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SHORT COMMUNICATION

Lipoxygenase activity in soybean is modulated by enzyme-substrate ratio

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Abstract The off-flavour development in soybean based food and oil industry is considered as a serious problem. In soybean three lipoxygenase isozymes namely LOX-1, LOX-2 and LOX-3 which contribute to about 1 % of storage protein have been reported and are the major culprits for the generation of volatile compounds causing the off-flavour. The present study showed that the 3 lipoxygenase isozymes isolated from defatted soybean flour exhibited inhibition potential by modulating the enzyme to substrate ratio. LOX-2 was the most inhibition prone enzyme. Defatting the flour may help in reducing off-flavour generation.

Keywords Soybean · Off- flavour · Defatting · Lipoxygenase isozymes · Inhibition

Abbreviations

LOX	Lipoxygenase
ESA	Enzyme specific activity
LS	Substrate solution
LA	Linoleic acid
LeA	Linolenic acid

There has been phenomenal expansion of soybean cultivation in India which has resulted in an increase in production leading to the growth of soy-based industries. Recently a great demand for soybean oil resulted in corresponding high yield of meal, a by- product of soybean oil. Although the fat-free (defatted) soybean meal is a primary, low-cost, source of protein, it is seldom being used in food, feed and pharmaceutical industry in India because of its objectionable flavour. Soybean oil is rich in polyunsaturated fatty acids which makes it nutritionally important but vulnerable to oxidation. Lipoxygenases (LOXs) (EC 1.13.11.12) are class of enzymes widely distributed in both plant and animal kingdoms, which oxidize unsaturated fatty acids containing a cis,cis 1,4 pentadiene system such as found in linoleic ($18:2^{\Delta 9,12}$) linolenic($18:3^{\Delta 9,12,15}$) and arachidonic ($20:4^{\Delta 5,8,11,14}$) acids(Hildebrand et al. 1988; Shibata and Axelrod 1995; Dahuja and Madaan 2003) . Once LOX has produced fatty acid hydroperoxides, a series of enzymatic reactions that further metabolize the hydroperoxides giving rise to a variety of oxygenated products called oxylipins (Blee 2002; Howe and Schilmiller 2002). One of the major oxylipin pathways is the hydroperoxide lyase pathway producing volatile aldehyde and alcohol compounds such as (2E) hexenal and (3Z) hexanal. These volatiles are associated with fishy, beany flavour development in soya based food products.

In soybean seeds, three distinct isoforms of LOXs have been described, based on differences in pH optima, substrate specificity and their product formation. Soybean seed isozymes are 94–97 kDa monomeric proteins with distinct isoelectric points ranging from about 5.7 to 6.4, and can be distinguished by marked difference in optimum pH, substrate preference, and product profile which are responsible for objectionable flavour composition (Siedow 1991; Mack et al. 1987). Kinetics of soybean lipoxygenases are related to the substrate availability and extraction procedures and it has been suggested that defatting inhibits

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lipoxygenase isozyme activity which indicates the possibility to modulate LOX activity by modifications of enzyme/substrate ratios and modifications of pH in the enzyme environment (Chedea et al. 2008).

Matured soybean seeds (cultivar P20) were ground to fine powder in a Cyclotec mill and defatting of full soybean was performed by extraction with hexane until the solvent became colourless. For LOX activity, the substrate LS solution of 10 mM was prepared by adding 35 μl of linoleic acid to 1.6 ml O_2 free double distilled water containing 25 μl Tween 20. The linoleic acid was dissolved by homogenization avoiding air bubbles, and facilitated by adding 0.5 N NaOH solution until the solution became clear. The final volume was brought to 10 ml by the addition of DD H_2O to yield a clear LS solution. The LOX activity for all standard solutions of soybean extracts was determined by a modified method of Axelrod et al. (1981). The time course of the peroxide formation was measured by the increase of absorbance at 234 nm using a Specord 200 spectrophotometer at 25^0C. For LOX-1 activity 0.2 M borate buffer pH 9.0, for LOX-2 activity 0.2 M sodium phosphate pH 6.8 and for LOX-3 activity 0.05 M sodium phosphate pH 7.1 were respectively used. To obtain the kinetic curves and determine the curve parameter, standard protocols were followed. Total protein content of the three extracts was determined by the Bradford method using gamma globulin as standard. The peroxide identification in all solvents and extracts was also checked using the potassium iodide test and was negative. The inhibition potential of lipoxygenase isozymes was carried out by isolating LOX-1 extracted by incubating defatted soya in 1:6 ratios with 0.2 M sodium acetate buffer, pH 4.5 for 1 h. After centrifugation at 16,000 g for 30 min, the pH was adjusted to 6.8 with NaOH solution. The LOX-2 and LOX-3 were extracted from defatted soya with 0.2 M phosphate buffer pH 6.8 in the same ratio.

The kinetics of soybean lipoxygenases is related to substrate availability and extraction procedures. Because of the specific kinetic behaviours of the three different LOXs found in mature crude soy seed extracts involved in off flavour generation one can modulate the inhibition of these isozymes during soybean processing. The main factors, which were considered as modulators of LOX catalysis, were the pH and the ratios of enzyme protein per substrate, as indicators of enzyme affinity in different environments (Chedea et al. 2008). Based on their observations the LOX isozymes were extracted from defatted soybean and compared their inhibition potential by determining the product formation. Figure 1a represents the kinetic curves registered at 234 nm for soya extract LOX-1 activity measured at pH 9.0 corresponding to the production of 13-HPOD. The data presented in the Table 1 represents comparatively the specific activities (ESA) corresponding to the progression curve of soy extract LOX-1, LOX-2 and LOX-3 with different E/LS ratios. In the case of LOX-1, a conventional shape of kinetic curve was obtained

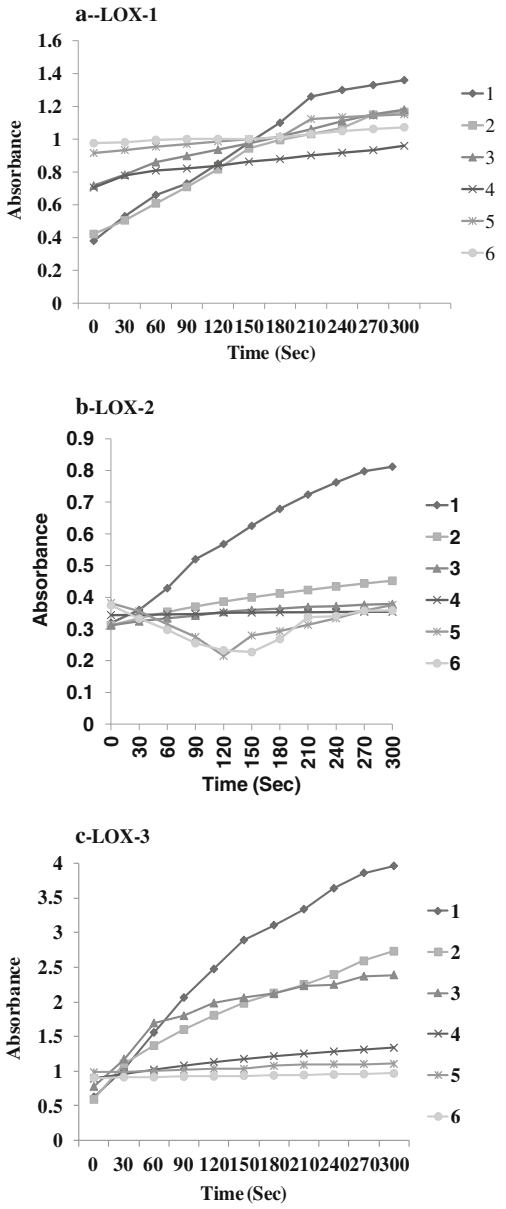

Fig 1 **a**–**c** Comparison of inhibition potential of lipoxygenase isozymes by defatting 1–6 represents different enzyme to substrate ratio as given in Table 1. **a** LOX-1 activity using different enzyme to substrate combination registered at 234 nm at pH 9.0. **b** LOX-2 activity using different enzyme to substrate combination registered at 234 nm at pH 6.8. **c** LOX-3 activity using different enzyme to substrate combination registered at 280 nm at pH 7.1

when the E/LS ratio of 0.10×10^{-3} μg/nanomole was used (Fig. 1a). But when this E/LS ratio was increased from 0.10 to 3.35×10^{-3} μg protein/nanomole the ESA decreased dramatically around 250 fold (from 17.33 to 0.07×10^{-3} AU/μg/s). The shape of the curve changed from conventional 'rectangular hyperbola' to 'plateau' shaped. The major reason behind this shape of curve is the initial burst phase rapidly decreased with increasing E/LS ratios and the plateau phase increased with increase in E/LS ratio. When the E/LS ratio was 2.5

Table 1 Effect of defatting on soybean LOX isozymes activities

Sl.No	Protein (μg)	E/LS (10^{-3} μg/nmole)	dA/dt (10^{-3} AU/s)	ESA(10^{-3} AU/μg/s)
LOX-1				
1	0.06±0.06	0.10±0.09	1.04±0.71	17.33
2	0.34±0.02	0.69±0.04	0.76±0.03	2.22
3	0.69±0.04	1.38±0.07	0.49±0.07	0.71
4	0.92±0.02	1.85±0.03	0.27±0.02	0.29
5	1.28±0.02	2.55±0.04	0.15±0.05	0.12
6	1.68±0.03	3.35±0.05	0.11±0.04	0.07
LOX-2				
1	0.12±0.04	0.23±0.07	0.51±0.16	4.40
2	0.46±0.04	0.93±0.08	0.13±0.03	0.29
3	0.85±0.01	1.70±0.02	0.07±0.02	0.08
4	1.67±0.02	3.35±0.04	0.01±0.02	0.00
5	2.54±0.02	5.09±0.04	−0.01±0.00	0.00
6	2.54±0.02	10.16±0.08	−0.01±0.00	−0.01
LOX-3				
1	0.46±0.04	0.23±0.02	3.66±0.18	7.95
2	0.59±0.01	0.30±0.01	2.42±0.04	4.10
3	0.91±0.05	0.46±0.03	1.73±0.04	1.90
4	1.64±0.01	0.82±0.01	0.44±0.02	0.27
5	1.64±0.01	3.31±0.04	0.11±0.02	0.07
6	1.64±0.01	6.56±0.04	0.06±0.03	0.04

The values are presented as Mean±SD of three independent experiments

(critical threshold) the behaviour of the enzyme started changing with almost no initial burst phase. These data demonstrated that the low E/LS ratio favours the catalytic reaction, while the excess of protein inhibits it. Figure 1(b) represents the kinetic curves registered at 234 nm for soya extract LOX-2 activity measured at pH 6.8 corresponding to the production of a mixture of 13 and 9 HPOD. In case of LOX-2 a conventional shape of kinetic curve was obtained when the E/LS ratio of 0.23×10^{-3} μg/nanomole was used. But when this E/LS ratio was increased from 0.23 to 10.16×10^{-3} μg protein/nanomole the ESA decreased dramatically to about 440 fold (from 4.40 to -0.01×10^{-3} AU/μg/s). In higher E/LS ratios, LOX-2 showed a nonconventional pattern having initial burst, a drifted lag phase and a plateau. But this was lifted towards the conventional shape containing an exponential burst phase followed by a plateau by down-regulating the E/LS ratio. Around 1.7 E/LS ratio (critical threshold) the initial burst phase started diminishing and plateau phase started increasing. The maximum change in shape of the curve was observed in case of LOX-2 with a typical mirror type relationship with enzyme to substrate ratio with enzyme specific activity. Figure 1(c) represents the kinetic curves registered at 280 nm for soya extract LOX-3 activity measurements. It is known that LOX-3 exhibits its maximal activity around pH 7.0 and displays a moderate preference for producing 9-HPOD (Ramadoss et al. 1978) but produces ketodiene. A conventional shape of kinetic curve was obtained in case of LOX-3 when the E/LS ratio of 0.46×10^{-3} μg/nanomole was used (Table 1). But when this E/LS ratio was increased from 0.46 to 6.56×10^{-3} μg protein/nanomole the ESA decreased almost around 50 fold (from 1.90 to 0.04×10^{-3} AU/μg/s). When the E/LS ratio was 0.23×10^{-3} μg/nanomole there was no plateau phase and only exponential burst phase was observed. The isozyme LOX-3 is inhibited the least among all the isozymes although with increase in E/LS ratio the decrease in ESA was observed. The shape of the curve started changing around E/LS of 0.8.

Chedea et al. (2008) have reported defatting as a simple operation by which the PUFA substrates are eliminated from seeds and can thereby modulate the LOX isozyme activity by manipulating pH as well as enzyme to substrate ratio in enzyme environment. Further, defatting mediated inhibition potential of these LOX isozymes was also compared. All the LOX isozymes (LOX-1, LOX-2, and LOX-3) isolated from defatted soybean exhibited inhibition potential by modulating the enzyme to substrate ratio. LOX-2 was found to be the most inhibition prone enzyme. This augurs well with the fact that LOX-2 can oxygenate the esterified unsaturated fatty acids in the membrane as well as free fatty acids (Maccarrone et al. 1994) and can contribute immensely for hexanal production. There is a specific mirror type relation between enzyme/substrate ratio and enzyme specific activity for LOX-2.

The present study was aimed to comparatively judge the lipoxygenase isozyme activity and their inhibition potential

by defatting. It was clearly evident that LOX-2 isozyme is the predominant isozyme for off flavour generation and is the most inhibition prone by defatting. This can be further followed by future inhibition studies on LOX-2 by natural antioxidants and bioactive compounds present in soybean thereby solving a serious problem of off-flavour in soybean oil and food processing industries.

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