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Stability study of the extracted mycelium bound lipase activity of *Aspergillus flavus*

(Kajian kestabilan aktiviti lipase yang diekstrak daripada miselium *Aspergillus flavus*)

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Key words: extracted mycelium bound lipase, protease, lipase, specific activity

Abstract

Specific activity of lipase extracted from the wet mycelium using 0.05 M Tris HCl pH 8.2 increased nearly 40 times higher than that of the dry mycelium. The activity and specific activity of extracted lipase kept at 4 °C decreased steadily during storage. The decrease in lipase activity could most probably be due to the co-existence of proteases that have the ability to deactivate the lipases. More than half of the proteases present in the extracted solution were successfully separated by acetone precipitation at 1.0:1.0 ratio (extraction solution:acetone). Addition of 1mM of ethylenediaminetetraacetic-acid-disodium salt (EDTA) and 1,10 phenanthroline (1mM to 8mM) successfully inhibited the metalloprotease activity and maintained the lipase activity for 32 days at 4 °C.

Introduction

Many researchers have reported the coexistence of lipase and protease during growth which affects the stability of lipase (Henriette et al. 1993; Pimentel et al. 1994; Guillou et al. 1995). There are many cases where free lipase is found to degrade rapidly by protease and lose its activity (Jonsson and Snygg 1974; Kumura et al. 1991). Many efforts have been made to overcome this problem. The addition of protease inhibitors is one of the techniques that has been widely used depending on the type of protease present (Stuer et al. 1986; Hoshino et al. 1992).

It is known that different species of *Aspergilli*, for instance *Aspergillus flavus*, has the ability to produce a diversified extracellular protease (Boer and Peralta 2000). *Aspergillus flavus* not only produce metalloprotease (Long et al. 1996; Mellon and Cotty 1996) but also a mixture of protease; metallo, serine and cystein depending on the substrate used during growth (Zhu et al. 1990). This paper describes the stability of the extracted lipases with and without the presence of protease inhibitors. In addition, this work investigated the possibility of using acetone to partially separate the lipase from the proteases.

Materials and methods

Fungus

Aspergillus flavus link (IMI 361648) was isolated from copra meal by Long et al. (1996). The spores were cultured on potato dextrose agar (PDA) for 7 days at 32 °C to obtain the vegetative form.

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Cultivation and medium

Aspergillus flavus spore suspension (average of 10^7 spores/cm^3) was added into 100 ml of medium consisting of 0.3% $(NH_4)_2SO_4$, 0.02% $CaCl_2$, 0.2% KH_2PO_4, 0.1% glucose, 2% yeast extract and 2% palm olein with the pH of 6.0. The mixture was subjected to 32 °C in horizontal shaker (200 rev/min) for 72 h.

Preparation of defatted mycelium bound lipase

Mycelium was filtered using Buchner funnel fitted with filter paper (Whatman No. 4) and washed several times with 1,000 ml of distilled water followed by 100 ml of hexane.

Extraction of defatted mycelium bound lipase

Hexane defatted mycelium was suspended in 0.05 M Tris HCl pH 8.2 (1 g/10 ml) and incubated at 30 °C for 30 min with agitation rate of 200 rev/min. Mycelium was removed by filtration through filter paper (Whatman No. 4).

Lipase activity assay

The activity of lipase was determined according to the method of Sugiura et al. (1975) with slight modifications by Long et al. (1996). Extracted lipase (1 ml) was added into 2.5 ml of 10% refined, bleached and deodorized (RBD) olein in 10% arabic gum, 2 ml 0.05 M Tris-HCl (pH 8.2), 0.5 ml 0.25 M $CaCl_2$ and 4 ml distilled water. The mixture was subjected to incubation at 50 °C and shaken at 200 rev/min for 30 min. The reaction was stopped by adding 45 ml of acetone:ethanol (1:1) and the activity was measured by titration with 0.05 M NaOH to pH 9.0.

Protease activity assay

Protease activity was measured according to Brock et al. (1982) with modification. An amount of 1 ml of 0.8% azocasein dissolved in 0.05 M Tris HCl pH 8.2 was preincubated at 40 °C for 10 min. Protease solution (1 ml) was added and incubated at 40 °C for 30 min with agitation rate of 150 rev/min. The reaction was stopped by adding 2 ml of 10% trichloroacetic acid (TCA) and allowed to stand at room temperature for 30 min and then centrifuged for 30 min at 9,000 g. The supernatant was removed and mixed with equal volumes of 1 M NaOH. The absorbance of the mixture was read at 450 nm. Control samples were assayed in the same manner except the enzyme was added at the end of incubation period.

Protein determination

Bradford method (1976) was used to measure the concentration of protein using BSA as the standard.

Acetone precipitation

Acetone precipitation was prepared as shown in *Figure 1*. Pellets obtained were dissolved in 0.05 M Tris HCl (pH 8.2) and analysed for lipase and protease activity.

Results and discussion

The effect of wet and dry mycelium (freeze dried for 3 h) on the extractability of lipase is shown in *Table 1*. The activity of lipase extracted from the wet mycelium was 30% higher than the dry mycelium. On the other hand, it was noted that the use of dry mycelium enhanced the extraction of protein by 30 times compared to wet mycelium. Freeze drying causes the lost of water and this increases the permeability of the cell membrane and cell wall, consequently enhancing the release of both intracellular and bound protein (Scopes 1987). Based on the results from this work, lipase from the wet mycelium was used as it could reduce lipase impurities in the next step of purification work.

The activity and specific activity of lipase extracted from wet mycelium were monitored for 36 days at 4 °C storage *(Figure 2)*. The lipase activity and its specific activity decreased as the storage time increased. Lipase activity reduced by 63% after 36 days of storage. The lost of

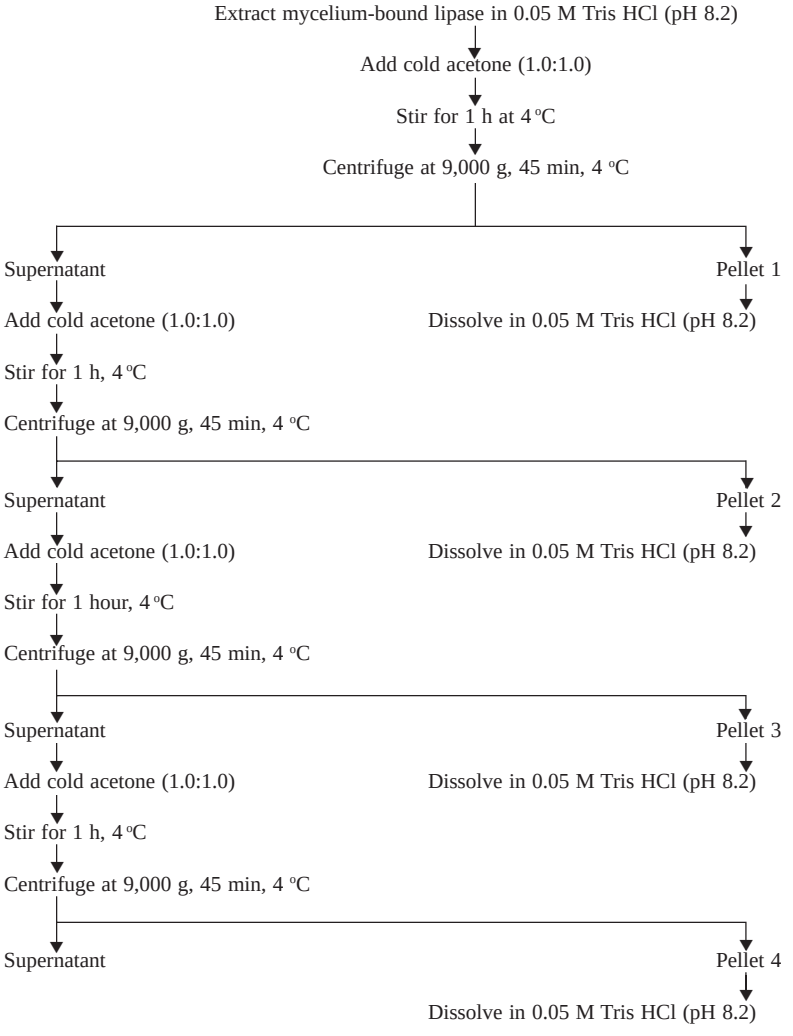

Figure 1. Pellets obtained were dissolved in 0.05 M Tris HCl (pH 8.2) and analysed for lipase and protease activity

Table 1. Effect of wet and dry mycelium on the extractability of mycelium bound lipase

	Lipase activity (U/ml)	Protein concentration (mg/ml)	Lipase specific activity (U/mg)
Dry mycelium	3.35 ± 0.11	1.50 ± 0.02	2.23 ± 0.11
Wet mycelium	4.80 ± 0.22	0.05 ± 0.002	88.36 ± 1.83

Each value is the mean of three readings ± SEM

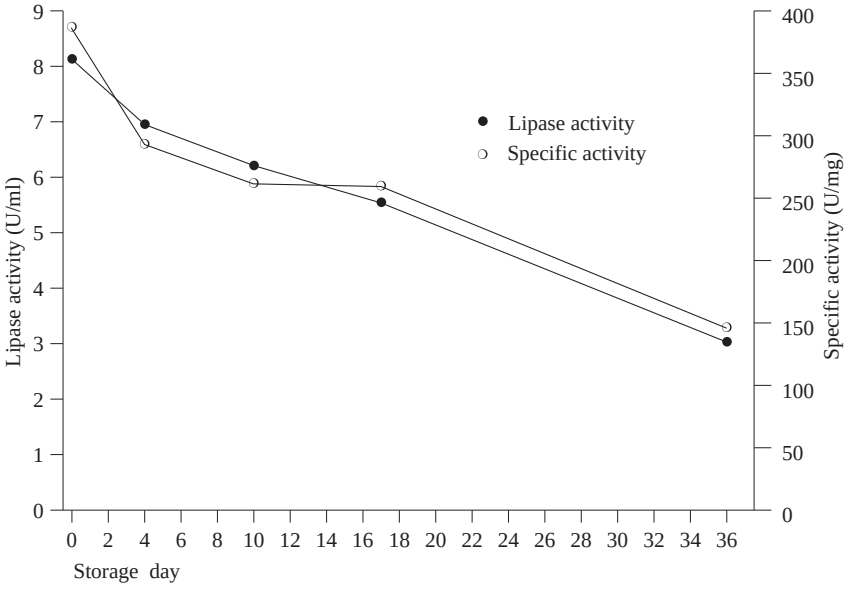

Figure 2. Activity and specific activity of extracted lipase during 36 days storage. Each value is the mean of three readings ± SEM

lipase activity could probably be because of the co-existence of protease in the extractant (Martinez et al. 1993; Long et al. 1996). The existence of proteases was detected in the extracted solution and its activity was stable during the 36 days of storage *(Figure 3).* It is a well known fact that many species of *Aspergilli* have the ability to produce diverse extracellular proteases (Boer and Peralta 2000) as compared to other fungi. Therefore, it is important to deactivate the protease so as to keep the lipase stable during storage.

Effort to use acetone to partially precipitate the protease was shown in *Table 2*. The first step of precipitation using 1.0:1.0 ratio (lipase solution:acetone) caused most of the protease to precipitate. The precipitated protein had proteolytic activity but contained no lipase activity *(Table 2).* At this stage, the specific activity of protease in the first pellet increased by five times. The subsequent step of acetone precipitation, (second pellet) however, caused both the lipase and remainder protease to precipitate. Basically, they share similar isoelectric point or molecular size which caused them to precipitate together. In addition, only the lipase activity was detected on the third pellet. There were no lipase and protease activities detected in the fourth pellet. Total separation of lipase from protease using different acetone ratio was unsuccessful. This result suggested that some of the proteases might have molecular sizes larger than the lipase or different isoelectric points causing it to precipitate at the beginning of acetone precipitation process (Scopes 1987) and some might share similar properties of lipase.

Since total separation of lipase from protease using different acetone ratio was unsuccessful, different types of protease inhibitor were thus added to the extractant to inhibit the protease activity and maintain the lipase activity. It was found that the activity of lipase was relatively stable at 4 °C for at least 32 days in the presence of both EDTA (1 mM) and 1,10 phenanthroline (1 mM, 4 mM, 8 mM) *(Figure 4).* However, the addition of phenylmethylsulfonyl fluoride (PMSF) was not able to maintain the lipase activity of the extractant. About 82% of the lipase activity lost during 32 days storage of extractant contained PMSF. The use of

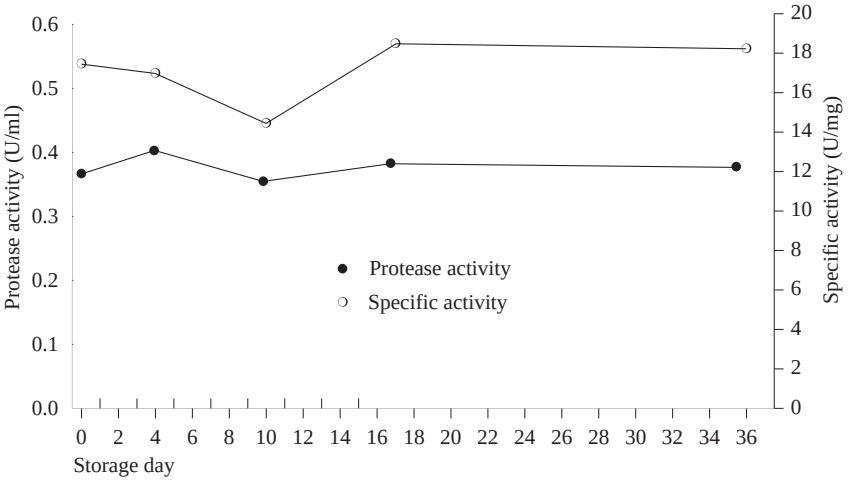

Figure 3. Activity and specific activity of extracted protease during 36 days storage. Each value is the mean of three readings ± SEM

Table 2. Activity and specific activity of lipase and protease at each acetone fraction

	Protein concentration (mg)	Lipase activity (U)	Lipase specific activity (U)	Protease activity (U)	Protease specific activity (U)
Crude lipase	6.216	2185.50	351.92	248.04	39.903
1st Pellet	0.627	0	0	138.09	220.24
2nd pellet	1.231	245.35	199.38	62.58	50.85
3rd pellet	1.243	146.40	117.76	0	0
4th pellet	0.261	0	0	0	0

Each value is the mean of three readings

protease inhibitor to maintain the lipase activity especially during purification process has been reported (Stuer et al. 1986; Hoshino et al. 1992). EDTA and 1,10 phenanthroline were found to be able to maintain and improved the specific activity of lipase from the extractant.

The effect of different protease inhibitors on the protease specific activity is shown in *Figure 5*. About 80% of the protease activity was successfully reduced in the presence of 1 mM EDTA and 1,10 phenanthroline (1 mM, 4 mM, 8 mM). However, the activities of proteases were not affected by serine protease inhibitor i.e. PMSF. The proteases responsible for inactivating the lipase were probably metalloprotease as indicated by the low activity of protease in the presence of EDTA and 1,10 phenanthroline. Previous worked by Zhu et al. (1990) and Mellon and Cotty (1996) have shown that EDTA and 1,10 phenanthroline inhibit the extracellular protease activity of *A. flavus* as observed in this study.

The effectiveness of EDTA as protease inhibitor was further tested on the lipase obtained after acetone precipitation. Lipase was first subjected to acetone precipitation at 1.0:1.0 ratio (lipase solution:acetone), centrifuged and the pellet discarded. The supernatant was further subjected to acetone precipitation at 1.0:3.0 ratio and the pellet was dissolved in 0.05 M Tris HCl/1 mM EDTA pH 8.2 . Lipase with better purity was obtained at 1.0:3.0 ratio (lipase solution:acetone) *(Table 3).* The purification fold of lipase increased by 2.6 times and an

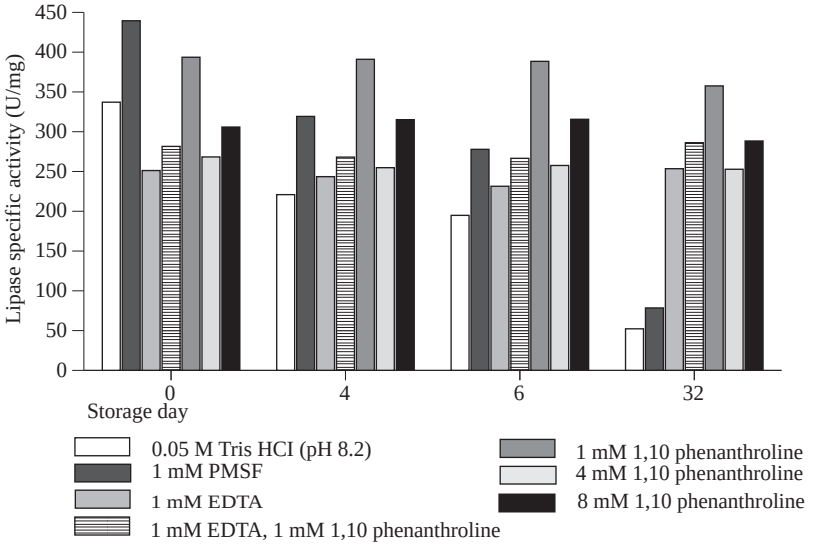

Figure 4. Specific activity of extracted lipase with protease inhibitor kept at 4 °C for 32 days

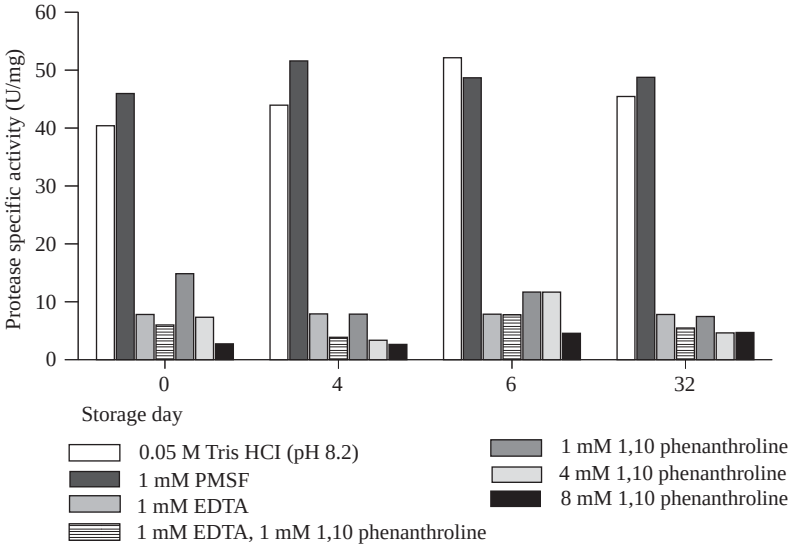

Figure 5. Specific activity of extracted protease kept at 4 °C for 32 days

Table 3. Activity, specific activity and activity yield of lipase after acetone precipitation

Purification step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Activity yield (%)	Purification fold
Crude lipase	208	2044.72	6.95	294.32	100	
Acetone precipitation (1.0:3.0)	80	1663.58	2.2	756.17	81.36	2.6

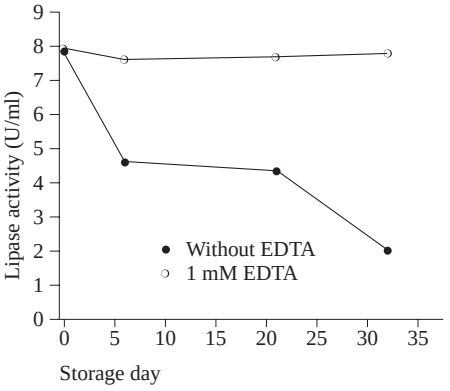

Figure 6. Storage stability of dissolved pellet containing lipase kept at 4 °C with and without 1 mM EDTA

amount of 68.3% of unwanted protein was successfully removed. The stability of lipase present in dissolved pellet (1.0:3.0) with and without EDTA was monitored for 32 days at 4 °C *(Figure 6)*. Control (without EDTA) showed a decline in lipase activity throughout the study which indicated that the remaining proteases left in dissolved pellet (1.0:3.0) were active. However, the activity of lipase was stable with the addition of 1 mM EDTA.

Conclusion

The use of wet mycelium is preferred as it increases the purification fold of extracted mycelium bound lipase by 40 times compared to dry mycelium. The use of acetone precipitation to totally separate the lipase from protease was unsuccessful as some of the protease might share similar molecular sizes or isoelectric points. Addition of 1 mM EDTA and 1,10 phenanthroline (1 mM, 4 mM, 8 mM) inhibited the metalloprotease activity and improved the stability of lipase for at least 32 days at 4 °C.

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Abstrak

Lipase yang diekstrak keluar daripada miselium basah menggunakan 0.05 M Tris HCl pH 8.2 telah menunjukkan aktiviti spesifik hampir 40 kali lebih tinggi daripada miselium kering. Aktiviti dan aktiviti spesifik lipase yang diekstrak daripada miselium yang disimpan pada suhu 4 °C, didapati menurun secara berterusan. Penurunan aktiviti ini mungkin disebabkan oleh kehadiran bersama protease yang didapati berkebolehan menyahaktifkan lipase. Pemendakan protein menggunakan aseton dalam nisbah 1.0:1.0 (larutan ekstrak:aseton) dapat memisahkan sebahagian besar daripada protease yang hadir di dalam larutan ekstrak. Kehadiran 1 mM ethylenediaminetetraacetic-acid-disodium salt (EDTA) dan 1,10 phenanthroline (1 mM hingga 8 mM) di dalam larutan ekstrak dan kehadiran kedua-dua bahan ini telah menstabilkan aktiviti lipase sehingga 32 hari pada suhu 4 °C.

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