Substrate blank	0.2	4.3 ± 0.1	6.9 ± 0.1	3.9 ± 1.4	1.2 ± 0.0
		0.4	3..4 ± 0.1	4.9 ± 0.1	3.5 ± 1.1	1.2 ± 0.01
		0.6	3.5 ± 0.1	4.6 ± 0.5	3.8 ± 1.1	1.4 ± 0.01
		0.8	3.6 ± 0.2	5.5 ± 0.14	3.3 ± 1.4	1.6 ± 0.0
		1	3.5 ± 0.1	5.2 ± 0.3	3.6 ± 1.5	1.6 ± 0.0
	Incubated at 45°C with AAE	0.2	1.85 ± 0.2	2.0 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
		0.4	1.2 ± 0.0	1.7 ± 0.0	0.6 ± 0.0	0.2 ± 0.1
		0.6	1.0 ± 0.0	1.7 ± 0.14	0.6 ± 0.0	0.4 ± 0.0
		0.8	1.0 ± 0.1	1.4 ± 0.0	0.65 ± 0.5	0.4 ± 0.0
		1	1.1 ± 0.14	1.3 ± 0.14	0.68 ± 0.4	0.4 ± 0.0

5.6. Effect of enriched DEAE-cellulose purified fractions of ragi AAE and FAE on the oxidative gelation of WSPs

In the present study, the relative viscosity of wheat WSP increased with increase in concentration (0.2 - 1.0%) compared to the ragi WSP, by the addition of hydrogen peroxide and peroxidase which indicates the high oxidative gelation of wheat WSP. This can be explained as the concentration of the polymers increase the rate of oxidative gelation property of the polymers increases, which in turn increases the intermolecular association and entanglement between the polymer chains and forms a three-dimensional network (Vinkx et al., 1991). The relative viscosities of WSPs of ragi and wheat increased marginally by the treatment of enriched AAE from 72 h ragi malt (Figure 67 & 68). Studies reported that as the content of acetyl groups increases, the gelling capacity of that polysaccharide decreases (Pippen et al., 1950).

In case of enriched FAE treated WSPs a marginal decrease in the relative viscosities of WSPs was observed (Figure 69a & b). This can be explained by the removal of ferulic acid groups, which are responsible in forming cross-links between arabinoxylan chains (form diferulic bridges) in the presence of free radical generating compounds (Rees, 1969; Grabber 1998a &b).

Studies showed that the presence of free ferulate competes with hydrogen peroxide (H_2O_2)/peroxidase thereby decreasing the effect of feruloylated arabinoxylan interference (Wang et al., 2002a). Earlier reports found that the feruloylated arabinoxylan solutions adopt more flexible conformations than non-feruloylated polymers due to the formation of a gel network by crosslinking the ferulic acid between arabinoxylan chains to form diferulic acids especially 8-5′ dimer in the case of wheat WSP (Izydorczyk and Biliaderis, 1992a; Izydorczyk and Biliaderis, 1995). This was attributed to the decrease in monomeric ferulic acid is much more than the increase of diferulic acids, but after a sufficient number of cross-links, the chain movement is impeded to prevent further cross-linking. The viscosity of arabinoxylans mainly depends on the concentration and molecular weight of the polysaccharide. The formation of diferulic acid bridges affects the viscosity of the polysaccharide solution by increasing its molecular weight (Dervilly-Pinel et al., 2001). Even, it is reported that the cross linking enhances the thermal stability of the gel network.

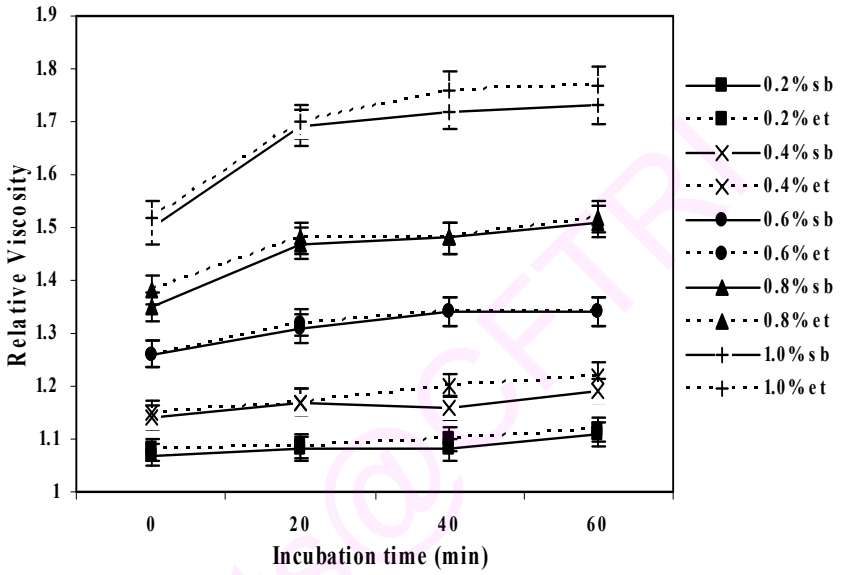


Figure 67. Effect of enriched DEAE-cellulose isolate of AAE from 72 h ragi malt on the oxidative gelation of wheat WSP with respect to increase in concentration (sb: substrate blank, et: enzyme treated)

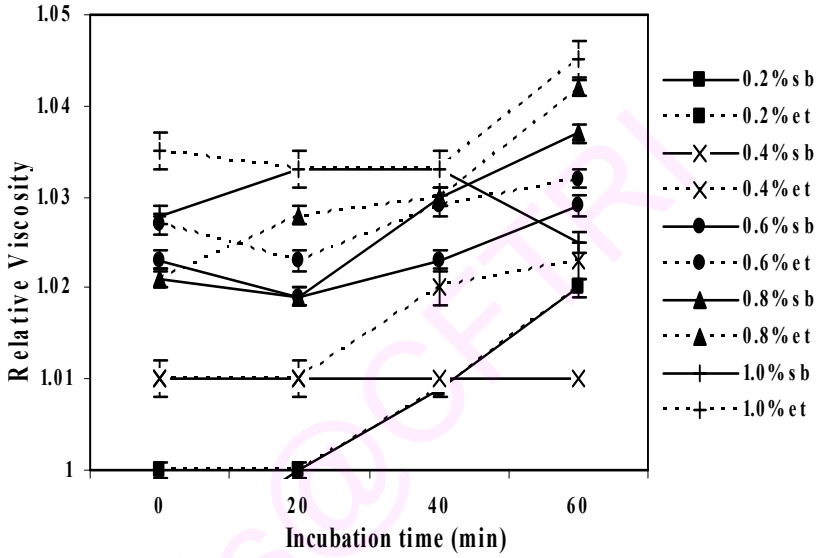
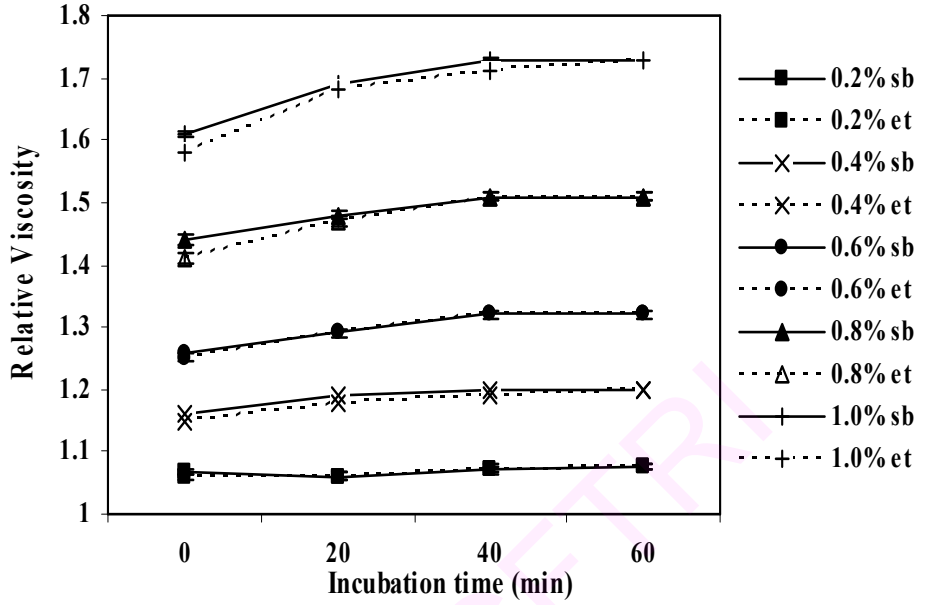
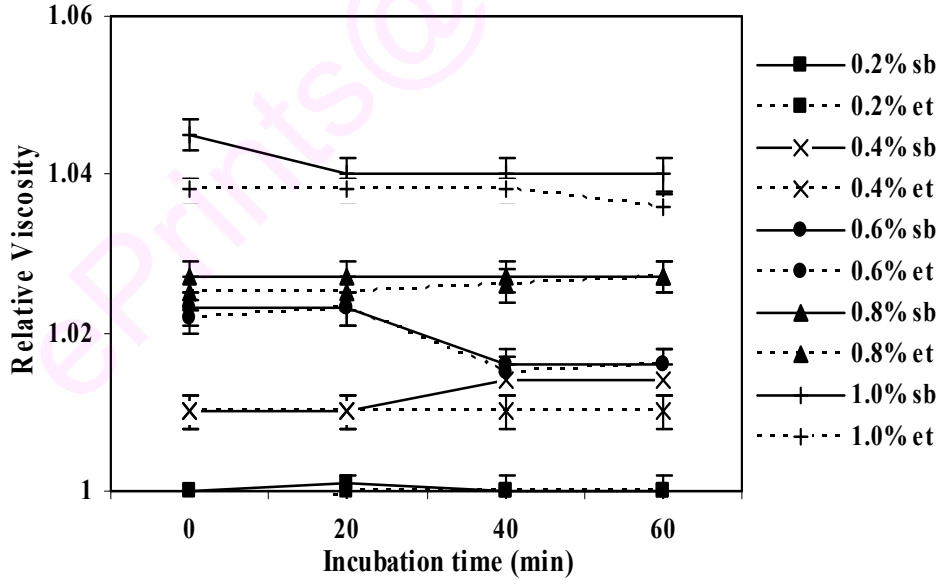


Figure 68. Effect of enriched DEAE-cellulose isolate of AAE from 72 h ragi malt on the oxidative gelation of ragi WSP with respect to increase in concentration (sb: substrate blank, et: enzyme treated)



(a)



(b)

Figure 69. Effect of enriched DEAE-cellulose isolate of FAE from 96 h ragi malt on the oxidative gelation of (a) wheat WSP and (b) ragi WSP with respect to increase in concentration (sb: substrate blank, et: enzyme treated)

5.6.1. Mechanism of oxidative gelation

The enzymatic treatment of WSPs (ragi and wheat) with enriched DEAE-cellulose purified FAE from 96 h ragi malt releases the ferulic acid esterified to the arabinose moiety of arabinoxylan (Figure 70). This prevents the cross-linking between the polysaccharide chains, which, in turn decreases the oxidative gelation of WSPs.

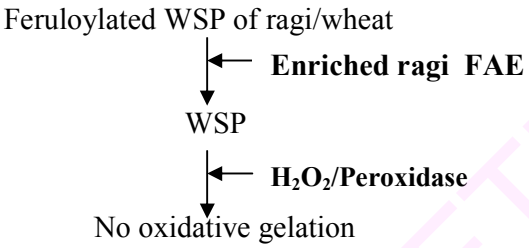


Figure 70. Schematic representation of the enzymatic deferuloylation of WSPs with enriched DEAE-cellulose purified fraction of FAE from 96 h ragi malt

According to the published literature, the mechanism of dimerization of ferulic acid linked to arabinoxylan of WSPs is a radical coupling reaction (Ralph et al., 1994), which may occur by two steps:

1. In the first step, the parent radical is generated by the attack of hydrogen of the hydroxyl group at the phenolic position. This result in the formation of a phenoxy radical, which is stabilized by delocalization of the unpaired electron on to the whole molecule essentially at C-4, C-5, C-8 positions of ferulic acid.
2. In the second step, the phenoxy radical monomers formed are coupled and the two unpaired electrons of the two monomers form a covalent linkage, connecting the two-arabinoxylan chains (Ralph et al., 1994). The structure of the dehydrodimer depend on the distribution of unpaired electrons. Coupling takes place at highest electron density region i.e. C-4, C-5 and C-8 positions of ferulic acid (Russel et al., 1999).

The distribution and relative amount of ferulic acid along the arabinoxylan backbone influence the gelling capacity of the arabinoxylan. As the content of ferulic acid increases the gel network becomes stronger resulting in a more stiffer gel (Izydorczyk and Biliaderis, 1992a).

Acetylated xylans have poor gelling capacity than the non-acetylated polymers. The presence of acetyl groups prevents the close packing of arabinoxylan polymer, thereby increasing their solubility. Deacetylation of these polymers in the presence of enriched ragi AAE might have resulted in chain-chain association of polysaccharides, perhaps leading to the formation of aggregate or a gel network (Bacon et al., 1975).

5.7. Role of enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt on the synergistic gelation of Xanthan and Locust bean gums (XG and LBG)

In the present study, the relative viscosity of the blend (XG + LBG) was much higher than the viscosities of individual XG and LBG solutions (Figure 71). LBG displaces the highly ordered conformation of XG for efficient binding to form heterotypic junctions (Zhan et al., 1993; Morris, 1996). The synergistic gelation of XG and LBG indicate the possible formation of thermally reversible gels of XG with LBG, which takes place by the intermolecular interactions between the side chains of XG and the mannan backbone of LBG (Rocks, 1971; Cairns et al., 1986). The interaction result in enhanced viscosity or gelation.

The treatment of XG with the enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt showed a slight decrease in the viscosity after 2 h of incubation, which could be due to the removal of acetyl groups from the XG. Studies showed that the presence of acetate groups stabilizes the ordered conformation of XG thereby reduces the synergistic interaction with LBG (Wang et al., 2002b). In the case of the blend (0.2 h, Enzyme treated XG + LBG), the relative viscosity was higher at 1 h, than control and 2 h, intervals. This can be explained, by the maximum deacetylation of XG after 1 h of enzyme treatment, which may lead to more synergism. As the deacetylation enhances the intermolecular synergistic interaction

between the flexible, destabilized xanthan chains with LBG resulting in increased hydrogen bonding in turn increase in the viscosity (Ojinnaka et al., 1998). The slight decrease in the viscosity of 2 h AAE treated XG and LBG blend may be due to the competition between these polymers and the liberated acetate ions for forming hydrogen bonding with water.

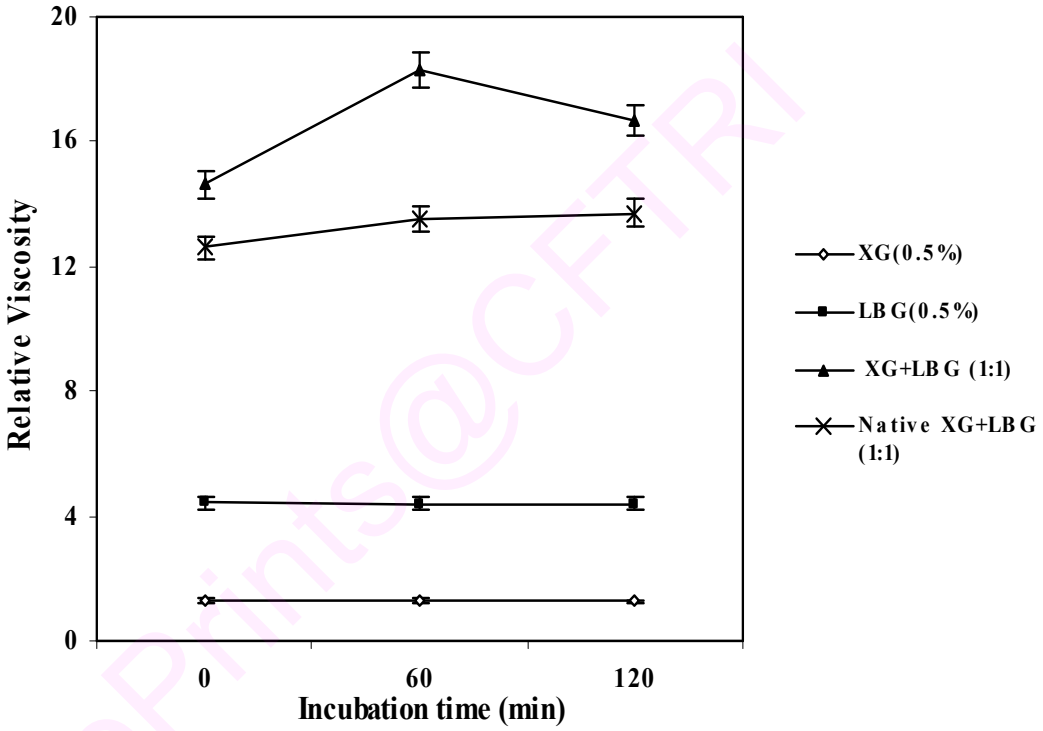
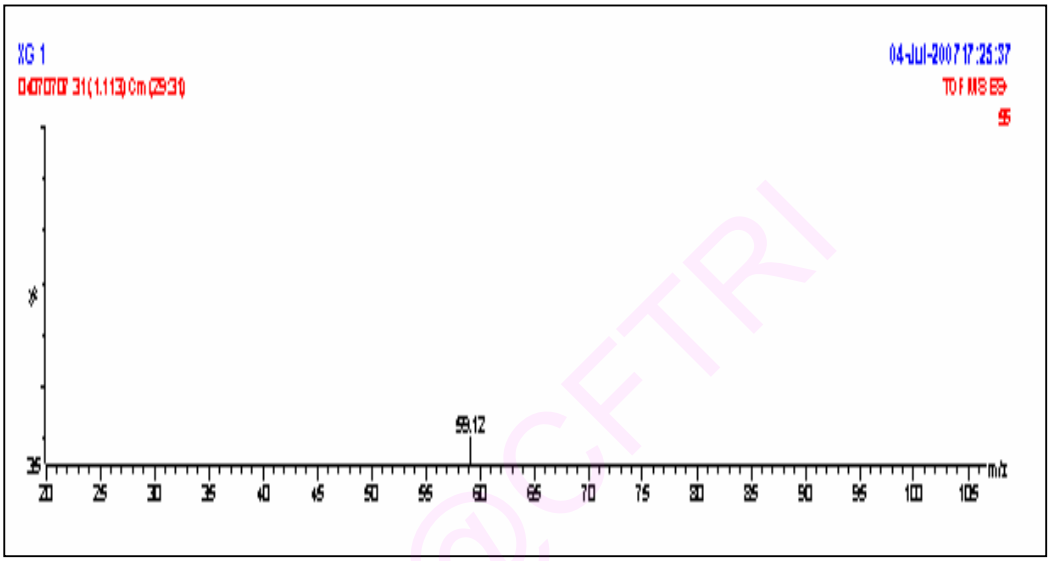


Figure 71. Effect of enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt on the gelation of XG & LBG blends [XG (0.5%), LBG (0.5%) and XG+LBG (1:1) are treated with AAE, Native XG+LBG (1:1) is untreated]

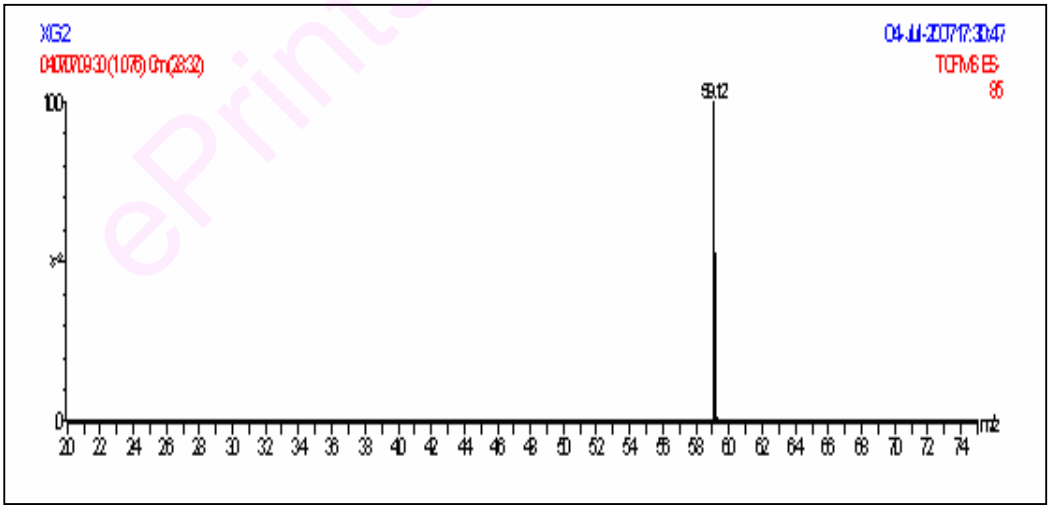
5.8. ESI-MS analysis of deacetylated xanthan gum by enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt

Analysis by ESI-MS confirmed the gradual deacetylation of XG by enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt (Figure 72). The formation of acetate ions by the enzymatic deacetylation of XG using this purified

enzyme increased with increase in incubation period (1 & 2 h) (Figure 72a & b). The signal at 59.12 *m/z* (ESI-negative mode) for acetate ion showed the deacetylation of XG by purified ragi AAE.



(a)



(b)

Figure 72. ESI-MS for the deacetylation of XG by enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt (a) XG 1 h and (b) XG 2 h

5.9. Possible gelling mechanism of xanthan and locust bean gum and the effect of enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt on their synergistic interaction

The kinetic and inhibitor (Eserine) studies indicated the possible presence of serine/histidine residues at the catalytic site of the purified AAE from ragi (Chapter 4a.3.9) which is in consensus with the catalytic triad ser-his-asp of esterases (Puchart et al., 2006; Levisson et al., 2007). The enzymatic hydrolysis of the ester bond requires two catalysis residues, a proton donar and a nucleophilic base (Figure 73). The catalytic nucleophile of the enzyme attacks the carbon atom of the ester (acetyl group present on the substituted inner mannose of xanthan). The release of deacetylated xanthan may facilitate the protonation with a proton donar present at the catalytic site of the enzyme. Acetyl group may then be transferred to the nucleophile thereby forming an acetyl-enzyme intermediate. Hydrolysis with a water molecule perhaps releases the acetyl group thereby restoring the ionization state of the catalytic residues of the enzyme (Puchart et al., 2006).

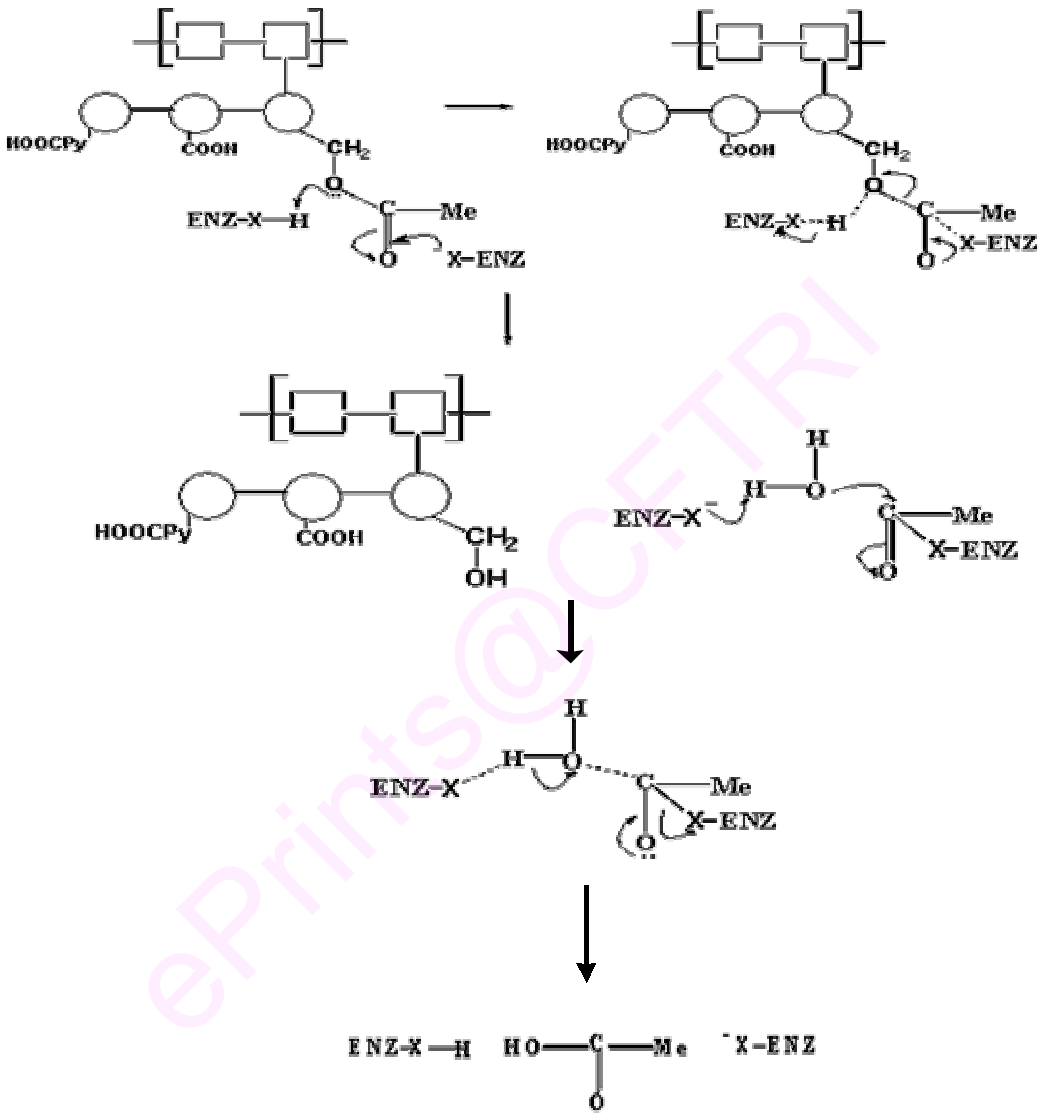


Figure 73. Possible mechanism of action of enriched on the deacetylation of xanthan gum (ENZ-X-H, proton donar; ENZ-X$^-$; Me, methyl group)

The acetyl groups in xanthan are shown to inhibit the interaction between xanthan and other polysaccharides such as galactomannans which are also involved in stabilizing the polymer, however the presence of pyruvate groups destabilizes the polymer (Tako et al., 1984; Lopes et al., 1992). Reports showed that acetate groups, which are linked to the inner mannose residues of the side chains, contribute to the intra molecular association with the backbone. The side chains become more flexible after deacetylation, therefore the intermolecular association between the side chains increases with the increase in concentration, and side chains are more liable to associate with galactomannan.

5.9.1. Possible mechanism of synergistic gelation of deacetylated xanthan gum and locust bean gum

In the present study, the incubation of xanthan (0.5%) with enriched fraction of AAE from 72 h ragi malt at 45°C for 1-2 h resulted in the deacetylation of xanthan gum which may form a disorderd helix which may able to form strong intermolecular association with the added LBG (0.5%). During the process of gelation of deacetylated XG with LBG, the side chains of the deacetylated xanthan gum (disordered helix) can form an intermolecular association/interaction connected either parallel or anti parallel to the LBG polymer which may be due to enhanced flexibility. In this mechanism, the hydrogen bonding and electrostatic interactions between the smooth regions (unsubstituted mannose backbone) of LBG and the side chain of xanthan play an important role in the association (Figure 74). These interactions are stronger in the deacetylated xanthan leading to synergistic gelation, which in turn increases the relative viscosity of the blend (Wang et al., 2002b). Studies showed that the viscosity of synergistic gel is maximum when both the polymers are present in 1:1 ratio (Copetti et al., 1997).

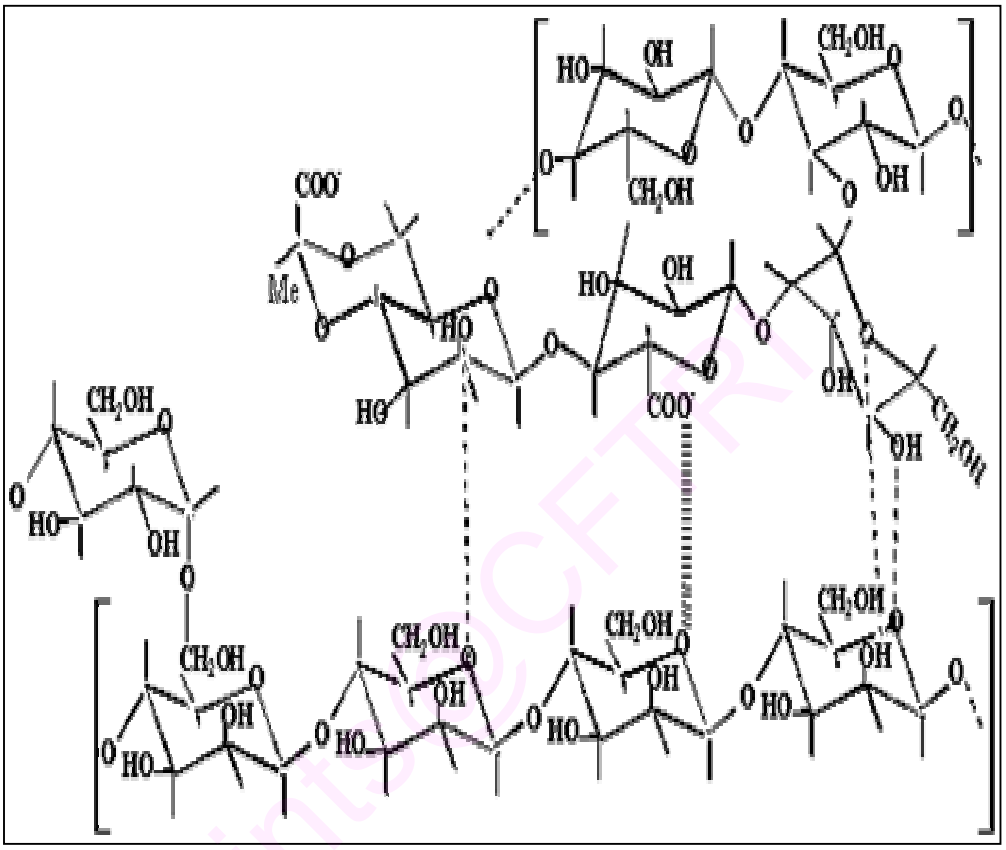


Figure 74. Possible intermolecular association of XG and LBG for synergistic gelation

Based on these conclusions, the nature of the gelation determined by the level of disorder induced in xanthan before mixing, and the level of interaction between xanthan and LBG is highly influenced by the presence and absence of acetyl groups on xanthan and the content of galactose residues of LBG (Copetti et al., 1997).

Conclusions

The above studies clearly indicated that esterases (AAE and FAE) from finger millet malt do play a role in modulating the functional characteristics of WSPs by

cleaving the acetyl and feruloyl groups of arabinoxylans respectively. Increase in the synergistic interaction of XG and LBG due to deacetylating XG by purified finger millet malt AAE, also gave an indication for the possible application of finger millet malt esterases in modulating the gelling properties of the above polysaccharides for various end uses.

SUMMARY AND CONCLUSIONS

The results obtained from the present investigation on 'Esterases from finger millet malt and their effect on the functionality of cereal non-starch polysaccharides' have been summarized with the following conclusions (Page and chapter numbers are given in the brackets as a ready reckoner)

Chapter 3. Isolation of esterases from ragi malt (AAE and FAE)

- 72 h ragi malt has showed maximum activity of acetic acid esterase (AAE). Conditions were optimized for the maximum extraction of AAE from 72 h ragi malt. Tris buffer extraction (pH 9.0, 75 mM) in the presence of GSH (100 mM), PVPP (0.5%), Triton X-100 (0.75%), $CaCl_2$ (6 mM) and $MgCl_2$ (4 mM) yielded maximum activity. **(page no. 67 - 73, Chapter 3a)**

- Temperature optima and thermal stability of crude extract of AAE from 72 h ragi malt was found to be 30°C. Polyhydroxy compounds such as mannitol, sorbitol and several amino acids were tested for their stabilizing effect on AAE from 72 h ragi malt, out of which glycine alone has some stabilization effect on the enzyme at 50°C. **(page no. 73 - 76, Chapter 3a)**

- 96 h ragi malt has showed maximum activity of ferulic acid esterase (FAE). For assaying FAE, substrates such as feruloyl glycerol and paranitrophenyl ferulate (PNPF) were synthesized and out of these two substrates, PNPF was found to be more suitable for routine spectrophotometric assay than feruloyl glycerol. Tris buffer extraction (pH 7.0, 50 mM) in the presence of GSH (25 mM), PVPP (1%) and Triton X-100 (1%) yielded maximum activity of FAE. **(page no. 77 - 86, Chapter 3b)**

- Temperature optima of crude extract of FAE from 96 h ragi malt and its coleoptiles were found to be 38°C and 40°C respectively and the enzyme was thermally stable at 30°C. **(page no. 86 - 87, Chapter 3b)**

Chapter 4. Purification of esterases (AAE and FAE) from ragi malt

- Tris buffer extracts of AAE and FAE were purified to apparent homogeneity by using ammonium sulphate fractionation (40 – 80%), anion exchange (DEAE-cellulose) and gel filtration (Sephacryl S-200 - AAE; Biogel P-30 - FAE) column chromatographies. In addition, AAE was finally purified on Phenyl-Sepharose CL-4B to remove associated carbohydrate. The fold purification and recovery of AAE by four-step purification was 34 and 0.36% respectively. The three-step purification of FAE has resulted in a fold purification and recovery of 3% and 22 respectively. **(page no. 88 - 94, AAE, Chapter 4a; 117 - 121, FAE, Chapter 4b)**

- The apparent homogeneity of these esterases was determined by protein, activity staining and by SDS-PAGE. The molecular weight of the native AAE was found to be 79.4 kDa by GPC and ESI-MS whereas the denatured enzyme was found to be 19.7 kDa on SDS indicating it to be a homotetramer. However, FAE was found to be a monomer with a molecular weight if 16.5 kDa as indicated by the similar molecular weight under both native (GPC and ESI-MS) and denatured (SDS) conditions. **(page no. 94 - 98, AAE, Chapter 4a; 121-125, FAE, Chapter 4b)**

Kinetic studies of esterases

- The pH and temperature optima of the purifed AAE were found to be 7.5 (Tris-HCl, 75 mM) and 45°C respectively. AAE is found to be stable in the pH range of 6.0 - 9.0 and in the temperature range of 30 - 40°C. **(page no. 99 - 102, Chapter 4a)**

- The pH and temperature optima of the purified FAE were found to be 6.0 and 45°C respectively. FAE is stable in the pH range of 5.5 - 9.0 and at a temperature of 30°C. **(page no. 126 - 129, Chapter 4b)**

- The activation energy of the hydrolysis of α-NA by AAE was found to be 7.29 kJ mol^{-1} whereas the hydrolysis of PNPF by FAE was found to be 4.08 kJ mol^{-1}. **(page no. 102 - 103, AAE, Chapter 4a; 129 - 130, FAE, Chapter 4b)**

- The apparent K_m and V_{max} of the purified AAE for α-NA (2.0 - 10 µg) were found to be 0.04 µM and 0.175 Uml^{-1} respectively. The apparent K_m and V_{max} of the purified FAE for PNPF (2.5 - 12.5 µg) were found to be 0.053 µM and 0.085 U ml^{-1} respectively. **(page no. 105 - 106, AAE, Chapter 4a; 132 - 133, FAE, Chapter 4b)**

- EDTA, citric acid and metal ions such as Fe^{3+}, Cu^{2+} increased the activity of purified AAE while Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Al^{3+} reduced its activity. Metal ions such as Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, oxalic and citric acids enhanced the FAE activity whereas it was completely inhibited by Fe^{3+}. **(page no. 106 - 107, AAE, Chapter 4a; 133 - 134, FAE, Chapter 4b)**

- Group specific reagents such as eserine and PCMB at 25 mM concentration completely inhibited the activity of purified AAE, while iodoacetamide showed very little inhibition (5%). Eserine is found to be a competitive inhibitor of AAE from ragi malt indicating the possible presence of serine residues in the active site pocket. PCMB and iodoacetamide inhibited the activity of purified FAE indicating the possible presence of cysteine residues in the active site pocket. Phenylmethylsulphonyl fluoride (0.0 - 50 mM) and eserine did not show any inhibitory effect on the purified FAE. **(page no. 107 - 108, AAE, Chapter 4a; 134 - 135, FAE, Chapter 4b)**

- Purified AAE was active on both small molecular weight synthetic substrates as well as water-soluble polysaccharides such as ragi, wheat, larch wood xylan and gum karaya. The activity was maximum with PNPA compared to other

substrates. ESI-MS and ^{1}H NMR spectra showed the gradual deacetylation of α-NA, PNPF and glucose pentaacetate to α-naphthol, PNP and glucose triacetate respectively by the action of purified ragi AAE. **(page no. 108 - 116, Chapter 4a)**

- Purified FAE was also active both on small molecular weight as well as polysaccharides showing similarity with AAE in its nature/mode of action. The maximum activity was found using the synthetic substrate PNPF. Ragi water-soluble preparation has shown maximum activity among the polysaccharides used. The ferulic acid liberated from water-soluble polysaccharides of wheat and ragi by the action of purified ragi FAE was authenticated by ESI-MS. **(page no. 135 - 139, Chapter 4b)**

Chapter 5. Role of esterases on the functionality of cereal water-soluble non-starch polysaccharides

- The IR spectra of water-soluble preparations of polysaccharides such as ragi, wheat, larch wood xylan and gum karaya showed medium-intensity absorption at 1200 cm^{-1} and 1735 cm^{-1} correspond to carbonyl stretching of an ester group indicated the presence of acetyl and feruloyl groups. WSPs from ragi and wheat had showed the presence of high content of bound ferulic acid than free ferulic acid. **(page no. 141, Chapter 5)**

- The relative viscosities of wheat and ragi WSPs [both enzyme(AAE&FAE) treated and untreated] increased with the increase in concentration (0.2 - 1.0%), decreased with increase in temperature (30 - 70°C). However, the relative viscosities of the esterase treated WSPs were marginally less than the untreated ones which, may be perhaps due to the removal of acetyl and feruloyl groups from WSPs. **(page no. 142 - 145, Chapter 5)**

- Foam stabilization effect of WSPs increased with increase in concentration (0.2 - 1.0%). The removal of acetyl and feruloyl groups by the enzymatic treatment (AAE/ FAE) decreased the foam stabilization capacity of polysaccharides, which can be attributed due to the decrease in the viscosity of the polysaccharide solution. **(page no. 145 - 149, Chapter 5)**

- Oxidative gelation (determined by the relative viscosities) of WSPs of ragi and wheat increased marginally by the treatment of enriched AAE from 72 h ragi malt, while FAE treated ones showed slight decrease in comparison with their respective controls. This can be explained by the removal of acetyl and ferulic acid groups, from the WSPs by these esterases. **(page no. 150 - 155, Chapter 5)**

- Xanthan gum (XG), deacetylated by enriched DEAE-cellulose purified fraction of AAE, showed improved gelation when blended with locust bean gum (LBG) in the ratio of 1:1 compared to the native xanthan gum indicating the effect of acetyl groups on the synergistic gelation of these blends. **(page no. 155 - 156, Chapter 5)**

- The formation of acetate ions (59.12 *m/z*) by the gradual enzymatic deacetylation of XG with enriched DEAE-cellulose purified fraction of AAE from 72 h ragi malt was analyzed by ESI-MS. **(page no. 156 - 157, Chapter 5)**

The above studies clearly indicated that both AAE and FAE are minor enzymes in the ragi malt, which can be isolated, purified to apparent homogeneity. Their kinetic properties are comparable with barley and microbial esterases. The enriched enzyme isolates of AAE and FAE from finger millet malt do have an effect in modulating the functional characteristics of WSPs such as viscosity, foam stabilization and oxidative gelation capacity due to the cleaving of the acetyl and feruloyl groups of arabinoxylans .These enzymes can be used for the modifications of

the properties of various feruloyl as well as acetyl polysaccharides from different sources. However these enzymes, in future can be expressed in microorganisms to have higher yields so that their properties and structure can be studied in much more detail, which will pave the way for their broad applications in the food industry.

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LIST OF PUBLICATIONS AND PATENT

Publications

1. **G. Madhavi Latha**, and G. Muralikrishna, 2007. Purification and partial characterization of acetic acid esterase from malted finger millet (*Eleusine corocana* Indaf-15). Journal of Agricultural and Food chemistry, 55(3*)*, 885 - 902.

2. **G. Madhavi Latha,** P.Srinivas and G. Muralikrishna, 2007. Purification and Characterization of ferulic acid esterase from malted finger millet (*Eleusine corocana* Indaf-15). Journal of Agricultural and Food Chemistry, 55, 9704 - 9712.

3. **G. Madhavi Latha** and G. Muralikrishna, 2007. Optimized procedure for the isolation of acetic acid esterase from malted finger millet (*Eleusine corocana* Indaf-15). Journal of Food Science and Technology (in press). .

4. **G. Madhavi Latha** and G. Muralikrishna, 2007. Effect of finger millet (*Eleusine coracana*, Indaf 15) malt esterases on the functional characteristics of non-starch polysaccharides" to be communicated to Food Hydrocolloids.

Patent

1. **G. Madhavi Latha** and G. Muralikrishna, 2005. A process for the preparation of acetic acid esterase from ragi malt. 237/NF/05-2336 DEL/2005.

PUBLICATIONS

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Partial Characterization of Acetic Acid Esterase from Malted Finger Millet (*Eleusine coracana,* Indaf-15)

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Acetic acid esterase (EC 3.1.1.6) cleaves the acetyl groups substituted at O-2/O-3 of the xylan backbone of arabinoxylans and is known to modulate their functional properties. To date, this enzyme from cereals has not received much attention. In the present study, acetic acid esterase from 72 h ragi malt was isolated and purified to apparent homogeneity by a four-step purification, i.e., ammonium sulfate precipitation, DEAE-cellulose, Sephacryl S-200, and phenyl-Sepharose column chromatography, with a recovery of 0.36% and a fold purification of 34. The products liberated from α-NA and PNPA by the action of purified ragi acetic acid esterase were authenticated by ESI-MS and ^{1}H NMR. The pH and temperature optima of the enzyme were found to be 7.5 and 45 °C, respectively. The enzyme is stable in the pH range of 6.0−9.0 and temperature range of 30−40 °C. The activation energy of the enzymatic reaction was found to be 7.29 kJ mol^{-1}. The apparent K_m and V_{max} of the purified acetic acid esterase for α-NA were 0.04 μM and 0.175 μM min^{-1} mL^{-1}, respectively. The molecular weight of the native enzyme was found to be 79.4 kDa by GPC whereas the denatured enzyme was found to be 19.7 kDa on SDS, indicating it to be a tetramer. EDTA, citric acid, and metal ions such as Fe^{+3} and Cu^{+2} increased the activity while Ni^{+2}, Ca^{+2}, Co^{+2}, Ba^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, and Al^{+3} reduced the activity. Group-specific reagents such as eserine and PCMB at 25 mM concentration completely inhibited the enzyme while iodoacetamide did not have any effect. Eserine was found to be a competitive inhibitor.

KEYWORDS: Acetic acid esterase; ragi; finger millet; purification; homogeneity

INTRODUCTION

The complex nature of plant cell walls and the structure of the individual polysaccharides have been the subject of many investigations (*1*−*3*). Hemicellulose is the second most abundant renewable polysaccharide in nature after cellulose (*4*). Hemicelluloses are composed of arabinoxylans, 1, 3/1, 4 β-D-glucans and glucomannans, etc. Cereal arabinoxylan is a β-1,4-linked D-xylose polymer which is generally substituted either at O-2/O-3 with arabinose and also sometimes with 4-*O*-methylglucuronic acid (*5*) in the side chains. Cinnamic acid derivatives such as ferulic acid and coumaric acids are esterified to the 5^I-OH group of arabinose side chains (*6*−*7*) whereas acetic acid is found to be esterified to the free hydroxyl groups present at the C-2 and C-3 positions of xylose residues present in the backbone (*8*). According to one school of thought, the presence of occasional acetyl groups in the xylan backbone contributes substantially to its solubility, while deacetylation leads to the formation of xylan aggregates (*9*). However, recent literature suggests that extensive acetylation does contribute to the insolubility of arabinoxylans (*10*), indicating the bifunctional nature of acetyl groups. Acetylated xylans have poor gelling properties (*11*). They are not degraded in the rumen by microbes wherein the degree of acetylation of arabinoxylans is believed to be one of the important factors (*12*, *13*).

Enzymatic hydrolysis of cereal arabinoxylans requires the participation of several hydrolytic enzymes. These are classified in to two groups based on the nature of the linkages that they cleave (*14*). The first group of enzymes is hydrolases involved in the hydrolysis of the glycosidic bonds of xylan. These include endoxylanases (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55) and α-D-glucuronidase (EC 3.2.1.1). The second group includes enzymes that cleave the ester linkages which include acetic acid esterases (EC 3.1.1.6) and cinnamic acid esterases (EC 3.1.1.73).

Acetic acid esterases (EC 3.1.1.6) hydrolyze the ester linkages of the acetyl groups present in the xylan side chains. Acetic acid esterases obtained from plant and animal sources differ from microbial acetyl xylan esterases (EC 3.1.1.72) such as *Trichoderma reesei*, *Aspergillus niger*, and *Schizophyllum commune* (*15*) with respect to their substrate specificity. Partial characterization of acetic acid esterase from barley malt was recently reported (*16*).

Finger millet (*Eleusine coracana*), also known as ragi, is an indigenous minor millet, rich in calcium and dietary fiber. It is extensively consumed by the south Indian rural population and

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is used both in the native and processed (malted) forms (*17*). Studies were reported from our group regarding (a) isolation, purification, and characterization of ragi amylases (*18*), (b) structure and functional relationship of alkali soluble arabinoxylans (*19*), and also (c) water extractable feruloyl polysaccharides and their antioxidant properties (*20*).

Both alkali soluble arabinoxylans (hemicelluloses) and water soluble feruloyl polysaccharides were proved to have an effect on the functionality of cereal nonstarch polysaccharides with respect to (a) foam stabilization, (b) gelling, and (c) viscosity (*21*). However, to date the effect of *O*-acetyl groups on the functionality of cereal water soluble nonstarch polysaccharides was not studied. Hence, a study was undertaken to purify and characterize *O*-acetic acid esterase from malted finger millet and explore its potential in modulating the functionality of various nonstarch polysaccharides which is one of the most important aspects in food science and technology. Very few attempts were made to purify and characterize this novel enzyme from plant sources, hence the present study.

MATERIALS AND METHODS

Materials. An authenticated variety of finger millet (*Eleusine coracana*, ragi, Indaf-15) was procured from the V.C. farm of the University of Agricultural Sciences, Bangalore, located at Mandya, Karnataka, India. Acrylamide, bis-acrylamide, ammonium persulfate (APS), *N*,*N*,*N*^{1}-tetramethylenediamine (TEMED), glycine, Tris-HCl, and reduced glutathione were obtained from Sisco Research Laboratories, Mumbai, India. Sephacryl S-200, phenyl Sepharose, BSA (bovine serum albumin), protein molecular mass standards, Comassie brilliant blue, α-NA (α-naphthyl acetate), α- naphthol, fast blue RR salt, polyvinylpolypyrrolidone (PVPP), Triton X-100, PNPA (*p*-nitrophenyl acetate), EDTA (ethylenediamine tetraacetate), esereine, PCMB (*p*-chloromercuric benzoate), and iodoacetamide were obtained from Sigma Chemical Company, St. Louis, MO. Protein molecular mass markers for SDS (sodium dodecyl sulfate) obtained from Genei, Bangalore, India. DEAE-cellulose was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Malting. Malting was carried as reported earlier (*22*). Ragi seeds (50 g) were cleaned and steeped for 16 h and germinated under controlled conditions on moist cloth at 25 °C in a B.O.D. incubator up to 72 h. Germinated seeds were taken and dried at 50 °C in an air oven for 12 h, and vegetative growth portions were removed (devegetated) by gentle manual brushing. Devegetated seeds were weighed powdered and used for the extraction of acetic acid esterase.

Enzyme Extraction. Malted ragi flour (72 h; 50 g) was extracted with 75 mM Tris-HCl buffer (1:7, pH 9.0, 350 mL) containing 25 mM reduced glutathione, 1% Triton X-100 (w/v), and 1% PVPP for 2 h at 4 °C, and supernatant was collected by centrifugation (7000*g*, 4 °C for 20 min) using a refrigerated centrifuge and dialyzed against the extraction buffer and used for further experiments.

Enzyme Assay/Activity. Acetic acid esterase activity was measured as described by Poutanen and Sundberg (*23*) using 1 mM α-NA dissolved in ethanediol as substrate. The reaction mixture containing acetic acid esterase and α-NA (made to a final assay volume of 1.0 mL with 75 mM Tris-HCl buffer, pH 9.0) was carried out for 30 min at 30 °C, and the reaction was stopped by adding 100 μL of 0.33 M H_2SO_4. One unit of acetic acid esterase activity is defined as the amount of enzyme required to liberate 1 μmol of α-naphthol min^{-1}. The reaction was monitored spectrophotometrically at 235 nm for the release of α-naphthol. The activity with PNPA was determined by monitoring photometrically the release of PNP at a wavelength of 400 nm (*24*), making use of PNP standard graph.

Protein Determination. The presence of protein was monitored in the column fractions measuring absorbance at 280 nm and quantified by Bradford method at 590 nm using BSA as standard (*25*).

Enzyme Purification. Crude enzyme preparation of acetic acid esterase from malted ragi was subjected to ammonium sulfate precipitation which resulted in 4 fractions, i.e., 0−20, 20−40, 40−60, and 60−80 fractions. Ammonium sulfate fractions of 40−60, 60−80 were pooled (80% activity) and loaded onto a DEAE-cellulose column (1.5 × 18 cm), pre-equilibrated with Tris-HCl buffer (5 × 28 mL, 20 mM, pH 8.5) at a flow rate of 18 mL h^{-1} and washed with the same buffer to remove unbound proteins. A linear NaCl gradient (0−0.6 M) in equilibrating buffer was used to elute the bound proteins, which were collected (1.5 mL each) and monitored for protein (280 nm) as well as acetic acid esterase activity. The active fractions from the anion exchange chromatography were concentrated and loaded on a Sephacryl S-200 column (0.6 × 90 cm) which was pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.0), and fractions (1.5 mL) were collected and monitored for protein and acetic acid esterase activity. The pooled and dialyzed active fractions obtained from Sephacryl S-200 were concentrated and further fractionated on a phenyl Sepharose CL-4B column which was pre-equilibrated with sodium phosphate buffer (5 mM, pH 6.5) containing 1 M $(NH_4)_2SO_4$ which was used to remove unbound proteins. A linear $(NH_4)_2SO_4$ gradient (1 M to 0 M) followed by 50% ethylene glycol in sodium phosphate buffer (5 mM, pH 6.5) was used to elute the bound proteins (2.5 mL) individually. Active fractions were monitored for protein and acetic acid esterase activity.

Purity Criteria. *Polyacrylamide Gel Electrophoresis (PAGE).* PAGE (12.5%) under native conditions was carried out to evaluate the purity of acetic acid esterase. Duplicate samples were run for simultaneous protein (silver staining) (*26*) and activity staining (*27*).

Acetic Acid Esterase Activity Staining. The gel after electrophoresis was incubated in activity staining solution (50 mM sodium phosphate buffer, 100 mL pH 7.2; α-NA dissolved in 1 mL of acetone, 10 mg; fast blue RR salt, 50 mg) in the the dark at 37 °C until dark gray or until black bands appeared. The stained gel was washed with water and fixed in 3% acetic acid (*28*).

Estimation of Molecular Weight (M_r). *GPC.* This was performed on a column (0.6 × 90 cm) of Sephacryl S-200 HR calibrated by using standard protein markers such as papain (12 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (1.50 kDa), and β-amylase (2.00 kDa). The molecular mass was calculated from a plot of V_e/V_o against the log of molecular weight.

SDS-PAGE. M_r values were estimated by SDS-PAGE by the method of Laemmli (1970) (*29*) using a 12% w/v acrylamide gel. Proteins were detected by silver staining. M_r values were estimated from a plot of log M_r versus mobility using the following protein standards such as lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1kDa), carbonic anhydrase (29 kDa), egg albumin (43 kDa), BSA (66 kDa), phosphorylase (97.4 kDa), and myosin (2.05 kDa).

Effect of pH. Acetic acid esterase activity was determined at various pH values using different buffers such as sodium acetate (pH 4.0−6.0), sodium phosphate (pH 6.0−8.0), and Tris-HCl (pH 7.0−9.0) at 75 mM concentrations. The maximum activity was taken as 100% and relative activity plotted against different pH values.

pH Stability. Stability of purified acetic acid esterase was carried out by preincubating the enzyme in different buffers such as glycine-HCl (pH 2.0−3.0), sodium acetate (pH 4.0−6.0), sodium phosphate (pH 6.0−8.0), and Tris-HCl (pH 7.0−9.0), followed by determining the residual activities at different time intervals. The original activity was taken as 100%, and relative activity was plotted against different time intervals.

Temperature Optima. Freshly purified enzyme (50 μL) was incubated with 1 mM α-NA in Tris-HCl buffer (pH 7.5, 75 mM) in a temperature range of 30−60 °C (with an interval of 5 °C) using a thermostatically controlled incubator. The optimum activity was taken as 100%, and relative activities were plotted against different temperatures.

Thermal Stability. Purified acetic acid esterase was preincubated in a temperature range of 30−80 °C for 15 min. The residual activity was estimated taking original activity as control (100%), and relative activity was plotted against different temperatures.

Measurement of Activation Energy. To determine the temperature dependence of acetic acid esterase activity, reaction rates/activities at a series of temperatures (30−60 °C) were determined. An Arrhenius plot was drawn taking the natural log of activity on *y*-axis and 1/*T* in K on *x*-axis. The activation energy was determined from the slope of

the plot using the Arrhenius equation:

$$\text{slope} = -E_a/R$$

where R is universal gas constant whose value is 8.314 J mol^{-1}.

Effect of Substrate Concentration. Different concentrations of α-NA (2−10 μg) in Tris-HCl buffer (75 mM, pH 7.5) was incubated with purified acetic acid esterase for 30 min at 45 °C, and activities were measured at every 5 min. Initial velocities (V_o) were calculated for all substrate concentrations and the K_m and V_{max} values were calculated from double reciprocal plot (*30*).

Effect of Metal Ions. Purified acetic acid esterase was incubated with 5 mM solution of salts of metal ions (chlorides of as Fe^{+3}, Cu^{+2}, Ni^{+2}, Ca^{+2}, CO^{+2}, Ba^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Al^{+3}, etc.) at 45 °C for 15 min, and residual activities was measured. The enzyme activities without metal ion were taken as control (100%), and relative activities were calculated.

Effect of Group Specific Reagents. Purified acetic acid esterase was incubated with 25 mM of PCMB, iodoacetamide, and eserine in Tris-HCl buffer (pH 7.5) at 45 °C for 15 min, and the residual activities were estimated. The enzyme activities without these chemicals were taken as 100%, and relative activities were calculated.

Enzymic Deesterification of Acetyl Substrates. Water soluble portions of larch wood xylan, gum karaya, and water extractable polysaccharides (0.5%, 1 mL) isolated from ragi and wheat were taken in Tris buffer (75 mM, pH 7.5) and incubated with purified ragi acetic acid esterase (0.1 mL). The reaction was allowed to proceed at 45 °C for 2 h and was stopped by boiling for 10 min and centrifuged at 15 000*g* for 30 min to separate the supernatant from the residue. The supernatant was analyzed by HPLC for acetic acid using Supelco-C610H ion exchange column at room temperature at 210 nm using orthophosphoric acid (0.05%) as the eluent with a flow rate of 0.5 mL min^{-1}. The retention time for acetate under these conditions was 19.865 min. The specific activities of purified ragi acetic acid esterase using various water soluble polysaccharide preparations were compared with small molecular weight synthetic substrates such as α-NA and PNPA.

IR Analysis of Water Soluble Polysaccharides. Freeze-dried water soluble polysaccharides of ragi, wheat, larch wood xylan, and gum karaya (2 mg each) were taken in KBr pellet discs (10 mg) and analyzed by infrared spectroscopy for the presence of acetyl groups.

ESI-MS Analysis. A 1 mg amount of α-NA and PNPA was dissolved in ethanediol and DMSO (0.5 mL), respectively, and incubated with purified acetic acid esterase at 45 °C for different time intervals (0−8 h). The reaction was stopped with methanol (0.3 mL) and centrifuged. The methanol layer which consists of products was analyzed on an ESI-MS Alliance Waters 2695 mass spectrometer using negative mode electrospray ionization. The capillary voltage was 3.5 kV, core voltage 100 V, source temperature 80 °C, dissolvation temperature 150 °C, core gas (argon) 35 L h^{-1}, and dissolvation gas (Nitrogen) 500 L h^{-1}.

^{1}H NMR Spectral Analysis. A 2 mg amount of α-NA or PNPA in solution was incubated with purified ragi acetic acid esterase for 8 h at 45 °C. Subsequently the reaction mixture was freeze-dried and dissolved in DMSO-d_6, and the products were analyzed by ^{1}H NMR using a Bruker 500 MHz spectrometer operating at 27 °C.

RESULTS AND DISCUSSION

Extraction of Acetic Acid Esterase. The 72 h malted ragi was found to be the best (0.380 μmol $min^{-1}gm^{-1}$ malt) compared to 24 (0.171), 48 (0.254), and 96 h (0.160) ragi malts with respect to the acetic acid esterase activity. Maximum enzyme activity was extracted with Tris-HCl buffer at pH 9.0 (10.21 μmol $min^{-1}gm^{-1}$ malt) compared to pH 7.0 (1.97), pH 7.5(1.31), pH 8.0 (1.8), and pH 8.5 (4.02). Various substances such as PVPP, reduced glutathione, and Triton X-100 were added to the extracting buffer to enhance the yield (*31*). PVPP was included to minimize the coextraction of phenolic compounds present in ragi malt and reduced glutathione to disrupt the disulfide bonds and Triton X-100 to extract membrane bound enzymes, if any.

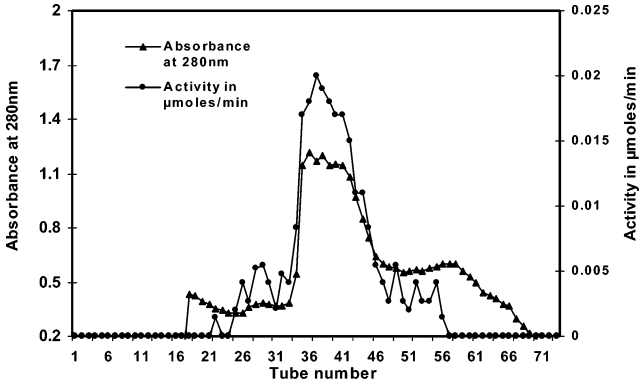

Figure 1. Elution profile of ragi acetic acid esterase (40–80% ammonium sulfate fraction) on a DEAE-cellulose column.

Acetic acid esterase and acetyl xylan esterase activities have been detected in a variety of microorganisms, particularly fungi such as *Penicillium purpurogenum* (*32*), *Trichoderma reesei* (*33*), *Ruminococcus flavefaciens* (*34*), *Streptomyces lividans* (*35*), and *Bacillus pumilus* (*36*), etc. Recently an acetic acid esterase from Barley malt (*16*) was isolated, purified, and partially characterized.

Purification of Acetic Acid Esterases. Crude Tris buffer extract (containing 1% PVPP, 1% Triton X-100, and 25 mM reduced glutathione) from 72 h ragi malt was subjected to ammonium sulfate precipitation and separated into four fractions (0−20, 20−40, 40−60, and 60−80%). Several chromatographic techniques, i.e., anion exchange, gel filtration, and hydrophobic interaction, were previously used to purify the acetic acid esterases from microbes. Since more than 80% of the activity was present in the 40−80% ammonium sulfate fraction, it was subjected to further purification on DEAE-cellulose column (**Figure 1**). This step was successful in removing the colored material, large amounts of unbound and contaminating proteins; in addition, it has also reduced the viscosity of the 40−80% fraction. The bound proteins were eluted with a linear gradient of NaCl (0−0.6 M). Acetic acid esterase was eluted as two peaks, one major (P-1) and one minor (P-2), at 0.27 and 0.43 M NaCl concentrations, respectively.

The DEAE-cellulose purified acetic acid esterase major peak (P-1) was further purified on a Sephacryl S-200 column (figure not shown). Acetic acid esterase was eluted as a single peak with a fold purification and recovery of 25.18 and 4.96%, respectively. However, on a native PAGE a large amount of carbohydrate streaking was observed along with minor contaminating protein bands (figure not shown). Most of the hydrophobically associated carbohydrate as well as contaminating minor proteins was removed by phenyl Sepharose column chromatography with a decreasing linear gradient of 1 M to 0 M $(NH_4)_2SO_4$. Acetic acid esterase was eluted with 50% ethylene glycol in sodium phosphate buffer (5 mM, pH 6.5) (figure not shown). The purified acetic acid esterase was obtained in 0.36% yield with fold purification of 34.

Recovery of the enzyme was significantly decreased after DEAE-cellulose column separation as well as after hydrophobic interaction chromatography, which might be due to (a) enzyme inactivation, and (b) removal of the carbohydrate which might be hydrophobically associated with the acetic acid esterase by stabilizing it. The overall scheme employed in the purification of acetic acid esterase from ragi malt is summarized in **Table 1**.

Table 1. Summary of the Purification of Acetic Acid Esterase from Ragi Malt

step	total activity[b]	total protein[c]	specific activity[d]	fold purification	% recovery
crude[a]	0.725	266.05	0.0027	1	100
40-80% $(NH_4)_2So_4$ fraction	0.59	15	0.04	14.8	81.2
DEAE-cellulo se	0.04	0.83	0.045	16.8	5.24
Sephacryl S-200	0.036	0.526	0.068	25.2	4.96
phenyl-Sepha rose 4B	0.0016	0.028	0.093	34.37	0.36

[a] 50 g scale (values are average of three independent experiments). [b] One unit is equivalent to 1 μmol of α-naphthol released min^{-1}. [c] Total protein is expressed in mg. [d] Specific activity is expressed in 1 μmol of α-naphthol released min^{-1} mg^{-1} of protein.

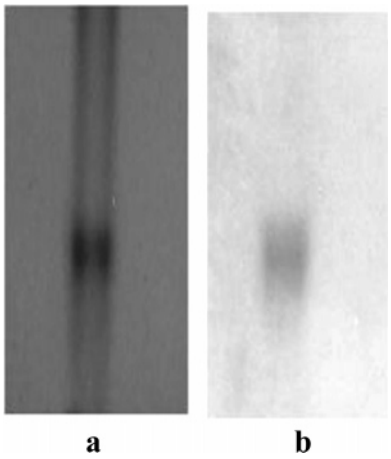

Figure 2. PAGE of purified ragi acetic acid esterase. (a) Protein staining. (b) Activity staining.

Criteria of Purity. The purity of acetic acid esterase was confirmed by SDS and native PAGE. The activity and protein bands coincide on native PAGE (**Figure 2**a,b). By SDS-PAGE a single subunit protein band with an estimated M_r of 19.7 kDa was observed (**Figure 3**a). The apparent molecular mass, determined under denaturing conditions, is comparable to the ones reported from the acetyl xylan esterases isolated from *Pencillium purpurogenum (32*), *Bacillus pumilus* (*36*) and acetic acid esterase from Barley malt (*16*). The estimated M_r of acetic acid esterase under nondenaturing conditions using Sephacryl S-200 was found to be 79.4 kDa (**Figure 3b**), indicating it to be a homotetramer similar to the one reported from acetyl xylan esterase of *Bacillus pumilus* (*36*).

pH Optima and Stability. Acetic acid esterase was found to have pH optima of 7.5 and 80% activity was retained between pH 6.0−9.0 (**Figure 4a**). The activity of acetic acid esterase in alkaline pH was more than the one observed in acidic pH. The activity has decreased both in acetate and phosphate buffers at lower pH compared to Tris-HCl buffer. However, the drop in activity in acetate buffer was much more pronounced in phosphate buffer. Most of the activity was retained in Tris-HCl buffer, indicating better stability of acetic acid esterase in Tris-HCl buffer. The enzyme activity has decreased drastically in the pH range 4.0−5.5 in acetate buffer and 6.0−7.0 in phosphate buffer, indicating the labile nature of the enzyme in acidic pH. The optimal pH of 7.5 is similar to the pH values of 8.0 reported for *Bacillus pumilus* (*36*), 7.7 for *Schizophyllum commune* (*37*), and 7.5 for *Streptomyces lividans* (*35*). The enzyme was stable over a broad pH range from 5.5 to 9.0, retaining about 80−90% activity after 2 h of incubation. (**Figure 4b**).

Temperature Optima and Stability. To determine the temperature optima of ragi acetic acid esterase, activities were determined at a temperature range of 30−80 °C (**Figure 5**). The optimum temperature was found to be 45 °C, and this was comparatively lower than the temperatures reported for microorganisms such as *Bacillus pumilus* (55 °C), *Streptomyces lividans* (70 °C), and *Thermoaerobacterium* species (80 °C) (*35*, *36*, *38*). The purified enzyme was thermally active at 30 °C. The stability of the enzyme decreased gradually, and at 60 °C about 40% of the activity was retained. Less than 5% of the activity was retained at 80 °C (**Figure 5**). Increase in the three-dimensional structure of the enzyme, which is maintained by a number of forces, mainly hydrophobic interactions and hydrogen bonds, will be disrupted which results in the denaturation of the protein and leads to inactivation of the enzyme.

Activation Energy. Activation energy, defined as the minimum energy required by the reactants in order to pass into a transition state, represents the half-way point where the bonds of the substrate are distorted sufficiently so that the conversion to products becomes possible. The activation energy of the reaction was calculated at the optimum pH of the enzyme (7.5), using α-NA as substrate. The energy of activation as calculated from the Arrhenius plot was found to be 7.29 kJ mol^{-1} (**Figure 6**).

Effect of Substrate Concentration. Effect of different substrate concentrations on the initial velocity was calculated, and the kinetic constants K_m and V_{max} were calculated from the double reciprocal plots (LB plot, Lineweaver and Burk, 1934 (**Figure 7**). The Michaeli constant K_m *v*alue of acetic acid esterase from ragi was found to be 0.40 μM for α-NA, and V_{max} was found to be 0.175 μM min^{-1} mL^{-1}. The K_m value of 0.40 μM determined for purified finger millet esterase is lower than the values reported for *B. pumilus* (1.54 mM) (*36*) and *F. succinogenes* (2.7 mM) (*39*), indicating a higher specificity for α-NA. K_m values for two acetyl xylan esterases from *Thermoanaerobacterium* sp. were determined using 4-methyl-umbelliferyl acetate at 0.45 and 0.52 mM, respectively (*38*). A K_m value of 25 mM for barley malt acetic acid esterase was reported using diacetin as the substrate (*16*). No information is available with respect to the detailed kinetics and substrate specificity of cereal/millet acetic acid esterases.

Effect of Metal Ions. A range of metal ions such as Fe^{+3}, Cu^{+2}, Ni^{+2}, Ca^{+2}, Co^{+2}, Ba^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, and Al^{+3} at 5 mM concentration were tested for acetic acid esterase activation/inhibition effects, and the results are given in **Table 2**. Metal ions such as Ni^{+2}, Ca^{+2}, Co^{+2}, and Ba^{+2} reduced the activity by 60−70%, while Mg^{+2}, Mn^{+2}, Zn^{+2}, Al^{+3} reduced the activity by 30−40% (**Table 2**). Similar concentrations of Fe^{+3}, Cu^{+2}, Cu^{+1}, and citric acid enhanced the enzyme activity. Oxalic acid did not show any significant effect on the enzyme activity. The inhibitory effect of Ca^{+2}, Co^{+2}, Ba^{+2}, Mg^{+2}, and Zn^{+2} was in accordance with the results reported for acetic acid esterase from *Schizophyllum commune* (*37*) and *Bacillus pumilus* (*36*) and with ferulic acid esterase from *Aspergillus awamori* (*40*). Fe^{+3} and Cu^{+2} increased the enzyme activity in accordance with a 10% increase in activity of feruloyl esterase of *Pencillium expansum* by Fe^{+3} ions (*41*).

The inhibitory and stimulatory effects of these ions may be important factors in the commercial exploitation of this enzyme where enzyme stability and activity are paramount.

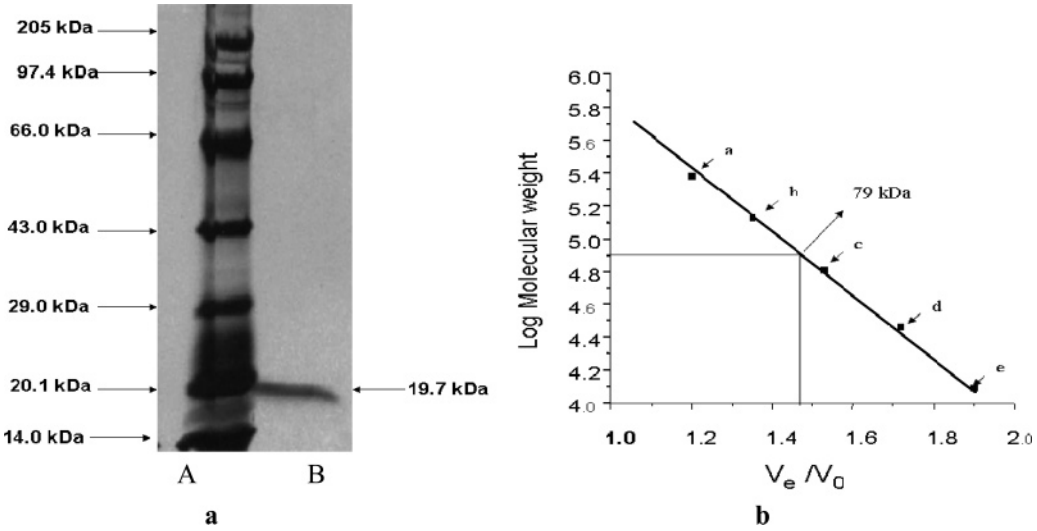

Figure 3. Molecular weight determination of purified ragi acetic acid esterase: (a**)** On SDS-PAGE: (A) Molecular weight markers: 205 kDa myosin; 97.4 kDa phosphorylase; 66.0 kDa bovine serum albumin; 43.0 kDa ovalbumin; 29 kDa carbonic anhydrase; 20.1 kDa soybean trypsin inhibitor; 14.0 kDa lysozyme. (B) Purified acetic acid esterase. (b**)** On Sephacryl S-200: a, β-amylase, 2.00 kDa; b, alcohol dehydrogenase, 1.50 kDa; c, bovine serum album, 66 kDa; d, carbonic anhydrase, 29 kDa; e, papain, 12 kDa. Purified ragi acetic acid esterase, 79.4 kDa.

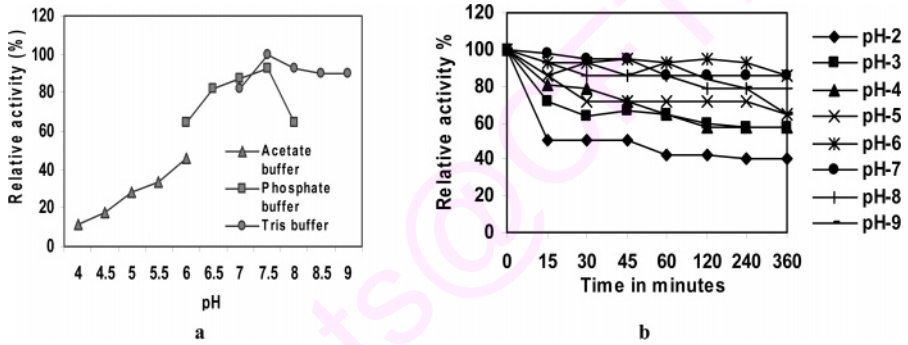

Figure 4. (a) pH optima and (b) pH stability of purified ragi acetic acid esterase.

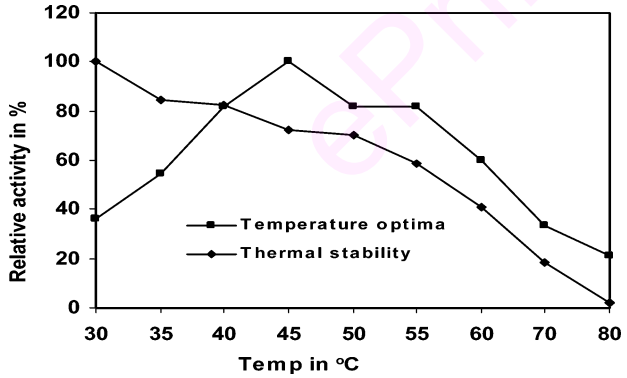

Figure 5. Temperature optima and thermal stability of purified ragi acetic acid esterase.

Effect of Specific Reagents on Acetic Acid Esterase Activity. Acetic acid esterase activity was determined in the chemicals such as PCMB, iodoacetamide, and eserine at 45 °C and 25 mM concentration. The acetic acid esterase was found to be completely inactivated by PCMB as well as eserine whereas iodoacetamide had showed almost negligible effect on the activity (data not shown). The inhibition of PCMB and eserine suggests the possible presence of amino acids such as cysteine or serine residues at the active site pocket. The pH optima (7.5) value of the acetic acid esterase also supports this statement, as the ionization values of these amino acids fall in this range. PCMB is known to form complexes with cysteine residues in the active site region of the enzymes. The effect of eserine on the enzyme kinetics of purified ragi acetic acid esterase showed that it is a competitive inhibitor (**Figure 7**). Eserine is a structural anaolgue of the amino acid serine which is known to be present in the active site of several of the esterases (*42*).

Enzymic Deesterification of Acetylated Substrates. The amount of acetylation achieved is determined by measurement of enzymically released acetic acid from water soluble polysaccharides and synthetic substrates. The specific activities of purified ragi acetic acid esterase using various water soluble polysaccharides and synthetic substrates is as follows: ragi, 0.027 U mg^{-1}; wheat, 4.44 U mg^{-1}; larch wood xylan, 0.061 U mg^{-1}; gum karaya, 0.282 U mg^{-1}; α-NA, 3.38 U mg^{-1}; PNPA, 5.39 U mg^{-1} (U is μM min^{-1}). The specific activity of purified ragi acetic acid esterase with respect to water soluble wheat preparation is higher than the other polysaccharide substrates. Acetic acid esterase from ragi was active both on small molecular weight substrates as well as polysaccharides as indicated by the present study.

IR Analysis of Water Soluble Polysaccharides. The IR spectra of water soluble preparations of polysaccharides such as ragi, wheat, larch wood xylan, and gum karaya showed

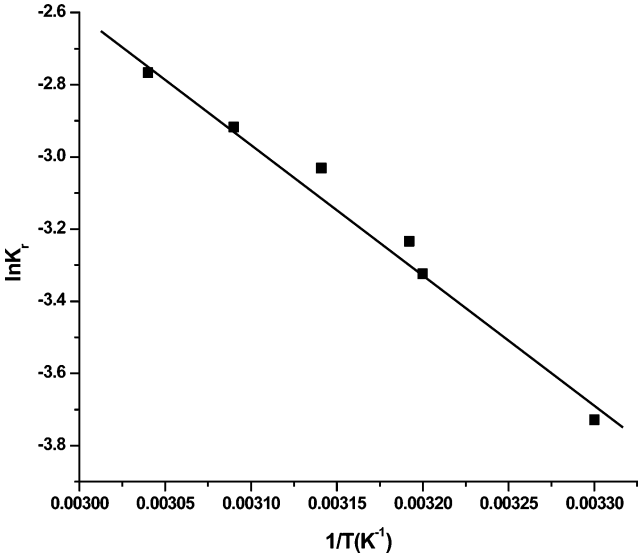

Figure 6. Arrhenius plot for the temperature dependence of acetic acid esterase using α-naphthyl acetate as substrate.

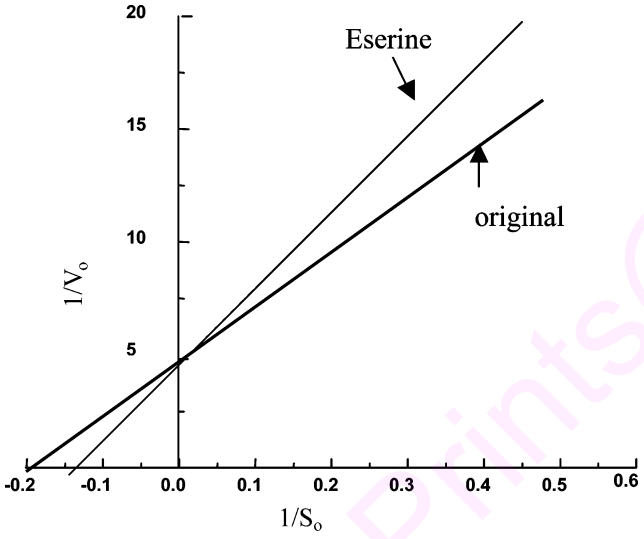

Figure 7. Determination of K_m and V_{max} of ragi acetic acid esterase by Lineweaver–Burk plot with and without inhibitor (substrate used: α-naphthyl acetate).

medium-intensity absorption at 1200 cm^{-1}, indicating the presence of acetyl groups (figure not shown).

Electro Spray Ion-Mass Spectrometry (ESI-MS). Analysis by ESI-MS confirmed the gradual deacetylation of α-NA and PNPA to α-naphthol and PNP, respectively, by purified ragi acetic acid esterase. The formation of PNP by the enzymatic deacetylation of PNPA using ragi acetic acid esterase increased with increase in incubation period (0−8 h). The ratio of signals at *m*/*z* for PNP and PNPA (a. 0.6:1.0, b.1.33:1.0, c. 5.0:1.0, d. 100%) showed the complete deacetylation of PNPA to PNP after 8 h of reaction (figure not shown). The *m*/*z* values of PNPA and PNP are 193.70 and 137.15, respectively. Similar results were observed with α-NA to α-naphthol. The *m*/*z* value of α-naphthol is 142.70 and showed complete deacetylation of α-NA (183.89, *m*/*z* negative mode). The amount of α-naphthol has increased concurrently with increase in the incubation time (figure not shown).

^{1}H NMR. The enzymatic deacetylation of α-NA and PNPA was confirmed by carrying out ^{1}H NMR experiments (figure not shown). The chemical shifts at 8.20−8.22 ppm were assigned to H-3 and H-5 protons, while those at 7.34−7.36 ppm were assigned to H-2 and H-6 protons in the aromatic ring of PNPA. The chemical shift values at 2.235 were assigned to acetyl protons of PNPA (figure not shown). The chemical shift values for these protons were shifted after enzymatic deacetylation of PNPA to PNP (8.022−8053 and 6.83−6.86 ppm). The unreacted PNPA showed a slight chemical shift value at 7.3 and 8.2 ppm for aromatic protons and at 2.2 for acetyl protons (figure not shown). In the case of α-NA the chemical shift values at 7.22−7.91 ppm were assigned for the aromatic protons of naphthol and 2.35 ppm was assigned for the protons of the acetyl group (*43*) (figure not shown). After enzymatic deacetylation, there is a change in chemical shift values in the nucleus of naphthol ring. The complete absence of acetyl protons and the presence of a carbonyl proton at 10.0 ppm indicates the complete deacetylation of α-NA to α-naphthol (figure not shown). The complete hydrolytic cleavage of acetyl group of α-NA and PNPA by ragi acetic acid esterase was found to be quite slow.

Table 2. Effect of Metal Ions, EDTA, and Citric Acid on Purified Ragi Acetic Acid Esterase

metal ions	relative activity (%)
control	100
Cu^{+2}	1130
Fe^{+3}	1000
Cu^{+}	728.2
Hg^{+2}	489.1
EDTA	293.4
citric acid	141.3
Mg^{+2}	82.6
Zn^{+2}	79.3
Mn^{+2}	77.1
Al^{+3}	69.5
Co^{+2}	50.0
Ba^{+2}	44.5
Ni^{+}	36.9
Ca^{+2}	36.5

The practical use of acetic acid esterases particularly from plant sources is limited because of the lack of sufficient knowledge regarding their isolation, purification, and kinetic properties. This particular study is an attempt in that direction for possible exploitation of malt enzymes in modulating the functional properties of cereal nonstarch polysaccharides in various food applications.

Conclusions. Ragi malt of 72 h gave maximum acetic acid esterase activity compared to other malts. The enzyme was purified to apparent homogeneity from 72 h malt by four-step purification with a recovery of 0.36% and a fold purification of 34. It was found to be a homotetramer with a molecular weight of 79.4 kDa. The pH and temperature optima of the enzyme were found to be 7.5 and 45 °C, respectively. The activation energy of the enzymatic reaction was found to be 7.29 KJ mol^{-1}. The apparent K_m and V_{max} of the purified acetic acid esterase were 0.04 μM and 0.175 μM min^{-1} mL^{-1}, respectively, with respect to α-NA. Acetic acid esterase from ragi malt is specific both for natural polysaccharides as well as synthetic substrates. Eserine was found to be a competitive inhibitor. The products liberated from α-NA and PNPA by the action of purified ragi acetic acid esterase were identified by ESI-MS and ^{1}H NMR.

ABBREVIATIONS USED

PVPP, polyvinylpolypyrrolidone; PCMB, *p*-chloromercuric benzoate; α-NA, α-naphthyl acetate; PNPA, *p*-nitrophenyl

acetate; PNP, *p*-nitrophenol; EDTA, ethylenediamine tetraacetate; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; MS, mass spectroscopy; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin.

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Supporting Information Available: Elution profile of ragi acetic acid esterase(III), MS and NMR spectra, and ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Purification and Characterization of Ferulic Acid Esterase from Malted Finger Millet (*Eleusine coracana*, Indaf-15)

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Ferulic acid esterase (EC 3.1.1.73) cleaves the feruloyl groups substituted at the 5′-OH group of arabinosyl residues of arabinoxylans and is known to modulate their functional properties. In this study, ferulic acid esterase from 96 h finger millet malt was purified to apparent homogeneity by three-step purification with a recovery of 3% and a fold purification of 22. The substrate *p*-nitrophenylferulate (PNPF) was synthesized and used to assay this enzyme spectrophotometrically. The products liberated from ragi and wheat water-soluble polysaccharides by the action of purified ragi ferulic acid esterase were identified by ESI-MS. The pH and temperature optima of the enzyme were found to be 6.0 and 45 °C, respectively. The pH and temperature stabilities of the enzyme were found to be in the range of 5.5–9.0 and 30 °C, respectively. The activation energy of the enzymatic reaction was found to be 4.08 kJ mol^{-1}. The apparent K_m and V_{max} of the purified ferulic acid esterase for PNPF were 0.053 μM and 0.085 unit mL^{-1}, respectively. The enzyme is a monomer with a molecular mass of 16.5 kDa. Metal ions such as Ni^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} and oxalic and citric acids enhanced the enzyme activity. The enzyme was completely inhibited by Fe^{3+}. Group specific reagents such as *p*-chloromercuric benzoate and iodoacetamide inhibited the enzyme, indicating the possible presence of cysteine residues in the active site pocket.

KEYWORDS: Ferulic acid esterase; ragi; finger millet; purification; homogeneity

INTRODUCTION

The cereal plant cell walls are a complex mixture of polysaccharides consisting of arabinoxylans, (1→3),(1→4)-β-D-glucans, and glucomannans in addition to large amounts of lignocellulosic complexes. Of these polysaccharides, arabinoxylans have received an increased level of attention due to their importance in foam stabilization, gelling, and bread making, due to their high viscosity and water holding capacity. Cereal arabinoxylan is a β-1,4-linked D-xylan polymer with arabinose residues present in the side chain covalently linked at either O-2 or O-3 of xylose residues of the backbone. These arabinose residues are further substituted with cinnamic acid derivatives such as ferulic and coumaric acids in an ester linkage (*1, 2*). These cinnamic acid derivatives are believed to influence the solution properties of arabinoxylans; however, it needs to be documented beyond ambiguity. Solution properties of arabinoxylans have to be addressed with and without ferulic acid, which requires enzymatic deesterification of cinnamic acid derivatives by using cinnamoyl esterases. Feruloyl esterases (FAEs, also known as ferulic acid esterases, cinnamoyl esterases, and cinnamic acid hydrolases; EC 3.1.1.73) are a subclass of the carboxylic acid esterases (EC 3.1.1.1) (*3*). Of several cinnamoyl esterases that have been reported, FAE are widely studied from microbial sources such as *Aspergillus niger* (*4*), *Aspergillus awamori* (*5, 6*), *Neocallimastix patriciarum* (*7*), *Fusarium* sp. (*8*), and bacteria like *Streptomyces olivochromogenes* (*9*), *Schizophyllum commune* (*10*), *Fibrobacter succinogenes* (*11*), *Butyrivibrio fibrisolvens* (*12*), and *Ruminococcus* sp., *Cellvibrio japonicus* (*Pseudomonas fluorescens*) (*13*). Cell wall solubilase activity of FAE was documented by the increased yields of β-glucans and pentosans isolated from the barley malt extract (*14*). These enzymes exhibit specificity depending on the linkage between the ferulic acid and the primary sugar (*4, 15*). Hence, there is considerable reason to utilize FAEs from cereals and their malts to modulate the functional properties of feruloyl arabinoxylans.

FAEs are assayed by using synthetic substrates such as methyl ferulate (*16*), ethyl ferulate (*11*), and feruloyl glycerol (*17*), wherein the liberated products are quantified by HPLC, which require expensive equipment, and not convenient for rapid analysis of a large number of samples from time to time. To circumvent this, a routine spectrophotometric assay is warranted which is quite handy in the isolation, purification, and characterization steps. A spectrophotometric substrate *p*-nitrophenyl ferulate (PNPF) was synthesized (*18*); the assay procedure was

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developed following the published literature (*19*) with a slight modification, and the synthesized substrate's structure was confirmed by both ^{1}H and ^{13}C NMR. This substrate models the major ferulic acid linkage to polysaccharides and provides a material with well-defined chemical properties.

Finger millet (*Eleusine coracana*), also known as ragi, is an indigenous minor millet, rich in calcium and dietary fiber. It is extensively consumed in south India and is used in both native and processed (malted) forms (*20*). Studies were reported from our group regarding the (a) isolation, purification, and characterization of ragi amylases (*21*), (b) structure and functional relationship of alkali-soluble arabinoxylans (*22*), (c) water extractable feruloyl polysaccharides and their antioxidant properties (*23*), and (d) isolation, purification, and partial characterization of ragi acetic acid esterase (*24*). However, to date, the effect of feruloyl groups on the functionality of cereal nonstarch polysaccharides attracted very little attention. Hence, this study was undertaken to isolate, purify, and characterize finger millet malt FAE with respect to its kinetic properties to delineate the role of ferulic acid moieties on the functionality of millet/cereal arabinoxylans in general as a long-term objective. In this work, we report the isolation, purification, and determination of the kinetic properties of ragi FAE by using synthesized artificial substrate, i.e., PNPF.

MATERIALS AND METHODS

Materials. An authenticated variety of finger millet (*E. coracana*, ragi, Indaf-15) was procured from V.C. farm of the University of Agricultural Sciences, Bangalore, located in Mandya, Karnataka, India. All chemicals were purchased from Sisco Research Laboratories (Mumbai, India) or Sigma Chemical Co. (St. Louis, MO). Protein molecular mass markers for SDS (sodium dodecyl sulfate) were obtained from Genei (Bangalore, India). DEAE-cellulose, Biogel P-30 was obtained from Pharmacia fine chemicals (Uppsala, Sweden). Precoated silica gel plates (0.25 mm layer thick) with an UV_{254} indicator were from Merck (Darmstadt, Germany).

Malting. Malting was conducted as reported previously (*25*). Ragi seeds (50 g) were cleaned and steeped for 16 h and germinated under controlled conditions on moist cloth at 25 °C in a BOD incubator up to 96 h. Germinated seeds were taken and dried at 50 °C in an air oven for 12 h, and vegetative growth portions were removed (devegetated) by gentle manual brushing. Devegetated seeds were weighed, powdered, and used for the extraction of FAE.

Enzyme Extraction. Malted ragi flour (96 h, 50 g) was extracted with 50 mM Tris-HCl buffer (1:7, pH 7.0, 350 mL) containing 25 mM reduced glutathione, 1% Triton X-100 (w/v), and 1% PVPP for 2 h at 4 °C, and supernatant was collected by centrifugation (7000*g* at 4 °C for 20 min) using a refrigerated centrifuge, dialyzed against the extraction buffer, and used for further experiments.

Protein Determination. The presence of protein was monitored in the column fractions by measuring absorbance at 280 nm and quantified by the Bradford method at 590 nm using BSA as a standard (*26*).

Substrate Preparation. PNPF was prepared according to the chemoenzymatic procedure described by Mastihubova et al. (*18*) with slight modification. The synthesis of PNPF involved a four-step reaction.

Acetylation. In the first step, acetylation of ferulic acid was carried out to prevent side reactions of the 4-hydroxy moiety (*27*). Ferulic acid (Sigma, 3 g) was acetylated with acetic anhydride (4.8 mL) in pyridine (5.25 mL). The reaction was monitored by TLC (using 5% methanol in chloroform) for completion (4 h), and the reaction was quenched with 95% ethyl alcohol to obtain crystals of 4-acetoxyferulic acid (3.6 g, mp 201–204 °C).

Chlorination. 4-Acetoxyferulate (3.2 g) was dissolved in benzene (62 mL); to this was slowly added thionyl chloride (6.2 mL, 68.5 mM), and the mixture was refluxed in an oil bath (95 °C) for 2 h. TLC was conducted (100% chloroform) to check the formation of 4-acetoxyferuloyl chloride. The solution was washed two or three times with toluene, to obtain small white plates of 4-acetoxyferuloyl chloride (2.8 g, mp 184–188 °C), which were stored in a vacuum desiccator over P_2O_5.

Coupling Reaction (Esterification). Coupling of 4-acetoxyferuloyl chloride with *p*-nitrophenol (PNP) was carried out using 4-(dimethylamino)pyridine (DMAP) (*28*). For this, 4-acetoxyferuloyl chloride (2.4 g) and PNP (2.1 g, 1.5 equiv) were dissolved in dry CH_2Cl_2 (111.4 mL) to which DMAP (295 mg, 0.25 equiv) and triethylamine (Et_3N) (1.16 mL, 0.85 equiv) had been added. The reaction mixture was stirred until the completion, for ~2 h as indicated by TLC, followed by flash chromatography on a silica gel column using $CHCl_3$ as the eluent, to remove traces of unreacted PNP, ferulic acid, and 4-acetoxyferuloyl chloride. The esterified 4-acetoxyferulate (*p*-nitrophenyl 4-acetoxyferulate) was eluted in the first quarter of the bed volume, which was evaporated, and preparative TLC (using chloroform as the eluent) was carried out to obtain the product in 99% purity.

*Selective Deacetylation (**18**).* In this step, the solvents CH_2Cl_2 and 2-propanol were predried over 3 Å molecular sieves. The esterified 4-acetoxyferulate (100 mg) was dissolved in CH_2Cl_2 (8 mL) and 2-propanol (4 mL), and lipase PS (465 mg) was added to the solution. The reaction mixture was incubated on a shaker (250 rpm) for 3 days at 40 °C or until the disappearance of the starting material. TLC was checked at every 6 h intervals for the selective deacetylation of esterified 4-acetoxyferulate to PNPF. The reaction was terminated by filtration of the enzyme, and the filtrate was concentrated by evaporation of the solvents. The product of each reaction was characterized by ^{1}H and ^{13}C NMR.

Enzyme Assay. *Spectrophotometric Assay Using PNPF (standard method used).* A stock solution of the substrate PNPF was prepared as described by Mastihuba et al. (*19*). Two milligrams of PNPF was dissolved in DMSO (50 μL) and Triton X-100 (50 μL), and the volume was made with Tris buffer (5 mL, 50 mM, pH 7.0). The reaction mixture containing FAE and PNPF [made to a final assay volume of 1.0 mL with 50 mM Tris-HCl buffer (pH 7.0)] was incubated at 37 °C for 60 min. Suitable enzyme and substrate blanks were maintained, as the substrate used is highly unstable. One unit of FAE activity is defined as the amount of enzyme required to liberate 1 μmol of PNP/h. The reaction was monitored spectrophotometrically at 400 nm for the release of PNP from PNPF, making use of the PNP standard graph.

HPLC Assay. One volume of ethyl ferulate [0.01 M ethyl ferulate in 0.5 mL of 50 mM Tris buffer (pH 7.0)] and 3 volumes of enzyme solution were incubated at 37 °C for 1 h. A 1 volume aliquot of the reaction mixture was withdrawn and mixed with 3 volumes of methanol to stop the reaction (*19*). The reaction mixture was mixed by vortexing and centrifuged at 7000*g* for 5 min, and the supernatant was separated from the residue. The released ferulic acid was monitored by HPLC analysis both at λ_{280} and at λ_{320} using the ferulic acid standard curve (0.01–0.1 mg).

Enzyme Purification. Crude enzyme preparation of FAE from 96 h malted ragi was subjected to ammonium sulfate precipitation, which resulted in four fractions, i.e., 0–20, 20–40, 40–60, and 60–80% fractions. Ammonium sulfate fractions of 40–60 and 60–80% were pooled (60% activity) and loaded onto a DEAE-cellulose column (2.25 cm × 25 cm), pre-equilibrated with Tris-HCl buffer (5 × 56 mL, 20 mM, pH 9.0) at a flow rate of 24 mL/h and washed with the same buffer to remove unbound proteins. A linear NaCl gradient (from 0 to 0.7 M) in equilibrating buffer was used to elute the bound proteins, which were collected (4.0 mL each) and monitored for protein (280 nm) as well as FAE activity. The active fractions from anion exchange chromatography were concentrated and loaded onto a Biogel P-30 column (0.7 cm × 100 cm) which was pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing NaCl (10 mM) and EDTA (1 mM), and fractions (2.0 mL) were collected and monitored for protein and FAE activity. The pooled and dialyzed active fractions obtained from Biogel P-30 were concentrated and used for further characterization after the homogeneity had been determined by native and SDS–PAGE.

Table 1. Chemical Shifts (δ) of Proton and Carbon Resonances of *p*-Nitrophenyl Ferulate (PNPF)

	chemical shifts
^{1}H NMR ($CDCl_3$)	3.96 (3H, OCH_3), 6.46 (H-A, d, J = 16 Hz), 6.97 (H-5, d, J = 8.2 Hz), 7.10 (H-2, d, J = 1.6 Hz), 7.16 (H-6, dd, J = 1.6 and 8.2 Hz), 7.38 (2H, H-2′,6′, d, J = 9 Hz), 7.84 (H-B, d, J = 16 Hz), 8.32 (2H, H-3′,5′, d, J = 9 Hz), 6.00 (1H, OH)
^{13}C NMR	56.08 (OCH_3), 109.90 (C-2), 113.39 (C-A), 115.66 (C-5), 122.49 (C-2′,6′), 123.80 (C-6), 125.24 (C-3′,5′), 126.20 (C-1), 146.58 (C-4′), 147.29 (C-3), 148.23 (C-B), 148.92 (C-4), 161.81 (C-1′), 164.90 (COO)

Purity Criteria. *Polyacrylamide Gel Electrophoresis (PAGE).* PAGE (12.5%) under native and denaturing conditions was carried out to evaluate the purity of FAE. The gel was taken for silver staining (*29*).

Determination of the FAE Activity by Diffusion of the Enzyme into an Agarose Gel. As the purified FAE was inactivated by running on PAGE, it was loaded in the agarose wells and the activity checked by diffusion. For detection of FAE activity, MUTMAC (5 mM) was incorporated in Tris buffer (50 mM, pH 7.0) into a 1 mm thick, 1.5% agarose gel cast on FMC gel-bonded agarose support film. Twenty micrograms of purified FAE was loaded into the wells after solidification. The gel was flooded with Tris buffer and illuminated with long-wavelength UV. FAE activity and the rate of diffusion of the enzyme were visible as light blue fluorescence against an intense green fluorescent background.

Estimation of Molecular Weight (M_r). *By Gel Permeation Chromatography (GPC).* This was performed on a column (0.7 cm × 100 cm) of Biogel P-30 calibrated by using standard protein markers such as aprotinin (7 kDa), lysozyme (12 kDa), and carbonic anhydrase (29 kDa). The molecular mass was calculated from a plot of V_e/V_o against the log molecular weight.

By SDS–PAGE. M_r values were estimated by SDS–PAGE (*30*) using a 12% (w/v) acrylamide gel. Proteins were detected by silver staining. M_r values were estimated from a plot of log M_r versus mobility using the following protein standards: lysozyme (14.3 kDa), carbonic anhydrase (29 kDa), egg albumin (43 kDa), BSA (66 kDa), and phosphorylase (97.4 kDa).

By ESI-MS. The molecular mass of purified ragi FAE was determined by ESI-MS. Purified ragi FAE (~5 μg) was placed in a 50% methanolic solution and subjected (10 μL) to ESI-MS with an Alliance Waters 2695 mass spectrometer using positive mode electrospray ionization.

Effect of pH. FAE activity was determined at various pH values using different buffers such as sodium acetate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 7.0–9.0) at a concentration of 50 mM. The maximum activity was taken as 100% and the relative activity plotted against different pH values.

pH Stability. The stability of purified FAE was carried out by preincubating the enzyme in different buffers such as glycine-HCl (pH 2.0–3.0), sodium acetate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 7.0–9.0), followed by determination of the residual activities at different time intervals (15 min, 30 min, 45 min, 1 h, 2 h, 3 h, and 4 h). The original activity was taken to be 100%, and relative activity was plotted against different time intervals.

Temperature Optima. Freshly purified enzyme (0.1 mL) was incubated with PNPF (1.0 mM) in sodium acetate buffer (pH 6.0, 50 mM) in a temperature range of 30–70 °C (with an interval of 5 °C) using a thermostatically controlled incubator. The optimum activity was taken to be 100%, and relative activities were plotted against different temperatures.

Thermal Stability. Purified FAE was preincubated in a temperature range of 30–70 °C for 15 min. The residual activity was estimated taking the original activity as the control (100%), and relative activity was plotted against different temperatures.

Measurement of Activation Energy. To determine the temperature dependence of FAE activity, reaction rates and/or activities at a series of temperatures (30–60 °C) were determined. An Arrhenius plot was drawn taking the natural log of activity on the y-axis and $1/T$ in kelvin on the x-axis. The activation energy was determined from the slope of the plot using the Arrhenius equation

$$\text{slope} = -E_a/R$$

where R is the universal gas constant (8.314 J mol^{-1}).

Effect of Substrate Concentration. Different concentrations of PNPF (2.5–12.5 μg) in sodium acetate buffer (pH 6.0, 50 mM) were incubated with purified FAE for 1 h at 45 °C, and activities were measured every 15 min. Initial velocities (V_o) were calculated for all substrate concentrations, and the K_m and V_{max} values were calculated from a double-reciprocal plot (*31*).

Effect of Metal Ions. Purified FAE was incubated with a 5 mM solution of citric acid, oxalic acid, EDTA, and salts of metal ions (chlorides of Fe^{3+}, Cu^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+},

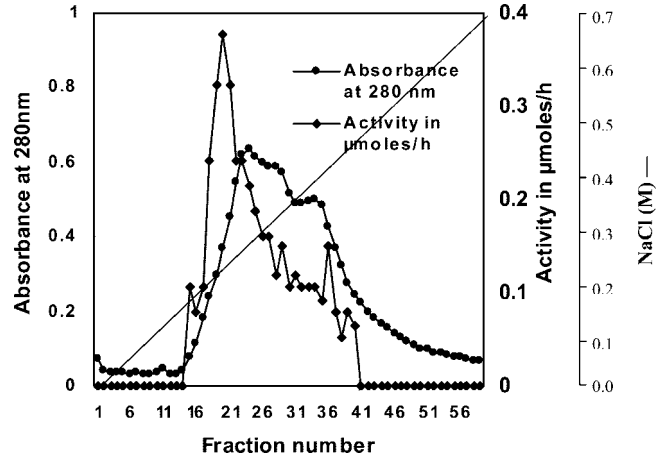


Figure 1. Elution profile of ragi FAE (40–80% ammonium sulfate fraction) on a DEAE-cellulose column.

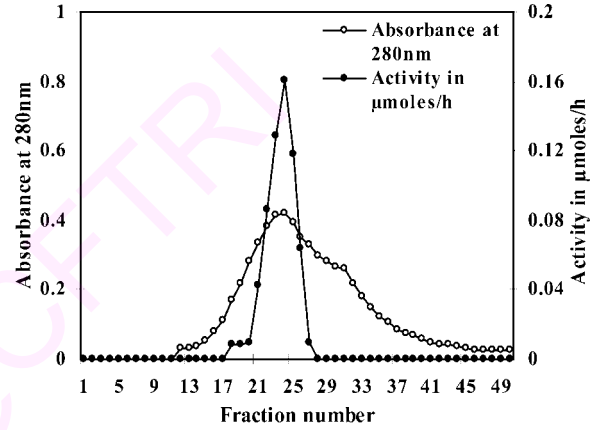
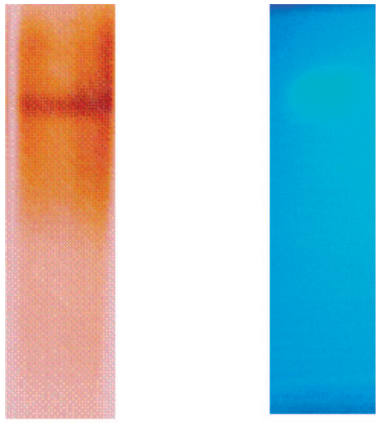


Figure 2. Elution profile of ragi FAE (DEAE-cellulose fraction) on a Biogel P-30 column.

Table 2. Summary of the Purification of FAE from Ragi Malt

step	total activity[b]	total protein[c]	specific activity[d]	fold purification	% recovery
crude[a]	107.5	88	1.22	1	100
40–80% $(NH_4)_2So_4$ fraction	64.40	4.2	15.3	12.5	59.9
DEAE-cellulose	12.56	0.6	20.9	17.13	11.68
Biogel P-30	3.22	0.12	26.8	21.9	2.99

[a] On a 50 g scale (values are averagea of three independent experiments). [b] One unit is equivalent to 1 μmol of PNP released/h. [c] Total protein is expressed in milligrams. [d] Specific activity is expressed in 1 μmol of PNP released h^{-1} (mg of protein)$^{-1}$.



a b

Figure 3. PAGE of purified ragi FAE: (**a**) protein staining and (**b**) activity staining.

Zn^{2+}, Al^{3+}, etc.) at 45 °C for 15 min, and residual activities were measured. The enzyme activities without respective compounds were taken as the control (100%), and relative activities were calculated.

Effect of Group Specific Reagents. Purified FAE was incubated with PCMB, iodoacetamide, and eserine (10–50 mM) in sodium acetate buffer (pH 6.0, 50 mM) at 45 °C for 15 min, and the residual activities were estimated. The enzyme activities without these chemicals were taken to be 100%, and relative activities were calculated.

Substrate Specificity. Water-soluble portions of water extractable polysaccharides (0.5%, 1 mL) isolated from ragi, wheat, and maize were placed in sodium acetate buffer (pH 6.0, 50 mM) and incubated with purified ragi FAE (0.1 mL). The reaction was allowed to proceed at 45 °C for 1 h and was stopped by boiling for 10 min, and the mixture was centrifuged at 15000*g* for 30 min to separate the supernatant from the residue. The supernatant was further phase separated with diethyl ether, and the ether layer was evaporated. The sample, after evaporation of ether, was taken in a minimum volume of methanol, which was further analyzed by HPLC for ferulic acid using a C_{18} column at room temperature at both λ_{280} and λ_{320} using the ferulic acid standard curve and a methanol/water/acetic acid mixture (7:2.5:0.5) as the eluent with a flow rate of 1.0 mL/min. The retention time for ferulate under these conditions was 17.8 min. The specific activities of purified ragi FAE using various water-soluble polysaccharide preparations were compared with those of low-molecular mass synthetic substrates such as PNPF and ethyl ferulate (~1 mM).

ESI-MS Analysis. Water-soluble portions of water extractable polysaccharides (0.5%, 1 mL) isolated from ragi, wheat, and maize were placed in sodium acetate buffer (pH 6.0, 50 mM) and incubated with purified ragi FAE (0.1 mL) at 45 °C for different time intervals (0–10 h). The reaction was stopped with methanol (0.3 mL) and the mixture centrifuged. The methanolic layer, which consists of ferulic acid, was subjected to ESI-MS with an Alliance Waters 2695 mass spectrometer using negative mode electrospray ionization. The capillary voltage was 3.5 kV, the core voltage 100 V, the source temperature 80 °C, the dissolvation temperature 150 °C, the core gas (argon) 35 L/h, and the dissolvation gas (nitrogen) 500 L/h.

RESULTS AND DISCUSSION

Extraction of Ferulic Acid Esterase. The 96 h malted ragi was found to be the best (0.480 unit/g of malt) compared to 24 (0.094 unit/g of malt), 48 h (0.25 unit/g of malt), and 72 h (0.15 unit/g of malt) ragi malts with respect to FAE activity. Enzyme activity was relatively higher in Tris-HCl buffer at pH 7.0 (0.4 unit/g of malt) than at pH 7.5 (0.3 unit/g of malt) and pH 8.0 (0.25 unit/g of malt). Various substances such as PVPP, reduced glutathione, and Triton X-100 were added to the extracting buffer to enhance the yield (*32*). PVPP helps in the absorption of phenolic compounds and acts as a stabilizer of many enzymes, thereby enhancing the enzyme recovery and activity (*33*). GSH is the smallest intracellular thiol (SH) molecule, which maintains cellular redox potential. It is the essential cofactor for many enzymes, which require thiol protection, and it helps to keep the active sites of the enzymes intact (*34*). FAE yield is increased by the exogenous addition of nonionic detergent Triton X-100 to the extraction medium, thereby confirming its hydrophobic association with the membrane (*17*).

Substrate Preparation. The synthesis of PNPF was carried according to the method cited by Mastihubova et al. (*18*) (figure not shown). The phenolic hydroxyl of ferulic acid was protected by acetylation prior to the formation of acid chloride to prevent polymerization. The selective deacetylation was a crucial step during the synthesis process, which was carried out using the commercial preparation of lipase obtained from *Burkholderia cepacia*. This enzyme does not possess feruloyl esterase activity and thus was used for effective deacetylation. The complete assignments of chemical shifts are given in **Table 1**. These assignments conform well to the literature values for PNPF (*18*).

Enzyme Assay. The assay conditions were optimized by examining the effect of PNPF or ethyl ferulate concentration on the rate of release of PNP or ferulic acid, respectively (*19*), using ragi FAE. Both substrates are not completely soluble in Tris buffer (50 mM, pH 7.0) but became completely soluble by prior addition of 50 μL each of DMSO and Triton X-100 (PNPF), while only methanol was used for ethyl ferulate. The emulsion systems of these substrates were stable only up to 4–5 h and, hence, were always prepared afresh for the enzyme assays.

Purification of Ferulic Acid Esterases. Crude Tris buffer extract (containing 1% PVPP, 1% Triton X-100, and 25 mM reduced glutathione) from 96 h ragi malt was subjected to ammonium sulfate precipitation and separated into four fractions (0–20, 20–40, 40–60, and 60–80%). The studies on barley malt extract showed FAE activity associated with the 40–60% ammonium sulfate fraction using methyl ferulate substrate (*35*). In this study, ~60% of the activity was present in the 40–80% ammonium sulfate fraction and was further taken for fractionation on a DEAE-cellulose column (**Figure 1**), which was successful in decreasing the viscosity as well as removal of the colored material, and large amounts of unbound contaminating proteins. The bound proteins were eluted with a linear gradient of NaCl (from 0 to 0.7 M) which yielded a major peak at a NaCl concentration of 0.23 M with a recovery of 11.7% which was further purified on a Biogel P-30 column (**Figure 2**) with a fold purification and recovery of 22 and 3.0%, respectively. Recovery of the enzyme was decreased after DEAE-cellulose column separation chromatography, which might be due to (a) enzyme inactivation or (b) removal of a synergistically acting enzyme, which might be associated with the FAE thereby stabilizing it. During gel filtration chromatography, NaCl (10 mM) and EDTA (1 mM) were added to the elution buffer to retain the activity of the enzyme. The overall scheme employed in the purification of FAE from ragi malt is summarized in **Table 2**.

Criteria of Purity. The purity of FAE was confirmed by SDS (**Figure 4a**) and native PAGE (**Figure 3a**). SDS–PAGE analysis of the purified ragi FAE gave a single band with an estimated molecular mass of 16.5 kDa (**Figure 4a**). An identical molecular mass was obtained by GPC (**Figure 4b**) and ESI-

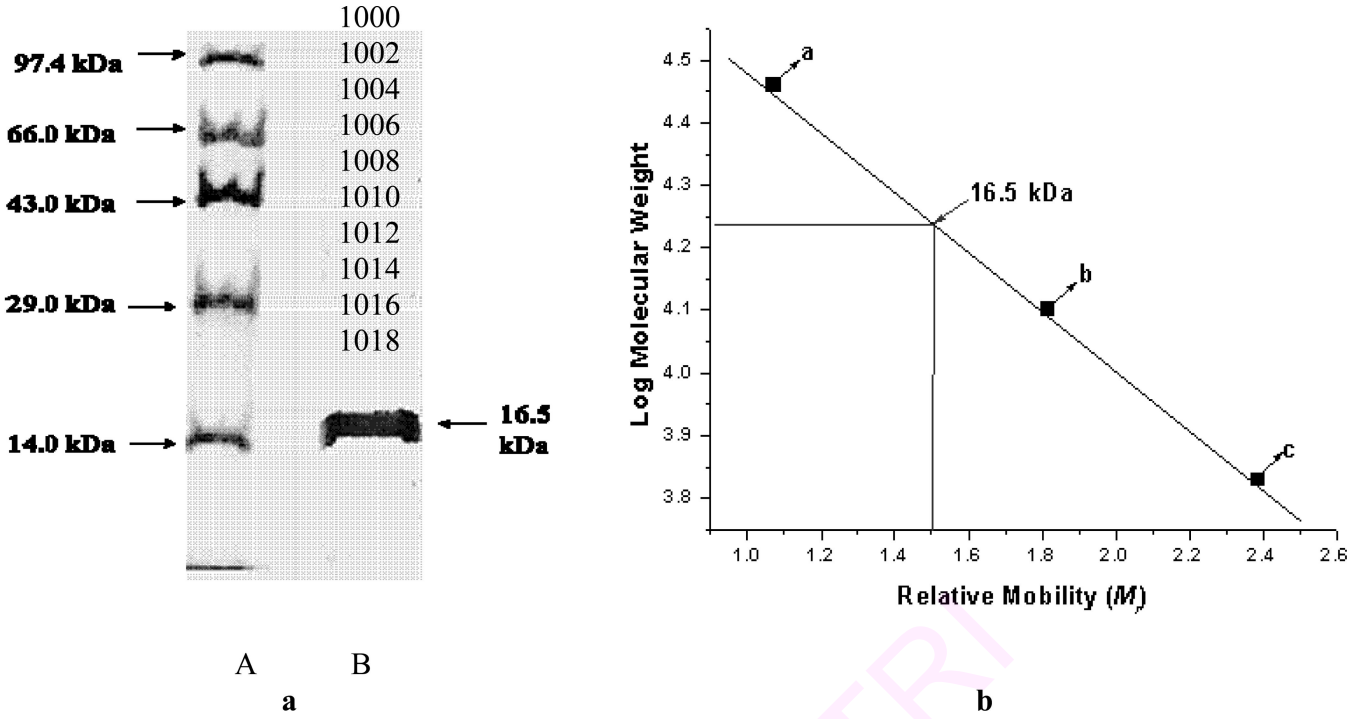


Figure 4. Molecular mass determination of purified ragi FAE (**a**) via SDS–PAGE and (**b**) on a Biogel P-30 column under nondenaturing conditions.

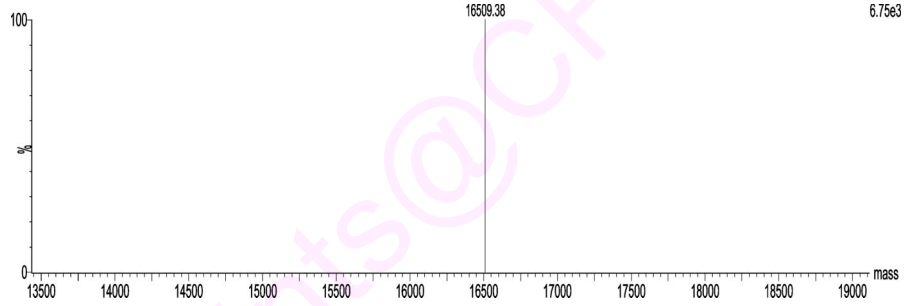


Figure 5. Molecular mass of purified ragi FAE by ESI-MS (positive mode).

MS (**Figure 5**), indicating the monomeric nature of the purified FAE. The apparent molecular mass, determined under denaturing conditions, is compared with the ones reported from the feruloyl esterase isolated from *Clostridium stercorarium* (33 kDa) (*36*), *A. awamori* (35 kDa) (*6*), *Penicillium pinophilum* (57 kDa) (*37*), *A. awamori* (75 kDa) (*5*), *Aureobasidium pullulans* (210 kDa) (*38*), and FAE from barley malt (138 kDa) (*39*). Microbial feruloyl esterase has exhibited varied molecular masses, ranging from 24 kDa (FAE-II from *Neocallimastix* strain MC-2) (*40*) to 132 kDa (FAE-I from *A. niger*) (*41*) and 210 kDa (*Aureobasidium pullulans*) (*38*). Three feruloyl esterases from *A. niger* have been characterized, and their estimated molecular masses are 132 kDa (FAE-I), 29 kDa (FAE-II), and 36 kDa (FAE-III) (*41, 42*).

Determination of the FAE Activity by Diffusion of Enzyme into an Agarose Gel. The activity of the purified enzyme was determined by using fluorescent substrate MUTMAC (**Figure 3b**). As the enzyme was inactivated immediately after being subjected to PAGE, the purified enzyme was loaded directly into the wells of agarose incorporated with substrate (MUTMAC) to check the activity. Green fluorescence was observed against a blue background indicated the cleavage of MUTMAC to fluorescent methylumbelliferone.

pH Optima and Stability. FAE was found to have a pH optimum of 6.0 and retained 55–60% of its activity between

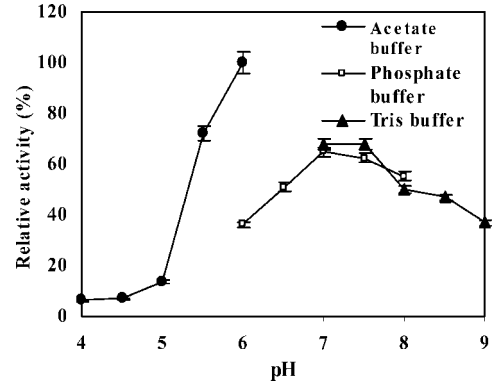


Figure 6. pH optima of purified ragi FAE.

pH 7.0 and 8.0 (**Figure 6**). Most of the activity was retained in acetate buffer, indicating better stability of FAE in this buffer. The enzyme activity has decreased drastically in the pH range of 4.0–5.0 in acetate buffer, indicating its labile nature at highly acidic pH. The pH optimum of the enzyme (6.0) is similar to the pH values reported for purified FAEs from *Streptomyces avermitilis* CECT 3339 (*43*), *Au. pullulans* (*38*), *Neocallimastix* strain MC-2 (*44*), *A. niger* (*42*), *A. awamori* (*5*), *Penicillium expansum* (*45*), *P. pinophilum* (*37*), and *S. olivochromogenes* (*9*), whereas it was lower compared

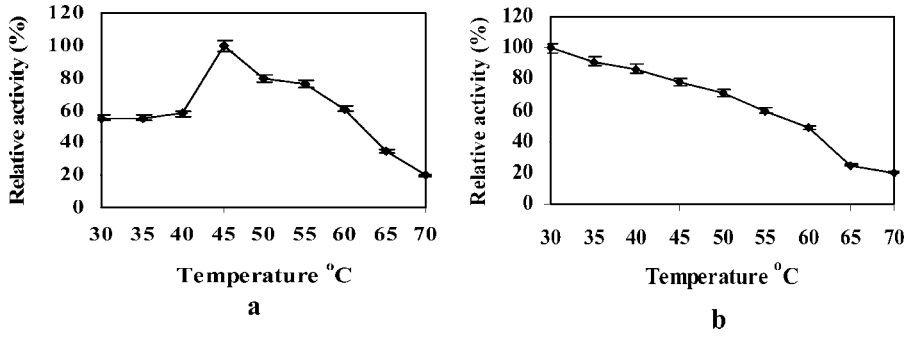


Figure 7. Temperature optima and thermal stability of purified ragi FAE.

to the values reported for *C. stercorarium* (*36*). Purified ragi FAE exhibited 100% stability in acetate buffer (pH 6.0, 50 mM). The enzyme was stable over a broad pH range from 5.5 to 9.0, retaining 80–90% activity after incubation for 2 h (figure not shown).

Temperature Optima and Thermal Stability. The temperature optimum of ragi FAE was found to be 45 °C (**Figure 7a**), similar to the one reported for *A. awamori* (*6*), and was comparatively lower than the ones reported for microorganisms such as *Au. pullulans* (60 °C) (*38*), *C. stercorarium* (65 °C) (*36*), *P. pinophilum* (55 °C) (*37*), *S. avermitilis* CECT 3339 (50 °C) (*43*), and *A. niger* (50 °C) (*46*) but higher than those reported from microorganisms such as *S. olivochromogenes* (30 °C) (*9*) and *P. expansum* (37 °C) (*45*). The purified enzyme was thermally stable at 30 °C, and its activity gradually decreased with the increase in temperature and lost 85% of its activity at 70 °C (**Figure 7b**). As the temperature increases, the three-dimensional structure of the enzyme which is maintained by a number of forces such as hydrophobic and hydrogen bonds will be disrupted, resulting in the denaturation of the protein and in turn inactivation of the enzyme.

Activation Energy. The activation energy of the reaction was calculated at the optimum pH of the enzyme (6.0), using PNPF as the substrate. The activation energy calculated from the Arrhenius plot was found to be 4.080 kJ mol^{-1} (figure not shown). Activation energy, defined as the minimum energy required by the reactants to pass into a transition state, represents the halfway point where the bonds of substrate are distorted sufficiently so that conversion to products becomes possible.

Effect of Substrate Concentration. The effect of different substrate concentrations on the initial velocity was calculated, and the kinetic constants, K_m and V_{max}, were calculated from the double-reciprocal plots (LB plot) (*31*) (**Figure 8**). The K_m value of FAE from ragi was found to be 0.053 μM for PNPF, and the V_{max} was found to be 0.085 unit mL^{-1}. The K_m value reported in this study is lower than the ones reported for *Au. pullulans* (50.2 μM) (*38*), *C. stercorarium* (40 μM) (*36*), and *P. pinophilum* (130 μM) (*37*) using methyl ferulate as the substrate. A K_m value of 0.46% for barley malt FAE was reported using feruloyl glycerol as the substrate (*39*). This indicates the higher specificity of ragi FAE for PNPF. No information is available with respect to the detailed kinetics and substrate specificity of cereal/millet FAEs.

Effect of Metal Ions. A range of metal ions such as Fe^{3+}, Cu^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Al^{3+} at 5 mM were tested for the FAE activation–inhibition effect, and the results are given in **Table 3**. Metal ions such as Mg^{2+}, Ca^{2+}, and Ba^{2+} and EDTA showed no visible effect on the purified FAE. Similar concentrations of Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{+}, Cu^{2+}, and oxalic and citric acid resulted in activation of the enzyme. The activity of purified ragi FAE was completely inhibited by 5 mM Fe^{3+}. The inhibitory effect of Fe^{3+} and the role of EDTA (no significant inhibition and activation) during the purification by GPC and its effect on the activity of FAE were in accordance with the results reported for feruloyl esterase from *C. stercorarium* (*36*). The inhibition of FAE is consistent with the phenolic acid esterases of *Neocallimastix* sp. (*40, 44*), *A. awamori* (*5*), *P. pinophilum* (*47*), and *P. expansum* (*45*). The inhibitory and stimulatory effects of these ions may be important factors in the commercial exploitation of this enzyme where enzyme stability and activity are paramount.

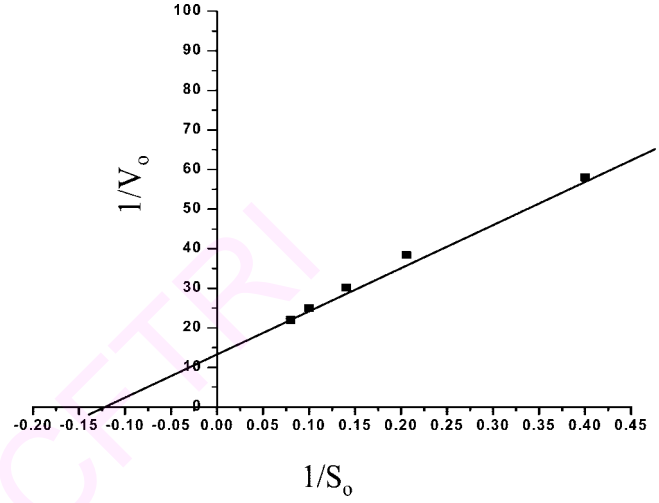


Figure 8. Determination of K_m and V_{max} values of ragi FAE with a Lineweaver–Burk plot (PNPF used as the substrate).

Table 3. Effect of Metal Ions, EDTA, and Citric Acid on Purified Ragi FAE

metal ion	relative activity (%)
control	100
Cu^{2+}	217
Fe^{3+}	0
Cu^{+}	199
EDTA	100
citric acid	119
oxalic acid	141
Co^{2+}	140
Mg^{2+}	100
Zn^{2+}	140
Ba^{2+}	100
Ni^{+}	140
Ca^{2+}	100

Effect of Specific Reagents on FAE Activity. FAE activity was determined in the presence of chemicals such as PCMB,

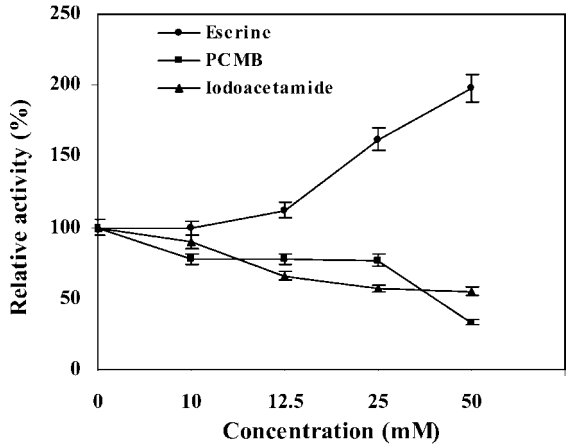


Figure 9. Effect of group specific reagents on the activity of purified ragi FAE.

Table 4. Substrate Specificity of Purified Ragi FAE

substrate	specific activity (units/mg of protein)
wheat	0.016
ragi	0.050
maize	0.0093
ethyl ferulate	3.0
PNPF	3.375

iodoacetamide, and eserine at 45 °C and at 10–50 mM. A PCMB concentration of 50 mM produced 67% inhibition (33% of relative activity), whereas 45% inhibition (55% of the relative activity) was observed with iodoacetamide. Both PCMB and iodoacetamide are specific cysteine residue modifiers and result in inactivation of the enzyme by forming complexes with cysteine present in the active site region of the enzyme (*48*). The pH optimum (6.0) of the FAE also supports this statement as the ionization values of these amino acids fall in this range. Eserine is a structural analogue of the amino acid serine and is involved in modifying histidine residues in the active site especially at pH 6.0 (*49*). In our study, eserine produced an increase in the activity (~98% more than the control) of the enzyme instead of inhibition (**Figure 9**).

Substrate Specificity. The substrate specificity of FAE was achieved by measuring the amount of ferulic acid enzymatically released from water-soluble polysaccharides and synthetic substrates. The specific activities of purified ragi FAE using various water-soluble polysaccharides such as ragi, wheat, maize, and synthetic substrates like PNPF and ethyl ferulate are listed in the **Table 4**. The specific activity of purified ragi FAE with respect to a ragi water-soluble preparation is higher than those of the other polysaccharides. FAE from ragi was active on both low-molecular mass substrates and polysaccharides as indicated in this study. The maximum substrate specificity was found using synthetic substrates, especially PNPF. The result obtained for purified ragi FAE was different from the one reported on FAE activity from barley malt (*35*), which indicates the specificity of ragi FAE toward synthetic substrates other than feruloyl polysaccharides. FAE isolated from various sources showed varied substrate specificity; for example, FAE from Cytolase M102 showed higher activities on both PNPF and ethyl ferulate, while that from *Trichoderma reesei* showed almost negligible activity on both (*19*).

Electrospray Ion Mass Spectrometry (ESI-MS). Analysis by ESI-MS confirmed the gradual deesterification of ragi and wheat water-soluble polysaccharides for the release of ferulic acid by purified ragi FAE. The amount of ferulic acid created by the enzymatic deesterification of the polysaccharides listed above using ragi FAE increased with an increase in time (0–4 h) and remained the same after 4 h, indicating the maximum release of ferulic acid within 4 h. The *m/z* value of released ferulic acid from ragi and wheat WSP was 191.70 (*m/z* negative mode) (figure not shown). The release of ferulic acid from polysaccharides, especially wheat bran, was reported (*50*) by a feruloyl esterase from *A. niger*.

The reports on the practical use of FAE particularly from plant sources are limited because of the lack of sufficient knowledge regarding their isolation, purification, and kinetic properties. The FAE purified and characterized in this study is the smallest one reported to date. This particular study is an attempt in that direction for possible exploitation of malt enzymes in modulating the functional properties of cereal nonstarch polysaccharides in various food applications.

ABBREVIATIONS USED

FAE, ferulic acid esterase; PVPP, polyvinylpolypyrrolidone; PCMB, *p*-chloromercuric benzoate; PNPF, *p*-nitrophenyl ferulate; PNP, *p*-nitrophenol; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; MS, mass spectroscopy; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; DMAP, (dimethylamino)pyridine; Et_3N, triethylamine; MUTMAC, methylumbelliferoyl *p*-trimethylammonium cinnamate chloride; GSH, reduced glutathione.

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Supporting Information Available: Scheme for the synthesis of PNPF, enzymatic release of ferulic acid from ragi WSP after 1, 2, and 4 h, and pH stability of purified ragi FAE. This material is available free of charge via the Internet at http://pubs.acs.org.

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Dear Dr Muralikrishna,

Your article entitled, `Optimized procedure for the isolation of acetic acid esterase from malted finger millet (*Eleusine corocna* Indaf-15) authored by Madhavi Latha has been accepted for publication in the Journal of Food Science and Technology with minor modification and the same will be published in one of the future issues of Journal of Food Science and Technology.

We wish you and your colleague all the best for sending this interesting paper to us for publication. And look forward to further quality contribution from you and your group and hope you will Achieve Greater Success and Accomplishment in the years to come**.**

With kind regards,

Yours sincerely,
(**N.S Mahendrakar**)