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

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SUMMARY

A study of the pancreatic lipase inhibitory activity of a 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) substrates. Protein at the final concentration of 100 μg/mL was able to inhibit 68.2 % pancreatic lipase on synthetic substrate and 60.0 % 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 the loss of 61.9 % activity. IC_{50} of this proteinaceous pancreatic lipase inhibitor was 73.1 μg/mL using synthetic substrate. This inhibitory protein was sensitive to pH, with the highest inhibitory activity at pH=8.0 and the lowest at pH=3.0. Protein was further analyzed using 10 % non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and, interestingly, it showed the presence of a single band of (61±2) kDa when stained with Coomassie brilliant blue. The isolated protein was finally crystallized to see its homogeneity by batch crystallization method. Crystals were well formed with distinct edges. The isolated protein showed good pancreatic lipase inhibitory activity.

Key words: fruit seed, *Litchi chinensis*, proteinaceous pancreatic lipase inhibitor, obesity treatment

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 saturated and polyunsaturated long-chain 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 is capable of reducing dietary fat absorption up to 30 % (*6*). As a saturated derivative of lipstatin, it is isolated from a Gram-positive bacteria *Streptomyces toxytricini* (*7*). Although orlistat has shown very promising results for obesity treatment, unfortunately it causes 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 that inhibits the activity of mammalian enzymes has long been known. Although the role of enzyme inhibitors in their original plant tissues has not been well elucidated, a report has described a system in which plant enzyme is inhibited by endogenous protein and it has been suggested that this protein plays its physiological role in an active regulatory mechanism in that tissue (*13*). Many enzyme inhibitors are

widely distributed in plants. Numerous components derived from plants including alcoholic and aqueous extracts, phytochemicals and 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 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 links to 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 from lychee (*16*), soybean (*17-19*) and sunflower (*20*). None of them investigated further the possibility of formulating pancreatic lipase inhibitor as a potential anti-obesity agent in view of the effect of pH and trypsin on the performance of pancreatic lipase inhibitor. The present study focuses on the new proteinaceous pancreatic lipase inhibitor isolated from the seeds of *Litchi chinensis* fruit and its potential in the development of anti-obesity agents. 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 Litchi chinensis seeds

The fruits of *Litchi chinensis* were purchased from Agricultural Produce Market Committee (APMC), Navi Mumbai, India. Seeds were separated from the fruits, washed thoroughly under running tap water, then twice with autoclaved distilled water and allowed to air dry for one week to completely remove the moisture. The air-dried seeds were pulverized using a mortar and pestle to obtain a coarsely crushed powder. Seed extract was prepared by adding 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 (model R-8C; REMI Laboratory Instruments, Vasai, India) at 806×*g* for 10 min and re-filtered using Whatman filter paper no. 1 (GE Healthcare Life Sciences, Chicago, IL, USA). It was then precipitated at 25 °C by gradual addition of 23.6 g ammonium sulphate salt (S D Fine-Chem Ltd., Boisar, India) to achieve 70 % saturation at and was allowed to stand at 4 °C overnight. This mixture was then centrifuged using a microcentrifuge (model RM 12C; REMI Laboratory Instruments) at 10 483×*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 for 72 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 Laboratories Pvt. Ltd., Panvel, India), mixed thoroughly and centrifuged using microcentrifuge (model RM 12C; REMI Laboratory Instruments) 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 °C until further use.

Determination of protein concentration

Modified protocol for Bradford microassay (*21*) was performed to estimate the concentration of protein isolated from *Litchi chinensis* seeds. In the assay 10 μL of protein were added to the 96-well microtitre plate (Tarsons Products Pvt. Ltd, Kolkata, India) and 200 μL of 1× Bradford reagent (SERVA Electrophoresis GmbH, 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 (model Readwell TOUCH™; Robonik, Ambernath, India). Standard curve of bovine serum albumin (BSA; Sigma-Aldrich, Merck, St. Louis, MO, USA) (1 mg/mL) was plotted using the absorbance values obtained at different dilutions ranging between 0 and 0.35 mg/mL. The total concentration of the protein isolated from *Litchi chinensis* seed was calculated from BSA standard curve using the following equation:

$$A=0.6667\gamma \qquad /1/$$

where A is the absorbance at 630 nm and γ is the concentration of protein in mg/mL.

Lipase activity assay using synthetic substrate

Lipase assay was performed by the method described by Winkler and Stuckmann (*22*) with slight modifications. The assay was carried out in triplicate, using a 96-well microtitre plate (Tarsons Products Pvt. Ltd). The pancreatic lipase enzyme solution (5 mg/mL) from porcine pancreas (Sigma-Aldrich, Merck) 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, Merck) dissolved in 200 μL of *N*,*N*-dimethyl formamide (S D Fine-Chem Ltd) and volume was made up to 10 mL by adding 0.1 M sodium phosphate buffer (pH=8.0). 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 30 min and the absorbance (*A*) was measured at 405 nm at 0 and 30 min. One unit of lipase activity is defined as the quantity releasing 1 nmol/min of free phenol from the substrate (*p*-nitrophenyl palmitate) in 0.1 M sodium phosphate buffer (pH=8.0) at 37 °C.

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) and porcine pancreatic lipase (type II). Porcine pancreatic lipase solution (2 mg/mL) was prepared in 200 mM Tris-HCl (Sigma-Aldrich, Merck) 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 with orbital power fan shaker (Neolab Instruments, Mumbai, India) at fixed position for 30 min at 37 °C. Reaction was stopped by adding 3 mL of 95 % ethanol (Labindia, Navi

Mumbai, India) followed by the addition of four drops of 0.9 % thymolphthalein indicator (S D Fine-Chem Ltd) prepared in 95 % ethanol and then the pancreatic lipase activity was assayed. Titration was carried out with 50 mM sodium hydroxide solution (EMPARTA®, Merck KGaA, Darmstadt, Germany) to obtain a light blue colour. One unit of enzyme hydrolyzed 1 μmol/min of fatty acid from a triglyceride at pH=7.7 and 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 substrates 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 of inhibition was calculated on the basis of enzyme activity (EA) values of the test and the inhibitor using the following equation:

$$\text{Inhibition} = \frac{\text{EA without inhibitor} - \text{EA with inhibitor}}{\text{EA without inhibitor}} \cdot 100 \qquad /2/$$

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 Asia Pvt. Ltd., New 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 as inhibition percentage.

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.

Effect of pH on the pancreatic lipase inhibitory activity of the Litchi chinensis seed protein

The stability of the inhibitory *Litchi chinensis* seed protein at final concentration of 100 μg/mL was studied at different pH values (3, 5, 7, 8 and 9). Solutions of different pH were prepared by adjusting the pH of autoclaved distilled water using 6 M HCl and 6 M NaOH solutions. Then, each of these solutions (500 μL) and inhibitory seed protein (500 μL) were mixed in the ratio 1:1 and 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 Eq. 2.

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

Non-reducing SDS-PAGE was performed using a slightly modified protocol of Laemmli (*24*), and electrophoresis was carried out using mini-PROTEAN Tetra cell electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA). 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 Coomassie brilliant blue staining (*25*), which consisted of staining the gel with 1 % solution of Coomassie brilliant blue R250 (Kemphasol, Thane, India), 50 % methanol (Ablychem Laboratories Pvt. Ltd), 10 % glacial acetic acid (S D Fine-Chem Ltd) and 39 % 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 the gel by cutting the defined part of the gel followed by centrifugation at 806×*g* (model R-8C; REMI Laboratory Instruments). The pancreatic lipase inhibitory activity (using synthetic substrate) of the protein isolated from the band was checked by the method described above.

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 (*26*). 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) were pipetted onto the light microscope (model MLM; Magnus Analytics, 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 at magnification of 40×.

RESULTS AND DISCUSSION

Lipase inhibitory activity of Litchi chinensis seed protein

Litchi chinensis seed protein at 100 μg/mL exhibited 68.2 % 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 the synthetic substrate and showed 60.0 % inhibition at 100 μg/mL. De Rezende Queiroz *et al.* (*16*) demonstrated the pancreatic lipase inhibitory activity only of ethanol seed extract of *Litchi chinensis*. In the present study, we have established the proteinaceous nature of pancreatic lipase inhibitor present in the seeds of *Litchi chinensis* by treating the precipitated protein with trypsin. The results in **Fig. 1** show 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.* (*27*) have also demonstrated the presence of trypsin-sensitive proteinaceous pancreatic lipase inhibitor in *Moringa* seed. It was found that *Moringa* seed protein lost the lipase inhibitory activity in the presence of trypsin. Hence, the study on the protection of the protein against trypsin inactivation was carried out and it was found that the protein was effective as a lipase inhibitor in the presence of trypsin inhibitors. Number of seed proteins has been investigated for pancreatic lipase inhibitory activity, but major work in this field was carried out with soybean seeds. Satouchi *et al.* (*17*) demonstrated the presence of lipase-inhibiting protein from lipoxygenase-deficient soybean seeds. Studies suggest that the inhibition of pancreatic lipase was caused not by direct interaction between the lipase and the inhibitor, but rather between the inhibitor and a substrate triglyceride emulsion. A crude inhibitor of 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, without causing significant destabilization of substrate emulsion. Gargouri *et al.* (*19*) have also isolated a protein from soybean seeds that inhibits pancreatic lipase. It was found to be highly surface-active and was able to penetrate monomolecular films of phospholipids and glycerides at high surface pressure. The ability of proteins to interact with lipids and

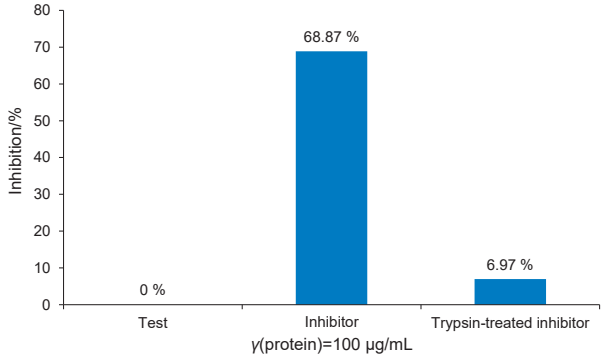

Fig. 1. Effect of trypsinization on the pancreatic lipase inhibitory activity of *Litchi chinensis* seed protein

to modify the quality of substrate-water interface is linked with inhibition of pancreatic lipase. Earlier, in 1977, Widmer (*28*) had isolated the pancreatic lipase effectors from soybean meal. Tani *et al.* (*29*) also purified and characterized proteinaceous inhibitor of lipase from wheat flour. Porcine pancreatic lipase was inhibited through direct interaction with proteinaceous lipase inhibitor. A kinetic study of lipase inhibition by proteins with dicaprin monolayers was carried out by Gargouri *et al.* (*30*). Experiments performed using lipid-protein film transfer showed that pancreatic lipase inhibition was due to the protein associated with lipid, and not because of direct protein-enzyme interaction in the aqueous phase. Chapman Jr (*20*) isolated and partially purified proteinaceous competitive inhibitor from confection and sunflower (*Helianthus annuus*) seeds with high oil content. IC_{50} value of 73.1 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.

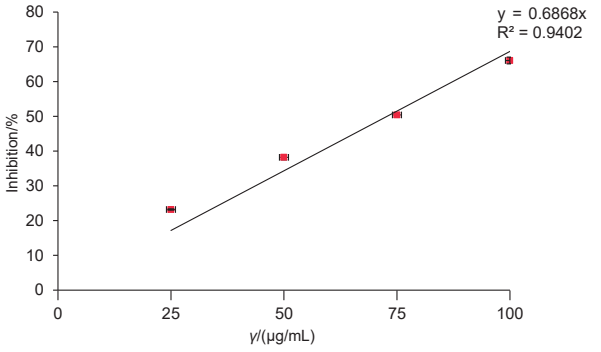

Fig. 2. Pancreatic lipase inhibitory activity of *Litchi chinensis* seed protein at various concentrations

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

The pH plays an important role in the functioning of a 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 given in **Fig. 3** clearly indicate the pH-sensitive nature of the proteinaceous lipase inhibitor, particularly towards lower pH. The results show 13, 23, 61, 70 and 59 % inhibition at pH=3, 5, 7, 8 and 9 in synthetic substrate. Interestingly, it is observed that although the enzyme retained its activity at pH values from 3 to 8, *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 the gut. Therefore, proper formulation is required that can resolve the issue of inactivation of proteinaceous inhibitor of *Litchi chinensis* at various pH values.

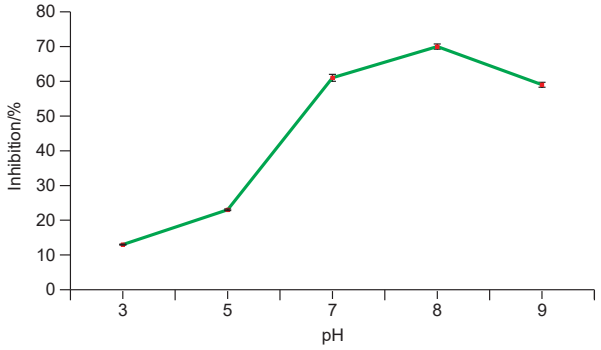

Fig. 3. Pancreatic lipase inhibitory activity of *Litchi chinensis* seed protein at various pH values

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 with Commassie brilliant blue staining solution (**Fig. 4**). It was surprising that a single major protein band appeared on the SDS-PAGE, possibly due to the extraction of protein at room temperature. This indicated the 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 the gel. The isolated band showed good pancreatic lipase inhibitory activity. Homogeneity of the isolated protein from the designated band was determined by protein crystallization. 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 indicated the homogeneity of the seed protein extracted from the fruit of *Litchi chinensis*.

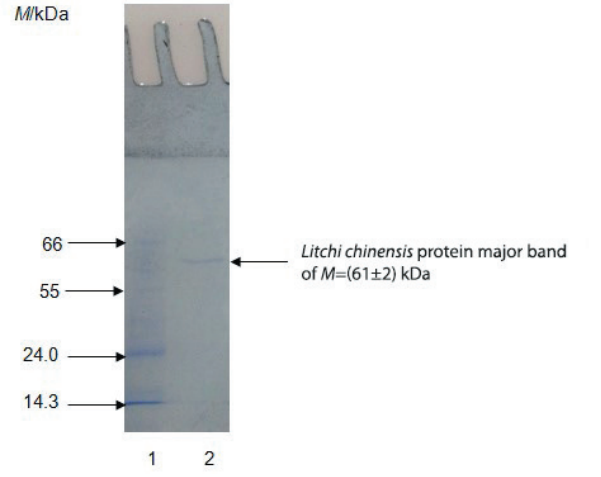

Fig. 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showing band of *Litchi chinensis* seed protein of approx. (61±2) kDa. Lane 1=molecular mass marker, lane 2=*L. chinensis* protein band

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

CONCLUSIONS

Pancreatic lipase inhibitors are interesting and relatively safer potential drugs 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, crystallization ability and inhibitory activity under various pH values. We 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 73.1 μg/mL. To the best of our knowledge, this is the first report where the *Litchi chinensis* seed protein has been confirmed as pancreatic lipase inhibitor.

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REFERENCES

1. Bell CG, Walley AJ, Froguel P. The genetics of human obesity. Nat Rev Genet. 2005;6:221-34. https://doi.org/10.1038/nrg1556
2. Kang JG, Park CY. Anti-obesity drugs: A review about their effects and safety. Diabetes Metab J. 2012;36(1):13-25. https://doi.org/10.4093/dmj.2012.36.1.13
3. Cawley J, Meyerhoefer C. The medical care costs of obesity: An instrumental variables approach. J Health Econ. 2012;31(1):219-30. https://doi.org/10.1016/j.jhealeco.2011.10.003
4. Thomson ABR, De Pover A, Keelan M, Jarocka-Cyrta E, Clandinin MT. Inhibition of lipid absorption as an approach to the treatment of obesity. Methods Enzymol. 1997;286:3-44. https://doi.org/10.1016/S0076-6879(97)86003-X

5. Tsujita T, Ninomiya H, Okuda H. p-Nitrophenyl butyrate hydrolyzing activity of hormone-sensitive lipase from bovine adipose tissue. J Lipid Res. 1989;30(7):997-1004.
6. Ballinger A, Peikin SR. Orlistat: Its current status as an anti-obesity drug. Eur J Pharmacol. 2002;440(2-3):109-17. https://doi.org/10.1016/S0014-2999(02)01422-X
7. Guerciolini R. Mode of action of orlistat. Int J Obes Relat Metab Disord. 1997;21 Suppl 3:S12-23.
8. Napier S, Thomas M. 36 Year old man presenting with pancreatitis and a history of recent commencement of Orlistat case report. Nutr J. 2006;5:Article no. 19. https://doi.org/10.1186/1475-2891-5-19
9. Birari RB, Bhutani KK. Pancreatic lipase inhibitors from natural sources: Unexplored potential. Drug Discov Today. 2007;12(19-20):879-89. https://doi.org/10.1016/j.drudis.2007.07.024
10. Mhatre SV, Bhagit AA, Yadav RP. Pancreatic lipase inhibitor from food plant: Potential molecule for development of safe anti-obesity drug. MGM J Med Sci. 2016;3(1):34-41. https://doi.org/10.5005/jp-journals-10036-1084
11. Mhatre SV, Bhagit AA Yadav RP. In vitro studies of some edible spices on pancreatic on lipase inhibitory activity. Indian Drugs. 2017;54(2):62-8.
12. Bustanji Y, Mohammad M, Hudaid M, Tawaha K, Al-Masri IM, Al-Khatib H, et al. Screening of some medicinal plants for their pancreatic lipase inhibitory potential. Jordan J Pharm Sci. 2011;4(2):81-8.
13. Fan SG, WU GJ. Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects. Bot Bull Acad Sin. 2005;46:273-92.
14. Upadhyay S, Yadav R, Gangan V, Kanekar Y. Compounds for treatment of lipase mediated disease. US 7355055-B2. 2008.
15. de la Garza AL, Milagro FI, Boque N, Campión J, Martínez JA. Natural inhibitors of pancreatic lipase as new players in obesity treatment. Planta Med. 2011;77(8):773-85. https://doi.org/10.1055/s-0030-1270924
16. de Rezende Queiroz E, Patto de Abreu CM, Alavarenga Rocha D, Simão AA, Alves Bastos VA, Botelho LNS, Braga MA. Anti-nutritional compounds in fresh and dried lychee fractions (Litchi chinensis Sonn.). Afr J Agric Res. 2015;10(6):499-504. https://doi.org/10.5897/AJAR2014.8750
17. Satouchi K, Kodama Y, Murakami K, Tanaka T, Iwamoto H, Ishimoto M. A lipase-inhibiting protein from lipoxygenase-deficient soybean seeds. Biosci Biotechnol Biochem. 2002;66(10):2154-60. https://doi.org/10.1271/bbb.66.2154
18. Satouchi K, Mori T, Matsushita S. Characterization of inhibitor protein for lipase in soybean seeds. Agr Biol Chem. 1974;38(1):97-101. https://doi.org/10.1271/bbb1961.38.97
19. Gargouri Y, Julien R, Pieroni G, Verger R, Sarda L. Studies on the inhibition of pancreatic and microbial lipases by soybean proteins. J Lipid Res.1984;25(11):1214-21.
20. Chapman Jr GW. A proteinaceous competitive inhibitor of lipase isolated from Helianthus annuus seeds. Phytochemistry. 1987;26(12):3127-31. https://doi.org/10.1016/S0031-9422(00)82455-3
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-54. https://doi.org/10.1016/0003-2697(76)90527-3
22. Winkler UK, Stuckmann M. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens. J Bacteriol. 1979;138(3):663-70.
23. Enzymatic assay of lipase (EC 3.1.1.3): Olive oil as substrate. St Louis, MO, USA: Sigma-Aldrich, Merck; 1999. Available from: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/lipase_olive_oil.pdf.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-5. https://doi.org/10.1038/227680a0
25. Kurien B, Scofield R, editors. Spicy SDS-PAGE Gels: Curcumin/Turmeric as an Environment-Friendly Protein Stain. Protein electrophoresis: Methods and protocols. Methods in molecular biology, vol. 869. Totowa, NJ, USA: Humana Press; 2012. https://doi.org/10.1007/978-1-61779-821-4
26. Rayment I. Small-scale batch crystallization of proteins revisited: An underutilized way to grow large protein crystals. Structure. 2002;10(2):147-51. https://doi.org/10.1016/S0969-2126(02)00711-6
27. Upadhyay S, Yadav RP, Ansari A, Rao H. Agent and composition comprising the same for inhibiting lipases and phospholipases in body fluid, cells and tissues. US 7368529 B2. 2007.
28. Widmer F. Pancreatic lipase effectors extracted from soybean meal. J Agric Food Chem. 1977;25(5):1142-5. https://doi.org/10.1021/jf60213a022
29. Tani H, Ohishi H, Watanabe K. Purification and characterization of proteinaceous inhibitor of lipase from wheat flour. J Agric Food Chem.1994;42(11):2382-5. https://doi.org/10.1021/jf00047a004
30. Gargouri Y, Pieroni G, Rivière C, Sugihara A, Sarda L, Verger R. Inhibition of lipases by proteins. A kinetic study with dicaprin monolayers. J Biol Chem. 1985;260(4):2268-73.