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Extraction, Purification and Characterization of Lipase Produced by a Local Isolate of *Staphylococcus aureus*

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Abstract

Background *Staphylococcus aureus* is a ubiquitous bacterium that is generating increasingly bad press coverage due to its propensity to adopt a pathogenic lifestyle in hospital and community settings. Lipases catalyze both the hydrolysis and synthesis of triacylglycerols. Many of these enzymes are characterized by stability at high temperatures and in organic solvents.

Objective Purification of the enzyme by using the conventional methods and characterization of lipase.

Methods Purification included: extraction of the enzyme, the precipitation of the enzyme by ammonium sulphate, dialysis, ionic exchange chromatography by using DEAE-Cellulose (Diethylaminoethyl-Cellulose), and gel filtration by using Sephacryl S-200. Equal amounts of purified lipase solution were mixed with PBS (Phosphate buffer sodium) solutions of different pH (4,5,... until 10) and incubated in a water bath at 37 ^{o}C for 30 minutes, then the lipase activity was estimated. The purified lipase was incubated at different degrees of temperature (5, 15, ...until 85 ^{o}C) for 30 minutes. The molecular weight was determined by gel filtration chromatography.

Results The results revealed that the crude enzyme solution had a total protein concentration of 21.3 mg/ml and an enzyme activity of 257 µmole/ml. The lipase was precipitated by ammonium sulphate with 50-75%. Then the protein concentration was 4.7 mg/ml while the enzyme activity was 812 µmole/ml. Revealed that the protein concentration was 2.3 mg/ml and enzyme activity was 1020 µmole/ml. This revealed that the protein concentration was 0.9 mg/ml and the enzyme activity was 1669 µmole/ml.

Conclusion Lipase was purified to a considerable homogeneity and the characterization experiments revealed that the enzyme showed considerable heat stability and was optimally active at alkaline pH.

Key words Lipase, ion exchange chromatography, gel filtration chromatography, molecular weight.

Lists of Abbreviations: DEAE-Sephadex = Diethylaminoethyl-Sephadex, KDa = Kilo Dalton, PBS = phosphate buffer saline, SDS-PAGE = Sodium dodecyl sulfate-polyacrylamide gel electrophoreses.

Introduction

The staphylococci are gram-positive spherical cells, usually arranged in grape like irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. Some are members of the normal flora of the skin and mucous membranes of humans; others cause suppuration, abscess formation, a variety of pyogenic infections, and even fatal septicemia. The pathogenic staphylococci often hemolyze blood, coagulate plasma, and produce a variety of extracellular enzymes and toxins. Staphylococci rapidly develop resistance to many antimicrobial agents and present difficult therapeutic problems [(1)].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. The many applications of lipases

include specialty organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses [2].

The objective of this study was the isolation and identification of S. aureus from different body sites and lesions and studying the optimal conditions of lipase production and purification of the enzyme by using the conventional methods and characterization of lipase in relation to its stability against different temperature, pH and determination of the enzyme molecular weight.

Methods

1. Extraction and purification of lipase

Purification steps of lipase included: extraction of the enzyme, the precipitation of the enzyme by ammonium sulphate, dialysis, ionic exchange chromatography by using diethylaminoethyl (DEAE-Cellulose), and gel filtration by using Sephacryl S-200.

a. Extraction of Lipase.

Staphylococcus aureus isolate was inoculated in 100 ml of Brain heart infusion broth and incubated for 24 hours at 37 ^{o}C. Afterwards, bacterial cells were harvested by cooling centrifugation at 5000 rpm at 4 ^{o}C for 15 minutes, and then the supernatant was collected in sterile test tubes. The protein concentration was determined by Bradford [3] while the enzyme unit is defined by the amount of enzyme that catalyses the reaction of µmol of substrate per minute and measured by katal (is that catalytic activity that will raise the rate of reaction by one mole/sec in a specified assay system) that the enzyme activity was determined by Bier [4].

b. Ammonium sulphate Precipitation.

Ammonium sulphate (13.4 gm) was dissolved gradually in 100 ml of the crude enzyme extract and was mixed in an ice bath with continuous stirring for 30 minutes to reach a saturation percentage of 0-25 percent; the solution was then centrifuged at 10000 rpm under cooling condition (4 ^{o}C) for 15 minutes. The precipitate was stored in the refrigerator at 4 ^{o}C, while the supernatant was collected in sterile test tubes; the total proteins concentration and lipase activity for the supernatant were measured according to their methods.

The same process was repeated with more amount of ammonium sulphate (14.9 gm) which was dissolved gradually in 100 ml of the supernatant [of saturation percentage (0-25%)] and the other steps were completed in the same manner to reach a saturation percentage of (25-50%) then protein concentration and lipase activity were also measured as mentioned earlier.

More amount of ammonium sulphate (16.4 gm) was dissolved gradually in 100 ml of the supernatant [of saturation percentage (25-50%)] and the other steps were completed in the same manner to reach a saturation percentage of 50-75% then the enzyme activity and the total protein concentration for the supernatant were also measured as before. The supernatant (50-75%) was saturated in the same sequence to reach the final saturation percentage of (75-97%) then the enzyme activity and the total protein concentration were also measured.

The stored precipitates remnants from each step of ammonium sulphate precipitation were dissolved in a minimum amount of 0.1 M PBS pH 7, and then lipase activity and the total protein concentration were measured.

c. Dialysis.

Dialysis tube was activated by immersing in boiling distilled water (D.W.) for few minutes, the precipitate solution (50 ml) resulted from ammonium sulphate fractionation (50-75%) was poured into the dialysis tube, and then dialysis tube was incubated in a baker containing 500 ml of phosphate buffer saline 0.1 M in the refrigerator at 4 ^{o}C for 24-48 hours. The sample was concentrated by embedding the dialysis tube within sucrose powder for 30 minute.

2. Ion exchange chromatography (DEAE-Cellulose)

a. Preparation and packing of the gel

It was prepared according to Schutte [5]. Sodium azide preserved DEAE-cellulose was filtered through Buchner funnel and washed with D.W.

several times. The pH of the exchanger was adjusted to 7 by washing it several times with 0.1 M phosphate buffer saline. The ion exchanger was then degassed under vacuum, and it was ready to be added to the column.

b. Preparation of DEAE-cellulose column

It was prepared according to Schutte *et al*., [5]. A column, with a diameter of 3 cm and a length of 8cm, was washed with Phosphate buffer saline (PBS). Leaving a small amount (about 5 ml) which is enough to fill the dead space to exclude air; DEAE-cellulose gel was poured onto the column with care to avoid bubbles. The column was left overnight for packing and it was equilibrated by adjusting its pH to approximately 7 through suspending in PBS (0.1M) over night.

3. Gel filtration chromatography (Sephacryl S - 200).

a. Preparation and packing of the gel.

Sephacryl S - 200 gel was prepared according to the instructions of the manufacturing company.

4. Characterization of purified lipase.

a. Determination of optimum pH for lipase stability.

Equal amounts of purified lipase solution were mixed with PBS solutions of different pH (4, 5, 6, 7, 8, 9, and 10) all test tubes were incubated in a water bath at 37 ^{o}C for 30 minutes, then the lipase activity was estimated according to the previous method and the relationship between activity and pH of the lipase solution was sketched.

b. Determination of optimum temperature for Lipase stability.

The purified lipase was incubated at different degrees of temperature (5, 15, 25, 35, 45, 55, 65, 75, and 85 ^{o}C) for 30 minutes, then lipase activity was estimated as mentioned before and the relationship between activity and temperature was sketched.

c. Determination of the molecular weight.

Molecular weight was determined by gel filtration chromatography according to Hu and Mobley [6].

Results

1. Extraction and purification of lipase.

a. Crude enzyme.

The results of the current study revealed that the crude enzyme solution had a total protein concentration of 21.3 mg/ml and an enzyme activity of 257 µmole/ml.

b. Ammonium sulphate fractionation.

The lipase was precipitated by ammonium sulphate with 50-75% saturation percentage as a first step of purification, the results of this step revealed that the protein concentration was 4.7 mg/ml while the enzyme activity was 812 µmole/ml.

c. Dialysis.

The results indicated nearly the same value of protein concentration and the specific activity of the enzyme of the previous step.

d. Ionic exchange chromatography by using DEAE-Cellulose.

The results of the current study showed that a prominent peak of protein resulted and it was around fraction number 20-30 and was characterized by a maximal enzyme activity as shown in fig. 1. The fractions were collected, protein concentration was 2.3 mg/ml and the enzyme activity was 1020 µmole/ml. The collected fractions with higher enzyme activity were dialyzed against sucrose to reach a final volume of 5 ml.

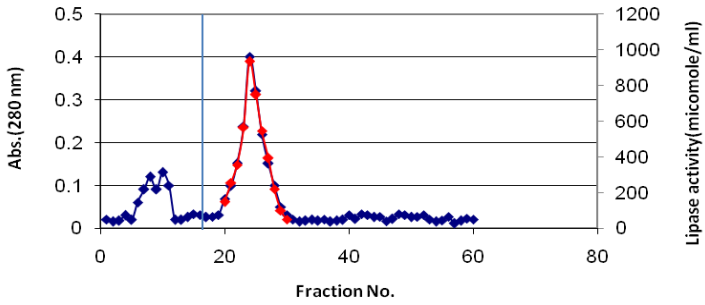

Fig. 1. Ion exchange chromatography for lipase through DEAE-Cellulosecolumn (3×8) cm. The column was calibrated with phosphate buffer saline PBS 0.1 M and pH 7.0, flow rate 40 ml/hour and 5 ml/fraction.

Table 1. Purification steps of lipase from local isolate of *Staphylococcus aureus*.

Purification step	Volume (ml)	Activity (µmole/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification fold
Crude extract	100	257	21.3	12.06	25700	100	1
Ammonium sulphate precipitation (50-75%)	50	812	4.7	172.8	40600	1.6	14.3
Ion exchange using DEAE-cellulose	10	1020	2.3	443.5	10200	0.4	36.8
Gel filtration using Sephadex S-200	5	1669	0.9	1854	8345	0.3	153.7

e. Gel filtration chromatography by using Sephacryl S-200 column.

The enzyme was further purified using gel filtration and the product of dialysis (5 ml solution) was applied to Sephacryl S-200 column (Fig. 2). This demonstrated that single peak of protein was observed with a concentration of 0.9 mg/ml and the fractions number 31 through 41 correlated with that peak were collected and showed an enzyme specific activity of 1669 µmole/ml.

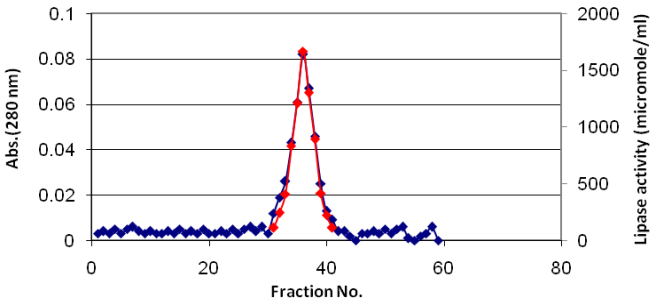

Fig. 2. The Purification of lipase by gel filtration chromatography using Sephacryl S-200 column (2x50) cm. The column calibrated with phosphate buffer saline PBS 0.1 M and pH 7.0, flow rate 60 ml/hour and 5 ml/fraction.

2. Characterization of purified lipase.

a. Determination of optimum pH for lipase stability.

For the characterization of lipase, pH dependence experiment was carried out by using several buffers with pH range from 5-10, enzyme activity was assayed. The result revealed that the activity had increased with increasing pH until pH 8; afterwards lipase activity had declined steadily (Fig. 3).

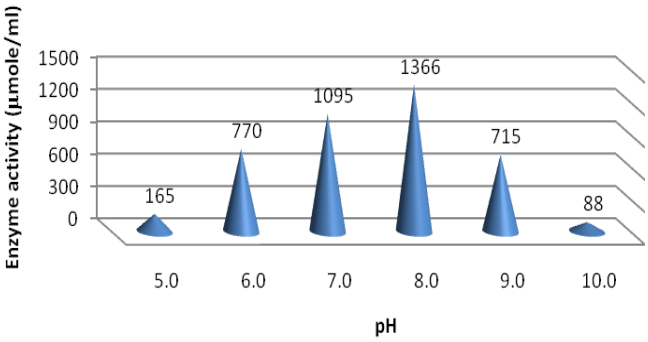

Fig. 3. Effect of different pH on lipase stability.

b. Determination of optimum temperature for lipase stability.

For determining the optimum temperature required for attaining full activity of the enzyme, temperature experiment had been performed. The purified lipase was incubated under different degrees of temperature from 10-70 ^{o}C for 30 minutes, and enzyme activity was then measured after the end of the incubation period, the result showed that lipase activity was stable as far as the temperature of incubation approached the forties Celsius but when the temperature of incubation increased, lipase activity decreased significantly (Fig. 4).

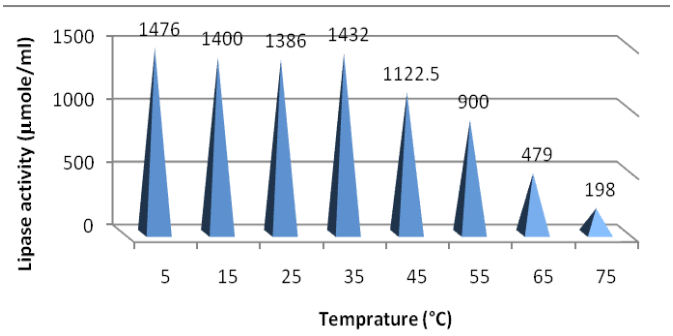

Fig. 4. Effect of different temperatures of incubation on lipase activity

c. Determination of the molecular weight of lipase using gel filtration chromotography (Sephacryl S-200).

The results of the current study revealed that when the four standards proteins of known molecular weight were eluted through the Sephacryl S-200 gel filtration column, they appeared within the elution buffer in a manner resembling their molecular weight starting from the highest to the lowest as shown in fig. 5.

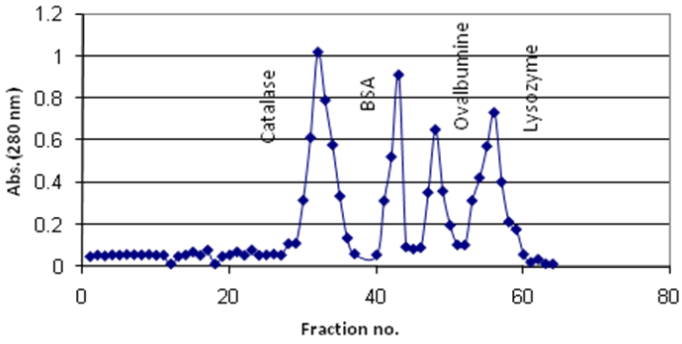

Fig. 5. Gel filtration chromatography for the four standard proteins using Sephacryl G-200 column (2x50) cm. The column calibrated with phosphate buffer saline PBS 0.1 M and pH 7.0, flow rate 60 ml/hour and 5 ml/fraction.

The construction of standard curve resembling the v_e/v_o values for the standard proteins against their relevant log molecular weight directed the way to determine the molecular weight of lipase which was about 110000 Dalton as shown in fig. 6.

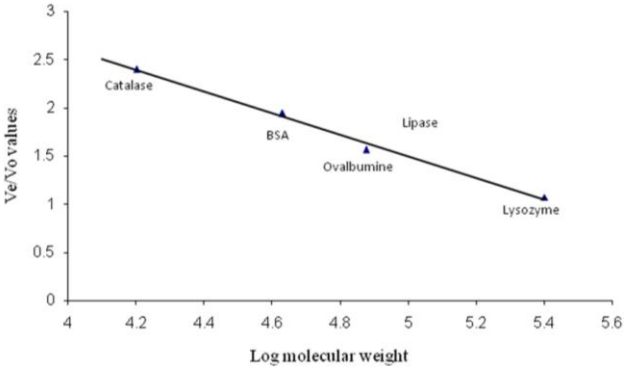

Fig. 6. Determination of the molecular weight of lipase

Discussion

In this study, the result of crude enzyme solution had a total protein concentration 21.3 mg/ml and an enzyme activity 257 µmol/ml.

In a marked disagreement with the results of the present study; Benattouche [7] found that the crude extract had a protein concentration of 67.44 mg and the lipase activity 37 unit/ml. Benjamin and Pandey [8] isolated and characterized three distinct forms of lipases from *Candida rugosa*. Three distinct forms of extra-cellular lipase were isolated by ammonium sulphate precipitation, dialysis, ultra filtration and gel filtration using Sephadex-200. The purification was 43-fold with specific activity 64.35 mg/ml.

In this study, the results revealed that the activity had increased with increasing pH until 8. Afterwards lipase activity had declined steadily.

In study of Akshalha [9], the effect of pH on lipase activity and stability were determined over a pH range of 5-9 and then residual activity was determined at pH 8.0.

The results of the present study are consistent with that of Zouaoui *et al*., [10] who stated that the pH stability of the lipase was determined by the activity retained at different pH from 3 to 10 after 1 h of incubation. The pH stability curve showed that the lipase was stable at pH 6 to 8. The stability data showed a decline in lipase activity below 6 and above 8.

Also, lipase activity was stable as far as the temperature of incubation approached the 40°C

but when the temperature of incubation increased, lipase activity decreased significantly (Fig. 4).

While Akshalha [9] mentioned that the maximum lipase activity was observed within the temperature range 30-40 ^{o}C, with different substrate, lipase from thermophilic strain *Pseudomonas putida* showed thermal stability up to 75 ^{o}C. In another study of Iftikhar *et al*., [11] the enzyme extract was incubated at different ranges of temperature and the effect of temperature on the activity of purified lipases was observed by incubation for one hour. After the end of incubation period, the result showed that lipases retained 80% of their activities at 25-30 ^{o}C and these results were nearly the same as those of the current study. Also similar to the results of the present study, the *Bacillus subtilis* lipase is most active at temperature between 30^{o}C and 50^{o}C and it retained more than 70% of its activity till 45 ^{o}C. The activity dropped rapidly above. The lipase from thermophilic *Bacilli* is relatively more stable at higher temperature. It is also mentioned that the thermal stability of lipases ranged from 20 ^{o}C to 60 ^{o}C.

The results of the current study revealed that the molecular weight of lipase obtained by gel filtration chromatography was around 110,000 dalton.

In the study of Saxena [12], lipase from strain of *S. aureus* has been purified by application of a multi-steps procedure involving ammonium sulphate precipitation and hydrophobic chromatography on phenyl-sepharose followed by gel filtration through sepharose. The molecular weight obtained by molecular sieving and electrophoresis in the presence of SDS (Sodium dodecyl sulfate) were 300 and 45 KDa. These findings are in marked disagreement with the results of the current study which may be due to application of different materials and methods for the estimation of the molecular weight. Moreover, it was found that the molecular weight of two novel lipases thermophilic *Bacillus thermocatenulatus* were of 16 and 43 KDa, respectively. The molecular weight of 16KDa is one of the smallest known of bacteria lipases [13].

In another approach to find out the approximate molecular weight of partially purified lipase, SDS-PAGE was run with protein marker. It was shown that the approximate molecular weight of the partially purified lipase is between 43 KDa and 29 KDa [14]. Other researchers found that the lipases were purified using a DEAE Sephadex A-50 column and preparative electrophoresis and purified enzyme from *A. repens and Eurotriumhebariorum* had molecular masses of 38 and 65 KDa, respectively as determined by SDS-BAGE [15].

In conclusion, *staphylococcus aureus* was isolated as one of the important causative agents of multiple body site infections. Lipase production was affected by incubation conditions and constituents of culture media indicating that this enzyme can be used by the bacteria in a versatile manner for the establishment of infection in various body sites. Traditional methods of purification can be of value for studying bacterial virulence factors and/or other products as those methods yielded results comparable to other studies that utilized different purification strategies and methods. It is evident that lipases have a wide practical application industry and medicine and are available with abroad range of properties depending on their source. With a molecular weight exceeding 100 kDa, it appeared that staphylococcal lipase is one of the biggest lipases that have been purified.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Asia Fadhil collected the sampling and analysis and discus the result and Dr. Amer Hani supervised the research.

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References

1. Jawetz, Melnick, and Adelberg's. Medical Microbiology. 25th ed. USA: McGraw-Hill Companies; 2010.
2. Rathi P, Saxena RK, Gupta R. A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. Process Biochem. 2001; 37: 187-92.
3. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-54.
4. Bier M. Lipases. In: Methods in Enzymology. 1955; 1: 103-7.
5. Schutte H, Buchholz R, Gotz P, et al. Methods in biotechnology. Britain: TJ International Ltd; 1997. p. 150- 3.
6. Hu LT, Mobley HLT. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect Immun. 1990; 58: 992-8.
7. Benattouche Z. Production, optimization and purification of lipase from *Pseudomonas aeruginosa*. Afr J Microbiol Res. 2012; 106: 4417-23.
8. Benjamin S, Pandey A. Isolation and characterization of three distinct forms of lipases from *Candida rugosa* produced in solid state fermentation. Braz Arch Biol Technol. 2001; 44(2): 213-21.
9. Akshatha KN. Purification and characterization of lipase from bacteria. J Res Sci Technol. 2011; 1(7): 19-27.
10. Zouaoui B, Bouziane A, Ghalem B. Production, optimization and purification of lipase from *Pseudomonas aeruginosa.* Afr J Microbiol Res. 2012; 6(20): 4417-23.
11. Iftikhar T, Niaz M, Jabeen R, et al. Purification and characterization of extracellular lipases. Pak J Bot. 2011; 43(3): 1541-5.
12. Saxena RK. Purification strategies for microbial lipase. J Microbiol. 2003; 52: 1-18.
13. Schmidt DC, Rua ML, Schmid RD. Two novel lipases from thermophilic *Bacillus thermocatenunulatus*: screening, purification, cloning, overexpression, and properties. Method Ezym. 1997; 284: 194-220.
14. Yapasan E, Caliskan A, Karadeniz H, et al. Electrochemical investigation of biomolecular interactions between platinum derivatives and DNA by carbon nanotubes modified sensors. Mater Sci Eng. 2010; 169(1-3): 169-73.
15. Kaminishi Y, Tanie H, Kimimoto M. Purification and characterization of lipase from Aspergillus repens and Eurotrium herbarium NU-2 used in "Katsuobushi" modeling. Fish Sci. 1999; 65: 274-78.

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