Characterization Of Partially Purified Indigenous Lipase From Germinated Bambara Groundnut (Voan Dzeia Subterrenea).

Ogueche, Nnamdi.Peter, Ikwuagwu, Emmanuel. Ogbonnaya, Onah, Benjamin. Emenike, Ugwu, Melvin Nnaemeka, Ogbu, Ambrose E.

Abstract: Lipase is an enzyme that catalyzes the hydrolysis of ester bonds in substrates such as phospholipids, triglycerides and cholesteryl esters. Lipase (E.C. 3.1.1.3) was extracted from the 5 days germinated bambara groundnut (Voandzeia subterrenea) using 0.1M phosphate buffer pH 7.8. The enzyme was purified using 60% ammonium sulphate saturation via precipitation followed by dialysis and column chromatography using carboxymethylcellulose. The partially purified lipase showed optimum activity at pH 10.0 and temperature of 60^0C. The K_m and V_{max} were observed to be 6mm and 1120 U/L respectively. Lipase from germinated voandzeia subterrenea has various industrial applications following its stability.

Keywords: Germination, characterization, lipase, purification

Introduction

Lipase (Triacylglycerol acylhydrolase) (E.C. 3.1.1.3) is an enzyme which catalyzes the hydrolysis of ester carboxyl bonds in acylglycerol to release free fatty acids and glycerol. It is capable of catalyzing esterification at water restricted environment. Generally, lipase has a catalytic triad which consists of amino acid: serine, histidine and aspartate or glutamate residues, but the character of each lipase may differ. The difference in lipase character is caused by the difference in its protein structure [1]. Lipases from microbial source can have their character influenced by its medium where it grows, while lipases from plant and animal sources are not influenced by their environment. Lipases from plant sources have specific properties, such as high affinity with triacylglycerol of the plant content. This property is not found in lipases from microbial sources [2]. Lipases have been isolated from many species of plants, animals, bacteria, fungi, and yeast. It have been widely used in the food and other industrial applications and thus there is an increasing demand in discovering new lipase sources having unusual characteristics to suit particular applications.

- *Ogueche, Peter Nnamdi is currently a lecturer in the Department of Human Biochemistry Nnamdi Azikiwe University, Awka, Nnewi Campus, Anambra State Nigeria Postal code: 435001. Email: pn.ogueche@unizik.edu.ng*
- *Ikwuagwu, Emmanuel Ogbonnaya is currently a lecturer in the Department of Biochemistry University on Nigeria, Nsukka, Enugu State, Nigeria.*
- *Onah, Benjamin. Emenike is currently a Ph. D student in the Department of Biochemistry University of Nigeria, Nsukka. Enugu State, Nigeria.*
- *Ogbu, Ambrose. E.; is currently a Ph .D student in the Department of Biochemistry University of Nigeria, Nsukka. Enugu State, Nigeria*

Lipases are one of the important groups of biocatalysts used in biotechnological applications [3]. [4] found that lipase from germinated seed, have higher activity, the highest activity being dependent on length of germination. Lipases have been foundto be present in seeds rich in oils [5]. Although there have been studies on lipases from germinated seeds especially groundnut seeds. Studies on lipases from germinated bambara groundnut are still limited. This study is aimed at purifying and characterizing the indigenous lipase of germinated bambara groundnut using olive oil as substrate.

Materials and Methods

Materials: Chloroforms used was a product of May and Baker Ltd, olive oil-Arista chemicals, triethanolamine hydrochloride crystal – M & M laboratory chemicals Ltd, Bovine Serum Albumin (BSA) - BDA England, Folin – ciocalteu-BDH England and Carboxymethyl cellulose – Sigma chemical company. Other chemicals used were of analytical grade.

Collection of plant materials:
Bambara groundnut seed (voandzeia subterrenea) were bought locally from Ogige Main Market Nsukka, Enugu State, Nigeria.

Methods

Germination of Bambara seeds:
Bambara groundnutseeds were immersed in water and viable seeds settled at the bottom. The viable seeds were imbibed in water for 10hrs and aerated for 2hrs followed by another 10hrs imbibition. At the end of the imbibition, the seeds were germinated on already prepared germination beds for 7 days. The beds were watered every two days [6].

Extraction of Lipase:
2, 3, 4, 5, 6 and 7 days germinated seeds as well as unimbibed seeds (day zero) were decoated and the roots removed. The seeds (51.5g each) were homogenized for 15minutes with cold 0.1M phosphate buffer (pH 7.8) using Philips blending machine (R2000) as described by [7]. After washing them severally with distilled water. The

homogenate was filtered using cheese cloth and the filtrate centrifuged for 10mins at 5000g. The supernatant containing the crude enzyme was collected and used as the crude extract while the pellets were discarded. Lipase activity was determined for each of the extracts as in the assay section below.

Purification of Lipase:
The crude lipase (100mls) was precipitated with ammonium sulphate by the method of [8] at various ammonium suphate saturation (10, 20, 30 – 100%). After addition of ammonium sulphate, crude lipase (100ml) was precipitated for 12hrs and resulting precipitate was collected by centrifugation at 5000g for 30mins at 4^0C. The precipitate was dissolved in 20mls of 0.1M phosphate buffer (pH 7.8). The lipase activity and protein content of this solution were then determined. Crude lipase (300mls) was then initially brought to 20% ammonium sulphate $(NH_4)_2SO_4$) saturation and was allowed to stand for 12hrs at 4^0C. This was centrifuged at 5000g for 30mins. The supernatant was made up to 60% ammonium sulphate saturation and allowed to stand for 12hrs at 4^0C. The precipitate was recovered by centrifugation at 5000g for 30mins. This was dissolved in 0.1M phosphate buffer (pH7.8) to a final volume of 50ml and dialyzed for 12hrs against the same buffer. The dialysate was applied into a gel filtration column ($2.5\times62cm^3$) with carboxymethyl cellulose and pre-equilibrated with 0.1M phosphate buffer (pH 7.8) and the eluting buffer of 5ml/min was collected. The protein content (λ = 250nm) and lipase activity (440nm) were monitored for every fraction (3ml). The fractions which had the highest lipase activity were pooled and used as partially purified enzyme.

Assay of Lipase Activity:
Hydrolytic activities of lipase were detected by the method of [9]Marseno et al., (1998). Olive oil 50% in isooctane (5ml) was mixed with 250µl lipase. It was then incubated in a shaking water bath (100 strokes/min) at 35^0C for 60mins. The resulting oil layer was taken (3ml) and added with 0.6ml of pyridine Cu-acetate (pH 6.0). After thoroughly mixing, the mixture was centrifuged at 750g for 5min and then read the absorbance of the oil layer at λ = 715nm. Oleic acid was used as a standard at concentration of 0, 2, 4, 6, 8 and 10mM. One unit of lipase activity was described as an amount of the enzyme which produced 1µmol fatty acid per min under standard conditions.

Protein Concentration:
Protein concentration in all enzyme extracts was determined as described by [10] with bovine serum albumin as a standard protein.

Effect of Temperature on lipase activity:
Optimum temperature of the partially purified lipase was determined by the hydrolysis assay method at different temperatures ranging from 25 – 90^0C at 5^0C intervals in a water bath using sodium acetate buffer pH 6.0

Effect of pH on Lipase Activity:
The lipase activity was measured by the hydrolysis assay method in pH range 3 – 12 at the interval of 5 using sodium acetate buffer pH 6.0.

Effect of substrate concentration on lipase activity: V_{max} and K_m values were calculated from lineweaver – Burk plot of the initial velocity data using different volumes of the substrate (olive oil).

Results and Discussion

Variation of protein and lipase activity with days of germination: The result of this study shows that germination started 2 days after imbibitions and was completed in 7 days. The result showed a steady increase in lipase activity from day zero through day 2 to the 5^{th}day of germination. Maximum lipase activity was detected at the 5th day and at pH 7.8 (table 1.0). The lipase activity started decreasing after day 5 through the 7^{th} day after imbibitions. This is possible because it has been found that lipase from germinated seeds has higher activity being dependent on the length of germination [4a]. In a similar study it was found that germination of cocoa bean had highest lipase activity at day 3 after imbibitions [11a]. During germination lipase activity increases in the endosperm to hydrolyse the lipids present in endosperm to release free fatty acid as the germination progresses.

Purification of lipase from germinated Bambara groundnut: Solid ammonium sulphate was used to precipitate the crude lipase extract in various concentrations (10% - 100%). The higher the concentration of ammonium sulphate, the more protein precipitated. The lipase activity likewise increased as the ammonium sulphate concentration increases and became relatively constant from 60% concentration. Consequently, two steps precipitation (20 – 40%, meaning discarding precipitate at 20% saturation and continuing precipitation with 40% saturation) were selected for the study. The 60% ammonium sulphate saturation and protein precipitated when dialyzed removed impurities from the sample raising the specific activity to 0.21U/mg with 4.38 purification fold. The purification fold of the lipase increased to 115.76after applying carboxylmethyl cellulose G-200 column chromatographic technique. The overall purification scheme is summarized in table 2.0. This result agrees with that of [12] who reported that 60% saturation was proved to be effective for maximum specific activity of lipase from Bacillus PumilusRK 31; and that of [11b] who also reported that lipase activity increased during solid ammonium sulphate precipitation with increasing ammonium sulphate concentration and became constant at the concentration of 60%.

Effect of temperature on lipase activity: In Figure 1.0, the optimum temperature of the enzyme was observed at 60^0C. The result of this research differs from that of cocoa bean lipase clone PBC 159 which had its optimum temperature at 40^0C [13] and lipase from Jatrophacurcas that was found to have its optimum temperature at 37^0C [14]. It also differs from that of lipase from white melon [15], and lipase from African oil seed [16] which had their temperature optimum at 30^0C. However, optimum temperature of the lipase agrees with that of lipase from Bacillus pumilus RK 31 [12b] which had its optimum temperature at 60^0C.

Effect of pH on lipase activity: In Figure 2.0, at pH 10.0, activity of the lipase was optimum which shows that lipase from germinated bambara groundnut is alkaline lipase. This finding was different from that of cocoa bean lipase of clone PBC 159 [13b] and lipase from the liver of carpcyprinuscarpio L. (1758) [17] which was reported to have pH optimum of 8.0. Lipase from germinated cocoa been was reported to have its optimum pH at 9.0 [11c]. Hence lipase from Bambara groundnut seeds seemed alkaline.

Effect of substrate concentration: In Figure 3.0 and 4.0, the kinetic values for lipase from germinated bambara groundnut were obtained using the Michaelis – Menten model. The values of K_m and V_{max}were observed to be 6mM and 1120 U/L respectively. Catalytic properties of liver lipase were found as K_m = 0.17mM and V_{max} = 2.6µmol/ml.dk [17b] and lipase from Bacillus pumilu RK31 was reported to have $K_m$of 1.83mMC^{-1} and V_{max} of 10.0mML^{-1} min^{-1} [12c]. Trichodermiviride lipase was found to have K_m of 1.14m$ML^{-1}$and V_{max} of 0.056m$ML^{-1}min^{-1}$ [18].

Conclusion: Crude lipase was extracted from germinated Bambara groundnut (Voandzeia subterrenea) and partially purified using 60% ammonium sulphate saturation, raising the enzyme specific activity to 0.21U/L with 4.38 purification fold. The enzyme showed optimum activity at pH 10.0 and temperature of 60^oC with kinetic constants Km and Vmax 6mM and 1120U/L respectively. This research contributes to the existing knowledge on lipase but needs to be researched further.

ACKNOWLEDGEMENTS

The authors are grateful to the staff and management of postgraduate laboratory, Department of Biochemistry, University of Nigeria, Nsukka. We are also grateful to our Redeemer industries, Catherine Rest House Road, OnuiyiNsukka, Enugu State of Nigeria for typing the manuscript.

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Table 1.0 *Variation of protein and lipase activity with days of germination.*

Days	Lipase activity
1	0.0123
2	0.134
3	0.197
4	0.256
5	0.442
6	0.315
7	0.201

Table 2.0: *Purification Table*

Purification step	Total volume (ml)	Total activity (u)	Total protein (mg)	Specific activity u/mg	Purification fold
Crude lipase	300	19.14	393.76	0.05	1.00
Ammonium sulphate precipitation	300	7.25	34.11	0.21	4.38
Dialysis	50	3.94	14.43	0.46	28.41
Carboxymethyl cellulose G-200	25	1.86	0.70	5.59	115.75

Figure 1.0 *Effect of Temperature on the lipase activity*

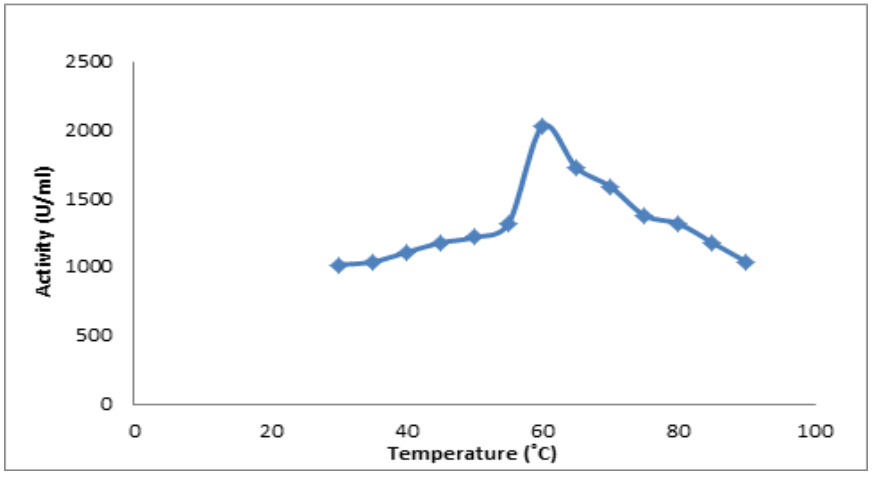

Figure 2.0 *Effect of pH on the lipase activity*

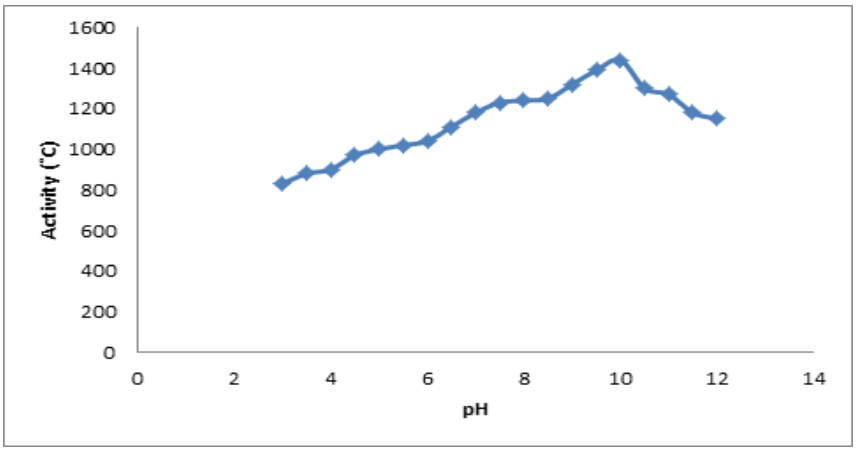

Figure 3.0 *Effect of Substrate concentration [S] on the lipase activity*

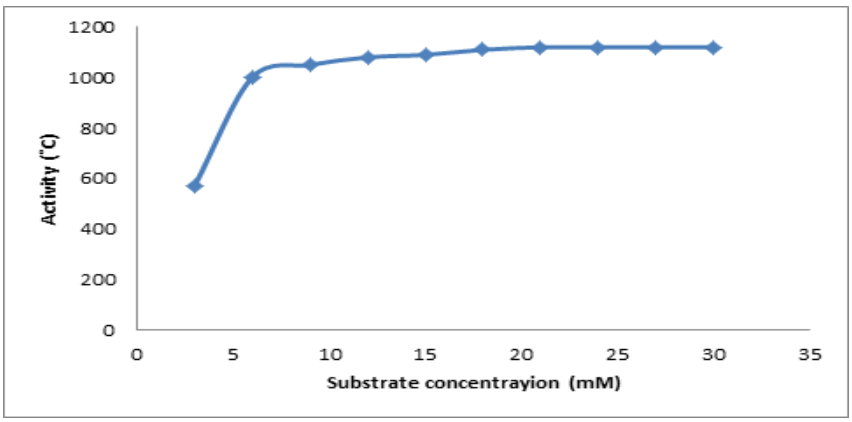

Figure 4.0 *Reciprocals v against [S] on the lipase activity*