UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OP LIPASE PRODUCED BY THERMOPHILIC PUNGI ISOLATED PROM POME

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CHARACTERIZATION OF LIPASE PRODUCED BY THERMOPHILIC FUNGI ISOLATED FROM POME.

By

MOHD. YUSOFF BIN ABD. SAMAD

Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies, Universiti Pertanian Malaysia

October 1990

Dedicated To My Parents -

Abd. Samad & Siti Zainab, and

those who pray for my success ! ! !

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Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfillment of the requirements for the degree of Master of Science.

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A plate assay to determine lipase activity was developed. Tween 80, used as a substrate with Victoria Blue B as the indicator seemed to be a reliable technique. Lipolytic activity was determined by the formation of the zone of intensification of the indicator colour after 24 hr. Intensity of the colour developed was greater than that of triolein dye system and clearer than the hydrolysis zone of tributyrin plate. Test using a commercial enzyme and growth media containing lipolytic activity showed that the zone of intensification increased with increased lipolytic activity. A linear relationship could be seen when log enzyme concentration was plotted against the diameter of zone of intensification. Using this technique primary

screening of lipolytic microorganisms could be conducted using the formation of zone of intensification around the colonies and mycelia.

Five fungi showing lipolytic activity on plate were isolated using plate assay. However, using broth culture only one fungus was found to produce lipase . The fungus was identified as Rhizopus rhizopodiformis, (strain S1 and S2). Strain S1 was found to produce maximum lipase yield in the basal medium containing of peptone and glycerol.

The enzyme was partially purified by means of acetone precipitation and chromatography on Sephadex G-100. The enzyme was purified about 9.7-fold and yielded 37%. The enzyme showed two bands on disc polyacrylamide gel electrophoresis. The optimum pH of the enzyme activity was 6.0 and the optimum temperature was 45 ^{o}C. The enzyme was most stable at pH 7.0 and could withstand temperature of up to 50 ^{o}C. The enzyme had a greater affinity to triglycerides with short chain fatty acids and it was shown to have 1, 3-positional specificity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat untuk mendapat Ijazah Master

PENCIRIAN LIPASE YANG DIHASILKAN OLEH KULAT TERMOFILIK YANG DIPENCIL DARI SISA BUANGAN KELAPA SAWIT (POME)

Oleh

MOHD. YUSOFF BIN ABD. SAMAD

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Kaedah plat asai untuk menentukan aktiviti lipase telah dihasilkan. Kaedah ini menggunakan Tween 80 sebagai substrat dan Victoria Blue B sebagai penunjuk. Teknik ini didapati boleh dipercayai. Aktiviti lipolitik ditentukan dengan pembentukan zon keamatan penunjuk warna selepas pengeraman plat selama 24 jam. Keamatan warna yang diperolehi adalah lebih tinggi daripada sistem pewarna triolein dan lebih terang daripada zon hidrolisis plat tributirin. Ujian menggunakan enzim komersial dan media pertumbuhan yang mengandungi aktiviti lipolitik menunjukkan zon keamatan bertambah dengan pertambahan aktiviti lipolitik. Hubungan yang linear boleh didapati bila log kepekatan enzim diplotkan berbanding dengan zon garispusat keamatan.

Penyaringan primer mikroorganisma-mikroorganisma lipolitik dengan menggunakan teknik ini boleh dijalankan melalui pembentukan zon keamatan di sekeliling koloni bakteria dan miselia kulat.

Dengan teknik plat asai ini lima jenis kulat dengan aktiviti lipolitik telah dipencilkan. Walau bagaimanapun, hanya dua jenis sahaja yang mengeluarkan lipase dalam kultur kaldu. Kulat-kulat ini telah dikenalpasti sebagai Rhizopus rhizopodiformis strain S1 dan strain S2. Strain S1 ini telah didapati mengeluarkan enzim lipase yang maksimum dalam media asas yang mengandungi pepton dan gliserol.

Enzim ini telah diseparatulenkan dengan menggunakan pemendakan aseton dan selanjutnya dengan proses kromatografi menggunakan gel Sephadex G-100. Enzim ini telah ditulenkan sebanyak 9.7 kali ganda dengan penghasilan sebanyak 37%. Dua jalur protein telah didapati pada cakra gel poliakrilamid. Enzim ini menunjukkan pH dan suhu optimum bagi aktiviti pada 6.0 dan 45^{o}C.

Enzim ini juga didapati sangat stabil pada pH 7.0 dan suhu sehingga 50^{o}C. Ia juga menunjukkan keafinan substrat yang tinggi kepada trigliserida yang mempunyai rantai asid lemak yang pendek. Kespesifikan kedudukan enzim ini menunjukkan ianya adalah pada kedudukan 1 dan 3 pada trigliserida.

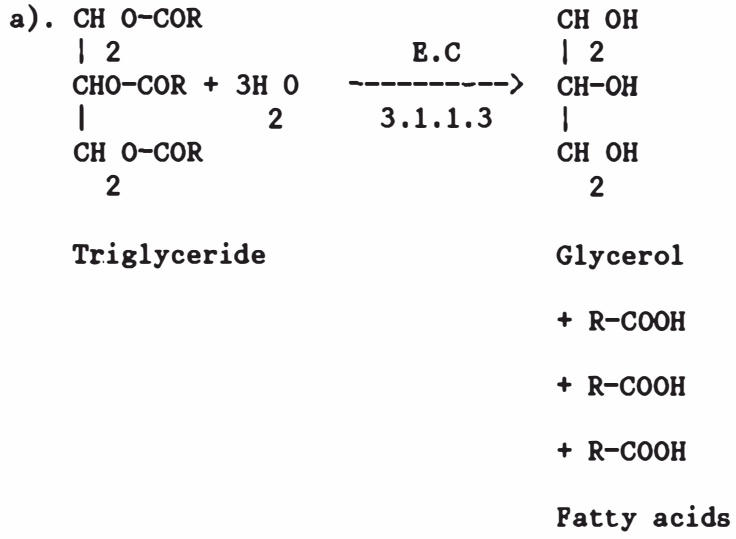
CHAPTER 1

INTRODUCTION

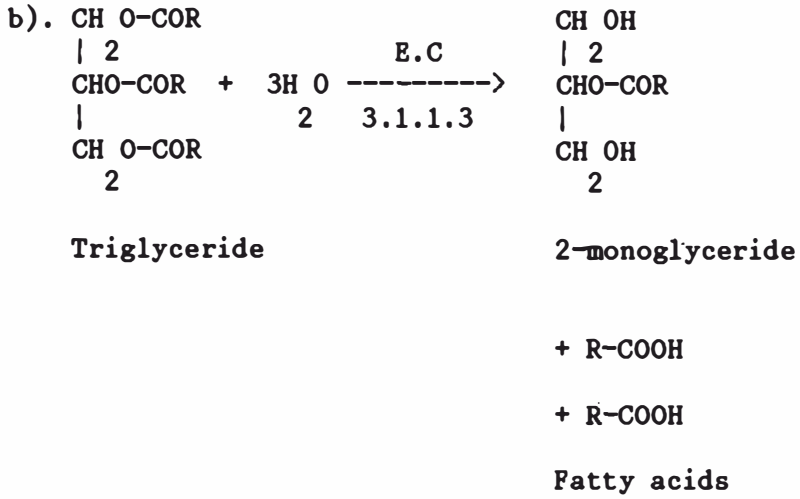
Lipolytic enzymes are indispensable for the biological turnover of lipids. These digestive enzymes are responsible for the transfer of lipid from one organism to another, such as from animal to plant or from one animal to the other. Within the organisms, they play a role in the deposition and mobilization of fats as well as in the metabolism of intracellular lipids. Therefore lipolytic enzymes are essential to the functioning of biological membranes (Brockerhoff and Jensen, 1974). Lipases, a major group of lipolytic enzymes, have recently received much attention from the academic and industrial sectors. In fact, lipases which are distributed in microorganisms such as yeasts, bacteria and fungi are potentially very important in industrial applications.

By definition, lipases are glycerol-ester-hydrolases (E.C.3.1.1.3) in the narrowest sense, i.e., long chain triglyceride acylhydrolases. They may or may not have positional specificity for the primary ester bonds. Usually the enzymes also hydrolyze di- and monoglyceride (Brockerhoff and Jensen, 1974). The action of lipases with water-insoluble substrates only occurs at water-oil interface. But this definition now becomes complicated as it is also known that some lipases can hydrolyze water-soluble substrates. However lipases can be

looked upon as the enzymes with a high activity towards water insoluble substrates. A complete reaction catalyzed by lipases is shown in Figure 1 below:



Non-specific reaction of lipase.



1,3 - positional specificity of lipase

Figure 1: Reactions of Lipases

Lipases are widely distributed in animals, plants and microorganisms. Many strains of bacteria, yeasts and fungi such as Staphylococcus sp (Vadehra and Harmon, 1969), Pseudomonas fragi (Nishio et al., 1987a), Chromobacterium (Yamaguchi et al.,1973), Alcaligens (Kokusho et al., 1982), Geotrichum candidum (Iwai et al., 1973), Rhizopus delemar (Iwai et al., 1966), Aspergillus niger (Pal et al., 1978), Humicola lanuginosa (Arima et al., 1972, Ibrahim et al., 1987a; Morinaga et al., 1986), Candida cyclindracea (Ota et al., 1968) and some other yeasts, molds or bacteria have been reported to be lipase-producing microorganisms.

Microbial lipases have always received more attention when compared to lipases from other sources. Microbial lipases can be produced in large quantity and microbes have short growth periods compared to plant and animal cells. Production of lipases in certain microbes can also be induced by addition of lipids and certain compounds. Based on a recent report, many microbial lipases have been successfully studied from academic and industrial viewpoints (Kilara, 1985). These microbial lipases were also successfully purified to homogeneity with the recent technical advances in the isolation of these enzymes through chromatographic and electrophoretical methods. With these preparations, the properties and reaction mechanisms of lipolysis are being steadily elucidated (Borgstrom and Brockman, 1984).

Fundamental studies for obtaining the optimum cultural condition for lipase production were carried out by several investigators (Chander et al., 1977, 1980, 1981; Chopra and Chander, 1983; Iwai et al., 1966). Some findings reported that the formation and synthesis of lipase are under feedback control of mono- and disaccharides and glycerol in the cultivation medium. Some microbial lipases are glycoprotein where the sugar moiety is thought to facilitate the passage of the enzyme through the microbial cell wall and into the growth medium (Kilara, 1985).

Previously, little studies on lipases have been carried out compared with the other hydrolytic enzymes such as amylases and proteases. However, recently, in view of increasing interest in biotechnology of oils and fats and their use in oleochemical industry, lipase-catalyzed hydrolysis or synthesis of lipid has become more than just an academic interest (Macrae, 1983a). Also, some other reactions catalyzed by lipase, such as synthesis of terpene alcohol esters (Iwai et al., 1980), interesterification of oils and fats (Macrae, 1983b), synthesis of glycerides (Tsujisaka et al., 1977) and optical resolution of racemic compounds (Kilara, 1985) have recently attracted much attention of both academic and industrial researchers. These bioconversions are expected to produce various value-added products from fats, oils, fatty acids and their related compounds.

In view of the potential biotechnological applications of lipases in the oleochemical industry, the exploitation of these enzymes will lead to the manufacturing of some useful products in the oil industry, especially in the oil palm modification in obtaining a valuable product such as cocoa butter substitute. In addition, the use of these enzymes in the modification of some essential oils by interesterification process will be very useful. Therefore this research was undertaken with the following objectives:

a) To develope a rapid and suitable plate assay media for detection of lipase activity as well as for screening lipase-producing microorganisms.

b) To screen and isolate thermostable lipase-producing microorganisms.

c) To study the effect of growth substrates for optimal lipase production.

d) To partially purify lipase from culture medium.

e) To characterize the partially purified lipase.

CHAPTER 2

LITERATURE REVIEW

Detection of Lipolytic Activity and Screening of Lipolytic Microorganism

Numerous reports regarding the methods for detection and screening of microorganisms with lipolytic activities have been presented by many researchers (Fryer et al.,1966; Karnetova et al.,1984; Oterholm and Ordal,1965). Substrates from triglycerides and polyoxysorbitan have always been employed for the detection of lipase activity. Sometimes the incorporation of chromogenic substances with the substrates gives easier detection as the zone of intensification or clearing that indicates lipolytic activity can be clearly seen.

Several substrates such as oils, triolein and tributyrin have been used in the agar after being emulsified to a certain extent. For example, Fryer et al. (1966) developed two double-layer techniques for detection of lipases in microorganisms. The two double-layer agar plates were developed in which the organisms grown on nutrient agar plate were overlaid with tributyrin agar or a thin layer of milk fat saturated with Victoria Blue. These techniques offer advantages such as the colonies can be isolated after detection, lipolysis can be followed and there is no toxicity due to the dye.

Many investigators (Adriana and Wouters, 1976; Jacks and Kircher, 1967) have employed chromogenic substrates such as fatty acid esters of 4-methylcoumarin (umbelliferone) for detection of lipase activity. Esterases or lipases released an alcohol (phenol, eosin, umbelliferon naphtol, etc.) from these compounds which were directly determined by fluorimetry. The oleic acid ester of 4-methyl umbelliferon was found to be a suitable substrate for the fluorimetric assay of the lipase produced by Geotrichum candidum (Adriana and Wouters,1976). However, the above method was only suitable for detection of isolated lipase and not suitable for screening of lipolytic organisms.

Karnetova and coworkers (1984) proposed a diffusion plate method. The substrate Tween 80 and indicators such as methylene blue, bromothymol blue and Nile blue (0.5-2.5%) were incorporated into the agar. Lipase activity was detected by the formation of light green zone on a dark blue background. A linear relationship between the logarithm of lipase activity and the diameter of the zone was found. In this method, the enzyme was placed in a well (8.5 mm diameter) and incubated at 28 ^{o}C for 20 hr.

Using tributyrin as a substrate sometimes creates a problem as zone of clearing due to lipase activity is difficult to be seen. Thus, Oterholm and Ordal (1965) have improved the method for isolation of lipolytic microorganisms as well as detection of lipolytic activity. The method using plating medium consisting

of 0.3% tributyrin and 1% agar was found to be a reliable technique for the detection of lipase activity as well as for screening for lipolytic microorganisms.

However, Mourey and Kilbertus (1976) developed a simple medium containing stabilized tributyrin for demonstrating lipolytic bacteria in foods and soils. The medium containing nutrient, tributyrin and polivinyl alcohol was emulsified then solidified with agar. Using this medium, isolation of microorganism with lipolytic activity shown by the clearance zone was easily done.

Detection of lipolytic activity and lipolytic microorganisms on agar plates is always based on using either tributyrin or Tween as a substrate. These substrates are considered to be unsuitable for detection of true lipase since they are also hydrolyzed by esterases. For that reason, Kouker and Jaeger (1987) developed a specific and sensitive plate for detection of bacterial lipases by adapting the method proposed by Hofelmann et al.(1983). They made use of nutrient with 2.5% olive oil and 0.001% Rhodamine B. The mixture was homogenised to an emulsion. Quantification of lipase activity was determined by the diameter of zone of fluorescence.

Production of Microbial Lipases

Many studies on cultural conditions for lipase production have been reported (Saiki et al., 1969; Alford and Smith, 1965;