Medical Journal of the
Islamic Republic of Iran

Volume 11
Number 3
Fall 1376
November 1997

EXTRACELLULAR LIPASE ACTIVITY CHARACTERIZATION OF SOME *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED FROM HUMAN INFECTIONS

MOHAMMAD HAJJARTABAR, NASSER GHAEMI*, NASRIN MOAZZAMI AND MAHSHID FAGHIHI-SHIRAZI**

*From the Department of Microbiology, School of Medical Sciences, Tarbiat Modarres University, the *Faculty of Sciences, Tehran University, Tehran, Islamic Republic of Iran and the**Food Research Institute, Vic 3030, Australia.*

ABSTRACT

Pseudomonas aeruginosa EF2, ATCC 9027 and ATCC 19660 were grown in a continuous culture under Tween 80 (polyoxyethylene sorbitan monooleate) limitation and optimum conditions (pH 6.5, 37°C at dilution rate of 0.05/h). Culture supernatants were carefully removed and stored at -20°C. To purify the lipases, the culture supernatant was reduced in volume to approximately 10 mL by an ultrafiltration unit. Excess salts were removed and extracellular lipase was purified. Biochemical characterization and SDS polyacrylamide gel electrophoresis suggested that lipase particles consisted of protein and carbohydrate–including lipopolysaccharide–with the major enzyme activity being lipase. Lipase activity was measured as the rate of standard olive oil (predominantly triolein) hydrolysis.

Characterization of the purified extracellular lipases was then investigated by hydrolysis activity, interesterification reactions and effect on the chemotaxis and chemiluminescence reactions on human peripheral blood neutrophils and monocytes. It was shown that lipase from the EF2 strain was the most effective enzyme used and monocytes were much more sensitive to lipases than neutrophils. Since monocytes are one of the most important cells of the host defence system, lipase activity of *Pseudomonas aeruginosa* may contribute to the pathogenesis of infections caused by this Gram-negative bacterium.
MJIRI, Vol. 11, No. 3, 229-236, 1997.

INTRODUCTION

Pseudomonas aeruginosa is one of the most important bacterial pathogens involved in nosocomial infections.[1-3] Patients with hematological malignancies and immunocompromised individuals are very susceptible to infection by this Gram-negative bacterium.[4,5] This opportunistic pathogen is capable of causing both chronic and acute infections. A prominent example is the chronic lung infection in cystic fibrosis (CF) which is regarded as the main cause of mortality in such patients.

However, this organism causes acute lung infections in other patients.[6-10] *Pseudomonas aeruginosa* currently accounts for about 10% of all Gram-negative bacteremias, and the mortality rate associated with *Pseudomonas aeruginosa* bacteremia is higher than that associated with other Gram-negative bacteremias.[5,11]

Furthermore, because of the seriousness of pseudomonas infections and their innate resistance to many antimicrobial agents, there has been considerable interest in elucidating the pathogenesis of *Pseudomonas aeruginosa* infections.[12] It has been shown that virulence of *Pseudomonas aeruginosa* is multifactorial. In fact, various extracellular products of this organism including enzymes and toxins contribute to this virulence.[13-15] Although extracellular lipase (triacylglycerol acylhydrolase EC 3.1.1.3) has been detected in most clinical strains of *Pseudomonas aeruginosa*, the exact pathogenic role of this extracellular enzyme of *Pseudomonas aeruginosa* is not clear.[16-18] Besides its clinical aspects, extracellular lipase of *Pseudomonas aeruginosa* is also of considerable attention in biotechnology as it specifically catalyses trans- and interesterification.[19-21]

This paper describes the characterization of extracellular lipase activity of three strains of *Pseudomonas aeruginosa* (EF2, ATCC 9027 and ATCC 19660) isolated from human infections. We further studied the effect of the purified lipases of the above strains on chemotaxis and chemiluminescence functions of human neutrophils and monocytes *in vitro*.

MATERIALS AND METHODS

Cultivation of the organisms

Three strains of *Pseudomonas aeruginosa* were isolated from nosocomial infections and identified as *Pseudomonas aeruginosa* EF2, ATCC 9027 and ATCC 19660. These strains were grown in continuous culture in an eleven laboratory fermenter (LH Engineering, 500 series) at 37°C according to the method of Gilbert et al.[22,23]

The medium contained Tween 80 (polyoxyethylene sorbitan monooleate) prepared by a vortex homogenizer as a 10% (V/V) emulsion in distilled water, 14 gr/L; KNO_3, 2.5 g/L; Na_2HPO_4, 1.5 g/L; ferric citrate, 25.0 mg/L (acidified with 2 mL concentrated HCl/L); $MgSO_4.7H_2O$, 0.4 g/L; $CaCl_2$, 25.0 mg/L; $MnSO_4.7H_2O$, 56.0 mg/L; $ZnSO_4.7H_2O$, 72.0 mg/L; $CuSO_4.5H_2O$, 62.5 mg/L; $CoCl_2.6H_2O$, 29.7 mg/L; $NaMoO_4.2H_2O$, 31.2 mg/L; KI, 20.7 mg/L; H_3BO_4, 7.3 mg/Land Agar 1.5% (V/V).

The pH of the medium was maintained at 6.5 by the automatic addition of 0.5 M-HCl or 2 M-KOH. The dissolved O_2 was measured using an electrode and maintained at > 20% air supply. Foam was controlled by the periodic addition of silicone antifoam RD emulsion. The ferric citric was added at a flow rate of approximately 2.5 mL/h. Temperature was controlled at 37°C automatically. Dilution rate was controlled automatically and fixed at 0.05/h. Steady state growth conditions were deemed to have been achieved following the passage of at least six culture volumes.

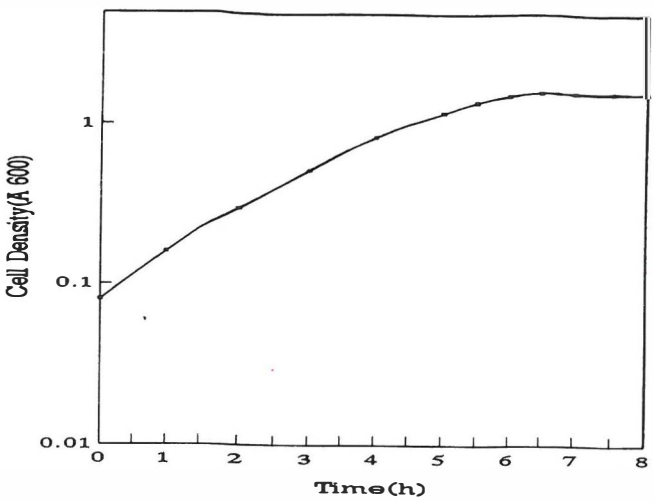

Fig. 1. *P. aeruginosa* EF2 grown in continuous culture under Tween 80 and optimum conditions (pH 6.5, 37°C at dilution rate of 0.05/h). Culture samples were taken at regular intervals over a 9 h period and assayed for growth by measuring OD_{600}.

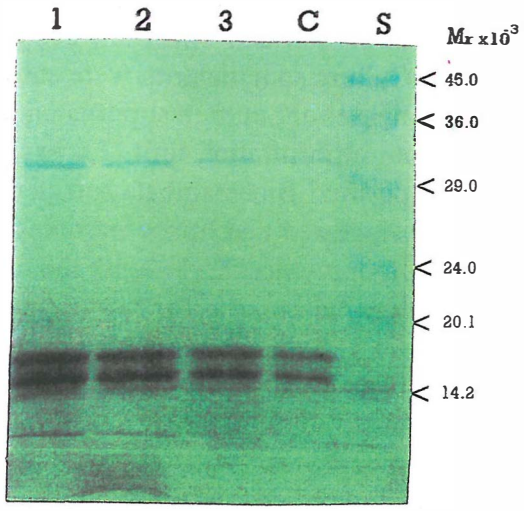

Fig. 2. SDS-analysis of lipases from a culture supernatant of *P. aeruginosa* EF2, ATCC 9027 and ATCC 19660 grown under Tween 80 limitation. Proteins were stained with brilliant blue R250 as described in *Methods*. Tracks: 1, EF2; 2, ATCC 9027; 3, ATCC 19660; S, M_r standards; C, control.

Determination of growth activity

Culture samples were taken at regular intervals over a 9 h period and assayed for growth activity by determination of the OD_{600} of each sample. At the maximum absorption, the culture supernatant was carefully removed by centrifugation at 10,000 g for 20 min and using a Pasteur pipette. The supernatants were

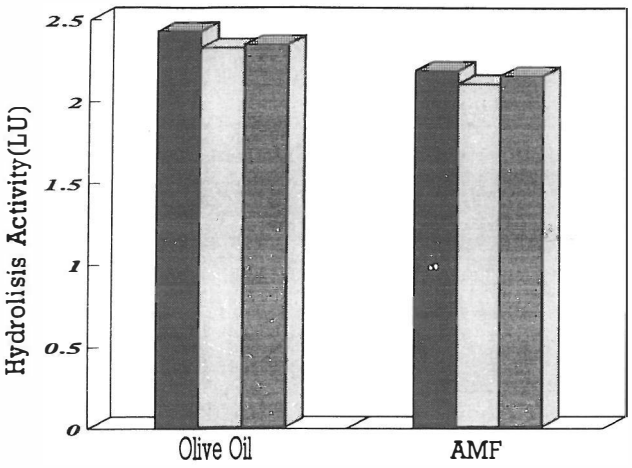

Fig. 3. Hydrolysis activity of the extracellular lipases using different substrates.

EF2 ATCC9027 ATCC19660

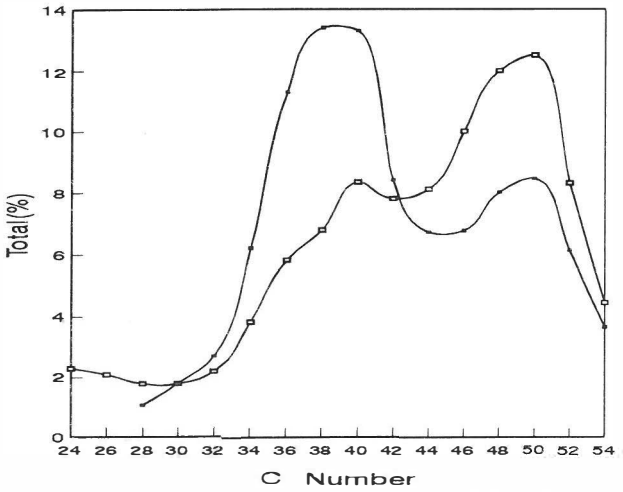

Fig. 4. Interesterification activity assayed by GC analysis as described in *Methods*. -□- AMF (0 h) -□- EF2.

stored at -20°C until required.

Purification of the lipases

To purify the lipases, the culture supernatants of each cultivation run were reduced in volume to approximately 10 mL by passage under nitrogen at 4°C through an ultrafiltration unit (202 Amicon). Excess salts were removed by passage through a desalting column (PD10-Pharmacia). The concentrated and desalted supernatants were analyzed by SDS-PAGE, as described previously,[22,23] and those which contained pure lipase were pooled and stored at -20°C.

Hydrolysis activity assays

Lipase activity was assayed titrimetrically at 37°C and pH 9.0 with a standard olive oil emulsion as substrate (Sigma). Lipase activity was expressed as lipase units (LU) where 1 LU was defined as the release of 1 μmol titratable fatty acid per min under the assay conditions used. The rate of fatty acid production due to the hydrolysis of olive oil (predominantly triolein) was measured from the rate at which a standard solution of NaOH (0.01 M NaOH) needed to be continuously added to the reaction mixture in order to maintain a constant pH. The reaction was carried out at pH 9.0 since preliminary experiments had indicated that this was the pH at which the fastest rate of hydrolysis was obtained and which also allowed the oleic acid produced by the reaction to be largely ionized. The NaOH (0.01 M NaOH) was made up from a standard Convol solution and stored at 4°C in a stopped bottle to minimize absorption of carbon dioxide.

The assay was carried out using an automatic titrator comprising a Metrohm 655 dosimat, Metrohm 632 pH-meter, Metrohm 614 impulsomat, E 64.9 magnetic stirrer, Electronic x,y recorder Kikadenki and a

Table I. Effect of *P. aeruginosa* lipases (EF2, ATCC 9027 and ATCC 19660) on neutrophil and monocyte chemotaxis.

Lipase	Neutrophils		Monocytes	
(8 LU/mL)	Heated	Nonheated	Heated	Nonheated
EF2	99±1	66±3	99.5±0.5	1.5±1.5
ATCC 9027	99±1	68±4	99.5±0.5	4.5±2.5
ATCC 19660	99±1	66.5±4.5	99.5±0.5	3.5±1.5

The cells were preincubated with various concentrations of lipases for 30 min and the neutrophil and monocyte responses to F-Met-Leu-Phe (10^{-6}M) were determined. Results are given as percentages of control cells response preincubated with buffer (mean±SD of two experiments). The responses of control neutrophils and monocytes to F-Met-Leu-Phe were 87±8 and 72±12 cells per field, respectively. Heat treatment of lipase was performed at 100°C for 10 min.

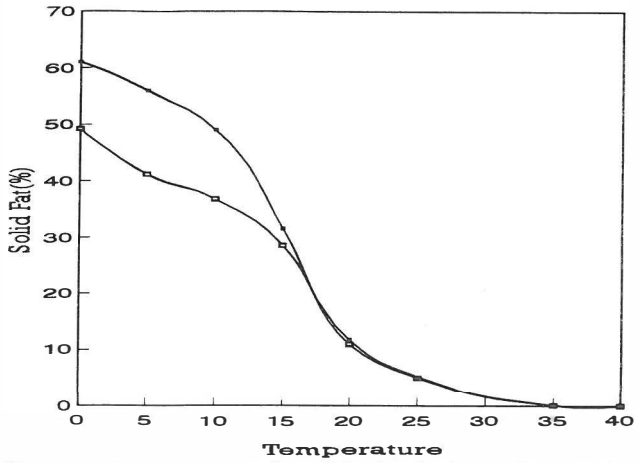

Fig. 5. Percentage of solid fat index after 8 h interesterification reaction of lipase EF2 on AMF using NMR. -□- AMF (0 h)-□- EF2.

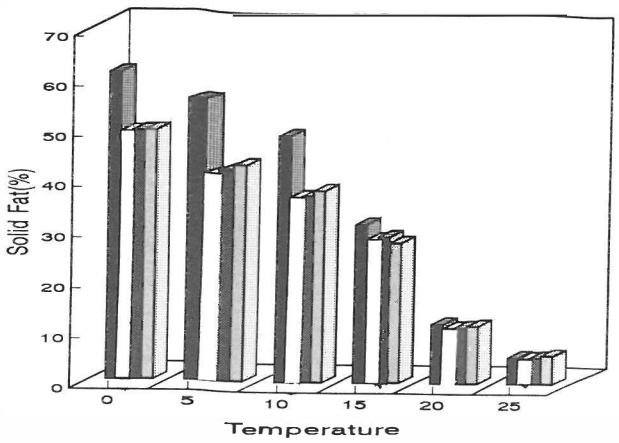

Fig. 6. Effect of the extracellular lipases on solid fat index of AMF after 8 h interesterification reaction using NMR. ■AMF (0 h) □ EF2 ▩ ATCC 9027 ▨ ATCC 19660.

Table II. Effect of *P. aeruginosa* lipases (EF2, ATCC 9027 and ATCC 19660) on neutrophil and monocyte chemiluminescence.

Lipase	Neutrophils		Monocytes	
(8 LU/mL)	Heated	Nonheated	Heated	Nonheated
EF2	100.5±1.5	78±4	99.5±0.5	0±0
ATCC 9027	100.5±1.5	79.5±6.5	99.5±0.5	1.5±0.5
ATCC 19660	100.5±1.5	79.5±8	99.5±0.5	1.0±1.0

The cells were preincubated with various concentrations of lipases and then stimulated with F-Met-Leu-Phe (10^{-5}M). Results are given as percentages of control cells response preincubated with buffer. The responses of control neutrophils and monocytes were 86-91×10^3 and 72-78×10^3 cpm per cells, respectively.

thermostat water bath. 5 mL of the substrate (standard olive oil from Sigma or standard Anhydrous Milk fat from FRI) was added to 95 mL of NaCl solution (0.89% W/V) and homogenized by a vortex homogenizer for 5 min while 0.76 gr of meyhall 30 was gently added to the mixture. 10 mL of this emulsion was stirred in a vessel and its temperature was held constant at 37°C. Then 9.98 mL of NaCl solution (0.89% W/V), 0.1 mL $CaCl_2$ (0.05 M) and 0.1 mL bile solution (0.1% W/V) was added to the emulsion gently while stirring and the pH was held constant at 9.0 (Na^+ and Ca^{2+} are fatty acid acceptors and suppress interfacial charge effects). Finally the reaction was started by adding 0.05 mL of the lipase to the above emulsion while the pH and temperature conditions were held constant. Automatic titration was allowed to continue for at least 5 min. During the reaction, the addition of 0.01 M NaOH to neutralize the fatty acid released by hydrolysis was recorded by the x,y-recorder and the hydrolysis activity of the lipase was calculated from the slope of the recorded line and the amount of lipase used by the following equation:[24]

$$A_h = \frac{t_{OH} \times S \times 1000}{M_E}$$

where A_h = hydrolysis activity of the lipase (μmol/ mL / min), t_{OH}= concentration of NaOH solution (M), S= slope of the recorded line (mL/min), and M_E=amount of the lipase added (mL).

The above experiments were performed by standard olive oil and AMF as different substrates independently and in duplicate manner.

Interesterification activity assays

The interesterification activity of the lipase was carried out in a batch system according to a method from NoVo A/S (1986).[25] 50 mL of olive oil (Sigma) was poured into a 200 mL flask and 1% (V/V) distilled water was added. This mixture was shaken vigorously and 10 mL was taken into another 50 mL flask, then N_2 gas was flushed into the flask for a few minutes and

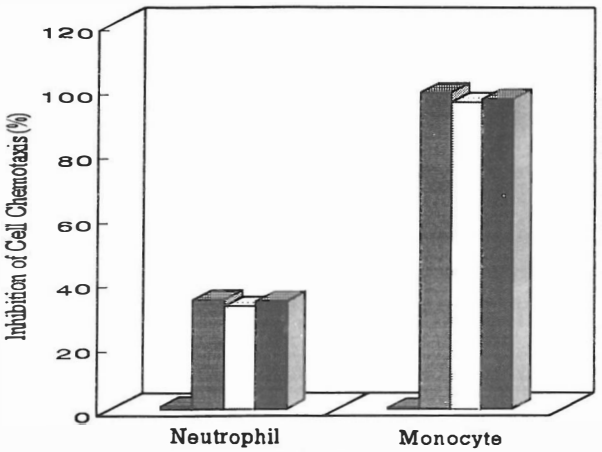

Fig. 7. Effect of the extracellular lipases at concentrations of 8 LU/mL on chemotaxis of neutrophils and monocytes towards F-Met-Leu-Phe. ■Heated ▨EF2 □ATCC 9027 ▩ATCC 19660.

finally 0.1 mL of the lipase was added to the mixture and the flask was incubated in a shaker at 0.9 rpm and 37°C for 8 hours. This procedure was also performed on AMF as another substrate. Appropriate controls were included in each experiment. The assays were performed in duplicates.

Determination of free fatty acids

The determination of free fatty acids of each reaction mixture was done according to the Australian Standard Method for determination of free fatty acids (AS 2300.8.4.).

5 gr of the sample was mixed with 50 mL of 95% ethanol into a 250 mL Erlenmeyer flask. After adding a few drops of phenolphthalein solution, the mixture was heated gently on a hot plate until the fat was dissolved in the solvent or until the mixture boiled. This solution was titrated immediately with 0.01 N NaOH until a faint pink color persisted for half a minute. For each series of the test 2 or 3 blanks (titration of the ethanol without sample) was done. Each experiment was performed in duplicates. The percentage of free fatty acids was calculated by the following formula:

$$\% \text{ FFA} = \frac{0.282 \times (\text{titer blank})}{\text{mass of the sample}}$$

GC analysis

The samples were analyzed on a gas chromatograph (Variant 3400 with FID detector). The samples were diluted to a solution of 5% in chloroform. 1 mL of this solution was injected to the gas chromatograph. The GC conditions were:

Instrument: Variant 3400 GC with Variant 8100 Autosampler

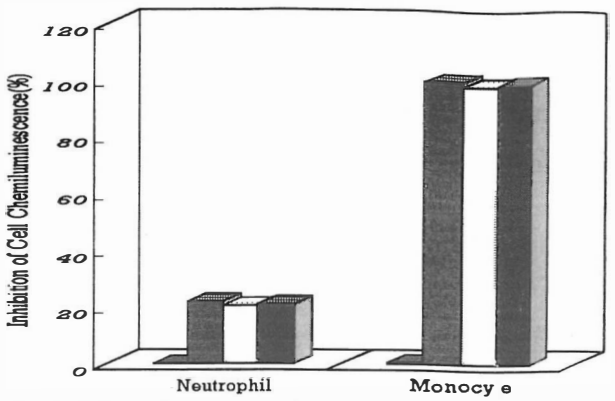

Fig. 8. Effect of the extracellular lipases at concentration of 8 LU/mL on chemotaxis of neutrophils and monocytes as described in *Methods*. ■Heated ▨EF2 □ATCC 9027 ▩ATCC 19660.

Integration System: Variant GC star (Version A2)

Column: SGE 12 M Polyamide coated fused silica capillary column (12QC3/HT5 0.1) i.d. 0.33 mm HT5-siloxane courborane copolymer

Detector: FISD 400°C

Carrier gas: Helium 4.2 mL/min

Sample: 1.0 µL injection

Determination of solid fat index

The determination of solid fat index of the sample was done by a nuclear magnetic resonance spectrometer (NMR). The samples were heated in the NMR-tubes for 30 minutes at 60°C and then put in ice for 90 minutes. After calibration of the NMR spectrometer, reading of the samples at different temperatures of the incubator was done in steps of 5°C and the tubes were tempered for 30 minutes before the readings were taken.

Neutrophils and monocytes

Neutrophils and monocytes from peripheral blood of healthy individuals were purchased from CSIRO-Australia. The purity of the cells was about 99%. The cells were suspended in appropriate buffers and media.

Chemotaxis assays

A chemotaxis chamber with a cellulose filter with 3 µm pore size (Sigma) for neutrophils and 5 µm pore size for monocytes was filled with 0.5 mL of the chemotactic peptide (F-Met-Leu-Phe 10^{-6} M) at a dilution of 1:20. The upper compartment of the chambers contained 0.5 mL of either neutrophil (10^6 / mL) or monocyte suspension (0.5×10^6 /mL). The cells have been preincubated with various concentrations of the lipases for 30 min at 37°C. The chambers were incubated at 37°C for 150 min for neutrophils and 90 min for monocytes. After incubation the filters were

removed, fixed on glass slides, stained with hematoxylin and mounted. The cells that had migrated through the filter to the other side were counted by direct microscopy in five random fields for neutrophils and 10 fields for monocytes on each filter. The assays were performed in duplicates. Appropriate controls were included in each experiment. Inactivation of the lipases were performed at 100°C for 10 min.

Chemiluminescence assays

A given volume of either neutrophil or monocyte suspension was preincubated with an equal volume of various concentrations of the lipases at 37°C for 60 min. After preincubation, the cells were stimulated with chemotactic peptide (F-Met-Leu-Phe 10^{-5} M) in scintillation glass vials. The reaction mixture contained 1 mL of (5×10^5 /mL) preincubated neutrophils or 1 mL of (2.5×10^5/mL) preincubated monocytes, 0.5 mL of chemotactic peptide (F-Met-Leu-Phe 10^{-5}M), 50 µL of luminol (5-amino-2-3dihydro-1,4 phthalazinedione 5×10^{-6} M) and 4.1 mL of Krebs Ringer solution. Thus the assay was performed in a total volume of 5.5 mL at ambient temperature in glass scintillation vials. A Beckman L 8000 scintillation counter placed under air-conditioned thermostat-controlled 21±1°C conditions was used. Reagents and vials were dark adapted before use, and the experiments were performed in duplicates under red light. Sequential 0.5 min counts were taken on each vial over a period of 90 min. Appropriate controls were included in each experiment.

RESULTS

Cultivation and purification

Pseudomonas aeruginosa EF2, ATCC 9027 and ATCC 19660 from various clinical samples were screened and grown in continuous culture under Tween 80 limitation and optimum conditions (pH 6.5, 37°C at dilution rate of 0.05/h). Culture samples were taken at regular intervals over an 8 h period and assayed for growth by measuring OD_{600} as described in *Methods*. Maximum growth for EF2, ATCC 9027 and ATCC 19660 strains occurred at 6.5, 7.5 and 7.0 h, respectively (Fig. 1). To purify the extracellular lipases, the culture supernatant from the maximum growth time was reduced in volume by ultrafiltration unit and SDS-PAGE was carried out as described in *Methods*. Results showed that the enzymes were approximately 99.0% pure with an M_r of approximately 29000 (Fig. 2).

Hydrolysis activities

Hydrolysis activity of the extracellular lipases was carried out using an automatic titrator and different substrate (standard olive oil or standard AMF) as described in *Methods*. Generally, results showed that the hydrolysis activity of the extracellular lipases EF2, ATCC 9027 and ATCC 19660 had higher activity on standard olive oil than AMF (Fig. 3). Also, extracellular lipase of EF2, ATCC 19660 and ATCC 9027 showed higher hydrolysis activity respectively (Fig. 3).

Interesterification activities

Interesterification activity of the extracellular lipases was assayed and followed by GC analysis. Results indicated that in standard olive oil after 8 h interesterification as described previously, total percentage of carbon numbers 50, 52 and 54 declined. Similar reactions using standard AMF as substrate showed that the total percentage of carbon numbers 36, 38 and 40 declined while the total percentage of carbon numbers 46, 48 and 50 increased (Fig. 4).

Determination of free fatty acids did not show any considerable variations in percentage of total free fatty acids in the samples before and after 8 h of interesterification reaction. Furthermore, the determination of solid fat index using the NMR spectrometer showed that the percentage of solid fat index in AMF declined after 8 h of interesterification (Fig. 5) These data indicate that interesterification had occurred as a result of ester exchange reactions. Apparently, the extracellular lipase of EF2 was more effective in declining the solid fat index in AMF than the lipase of the two other strains used (Fig. 6).

Effect on neutrophil and monocyte chemotaxis

Human peripheral blood neutrophils and monocytes were preincubated with various concentrations of the purified lipases for 30 min and then chemotaxis function of the cells was assayed as described in *Methods*. Results are shown in Table I. Generally, heat treatment of lipases at 100°C for 10 min almost abolished the inhibitory effect on neutrophil and monocyte chemotaxis, while lipases at concentrations of 16 LU/mL inhibited neutrophil and monocyte chemotaxis to F-Met-Leu-Phe by approximately 50 and 100%, respectively. Also, the lipases at concentrations of 8 LU/mL inhibited the chemotaxis of neutrophils and monocytes towards F-Met-Leu-Phe by approximately 30 and 100%, respectively (Fig. 7).

The results showed that the inhibitory effect of the extracellular lipase from the EF2 strain was higher than the two other strains used. Also, monocytes were more sensitive to the lipases than neutrophils.

Effect on neutrophil and monocyte chemiluminescence

According to described *Methods*, human peripheral

blood neutrophils and monocytes were preincubated with various concentrations of the purified lipases for 60 min. Results were calculated as percentage of control cells response (preincubated with buffer) and are shown in Table II. The lipases at concentrations of 8 LU/mL inhibited neutrophil and monocyte chemiluminescence responses by approximately 22 and 100%, respectively (Fig. 8). Preincubation of the cells with heat treated lipases (100°C, 10 min) almost had no inhibitory effect on neutrophil and monocyte chemiluminescence responses. Also, preincubation of neutrophils with the lipases at concentrations of 4, 2 and 1 LU/mL resulted in approximately 12, 5 and 4%, respectively, while preincubation of monocytes with the same concentrations resulted in 95, 48 and 32%, respectively, of the chemiluminescence response of control cells (preincubated with buffer). The results showed that monocyte chemiluminescence response is considerably more sensitive to the lipases than the same response of the neutrophils (Fig. 8).

DISCUSSION

Three strains of *Pseudomonas aeruginosa* were isolated from nosocomial infections and identified as *Pseudomonas aeruginosa* EF2, ATCC 9027 and ATCC 19660. According to the method of Gilbert et al. (1991)[22,23] *Pseudomonas aeruginosa* EF2, ATCC 9027 and ATCC 19660 were grown in a continuous culture under Tween 80 and optimum conditions (pH 6.5, 37°C at dilution rate of 0.05/h). Extracellular lipases from culture supernatants at maximum growth rate were purified. These results showed that maximum lipase activity had probably occurred in the late logarithmic growth phase which is in accord with previous works.[22,23,26] SDS-PAGE of the purified lipases revealed an active extracellular enzyme of approximately M_r 29000, and apparently there was no difference between the lipases of the three strains.[27]

Hydrolysis activity on different substrates (standard olive oil and AMF) showed that the kind of substrate is effective in the rate of this activity. Apparently the kind of lipase used was effective in the rate of the reaction too. These properties, including the molecular weight (29K) and the ability to hydrolyze trioleoylglyceral, clearly distinguished the purified enzymes as true lipases from both phospholipase C and esterase.[26]

Interesterification reactions using purified lipases revealed variations in carbon numbers of the substrates which is related to ester exchanges in the molecules. This conclusion was confirmed by the results of GC analysis of free fatty acids and solid fat index of the substrates before and after interesterification reactions. The decline of solid fat content of the samples after interesterification and no change of free fatty acids of the same samples showed that there should be an exchange in the fat content from saturated to unsaturated. Thus lipases are suitable enzymes promoting interesterification reactions which have several advantages over the nonenzymatic methods, including selective and positional specific fatty acid exchange and milder reaction conditions.

The data on the effect of purified lipases on human neutrophil and monocyte chemotaxis and chemiluminescence functions demonstrated that the lipases exhibited a more profound inhibitory effect on monocytes than on neutrophils. Lipases at concentrations of 8 LU/mL inhibited monocyte chemotaxis and chemiluminescence functions completely, while this concentration of the same lipases had almost less than 1/3 of the above effect on neutrophils.

On the other hand, lipases at lower concentrations (1 and 2 LU/mL) had almost no effect on neutrophil functions. The results showed that monocytes are much more sensitive to lipases than neutrophils. It is not clear why lipase is significantly effective on monocytes. One possibility could be that the affinity of lipase binding to monocytes is higher than that of neutrophils. It is known that bacterial products interacting with membrane lipid components exert quite different effects on human neutrophils and monocytes.[28,29] However, further studies are required to clarify the mechanisms involved in the differential effects of the lipases on neutrophils and monocytes.[30,32]

It has been shown that most clinical isolates of *Pseudomonas aeruginosa* produce lipase. However, the role of lipase in the pathogenesis of *Pseudomonas aeruginosa* infection is not known.[16-18] Perhaps the inhibitory role of extracellular lipase on the functions of monocytes (macrophages), a significant cell of the host defence system, may contribute to the pathogenesis of infections caused by *Pseudomonas aeruginosa*.

REFERENCES

1. Hugh R, Gilardi GL: Pseudomonas. In: Lennette EH, Balows A, Hausler WJ Jr, and Truant JP (eds.), Manual of Clinical Microbiology. 3rd ed, Washington DC: Am Soc Microbiol, pp. 288-317, 1980.
2. Pollack M: The virulence of *Pseudomonas aeruginosa*. Rev Infect Diseases 6(3): S617-S626, 1984.
3. Gilardi GL: Medical microbiology. In: Sabath LD, (ed.), *Pseudomonas aeruginosa*: The Organism, Diseases it Causes and their Treatment. Bern, Switzerland: Hans Huber Publishers, pp. 25-30, 1980.

4. Edmonds P, Suskind RR, MacMillan BG, Holder IA: Epidemiology of *Pseudomonas aeruginosa* in a burns hospital: evaluation of serological, bacteriophage and pyocine typing methods. Appl Microbiol 24: 219, 1972.
5. Neu HC: Clinical role of *Pseudomonas aeruginosa*. In: Gilardi GL, (ed.), Glucose-Nonfermenting Gram-Negative Bacteria in Clinical Microbiology. West Palm Beach, Florida: CRC Press, pp. 83-104, 1978.
6. Clarke PH: *Pseudomonas aeruginosa*, an opportunist pathogen. In: Gacesa P, Russell NJ, (eds.), *Pseudomonas* Infection and Alginates: Biochemistry, Genetics and Pathology. London: Chapman and Hall, pp. 1-13, 1990.
7. Doggett RG, Harrison GM: *Pseudomonas aeruginosa*: immune status in patients with cystic fibrosis. Infec Immun 6(4): 628-635, 1972.
8. Hoiby N: Microbiology of lung infections in cystic fibrosis patients. Acta Paediatr Scand Suppl 301: 33-54, 1982.
9. Hoiby N, Doring G, Schiotz PO: Pathogenetic mechanisms of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis patients. Antibio Chemother 39: 60-62, 1987.
10. Winkler UK, Wingender J, Jaeger KE: *Pseudomonas aeruginosa* infections of the respiratory tract in cystic fibrosis patients. Klin Wolchenschr 63: 490-498, 1985.
11. Nicas TI, Iglewski BH: Toxins and virulence factors of *Pseudomonas aeruginosa*. In: Sokatch JR, (ed.), The Biology of Pseudomonas. Orlando: Academic Press, pp. 195-213, 1986.
12. Richmond MH: Resistance of *Pseudomonas aeruginosa* to antibiotics. In: Sabath LD, (ed.), *Pseudomonas aeruginosa*: The Organism, Diseases it Causes and their Treatment. Bern, Switzerland: Hans Huber Publishers, pp. 176-192, 1980.
13. Gacasa P, Russell NJ: Pseudomonas Infection and Alginates: Biochemistry, Genetics and Pathology. London: Chapman and Hall, pp. 29-50, 1990.
14. Homma JY: Roles of exoenzymes and exotoxins in the pathogenicity of *Pseudomonas aeruginosa* and the development of a new vaccine. Jap J Exp Med 50: 149-165, 1980.
15. Wretlind B, Heden L, Sjoberg L, Wadstorm T: Production of enzymes and toxins by hospital strains of *Pseudomonas aeruginosa* in relation to serotype and phage-typing pattern. J Med Microbiol 6: 91-100, 1973.
16. Wohlfarth S, Winkler UK: Chromosomal mapping and cloning of the lipase gene of pseudomonas and cloning of the lipase gene of *Pseudomonas aeruginosa*. J Gen Microbiol 134 (2): 433-440, 1988.
17. Jaeger KE, Kinscher DA, Koenig B, Koenig W: Extracellular lipase of *Pseudomonas aeruginosa* biochemistry and potential role as a virulence factor. In : Holby N, Pederson SS, (eds.), Cystic Fibrosis: Basic Clinical Research. Amsterdam: Elsevier, pp. 113-119, 1992.
18. Finkelstein AE, Strawich ES, Sonnino S: Characterization and partial purification of a lipase from *Pseudomonas aeruginosa*. Biochem Biophys Acta 206: 380-391, 1970.
19. Silver S, Chakrabarty AM, Iglewski B, Kaplan S: Pseudomonas biotransformation: pathogenesis and evolving biotechnology. Washington DC, Am Soc Microbiol, pp. 23-24, 1990.
20. Rogalska E, Cudrey C, Ferrato F, Verger R: Stereoselective hydrolysis of triglycerides by animal and microbial lipases. Chirality 5 (1): 24-30, 1993.
21. Yamane T: Enzyme technology for the lipids industry : an engineering overview. J Am Oil Chemists Soc 64: 1657-1662, 1987.
22. Gilbert EJ, Cornish A, Jones CW: Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. J Genet Microbiology 137: 2223-2229, 1991.
23. Gilbert EJ, Drozd JW, Jones CW: Physiological relation and optimization of lipase activity in *Pseudomonas aeruginosa* EF2. J Genet Microbiology 137: 2215-2221, 1991.
24. Pronk W, Kerkhof PJAM, Van Helden C, Van'I Riet K: The hydrolysis of triglycerides by immobilized lipase in a hydrophilic membrane reaction. Biotechnology Bioengineering 32: 512-518, 1988.
25. Novo Industry A/S: Novo method for the determination of lipase batch interesterification activity analytical method. AF 206: 1-12, 1986.
26. Stuer W, Jaeger KE, Winkler UK: Purification of extracellular lipase from *Pseudomonas aeruginosa*. J Bacteriology 168 (3): 1070-1074, 1986.
27. Jaeger KE, Adrian FJ, Meyer HE, Hancock REW, Winkler UK: Extracellular lipase from *Pseudomonas aeruginosa* is an amphophilic protein, enzyme purification, characterization and N-terminal protein sequence comparison with other pseudomonas spp. lipases. Biochem Biophys Acta 1120 (3): 315-321, 1992.
28. Kharazmi A, Bibi Z, Neilsen H, Hoiby N: Effect of *Pseudomonas aeruginosa* rhamnolipid on human neutrophil and monocyte function. Acta Pathol Microbiol Immunol Scand 97: 1068-1072, 1989.
29. Wilkinson PC: Inhibition of leukocyte locomotion and chemotaxis by lipid-specific bacterial toxins. Nature 255: 485-487, 1975.
30. Jaeger KE, Kharazmi A, Hoiby N: Extracellular lipase of *Pseudomonas aeruginosa*: biochemical characterization and effect on human neutrophil and monocyte function *in vitro*. Microb Path 10 (3): 173-182, 1991.
31. Kharazmi A, Fomsgaard A, Conrad RS, Galanos C, Hoiby N: Relationship between chemical composition and biological function of *Pseudomonas aeruginosa* lipopolysaccharide: effect on human neutrophil chemotaxis and oxidative burst. J Leukocyte Biol 49: 15-20, 1991.
32. Schimpff SC, Greene WH, Young VM, Wiernik PH: Significance of *Pseudomonas aeruginosa* in the patient with leukemia or lymphoma. J Infect Dis 130 (suppl): S24-31, 1974.