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ORIGINAL ARTICLE

Two isoforms of lipoxygenase from mature grains of pearl millet [*Pennisetum glaucum* (L.) R. Br.]: purification and physico-chemico-kinetic characterization

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Abstract This study describes the partial purification and characterization of lipoxygenase (LOX) from pearl millet mature grains of inbred HBL 0843-2. Two isoforms of LOX, i.e., LOX 1 and LOX 2, were purified using ammonium sulphate fractionation, gel filtration chromatography and ion exchange chromatography near homogeneity to 56 and 40 folds with yield of 28 and 24%, respectively. LOX 1 and LOX 2 having molecular masses of approximately 85 and 79 kDa, respectively were purified. LOX 1 and LOX 2 exhibited maximum activity at pH 4.5 and 4.8, respectively at 25 °C temperature. Both the isoforms, which showed thermostability up to 35 °C when incubated for 30 min, were stable at a pH range of 7–7.8. LOX 1 and LOX 2 had apparent K_m value of 0.86 and 0.57 μM, respectively. Ascorbic acid and vitamin E inhibited 66–78 and 61–69% activity of LOX 1 and LOX 2, respectively but Na^+, Zn^{2+} and K^+ strongly inhibited the activity of these isozymes. The present information about lipoxygenase enzyme might be valuable in drafting the strategies for its inactivation, which in turn can obstruct the LOX damaging effects on food products during processing and storage.

Keywords Lipoxygenase · Purification · Pearl millet · Isoforms · Characterization

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Introduction

Pearl millet [*Pennisetum glaucum* (L.) R.Br.], commonly known as *Bajra*, is the fourth most important crop in India after rice, wheat and sorghum. Being highly nutritious especially in terms of energy (2900 kcal/kg), it is primarily used for human consumption mainly in Asia and Africa (Singh et al. 2014). Being free from major anti-nutrients, it is one of the cheapest sources of protein, iron and zinc among the cereals and the pulses (Rai et al. 2013; Anonymous 2015). Besides being a nutrients rich crop, it is well known for its adaption to drought, low soil fertility and acidic soil (Khairwal and Yadav 2005). Moreover, it is also able to yield more than any cereal crop with low or no fertilizer input. Currently, India is producing 9.60 million tonnes of pearl millet with a productivity of 11.61 q/ha (Anonymous 2015). The five states namely, Rajasthan, Maharashtra, Gujarat, Uttar Pradesh and Haryana contribute about 92% of its total production in India. Although it is primarily grown as a food grain crop but it is also a major component of ruminant and poultry feed, especially in dry areas (Singh et al. 2014). It is also a good forage crop because of lesser hydrocyanic acid content than sorghum, as its green fodder is rich in protein, calcium and phosphorus, which are essential for the strong bones. Despite its nutritional quality, the pearl millet consumption has declined by 73.4 and 71.8% in rural and urban areas, respectively (NRAA 2012). Many factors like subsidized and cheaper availability of rice and wheat and an increase in per capita income can be held responsible for this decline but short storability of pearl millet flour is the major cause for its poor utilization by the people and in food industries too. Fast deterioration of pearl millet flour can be attributed to high fat content, containing high polyunsaturated fatty acids (PUFA), which make it more

susceptible to rancidity due to lipolysis and subsequent oxidation of PUFA (Singh et al. 2014). Linoleic acid is found as the major fatty acid (40.1–47.4% of the total fatty acids) in different pearl millet varieties. Moreover, few enzymes, which are essential in the metabolism of plants, remain active even after harvesting and lead to detrimental changes in quality attributes such as colour, flavour, texture and nutritional value of its products. Lipoxygenases (LOX; linoleate oxygen oxidoreductase; EC 1.13.11.12), the enzymes found in all forms of life, catalyze the oxidation of PUFA and lipids containing a *cis*,*cis*-1,4-pentadiene structure (Wennman et al. 2016). This reaction produces lipid hydroperoxides that decompose and form secondary oxidation products, which can react with chlorophylls, carotenoids, ascorbic acid, phenols, α-tocopherol, etc. and cause alteration of colour and organoleptic properties (Siedow 1991; Li et al. 2007; Zilic et al. 2010). LOX has been found responsible for the generation of off-flavour due to its action on PUFA, especially on linoleic and linolenic acid, resulting in deterioration of flavour and quality (Dahuja and Madaan 2004; Wang et al. 2014; Mandal et al. 2014; Chen et al. 2016). Pearl millet has the high activity of LOX, therefore, the present study was carried out to evaluate its physico-chemical and kinetic properties, which could help in understanding its role in foul smell generation *vis-a-vis* shelf life of flour.

Materials and methods

Plant material

The mature grains of pearl millet inbred HBL 0843-2 were harvested from the field of Bajra Section of the Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar. All the chemicals used in estimation and purification of LOX were of high purity analytical grade.

Extraction and purification of LOX

Unless otherwise stated, all procedures during the extraction and purification of the enzyme were carried out at 0–4 °C temperature. The method described by Babitha et al. (2004) was followed for extraction of LOX with slightly modifications. Eight-gram mature grains were hand homogenized with acid treated sand in a prechilled pestle and mortar in a medium containing 0.2 M phosphate buffer (pH 7.5). The homogenate was filtered through four layers of cheese cloth and centrifuged at 15,000 rpm for 30 min in a refrigerated centrifuge to get crude extract. LOX in the crude extract was precipitated at 25–65% saturation of $(NH_4)_2SO_4$. The precipitates obtained after centrifugation at 15,000 rpm for 15 min were resuspended in 0.2 M phosphate buffer (pH 7.5) and dialysed for 24 h against same buffer with repeated changes of buffer. The dialysed $(NH_4)_2SO_4$ fraction was concentrated against solid sucrose. The concentrated $(NH_4)_2SO_4$ fraction (2 ml) was carefully layered over top of the Sephadex G-100 column (100 × 1.7 cm) pre-equilibrated with 0.2 M phosphate buffer (pH 7.5) and subsequently eluted with same buffer. Each fraction of 3 ml was collected at a flow rate of 12 ml per hour. The fractions with highest LOX activity were pooled and concentrated against solid sucrose. Finally, the concentrate was placed on top of the DEAE-cellulose column (60 × 3 cm) pre-equilibrated with 0.2 M phosphate buffer (pH 7.5). The column was eluted with 0.2 M phosphate buffer (pH 7.5) followed by a linear gradient of 0–0.5 M KCl in the same buffer. Each fraction of 3 ml was collected and analysed for protein (A_{280}) and enzyme activity. Fractions showing the enzymatic activity were pooled and concentrated using solid sucrose and stored at 4 °C for further studies.

Enzyme assay

LOX activity in crude extract and in the preparations during purification process was assayed by the method of Babitha et al. (2004). The reaction mixture (2.66 ml) contained linoleic acid solution in methanol (7.5 mM), phosphate buffer (0.05 M and pH 7.5) and enzyme preparation (0.1 ml). Reaction was started by the addition of enzyme, and the increase in absorbance was measured at room temperature for 2 min at 234 nm wavelength. LOX activity was measured by monitoring the formation of conjugated dienes from linoleic acid. One unit of enzyme is defined as the amount of enzyme that catalyses the formation of 1 μmol of product per minute at 234 nm wavelength.

Protein estimation

Protein in various fractions during purification was detected by measuring the absorbance at 280 nm wavelength. Quantitative estimation of protein at each step of purification was done by following the method of Bradford (1976).

Characterization of LOX

Determination of molecular mass of the purified LOX

Molecular mass of the purified LOX was estimated using Sephadex G-100 column, which was calibrated with the standard protein markers (2 mg/ml each), i.e., aprotinin (8 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (45.0 kDa), glutamate

dehydrogenase (60 kDa), alcohol dehydrogenase (100 kDa) and myosin (220 kDa). Void volume was calculated using blue dextran. The log molecular mass of each standard protein was plotted against elution volume and the molecular mass of the purified enzyme was calculated from the calibration graph. Polyacrylamide gel electrophoresis (10% gel) in the presence of SDS was used to determine the subunit molecular masses using the method of Laemmili (1970).

Determination of optimum pH for activity

To study the effect of pH, all the components of reaction mixture except buffer were kept same as explained in enzyme assay. Buffers of different pH values were used. The 0.05 M citrate and 0.2 M potassium phosphate buffers were used for the pH range of 3.0–6.0 and 6.1–8.0, respectively.

Determination of optimum temperature and thermostability

The effect of temperature on LOX activity was evaluated at temperature ranging from 10 to 60 °C. All the constituents of the reaction mixture were maintained at appropriate temperature in a water bath before starting the reaction. Thermostability of the purified enzyme was tested by measuring the residual activity after incubating the enzyme for 30 min in the temperature range of 25–65 °C.

Determination of apparent K_m

The activity of purified preparation was measured using linoleic acid as the substrate at final concentration varying from 0.3 to 2.88 mM. The value of K_m for linoleic acid was determined using the method of Lineweaver and Burk (1934).

Effect of chemical compounds

Activity of purified LOX was measured in the presence of ascorbic acid, benzoic acid, sodium azide, trolox, vitamin E and EDTA at different concentrations in the range of 0.01–5 mM.

Effect of metal ions

The effect of monovalent ions Na^+ and K^+, divalent ions Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+}, and trivalent ions Fe^{3+} at 10 mM concentration was determined. The residual activity was calculated by taking the control activity as 100%.

Results and discussion

Purification of LOX

LOX from pearl millet mature grains was purified using a three steps purification procedure. The specific activity in crude extract was 4.9 units per mg protein, while in $(NH_4)_2SO_4$ fraction, it increased to 60.4 units per mg protein with a recovery of 81% (Table 1). The size exclusion chromatography using Sephadex G-100 led to 20.9 folds purification with 56% recovery (Fig. 1a). During ion exchange chromatography using DEAE-cellulose as the matrix, two isozymes (LOX 1 and LOX 2) were purified with 56 and 40 folds purification, respectively (Fig. 1b). LOX 1 had specific activity of 274.2 units per mg protein, while LOX 2 had specific activity of 195.3 units per mg protein. The overall recovery of LOX 1 and LOX 2 was 28 and 24%, respectively. The achieved fold purification was comparable with LOX from wheat grains (Hsieh and McDonald, 1984). Using ammonium sulfate fractionation, Szymanowska et al. (2009) purified LOX by 21 folds.

Physico-chemical and kinetic properties

Molecular mass

The molecular masses of purified LOX 1 and LOX 2 as determined from gel filtration using Sephadex G-100 were 85 and 79 kDa, respectively (Fig. 2). Monomeric nature of purified LOX 1 and LOX 2 was established by the presence

Table 1 Summary of purification of LOX from grains of pearl millet inbred HBL 0843-2

Fraction	Volume (ml)	Total activity (units)	Protein (mg)	Specific activity (units/mg protein)	Fold purification	Recovery (%)
Crude extract	150	4500	905	4.9	1	100
$(NH_4)_2SO_4$(25–65%) saturation)	15	3630	60.1	60.4	12.3	81
Sephadex G-100	30	2616	25.5	102.6	20.9	56
DEAE-cellulose (LOX-1)	15	1275	4.7	274.2	55.9	28
DEAE-cellulose (LOX-2)	24	1086	5.6	195.3	39.8	24

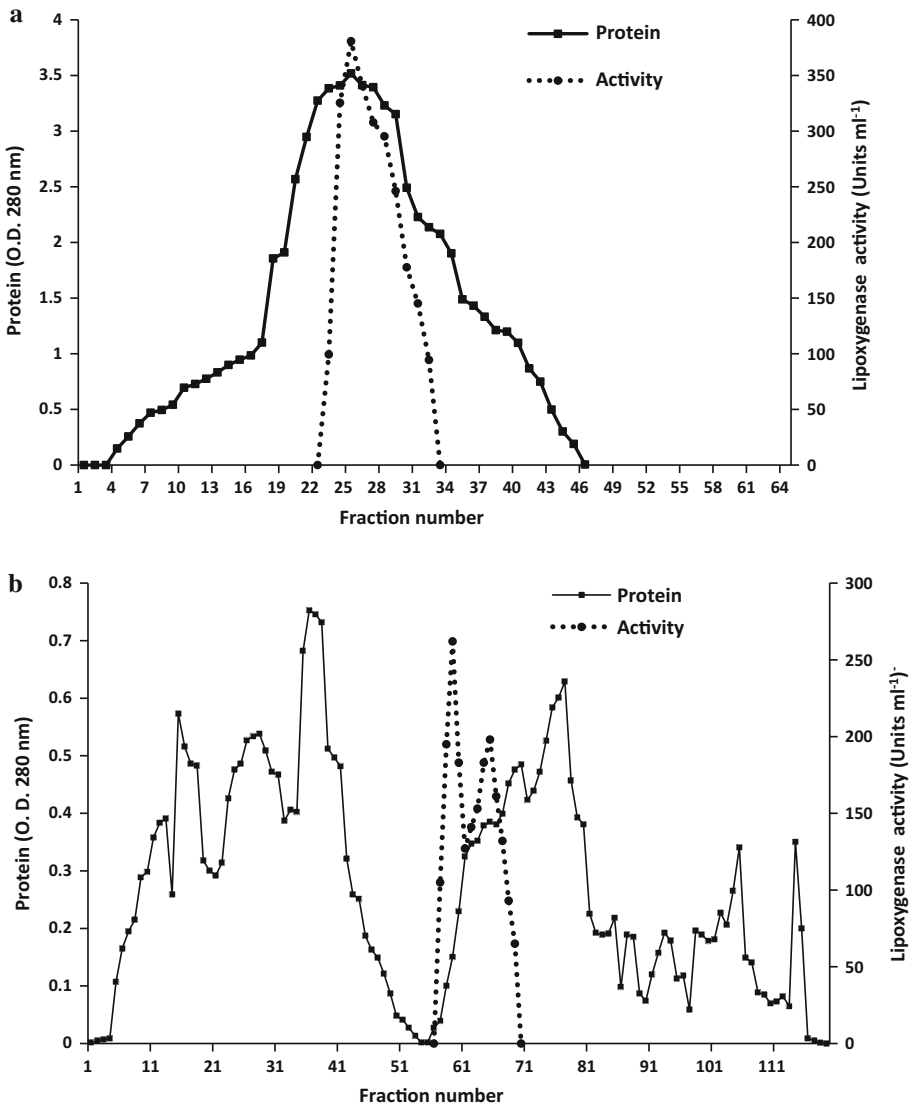

Fig. 1 **a** Elution profile of LOX on Sephadex G-100 column. **b** Elution profile of LOX 1 and LOX 2 on DEAE-cellulose column

of a single band on SDS-PAGE. The monomeric nature of LOX was in accordance with LOX extracted from germinating barley (Yang et al. 1993), durum wheat endosperm (Hsieh and McDonald, 1984), ungerminating barley (Peter et al. 1991), wheat germ (Shiiba et al. 1991), durum wheat semolina (Barone et al. 1999), tomato (Catherine et al. 1998), *Cichorium intybus* (Daglia et al. 2005), rice (Ratachatachaiyos and Theerakulkait, 2009) and *Pleurotus ostreatus* (Abdullah et al. 2014). The molecular mass of purified LOX from banana leaf was 85 kDa (Kuo et al. 2006). According to Babitha et al. (2004), LOX isozymes in pearl millet seedlings had molecular mass 83, 77 and 73 kDa for LOX 1, 3 and 6, respectively.

pH optima for activity

Enzyme activity as determined over a pH range of 3.0–7.0 increased from 83 units per mg protein at pH 3.0–270.3 units per mg protein at pH 4.5 for LOX 1, while the activity of LOX 2 increased gradually from 56 units per mg at pH 3.0 to maximum activity of 197 units per mg protein at pH 4.8. Thereafter, the activity declined gradually with the increase in pH. The results clearly indicated the value 4.5 and 4.8 as the optimum pH for purified LOX 1 and LOX 2, respectively (Fig. 3a), which are in conformity with the report for the enzyme from durum wheat endosperm having maximum activity at pH 4.8 (Hsieh and McDonald 1984). The purified LOX of *Pleurotus ostreatus* was found stable over a pH range of 5.0–8.5. However, for the reaction using linoleic acid substrate, the major optimum pH was 8.0 with a minor optimum at pH 4.5 (Abdullah et al. 2014). The pH stability of LOX from pearl millet grain was almost similar to that of the enzyme purified from *Pleurotus ostreatus* (Abdullah et al. 2014).

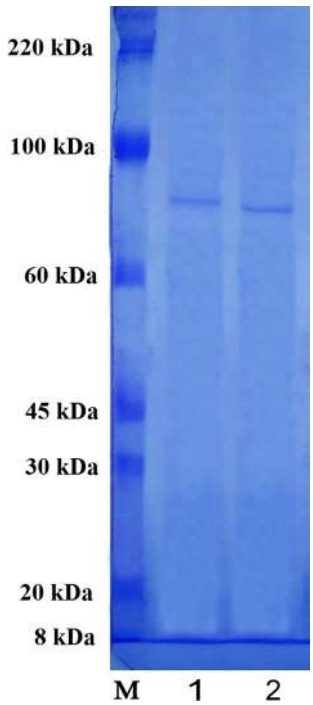

Fig. 2 SDS-PAGE pattern of purified LOX 1 and LOX 2 purified from grains of pearl millet inbred HBL 0843-2. *Lane M*: Standard Protein Markers, *Lane 1*: Purified LOX 1, *Lane 2*: Purified LOX 2

Temperature optima for activity and thermostability

The LOX showed maximum activity at 25 °C and lesser activity was observed at either side of this temperature (Fig. 3b). Generally, the loss in activity was severe at temperature above 30 °C since high temperature initiates unfolding processes that damage the active site. Lesser activity at low temperature might be attributed to decreased substrate or product diffusion rate (Wang et al. 2014). The present study results are in accordance with the earlier reports on LOX purified from pearl millet seedlings (Babitha et al. 2004) and rice (Ratachatachaiyos and Theerakulkait, 2009) who reported 25 °C an optimum temperature for activity of enzymes. However, Barone et al. (1999), Jacobo-Velazquez et al. (2010) and Altunkaya and Gokemen (2011) purified LOX from wheat, avocado and lettuce, respectively at 60 °C temperature. The isoforms purified in present study were stable at 30 °C with a pre-incubation period of 30 min, while they underwent rapid inactivation at higher temperature (Fig. 3c), however, the LOX enzyme purified from *Pleurotus ostreatus* was stable up to 45 °C (Abdullah et al. 2014), from olive pulp up to 60 °C (Lorenzi et al. 2006) and from sweet corn up to 50 °C (Theerakulkait and Barrett 1995). Overall, it appears that optimum reaction temperature for LOX was fairly broad due to high occurrence (~66%) of α-helices in 3D structure (Gardner 1996).

K_m values

Both, LOX 1 and LOX 2 followed typical Michaelis–Menten kinetics with K_m value of 0.86 and 0.57 μM for linoleic acid, respectively (Fig. 3d, e). Similar hyperbolic saturation curves for the substrates have been reported for the enzymes isolated from germinating barley (Yang et al. 1993), durum wheat endosperm (Hsieh and McDonald 1984), ungerminating barley (Peter et al. 1991), wheat germ (Shiiba et al. 1991), durum wheat semolina (Barone et al. 1999), *Cichorium intybus* (Daglia et al. 2005), rice (Ratachatachaiyos and Theerakulkait 2009) and *Pleurotus ostreatus* (Abdullah et al. 2014). The observed K_m value of isozymes from pearl millet grains was higher as compared to the enzymes from olive pulp (Lorenzi et al. 2006) and lettuce (Altunkaya and Gokemen 2011). Wide variation in K_m value might be attributed to the diversity of methods used in measuring the enzyme activity, difference in pH, degree of the enzymatic preparation purity, the nature of isozymes and their sources.

Effect of metal ions

The effect of various metal ions on LOX activity was studied. The data presented in Table 2 indicate that various metal ions differentially affected the activity of both the isoforms which was increased by Ca^{2+} and Mn^{2+} but strongly inhibited by Na^{+}, Zn^{2+} and K^{+}. Earlier, Koch (1986) asserted that divalent ions such as calcium, magnesium and manganese acted as activators for the LOX, while ions such as iron, copper and zinc inhibited its activity. Activation of LOX by Ca^{2+} has also been earlier reported in pearl millet seedlings and rice (Babitha et al. 2004; Ratachatachaiyos and Theerakulkait 2009). Fe^{3+} is considered essential for LOX activity in most of the plants (Baysal and Demirodoven 2007; Wennman et al. 2016). Zn^{2+}, Na^{+} and K^{+} inhibited the activity of both the isozymes to same extant. The inhibition of LOX by Zn^{2+}, Na^{+} and K^{+} has also been reported for aromatic rice and pearl millet seedlings (Ratachatachaiyos and Theerakulkait 2009; Babitha et al. 2004).

Effect of chemical compounds

The modulation of LOX 1 and LOX 2 activities by different compounds was studied. Vitamin E, ascorbic acid and trolox were found to inhibit LOX activity (Table 3). It has already been established that peroxyl radical

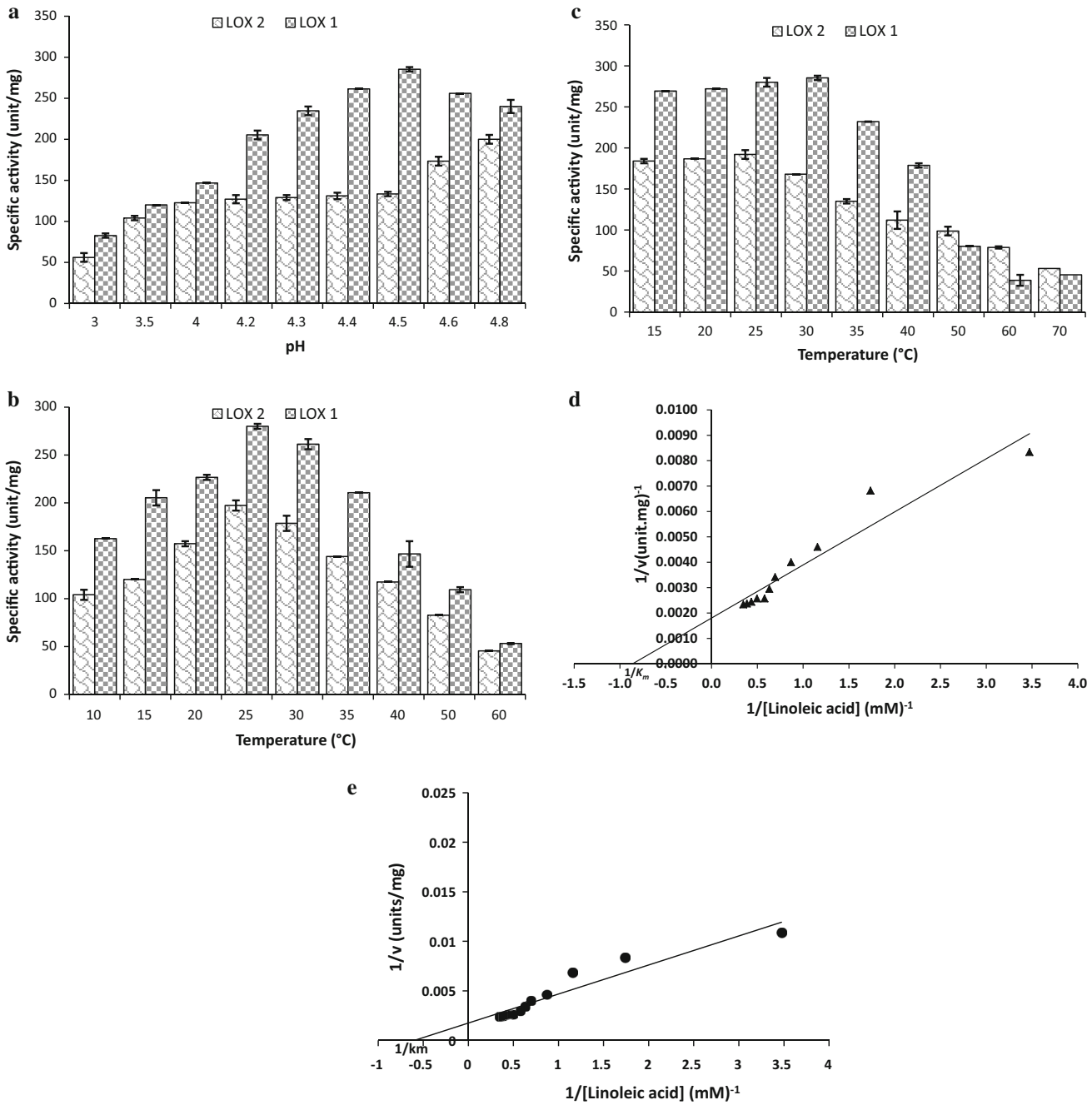

Fig. 3 Characterization of different physico-chemico-kinetic properties of purified LOX 1and LOX 2. **a** Effect of pH on the activity of purified LOX 1and LOX 2. **b** Effect of temperature on the activity of purified LOX 1 and LOX 2. **c** Effect of temperature on the stability of purified LOX 1 and LOX 2. **d** Lineweaver–Burk plot showing K_m value for LOX 1 as a function of linoleic acid concentration. **e** Lineweaver–Burk plot showing K_m value for LOX 2 as a function of linoleic acid concentration

intermediates are produced during LOX catalysed conversion of PUFA to hydroperoxides. The externally added antioxidants quench these radicals and thereby inhibit the activity of LOX (Zhou et al. 2005). The present results are supported by the earlier observed negative impact of these compounds on LOX from rice, pearl millet seedlings and pea (Ratachatachaiyos and Theerakulkait 2009; Babitha et al. 2004; Szymanowska et al. 2009). Sodium azide has no effect on purified LOX 1 and LOX 2 from pearl millet grains, which is in accord with the results obtained from pearl millet seedlings and potato tubers (Babitha et al. 2004; Galliard and Phillips 1971). Antioxidants like vitamin E and C, BHA and BHT have been reported as potent inhibitors of LOX in sweet corn germs (Theerakulkait and

Table 2 Effect of chemical compounds on the activities of purified LOX 1 and LOX 2

Compounds	% inhibition					
	LOX 1			LOX 2		
	Concentration (mM)			Concentration (mM)		
	0.1	1	5	0.1	1	5
Sodium azide	2	2	2	3	3	3
EDTA	3	7	21	3	11	25
Benzoic acid	3	20	38	7	30	43
Vitamin E	46	53	64	62	66	66
Ascorbic acid	23	44	58	56	61	69
Trolox	25	35	63	25	41	60

Table 3 Effect of various metal ions on activities of purified LOX 1 and LOX 2

Metal ions (10 mM)	Activity (% control activity)	
	LOX 1	LOX 2
Na^{+}	19	21
K^{+}	22	28
Mg^{2+}	91	100
Mn^{2+}	90	99
Zn^{2+}	21	31
Ca^{2+}	100	109
Fe^{3+}	97	101

Barrett 1995) and aromatic rice (Ratachatachaiyos and Theerakulkait 2009). LOX 1 showed more sensitivity toward inhibitors as compared to LOX 2. Both the isozymes were moderately inhibited by benzoic acid and EDTA.

Conclusion

The two isoforms of LOX from pearl millet grains were purified and characterized. Being cheaper, safer and easily available, vitamin E and ascorbic acid can be more reliable and potent inhibitors for lipoxygenase as compared to trolox, thus, can successfully be used in protecting the food from oxidation. The results presented herein can be helpful in proposing the strategies concerning the inactivation of LOX, which can prolong shelf-life of the products. Hence, the characterization of this enzyme might be useful in preventing the LOX detrimental effects on food quality during processing and storage.

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