BUFFALO MILK LYSOZYME

A DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN

DAIRYING

(ANIMAL BIOCHEMISTRY)
TO THE KURUKSHETRA UNIVERSITY,
KURUKSHETRA

BY

VEENA KUMARI

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

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Dr.M.P.Mathur, Ph.D.,
SCIENTIST S-1

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Dated the 10th May, 1979

This is to certify that the work reported in the dissertation entitled "BUFFALO MILK LYSOZYME" was carried out by Miss Veena Kumari, under my supervision, in partial fulfilment of her M.Sc.Dairying (Animal Biochemistry) course.

M P Mathur

(M.P.MATHUR)

ACKNOWLEDGEMENT

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I express my heartfelt thanks and gratitude to my friends for their day-to-day help and cooperation.

Veena Kumari

(VEENA KUMARI)

C O N T E N T S

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Milk, produced under normal conditions by healthy animals, contains a variety of enzymes. Mammary glands are probably the site of production of these enzymes. Shahani et al.(1973) and Whitney (1958) reported that bovine milk contained over 20 enzymes, produced by mammary gland. The principal enzymes are catalase, peroxidase, xanthine oxidase, alkaline phosphatase, amylase, protease, lipase and aldolase. Some of these enzymes are concerned with the synthesis of compounds like lactose, fat and protein, while others are associated with certain aspects of milk technology.

A number of other enzymes also occur in minor quantities, however, relatively little or no definitive information is available concerning their presence, role or significance. Lyso--zyme is one such enzyme. Due to its ability to act as an anti-bacterial factor(s) in bovine milk, it has stimulated the interest of chemists and bacteriologists.

Several investigation concerning food application of lysozyme have been known. It has been shown that lysozyme can cause destabilization of casein micelles in a manner similar to the action of rennin. It hastens the digestion of milk proteins by pepsin. Eggs with a high lysozyme content maintained quality, foam stability, fertility, and hatchability better than eggs

with a low lysozyme content. Lysozyme has also been used for preserving fresh vegetables, fish, meat, fruits, sake and sea foods, and preventing butyric acid blowing in semi-hard cheeses. It has also been shown to be used in treatment of sub-clinical mastitis. Due to its wide applicability, it becomes imperative that its level and properties in milk of different species be properly investigated, so as to enable us to exploit its potential. Several workers have either detected or isolated this enzyme from the milk of donkey, mare, camel, human, rat, cat, rabbit, dog, ilama, pig, rhesus monkey, sheep, cow and guinea pigs.

In India, since buffalo is the major milk producer followed by cow and goat and as there is absence of literature on buffalo milk lysozyme, it was considered desirable to initiate studies on buffalo milk. In the present study, the distribution of lysozyme among the available three species namely, cow, buffalo and goat, and preliminary studies on buffalo milk lysozyme were carried out. An attempt has been made to isolate lysozyme from buffalo milk in a pure form and to study some of its properties.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Lysozyme is the name given to the enzyme which lysis the cell wall of certain bacteria. The Commission on Enzyme Nomenclature of International Union of Biochemistry (IUB) designed lysozyme as E.C.3.2.1.17, systematic name, mucopeptide N-acetylmuramyl hydrolase, recommended trivial name mucopeptide-gluco hydrolase or lysozyme but not muramidase.

Fleming (1922) discovered a substance in nasal mucus and body secretions, which was readily capable of dissolving certain bacteria. Because of its resemblence with enzyme, he named it lysozyme. Earlier it was studied by bacteriologists because of its lytic activity against bacteria. Following successful crystallization of Hen egg-white lysozyme (EWL) by Abraham and Robinson(1937), the enzyme began to attract the attention of protein chemists.

2.1 OCCURENCE

Lysozyme is widely distributed in nature. Goudswaard et al. (1978) detected lysozyme in a number of body fluids e.g. nasal mucus, salvia, exudates from infections, in the extracts from kidney, spleen, liver, lung and lymph. Jolles and Jolles(1967) reported the presence of lysozyme in human tears. It is also present in plants like cabbage, turnip, cauliflower and in high concentration in cartilage. Its presence in certain bacteria has also been reported.

Lysozyme has either been detected or isolated in milk from a number of species - rat, sow, cow, goat, ewe, human, mare, donkey, camel, duck, baboon and guinea pig (Bordet,1928; Rosenthal and Leiberman, 1931; Prickett et al.,1933 and Cattaneo and Vergano, 1948). Lysozyme like particles was also reported in rat mammary tissue by Greenbaum et al.(1960).

2.2 ASSAY METHODS

The Commission on Enzyme of IUB has recommended that lysozyme assay should be based wherever possible on the initial reaction rate measurement. General procedure involves the preparation of cell suspension of heat killed micrococcuslysodeikticus cells in an appropriate buffer, mixing of the enzyme solution and measuring the decrease in optical density (OD) in a spectrophotometer.

Shahani et al.(1962) modified the method of Smoleilis and Hartsell (1949) for egg, to adapt it to milk. The procedure includes preparation of cell suspension in Sorenson 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.

Chandan et al.(1965) modified the above method. They prepared cell suspension in 0.067 M 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 half millilitre of cell suspension and 0.5 ml of 0.3 M sodium chloride solution is mixed with 1 ml of whey. This 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 hrs. The % T change 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 minute was calculated.

Gary et al.(1977) modified the method developed by Katz (1972) for assay of lysozyme activity. The method consists in preparation of 30 mg/ml cell suspension in 0.066 M 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.

2.3 DISTRIBUTION AMONG SPECIES

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. Lysozyme content in four species of cows namely, Holstein, Jersey, Guernsey and Brown Swiss was 11, 5, 15 and 21 µg/100 ml, respectively. Chandan et al.(1968) determined lysozyme content in human, cow, goat, ewe and sow's milk and observed it to be 40000, 13, 25, 10 and zero µg/100 ml, respectively. Chandan et al.(1964) determined lysozyme content of 105 samples of human milk and 12 samples of human colostrum. The lysozyme concentration varied from 3-300 mg/100 ml for milk with an average of 39 mg/100 ml, and from 9-102 mg/100 ml for colostrum, averaging 46 mg/100 ml. Rao and Belvady (1973) observed lower lytic activity in human milk colostrum than milk samples obtained between 1 and 12 months of lactation.

Hankiewicz and Swierczek (1974) determined lysozyme activity in body fluids of adults and children by diffusion on agrose gel. These workers have also reported that average activity of 21 human milk samples (3-4 days post partum) was 65.0 ± 10.2 mg/litre and that in serum 9.8 ± 2.9 mg/litre.

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 ug/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.

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.

2.4 ISOLATION OF MILK LYSOZYME

Many workers attempted to isolate and purify lysozyme from milk of a number of species so as to study its physiolo-gical, nutritional and technological significance.

Parry et al.(1964) purified lysozyme from human milk to 400 times. They also isolated it from bovine milk. The steps included are - preparation of acid whey, adsorption on

Amberlite IRC-50, fractional precipitation with $(NH_4)_2SO_4$ and chromatography on Sephadex G-50, purification was upto the extent of 36,000 folds. Jolles and Jolles(1967) purified lysozyme from human tears, tissue secretions and milk using ion-exchange chromatography on Amberlite and found similar amino acid sequence. 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. Using chromatography and CM-cellulose, 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 rechromatography on Amberlite, but the recovery was poor. Izaka et al.(1971) isolated lysozyme from human placenta with a purification of 1550 folds. 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. The enzyme obtained was homogeneous as confirmed by polyacrylamide gel electrophoresis.

Gary et al.(1977) developed a method to separate lysozyme from hen egg, white, human blood, human and goat 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 and also had good stability and fast flow rate.

2.5 KINETICS AND OTHER STUDIES

Lysozyme is the first enzyme in which it is possible to understand the enzymatic activity on the basis of the three-dimensional fine structure of the molecule.

2.5.1 Molecular weight and structure

Parry et al.(1967) determined molecular weight of lyso-zyme from bovine milk. Their calculated value was 16200 as compared to 16000 obtained from sedimentation equilibrium method. They determined amino acid sequence of carboxymethylated deri-vative and found it to be containing 123 ± 2 amino acid residues. In 1969 the same workers reported molecular weight to be 15000 ± 600, obtained from sedimentation velocity. Similar results were observed by Buss (1969) for baboon milk. These results were different from those reported by Rao and Belvady (1973) which was 13570 for human milk.

Human milk lysozyme contains 5-6 lysine, one histidine, 11-Arg, 18-Asp, 6-threonine, 6-7 serine, 9 glutamine, 11-12 glycine, 6-Cys, 12-Ala, 7-Val, 2-Met, 5-isoleucine, 8-Leu, 5-Tyr, 5-6 Try, 2-Phen, and 18 ± 1 NH_4 (Parry et al., 1967). Whereas Jolles and Jolles (1967) determined primary structure of human milk lysozyme and showed it to be containing 124 ± 3 amino acid residues of which 11-12 Arg, 5-Lysine and Valine as C-terminal. But in 1969 they found it to be containing 129 amino acids.

Kirshenbaum (1977) also determined the amino acid sequence of lysozyme from cow, sheep, pig, mare, kangroo, chimpanze, baboon and guinea-pig milk.

2.5.2 Optimum pH

Lysozyme shows lytic activity in a wide range of pH from 3 to 9. Lysozyme is also stable in lower pH region. Chandan et al.(1965), Parry et al.(1969) reported optimum pH for HML, BML and 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.

2.5.3 Isoelectric point and sedimentation constant

Chandan et al.(1975) determined sedimentation coefficient for BML and HML to be 2.0 S and 1.35 S and isoelectric point to be pH 9.5 and 11.0, respectively.

2.5.4 Effect of temperature

Lysozyme shows lytic activity in a wide range of temperatures. This has been found to be quite heat stable. Shahani et al.(1962) found HML and BML to be stable to LTLT pasteurization. Kisza et al.(1977), Ford et al.(1977) and Evans et al. (1978) also observed similar results. Jolles and Jolles (1961, 1967) heated HML at pH 7.5 for one min at 100°C and observed 30% loss of activity. Jauregui (1975) reported that mare milk lysozyme was stable at acidic and neutral pH, but labile at alkaline pH. Kimura et al.(1970) showed HML to be less heat stable as compared to EWL. Eitenmiller et al.(1976)

showed BML to be more stable than HML at pH 4.0, but more labile at pH 7.0 and 9.0. Gary and Kroger (1978) found BML to be sensitive to heat.

Ruegg et al.(1977) studied thermal transition by differential scanning colorimeter (DSC) and found that conformational changes started at temperature as low as 45°C and it was concluded that thermal stability observed after heat treatment of milk should be due to renaturation and not due to high temperature.

Kisza et al.(1977) reported that there was no loss of lytic activity when milk was stored at 4°C for $\leq$ 72 hrs, but lytic activity decreased when stored at 18°C. Similar results were observed by Shahani et al.(1962) for bovine milk.

2.5.5 Denaturation by organic and inorganic compounds

Lysozyme is denatured by urea, guanidine-hydrochloride, LiCl and $LiClO_4$. Guanidine is very strong denaturant for lysozyme. Hamaguchi and Kurono (1963) studied denaturation by guanidine-hydrochloride by measuring optical rotatory properties, viscosity and U.V. absorption, and demonstrated that denaturation followed first order. Greene and Pace (1974) reported that lysozyme is very resistant to urea. It is denatured only to a very small extent, even in the presence of 9 or 10 M urea.

2.5.6 Digestibility of lysozyme by protease enzymes

Fujimaki et al.(1973) roasted lysozyme at 100-300°C and

studied changes occuring and observed that decomposition of amino acids started at 150-180°C. Tryptophan, sulphur containing basic and β-hydroxy amino acid decomposed easily as compared to acidic, aormatic and alkyl amino acids. There was cleavage of peptide bonds by protein bound water. Hayase et al.(1975) studied in vitro digestion of roasted lysozyme by pancreatin, trypsin, papain and protease. There was complete digestion when lysozyme was roasted at 180°C for 20 min, but the residue became water insoluble when roasted at $\geq$ 210°C for 20 min. It was considered to be partially based upon the decomposition and recemization of amino acid residues in lysozyme.

2.5.7 Role of different amino acids in the lytic activity

It has been reported that Tryptophan-62 is involved in the lytic activity of lysozyme as reported by a number of workers. Imito et al.(1975) observed higher Vmax value for Try-62 oxidized lysozyme than for native lysozyme, when the activity was studied towards glycol-chitin and bacterial cell wall. It was concluded that Try-62 was necessary for the lytic activity, so higher Vmax value was obtained. Friend et al. (1975) obtained similar results. Chandan et al.(1975) selectively modified lysine, tryptophan and tyrosine in BML and HML, to study the role of these amino acids in the lytic activity of lysozyme. They observed that acetylation of lysine residues reduced the lytic activity, which contributed to the total basicity and had no direct function in enzyme activity.

Oxidation of tryptophan with N-bromosuccinamide and inhibition with N-acetyl glucosamine and histidine indicated that tryptophan was involved in the substrate binding site of HML, but not of BML.

2.5.8 Effect of lysozyme on bacterial growth

Lysozyme behaves as an antibiotic. It causes lysis of certain bacteria and inhibits the growth of microorganisms. Several gram positive and gram negative bacteria are susceptible to purified milk lysozyme (Shahani, 1970).

Wolin and Kosikowski (1955) measured antibacterial properties of cow's milk using lyophilized milk tablets in place of saturated discs. This produced two inhibition zones, primary and secondary. Primary zone varied seasonally to larger extent as compared to secondary zone which caused disappearance of bacterial colonies.

Addition of more than 0.012% of lysozyme to milk, decreased the total as well as coliform bacterial counts (Ferlazzo et al.,1960). When NaCl is added to BML and HML, it accelerated lytic activity of both live and U.V. killed cells of Micrococcus-lysodeikticus and Sarcinalutea, but impaired the lysis of other bacterias sensitive to it. However, in case of live and actively growing bacteria, addition of either NaCl or EDTA improved lysis by milk lysozyme and EWL (Vakil et al.,1969).

Bovine milk lysozyme does not show lytic activity in the absence of salt e.g. NaCl, but HML and EWL do, Parry et al. (1965).

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2.5.9 Factors affecting lysozyme level in milk

Lysozyme content in milk is affected by a number of factors. It varied from species to species, breed to breed, seasonally and for an animal from one milking to another and from different quarters (Shahani et al.,1962). Lactation period upto 13 weeks has no effect on lysozyme concentration in bovine milk. However, Kospakov(1975) observed that lysozyme content increased with advancement of lactation in camel's milk. Todorov(1972), Gotze et al.(1974), Schollenberger et al.(1976) and Goudswaard et al.(1978) found that lysozyme content in milk increased during udder infection.

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

Lysozyme when added to cheese milk before the incorporation of rennet, prevents, late blowing of cheese. Pulay and Krasz (1966) added 0.2% (V/V) EWL or 0.001% (W/W) egg-white along with EWL to the pasteurized milk. They observed that egg-white alongwith EWL 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) and Wasserfall et al.(1976).

Wasserfall et al.(1976) also showed that addition of dried egg-white was ineffective against late blowing of cheese and lysozyme had no effect on the process of manufacture of cheese and it can be used in place of nitrate in preventing late blowing of cheese.

2.5.11 Comparison of lysozyme and α-lactalbumin

α-lactalbumin has no separate activity in lactose synthetase assay, but acts as a modifier protein. Amino acid sequence of lysozyme from bovine milk has been shown to be very much similar to α-lactalbumin by Brew et al.(1967).

Krigbaum and Kugler (1969) showed that lysozyme was prolate whereas α-lactalbumin was oblate, so it is concluded that these have different molecular conformation in solution and lysozyme undergoes very little changes in solution. Both these proteins have approximately equal molecular weights and the effective molecular weight of α-lactalbumin, in solution is larger than that for lysozyme which is due to difference in conjugation and hydration.

Sharma and Bigelow (1974) observed that both α-lactalbumin and lysozyme respond similarly to denaturants, which indicates that these may have similar three dimensional structure. Hill et al.(1974) also reported α-lactalbumin and lysozyme to be related to each other with respect to their evolution and three-dimensional structure.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Collection of Milk

Pooled raw buffalo milk samples and individual samples of buffalo, cow and goat milk were collected from the herd maintained by the Institute. Collection was done twice, in the morning and in the evening and milk assayed for enzymatic activity.

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-white 3 x crystallized, dialyzed and lyophilized, was obtained from Sigma Chemical Co., U.S.A.

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

(d) CM-Cellulose:- It was obtained from Sigma Chemical Company. Equilibrated in phosphate buffer pH 6.2 for overnight before use.

(e) Sephadex G-50:- Sephadex G-50 used was a product of Pharmacia Uppsala, Sweden, and swollen in 0.1 M NaCl -0.1 M 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 standard curve.

3.4 Enzyme unit

One unit of enzyme activity, used in the purification procedure, was defined as decrease of 0.001 O.D. in Coleman 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.

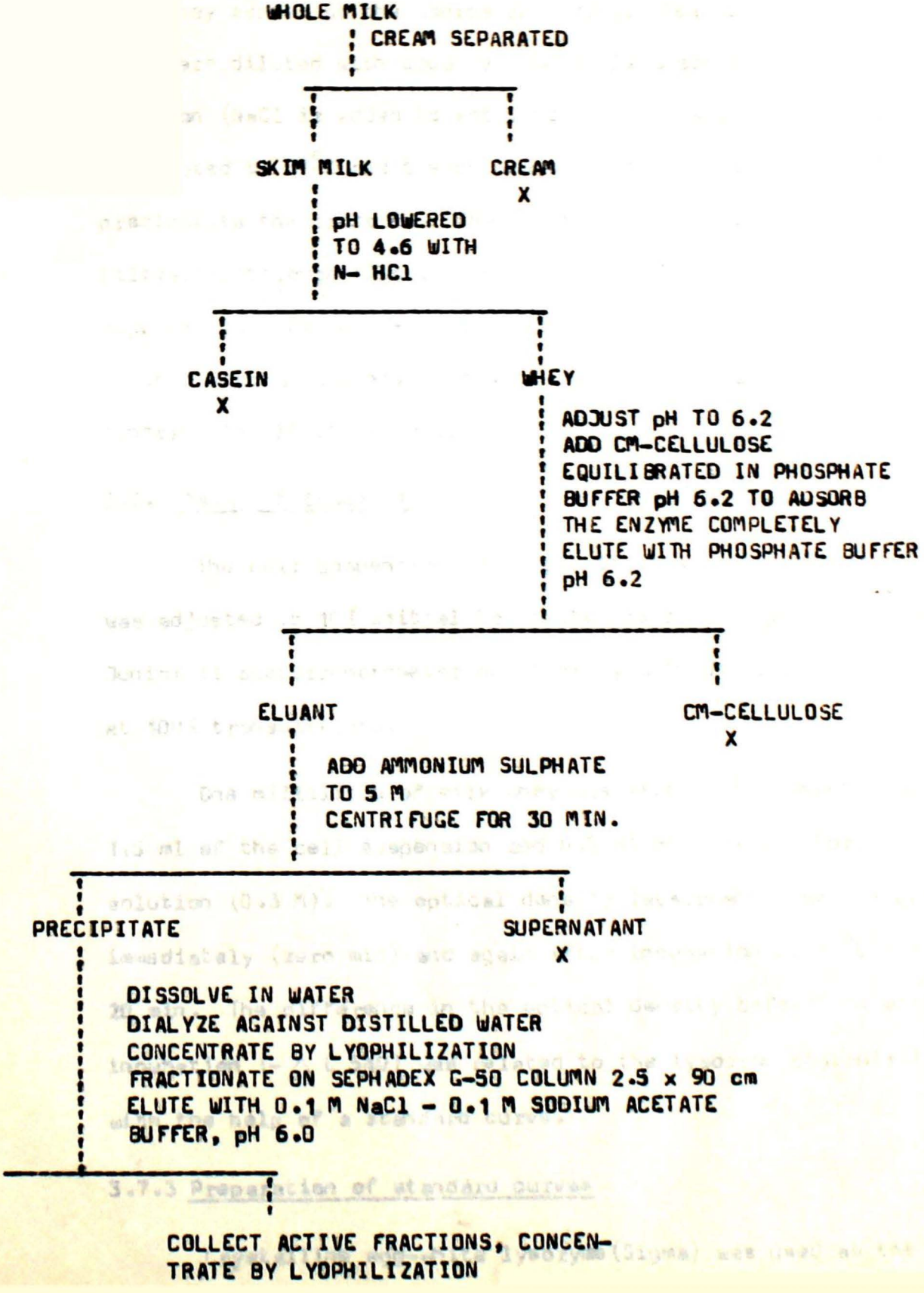
3.6 Purification of enzyme

The purification of enzyme was done by the method of Chandan et al.(1965) using CM-cellulose instead of Amberlite with some modification. The details are given in flow sheet on page 18.

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).

FLOW SHEET FOR PURIFICATION PROCEDURE



3.7.1 Preparation of whey for the assay

For spectrophotometric assay method of lysozyme, clear milk whey serves as the source of enzyme. Ten millilitres of milk were diluted with equal volume of 0.5% sodium chloride solution (NaCl is added to activate the enzyme). The mixture was heated to 37°C and the pH was adjusted to 4.6 with 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 IN-sodium hydroxide. The resulting solution is then used as the enzyme source. The total volume is kept constant in all the samples.

3.7.2 Assay of lysozyme

The cell suspension (100 mg%) in phosphate buffer (pH 6.2) was adjusted to 10% initial transmittance at 540 mμ in a Coleman Junior II spectrophotometer Model 6/20, 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 0.3 ml of sodium chloride solution (0.3 M). 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 ($-\triangle$ E 540) 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

FIG. I STANDARD CURVE FOR LYSOZYME CONCENTRATION IN MILK

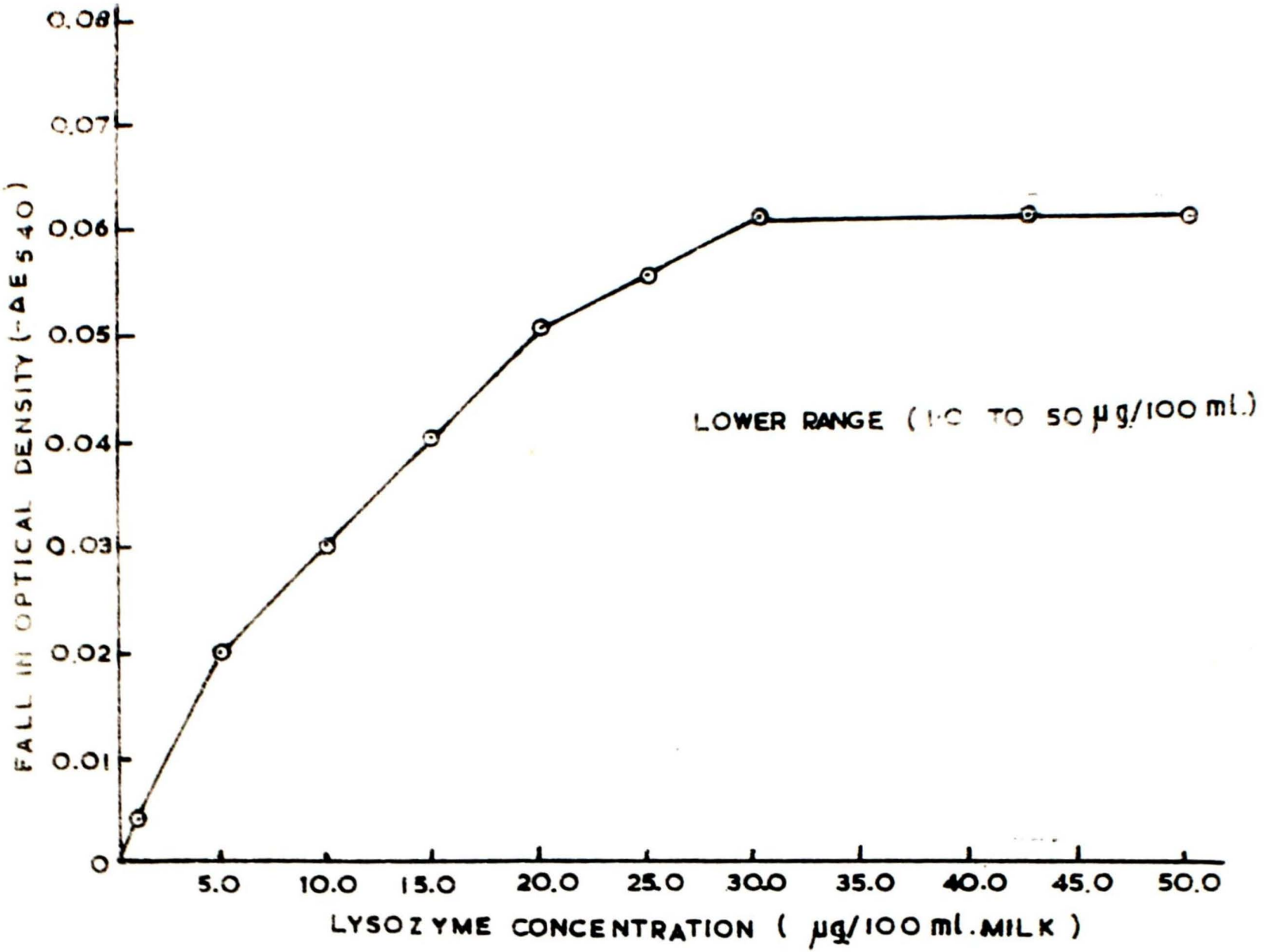
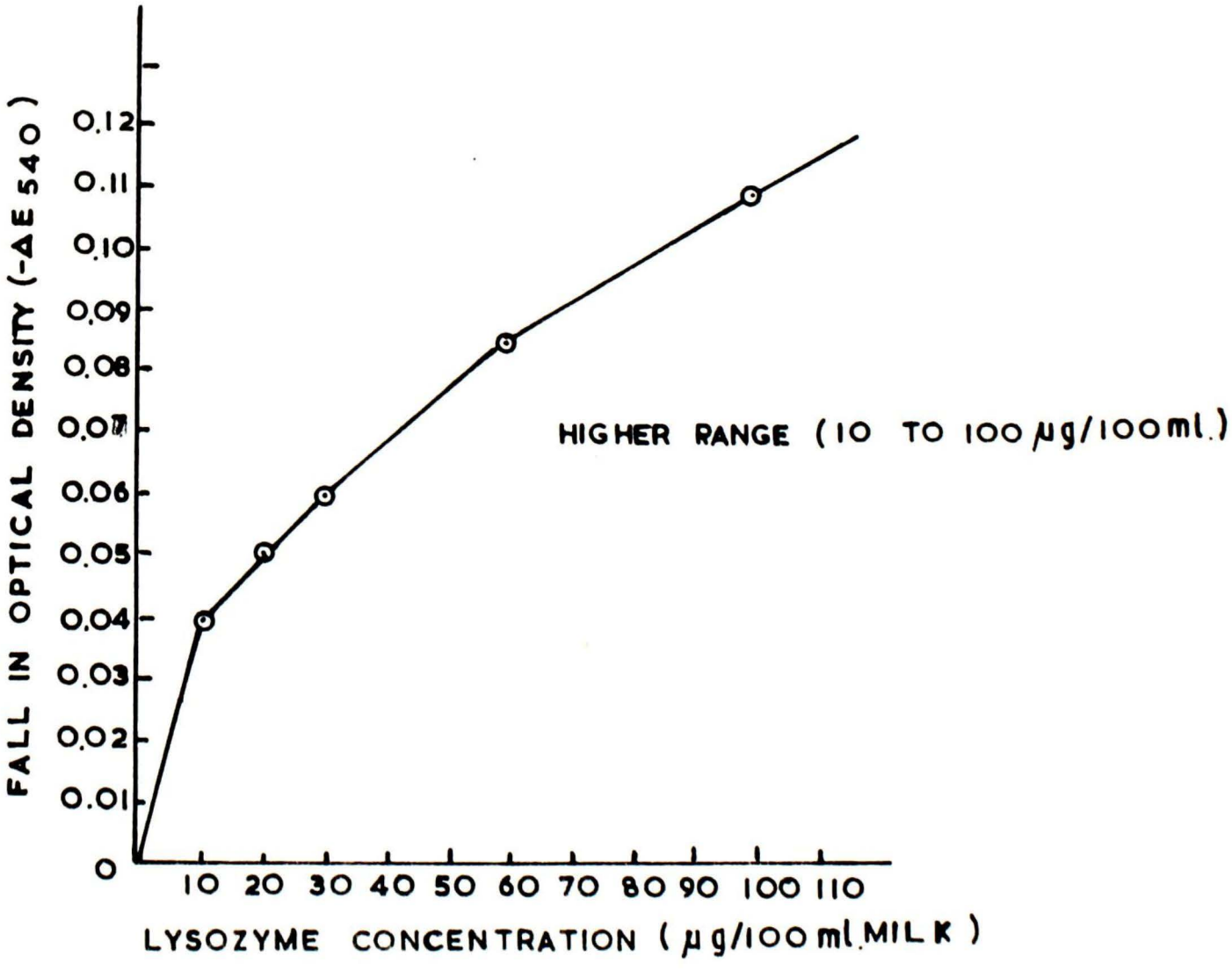


FIG. II STANDARD CURVE FOR LYSOZYME CONCENTION IN MILK.



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 (Figs.I,II) whole milk was used in which the possible inherent lysozyme activity was destroyed by boiling. Varying concentrations of lysozyme (1 to 50 µg, and 10 to 100 µg/100 ml) were added to this and a clear whey was prepared for use as the enzyme source. The cell suspension (100 mg%) in phosphate buffer pH 6.2 was taken and adjusted to 10% initial transmittance at 540 mu with distilled water set at 100% transmittance. The curves were prepared by plotting $-\Delta E$ 540 values against the concentration of lysozyme.

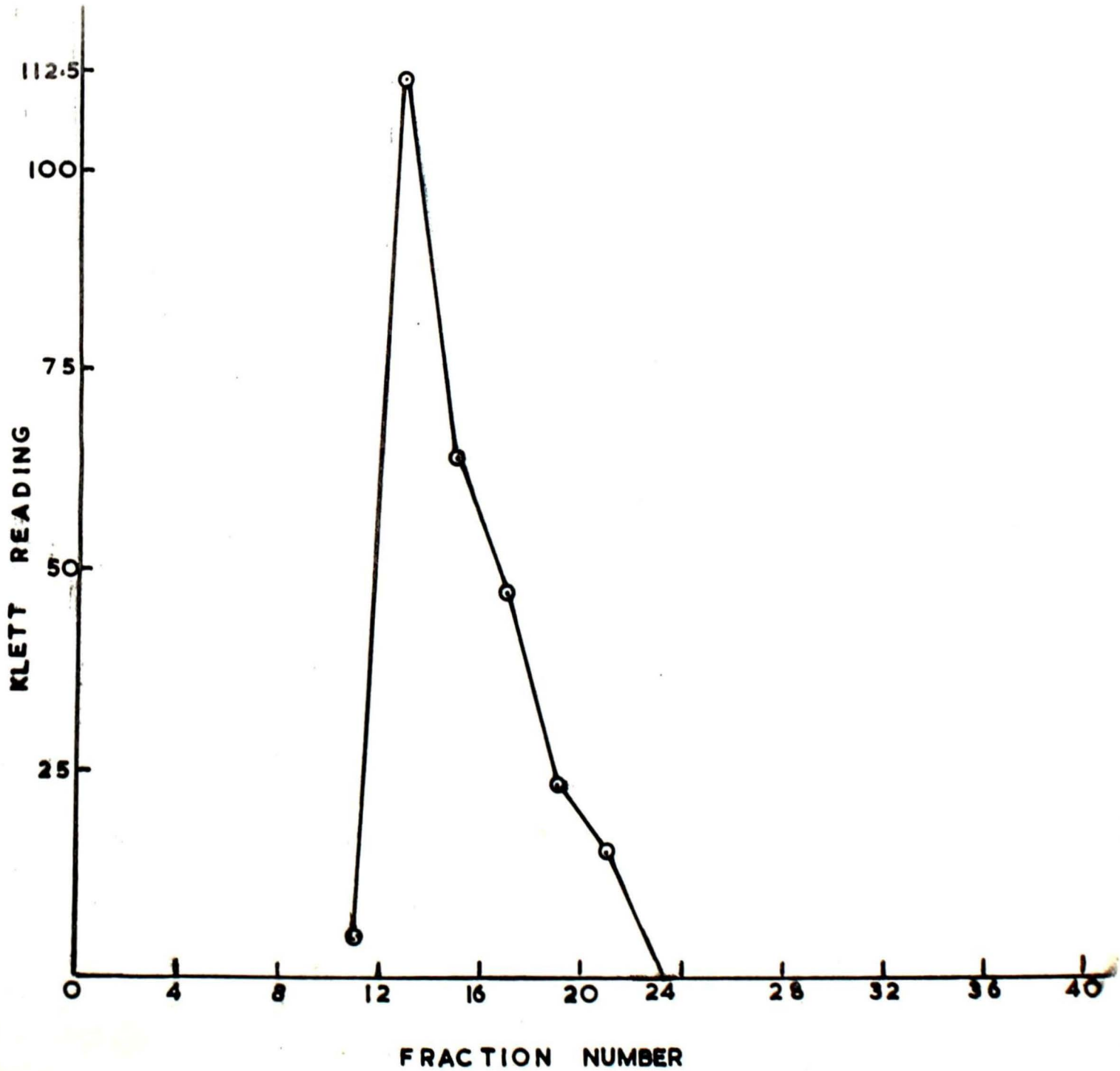
3.8 Gel filtration

(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 (0.1 M NaCl-0.1 M sodium acetate buffer pH 6.0) overnight and poured into a glass column 2.5 x 90 cm. The gel was allowed to settle for sometime and the column was flushed with the eluting buffer for 2-3 hours prior to the application of sample.

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

(c) Filtration procedure:- Before applying the sample, the column was allowed to drain until the buffer surface came in level with the gel surface. The samples dissolved in buffer were

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



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. Ten ml of eluant was collected in each tube.

(d) Analysis of fractions:- The protein was determined in each ten ml fraction and Klett reading was plotted on graph paper against the fraction number. 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 dissolving 18 gm cyanogum in 100 ml of Tris glycine buffer (pH 9.5) which was prepared by dissolving 6 gm of Tris buffer and 28.8 gm of glycine per litre of distilled water (Gary et al.,1977).

To 13.5 ml of this solution, 15 ml of Tris-glycine buffer (pH 9.5) was mixed. The base NN'N'N'-Tetramethylene diamine (TEMED) 0.045 ml was added with stirring, as a catalyst accelera-tor. The solution was then filtered and polymerization was initiated in it by addition of 1.5 ml of freshly prepared ammo-nium per-sulphate (15 mg/ml) (Weber et al.,1972). The material was then transferred to gel tube. The tubes were held perpendi-cularly during their filling and polymerization 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 polymerization 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 suction effect.

(b) Electrophoretic assembly:- The tubes were fixed vertically between upper and lower electrode reservoir which had 12 holes each fitted with a rubber gammet and an electrode fitted in the centre of vessel. Each tube was inserted into one of the holes in upper electrode reservoir. Any unused hole was sealed with rubber stopper. The upper and the lower chambers were filled with same buffer (Tris-Glycine pH 9.5).

(c) Preparation and application of samples:- Purified lysozyme and standard lysozyme (2 mg/ml) were dissolved in buffer solution (40 gm sucrose and 21 g urea added to 100 ml of 2% Na_2CO_3 solution in 0.1 N - NaOH). One hundred to one hundred fifty µl of protein solution (200-300 µg) was layered directly on the top of each gel 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 acrylamide gel rod:- After the run, the buffer solution in the upper tank was discarded and gel tubes systematically detached. The gel rod was then ejected from its glass tube with the help of fine needle. The gel rods were

then put into staining bath containing 250 mg Amido black in 100 ml of methanol-acetic acid mixture (10:1).

The staining was completed within one hour and subse--quently washed with water. The back-ground staining was removed by washing the gel rods with methanol, acetic acid: water (50:75:850) solution with few changes. The stained protein band became distinct with additional washings.

(e) SDS-polyacrylamide gel electrophoresis:- The SDS-polyacry--lamide gel electrophoresis was performed by adding sodium lauryl sulphate (SDS) to the polyacrylamide gel, the gel buffer (Tris-glycine pH 9.5) contained 2% sodium lauryl sulphate and 1% 2-mercaptoethanol.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Lysozyme content of buffalo, cow and goat milk

Fresh milk samples were collected from the Institute herd and assayed for lysozyme within an hour of milking. The lysozyme activity of milk of different species are presented in Table 1. On an average it was observed that cow milk showed maximum activity followed by buffalo and goat. However, Chandan et al. (1965) reported that bovine milk contained very low concentration of lysozyme (13 µg/100 ml) while human milk showed 39 mg/100 ml.

Table 1. Lysozyme content of buffalo, cow and goat milk.

Lysozyme concentration (µg/100 ml)

Species	No. of animals	Range	Average	Remarks
Buffalo	32	0 - 100.00	15.21	Two samples with zero lysozyme content, average of 30 samples shown.
Cow	45	1.25- 209.09	18.09	-
Goat	24	0 - 15.00	7.99	One sample with zero lysozyme content, average of 23 samples shown.

In an earlier communication, Shahani et al.(1962), on the basis of 67 cows of four different breeds reported variation between 0 and 260 µg/100 ml. The average lysozyme content of

Brown Swiss, Guernsey, Holstein and Jersey was 21, 15, 11 and 5 µg/100 ml, respectively. It was, therefore, observed that lysozyme content varies from breed to breed. Further they also reported the variations in lysozyme activity from milking to milking.

In present work, on an average it was observed that Murrah buffaloes showed 15.21 µg/100 ml, while for cow - Brown Swiss (21.875), Holstein (57.32), Tharparkar (3.125), Sahiwal (31.33), Karan Swiss (5.94) and Jersey (3.17) µg/100 ml.

For Goat, Alpine showed 35.62 µg/100 ml while cross-bred animals showed 7.37 µg/100 ml. However, these results are shown species-wise in Table 1.

4.2 Distribution of lysozyme in buffalo milk from different quarters

In order to determine the distribution of lysozyme content in milk from different quarter, samples of five buffaloes were collected and analysed for lysozyme content. The results presented in Table 2 reveal that lysozyme activity in milk from different quarters followed no definite pattern of distribution and showed a random distribution of lysozyme in different quarters of the udder. Similar results were obtained for cow milk by Shahani et al.(1962).

Table 2. Distribution of lysozyme in buffalo milk from different quarters

Lysozyme concentration (µg/100 ml)

Animal No.	Right-rear	Right-front	Left-rear	Left-front	Average
1	3.70	5.00	5.00	17.50	7.80
2	3.70	7.50	7.50	10.75	7.36
3	10.00	3.75	5.00	5.00	5.93
4	5.00	5.00	5.00	6.25	5.31
5	5.00	5.00	2.50	2.30	3.75

4.3 Effect of heat treatment on buffalo milk lysozyme

Studies were conducted, to determine the stability of buffalo milk lysozyme, at two different conventional pasteuri-zation temperatures. The results are reported in Table 3. An average of 58.34% destruction was observed in case of HTST pasteurization, while for LTLT, 71.12% destruction was observed. When milk was boiled for 10 min, 94.5% enzyme activity was destroyed. From the data it can be observed that enzyme is quite heat stable. However, heating for a longer time destroyed the lysozyme activity as indicated by LTLT pasteurization.

Shahani et al.(1962) also showed a destruction of 59.1% HTST pasteurization for cow milk which is similar to our value of 58.34% for buffalo milk as shown in Table 3. However, the percent destruction in LTLT pasteurization was more than double for buffalo milk when compared to cow milk as reported by Shahani et al. (1962) and Kuncewicz & Kisza (1976) which is about 31%.

Table 3. Effect of heat treatments on buffalo milk lysozyme

Lysozyme concentration (µg/100 ml)

Sample Number	Raw milk	Boiled milk	HTST 74°C for 16sec.	LTLT 63°C for 30 min
1	5.00	0	3.75	3.00
2	10.00	1.25	3.75	2.50
3	7.50	0	3.75	2.50
4	12.50	0	3.75	2.50
5	10.00	1.25	3.75	2.50
Average	9.00	0.50	3.75	2.60
Destruction %	0	94.50	58.34	71.12

4.4 Variation of lysozyme content in buffalo milk with respect to lactation period

An attempt was made to determine the effect of stage of lactation on lysozyme activity of milk. Four animals in each group representing early, middle and late lactation were selected. The lysozyme activity of milk from these animals was determined for three consequtive days. The values obtained are reported in Table 4. On an average lower values of lysozyme were obtained in early lactation period as compared to middle and late lactation.

Table 4

Variation of lysozyme content in buffalo milk with respect to lactation period

Lysozyme concentration (µg/100 ml)

Lactation period	Time in days	Animal Number	Lysozyme content of samples taken	Average of samples	Overall range
Early	31	378	3.75,2.50,1.25	2.50	1.25 - 3.75
	34	379	2.50,2.50,3.75	2.91	
	44	380	2.50,2.50,2.50	2.50	
	61	377	2.50,2.50,3.75	2.91	
Middle	105	372	3.75,7.50,3.25	4.58	1.25 - 40.00
	105	358	5.00,5.00,6.00	5.33	
	145	351	5.00,35.00,40.00	26.60	
	186	680	1.25,5.00,7.50	4.58	
Late	273	118	5.00,6.50,2.50	4.66	1.25 - 10.00
	273	178	3.75,8.25,1.25	4.41	
	274	290	7.50,5.00,2.50	5.00	
	314	579	4.00,10,00,2.50	5.50	

Another set of experiments on three animals of middle lactation showed that the lysozyme content increases during late lactation (Table 5).

Four animals of early lactation group were selected and lysozyme content of milk was determined. It was observed that in all the four cases lysozyme content increased with the advance in stage of lactation (Table 6).

Shahani et al.(1962) also observed that lactation stage had no effect on lysozyme content of bovine milk, but Rao and Belvady(1973) observed an increase in lysozyme activity with the duration of lactation in human milk.

Gotze et al.(1974) observed an increase of milk lysozyme from second to sixth month of lactation in cow milk. Similar results were also observed by Kospokov (1975) with Camel's milk.

4.5 Effect of storage at various temperatures on lysozyme stability

In order to determine the effect of storage of milk on lysozyme activity, three different temperatures - 6°C, 21°C and 37°C were selected. The milk samples were stored at these temperatures and lysozyme activity was determined after 14 hrs, 38 hrs and 48 hrs. The results are presented in Table 7.

A perusal of Table 7 shows that when milk was stored at 37°C, 76-100% destruction was observed after 14 hrs, 38 hrs

Table 5

Variation of lysozyme content in buffalo milk with respect to middle and late lactation

Lysozyme concentration (µg/100 ml)

Animal Number	Middle Lactation				Late Lactation				
	1	2	3	Average	1	2	3	4	Average
372	3.75	7.50	2.5	4.58	15.00	30.00	25.00	30.00	25.00
351	5.0	35.0	40.0	26.60	300.0	309.09	263.60	286.35	289.76
358	5.0	5.0	6.0	5.33	110.00	92.00	83.00	83.00	92.00
680	1.25	5.0	7.5	4.58	-	-	-	-	-

Table 6

Variation of lysozyme content in buffalo milk with respect to lactation

Lysozyme concentration (µg/100 ml)

Animal No.	EARLY LACTATION				MIDDLE LACTATION					LATE LACTATION				
	1	2	3	Average	1	2	3	4	Average	1	2	3	4	Averag
377	2.50	2.50	3.75	2.75	5.00	5.00	5.00	5.50	5.12	10.0	9.50	10.00	9.75	9.82
378	3.75	2.50	1.25	2.50	7.50	6.00	7.50	8.00	7.25	7.50	8.00	8.00	8.25	7.93
379	2.50	2.50	3.75	2.75	10.00	11.25	8.75	10.00	10.00	12.50	12.75	12.50	12.25	12.50
380	2.50	2.50	2.50	2.50	12.50	10.00	10.75	10.25	10.87	15.00	14.75	14.50	15.25	14.87

Table 7

Effect of storage at various temperature and time on lysozyme content of buffalo milk

Lysozyme concentration (µg/100 ml)

Sample Number	Lysozyme concentration at zero hour	14 hours			38 hours			48 hours		
		6°C	21°C	37°C	6°C	21°C	37°C	6°C	21°C	37°C
1	7.50	6.00	5.00	0	6.00	3.75	0	1.25	0	0
2	6.00	5.00	4.50	1.25	6.00	3.75	0.50	2.50	0	0
3	45.00	40.00	42.00	52.00	30.00	92.00	0.25	30.00	0	0
4	40.00	40.00	30.00	3.50	5.00	1.25	0.50	8.75	16.00	0
5	53.00	50.00	28.00	1.25	40.0	67.0	0	35.00	0	1.25
6	7.50	6.00	3.75	0	1.25	0	0	2.50	0	0
		PERCENTAGE DESTRUCTION								
		20.00	33.34	100.00	20.00	50.00	100.00	83.34	100.00	100.00
		16.67	25.00	79.17	0	37.50	91.67	58.34	100.00	100.00
		11.12	6.70	–	33.33	–	99.45	33.34	100.00	100.00
		0	25.00	91.25	87.50	68.88	98.75	78.13	–	100.00
		5.66	47.17	76.30	24.53	–	100.00	33.96	100.00	97.64
		20.0	50.00	100.00	83.33	100.00	100.00	66.67	100.00	100.00

and 48 hrs. With the increase of time, percent destruction increases at 21°C, showing no activity after 48 hrs of storage. On storage of samples in refrigerator (6°C) conflicting results were obtained between different samples on the percent destruction of lysozyme. However, upto 20% destruction of enzyme activity was observed at this temperature between various samples upto 14 hrs. This is different from that reported by Kuncewicz and Kisza (1976) that there was no loss of lytic activity of human milk when stored at 4°C for ≤ 72 hrs. On further storing the buffalo milk samples, activity declined by 87%. In general, it was concluded that higher the storage temperature, the faster was the inactivation and also longer the storage time, the greater the loss of enzymatic activity.

4.6 Purification of lysozyme from buffalo milk

Lysozyme was purified by the procedure adapted by Chandan et al.(1965) with some modifications. CM-cellulose was used in place of Amberlite IRC-50, as an ion-exchanger. The specific activity and purification of lysozyme at each step are given in Table 8. With progressive stages of purification, the enzyme preparation had a higher specific activity. The enzyme obtained after chromatography and concentration by lyophilization, showed a range of 5.4 to 142 fold purification when compared with the specific activity of whey (not shown in table).

Table 8

Summary of purification procedure (based on an average of ten trials)

No.	Purification step	Volume (ml)	Units/mg protein	mg protein/ml	Total protein	Total units	Specific activity (units/mg protein)	Fold Purification
1.	Whole milk	4000	-	32.28	-	-	-	-
2.	Skim milk	3600	-	30.10	-	-	-	-
3.	Whey	3120	31.60	3.60	11232.00	98592.0	8.77	-
4.	CMC-Eluant	300	54.17	1.61	483.0	16251.0	33.64	3.83
5.	Ammonium sulphate (5M) precipitate	30	60.00	0.88	26.40	1800.0	68.18	7.77
6.	Sephadex G-50-Eluant-lyophilized fraction	5	45.00	0.122	0.610	225.0	368.85	42.05

Whey was taken as the starting material because all of the enzyme activity is present in it. The extent of purification will be more if it is taken on the basis of average milk specific activity. An average of 42 fold purification could be achieved, however, in one trial 142 fold purification could be achieved.

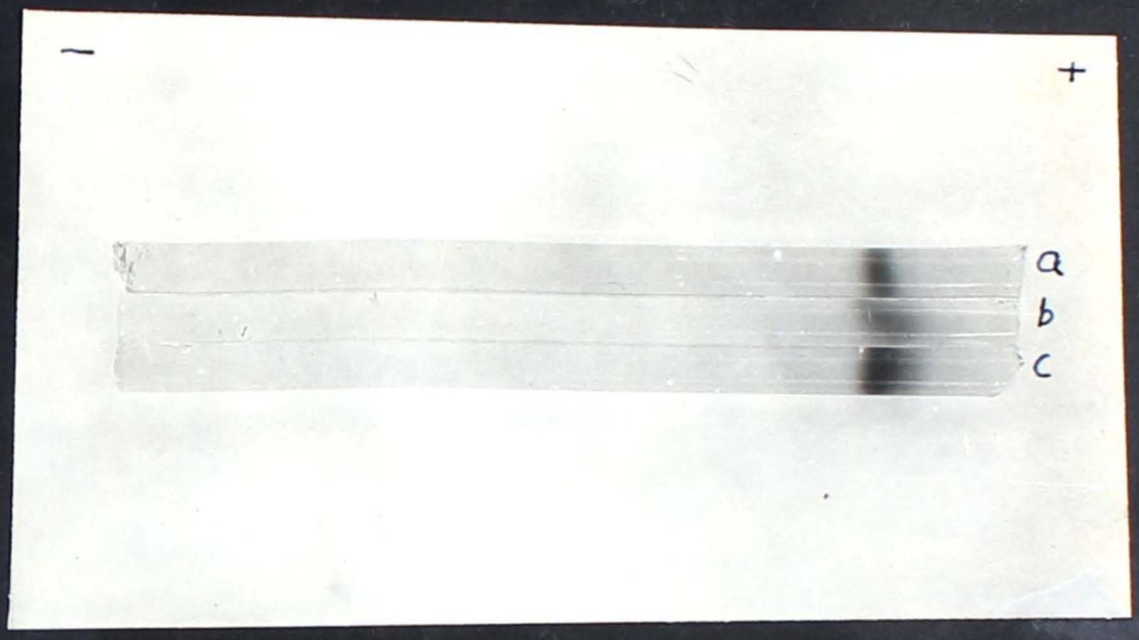
The recovery of lysozyme was poor. Lesser purification and poor recovery can be attributed to the use of CM-cellulose in place of Amberlite IRC-50 as an ion-exchanger. This may be either due to the enzyme not being adsorbed by CM-cellulose or not being eluted completely. Other factors which might have contributed to lesser purification fold and poor recovery includes amount of starting material, whole or skim milk, initial activity of lyso--zyme and the experimental conditions.

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 4 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.

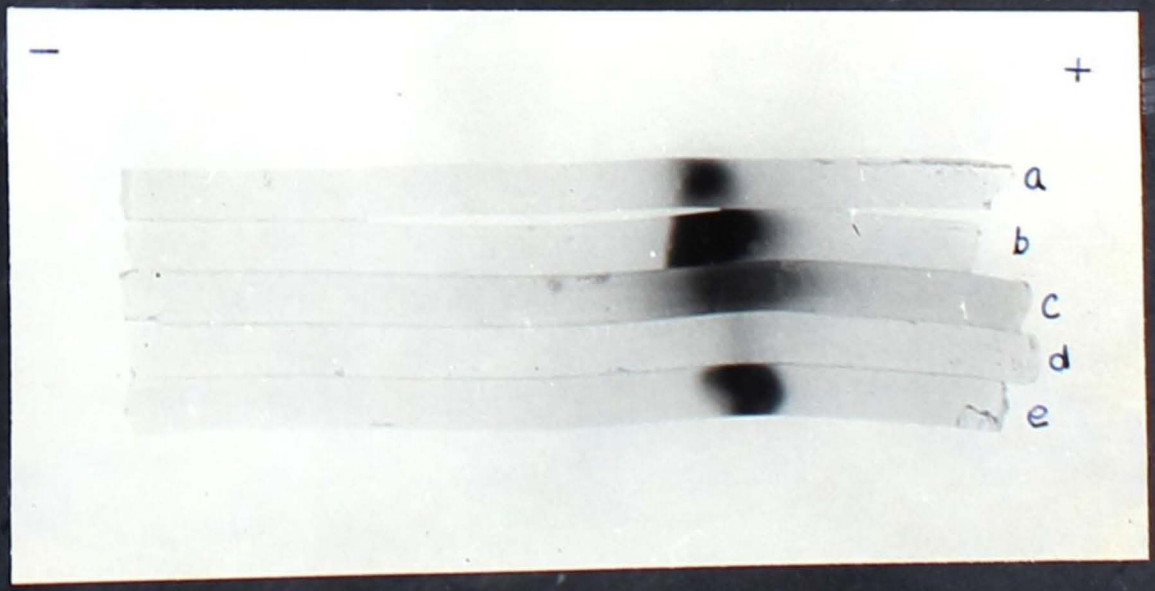
4.7 Electrophoresis of purified enzyme

Samples of isolated buffalo milk lysozyme and egg-white lysozyme as standard were subjected to polyacrylamide dis: gel-

Fig IV Polyacrylamide disc gel electrophoresis of Lysozyme.



(A) In the presence of Sodium lauryl sulphate
(a) Standard (EWL) (b) 142 Fold purified (BML)
(c) 18 Fold purified (BML)



(B) In the absence of sodium lauryl sulphate
(a) Standard (EWL) (b) 142 Fold purified
(c) 18 Fold purified (d) 16 Fold purified
(BML), (e) 42 Fold purified (BML)

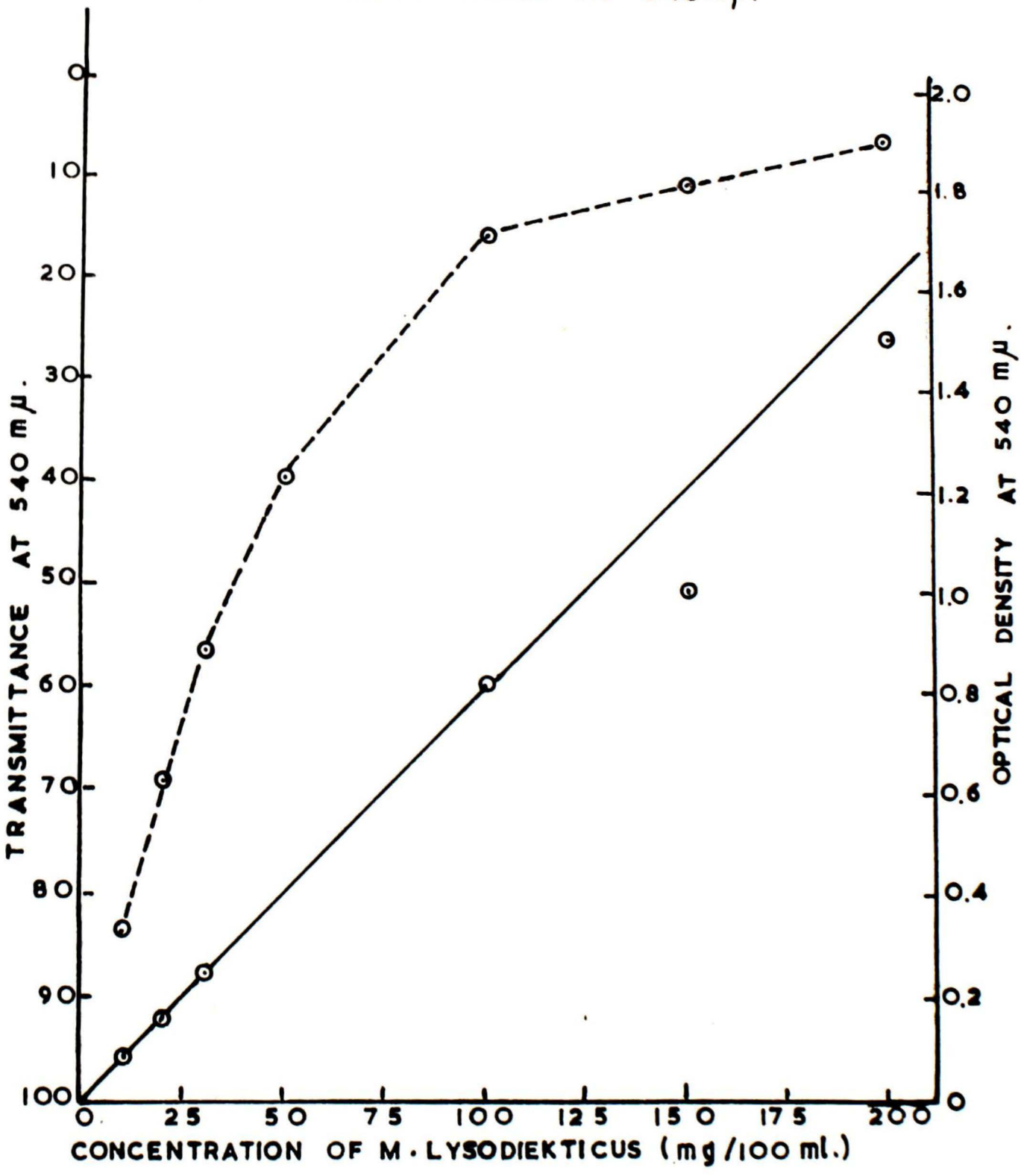
electrophoresis using Tris-Glycine buffer (pH 9.5). Fig.IV presents the photograph, for polyacrylamide disc electrophoresis pattern, of lysozyme purified to different folds (142,18,16 and 42). The presence of single zone at pH 9.5 by enzyme indicates the homogeneity of enzyme. It was further observed that the mobility of egg-white lysozyme run as a control compares well with the different samples. However, three of the samples(142,18 and 42 fold purified) showed denser band as compared to 16 fold purified. This may be due to the fact that amount of protein applied is more in these cases.

When the sample purified to 142 fold and 18 fold were applied at pH 9.5 using SDS-polyacrylamide gel electrophoresis along with egg-white lysozyme only one band was observed. The presence of one band in SDS-electrophoresis might indicate towards a lesser possibility of monomers of different molecular weight. However, no such report is observed in literature and it needs further experimentation. Migration of both proteins (egg-white and buffalo milk lysozyme) as a single band towards the cathode indicate them to be basic proteins. It was further observed that mobility of these protein with SDS is slightly faster than without SDS.

4.8 Kinetic studies of milk lysozyme

An enzyme having 42 fold purification (2.0 mg/ml) was used for kinetic studies.

FIG. V PERCENT TRANSMITTANCE AND ABSORBANCE AT VARIOUS CONCENTRATIONS OF MICROCOCCUS LYSODIEKTICUS CELLS AT 540mμ.



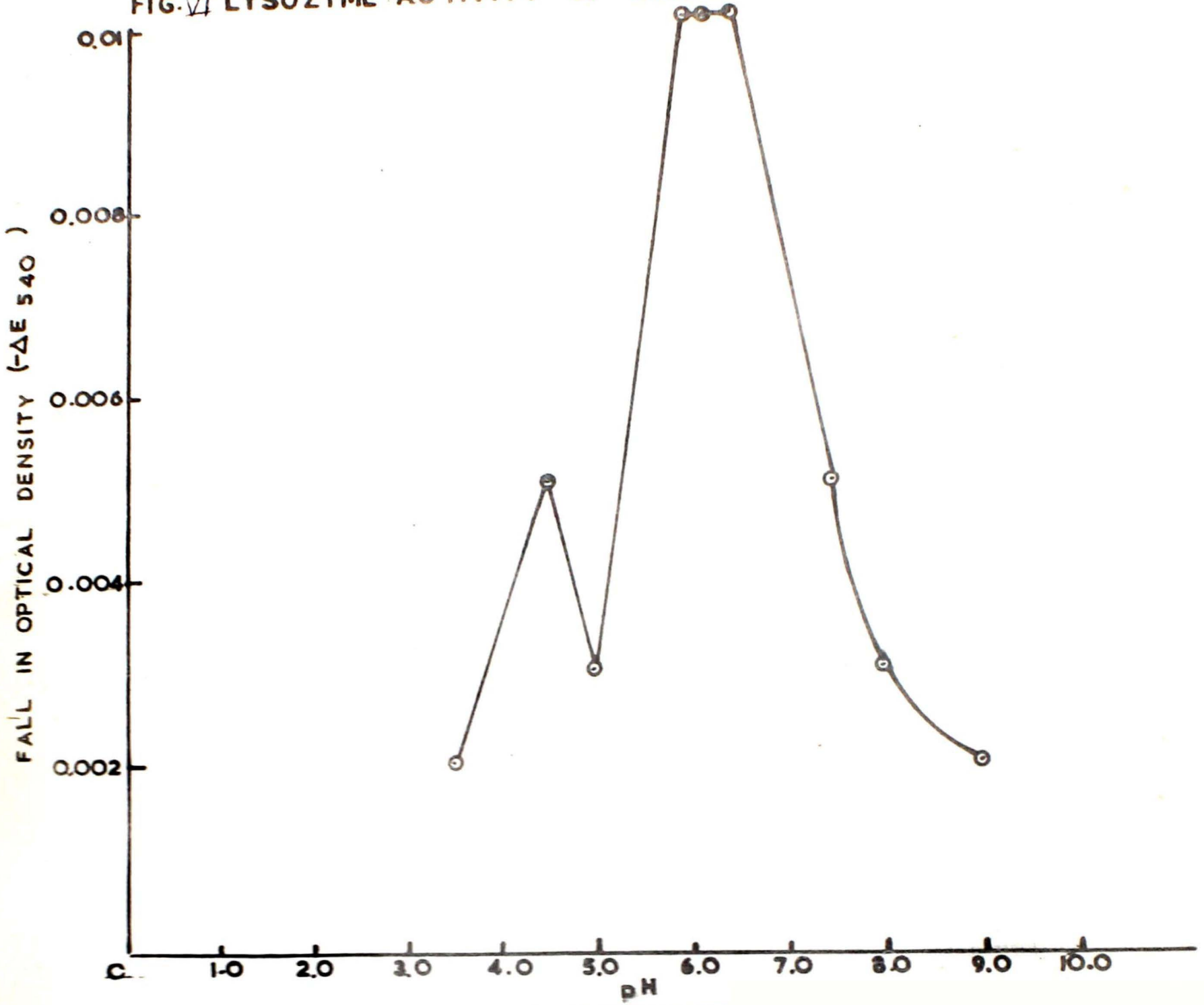
density between O.D. at zero minute and after 20 minutes is compared on a standard curve previously drawn, which is, however, not a straight line (Fig.I and II). A straight line can only be obtained between Δ %T per min and enzyme concentration in µg or between absorbance at various enzyme concentration and not if fall in O.D. ($-\Delta$ E 540) is taken as the criteria of enzyme estimation.

The determination of the dissolution of bacterial cells by lysozyme is a poor representation of true enzymatic reaction, due to the complex system involved and, therefore, its application to the study of kinetics should be observed with caution. This limitation cannot be overcome until a method can be deviced to measure the appearance of end products or disappearance of substrate, employing a pure well defined substrate. Due to this limitation, turbidimetric lysozyme assay do offer a rapid quanti--tative method which is readily applicable, to assay the enzyme from other sources also. However, it is important to use lyso--zyme concentration ranging from 0.1 µg to 10 µg and measurement of the initial reaction velocities.

4.8.2 Effect of pH on lysozyme activity

Enzyme preparation was assayed over the pH range of 3 to 9. The effect of pH on lysozyme activity is shown in Fig. VI. A small minor peak and one broad peak (pH 6.0-6.5) is obtained which revealed an optimum pH between 6.0 and 6.5. The activity declined above pH 6.5. Chandan et al.(1965) had also observed

FIG. VI LYSOZYME ACTIVITY OF BUFFALO MILK AT DIFFERENT p^H LEVELS.



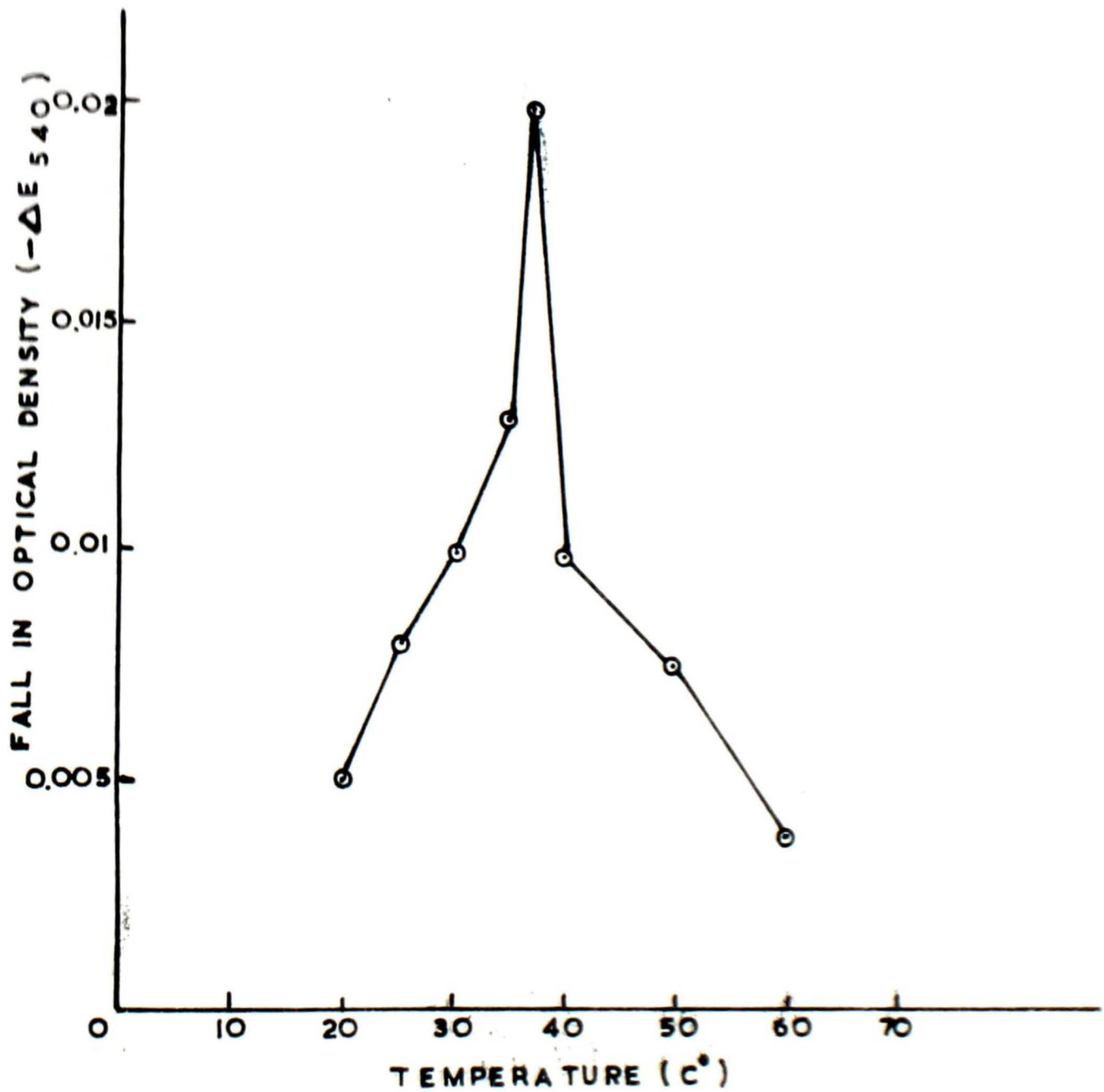
that in general both BML and EWL showed activity in a wide range of pH (3.5 - 9.5) with a maximum activity at pH 7.9 for BML and pH 6.2 for EWL. The two lysozymes obtained from different sources possessed different pH optima. Parry et al.(1969) observed that human milk lysozyme exhibited a single pH optimum at pH 6.35 which compered favourably with that obtained for egg-white lysozyme. In present experiment, the optimum pH for buffalo milk lysozyme was found to be between 6.0 - 6.5, which is similar to that for HML and EWL.

The presence of another small peak at pH 4.5 obtained on the basis of three trials indicates that the buffalo milk lyso--zyme might have two pH optima. However, this needs further study.

4.8.3 Effect of temperature on lysozyme activity

Enzyme activity was noted at different incubation tempera--tures, ranging from 20° to 60°C and the results are presented in Fig.VI. It indicates that the optimum temperature for enzyme activity was 37°C. The activity declined sharply after 37°C. The probable reason for low activity at high temperature was denatura--tion of enzyme. This optimum temperature was same as that reported by Shahani et al.(1962) and is the optimum temperature for most of the milk enzymes.

FIG. VII LYSOZYME ACTIVITY OF BUFFALO MILK AT DIFFERENT TEMPERATURES.



CHAPTER 5

SUMMARY

5. SUMMARY

1. Lysozyme activity differs in the milk of three species, namely cow, buffalo and goat. On an average it was observed that cow milk showed maximum activity followed by buffalo and goat. Variation within breeds of cow and goat and amongst the samples of three species were also significant.

2. Lysozyme activity in buffaloes' milk from different quarters followed no definite pattern of distribution and showed a random distribution of enzyme in different quarters of udder.

3. On an average 58.34% and 71.12% destruction of lysozyme activity was observed during HTST and LTLT pasteurization, respectively.

4. It was observed that lysozyme content increases with the advancement in stages of lactation.

5. When milk was stored at 6^{o}, 21^{o} and $37^{o}C$ for 14, 38 and 48 hrs, it was observed that lysozyme activity decreased with increase in storage time and temperature of milk.

6. Lysozyme was purified to the extent of 42 fold, on an average, using CM-cellulose and chromatography on Sephadex G-50. However, purification upto 142 fold was also achieved in one trial. Use of cation exchanger like CM-cellulose in place of Amberlite IRC-50 does not improve the purification of enzyme.

7. Both polyacrylamide and SDS-gel electrophoresis of purified buffalo milk lysozyme gave single band indicating homogeneity of sample.

8. Kinetic studies carried out with partially purified enzyme reveals that it was not possible to obtain an optimum substrate concentration for this enzyme. The optimum pH for buffalo milk lysozyme was found to be between 6.0 - 6.5. The optimum tempera-ture for enzyme activity was 37^{o}C, which is the optimum tempera-ture for most of the milk enzymes.

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