PRELIMINARY STUDIES ON GOAT MILK LYSOZYME

BY

ANITA MEHTA

A DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
(ANIMAL BIOCHEMISTRY)
TO THE
KURUKSHETRA UNIVERSITY
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1980

DIVISION OF DAIRY CHEMISTRY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL (HARYANA)

REGD. NO. 78-DK-63

DEDICATED TO

MY PARENTS AND SISTER, MRS USHA VIJ

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Dated the 26th September, 1980

CERTIFICATE

This is to certify that the work reported in the dissertation entitled "GOAT MILK LYSOZYME", was carried out by Ms. Anita Mehta, under my guidance and supervision in partial fulfilment of her M.Sc. Dairying (Animal Biochemistry) course.

M P Mathur
(M.P.MATHUR)

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Anita Mehta

26th Sept. 1980 (ANITA MEHTA)

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CHAPTER I

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INTRODUCTION

1.0 INTRODUCTION

Lysozyme (Mucopeptide N-acetyl muramyl hydrolase EC-3.2.1.17) is widely distributed in nature. It has been detected in number of body fluids, e.g., nasal mucus, saliva, exudates from infections, in extracts from kidney, spleen, liver, lung and lymph. It is also present in certain plants and bacteria. Lysozyme has also been detected or isolated in milk of human, bovine and of several other species including buffalo and goat.

Lysozyme plays a significant role in the inherent anti-bacterial property of milk. It occurs in very small quantity in milk as do other enzymes. In recent years several investigations concerning food application of lysozyme have been known. Rapid gain in body weight of pre-mature infants was observed by administration of egg white lysozyme (Shahani, 1966).

Bakhri and Wolfe (1971) showed that lysozyme can cause destabilisation of casein micelles in a manner similar to the action of rennin. Panfil - Kuncewicz and

Kisza (1973) reported that added lysozyme hastens the digestion of milk proteins by pepsin. Lysozyme has also been used in the preservation of vegetables, fish, meat, fruits, sake and sea foods, and in preventing butyric acid blowing in semi hard cheeses (Matsuoka, 1971).

Although in India buffalo is the major milk producer followed by cow, goat contributes an appreciable amount of milk to the milk industry in certain parts of the country, it is generally accepted that the composition of milk of all species is ideally suitable for the growth pattern of the young of the species, but goat milk has been found entirely suitable for youngsters of other species which grow at approximately the same rate as kids. Further, goat milk products (cheese and butter) are becoming popular in European countries.

Very few, if any, data exists on the lysozyme from goat milk. Therefore, preliminary studies were initiated with a view to gather information on the lysozyme content in the milk of various breeds of goat, and to determine the effects of heat treatment and homogenisation on lysozyme activity. An attempt was as well made to purify the enzyme and conduct kinetic studies with it.

CHAPTER II

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

The presence of natural antibacterial factor(s) in fresh bovine raw milk was first reported by Kitasato in 1889 and year later by Fokker. The enzyme recognised as a lysozyme, should be a basic protein of molecular weight upto 30,000 and should be active against Micrococcus lysodeikticus. The Commission on Enzyme Nomenclature of the International Union of Biochemistry has given the following designation to lysozyme: E.C.3.2.1.17; systematic name mucopeptide N-acetyl muramyl hydrolase; recommended the name mucopeptide glucohydrolase.

Later, a substance detected in the nasal mucus and body secretions by Fleming in 1922 was named as lysozyme because it was capable of dissolving certain bacteria and it resembled lysozyme. After the crystallization of hen egg white lysozyme (EWL) (Abraham and Robinson, 1937), it became very popular with the protein chemists.

2.1 Occurrence - Lysozyme has a very wide occurrence. It is present in various species of plant and animal kingdom. Jolles and Jolles (1967) reported presence of lysozyme in human tears. It is present in certain plants like cabbage, turnip, cauliflower and also in bacteria. It has been detected in a number of body fluids, e.g., nasal mucus, saliva, exudates from infections, in the extracts from kidney, spleen, liver, lung and lymph (Goudswaard et al., 1978). Lysozyme like particles were also reported in rat mammary tissue by Greenbaum et al. (1960). Lysozyme has been detected in milk from a number of species - rat, sow, cow, goats, ewe, mare, camel, duck, human, baboon, donkey and guinea pig (Bordat, 1928; Rosenthal and Leiberman, 1931; Prickett et al., 1933 and Cattaneo and Vergano, 1948).

Shahani et al. (1962) determined lysozyme activity in raw bovine milk and observed it to be varying from 0 - 260 µg/100 ml with an average of 13 µg/100 ml. Chandan et al. (1968) determined lysozyme content in human, cow, goat, ewe and sows milk and observed it to be 40000, 13, 25, 10 and zero µg/100 ml, respectively.

Rao and Belvady (1973) observed lower lytic activity in human milk colostrum than milk samples obtained between 1 and 12 months of lactation.

Henkiewicz and Swierczek (1974) determined lysozyme activity in body fluids of adults and children by diffusion on agarose gel. These workers have also reported that average activity of 21 human samples (3-4 days post-partum) was 65.0 ± 10.2 mg/litre and that in serum 9.8 ± 2.9 mg/litre. Schollenberger et al. (1976) determined lysozyme level of jugular blood serum of leukaemic and healthy cows. Mean values with standard deviation in blood serum of leukaemic and healthy cows respectively were 0.62 ± 0.40 and 1.27 ± 1.14 µg/ml. Lysozyme was present in the milk of 8 leukaemic and 3 healthy cows, the values were 0.82 ± 0.45 µg/ml and 0.37 ± 0.22 µg/ml.

Korhonen (1977) reported lysozyme level in bovine colostrum as 0.40 µg/ml. Kuncewicz and Kisza (1976) reported it to be ranging from 0.23 to 0.29 µg/ml and 37 - 91 µg/ml for cow and human colostrum, respectively.

They reported lysozyme content for human and cow milk to be 15 - 60 µg/ml and 0.12 - 0.84 µg/ml, respectively.

Gibbs et al. (1979) observed that in drip breast milk Ig A, lysozyme and macrophage conc. and Chemical composition was similar to those of pooled mature expressed human milk except for a lower fat content.

2.2 Lysozyme Isolation from Milk:

There are various methods reported for the isolation and purification of milk lysozyme from various species.

Parry et al. (1964) separated lysozyme from human and bovine milk (in human milk purified to 400 times). The various steps included preparation of acid whey, adsorption on Amberlite IRC-50, fractional precipitation with $(NH_4)_2SO_4$ and chromatography on Sephadex G-50. The purification achieved was 36,000 folds. Jolles and Jolles (1967) purified lysozyme from human tears, tissue secretions and milk using ion exchange chromatography on Amberlite and found them to have

similar amino acid sequence. Subsequently, in 1969, they also purified the enzyme from human, cow and goat milk obtaining high yield and recovery. Mouton and Jolles (1969) purified lysozyme from normal and abnormal tissues or secretions. Kimura et al. (1970) isolated lysozyme to the extent of 10 - 20 mg from human milk.

Jauregui (1971) purified lysozyme from human and mare milk using biogel CM-30 for adsorption as well as chromatography and re-chromatography on Amberlite but the recovery was poor. Rao and Belvady (1973) separated a basic protein from human milk by $(NH_4)_2SO_4$ precipitation, acid extraction and Sephadex G-15 and G-75 filtration.

Gary et al. (1977) developed a method to separate lysozyme from hen egg white, human blood, human and goats milk, and also from some foods and biological tissues using deaminated chitin chromatography. Recovery was found to be more than 99%. This method was found to be highly specific. Deaminated chitin had higher capacity for lysozyme, good stability and fast flow rate.

2.3 Assay of lysozyme activity:

Lysozyme is detected by its activity against heat killed *Micrococcus lysodeikticus* cells by measuring the decrease in optical density (O.D) in a Spectrophotometer. The Commission on enzyme of I.U.B. has recommended that lysozyme assay be based on the initial reaction rate measurement.

Shahani *et al.* (1962) modified the method of Smoleilis and Hartsell (1949) for egg, to adapt to milk. The procedure includes preparation of cell suspension in Sorensen buffer (pH 6.2), so as to yield 10 or 30% transmittance (T) at 540 mμ in a Coleman Spectrophotometer, with distilled water set at 100% T. Three millilitre of whey is mixed with 3 ml. of cell suspension, % T is recorded immediately after mixing and after incubation at 37°C for 20 min. The decrease in O.D. is related to lysozyme concentration.

The above method was later modified by Chandan *et al.* (1965). They prepared cell suspension in 0.067M sodium phosphate buffer so as to give an initial transmittance of approximately 10%, when diluted 1:1 with enzyme solution. Increase in transmittance

was taken per min. at room temperature (approx. 25°C) in a Beckman DB Spectrophotometer. Later on, Parry et al. (1965) used a preparation of 50 mg % cell suspension in M/15 phosphate buffer (pH 6.2). One and a half millilitre of cell suspension and 0.5 ml of 0.3M sodium chloride solution is mixed with 1 ml of whey. The mixture is stirred and rate of clearance of cell suspension is measured in Beckman DB Spectrophotometer at 540 mμ.

Matusevich et al. (1969) developed a method for large scale laboratory trials. In this method, 10 ml of suspension showing 40-42% transmittance are added to 1.0 ml whey, after thorough mixing transmittance is measured in a nephelometer/photocolorimeter using green filter immediately after mixing and after incubation at 37.5°C for 2 hr. The change in percent transmittance serves as a measure of lysozyme activity.

Rao and Belvady (1973) measured lytic activity of basic protein, isolated from human milk. The solution was prepared such that one ml contained 2-20 μg of basic protein. A cell suspension, 35 mg/100 ml in phosphate buffer (pH 6.2) containing 0.1% NaCl was prepared.

Protein solution (0.1 ml) was added to 3 ml of suspension and decrease in O.D. at 540 mμ after 3 min. was taken and average decrease in O.D. per μg of lysozyme per min. was calculated.

Gary et al. (1977) proposed a method which consists in preparation of 30 mg/ml cell suspension in .066M phosphate buffer (pH 7.0). Ten μl of enzyme solution is mixed with 3 ml of cell suspension. Optical density is read at 450 nm. in Spectronic 20 Spectrophotometer (Bausch and Lomb, Inc. Rochester, N.Y.) set at 0.5 absorbance optical density is taken every 30 sec. for 2 min. A decrease in absorbance of 0.001/min. was taken as one unit of enzyme activity.

2.4 Other Studies on Milk Lysozymes:

2.4.1. Molecular weight determination and structure -

Jolles and Jolles (1967) determined primary structure of human milk lysozyme and showed it to be containing 124 ± 3 amino acids. But in 1969 they found it to be containing 129 amino acids.

Rao and Belvady (1973) reported the molecular

weight of human milk lysozyme to be 13570. Kimura et al. (1970) reported molecular weight of human milk lysozyme to be 14000 - 15000 by Sephadex G-50 gel filtration. Molecular weight of the bovine milk was determined by Parry et al. (1967) . Their calculated value was 16200 in contrast to 16000 obtained from sedimentation equilibrium method. In 1969, the same workers obtained the molecular weight 15000± 600 by Sedimentation method.

The amino acid sequence of the lysozymes obtained from the milk of baboon, guinea pig, chimpanzee, cow, sheep, pig, mare and kangaroo were determined by Kirshenbaum (1977).

2.4.2 Effect of temperature - Jolles and Jolles (1961, 1967) heated human milk lysozyme (HML) at pH 7.5 for one min. at 100°C and observed 30% loss of activity. Jauregui (1975) observed that mare milk lysozyme was stable at acidic and neutral pH but labile at alkaline pH. Kimura et al. (1970) showed human milk lysozyme (HML) to be less heat stable than egg white lysozyme (EWL). Shahani et al. (1962) found that human milk lysozyme (HML) and bovine milk lysozyme (BML) were stable at LTLT pasteurisation treatment. Kisza et al. (1977), Ford et al.

(1977) and Evans et al. (1978) also observed similar results.

Eitenmiller et al. (1976) showed bovine milk lysozyme (BML) to be more stable than human milk lysozyme (HML) at pH 4.0. But more labile at pH 7.0 and 9.0. Gary and Kroger (1978) found bovine milk lysozyme (BML) to be sensitive to heat. Panfil - Kuncewicz and Kisza (1977) reported some loss in lysozyme at 18°C, when human milk was held for $\angle$ 72 hours at 4°C. Samples of human milk treated at 63°C for 30 min., 72°C for 16 sec. or 100°C for 1 min. contained 30.7, 26.9 and 0 µg/ml Vs 44 µg/ml lysozyme in the unheated sample.

Ruegg et al. (1977) adopted differential scanning colorimetric (DSC) technique to study thermal transitions of lysozyme. The technique revealed conformational changes starting at temp. as low as 45°C.

2.4.3 Optimum pH - Chandan et al. (1965) reported that the bovine and egg white lysozymes were active over the pH range of 3.5 - 9.5 with a minimum activity at pH 7.9 for the bovine enzyme and at pH 6.2 for the egg white enzyme. The bovine lysozyme was more active in

the basic pH range in comparison with egg white lysozyme. Parry et al. (1969) reported optimum pH for bovine milk lysozyme (BML), human milk lysozyme (HML) and egg white lysozyme (EWL) to be 6.35, 7.9 and 6.2 respectively. Kimura et al. (1970) determined optimum pH to be 6.0 and 8.0 for HML and EWL respectively in Tris-Maleic acid buffer.

2.4.4 Sedimentation coefficient and Isoelectric point - Sephianopoulos et al. (1962) reported sedimentation coefficient with S^{0}_{20} , W value of 1.91 S for egg white lysozyme. Chandan et al. (1965) found sedimentation coefficient from ultracentrifugal data to be 2.00 S. Chandan et al. (1965) determined isoelectric point of bovine milk lysozyme to be around pH 9.5 and that of egg white lysozyme in the range 10.5 - 11.0.

2.4.5 Antibacterial properties of lysozyme - Lysozyme checks the growth of many gram positive and gram negative strains of bacteria (Shahani, 1970. Addition of NaCl or EDTA improved lysis by milk lysozyme and egg white lysozyme (EWL) (Vakil et al., 1969). Bovine milk lysozyme does not show lytic activity in the absence of

salt, e.g., NaCl, but human milk lysozyme (HML) and egg white lysozyme (EWL) do (Parry et al., 1965).

Brock (1979) studied antimicrobial activities of lysozyme in milk and colostrum. Korhonen (1980) observed that samples of milk and colostrum of Finnish Ayrshire cows at 1-14 days of lactation inhibited the test organism B. stearothermophilus; concentrations of lysozyme were positively related with the inhibition zone diameter, obtained with incubation for 4.5 h at 55°C.

Wasserfall et al. (1979) observed that egg white lysozyme (at a concn. of 500 U/ml) was able to kill 99% of 5 x 10^5 resting vegetative cells of C. tyrobutyricum within 24 h. of incubation at 25°C. Spores were completely resistent to lysozyme.

2.4.6 Digestibility of lysozyme by protease enzymes - Fujimaki et al. (1973) studied changes occurring during roasting of lysozyme at 100 - 300°C and observed that decomposition of amino acids started at 150 - 180°C. Acidic, aromatic and alkyl amino acids decomposed slowly as compared to Tryptophan and sulphur containing basic and

β-hydroxy amino acids. Hayase et al. (1975) studied digestion of roasted lysozyme by papain, protease, trypsin and pancreatin, when roasted at 180°C for 20 min; there was complete digestion of lysozyme, but the residue became water insoluble when roasted at 210°C for 20 min. It was considered to be partially based upon the decomposition and racemisation of amino acid residues in lysozyme.

2.4.7 Comparison of lysozyme and α-lactalbumin -

Brew et al. (1967) demonstrated similarity between the amino acid sequence of a major section of the molecule of egg white lysozyme (EWL) and α-lactalbumin. On this basis, they proposed that the genes for EWL and α-lactalbumin are derived from a common ancestor.

Krigbaum and Kugler (1969) showed that lysozyme was prolate whereas α-lactalbumin was oblate; hence, it was suggested that they have different molecular conformation in solution. Though, both have almost equal molecular weights, the effective molecular weight of α-lactalbumin in solution is larger than that of lysozyme which is, perhaps, due to difference in conjugation and hydration. Hill et al. (1974) and Sharma

and Bigelow (1974) reported that lysozyme and α-lactalbumin are related due to theirhaving a similar three dimensional structure.

Hopp (1979) undertook detailed immuno-chemical analysis of the α-lactalbumin molecule and compared it with lysozyme. The antigenic determinants, several of which are located around the disulfide bridges, share certain characteristics with antigenic determinants identified on the other proteins. Immunological findings for lysozyme and α-lactalbumin were similar.

2.4.8 Role of added lysozyme in the manufacture of semi hard cheese -

The late blowing of cheese is prevented by the addition of lysozyme before incorporating rennet. Pulay and Krasz (1966) added 0.2% (v/v) egg white lysozyme(EWL) or 0.001% (w/w) egg white along with EWL to the pasteurised milk. They observed that egg white along with egg white lysozyme was more effective than EWL alone. Egg white through its avidin and conalbumin components, bind biotin and ferric ions respectively. Similar findings were reported by Koterska et al. (1972). Wasserfall et al.

(1976, 1979) reported that lysozyme prevents late blowing of Edam cheese. Bottazzi and Battistotti (1979) also observed that late blowing of Grana cheese could be controlled by lysozyme.

CHAPTER III

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Collection of milk samples:

Pooled and individual raw goat milk samples were collected from the goat herd maintained by the Institute. The samples were assayed for enzymatic activity immediately after collection.

3.2 Chemicals needed:

a) Micrococcus lysodeikticus - Dried cells of Micrococcus lysodeikticus were obtained from Sigma Chemical Company, U.S.A.

b) Lysozyme Grade-I - Lysozyme (muramidase, mucopeptide N acetyl muramyl hydrolase) from hen egg white3x crystallised, dialysed and lyophilised, was obtained from Sigma Chemical Company, U.S.A.

c) Phosphate buffer - Phosphate buffer (pH 6.2) was prepared by mixing 81.5 ml of monobasic sodium hydrogen phosphate (0.2M) and 18.5 ml of dibasic sodium hydrogen phosphate (0.2M) and making the volume to 200 ml. The pH was adjusted to 6.2, if necessary.

d) "Amberlite "resin IRC-50(H) Standard grade - It was obtained from BDH Chemicals Ltd., Poole, England.

e) "Amberlite" resin CG-50(H) Chromatography Type-I (110-200 mesh) - It was obtained from BDH Chemicals Ltd., Poole, England.

f) Sephadex G-50 - Sephadex G-50 used was a product of Pharmacia Uppsala, Sweden, and swollen in 0.1M NaCl-0.1M sodium acetate buffer (pH 6.0).

3.3 Protein Estimation:

Protein was estimated by the method of Lowry et al. (1951) with egg albumin as reference protein for drawing standard curve.

3.4 Enzyme Unit:

One unit of enzyme activity, used in the purification procedure, was defined as a decrease of 0.001 in O.D. in Carlzeiss Jena Spectrophotometer per twenty minutes per ml of enzyme solution used.

3.5 Specific activity:

It was defined as the units of lysozyme per mg of protein.

3.6 Purification of enzyme:

The purification of enzyme was done by the method of Chandan et al. (1965) using Amberlite IRC-50 and also Amberlite CG-50. The details are given in the flow sheet.

3.7 Assay of lysozyme activity in milk:

Lysozyme activity in milk was assayed as per the method of Shahani et al. (1962) incorporating the modifications suggested by Parry et al. (1965).

3.7.1 Preparation of whey for the Assay:

For Spectrophotometric assay of lysozyme, clear milk whey served as the source of enzyme. Whey was prepared in the following manner. Ten millilitres of milk were diluted with equal volume of 0.5% NaCl solution (NaCl is added to activate the enzyme). The mixture was warmed to 37°C and the pH was adjusted to 4.6 with 1 N HCl to precipitate the proteins. The precipitate was removed by filtration through filter paper. The pH of the clear whey was adjusted to 6.2 with 1 N sodium-hydroxide. The resulting solution is then used as the enzyme source. The total volume is kept constant in all the samples.

FLOW SHEET FOR PURIFICATION PROCEDURE

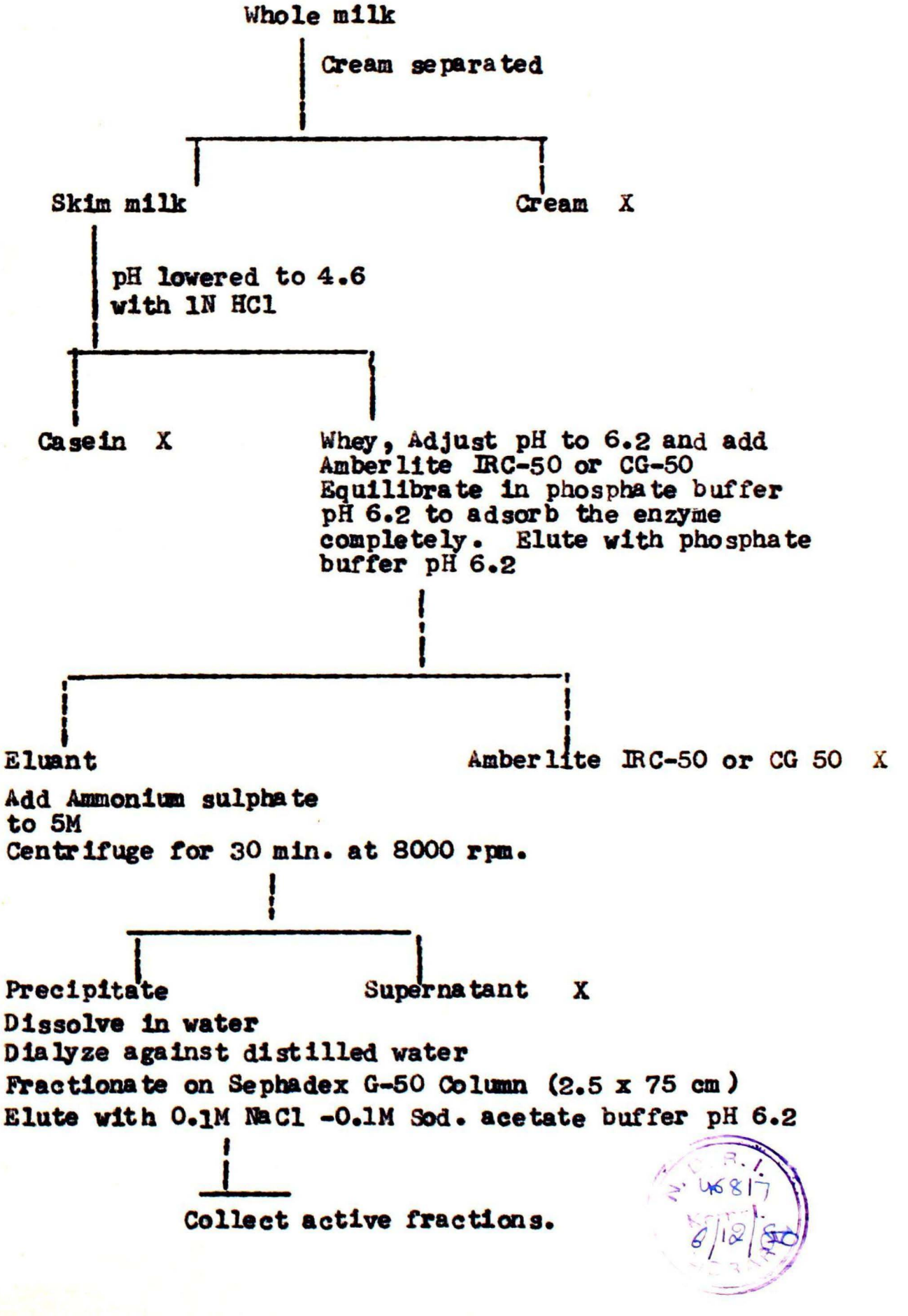
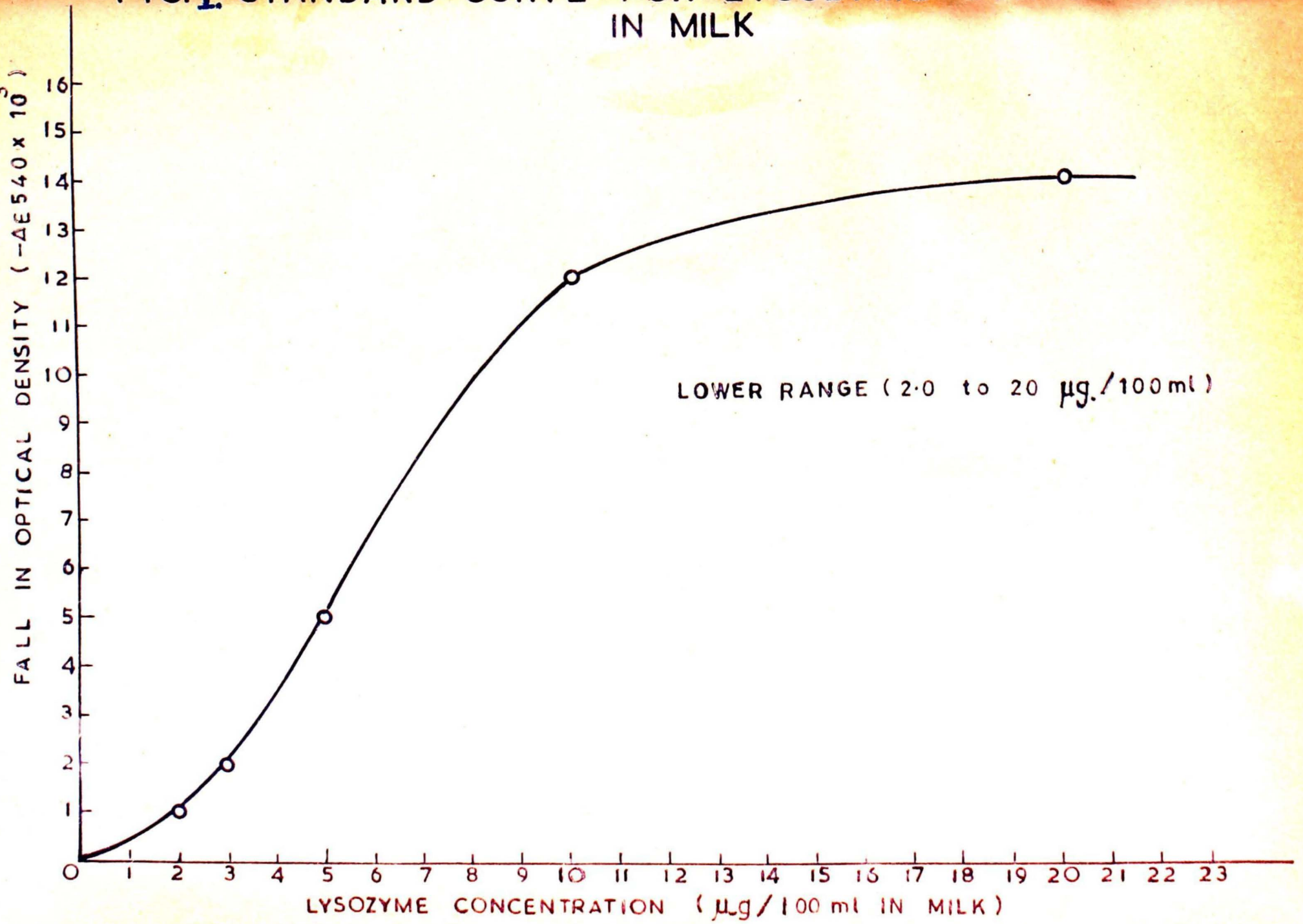


FIG. 1. STANDARD CURVE FOR LYSOZYME CONCENTRATION IN MILK



3.7.2 Assay of lysozyme:

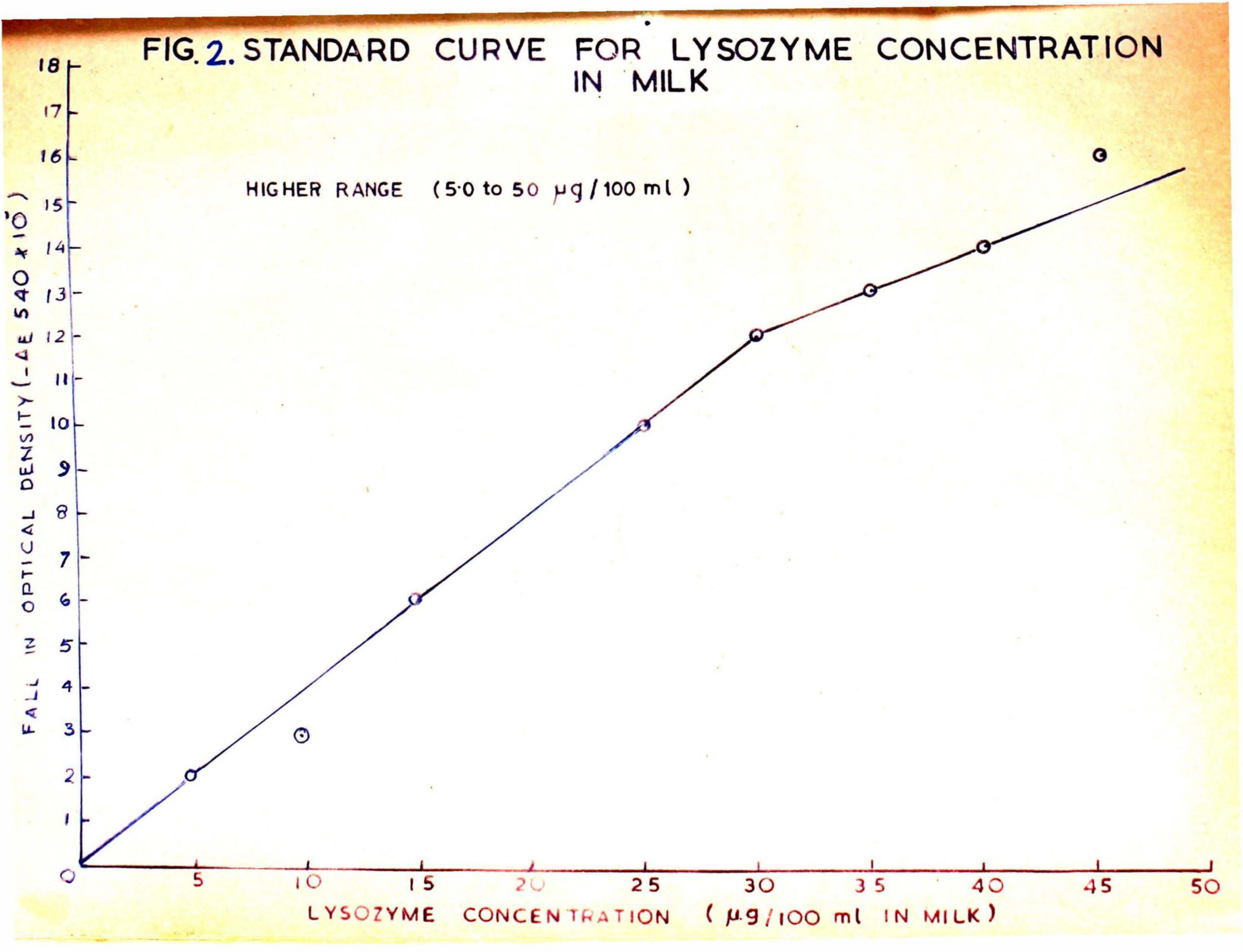
The cell suspension (20 mg % approx.) in phosphate buffer (pH 6.2) was adjusted to 30% initial transmittance in Carlzeiss Jena Spectrophotometer at 540 nm with distilled water set at 100% transmittance.

One millilitre of milk whey was mixed with a mixture of 1.5 ml of the cell suspension and 0.5 ml of sodium chloride solution (0.3M). The optical density (absorbance) was recorded immediately (zero min.) and again after incubation at 37°C for 20 min. The difference in the optical density before and after incubation ($-\Delta E540$) was related to the lysozyme concentration with the help of a standard curve.

3.7.3 Preparation of standard curves:

Crystalline egg white lysozyme (Sigma) was used as the reference enzyme in the preparation of standard curves, and all the results are expressed in terms of egg white lysozyme. For the preparation of standard curves (Fig. I, II) whole milk was used in which the possible inherent lysozyme activity was destroyed by boiling. Two ranges of lysozyme concentration viz. (2 to 20 µg) and 5 to 50 µg/100 ml) were separately added

FIG. 2. STANDARD CURVE FOR LYSOZYME CONCENTRATION IN MILK



to milk and a clear whey was prepared for use as the enzyme source. The cell suspension (20 mg %) in phosphate buffer PH 6.2 was taken and adjusted to 30% initial transmittance at 540 nm with distilled water set at 100% transmittance. The curves were prepared by plotting $-\Delta E540$ values against the concentration of lysozyme.

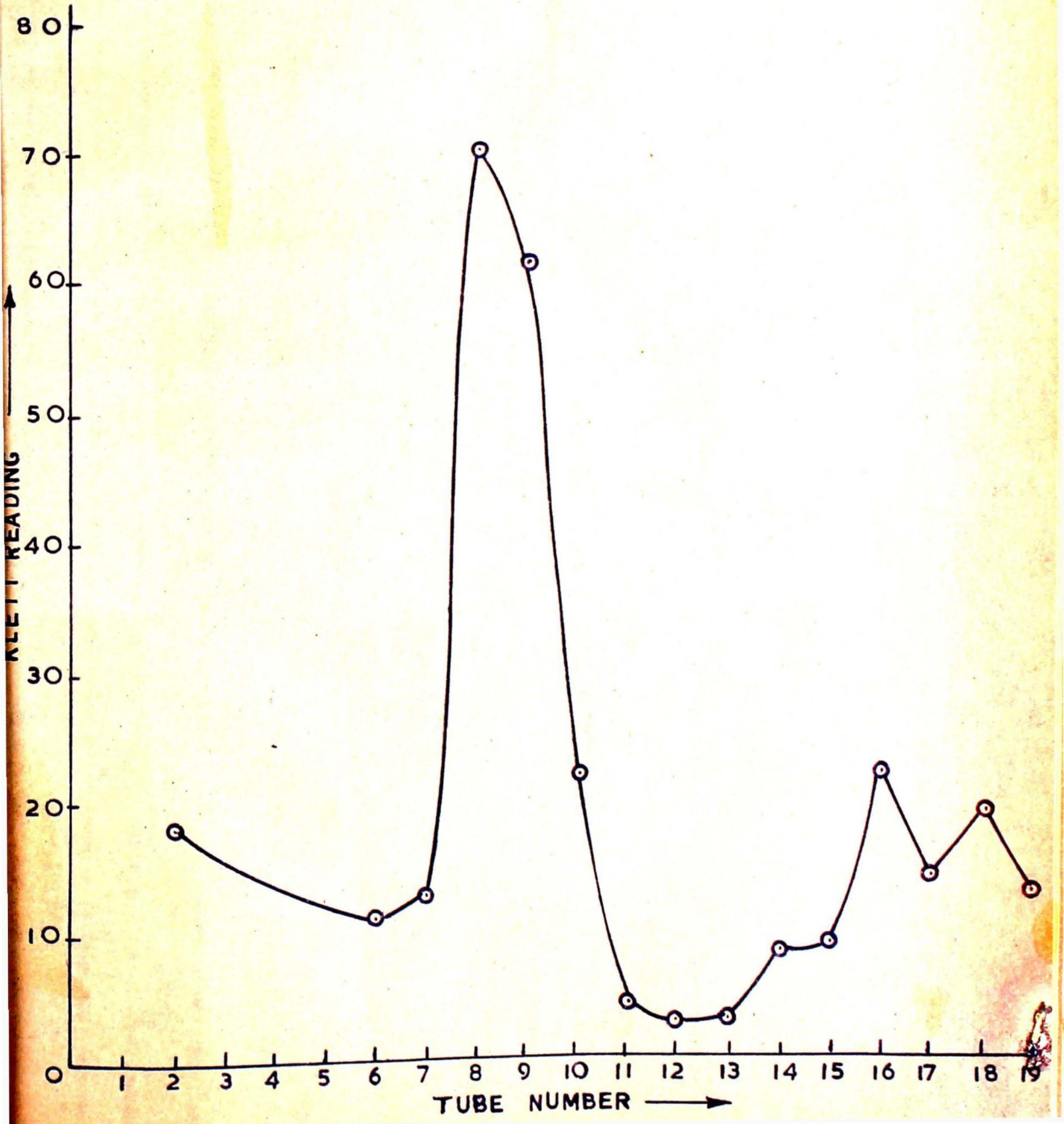
3.8 Gel Filtration:

a) Preparation of Gel column - The dry gel (G-50) was soaked in distilled water for 24 hours. The fines were decanted and gel was suspended in the eluting buffer (0.1M NaCl - 0.1M sodium acetate buffer pH 6.0) overnight and poured into a glass column 2.5 x 75 cms. The gel in the column was allowed to settle for 2-3 hours prior to the application of the sample.

b) Preparation of the sample - The lyophilised sample was dissolved in minimum quantity of phosphate buffer (pH 6.2) and transferred to the gel bed for seiving.

c) Filtration procedure - Before applying the samples the column was allowed to drain until the

FIG. 3. ELUTION PATTERN OF LYSOZYME ON SEPHADEX G-50.



buffer surface came in level with the gel surface. The sample dissolved in buffer was carefully layered over the surface of the gel and the column was allowed to drain until the sample passed into the top layer of gel bed. Subsequently, the column was filled with the eluant and flow rate (1 ml/min.) was adjusted. Five ml of eluant was collected in each tube.

d) Analysis of fractions -

The protein content in each fraction was determined by Lowry's method. The absorbance of the developed blue colour was taken in a Klett-Summerson Photoelectric Colorimeter using red filter. Fractions representing individual peak or peaks were pooled together, concentrated and analysed for lysozyme activity (Fig. III).

3.9 Polyacrylamide gel electrophoresis:

The electrophoresis was carried out in BIO-RAD disc electrophoresis apparatus according to the standard method.

a) Preparation of acrylamide monomer - Stock acrylamide gel was prepared by mixing 8 g cyanogum-41

and 28 g urea and volume was made upto 100 ml by addition of Tris-citrate buffer (pH 8.65). The base (0.1 ml) N'N'N'N' - Tetramethylene diamine (TEMED) was added with stirring, as a catalyst accelerator. The solution was then filtered and polymerisation was initiated by addition of 0.1 ml of freshly prepared ammonium per sulphate (100 mg/ml). The material was then transferred to gel tube. The tubes were held prependicularly during their filling and polymerisation with their lower ends closed by means of polythene caps. The tubes were filled upto mark, 10 mm from the top and each tube was then overlayered with distilled water. These steps were completed within 10 min., from the addition of per sulphate catalyst to monomer solution. The polymerisation is completed within 20-25 minutes. The caps were removed from the lower ends of the gel tubes with care to avoid disturbing the gel by the effect of suction.

b) Electrophoretic apparatus - The tubes were fixed vertically between upper and lower electrode reservoir which had 12 holes each fitted with a rubber gammet and an electrode fixed in the centre of vessel. Each tube was inserted into the respective holes in upper

electrode reservoir. Unused holes were sealed with rubber stoppers. The upper and lower chambers were filled with the same buffer. The buffers used were of pH 7.9 (NaOH-boric acid), pH 9.5 (Tris-glycine), pH 10.0 (NaOH-glycine), pH 10.6 (NaOH-glycine).

c) Preparation and application of samples - Purified lysozyme and standard lysozyme (2 mg/ml) were dissolved in buffer solution (veronal buffer, pH 8.6). One hundred to one hundred fifty µl. of protein solution (200 - 300 µg) was layered directly on the top of each tube by means of a micropipette and a current of about 4 mA per tube was run and after 8 hours, the current was stopped.

d) Staining and destaining of the gel rod - After the run, the buffer solution in the upper tank was discarded and gel tubes were systematically detached. The gel rod was then ejected from its glass tube with the help of a fine needle. The gel rods were then put into staining bath containing 0.1% Amido black in 7% acetic-acid.

The staining was completed within one hour and subsequently washed with water. The background staining was removed by washing the gel rods with 7% acetic acid solution with few changes. The stained protein band became distinct with additional washings.

CHAPTER IV

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

4.1 Lysozyme content of different breeds of goat milk:

Fresh milk samples were collected from the Institute herd and assayed for lysozyme within an hour of milking. The lysozyme activity of different breeds of goat are presented in Table-1. It was observed that on an average Alpine had the highest lysozyme content (34.4 µg/100 ml milk) among the various breeds of goat followed by Beetal. The other breeds including triple cross had lysozyme content in the range of 3.9 - 8.0 µg/100 ml. Earlier (Veena Kumari, 1979) values reported from this laboratory for lysozyme in cow, buffalo and goat were 18.01 µg/100 ml, 15.21 µg/100 ml and 7.99 µg/100 ml, respectively. Chandan et al. (1965) reported that bovine milk contained very low concentration of lysozyme (13.0 µg/100 ml) while human milk showed 39 mg/100 ml.

From the above, it is clear that lysozyme content of goat milk varied from breed to breed.

Table-1 Lysozyme content of different breeds of goat milk.

Lysozyme concentration (µg/100 ml)

Breed	No.of animals	Range	Average	Remarks
Alpine	5	10.0 - 50.0	34.4	-
Beetal	10	2.0 - 20.0	8.5	One sample with zero lysozyme content - average of 9 samples shown.
Alpine-Beetal Saanen cross	10	2.0 - 10.0	3.9	Two samples with zero lysozyme contents. Average of 8 samples shown.
Alpine-Saanen Beetal cross	4	2.0 - 20.0	6.5	-
Saanen-Beetal cross	6	5.0 - 10.0	8.0	-

4.2 Distribution of lysozyme in goat milk from different teats:

For determining the distribution pattern of

lysozyme content in milk from different teats of goat, samples of milk from twelve different goats were collected and analysed for lysozyme content. The results presented in Table-2 reveal that lysozyme activity in milk from different teats showed that nine out of twelve animals showed higher lysozyme content in the left teat. No definite pattern of lysozyme distribution in different teats was observed in buffalo milk (Veena Kumari, 1979). Similar results were obtained in cow milk (Shahani et al., 1962).

Table-2 Distribution of lysozyme in goat milk from different teats.

Animal No.	Lysozyme concentration (µg/100 ml)		
	Left teat	Right teat	Average
1.	8.35	5.00	6.65
2.	9.20	4.20	6.70
3.	7.00	3.33	5.18
4.	10.00	3.33	6.70
5.	8.35	7.53	7.93
6.	4.00	13.30	8.90
7.	8.30	4.16	6.25
8.	8.35	1.66	5.00
9.	3.33	5.86	4.60
10.	5.00	9.20	7.10
11.	5.86	4.10	5.02
12.	15.20	13.30	14.26

4.3 Effect of heat treatment of goat milk on its lysozyme activity:

Goat milk was pasteurized according to HTST and LTLT methods and lysozyme activity in such variously treated milk was determined. Data included in Table-3 would indicate that HTST pasteurisation of milk resulted in an average destruction of 64.5% in lysozyme activity while in the case of milk pasteurized by LTLT method 70.46% enzyme activity was destroyed. On boiling the milk for 10 min. 95.34% enzyme activity was destroyed. In comparison to the above data buffalo milk showed an average of 58.34% destruction (HTST) and 71.12% destruction (LTLT) (Veena Kumari, 1979), while Shahani et al. (1962) reported destruction of 59.1% for HTST pasteurized milk and for LTLT pasteurized milk, the percent destruction was more than double for buffalo milk when compared to cow milk. Kuncewicz and Kisza (1976) observed that about 31% enzyme activity was destroyed by LTLT pasteurisation.

4.4 Effect of homogenisation of goat milk on its lysozyme activity:

An attempt was made to determine the effect of homogenisation on goat milk lysozyme. For this, ten

Table-3 Effect of heat treatments of goat milk on its lysozyme activity

Lysozyme concentration (µg/100 ml)

Sample No.	Raw milk	Boiled milk	HTST 74°C for 16 sec.	LTLT 68°C for 30 min.
1.	6.00	0.00	2.50	1.50
2.	9.00	0.75	4.60	2.00
3.	8.50	0.00	3.00	2.00
4.	11.50	1.20	3.50	4.00
5.	11.00	1.15	4.00	4.00
6.	8.00	0.00	2.00	2.00
7.	8.00	0.60	2.50	2.00
8.	7.00	0.00	2.50	3.00
Average	8.62	0.46	3.07	2.56
% Destruction	0.00	95.34	64.50	70.46

pooled milk samples were taken and these were assayed for lysozyme activity in raw and homogenised milk. The homogenisation was done at 600 - 700 lbs per sq. inch using hand homogeniser. The results are given in Table-4. No definite effect of homogenization on the lysozyme activity could be observed in the ten samples analysed. However, in at least five samples, the lysozyme activity seemed to have increased to various degrees, as a result of homogenization.

Table-4 Effect of homogenization on lysozyme concentration (µg/100 ml) in goat milk

Sr.No.	Raw milk	Homogenised milk
1.	10.0	10.0
2.	10.0	15.0
3.	10.0	10.0
4.	15.0	20.1
5.	5.0	5.0
6.	5.0	7.5
7.	7.5	5.0
8.	7,5	10.0
9.	10.5	12.0
10.	10.0	5.0

4.5 Purification of lysozyme from goat milk:

Lysozyme was purified by the procedure adopted by Chandan et al. (1965) with some modifications. Amberlite IRC-50 and Amberlite CG-50 were used as ion exchangers. Milk whey was prepared from skim milk. Subsequently, ammonium sulphate fractionation was carried out followed by chromatography on Sepharose G-50. The flow diagram of the purification procedure is given on page 21.

4.5.1 Preparation of whey - Although lysozyme could be adsorbed on Amberlite column directly from skim milk, a higher yield was expected when whey was used instead. Skim milk obtained from fresh goat milk was treated with 5N HCl to pH 4.6 and the casein precipitated at its isoelectric point was allowed to settle. The resulting whey was filtered through cheese cloth and adjusted to PH 6.2 with 5N NaOH.

4.5.2 Adsorption of lysozyme on Amberlite IRC-50/CG-50 - The whey was cooled and then passed through Amberlite IRC-50/CG-50 column containing resin at the rate of 5.5 g/litre. The rate of flow was adjusted. The eluted whey possessed negligible lysozyme activity, indicating that most of the enzyme was adsorbed on the resin.

4.5.3 Elution from Amberlite IRC-50/CG-50 - The whey was discarded and the resin was removed from the column and washed with deionised water until the decantant was clear. Then the resin was washed with phosphate buffer (pH 6.2) and the eluant thus collected contained whole of lysozyme in it.

4.5.4 Fractional precipitation with ammonium sulfate - The eluant from theAmberlite column was chilled and ammonium sulfate was added while stirring till its concentration was 5M (about 90% saturation). The precipitate was collected by centrifugation, dissolved in distilled water and dialysed against distilled water for about four hours.

4.5.5 Sephadex Fractionation - For chromatography some of the primary material was applied on the Sephadex G-50 column and eluated with 0.1M NaCl - 0.1M sodium acetate buffer (pH 6.0). Most of the contaminating protein was eluted in the first peak after the first column volume and the fractions containing lysozyme activity were pooled together and the enzyme was precipitated with 5M ammonium sulfate dissolved in distilled water and then dialysed for four hours against distilled water.

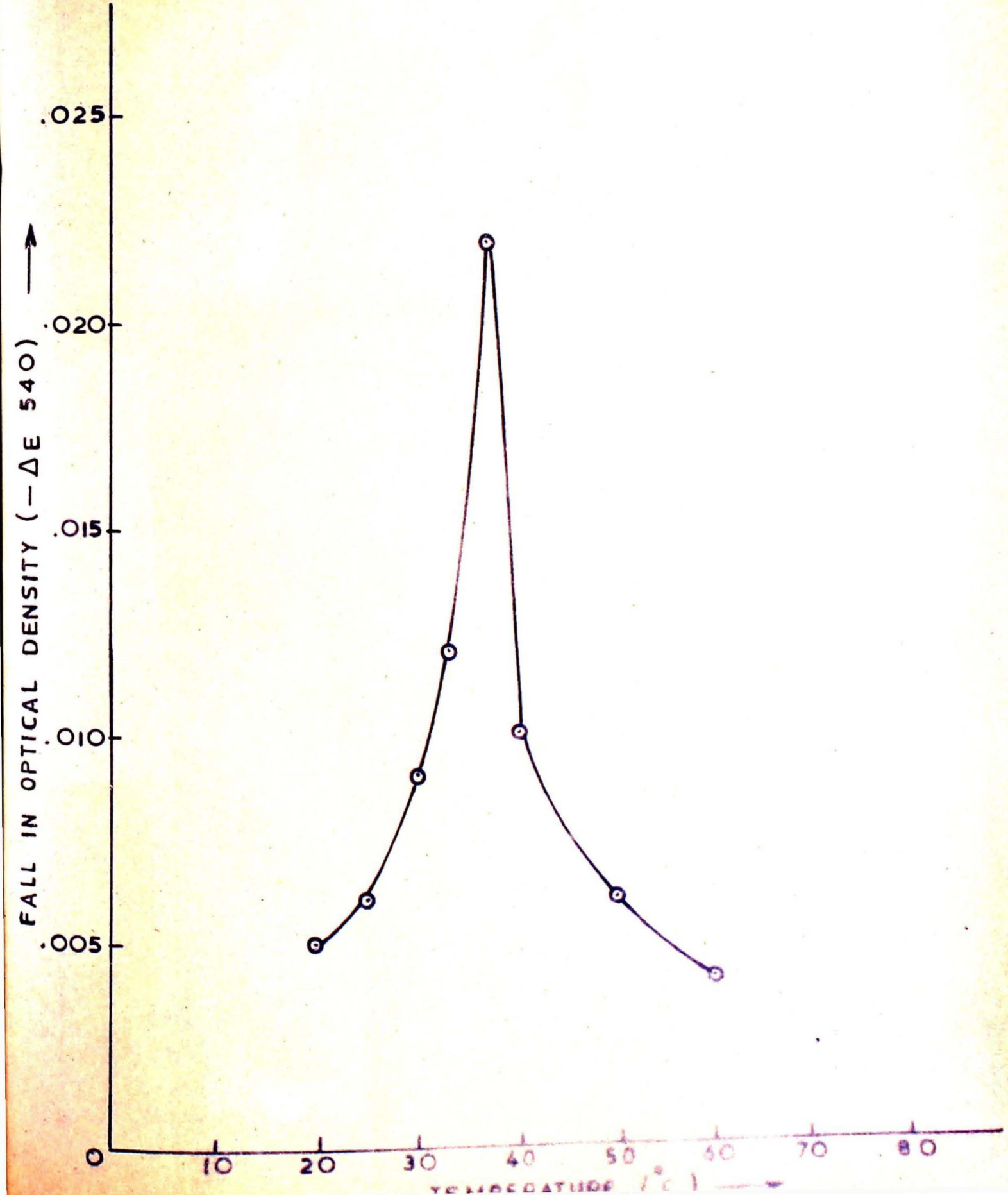
The specific activity and fold purification of the enzyme at each step of purification are given in Table-5. With progressive stages of purification, the enzyme preparation had a higher specific activity. An average of 77 fold purification could be achieved compared to the initial lysozyme activity in whey. However, in one trial, 150 fold purification could be achieved. This was the case when Amberlite IRC-50 was used as cation exchanger in place of Amberlite CG-50 which yielded poor recovery. Poor recovery of the enzyme in the present work was attributed to the various experimental conditions like less amount of starting material, use of whole or skim milk and lower initial activity of the enzyme.

Chandan et al. (1965) isolated lysozyme from bovine milk which showed a purification of 36,000 fold on milk protein basis and 1,000,000 fold on whole milk basis. The starting material was 60 gallons as compared to 5 litres used for each trial in our experiments. Parry et al. (1969) purified lysozyme from human milk which showed a purification of 195 fold, on milk protein basis. Gary et al. (1977) purified lysozyme from goat milk, human milk and egg white using squid dech because of its affinity for lysozyme. They obtained 7, 36 and 16 fold purification. Buffalo milk lysozyme was

Table-5 Summary of purification procedure on the basis of ten trials using Amberlite IRC-50

Purification step	Volume	Units/mg protein	Mg protein per ml.	Total protein	Total units	Specific activity	Fold purification
1. Whey (litres)	5.0	2.99	4.07	20350.00	14997.95	0.737	-
2. Eluant(ml)	5.0	5.97	0.45	226.20	2985.84	13.200	17.8
3. Ammonium sulfate ppt (ml)	80.0	8.00	0.20	16.00	640.00	40.000	54.7
4. Sephadex G-50 Eluant lyopholised fractions (ml)	7.0	7.99	0.14	0.98	55.98	57.130	77.1

FIG. 4. EFFECT OF TEMPERATURE ON LYSOZYME ACTIVITY.



purified using CM cellulose to the extent of 46 fold (Veena Kumari, 1979).

4.6 Kinetic studies with goat milk lysozyme:

An enzyme preparation having 77.1 fold purification was used for kinetic studies.

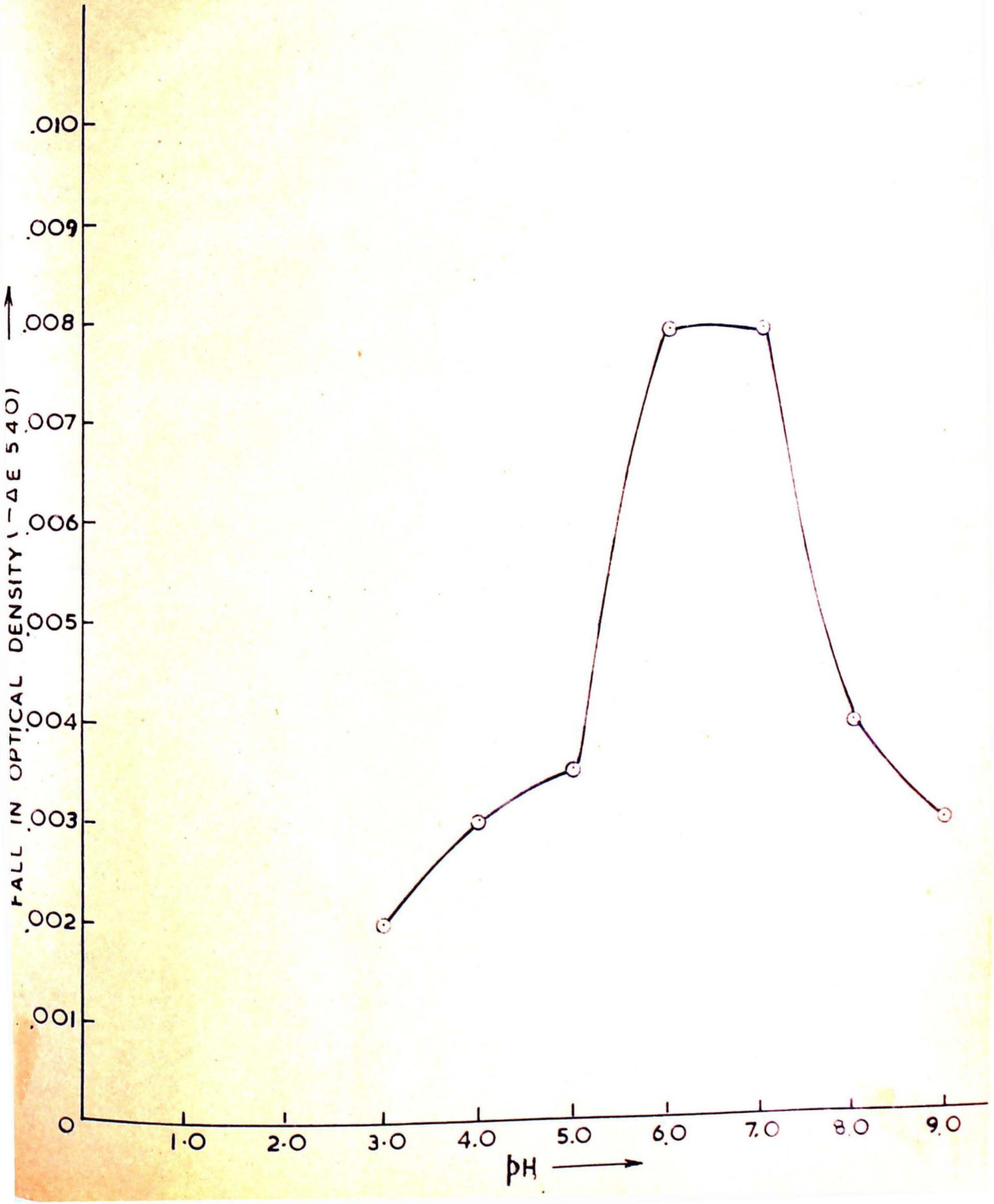
4.6.1 Effect of temperature on lysozyme activity -

Effect of different temperatures on the goat milk lysozyme activity was studied. A range of 20 - 60° was selected and the results are presented in Fig. IV. It can be seen that the optimum temperature for the enzyme activity was at 37°C after which the activity sharply declined. At high temperatures, the enzyme probably gets denatured, thus showing low activity. The optimum temperature obtained in the present study is similar to that reported by Shahani et al. (1962) and is the optimum temperature for most of the other milk enzymes.

4.6.2 Effect of pH on lysozyme activity - The

enzyme preparation was assayed in the pH range of 3 to 9. The Fig. V depicts the effect of pH on lysozyme activity. The maximum activity was observed between pH 6.0 and 7.0 which means that enzyme has a pH optimum between 6.0 and 7.0

FIG. 5. EFFECT OF pH ON LYSOZYME ACTIVITY.



The activity declined above pH 7.0. Chandan et al. (1965) had also observed that in general both BML and EWL showed activity in a wide range of pH (3.5 - 9.5) with a maximum activity at pH 7.9 for BML and pH 6.2 for EWL. The two lysozymes obtained from different sources possessed different pH optima. Parry et al. (1969) observed that human milk lysozyme exhibited a single pH optimum at pH 6.35 which compared favourably with that obtained from egg white lysozyme.

4.7 Electrophoresis of purified enzyme:

The partially purified lysozyme (to the extent of 77.1 folds) as well as crystalline egg white lysozyme were subjected to polyacrylamide disc gel electrophoresis. Mobility of goat milk lysozyme towards the cathode indicated its basic nature.

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CHAPTER V

SUMMARY

5.0 SUMMARY

1. Lysozyme content of goat milk varied from breed to breed. It was observed, on an average that, Alpine had the highest lysozyme content followed by Beetal, Saanen-Beetal cross, Alpine-Saanen-Beetal and Alpine-Beetal-Saanen.

2. Distribution pattern of lysozyme content in milk from different teats of goat showed that on an average, left teat had a higher lysozyme content than the right one.

3. An average destruction of 64.5% and 70.46% of lysozyme activity was observed during HTST and LTLT pasteurisation of milk, respectively.

4. No significant effect of homogenisation of milk was found on the lysozyme activity, though in a few cases there was a slight increase in the enzyme activity.

5. Purification with Amberlite IRC-50 as a cation

exchanger was achieved to the extent of 77.1 folds. However, purification upto 150 folds was also achieved in one trial. No improvement in fold purification was found when Amberlite CG-50 was used in place of Amberlite IRC-50.

6. Kinetic studies with partially purified goat milk lysozyme reveal that whereas the optimum temperature for the enzyme activity was 37°C, the optimum pH range was between 6.0 - 7.0

7. Goat milk lysozyme behaves as a basic protein due to its electrophoretic mobility towards cathode in polyacrylamide disc gel electrophoresis.

CHAPTER

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