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CHARACTERIZATION OF HORSE GRAM (*DOLICHOS BIFLORUS*) LIPOXYGENASE

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Lipoxygenases LOX, (EC: 1.13.12.11) of horse gram (HG) (*Dolichos biflorus)* was purified to electrophoretic homogeneity and its partial characterization was made using UV CD spectrum. The purification and electrophoretic analysis of horse gram lipoxygenase has revealed that it is a monomeric protein with a molecular mass of 95±5 KDa. This enzyme showed optimum activity at pH 6.8 in between the temperature of 25-35°C, and had K_M values for Linolenic and Arachidonic acids as 9.95 and 15.5µmoles, respectively. This protein on the far UV CD absorption the spectra, showed a negative dip at 208 and 222nm to indicate the presence of α-helix and β strands and near UV CD spectra showed maximum absorption at 278, 282 and 292nm to decide about the contribution of aromatic amino acids towards the tertiary structure of horse gram lipoxygenase. The thermo stability of this protein was observed on increasing the effect of temperature at pH values of 6.8 and 9.0. The inflection point at 40°C at 222nm indicated more thermo stability of this protein at pH 6.8. The isoelectric point of this enzyme was determined as 5.85 using the chromatofocussing. In summary a lipoxygenase of HG purified to electrophoretic homogeneity has showed 95±5KDa molecular mass and had α-helix and β pleated structures with a pH optimum of 6.80 and a pI value of 5.85. This enzyme showed preferred activity towards linolenic acid.

Keywords: Arachidonic acid; Circular dichroism; Horse gram; Linoleic acid; Lipoxygenases.

Introduction

Horse gram (*Dolichos biflorus*) is the unexploited legume of the tropics and sub-tropics grown mostly under dry land agriculture. Horse gram is an excellent source of iron and molybdenum. Horse gram seeds have higher trypsin inhibitor and haemagglutinin activities and polyphenols. A large population in rural areas of southern India consumes the Horse gram seed as a whole seed, sprouts or whole meal. This source is also having the enzyme lipoxygenase.

Lipoxygenases (Lox, EC: 1.13.12.11), a family of non-heme iron containing fatty acid dioxygenases, are widely distributed in both plants and animals[1,2] and they catalyses the regio-stereo specific dioxygenation of polyenoic fatty acids containing a 1z, 4z, pentadiene system[3]. Lox reactions can intiate the synthesis of signaling molecules and are involved in inducing structural or metabolic changes in the cell[4]. Lox were purified to electrophoretic homogeneity and characterized to their chemical and enzymatic properties[5] and over the years more of the isoforms of lox were discovered.

Among the isolated Lipoxygenases of many plants the well-characterized Lox was from soybean seeds. The Isozymes identified and purified in soybean were Lox1, Lox2, and Lox3.These three were distinguished by the analysis of pH optimum, substrate specificity, product formation, kinetic parameters and enzyme stability[6-8]. The function of the lox is both regio and stereo specific[9]. The literature on Lox suggests that they are induced in early stages of barley, cucumber and soybean seed germination, and during their leaves senescence [10,11].

In plants linolenic and linoleic acids are the most common substrates for Lox. These two molecules are oxygenated in the presence of Lox. For example in case of linoleic acid the incorporation of oxygen can lead to the formation of two possible products, 9-or 13-hydro peroxy fatty acids[12].The plant products of lox path way have several diverse functions; in addition, lox has been associated with a number of developmental stages[13] and mobilization of stored lipids from seeds during germination[14]. Lox is present as a storage protein during vegetative growth[15]. Also a novel degradative mechanism initiated by a 13-Lox during germination uses etherified fatty acids as substrates[14].So the amount of oxygenation of lipids proportionately increases during germination. Hence, lox plays an important role in the formation of flavor or aroma in many plants[16].

In spite of many reports on lox in higher plants, the enzymatic properties of these components except for those of soyabean were not studied much, because most loxs are unstable during and after purification. The horse gram seed contain lox and its expression was different from other seeds. To determine and characterize horse gram lox (HGL), experiments were conducted in our laboratory and results are discussed.

Materials and Methods

Horse gram seeds were obtained from A.P.State Seed Corporation, Tirupati, Arachidonic acid, linoleic acid, Sephadex G-150, DE-52, Linoleate linked agarose, PBE-94, Phenyl methyl sulfonyl fluoride (PMSF), EDTA, Acryl amide, Bis-acrylamide, Coomassie brilliant blue, Lauryl sulphate (SDS) and protein size markers were procured from Sigma Chemicals Co., St. Louis, MO, USA. All other chemicals were reagent grade procured from indigenous companies.

Enzyme assay: Lox activity was measured spectophotometrically using Jasco V-530 UV-VIS spectrophotometer using temperature control peltier at 25°C by measuring the absorbance change at 234nm. The reaction mixture contained 100mM phosphate buffer, pH 6.8, approximately 0.1µg of enzyme and the final concentration of substrate was 80µM. One unit of enzyme activity is defined as the amount of µmoles of hydro peroxide formed per minute at 25°C per mg of protein.

Germination condition of seeds: The horse gram seeds sterilized with mercuric chloride were washed in sterile water and allowed to imbibe in water for 5 hours. These seeds were spread on Petri plates for germination with two layers of Whatmann paper towels support.

Lox purification: Purification of HGL was performed at 4°C. After 72 h (3day old) of germination a 10% homogenate was prepared in 100mM phosphate buffer, pH 6.8, containing 2mM Sodium meta bi sulphite, 2mM ascorbic acid, 1m MEDTA, 0.1mM PMSF and 10% sucrose, with a porter Elvejhem homogenizer and was filtered through four layers of cheese cloth. The filtrate was centrifuged at 10,000xg for about 30 minutes and the supernatant was considered as crude enzyme. The supernatant was fractionated with ammonium sulphate to bring the concentration to 45-60 %. The active pellet obtained by centrifugation at 16,000xg was redissolved in 20mM Tris-HCl buffer, pH 7.0 containing 1mM EDTA, 10% glycerol and dialyzed overnight at against 150 volumes of same buffer with four changes and centrifuged at 10,000xg for few minutes and inactive pellet was discarded.

The active supernatant was subjected to anion exchange column chromatography, DE-52, which was previously equilibrated with the Tris-HCl buffer, pH7.0 containing 1mM EDTA. After washing the column with same buffer, the bound protein was eluted with linear NaCl gradient (0.0-0.5M) at a flow rate of 1 ml per minute and the fractions containing more Lox activity were pooled and precipitated with ammonium sulphate (90%). Then the precipitate was redissolved in minimum volume of Tris-HCl buffer, pH 7.0 and was applied on to the Sephadex G-150 column, after equilibration with the same buffer. The protein was eluted at a flow rate of 20 ml per hour and the fractions containing high lox activity were pooled and concentrated with amicon filters. The concentrated protein was further applied on to affinity column (Linoleate linked agarose), which was previously equilibrated with 5mM phosphate buffer, pH 6.8 and the bound protein was eluted with a linear salt gradient of 0-0.2M NaCl using the same buffer. The eluted fractions, which showed maximum activity, were pooled and concentrated by ultra filtration to minimal volume and dialyzed overnight. Later the protein was subjected to pH 7.0 equilibrated chromatofocussing column PBE-94, after creation of the gradient with polybuffer 74 (1:8) pH4.0, and eluted with gradient buffer pH 4.0. The pH of active lox fractions was determined by using Elico pH meter and the pI values were determined for pooled protein fractions.

Gel electrophoresis: Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was used to determine the Lox purity. Protein concentration was determined and calculated according to the method of Lowry *et al*[17]. A 12% gel was performed according to the method of Laemmli[18] and 8% native gel was used (without SDS) according to the method of Hydeck and Schewe[19] for activity staining. Protein size markers were used to determine the molecular weight.

Effect of pH and Temperature: The optimum pH for Lox activity was carried out using linoleic acid as substrate at 25°C, spectrophotometrically in the pH range between 2.0-11.0 using Glycine-HCl, Acetate buffer, Phosphate buffer, Borate buffer, Glycine-NaOH buffers. The effect of ionic strength on enzyme activity was done using different concentrations of sodium phosphate (pH 6.8) from 20-100mM. Thermophilicity of lox was determined by preincubated standard assay mixture from 5 to 80°C for 10 minutes at the indicated temperature.

Kinetic parameters: The kinetic constants of HGL activity were measured under standard assay conditions using substrate concentrations in the range of 1-100µM for both linoleic and arachidonic acids. All determinations were done in duplicate and respective kinetic parameters were calculated from Line-Weaver Burk (LB) plot.

Storage stability: Stability of HGL was tested for 5 days of storage at 4°C and 25°C separately, in the absence and

presence of 1mM β-mercapto ethanol and ascorbic acid (200ppm) using standard assay conditions.

Circular Dichroism: These experiments were carried out in Jasco J-715 polarimeter attached with Jasco PTC-348 WI, calibrated solution of (+)-10-camphorsulphonic acid. Far UV CD spectrum from 200 to 250 nm was recorded using 0.1 cm light path quartz cuvettes under nitrogen flow. Near UV CD spectrum from 250-300 nm were recoded using 1.0 cm light path quartz cuvettes. A special acquisition spacing of 0.1 nm was used and each spectrum was averaged 4 times and smooth curve was made. Protein concentration of 60μg/ml for far UV and 0.5mg/ml for near UV regions was used in 20mM phosphate buffer at pH6.8. Thermal stability was probed by following ellipticity change at 220nm, a range of temperature between 10-85°C at pH6.8 and also at pH9.0. Using peltier setup attached to the spectropolarimeter varied the temperature.

Serological studies: Antiserum was prepared against purified HGL in male New Zealand white rabbit. Immunodiffusion was carried out according to ouchterlony procedure [20] for cross reactivity of antibody and antigen. The wells were made on gels and the central well was loaded with lox antiserum and encircled wells were loaded with lox from different sources.

Results and Discussion

A multitude of plant sources were screened for their lox activities [21]. The higher lox activity was observed in soybean, black gram, cowpea, field bean and potatoes. But no study was made on the characterization of horse gram lox. Therefore, horse gram seeds were selected as source of lox. The Horse gram lox (HGL) was purified and partially characterized from germinating seedlings. During germination, the activity of HGL showed a similar pattern often found in other plants. The HGL activity was increased during germination and reached to maximum on day 3 and thereafter decreased gradually. This preliminary data will be presented elsewhere.

Purification of Lox: Lox extracted from 3 day old germinated seedlings of horse gram had a specific activity of 0.073 units /mg protein (Table 1) and was fractionated by using ammonium sulphate to 45-60%. This fraction contained a specific activity of 0.33-units/mg proteins. The dialysate of ammonium sulfate fraction upon loading onto anion exchanger, DE-52, column, the lox was bound to the column material. The bound protein on elution resolved in to two protein peaks one with major activity of 1.07 units/ mg protein and the other peak with minor activity of 0.09 units / mg protein. Among the two, major protein peak was collected, concentrated, and then subjected to sephadex G-150 column chromatography. Active fractions of this were collected as single broad peak and found to contain the specific activity of 3.23 units / mg protein. Further the pooled protein was loaded on to linoleate linked agarose chromatography (Affinity chromatography) column to purify Lox. Lox was eluted at 0.08 M salt concentration as single peak (Fig. 1). The affinity- purified protein had a specific activity of 6.0 units /mg protein and a purification fold of 82.14.This recovery of lox activity was low as compared to that of rice 280 units[22], cow pea 1568 units[23] and potato 140- 160 units[9]. The reason for this low specific activity could be explained by the unstable nature of Lox protein during purification though it was conducted at 4°C.

Localization of Lox Isozymes : The three day old germinating seedlings of Horse gram were crushed in the phosphate buffer, pH 6.8 and centrifuged at 10,000 Xg for 20 minutes to remove the cell debris. The collected supernatant was subjected to native gel electrophoresis and stained with O-diansidine HCl. On native gel electrophoresis at three places the pink color stain was observed. This indicated the presence of three isozymes of Lox in Horse gram (Fig. 2). This observation was coinciding with the earlier reports of that of multiple lox isozymes involved in cell growth and development and further their existence in several isozyme forms at different parts of the same plant [10,22].

Determination of Isoelectric point: To separate the isoenzymes and determine the pI value, the affinity-purified Lox protein was subjected to PBE-94 chromatofocussing column. On chromatofocussing HGL was eluted into two peaks, one at pI value 5.85 and other at pI 5.55. The pI values found for HGL are in agreement with dry English pea lox having pI values of 5.8 and 5.82, and dry pea lox, pI values of 4.05, 4.20, 5.82 and 6.25[24] and soybean isoenzymes pI values, 5.68, 6.25, 6.12 for lox1, lox2 and lox3, respectively[5]. However, the third isozyme as found in native gel may be lost at DE-52 chromatography, since the third protein band was not appeared after this chromatography.

SDS-PAGE: The affinity purified HGL was subjected to 12 % SDS-PAGE. This electrophoretic pattern showed single band on gel and its molecular mass was found to be approximately 95±5KDa (Fig. 3).

Molar extinction coefficient: The Molar extinction coefficient, εM, was calculated by using Beer-Lamberts law at a wavelength of 280 nm and equals to $1.53 \times 10^5 M^{-1} Cm^{-1}$. This value is comparable to that of soybean lox $1.4 \times 10^5 M^{-1} Cm^{-1}$ [6] and duram wheat semolina lox $1.3 \times 10^5 M^{-1} Cm^{-1}$ [25].

Profiles of pH and Temperature: To determine the pH optimum of HGL, the HGL activity was tested using

Table 1. Purification profile of Horse gram lipoxygenase.

S. No.	Method	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	% Yield
1.	Crude enzyme	15375	1130	0.073	1.0	100
2.	45-60% Ammonium sulfate precipitation	1921	780	0.38	5.2	69
3.	Anion exchange chromatography	325	Peak 1 456 Peak 2 40	1.4 0.12	19.1	58
4.	Sephadex G-150 chromatography	50	222	4.44	60.8	48
5.	Linoleate linked Agarose Affinity Chromatography	15	90	6.0	82.14	40

Table 2. Horse gram lipoxygenase substrate specificity analysis.

Substrate	Km µmol	Vmax µmol/min	Km/Kcat s^{-1} $microM^{-1}$
Linolei acid	9.95	0.1799	17.170
Arachidonic acid	15.54	0.226	14.577

various buffers and the maximum activity was observed at pH 6.8 (Fig. 4A). The pH results of HGL are closely related to lox of barley[26], wheat flour[27], soybean[6], and duram wheat semolina[25]. However, considerable activity was noted at pH range of 4.5-9.0 and less activity was observed below pH of 4.5 and above pH of 9.0. Many investigators classified plant lox in to two forms based on their pH optima. Type 1 loxs are characterized at high pH optima, at pH 9.0 and above, while type 2 lox are with optimum pH about 7.0[28]. Almost all loxs, except soybean seed lox1 have optimum activities in between pH 6.5 and 7.0[16]. On testing of this enzyme using temperature variation, maximum lox activity was observed in between 25 and 35°C (Fig. 4B).

Kinetic parameters: Kinetic characterization of HGLwas carried out in relation to two substrates: linoleic acid (LA, 18:2) and arachidonic acid (AA, 20:4) using phosphate buffer pH 6.8. The values of Km and Kcat of HGL are given in Table 2. The Km values of LA and AA are 9.95µM and 15.54 µM respectively, which indicate that high enzyme affinity for both the substrates; however the Kcat/ Km ratio showed that the linoleic acid was the preferred substrate. These are in correlation with Km values of germinating barley lox1 and lox2, 13 µM and 19 µM[29] and L1, L2 of soybean lox 12 and 16 µM[6], and duram wheat lox[25] 11.25 µM and 16.47 µM with LA and AA, respectively.

Storage stability: Storage stability of HGL was determined at 25°C and at 4°C, in the presence of 1mM β-mercaptoethanol and ascorbic acid (Fig. 5). The HGL was stable at 4°C storage after 120 h (5 days) and at 25°C a 50% loss of activity was observed after 24h storage. Incubation with reducing agent, 1mM β-mercaptoethanol and ascorbic acid resulted in the inhibition of the enzyme activity at 4°C.This was probably occurred due to hydro peroxide ion release by Fenton – like reaction[30], in the presence of β-mercaptoethanol / ascorbic acid storage.

Circular dichroism studies: The structural analysis of HGL was performed by using UV CD spectra far and near UV regions. On spectral analysis in far UV CD spectra the HGL showed a negative dip at 208 and at 222nm, (Fig. 6A) indicating the existence of secondary structure with significant α-helix and β-strands. Near UV CD was also recorded to HGL to see the environment of presence of aromatic residues. The near UV CD spectrum showed absorption maxima at 278, 282 and 292 nms indicating good contribution of aromatic residues towards tertiary structure (Fig. 6B). Thermal stability of HGL was also investigated at the pH values, 6.8 and 9.0 by measuring the dichroic activity at 222nm as a function of temperature (Fig. 7A and B). A Sigmoidal decrease in ellipticity at 222nm was observed with increase in temperature. The inflection points at 40°C at pH 6.8 and at 35°C at pH 9.0 were observed, which indicates that HGL is thermally stable at pH 6.8 than at pH 9.0. These studies conclude that the HGL has

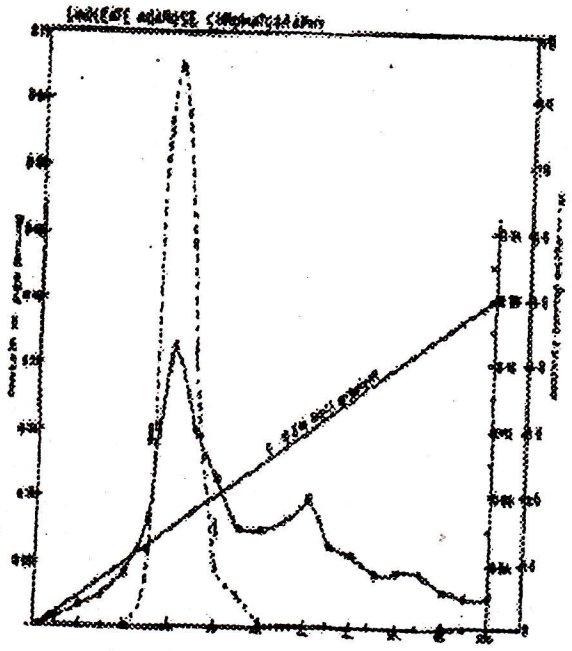

Fig.1. Affinity Chromatography profile of Horse gram lipoxygenase.

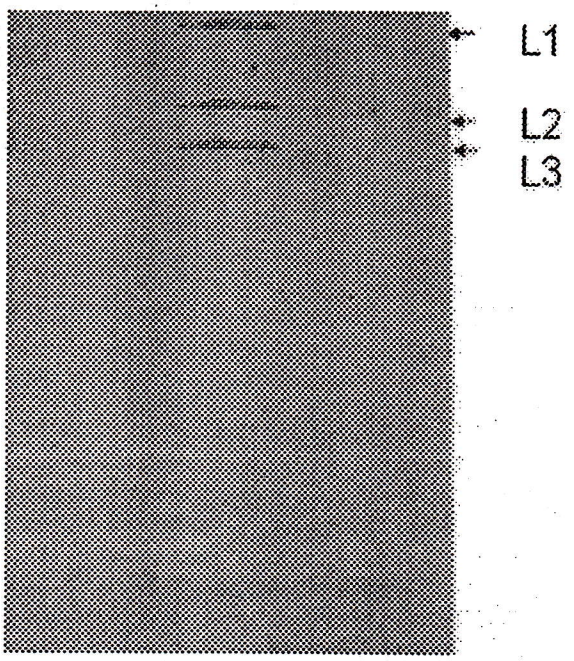

Fig. 2. Native gel electrophoresis stained with O-diansidine Hydrochloride, showing isozymes of Horse gram lipoxygenase.

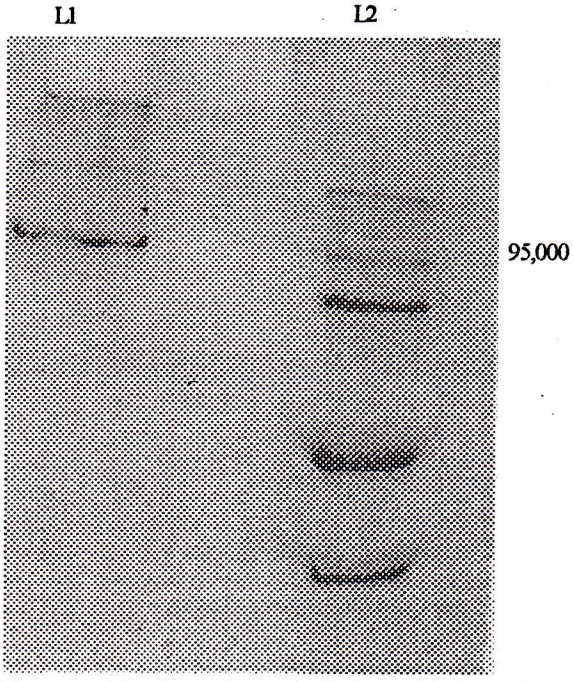

Fig. 3. SDS-PAGE analysis of Affinity purified protein of Horse gram lipoxygenase;Lane 1: Protein of Horse gram lipoxygenase; Lane 2: Protein markers.

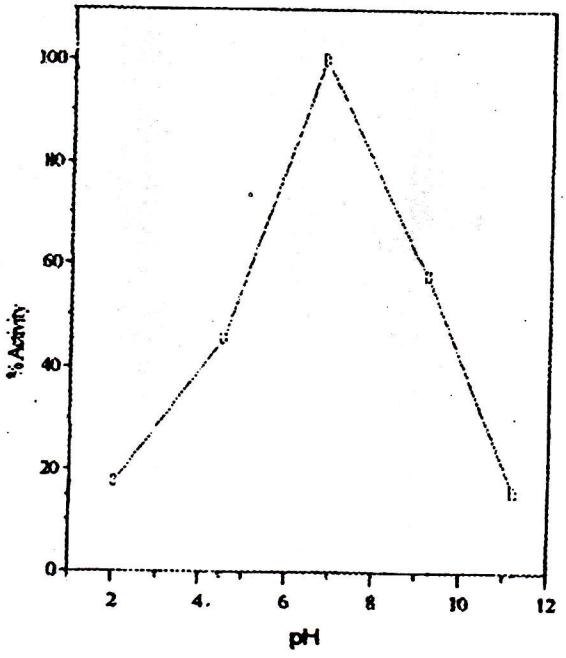

Fig. 4A. Effect of pH on purified HGL activity.

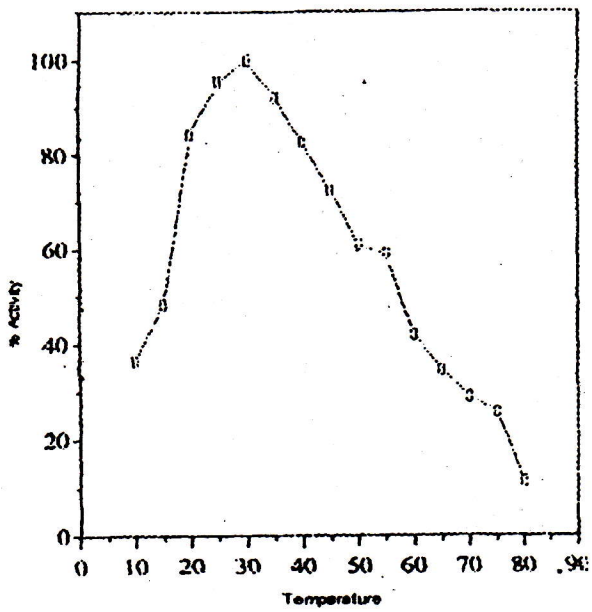

Fig.4B. Effect of temperature on purified HGL activity.

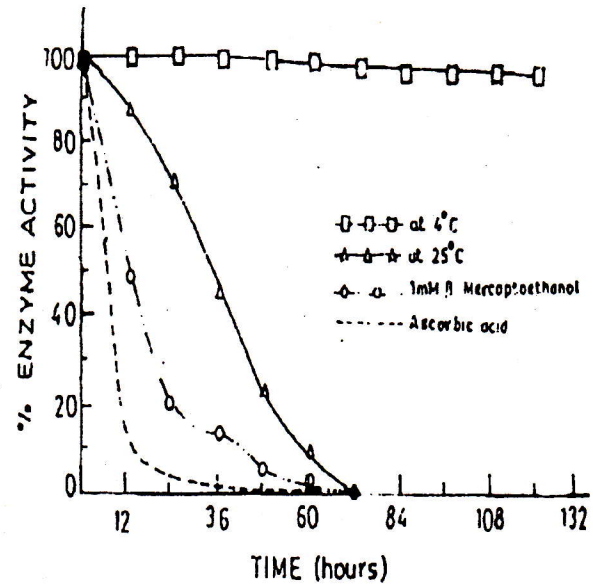

Fig. 5. Analysis of storage stability of horse gram lipoxygenase in various conditions.

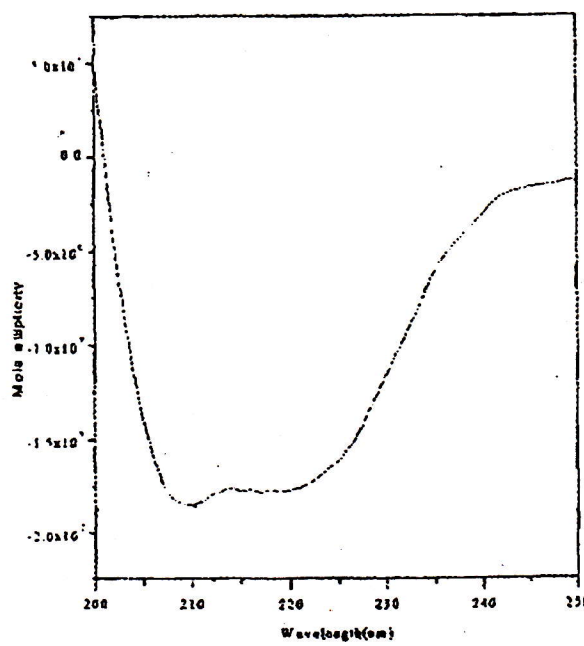

Fig. 6A. Far UV CD spectrum analysis of HGL.

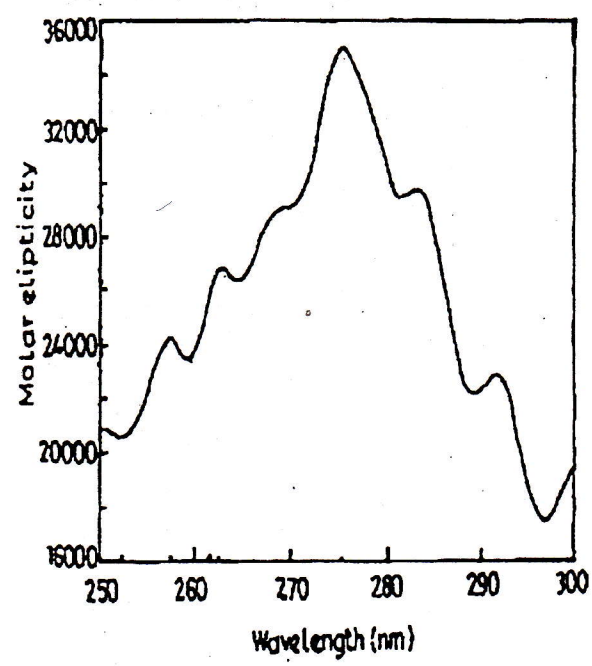

Fig. 6B. Near UV CD spectrum analysis of HGL

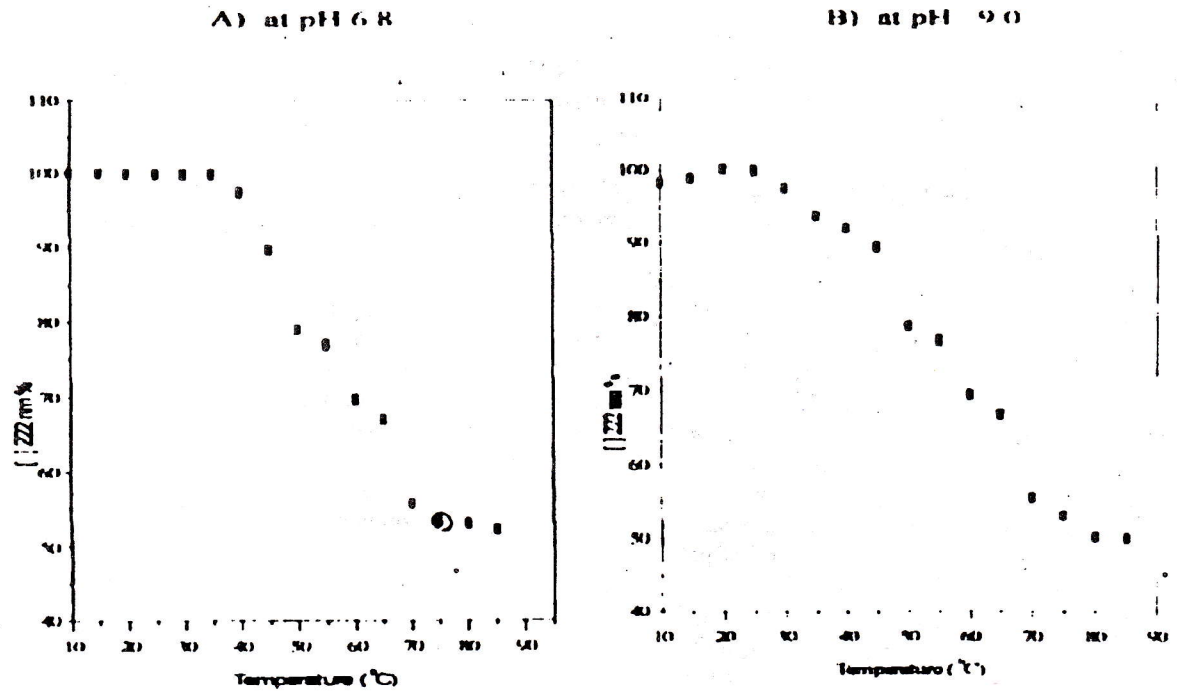

Fig. 7. Temperature dependence of horse gram lipoxygenase on α-helix content at A. pH 6.8 and B. pH 9.0

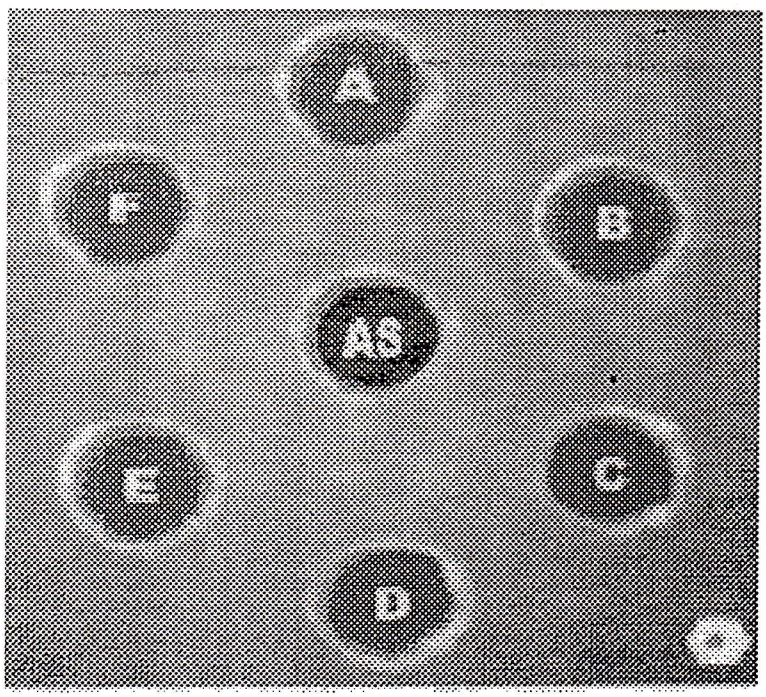

Fig. 8. Ouchterlony double immunodiffusion studies to determine the protein sequence homology of various plant lipoxygenases. The central well is loaded with AS-Antiserum and Encircled wells were filled with A-Horse gram, B-Green gram, C- Red gram, D- Bengal gram E- Black gram, and F- Soybean.

secondary and tertiary structures and these structures are more stable at pH 6.8 at 40°C.

Immunodiffussion: The sequence relationships of HGL were determined using the immunological diffusion studies. In this procedure the antiserum raised against purified HGL on immuno double diffusion (Fig. 8) showed precipitin bands with green gram, red gram, Bengal gram and soybean loxs. This indicates that the purified HGL protein has sequence homology with all the gram seed proteins.

In literature loxs were classified in to two types depending upon the pH optima. Type 1 loxs were characterized by pH optima at 9.0 and type 2 lox at pH 7.0[31].Almost all loxs except soybean lox-1 have optimal activities in between pH 6.8 and 7.0 [32]. Soybean lox-3 and lox-2 exhibits maximum activity at pH 7.0 and 6.8, respectively [6]. Present study shows that the HGL has pH optima at 6.8 and pI value of 5.85. Based on these coinciding results the HGL may be considered as related to soyabean Lox-1. However, it requires further studies to confirm the HGL is type 1 or type 2.

Acknowledgements

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