UNIVERSITI PUTRA MALAYSIA

SCREENING, PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR LIPASE PRODUCED BY *Pediococcus acidilactici* UB6 ISOLATED FROM MALAYSIAN FERMENTED FOODS

YAP SIA YEN

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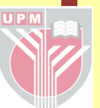

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By

YAP SIA YEN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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SCREENING, PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR LIPASE PRODUCED BY *PEDIOCOCCUS ACIDILACTICI* UB6 ISOLATED FROM MALAYSIAN FERMEMTED FOODS

By

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June 2007

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Faculty : Biotechnology and Biomolecular Sciences

The extracellular lipase produced by Lactic Acid Bacteria (LAB) has not been studied extensively, although the intracellular lipolytic capability of LAB isolated from fermented foods has been reported. Thus, the present work was conducted to screen, characterize and purify the extracellular lipase produced by 41 Lactic Acid Bacteria (LAB) isolated from Malaysian fermented foods. The lipase producer was determined by using qualitative and quantitative methods. For qualitative method, all tested LAB isolate demonstrated blue colour colonies on the Nile blue sulphate agar plate. Thus, the extracellular lipase activity of LAB was further quantified by using both titration and spectrophotometric assay methods. All tested LAB isolates exhibited lipolytic activity when assayed with 50 mM Tris-HCl, pH 8.0 buffer with UB6 isolate as the highest extracellular lipase producer. Only 38 isolates of LAB demonstrated lipolytic activity when assayed with 50 mM sodium acetate, pH 4.5 buffer by using titration assay method

with GP13 as the highest extracellular lipase producer. However, UB6 was selected for further studies as it exhibited lipolytic activity under both alkaline and acidic assay conditions. The UB6 isolate was designated as *Pediococcus acidilactici* UB6 based on both phenotypic biochemical tests and API test kit. The optimum alkaline assay condition for titration method was: 150 rpm of agitation, 20 min of incubation time, 5% (w/v) gum Arabic, 500 µl olive oil and 100 µl of cell free supernatant (CFS). The same optimum assay condition was obtained for the spectrophotometric method, except 20 µl of p-NP palmitate and 300 µl of CFS was used in the assay mixture. For the growth study, the maximum production of extracellular lipase was detected after 15 h incubation, which was occurred at the late log phase.

The crude extracellular lipase UB6 was characterized on the basis of pH and buffer types, temperatures and substrates specificity. The optimum activity was attained when lipase assay was performed with 50 mM Tris-HCl, pH 8.0 buffer at 37ºC for both titration and spectrophotometric assay methods. However, the optimum temperature was shifted to 40ºC when assayed with 50 mM sodium acetate, pH 5.0 buffer for titration method. Generally, the crude extracellular lipase UB6 exhibited broad substrate affinity. However, the preference was towards the long chain fatty acids. For temperature stability study, the crude extracellular lipase UB6 was able to retain 100% activity after being incubated at 40ºC for 1 h. Conversely, the lipolytic activity decreased dramatically when temperature was above 50ºC. For storage study, the lipolytic activity remained 70% after being kept at –20, 0 and 4ºC for 9 weeks, respectively. However, after being stored at 8, 15, 30 and 37ºC for 9 weeks, the lipolytic activity was remained at 60%, 55%, 50% and

40%, respectively. The lipase activity was not significantly affected by Proteinase K, however, it was affected greatly by β-chymotrypsin, α-chymotrypsin, trypsin, papain and lysozyme. The extracellular lipase UB6 was stable in 0-1% (w/v) NaCl.

The extracellular lipase UB6 was purified to apparent homogeneity by using 4 steps purification procedure comprising of 0-100% ammonium sulphate precipitation, anion-exchange Source 30 Q chromatography, packed Superose 12 gel filtration chromatography and Concanavilin A (Con-A) affinity chromatography. The extracellular lipase UB6 was successfully purified to apparent homogeneity with 3.23% overall recovery and 136 purification fold. The molecular mass of both purified unbound and bound Con-A lipase active fractions was estimated to be 28,155 and 32,000 Da by Superose 12 gel filtration chromatography and Glycine sodium dodecyl sulphate polyacylamide gel electrophoresis, respectively, whereas, the isoelectric points of both lipase active fractions were estimated to be pI 3.5-5.2 (acidic) and pI 8.4 (alkaline). Both purified unbound and bound to Con-A lipase active fractions contained 60% and 71% of hydrophobic amino acids at N-terminal. In addition, the maximal activity for both purified Con-A fractions were detected at pH 4.0 and pH 8.0, respectively. As for substrate affinity, both purified Con-A fractions exhibited higher affinity towards long chain fatty acids.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENYARINGAN, PENCIRIAN DAN PENULENAN LIPASE EKSTRASEL YANG DIHASILKAN OLEH PENCILAN *PEDIOCOCCUS ACIDILACTICI* UB6 DARI MAKANAN TERTAPAI MALAYSIA

Oleh

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Lipase ekstrasel yang dihasil oleh Bakteria Laktik Asid (BLA) belum dikaji dengan terperinci, walaupun keupayaan sifat lipolitik intersel bagi BLA yang dipencil dari makanan tertapai telah dilaporkan. Oleh itu, kajian ini dijalankan untuk menyaring, menciri dan menulen lipase ekstrasel yang dihasilkan oleh 41 BLA terpencil daripada makanan tertapai Malaysia. Penghasil lipase telah ditentu dengan menggunakan kaedah kualitatif dan kuantitatif. Bagi kaedah kualitatif, kesemua pencilan BLA mempamerkan koloni berwarna biru di atas piring agar "Nile blue sulphate". Oleh demikian, aktiviti lipase ekstrasel bagi kesemua pencilan BLA dikuantifikasikan dengan menggunakan kedua-dua kaedah pentitratan dan spektrofotometrik. Kesemua pencilan menunjukkan aktiviti lipolitik apabila diasaikan dengan larutan penimbal 50 mM Tris-HCl, pH 8.0 dengan pencilan UB6 sebagai penghasil lipase ekstrasel yang tertinggi. Hanya 38 pencilan BLA mendemostrasikan aktiviti lipolitik apabila diasai dengan larutan penimbal

50 mM sodium asetat, pH 4.5 melalui kaedah pentitratan dengan pencilan GP13 sebagai penghasil lipase ekstrasel yang tertinggi. Walaubagaimanapun, UB6 telah dipilih untuk kajian seterusnya memandangkan ia mendemonstrasikan aktiviti lipolitik dalam keadaan asid dan alkali. Pencilan UB6 telah dinamakan sebagai *Pediococcus acidilactici* UB6 berdasarkan kepada kedua-dua ujian biokimia fenotipik dan API. Bagi kaedah pentitratan, keadaan optimum bagi asai berakali ialah: goncangan pada 150 rpm, masa pengeraman selama 20 min, gum Arabik sebanyak 5% (w/v), 500 µl minyak zaitun dan 100 µl supernatan sel bebas (SSB). Keadaan optimum bagi asai diperolehi untuk kaedah spektrofotometrik, kecuali 20 µl pNP-palmitik dan 300 µl SSB digunakan dalam campuran asai. Bagi ujikaji pertumbuhan, penghasilan maksimum bagi lipase ekstrasel telah dikesan selepas 15 jam pengeraman, iaitu berlaku pada fasa lewat log.

Lipase ekstrasel UB6 yang tidak tulen telah dicirikan berasaskan pH dan jenis larutan penimbal, suhu dan spesifisiti substrat. Aktiviti optimum diperolehi apabila asai lipase dijalankan dengan larutan penimbal 50 mM Tris-HCl, pH 8.0 pada suhu 37ºC bagi kedua-dua kaedah pentitratan dan spektrofotometrik. Manakala, suhu pengeraman optimum teranjak ke 40ºC apabila diasai dengan kaedah pentitratan dengan menggunakan larutan penimbal 50 mM sodium asetat, pH 5.0. Secara amnya, lipase ekstrasel UB6 tidak tulen memaparkan afiniti substrat yang luas. Walaubagaimanapun, keutamaan adalah terhadap rantai asid lemak panjang. Bagi kaji kestabilan suhu, ektrasel lipase UB6 tidak tulen dapat mengekalkan 100% aktivitinya selepas dieram pada 40ºC selama 1 jam. Sebaliknya, aktiviti lipase berkurangan secara dramatik apabila suhu melebihi 50ºC. Untuk kajian penyimpanan, aktiviti lipolitik UB6 adalah 70% setelah disimpan pada –20,

0 dan 4ºC selama 9 minggu. Manakala selepas disimpan pada 8, 15, 30 dan 37ºC untuk selama 9 minggu, aktiviti lipase tertinggal pada 60%, 55%, 50% dan 40% masng-masing. Aktiviti lipase tidak dipengaruhi oleh proteinase K. Malah, ia dinyahaktifkan oleh β-kimotripsin, α-kimotripsin, tripsin, papain dan lisozim. Lipase ekstrasel UB6 stabil dalam keadaan 0-1% (w/v) NaCl.

Lipase ekstrasel UB6 ditulenkan ke tahap kehomogenan yang nyata dengan menggunakan 4 langkah penulenan terdiri daripada 0-100% pemendakan ammonium sulfat, kromatografi penukaran anion Source 30 Q, kromatografi penurasan gel Superose 12 dan kromatografi afiniti Concanavilin A (Con-A). Lipase ekstrasel UB6 telah berjaya ditulenkan ke tahap kehomogenan yang nyata dengan pemulihan keseluruhan 3.23% dan 136 kali ganda penulenan. Berat molekul bagi kedua-dua fraksi aktif lipase yang terikat dan tidak terikat Con-A dianggar sebanyak 28,155 dan 32,000 Da dengan menggunakan kromatografi penurasan gel Superose 12 dan gel elektroforesis glysine poliakrilamid sodium dodesil sulfat masing-masing, manakala titik isoelektrik bagi kedua-dua fraksi aktif lipase dianggar sebagai pI 3.5- 5.2 (berasid) dan pI 8.4 (beralkali). Kedua-dua fraksi aktif lipase yang terikat dan tidak terikat Con-A yang tulen mengandungi 60% dan 71% asid amino hidrofobik pada terminal N. Aktiviti maksimum untuk kedua-dua fraksi Con-A yang tulen dikesan pada pH 4.0 dan pH 8.0 masing-masing. Untuk afiniti substrat, kedua-dua fraksi Con-A yang tulen memaparkan afiniti lebih tinggi kepada rantai asid lemak panjang.

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I certify that an Examination Committee met on 7th June 2007 to conduct the final examination of Yap Sia Yen on her Master of Science thesis entitled "Screening, Purification and Characterization of Extracellular Lipase Produced by *Pediococcus acidilactici* UB6 Isolated From Malaysian Fermented Foods" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

YAP SIA YEN

Date: 15 JUNE 2007

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LIST OF ABBREVIATIONS

2DE	2-Demension Electrophoresis
BSA	Bovine Serum Albumin
C	Carbon chain
CBB	Coomassie Brilliant Blue
CFR	Code of Federal Regulation
CFS	Cell Free Supernatant
CFU	Colony forming unit
Con-A	Concanavilin A
cv	Column volume
E.C	Enzyme Commission
FFA	Free Fatty Acid
FPLC	Fast Protein Liquid Chromatography
g	Gram
g	G-force
GRAS	Generally Recognized As Safe
h	hours
H_2O_2	Hydrogen Peroxide
HCl	Acid hydrochloride
IEF	Isoelectric focusing
IEF-PAGE	Isoelectric Focusing-Polyacrylamide Gel Electrophoresis
kDa	Kilo Dalton
K_m	*Michaelis* constant

KOH	Potassium Hydroxide
L	Liter
LAB	Lactic Acid Bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
M	Molar
mA	Milliampere
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
MRS	De-Man, Ragosa and Sharpe
MW	Molecular weight
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide Hydrogen or Reduced NAD
NaOH	Sodium Hydroxide
nmol	Nano mole
ºC	Degree Celsius
OD	Optical density
P.	*Pseudomonas*
PAG	Polyacylamide gel
PAGE	Polyacrylamide gel electrophoresis
Pd.	*Pediococcus*

pI	Isoelectric point
p-NP	p-nitrophenol
PUFA	Polyunsaturated Fatty Acid
PVDF	Polyvinyllidene difluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sp.	Species
spp.	Sub species
TAG	Triacylglycerol
TCA	Trichloroacetic acid
TPC	Total plate count
U	Unit
U.S.	United State
UV	Ultra violet
V	Volt
V	Voltage
v/v	Volume/volume
V_{max}	Maximum velocity
W	Watt
w/v	Weight/volume
µg	Microgram
µl	Microliter
µM	Micromolar