A Study of Production and Partial Purification of Lipase Enzyme from Milk

K. Nivedha[1], Y. Shanmugapriya[1], N. G. Ramesh Babu[1] and N. Saravanan[1]
Department of Biotechnology, Adhiyamaan College of Engineering,
Hosur-635 109, Tamil Nadu, India

Abstract - Lipases (triacylglycerol acyl hydrolases) are pervasive enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Lipases have been isolated from many species but the majority of the industrial lipases are of microbial origin. The nutritional value of milk has been suspected due to the elevated content of hypercholesterolemic saturated fatty acid moieties. Hence, there is a growing appeal for healthier milk. Lipase mediated enzymatic modification is drawing interest among the other methods carried out to reduce the fat content as they display high specificity and high efficiency. They present a natural image and can be conducted under mild conditions. 1.1 % of fat got utilized by partially purified Lipase enzyme in our study. Thus, the upshot of activation of partially purified lipase enzyme obtained from cow milk is a promising application in food industry

1. INTRODUCTION

Milk is a butterfat globule within a water-based fluid that contains dissolved carbohydrates and protein aggregates with minerals [1]. Each fat globule is composed almost entirely of triacylglycerols and is surrounded by a membrane consisting of complex lipids such as phospholipids, along with proteins. The fat content of milk varies from animal source to the other. When we compared cow, goat and buffalo milk, high fat content was present in buffalo milk.

From the health and nutrition point of view, nutrition intake between whole milk drinkers and skimmed or low fat drinkers is different. The whole milk drinkers were more likely to choose foods that were less micronutrient-dense, which resulted in their less healthful diets [2]. Although milk fat is attractive for its sensory and physical properties, its nutritional value has been questioned due to the high content of hyper-cholesterolemic saturated fatty acid moieties. Modification of the fatty acid profile of milk fat can be achieved in two ways, 1. In farm practices (animal genetic selection and change of the feeding regime) and 2. Post-farm processes (chemical inter-esterification, enzymatic modification, etc,). As In farm modifications are time consuming, difficult to control and could affect other nutrients in milk, direct post farm processes have attracted more interest. Lipases are one of the important groups of biocatalysts used in biotechnological applications [3]. Lipases extracted from microorganisms are used in various industries. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. This study aims to compare and study the production of lipase enzyme from various sources of milk and its application from the partially purified lipase enzyme.

2. MATERIALS AND METHODS

Milk samples were collected from a cow, buffalo and goat as shown in Figure 1.

Figure 1: Milk samples collected from Cattle

The milk samples collected were serially diluted to 10^{-3}. Nutrient broth culture was prepared and centrifuged at 5000 RPM for 5 minutes at 12 °C in a centrifuge (Remi RM) to separate lipase (the enzyme of interest). Only the supernatant of centrifuge vial was taken out which contains Lipase along with the media.

2.1 Estimation of protein by Lowry method

The protein (enzyme) separated using centrifuge was estimated using the Lowry method. The test milk sample of Cow, Goat and Buffalo taken were 0.2 mL each and estimation was done using a calorimeter (ELICO CL 63) at 660 nm.

2.2 Quantitation of Lipase

Quantitation of lipase was done using olive oil as the substrate. The lipase enzyme solution taken was 1 mL and the formula applied for quantitation was [4],

$$\text{Units/ml of enzyme} = \frac{(\text{NaOH}) \times (\text{Molarity of NaOH}) \times (1000) \times (\beta) \times (df)}{(\alpha)}$$

where,

NaOH = Volume (in milliliters) of Reagent E used for Test minus volume (in milliliters) of Reagent E used for Blank. (Reagent E-50 mM Sodium Hydroxide Solution-Standardized)
1000 = Conversion factor from milli equivalent to micro equivalent.
β = Time conversion factor from 30 minutes to 1 hour (Unit Definition)

df = Dilution factor
α = Volume (in milliliter) of enzyme used

2.3 Specific activity of Lipase

$$\text{Specific activity} = \frac{\text{Lipase enzyme activity}}{\text{Total protein extracted}} \text{ U/mg}$$

2.4 Acetone precipitation

Partial purification was done using acetone precipitation method. The partially purified enzyme extract was centrifuged and the pellets were collected. The pellets were solubilized by using 200mM Tris HCl buffer and the partially purified enzyme extract was collected in an Eppendorf.

After acetone precipitation, the protein estimation and Lipase assay were done so as to compare the specific activity of Lipase before and after partial purification and also to find the yield percentage and purification fold. For protein estimation after the partial purification the volume of test sample taken was 0.1mL and for lipase assay the test sample taken was 0.3mL.

2.5 Determination of fat in cow milk by Rose-Gottlieb method

Rose Gottlieb uses ammonia to break down lipo-protein bonds in milk allowing fat content to get dissolved by ether. 50 mL milk was weighed in a flask and 15mL ammonia was added and mixed. 10 mL diethyl ether and 10 mL petroleum ether were added and the mixture was shaken for 30 minutes. The layers were separated using a separating funnel. The ether layer containing fat content was separated from the flask. Ether was added to the flask and shaken again. Ether extracted was added to the fat extract collected before. This was repeated once again for efficient separation and extract collected was dried and weighed. The weight obtained was compared to the initial weight of the sample to determine fat content (%) of sample [5].

$$\text{Fat \% (w/w)} = \frac{\text{Weight of extract (fat)} \times 100}{\text{Weight of milk}}$$

Utilization by partially purified lipase enzyme=

(fat% before treatment) - (fat% after treatment)

3. RESULTS AND DISCUSSION

Isolates were obtained from tributyrin half strength agar media and observed for zone formation. Among the samples collected, cow milk showed maximum zone formation and number of colonies compared to goat milk and buffalo milk as shown in figure 2.

Figure 2: Lipase producing bacteria from cow, buffalo and goat milk grown on TBA

3.1 Estimation of Protein by Lowry's Method

The enzyme present in the inoculum were collected by centrifugation and subjected to protein estimation by Lowry's method. By plotting the results by taking concentration of protein in µg as x-axis and O.D at 660 nm as y-axis, we obtain the following standard graph has been obtained as shown in Figure 3.

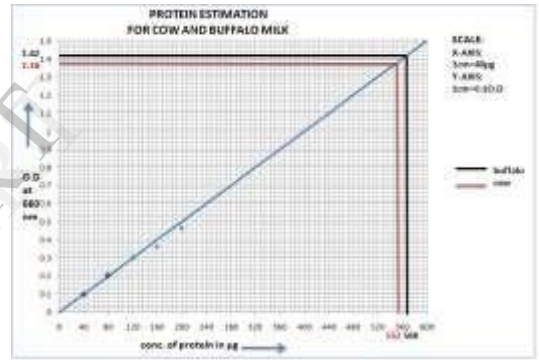

Figure 3: Protein estimation for cow and buffalo milk.

From the values of O.D obtained from the test solutions of Cow milk sample and Buffalo milk sample (1.42 O.D for Cow milk and 1.38 O.D for Buffalo milk) and the standard graph plotted, the values of the concentration of protein present in cow milk and buffalo milk were obtained as 568 µg and 552 µg respectively.

3.2 Protein Estimated

3.2.1 In cow milk

O.D value 1.38 corresponds to 552 µg of protein. 0.2 mL of protein test sample was taken; this contains 552 µg of protein. Therefore, 1mL of protein test sample contains 2760µg or 2.76 mg of protein per mL of crude enzyme extract.

3.2.2 In buffalo milk

O.D value 1.42 corresponds to 568 µg of protein. 0.2 mL of protein test sample was taken; this contains 568 µg of protein. Therefore, 1mL of protein test sample contains 2840µg or 2.84 mg of protein per mL of protein test sample.

3.3 Quantitation of Lipase

For determining the Lipase activity, the enzymatic

assay of Lipase was done using olive oil as substrate and the end point, a light blue color is obtained. The results obtained are:
Units / mL enzyme (cow milk) = 55 U/mL
Units / mL enzyme (buffalo milk) = 40 U/mL

3.4 Specific Activity of Lipase
Specific activity of lipase in cow milk sample =19.9275 U/mg
Specific activity of lipase in buffalo milk sample =14.0845 U/mg

The specific activity of lipase is more in cow‘s milk when compared to buffalo‘s milk. Therefore the Lipase enzyme produced from cow‘s milk was taken for partial purification.

3.5 Partial Purification

Partial purification was done by acetone precipitation method as shown in Figure 4.

Figure 4: Acetone precipitation

3.6 Estimation of Protein after Acetone Precipitation

By plotting results by taking concentration of partially purified protein (µg) on the x-axis and O.D (660 nm) on the y-axis, we obtained the following standard graph shown in Figure 5.

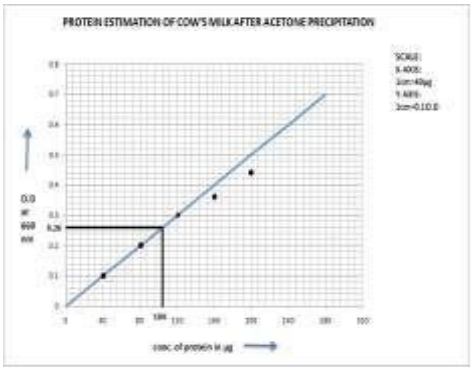

Figure 5: Protein estimation of cow milk after acetone precipitation.

From O.D values obtained from test solution of partially purified enzyme extract of cow milk sample and standard graph plotted, concentration of partially purified protein was obtained as 104 µg.

3.6.1 Protein Estimated

O.D value 0.26 corresponds to 104 µg of protein. 0.1mL of protein test sample was taken; this contains 104 µg of protein. Therefore, 1ml of protein test sample contains 1040 µg or 1.04 mg of protein per mL of partially purified enzyme extract.

3.7 Quantitation of Lipase

For determining the lipase activity in the partially purified enzyme extract of cow‘s milk, the enzymatic assay of lipase was done using olive oil as substrate.
Test solution = 4.9 mL
Blank solution = 4.6 mL
NaOH = 0.3 mL
Units / mL enzyme = 100U/mL

3.8 Specific Activity after Acetone Precipitation

Specific activity of lipase in partially purified enzyme extract in Cow milk sample = 96.153 U/mg.

The comparison of various parameters before and after Acetone Precipitation is shown in table 1.

Table 1: Comparison of various parameters before and after Acetone Precipitation

Acetone Precipitation	Lipase Activity (U/mL)	Protein Estimation (mg)	Specific Activity of Lipase (U/mg)
Before Acetone Precipitation	55	2.76	19.9275
After Acetone Precipitation	100	1.04	96.153

3.9 Application of Lipase Enzyme in the Defatting of Milk

The fat content of test milk sample was estimated by Rose Gottlieb method.

Figure 6: Separating funnel showing separated layers of ether

Fat % before treatment = 3 %
Fat % after treatment = 1.9 %
Fat utilization by partially purified lipase enzyme = 1.1%

3.10 Discussion

The factors which affect the lipase activity are the concentration of the substrate and the size of the fat molecule. The size of the fat molecules found in goat's milk are only a fraction of the size of the fat globules found in cow milk.

This means that fat molecules in goat's milk are broken down easily and goat milk lacks agglutinin, which causes fat globules of cow milk to cluster when cooled. Goat's milk contains higher amount of medium chain triglycerides or MCT's. These may be the reasons why the microbial activity of lipase producing bacteria is nil in goat milk, and maximum in cow milk [6].

Hydrolysing the protein and estimating the amino acids will give the exact quantification of protein. The method developed by Lowry gives a moderately constant value and hence widely followed because of is its sensitivity, 100-fold more sensitive than the Biuret assay. It can be employed to estimate low protein concentration. Hence this method was applied for protein estimation.

Most of the microbial lipases are extracellular and the fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or by filtration. The cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents. About 80% of the purification schemes attempted thus far have used precipitation step, with 60% of these using ammonium sulphate and 35% using ethanol, acetone or an acid (usually Hydrochloric acid) followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography. Precipitation is usually used as a fairly crude separation step, often during the early stages of the purification procedure, and is followed by chromatographic separation. Increase in lipase activity depends on the concentration of ammonium sulfate solution used [7]. Large quantities of sample can be handled, and this step is less affected by interfering non-protein materials than chromatographic methods. In comparison to other techniques which give lower yields (60–70%), precipitation methods often have high average yield (87%) [8].

The Rose-Gottlieb method of estimating fat in milk and milk products could replace the Gerber method, especially for milk products high in Solid Not Fat (SNF), if rendered less time-consuming and less exposed to fire risks. Hence, this method was employed for defatting of milk [9].

4. CONCLUSION

Though milk is considered to have several nutritional values, the fat content present in it causes many health hazards. Therefore removal of fat content from milk has assumed importance in the diary sector. Lipase enzyme which is used widely for defatting of milk was isolated from various sources of cow milk. Cow milk showed maximum specific activity of lipase compared to buffalo and goat milk. The sample showing maximum specific activity of lipase (cow milk) was subjected to partial purification. The partially purified enzyme was employed in the defatting of milk and the reduction in the fat content after the defatting was calculated.

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