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Lipase Enzyme Kinetics with the Addition of Avocado Seed Extract as an Inhibitor in Olive Oil

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Abstract: Free fatty acids are compounds resulting from the hydrolysis of triglycerides catalyzed by the enzyme lipase. Free fatty acids harm health if they enter the body in large amounts. Avocado seed extract can reduce free fatty acid levels by inhibiting lipase activity. This research is a type of true-experimental research that aims to determine the effect of variations in substrate concentration on lipase enzyme activity with the addition of avocado seed extract and to determine the impact of the addition of avocado seed extract as an inhibitor on lipase enzyme kinetics. Lipase activity was determined based on the amount of oleic acid formed using Cu acetate pyridine reagent by Uv-Vis spectrophotometry at a wavelength of 715 nm. Lipase enzyme kinetics were determined using the Lineweaver-Burk curve equation, a transformed form of the Michaelis-Menten equation. The results showed that 60% substrate concentration was the most effective in reducing lipase enzyme activity by 60%. In addition, the addition of avocado seed extract proved to act as a competitive inhibitor, with the K_M value decreasing from 0.014 M to 0.004 M and V_{maks} remaining relatively stable at 23.2 µM. This shows that avocado seed extract effectively reduces lipase enzyme activity, which has implications for reducing FFA levels in olive oil. Thus, this study concludes that avocado seed extract has great potential as a natural inhibitor agent in reducing lipase enzyme activity.

Keywords: Avocado Seed; Free Fatty Acid; Inhibition; Kinetics; Lipase Enzyme.

Introduction

As an agricultural country, Indonesia allows vast farmland to grow various plants, including avocados [1]. Avocados consist of parts, namely pulp, seeds, and skin. The avocado pulp is the part that is most often utilized, such as fresh juice or skin. Most often utilized, such as making fresh juice or mixing with other healthy foods such as salads. Different parts of the avocado, namely the skin and seed, are less utilized, so they are often underutilized. Parts that are less utilized, so they usually accumulate into waste [2]. Avocado seeds contain beneficial carbohydrates, proteins, minerals, and secondary metabolite compounds. Avocado seeds extracted with methanol solvent contain secondary metabolite compounds, including alkaloids, flavonoids, saponins, tannins, triterpenoids, and steroids [3]. According to [4], the results of methanol extracts from avocado seed waste contain alkaloid, tannin, and saponin compounds that can reduce free fatty acid (FFA) levels.

Free fatty acids are fatty acids that are not bound as triglycerides. Moisture in the oil produced during the frying process results in the oil hydrolysis, resulting in high amounts of free fatty acids. Improper or too long oil storage can cause the triglyceride bonds in the oil to break, resulting in glycerol and free fatty acids [5]. High concentrations of free fatty acids in oil can adversely affect the body when consumed, such as poisoning, fat accumulation in blood vessels, diarrhea, and cancer [6]. Free fatty acids can also be formed through triglyceride hydrolysis, catalyzed by lipase enzymes [7]. Lipase enzymes function as biocatalysts, meaning compounds that increase the rate of enzymatic reactions.

Enzyme activity can be affected by inhibitors. The inhibitor is a compound that binds to an enzyme by attacking the active side to decrease enzyme activity [8]. The Lineweaver-Burk enzyme kinetics equation shows how inhibitors can affect lipase activity, a modified version of the Michaelis-Menten equation. The Michaelis-Menten method describes the relationship between the maximum reaction rate (V_{max}) and the Michaelis-Menten constant (K_M) [9]. Through the Michaelis-Menten equation, the Lineweaver-Burk Plot graph is represented, where through this plot, it can be seen whether the enzyme inhibitor is competitive or non-competitive. If the inhibitor is competitive, the K_M value obtained is different. The V_{max} value is the same or has similarities, so the Lineweaver-Burk plot shows lines that intersect on the y-axis, while if the inhibitor is non-competitive, the K_M value obtained is the same or not much different [10]. The Vmax value is different, and the Lineweaver-Burk plot will show lines that intersect on the x-axis [11].

Based on the background presented, avocado seed extract contains secondary metabolite compounds that inhibit the formation of free fatty acids catalyzed by the lipase enzyme on olive oil substrates. Then, this study was conducted to determine the kinetics of lipase enzymes by adding avocado seed extract as a lipase enzyme inhibitor on olive oil substrates.

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Research Methods

Tools and Materials

In this study, several tools were used, UV-Vis spectrophotometer (Shimadzu 1800), vacuum rotatory evaporator, test tube (Iwaki), measuring flask (pyrex), measuring cup (pyrex), analytical balance, pH meter, test tube (pyrex), spatula, funnel. The materials used were avocado seed, Borges brand olive oil, Novozymes brand lipase enzyme, technical methanol, Cu acetate (Merck), pyridine (Merck), and isoctane (Merck).

Avocado Seed Extraction

Avocado seed samples were cleaned and washed with running water and then cut into small pieces to facilitate the drying and grinding. Avocado seeds are dried by aerating and made into simplistic powder by pureeing and sieving using a blender. Avocado seed simplisia was extracted using the maceration method. Avocado seed simplisia, as much as 200 g, was put into a glass jar and then filled with 800 ml of methanol until the sample was submerged. Maceration was carried out for 72 hours. Furthermore, the maceration results were evaporated with an evaporator at 50^{o}C [4].

Lipase Activity of Substrate Concentration Variation

A 0.5 ml lipase enzyme was added with variations of olive oil concentrations of 40, 50, 60, 70 and 80% (v/v), 5% avocado seed extract, then incubated for 1 hour at 25^{o}C. After that, the oil layer was taken in as much as 3 ml and added with 0.4 ml Cu-acetate pyridine pH 5.6. Then, it was vortexed until homogeneous. After homogeneous, the solution was centrifuged for 15 minutes at 4000 rpm. Then, the absorbance was read using a spectrophotometer with a wavelength of 715 nm [12].

Kinetics of Lipase Enzyme with Addition of Avocado Seed Extract

The determination of lipase enzyme kinetics (Vmax and KM) is based on a graphical plot of the relationship between 1/[S] and 1/V where [S] is the substrate concentration and V is the enzyme activity, and then the Vmax and KM values are determined based on the Lineweaver-Burk equation as follows

- That from the equation 1/V = 1/Vmax + Km/Vmax(1/[S])
- If 1/V = Y and 1/[S] = X, the formula can be Y = a + bX, so a = 1/Vmax and b = Km/Vmax.

Thus, if the price of 1/Vmax is known, then the value of Vmax is obtained, and the value of Km will also be obtained from the equation b = Km/Vmax [13].

Free Fatty Acid Content Assay

Free fatty acids were determined by adding 5 grams of olive oil to 10 ml of 96% alcohol and then dropping five drops of PP indicator into the mixture. The mixture was then shaken and titrated with 0.1 N KOH until a pink colour appeared, which did not disappear within 10 seconds [14].

$$\text{FFA\%} = \frac{\text{v KOH (mL)} \times \text{N KOH} \times \text{BM Oleic Acid}}{\text{Sample weight (g)}} \times 100\%$$

Results and Discussion

Avocado seed extraction was carried out using a maceration method using a methanol solvent, giving a yield of 8.76%, and the extract obtained was oily. This study used a cold extraction method, namely maceration, to avoid heating, which could damage the target compounds in the sample [15]. The maceration process lasts several days, and the sample is soaked in a methanol solvent. The avocado seed extracts obtained were tested for secondary metabolite compounds using LC-MS (Liquid Chromatography-Mass Spectrometry) instruments, and 101 active compounds were identified, as shown in Figure 1.

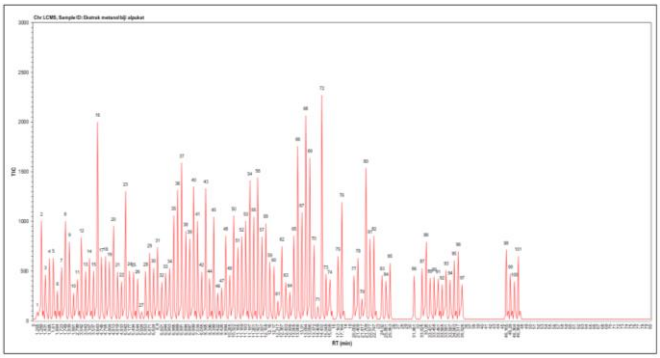

Figure 1. Chromatogram LC-MS Avocado Seed Extraction

Based on the secondary metabolite compounds identified, there are compounds with the potential to inhibit lipase, with the highest percentage composition contained in avocado seed extract, as in Table 1.

Table 1. Results of Identification of Secondary Metabolite Content of Avocado Seed Extract Through LC-MS Analysis

Compound Result	RT (Min)	Composition (%)
Caffeic acid	4.643	2.70476
Kaempferol	10.322	1.42961
Quercetin	11.427	1.94858

Based on the research results, it was found that caffeic acid, kaempferol, and quercetin compounds have the potential to be lipase inhibitors. The compounds were found in high amounts, and the three compounds are suspected of inhibiting lipase. Through docking analysis, it is predicted that there is a hydrogen bond between the hydroxyl group on caffeic acid and the polar group of lipase, causing lipase inhibition [16]. Research by Li et al. (2020) related to kaempferol compounds as lipase inhibitors showed a competitive inhibition type, where kaempferol competes with the substrate to bind to the active side of the enzyme so that enzyme activity is inhibited and can reduce fat absorption [17]. Quercetin compound is one of the suitable inhibitors for lipase. Several other studies that have been conducted related to quercetin as a lipase inhibitor show more potent lipase inhibition compared to rutin, luteolin,

catechin, and hesperetin. According to Junyoung (2019), quercetin inhibited potent lipase compared to kaempferol and quercitrin. Quercetin binds to the non-competitive domain of the lipase enzyme and inhibits enzyme activity [18].

Effect of Substrate Concentration Variation on Lipase Activity with the Addition of Avocado Seed Extract

One of the factors that affect enzyme activity is the substrate concentration. Lipase enzymes catalyze triglyceride hydrolysis, producing free fatty acids and glycerol and producing free fatty acids and glycerol. The high free fatty acid content in oil can cause negative effects on the body when consumed, for example, poisoning, diarrhoea, fat deposition in blood vessels, and cancer [6]. Inhibition of free fatty acids can be done by adding avocado seed extract as an inhibitor, which can be identified based on decreased lipase activity.

Table 2. Lipase activity with and without avocado seed extract at various substrate concentrations

Substrate Concentration (%)	Lipase Activity (U/ml) Without avocado seed extract	Lipase Activity (U/ml) With avocado seed extract	Decrease in Lipase Activity (%)
40	8.4	3.4	58
50	9.12	3.9	57
60	11.2	4.5	60
70	11.6	5.55	52
80	11.9	5.85	51

The effect of olive oil substrate concentration: Increasing the substrate concentration causes the enzyme reaction speed to increase, increasing the enzyme activity value. The addition of the same inhibitor concentration in the substrate concentration variation shows a difference in lipase activity compared to without the addition of avocado seed extract. The research supports this [11] with Zn (II) Catechin as an inhibitor with the same amount of lipase can inhibit lipase enzymes with varying concentrations of p-nitrophenyl substrate and in the research [19] with the addition of methanolic extract of papaya leaves in the same amount can also inhibit lipase enzyme at various substrate concentrations. The decrease in olive oil substrate concentration is directly proportional to the increase in lipase enzyme activity. The increase in enzyme reaction speed or enzyme activity will be more minor after reaching a point where increasing substrate concentration will only slightly increase enzyme activity, and this situation is called the maximum point. The percentage decrease in lipase enzyme activity by avocado seed extract as influenced by substrate concentration is shown in Figure 2.

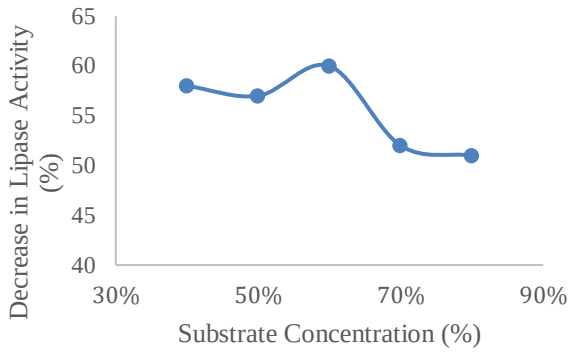

Figure 2. Percentage decrease in lipase activity at various substrate concentrations

The olive oil substrate concentration on increased lipase enzyme activity is shown in Figure 2. Increasing the substrate concentration causes the enzyme reaction speed to expand to obtain an increasing graph. The addition of the same inhibitor concentration at various substrate concentrations shows a difference in lipase activity compared to without the addition of avocado seed extract.

Lipase Enzyme Kinetics with the Addition of Avocado Seed Extract

The Lineweaver-Burk equation, modified by the Michaelis-Menten equation, determined Lipase enzyme kinetics. The parameters specified in this enzyme kinetics are K_M (Michaelis-Menten Constant) and V_{max}, or maximum reaction speed [20]. Determination of V_{max} and K_M is done by using the relationship graph between 1/initial reaction rate ($1/V_0$) as the y-axis and 1/Substrate concentration (1/S) as the x-axis, as shown in Figure 3. to obtain a K_M value of 0.014 M and a V_{max} of 23.1 µM in the lipase enzyme without the addition of avocado seed extract. The lipase enzyme added with avocado seed extract showed a K_M value of 0.004 M and a V_{max} of 23.2 µM.

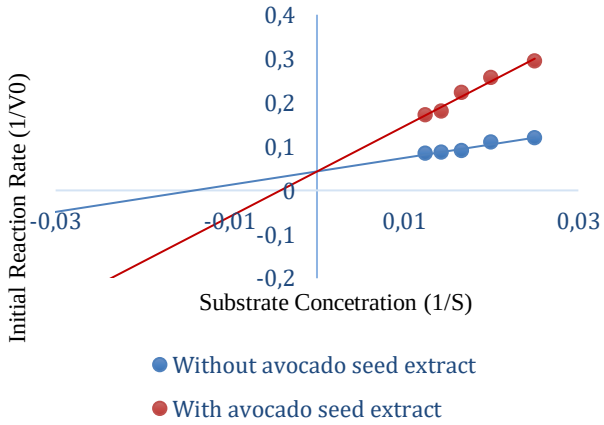

Figure 3. Lineweaver-Burk Graph of Inhibition Reaction of Lipase Enzyme Activity by Avocado Seed Extract

All enzymes are bound as enzyme-substrate complexes at the maximum reaction rate (V_{max}). The V_{max} value without the addition of avocado seed extract is higher than that with the addition of avocado seed extract, meaning that the enzyme reaction rate to form the enzyme-

substrate complex is faster due to the absence of an inhibitor. The K_M value indicates the concentration of olive oil substrate when the lipase enzyme reaches half its maximum speed. A small K_M value indicates the enzyme-substrate complex has a low affinity for the substrate. Enzyme inhibition is divided into competitive, non-competitive, and uncompetitive. Competitive inhibition decreases the K_M value, but the V_{max} value does not change, while non-competitive inhibition decreases the Vmax value [21]. Based on the results obtained, the K_M value decreases significantly while the V_{max} value is similar or does not change until the lines formed intersect the Y-axis. Through this, it is known that the type of inhibition occurs is competitive. Competitive inhibition is an enzyme inhibition that binds to the enzyme at the active site location so that the inhibitor competes with the substrate [22].

Free Fatty Acid Content Assay

The measurement results of free fatty acid (FFA) content without the addition of avocado seed extract and the addition of avocado seed extract as a lipase enzyme inhibitor are shown in Table 3. Based on the data obtained, it is known that the higher the substrate concentration added, the more free fatty acids are formed. In this study, the measurement of free fatty acids was carried out using the alkalimetric titration method. Alkalimetry is the determination of compound levels with the principle of neutralization reaction due to the reaction between hydrogen ions derived from acid compounds in olive oil and hydroxide ions derived from the base (KOH) used as a titrant.

Table 3. FFA levels with and without avocado seed extract at various substrate concentrations

Substrate Concentration (%)	FFA content (%) Without avocado seed extract	FFA content (%) With avocado seed extract	Decrease in FFA content (%)
40	0.42	0.33	21.43
50	0.48	0.37	22.9
60	0.6	0.45	25
70	0.71	0.51	28.17
80	0.73	0.56	23.3

Free fatty acids (FFA) are fatty acids that are not bound to triglycerides. Free fatty acids result from the hydrolysis of triglycerides catalyzed by lipase enzymes. Adding avocado seed extract at each concentration can reduce free fatty acid levels. So, it can be proven that the compounds in avocado seed extract can inhibit the formation of free fatty acids catalyzed by lipase enzymes. This is supported by the research [18], which shows that lipase inhibitors can reduce fat absorption and increase fat excretion in rats.

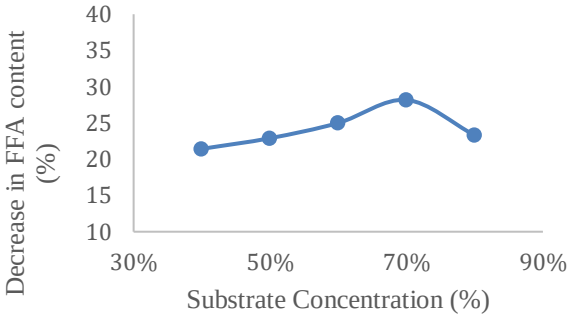

Figure 4. Percentage reduction in FFA levels at various substrate concentrations

The decrease in free fatty acid (FFA) levels before and after adding avocado seed extract in Figure 4 shows the maximum point is at 70% substrate concentration. This can occur because avocado seed extract, which is a competitive inhibitor, binds to the lipase enzyme on the active side and competes with the substrate (olive oil) so that the enzyme cannot bind to the substrate, which results in the formation of fatty acids as an inhibited product [21].

Conclusion

This study shows that adding avocado seed extract as a competitive inhibitor can significantly inhibit the lipase enzyme activity in olive oil. Based on the experimental results, 60% substrate concentration was the most effective, with a 60% decrease in enzyme activity, and the best substrate concentration in reducing free fatty acid (FFA) was at 70% concentration, with a reduction in ALB levels by 28%. Enzyme kinetics results showed that adding avocado seed extract caused a decrease in K_M value from 0.014 M to 0.004 M. At the same time, V_{maks} remained stable at 23.2 µM, indicating that the inhibition mechanism is competitive.

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