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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.

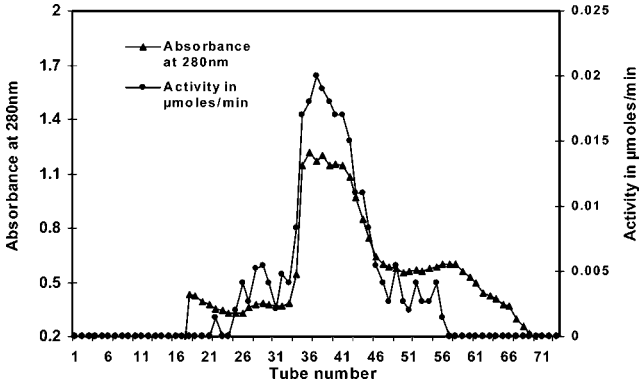

Figure 1. Elution profile of ragi acetic acid esterase (40–80% ammonium sulfate fraction) on a DEAE-cellulose column.

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.

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**).

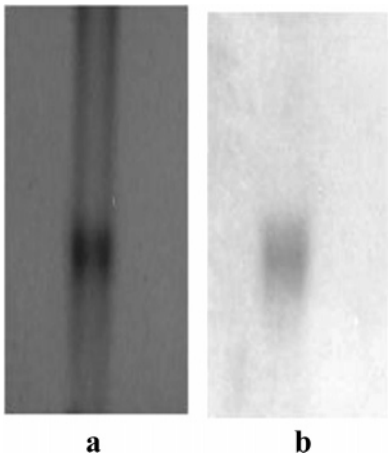

Figure 2. PAGE of purified ragi acetic acid esterase. (a) Protein staining. (b) Activity staining.

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.

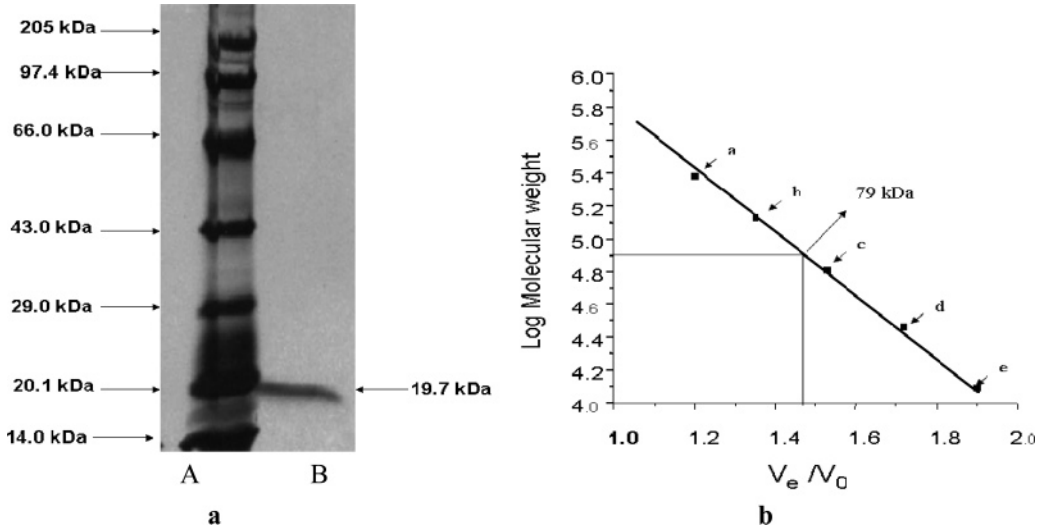

Figure 3. Molecular weight determination of purified ragi acetic acid esterase: (a**)** On SDS-PAGE: (A) Molecular weight markers: 205 kDa myosin; 97.4 kDa phosphorylase; 66.0 kDa bovine serum albumin; 43.0 kDa ovalbumin; 29 kDa carbonic anhydrase; 20.1 kDa soybean trypsin inhibitor; 14.0 kDa lysozyme. (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.

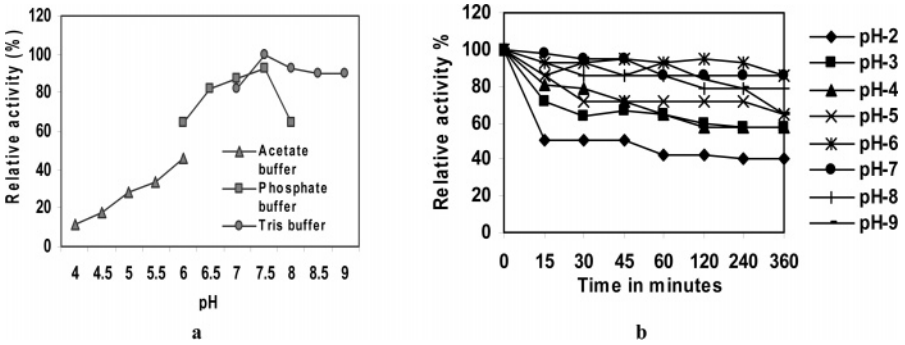

Figure 4. (a) pH optima and (b) pH stability of purified ragi acetic acid esterase.

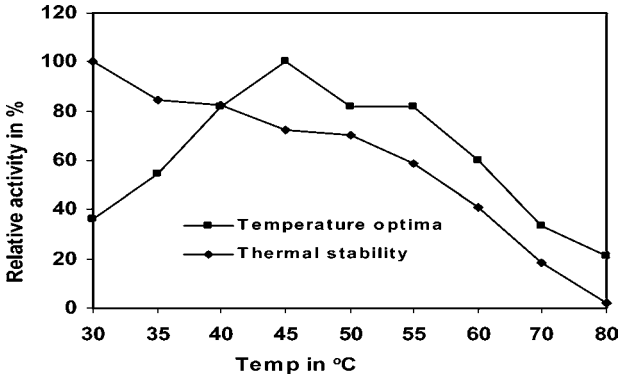

Figure 5. Temperature optima and thermal stability of purified ragi acetic acid esterase.

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

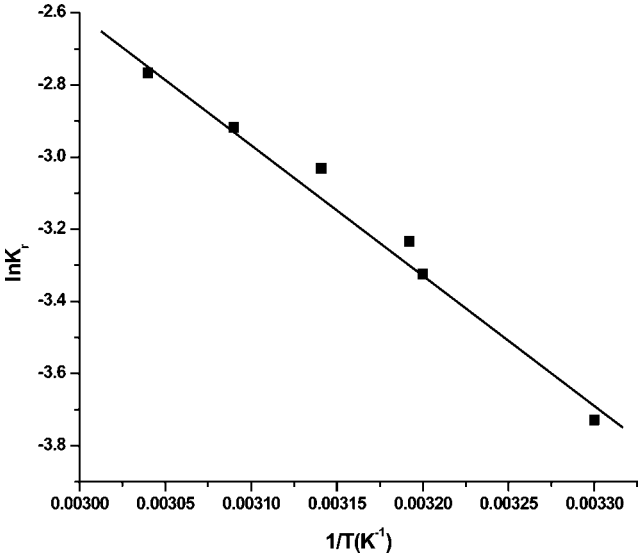

Figure 6. Arrhenius plot for the temperature dependence of acetic acid esterase using α-naphthyl acetate as substrate.

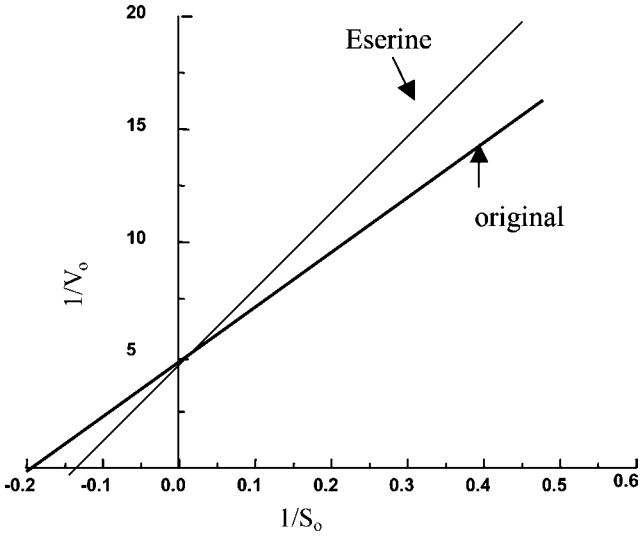

Figure 7. Determination of K_m and V_{max} of ragi acetic acid esterase by Lineweaver–Burk plot with and without inhibitor (substrate used: α-naphthyl acetate).

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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