

Research Article

Characteristics of purified indigenous lipase from germinated cocoa bean using phenyl sepharose

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Abstract

This study was carried out with lipase of germinated cocoa bean (*Theobroma cacao L.*) purified on hydrophobic interaction chromatography of phenyl sepharose as support matrix to find out the lipase characteristics. The results of the research show that the lipase purification had a recovery of 20.58%. The lipase was purified about 115 fold and had a molecular weight of 36 kDa. Optimum temperature of the lipase activity was 38^oC and temperature stability was 30-40^oC. This cocoa bean lipase was alkaline lipase which had optimum pH of 9 and stable at pH range of 7 to 10. The lipase activity increased with addition of CaCl₂ 1mM, MgCl₂ 10mM and PMSF 1-10 mM. However, the activity was inhibited when CuCl₂, HgCl₂, FeCl₃, AlCl₃, EDTA and DMSO were added, while NaCl did not affect the enzyme activity. Substrate specificities of the germinated cocoa bean lipase were stearic, oleic and palmitic acids. Its region-specificity was at *sn*-1.3.

Keywords: enzyme activity, germination, purification, phenyl sepharose, characteristics, *Theobroma cacao L.*, Indonesia.

Introduction

Lipase (EC 3.1.1.3) is an enzyme which catalyses the hydrolysis reaction of ester carboxyl bonding in acylglycerol and is capable of esterification at low water content. Generally, lipase has a catalytic triad which consists of amino acid: serine, histidine and aspartate or glutamate, but the character of each lipase can differ. The difference in lipase character is caused by the difference in its protein structure [1]. Lipase character of microbes can be influenced by its medium where it grows, while lipase character of plants and animals are not influenced by their environment. Lipase from bean has specific properties, such as it has high affinity with fatty acid of the bean content. This property is not found in lipase of microbes [2].

Different kinds of bean giving different characters of lipase, such as lipase from peanut beans (*Arachis hypogaea L.*) has optimum pH of 8.5 [3], while lipase from linseed (*Linum usitatissimum L.*) optimum at pH 4.7 [4]. Moreover, lipase from bay laurel (*Laurus nobilis L.*) has the same

optimum pH of 8 with the African oil bean (*Pentacletra macrophylla* Benth), but the optimum temperature of these lipases are different, that is 50°C and 30°C, respectively [5, 6]. Due to these differences, lipase from beans should be characterized to find out their optimum activities.

Cocoa beans have high fat content of about 30-32% of wet beans or > 50% in dry beans. Cocoa fat is composed of POST, StOST and POP (P is palmitic acid, O is oleic acid, St is stearic acid) as main triglycerides, giving 70%-80% of total fat content [7]. Some research has been performed related to lipase from cocoa bean. Samsumoharto [8] has isolated lipase from ungerminated cocoa bean clone PBC 159. In a previous study, it was found that lipase from germinated bean has higher activity than that of ungerminated bean [9]. The highest activity was dependent on length of germination, for hydrolysis activity was at 3 days while for esterification activity was at 4 days. Although there have been many studies on cocoa beans, studies on lipase character of germinated cocoa beans are still limited. This study is aimed to characterize the indigenous lipase of germinated cocoa bean purified using phenyl sepharose.

Materials and Methods

Materials

Cocoa beans of *Klon Kakao* Malaysia 4 (KKM4) were harvested from the cocoa plantation of PT. Pagilaran at Samigaluh, Kulon Progo Regency, Yogyakarta. Sucrose, CaCl₂, olive oil, fatty acid kit, pyridine, Cu-acetic, oleic acid and polyphenylpolypyrrolidone (PVPP) were from Sigma Co., USA. Plate of TLC Silica gel F, NaH₂PO₄, Na₂HPO₄, n-hexane, diethylether, petroleum ether and isooctane were from Merck, Germany, whereas Phenyl Sepharose was from Tosoh Corporation, Japan. Ethylenediaminetetraacetic acid (EDTA), Phenylmethyl-sulfonylfluoride (PMSF) and Dimethyl sulfoxide (DMSO) were purchased from Sigma Co., USA.

Bean germination and lipase extraction

The bean germination and lipase extraction were carried out by the method of Abigor, *et al* [10] that was slightly modified [11]. The 4-days-germinated cocoa beans were peeled and 50 g of them was added with 4 g of PVPP, and then were scraped. The scraped cocoa powder was added with 150 ml of 0.15 M phosphate buffer pH 7.5 which contained 0.6 M sucrose and 1 mM of CaCl₂. The mixture was homogenized using homogenizer of Nissei AM-10 for 10 minutes at 10,000 rpm followed by centrifugation for 30 minutes at 5000 g and 4°C. The supernatant was described as crude lipase and used for the following study.

Purification of lipase

All of purification processes were carried out at temperature of 4°C. Before purification, lipase was precipitated with ammonium sulphate by the method of Doonan [12] at various ammonium sulphate saturations (20, 40, 60 and 80%). After addition of ammonium sulphate, crude lipase (100 ml) was precipitated for 1 h and resulting precipitate was collected by centrifugation at 5000 g for 30 min (4°C). The precipitate was dissolved in the smallest possible volume of phosphate buffer (pH 7.5). The lipase activity and protein content of this solution were then examined.

The concentrated enzyme solution was loaded onto a hydrophobic interaction chromatography column of phenyl sepharose (3x10 cm) according to the method of Mhetras, *et al* [13]. The column was equilibrated with 0.15 M phosphate buffer pH 7.5 containing ammonium sulphate at selected saturation degree (buffer A). Before using phenyl sepharose was soaked overnight in the same buffer. Elution of un-bound proteins was performed with the same buffer at flow rate of 0.5 ml/min and 2 ml fractions were collected. The lipase was then eluted using phosphate buffer pH 7.5 containing gradient ammonium sulphate concentration (buffer A) to 0% in a total volume of four times of the column volume at flow rate of 0.5 ml/min. The protein content ($\lambda= 280$ nm) and its

activity were monitored for every fraction (2 ml). The fractions which had lipase activity were pooled and used for the next study.

SDS-PAGE

The protein of every purification step was applied to SDS-PAGE by the method of Laemmli [14] on a 10% gel. The gel was stained with Coomassie Brilliant Blue R250. Protein marker used was the Full-Range MW Marker and purchased from GE Healthcare, USA.

Effect of temperature on activity and stability of lipase

Optimum temperature of purified lipase was determined by the hydrolysis assay method at various temperatures (30, 35, 40, 45 and 50 °C). Thermal stability of the enzyme was carried out by soaking the enzyme solution in water-bath at 30-70 °C for 60 min and after that put them in ice-bath immediately. The residual activity was then measured by the hydrolysis assay method at the optimum temperature.

Effect of pH on activity and stability of lipase

The lipase activity was measured by the hydrolysis assay method in pH range of 4-10. The pH stability was observed by the method of Mayordomo, *et al* [15]. Buffer of 1.35 ml was added to 0.15 ml lipase solution and then incubated for 30 min at room temperature. The residual lipase activity was detected by the hydrolysis assay method at the optimum pH condition. The 0.15 M of universal buffer [16] was used for all pH ranges.

Effect of metal ions and inhibitors

The effect of metal ions (NaCl, CaCl₂, MgCl₂, CuCl₂, HgCl₂, FeCl₃, AlCl₃) and inhibitors (EDTA, PMSF, DMSO) on the purified lipase activity were analyzed by the hydrolysis assay method. Final concentration of the metal ions and inhibitors in the reaction mixture was adjusted to 1 and 10 mM.

Substrate specificity

Substrate specificities were detected by the methods of hydrolysis and esterification assay. The hydrolysis activity was carried out on various substrates (tricaprilin, trilaurin, triolein, tripalmitin and tristearin), whereas esterification activity was examined by reacting glycerol with various fatty

Regio-specificity

Lipase region-specificity was detected by utilizing the enzyme for 24 h esterification of glycerol and oleic acid. The results were compared to that result of pancreatic lipase which was known as having region-specificity of sn-1.3. The esterification products were spotted onto a plate of TLC silica gel F and developed with a mixture of petroleum ether: diethyl ether: acetic acid = 70:30:1. Triolein, 2-monoolein and oleic acid were used as standards. The results were visualized using iodine vapor.

Enzyme assay

Hydrolysis activities of lipase were detected by the method of Marseno *et al* [17]. Olive oil 50% in isooctane (5 ml) was mixed with 250 µl lipase. It was then incubated in a shaking water-bath (100 strokes/min) at 35 °C for 60 min. The resulting oil layer was taken (3 ml) and added with 0.6 ml of pyridin Cu-acetate (pH 6). After thoroughly mixing, the mixture was centrifuged at 750 g for 5 min and then read the absorbance of the oil layer at $\lambda=715$ nm. Oleic acid was used as a standard at concentration of 0, 2, 4, 6, 8 and 10 mM. One unit of lipase activity was described as an amount of the enzyme which produced 1 µmol fatty acid per min under standard conditions.

Esterification activities were detected by the method of Watanabe, *et al* [18]. Glycerol and fatty acid (in ratio of 1:3 mol/mol) were mixed with lipase 10% and then incubated in a shaking water-

bath (100 strokes/min) at 40°C for 6 h. The residual fatty acid was measured by the method of Marseno, *et al* [17] as described above. One unit of esterification activity was described as an amount of lipase which catalyzed 1 μ mol fatty acid per min under the standard conditions.

Protein analysis

Protein was detected by the method of Lowry [19] with bovine serum albumin as a standard protein.

Results and Discussion

Purification of germinated cocoa bean lipase on phenyl sepharose

Figure 1 shows the results of ammonium sulphate precipitation of lipase in various concentrations. The higher the concentration of ammonium sulphate was added, the more protein precipitated. The lipase activity likewise increased with increasing the ammonium sulphate concentration and became constant at concentration of 60%. At 80% ammonium sulphate the enzyme activity was the lowest, which may due to the structural conformation changes. Based on these data, two steps precipitation (20-40%, which means discarding precipitate at 20% saturation and continuing precipitation with 40% saturation) were selected for the next study.

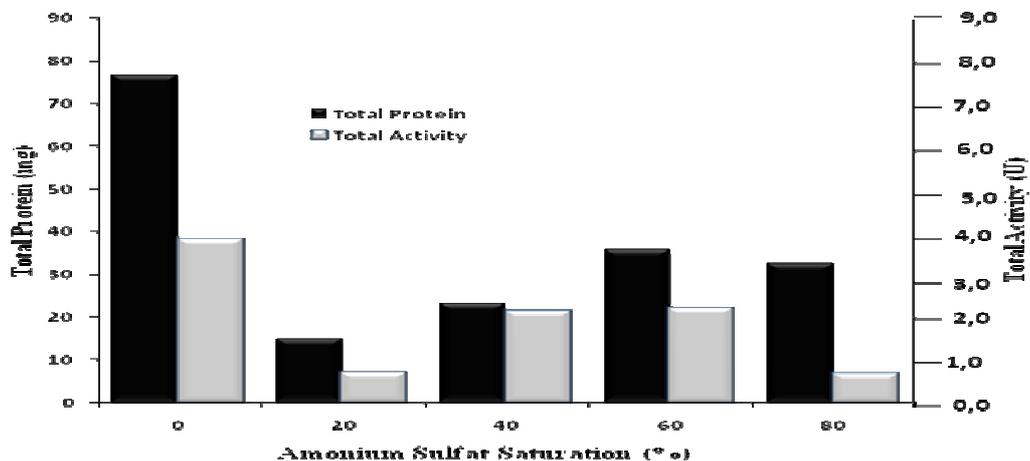


Figure 1. Precipitation of Germinated Cocoa Bean Lipase with Ammonium Sulphate.

Purification of the enzyme on phenyl sepharose column equilibrated with buffer A containing 40% ammonium sulphate made the un-bound protein impurities extensively washed out leaving the enzyme protein bound to the matrix (Fig. 2). Elution with gradient concentration of ammonium sulphate from 40% to 0% gave one peak of protein eluted at concentration near 0% which coincided with the lipase activity. This result was the same as that of potatoe lipase purified on octyl sepharose [20]. These purification steps gave a recovery of 20.58% with specific activity of 5.59 U/mg or purified approximately 115 fold (Table 1). This purified lipase gave a single band on SDS-PAGE (Fig 3) with MW of 36 kDa, which was different with that of ungerminated cocoa bean clone PBC 159 having a MW of 55-66 kDa [21].

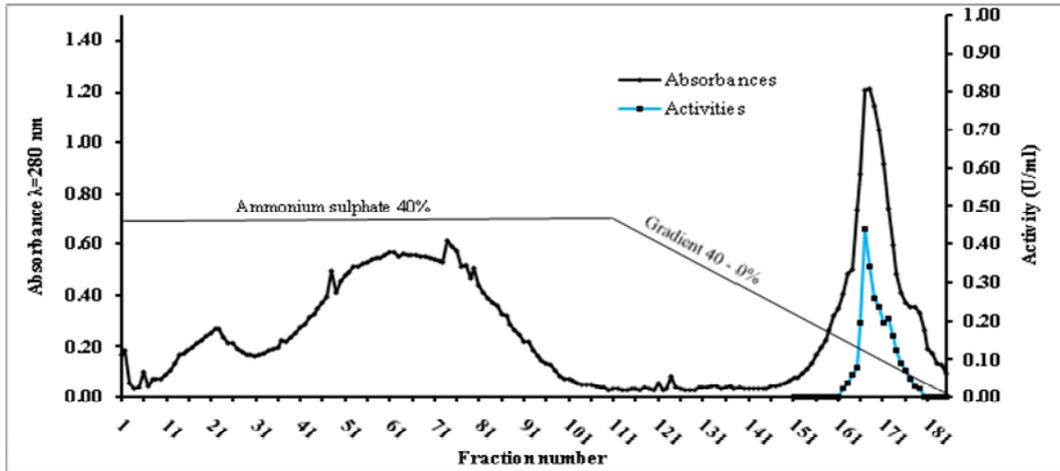


Figure 2. Purification of Lipase of Germinated Cocoa Bean on Phenyl Sepharose.

Table 1. Purification Results of Cocoa Bean Lipase.

Purification Step	Total Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude lipase	19.14	393.76	0.05	100.00	1.00
Ammonium sulphate Precipitation (20-40%)	7.25	34.11	0.21	37.91	4.38
Phenyl Sepharose	3.94	0.70	5.59	20.58	115.08

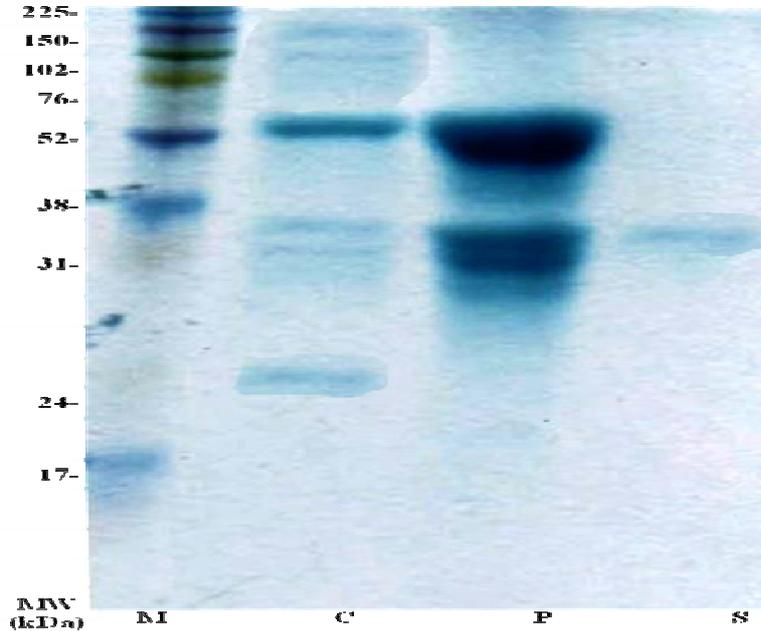


Figure 3. SDS-PAGE Profile on 10% gel of Each Purification Step of Germinated Cocoa Bean Lipase.

M = Marker, C = Crude lipase, P = lipase precipitated in ammonium sulfate, S = lipase purified on phenyl sepharose.

Nevertheless, it also showed a discrepancy with that MW of sunflower lipase (22 kDa) [22] and castor bean lipase (58 kDa) [23]. However, MW of the enzyme was similar with that of canola bean lipase (34 kDa) [24].

Effect of temperature on activity and stability of germinated cocoa bean lipase

As shown in Fig 4a, the optimum temperature was achieved at 38°C. This finding was similar to that of cocoa bean lipase Clone PBC 159 (40°C) [21], or lipase from *Jatropha curcas* (37°C) [10] and from castor bean (40°C) [25]. However optimum temperature of the lipase was different with other lipase, such as white melon [26], African oil seed [6] or California laurel [27], which were all 30°C, olive fruit (35°C) [28], bay laurel beans or sun flower beans (both 50°C) [22].

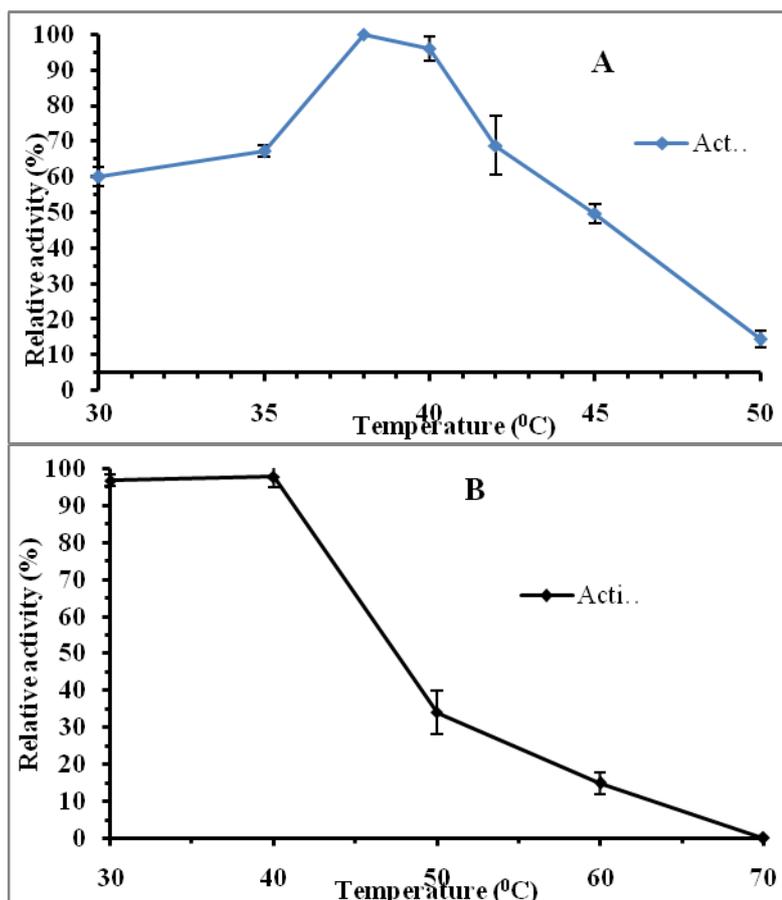


Figure 4. Effect of Temperature on Activity and Stability of Lipase of Germinated Cocoa Bean.

Lipase of germinated cocoa bean was stable up to 40°C (Fig 4b). The activity was then decreased when temperature increased. At 50°C, the remaining activity turned into 66%, while at 70°C the activity was lost.

Effect of pH on activity and stability of lipase of germinated cocoa bean

At pH 9, activity of the lipase was optimum (Fig 5a). This seemed that lipase from germinated cocoa bean was alkaline lipase. This result was slightly different with the cocoa bean lipase of Clone PBC 159 reported by Samsumoharto [21], which had pH optimum of 8.0. However, this lipase had the same optimum pH with that of peanut [29], rape and mustard [30].

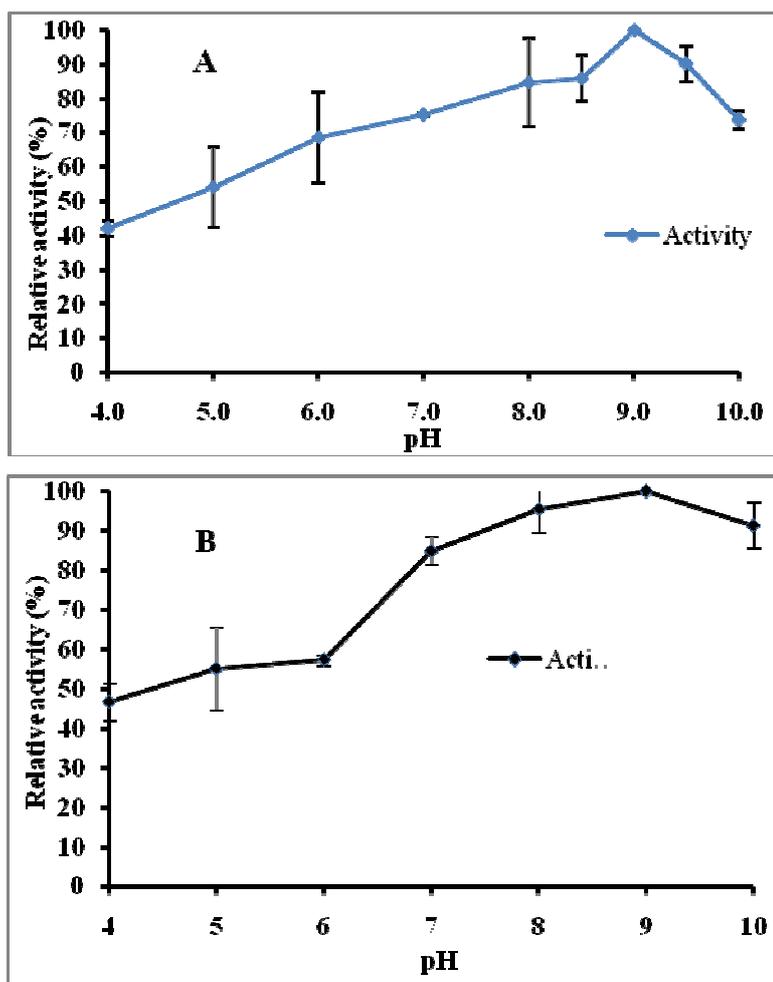


Figure 5. Effect of pH on Activity and Stability of Lipase of Germinated Cocoa Bean.

The lipase was stable at pH 7-10 (Fig 5b). At pH 7 its activity decreased by 15%, whereas at pH 4 it decreased up to 54%. The same results were also found in lipase of other beans, such as white melon lipase, which was stable at pH 8-9 [26]. This result shows that lipase in this study was more stable in a wide range of pH compared to that of white melon lipase.

Effect of metal ions and inhibitors on activity of lipase of germinated cocoa bean

It was found out that NaCl did not affect lipase activity and its activity tended to decrease in the concentration of 10 mM (Fig 6). While CaCl₂ 1 mM could increase lipase activity of cocoa bean germination, but when the concentration was increased to 10 mM, the activity precisely decreased. In contrast to MgCl₂, at 1 mM it did not affect lipase activity on the contrary at 10 mM lipase activity increased. Moreover, HgCl₂, CuCl₂, FeCl₂, and AlCl₃ at 1 mM inhibited lipase activity and the inhibition increased when concentration was increased to 10 mM. The highest inhibitions were HgCl₂ and CuCl₂. This indicated that there was a sulphhydryl group in its active site [3, 6]. These results were not surprisingly since many bean lipases were usually inhibited by heavy metal.

EDTA 1 mM inhibited the activity of lipase and the inhibition increased when the concentration was increased to 10 mM (Fig 6). EDTA is a kind of metal chelator which enables to bond metal needed by lipase as co-factor, so that the formation of complex enzyme-substrate is inhibited [6]. This result shows that cocoa beans lipase was a metalloenzyme. The data were differed with that lipase of olive fruit [28] and linseed [4], which its activities were not inhibited by EDTA.

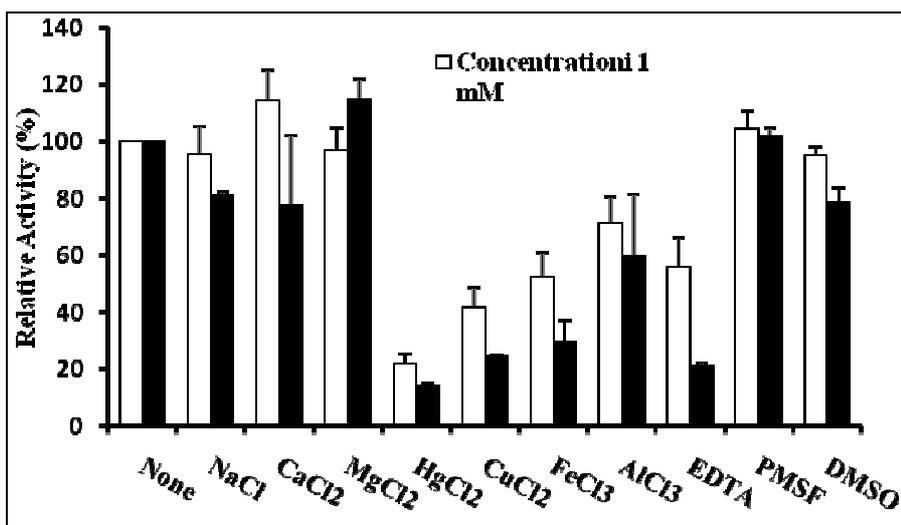
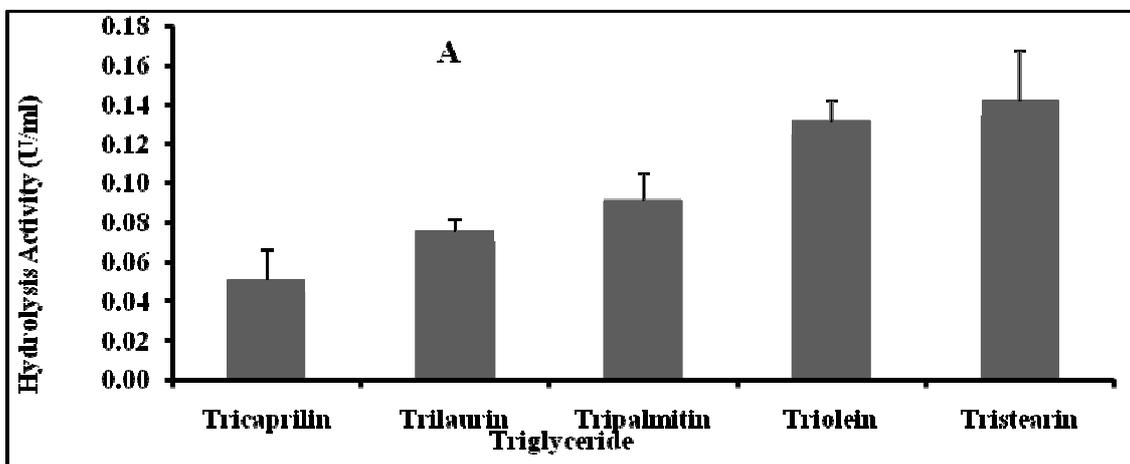


Figure 6. Effect of Metal and Inhibitor to Lipase Activity of Cocoa Bean Germination.

Different with that of EDTA, PMSF was slightly increased the lipase activity. This indicated that serine might not function in the active site. This result was dissimilar with the data reported by Mhetras, *et al* [13] that lipase of *Aspergillus niger* NCIM 1207 was also inhibited by PMSF 5 mM up to 82% inhibition. Moreover, Samsumoharjo reported [21] that diisopropyl frourophosphate (DFP) (serine inhibitor) of 0.2 μM inhibited 90% of the activity of cocoa bean lipase clone PBC 195. DMSO inhibited lipase activity of germinated cocoa bean. The more the concentration of DMSO was, the more activity was inhibited. The inhibition of DMSO showed that histidine and aspartate play a role on catalytic site of lipase of germinated cocoa bean.

Substrate specificity of germinated cocoa bean lipase

As shown in Fig 7A, the enzyme hydrolyzed tristearin at the highest activity then followed by triolein and tripalmitin. Lipase activity was the lowest in tricaprilin as a substrate. Furthermore, esterification activity of the enzyme found the highest if stearic acid was used as a substrate, followed by oleic acid and palmitic acid (Fig 7B). Fatty acids of cocoa bean composed by palmitic acid 24.4%, stearic acid 35.4% and oleic acid 38.1%. This indicated that lipase activity of germinated cocoa bean was specific for main fatty acids found in cocoa beans. The result was different with that reported by Samsumoharto [21] using cocoa bean lipase of clone PBC 159 that the longer the chains of fatty acid were, the lower its affinity was. While lipase isolated from California-laurel is selective to lauric acid, since the beans contained 60%-70% of lauric acid [27].



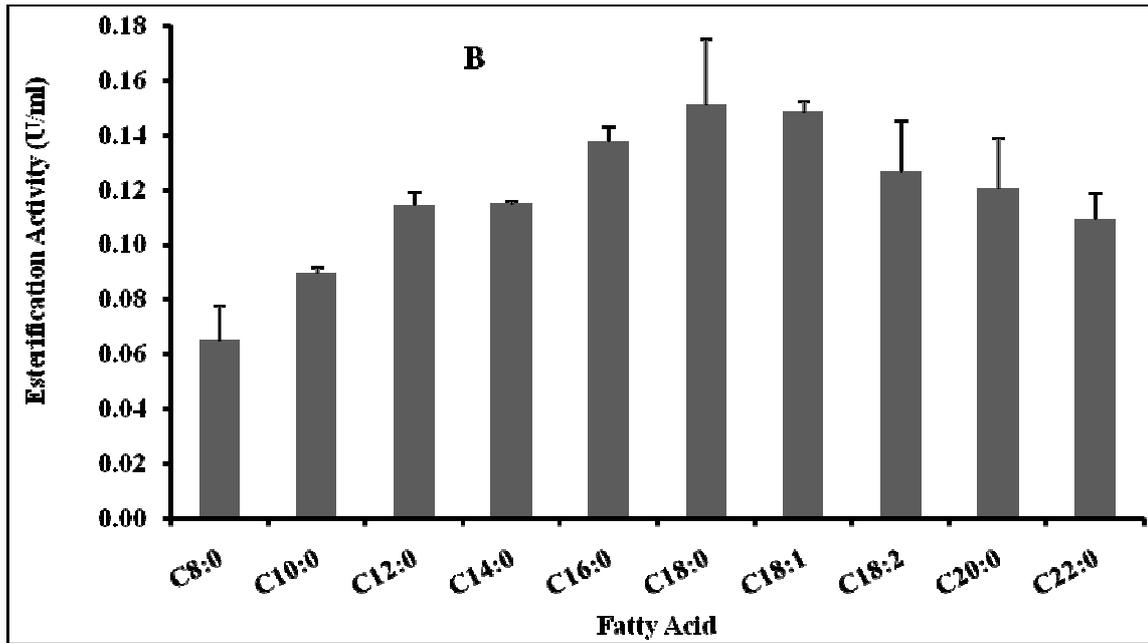


Figure 7. Hydrolysis (A) and Esterification (B) Activity of Cocoa Bean Lipase in Various Substrates.

Regio-specific lipase of cocoa bean germination

Regiospecific properties of lipase were detected by esterification of oleic acid and glycerol. As a comparison, pancreatic lipase known to have regiospecific *sn*-1,3 was used. The results of visualization on plate of TLC with iod vapor are presented in Figure 8. The results show that esterification using lipase of germinated cocoa bean gave 4 bands that were; band I glycerol, band II monoolein, band III diolein, band IV oleic acid. There was no band of triolein. Comparing with the result of pancreatic lipase esterification, there was similar results for these two kinds of enzyme. This means that band II was 1-monoolein or 3-monoolein while band III was 1,3-diolein. From these data, it could be concluded that lipase of germinated cocoa bean had a region-specificity of *sn*-1,3. Hassanian and Mukherjee [30] also reported that lipase of rape and mustard beans had a region-spesificity of *sn*-1,3. On the other hand, lipase of potatoes had a region-specificity at *sn*-2 [20].

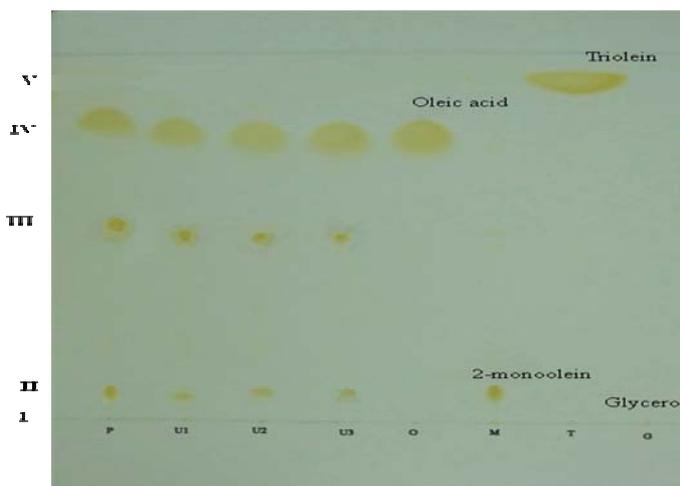


Figure 8. TLC pattern of esterification activity of glycerol and oleic acid developed in a solvent mixture of petroleum ether : diethyl ether : asetat acid = 70:30:1.

Notes: P = Catalisator of pancreatic lipase, U1, U2, and U3 = Catalisator of cocoa beans lipase at each repeated test. Standards : O = oleic acid, M = 2-monoolein, T = triolein, and G = glycerol

Conclusions

Indigenous lipase of cocoa beans was purified up to 115.08 times using a hydrophobic interaction chromatography column on phenyl sepharose, giving a recovery of 20.58% and MW of 36 kDa. Optimum temperature for lipase activity was 38°C and the enzyme was stable at 30-40°C. Indigenous lipase of germinated cocoa bean was an alkaline lipase having optimum pH of 9 and stable at pH of 7-10. The activity increased with addition of 1 mM CaCl₂ and 10 mM MgCl₂, but it decreased with addition of 10 mM CaCl₂. NaCl did not influence the lipase activity, while HgCl₂, CuCl₂, FeCl₃, and AlCl₃ at the concentration of 1 mM inhibited lipase activity. The enzyme presumably was metalloenzyme; histidine and aspartate might play role in catalytic activity; whereas serine might not function in the active site. Lipase had specific substrates of stearic acid, oleic acid, and palmitic acid. It had regiospecific of *sn*-1,3.

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