JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

G. MADHAVI LATHA,[†] P. SRINIVAS,[‡] AND G. MURALIKRISHNA*,[†]

Department of Biochemistry and Nutrition and Department of Plantation Products, Spices and Flavour Technology, Central Food Technological Research Institute, Mysore 570 020, Karnataka, India

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

* To whom correspondence should be addressed. E-mail: krishnagm2002@yahoo.com. Fax: +91-821-2517233. Telephone: +1-821-2514876.

[†] Department of Biochemistry and Nutrition, Central Food Technological Research Institute.

[‡] Department of Plantation Products, Spices and Flavour Technology, Central Food Technological Research Institute.

10.1021/jf071918d CCC: $37.00
Published on Web 10/19/2007

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}).

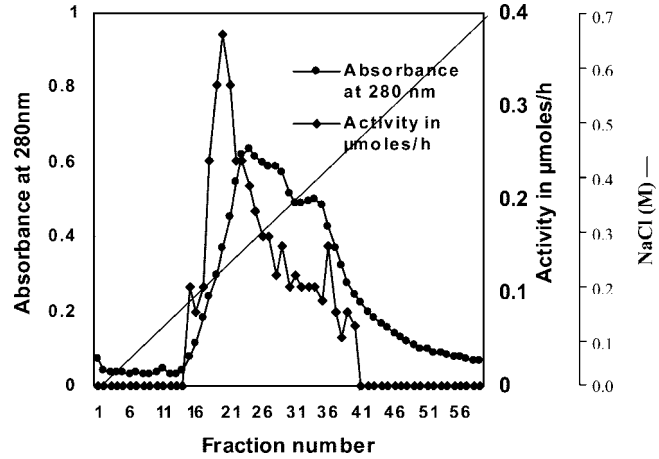

Figure 1. Elution profile of ragi FAE (40–80% ammonium sulfate fraction) on a DEAE-cellulose column.

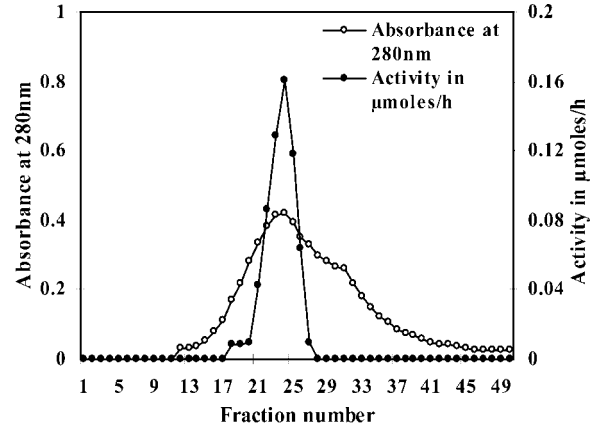

Figure 2. Elution profile of ragi FAE (DEAE-cellulose fraction) on a Biogel P-30 column.

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)$^{-1}$.

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+},

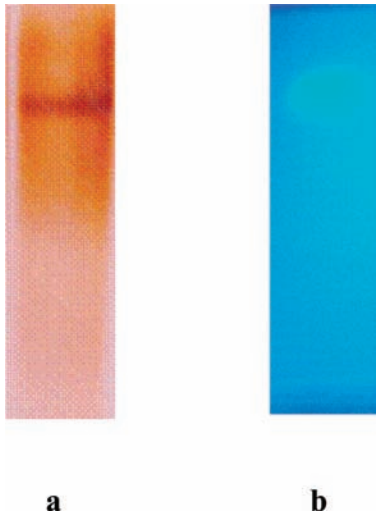

Figure 3. PAGE of purified ragi FAE: (**a**) protein staining and (**b**) activity staining.

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-

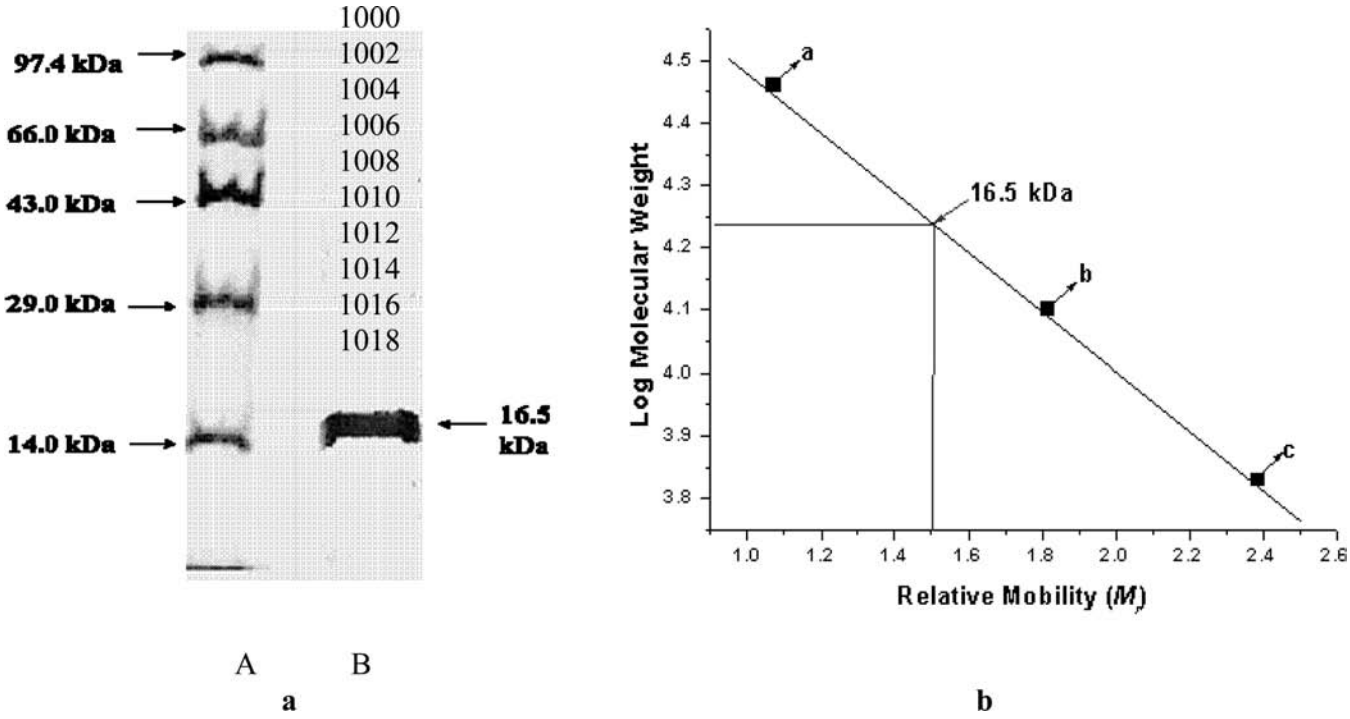

Figure 4. Molecular mass determination of purified ragi FAE (**a**) via SDS–PAGE and (**b**) on a Biogel P-30 column under nondenaturing conditions.

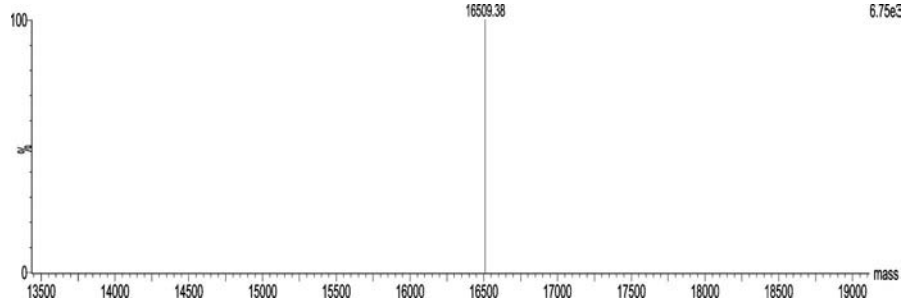

Figure 5. Molecular mass of purified ragi FAE by ESI-MS (positive mode).

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

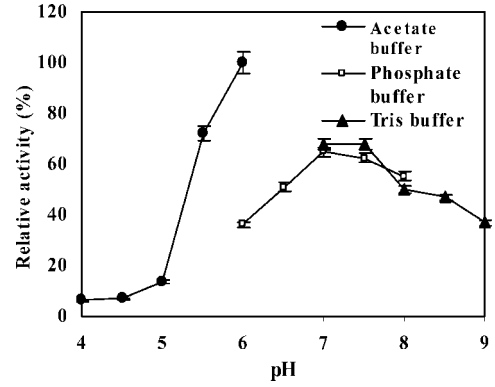

Figure 6. pH optima of purified ragi FAE.

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

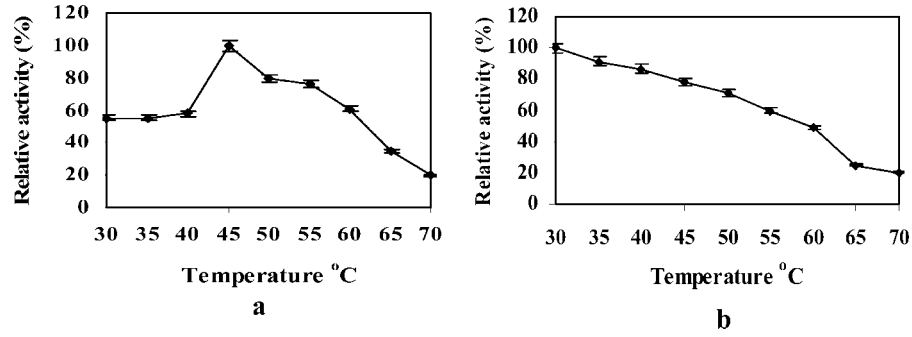

Figure 7. Temperature optima and thermal stability of purified ragi FAE.

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+},

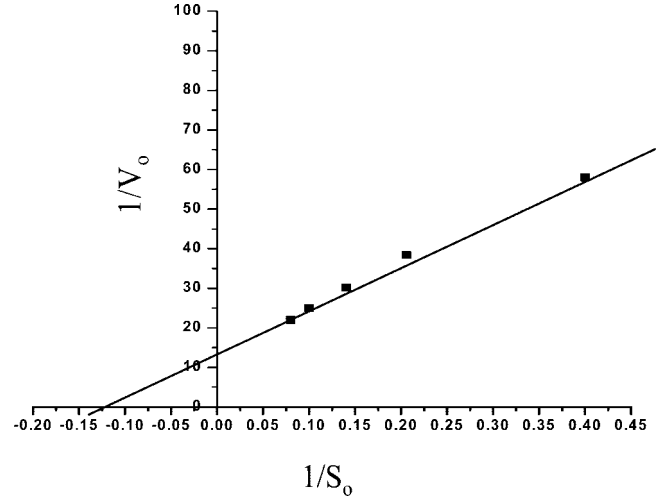

Figure 8. Determination of K_m and V_{max} values of ragi FAE with a Lineweaver–Burk plot (PNPF used as the substrate).

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

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.

Effect of Specific Reagents on FAE Activity. FAE activity was determined in the presence of chemicals such as PCMB,

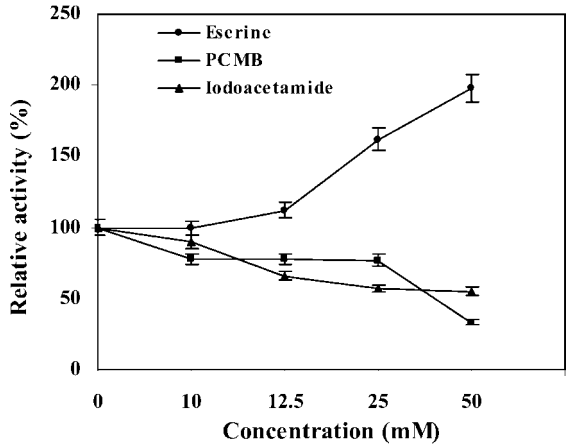

Figure 9. Effect of group specific reagents on the activity of purified ragi FAE.

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.

ACKNOWLEDGMENT

We thank Dr. V. Prakash F.R.SC, Director CFTRI, for his keen interest in this work and encouragement.

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.

LITERATURE CITED

(1) Mueller-Harvely, I.; Hartley, R. D.; Harris, P. J.; Curzon, E. H. Linkage of p-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr. Res.* **1986**, *148*, 71–85.

(2) Smith, M. M.; Hartley, R. D. Occurence and nature of ferulic acid substitution of cell wall polysaccharides in graminaceous plants. *Carbohydr. Res.* **1983**, *118*, 65–80.

(3) Williamson, G.; Kroon, P. A.; Faulds, C. B. Hairy plant polysaccharides: A close shave with microbial esterases. *Microbiology* **1998**, *144*, 2011–2023.

(4) Kroon, P. A.; Williamson, G. Release of ferulic acid from sugar-beet pulp by using arabinanase, arabinofuranosidase and an esterase from *Aspergillus niger*. *Biotechnol. Appl. Biochem.* **1996**, *23*, 263–267.

(5) McCrae, S. L.; Leith, K. M.; Gordon, A. H.; Wood, T. M. Xylan degrading enzyme system produced by the fungus *Aspergillus awamori*: Isolation and characterization of a feruloyl esterase and a p-coumaroyl esterase. *Enzyme Microb. Technol.* **1994**, *16*, 826–834.

(6) Koseki, T.; Furuse, S.; Iwano, K.; Sakai, H.; Matsuzawa, H. Purification and characterization of feruloyl esterase from *Aspergillus awamori*. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 2032–2034.
(7) McSweeney, C. S.; Dulieu, A.; Katayama, Y.; Lowry, J. B. Solubilization of lignin by the ruminal anaerobic fungus *Neocallimastix patriciarum*. *Appl. Environ. Microbiol.* **1994**, *60*, 2985–2989.
(8) Topakas, E.; Stamatis, H.; Mastihubova, M.; Biely, P.; Kekos, D.; Macris, B. J.; Christakopoulos, P. Purification and characterization of a *Fusarium oxysporum* feruloyl esterase (FoFAE-I) catalyzing transesterification of phenolic esters. *Enzyme Microb. Technol.* **2003**, *33* (5), 729–737.
(9) Faulds, C. B.; Wiliamson, G. The purification and characterization of 4-hydroxy-3-methoxy cinnamic (ferulic) acid esterase from *Streptomyces olivochromogenes*. *J. Gen. Microbiol.* **1991**, *137*, 2339–2345.
(10) Mackenzie, C. R.; Bilous, D. Ferulic acid esterase activity from *Schizophyllum commune*. *Appl. Environ. Microbiol.* **1988**, *54*, 1170–1173.
(11) McDermid, K. P.; MacKenzie, C. R.; Forsberg, C. W. Esterase activities of *Fibrobacter succinogenes* subsp. *Succinogenes* S85. *Appl. Environ. Microbiol.* **1990**, *56*, 127–132.
(12) Dalrymple, B. P.; Swadling, Y.; Cybinski, D. N.; Xue, G. P. Cloning of a gene encoding cinnamoyl ester hydrolase from the ruminal bacterium *Butyrivibrio fibrisolvens* E14 by a novel method. *FEMS Microbiol. Lett.* **1996**, *143*, 115–120.
(13) Ferreira, L. M. A.; Wood, T. M.; Williamson, G.; Faulds, C. B.; Hazlewood, G. P.; Gilbert, H. J. A modular esterase from *Pseudomonas fluorescens* subsp. Cellulose contains a noncatalytic cellulose-binding domain. *Biochem. J.* **1993**, *294*, 349–355.
(14) Bamforth, C. W.; Moore, J.; McKilop, D.; Williamson, G.; Kroon, P. A. Enzymes from barley which solubilise β-glucan. *EBC Congress* **1997**, *1*, 75–82.
(15) Ralet, M. C.; Faulds, C. B.; Williamson, G.; Thibault, J. F. Degradation of feruloylated oligosaccharides from sugar-beet pulp and wheat bran by ferulic acid esterases from *Aspergillus niger*. *Carbohydr. Res.* **1994**, *263*, 257–269.
(16) Borneman, W. S.; Hartley, R. D.; Morrison, W. H.; Akin, D. E.; Ljungdahl, L. G. Feruloyl and p-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 345–351.
(17) Humberstone, F. J.; Briggs, D. E. Extraction and assay of ferulic acid esterase from malted barley. *J. Inst. Brew.* **2000**, *106*, 21–29.
(18) Mastihubova, M.; Mastihuba, V.; Kremnicky, L.; Willett, J. L.; Cote, G. L. Chemoenzymatic preparation of novel substrates for feruloyl esterases. *Synlett* **2001**, *2001* (10), 1559–1560.
(19) Mastihuba, V.; Kremnicky, L.; Mastihubova, M.; Willett, J. L.; Cote, G. L. A spectrophotometric assay for feruloyl esterases. *Anal. Biochem.* **2002**, *309*, 96–101.
(20) Subba Rao, M. V. S. S. T.; Muralikrishna, G. Non starch polysaccharides and phenolics from native and malted ragi (*Eleusine coracana*). *Food Chem.* **2001**, *72*, 187–192.
(21) Nirmala, M.; Muralikrishna, G. Three alpha amylases from malted finger millet (*Eleusine coracana*, Indaf-15) purification and partial characterization. *Phytochemistry* **2003**, *62*, 21–30.
(22) Subba Rao, M. V. S. S. T.; Muralikrishna, G. Structural analysis of arabinoxylans isolated from native and malted finger millet. *Carbohydr. Res.* **2004**, *339*, 2457–2463.
(23) Shyama Prasad Rao, R.; Muralikrishna, G. Water soluble feruloyl arabinoxylans from rice and ragi: Changes upon malting and their consequence on antioxidant activity. *Phytochemistry* **2006**, *67* (1), 91–99.
(24) Madhavi Latha, G.; Muralikrishna, G. Purification and partial characterization of acetic acid esterase from malted finger millet (*Eleusine corocana*, Indaf-15). *J. Agric. Food Chem.* **2007**, *55* (3), 885–902.
(25) Nirmala, M.; Subba Rao, M. V. S. S. T.; Muralikrishna, G. Carbohydrates and their degrading enzymes from native and malted finger millet (*Eleusine coracana*, Indaf-15). *Food Chem.* **2000**, *69*, 175–180.
(26) Bradford, M. M. A rapid sensitive method of quantitative microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(27) Hatfield, R. D.; Helm, R. F.; Ralph, J. Synthesis of methyl 5-O-trans-feruloyl-α-L-arabinofuranoside and its use as a substrate to assess feruloyl esterase activity. *Anal. Biochem.* **1991**, *194*, 25–33.
(28) Lu, F.; Ralph, J. Facile synthesis of 4-Hydroxycinnamyl p-Coumarates. *J. Agric. Food Chem.* **1998**, *46*, 2911–2913.
(29) Wray, W.; Boulikas, T.; Wray, V. P.; Hancock, R. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **1981**, *118*, 197–203.
(30) Laemmli, U. K. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **1970**, *227*, 680.
(31) Lineweaver, H.; Burk, D. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **1934**, *56*, 658–666.
(32) Madhavi Latha, G.; Muralikrishna, G. A process for the preparation of acetic acid esterase from ragi malt. Patent 2336/DEL, **2005**.
(33) McMurrough, I.; Madigan, D.; Smyth, M. R. Adsorption by polyvinylpolypyrrolidone of catechins and proanthocyanins from beer. *J. Agric. Food Chem.* **1995**, *43*, 2687–2691.
(34) Pompella, A.; Visvikis, A.; Paolicchi, A.; De Tata, V.; Casini, A. F. The changing faces of glutathione, a cellular protangonist. *Biochem. Pharmacol.* **2003**, *66*, 1499–1503.
(35) Bartolome, B.; Garcia-Conesa, M. T.; Williamson, G. Release of the bioactive compound ferulic acid from malt extracts. *Biochem. Soc. Trans.* **1996**, *24*, 379S.
(36) Donaghy, J. A.; Bronnenmeier, K.; Soto-Kelly, P. F.; McKay, A. M. Purification and characterization of an extracellular feruloyl esterase from the thermophilic anaerobe *Clostridium stercorarium*. *J. Appl. Microbiol.* **2000**, *88*, 458–466.
(37) Castanares, A.; Wood, T. M. Purification and characterization of a feruloyl/p-coumaroyl esterase from solid-state cultures of the aerobic fungus *Penicillium pinophilum*. *Biochem. Soc. Trans.* **1992**, *20*, 275S.
(38) Rumbold, K.; Biely, P.; Mastihubova, M.; Gudelji, M.; Guebitz, G.; Robra, K. H.; Prior, B. A. Purification and properties of feruloyl esterase involved in lignocellulose degradation by *Aureobasidium pullulans*. *Appl. Environ. Microbiol.* **2003**, *69*, 5622–5626.
(39) Humberstone, F. J.; Briggs, D. E. Partial purification of ferulic acid esterase from malted barley. *J. Inst. Brew.* **2002**, *108* (4), 439–443.
(40) Borneman, W. S.; Ljungdahl, L. G.; Hartley, R. D.; Akin, D. E. Isolation and characterization of p-coumaryl esterase from anaerobic fungus *Neocallimastix* strain MC-2. *Appl. Environ. Microbiol.* **1991**, *57*, 2337–2377.
(41) Faulds, C. B.; Williamson, G. Ferulic acid esterase from *Aspergillus niger*: Purification and partial characterization of two forms from a commercial source of pectinase. *Biotechnol. Appl. Biochem.* **1993**, *17*, 349–359.
(42) Faulds, C. B.; Williamson, G. Purification and characterization of a ferulic acid esterase (FAE III) from *Aspergillus niger*: Specificity for phenolic moiety and binding to microcrystalline cellulose. *Microbiology* **1994**, *140*, 779–787.
(43) Garcia, B. L.; Ball, A. S.; Rodriguez Perez-Leblic, M. L.; Arias, M. E.; Copa-Oatino, J. L. Production and characterization of ferulic acid esterase activity in crude extracts by *Streptomyces avermitilis* CECT 3339. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 213–218.
(44) Borneman, W. S.; Ljungdahl, L. G.; Hartley, R. D.; Akin, D. E. Purification and partial characterization of two feruloyl esterases from anaerobic fungus *Neocallimastix* strain MC-2. *Appl. Environ. Microbiol.* **1992**, *57*, 3762–3766.
(45) Donaghy, J. A.; Mckay, A. M. Purification and characterization of a feruloyl esterase from the fungus *Penicillium expansum*. *J. Appl. Microbiol.* **1997**, *83*, 718–726.
(46) Kroon, P. A.; Faulds, C. B.; Williamson, G. Purification and characterization of a novel esterase induced by growth of *Aspergillus niger* on sugar-beet pulp. *Biotechnol. Appl. Biochem.* **1996**, *23*, 255–262.

(47) Castanares, A.; McCrae, S. I.; Wood, T. M. Purification and properties of a feruloyl/p-coumaryl esterase from the fungus *Pencillium pinophilum*. *Enzyme Microb. Technol.* **1992**, *4* (11), 875–884.

(48) Bell, J. E.; Bell, E. T. *Proteins and Enzymes*; Prentice- Hall, Inc.: Englewood Cliffs, NJ, 1998; pp 156–157.

(49) Kim, S.; Lee, S. B. Thermostable esterase from a thermoacidophilic archaeon: Purification and characterization for enzymatic resolution of a chiral compound. *Biosci., Biotechnol., Biochem.* **2004**, *68* (11), 2289–2298.

(50) Faulds, C. B.; Williamson, G. Release of ferulic acid from wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 1082–1087.

Received for review June 28, 2007. Revised manuscript received September 14, 2007. Accepted September 17, 2007. GML acknowledges Council of Scientific and Industrial Research (C.S.I.R). New Delhi for the grant of Junior and Senior Research fellowships.

JF071918D