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Proteinaceous Pancreatic Lipase Inhibitor from the Seed of *Litchi chinensis*

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Summary

A study on the pancreatic lipase inhibitory activity of protein from the seed of *Litchi chinensis* was carried out. Protein was isolated by 70 % ammonium sulphate precipitation followed by dialysis. Lipase inhibitory activity of the protein was evaluated using both synthetic (*p*-nitrophenyl palmitate) and natural (olive oil) substrate. Protein at the final concentration of 100 µg/mL was able to inhibit pancreatic lipase with 68.15 % inhibition on synthetic substrate and 60 % inhibition on natural substrate.Proteinaceous nature of the inhibitor was determined using trypsinization assay. Pancreatic lipase inhibitory protein was sensitive to 0.05 % trypsin treatment with loss of 61.9 % activity. IC_{50} of this proteinaceous pancreatic lipase inhibitor was 73.099 µg/mL using synthetic substrate. This inhibitory protein was sensitive to pH with highest inhibitory activity at pH = 8.0 and lowest at pH = 3.0. Protein was further analyzed on 10 % non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and interestingly it showed the presence of a single band of ( ) kDa stained by Commassie brilliant blue. Protein band was further isolated from gel which showed 98.29 % purity on high-performance liquid chromatography. Isolated protein was finally crystallized to see the homogeneity of protein by batch crystallization method. Crystals were well formed with distinct edges. Isolated protein showed good pancreatic lipase inhibitory activity.

Keywords: pancreatic lipase inhibitor, fruit seed, *Litchi chinensis*, proteinaceous pancreatic lipase inhibitor

Introduction

Obesity is a global health concern, widely recognised as the largest and fastest growing public health problem in the developed and developing world associated with high morbidity and mortality (*1*). Numerous drugs have been accepted for the treatment of obesity but most of them have been discontinued as they exhibit a lot of adverse effects (*2*).Various basic mechanisms have been considered for anti-obesity strategy but these entail high costs and serious complexities (*3*).

Pancreatic lipase is a prime lipid digesting enzyme that removes fatty acids from the α and α′ positions of dietary triacylglycerols, yielding lipolytic product β-monoglyceride and long chain saturated and polyunsaturated fatty acids. Therefore inhibition of pancreatic lipase is an interesting advancement towards the discovery of potent anti-obesity agents for the management of obesity (*4*,*5*). Orlistat is well known to inhibit pancreatic and gastrointestinal lipases and it is capable of reducing dietary fat absorption upto 30 % (*6*). Orlistat, a saturated derivative of lipstatin, is isolated from a Gram-positive bacteria *Streptomyces toxytricini* (*7*). Although orlistat has displayed very promising results for obesity treatment, unfortunately it is associated with a number of unpleasant gastrointestinal side effects (*8*). Natural products provide an ample scope for the discovery of pancreatic lipase inhibitors that can perhaps be developed into anti-obesity drugs (*9*-*11*). Currently, the potential of developing successful and targeted natural products for the safe management of obesity is still largely unexplored (*12*). The existence of plant protein which inhibits the activity of mammalian enzymes has long been known. Although the role of enzyme inhibitors in their original plant tissues have not been well elucidated, a report has described system in which plant enzyme is inhibited by endogenous protein and it has been suggested that this protein in plant plays its physiological role in an active regulatory mechanism in those tissue (*13*). Many enzyme inhibitors are widely distributed in plants. Numerous components derived from plants extracts including extracts, phytochemicals, bioactive compounds, have been investigated for pancreatic lipase inhibitory activity (*14*,*15*) but could not be explored as anti-obesity agents due to many reasons including limited study in the direction of their potential. Fruit seed extracts showed properties that are beneficial to health and could be used as an alternative approach in managing risk factors and associated linkages of obesity. Reports on proteinaceous lipase inhibitors are limited. Few scientific reports are available in public domain on the proteinaceous pancreatic lipase inhibitory activity of seeds like Lychee (*16*), Soyabean (*17*-*19*), Sunflower (*20*). None of them have proceeded further to investigate the possibility to formulate pancreatic lipase inhibitor as potential anti-obesity agent in view of the effect of pH and trypsin on the performance of pancreatic lipase inhibitor. Present study focuses on the new proteinaceous pancreatic lipase inhibitor isolated from the seeds of *Litchi chinensis* fruit and its potential in development of anti-obesity agent. In this study we have reported novel proteinaceous pancreatic lipase inhibitor from

the seeds of *Litchi chinensis*. Protein at the concentration of 100 µg/mL was able to inhibit porcine pancreatic lipase *in vitro* both in synthetic and natural substrate.

Materials and Methods

Isolation of protein from seed of Litchi chinensis

The fruits of *Litchi chinensis* were purchased from Agricultural Produce Market Committee (APMC) Market, Navi Mumbai, India. Seeds were isolated from the fruits, washed thoroughly under running tap water and then twice with autoclaved distilled water and allowed to air dry for 1 week to completely remove the moisture. The air dried seeds were pulverized well using a mortar and pestle to obtain a coarsely crushed powder. Seed extract was prepared by measuring 5 g of powder in 50 mL of autoclaved distilled water and the mixture was kept at room temperature (25 °C) for 24 h. The seed extract was then filtered through a normal sieve and centrifuged (Remi Lab World, model R-8C, Vasai, India) at 806×*g* for 10 min and re-filtered using Whatman filter paper no.1. It was then precipitated by gradual addition of 3.6 g of 70 % ammonium sulphate salt (S D Fine-Chem Limited, Boisar, India) in an ice bath, and it was allowed to stand at 4 ºC overnight. This mixture was then centrifuged using a microcentrifuge (Remi Lab World, model RM 12C, Vasai, India) at 10483×*g* for 20 min. The supernatant was discarded and pellet was reconstituted in autoclaved distilled water. It was then dialyzed in autoclaved distilled water using a cellulose dialysis membrane (Himedia, Mumbai, India) with molecular mass cut off of 12 kDa for72 h with three changes of dialysate at interval of 24 h. To increase the concentration of the protein it was then precipitated using 50 % acetone (Ablychem, Panvel, India), mixed thoroughly and centrifuged using microcentrifuge (Remi Lab World, model RM 12C, Vasai, India) at 7280×*g* for 15 min. The pellet obtained after centrifugation was reconstituted in 1 mL of autoclaved distilled water, and stored at 2-8 ºC until further use.

Determination of protein concentration

Modified protocol for Bradford's microassay (*21*) was performed to estimate the concentration of protein isolated from seeds of *Litchi chinensis*. In the assay 10 µL of protein were added to the 96-well microtitre plate and 200 µL of 1× Bradford reagent (SERVA, Heidelberg, Germany) were added. The plate was incubated at room temperature (25 °C) for 5 min and absorbance (*A*) was measured at 630 nm using an ELISA plate reader (Robonik, model Readwell touch, Ambernath, India). Standard curve of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) (1 mg/mL) was plotted using the absorbance values obtained at different dilutions ranging between 0 – 0.35 mg/mL. The total concentration of the protein isolated from *Litchi chinensis* seed was calculated using the BSA standard curve's equation $y = 0.667x$.

Lipase activity assay using synthetic substrate

Lipase assay was performed by method described by Winkler and Stuckmann (*22*) with slight modification. Assay was carried out in triplicate format, using a 96-well microtiter plate (TARSONS Product (P) Ltd, Kolkata, India). The pancreatic lipase enzyme solution (5 mg/mL) from porcine pancreas (Sigma-Aldrich, St. Louis, MO, USA) was prepared in 0.1 M sodium phosphate buffer (pH = 8.0) and stored at 2 – 8 °C until usage. The substrate used in this assay was 4.5 mg of *p*-nitrophenyl palmitate (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 200 µL of N,N-dimethyl formamide (S D Fine-Chem Limited, Boisar, India) and volume was made up to 10 mL by adding 0.1 M sodium phosphate buffer (pH = 8.0), prepared by mixing the two stock solutions of sodium dihydrogen phosphate (S D Fine-Chem Limited, Boisar, India) and disodium hydrogen orthophosphate (S D Fine-Chem Limited, Boisar, India). The reaction mixture (10 µL of pancreatic lipase, 40 µL of 0.1 M sodium phosphate buffer (pH = 8.0) and 150 µL of *p*-nitrophenyl palmitate solution) was incubated at 37 °C for 30minand the absorbance (*A*) was measured at 405 nm at 0 and 30 min. One unit of lipase is defined as that quantity releasing 1 nm of free phenol from the substrate (*p*-nitrophenyl palmitate) per mL per min in 0.1 M sodium phosphate buffer pH 8.0 at 37 °C for 30 min.

Lipase activity assay using natural substrate

Lipase assay was performed using slightly modified titrimetric method (*23*) with olive oil (Research-Lab Fine Chem Industries, Mumbai, India). Porcine pancreatic lipase (type II) inhibitory activity was measured by titrimetric method using olive oil (Research-Lab Fine Chem Industries, Mumbai, India) as a substrate. Porcine pancreatic lipase solution (2 mg/mL) was prepared in 200 mM Tris-HCl (Sigma-Aldrich, St. Louis, MO, USA) buffer (pH = 7.7). To determine the lipase activity, autoclaved distilled water (2.5 mL), Tris-HCl buffer (1 mL), olive oil (3 mL) and pancreatic lipase enzyme (0.5 mL) were mixed thoroughly and incubated in an incubator cum orbital shaker (Neolab, Mumbai, India) for 30 min at 37 °C. Reaction was stopped by adding 3 mL of 95 % ethanol (Lab India, Navi-Mumbai, India) followed by the addition of 4 drops of 0.9 % thymolphthalein indicator (S D Fine-Chem Limited, Mumbai, India) prepared in 95 % ethanol and then the pancreatic lipase activity was assayed. Titration was carried out with 50 mM sodium hydroxide solution (MERCK EMPARTA, Darmstadt, Germany) to obtain a light blue color. One unit of enzyme hydrolyzed 1 µL of fatty acid from 1 triglyceride in 1 hour at pH=7.7 at 37 °C.

Measurement of lipase inhibitory activity

To determine the pancreatic lipase inhibitory activity, the seed protein at final concentration of 100 µg/mL was preincubated in both synthetic and natural substrate and after completion of reaction *in vitro*, inhibition percentage was calculated by determining the enzyme activity (U) using absorbance (*p*-nitrophenyl palmitate) and titrimetric value (olive oil). Percentage inhibition was calculated on the basis of enzyme activity values of the test and the inhibitor using formula, percentage inhibition (%) = Activity of enzyme without inhibitor – Activity of enzyme with inhibitor / Activity of enzyme without inhibitor x 100. Enzyme activity without the presence of inhibitor was 1 and 50 U/mL on synthetic and natural substrate, respectively.

Effect of trypsinization on pancreatic lipase inhibitory activity

The seed protein of *Litchi chinensis* was treated with 0.05% trypsin (Genetix Biotech Pvt Ltd, Delhi, India) to study the effect of trypsin on the activity of pancreatic lipase inhibitor. The solution of protein (500 µg/mL) and trypsin in the ratio of 1:1 was incubated at 37 °C for 2 h, followed by estimation of pancreatic lipase inhibitory activity of *Litchi chinensis* protein expressed in terms of percentage inhibition).

Determination of IC_{50} value

IC_{50} value of the *Litchi chinensis* seed protein was measured using linear regression at concentrations of 25, 50, 75 and 100 µg/mL. Pancreatic lipase activity was assayed as per the above stated protocol and inhibition percentage was plotted against concentration. The concentration at 50 % inhibition was determined and expressed in µg/mL.

Pancreatic lipase inhibitory activity of the Litchi chinensis seed protein at various pH

Effect of pH on the pancreatic lipase inhibitory activity of *Litchi chinensis* seed protein at final concentration of 100 µg/mL was studied at different pH values (3, 5, 7,8and 9). The different pH solutions were prepared by adjusting the pH of autoclaved distilled water using 6M HCl and 6M NaOH solutions. Then each of these solutions (500 µL)and inhibitory seed protein (500 µL) were mixed in the ratio of 1:1and preincubated at 37 °C for 30 min. Lipase inhibition assay was performed using synthetic substrate described earlier. A volume of 40 µL of this reaction mixture was added to 10 µL of enzyme followed by 150 µL of the substrate as per the protocol for lipase assay. Each reaction was performed in triplicate. The reaction mixture was incubated at 37 °C for 30 min and then the absorbance (*A*) was measured at 405 nm. The enzyme inhibition was expressed in percentage using formula, percentage inhibition (%) = Activity of enzyme without inhibitor – Activity of enzyme with inhibitor / Activity of enzyme without inhibitor x 100.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of isolated Litchi chinensis seed protein

Non reducing SDS-PAGE was performed using Bio-Rad standard protocol for mini protean tetra cell electrophoresis (*24*). Standard discontinuous non reducing gel with 10 % resolving gel and 4 % stacking gel was used. Sample loading volume was standardized to 25 µL and the voltage was maintained at 120 V. The gel was stained by modified method of Commassie brilliant blue staining (*25*) which consisted of staining the gel with 1%solution of Commassie brilliant blue R250 (Kemphasol, Thane, India), 50 % methanol (Ablychem), 10 % glacial acetic acid (S D Fine-Chem Limited) and 40 % distilled water for 4-5 h, followed by destaining the gel with a solution of 40 % methanol, 10 % acetic acid and 50 % distilled water until the bands were visible. The destaining was then stopped and the gel was stored in 5 % acetic acid for further analysis. Protein band of (61 ± 2) kDa was isolated from gel by cutting the defined part of gel followed by centrifugation at 806×*g* (Remi Lab World, model R-8C, Navi-Mumbai, India).The pancreatic lipase inhibitory activity (using synthetic substrate)of protein isolated from the band was checked by above described method .

High-performance liquid chromatography analysis of the protein band isolated from Litchi chinensis

High-performance liquid chromatography (HPLC) technique was used to determine the purity of the protein isolated from defined band of non-reducing SDS-PAGE. Separation was achieved using (Shimadzu HPLC model LC-2010CHT, Tokyo, Japan) C18 column with the cut-off of 5 µm. The isocratic mobile phase was pumped at a flow rate of 1 mL/min and it consisted of acetonitrile (Sisco Research Laboratories, Mumbai, India)and HPLC grade water (Merck, Darmstadt, Germany) at the volume ratio of 60:40 with 0.1 % freshly prepared formaldehyde (Thomas Baker, Mumbai, India), filtered using a 0.45 µm filter (Merck Millipore, Billerica, Massachusetts, United States) and sonicated for 15 min using a Soltec sonicator (model SONICA 2200MH S3, Milano, Italy) The injection volume was 20 µL and the wavelength for UV detection was 280 nm. The chromatogram of protein band isolated from the gel was compared to the

chromatogram of seed extract obtained post dialysis to determine the protein purity in percentage. Protein purity was determined using the formula, Area of specific peak /Total area of peaks x 100.

Crystallization of the pure Litchi chinensis seed protein

To assess the homogeneity of the isolated protein, crystallization was carried out by commercial kit (Protein Crystallization Starter Kit, Jena Bioscience, Jena, Germany)using the batch-method for crystallization which has a similar pipetting strategy as the hanging-drop method (2). About 4 µL of the premixed batch precipitant solution containing (30 % (*m/V*) PEG 5000-MME, 1 M NaCl and 50 mM sodium acetate; pH = 4.4) was pipetted onto the light microscope (model MLM, Magnus, New Delhi, India) slide. Then 2 µL (1.2 µg) of *Litchi chinensis* seed protein solution were added onto the precipitant solution drop and the formation of crystals was observed under the microscope.

Results and Discussion

Lipase inhibitory activity of Litchi chinensis seed protein

Litchi chinensis seed protein at 100 µg/mLexhibited 68.15 % of pancreatic lipase inhibition in synthetic substrate. This indicates that *Litchi chinensis* is a potential source of pancreatic lipase inhibitor. Result of pancreatic lipase inhibition using olive oil as natural substrate was also similar to synthetic substrate and showed 60 % inhibition at 100 µg/mL of inhibitor on natural substrate olive oil.

Ethanol extract of seed of *Litchi chinensis* was previously investigated for pancreatic lipase inhibition by Queiroz *et al.* (*16*). Investigators have demonstrated the pancreatic lipase inhibitory activity of ethanol seed extract of *Litchi chinensis* only. In the present study we have established the proteinaceous nature of pancreatic lipase inhibitor present in seed of *Litchi chinensis* by treating precipitated protein with trypsin. The results in Fig. 1 showed that trypsin significantly affected the lipase inhibitory activity *of Litchi chinensis* seed protein with residual activity of only 6.97 % in comparison with the untreated seed protein which displayed 68.87 % pancreatic lipase

inhibition, indicating that it was responsible for the lipase inhibitory activity. Upadhyay *et al*. have also demonstrated the presence of trypsin sensitive proteinaceous pancreatic lipase inhibitor in *Moringa* seed (*27*). It was found that *moringa* seed protein lost the lipase inhibitory activity in the presence of trypsin. Hence the study on protection of the protein against trypsin inactivation was carried out and it was found that the protein was effective as a lipase inhibitor in presence of trypsin inhibitors.

Number of seed proteins has been demonstrated for pancreatic lipase inhibitory activity but major work in this field was carried out for soybean seeds. Satouchi *et al*. have demonstrated the presence of lipase-inhibiting protein from lipoxygenase deficient soybean seeds (*17*). Studies suggested that the inhibition of pancreatic lipase was caused not by direct interaction between lipase and the inhibitor, but rather between the inhibitor and a substrate triglyceride emulsion. A crude inhibitor for pancreatic lipase was also extracted from soybean seeds (*18*). As the concentration of the inhibitor increased, the activity of lipase decreased curvilinearly. It was observed that the presence of protein like bovine serum albumin in the reaction mixture enhanced inhibition even at low inhibitor concentration. After the addition of the inhibitor, the activity of lipase enzyme was immediately inhibited, thus the inhibitor failed to cause significant destabilization of substrate emulsion. Gargouri *et al*. have also isolated a protein that inhibits pancreatic lipase from soybean seeds (*19*). It was found to be highly surface-active and possessed the ability to penetrate monomolecular films of phospholipids and glycerides at high surface pressure. The ability of proteins to interact with lipids and to modify the quality of substrate-water interface is linked with inhibition of pancreatic lipase. Earlier in 1977, Widmer had isolated the pancreatic lipase effectors from soybean meal (*28*). Tani *et al*. have also purified and characterized proteinaceous inhibitor of lipase from wheat flour (*29*). Porcine pancreatic lipase was inhibited through direct interaction with proteinaceous lipase inhibitor. A kinetic study of lipases inhibition by proteins with dicaprin monolayers was carried out by Gargouri *et al*. (*30*). It was observed that pancreatic lipase inhibition was due to the protein associated with lipid, and not because of direct protein-enzyme interaction in the aqueous phase, when experiments were performed using lipid-protein film transfer.

Chapman isolated and partially purified proteinaceous competitive inhibitor from confectionary and high oil type sunflower (*Helianthus annuus*) seeds (*31*).

IC_{50} value, 73.099 μg/mL of seed protein isolated from *Litchi chinensis* was determined by linear regression method using *p*-nitrophenyl palmitate (Fig. 2). This protein showed good inhibitory activity.

Pancreatic lipase inhibitory activity of the Litchi chinensis seed protein at various pH

The pH plays important role in the functioning of protein. As drug target for pancreatic lipase inhibitor is in alimentary canal, effect of pH on pancreatic lipase inhibitory protein (at the final concentration of 100 μg/mL) extracted from *Litchi chinensis* was studied. Results presented in Fig. 3 clearly indicate the pH sensitive nature of the proteinaceous lipase inhibitor, particularly towards lower pH. The results show 13 % inhibition at pH = 3, 33 % inhibition at pH = 5, 61 % inhibition at pH = 7, 70 % inhibition at pH = 8 and 59 % inhibition at pH = 9 in synthetic substrate. Interestingly, it is observed that although the enzyme retained its activity at pH values from 8 to 3, *Litchi chinensis* seed protein showed maximum pancreatic lipase inhibition at pH = 8 and lost more than 50 % inhibition at pH = 3.

Results presented here indicate that protection of natural proteinaceous inhibitor of *Litchi chinensis* at various pH is very important for significant inhibition of pancreatic lipase in gut. Therefore, proper formulation is required that can resolve the issue of inactivation of proteinaceous inhibitor of *Litchi chinensis* at various pH values.

Molecular mass determination of pancreatic lipase inhibitory protein

In order to see the profile of precipitated protein fraction of *Litchi chinensis*, non-reducing SDS-PAGE was performed. Interestingly, ammonium sulphate precipitated fraction showed single band of (61 ± 2) kDa protein stained by Commassie brilliant blue staining solution (Fig. 4). It was

surprising that a single major protein band appeared on SDS-PAGE, possibly due to the extraction of protein at room temperature. This indicatedthe homogeneous nature of the pancreatic lipase inhibitory protein extracted from the seeds of *Litchi chinensis*.

The (61 ± 2) kDa protein band was finally isolated from *Litchi chinensis*. Isolated band showed good pancreatic lipase inhibitory activity. Purity and homogeneity of the isolated protein from designated band was carried out through high-performance liquid chromatography analysis and protein crystallization.The HPLC chromatogram of *Litchi chinensis* seed extract (Fig. 5) showed the presence of very few peaks along with the major peak of protein at the retention time of 2.946 min. This was confirmed by the HPLC chromatogram of the band isolated from the SDS-PAGE gel (Fig. 6), which showed the presence of one major peak with retention time of 2.947 min and a peak area of 1109957 mAU. The purity of the precipitated protein fraction loaded on SDS-PAGE was 76.02 %, whereas the purity of the protein (in %) isolated from the band was 98.29 %.

Pure, homogeneous protein is the most critical prerequisite for protein crystallization. Isolated pancreatic lipase inhibitory protein was successfully crystallized, showing the formation of crystals with distinct edges. This confirmed the purity and homogeneity of the seed protein extracted from the fruit of *Litchi chinensis*.

Satouchi *et al*. have demonstrated the presence of 56-kDa lipase-inhibiting protein from lipoxygenase deficient soybean seeds (*17*). They also showed that the molecular mass of a main peak of inhibitor was estimated to be around 80 kDa using column chromatography (*18*). Gargouri *et al.* have isolated type A protein from soybean seeds with molecular mass of 70 kDa that inhibits pancreatic lipase (*19*). Protein isolated from moringa seed by Upadhyay *et al.* was purified and characterized using SDS-PAGE and LC-MS techniques. Low molecular mass protein was identified as pancreatic lipase inhibitor (*27*). Present study demonstrates the wide range pH inhibitory activity of a novel and efficient (61±2) kDa proteinaceous pancreatic lipase inhibitor.

Conclusion

Pancreatic lipase inhibitors are interesting and relatively safer drug target for the management of obesity. Biomolecules from natural origin can be exploited as a new source for the discovery of good candidates for designing safer anti-obesity drugs for long-term use. In this study we have investigated the pancreatic lipase inhibitory activity of the protein isolated from the seeds of *Litchi chinensis* fruit by evaluating its potential, efficacy (IC_{50}), homogeneity, purity, crystallization ability and effect of pH on inhibitory molecule. We have identified a protein with (61±2) kDa which inhibited pancreatic lipase. *Litchi chinensis* seed protein showed good potential for pancreatic lipase inhibition and proved to be an efficient source of pancreatic lipase inhibitor with an IC_{50} of 73.099 µg/mL. To the best of our knowledge, this is the first report where the *Litchi chinensis* seed protein is confirmed as pancreatic lipase inhibitor.

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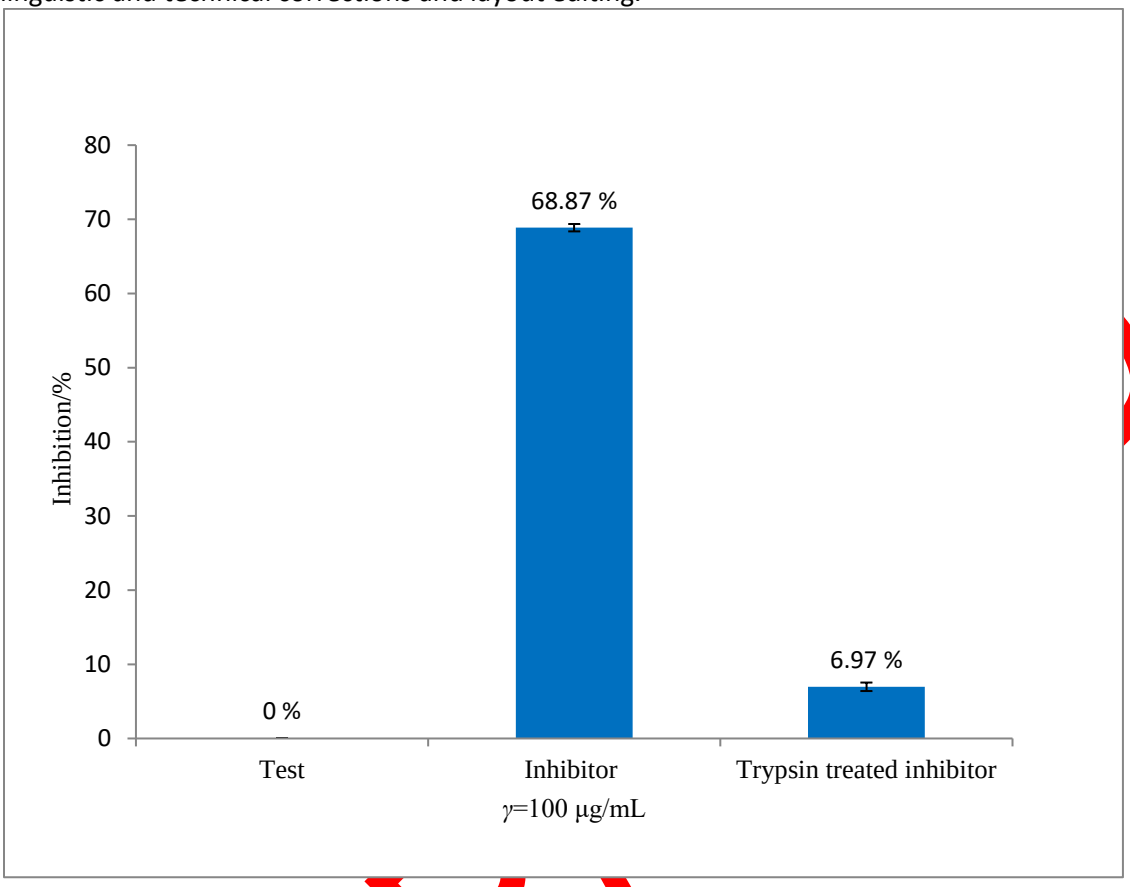

Fig. 1. Effect of trypsinization on the pancreatic lipase inhibitory activity of *Litchi chinensis* seed protein

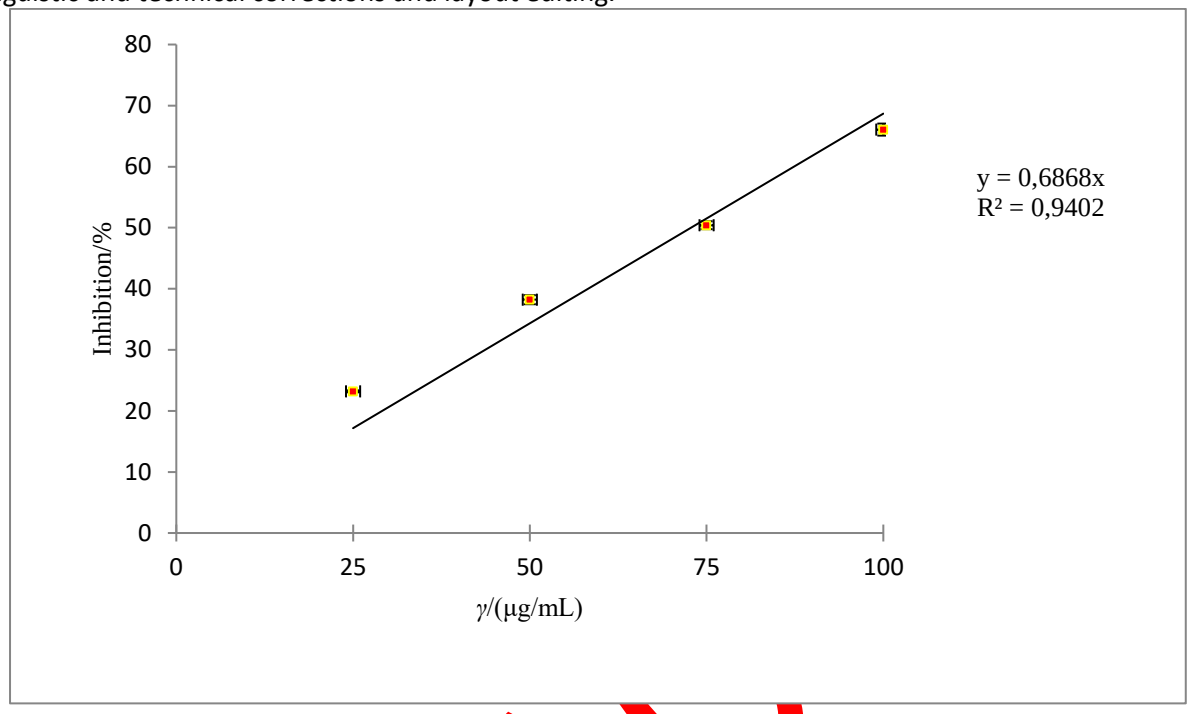

Fig. 2. Pancreatic lipase (PL) inhibitory activity of *Litchi chinensis* seed protein at various concentrations

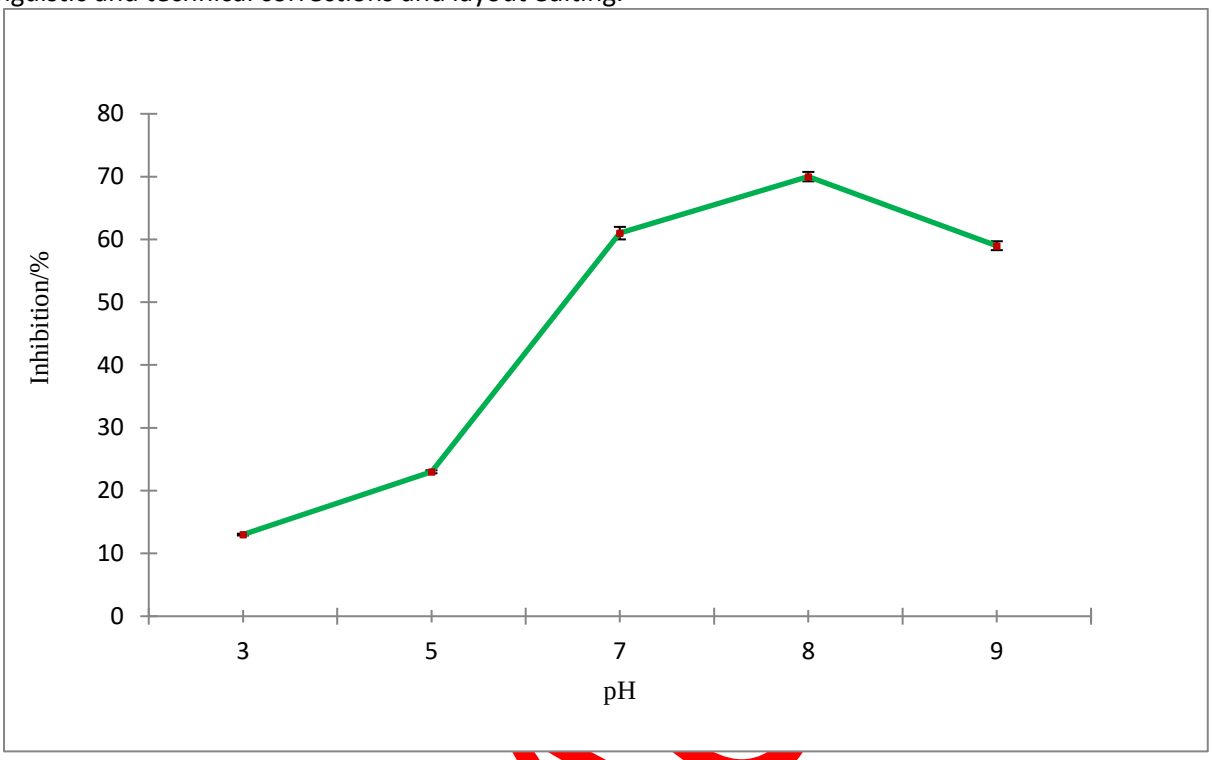

Fig. 3. Effect of pH on the *Litchi chinensis* seed protein

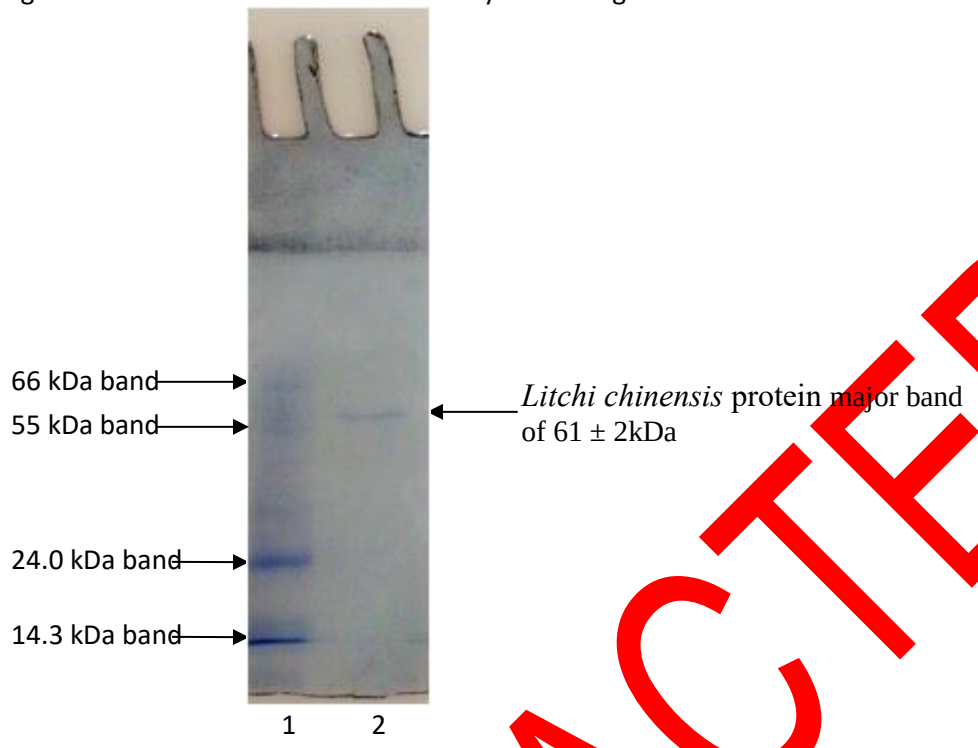

Fig. 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis gel (10 %) showing band of *Litchi chinensis* seed proteinof approx. (61 ± 2) kDa. Lane 1=molecular mass marker, lane 2 = *Litchi chinensis* protein band

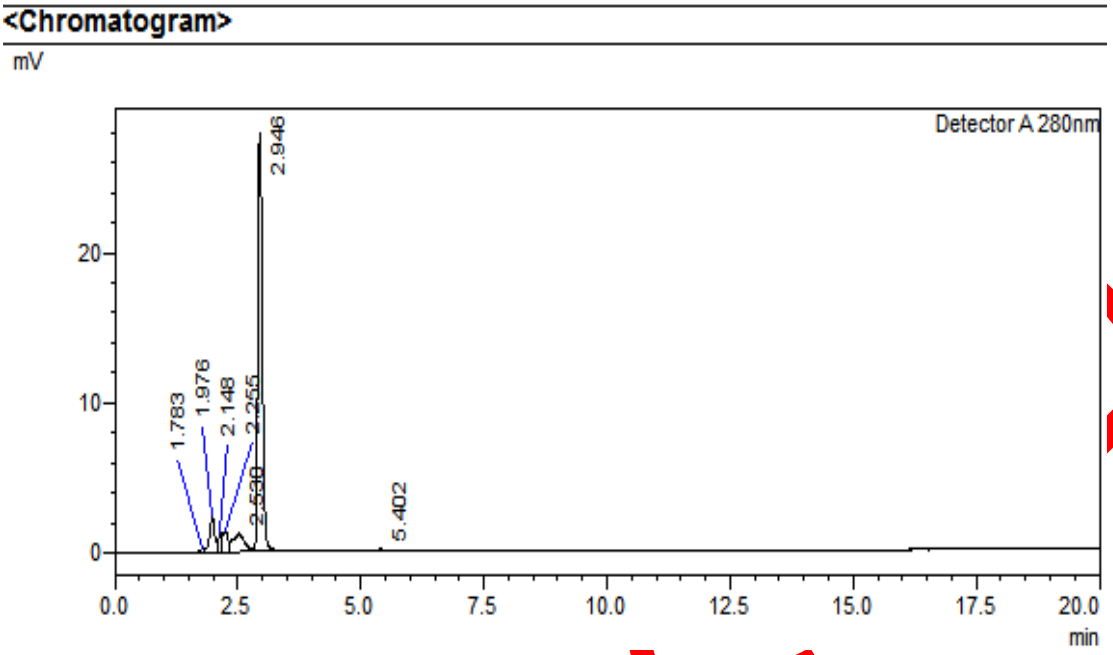

Fig. 5. HPLC chromatogram of the *Litchi chinensis* seed extract

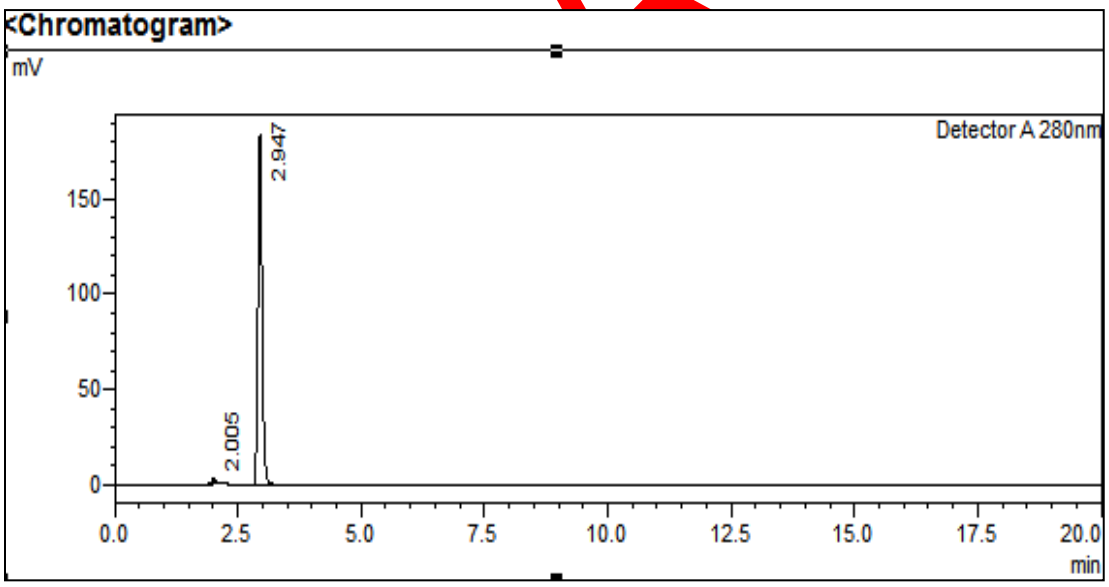

Fig. 6. HPLC chromatogram of the protein band isolated from *Litchi chinensis* for the confirmation of purity