Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 [7] June 2014: 06-11

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Purification and Characterization of Hydroperoxidelyase from soybean (*Glycine max*)

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ABSTRACT
A fatty acid hydroperoxidelyase (HPL) enzyme from soybean (Glycine max) seeds was purified by ammonium sulphate precipitation followed by ion exchange chromatography on DEAE-Cellulose and DEAE-Sephacel. HPL showed specific activity of 161U/mg with fold purification of 16.4 and enzyme yield was found to be 9.06%. The purified enzyme on SDS-PAGE showed a prominent band of ~53KDa. Kinetic studies were carried out for the HPL enzyme which revealed Vmax and K_m value of 275.48 x 10^{-3} ΔOD/min and 25μm respectively. Natural antioxidants of soybean such as α-tocopherol, genistein, glycetein, daidzein and ascorbic acid were subjected under In vitro studies to analyse inhibitory effect on the enzyme. Among all the natural antioxidants, ascorbic was found to be potent inhibitor with IC^{50} value 39.25 μm.
Key words:hydroperoxide lyase, soybean, ascorbic acid, DEAE cellulose, kinetic studies and inhibition

Received 29.04.2014 **Revised 12.05.2014** **Accepted 09.06.2014**

INTRODUCTION

Soybean is one of the most valuable crops in the word not only as oil seed crop but also as a good source of protein for the human diet, it is one of the important ingredients in many processed foods and is rich in linoleic and α-linolenic acid[1]. Fatty acid hydroperoxidelyase (HPO lyase) is an enzyme that cleaves a C-C bond in the hydroperoxides (HPOs) of polyunsaturated fatty acids to generate aldehydes and omega-oxo-acids. This enzyme is widely distributed and is involved in the biosynthesis of volatileC_6 and C_9aldehydes and alcohols [2] [3],which are the important contributors to the characteristics flavors of fruits, vegetables and green leaves, because of their "fresh green" odour, they are widely used as food additives, for example, to restore food freshener after sterilization process and their short chain aldehydes also play an important role in would healing and pest resistance[4].One of the hydroperoxide product originating from linolenic acid, 2Z-hexenal, is an effective fungicide and bactericide in vitro [5]. The other product, 12-oxo-9Z-dodecenoic acid, can undergo isomerization to 12-oxo-10E-dodecenoic acid, which has been proposed as the plant wound hormone traumatin which stimulate cell division near the wound site, resulting in the formation of a protective callus around the wound [6]. Apart from HPL involvement in these beneficial pathway, it also play an important role in lipoxygenase (LOX) mediated oxylipin pathway,where it utilizes Lox catalysed products such as fatty acid hydroperoxides as a substrate to produce volatile aldehyde and alcoholic compounds such as 2E hexenal and 3Z hexenal and these volatiles are associated with fishy,beany flavour development in soy based food products and also reduces the storability of the soybean and inhibit the germination[7]. Hydroperoxidelyase is membrane bound enzyme localized mainly in chloroplast lamellae [8] and was first suspected in banana [9].HPL presence was later demonstrated in various plant leaves and fruits. The hydroperoxidelyase was reported from soybean leaves, seeds and seedling [10], other plants liketea leaves [11], bell pepper fruits [12], tomato leaves [13], cucumber [14], sunflower [15], tomato fruit[16] and pea seedlings [17]. Several procedures were employed to purify the enzyme, many of the researchers used either Triton X-100 [18],[19] or Tween 20 [8] to solubilize lyase from either whole tissue homogenate or cellular particulate fractions and to enhance the formation of HPL catalyzed product such as(E)-2-nonenal and 9-oxononanoic acid from linoleic acid[20].

The report on purification and characterization of HPL from soybean is limited. Therefore in the present study an attempt has been made to purify HPL enzyme and to study inhibitory effects of naturally occurring antioxidants on HPL.

MATERIALS AND METHOD

Plant material: 14 varieties of soybean (Table1) varying in germination % were obtained by Division of Seeds Science and Technology, IARI, New Delhi. Among all the genotypes of soybean P-884 showed very high HPL activity was used for purification of the enzyme.

Table (1): HPL activity in soybean (*glycine max*) genotypes

Line	Name of the Genotype	Seed coat colour	HPL activity
P1	P-218	Yellow	14.79±0.86
P2	P-222	Yellow	11.53±1.02
P3	P-241	Yellow	9.34±0.17
P5	P-732	Yellow	32.98±1.08
P8	P-876	Yellow	11.95±0.35
P9	P-884	Yellow	40.34±1.37
P10	P-898	Yellow	35.44±1.79
G2	DS-74	Black	6.68±0.83
G9	G-2253	Black	6.06±1.60
G14	G-2651	Black	6.85±0.02
G17	M-1090	Black	8.05±0.71
G18	M-11913	Black	4.30±0.28
G20	TGX444-422	Black	0.39±0.08

HPL activities were expressed as $\Delta OD min^{-1} mg^{-1}$ protein and experiment was carried out in triplicates with $P<0.05$.

Enzyme extraction: *crude extract*. A temperature of 4^0C was maintained throughout the enzyme extraction procedure.10g of overnight soaked (in distilled water) soybean seeds were homogenized with pestle and mortar in 100 ml ice-cold buffer A(buffer A consisting of 150mM Tris-HCl (pH-8.5),0.3% (w/v) CHAPS, 3mM DTT, 3mM EDTA and 30% w/v glycerol) and centrifuged at 12000g for 30min. the supernatant was filtered through muslin cloth and filtrate was used as a crude extract.

Partial HPL purification

Ammonium sulphate was added to the crude extract to bring into 50% saturation, and was stirred for 30 min. After centrifugation at 12000g for 30 min, additional ammonium sulphate was added to the resulting supernatant to achieve 75% saturation; the mixture was stirred for 20 min and centrifuged at 12000g for 10 min. The pellet was dissolved in a small volume of buffer A. This fraction was dialyzed against buffer B (composed of three fold dilution of buffer A) for 12 h with three changes of buffer B, and then centrifuged at 12000g for 10 min, the resulting extract was loaded on aDEAE cellulose column (0.75cm×25cm) (Sigma, India) equilibrated with buffer B. The column was washed with buffer B at the flow of 0.5ml/min. Active fractions (5ml) from anion chromatography were pooled after monitoring for OD at 280nm and assayed for HPL activity, then the active fractions were reloaded onto a DEAE-Sephacel column (Sigma, India) and eluted as described above.

Enzyme activity measurements*:* A typical assay consisted of 2600µl of assay mixture [3.5mg of NADH and 6.6mg of Yeast alcohol dehydrogenase in a 50ml of 100mM sodium phosphate buffer (pH 6.0)] and 200 µl of enzyme extract.Aldehydes and ω-oxoacids, the products of hydro peroxide lyase served as the substrate for the enzyme yeast alcohol dehydrogenase that uses NADH as its cofactor. Activity was determined at 4^0C by monitoring decrease in the absorbance due to oxidation of NADH to NAD^+ at 340nm by the NADH-coupled enzyme assay as described by Vick [21]. The difference in absorbance was recorded for every 30 second interval for 6 minutes. The hydroperoxidelyase activity was expressed as ΔOD/min/mg of protein. The molecular mass of the purified hydroperoxidelyase was determined by SDS PAGE (Leammli, 1970) using high range molecular mass standards (G-bioscience India). Proteins were stained with Coomassie Brilliant Blue R250.

Substrate Preparation

Lipoxygenase solution and 2. 8mM Linoleic acid was prepared by dissolving 60mg of lipoxygenase in 6ml of 10mM borate buffer pH 8.9 and 21µl of linoleic acid in 2 ml of distilled water respectively and 20 µl of Tween 20 and 300µl of 0.5N NaOH were added. Final volume was made to 10ml by adding double distilled Water.35.5 ml of double distilled water and 7.5 ml of 8mM Linoleic acid was added to 3ml of

lipoxygenase solution and was subjected to stirring for 20 minutes to supplycontinuous oxygen to form a substrate called fatty acid hydroperoxide.

Inhibitor assay and kinetic studies

Enzyme inhibition experiments were carried out by pre incubating the 0.2 ml of enzyme with 10 µl of inhibitor compounds with different concentration (Table 3) and 2.6ml of Sodium phosphate buffer (100mM; pH 6.0), after 10 min, 10 µl of hydroperoxide fatty acid solution (substrate) was added and effects of potential inhibitors on purified HPL activity was examined by measuring the decrease in absorbance at 340nm as previously explained in NADH-coupled enzyme assay. Kinetic study of HPL was done by measuring the purified enzyme activity at different substrate (hydroperoxide fatty acid) concentration. Lineweaver-Burk Plot was drawn as 1/V at Y axis against 1/[S] at X axis. From the plot Vmax and Km value were calculated. Km and V max were determined in triplicate conditions at 340nm.

RESULTS AND DISCUSSION

Purification of HPL:

Table 2 indicates that, from a crude extract of soybean mature seeds containing 2175 mg of protein, only 206.5 mg of protein could recovered by 50-75% ammonium sulphate precipitation. All the eluted fractions from DEAE cellulose column were analyzed for HPL activity and protein content. An early single peak of HPL activity was obtained and fractions having HPL activity were pooled together and specific activity of 161.09 U/mg proteins was determined with a purification fold of 16.47 and9.0% of protein recovery was observed. To ensure the activity of the enzyme, HPL activity was measured throughout the purification step by spectrophotometric method and protein content in each fraction were also regularly monitored. During the purification procedure it was observed that the activity of the enzyme was lost considerably on storage at 4°C within one day. Our earlier experiments with 0.1 M Tris-Cl buffer having TritonX-100 showed that enzyme activity was lost at a faster rate (data not shown). Hence the buffer was changed to 0.15M Tris-Cl (pH 8.5) with CHAPS to achieve improved stability of the enzyme. The instability of tomato HPL on storage at 4°C and 20°C has been reported by Suurmeizer [16], they found that, HPL activity was retained at - 80°c without any additives for several months. However more studies are required to study stability of the enzyme during storage. HPL was purified from strawberry showed 21 fold purification with only 1.5% yield using ammonium sulphate precipitation followed by chromatography on Sephacel S-300 followed by DEAE-Sepharose [22]. Horonsta*jet.al.*[17] found poor recovery of about (3%)and10 fold increase in specific activity using DEAE–toyopearl 650Manion exchange chromatography followed by PEG 6000 precipitation and chromatography of the resuspended PEG pellet on hydroxylapatite chromatography, in addition to this, they also noticed in pea seedlings that, purified HPL lost its stability during storage in absence of DTT. Two HPL isozymes having substrate specificities to 9-hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid were purified from cucumber showed 80 and 82 fold purification respectively [23] and observed relative enzyme stability at 4°C for more than 3 weeks with loss of its activity within 2 min at 50°C. Two isozymes of HPL purified from sunflowershowed166 to 192 fold purification using combination of ion-exchange, hydrophobic interaction and gel filtration chromatography to 166 and 192 fold respectively [15]. Difference in the fold purification value for the single HPL enzyme might be attributed to existence of different isoforms of the enzyme and their spatial and temporal distribution in the plant, it also depends on the type of ion exchange material used in combination with different suitable chromatographic methods inturn leads to variation in the fold purification values.

Table (2): Purification of HPL from soybean

	Total Protein (mg)	Total activity (ΔA/min.× 10^{-3})	Specific activity(ΔA/min./ mg protein × 10^{-3})	Yield purification (%)	Fold purification
Crude	2175.00	21271.50	9.78	100.00	1
$(NH_4)_2SO_4$ 0-50 % supernatant	1584.00	15400.00	9.72	72.39	0.99
$(NH_4)_2SO_4$ 50 -75% pellet	206.60	3820.00	18.48	17.95	1.88
DEAE cellulose	25.00	2893.75	115.75	13.60	11.83
DEAE sephacel	11.97	1928.25	161.09	9.06	16.47

Electrophoresis of purified protein on SDS-PAGE showed a major band of ~53kDa (Fig.1). Similar kind of result was reported from sunflower that, purified HPL protein was appeared as a major band at 53kDa on SDS-PAGE also further analyzed for its nature of occurrence in the cell and found that purified protein was tetrameric in nature [15]. HPL with molecular weight of 55kDa was also reported from cucumber and [23] bell pepper [12] and two HPL isozymes from tea leaves showed molecular weight of 53kDa and 55 kDa[11].

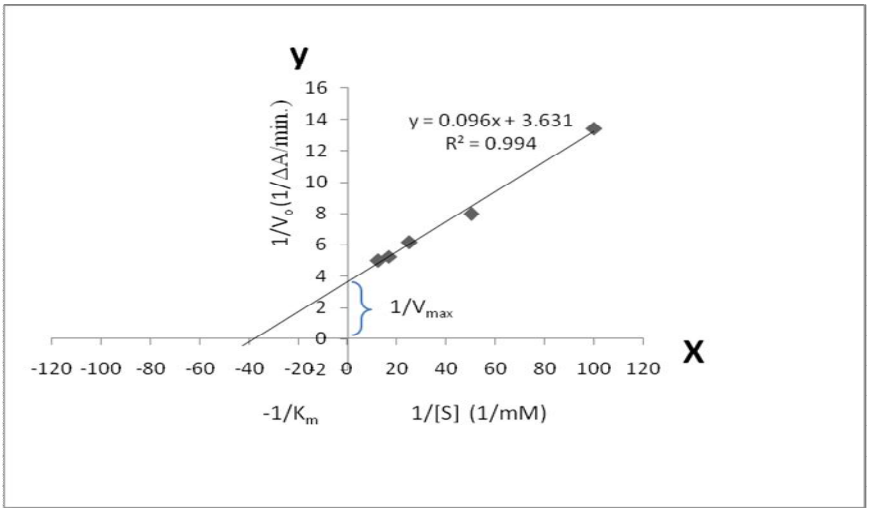

Figure (1): Lineweaver- Burk plot for determination of K_m and V_{max} for purified hydroperoxide lyase from soybean

Kinetic studies In order to determine the K_m and V_{max} of HPL,its activity was assayed using different substrate concentration. Based on the data shown in Table 4,Vmax and Km values were derived from Line Weaver-Burk plot(Fig.2). The V_{max} and $K_m$for purified HPL was found to be 275.45 x 10^{-3} ΔOD/ min and 25µM respectively. α-LNA13(Z,E,Z-HPO) was reported to be the best substrate for green bell pepper HPL with Km value of 9.8µm as compared to LA13(Z,E)HPO and γ-LNA(Z,Z,E)-HPO [17]. HPL isolated from strawberry had Km value with 13-HPOT of 62µM and Vmax of 4.3µm/min, and $k_m$value with 13-HPOD 102µM and Vmax of 0.3mM/min and observed that 13HPOT as a better substrate for strawberry HPL [22]. Kinetic studies have shown that HPL from pea seeds had a slightly greater affinity for 9-hydroperoxy-linoleic acid followed by 13-hydroperoxy-linoleic acid [17]. Gardner et al. [10] reported that, HPL from soybean seedlings had k_m values of about 200µM and 120µM for LA13 (Z,E)-HPO and α-LNA 13 (Z,E,Z)-HPO respectively and these k_m values are different from the values obtained for HPL from soybean chloroplasts (650µM and 430µM respectively). The large difference in substrate specificity and kinetic parameter of HPL indicate the possibility that, various types of HPL exist in plant kingdom [12].

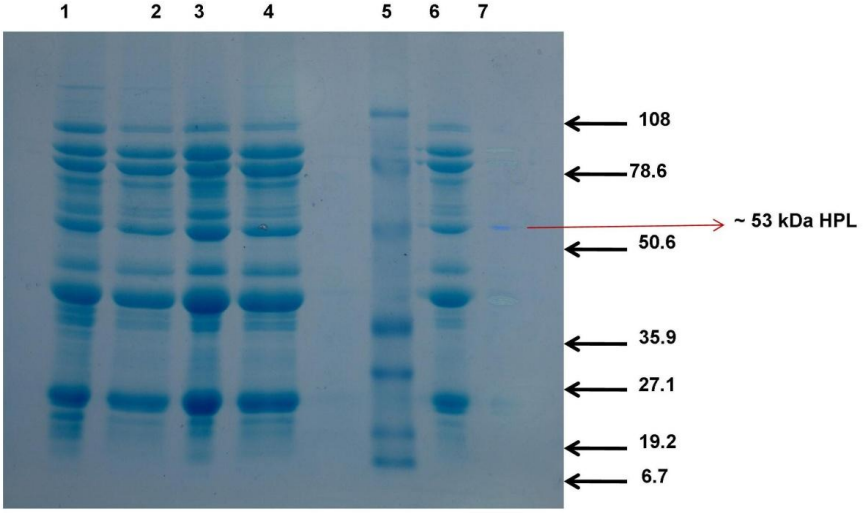

Figure (2):SDS-PAGE profile of protein fractions during purification of HPL:
Lanes: 1- Crude extract, 2- Ammonium sulphate (0-50%) pellet, 3- Ammonium sulphate (0-50%) supernatant, 4- Ammonium sulphate (50-75%) pellet, 5- Molecular weight marker, 6- DEAE- Cellulose fraction and 7- DEAE- Sephacel fraction.

Table (4): HPL activity at different concentration of HPL substrate (fatty acid hydroperoxide)

1/[S] (1/µM)	1/V(1/ΔOD/min.)
12.5	4.99±0.32
16.66	5.25±0.11
25	6.15±0.27
50	7.98±0.06
100	13.44±0.09

Hydroperoxide lyase activity was measured at 340nm with different concentration of fatty acid hydroperoxide and experiment was carried out in triplicates with *P<0.05.*

Inhibitor studies

The effect of isoflavones and free radical scavenging antioxidants like alpha tocopherol and ascorbic acid on the activity of purified HPL were studied. Ascorbic acid was found to be the most potent inhibitor with IC50 value 39.35µM and among the isoflavones,genestien showed maximum inhibition with IC50 value 146.79µM followed by α-tocopherol, glycetein and diazein (Table 3).Shibata et al [12] observed potent inhibition of HPL activity by natural antioxidants such as α-tocopherol (0.1mM) and mild inhibition of HPL by ascorbic acid (0.2mM).

Table (3): Inhibition studies of the purified HPL enzyme using natural antioxidants

Name of Inhibitor	IC^{50} value for inhibition of hydroperoxide lyase (µM)
α-Tocopherol	220.00±0.08
Genistein	146.79±0.05
Glycitein	404.31±0.16
Daidzein	529.66±0.24
Ascorbic acid	39.35±0.28

Hydroperoxide lyase activity was measured at 340nm after 10 min of incubation with the above inhibitors and Experiments were conducted in triplicates and $P<0.05$.

CONCLUSIONS

In the present study HPL has been purified to 16 fold and SDS PAGE showed the molecular weight to be ~53KDa. Ascorbic acid was found to be the most potent inhibitor. Among the isoflavones, genestien showed maximum inhibition. However more studies are required to find out substrate specificity of HPL.

REFERENCES

1. Masuda,T., Goldsmith, P.D.(2009). World Soybean Production: Area Harvested, Yield, and Long-Term Projections. International Food and Agribusiness Management Review.,12 (4):145-164.
2. Hatanaka, A., Kajiwara, T. and Sekiya, J. (1987) Biosyntheticpathway for Q-aldehyde formation from linolenic acid in greenleaves. Chem. Phys. Lipids 44: 341-361.
3. Gardner, H.W., (1991) recent investigation into lipooxygenase pathway of plants. Biochem. Biophys. Acta.1084:221-239.
4. Noordermeer, M.A., Veldink, G.A. and Vliegenthart J.F. G. (2001) Fatty Acid Hydroperoxide Lyase: A Plant Cytochrome P450 Enzyme Involved in Wound Healing and Pest Resistance. ChemBiochem, 2:494 -504
5. Croft KPC, JuttnerF,Slusarenko AJ (1993).Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with Pseudomonas syringaepv-Phaseolicola. Plant Physiol. 101: 13–24.
6. Zimmerman, D.C., Coudron, C.A. (1979). Identification of traumatin, a wound hormone, as 12-oxo-trans-10-dodecenoic acid. Plant Physiol.,63:536–541.
7. Gardner,H.W., Dornbos,D.L.and Desjardins, A.E. (1990). Hexanal, trans-2-hexenal, and trans-2-nonenal inhibit soybean (Glycine max) seed germination. J. of Agric. & Food Chem.,38:1316-1320
8. Evans, C.D., List, G.R., Moser, H.A. and Cowan, J.C.(1973). Longterm storage of soybean and cotton salad oils. *J.Am. oil. Chem. Soc*.50: 218-222.
9. Tressel, R., Drawert, F. (1973). Biogenesis of banana volatiles. J. Agric. Food Chem., 21(4):560-565.
10. Gardner, H.W., Weisleder, D., Plattner, R.D. (1991).Hydroperoxidelyase and other hydroperoxide metabolizing activity in tissues of soybean, Glycine max.Plant Physiol.,97:1059-1072.
11. Matsui, K., Toyot.H.,Kajiwara,T., Kakuno, T. and Hatanaka, A.(1991). Fatty acid hydroperoxide cleaving enzyme, hydroperoxidelyase, from tea leaves. Phytochem., 30: 2109-2113.
12. Shibata, Y., Matsui, K.,Kajiwara, T. and Hatanka, A. (1995). Purification and properties of fatty acid hyderoperoxide lyase from green bell paper fruits. *Plant Cell Physio*. 36(1):147-156.

13. Fauconnier, M.L., Perez, A.G., Sanz, C, and Marlier, M. (1997). Purification and characterization of tomato leaf (Lycopersiconesculentum Mill.) hydroperoxidelyase. *J. Agric. Food Chem*., 45:4232-4236.
14. Phillips, D. R. and Galliard,T. (1978). Flavour Biogenesis Partial Purification and Properties of A Fatty Acid Hydroperoxide Cleaving Enzyme from Fruits of Cucumber. *Phytochemistry* .,17 : 355-358.
15. Itoh, A., Vick, B.A.(1998). The purification and characterization of fatty acid hydroperoxidelyase in sunflower. Biochem. *Biophys. Acta*., 1436:531-540.
16. Suurmeijer,C.N., Perez-Gilabert, M., van Unen, D.J., van der Hijden, H.T.,Veldink, G. A., Vliegenthart, J. F. (2000). Purification, stabilization and characterization of tomato fatty acid hydroperoxidelyase. *Phytochemistry*.,53: 177-185.
17. Hornostaj, A.R., Robinson, D.S. (2000).Purification of hydroperoxidelyase from pea seeds. *Food Chemistry*.,71:241-247.
18. List, G. R., Mounts, T.L., Warner, K. and Heakin, A.J. (1978). Steam refined soybean oil: effect of refining and degumming methods on oil quality. 55: 277-279.
19. Warner, K. and Mounts, T.L. (1984). Flavor and oxidative stability of hydrogenated and dehydrogenated soybean oils: efficacy of plastic packaging. *J.Am. oil. Chem. Soc.* 61: 548-551.
20. Mounts, T.L., Warner, K. and List, G.R. (1981). Flavor and oxidativestability of hydrogenated and unhydrogenated soybean oils: Effects of teritiary butyl hydroquinone. *J. Am. oil. Chem*. Soc.58:792-794.
21. Vick, B. A. (1991). A spectrophotometric assay for hydroperoxidelyase. Lipids. 26: 315-320.
22. Pérez, A.G., Sanz,C.,Olias,R. and Olias.J.M.(1999). Lipoxygenase and hydroperoxidelyase activities in ripening strawberry fruits. *J. Agric. Food Chem*., 47:249-253.
23. Hornostaj,A.R.,Robinson, D.S.(1999). Purification of hyderoperoxidelyase from cucumbers. *Food Chemistry*., 66:73-180.

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