In vitro study of effective factors for the inhibitory assay on pancreatic lipase

Tran The Huan[1], Ho Thi Thu Trang[1], Cao Thi Cam Nhung[1], Ho Hoang Nhan[1], Tran Thai Son[1*]
(1) Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Hue University

Abstract

Background: Pancreatic lipase is one of the safest targets of anti-obesity drugs. To date, orlistat is the only pancreatic lipase inhibitor approved for the long-term treatment of obesity. Therefore, there is an elevated need to find new drugs for this disease. Determining the factors affecting the test to evaluate pancreatic lipase inhibitory activity in order to build a standard assay procedure is necessary. This will make it much easier for researchers to find novel compounds that inhibit the enzyme. **Materials and method:** The current study investigated the factors influencing pancreatic lipase activity and evaluated the enzyme inhibition of orlistat by spectrophotometric method at 405 nm using p-nitrophenyl palmitate as a substrate. **Results:** With the optimized conditions, the test to evaluate pancreatic lipase inhibitory activity of orlistat gave results similar to those published by other authors. **Conclusion:** The methodology of this work should be applied in the studies looking for new effective drugs to treat obesity.

Keywords: *obesity, orlistat.*

1. INTRODUCTION

Currently, obesity is one of the global health problems. According to the World Health Organization, the worldwide prevalence of obesity has nearly tripled since 1975 [1]. Obesity is a risk factor for a wide range of non-communicable diseases including type 2 diabetes, cardiovascular disease, hypertension and stroke, various forms of cancer as well as mental health [2]. Furthermore, obesity is a known cause of impaired respiratory function and may put the group of patients with this condition at an increased risk for more serious clinical outcomes if they become infected with SAR-CoV-2. Obese patients are three times more likely to be hospitalized for COVID-19 [2, 3].

One of the goals of obesity management is the development of substances that inhibit the digestion and absorption of nutrients. Inhibition of pancreatic lipase and reduction of fat absorption are attractive approaches for exploring potential agents in the treatment of obesity. Currently, orlistat is the only pancreatic lipase inhibitor approved for clinical use in Europe [4]. This medication is capable of reducing dietary fat absorption by up to 30%, whereas most other obesity treatments have central nervous system effects [5]. Clinical use of orlistat has been associated with some mild to moderate gastrointestinal adverse effects [4, 5]. The current research trend is to search for new pancreatic lipase inhibitors that are safer for patients [6].

At present, there are two commonly used assays to evaluate pancreatic lipase inhibitory activity: spectrophotometry using the substrate triolein and the substrate p-nitrophenyl palmitate (p-NPP). Among them, the method using substrate p-NPP is more commonly used than the other. The search for new anti-obesity drugs that inhibit pancreatic lipase requires a high reliability and accuracy assay to evaluate the inhibitory activity of this enzyme. Determining the factors affecting the test is needed to find the most optimal parameters. Some research evaluated experimental factors influencing the hydrolysis rate in lipase assay. These factors are emulsifiers, incubation time, assay temperatures, buffers and pH, organic co-solvents, additives, and enzyme storage conditions [7]. Therefore, this study investigated the factors influencing pancreatic lipase enzyme activity by spectrophotometry method: measuring the absorbance at 405 nm of p-nitrophenol (p-NP) formed from the hydrolysis of the p-NPP substrate, thereby proposing optimal conditions for the assay to evaluate the inhibitory activity of this enzyme.

2. EXPERIMENTAL

2.1. Materials and Equipment

Materials: porcine pancreatic lipase, type II (L-3126); substrate of p-NPP; orlistat; and other chemicals were purchased from Merck Millipore (Burlington, Massachusetts, United States), and

Corresponding author: Tran Thai Son, email: ttson@huemed-univ.edu.vn
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Sigma-Aldrich (St. Louis, Missouri, United States).

Equipment: Clinical Microplate Reader Touch Screen EMR-500 (Labomed Inc., Los Angeles, United States), Refrigerated Centrifuge Z206A (Hermle Labortechnik, Wehingen, Germany)

Data processing using Microsoft Excel 2021 software (Microsoft, Redmond, Washington, United States).

2.2. Methods

Spectrophotometry using substrate p-NPP is a commonly used assay to evaluate pancreatic lipase activity. It was assessed by measuring the absorbance at 405 nm of p-NP formed from the hydrolysis of the p-NPP substrate (Fig. 1).

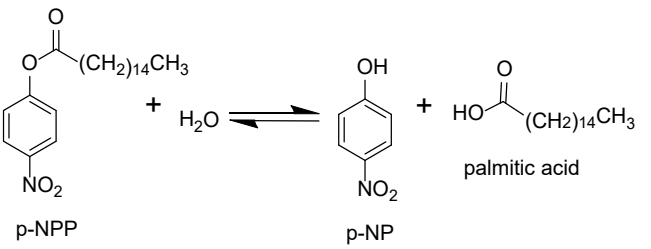

Fig. 1. The hydrolysis of p-nitrophenyl palmitate

Investigation of effective factors on enzyme activity

The effects of *pH (including buffer solution), temperature, incubation time,* and *hydrolysis time* were studied on the hydrolysis rate as well as the stability of the lipase enzyme. The assay was performed using a Clinical Microplate Reader Touch Screen EMR-500 (Labomed Inc., Los Angeles, United States) at 405 nm on a 96-well plate. The final 200 µL reaction mixture in each well contains 1 mg/ml of pancreatic lipase enzyme solution; 166.7 µM p-NPP. The factors were investigated in turn by independently changing the factor that should be evaluated and the remaining factors kept fixed, considering the influence of this change through the rate of enzymatic hydrolysis and relative rate of hydrolysis. The initial hydrolysis rate was determined as the slope of the linear cross-section of the absorbance over time, recorded every 1 min (based on the slope value, K, the slope of the hydrolysis rate against the timeline is calculated as absorbance over time). The relative hydrolysis rate is the ratio of the slope K at a given case to the case with the highest hydrolysis rate when examining a given element.

Investigation of enzyme inhibitory activity

In this study, the pancreatic lipase inhibitory activity of orlistat, which is commonly used as a reference standard, was tested by photometric method with p-NPP substrate. Based on the factors investigated, the experiment was conducted as follows: each solution consisting of 50 mM Tris-HCl buffer pH 8.0, orlistat, and 10 mg/ml pancreatic lipase enzyme was added one by one. The mixture of these solutions was then mixed well and incubated for 10 min at 37 °C. After that, p-NPP substrate solution was added to the mixture and mixed well. Continue to incubate the mixture for 7 minutes at 37 °C. Then the absorbance of the solution was measured at 405 nm. The control was made in the same way as the sample, replacing the sample solution (orlistat) with 10% DMSO. The blank was the solution with no enzyme added.

The percentage of pancreatic lipase inhibition (I%) was calculated by equation (1):

$$I\% = [(\Delta A_0 - \Delta A)/ A_0] \times 100 \quad (1)$$

Where: ΔA_0 and ΔA were the absorbance difference of the control solution and the test sample compared to the blank, respectively.

A linear regression equation showing the correlation between the logarithm of test substance concentration (µM) and the percentage of pancreatic lipase inhibition was built, from which the IC_{50} value of the test sample would then be obtained.

3. RESULTS

3.1. Factors affecting the enzymatic activity of pancreatic lipase

Buffer solution and pH

The investigation of the influence of the buffer on enzyme activity was performed on two systems, Tris-HCl and potassium phosphate (KP) with different pH values used in published works [8-14]. The results are shown in Table 1.

Table 1. Slopes of absorbance against time linear graphs in various buffer solutions.

Buffer	Slope
KP pH 7.0	0.0272
KP pH 7.5	0.0431
KP pH 8.0	0.0574
Tris-HCl pH 7.0	0.0217
Tris-HCl pH 7.5	0.0590
Tris-HCl pH 8.0	0.0764
Tris-HCl pH 8.5	0.0635
Tris-HCl pH 9.0	0.0571

Among the buffer systems tested, Tris-HCl buffer at pH 8.0 was measured for the highest pancreatic lipase activity, with a slope K value of 0.0764.

Temperature

This study investigated the influence of experimental temperature conditions on pancreatic lipase enzyme activity at 2 levels, room temperature (25 °C) and physiological temperature (37 °C) (Table 2).

Table 2. Slopes of absorbance against time linear graphs at different temperature conditions and incubation times.

Time (min)		5	10	15
Slope	25 °C	0.0766	0.0758	0.0753
	37 °C	0.0750	0.0758	0.0762

The study selected a test temperature of 37 °C in accordance with the results of the investigation and the natural conditions of the enzyme.

Incubation time

Results of IC_{50} determination of orlistat, when incubated for different time periods, are shown in Table 3, in which the selected incubation time was 10 min.

Table 3. IC_{50} values at different incubation times

Incubation time before adding substrate (min)	R^2	IC_{50} (µM)
0	0.9458	0.13
5	0.9415	0.13
10	0.9776	0.14
15	0.9662	0.13

Hydrolysis time

Based on a curve showing the rate of a hydrolysis reaction over a complete progression from the initial stage of the reaction until the reaction slows down and ceases, clearly define the linear range of the hydrolysis rates over time. The absorbance against time at 405 nm every 1 min for 20 min was measured, and the results as shown in Fig. 2 and Table 4 were obtained, from which 7 min was the selected measuring interval.

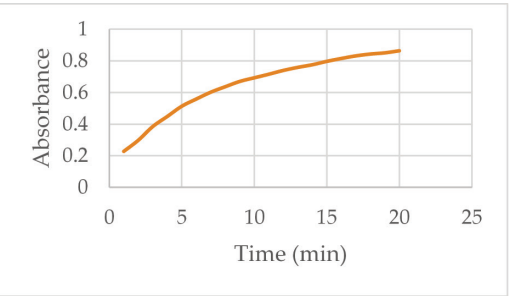

Fig. 2. The absorbance over time of p-nitrophenol from the hydrolysis of the p-nitrophenyl palmitate substrate.

Table 4. R^2 values of the graph showing absorbance against time at every minute.

Time (min)	R^2	Time (min)	R^2
5	0.9968	13	0.9448
6	0.9918	14	0.9380
7	0.9868	15	0.9332
8	0.9803	16	0.9293
9	0.9745	17	0.9256
10	0.9663	18	0.9210
11	0.9580	19	0.9150
12	0.9514	20	0.9096

3.2. Pancreatic lipase inhibitory activity

Applying the conditions of time, pH, and temperature that were investigated above, the study evaluated the pancreatic lipase inhibitory activity of orlistat obtained IC_{50} values as presented in Table 5.

Table 5. Pancreatic lipase inhibitory activity of orlistat obtained from this study and reported by other authors.

No.	Study	IC_{50} (µM)
1	Current study	0.14
2	Itoh et al. (2019) [15]	0.10
3	Patil et al. (2015) [16]	0.15
4	Dechakhamphu Ananya & Wongchum Nattapong (2015) [17]	0.16

4. DISCUSSIONS

In the present study, pH, temperature, incubation time, and hydrolysis time were significantly affected by the inhibiting activity of pancreatic lipase. The most optimal conditions selected to conduct the assay were 10 min incubation at 37 °C in Tris-HCl buffer pH 8.0 and measured the absorbance 7 min from the start of the reaction.

The pH of the buffer solution greatly affects the enzyme activity. Enzymes usually only exhibit their maximum activities within a certain pH range [18]. Outside this range, their activities are significantly reduced. Therefore, the choice of buffer solution is very important. The results showed that the hydrolysis rate was highest and the enzyme was stable at pH 8.0 in Tris-HCl buffer. Furthermore, pH 8.0 is close to the physiological condition in the duodenum where pancreatic lipase is active in humans [19]. While buffering at pH 8.5 or 9.0, the strongly alkaline environment itself can cause spontaneous hydrolysis of p-nitrophenyl esters; in addition, this condition is different from the physiological pH of the human body. Therefore, Tris-HCl buffer pH 8.0 was selected to conduct the assay. On the other hand, potassium phosphate pH 8.0 also give the highest pancreatic lipase activity with a slope K value of 0.0574. The results showed that the hydrolysis rate increases proportionally to the pH of the buffer system. We need to do more investigations at higher pH to determine the influence of the pH of KP on enzyme activity.

Enzyme activity is temperature-dependent. Many lipase inhibition assays were performed at physiological temperature (37 °C) [20-22], while others were at 25 °C [9, 23]. Enzyme incubation at 37 °C for 5, 10, and 15 min compared with incubation at 25 °C for the same time interval showed that the hydrolysis rate, as well as the stability of the enzyme, were not significantly different ($p>0.05$). Therefore, the study selected a test temperature of 37 °C under the results of the investigation and the natural conditions of the enzyme.

The incubation time in the pancreatic lipase inhibitor test is the period in which the inhibitor and enzyme are incubated in a buffer solution before the substrate is added to ensure that the inhibitor can interact with the enzyme prior to the test. The test solutions are stored at low temperatures so incubation will warm the test sample, ensuring that the reaction in the test is initiated at the chosen physiological temperature. Results of IC_{50} determination of orlistat, when incubated for different time periods, are shown in Table 3. These IC_{50} values obtained did not have a statistically significant difference ($p>0.05$). However, the R^2 value of the regression equation representing the logarithmic percentage inhibition of orlistat concentration when incubated for 10 minutes was higher than that without incubation and incubation for 5 minutes and 15 minutes. Therefore, the selected incubation time was 10 min.

From Fig. 2, it could be seen that the reaction followed a linear relationship at the beginning, then due to substrate depletion, the reaction slowed down and finally ceased. Based on Table 4, the linearity of the graph showing absorbance against time was decreased, which was demonstrated by the decreasing value of R^2. The measurement time was chosen as the time at which the reaction rate remained linear and the amount of formed product was sufficient to ensure accurate detection by the photometer [24]. On that basis, 7 min was the selected measuring interval, at which $R^2 = 0.9868$ and the absorbance of the sample with enzyme was in the range of 0.6-0.8 in accordance with the range of measuring linearity of the equipment. The results obtained from measuring at a time when the absorbance is too low would be liable to errors.

The experimental conditions employed for assessing pancreatic lipase inhibitory activity in this research exhibit certain resemblances and features when compared to the three referenced studies as presented in Table 6. Firstly, the utilization of p-NPP as the substrate in this study shares a common coloration mechanism with the p-nitrophenyl butyrate substrate employed in the investigations conducted by Patil et al. (2015) [16] and Ananya et al. (2015) [17]. Secondly, all four studies, including the present one, employed the Tris-HCl buffer system. While this study maintained a pH of 8.0, Itoh et al. (2019) [15] employed a similar pH value, and the remaining two studies were conducted at a pH of 7.0. Thirdly, all the tests were incubated at 37 °C, except for Patil et al. (2015), which did not specify the incubation temperature. In this study and the investigation by Patil et al. (2015), the incubation period was 10 minutes, whereas the other two studies utilized longer incubation times. Regarding the measurement of absorption, p-NP pigment exhibits its maximum absorption at approximately 410 nm within a nearly neutral pH range [25]. Patil et al. (2015) performed absorption spectroscopy at this wavelength using a UV-vis spectrophotometer. However, this instrument measures samples one by one in succession, resulting in deviations due to the time difference between measurements and

consuming a substantial amount of time. To address this issue, the present research employed an Elisa machine capable of simultaneously measuring 96 wells at an accelerated rate. Nonetheless, conventional Elisa machines only measure specific wavelengths. Consequently, the study opted to measure absorption spectroscopy at 405 nm on the Elisa machine, which closely aligns with the maximum absorption of p-NP. This wavelength selection is also consistent with the research approach employed by Ananya et al. (2015). Notably, Itoh et al. (2019) conducted their study using the substrate 4-Methyl-umbelliferyl oleate, which operates under a distinct mechanism of action compared to the other three studies. Consequently, the marker determination was performed using a different method and a different type of instrument.

Table 6. Comparison of conditions for performance testing of published works

No	Study	Substrate	Buffer	t°C	Incubation time	Wave-length	Machine
1	Current study	p-nitrophenyl palmitate	Tris-HCl 8.0	37 °C	10 mins	405 nm	Elisa
2	Itoh et al. (2019) [15]	4-Methyl-umbelliferyl oleate	Tris-HCl 8.0	37 °C	30 mins	355 nm and 460 nm	Multi-label counter
3	Patil et al. (2015) [16]	p-nitrophenyl butyrate	Tris-HCl 7.0	---	10 mins	410 nm	UV-Vis Spectro-photometer
4	Dechakhamphu Ananya & Wongchum Nattapong (2015) [17]	p-nitrophenyl butyrate	Tris-HCl 7.0	37 °C	15 mins	405 nm	Elisa

---: not mentioned

Orlistat is currently the only pancreatic lipase inhibitor licensed for the treatment of obesity, and it is also commonly used as a control in the assays evaluating pancreatic lipase inhibition. The data in Table 5 shows that the inhibitory activity of orlistat obtained from this study was similar to those published by other authors. This shows that with the parameters of conditions affecting pancreatic lipase activity drawn from this study, the results of enzyme inhibition activity could be reliable and these conditions could be applied in the assays evaluating enzyme activity in search of novel pancreatic lipase inhibitors for use as anti-obesity drugs.

4. CONCLUSION

The study investigated the factors affecting pancreatic lipase activity and proposed optimal parameters for testing pancreatic lipase inhibitory activity under available research conditions. The test method applying the conditions drawn from this study gave the results of evaluating the pancreatic lipase inhibitory activity of orlistat - a commonly used reference standard in the assays to evaluate the pancreatic lipase inhibitory activity – in agreement with the statement of other authors. Thus, the experimental condition parameters obtained from this publication should be used in studies looking for novel substances with pancreatic lipase inhibitory activity.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. WHO. Obesity and overweight: WHO; 2021 [01/05/2022]. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.
2. WHO. Obesity: WHO; 2021 [01/05/2022]. Available from: https://www.who.int/news-room/facts-in-pictures/detail/6-facts-on-obesity.
3. Albashir AAD. The potential impacts of obesity on COVID-19. Clin Med (Lond). 2020;20(4):109-13.
4. Buchholz T, Melzig MF. Polyphenolic Compounds as Pancreatic Lipase Inhibitors. Planta Med. 2015;81(10):771-83.
5. Lucas KH, Kaplan-Machlis B. Orlistat--a novel weight loss therapy. Ann Pharmacother. 2001;35(3):314-28.
6. Mohamed GA, Ibrahim SRM, Elkhayat ES, El Dine RS. Natural anti-obesity agents. Bulletin of Faculty of Pharmacy, Cairo University. 2014;52(2):269-84.
7. Cam-Van T. Vo, Nhan V. H. Luu, Thoai T. H. Nguyen, Truc T. Nguyen, Bach Q. Ho, Thuong H. Nguyen, Thanh-Dao Tran & Quoc-Thai Nguyen (2022) Screening for pancreatic lipase inhibitors: evaluating assay conditions using p-nitrophenyl palmitate as substrate, All Life, 15:1, 13-22
8. Ha MT, Tran MH, Ah KJ, Jo KJ, Kim J, Kim WD, et al. Potential pancreatic lipase inhibitory activity of phenolic constituents from the root bark of *Morus alba* L. Bioorg Med Chem Lett. 2016;26(12):2788-94.
9. Zhao T, Yan GR, Pan SL, Wang HY, Hou AJ. New isoprenylated 2-arylbenzofurans and pancreatic lipase inhibitory constituents from *Artocarpus nitidus*. Chem Biodivers. 2009;6(12):2209-16.
10. Mhatre SV, Bhagit AA, Yadav RP. Proteinaceous Pancreatic Lipase Inhibitor from the Seed of *Litchi chinensis*. Food technology and biotechnology. 2019;57(1):113-8.
11. Jang DS, Lee GY, Kim J, Lee YM, Kim JM, Kim YS, et al. A new pancreatic lipase inhibitor isolated from the roots of *Actinidia arguta*. Arch Pharm Res. 2008;31(5):666-70.
12. Wang S-m, Huang AHC. Inhibitors of Lipase Activities in Soybean and Other Oil Seeds. Plant Physiology. 1984;76(4):929–34.
13. Sridhar SN, Ginson G, Venkataramana Reddy PO, Tantak MP, Kumar D, Paul AT. Synthesis, evaluation and molecular modelling studies of 2-(carbazol-3-yl)-2-oxoacetamide analogues as a new class of potential pancreatic lipase inhibitors. Bioorg Med Chem. 2017;25(2):609-20.
14. Lee EM, Lee SS, Chung BY, Cho JY, Lee IC, Ahn SR, et al. Pancreatic lipase inhibition by C-glycosidic flavones Isolated from *Eremochloa ophiuroides*. Molecules. 2010;15(11):8251-9.
15. Itoh K, Matsukawa T, Murata K, Nishitani R, Yamagami M, Tomohiro N, et al. Pancreatic Lipase Inhibitory Activity of *Citrus unshiu* Leaf Extract. Natural Product Communications. 2019;14(9):1934578X19873439.
16. Patil SG, Patil MP, Maheshwari VL, Patil RH. In vitro lipase inhibitory effect and kinetic properties of di-terpenoid fraction from *Calotropis procera* (Aiton). Biocatalysis and Agricultural Biotechnology. 2015;4(4):579-85.
17. Dechakhamphu A, Wongchum N. Screening for anti-pancreatic lipase properties of 28 traditional Thai medicinal herbs. Asian Pacific Journal of Tropical Biomedicine. 2015;5(12):1042-5.
18. Bao VVQ, Trung NT. Obtaining and investigating some properties of ficin products from fig (*Ficus auriculata* L). Hue University Journal of Science: Agriculture and Rural Development. 2018;127(3A):139-49.
19. Carrière F RC, Lopez V, De Caro J, Ferrato F, Lengsfeld H, De Caro A, Laugier R, Verger R. The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test meals. Gastroenterology. 2000;119(4):949-60.
20. Kim YM, Lee EW, Eom SH, Kim TH. Pancreatic lipase inhibitory stilbenoids from the roots of *Vitis vinifera*. Int J Food Sci Nutr. 2014;65(1):97-100.
21. Noorolahi Z, Sahari MA, Barzegar M, Ahmadi Gavlighi H. Tannin fraction of pistachio green hull extract with pancreatic lipase inhibitory and antioxidant activity. J Food Biochem. 2020;44(6):e13208.
22. Sridhar SNC, Palawat S, Atish T. Paul. Design, synthesis, biological evaluation and molecular modelling studies of indole glyoxylamides as a new class of potential pancreatic lipase inhibitors. Bioorganic Chemistry. 2019;85:373-81.
23. Kang HS, Kim JP. Ostalactones A-C, β- and ε-Lactones with Lipase Inhibitory Activity from the Cultured Basidiomycete *Stereum ostrea*. J Nat Prod. 2016;79(12):3148-51.
24. Hans B. Enzyme assays. Perspectives in Science. 2014;1(1):41-55.
25. Syedd-León, R., Sandoval-Barrantes, M., Trimiño-Vásquez, H., Villegas-Peñaranda, L., & Rodríguez-Rodríguez, G. (2020). Revisiting the fundamentals of p-nitrophenol analysis for its application in the quantification of lipases activity. A graphical update. Uniciencia, 34(2), 31-43.