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Partial purification and characterization of fatty acid esterase from pearl millet

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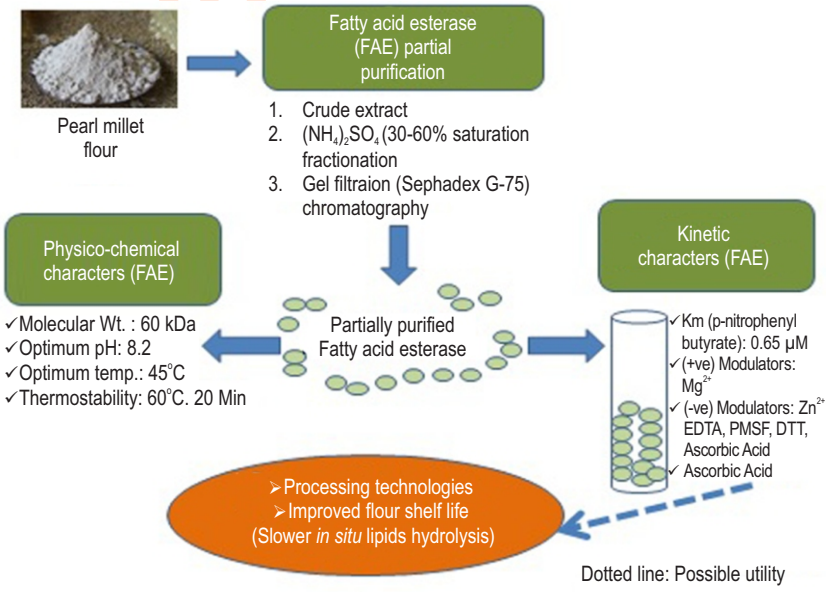
Abstract

Aim: The present study aimed to identify a negative modulator of lipolytic enzyme fatty acid esterase (FAE) for exploring possibilities of increasing shelf life of pearl millet flour through arresting *in-situ* hydrolysis of lipids.

Methodology: FAE was partially purified from flour of pearl millet hybrid HHB 234 by $(NH_4)_2SO_4$ fractionation (30-60 % saturation) and gel filtration chromatography using Sephadex G-75. The enzyme was characterized for physico-chemical properties viz., molecular mass, optimum pH, optimum temperature and thermal stability and kinetic properties viz., K_m value and effect of modulators.



Results: Crude extract contained 1008 units of activity and 421 mg proteins resulting in to specific activity of 2.4 units mg^{-1} protein. The enzyme was purified 10.7 fold with a recovery of 21.52% and specific activity of 25.7 units mg^{-1} protein by ammonium sulphate fractionation followed by gel filtration. The molecular weight of purified enzyme preparation was 60 kDa, as determined by gel filtration through Sephadex G-75. The enzyme exhibited maximum activity at pH 8.2 and 45°C. The enzyme was stable up to 60°C for 20 min and showed K_m value of 0.65 µM for p-NPB. At 10 mM concentration, Mg^{2+} and Zn^{2+} altered the activity positively by 54% and negatively by 42% whereas EDTA, DTT, PMSF, ascorbic acid inhibited the activity by 75, 68, 50 and 48%, respectively.

Interpretation: Partially purified lipolytic enzyme FAE from pearl millet flour was strongly inhibited by ascorbic acid. This novel information might be useful in developing processing technologies for improving shelf life of flour.

Key words: Characterization, Fatty acid esterase, Pearl millet, Purification, Shelf life

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Introduction

Food security has always been a key priority due to increasing demand for food with ever increasing population. Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important coarse grain cereal and forage crop of the arid and semi-arid tropics of the Indian subcontinent and several African regions. It is a central component of the food and fodder security of the rural poor residing in these areas. Nutritional profile of pearl millet is better than many other cereals. Pearl millet is the cheapest source of energy, protein, Fe and Zn among all cereals and pulses (Rai *et al.* 2013). Its proteins are gluten free, thus safe to consume by celiac patients (Sarita and Singh, 2016). Antioxidant activity of pearl millet hybrids and varieties is higher than that of fine cereals (Berwal *et al.,* 2018). Dietary fibre content in pearl millet has been reported to vary by 10-11% (Iswarya and Narayanan, 2017). Pearl millet is also a good source of polyunsaturated fatty acid- rich storage lipids (Lai and Varriano-Marston, 1980a), which ranges from 4.1 to 7.5% (Goyal *et al.,* 2017). Despite being nutritionally rich, its potential use in food industries has remained low because of short shelf life of flour owing to rapid development of rancidity.

High lipids content is one of the important factors responsible for the development of rancidity (Goyal *et al.,* 2017). Development of hydrolytic rancidity has been strongly correlated with high activities of lipolytic enzymes esterase (Goffman and Bergman, 2003) and lipase (Rose and Pike, 2006). *In-vitro* and *in-situ* activities of fatty acid esterase (FAE) and *in-situ* lipase of pearl millet are strongly correlated (Bajaj *et al.,* 2016). Strong correlation between activities of FAE and lipase indicates possibilities of presence of both on a single protein has been found in wheat (Fadıloglu and Soylemez, 1996). Lipolytic activities in pearl millet are concentrated in the germ, pericarp, aleurone and subaleurone layers of pearl millet (Lai and Varriano-Marston, 1980b). Attempts through developing processing technologies for improving shelf life of its flour invariably involve reduction in lipid content and activities of lipolytic enzymes or inactivating them. Traditionally, pearl millet is decorticated by hand pounding to reduce both. However, besides being a cumbersome process de-cortication at domestic level also results in loss of nutrients (Tiwari *et al.,* 2012). Refrigeration (Varsha and Narayanan, 2017), though causes suboptimal functioning of lipolytic enzymes and prolongs shelf life but has not gained favour among the industries.

Many modern grain processing techniques, for inactivating lipolytic enzyme(s) in flour such as steaming at 97°C for 12 min by steam-jacketed paddle conveyer (Bookwalter *et al.*, 1987), blanching at 98°C for 1 min (Chavan and Kachare, 1994), dry heat treatment for few hours (Kachare and Chavan, 1992), toasting at 120°C for 16 hr (Nantanga *et al.,* 2008), boiling for 15 min (Nantanga *et al.,* 2008), microwave treatment for 80 sec at 18% grain moisture (Yadav *et al.,* 2012). However, such processes have either of these inherent limitations and lack wider applicability at commercial level higher cost of operation (steam-jacketed paddle conveyer), loss of certain phytonutrients (blanching), change of colour of flour or products (dry heat treatment/toasting/boiling), susceptibility of products to auto oxidation of lipids (boiling) or ineffective control of auto-oxidation of lipids (microwave treatment) which lowers the nutritional value (Randhir *et al.,* 2008). Modulators as extrinsic factors, metal ions or organic compounds of known as well as unknown function have often been employed either to identify their role as cofactor in catalysis, identification of amino acid residues in active site, or stabilizer/de-stabilizer of intra and inter subunit interactions (Zeng *et al.,* 2014; Jensen *et al.,* 2016) so as to change catalysis favourably.

Negative modulation of lipids hydrolyzing or oxidizing enzymes native to grains by pre treatment of grains and/or fortification of flour with food grade additives may prove to be an effective approach for decreasing their activities in flour and thus slowing down the reactions leading to minimal production of undesirable metabolites viz., free fatty acids, hydroxyperoxides, volatile aldehydes/ketones etc. Thereby improving the shelf life of flour. However, none of such reports are available on food grade additives to suppress lipolytic enzymes in pearl millet grains/flour. In view of the foregoing discussion, the present investigation was conducted to identify negative modulator of FAE. Gaining knowledge of its physico-chemical and kinetic properties is a pre-requisite for identifying modulator(s) of enzyme activity which necessitates purification of FAE.

Materials and Methods

Grain sample and preparation of flour: Mature grains of pearl millet hybrid HHB 234 were used for the study. Flour of 0.8 mm particles was prepared in Cyclotec machine (Foss Analytical AB, Sweden). Flour was kept in a desiccator to bring it to room temperature before preparing of crude extract.

Purification of FAE: Throughout extraction and purification, all operations were carried out at temperature between 0 and 4°C maintained in a cold lab (LKB, Sweden).

Crude extract: Twenty gram of flour was hand homogenized in a pre-chilled pestle and mortar in 200 ml of 0.1M phosphate buffer (pH 8.0). The homogenate filtered through four layers of cheese cloth was centrifuged at 10000 rpm for 20 min in a refrigerated centrifuge at 4°C.

$(NH_4)_2SO_4$ fractionation and dialysis: The supernatant was fractionated using ammonium sulphate $(NH_4)_2SO_4$ up to 30% saturation. For further enzyme purification, the supernatant was again fractionated with $(NH_4)_2SO_4$ raising saturation to 60% which resulted in precipitation of esterase. The precipitates were dissolved in 12.5 ml of 0.1M sodium phosphate buffer (pH 8.0). The resulting solution was dialyzed using properly washed dialysis membrane (Flat width 25 mm, molecular weight cut-off limit 14000, Sigma Aldrich, USA) against the same buffer for 24 hr. Every eight hrs, the used dialysis buffer was replaced with fresh buffer. Fifteen ml of $(NH_4)_2SO_4$ fraction recovered after dialysis was concentrated against solid sucrose to 3 ml which was further used for gel filtration chromatography.

Gel filtration chromatography: Gel filtration column was prepared as follows: Sephadex G-75 (8. 50 g) (particle size 40-120µ, Bed volume 12-15 ml, Pharmacia Fine Chemicals, Upasala) was suspended in 300 ml of 0.1M phosphate buffer (pH 8.0) in a beaker and allowed to swell at room temperature for 8 hrs with intermittent shaking. Excessive buffer was decanted from the gel suspension. The suspension was poured carefully into an LKB glass column (length 100 cm x diameter 1.7 cm) and allowed to settle by gravity. After complete sedimentation of Sephadex in the column, the effective length of column was 67 cm.

The void volume was calculated by passing 1.5 ml of blue dextran solution (2 mg ml^{-1}) through the column as per instructions given in the Technical Bulletin of the manufacturer (Sigma-Aldrich, USA). The concentrated $(NH_4)_2SO_4$ fraction was carefully layered over the top of the Sephadex G-75 bed pre-equilibrated with 0.1M phosphate buffer (pH 8.0). The column was subsequently eluted with same buffer. The fractions (3 ml) were collected at a flow rate of 12 ml hr^{-1}. These fractions were analyzed for protein and FAE activity. The fractions with substantial FAE activity were pooled and concentrated using solid sucrose.

Protein estimation: Protein in various fractions during elution of column was monitored by measuring the absorbance at 280 nm on a spectrophotometer (Thermo Scientific, USA, Model Evolution 201). Quantifying of protein in crude extract was done following the method of Bradford (1976) $(NH_4)_2SO_4$ fraction and gel filtered fractions and all measurements were made in duplicates.

FAE assay: FAE activity in crude extract and in the preparations during purification process was assayed by the method of Winkler and Stuckman (1979). The assay mixture (1 ml) contained 900 µl of 1:20 dilution of the substrate solution A (0.3 % p-NPB prepared in isopropanol) in solution B (0.1% Gum acacia solution prepared in double distilled water), 50 µl of 0.1 M sodium phosphate buffer (pH 8.0) and 50 µl of enzyme preparation. The reaction was started by adding enzyme. Released p-nitrophenol (p-NP) was immediately determined by measuring the absorbance at 410 nm at room temperature at an interval of 15 sec for two and a half min. The linear portion of change in absorbance was taken into account for calculating enzyme activity. One unit of esterase activity was defined equal to 0.1 change in O.D./min. All assays were performed in duplicates.

Characterization of FAE

Molecular weight: The molecular weight of purified enzyme was estimated by gel filtration chromatography. The column was calibrated with the following standard protein markers (2 mg ml^{-1} each): cytochrome C (12.4 kDa), carbonic anhydrase (29.0 kDa), bovine serum albumin (66.0 kDa), alcohol dehydrogenase (150.0 kDa) and beta-amylase (200 kDa). The log molecular weight of each standard protein was plotted against elution volume and the molecular weight of purified enzyme was calculated from the calibration graph.

Optimum pH: The optimum pH value for FAE was determined by assaying enzyme activity using assay buffer of pH values ranging from 6.0-9.0. For maintaining pH range 6.0 to 8.0, 0.1 M phosphate buffer was used and for pH above 8.0, 0.1 M Tris HCl buffer was used. For determining optimum pH for activity, 100 µl of purified enzyme was preincubated with 100 µl each of appropriate buffer of different pH values separately for 1 hr at 27°C. The enzyme activity was measured as mentioned earlier.

Optimum temperature and thermal stability: The optimum temperature for the enzyme was determined by measuring the enzyme activity at temperatures ranging from 20 to 65 °C. Except for the enzymes, all constituents of the reaction mixture were maintained at appropriate temperature in a water bath before starting the reaction. Enzyme activity was then determined as outlined earlier. Thermo stability of purified enzyme was tested by measuring the residual activity after incubating 100 µl of enzyme for 20 min at temperature ranging from 20 to 90°C in a water bath.

K_m value: The activity of purified preparation was measured using p-NPB as substrate at a final concentration varying from 0.3 to 2.88 µM. The K_m value was determined from X-intercept on Line weaver-Burk plot of the reciprocal data drawn on Microsoft office Excel.

Effect of modulators: The effect of monovalent ions Na^+ and K^+, divalent ions Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and trivalent ions Fe^{3+} and Al^{3+} was determined by carrying out enzyme assay at different concentrations of these metal ions (1, 5 and 10 mM). The chloride salts of all metals were used for the experiment and the residual activity was calculated taking activity of control as 100%. The activity of purified preparation was also measured in the presence of following compounds at different concentrations (1 to 10 mM): EDTA, PMSF, β-mercaptoethanol (β-ME), dithiothreitol (DTT), sodium dodecyl sulfate (SDS) and ascorbic acid. The percent of inhibition was expressed as relative to the enzyme activity without inhibitor.

Statistical analysis: Standard deviations were analysed using Microsoft Office Excel 2007 software.

Results and Discussion

Purification of esterase: FAE from pearl millet flour was partially purified by $(NH_4)_2SO_4$ fractionation (30-60 % saturation) and using a two steps purification procedure. The crude extract in a total volume of 180 ml displayed 1008 units of activity and 421 mg protein resulting in to specific activity of 2.4 units mg^{-1} protein (Table 1). Specific activity increased to 6.8 units mg^{-1} protein in $(NH_4)_2SO_4$ fraction with a recovery of 43.15 %. Elution profile of the enzyme through Sephadex G-75 is depicted in (Fig. 1). FAE was eluted after the major protein peak in twelve fractions numbered as 26-37. Rest of the fractions showed enzyme activity in traces. The active fractions were pooled. Total volume of pooled fractions was 36 ml. The results presented in Table 1 showed that 50.1 % of the total activity present in the $(NH_4)_2SO_4$ fraction was lost. Finally, 21.52 % of the total activity present in crude extract was recovered with specific activity equivalent to 25.7 units mg

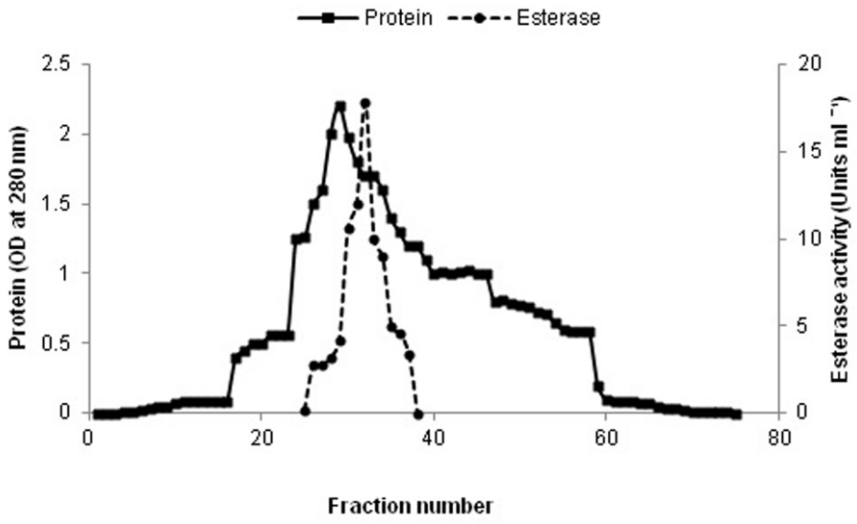

Fig. 1: Elution profile of fatty acid esterase on Sephdex G-75 column.

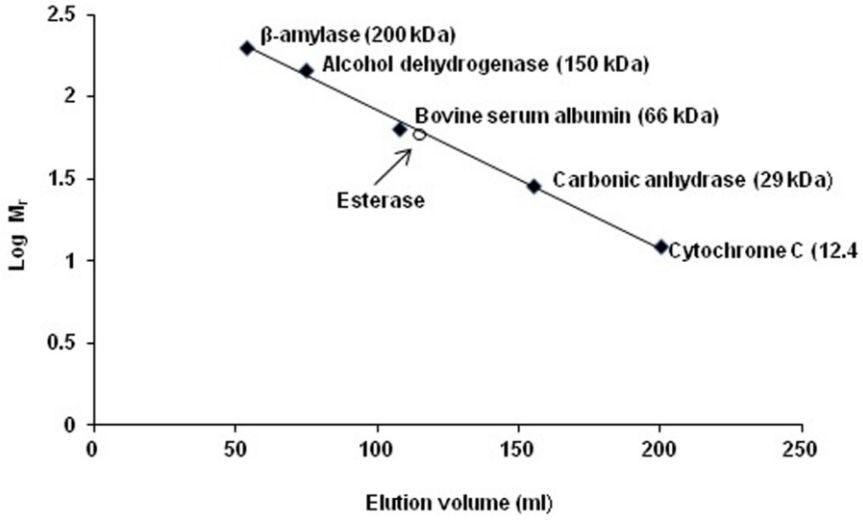

Fig. 2: Determination of molecular weight of purified fatty acid esterase by gel filtration through Sephadex G-75.

proteins^{-1}. Thus, 10.7 fold purification was achieved. The achieved purification of FAE from pearl millet in terms of folds and specific activity was less compared to carboxyl esterase (Upadhya *et al.,* 1985), ferulic acid esterase (Latha *et al.,* 2007) and acetic acid esterase (Latha and Muralikrishna, 2007) purified from germinated finger millet because during the present investigation ion exchange chromatography was not carried out as has been followed by the investigators mentioned above.

Physico-chemical and kinetic properties: The purified enzyme was characterized for the following physico-chemical and kinetic properties.

Molecular weight: The molecular weight of partially purified FAE from pearl millet, determined by gel filtration was 60 kDa (Fig. 2). The size of pearl millet FAE purified during the present investigation was closer to that of FAE I and FAE II fractions (50-64 kDa) purified from yam (*Dioscorea batatas*) (Hou *et al.,* 1999), carboxyl esterase of finger millet (70 kDa) (Upadhya *et al.,* 1985) and carboxyl esterase of *Tribolium castaneum* (60 kDa) (Haubruge *et al.,* 2002). Humbertson and Briggs (2002) reported that molecular weight for esterase purified from barley varied between 22 to 158 kDa indicating the presence of multiple isoforms. According to Latha and Muralikrishna (2007) finger millet acetic acid esterase had a molecular mass of 79.4 kDa in

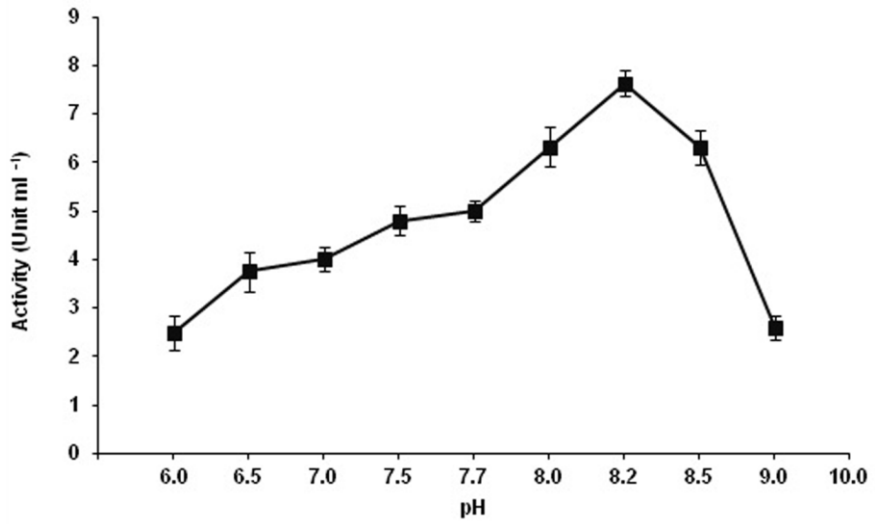

Fig. 3: Effect of pH on the activity of purified fatty acid esterase.

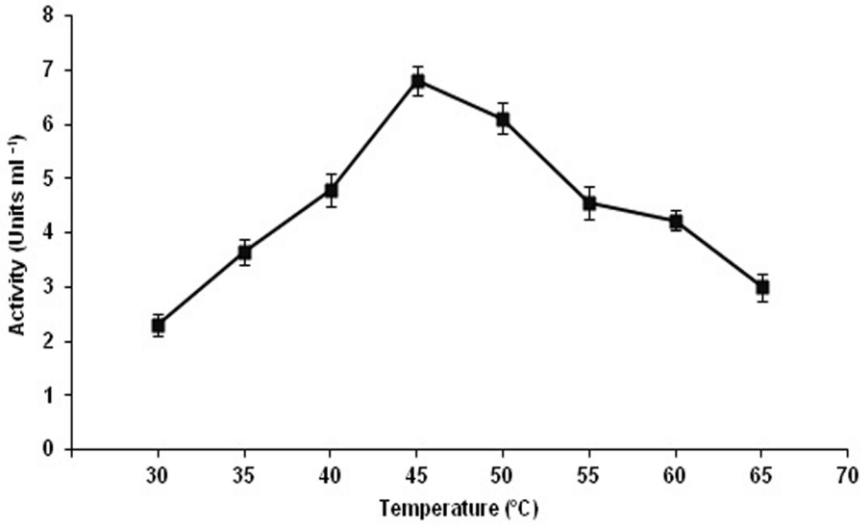

Fig. 4: Effect of temperature on the activity of purified fatty acid esterase.

native form whereas denatured enzyme was found to be a homo-tetramer having molecular weight of 19.7 kDa for each subunit.

pH optimum: The effect of pH on FAE activity is depicted in (Fig. 3). Enzyme activity increased gradually from 2.48 units ml^{-1} at pH 6.0 to 6.32 units ml^{-1} at pH 8.0. Thereafter, the activity increased sharply and reached to a level of 7.64 units ml^{-1} at pH 8.2. Thereafter, the activity started decreasing rapidly with gradual increase in pH till 9.0. Thus optimum pH for obtaining maximum activity of esterase was 8.2. Staubmann *et al.* (1999) also reported pH optimum for butyrate dependent esterases isolated from *Jatropha curcas* seeds to be 8.0 for isoform JEA and 7.5 for the isoform JEB. Many of the plant esterases show optimum pH between 7 and 8 (Chandrashekharaiah *et al.*, 2011; Bhavith *et al.*, 2014; Kantharaju and Murthy, 2014). The pH of water extract of fresh and stored pearl millet flour varies from 6.67 to 6.25 (Goyal *et al.,* 2015). The present investigation showed (Fig. 3) that *in-vitro* activity of FAE at pH within this (6.67 to 6.25) range was approximately 40-45 % of that exhibited at pH 8.2 (optimum pH). Thus FAE is likely to be much less active in stored flour. These results corrborates with the earlier results of Bajaj *et al.* (2016) who demonstrated that *in-situ* activity of FAE in pearl millet flour is

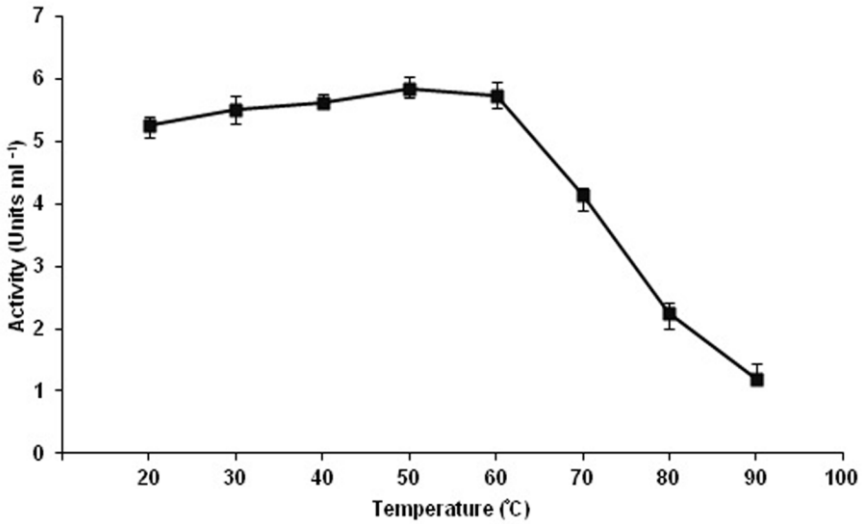

Fig. 5: Effect of temperature on the stability of purified fatty acid esterase.

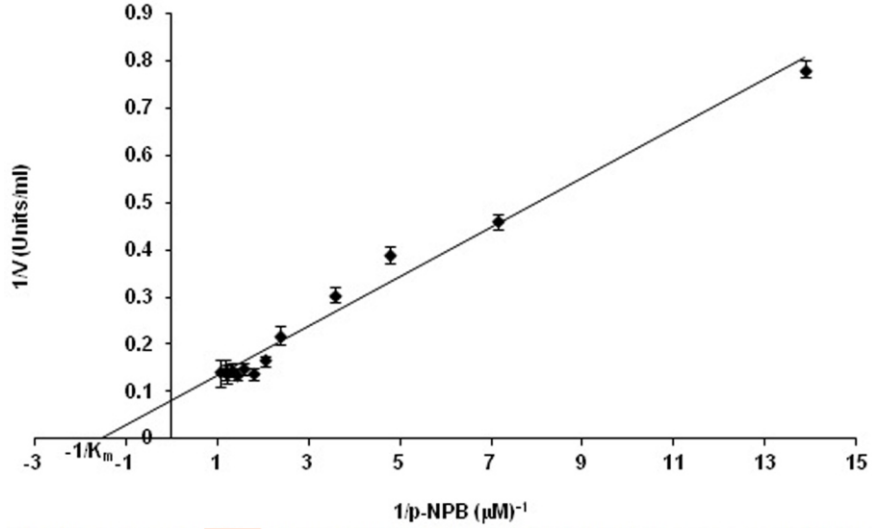

Fig. 6: Linewaever-Burk plot showing km value of purified fatty acid esterase as a function of p-NPB concentration.

about 10 folds less than the *in-vitro* activity. Nevertheless, *in-situ* hydrolysis of lipids might not get affected significantly by minor change in the pH of flour.

Temperature optimum and thermo stability: Profile of changes in activity in response to varying assay temperature is presented in (Fig. 4). The activity of esterase increased rapidly with increasing temperature from 30 °C and reached to a maximum value of 6.8 units ml^{-1} at 45°C. The activity started declining gradually with rise in temperature beyond 45°C. At 60°C esterase lost about 39 % of its activity. Thus, the purified enzyme was optimally active at 45°C which is higher than exhibited by yam tuber esterase (Hou *et al.,* 1999).The optimum temperature of pearl millet FAE was lower than that (50°C) of isoform JEA and nearly equal to that of isoform JEB of FAE from *Jatropha curcas* L. (Staubmann *et al.,* 1999). Esterase from finger millet - (Latha *et al.,* 2007; Latha and Muralikrishna, 2007), *Caesalpinia mimosoides* (Bhavith *et al.,* 2014), *Tamarindus indica* (Kantharaju and Murthy, 2014) and soybean (Barros and Macedo (2015) have also been found optimally active at temperature near 45°C. Enzyme normally exhibit optimum temperature ranging between 25 to 37°C. A high optimum temperature implies that the flour

Table 1: Summary of purification of FAE from pearl millet flour

Fraction	Volume (ml)	Total activity (units)*	Protein (mg)	Specific activity (units mg^{-1} protein)	Fold purification	Recovery (%)
Crude extract	180	1008	421	2.4	–	100
$(NH_4)SO_4$ fraction (30-60% Saturation)	15	435	64	6.8	2.8	43.15
Gel filtration (Sephadex G-75)	36	217	8.43	25.7	10.7	21.52

*One unit is equal to 0.1 change in O.D./min.

Table 2: Effect of various metal ions on the activity of purified FAE

Metal ion	Activity (% of control)*		
	Concentration (mM)		
	1	5	10
Mg^{2+}	106 ± 4	122 ± 4	154 ± 5
N^{a+}	101 ± 3	111 ± 2	141 ± 6
Al^{3+}	103 ± 0	115 ± 0	130 ± 2
Ca^{2+}	105 ± 2	107 ± 1	115 ± 3
Mn^{2+}	101 ± 2	105 ± 0	110 ± 2
Fe^{3+}	93± 1	88 ± 3	81 ± 1
Co^{2+}	86 ± 2	80 ± 4	77 ± 2
Cu^{2+}	89 ± 2	75 ± 2	70 ± 2
Zn^{2+}	80 ± 2	69 ± 1	58 ± 9

Values represent mean of two determinations ± SD; *Activity in control was 25.74 units mg $proteins^{-1}$ and *50 ul extract was pre incubated with metal ions for 20 min before measuring activity at 410 nm

stored at high temperature prevailing during summer will get deteriorated faster. In fact comparatively faster increase in fat acidity in pearl millet flour stored at higher temperature is indicative of rise in *in-situ* activity of lipolytic enzymes with increasing temperature (Lai and Varriano-Marston, 1980b; Kadlag *et al.,* 1995). The enzyme purified during present investigation was thermally stable till 60°C and (Fig. 5) at 90°C the enzyme lost its activity by 81.3%. Similar level of activity of ferulic acid esterase was, however, lost at 70°C (Latha *et al.,* 2007). Almost identical stability behaviour has also been reported for Caesalpinia seed esterase (60°C) (Bhavith *et al.,* 2014). Hou *et al.* (1999) found thermo stability of FAE at temperatures below 50°C from yam (*Dioscorea batatas*) tuber. At 60°C, about 60% of acetic acid esterase activity was decreased (Latha and Muralikrishna, 2007). Esterases of *Tamarindus indica* seed were even labile at temperatures below 40°C (Kantharaju and Murthy, 2014). Thus, pearl millet FAE was comparatively more thermostable. Rapid decline of *in-vitro* activities of the enzymes viz., peroxidase, lipoxygenase and polyphenol oxidase in pearl millet flour stored at 37°C (Goyal *et al.,* 2015) shows *in-situ* instability of these enzymes. On the contrary, statistically similar levels of *in-vitro* as well as *in-situ* activities of FAE and *in- situ* activity of lipase in control and 10 day stored flour at the identical temperature (Bajaj *et al.,* 2016), clearly indicates stability of these enzymes, continuous release of fatty acids making the flour rancid. Stability of purified pearl millet FAE till 60 °C might be the result of architecture of the enzyme which needs further investigations.

K_m value: Esterases comprise a diverse group of hydrolases and many of them show activity with wide range of substrates like phenyl acetate, naphthyl acetate, naphthyl propionate, p-nitrophenyl butyrate (p-NPB), nitrophenyl ferulate, naphthyl myristate, etc. (Latha and Muralikrishna, 2007; Xin and Hui-Ying, 2013; Jensen *et al.,* 2016) During the present investigation only one substrate *i.e.,* p-NPB was used. Pearl millet FAE followed typical Michaelis–Menten kinetics with K_m value of 0.65 µM for p-nitrophenyl butyrate (Fig. 6). Using identical substrate Staubmann *et al.* (1999) reported K_m value of 0.02 mM and 0.07 mM of two isoform of the enzyme from *Jatropha curcas* L. seeds. K_m value equivalent to 0.11 mM has been reported for *Caesalpinia mimosoides* esterase using 1-naphthyl acetate as substrate. Jensen *et al.* (2016) found K_m value of 1.07 mM p-NPB for carboxyl esterase from *Pseudoalteromonas arctica*. Finger millet ferulic acid esterase and acetic acid esterase showed K_m value 0.053 and 0.40 µM for their respective substrates (Latha and

Table 3: Effect of chemical compounds on the activity of purified FAE

Inhibitors	Activity (% inhibition)		
	Concentration (mM)		
	1	5	10
EDTA	28 ± 2	51 ± 3	75 ± 4
PMSF	21 ± 1	34 ± 3	50 ± 0
SDS	18 ± 1	30 ± 2	34 ± 2
β-ME	15 ± 1	18 ± 0	22 ± 1
DTT	33 ± 2	59 ± 3	68 ± 3
Ascorbic acid	26 ± 2	40 ± 1	48 ± 0

Values represent mean of two determinations ± SD; *Activity in control was 25.74 units mg protein^{-1} and *50 ul extract was pre incubated with inhibitors for 20 min before measuring activity at 410 nm

Muralikrishna, 2007; Latha *et al.,* 2007). Yet higher K_m value of 3.62 mM p-nitrophenyl ferulate for feruloyl esterase from *Aspergillus usamii* has also been reported by Yin *et al.* (2015). Wide variation in the K_m value of esterases is related to diversity of sources, degree of purity of enzyme preparation, methods and type of substrates used. K_m values in µM range for p-NPB for pearl millet FAE point out towards high affinity for substrate might be partly responsible for rapid hydrolysis of lipids in stored pearl millet flour.

Effect of modulators: Data presented in Table 2 showed that activity of purified esterase was stimulated by Mg^{2+}, Na^{+}, Al^{3+}, Ca^{2+}, and Mn^{2+} in the decreasing order of their influence whereas Fe^{3+}, Co^{2+}, Cu^{2+} and Zn^{2+} inhibited with increasing deleterious order. Irrespective of stimulatory or inhibitory effect of these metal ions, the activity increasingly affected with changing concentration from 1 to 10 mM. For example, the presence of Mg^{2+}, Na^{+}, Al^{3+}, Ca^{2+} and Mn^{2+} in the assay mixture at 10 mM increased the activity by 54, 41, 30, 15 and 10 %, respectively. Similarly at 10 mM concentration, Fe^{3+}, Co^{2+}, Cu^{2+} and Zn^{2+} inhibited the activity by 19, 23, 30 and 42 %, respectively. Thus, Mg^{2+} and Zn^{2+} which altered the activity positively by 54 % and negatively by 42 %, were the most potent stimulator and inhibitor of the purified pearl millet FAE. The effect of these metals on the activity of purified esterases from different plant or microbial sources reported in literature is not equivocal (Staubmann *et al.,* 1999; Latha and Muralikrishna, 2007; Latha *et al.,* 2007; Barros and Macedo, 2015; Hu *et al.,* 2018). Data on inhibition of esterase by different compounds is presented in Table 3. FAE activity was highly inhibited by the metal chelator, EDTA. For investigating the essentiality for metal ion cofactors, the effect of EDTA was examined. At the highest concentration of EDTA (10 mM), 75 % inhibitory effect was visible on the activity, while at 1 mM EDTA, the activity decreased to 28 % of the control.

A strong inhibitory effect at lowest concentration 1 mM otherwise observed at 10 mM would have indicated absolute requirement of metal ions as cofactor for the activity. The observed stimulatory and inhibitory effects of metal ions showed stability of the enzyme. The modest inhibitory effect encountered with EDTA suggests that certain metals can have a stabilising effect on the structure of the enzymes. Al^{3+} and Co^{2+} are not constituents of pearl millet. Abundance of major nutrients Mg^{2+} and Na^{+} (both stimulators of FAE) and deficiency in micronutrient Zn^{2+} (strongest inhibitor of FAE) in pearl millet might be favourable for stability of FAE in flour. Inhibition of enzyme by phenylmethylsulfonyl fluoride is in accordance with the earlier reports on negative impact of this compound on extracellular esterase from *Salimicrobium sp.* LY19 (Xin and Hui-Ying, 2013). Inhibition of esterase enzymes by PMSF at all concentrations suggests that serine residues are essential for its catalytic function. The disulphide bond reducing agents beta-mercaptoethanol (β-ME) and dithiothreitol (DTT) showed concentration dependent inhibitory effect on the enzyme activity though β-ME was comparatively less effective. At 5 mM concentration, β-ME and DTT inhibited the activity by 18 and 59 %, respectively (Table 3). Many of the esterases contain multiple cysteine residues which are capable of forming disulphide bridges (Jensen *et al.,* 2016). The effect of reducing agent DTT on the activity of this enzyme may be a consequence of destabilisation of disulphide bonds, which indicates that cysteine residues may be necessary for the activity and/or stability of enzyme by forming disulphide bridges. Studies of Yin *et al.* (2015) have shown that disulfide bridges contribute significantly to the thermostability of feruloyl esterase.

The observed thermostability (motioned above) of pearl millet FAE might be the result of disulphide bridges. Sodium dodecyl sulphate (SDS), an anionic surfactant, which can destroy the non-covalent bonds between enzyme molecules and change the conformation of enzyme also, inhibited FAE activity significantly in a concentration dependent manner (Table 3). Inhibition of enzyme activity by SDS is in accordance with the observed negative impact of this compound on esterase from *Stenotrophomonas maltophilia* (Gao *et al.,* 2019). Ascorbic acid also showed detrimental effect on the purified pearl millet esterase (Table 3). At 5 and 10 mM, it caused 42 and 48 % reduction in its activity. The ability of ascorbic acid to inhibit the enzyme appears due to lowering of pH of the reaction medium. Final pH of the reaction mixture

adding 3 and 10 mM ascorbic acid was 5.32 and 4.09. The observed significantly lower levels of activity recorded at pH below the optimum (Fig. 3) supports this hypothesis.

Among the negative modulators identified, ascorbic acid is a known antioxidant and is used frequently as a safe food additive. Its use in developing processing technologies such as steeping of grains in ascorbic acid or fortification of flour with ascorbic acid for slowing down *in-situ* hydrolysis of lipids through inactivating lipolytic activities for improving shelf life of flour might be explored. Simultaneous inhibition of pearl millet lipoxygenase by ascorbic acid (Sharma and Chugh 2017) might also delay oxidation of unsaturated fatty acids.

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Add-on Information

Authors' contribution: S. Bajaj: Execution of the experiment; **L.K. Chugh:** Planning of the experiment, Interpretation of data and Discussion of results; **P. Goyal:** Writing of Manuscript; **A. Kumar:** Statisitical analysis and Review of Manuscript

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