BUFFALO MILK LACTOPEROXIDASE: ISOLATION, PURIFICATION AND CHARACTERIZATION

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
(DAIRYING)
IN
DAIRY CHEMISTRY

BY

RAJESH KUMAR

DIVISION OF DAIRY CHEMISTRY
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL – 132001 (HARYANA), INDIA

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Dedicated to my
beloved parents

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RAJESH KUMAR

A thesis submitted to the National Dairy Research Institute (Deemed University), Karnal in partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY
(DAIRYING)
IN
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Approved by

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CERTIFICATE

This is to certify that the thesis entitled ***BUFFALO MILK LACTOPERROXIDASE : ISOLATION, PURIFICATION AND CHARACTERIZATION"*** submitted by ***Mr. Rajesh Kumar*** in partial fulfilment of the requirement for the award of the degree of ***DOCTOR OF PHILOSOPHY*** (Dairying) in **Dairy Chemistry** of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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Rajesh Kumar

(**RAJESH KUMAR**)

C O N T E N T S

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LIST OF ABBREVIATIONS

A°	Angstrom (1 x 10^{-10} metres)
ABTS	2,2'azinobis (3-ethyl benzthiazoline - 6-sulfonic acid)
Bis	N,N-methylene-bis acrylamide
CM	Carboxy methyl
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (ß-aminoethyl ether) N,N,N',N'- tetra acetic acid
FPLC	Fast protein liquid chromatography
IEF	Isoelectric focusing
Ig	Immunoglobulin
IgG1	Immunoglobulin-G1
Kd or Kav	Distribution coefficient
kDa	Kilodalton
Lf	Lactoferrin
mM	Millimolar
uM	Micromolar
NANA	N-acetyl neuraminic acid
NEM	N-ethyl maleimide
PAGE	Polyacrylamide gel electrophoresis
PBSG	Phosphate buffer saline containing gelatin
pI	Isoelectric point
ppm	Parts per million
SDS	Sodium dodecyl sulphate
SIgA	Secretory Immunoglobulin A
TEMED	N,N,N',N'-tetra methylene diamine
TMBZ	3,3',5,5'-tetramethylbenzidine
Tris	Trihydroxy methyl amino methane
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Milk is a rich source of a variety of enzymes and about fifty enzymatic activities have been detected in bovine milk. Peroxidase activity was first reported in bovine milk by Hanssen (1924) and almost twenty years elapsed before a significant purification and more detailed chemical characterizations of this enzyme could be made (Theorell and Akesson, 1943). It is the most abundant enzyme in bovine milk and exhibits important physiological and technological functions.

The enzyme lactoperoxidase (EC 1.11.1.7) is widely distributed in various biological secretions and body tissues of mammals. It is present in milk of different species in varying concentrations as well as between colostrum and milk in the same species (Reiter, 1981a). Lactoperoxidase acts in a system together with thiocyanate and hydrogen peroxide by catalyzing the peroxidation of thiocyanate to the putative antimicrobial hypothiocyanite ion (Aune and Thomas, 1977). As such, lactoperoxidase serves as a component of the biological defense systems of mammals.

Bovine lactoperoxidase is a glycoprotein of 78 kDa molecular mass, possessing a heme group and a calcium ion (Rombauts et al., 1967; Carlstrom, 1969b; Booth et al., 1989). Structural studies reveal that it is a single polypeptide chain of 612 amino acid residues (Cals et al., 1991). The amino acid sequence of bovine lactoperoxidase indicates a striking similarity of 55, 54 and 45 per cent with the corresponding peptide segments of human myeloperoxidase, eosinophilperoxidase and thyroperoxidase, respectively (Cals et al., 1991).

Presently, with the increased public interest in natural foods, it is pertinent to consider "natural antimicrobial systems" with a view to assess their possible utility in food preservation. Although the antimicrobial properties of milk have been known for many years, it is only recently that improved purification techniques have enabled to characterize the various inhibitory systems present in milk. The antimicrobial property of lactoperoxidase gives it a potential use in different commercial applications like pharmacology as well as food and agriculture industry.

Various workers have investigated the physico-chemical properties of bovine lactoperoxidase (Polis and Shmukler, 1953; Rombauts *et al.*, 1967; Carlstrom, 1969a,b,c; Paul *et al.*, 1980; Hernandez *et al.*, 1990). Also, a few reports on purification and properties of this enzyme from goat, sheep (Allen and Morrison, 1966; Lukat *et al.*, 1993) and human milk (Langbakk and Flatmark, 1989) are available.

During the last decade, there have been some efforts to study the structural aspects of bovine lactoperoxidase by various physical techniques including circular dichroism (Sievers, 1980), electron spin resonance (Sievers *et al.*, 1983), resonance Raman (Kitagawa *et al.*, 1983) and magnetic resonance spectroscopy (Behere *et al.*, 1985; Goff *et al.*, 1985; Thanabal and Lamar, 1989; Lukat *et al.*, 1993). The efforts to obtain suitable crystals of bovine lactoperoxidase have not been successful so far (Paul and Ohlsson, 1985). Thus, the structural information on lactoperoxidase is still not available. The

X-ray crystallographic studies on lactoperoxidase molecule will be of vital importance so as to reveal the very specific information about this protein molecule which can be used as a fundamental basis for future applications.

In India, buffalo milk contributes to about 50 per cent of its total milk production. However, no reports are available on the characterization of lactoperoxidase from buffalo milk. Since the enzyme is a part of the important natural inhibitory system of milk, the information generated in relation to its biochemical and structural properties will be useful to elucidate the function and mechanism of its action. Henceforth, the work has been initiated on this important biological active minor milk protein.

CHAPTER 2

REVIEW OF LITERATURE

2. *REVIEW OF LITERATURE*

2.1 *OCCURRENCE*

Peroxidase is a mammalian enzyme occurring widely in various biological secretions. Lactoperoxidase is the trivial name given to milk peroxidase. The presence of peroxidase has been demonstrated in the salivary, lacrimal, harderian and mammary glands but not in any other bovine tissue (Morrison and Allen, 1963; Morrison *et al.*, 1965; Morrison and Allen, 1966) and these peroxidases have been found to be immunologically and chemically similar (Morrison and Steele, 1968). The salivary peroxidase is also referred to as lactoperoxidase because of above mentioned similarity. Lactoperoxidase is present in the salivary glands of pig (Morrison and Steele, 1968), monkeys, rats, guinea pigs and hamsters (Tenovuo, 1985) and the peroxidase activity has also been detected in rat lacrimal gland (De, 1992).

Various human secretions have been shown to possess peroxidase activity such as saliva, tears, cervical mucus (Shindler *et al.*, 1976) and milk (Gothefors and Marklund, 1975; Langbakk and Flatmark, 1989). Other peroxidases in the human body include neutrophil myeloperoxidase, eosinophil peroxidase, glutathione peroxidase, thyroid peroxidase and uterine peroxidase (Tenovuo, 1985).

2.2 *REACTION MECHANISM OF LACTOPEROXIDASE*

2.2.1 *REACTION WITH HYDROGEN PEROXIDE*

Studies on the basic understanding of the physical chemistry of the reactions of peroxidases with hydrogen peroxide began 50 years ago with the pioneering work of Hugo Theorell, Karl Gustav Paul, Britton Chance and their colleagues (reviewed by Chance, 1951; Chance *et al.*, 1984). The reactions are complex and follow different pathways depending upon the concentration of hydrogen peroxide and whether or not exogenous electron donors are present (Kohler and Jenzer, 1989). The major intermediates for lactoperoxidase are ferric peroxidase (the native enzyme in its ground oxidation state), compound I, compound II, compound III and ferrous peroxidase. The following discussion is based on the reactions which generate these intermediates and the resulting state of the heme group is as follows:

In the absence of exogenous electron donors:

[Ferric enzyme] > [H_2O_2]:

$$\text{Ferric enzyme} + H_2O_2 \longrightarrow \text{Compound 1} + H_2O \quad (1)$$

$$H^+ + \text{Compound 1} + e \longrightarrow \text{Compound II} \quad (2)$$

$$\text{Compound II} + e \longrightarrow \text{Ferric enzyme} \quad (3)$$

[Ferric enzyme] < [H_2O_2]:

$$\text{Compound II} + H_2O_2 \longrightarrow \text{Compound III} + H_2O \quad (4)$$

$$\text{Compound III} \longrightarrow \text{Ferrous enzyme} + O_2 \quad (5)$$

$$\text{Ferrous enzyme} + H_2O_2 \rightarrow \text{Compound II} + H_2O \quad (6)$$

In the above scheme, e represents an electron. In the absence of exogenous electron donors, this electron may be supplied by impurities in the enzyme preparation and by oxidizable groups within the enzyme. In reaction (1), both oxidizing equivalents of the peroxide are transferred to the heme. One oxidation equivalent is stored as a porphyrin-centred, cation radical and the second as a low-spin oxyferryl center, $Fe^{4+} = O$ (Kohler and Jenzer, 1989). The cation radical is reduced by the addition of an electron to compound I to form compound II in reaction(2) (Makino *et al.*, 1986; Sitter *et al.*, 1985; Terner *et al.*, 1985). The oxyferryl center retains a single bond between iron and oxygen, Fe^{4+}-$O^{\cdot}$ (Chance *et al.*, 1984). In this reaction, the enzyme also takes up a single proton. The actual site occupied by this proton is a matter of controversy (Yamada and Yamazaki, 1974; LaMar *et al.*, 1983). Addition of an electron to the Fe^{4+}-$O^{\cdot}$ [reaction(3)] regenerates the ferric enzyme.

At high concentrations of peroxide and in the absence of exogenous electron donors, compound II may react with peroxide to generate compound III, reaction (4). In compound III, the iron has been reduced to the ferrous state and an adduct with dioxygen is formed, Fe^{2+}-O_2 (Dunford and Stillman, 1976). Dissociation of dioxygen from compound III, reaction (5), generates the ferrous enzyme in which the heme group has the same structure as ferric enzyme but with iron in lower oxidation state. The ferrous enzyme can be oxidized by peroxide to regnerate compound II, reaction (6). Reactions (4), (5) and (6) may be accompanied by irreversible enzyme inactivation by cleavage of the heme group (Jenzer *et al.*, 1986, 1987). The net

result of reactions (4), (5) and (6) is the catalytic degradation of 2 molecules of peroxide to 2 molecules of water and 1 molecule of dioxygen (Kohler *et al.*, 1988).

2.2.2 REACTIONS IN THE PRESENCE OF ONE ELECTRON DONORS

The following reaction mechanism describes the one electron reductions:

$$\text{Compound I} + \text{AH} \longrightarrow \text{Compound II} + \text{A}^\circ \quad (7)$$

$$\text{Compound II} + \text{AH} \longrightarrow \text{Ferric enzyme} + \text{A}^\circ \quad (8)$$

$$2\text{A}^\circ \longrightarrow \text{A2} \quad (9)$$

where, AH represents a broad range of donors and A° is a free radical. The half life of compound I is 0.2 S (Kimura and Yamazaki, 1979). Thus, in the presence of one electron donors and low concentrations of peroxide, compound II is the intermediate which will be observed under steady state conditions.

2.2.3 REACTIONS IN THE PRESENCE OF TWO ELECTRON DONORS

In this reaction mechanism, halide ions (Cl^-, Br^-, I^-) and the thiocyanate ion (SCN^-) are oxidized by compound I by a direct two electron transfer of oxidizing equivalents (Morrison and Schonbaum, 1976; Thomas, 1985). The net reaction is:

$$\text{Compound I} + \text{X} \longrightarrow \text{Ferric enzyme} + \text{XO} \quad (10)$$

where, X represents the halide or the thiocyanate ion and

XO is the oxidized product. Equation 10 describes the net reaction for these complex, two electron transfers. The detailed mechanisms vary depending upon the particular peroxidase and the particular donor.

2.3 LACTOPEROXIDASE ASSAY

Based on reaction mechanism mentioned earlier (2.2.2), several spectrophotometric peroxidase assays have been used. It involves the one electron oxidation of aromatic electron donors which generate coloured products. It is, however, difficult to compare reported results since there are no standardized peroxidase units upon which such comparisons may be based.

Some typical donors that have been utilized in peroxidase assay are o-phenylenediamine (Wallerstein *et al.*, 1947), p-phenylenediamine (Aurand *et al.*, 1956), N,N-dimethyl-p-phenylene- diamine hydrochloride (Sastry *et al.*, 1985), pyrogallol and guiacol (Chance and Maehly, 1955), o-aminophenol (Murty *et al.*, 1984), 0-dianisidine (Lundquist and Josefsson, 1971), ABTS [2,2'-azino-di- (3-ethylbenzthiazoline-6-sulfonic acid)] (Shindler and Bardlsey, 1975; Shindler *et al.*, 1976) and TMBZ (3,3', 5,5'- tetramethyl benzidine) (Beutler, 1988).

The one electron peroxidations of phenol, catechol, and hydroquinone generate free radicals which bind to proteins and to DNA (Smith *et al.*, 1989). These donors are *in vivo* metabolites of benzene. Their subsequent peroxidations have been suggested to play a role in benzene toxicity and carcinogenicity. Free radicals are also generated by one-electron peroxidations of

Dopamethylesters (dihydroxyphenyl-alanine methyl ester) and 6-hydroxy-Dopa (trihydroxypheny-lalanine) (Metodiewa *et al.*, 1989b), norepinephrine (Metodiewa *et al.*,1989a) and benzidine (Josephy, 1985).

ABTS has been demonstrated to be an ideal reagent for peroxidase studies being relatively non-toxic when compared with some above mentioned electron donors and form a well-defined reaction product with high absorbance (Bardsley, 1985).

Based on the method originally developed for measurement of peroxidase activity in cervical mucus (Shindler *et al.*, 1976), using ABTS as chromogen, several workers have reported the procedure for peroxidase assay in other biological secretions (Putter and Becker, 1983; Bardsley, 1985; Bjorck and Mullan, 1993).

A two-electron donor thiocyanate ion has also been used for peroxidase measurement (Mansson-Rahemtulla *et al.*, 1986). Pruitt *et al.* (1990) have reported a detailed kinetic comparison between different peroxidases and the use of various electron donors for the assay of peroxidase activity.

2.4 MILK PEROXIDASE ACTIVITY

Milk of all species tested so far show peroxidase activity (Reiter and Harnulv, 1984). Lactoperoxidase is mainly present in serum phase and practically absent from fat phase (Sharma and Ganguli, 1971; Djordjevic *et al.*, 1974). Variation of lactoperoxidase activity in milk is influenced by factors such

as breed, season, feed and especially, stage of lactation (Kiermeier and Kayser, 1960a; Korhonen et al., 1978). In addition to it, activity varies from day-to-day and from individual to individual but does not depend significantly on the health of udder (Kiermeier and Kayser, 1960a; Kiermeier and Kuhlmann, 1972).

The concentration of lactoperoxidase is low in bovine colostrum and increases rapidly to reach a peak at 4 to 5 days postpartum. Further, it declines rapidly afterwards to reach a constant rather high plateau during the lactation (Reiter, 1985). In contrast to bovine milk, human peroxidase is highest in colostrum and declines rapidly within one week (Kiermeier and Kuhlmann, 1972). Gothefors and Marklund (1975) concluded that human milk peroxidase activity was due primarily to lactoperoxidase and not myeloperoxidase. However, Moldoveanu et al. (1982) and Hashinaka and Yamada (1986) found that peroxidase enzyme in human milk was myeloperoxidase. Langbakk and Flatmark (1984,1989) confirmed that human colostrum contains lactoperoxidase. Further, Pruitt et al. (1991) reported that presence of enzyme activity in human milk is attributable to both human lactoperoxidase and myeloperoxidase with former accounting for 62 per cent while latter 38 per cent of total peroxidase activity.

An inter study comparison on lactoperoxidase activity of milk is difficult because of various chromogens used for its assay.Reiter (1985) reported a comparative data based on ABTS units from milk of diffeent species (Table 1) and showed that

Table 2.1 Peroxidase activity (mu/ml) assayed with ABTS as electron donor (Reiter, 1985; Harnulv and Kandasamy, 1982)

Source	Mean activity
Bovine (3-30 weeks)	1,422
Buffalo	900
Human: 1 day	700
2 to 10 days	314
Guinea pig	22,000
Sow	1,700
Goat	2,550
Rabbit	800
Mouse	2,000

guinea pig is far richer in lactoperoxidase than cow's milk. Higher peroxidase activity in buffalo milk than cow's milk have been reported (El-Hagarawy, 1959; Riffaat *et al.*, 1971), while Sharma and Ganguli (1971), Reiter and Harnulv (1982) observed lower peroxidase activity in buffalo milk than cow's milk.

Djordjevic *et al.* (1974) reported that the peroxidase activity of whey is 2/3 that of original milk and 82.4 per cent as compared to skim milk. A wide variability in peroxidase activity of bovine whole milk, skim milk and acid whey has been observed using ABTS as electron donor and showed a decrease in peroxidase activity on separation of whole milk to skim milk. Further decrease of peroxidase activity is more in acid whey than

rennet whey. (Yoshida, 1988a,b; Yoshida and Xiuyun 1991a,b)

2.5 *ISOLATION AND PURIFICATION OF LACTOPEROXIDASE*

Biological properties of antimicrobial whey proteins such as lactoperoxidase and lactoferrin have aroused a considerable interest among scientists to develop procedures for isolation and purification of these proteins on laboratory scale. Latoperoxidase and lactoferrin constitute 1.5 and 0.5 per cent of total whey proteins, respectively. Ion-exchange chromatography is the most favoured method for the isolation of these antimicrobial proteins because of their relatively high pI values. Methods have also been developed to make the extraction of these minor milk proteins a commercially viable process.

Lactoperoxidase (EC 1.11.1.7) was first isolated from milk in crystalline form by Theorell and his co-workers (Theorell and Akeson, 1943; Theorell and Paul, 1944). Polis and Shmukler (1953) isolated and crystallized the enzyme by salt fractionation and displacement chromatography from rennet whey and found two lactoperoxidase of absorbancy ratio A_{412}/A_{280} (Rz) as 0.9 and 0.77. Later, a method was developed (Morrison *et al.*, 1957; Morrison and Hultquist, 1963) employing a carboxylic acid resin in the ammonium or sodium form to adsorb preferentially lactoperoxidase from rennet whey or skim milk. The crude protein obtained from the resin was then chromatographed on the cation-exchange resin and then through a Sephadex G-100 column. The Rz value of preparation obtained was 0.91-0.95.

In another study of the heterogeneity of lactoperoxidase,

Carlstrom (1965) used a cation-exchange resin, carboxy methyl cellulose and Sephadex G-200 to isolate milk peroxidase from rennet whey. Chromatography of the purified enzyme on DEAE-Sephadex A-50 resulted in five active fractions. The major portion of the enzyme was found in fractions 1 and 2, while relatively little was found in fractions 3, 4 and 5. Rechromatography of fractions 1 and 2 showed that fraction 1 was homogeneous but 2 was slightly contaminated with fraction 1. The absorbancy ratio of fraction 1 and 2 was 0.96 and 0.85, respectively. The iron content and specific activity of these two major fractions were the same.

Rombauts *et al.* (1967) further modified the method of Morrison and Hultquist (1963) by substituting for Sephadex fractionation, a chromatographic procedure using an intermediate base anion-exchange resin.

Thorell and Johansson (1971) isolated lactoperoxidase from cow milk by adsorption with CM Sephadex C-50 and purified the eluate fractions of Rz value more than 0.5 to 0.8 or higher using $(NH_4)_2SO_4$ precipitation. Essentially same procedure has been used for preparation of lactoperoxidase from buffalo milk (Moodbidri *et al.*,1976) and obtained a yield of 2 mg/L comparable to that from cow milk.

Rule *et al.* (1976) purified the commercially available lactoperoxidase preparations with increased specific enzyme activity and absorbancy ratio using isoelectric focusing.

Paul *et al.* (1980) reported the isolation procedure of lactoperoxidase from rennet whey using cation exchanger CG-50-NH_4^+

and purified on phenyl-Sepharose® and finally from CM-52 using 10-130 mM sodium phosphate gradient. Yield of purified lactoperoxidase of absorbancy ratio 0.9 or higher was 7-10 mg/L.

Zhao *et al.* (1980) purified crude lactoperoxidase from cow's milk by CM-11 column chromatography and recovered 10 mg purified lactoperoxidase from 2 L milk.

Jin *et al.* (1981) isolated lactoperoxidase from fresh skim milk by CM Sephadex C-50 and precipitated the eluate fractions showing peak absorbance at 412 nm, with $(NH_4)_2SO_4$ salting out and finally purified by CM-cellulose chromatography using linear gradient of 0.05-0.40 M NaCl. Yield of the enzyme protein obtained was 3.26-3.88 mg from 5 kg fresh milk. Specific activity of the enzyme after CM Sephadex C-50, $(NH_4)_2SO_4$ and CM-cellulose treatment was 206, 576 and 2363 u/mg, respectively.

Zhao *et al.* (1982) reported the isolation of lactoperoxidase from milk by adsorption onto CM-Sephadex and then fractionated with $(NH_4)_2SO_4$ and the crude enzyme was finally purified using CM-cellulose chromatography. Yield of the purified enzyme of absorbancy ratio more than 0.9 was greater than 9 mg/L milk.

Langbakk and Flatmark (1984) partly purified lactoperoxidase from human milk colostrum using hydrophobic interaction chromatography on phenyl-Sepharose Cl-4B, with an apparent recovery of 45 per cent.

Buzila *et al.* (1984) simultaneously prepared the active components from human milk using Amberlite CG50 II. Lactoperoxidase

was eluted from Amberlite by 1 M K_2HPO_4 and purified by gel filtration on Sephadex G-100. Yield of lactoperoxidase obtained was 0.1 per cent of total whey proteins.

Goff *et al.* (1985) isolated lactoperoxidase from cow's milk using BioRex-70 resin and then salted out by $(NH_4)_2SO_4$. The crude enzyme preparation was purified using CM-52 chromatography with 50 to 150 mM NaCl gradient and finally purified by gel filtration on Sephadex G-100. The purified enzyme preparation obtained was of absorbancy ratio 0.91.

Prieels and Peiffer (1986) patented the process of isolation of lactoperoxidase and lactoferrin from milk or whey with acidic polysaccharides such as alginates and carrageenans.

Denisova *et al.* (1986) reported a method of isolation and purification of lactoperoxidase from cow's milk by binding to CM-Sephadex, ultrafiltration of eluate, salting out with $(NH_4)_2SO_4$ and isoelectric focusing in a borate-polyol system. This method produced highly purified, active lactoperoxidase preparation within relatively short period of time.

Ekstrand and Bjorck (1986) described the process of purification of antibacterial components lactoperoxidase, lactoferrin and lysozyme in whey from human and cow's milk by fast protein liquid chromatography using 0.01 M imidazole-HCl (pH 7.0) buffer containing 0-1 M NaCl.

Langbakk and Flatmark (1989) confirmed that human colostrum contains lactoperoxidase constituting 0.004 per cent

of total protein in crude colostrum. An apparent 32 fold purification with a recovery of 7 per cent was obtained by multistep proceudre. Using chromatography on an immunoaffinity column, an apparent 1,450 fold purification was obtained in a single step with 21 per cent recovery of protein.

Burling (1989) patented the process for preparation of lactoperoxidase and lactoferrin from skim milk or whey using strong cation exchanger such as S-Sepharose. Lactoperoxidase and lactoferrin were eluted at 0.3 M and 0.9 M NaCl, respectively. Yield of the two proteins of purity > 90 per cent was 1 kg lactoperoxidase and about 1.5 kg lactoferrin from 65 m^3 whey.

Frankinet (1989) patented the process for recovering the metalloproteins including lactoperoxidase, lactoferrin and transferrin from whey by adsorption on porous silica coated with crosslinked polysaccharides and obtained high yield.

Hernandez *et al.* (1990) described an isolation procedure of lactoperoxidase amenable to scaling up for production on a technological scale. The procedure is essentially that given by Paul *et al.* (1980) for isolation of basic proteins from whey, except using cheese whey concentrate instead of acid whey as source material and purifying by reverse phase-high performance liquid chromatography.

Dionysius *et al.* (1991) standardized the parameters for separation of lactoperoxidase and lactoferrin from rennet whey and acid whey and obtained maximum recovery using CM-Sephadex

resin with 60 min binding time. Further, pH in the range 5 to 9 gave good recoveries of both lactoperoxidase and lactoferrin. However, conductivity of whey significantly affected the adsorption of lactoperoxidase but not lactoferrin. Cheese whey yielded better recoveries of lactoperoxidase than rennet and acid wheys, but lactoferrin recovery was largely unaffected by whey type.

Yoshida and Xiuyun (1991a) reported the method for isolation of lactoperoxidase from acid whey using CM-cation exchange chromatography with a yield of 41 mg/L. However, using SP-cation exchange chromatography, yield of the lactoperoxidase, was 33.7 mg/L and 57.4 mg/L from rennet whey and acid whey, respectively (Yoshida and Xiuyun, 1991b).

Cals *et al.* (1991) reported the purification of bovine lactoperoxidase from rennet lactoserum by adsorption on CM-Sepharose, eluting stepwise with 80 mM ammonium acetate at pH 8.5 and 10.5, respectively. Elution by later fractionated on FPLC by two steps, in the first step using Mono Q column, 25 mM trimethyl- amine, pH 10.5, 0-0.6 M NaCl gradient gave three fractions: A (main), B and C in order of elution. Second step using Mono S column, 50 mM ammonium acetate, pH 8.0, 0.2-0.3 M NaCl gradient, fractionated A into subfractions A_1 (main) and A_2 in order of elution.

2.6 PROPERTIES OF LACTOPEROXIDASE

2.6.1 ELECTROPHORETIC PATTERN OF LACTOPEROXIDASE

Various reports on electrophoretic behaviour of lactoperoxidase (Groves, 1971; Paul and Ohlsson, 1985) are delineated below :

Polis and Shmukler (1953) separated lactoperoxidase into two fractions A and B, by means of displacement chromatography and free zone electrophoresis and observed isoelectric point to be 8.0 for slow component in 0.01 u phosphate buffer, while 9.2 for fast components in 0.10 u phosphate buffer.

Morrison and Hultquist (1963) suggested that heterogeneity of lactoperoxidase might result from proteolytic activity since casein is precipitated by rennet or that it represents a chemically different protein produced by genetically different animals.

Groves (1971) observed differences in gel electrophoretic patterns of lactoperoxidase at pH 9.1 from individual cows.

Carlstrom (1965) suggested that since both pooled milk and that from single cows gives lactoperoxidase that can be fractionated into several fractions, therefore, either the cow produces several peroxidases or there is one native peroxidase that is converted into several others during isolation.

Swope *et al.* (1966) found that starch gel electrophoresis of a whey fraction enriched in lactoperoxidase showed only one band with peroxidase activity at both acid and alkaline pH values.

Rombauts *et al.* (1967) observed five bands with peroxidase activity when lactoperoxidase was subjected to disc electrophoresis at an acid pH value. The major fraction corresponded to the band with the greatest mobility while the other four bands varied in relative concentration. It was thought that the slower moving bands were not aggregates of increasing degrees of polymerization but rather the multiple components were

artifacts of electrophoretic procedure since on subjecting the major band again to electrophoresis all five zones were obtained. The multiple bands did not appear to result from differential oxidation states or altered ligands of hemoproteins during polymerization of sample in the gel since samples layered in high density solution also gave five bands. A single band was obtained under conditions where spacer and sample gels were omitted and enzyme was layered directly onto a 15 per cent gel.

Carlstrom and Vesterberg (1967) separated the lactoperoxidase into six subcomponents by isoelectric focusing method but found no difference in patterns for preparation of lactoperoxidase made with or without rennet in the isolation procedure. Isoelectric point of subcomponents obtained was 9.80, 9.69, 9.68, 9.49, 9.31 and 9.16, respectively.

Calrstrom (1969a,b,c) using ion exchange chromatography, moving boundary electrophoresis, disc gel electrophoresis and isoelectric focusing, made further studies on the heterogeneity of lactoperoxidase prepared from pooled milk and the milk of individual cows. He found that the proportion of certain fractions of the enzyme varied among individual cows. He was unable to explain the heterogeneity on the basis of differences between the A and B fractions containing four and six subfractions, respectively, in amino acid analysis and molecular weight studies. However, presented evidence suggest that the major fraction A is derived from fraction B by loss of carbohydrate groups and that subfractions within each of the major fractions are derived by successive deamination of asparagine and/or glutamine residues.

Nichol *et al.* (1987) observed only one major band by disc electrophoresis of lactoperoxidase at pH 4.3, on staining with leucomalachite green.

Cals *et al.* (1991) reported that electrophoretic pattern of lactoperoxidase A_2 at pH 4.3 give two major and two minor fractions on staining for peroxidase activity by ABTS and H_2O_2. Also, they observed that the minor fractions have lowest mobility while the most abundant has highest.

2.6.2 IRON CONTENT, CALCIUM CONTENT AND MOLECULAR WEIGHT

Theorell and Padersen (1944) and Theorell and Paul (1944) reported that lactoperoxidase preparation of absorbancy ratio (A_{412}/A_{280}) 0.77 contain an iron content of 0.07 per cent and is of molecular weight 92,700 Da, calculated on the basis of sedimentation velocity. The corresponding values reported by Polis and Shumukler (1953) are 0.90, 0.069 per cent and 82,000 Da on the basis of light scattering. Morrison and Hultquist (1963) and Rombauts *et al.* (1967) gave these values to be 0.95, 0.0729 per cent and 77,500 Da calculated from sedimentation equilibrium. Carlstrom(1969b) reported the fractions A and B of lactoperoxidase with absorbancy ratio and iron content as 0.92 and 0.0747 per cent and 0.92-0.98 and 0.068-0.0709 per cent, respectively and calculated the molecular weight for fraction A as 76,500 Da by sedimentation equilibrium and 74,800 Da from iron content, while the corresponding values for fraction B are 78,000 Da and 78,800 to 82,100 Da. Groves (1971) reported that molecular weight calculations based on iron content are in good agreement with the physical measurements.

Molecular weight calculated from Ferguson plots of the migration rates in gels of different acrylamide concentrations gave the value 77,500 Da (Sievers, 1981) and 77,000 ± 2,000 Da (Mansson-Rahemtulla *et al.*, 1988). From human colostrum, Langbakk and Flatmark (1989) estimated the molecular weight of lacto- peroxidase as 80,000 Da based on SDS-PAGE and size exclusion-HPLC.

Booth *et al.* (1989) reported that lactopeoxidase contain an equimolar quantity of calcium and iron using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). The ratio of calcium to iron was calculated as 0.83 ± 0.08 without any treatment and 0.92 ± 0.06, 1.07 ± 0.03 on dialysis against diethylene-triamine penta acetic acid (DTPA) and ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA), respectively. However, with guanidine hydrochloride and EGTA a value of 0.83 ± 0.35 in supernatant and 0.10 ± 0.14 in precipitate was reported.

Lukat *et al.* (1993), using atomic absorption spectroscopy (AAS) reported that in the absence of chelators, native enzyme contains additional calcium with calcium to heme ratio for three independent Gurnesey lactoperoxidase preparations as 1.6, 1.4 and 1.5.

2.6.3 CARBOHYDRATE COMPOSITION

Rombauts *et al.* (1967) found that lactoperoxidase is a glycoprotein in which 16 of carbohydrate residues are glucosamine and 10 are galactosamine. Also, reported that

neutral carbo- hydrates are present at a level of 1.5 per cent but has no acetyl neuraminic acid. The enzyme contains 10 acetyl residues per mole which could be present as acetylated hexosamines and possibly acetylated N-terminal groups.

Carlstrom (1969b) reported that 14 of carbohydrate residues to be glucosamine and 4 to be galactosamine; neutral sugars constitute 5.37 per cent with no sialic acid present. Further the carbohydrate content of fraction A was found to be lower than that of B.

Mansson-Rahemtulla *et al.* (1988) in a recent report based on high performance liquid chromatography (HPLC) showed lactoperoxi- dase contains 10.7 per cent total sugars in accordance with the earlier reports (Rombauts *et al.*, 1967; Morrison and Steele, 1968; Carlstrom, 1969b) and reported the lactoperoxidase carbohydrate components with galactose constituting 0.4 per cent, mannose 0.9 per cent, fucose 0.19 per cent, glucose 0.56 per cent, glucosamine 2.70 per cent and 0.90 per cent galactosamine, indicating the majority of oligosaccharides are of high mannose type. Also, demonstrated that the side chains are N-linked to polypeptide, while small amounts of glucose detected by the sensitive HPLC method may be contaminants.

2.6.4 AMINO ACID COMPOSITION

The various reports (Morrison and Steele, 1968; Carlstrom, 1969b; Mansson-Rahemtulla *et al.*, 1988; Cals *et al.*, 1991) on the aminoacid composition of bovine lactoperoxidase are

reasonably consistent. Pruitt and Kamau (1991) regrouped the data given by Carlstrom (1969b) as presented in Table 2.2.

Table 2.2 Amino acid composition of bovine lactoperoxidase

Non-polar side chains		Polar side chains	
Alanine	37	Asparagine + glutamine	62
Cystine	8	Serine	33
Cysteine	0	Threonine	32
Glycine	40	Tyrosine	15
Isoleucine	27		-
Leucine	68	Total	= 142
Methionine	8		
Phenylalanin	30		
Proline	42		
Tryptophan	15		
Valine	28		
Total	= 303		

Basic side chains		Acidic side chains	
Arginine	37	Asparagine + Aspartic acid	71
Lysine	34	Glutamine + Glutamic acid	61
Histidine	14		-
Total	= 85	Total	= 132
		Aspartic acid + Glutamic acid	= 70

2.6.5 EFFECT OF ACTIVATORS AND INHIBITORS

Morrison (1970) reported that peroxidase being a hemoprotein interact with usual hemoprotein ligands, such as cyanide, fluoride and azide which, in turn, compete with peroxide and hence inhibit the enzyme activity.

Shindler *et al.* (1976) reported the inhibition of peroxidase isolated from human cervical mucus by several compounds such as N-ethylmaleimide, iodoacetamide, sodium di-ethyl-dithiocarbamate, 2,2'-bipyridyl; 1,10-phenanthroline; hydroxylamine-hydrochloride, ethylenediamine tetra-acetic acid; cupric sulphate; potassium cyanide; sodium azide; potassium borohydride and sodium dithionite.

Geike and Prasher (1976) in a simple screening test for detection of lactoperoxidase inhibitor reported aniline, 3-toluidines, mercuric chloride, 5-quinones, resourcinol and phloroglucinol to be inhibitory to bovine milk peroxidase.

Mansson-Rahemtulla *et al.* (1988) reported that salivary peroxidase is more sensitive to azide than lactoperoxidase, while the sensitivity of these two peroxidases to cyanide is comparable.

Tenovuo *et al.* (1982) reported that myeloma IgG, colostral SIgA, colostral lactoferrin and iron saturated lactoferrin have most protective effect on lactoperoxidase activity while IgM is least effective and α -lactalbumin and human serum albumin do not exhibit any protective effect.

Hulea *et al.* (1989) reported that lactoperoxidase was slightly activated by complexing to lysozyme while IgA and IgM were inhibitory for peroxidase. IgG and ribonuclease have no effect on enzyme activity.

2.6.6 STABILITY OF LACTOPEROXIDASE

2.6.6.1 Heat

Lactoperoxidase is one of the most stable enzymes in milk and was used in storch test for flash pasteurization. Thermal inactivation of lactoperoxidase has been reported by various workers as follows:

Pien (1945) demonstrated that at 80°C, 3.5 min are required for complete inactivation of lactoperoxidase.

El-Hagarawy (1959) observed that thermal stability of buffalo milk peroxidase is more than that of cow's milk during heating at 60° to 70°C for 30 min.

Heat inactivation of lactoperoxidase in milk is maredkly sensitive to temperature changes around 80°C, but much less effected by variation in heating time at a particular temperature (Kiermeier and Kayser, 1960b,c; Woerner, 1961) and some restoration of peroxidase activity occur after storage depending on the temperature of inactivation. The more severe the heat treatment, the smaller the degree of subsequent regeneration. However, storage at 4°C for 24 h yielded no restoration of activity following heating at a holding time of

15 sec at temperatures between 65° and 80°C. Milk peroxidase may also be inactivated by microbial growth (Kiermeier and Kayser, 1960c).

Jankoff and Pronadanski (1962) in a study on peroxidase from buffaloes', ewes' and goats' milk found buffalo milk peroxidase to be most heat stable and that from ewes' milk least heat stable, being inactivated in 6 sec at 82.8°C.

Djordjevic *et al.* (1974) reported that pasteurization of the milk at 63°C for 30 min reduced the total peroxidase activity by 32 per cent; heat treatment at 75°C for 15 sec caused a 2/3 reduction of activity, while a temperature of 80°C for 5 sec completely inactivated the enzyme.

Monget and Laviolette (1978) demonstrated that the lactoperoxidase was completely destroyed by heating at 80°C for 20 sec.

Shidlovskya (1982) reported the complete inactivation of lactoperoxidase by heating at 80°C for more than 20 sec.

Griffiths (1986) revealed in laboratory studies a reduction of lactoperoxidase activity by about 40 per cent on heating at 80°C for 15 sec. However, using plate heat exchanger, the enzyme activity was completely destroyed within 5 sec at 80°C. The enzyme appeared to be sensitive to temperatures above 75°C and has a Z-value of 5.4°C.

Hernandez *et al.* (1990) reported that thermal inactivation of lactoperoxidase follow first order kinetics and calculated

the activation energy for heat denaturation of lactoperoxidase in milk and whey as 800 kJ/mol and 1030 kJ/mol, respectively. Further, he calculated the Arrhenius energy of lactoperoxidase thermal inactivation in cheese permeate as 426 kJ/mol, which increased to 603 kJ/mol on addition of ß-lactoglobulin and still increased to 810 kJ/mol on addition of ß-lactoglobulin and caseinate in cheese permeate. It was also reported that the presence of calcium increases the thermostability of lactoperoxidase.

Sato *et al*. (1992) speculated that mechanism of enhancement of thermostability of lactoperoxidase with monovalent cations is different in its mechanism from that of calcium.

Olszewski and Reuter (1992) studied the effect of temperature and time on reaction kinetics of lactoperoxidase inactivation and the influence of heating conditions on its reactivation. Inactivation follows 1.5 order reaction and in the temperature range where 30 to 80 per cent inactivation occurs, calculated the Arrhenius energy of 634 kJ/mol and Z-value of 3.7°C. Further, the amount of lactoperoxidase which is able to regenerate during storage depends on both temperature and time of heating.

2.6.6.2 pH

Carlstrom (1969a) reported that deamidation of lactoperoxidase by glycine at pH 10.3 for 48 h at room temperature did not inactivate lactoperoxidase.

Maquire *et al.* (1971) indicated in the kinetics studies of compound I formation that lactoperoxidase was stable when stored at pH 7.0 but deactivated by storage at pH 3.0.

Kimura and Yamazaki (1978) showed that some denaturation of lactoperoxidase occurs at pH 4.0.

Hernandez *et al.* (1990) reported that milk adjusted to different pH values from 5.3 to 7.0 showed lowest thermostability at pH 5.3.

Sato *et al.* (1992) reported that lactoperoxidase is stable over the pH range 4 to 9, but completely denatured at pH 2.6. The denaturation at pH 2.0 is partially reversible depending on incubation time. Although lactoperoxidase is stable at pH higher than 4.0 but heated lactoperoxidase is stable only in the pH range 5.6 to 7.7.

2.7 NON-HEME LACTOPEROXIDASE

Allen and Morrison (1963) reported that crude bovine lactoperoxidase preparations contained hemin-free polypeptides which reacted with anti-lactoperoxidase immune serum. No further studies of this non-heme lactoperoxidase were published until the work of Dumontet and Rousset (1983). These workers reported isolation by cation exchange chromatography of comparable amounts of lactoperoxidase and non-heme lactoperoxidase from bovine whey. The two forms gave a single line on immunodiffusion using anti-lactoperoxidase antibodies. They had the same apparent molecular weight in velocity sedimentation on sucrose gradients, similar amounts of carbohydrate, and

similar peptide maps after limited proteolysis by substilisin or trypsin. The non-heme lactoperoxidase gave no spectrum in the Soret region and was devoid of enzymatic activity. These authors also found that non-heme lactoperoxidase in goat, sheep and human milk. Ekstrand and Bjorck (1986) further analysed the non-heme lactoperoxidase prepared according to Dumontet and Rousset (1983) on FPLC and found this fraction coincided with lactoferrin peak. On comparison of amino acid composition of this non-heme fraction with that of lactoperoxidase (Carlstrom, 1969b) and lactoferrin (Castellino *et al.*, 1970), the Metzer indices (Metzer *et al.*, 1968) were determined. Between lactoperoxidase and non-heme lactoperoxidase, the Metzer indices were found to be 11.7, while it was 4.4 between lactoferrin and non-heme lactoperoxidase. It was concluded that non-haem lactoperoxidase is identical with lactoferrin, however, its origin and biological significance are unknown.

2.8 MOLECULAR STRUCTURE OF LACTOPEROXIDASE

2.8.1 PRIMARY STRUCTURE

Sievers (1981) reported that the lactoperoxidase exists as a single chain with leucine as the N-terminal amino acid. Recently, the sequence of cDNA encoding the entire bovine lactoperoxidase has been reported (Dull *et al.*, 1990). Using a conventional strategy, Cals *et al.* (1990) sequenced bovine lactoperoxidase and reported that it is a single peptide chain containing 612 amino acid residues, including 15 half cystines and 4 or 5 potential N-glycosylation sites. The structure of lactoperoxidase is stabilized by eight disulfide bonds

(Carlstrom,1969b). Reduction of disulfide bonds destroys enzymatic activity (Mansson-Rahemtulla *et al.*, 1988).

2.8.2 SECONDARY AND TERTIARY STRUCTURE

Sievers (1980) in a detailed study of the far-ultraviolet circular dichroism spectrum of lactoperoxidase indicated that the molecule contains 65 per cent ß-structure, 23 per cent α - helix and 12 per cent unordered structure. Paul and Ohlsson (1985) on the basis of sedimentation analyses suggested that the molecule has an ellipsoidal form in solution. The thermodynamic properties of lactoperoxidase in solution have been reported to be similar to those of other small, globular proteins (Pfeil and Ohlsson, 1986).

On the basis of properties and amino acid composition of lactoperoxidase, Pruitt and Kamau (1991) made some inferences about its secondary and tertiary structure. The enzyme contains 50 per cent non-polar side chains and the fact that the hydrophobic chromatography is an effective means for purification indicates that these non-polar side chains exist in part as exposed clusters on the surface of the molecule. The molecule also has exposed positive charges on its surface as indicated by its affinity for negatively charged ion-exchange resins and consistent with the amino acid composition which shows that number of basic side chains exceeds the number of acidic side chains by fifteen.

These hydrophobic and charged clusters on the surface of lactoperoxidase provide a mosaic which gives the molecule strong

affinity for many different kinds of surfaces. It is strongly adsorbed to glass (Honka *et al.*, 1982), enamel (Pruitt and Adamson, 1977), bacterial cell surfaces (Pruitt *et al.*, 1979) and to other proteins (Tenovuo *et al.*, 1982).

Adsorbed lactoperoxidase retains its enzymatic activity, thus the surface binding sites and the heme group must be in separate locations on lactoperoxidase surface (Pruitt and Kamau, 1991). Pfeil and Ohlsson (1986) studied the thermal unfolding of lactoperoxidase using differential scanning calorimetry and optical methods. It was observed that lactoperoxidase has at least two structural domains of different thermal stabilities and that heme group is located in the less stable domain.

2.8.3 HEME STRUCTURE

Sievers (1979) isolated and identified protoheme 1X (1,3,5, 8-tetramethyl-2, 4-divinylporphyrine-6, 7-dipropionic acid) from a pronase digest of bovine lactoperoxidase. She concluded that protoheme 1X was the prosthetic group in lactoperoxidase and found no evidence that heme was covalently bound to the apoenzyme.

However, recent reports (Nichol *et al.*, 1987; Thanbal and LaMar, 1989; Modi *et al.*, 1990) do provide evidence that the prosthetic group in bovine lactoperoxidase is covalently attached. The suggested structure is shown in Fig. 2.1. The modification of protoheme IX is that the heme group is covalently bound to the peptide backbone via a disulfide bond at the 8-CH_3 group. The Fe^{3+} is held in the porphyrin ring by four bonds to

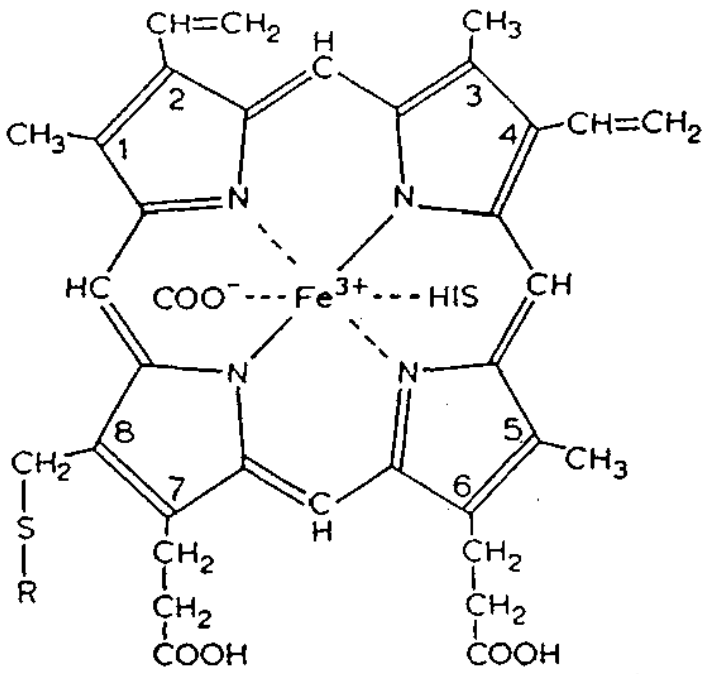


Fig. 1. Structure of the lactoperoxidase heme. The heme group is proposed to be bound to the apoenzyme by a disulfide bond linking the 8—CH_2 group to a cysteinyl residue. The proximal and distyl axial ligands are proposed to be a histidyl residue and a carboxyl group.

nitrogen. The fifth and sixth axial ligands are thought to be a histidyl residue and a carboxylate ion, respectively (Sievers, 1980; Sievers *et al.*, 1983).

Further, Nichol *et al.* (1987) reported that the unusual visible spectrum of lactoperoxidase is largely due to the nature of the axial ligands binding to iron in lactoperoxidase heme rather than to the nature of lactoperoxidase heme itself. Behere *et al.* (1985) also showed that the possible presence of positively charged groups in the heme environment which interact with axial ligands may be of relevance.

2.8.4 CRYSTALLIZATION

Crystallization has long been one of the stages in the production of highly purified protein. Since 1950, there has been rapid development in the field of X-ray crystallography and its application in the study of macromolecules. With the advancement in the field of biological sciences, the demand for protein crystals is increasing to acquire the knowledge of their three dimensional structures. The growth of protein crystals of suitable size and homogeneity, which diffract to high resolution and reproducible production, is still a rate limiting step in protein crystallography.

The word 'crystal' is derived from Greek word meaning ice or 'frozen water'. Crystals are solid in which there is a well defined, long-range, three-dimensional molecular order. There are relatively strong intermolecular forces between the molecules in close promixity and thus a state of minimum free

energy is achieved. The spontaneous transition of such complex asymmetrical objects as protein macromolecules in a state with small degrees of freedom and arrangement in a fixed lattice should be accompanied by a decrease in the entropy. However, the simultaneous occurrence of new stable interactions result in a net decrease in the free energy of the system, which is also a motive force for the ordering process. Hydrogen and electrostatic bonds as well as hydrophobic interactions between different protein molecules take part in the maintenance of the structure of the protein crystal lattice.

The changes in protein conformation and lattice-packing arrangements result in different crystal structures. Conversely, the intermolecular contacts can influence the local conformation of polypeptide chain. Protein crystals, however, invariably have a large solvent content, with typical values ranging from 30 to 78 per cent solvent (Matthews, 1968). Thus, there are rather large interstitial spaces between molecules and only a few areas of contact between them.

A number of methodologies, possessing a broad sphere of applicability, which aid in shortening the time in the search for conditions of crystallization have been developed and formulated (Blundell and Johnson, 1976; McPherson, 1982; Ducruix and Giege, 1990). Several automated systems for protein crystallization based upon the vapour diffusion or the microbatch method have been developed (Cox and Weber, 1987; Ward *et al.*, 1988; Jones *et al.*, 1989; Chayen *et al.*, 1990 and Rubin *et al.*, 1991).

Although long back a few attempts were made on preparation of bovine lactoperoxidase crystals (Polis and Shmukler, 1953; Morrison *et al.*, 1957), however, no X-ray crystallographic investigations have been conducted on lactoperoxidase from milk of any species.

2.9 LACTOPEROXIDASE SYSTEM

2.9.1 MODE OF ACTION

Antimicrobial activity of hemoprotein peroxidase enzyme is due to their ability to catalyze H_2O_2-dependent oxidation of halide ions or thiocyanate (SCN^-) to yield halogens or other oxidizing agents related to halogens. The halides and SCN^- differ in their ease of oxidation and peroxidases differ in their ability to catalyze oxidation of these ions (Thomas, 1985).

In the presence of H_2O_2, lactoperoxidase catalyzes the oxidation of thiocyanate to non-inhibitory end products such as SO_4^{2-}, CO_2 and NH_3 (Oram and Reiter, 1966). In the course of this oxidation, however, more or less inhibitory intermediates form, e.g. hypothiocyanate ($OSCN^-$) (Aune and Thomas, 1977) or higher oxyacids O_2SCN^- and O_3SCN^- (Pruitt *et al.*, 1982). The oxidizing agents formed in the reaction make an electrophilic attack on microbial components, resulting in chemical modification of essential enzymes, transport systems and other functional components (Thomas, 1985).

Sulfhydryl groups are especially susceptible to electrophilic attack, and are usually present in higher amounts than

other easily oxidizable groups (Thomas, 1985). Thus, hexokinase are totally inhibited and aldoses and 6-phosphogluconate dehydro- genase are partially inactivated by LP-system (Thomas and Aune, 1978). However, Law and John (1981) showed that energy transducing D-lactate dehydrogenase is also inhibited by this system, even though its active site does not contain -SH groups. The lactoperoxidase system also causes lesions in the cytoplasmic membrane, causing the leakage of potassium ions, amino acids and polypeptides from the cell. Sugar and amino acid transport system are also inhibited as are the synthesis of DNA, RNA and proteins (Reiter and Harnulv, 1984).

Thus, peroxidase catalyzed oxidation of halides or thiocyanate conserves the oxidizing power of hydrogen peroxide in forms that react more rapidly and for which the target cells may have no defence (Thomas, 1985).

2.9.2 BIOLOGICAL SIGNIFICANCE

2.9.2.1 Human milk

Since human milk contains myeloperoxidase, lactoperoxidase, halides and thiocyanate, some of reaction products potentially generated may be such as Cl_2, OCl^- from Cl^-, Br_2, OBr^- from Br^-, $[SCN]_2$, $OSCN^-$ from SCN^- and I_2, I_3, OI^- from I^- (Thomas, 1985). There have been no reports of measurement of these substances in human milk (Pruitt and Kamau, 1991). They, however, reported that oxidized forms of SCN^- are probably the major oxidation products. Both myeloperoxidase and lactoperoxidase catalyzes the peroxidation of this ion. The SCN^- competes very effectively

with Cl^- for available oxidizing equivalents of myeloperoxidase compound I and the concentration of Br^- and I^- are far less than SCN^- (Pruitt and Kamau, 1991).

The oxidized forms of SCN^- kill or inhibit the growth and metabolism of many different species of microorganisms (Pruitt and Reiter, 1985) so that lactoperoxidase system in human milk could function as an antibody independent defence mechanism. Reiter (1981b) reported that human milk will kill *Escherichia coli* on supplementation with SCN^- and H_2O_2 to concentration of 0.22mM. Pruitt *et al.* (1991) showed that human milk with a high intrinsic peroxidase activity will kill *E. coli* and *Salmonella typhimurium* when it is supplemented with SCN^- and H_2O_2 to concentration of 3 mM. The biological significance of these experiments is not clear (Pruitt and Kamau, 1991). However, anti-bacterial properties of the human milk peroxidase system has been demonstrated only on exogenous addition of thiocyanate and hydrogen peroxide to concentrations not normally found *in vivo*. Carlsson (1987) reported that the most important function of the human milk peroxidase system is to protect the mammary gland from the toxic products of aerobic metabolism.

2.9.2.2 *Bovine milk*

Although lactoperoxidase is the most abundant enzyme in bovine, it is the concentration of SCN^- and H_2O_2 in fresh bovine milk from healthy cows, which limits the generation of inhibitory levels of $HOSCN + OSCN^-$ *in vivo*.

When H_2O_2 and SCN^- are added *in vitro* to bovine milk, whey or growth media containing lactoperoxidase, the systems inhibit various mastitis causing bacteria (Mickelson and Brown, 1985; Marshall *et al.*, 1986; Sandholm *et al.*, 1988). Some of these microorganisms are human pathogens and can be shed into milk from udders of infected cows. Banks and Board (1985) reported that lactoperoxidase system is also inhibitory against enterococci and enterobacteriaceae which cause gastroentritis and are associated with high infant morbidity and mortality, especially in developing countries. Thus, the lactoperoxidase system may provide an *in vivo* defence mechanism against udder infections in the cow, and may protect the newborn from gastroenteritis (Reiter, 1985). Based on these observations, Pruitt and Kamau (1991) reported that protective effects of bovine milk consumption would be less significant for human infants than they would be for newborn calves as the saliva of human infants has high concentration of salivary peroxidase which retains its activity in the gastric juice, while calf saliva contains very little peroxidase activity (Gothefors and Marklund, 1975).

2.9.3 PRACTICAL APPLICATIONS

2.9.3.1 Preservation of milk

The most widely recommended industrial application of lactoperoxidase system in food production is in the dairy industry for the preservation of raw milk during storage and/ or transportation to processing plants. The proper use of lactoperoxidase system in milk preservation poses no known health hazard and can help to improve milk production especially in

developing countries where refrigeration is not available (IDF, 1983, 1986; Codex Alimentarius Commission, 1988). Further, if lactoperoxidase system is activated prior to application of approved thermal processes, the shelf-life of dairy products may be extended significantly (Pruitt and Kamau, 1991).

Beside that, lactoperoxidase system has also been exploited as an additional means of controlling the growth of spoilage bacteria and certain pathogenic bacteria and should be viewed as complementary to refrigeration (Pruitt and Kamau, 1991).

2.9.3.1.1 Control of spoilage bacteria

The antibacterial activity of lactoperoxidase system in milk against psychrotrophic organisms has been widely investigated (Bjorck *et al.*, 1975; Bjorck, 1978; Reiter *et al.*, 1976). Using a glucose/glucose oxidase system to generate H_2O_2 and supplementing milk with 0.17 to 0.26 mM SCN^-, Bjorck *et al.* (1975) demonstrated that the lactoperoxidase system is bacteri- cidal against *Pseudomonads* and *E. coli*. Reiter *et al.* (1976) observed that bactericidal effect against *E. coli* is increased on raising the SCN^- concentration from 0.015 to 0.15 mM and is reduced when initial inoculum is increased.

Apart from controlling psychrotrophic spoilage bacteria, the lactoperoxidase system has been widely investigated for its potential to control mesophilic spoilage bacteria, especially in bovine milk (Bjorck *et al.*, 1979; Reiter and Harnulv, 1982; Zajac *et al.*,1983). Preservation of buffalo milk by the

lactoperoxidase system has also been investigated both at ambient and refrigeration temperatures (Thakar and Dave, 1986; Chakraborty *et al.*, 1986). For bacteria that survive the initial bactericidal activity of the lactoperoxidase system, there is an extended lag phase or recovery period. The length of this lag period is highly temperature dependent, being much longer at cold storage than at high temperatures (Bjorck *et al.*, 1979; Zajac *et al.*, 1983). On an average, this lag period is about 8 h at ambient temperatures (22° to 37°C). After recovery, most bacteria resume normal growth. Thus, the lactoperoxidase system can prevent spoilage of milk during short storage or transportation periods at ambient temperatures (Pruitt and Kamau, 1991).

2.9.3.1.2 Control of pathogenic bacteria

Certain food borne pathogens are capable of growth and/or toxin production under cold storage as well as at normal growth temperature. These pathogens include *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Bacillus cereus* and *Salmonella typhimurium* (Palumo, 1986). Although most of these pathogens are destroyed by proper pasteurization, their presence in milk poses a health hazard (Smith *et al.*, 1983).

The lactoperoxidase system is both bactericidal and bacteriostatic against *S. typhimurium*, a well recognized food-borne pathogen (Reiter *et al.*, 1976; Wray and McLaren, 1987). The rough strains of *Salmonella* are more susceptible to the lactoperoxidase system than the smooth strains (Purdy *et al.*, 1983; Wray and McLaren, 1987). The bactericidal effect against

C. jejuni in milk has also been demonstrated (Potter *et al.*, 1984; Barret, 1986). The lactoperoxidase system is both bacteriostatic and bactericidal against *S. aureus* in milk (Kamau *et al.*, 1990a) and *L. monocytogenes* (Dennis and Ramet, 1989; Earnshaw and Banks, 1989; Kamau *et al.*, 1990a). Dennis and Ramet (1989) observed that the initial bactericidal effect against *L. monocytogenes* are more pronounced at 15°C than at 4°C. Kamau *et al.* (1990b) reported that exposure to lactoperoxidase system prior to thermal processing greatly enhances the thermal inactivation of *L. monocytogenes* and *S. aureus* in milk.

2.9.3.2 Milk replacers

Bovine milk contains an array of antibacterial agents which are known to give neonate vital protection against various infectious agents before its own defence system are developed (Reiter *et al.*, 1981). Because many of these factors are denatured by heat treatments used in milk replacer manufacture (Ford *et al.*, 1977), Commercial products, unless specifically produced, generally do not contain antimicrobial protein in active form.

2.9.3.2.1 Calf milk replacer

Another application of lactoperoxidase system is in calf milk replacers as a substitute for low dose antibiotics. The improved performance of calves against scouring and enteric disease on feeding lactoperoxidase activated raw milk has been demonstrated by Reiter *et al.* (1981). Recently, Still *et al.* (1990) reported a trial in which the lactoperoxidase system and

lactoferrin have been used successfully to treat induced enterotoxin Colibacillocis in calves.

Consequently, calf milk replacers containing an activated lactoperoxidase system are marketed in several countries in which the use of feed antibiotics is restricted, e.g., Denmark, Sweden, Finland and Norway (Bjorck, 1991).

2.9.3.2.2 Improved human milk replacers

There is interest in the addition of the antimicrobial proteins of bovine milk to infant formulae. However, published data are not available and the benefits, if any, are speculative at this stage (Bjorck, 1991). Bank and Board (1985) reported that milk powders containing an active lactoperoxidase system may have an advantage as far as protecting the health of the newborn when formula feeds are reconstituted with contaminated water under unsanitary conditions.

The availability of lactoperoxidase through large scale isolation from skim milk or whey has opened up several applications for the antibacterial effects of the lactoperoxidase system. Of great interest are its use in pharmacology by incorporating the ingredients necessary to activate the system in toothpastes or contact lens solution. In the future, it is likely that several other applications will be developed.

CHAPTER 3

MATERIALS AND METHODS

3. *MATERIALS AND METHODS*

Pooled milk samples were collected from the Murrah breed of buffaloes and crossbred cows maintained at National Dairy Research Institute, Karnal. The samples were skimmed using Alfa-Laval cream separator.

3.2 *PREPARATION OF WHEY SAMPLES*

3.2.1 *RENNET WHEY*

To the skim milk (3.1), Meito rennet (Meito Sangyo, Japan) was added at the rate of 20 mg per litre and incubated at 30°C for 30 minutes. After setting, the curd was cut into small cubes and cooked by raising the temperature to 37°C. The whey was filtered using cheese cloth and passed through Whatman No.1 filter paper and clear whey was obtained.

3.2.2 *ACID WHEY*

Skim milk (3.1) was diluted in the ratio 1:1 by distilled water. Diluted milk was acidified to pH 4.6 at 20°C using 2 N HCl. The precipitated casein was removed by passing through cheese cloth. Further, the clear whey was obtained by filtration through Whatman No. 1 filter paper.

3.3 *ISOLATION AND PURIFICATION OF LACTOPEROXIDASE*

3.3.1 *LACTOPEROXIDASE ASSAY*

Measurement of peroxidase activity was carried out using

method given by Shindler *et al.* (1976) and incorporating modifications from Pruitt *et al.* (1990).

3.3.1.1 *Reagents*

(a) Phosphate buffer (stock solution)

(i) Di-sodium hydrogen phosphate solution ($Na_2HPO_4.2H_2O$) (0.2 M)

35.6 g of $Na_2HPO_4.2H_2O$ was dissolved in distilled water and the volume was made up to one litre.

(ii) Sodium dihydrogen phosphate solution ($NaH_2PO_4.H_2O$) (0.2 M)

27.6 g of $NaH_2PO_4.H_2O$ was dissolved in distilled water and the volume was made to one litre.

(b) Phosphate buffer (0.1 M, pH 6.0)

To 87.7 ml of solution a(i), added 12.3 ml of solution a(ii) and diluted it by 1:1 with distilled water.

(c) Phosphate buffer (0.1 M, pH 7.0)

To 39 ml of solution a(i), added 61 ml of solution a(ii) and diluted it by 1:1 with distilled water.

(d) Phosphate buffer saline containing gelatin (PBSG)

Dissolved 0.1 g gelatin (Loba) and 0.8875 g sodium chloride in 100 ml of phosphate buffer (pH 7.0, 0.1 M).

(e) ***Acetate buffer (stock solutions)***

(i) **Acetic acid (0.2 M)**

Diluted 11.55 ml of glacial acetic acid to 1,000 ml with distilled water.

(ii) **Sodium acetate solution (0.2 M)**

Dissolved 27.2 g of sodium acetate trihydrate in distilled water and made up the volume to 1,000 ml.

(f) ***Acetate buffer (0.1 M, pH 4.4)***

Mixed 30.5 ml of solution e(i) with 19.5 ml of solution e(ii) and made up the volume to 100 ml.

(g) ***ABTS solution (1 mM)***

Fifty five mg of 2,2'-azinobis (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS, Sigma) was dissolved in 100 ml of 0.1 M phosphate buffer, pH 6.0 or 0.1 M acetate buffer, pH 4.4.

(h) ***Standardizationof hydrogen peroxide (H_2O_2) solution (3.2mM)***

The standardization of hydrogen peroxide solution was carried out by potassium permagnate titration as described below:

(i) **Standardization of potassium permagnate**

(A) **Oxalic acid solution (N/20):** 3.2 g hydrated oxalic acid

was dissolved in distilled water and made up the volume to 250 ml.

(B) **Potassium permagnate ($KMnO_4$) solution (N/20)**: 0.8 g of $KMnO_4$ was dissolved in distilled water and made up the volume to 250 ml.

The strength of $KMnO_4$ solution prepared was determined by titration against standard oxalic acid solution in the presence of diluted H_2SO_4 at 60-70°C.

(ii) Standardization of H_2O_2 solution

1.1 ml of H_2O_2 solution 30 per cent (v/v) (Merck) was diluted with distilled water and made up the volume to 100 ml. The strength of diluted H_2O_2 solution was determined by titration against standard $KMnO_4$ solution.

(iii) H_2O_2 solution (3.2 mM)

The H_2O_2 solution of known strength (ii) was further diluted with distilled water to a final concentration of 3.2 mM.

3.3.1.2 Sample preparation

Samples of milk or whey were generally diluted 1:250 with PBSG while for treated samples appropriate dilution was carried out.

3.3.1.3 Procedure

Pipetted 3.0 ml of 1 mM ABTS solution into cuvette, to it

added 0.1 ml of peroxidase sample, mixed the contents using teflon lid and placed the cuvette in the spectrophotometer (Spectronic 21 D). Adjusted the absorbance to zero. To another cuvette containing 3 ml of 1mM ABTS solution and 0.1 ml peroxidase sample, added 0.1 ml of 3.2 mM H_2O_2 solution, mixed the contents immediately. Now placed the cuvette in the spectrophotometer and started measuring the absorbance at 412 nm as a function of time for 2 to 3 minutes at 10 sec interval using stop watch.

3.3.1.4 *Unit of activity*

One unit of lactoperoxidase activity is defined as that amount of enzyme catalyzing the oxidation of 1 umole of ABTS per minute at 20°C (molar absorption coefficient 32,400 M^{-1} cm^{-1}).

3.3.1.5 *Calculation*

The activity in units/ml was calculated as follows:

$$\frac{\text{Change in absorbance at 412 nm } (\Delta A_{412}) \times \text{assay volume, V (ml)}}{\text{Extinction coefficient, } \varepsilon \text{ (1mmol}^{-1}\text{cm}^{-1}\text{)} \times \text{sample volume, v(ml)} \times \text{time, t(min)}}$$

The enzymatic reaction of lactoperoxidase with ABTS as chromogenic substrate takes place as follows:

$$2\ ABTS + H_2O_2 \xrightarrow{LPO} 2\ ABTS^{\cdot} + 2H_2O \qquad \ldots\ldots\ldots\ 1$$

Since the degradation of one mole of H_2O_2 yields two moles

of oxidized ABTS (Reaction 1). Therefore, the stoichiometric coefficient $\nu i = 2$ was taken in the denominator of the above relation. Using the above stated volumes, the relation becomes:

$$\frac{\Delta A_{412}/\text{min} \times 3.2}{32.4 \times 0.1} \times \frac{1}{2}$$

$= 0.4938 \ \Delta A412/\text{min}$ Units (u) of lactoperoxidase per ml of the sample

3.3.2 PROTEIN ESTIMATION

Protein determination in samples of milk, whey and crude lactoperoxidase was carried out according to the method of Lowry *et al.* (1951) as given below and absorbance at 280 nm was used to monitor protein in the column eluates (Yoshida and Xiuyun, 1991a).

3.3.2.1 Reagents

(a) Copper sulphate solution (1% w/v)

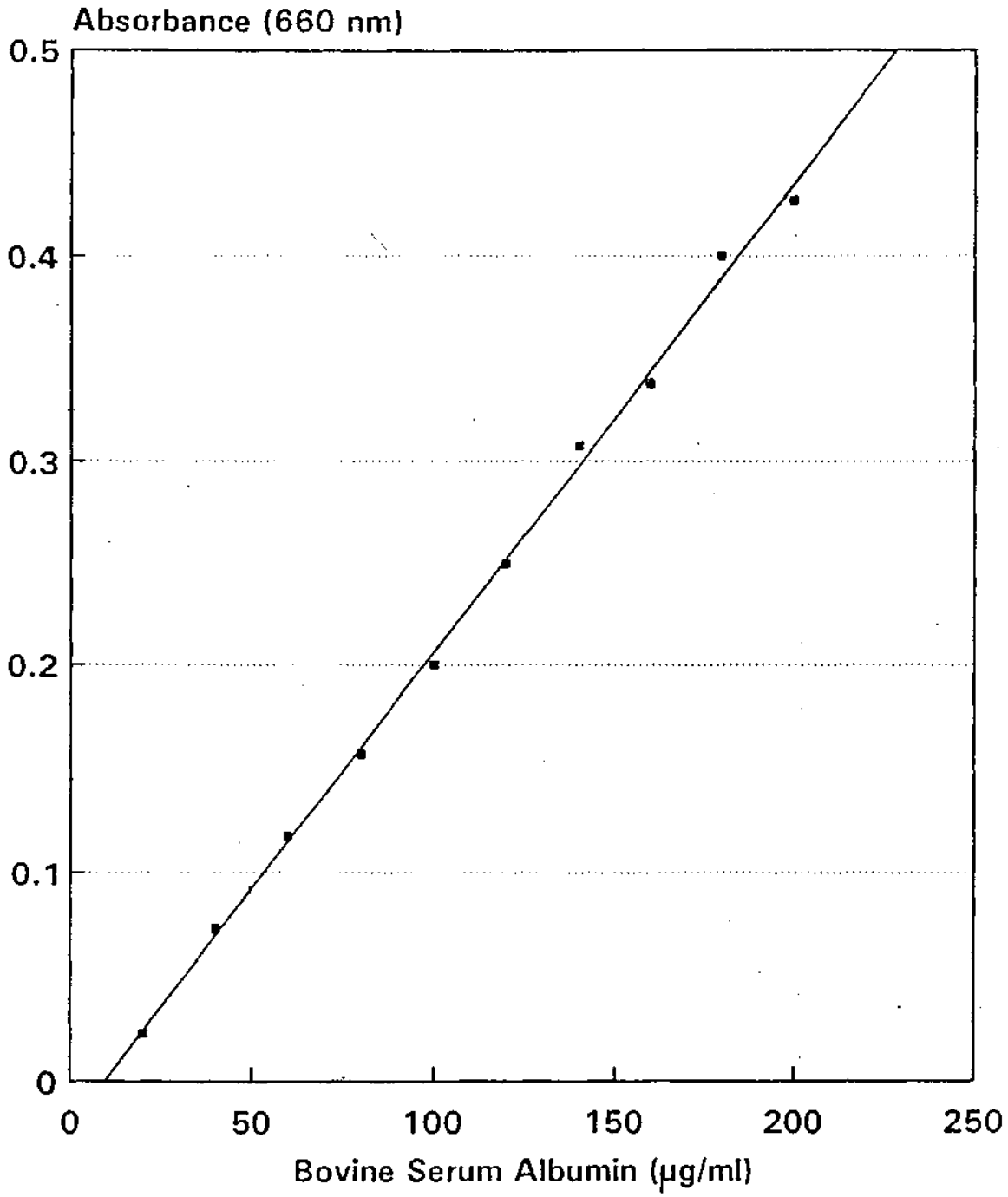
Dissolved 1.0 g of copper sulphate in distilled water and made up the volume to 100 ml.

(b) Sodium potassium tartrate solution (2% w/v)

Dissolved 2.0 g of sodium potassium tartrate in distilled water and made up the volume to 100 ml.

FIG. 3.01

Standard curve for protein determination



(c) Sodium hydroxide solution (0.2 M)

Dissolved 8.0 g of sodium hydroxide pellet in distilled water and made up the volume to one litre.

(d) Sodium carbonate solution (4% w/v)

Dissolved 4.0 g of sodium carbonate solution in distilled water and made up the volume to 100 ml.

(e) Alkaline reagent

To 49 ml of reagent (c) added 49 ml of reagent (d). Then added 1 ml of reagent (a) followed by 1 ml of reagent (b). This reagent was prepared freshly when required.

(f) Folin's reagent

To 10 ml of Folin and Ciocalteau's phenol reagent (Loba), added 10 ml of distilled water.

3.3.2.2 ***Procedure***

To 0.5 ml of suitably diluted sample, added 2.5 ml of alkaline reagent (e). Mixed the contents rapidly and allowed to stand for 10 min. Thereafter, 0.25 ml of Folin reagent (f) was added, mixed immediately and allowed to stand at room temperature for 30 min. The blue colour developed was measured by taking absorbance at 660 nm on Spectronic 21 D spectrophotometer against a blank of 0.5 ml distilled water processed under

identical conditions as sample. Protein content of sample was determined from a standard curve of bovine serum albumin (20 to 200 µg) (Fig. 3.01).

3.3.3 PREPARATION OF LACTOPEROXIDASE

Lactoperoxidase preparation from buffalo skim milk, rennet whey and acid whey was carried out following the method given by Goff *et al.* (1985) with certain modifications.

3.3.3.1 Buffer solutions

I. *Acetate buffer (stock solutions)*

A. Acetic acid solution (0.5 M)

Dilute 29.0 ml of concentrated glacial acetic acid to 1,000 ml with distilled water.

B. Sodium acetate solution (0.5 M)

Dissolved 68 g of sodium acetate trihydrate in distilled water and made up the volume to one litre.

(a) Acetate buffer (0.5 M, pH 6.8)

It was prepared by adjusting the pH of solution B to 6.8 with solution A.

(b) Acetate buffer (0.02 M, pH 6.8)

Diluted the solutions A and B to 20 mM and adjusted the pH of solution B with solution A to 6.8.

(c) Acetate buffer (0.005 M, pH 6.8)

Diluted the 20 mM acetate solution to 5 mM solution with distilled water and adjusted the pH to 6.8 with 5 mM acetic acid.

II. Phosphate buffer

(i) Phosphate buffer (10 mM, pH 6.8)

Diluted the stock solutions (3.3.1.1 a) with distilled water to 10 mM disodium hydrogen phosphate and 10 mM sodium dihydrogen phosphate solution. Mixed these two solutions to a pH 6.8.

(ii) Phosphate buffer (5 mM, pH 6.8)

Diluted the 10 mM phosphate buffer (pH 6.8) by 1:1 with distilled water and adjusted the pH to 6.8 with 5 mM sodium dihydrogen phosphate.

(iii) Sodium chloride solutions (0.1 M, 0.15 M, 0.20 M)

Sodium chloride solutions of molarity 0.1 M, 0.15 M and 0.20 M were prepared by dissolving 5.85 g, 8.775 g and 11.7 g, respectively in 1,000 ml phosphate buffer (10 mM, pH 6.8) for each concentration.

3.3.3.2 Isolation

(a) Cation exchange chromatography

Ten litres of fresh raw skim milk (3.1), rennet whey (3.2.1) and acid whey (3.2.2) was taken as source material for

lactoperoxidase preparation. The protein content and peroxidase activity of the sample was measured. Weakly acidic cation exchanger Amberlite CG-50-NH_4^+ resin (equilibrated with 5 mM sodium acetate buffer, pH 6.8) was added @ 22 g per litre of the sample. The sample-resin mixture was stirred for one hour at 4°C and thereafter allowed the resin to settle for half an hour. The sample was then decanted taking care to prevent the loss of resin. The sample-resin mixture was then transferred to a Buchner funnel, layered with Whatman No.1 filter paper and fitted to a vacuum flask. Under gentle vacuum, the resin was washed with (about one litre) distilled water and sodium acetate buffer (20 mM, pH 6.8) (about two litres), till the absorbance of eluate was less than 0.02 at 280 nm.

(b) ***Ammonium sulphate precipitation***

After washing the resin, the elution of bound protein was carried out with 500 mM sodium acetate buffer, pH 6.8 (about 2 to 3 litres) over a Buchner funnel under a gentle vacuum. Measured the enzyme activity in the eluate. Lactoperoxidase was salted out from acetate buffer eluate by addition of ammonium sulphate at the rate of 53 g per 100 ml solution, while stirring it at 4°C. The stirring was continued over a period of 20 h. The enzyme was collected by centrifugation at 10,000 rpm for 30 minutes at 0°C (Kubota centrifuge). The precipitate was taken up in 25 to 30 ml of 5 mM sodium phosphate buffer, pH 6.8 and dialyzed overnight against two changes of the same buffer. The dialyzed lactoperoxidase solution was centrifuged at 10,000 rpm for 30 minutes at 0°C (Kubota centrifuge) to remove any particulate and clear brown supernatant was collected. The

peroxidase activity and protein content of crude lactoperoxidase preparation was determined. The purity index of the preparation (expressed as $R_z = A_{412}/A_{280}$) was determined by measuring the absorbance at 412 nm and 280 nm using Spectronic 21 D spectrophotometer.

3.3.3.3 *Purification*

(a) Cation exchange chromatography

For purification of crude lactoperoxidase, 3.0 cm x 10.0 cm column was packed with cation exchanger CM Sephadex C-50 (Pharmacia), previously equilibrated with 10 mM sodium phosphate buffer, pH 6.8. About 25 to 30 ml of crude lactoperoxidase obtained was allowed to enter the column at a very slow rate in order to obtain a concentrated band of the material at the top of the column. The column was then washed with 100 ml of 10 mM sodium phosphate buffer, pH 6.8. Thereafter, it was washed with three column volumes of 10 mM sodium phosphate buffer, pH 6.8 containing 100 mM NaCl. A linear gradient of 100 to 150 mM NaCl in 10 mM sodium phosphate buffer (pH 6.8) was applied to the coloumn. The elution of lactoperoxidase was obtained with a second gradient of 150 to 200 mM NaCl in 10 mM sodium phosphate buffer (pH 6.8). The eluate was collected from the column at a flow rate of 60 ml per hour using peristaltic pump (Pharmacia) in 10 ml fractions. It was assayed for peroxidase activity and the absorbance was measured at 412 as well as 280 nm. The fractions of R_z value 0.79 or higher were pooled and dialyzed against distilled water. The dialyzate was concentrated to about 10 ml by lyophilization. The activity as well as protein concentration of the purified preparation was measured.

(b) Gel permeation chromatography

A final purification step employing gel filtration on Sephadex G-100 was used to remove traces of contaminating proteins. A column of 100 cm x 2.5 cm was packed with Sephadex G-100 (32 g Sephadex G-100, previously equilibrated with 0.1 M phosphate buffer, pH 6.8). The concentrated sample was loaded into the column carefully. Lactoperoxidase was eluted with 0.1 M phosphate buffer (pH 6.8). The eluate was collected at a flow rate of 35 ml per hour using peristaltic pump (Pharmacia) in 10 ml fractions. It was assayed for peroxidase activity. The absorbance of the eluted fractions was measured at 412 nm and 280 nm, respectively. Brownish colour lactoperoxidase fractions with R_z value 0.90 or higher were pooled and their activity as well as protein concentration was determined. Purified enzyme was dialyzed against distilled water and lyophilized. It was stored in deep freeze in a desiccator.

(c) Fast protein liquid chromatography (FPLC)

To further assess the purity of lactoperoxidase preparation obtained after gel filtration, the size exclusion fast protein liquid chromatography (Pharmacia) was performed.

The column of Superose 12 pg (1.6 cm x 90 cm) was programmed for elution. Six mg of purified lactoperoxidase was dissolved in 0.5 ml of sodium phosphate buffer (0.1 M, pH 7.0) and injected to the column of Superose 12 pg. The elution of lactoperoxidase was carried out with phosphate buffer (0.1 M, pH 7.0) at a flow rate of 0.4 ml/min, regulated with peristaltic pump (Pharmacia)

in 2.0 ml fractions. The absorbance of fractions was measured at 412 and 280 nm with spectrophotometer. The enzyme activity of the fractions was also estimated.

3.4 CATALYTIC PROPERTIES OF LACTOPEROXIDASE

3.4.1 pH OPTIMA

The effect of pH on peroxidase activity of purified enzyme preparation was determined using ABTS as chromogenic substrate, dissolved in 0.1 M acetate buffer (pH 4 to 5) and 0.1 M phosphate buffer (pH 6 to 8) to a final concentration of 1 mM.

3.4.2 pH STABILITY

3.4.2.1 Solutions

(a) Citrate-phosphate buffer (Stock solutions).

A. Citric acid (0.1 M)

21.01 g of citric acid was dissolved in one litre distilled water.

B. Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) (0.2 M)

Dissolved 35.6 g $Na_2HPO_4.2H_2O$ in distilled water and make up the volume to one litre.

Citrate phosphate buffer (0.1 M, pH 3.0, 4.0, 5.0, 6.0, 7.0)

Mixed the solution A to solution B in such a proportion to

adjust the pH to 3.0, 4.0, 5.0, 6.0 and 7.0, and dilute each solution by 1:1 with distilled water.

(b) Glycine-NaOH buffer (stock solution)

A. Glycine solution (0.2 M)

Dissolved 15.01 g of glycine in distilled water and make up the volume to one litre.

B. Sodium hydroxide (NaOH) solution (0.2 M)

Dissolved 8.0 g of NaOH pellets in distilled water and make up the volume to one litre.

Glycine-NaOH buffer (0.1 M, pH 8.0, 9.0, 10.0)

Mixed the solution A to solution B in such a proportion to adjust the pH to 8.0, 9.0 and 10.0, dilute each solution by 1:1 with distilled water.

3.4.2.2 Procedure

The pH stability of buffalo lactoperoxidase of specific activity 225 U/mg was studied over a pH range of 3 to 10. The 2.0 ml incubation mixture consisted of lactoperoxidase at concentration of 50 ug/ml in citrate-phosphate buffer (pH 3 to 7) and glycine-NaOH buffer (pH 8 to 10) at 25°C for 24 hours.

3.4.3 EFFECT OF CHEMICAL SUBSTANCES

Lactoperoxidase preparation was exposed to different

chemical substances such as sodium azide (SRL), potassium cyanide (BDH), 1,10-phenanthroline (SRL), N-ethylmaleimide (Sigma), iodo- acetamide (Koch Labs,England), hydroxylamine-hydrochloride (SRL), ethylenediaminetetraacetic acid (EDTA) (SRL), mercuric chloride ($HgCl_2$)(SRL), ethylene glycol-bis (ß-aminoethyl ether) N,N,N',N'- tetraacetic acid (EGTA) (Sigma) and copper sulphate ($CuSO_4.5H_2O$) (Loba) for a fixed period and measured the residual enzyme activity. The 2.0 ml incubation mixture consisted of chemical compound at concentration of 0.5 to 10 mM (except EGTA and $CuSO_4$, 10-50 mM each) and lactoperoxidase (10 ug/ml) in 0.01 M phosphate buffer (pH 7.0) at 25°C for 10 minutes. Residual enzyme activity was measured using ABTS as substrate at pH 6.0.

3.4.4 EFFECT OF WHEY PROTEINS

3.4.4.1 Solutions

(a) Phosphate buffer saline (PBS), pH 7.2

Dissolved 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 500 ml distilled water and make up the volume to one litre.

(b) Tris-HCl buffer (0.05 M, pH 8.4)

A. Tris (hydroxymethyl) amino methane solution (0.2M) Dissolved 24.2 g Tris (Sigma) in 1000 ml of distilled water.

B. 0.2 M HCl

Tris-HCl buffer (0.05 M, pH 8.4)

To 50 ml of A, added 16.5 ml of B and diluted to a total of 200 ml. This solution was further diluted 1:1 by distilled water.

(c) Sodium chloride solutions (0.2 M and 0.5 M)

These solutions of concentration 0.2 M and 0.5 M were prepared by dissolving 11.7 g and 29.25 g NaCl, each in 1000 ml Tris-HCl buffer (0.05 M, pH 8.2).

(d) 0.1 M citric acid solution

Dissolved 21.01 g citric acid in one litre of distilled water.

3.4.4.2 Preparation of buffalo lactoferrin

Lactoferrin was isolated from buffalo colostrum by the method of Law and Reiter (1977). The protein was bound to CM Sephadex C-50 and lactoferrin was eluted with 0.5 M NaCl in 50 mM Tris-HCl buffer. The crude lactoferrin obtained was dialyzed against distilled water and rechromatographed on CM Sephadex C-50, previously equilibrated with 50 mM Tris HCl buffer (pH 8.0). The column was washed with 0.2 M NaCl in 50 mM Tris HCl buffer (pH 8.0) and then eluted with a linear gradient of 0.2 to 0.5 M NaCl in 50 mM Tris-HCl buffer. The protein was dialyzed and freeze dried. The lactoferrin thus obtained was further purified by gel filtration on Sephadex G-100 and the purified lactoferrin obtained was used for studies.

3.4.4.3 *Preparation of apo lactoferrin*

The procedure of Masson and Herrmans (1968) was followed. Purified lactoperoxidase (1%) was extensively dialyzed against excess of 0.1 M citric acid with regular changes after every six hours. Then it was dialyzed against distilled water at 4°C. The colourless apo lactoferrin was lyophilized.

3.4.4.4 *Preparation of γ-globulins (total Igs)*

The γ-globulins from buffalo colostrum was prepared according to the method of Heide and Schwick (1967).

Buffalo colostrum was diluted 1:6 with distilled water and acid whey was prepared using HCl. To 500 ml of colostral whey, ammonium sulphate was added slowly at 4°C to a final saturation of 33 per cent, while continuously stirring for 2 hours. Then the solution was kept for 1 hour and centrifuged at 10,000 rpm for 30 min at 4°C (Kubota centrifuge). The precipitates were collected and dissolved in 100 ml distilled water and reprecipitated with ammonium sulphate to 33 per cent saturation. The above procedure was repeated twice and finally collected the precipitates in 20 ml distilled water, dialyzed against distilled water and freeze dried. This preparation was used for studies.

3.4.4.5 *Preparation of whey protein isolate*

The acid whey prepared (section 3.2.2) was dialyzed against distilled water and freeze dried. This preparation was used for studies.

3.4.4.6 *Other whey proteins*

Alpha-lactalbumin (Sigma), ß-lactoglobulin (Sigma) and bovine IgG_1 (Miles) were obtained for experimental work.

3.4.4.7 *Procedure*

The effect of immunoglobulin (bovine IgG_1, buffalo Igs), buffalo lactoferrin and apolactoferrin, alpha-lactalbumin, ß-lactoglobulin and whey protein isolate on lactoperoxidase was achieved by timed incubation at 4°C or 25°C. The 2.0 ml incubation mixture consisted of added protein at concentration of 5 mg/ml and buffalo lactoperoxidase at 2 µg/ml in PBS. This mixture was incubated at 4°C for 12 hrs and 25°C for 4 hrs. The change in lactoperoxidase activity during incubation was compared with reference tubes containing lactoperoxidase alone. The measurement of peroxidase activity was carried out at pH 6.0 using ABTS as chromogenic substrate.

3.4.5 *EFFECT OF COLD STORAGE*

Buffalo milk was stored at -20°C, 0°C and 4°C for one week. The enzyme activity of the samples was measured after regular interval of 24 h.

3.4.6 *EFFECT OF HEAT TREATMENT*

3.4.6.1 *Kinetics of heat inactivation*

(a) *Order of heat inactivation*

To determine the order of heat inactivation of lactoperoxidase, 2 ml each of buffalo milk and buffalo milk + lactoperoxidase (200 µg/ml) was heated in test tubes (12 mm x 100 mm) at 71°C in water bath. It took around 3 min to reach the set

temperature. This time period was taken as zero time and heat treatment was conducted for 0, 5, 10, 20 and 30 min. The residual enzyme activity was measured. From the plot of ln (residual activity) vs. time, the slope of the lines was determined.

(bi) Milk, rennet whey and neutralized acid whey

Two ml of buffalo skim milk/rennet whey/neutralized acid whey (pH 6.8) was taken in test tubes (12 mm x 100 mm) and immersed in water bath set at desired temperature. The contents of the tubes reached the set temperature in around 3 min. The zero time was taken as 3.0 min after the moment of immersion and tubes were transferred from the water bath at 0, 5, 10, 20 and 30 min to a container with melting ice. The samples were immediately analysed for peroxidase activity.

(bii) Lactoperoxidase in buffer

Lactoperoxidase preparation (50 μg/ml) equilibrated overnight at 4°C with acetate buffer (0.1 M, pH 6.0). Then its thermal inactivation behaviour was studied in a similar way as described in (b_i). Enzyme activity was estimated using 0.1 M acetate buffer, pH 4.4

(c) Computation of kinetic and thermodynamic parameters

The decrease of enzymic activity resulting from heat treatment follows first order reaction kinetics:

$$\frac{da}{dt} = -ka$$

or, after integration

$$\ln (a/a_o) = - kt$$

where, a is the activity at time t and

a_o the activity at t=0.

A plot of $\ln(a/a_o)$ vs.time is a straight line with slope -k. The reaction constant, k, is temperature dependent. The Arrhenius or activation energy, A of this reaction can be calculated from the temperature dependence of k using the relation:

$$A = RT^2 (d \ln kT/dT)$$

where T is the absolute temperature and R the gas constant. After integration, the result is:

$$\ln kT = (\triangle A/R) (1/T) + \text{constant}$$

A plot of ln kT against 1/T is a straight line with slope A/R. It is assumed that A is the temperature independent in the temperature interval studied.

3.4.6.2 Effect of temperature of heat treatment

The thermostability of lactoperoxidase in buffalo milk was determined by taking 2.0 ml of fresh buffalo milk in a test tube (12 mm x 100 mm) and placed in the water bath at set temperature (70 to 85°C). It took around 3 min to reach the required temperature. This time period was taken as zero time. Heat treatment was conducted for 30 sec and immediately placed the tubes in a melting ice container. The residual enzyme activity of the samples was analysed.

3.4.6.3 *Effect of pH*

The effect of pH on thermostability of lactoperoxidase in whey (acid/rennet) was determined. Both rennet whey (3.2.1) and acid whey (3.2.2) were adjusted to pH 4.6, 5.5, 6.0, 6.8 and 7.5 and centrifuged at 2,000 rpm for 10 min (Kubota centrifuge). The clear supernatant whey sample was taken. Two ml of sample was taken in test tube (12 mm x 100 mm) and heat treated at 72°C in water bath. It took around 3 min to reach the set temperature. This time period after immersion of tubes in water bath was taken as zero time and heated for 0, 10, 20 and 40 min. Tubes were immediately placed in a melting ice container and measured the residual enzyme activity.

3.4.6.4 *Effect of salts*

Lactoperoxidase (50 µg/ml) in acetate buffer (0.1 M, pH 6.0) was heated in test tubes (12 mm x 100 mm) at 71°C for 0, 5, 10, 20 and 30 min in the presence of 100 mM of each salt [sodium chloride (NaCl), potassium chloride (KCl), barium chloride ($BaCl_2$), manganese chloride ($MnCl_2$), calcium chloride ($CaCl_2$), sodium sulphate (Na_2SO_4), magnesium sulphate ($MgSO_4$), potassium sulphate (K_2SO_4)] and assayed for enzyme activity using acetate buffer (0.1 M, pH 4.4).

3.5 *PHYSICO-CHEMICAL PROPERTIES OF LACTOPEROXIDASE*

3.5.1 *SPECTRAL ANALYSIS*

Absorbance spectra of purified lactoperoxidase preparations from buffalo skim milk, rennet whey and acid whey at

concentrations of 0.7, 0.6 and 0.4 mg/ml in 0.1 M phosphate buffer (pH 7.0) was scanned over 200 nm to 600 nm using double beam spectrophotometer (JASCO).

3.5.2 DISC GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed by the method of Groves (1975) using LKB disc gel electrophoresis unit.

3.5.2.1 SOLUTIONS

(a) Electrode buffer (pH 5.0)

62.4 g of ß-alanine (E. Merck) was dissolved in distilled water, to it added 8.0 ml of glacial acetic acid and made the volume to two litres by distilled water.

(b) Running gel buffer (pH 4.3)

To 48 ml of 1 N potassium hydroxide solution, added 4.0 ml of N,N,N'N'-tetramethyl ethylenediamine (TEMED) and 17.2 ml of glacial acetic acid, finally made up the volume to 100 ml with distilled water.

(c) Stacking gel buffer (pH 6.7)

To 48 ml of 1 N potassium hydroxide solution, added 0.46 ml TEMED and 2.87 ml of glacial acetic acid. Made up the final volume to 100 ml with distilled water.

(d) Acrylamide stock

30 g of acrylamide (Sigma) and 0.8 g of N,N'-methylene bisacrylamide (Bis) (Spectrochem) was dissolved in distilled water and made up the volume to 100 ml. The solution was filtered using Whatman No.1 filter paper and clear solution was stored in a dark bottle at 4°C.

(e) Stacking gel stock

10 g of acrylamide (Sigma) and 2.5 g Bis (Spectrochem) was dissolved in distilled water and made up the volume to 100 ml. The solution was filtered using Whatman No. 1 filter paper and clear solution was stored at 4°C in a dark bottle.

(f) Ammonium persulphate solution

Dissolved 0.15 g ammonium persulphate in distilled water and made up the volume to 50 ml with distilled water.

(g) Resolving gel solution (7.5% acrylamide)

To 8.0 ml of acrylamide stock, added 4.0 ml of running gel buffer (pH 4.3) and 19.0 ml of distilled water. This solution was then degassed under vacuum.

(h) Stacking gel solution (2.5% acrylamide)

To 2.0 ml of stacking gel buffer (pH 6.7), added 4.0 ml of stacking gel solution and 9.2 ml of distilled water. This 15.2 ml stacking gel solution was degassed under vacuum.

(i) Protein fixing and staining solution

Dissolved 1.0 g of Coomassie Brilliant Blue R-250 in a solution of 227 ml methanol and 46 ml glacial acetic acid. Then made the volume to 500 ml with distilled water. The staining solution was filtered through Whatman No.1 filter paper.

(j) Destaining solution

Mixed 300 ml of methanol with 100 ml of glacial acetic acid and made the volume to one litre with distilled water.

(k) Lactoperoxidase staining

The staining of peroxidase on polyacrylamide gel was carried out using 3,3',5,5'-tetramethylbenzidine (TMBZ)-hydrogen peroxide (H_2O_2) as a stain following the method of Thomas *et al.* (1976).

A 6.3 mM TMBZ (Sigma) solution was freshly prepared in methanol. Immediately before use, 3 parts of TMBZ solution were mixed with 7 parts of 0.25 M sodium acetate, pH 5.0. The gels were immersed in this mixture at room temperature in the dark. After 2 h with occasional mixing at every 10 to 15 min, H_2O_2 was added to a final concentration of 3.0 mM. The staining was visible within 3 min and increased in intensity over next 30 min. Thereafter, the gels were placed in a 3:7 mixture of isopropanol and 0.25 M sodium acetate buffer, pH 5.0. The acetate buffered 30 per cent isopropanol solution was replaced once or twice with fresh solution to remove any precipitated TMBZ. Gels were

photographed after 1 h of placing them in buffered 30 per cent isopropanol solution.

3.5.2.2 *Sample preparation*

To 1 mg of purified lactoperoxidase, added 0.3 ml of stacking gel buffer (pH 6.7), 0.02 ml glycerol and 0.01 ml of 0.2 per cent methyl green as tracking dye.

3.5.2.3 *Casting the gels*

Dry glass tubes of 120 mm in length and ? mm internal diameter were used. The lower end of the tubes was closed with parafilm 'M' Laboratory Film (American Can Company, Greenwich) and held vertical in the gel casting rack (Canalco, U.S.A.).

To the degassed 31 ml resolving gel solution, added 1.0 ml of one per cent ammonium persulphate solution and mixed gently. It was then slowly filled into the tubes with the help of a syringe taking care to avoid entraping air bubbles in the gel. Gel was filled upto 100 mm length. After polymerization of separating gel, stacking gel was added over the resolving gel upto a height of 115 mm.

3.5.2.4 *Electrophoresis assembly*

The para film was removed and the tubes were held vertically in the holes fitted with rubber gammets of the upper reservoir of the electrophoretic cell Model 150 A (BIO-RAD Laboratories, U.S.A.). The lower and upper reservoir were filled with

electrode buffer (pH 4.3), with both ends of gel dipping in electrode buffer. The electrophoretic assembly was connected to power supply and a pre-run was given at 60 mA for one hour.

3.5.2.5 *Sample application*

Sample was applied to the gel with the micro-syringe

3.5.2.6 *Power setting and time*

The electrophoresis was run at 5 mA per tube for 2 h at 10°C maintained with the Multi Temp Cooling Unit (LKB, Sweden).

3.5.2.7 *Lactoperoxidase staining*

Immediately after the electrophoresis, the gels were taken out of the tubes by carefully injecting water into the space between the walls of the tube and the gel. Lactoperoxidase staining on the gel was performed by TMBZ-H_2O_2 stain.

3.5.2.8 *Protein fixing and staining*

For protein staining, the gels were placed into the staining solution for one hour.

3.5.2.9 *Destaining*

After staining, the gels were rinsed with distilled water and immersed in the destaining solution. The destaining solution was continued to change till clear background obtained.

3.5.3 DETERMINATION OF MOLECULAR WEIGHT OF LACTOPEROXIDASE

3.5.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of lactoperoxidase was determined by polyacrylamide gel electrophoresis as described by Laemmli (1970), with some modifications for slab gel electrophoresis from the standard curve drawn by plotting the electrophoretic mobility of marker proteins against their molecular weight (Fig. 3.02). The equipment used was 2001-001 vertical gel electrophoresis unit (LKB Produkter AB, Sweden) and power supply unit (LKB 2301 Macro Drive 1)

(a) Solutions

(i) Tris-glycine electrode buffer (pH 8.3, 0.025 M tris, 0.192 M glycine):

Dissolved 15.15 g tris, 72 g glycine and 5 g SDS in about four litres of distilled water. Adjusted the pH to 8.3 and made the volume to five litres with distilled water.

(ii) Tris-SDS stock solution (pH 8.8, 0.75 M tris, 0.2%SDS):

Dissolved 90.8 g tris and 2 g SDS in about 800 ml distilled water.Adjusted the pH to 8.8 with 2 N HCl and made the volume to 1000 ml with distilled water.

(iii) Tris-SDS stock solution (pH 6.8, 0.25 M tris, 0.2% SDS):

Dissolved 30.25 g tris and 2 g SDS in about 800 ml distilled

water.Adjusted the pH to 6.8 with 2 N HCl and made the volume to 1000 ml with distilled water.

(iv) Sample buffer (pH 6.8, 0.0625 M tris):

Dissolved 2 g SDS and 2 mg bromophenol blue as tracking dye in about 40 ml distilled water. To it added, 25 ml of tris-SDS stock solution (pH 6.8), 5 ml of 2-mercaptoethanol and 10 ml glycerol and finally made up the volume to 100 ml with distilled water.

(v) Acrylamide stock:

30 g acrylamide (Sigma) and 0.8 g N, N'-methylene-bis-acrylamide (Spectrochem) was dissolved in distilled water and made up the volume to 100 ml. It was filtered through Whatman No.1 filter paper and kept in dark bottle at 4°C.

(vi) Ammonium persulphate solution:

Dissolved 0.1 g ammonium persulphate in distilled water and made up the volume to 10 ml with distilled water. This solution was prepared freshly every time.

(vii) Resolving gel solution:

To 8.6 ml acrylamide stock solution, added 20 ml tris-SDS stock solution (pH 8.8) and 1.5 ml ammonium persulphate solution and made the volume to 40 ml with distilled water.

(viii) Fixing and staining solution:

Dissolved 1.0 g Coomassie Brilliant Blue R-250 in a solution of 227 ml methanol and 46 ml glacial acetic acid. Then made up the volume to 500 ml with distilled water. The staining solution was filtered through a Whatman No.1 filter paper.

(ix) Destaining solution:

Mixed 300 ml of methanol with 100 ml of acetic acid and made up the volume to one litre with distilled water.

(b) Preparation of sample

One to two mg of finally purified buffalo milk lactoperoxidase was dissolved in 0.4 ml of sample buffer in an eppendorf tube. Similarly, standard proteins Amyloglucosidase (97 kDa), Buffalo lactoferrin (78.5 kDa), Bovine serum albumin (68 kDa), chicken egg ovalbumin (45 kDa) and α-lactalbumin (12.5 kDa) were dissolved in sample buffer to a final concentration of 5 ug per ul of sample buffer. The lactoperoxidase sample as well as standard protein solutions were placed in a boiling water bath for 3 min for complete denaturation of proteins. Then these were cooled to room temperature and refrigerated.

(c) Casting of gels

A dry glass plate was placed on the inverted casting stand (LKB) with the short sides extending, greased spacers (1.5 mm) were placed along each of the short sides and covered with a

second glass plate, forming a sandwich. Slided a clamp over the edge of the sandwich, tightened a screw and repeated the process with the second clamp. The glass plate set was placed on the flat surface, loosened the screws and slided the clamps as down as possible and then hand tightened all screws. The sandwich of glass plates were turned upside down and applied a trace amount of celloseal (LKB) to the edges of the plates. The casting stand was turned right side and slided the sandwich fully on the silicone rubber inserts into the casting stand. The cams were inserted into each side of the casting stand and turned them 180°. The comb was inserted on into the top of the glass plate sandwich. The casting stand containing the gel mould was levelled.

40 ml of resolving gel solution was degassed and to it added 0.1 ml of N,N,N',N'-tetramethylenediamine (TEMED). Mixed it gently taking care to avoid incorporation of air in the gel solution. The gel solution was filled in the gel mould by the side of the spacer with the help of a syringe till the solution touched the teeth of the comb. After removing the trapped air bubbles, the gel mould was filled to 2 mm from the top. Carefully overlayed the gel surface with distilled water and allowed to polymerize for one hour at room temperature. The comb was removed and the wells were rinsed with distilled water. Filled the wells with electrode buffer by a syringe.

(d)Mounting gels in the upper buffer reservoir

Applied cello-seal to the rubber gaskets and fitted them over the two moulds till their cover ends extended upto the top edge of the clamps. The upper buffer reservoir was lowered over the mould till it was fully seated over the gaskets. The reservoir was pressed firmly and the cams were placed through the camming holes on each side and rotated the cams through 180°. Holding the reservoir, lifted the mould out of casting stand.

(e) Assembling the electrophoresis unit

An electrode was placed into lower reservoir and the gel mould with upper reservoir was fitted into this reservoir. The electrode buffer was filled into the upper as well as lower reservoir and covered the electrophoresis unit with safety lid. It was attached to a power supply unit (LKB, Macro Drive 1). The pre-run was given at 100 mA for one hour.

(f) Sample application

With the help of a microsyringe, samples were carefully layered through the buffer, in the wells to give a uniform layer at the bottom of wells. Ten ul of each sample was applied.

(g) Power setting and time

The electrophoresis was run at 60 mA for 1.5 h when the tracking dye had reached the bottom of the gel.

(h) Dismantling the gel mould

Immediately after electrophoresis, the power supply was switched off and removed the safety lid. The upper buffer reservoir and the gels were lifted out of the lower buffer reservoir and electrode buffer from upper reservoir was decanted. The cams were loosened and the gel mould was pulled out of the reservoir. The clamps were removed and slided the spacers partially out of the glass plate sandwich. The spacers were twisted to pry apart the glass plates.

(i) Fixing and staining

Gel was gently placed into the staining solution and kept it there for one hour.

(j) Destaining

The staining solution was decanted off and the gel rinsed with destaining solution. Then the gel was placed in the destaining solution, with several changes, until the background was clear.

3.5.3.2 Gel filtration

Molecular weight of lactoperoxidase was determined by gel filtration on Sephadex G-100 column (Whitaker, 1963). The Kav values of purified lactoperoxidase sample and marker proteins was determined and from the plot of Kav values of marker proteins and their molecular weights (Fig. 3.03), the molecular weight of lactoperoxidase was calculated.

Fig. 3.03
Standard curve for determination of molecular weight by Gel filtration

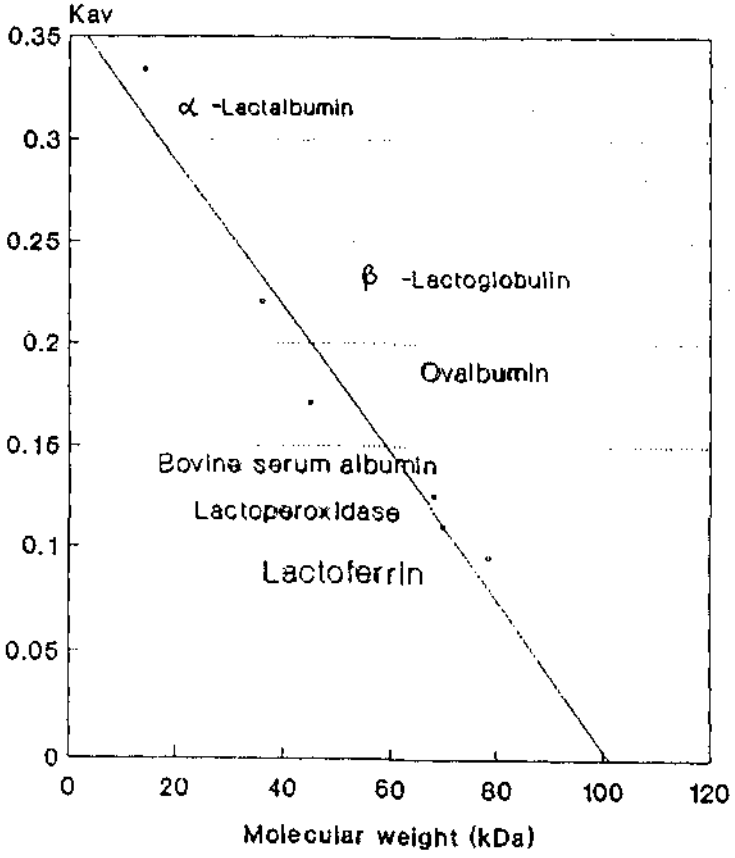
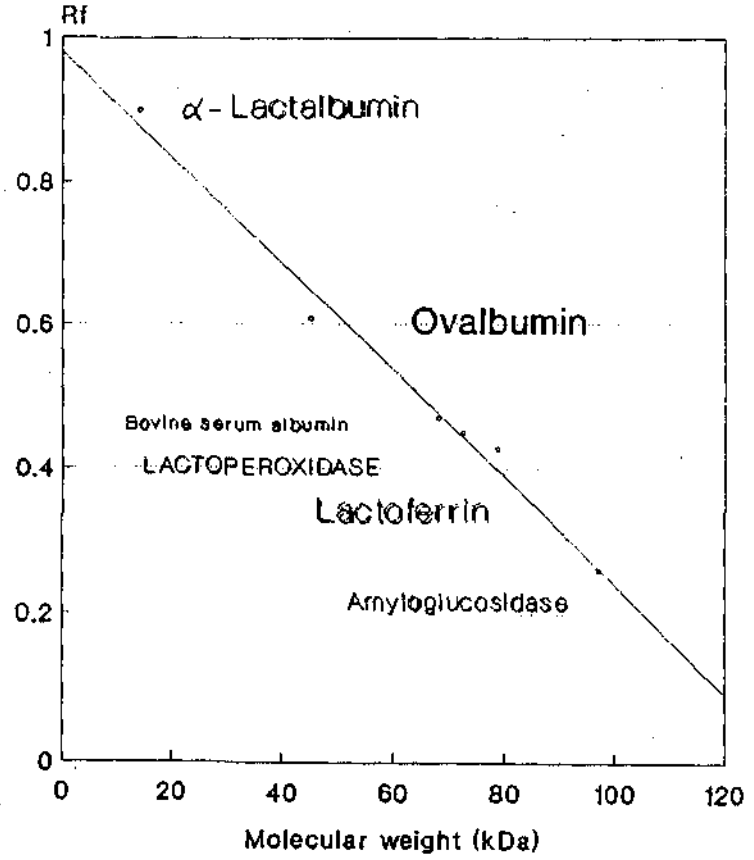


Fig. 3.02
Standard curve for determination of molecular weight by SDS-PAGE



(a) Packing of the column

32 g of Sephadex G-100 (Pharmacia) was soaked in distilled water for 48 h and then in 0.1 M sodium phosphate buffer, pH 7.0 for 12 h. It was packed into the column 1.6 x 100 cm and equilibrated overnight to a bed height of 80 cm.

(b) Calibration of the column

The column was calibrated for void volume (V_o) and total volume (V_t) using blue dextran and bromophenol blue, respectively. The flow rate of the column was regulated to 35 ml per hour using peristaltic pump (Pharmacia). The eluent was monitored with UVICORD (LKB) at 280 nm and recorded on REC-482 recorder (Pharmacia). Ten mg of each marker protein buffalo lactoferrin, 78 kDa; bovine serum albumin, 68 kDa; chicken egg ovalbumin, 43 kDa; ß-lactoglobulin, 36 kDa and α-lactalbumin, 12 kDa) was applied to the column and eluted with 0.1 M, sodium phosphate buffer, pH 7.0. The elution volume (V_e) of each marker protein was determined.

(c) Application and elution of lactoperoxidase

Ten mg of purified lactoperoxidase dissolved in one ml of 0.1M sodium phosphate buffer, pH 7.0 was applied to the calibrated column and determined its elution volume (V_e).

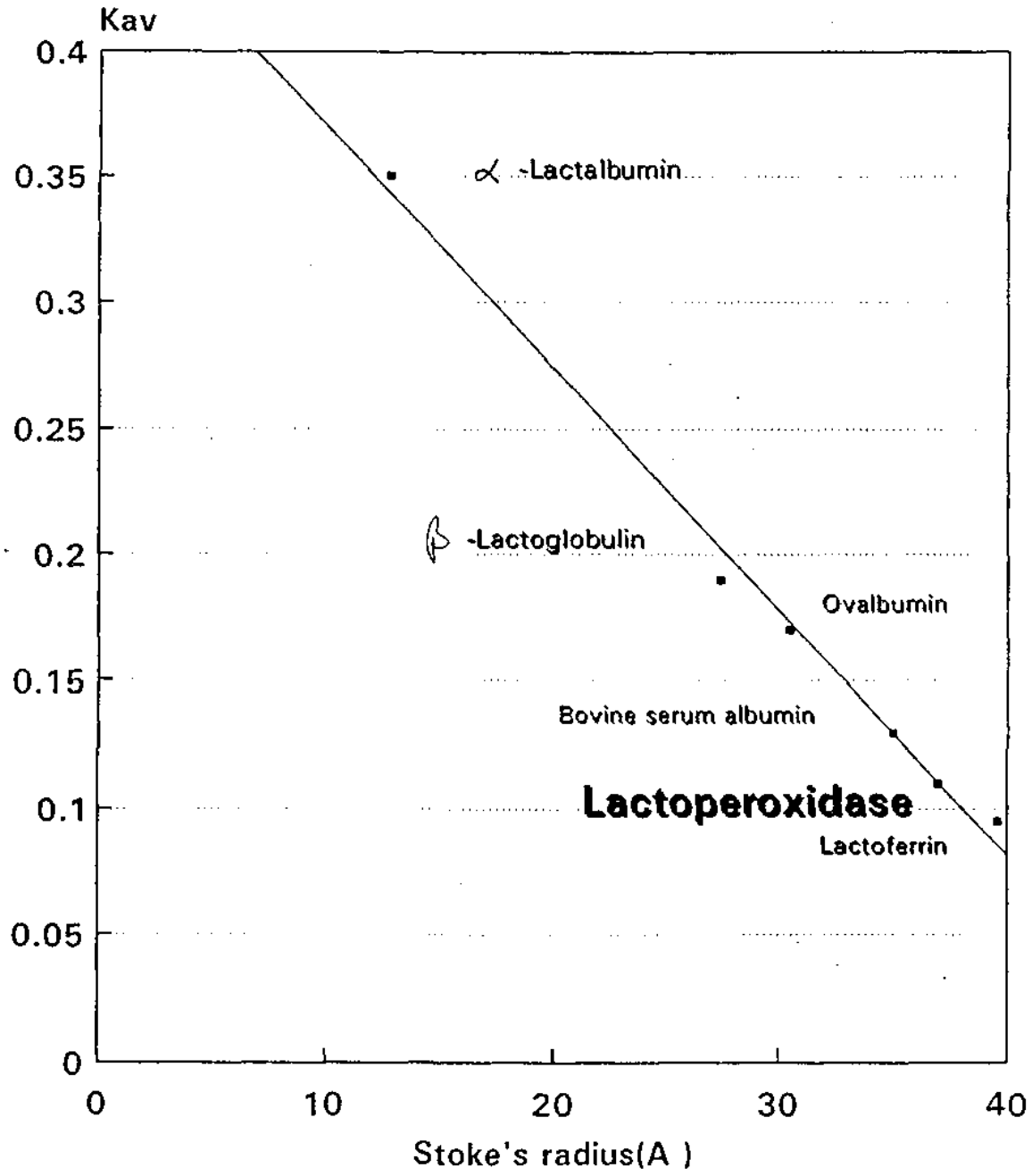
(d) Determination of K_{av}

The K_{av} value of lactoperoxidase sample and marker proteins was determined using following relationship:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

FIG. 3.04

Standard curve for determination of Stoke's radius



3.5.3.3 Determination of Stoke's radius of lactoperoxidase

Stoke's radius of purified lactoperoxidase was determined by chromatography on Sephadex G-100 (3.5.3.2) and was calculated from the plot of Kav values versus Stoke's radii of standard marker proteins [α-lactalbumin (12.8 A°), ß-lactoglobulin (27.4 A°), ovalbumin (30.5 A°), bovine serum albumin (35.0 A°) and lactoferrin (40.2 A°) (Fig. 3.04).

3.5.4 DETERMINATION OF ISOELECTRIC POINT OF LACTOPEROXIDASE

Isoelectric point of purified lactoperoxidase from buffalo milk was determined by analytical electrofocusing in thin layers of polyacrylamide gels (LKB) from calibration curve (Fig. 3.05).

3.5.4.1 Solutions

(a) Acrylamide solution (29% w/v)

Dissolved 29.1 g acrylamide (Sigma) in 75 ml of distilled water. Stirred until clear solution formed. Then made up the volume to 100 ml with distilled water. Filter through Whatman No.1 filter paper and stored it in a dark bottle at 4°C for maximum one week.

(b) N,N'-methylene bis-acrylamide (Bis) (0.9% w/v)

Dissolved 0.9 g Bis in 100 ml of distilled water stirring under gentle heating. Allowed the solution to cool to room

temperature and filtered it using Whatman No.1 filter paper. Stored it in a dark bottle at 4°C for maximum period of one week.

(c) Ammonium persulphate solution (1% w/v)

Dissolved 0.1 g ammonium persulphate in 10 ml of distilled water. This solution is prepared freshly every time.

(d) Glycerol solution (87% w/v)

Diluted 87 ml of glycerol to 100 ml with distilled water.

(e) Electrode solutions

(i) Cathode solution (1 M NaOH): Dissolved 40 g sodium hydroxide (NaOH) in one litre of distilled water.

(ii) Anode solution (1 M H_3PO_4): Added 67.3 ml of 15.7 M phos- phoric acid (H_3PO_4) to 900 ml of distilled water and diluted it further to one litre by distilled water.

(f) Fixing solution

Dissolved 17.3 g sulphosalicylic acid and 57.5 g trichloro-acetic acid in distilled water and diluted it to 500 ml.

(g) Staining solution

Dissolved 0.46 g of Coomassie Brilliant Blue R-250 in 400 ml of destaining solution and filtered it through Whatman No.1 filter paper.

(h) Destaining solution

Mixed 500 ml ethanol with 160 ml acetic acid and made up the volume to 2 litres with distilled water.

(i) Preserving solution

To 500 ml of destaining solution, added 50 ml of glycerol.

(j) Ampholyte reagent

pH gradient of 3.5-9.5 was obtained using LKB 1802 Ampholine (pH 3.5-10.0).

3.5.4.2 *Preparation of standard protein and lactoperoxidase sample solution*

0.5 mg of purified and extensively dialyzed lactoperoxidase from buffalo milk was dissolved in 100 ul of distilled water.

Standard proteins (Trypsin inhibitor from Soybean, pI 4.55; Carbonic anhydrase B from bovine erythrocytes, pI 5.65; Myoglobin from horse heart, pI 6.8/7.2) and methyl red as tracking dye from isoelectric focusing marker kit (Sigma) were dissolved in distilled water each to a concentration of 500 ug per ul.

3.5.4.3 *Preparation of gels*

(i) Mounting the mould

The mould used for making gels that fit LKB multiphor-II

consists of 1 mm supporting glass plate, a 2 mm thick rubber gasket and two 3 mm thick glass plates, all of which clamped together in such a way that supporting glass plate (1 mm) with silicone lubricated rubber gasket placed over it, is sandwiched between two glass plates of 3 mm thick.

(ii) Preparation of gelling solution (5% acrylamide, 3% Bis)

The stock solution used for a pH gradient 3.5-9.5 was prepared by mixing 10 ml acrylamide solution with 10 ml of Bis stock solution, 7 ml of 87 per cent glycerol and 3 ml of pH 3.5-10 (LKB 1802) ampholine. Made up the volume to 60 ml with glass distilled water and degassed the solution for 10 min.

(iii) Filling the mould

To the 60 ml of gelling solution, 1.5 ml of one per cent (w/v) ammonium persulphate solution was added and complete gelling solution was mixed by swirrling the flask taking care to avoid reuptake of air. Then the open end of rubber gasket was slightly lifted and the gelling solution was loaded to the mould, while keeping the mould in slightly tilted position, in order to fill it completely without trapping air bubbles. Then the mould was sealed and made air tight by matching the free ends of rubber gasket and applying the remaining clamp. Polymerization was completed in one hour by placing the mould at room temperature. After polymerization, the mould was stored at 4°C for 30 min without clamps in order to facilitate dismantling.

(iv) Dismantling the mould

The mould was placed flat on the table with the thin supporting glass plate facing downwards. The rubber gasket was partly removed and a thin wetted spatula was inserted between the two glass plates surrounding the gel. The covering thick plate was loosened by gently twisting a broader spatula inserted between two glass plates surrounding the gel. Air is penetrated through the channel made by thin spatula. The covering thick glass plate was lifted off only after totally separating from the gel surface. Finally, the rubber gasket was removed and the edges of the gel were wiped with a folded piece of filter paper.

3.5.4.4 *Setting up of analytical electrofocusing*

Connected the Multiphor II (LKB 2117) to the Multitemp II thermostatic circulator (LKB 2219) and set the temperature to 4°C. Levelled the Multiphor using the three levelling feet. Now placed the template on the cooling plate of the Multiphor with a thin film of light paraffin oil in between. Avoided traping the air bubbles. Poured some light paraffin oil in between along the long edges of the template. Aligned the long edges of the template and the thin glass plate supporting the acrylamide gel and slowly lowered the latter until it rests on the template. Now put the electrode strips soaked in the proper electrode solutions on the gel as indicated by the template and cut off the protruding parts.

3.5.4.5 *Sample application*

The sample was applied on the gel surface by soaking into

rectangular pieces of suitable filter paper (Whatman 3 MM, paper) or as a droplet of 10 ul size.

3.5.4.6 *Running the gel*

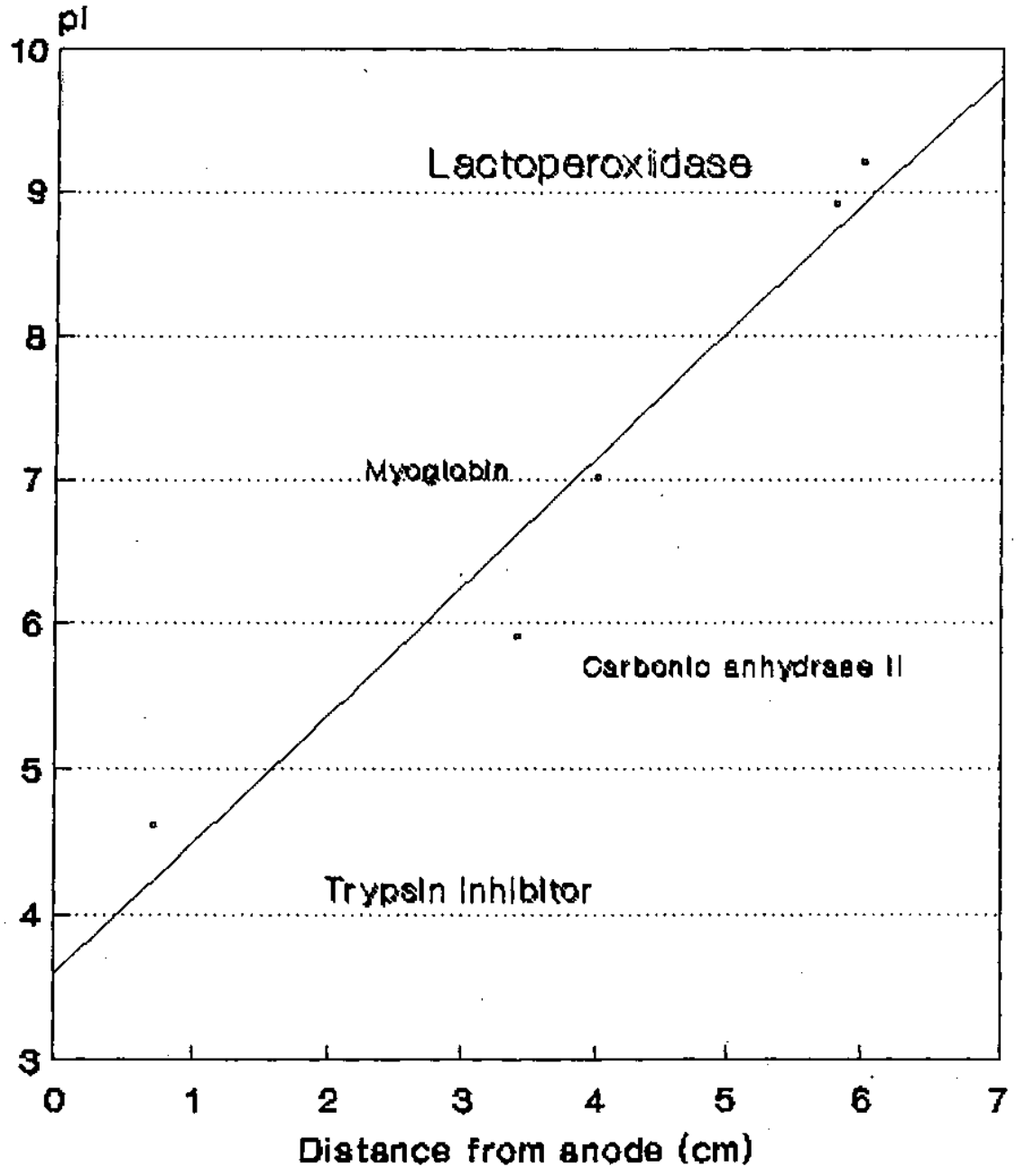
Moveable electrodes were placed on the gel in such a way that the platinum wires matched the electrode strips. Then placed the safety lid back on Multiphor II and connected to a constant wattage power supply (LKB 2297, Macro Drive 5). The gels were run under a constant power of 25 W at 4°C. The filter paper strips were removed from the gel surface half an hour after run. In 1.5 h, the focusing is completed for wide range pH gradient 3.5-9.5, and the progress of electrofocusing was followed by using methyl red as marker dye, which on applying cathodically changed from light yellow to pink in acid range.

3.5.4.7 *Fixing, staining and destaining*

On completion of electrofocusing, the glass plate supporting the gel was carefully lifted and placed in the fixing solution for 1 h. Then the gel was placed in destaining solution for 15 to 30 min in order to wash out the remainder ampholine and adjusted the pH of the gel to match that of the staining solution. Thereafter, the gel was stained by leaving it in the staining solution for 10 min at 60°C, with the lid in place. Now removed the excess stain from the gel by immersing it in the destaining solution and continued changing the destaining solution till the gel background becomes clear.

Fig. 3.05

Standard curve for determination of Isoelectric point(pI) of lactoperoxidase



3.5.4.8 *Preservation of stained gels*

Immersed the fully destained gel in destaining solution containing 10 per cent (v/v) glycerol for one hour. Then soaked a cellophane sheet (LKB 2117-103 accessory kit) in the same solution for a few minutes and wrap it around the gel and the supporting glass plate, while avoiding trapped air. Allowed the wrapped gel to dry at room temperature.

3.5.5 *DETERMINATION OF IRON AND CALCIUM CONTENT OF BUFFALO LACTOPEROXIDASE*

3.5.5.1 *Preparation of sample*

Exactly 10 mg of purified lactoperoxidase was weighed and digested with triacid mixture of nitric acid (HNO_3), perchloric acid ($HClO_4$) and sulphuric acid (H_2SO_4) in the ratio 10:3:1, respectively over hot plate till clear solution was obtained. This clear solution was diluted to 5 ml with glass distilled water and determination of calcium and iron was performed using atomic absorption spectrophotometer (Philips PU-9100 X).

(a) Iron

The concentration of iron in the sample was determined from the standard curve of iron (Fig. 3.06). For preparation of standard curve, a stock solution of iron containing 100 ug per ml was prepared by dissolving 0.1775 g ammonium ferrous sulphate $(NH_4)_2FeSO_4.6H_2O$ (Glaxo) with glass distilled water and made up the volume to 250 ml. Aliquots of 2.0,4.0,8.0,10.0 and 12.0 ppm

Fig. 3.07
Standard curve for determination of calcium in lactoperoxidase

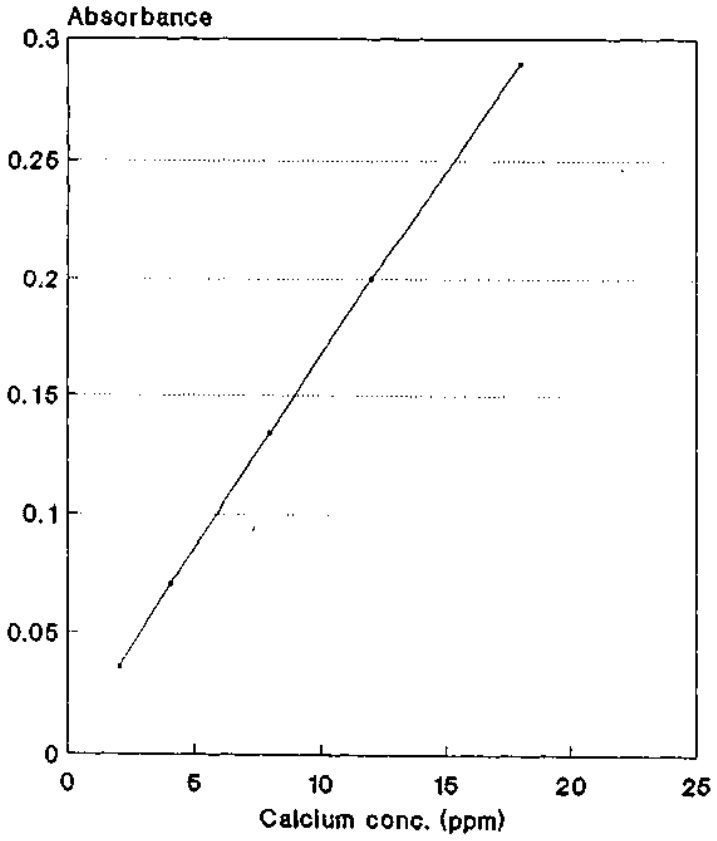
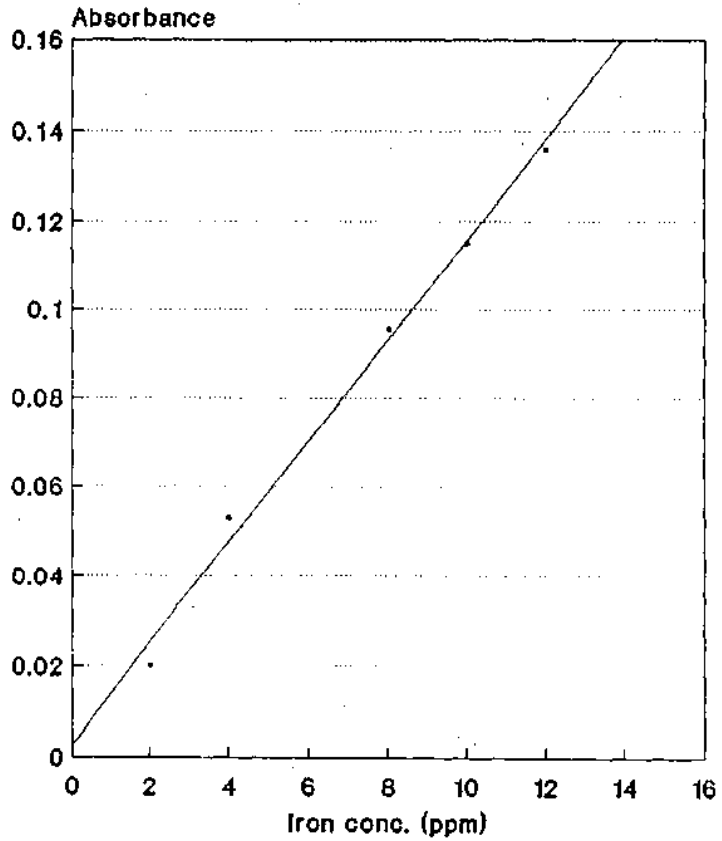


Fig. 3.06
Standard curve for determination of iron in lactoperoxidase



of iron were prepared. Measured the absorbance of standard iron solutions as well as sample using atomic absorption spectrophotometer (Philips PU 9100 X).

(b) Calcium

The calcium content in the sample was determined from the standard curve of calcium (Fig. 3.07). For standard curve preparation, 0.2769 g of calcium chloride ($CaCl_2.2H_2O$) (S. Merck) was dissolved in glass distilled water resulting in concentration of 100 ppm per ml. Aliquots of 2.0, 4.0, 8.0, 12.0 and 18.0 ppm per ml containing 0.1 per cent (w/v) strontium chloride ($SrCl_2$) (Sigma) as releasing agent, were prepared. Measured the absorbance of sample containing 0.1 per cent (w/v) $SrCl_2$ and those of standard calcium solutions using atomic absorption spectrophotometer (Philips PU 9100 X).

3.5.6 CARBOHYDRATE ANALYSIS

3.5.6.1 Hexoses

The hexoses content of lactoperoxidase was determined by the method of Dubois *et al.* (1956) using phenol-sulphuric acid reagent.

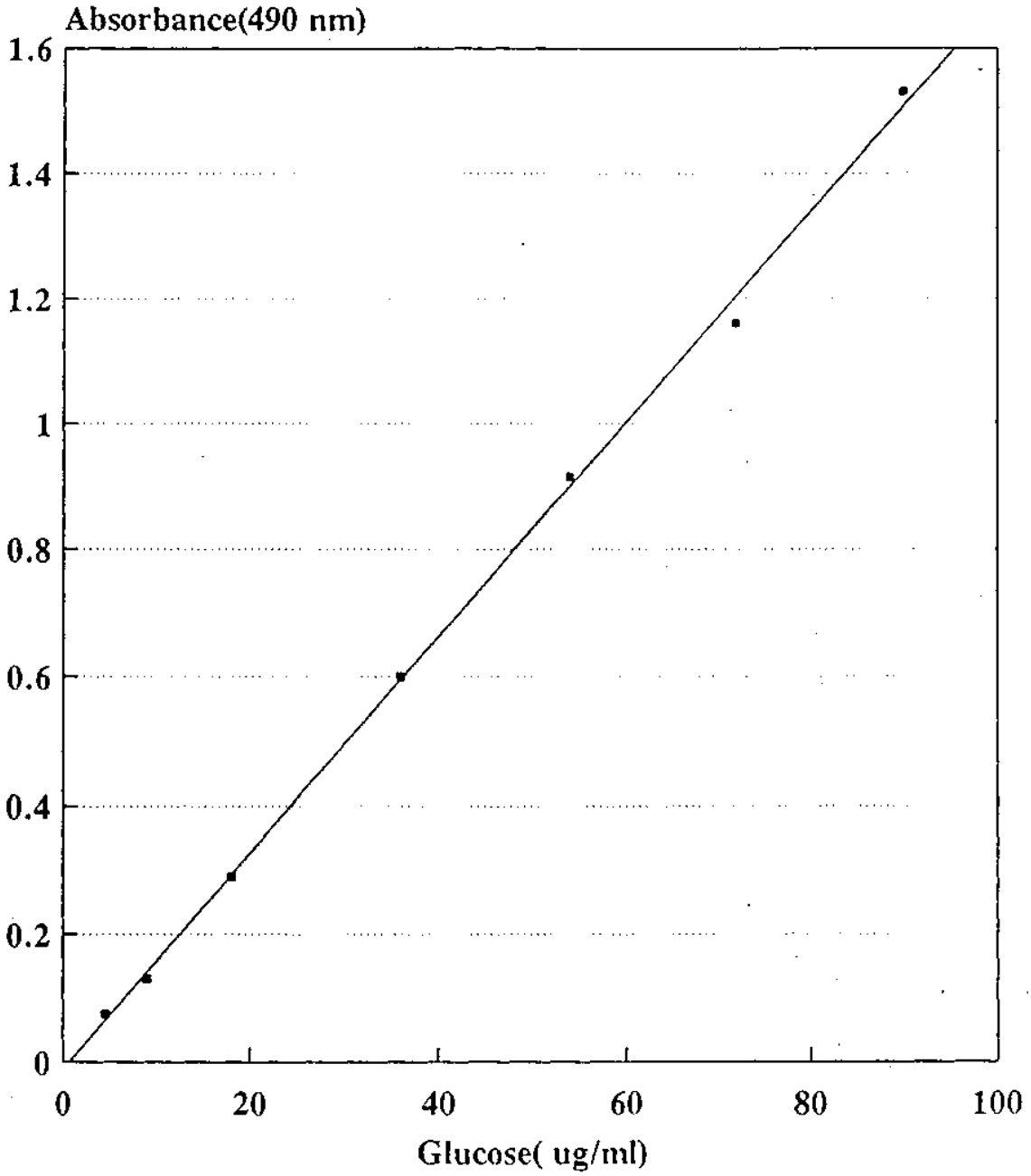
(a) Reagents

(i) Phenol solution (5%, w/v)

Dissolved 5.0 g phenol (S. Merck) in distilled water and made up the volume to 100 ml.

FIG.3.08

Standard curve for determination of hexoses in lactoperoxidase



(ii) Concentrated H_2SO_4

(b) Procedure

Standard glucose (Glaxo) solutions of concentration 9-180 ug/ml were prepared from stock 2 mM glucose solution by appropriate dilution. Purified lactoperoxidase was taken at a concentration of 5 mg/ml and 10 mg/ml. Mixed 0.4 ml of sample/ standards/control (reagent blank) solution with 0.4 ml of reagent (i). Added 2.0 ml of reagent (ii) rapidly and directly to the solution surface without touching the sides of the tube. Leave the solutions undisturbed for 10 min before shaking vigorously and determined the absorbance at 490 nm after a further 30 min against reagent blank. The concentration of hexoses in lactoperoxidase sample was determined from glucose standard curve (Fig. 3.08).

3.5.6.2 *N-acetyl neuraminic acid*

NANA was estimated by thiobarbituric acid method of Warren (1959).

(a) Reagents

(i) Sodium metaperiodate solution

Dissolved 4.3 g of sodium metaperiodate (Sigma) in 4.0 ml distilled water, added 58 ml concentrated orthophosphoric acid and made upto 100 ml with distilled water.

(ii) Sodium arsenite solution

Dissolved 10 g sodium arsenite (Sigma) and 7.1 g of sodium sulphate in 0.1 M H_2SO_4 (prepared by adding 5.7 ml concentrated H_2SO_4 to one litre with distilled water) to a total volume of 100 ml.

(iii) Thiobarbituric acid solution

Dissolved 1.2 g 2-thiobarbituric acid (Sigma) and 14.2 g sodium sulphate in water to a total volume of 200 ml.

(iv) Redistilled cyclohexanone

(b) Procedure

Stock solution of N-acetyl neuraminic acid (NANA) (Sigma) was prepared by dissolving 1 mg NANA in 10 ml distilled water (100 ug/ml). Lactoperoxidase sample was prepared by dissolving 7 mg in 0.5 ml of 0.1 M H_2SO_4 and keeping it at 80°C for one hour. To 0.2 ml of sample/standard/control (distilled water), added 0.1 ml of reagent(i) and mixed well. Leave the solution at room temperature for 20 min. Then added 1.0 ml of reagent(ii) and shook the tubes vigorously to expell the yellow coloured iodine. Again leave for a further 5 min at room temperature. Now added 3.0 ml of reagent(iii), shook the tubes, placed marble on tubes and heated at 100°C for 15 min. Then cooled the tubes rapidly at room temperature. Extracted the chromophore into reagent(iv) by vigorous shaking. Centrifuged the solutions using bench centrifuge for a few minutes in order to properly separate two layers. Determined the absorbance of upper cyclohexanone layer at 549 nm. The concentration of NANA in lactoperoxidase sample was determined from the standard curve (Fig. 3.09).

3.5.6.3 Hexosamine determination

Hexosamine was determined by the method of Belcher *et al.* (1954).

(a) Reagents

(i) Acetyl acetone reagent

Acetyl acetone was freshly prepared by adding 0.2 ml of acetyl acetone to 4.8 ml of 0.625 M sodium carbonate (Na_2CO_3) solution.

(ii) Ehrlich's reagent

It was prepared by dissolving 1.6 g of p-dimethyl amino benzaldehyde (recrystallized as the hydrochloride) (Sigma) in 30 ml concentrated HCl.

(b) Sample preparation

8 mg of dried lactoperoxidase was taken in a glass vial and to it added 0.5 ml of 3 N HCl. The vial was sealed and kept at 100°C for 5 h. Then the hydrolyzate was poured into small petri plate and kept in vacuum oven at 20 psi and 50°C and dried in the presence of sodium hydroxide pellets. The dry product was dissolved in 0.5 ml distilled water and centrifuged.

Fig. 3.10
Standard curve for the determination of hexosamine content in lactoperoxidase

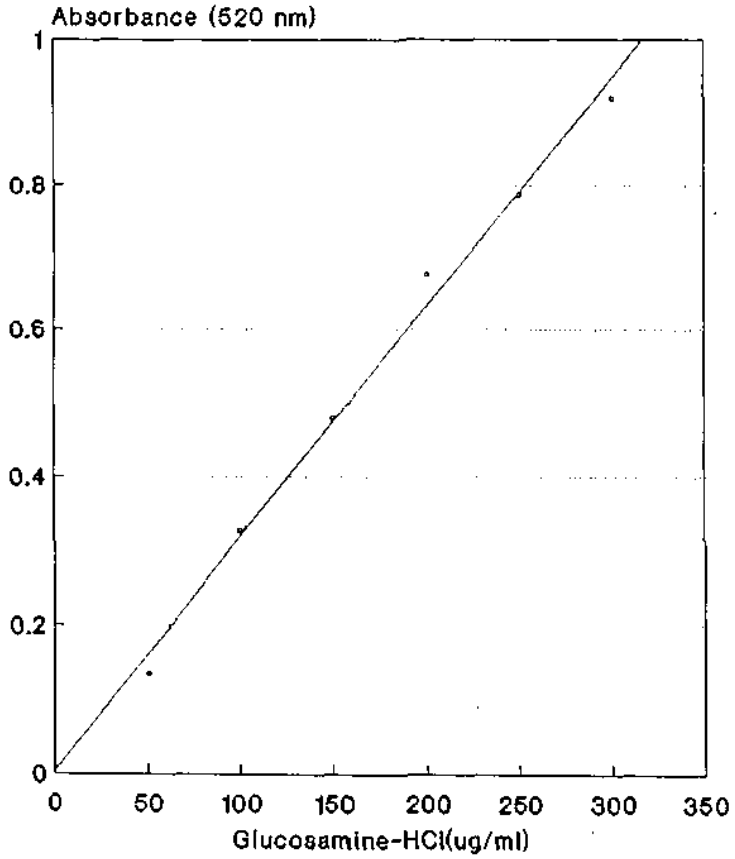
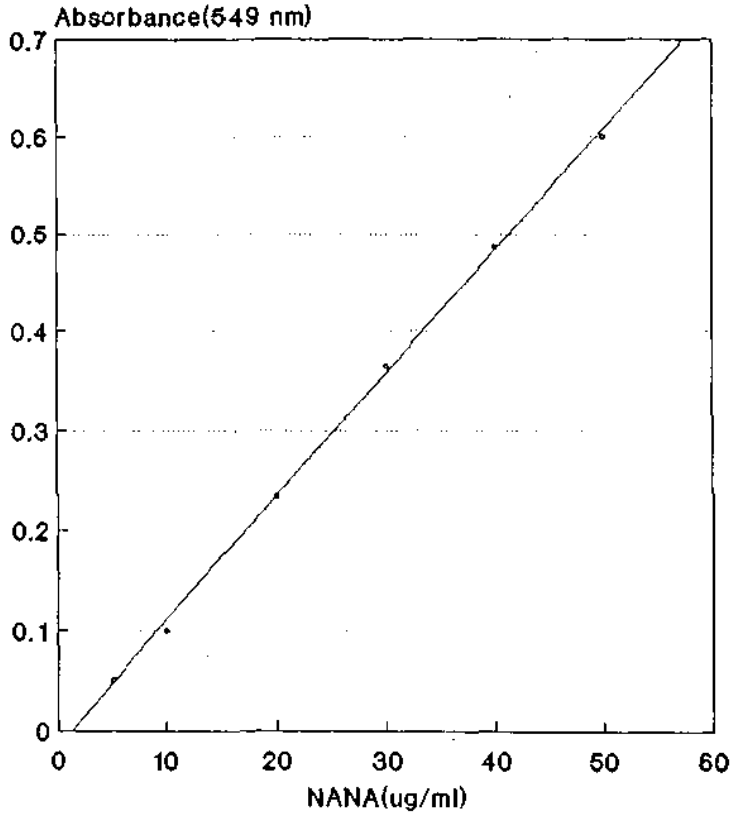


Fig. 3.09
Standard curve for determination of N-acetyl neuraminic acid (NANA) content in lactoperoxidase



(c)Standard solution

Standard stock glucosamine hydrochloride solution (Sigma) was prepared to a concentration of 1 mg per ml. It was diluted to standard solutions of concentration 50 to 300 ug/ml.

(d) Procedure

To 0.2 ml of sample/standard/control (distilled water), added 0.2 ml of acetyl acetone reagent and 0.4 ml of distilled water. Shake the tubes vigorously, cap with glass marbles to minimize evaporation and heat in a boiling water bath for 10 min. Then cooled to room temperature and added 1.0 ml of ethanol, mixed carefully and maintained the tubes at 75 ± 2°C for 5 min in a water bath. Added 0.2 ml of Ehrlich's reagent and heated at 75°C for 30 min, cooled the tubes to room temperature and added 1.0 ml of 95 per cent ethanol. Determined the absorbance at 520 nm after 30 min (pink colour). The hexosamine concentration in lactoperoxidase sample was determined from the standard curve (Fig. 3.10).

3.6STRUCTURAL PROPERTIES

3.6.1 CRYSTALLIZATION

Crystallization of macromolecules basically involves bringing the macromolecules slowly towards a state of minimum solubility. Various factors, like ionic strength, pH, counter ions, temperature and organic solvents can be altered to bring the macromolecules to a state of minimum solubility.

Purified buffalo lactoperoxidase was concentrated by freeze drying and used for crystallization. The precipitants tried out for crystallization included ethanol and dipotassium hydrogen phosphate. The precipitants were used with phosphate buffer and Tris-HCl buffer, with different variables like pH, ionic strength and protein concentration. The methodology used for crystalli- zation is presented below.

3.6.1.1 Microdialysis method

The procedure given by Zeppezauer (1971) was followed for microdialysis.

(a) Parameters

(i) Ionic strength and pH

K_2HPO_4 solution was used by varying the ionic strength from 1.4 to 2.4 M, at an interval of 0.2 unit. For each concentration of K_2HPO_4 solution, the pH was varied between 7.0-9.0, at an interval of 0.4 unit.

(ii) Ethanol

To 0.1 M sodium phosphate buffer with pH varying between 7.0-9.0, at an interval of 0.4 unit, ethanol was added at a concentration of 8-16 per cent (at 2 unit interval). At each concentration of ethanol, the pH of phosphate buffer ranged between 7.0-9.0.

(iii) Lactoperoxidase concentration

It was varied between 10-30 mg/ml for each concentration of K_2HPO_4 solution (i) and ethanol (ii).

(b) Procedure

In this method, polyethylene tube with a dialysis membrane (cut off 12,000-14,000 MW) placed at its horizontal end pushed into a glass capillary tubing (diameter less than 5 mm). The dialysis membrane, placed between the polyethylene tube and glass capillary, get hermatically sealed. The protein solution was added into the glass capillary. The set up with polyethylene tube's end (cut at an angle of 45°) dipping in the precipitant was placed in a screw capped vials containing precipitant and left undisturbed at 4°C.

3.6.1.2 Vapour diffusion technique

The methodology given by Davies and Segal (1971) was followed for vapour diffusion technique.

Parameters

(i) Ethanol

To sodium phosphate buffer (0.01 M, pH 8.2), and Tris-HCl buffer (0.01 M, pH 8.2), ethanol concentration was varied between 8 to 12 per cent at 2 unit interval.

(ii) Protein concentration

It was varied between 10-30 mg/ml for each concentration of ethanol in sodium phosphate buffer and Tris-HCl buffer.

(a) Hanging drop experiment

Crystallization using this method was performed using tissue culture plates of 24 wells. A droplet of protein solution, about 20 µl (10-30 mg/ml) was placed in a siliconized coverslip and hung face downwards in a reservoir filled with the precipitant. The coverslips were further secured by means of silicone grease. This set up was left undisturbed at 4°C.

(b) Sitting drop experiment

The protein solution about 20 ul (10-30 mg/ml) was placed in a depression in a plastic bridge which was placed in a petri dish. The crystallizing agent which was the buffer 0.01 M Tris-HCl buffer (pH 8.2) or 0.01 M phosphate buffer (pH 8.2) containing ethanol was poured in the petri dish and surrounded the plastic bridge containing the protein solution. The dish was sealed and left undisturbed at 4°C.

3.6.2 X-RAY DIFFRACTION AND DATA COLLECTION

The data collection of the lactoperoxidase crystals was carried out using synchrotron radiation (λ = 0.92 A°) with the help of the Mar Research Imaging Plate Scanner.

CHAPTER 4

RESULTS AND DISCUSSION

4. *RESULTS AND DISCUSSION*

4.1 *LACTOPEROXIDASE ASSAY*

Standardization of assay was carried out using ABTS as chromogenic substrate. The reaction mixture included 1 mM ABTS (in 0.1 M sodium phosphate buffer, pH 6.0) and 0.1 mM H_2O_2. The concentration of lactoperoxidase was varied from 0.1 to 8.0 µg/ml. It was observed that a linear relationship exists between change in absorbance per minute and lactoperoxidase concentration upto 6.0 µg/ml (Fig. 4.01). The corresponding change in absorbance per minute was found to range between 0.007 to 0.260. Thus, for lactoperoxidase assay the dilution of samples was carried out with 0.1 M PBSG (pH 7.0) in such a way that change in absorbance per minute lies in the range mentioned above (Fig. 4.02 ; Appendix I).

4.1.1 *LACTOPEROXIDASE ACTIVITY OF MILK*

With a view to have a comparative study on peroxidase activity of cow and buffalo milk, forty samples of each were analysed at pH 6.0. The average peroxidase activity of buffalo and cow milk was observed to be 7.38 ± 2.18 units/ml and 5.76 ± 1.40 units/ml, respectively.

A 24 per cent higher peroxidase activity has been observed in buffalo milk than cow milk. Although it is difficult to have an interstudy comparison on lactoperoxidase activity of milk because of different chromogenic substrates used for assay. However, there is one report on interspecies comparison on

FIG. 4.01

Effect of Protein Concentration on Lactoperoxidase Assay

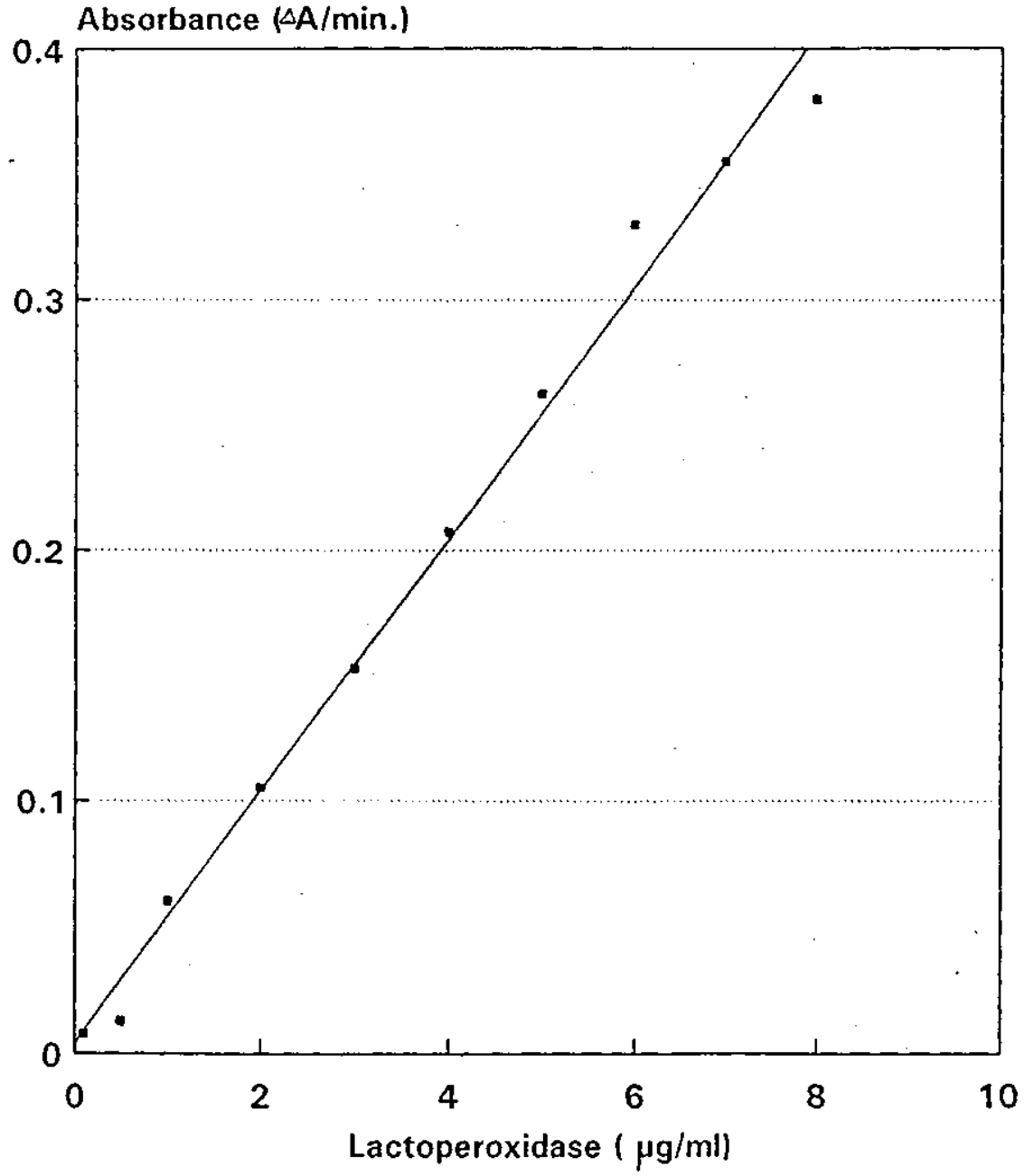
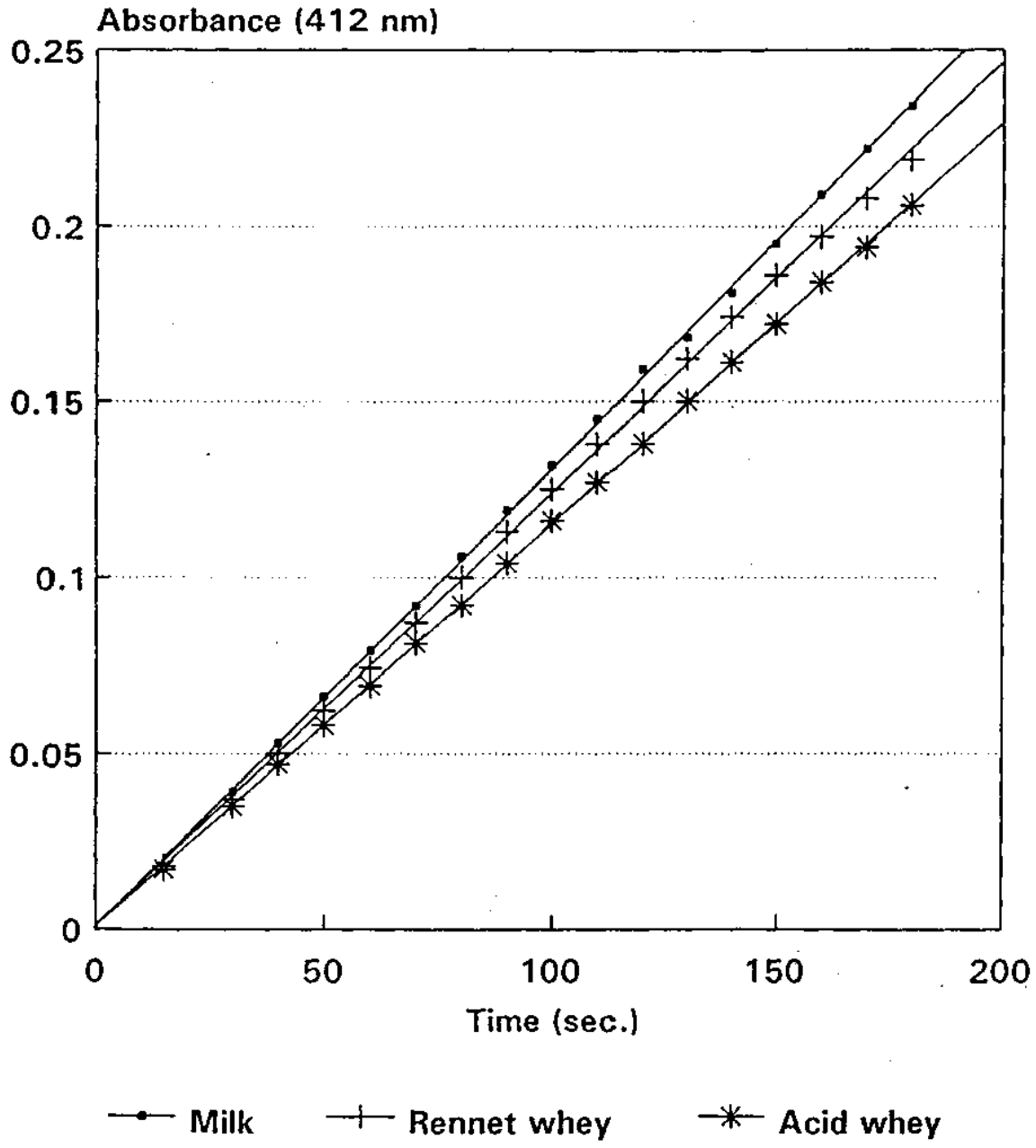


FIG. 4.02

Lactoperoxidase assay of buffalo milk, rennet whey and acid whey



Dilution (1:250)

peroxidase activity of cow and buffalo milk using ABTS as substrate (Harnulv and Kandasamy, 1982) showing higher peroxidase activity in cow milk (1.2 U/ml) than buffalo milk (0.90 U/ml). The differences in peroxidase activity might be due to differences in feed, season and particularly stage of lactation. Further, the differences in absolute values of ABTS units might be due to differences in assay conditions.

4.1.2 *DISTRIBUTION OF LACTOPEROXIDASE BETWEEN DIFFERENT MILK FRACTIONS*

Ten samples each of cow and buffalo milk were analysed for peroxidase activity distribution in whole milk, skim milk, rennet whey and acid whey.

On separation of whole milk, peroxidase activity was found to increase slightly both in cow and buffalo milk samples (Table 4.1). Yoshida and Xiuyun (1991a) also observed an increase in lactoperoxidase activity on skimming of whole milk. However, contrary to this observation, a decrease of peroxidase activity has also been reported (Djordjevic *et al.*,1974; Yoshida, 1988a,b; Yoshida and Xiuyun, 1991a). Increase in peroxidase activity on removal of milk fat might be due to effective increase in concentration of lactoperoxidase which indicates that lactoperoxidase mainly reside in the serum phase of milk in accordance to earlier reports (Sharma and Ganguli, 1971; Djordjevic *et al.*, 1974)

On preparation of rennet whey and acid whey from skim milk, about 10 to 15 per cent loss of activity was observed (Table 4.1).

Table 4.1 Changes in lactoperoxidase activity on separation of milk to whey

Source (n)	Assay pH	Peroxidase activity			
		Whole milk	Skim milk	Rennet whey	Acid whey
Buffalo (10)	4.4	1.371 ± 0.262	1.495 ± 0.129	1.252 ± 0.152	1.182 ± 0.627
	6.0	7.315 ± 0.134	7.720 ± 0.109	6.913 ± 0.188	6.786 ± 0.114
Cow (10)	4.4	1.263 ± 0.227	1.355 ± 0.160	1.114 ± 0.245	1.095 ± 0.163
	6.0	5.724 ± 0.274	5.973 ± 0.147	5.251 ± 0.196	5.135 ± 0.180

The difference between rennet whey and acid whey was not significant. Djordjevic *et al.* (1974) reported that lactoperoxidase activity of whey is 82.4 per cent as compared to skim milk, while Yoshida and Xiuyun (1991b) reported 11 per cent decrease of peroxidase activity. Yoshida (1988a,b) reported the decrease of peroxidase activity on preparation of acid whey from skim milk to be 66 per cent and 36.5 per cent (Yoshida and Xiuyun, 1991a,b).

4.2 *ISOLATION AND PURIFICATION OF LACTOPEROXIDASE*

Lactoperoxidase purification from buffalo milk as well as rennet whey and acid whey involved essentially four steps, viz. cation exchange chromatography using weakly acidic cation exchanger Amberlite CG-50-NH_4^+; ammonium sulphate precipitation of the eluate at 85 per cent saturation; purification on cation exchanger CM Sephadex C-50 using linear gradient of 150-200 mM NaCl and final purification by gel filtration chromatography on Sephadex G-100. Twenty trials were conducted for purification of lactoperoxidase. The summary of purification of lactoperoxidase from skim milk, rennet whey and acid whey is presented in Tables 4.2, 4.3 and 4.4, respectively. The purity of the protein was monitored during successive steps on the basis of specific activity and R_z value (A_{412}/A_{280}).

4.2.1 *ISOLATION*

4.2.1.1 *Cation exchange chromatography*

The average specific activity of lactoperoxidase was found to be 0.29, 0.83 and 1.33 units/mg in skim milk, rennet whey and

FIG. 4.03

Elution profile of lactoperoxidase on CM Sephadex C-50 chromatography from skim milk

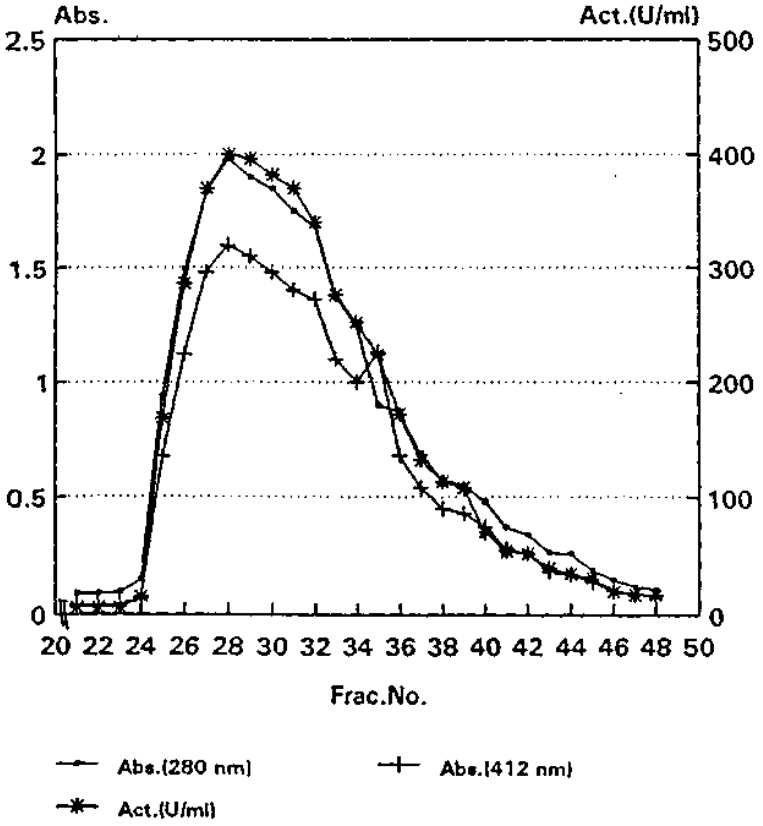


FIG. 4.04

Gel filtration of lactoperoxidase from skim milk

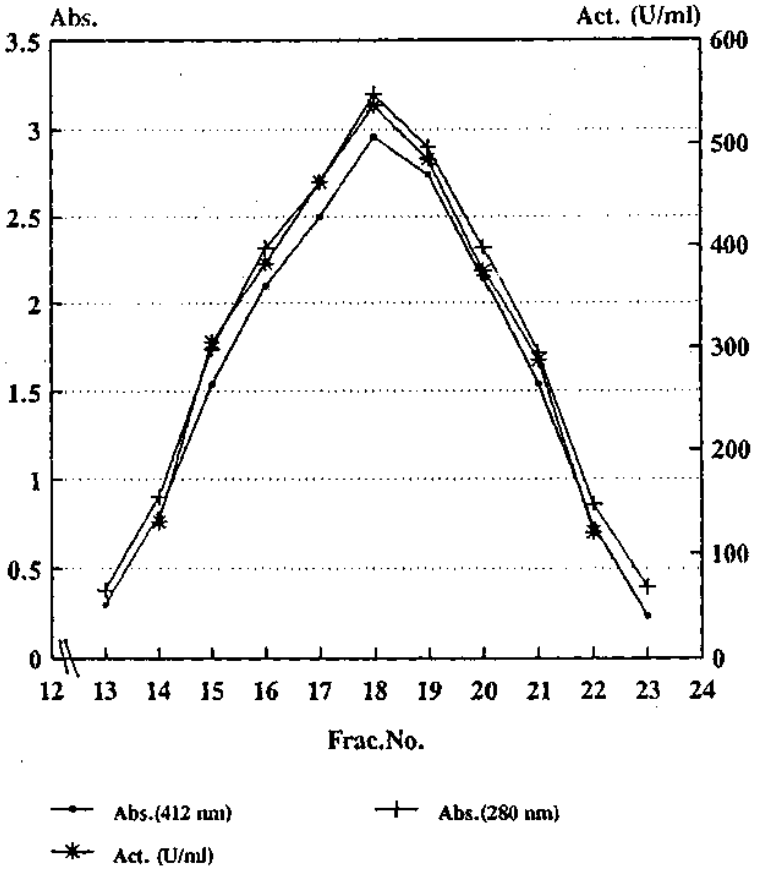


Table 4.2 Summary of purification of lactoperoxidase from buffalo skim milk

Purification steps	Total volume (ml)	Total activity (umol/min)	Total protein (mg)	Specific activity of protein ($umol\ min^{-1} mg^{-1}$)	Purification factor (based on sp.act.)	Rz-value (A_{412}/A_{280})	Per cent Recovery	
							Based on total activity of milk	Based on total protein of milk
Skim milk	10,000	98,200	3,40,000	0.288				
Crude eluate	2,650	72,000	576.8	125	433		73.32	0.169
85% ammonium sulphate fractionation	27.5	59,824	370	161	561	0.45	60.92	0.109
CM Sephadex C-50 eluate (pooled fractions 25-44)	200	36,714	147	250	868	0.80-0.85	37.40	0.043
Sephadex G-100 eluate (pooled fractions 14-22)	90	31,192	120	260	902	0.90-0.95	31.76	0.035

FIG. 4.05

Elution profile of Lactoperoxidase
on CM Sephadex C-50 chromatography
from rennet whey

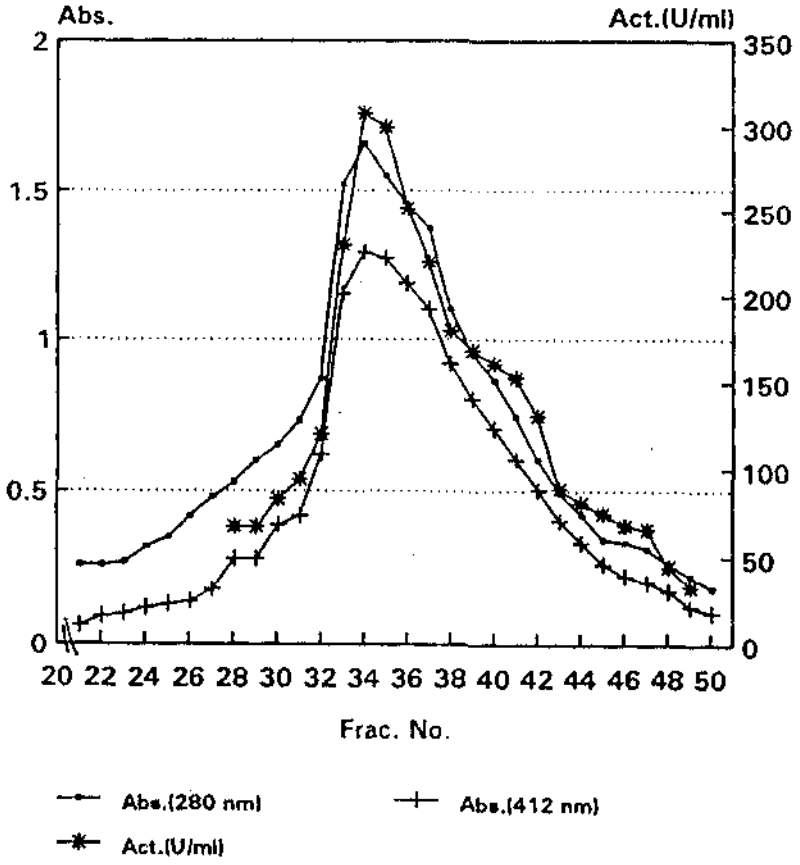


FIG. 4.06

Gel filtration of lactoperoxidase
from rennet whey

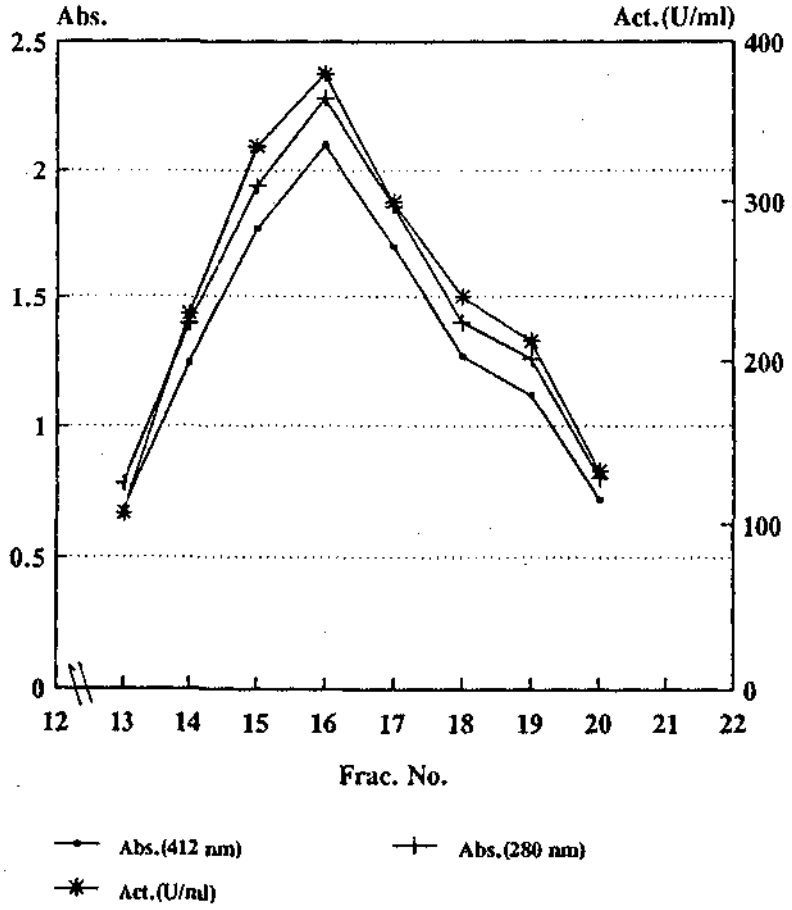


Table 4.3 Summary of purification of lactoperoxidase from rennet whey

Purification steps	Total volume (ml)	Total activity (umol/min)	Total protein (mg)	Specific activity of protein ($umol\ min^{-1} mg^{-1}$)	Purification factor (based on sp.act.)	Rz-value (A_{412}/A_{280})	Per cent Recovery			
							Based on total activity of		Based on total protein of	
							Milk	Rennet whey	Milk	Rennet whey
Skim milk	15,000	1,33,000	4,69,000	0.283						
Rennet whey	10,000	75,960	92,000	0.825	2.9		57.11		19.62	
Crude eluate	2,800	58,896	561	105	371		44.30	77.53	0.120	0.610
85% ammonium sulphate fractionation	23	39,871	262	152	537	0.46	29.98	52.50	0.056	0.285
CM Sephadex C-50 eluate (pooled fractions 29-45)	170	26,795	108	248	876	0.80-0.88	20.15	35.30	0.023	0.119
Sephadex G-100 eluate (pooled fractions 14-20)	70	23,466	94	249	880	0.90-0.93	17.64	30.89	0.020	0.102

FIG. 4.07

Elution profile of lactoperoxidase
on CM Sephadex C -50 chromatography
from acid whey

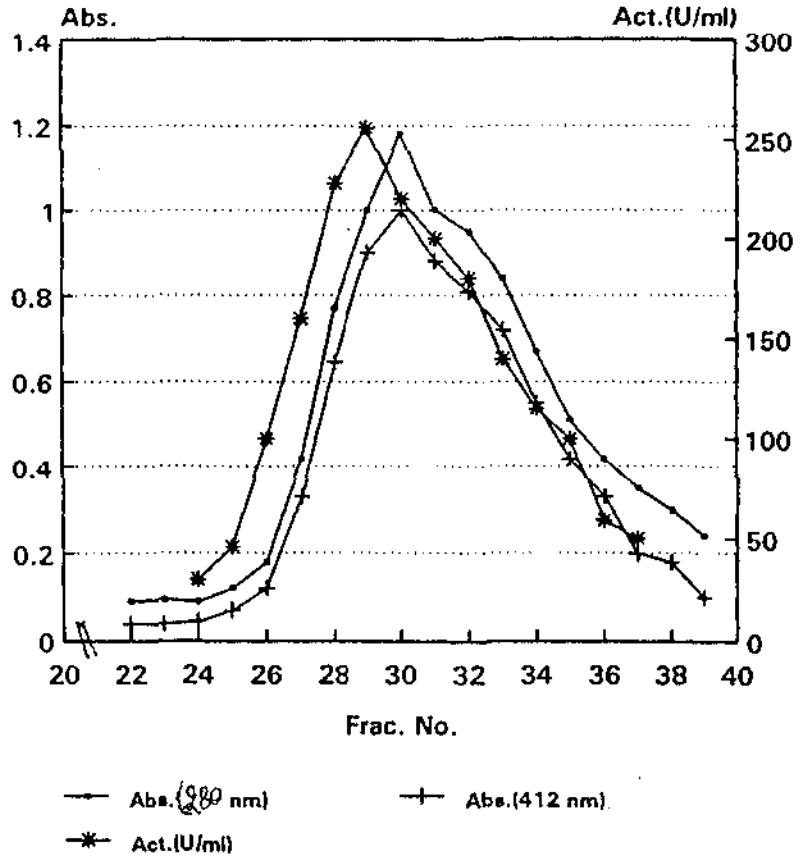


FIG. 4.08

Gel filtration of lactoperoxidase
from acid whey

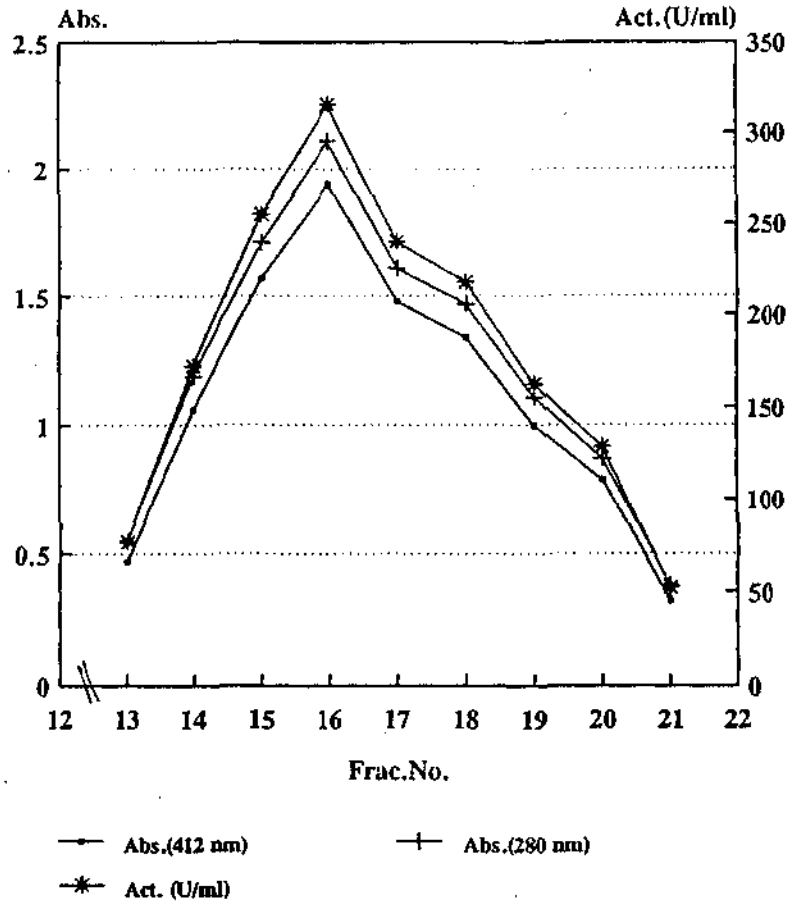


Table 4.4 Summary of purification of lactoperoxidase from acid whey

Purification steps	Total volume (ml)	Total activity (umol/min)	Total protein (mg)	Specific activity of protein ($umol\ min^{-1}\ mg^{-1}$)	Purification factor (based on sp.act.)	Rz-value (A_{412}/A_{280})	Per cent Recovery			
							Based on total activity of		Based on total protein of	
							Milk	Acid whey	Milk	Acid whey
Skim milk (1:1 dilution)	17,000	88,400	2,97,500	0.297						
Acid whey	10,000	41,000	30,700	1.335	4.5		46.38		10.32	
Crude eluate	2,400	32,140	314.8	115	387		36.36	78.39	0.106	1.025
85% ammonium sulphate fractionation	25	26,729	193.7	138	464	0.50	30.24	65.19	0.065	0.630
CM Sephadex C-50 eluate (pooled fractions 26-36)	110	17,819	73	243	818	0.85-0.87	20.16	43.46	0.024	0.238
Sephadex G-100 eluate (pooled fractions 14-21)	80	13,812	55.9	247	831	0.90-0.95	15.62	33.68	0.0188	0.182

acid whey, respectively. After stirring the sample with cation exchanger Amberlite CG-50-NH_4^+ for one hour, the binding of lactoperoxidase to the resin was found to range from 80 to 85 per cent on the basis of peroxidase activity measurement in spent skim milk or whey (acid/rennet). Recovery of lactoperoxidase activity in the eluate varied between 70 to 75 per cent of total activity of skim milk, rennet whey or acid whey. The specific activity of eluate obtained was 125, 105 and 115 units/mg from skim milk, rennet whey and acid whey, respectively.

4.2.1.2 Ammonium sulphate precipitation

Lactoperoxidase was salted out from the eluate at 85 per cent saturation of ammonium sulphate. The grey slimy precipitates were collected in 5 mM sodium phosphate buffer (pH 6.8) and dialyzed against the same. The R_z value of crude lactopeoxidase preparation was found in the range of 0.4 to 0.6, whereas its specific activity was observed to be 161, 152 and 138 units/mg, with the corresponding purification factor of 561, 537 and 464 for skim milk, rennet whey and acid whey, respectively. The purity of the brown coloured lactoperoxidase solution, determined on the basis of specific activity and R_z value was found to be about 50 per cent.

4.2.2 PURIFICATION

4.2.2.1 Cation exchange chromatography

Purification of crude lactoperoxidase was carried out using cation exchanger CM Sephadex C-50. On washing with 10 mM

sodium phosphate buffer (pH 6.8) and then with same buffer containing 0.1 M NaCl, the peroxidase negative extraneous proteins were removed. The elution of the lactoperoxidase was carried out using linear gradient of 150 to 200 mM NaCl as depicted in Figs. 4.03, 4.05 and 4.07. Those fractions having R_z value about 0.8 or higher were pooled. The specific activity of the enzyme preparation at this stage was 250, 248 and 243 units/mg with a purification factor of 868, 876 and 818 for skim milk, rennet whey and acid whey, respectively.

4.2.2.2 Gel filtration

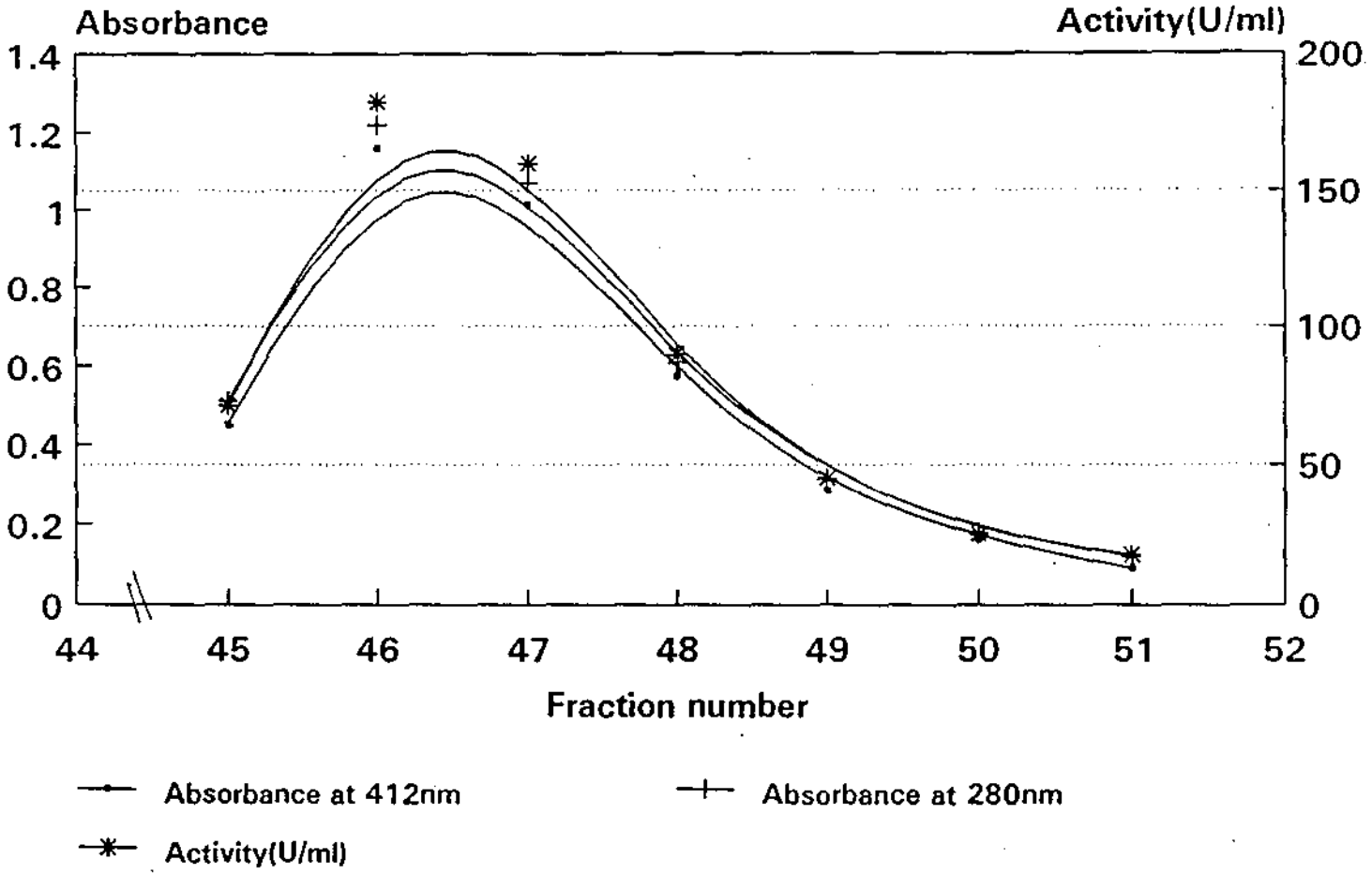
Lactoperoxidase solution obtained after cation exchange chromatography was dialyzed against distilled water and then concentrated to a volume of 10 to 15 ml by lyophilization. This was then subjected to gel filtration on Sephadex G-100 to remove traces of contaminants. The enzyme was eluted with 0.1 M sodium phosphate buffer (pH 6.8). The elution profiles are presented in Figs. 4.04, 4.06 and 4.08. The R_z value of the central cut of the peak was 0.9 or higher and the specific activity of finally purified enzyme was 260, 249 and 247 units/mg for skim milk, rennet whey and acid whey, respectively. Based on specific activity of milk, the purification factor was observed to be 902, 880 and 831 for skim milk, rennet whey and acid whey, respectively.

4.2.2.3 Fast protein liquid chromatography (FPLC)

The purity of lactoperoxidase isolated from skim milk was reassessed after gel filtration step by size exclusion fast

FIG. 4.09

Elution Profile of Lactoperoxidase on FPLC



protein liquid chromatography. As shown in Fig. 4.09, the enzyme was eluted as a single peak. The R_z value and specific activity were also observed to be similar to that obtained after gel filtration step.

4.2.3 RECOVERY OF LACTOPEROXIDASE

4.2.3.1 Skim milk

Recovery of finally purified lactoperoxidase from skim milk based on total activity and total protein content was 31.8 per cent and 0.035 per cent, respectively. The average protein content from six batches was found to be 10.01 ± 1.87 mg per litre of milk with specific activity ranging from 240-270 units/mg.

4.2.3.2 Rennet whey

The activity recovered in final preparation from rennet whey based on total activity of milk and whey was found to be 17.6 and 30.89 per cent, respectively. The protein content was recovered to a level of 0.02 per cent and 0.102 per cent based on total protein content of milk and whey, respectively. The average content of lactoperoxidase recovered from four preparations was found to be 9.0 ± 1.7 mg per litre of whey with specific activity ranging from 236-256 units/mg.

4.2.3.3 Acid whey

Using acid whey, the per cent activity recovered in purified enzyme preparation based on total activity of milk and

whey was found to be 15.6 and 33.68, respectively. The protein content of final preparation was 0.0188 and 0.1820 per cent based on total protein content of skim milk and acid whey, respectively. The specific activity of four individual preparations was found to range between 220 to 255 units/mg with the average enzyme level of 9.5 ± 1.5 mg per litre of acid whey.

The yield of lactoperoxidase obtained from buffalo milk was found to be of similar order irrespective of its method of preparation, i.e., either from skim milk or from rennet whey or acid whey. However, the yield obtained is higher than that reported earlier (Moodbidri *et al.*, 1976). Different reports are available in the literature regarding the yield of lactoperoxidase from bovine milk and whey (Paul *et al.*, 1980; Zhao *et al.*, 1980; Jin *et al.*, 1981; Zhao *et al.*, 1982; Burling, 1989; Yoshida and Xiuyun, 1991a,b; Hernandez *et al.*,1990). The difference in yield reported by various workers could be attributed to the differences in methodology adopted for lactoperoxidase preparation and the sample variations.

The specific activity of lactoperoxidase from skim milk, rennet whey and acid whey was found comparable. However, Hernandez *et al.* (1990) reported that the specific activity of lactoperoxidase from acid whey was lower than that of rennet whey.

Taking R_z as criteria of purity index, the purity of lactoperoxidase preparation obtained from skim milk, rennet whey and acid whey was found to be similar at different stages of preparation on comparison to the values reported in the

literature (Morrison and Hultquist, 1963; Rombauts *et al.*, 1967; Carlstrom, 1969a; Paul *et al.*, 1980). Purification based on specific activity was 902 fold from skim milk, 880 fold from rennet whey and 831 fold from acid whey. Dumontet and Rousset (1983) reported a 1000-fold purification of lactoperoxidase from rennet whey.

4.3 *CATALYTIC PROPERTIES*

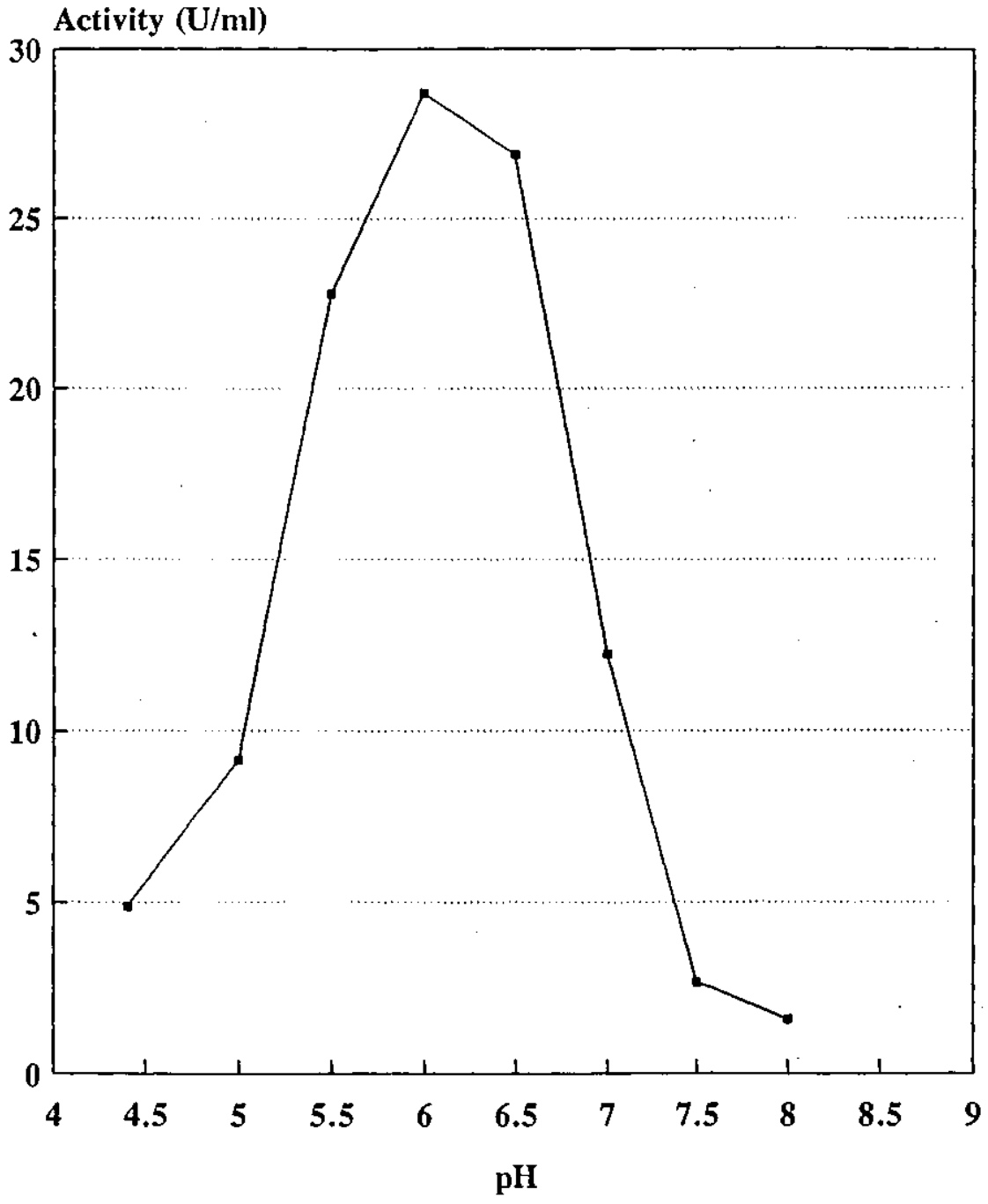
4.3.1 *pH OPTIMUM*

The effect of pH on lactoperoxidase activity was studied using 2,2'-azinobis (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) as chromogenic substrate 0.1 M acetate buffer (pH 4.0-5.0) and 0.1 M phosphate buffer (pH 6.0-8.0) at the lactoperoxidase concentration of 4 µg/ml.

As shown in Fig. 4.10, it is clear that highest peroxidase activity was observed at pH 6.0. Further, the activity observed at pH 6.0 was six times higher than that at pH 4.4 which is in accordance to Pruitt *et al.* (1991). The peroxidation of ABTS have been reported to have a complex dependence on pH (Bardsley, 1985). However, Hernandez *et al.* (1990) showed a broad pH optima for lactoperoxidase activity between pH 5.1 and 6.5, using ABTS as chromogenic substrate. With other substrates, different pH optimum values have been reported for lactoperoxidase activity. Rozental (1952) reported the pH optimum for bovine lactoperoxidase as 8.3 using guaicol as substrate, while El-Hagarawy (1954) observed the corresponding values as 6.5 and 7.0 for cow and buffalo milk, respectively, using p-phenylenediamine as substrate.

FIG. 4.10

Effect of pH on lactoperoxidase activity



4.3.2 pH-STABILITY

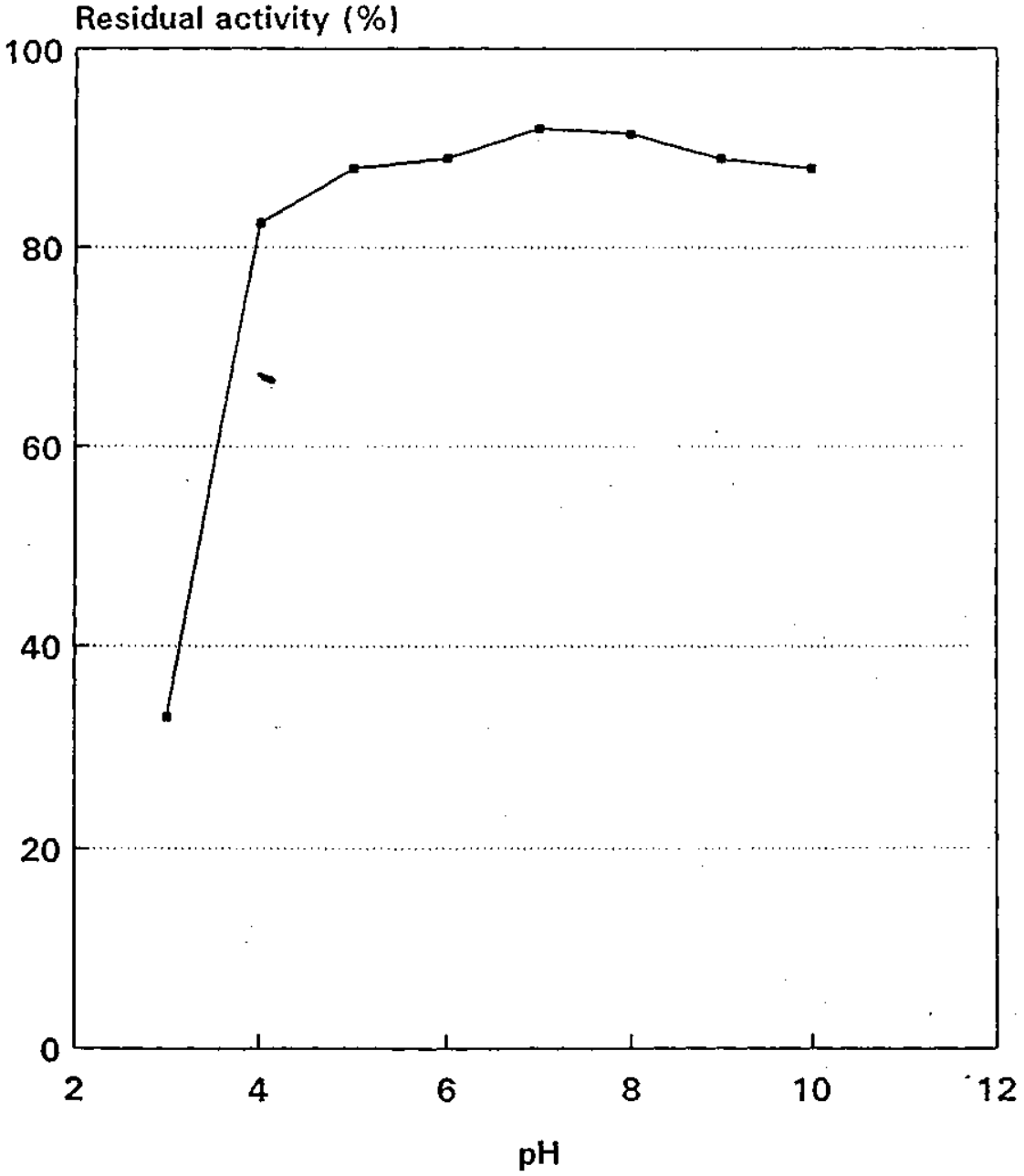
The pH stability of purified buffalo lactoperoxidase of specific activity 225 units/mg was studied over a pH range of 3.0 to 10.0, using citrate phosphate buffer (pH 3-7) and glycine-NaOH buffer (pH 8-10). The concentration of lactoperoxidase in each buffer was taken as 50 µg/ml. The enzyme solution was incubated at 25°C for 24 h and the enzymatic activity was measured using ABTS as chromogenic substrate at pH 6.0.

As shown in Fig. 4.11 after 24 h interval, the loss of activity was maximum at pH 3.0, relative to that for pH 4.0 to 10.0. The residual activity found at pH 4.0 was 83 per cent, while in the pH range 5-10, the lactoperoxidase was found to be relatively stable.

Maquire *et al.* (1971) observed that bovine lactoperoxidase is stable at pH 7.0 but deactivated at pH 3.0. Kimura and Yamazaki (1979) also reported that some denaturation of lactoperoxidase occurs below pH 4.0. Carlstrom (1969a) showed that bovine lactoperoxidase does not inactivate at pH 10.3 for 48 h at room temperature. Recently, Sato *et al.* (1992) reported that the bovine lactoperoxidase is completely denatured at pH 2.6 while relatively it is stable between pH range 4.0 to 9.0.

The results obtained in the present study are in general agreement to the earlier reports. The loss of lactoperoxidase activity at low pH might be due to denaturation of lactoperoxidase (Sato *et al.*, 1992).

FIG.4.11 pH stability of buffalo lactoperoxidase



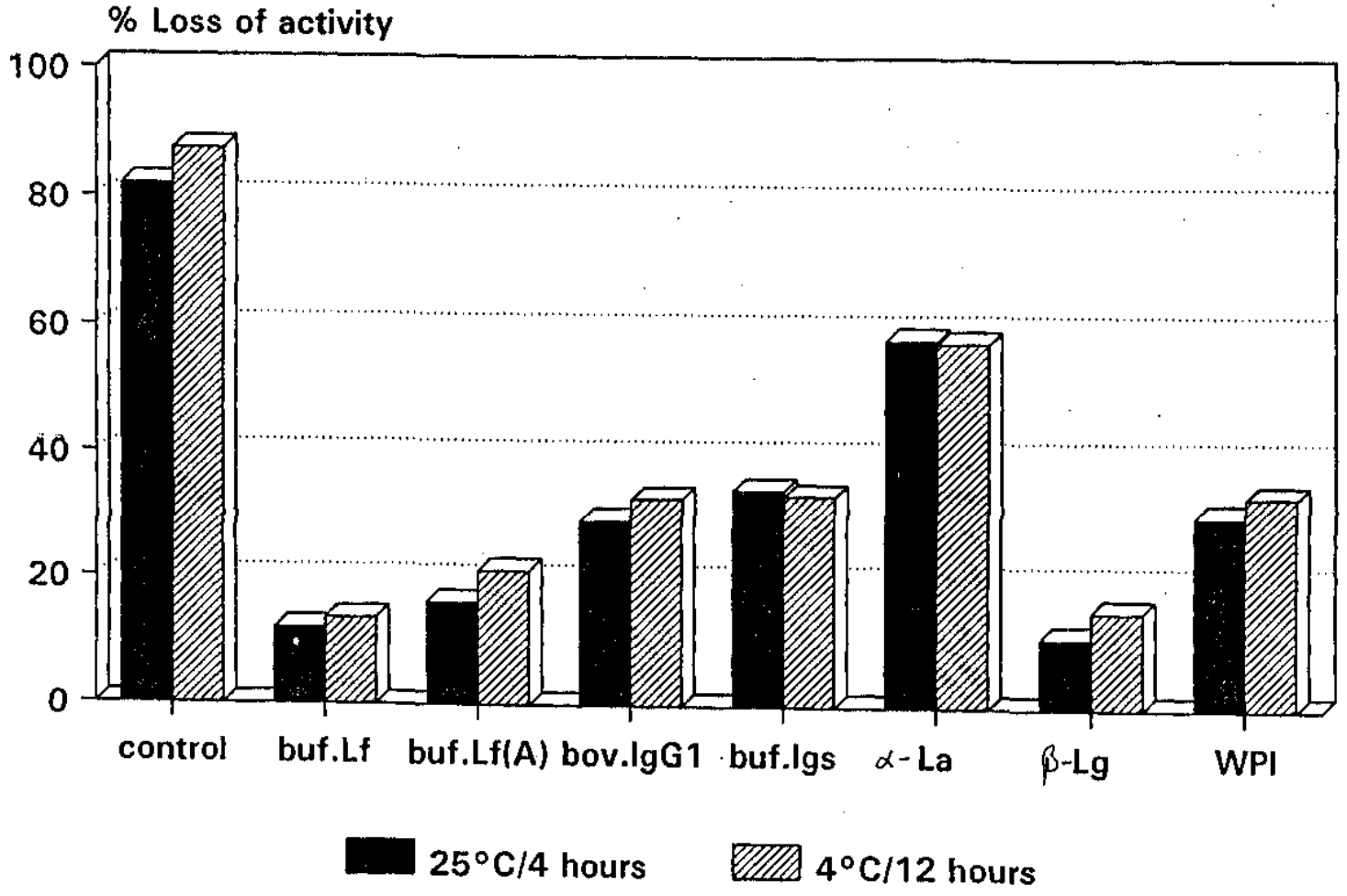
4.3.3 *EFFECT OF WHEY PROTEINS*

Different whey proteins were studied for their effect on peroxidase activity. Bovine IgG_1, buffalo Igs, buffalo lactoferrin and apolactoferrin, alpha-lactalbumin, ß-lactoglobulin and whey protein isolate, each at a concentration of 5 mg/ml were incubated with buffalo lactoperoxidase (2 µg/ml) in PBS (pH 7.2) at 25°C for 4 h and 4°C for 12 h. Lactoperoxidase with no added protein in PBS (pH 7.2) was also run under identical conditions.

Results as expressed in Fig. 4.12 clearly indicate that added protein protected the lactoperoxidase from inactivation. The effect of various types of proteins on lactoperoxidase activity was different during incubation period, both at low and high temperature. Amongst the various proteins studied, ß-lactoglobulin and lactoferrin (both metal and apoform) showed the greatest protection toward lactoperoxidase activity. Further, the prevention of inactivation obtained with samples containing immunoglobulins (bovine IgG_1 and buffalo Igs) and whey protein isolates was similar, while alpha-lactalbumin gave least protection to lactoperoxidase.

Tenovuo *et al.* (1982) reported that colostral SIgA, myeloma IgA_1, normal IgG and macroglobulinemic IgM provided protection to the bovine lactoperoxidase from inactivation. It was also reported that colostral lactoferrin prevented the loss of lactoperoxidase activity, with Fe^{3+} saturated lactoferrin being more effective activator than normal or apolactoferrin. Alpha-lactalbumin and human serum albumin did not exhibit any protective effect on lactoperoxidase. Hulea *et al.* (1989)

FIG. 4.12 **Effect of whey proteins on lactoperoxidase activity**



observed that lactoperoxidase was slightly activated by complexing to lysozyme, while IgA and IgM were inhibitory for the peroxidase, further IgG and ribonuclease did not exhibit any effect on the enzyme activity.

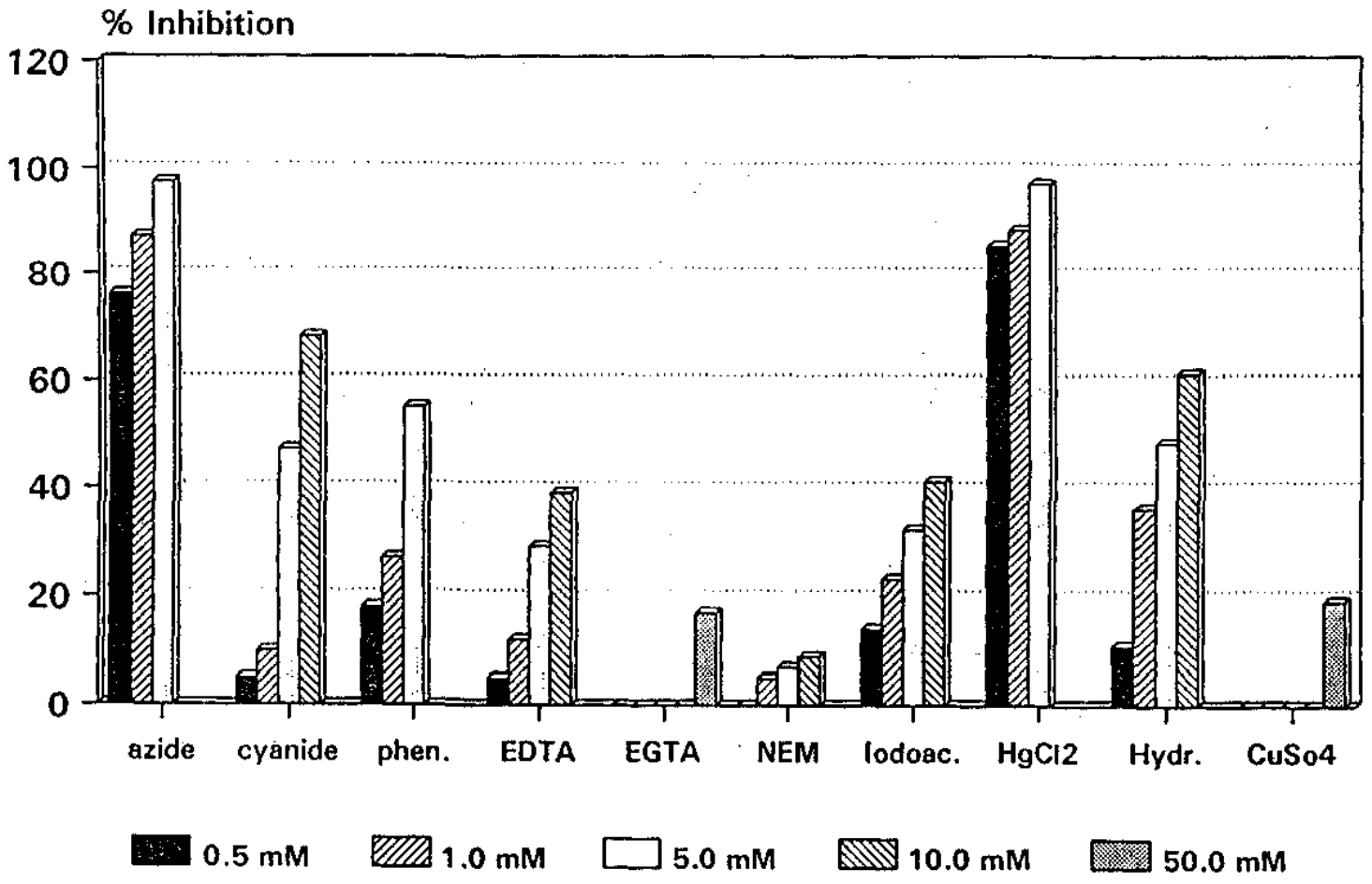
4.3.4 EFFECT OF CHEMICAL SUBSTANCES

Various chemical substances were studied for their effect on buffalo lactoperoxidase. These included metal complexing agents namely sodium azide, potassium cyanide, 1,10-phenanthroline (OP), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis- (ß-aminoethyl ether), N,N,N',N'-tetraacetic acid (EGTA) and chemical modifying reagents such as N-ethylmaleimide (NEM), mercuric chloride ($HgCl_2$), copper sulphate ($CuSO_4.5\ H_2O$) iodoacetamide and hydroxylamine-hydrochloride. These compounds were incubated with buffalo lactoperoxidase (10 µg/ml) at concentrations varying between 0.5 mM-10 mM (except $CuSO_4$ and EGTA, 10 mM - 50 mM each) in 0.01 M phosphate buffer (pH 7.0) at 25°C for 10 minutes.

Results as presented in Fig. 4.13 clearly indicate that buffalo lactoperoxidase is highly sensitive to sodium azide, resulting in complete inhibition at 5 mM concentration compared to potassium cyanide giving 50 per cent inhibition at 5 mM concentration. 1,10-phenanthroline resulted in 18 per cent inhibition at 0.5 mM, which increased to 55 per cent at 5 mM concentration. Further, lactoperoxidase was observed to be more sensitive to EDTA than EGTA.

Tenovuo and Kurkijarvi (1981) reported that immobilized

FIG.4.13 **Effect of chemical substances on lactoperoxidase activity**



lactoperoxidase retained about 40 per cent of their initial activity at cyanide concentration of 0.5 mM, while soluble lactoperoxidase retained only 10 per cent activity. Mansson-Rahemtulla *et al.* (1988) found that half of bovine lactoperoxidase activity lost on exposing lactoperoxidase (6 µg/ml) to azide(2.4 ± 0.9 mM) and cyanide (3.2 ± 1.2 mM) for 30 min. Further, the sensitivity of lactoperoxidase to azide has been reported to be lesser than salivary peroxidase, while to cyanide it is comparable. Shindler *et al.* (1976) reported that cervical mucus peroxidase is strongly inhibited by cyanide than azide.

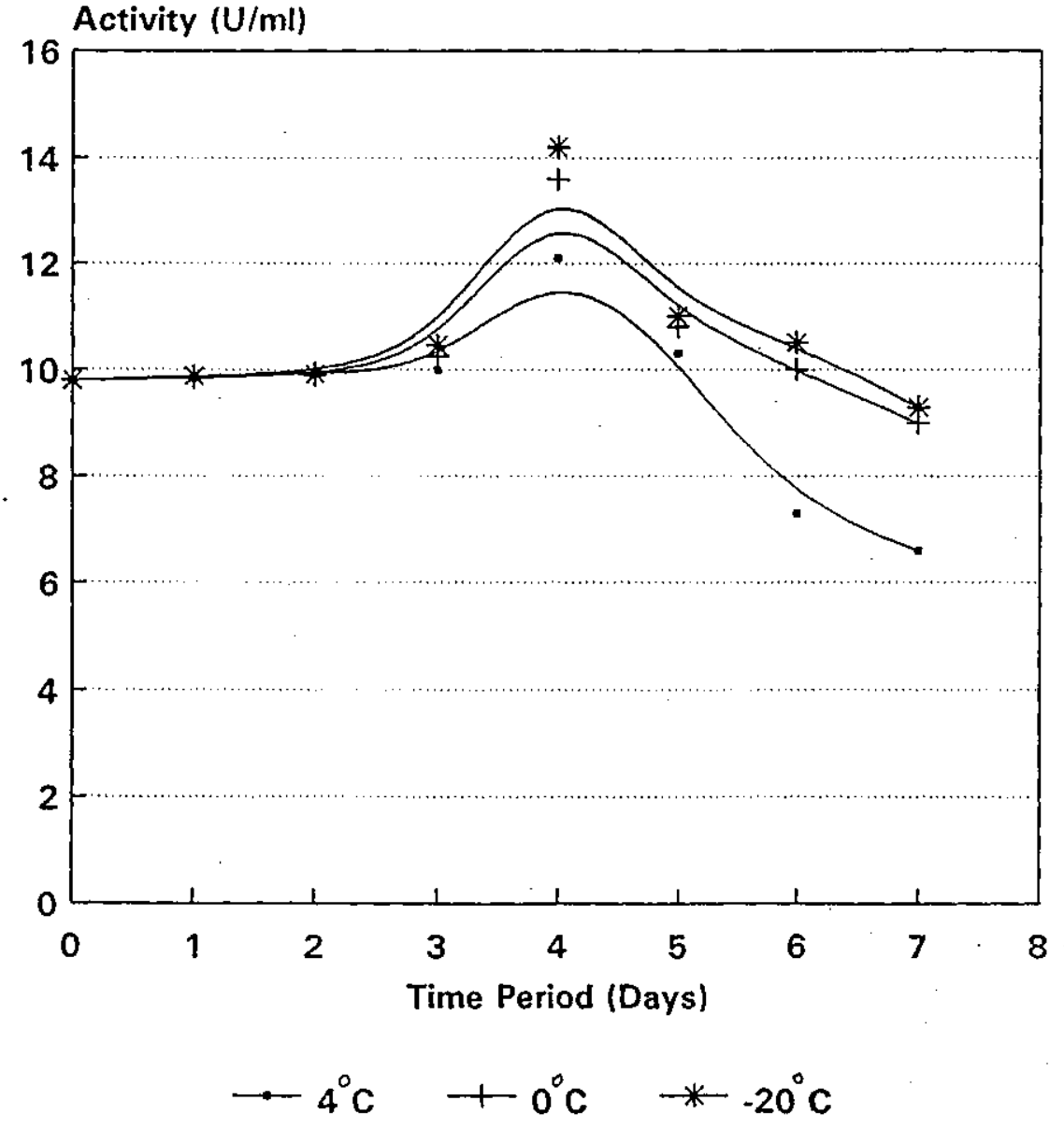
The inhibition caused by azide and cyanide has been reported to be due to the fact that both of these compounds block the oxidation of peroxidase to the reactive intermediate compound-I by binding to the iron atom in the prosthetic group (Paul and Ohlsson, 1985).

Amongst the compounds reacting with thiol group and causing chemical modification, $HgCl_2$ has been observed to be strongest inhibitor of buffalo lactoperoxidase, resulting in 85 per cent inhibition at 0.5 mM concentration. Relatively, N-ethylmaleimide and $CuSO_4$ were observed to be least effective. The inhibition caused by iodoacetamide and hydroxylamine hydrochloride was observed to be nearly of similar order.

Geike and Prasher (1976) detected Hg as inhibitor of bovine lactoperoxidase. The inhibition of buffalo lactoperoxidase with compounds reacting with thiol group and causing chemical

FIG. 4.14

Effect of Low Tempertature on Lactoperoxidase Activity of Buffalo Milk



modification was observed to be of similar order as that reported for cervical mucus peroxidase (Shindler *et al.*, 1976) with minor differences. Makinen and Makinen (1982) reported that p-chloro mercuribenzoate (0.03 mM), N-ethylmaleimide (50 mM) and 5,5'-dithiobis (2-nitrobenzene sulfonate) (1 mM) caused only slight or no inhibition of bovine lactoperoxidase.

4.3.5 EFFECT OF LOW TEMPERATURE

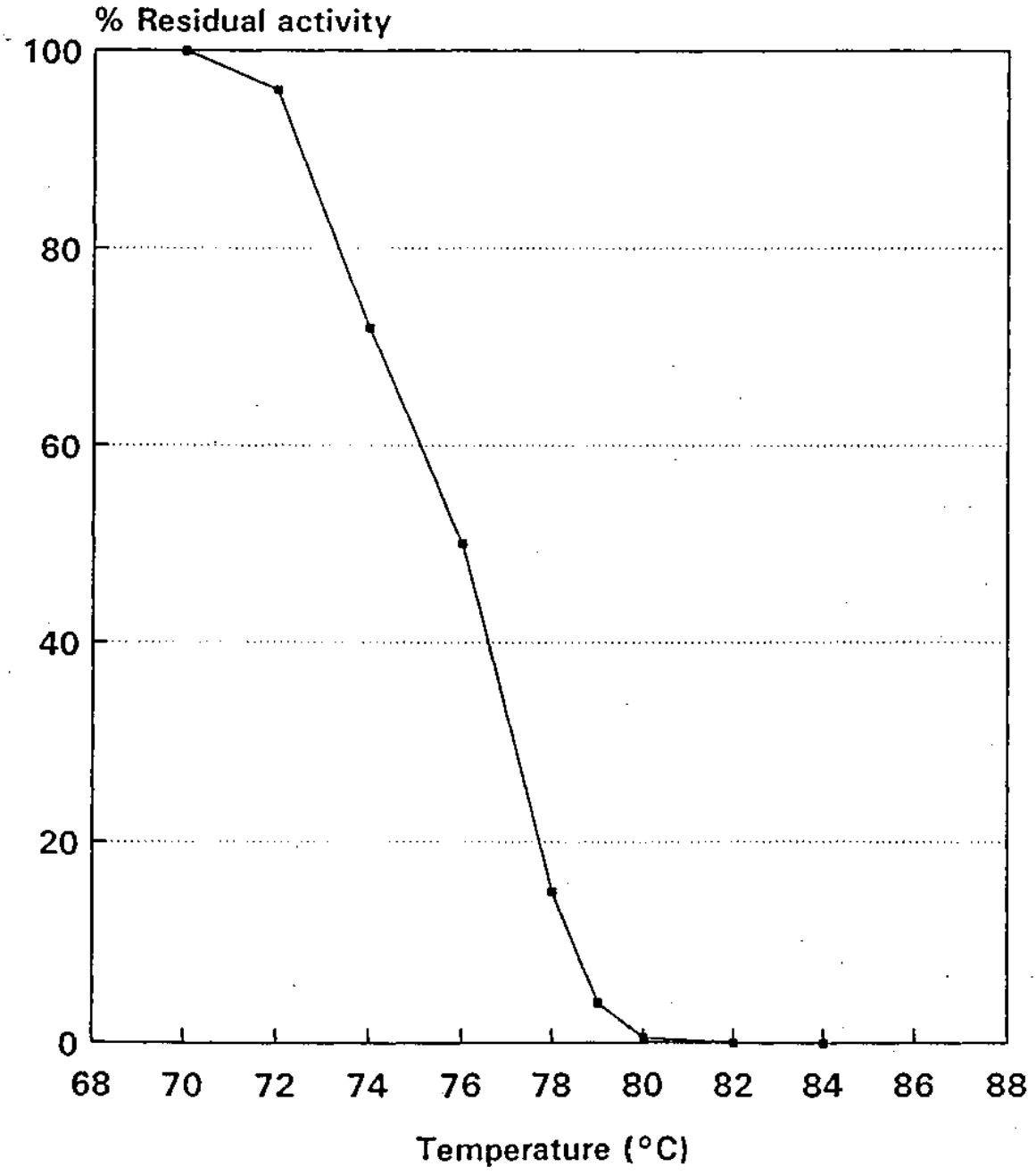
Lactoperoxidase activity of buffalo milk stored at 4°, 0° and -20°C for a period of one week was monitored at 24 h interval. As shown in Fig. 4.14, lactoperoxidase activity remained unchanged up to three days. However, an apparent rise in peroxidase activity was observed on fourth day. Further, the rise being more at -20°C and 0°C than that at 4°C. After one week of storage, the peroxidase activity at 4°C dropped by 30 per cent of initial activity, while at -20°C and 0°C the change in peroxidase activity was relatively less. Djordjevic *et al.* (1974) also reported an apparent increase in peroxidase activity of bovine milk stored at 3°C and -20°C.

4.3.6 THERMOSTABILITY OF LACTOPEROXIDASE

4.3.6.1 Effect of temperature

Buffalo milk was subjected to heat treatment from 70° to 85°C for 30 sec. As shown in Fig. 4.15, heat inactivation of lactoperoxidase in milk has been observed to be markedly sensitive at temperature around 80°C, resulting in complete inactivation at 80°C. Results obtained in the present study are in accordance with the earlier reports on heat sensitivity of

FIG. 4.15 Thermal stability of buffalo lactoperoxidase



lactoperoxidase in bovine milk (Kiermeier and Kayser, 1960b; Woerner, 1961; Monget and Laviolette, 1978; Pien, 1945; Shidlovskaya, 1982; Griffiths, 1986).

4.3.6.2 Effect of pH

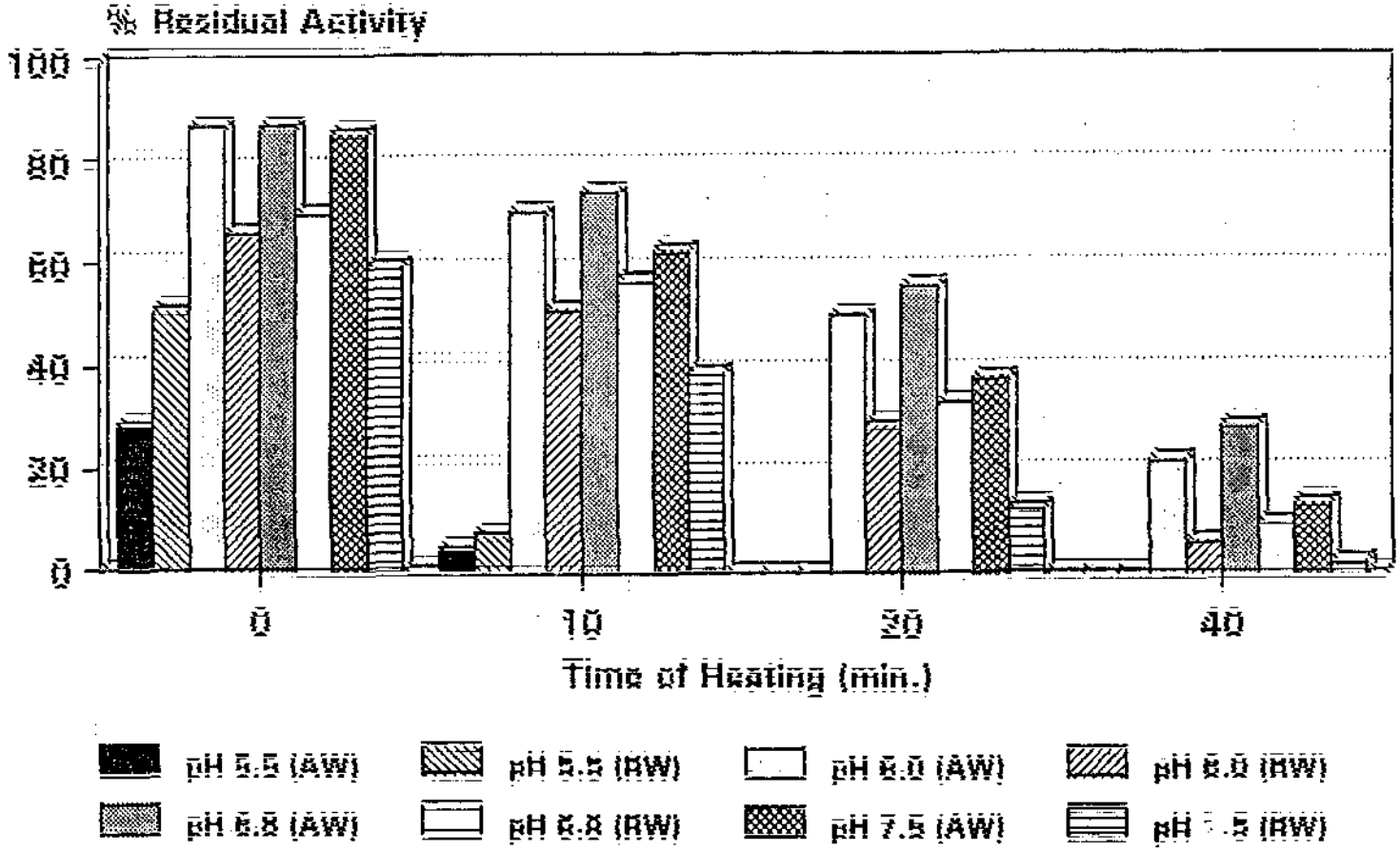
Both rennet whey and acid whey, adjusted to pH 4.6, 5.5, 6.0, 6.8 and 7.5 were subjected to heat treatment at 72°C for 0, 10, 20 and 40 minutes and measured for their residual lactoperoxidase activity. As shown in Fig. 4.16, in both acid whey and rennet whey, at pH 6.0 and 6.8, the thermostability of lactoperoxidase was found to be similar. However, relatively greater decrease was observed at pH 7.5. The heat sensitivity of lactoperoxidase was found to be higher towards acidic pH. The total inactivation of lactoperoxidase activity occurred in 10 minutes at pH 5.5, while at zero time the complete inactivation occurred at pH 4.6.

Hernandez *et al.* (1990) reported that lactoperoxidase is markedly sensitive to heat at acidic pH. However, the thermal inactivation curve at pH 6.0 and 7.0 is superimposable. Sato *et al.* (1992) observed that heated lactoperoxidase is stable in the pH range of 5.6-7.7. Thus, the results obtained in the present study are in general agreement to the earlier reports.

4.3.6.3 Effect of type of whey

As shown in the Fig. 4.16, it is clear that at all the pH values, the thermostability of lactoperoxidase in acid whey has been observed to be greater than that of rennet whey. The difference of thermostability in rennet whey and acid whey might

FIG. 4.10 **Effect of pH on thermostability of lactoperoxidase in whey**



RW = Rennet Whey
AW - Acid Whey

be attributed to their difference in ionic concentration/ionic strength. Hernandez *et al.* (1990) and Sato *et al.* (1992) reported that ionic concentration especially of calcium and sodium ions have a marked stabilizing effect on lactoperoxidase activity. Since the acid whey has a higher ionic strength than rennet whey, therefore, this might be the contributing factor towards greater thermostability of lactoperoxidase in acid whey than rennet whey.

4.3.6.4 Kinetics of thermal inactivation of buffalo lactoperoxidase activity

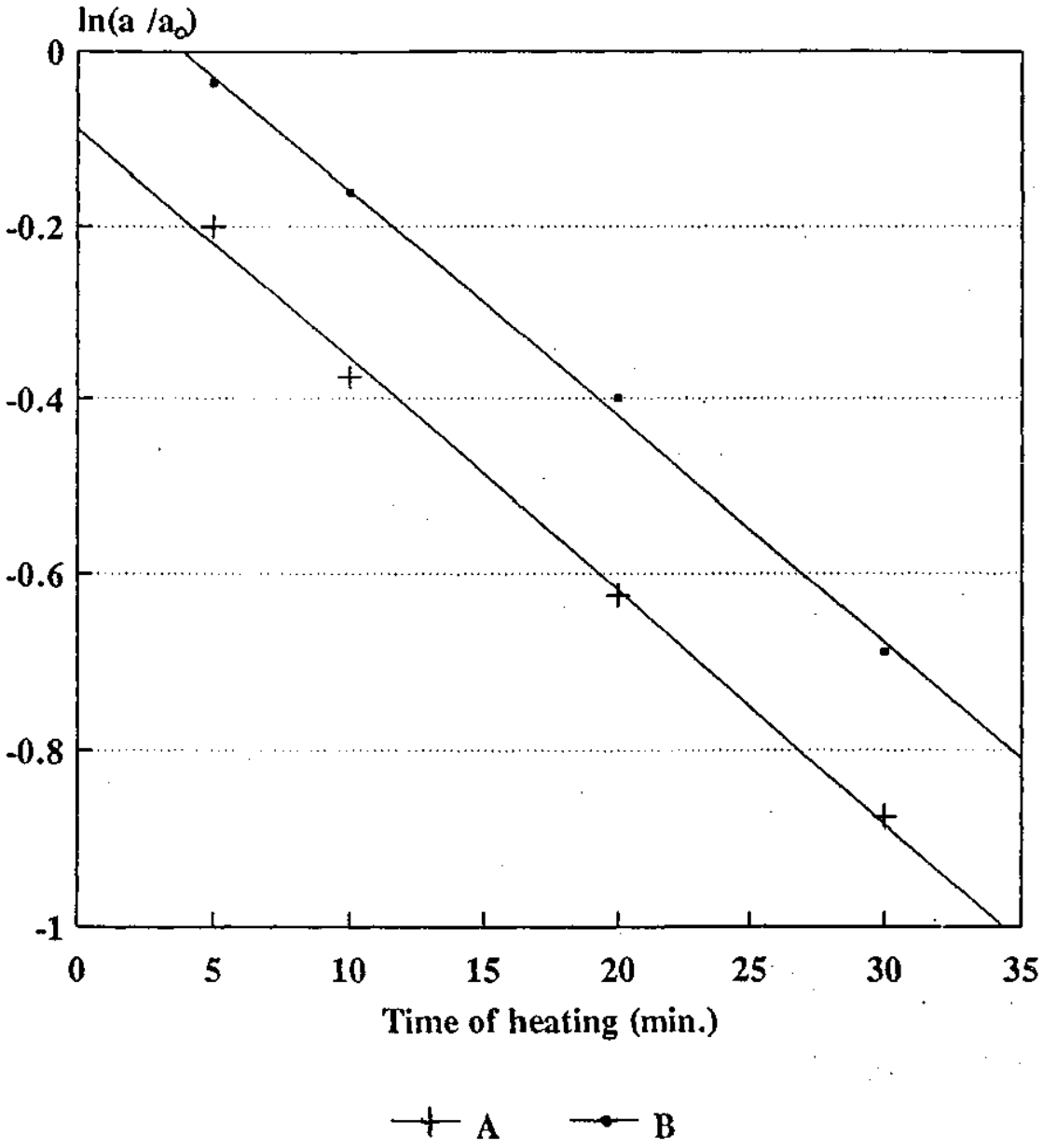
Buffalo milk and buffalo milk + lactoperoxidase (200 ug/ml) was heated at 71°C for 5, 10, 20 and 30 minutes. As shown in Fig. 4.17, the plots of $\ln(a/a_o)$ vs. time were linear and had the same slope. It proves that the reaction is of first order kinetics.

(a) Thermal inactivation of lactoperoxidase in buffalo milk

Heat inactivation of lactoperoxidase in buffalo milk was determined by heating skim milk at 69, 70, 71 and 72°C for 5, 10, 20, 30 and 40 minutes and measuring the residual lactoperoxidase activity with ABTS as chromogenic substrate at pH 6.0. The velocity constants obtained from plot of $\ln(a/a_o)$ vs. time (Fig. 4.18) are presented in Table 4.5 (Appendix II).

Rennet whey and neutralized acid whey (pH 6.8) were also heat treated at 70, 71, 72 and 73°C for 5, 10, 20, 30 and 40 minutes and remaining activity was measured. The velocity

FIG. 4.17 **Order of heat inactivation of Lactoperoxidase in milk**



A: Milk
B: Milk + Lactoperoxidase (200 mg/l)

Fig. 4.18

Rate of inactivation of Lactoperoxidase in buffalo milk

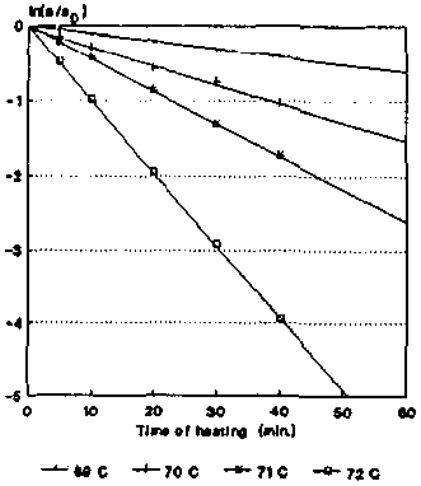


Fig. 4.19

Rate of inactivation of Lactoperoxidase in rennet whey

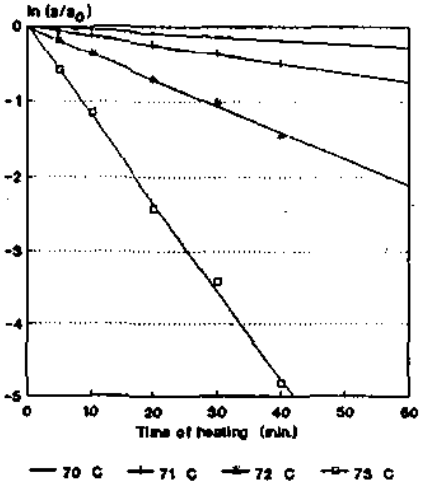


Fig. 4.20

Rate of inactivation of Lactoperoxidase in acid whey (pH 6.8)

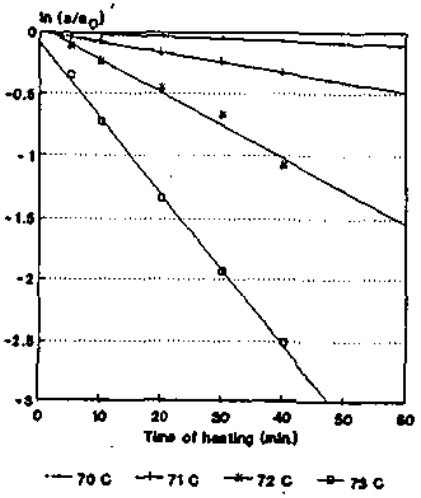


Fig. 4.21

Rate of inactivation of Lactoperoxidase in acetate buffer (0.1 M ,pH 6.4)

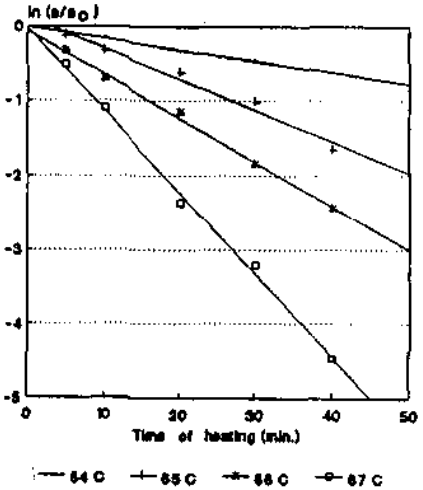
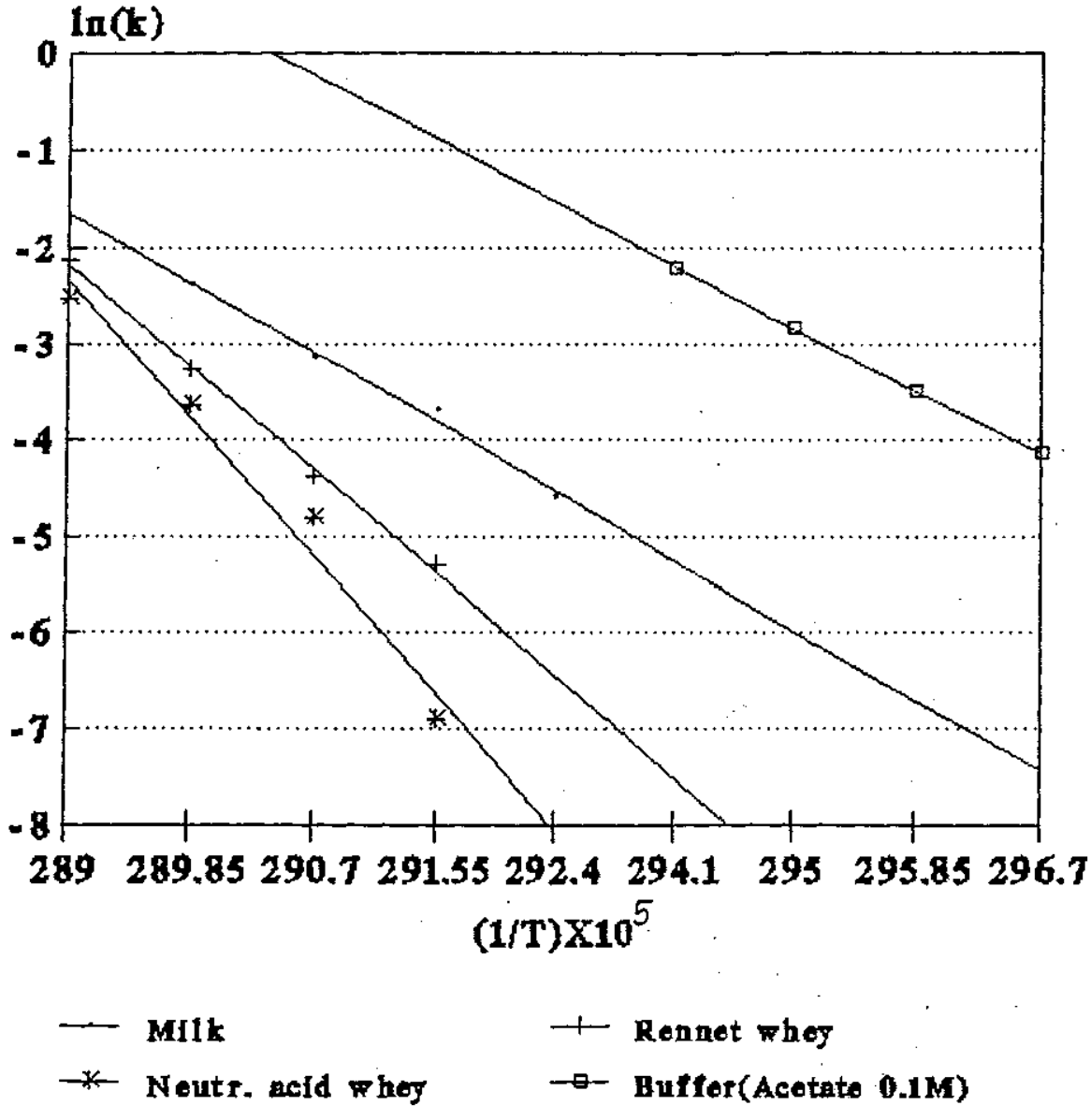


Fig. 4.22

Arrhenius Plots of Lactoperoxidase Inactivation in Milk,Rennet whey, Neut.acid whey and acetate buffer



constants determined from their respective plots of $\ln(a/a_o)$ vs. time (Figs. 4.19 and 4.20) are presented in Table 4.5 (Appendix II).

The activation energy for heat denaturation of lactoperoxidase in skim milk, rennet whey and neutralized acid whey was determined from the Arrhenius plots, i.e., the plots of their velocity constants ln(k) vs. reciprocal of absolute temperature (I/T) (Fig. 4.22).

The activation energy for heat denaturation of lactoperoxidase in skim milk, rennet whey and neutralized acid whey has been found to be 710.25 kJ/mol, 1031.90 kJ/mol and 1222.16 kJ/mol, respectively.

Various investigators have reported the activation energy for heat denaturation of bovine milk lactoperoxidase ranging from 634 to 800 kJ/mol [797 kJ/mol (VanEck, 1911); 776 kJ/mol (Zilva, 1914); 711 kJ/mol & 648 kJ/mol (Woerner,1963); 800 kJ/mol (Hernandez *et al.*, 1990) and 634 kJ/mol (Olszewski and Reuter, 1992)]. For rennet whey, Arrhenius activation energy has been reported to be 839 kJ/mol (Woerner, 1961) and 1030 kJ/mol (Hernandez *et al.*, 1990).

The activation energy for heat denaturation of lactoperoxidase in buffalo milk and rennet whey are observed to be closer to that for bovine milk and whey (Woerner, 1963; Hernandez *et al.*, 1990). However, no data is available on the thermal stability of bovine lactoperoxidase in neutralized acid whey. It was found that activation energy required for

Table 4.5 Velocity constants and Arrhenius activation energy for inactivation of lactoperoxidase in different media

Medium	Temperature (°C)	Velocity constant k (min^{-1})	Arrhenius activation energy A (kJ/mol)
Milk	69	0.010	710.25
	70	0.025	
	71	0.043	
	72	0.0929	
Rennet whey	70	0.005	1031.90
	71	0.0124	
	72	0.0385	
	73	0.1192	
Acid whey (pH 6.8)	70	0.001	1222.16
	71	0.008	
	72	0.026	
	73	0.080	
Lactoperoxidase (0.1 M acetate buffer, pH 6.4)	64	0.0156	610.00
	65	0.030	
	66	0.0593	
	67	0.110	

denaturation of lactoperoxidase in neutralized acid whey is much greater than that for rennet whey. The differences in thermostability of lactoperoxidase found between whey and milk as well as between rennet whey and acid whey might be due to differences in ionic composition or strength. Hernandez *et al.* (1990) reported that Ca^{2+} plays an important role on the thermal-stability behaviour of lactoperoxidase and that proteins, notably ß-lactoglobulin, also may have an effect on it. Sato *et al.* (1992) also observed that calcium and sodium salts have stabilizing effect during heating of lactoperoxidase.

(b) Thermal inactivation of buffalo lactoperoxidase in buffer

Purified buffalo lactoperoxidase at a concentration of 50 ug/ml in 0.1 M sodium acetate buffer (pH 6.0) equilibrated overnight at 4°C was subjected to heat treatments at 64, 65,66 and 67°C for 5, 10, 20, 30 and 40 minutes. The velocity constants (k) determined from the plot of $\ln(a/a_o)$ vs. time (Fig. 4.21) are given in Table 4.5. The activation energy for heat denaturation of lactoperoxidase in acetate buffer was calculated from Arrhenius plot (Fig. 4.22) as 610 kJ/mol.

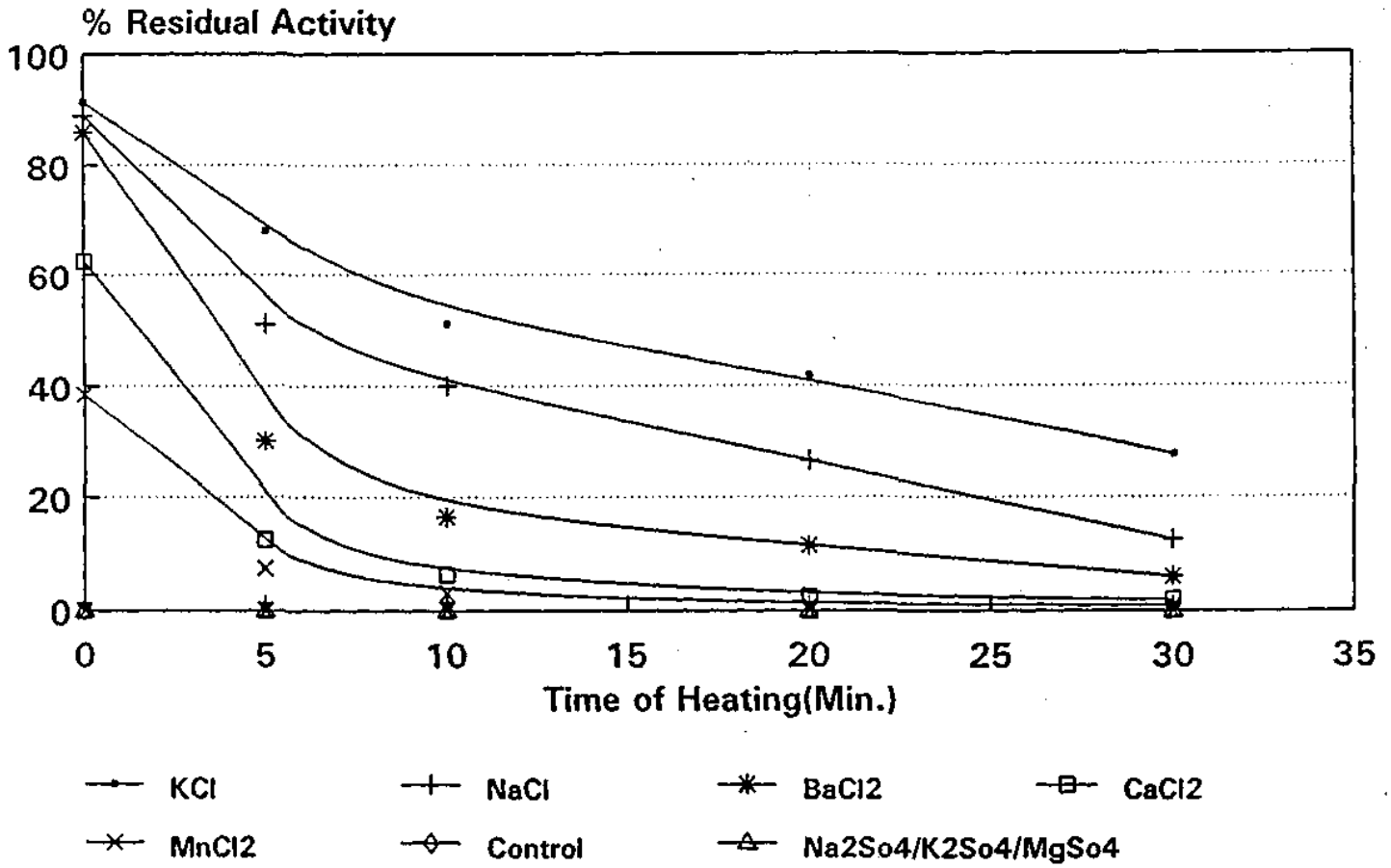
Bardsley (1985) found lower denaturation temperature and two stage thermal denaturation kinetics, instead of first order reaction for lactoperoxidase in 0.1 M phosphate buffer, pH 7.0. However, Hernandez *et al.* (1990) reported the first order thermal inactivation of lactoperoxidase and reported the Arrhenius activation energy of 602 kJ/mol. The value observed for Arrhenius activation energy for buffalo lactoperoxidase is quite close to that reported in the literature.

4.3.6.5 Effect of salts on thermal inactivation of lactoperoxidase

The effect of salts on heat inactivation of lactoperoxidase is shown in Fig. 4.23. The heat inactivation in the presence of salts seemed to induce some heat stabilization of lactoperoxidase structure as measured by loss of initial activity. The protective effect of salts on lactoperoxidase activity seems to depend on the nature of salts. Amongst the salts investigated, a series ranking most to least effective was as follows: KCl > NaCl > $BaCl_2$ > $CaCl_2$ > $MnCl_2$. At the same concentration, salts of Na_2SO_4, K_2SO_4 and $MgSO_4$ did not exhibit any protective action on lactoperoxidase activity. Further, the enhancement of thermostability of buffalo lactoperoxidase has been observed more by monovalent than divalent cations. Moreover, the monovalent anions seem to provide thermostability to lactoperoxidase, while divalent anions are ineffective. Zilva (1914) observed that addition of salts to milk gives protective effect to lactoperoxidase from heat inactivation. However, it was observed that their influence is specific and not solely dependent on valency of the ion. Hernandez *et al.* (1990) and Sato *et al.* (1992) reported that thermal stability of bovine lactoperoxidase increases with increase in calcium chloride concentration. Sato *et al.* (1992) speculated that the mechanism of thermal enhancement with monovalent cations is different than that of calcium.

Results obtained in the present study corroborate the earlier reports. Besides, it also reveals that both cations and anions have specific role in the stabilization of lactoperoxidase during heat treatment.

FIG. 4.23 **Effect of salts on thermostability of Lactoperoxidase**



4.4 PHYSICO-CHEMICAL PROPERTIES

4.4.1 SPECTRAL ANALYSIS

The absorption spectra of purified lactoperoxidase isolated from buffalo skim milk, rennet whey and acid whey at concentrations 0.7, 0.6 and 0.4 mg/ml in 0.1 M phosphate buffer (pH 7.0) was recorded with double beam spectrophotometer (JASCO).

As shown in Fig. 4.24, the absorption spectra of lactoperoxidase preparation has two peaks, one at 280 nm and another in the soret region at 412 nm. It was found that irrespective of method of preparation, the absorption spectra of lactoperoxidase was similar.

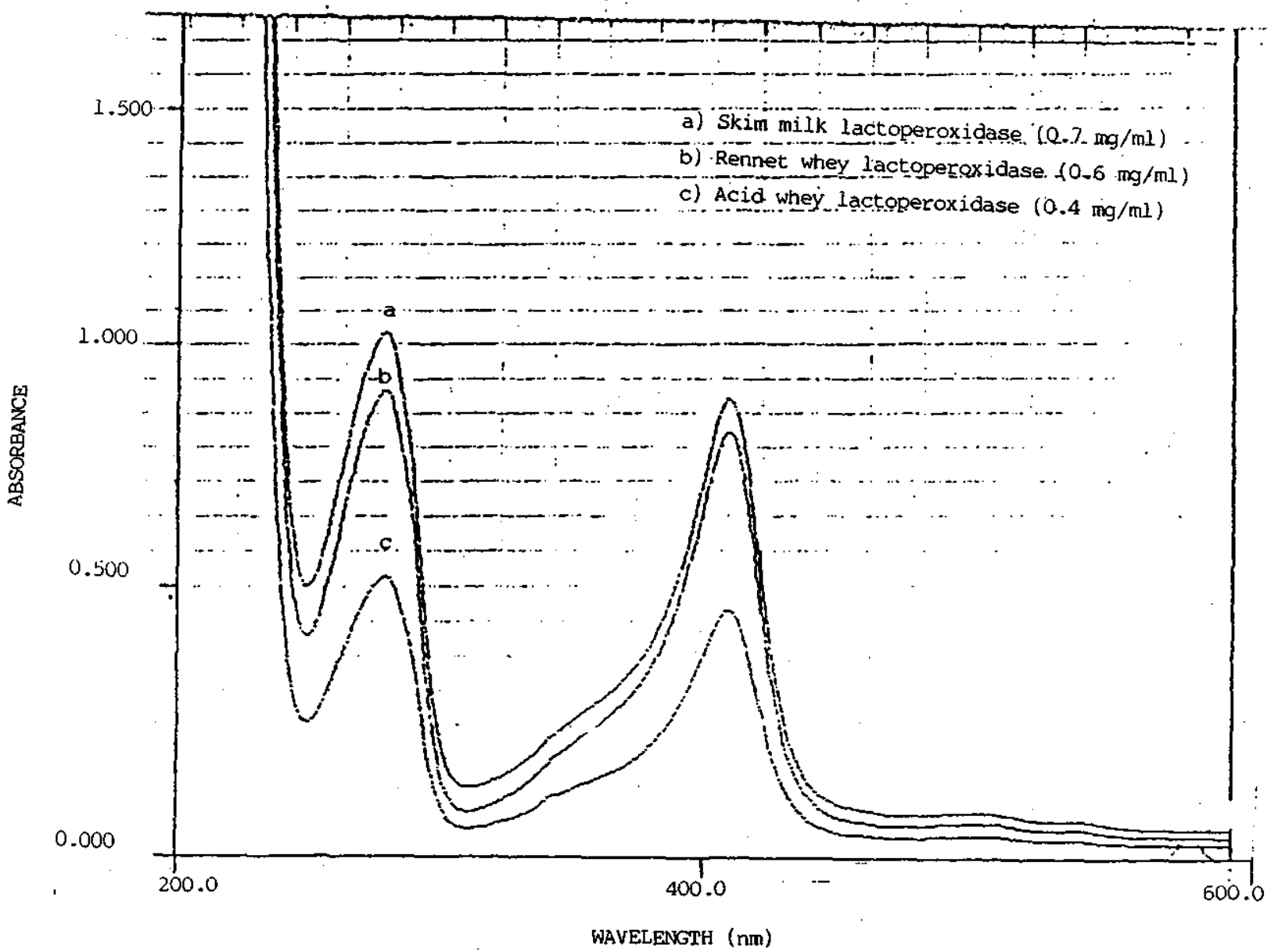
Results obtained are in accordance to that reported for bovine lactoperoxidase (Morrison *et al.*, 1957; Carlstrom, 1969c; Nichol *et al.*, 1987; Hernandez *et al.*, 1990). However, Hernandez *et al.* (1990) reported the absorption spectra of lactoperoxidase prepared from acid whey to be irregular with diminished absorption in the soret region.

4.4.2 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The homogeneity of purified buffalo lactoperoxidase was ascertained by PAGE under acidic conditions (pH 4.3) following the method of Groves (1975).

As shown in the Plate 4.1, a single band of buffalo

FIG. 4.24 ABSORPTION SPECTRA OF BUFFALO LACTOPEROXIDASE IN PHOSPHATE BUFFER (0.1 M, pH 7.0)



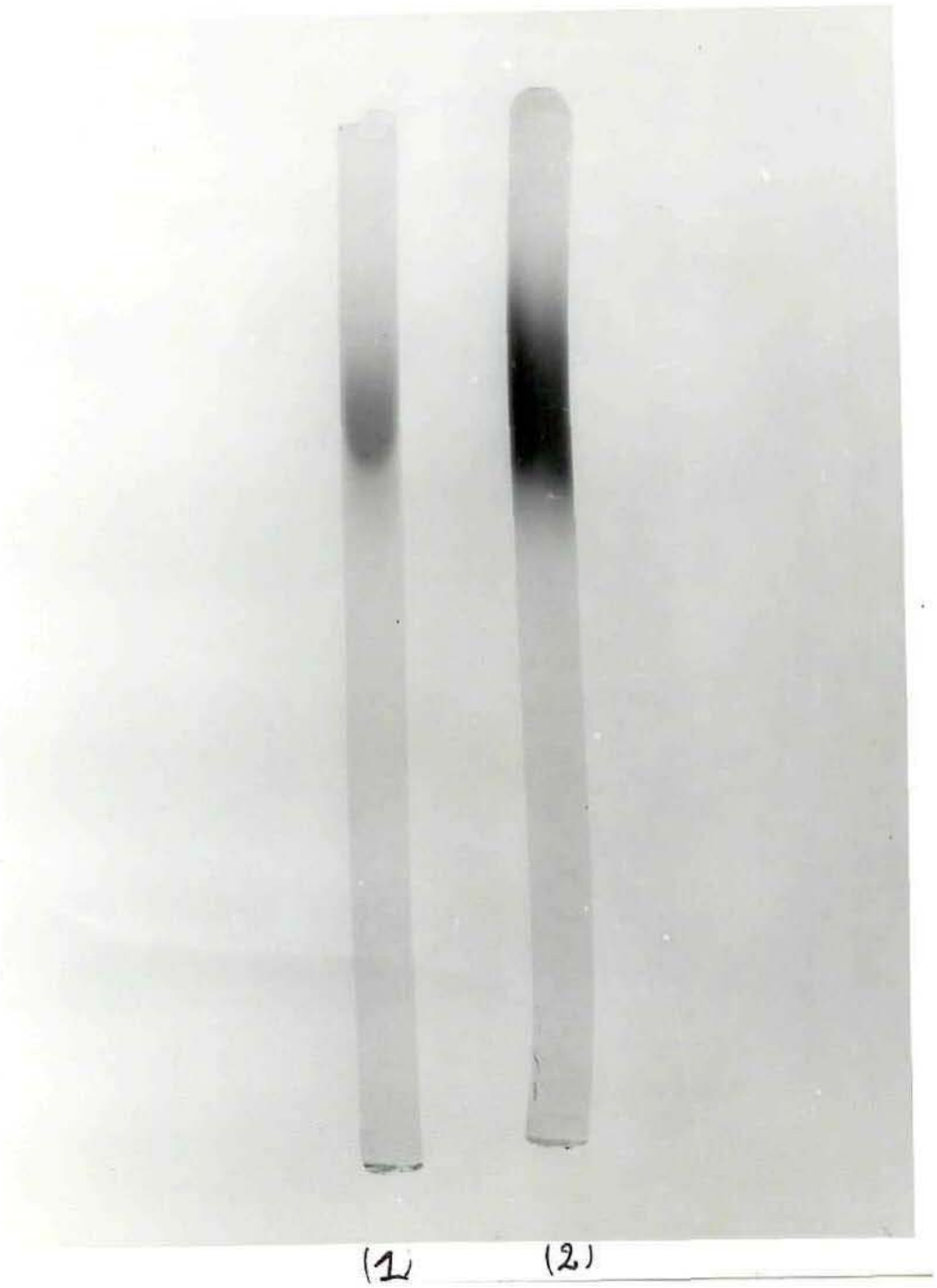


Plate 4.1 PAGE pattern of purified buffalo lacto-peroxidase sample
Lane 1 (1 µl), Lane 2 (5 µl)
(Coomassie brilliant blue R-250 stained gel)

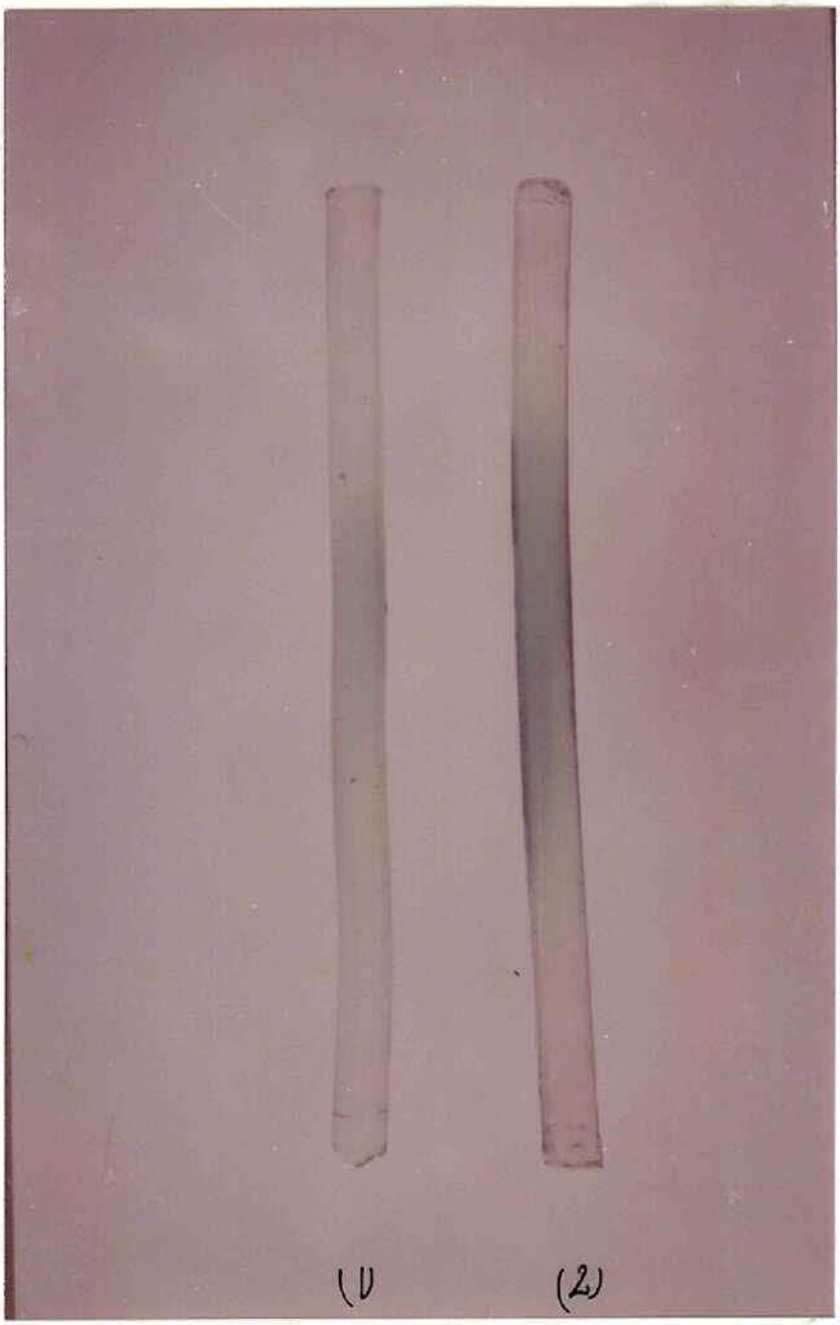


Plate 4.2 PAGE pattern of purified buffalo lactoperoxidase
Lane 1 (1 µl), Lane 2 (5 µl)
(TMBZ - H_2O_2 stained gel)

lactoperoxidase was detected on staining the gel with Coomassie Brilliant Blue R-250. However, on staining the gel for enzyme activity using 3,3',5,5'-tetramethyl benzidine (TMBZ) as chromo- genic substrate, a broader zone was detected (Plate 4.2).

Results obtained in the present study corroborate the findings of Nichol *et al.* (1987). Buffalo lactoperoxidase preparations were observed to be electrophoreti- cally homogeneous, unlike the reports from various workers that bovine lactoperoxidase shows electrophoretic heterogeneity (Rombauts *et al.*, 1967; Carlstrom, 1969a,b,c; Cals *et al.*, 1991). Rombauts *et al.* (1967) reported that multiple bands were the result of artifacts of electrophoretic procedure, while Carlstrom (1969a) suggested that partial deamidation and/or proteolysis contribute to heterogeneity.

4.4.3 ISOELECTRIC POINT

Isoelectric point of purified buffalo lactoperoxidase was determined on polyacrylamide gels using ampholyte(pH3.5-9.5).

The results are shown in Plate 4.3. Buffalo lactoperoxidase was found to resolve into two closely spaced zones. The pI values were calculated from the standard curve (Fig. 3.05) as 8.85 and 9.1.

Polis and Shmukler (1953) reported the isoelectric point of bovine lactoperoxidase by moving boundary electrophoresis as 8.0 in 0.01 μ phosphate buffer and 9.2 in 0.1 μ phosphate buffer for slow and fast bands, respectively. However, Carlstrom and

Plate 4.3 IEF pattern of purified buffalo lacto-peroxidase in polyacrylamide gel.

Lane 1 (Trysin inhibitor from soybean, pI 4.55)

Lane 2 (Carbonic anhydrase B from bovine erythrocytes, pI 5.65)

Lane 3 (Myoglobin from horse heart, pI 7.2)

Lane 4 (Buffalo lactoperoxidase sample)

Vesterberg (1967), on isoelectric focusing in column, separated the lactoperoxidase into six subcomponents with their pI 9.8, 9.69, 9.68, 9.49, 9.31 and 9.16, respectively. Recently, De (1992) electrofocused bovine lactoperoxidase in the Phast system and revealed that it has a distinctly basic pI and focused at the cathode.

The results obtained in the present study indicate that buffalo lactoperoxidase is a basic protein, which is in agreement to earlier reports.

4.4.4 MOLECULAR WEIGHT

The molecular weight of buffalo lactoperoxidase was determined by gel filtration chromatography on Sephadex G-100 and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The results obtained are presented in Table 4.6 and Plate 4.4. On SDS-PAGE, it has been observed that lactoperoxidase migrated as a sharp single band with its relative mobility higher than that of buffalo lactoferrin and lower than bovine serum albumin. On gel filtration, the elution volume of lactoperoxidase was found to be less than buffalo lactoferrin and more than bovine serum albumin.

Table 4.6 Molecular weight of buffalo lactoperoxidase

Method	Molecular weight (kDa)
Gel filtration chromatography	70.5
SDS-PAGE	73.0

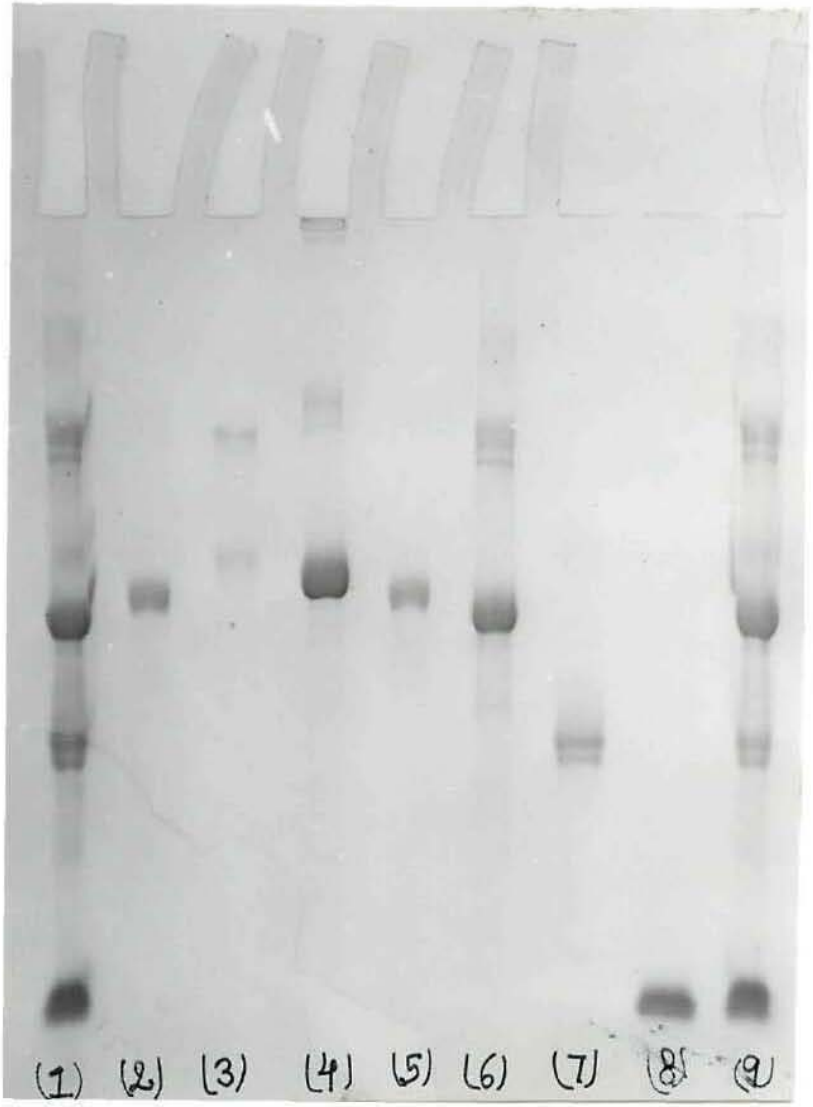


Plate 4.4 SDS-PAGE pattern of purified buffalo lactoperoxidase.

Lane 1 & 9 (97, 68, 45, 14 kDa)

Lane 3 (97 kDa), Lane 4 (78.5 kDa),

Lane 6 (68 kDa), Lane 7 (45kDa),

Lane 8 (14 kDa)

Lane 2 & 5: Lactoperoxidase sample

Based on sedimentation equilibrium, iron content and amino acid content, the molecular weight of bovine lactoperoxidase has been reported to be ranging between 76,000-82,000 daltons (Rombauts *et al.*,1967; Carlstrom, 1969b; Polis and Shmukler, 1953). With SDS-PAGE (5-15% gel concentration), the molecular weight of bovine lactoperoxidase has been reported to be 75,000 ± 2,000 daltons (Sievers, 1981; Mansson-Rahemtulla *et al.*, 1988).

The molecular weight of buffalo lactoperoxidase is observed to be similar to that reported for bovine lactoperoxidase. Further, the close similarity between the molecular mass values obtained for the purified buffalo lactoperoxidase by SDS-PAGE and gel filtration indicates that the peroxidase exists as a single polypeptide chain similar to that shown for bovine lactoperoxidase (Sievers, 1981).

4.4.5 STOKE'S RADIUS

The Stoke's radius of buffalo lactoperoxidase was determined by gel filtration chromatography on Sephadex G-100. The Kav values of marker proteins (alpha-lactalbumin, ß-lactoglobulin, ovalbumin, bovine serum albumin and lactoferrin) was calculated from their elution volume (Ve). From the standard curve (Fig. 3.04), the Stoke's radius of buffalo lactoperoxidase was calculated as 37 A° . Thus, the Stoke's radius of buffalo lactoperoxidase has been observed to be close to that reported for bovine lactoperoxidase (Shimazaki and Sukegawa, 1982).

4.4.6 IRON AND CALCIUM CONTENT

Purified buffalo lactoperoxidase with R_z value of 0.91 was used for the estimation of iron and calcium content. After digestion of the sample with triacid mixture, it was diluted with glass distilled water and the absorbance was measured by atomic absorption spectrophotometer (Philips). The quantity of iron and calcium was determined from their respective standard curves (Figs. 3.06 and 3.07).

The average of three trials conducted for the determination of iron and calcium content was found to be 0.071 ± 0.011 and 0.059 ± 0.016 per cent, respectively. Taking the molecular weight of buffalo lactoperoxidase as 73 kDa, nearly equimolar level of iron and calcium atom per mole of lactoperoxidase molecule was found.

Various workers have studied the iron and calcium content of lactoperoxidase from bovine milk. Iron content has been reported to a level of 0.0755 per cent (Theorell and Akeson, 1943), 0.0690 per cent (Polis and Shmukler, 1953), 0.0729 per cent (Rombauts *et al.*,1967) and 0.068-0.0709 per cent (Calstrom, 1969b) in different studies. Booth *et al.* (1989) reported equimolar quantity of calcium and iron in lactoperoxidase molecule, while Lukat *et al.* (1993) observed that bovine lactoperoxidase contain 1.5 ± 0.1 calcium ions per heme unit. The results obtained in the present investigation are in agreement to the earlier reports.

4.4.7 *CARBOHYDRATE ANALYSIS*

The purified buffalo milk lactoperoxidase was analysed for hexoses by phenol-sulphuric acid method (Dubois *et al.*, 1956), hexosamine by the procedure of Belcher *et al.*(1954) and N-acetyl-neuraminic acid content according to Warren (1959).

Results as given in Table 4.7 indicate that the hexoses (neutral sugar) content found for buffalo milk lactoperoxidase is considerably lower than that reported for bovine lactoperoxidase. Using spectrophotometric method, with standard curve of glucose, Rombauts *et al.* (1967) reported 1.5 per cent neutral sugar, while with mannose standard curve, Carlstrom (1969b) reported 5.37 per cent neutral sugar. Mansson-Rahemtulla *et al.* (1988) following HPLC method reported the level of hexoses to be 7.0 per cent with mannose constituting 5.9 per cent.

Table 4.7 Carbohydrate composition of buffalo milk lactoperoxidase (µg/mg)

Hexoses	10
Hexosamine	15
N-acetyl neuraminic acid (NANA)	0.77

The hexosamine content of buffalo lactoperoxidase (Table 4.7) was found to be lower than that reported for bovine lactoperoxidase. Rombauts *et al.* (1967) reported the total

hexosamine content of bovine lactoperoxidase by amino acid analyser as 5.475 per cent, while Carlstrom (1969b gave the corresponding value as 4.1 per cent. Mansson-Rahemtulla *et al.* (1988) using HPLC method reported the total hexosamine content as 3.65 per cent, with glucosamine constituting 75 per cent.

NANA content determined from buffalo lactoperoxidase (Table 4.7) corresponds to 0.19 moles of NANA per mole of lactoperoxidase molecule which lies well within the range (0.17 to 0.73 moles of NANA per mole of lactoperoxidase) of bovine lactoperoxidase subcomponents (Carlstrom, 1969b). However, Rombauts *et al.* (1967) showed that bovine lactoperoxidase does not contain any neuraminic acid.

4.5 *STRUCTURAL PROPERTIES*

4.5.1 CRYSTALLIZATION

Various sets of conditions and methodologies were used for the crystallization of lactoperoxidase as described earlier (section 3.6.1). The crystals were observed with two morphologies.

As shown in Plate 4.5, using microdialysis method, under the conditions of 0.1 M phosphate buffer (pH 8.2) containing 10 per cent ethanol, the crystals observed were very large in two dimensions but extremely thin in the third dimension (i.e. 0.5 x 0.5 x 0.01 mm^3). However, these crystals did not diffract.

The crystals with another morphology (Plate 4.6) of needle like shape showed good extinctions. These were grown under the

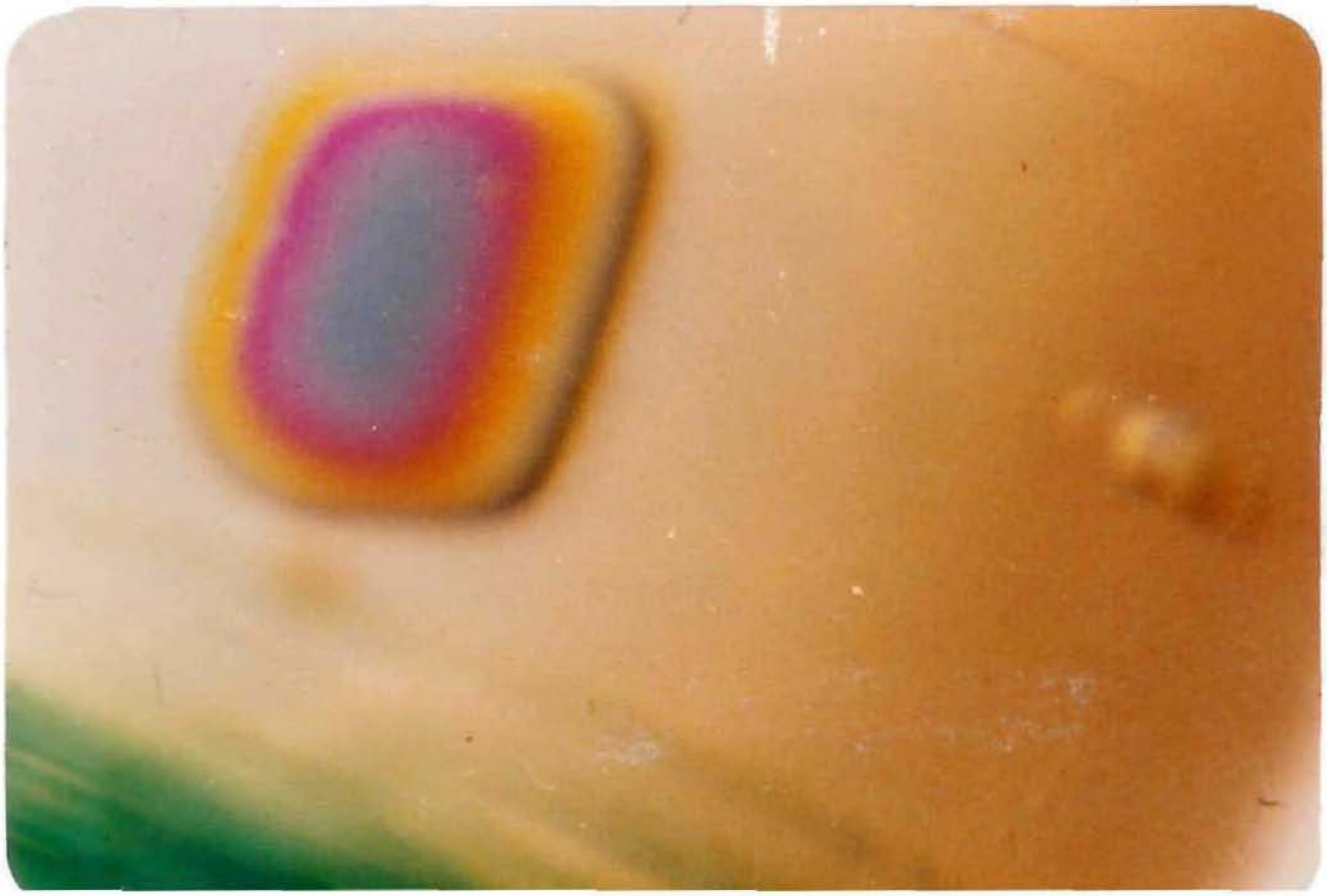


Plate 4.5 Crystals grown in 0.1 M phosphate buffer (pH 8.2) containing 10% ethanol by microdialysis method.

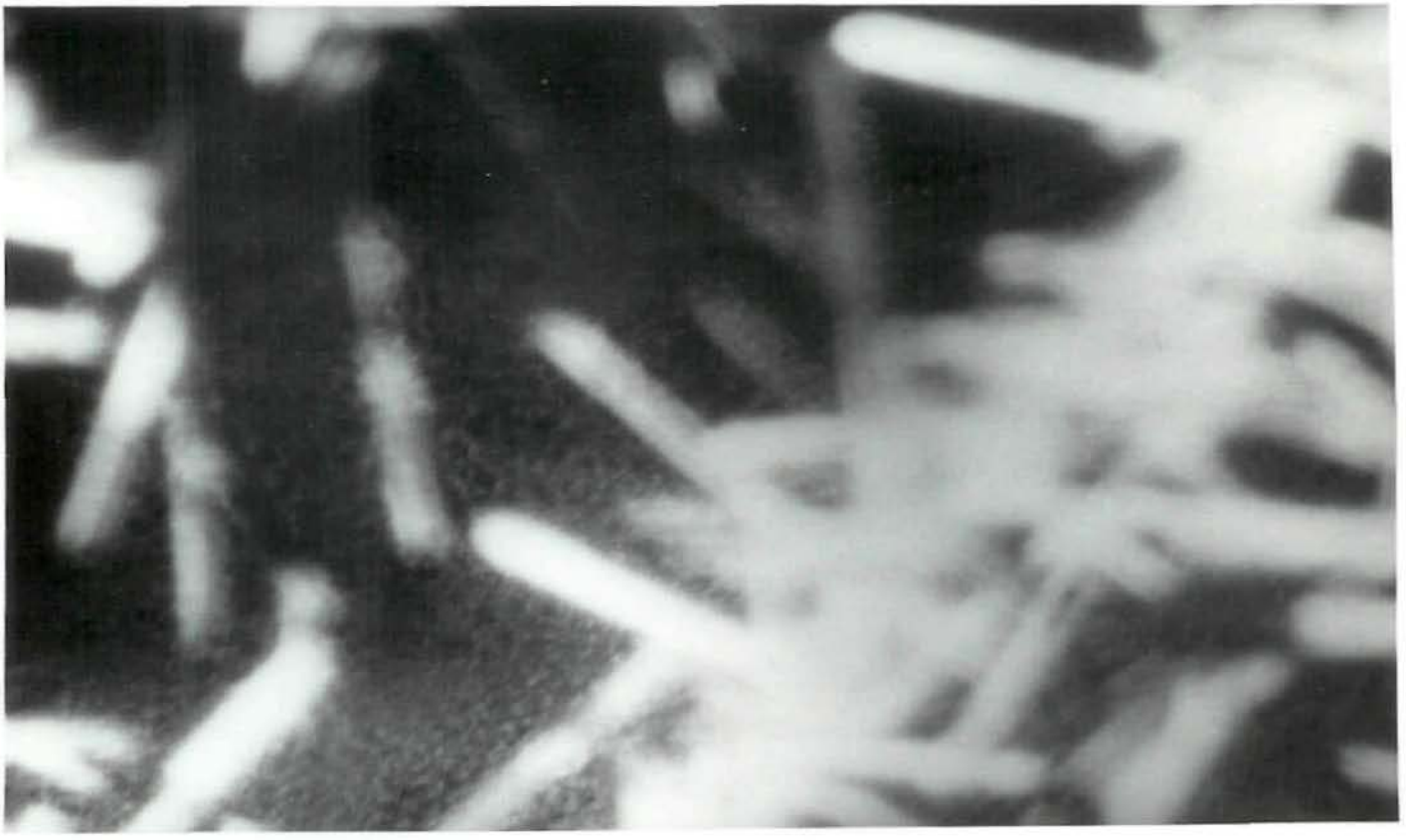


Plate 4.6 Crystals grown in 0.01 M phosphate buffer (pH 8.2.) containing 10% ethanol by sitting drop vapour diffusion technique.

conditions of 0.01 M phosphate buffer (pH 8.2) containing 10 per cent ethanol with sitting drop vapour diffusion technique, in 10 days at 4°C. A crystal with dimensions 0.40 x 0.20 x 0.07 mm³ was used to collect 64 images with $\Delta\phi = 1°$. The data limits extended beyond 3.5 A° resolution.

4.5.2 *MOUNTING OF THE CRYSTALS AND DATA COLLECTION*

A crystal of dimensions 0.4 x 0.2 x 0.07 mm³ was used for data collection using synchrotron radiation (λ = 0.92 A°) and the Mar Research imaging plate scanner.

4.5.2.1 *Imaging Plate Scanner*

The imaging plate scanner (Plate 4.7) of the following specifications was used:

plate diameter	- 180 mm
pixel size	- 150 µm x 150 µm
number of pixels	- 1200 x 1200
sensitivity	- 1 photon/ADC unit at 8 KeV
intrinsic noise	- 2 to 3 photons equivalent
dynamic range	- Approx. 1:128000
scanning time	- Approx. 70 seconds
scan and erase cycle time	- Approx. 90 seconds
minimum distance from crystal to plate analyser	- 65 mm
angular acceptance	- 110 degrees
outside dimensions of the scanner	- Approx. 85 x 40 x 87 cm

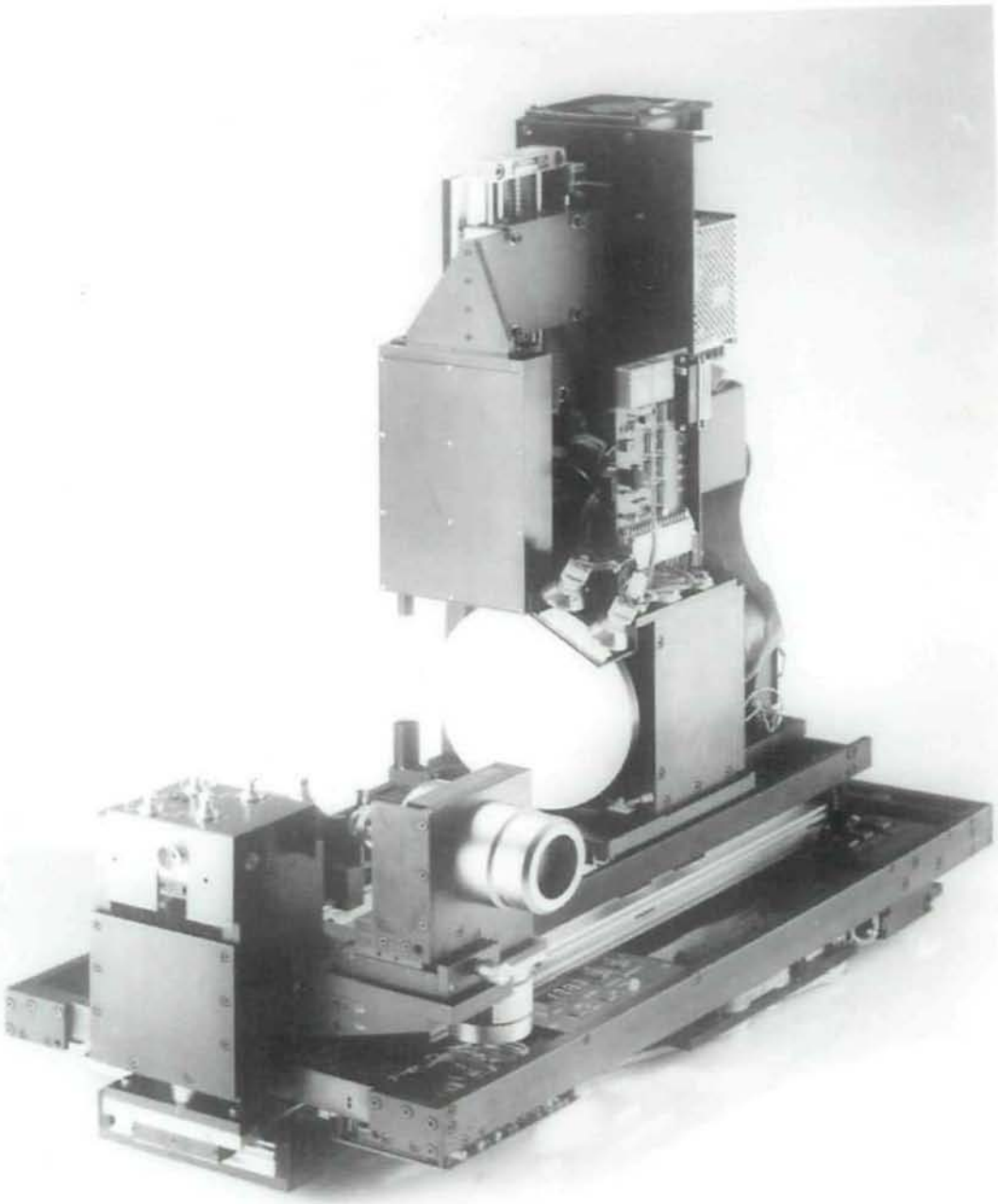


Plate 4.7 Imaging plate scanner.

The crystallographic cradle consists of a phi-axis, a collimator system with continuously variable x/y-slits and ionization chambers to monitor the primary beam intensity. A local beam shutter allows accurate computer control of the exposure time.

The detection system is based on the property of imaging plates (Fuji Photo Film Co. Ltd.) to store X-ray intensities as latent images. The imaging plates consist of an amorphous layer (150 μm) of barium europium halides (BaFBr: Eu 2+ -X).

Irradiation with X-rays creates so called colour centers of F-centers which are metastable states of trapped electrons in the BaFBr : EuX-phosphor. An excitation energy of 6 eV is required to create a colour center. Upon absorption of a 2 eV photon of red He-Ne light, the metastable electron returns to its ground state under emission of blue light (4 eV). The intensity of this blue stimulated luminescence is proportional to the number of absorbed X-rays.

The scanner consists of a circular imaging plate which is part of the X-ray diffraction set up and consists of a collimator, ϕ-axis and translational stage.

After exposure, the plate is rotated and scanned by a reading head in a record player like scanner. The image is transferred to a storage disk for further processing. At the end of the scan, the residuals of the image are erased by illumination with intense visible light. The plates can be exposed again in order to collect a new diffraction pattern. The

whole process takes place fully automatically under computer control.

The hardware configuration of the system consists of:

- the scanner itself.
- a translational stage with a variable plate to crystal distance of 65 to 400 mm.
- a collimator system with continuously variable slits.
- an ionization chamber system for measuring the primary beam intensity
- a ϕ -axis system.
- a cardanic system for easy and accurate alignment in the X-ray beam
- a CCD camera for centering the crystal

The scanner software package for the data acquisition is responsible for the control of the read-out-erasure cycle and display of the collected data. The program also carries out correction of the collected images, transformation of polar coordinates into cartesian coordinates, display for inspection purpose and storage on to mass storage devices.

4.5.2.2 *Synchrotron source*

The synchrotron radiation has been widely used by protein crystallographers in recent years. The availability, performance and number of beam-lines has steadily increased the subject on a more or less regular basis. In EMBL, where the synchrotron beam-line is available it has five synchrotron beams: two for

protein crystallography, two for small angle scattering and one for EXAFS. These lines are situated on the DORIS storage ring at the DESY site (Fig. 4.25).

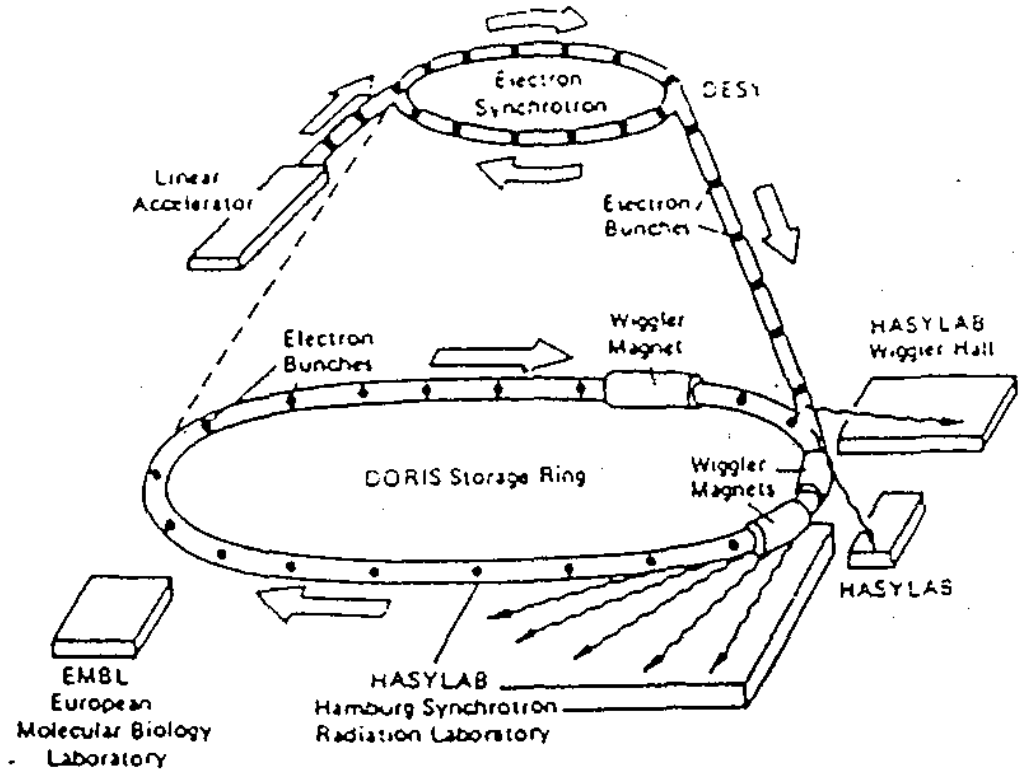
The two EMBL lines for protein crystallography are called for historical reasons XII and X31 line. XII is situated in the EMBL building and takes X-rays from the circulating positrons. It is thus only available during parasitic user time. XII is the high intensity line for protein crystallography.

The XII line consists of a bent Frankuchen triangular Ge(III) crystal, segmented flat quartz mirrors, slits, and a moveable bench with a mount for either rotation camera or a 2-D detector. All moveable elements are remotely controlled from a computer. The distance from source to the central mirror is 22.5 m and from central mirror to focus 4 m so that demagnification is roughly 5:1. The monochromator is bent to achieve focusing in the horizontal plane. The available wavelength is 0.7-2.3 A°. Changing the wavelength requires changing the take-off angle by rotating the whole bench with collimators and detectors. Vertical focusing is carried out by bending the bench on which the mirror segments have been prealigned.

The dimensions of the focused beam are about 1.2 x 0.7 mm^2. An image plate detector was used for data collection. The whole crystallographic set up on the bench can be optimally oriented into the beam by a fully automatic procedure taking 1-2 minutes.

The intensity of the beam-line allows data collection at roughly 400 X the speed on a conventional source. This makes X

Fig.4.25 Synchrotron beam lines in DESY.



II one of the most intense beam lines currently available for protein crystallography.

The X 31 beam-line is in the HASYLAB. The line takes radiation generated by the electrons and is available in both main and parasitic mode. X 31 consists of a channel cut Si(III) crystal monochromator, double focusing segmented torodial mirrors, slits and the same type of automatically aligned crystallographic cradle with image plate as on XII. All elements are mounted on movable benches and their movement remotely controlled by computer. The distance from source to mirror center is 17 m and is equal to the distance between mirrors and focus. Thus, the demagnification is 1:1. The line is characterised by easy wavelength tunability, but has relatively large focal dimensions. The image plate detector is mounted on it.

The overall intensity on crystal is some 30X weaker on X31 in comparison to XII. One advantage of this line is its high positional stability resulting from the large focus. In addition, the wavelength can be very easily tuned, involving only a rotation of the monochromator crystal with the outgoing beam remaining parallel and merely undergoing a small vertical shift. The property coupled with the narrow wavelength band pass of the Si (III) channel cut crystal makes the line ideal for multiple wavelength anomalous scattering studies.

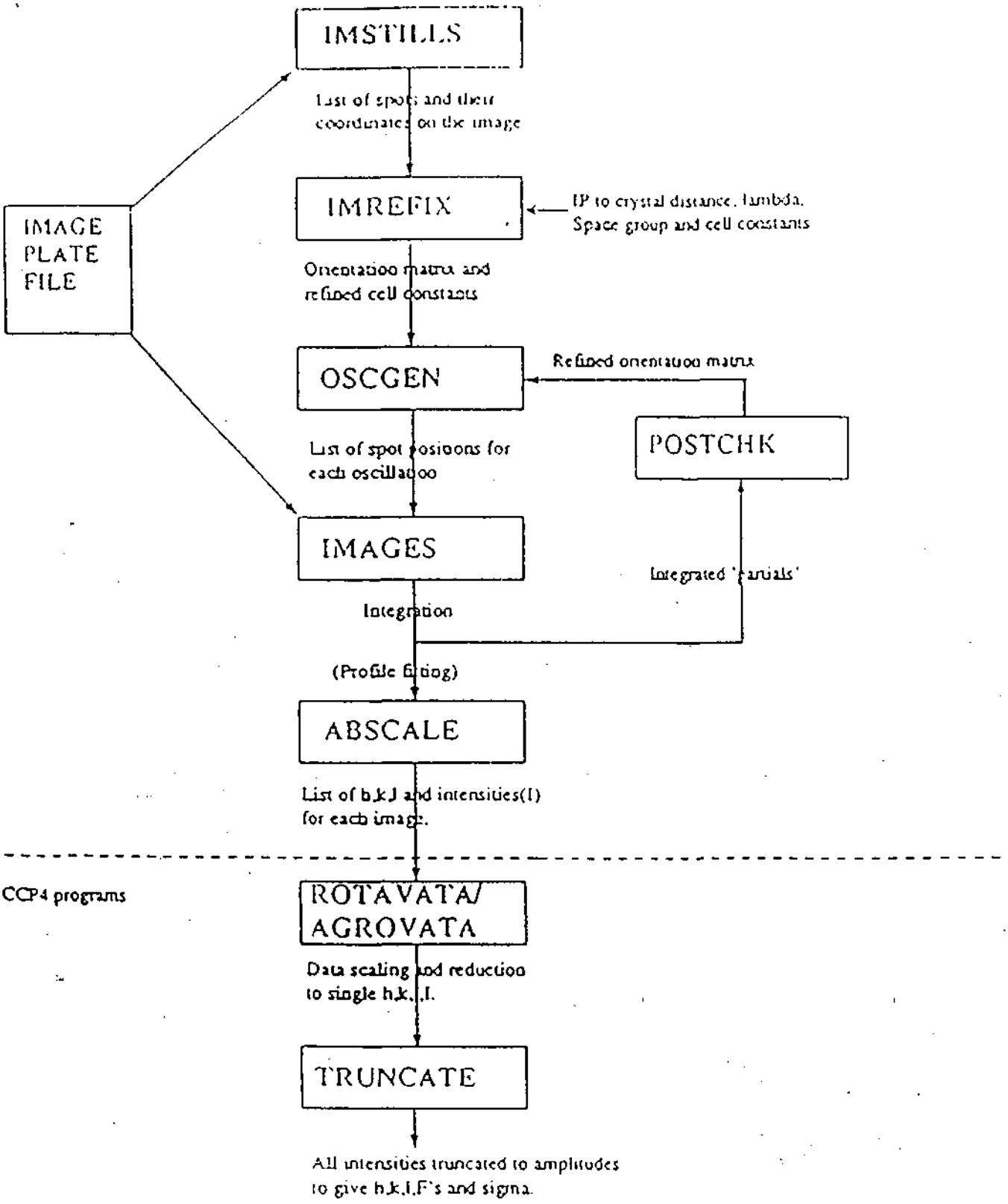
The first advantage of synchrotron radiation is simply its high intensity. This means that data collection on protein crystal can proceed much more rapidly. For crystals of good

quality and high diffracting power, this may not be a major advantage, especially with the developments in recent years of 2-D detectors and imaging plates which optimally exploit conventional X-ray sources in the home laboratory. Clearly, the synchrotron radiation is essential for difficult problems, particularly if the crystals are small and/or the unit cell is large as is the case in present study.

A further advantage of synchrotron radiation is the increased effective life time of the average protein crystal. Crystals do, of course, suffer rapid and dramatic radiation damage in a synchrotron beam. X-ray damage to protein crystals appears to have two components, firstly, primary damage occurs through the direct effect of the energy dispersed in photon absorption at X-ray energies. An additional and apparently in many cases more important effect is the secondary decay of the crystal, which continues even after the irradiation of the crystal is stopped. This is assumed to occur by the attack of free radicals and ions released after the absorption of photons. This damage can be avoided if the rate of data collection exceeds the rate at which damage proceeds.

A property unique to synchrotron radiation is its wide spectrum. For conventional sources, work is in practice restricted to a small number of wavelengths, characteristic of the metal used in the anode target, usually copper or molybdenum. Any studies away from these lines have vastly reduced intensity available. In contrast, synchrotrom radiation can cover a very wide area of the spectrum, certainly spanning the 0.5 A°-2.5 A° range of the most interest to crystallographers. The first

Fig.4.26 IMAGE PLATE DATA PROCESSING



advantage of this is the use of short wavelength around 0.8 A° - 1.0 A°. For protein crystals, use of molybdenum radiation on a conventional source usually gives too low an intensity to be useful. Use of synchrotron radiation in this range minimizes error arising from absorption effects, probably the largest single source of systematic error in data collection. Since users have also claimed reduced damage at shorter wavelength.

The tunability of the X-ray beam can also be exploited in multiwavelength measurements of anomalous X-ray scattering to solve the phase problem. This is effective if there is a heavy atom such as a metal, in the structure, if only a single isomorphous derivative is available or indeed if only a single non-isomorphous derivative can be found.

4.5.2.3 Collection of data

The data were collected on XII beam line. XII beam line station had image plate scanners from MAR RESEARCH. The storage ring was operating at 1.85 GeV and about 70 mA. The crystals were mounted in the capillary (0.5 mm diameter) with crystallization buffer and it was sealed from both sides by bee's wax. The capillary was mounted on a goniometer head with the help of plasticine. The diffraction extended to 3.2 A° resolution. From one crystal, native data upto 3.5 A° were collected. The crystal to detector distance was 350 mm. At a wavelength of 0.92 A°. 64 images were collected with a rotation range of 1.0° per image. Some typical images are shown in plate 4.8.

The data processing was done according to the flow chart in Fig. 4.26 and MOSFLM and CCP4 packages were used for

Table 4.8 **PRELIMINARY CRYSTALLOGRAPHIC DATA FOR BUFFALO LACTOPEROPEROXIDASE**

Crystallization	0.01M Phosphate buffer (pH 8.2)+ 10 % ethanol 10 days at 4°C
Crystals	Orthorhombic
Dimensions	0.4 X 0.2 X 0.07 mm^3
Space group	$P2_12_12_1$
Unit cell constants	a=116.88 Å, b=103.19 Å, c=62.25 Å
V	$7.51x10^5$ $Å^3$
Z	1
Solvent content	52 %
Data collection	At EMBL, DESY
Source	Synchrotron beam line XII
Wavelength	0.92 Å
Detector	Image Plate (Mar Research)
Resolution	3.5 Å
R_{merge}	5.5 %

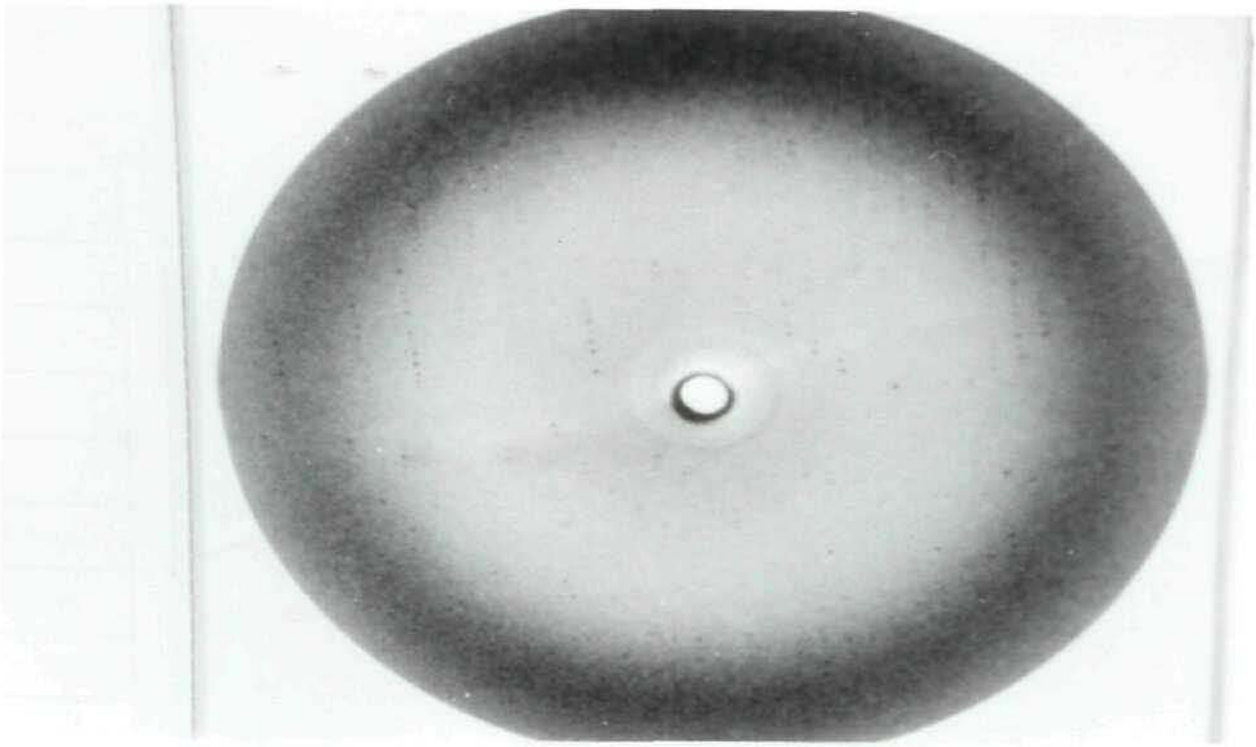
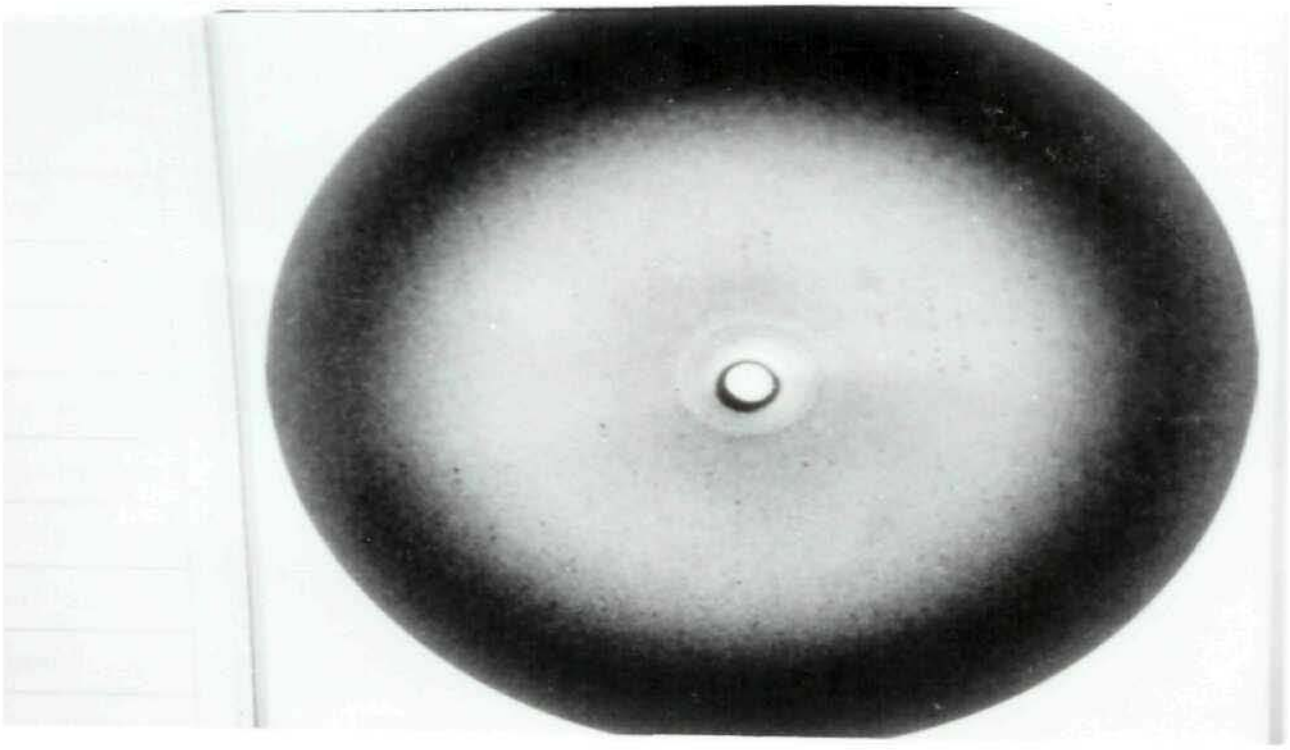


Plate 4.8 Diffraction pattern of buffalo lactoperoxidase crystals at 1° rotation using imaging plate scanner with synchrotron radiation (λ = 0.92 A°)



processing the data. The image files were the inputs. The spots were read and listed with their coordinates using the IMPREFIX. It determined the refined cell constants.

The preliminary data and other detailes are given in Table 4.8.Assuming one molecule of lactoperoxidase with molecular weight around 73,000 Da, a value of Vm = 2.57A^{o3}/Da was obtained, which lies well within the range of values obtained for other protein crystals (Matthews, 1968). This implies that asymmetric unit contains one molecule with a solvent content of approximately 52 per cent. The intensity data were integrated using the program DENZO (Otwinosla, 1991). The completeness of native data is > 80 per cent up to 3.5 A° resolution and R_{merge} is 5.5 per cent.

CHAPTER 5

SUMMARY AND CONCLUSION

5. *SUMMARY AND CONCLUSION*

1. Lactoperoxidase was isolated from buffalo skim milk as well as whey (both rennet whey and acid whey), using cation exchanger CG-50-NH_4^+. The purification of the lactoperoxidase was carried out in three steps, viz. ammonium sulfate precipitation (85% saturation), rechromatography on CM Sephadex C-50 using linear NaCl gradient (150-200 mM) and finally gel permeation chromatography on Sephadex G-100.

2. The purity of the enzyme was monitored during successive steps based on specific activity and R_z value. In different preparations of lactoperoxidase, a purification factor of 902, 880 and 831 was observed for skim milk, rennet whey and acid whey, respectively. The R_z value of the purified enzyme was found in the range of 0.90-0.95. FPLC of the buffalo lactoperoxidase (obtained after gel filtration step) on Superose 12 pg again gave a single peak with no further increase in specific activity and R_z value.

3. The recovery of lactoperoxidase preparation from six batches of skim milk was found to be 10.01 ± 1.87 mg per litre with the average specific activity of 255 ± 15 units per mg. The average lactoperoxidase content obtained from four trials each of rennet whey and acid whey was observed to be 9.0 ± 1.7 and 9.5 ± 1.5 mg per litre with the corresponding specific activity of 246 ± 10 and 240 ± 15 units per mg, respectively.

4. Two peaks of purified lactoperoxidase were observed, one in the ultraviolet region at 280 nm and another characteristic peak in the Soret region at 412 nm. The absorption spectra of lactoperoxidase was found to be similar, irrespective of its source of preparation, namely skim milk, rennet whey or acid whey.

5. Standardization of lactoperoxidase assay was carried out using ABTS as chromogenic substrate. A linear relationship was observed between lactoperoxidase activity and enzyme concentration (0.1 to 6.0 µg/ml).

6. The pH optima of buffalo lactoperoxidase was found to be 6.0, using ABTS as chromogenic substrate.

7. The lactoperoxidase in milk was found to be present mainly in serum phase. On preparation of rennet whey or acid whey from skim milk, a loss of 10 to 15 per cent of lactoperoxidase activity was observed.

8. Buffalo milk was found to contain a higher level of lactoperoxidase activity as compared to cow milk. The average peroxidase activity of buffalo milk and cow milk was observed to be 7.58 ± 2.38 and 5.76 ± 1.40 units/ml, respectively.

9. The stability of buffalo lactoperoxidase was studied in the pH range 3 to 10. It was found to be stable in the pH range 5 to 10. However, the enzyme showed relatively greater sensitivity towards lower pH.

10. Amongst the metal complexing agents and chemical modifying substances, sodium azide and mercuric chloride were found to be potent inhibitors of buffalo lactoperoxidase. The inhibition caused by other chemical substances was in the following order: hydroxylamine-hydrochloride = cyanide > 1,10-phenanthroline > iodoacetamide > EDTA > EGTA = $CuSO_4$.

11. Various whey proteins were found to give protective action to lactoperoxidase from inactivation. Lactoferrin and ß-lactoglobulin provided maximum protective action to lactoperoxidase activity followed by apolactoferrin, immuno- globulins (IgG_1 and Igs) and whey protein isolate. However, α -lactalbumin was found to be relatively less effective.

12. During storage of milk at low temperatures (-20°, 0° and 4°C), an apparent rise in peroxidase activity was observed after 4 days.

13. On heat treatment of buffalo milk, lactoperoxidase was found to be most heat labile in the temperature range 75° to 80°C with its complete inactivation occurring at 80°C.

14. Thermostability of lactoperoxidase was found to be greatly affected by pH. Acid whey and rennet whey adjusted to pH 6.0 and 6.8 showed relatively higher thermostability. However, at still lower pH values (4.6 and 5.5), it showed greatest heat lability.

15. Type of whey was also observed to affect the thermostability of lactoperoxidase. The stability was found to be higher in acid whey than rennet whey (each at pH 5.5, 6.0, 6.8 and 7.5).

16. Heat inactivation of lactoperoxidase in milk was found to be first order reaction.

17. The rate constants for heat inactivation of lactoperoxidase in buffalo milk, rennet whey, neutralized acid whey and in acetate buffer (0.1 M, pH 6.0) were determined. Arrhenius activation energy was found to be of the order of 710.25, 1031.90, 1222.16 and 610.00 kJ/mol for skim milk, rennet whey, neutralized acid whey and acetate buffer (0.1 M, pH 6.0), respectively.

18. The effect of salts on the thermostability of lactoperoxidase showed that monovalent cations (Na^+, K^+) provided greater stability to lactoperoxidase during heating than divalent cations (Ba^{+2}, Ca^{+2}, Mn^{+2}). Further, the monovalent anion (Cl^-) had a thermostabilizing effect, while divalent anion (SO_4^{-2}) was found to be ineffective.

19. On polyacrylamide gel electrophoresis, a single band of lactoperoxidase was detected both with protein and enzyme staining.

20. On SDS-PAGE, a sharp single band of lactoperoxidase was observed corresponding to a molecular weight of 73 kDA,

while on gel filtration, the molecular weight was calculated as 70.5 kDa.

21. The Stoke's radius of buffalo lactoperoxidase determined by gel filtration on Sephadex G-100 was found to be 37 A°.

22. Isoelectric focusing of buffalo lactoperoxidase on thin polyacrylamide gels, resolved the molecule into two closely spaced zones, corresponding to the isoelectric point of 8.85 and 9.10, respectively.

23. The calcium and iron content of buffalo lactoperoxidase was found to be 0.059 ± 0.016 and 0.071 ± 0.011 per cent, respectively.

24. Buffalo lactoperoxidase was observed to be a glycoprotein containing hexoses, hexosamine and N-acetyl neuraminic acid to a level of 10, 15 and 0.77 ug/mg, respectively.

25. Lactoperoxidase crystals grown under the conditions of 0.1 M sodium phosphate buffer (pH 8.2) + 10 per cent ethanol, were not found suitable for diffraction.

26. The thin colourless needle shaped crystals of lactoperoxidase grown under the conditions of 0.01 M sodium phosphate buffer (pH 8.2) + 10 per cent ethanol (with sitting drop vapour diffusion technique) in 10 days at 4°C were used for collecting the diffraction data. The crystals were found to be stable in X-ray beam and the native data upto 3.5 A° resolution was collected.

27. The crystals obtained belonged to the orthorhombic space group $P2_12_12_1$ with cell dimensions a = 116.88 A°, b = 103.19 A°, c = 62.25 A° and V = 7.51 x 10^5 $A°^3$. The asymmetric unit contains one molecule with a solvent content of 52 per cent.

The biochemical and structural characterization of buffalo lactoperoxidase have been carried out. Though we are not in a position to answer the structure function relationship of this biomolecule, but surely we have initiated the process of structure determination of lactoperoxidase by getting it crystallized for the first time with data collection up to 3.5 A° resolution. The structure solution of buffalo lactoperoxidase is in progress.

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APPENDIX

LACTOPEROXIDASE ASSAY

(a) Skim milk (dilution 1:250, 0.1 M PBSG, pH 7.0)

Time (sec.)	Absorbance (A)	Change in absorbance per minute ($\triangle$A/min.)
15	0.018	
30	0.039	
40	0.053	
50	0.066	
60	0.079	0.0790
70	0.092	
80	0.106	
90	0.119	
100	0.132	
110	0.145	
120	0.159	0.0795
130	0.168	
140	0.181	
150	0.195	
160	0.209	
170	0.222	
180	0.234	0.0780

Average = 0.07883

Calculation

Lactoperoxidase activity = 0.4938 x $\triangle$ A/min x dilution factor

= 0.4938 x 0.07883 x 250/1

= 9.73 units/ml

The lactoperoxidase activity of given sample is 9.730 U/ml

contd.....

(b) Rennet whey (dilution 1:250, 0.1 M PBSG, pH 7.0)

Time (sec.)	Absorbance (A)	Change in absorbance per minute ($\triangle$A/min.)
15	0.018	
30	0.037	
40	0.050	
50	0.062	
60	0.074	0.074
70	0.087	
80	0.100	
90	0.113	
100	0.125	
110	0.138	
120	0.150	0.075
130	0.162	
140	0.174	
150	0.186	
160	0.197	
170	0.208	
180	0.219	0.073

Average = 0.074

Calculation

Lactoperoxidase activity = 0.4938 x 0.074 x 250/1

= 9.139 units/ml

The lactoperoxidase activity of given rennet whey sample is 9.135 U/ml

contd.....

contd.... Appendix I

(c) Acid whey (dilution 1:250, 0.1 M PBSG, pH 7.0)

Time (sec.)	Absorbance (A)	Change in absorbance per minute (ΔA/min.)
15	0.017	
30	0.035	
40	0.047	
50	0.058	
60	0.069	0.069
70	0.081	
80	0.092	
90	0.104	
100	0.116	
110	0.127	
120	0.138	0.069
130	0.150	
140	0.161	
150	0.172	
160	0.184	
170	0.194	
180	0.206	0.0686
		Average = 0.0688

Calculation

Lactoperoxidase activity = 0.4938 x 0.0688 x 250/1

= 8.49 units/ml

The lactoperoxidase activity of given acid whey sample sample is 8.490 U/ml

RATE OF INACTIVATION OF LACTOPEROXIDASE IN DIFFERENT MEDIA

Sample	Time of heating (min.)	Temperature (°C)				
		69	70	71	72	73
		Activity left (a/a_o)				
Skim milk	5	0.9448 (-0.0567)**	0.8750 (-0.1331)	0.8042 (-0.2178)	0.6130 (-0.4890)	-
	10	0.9019 (-0.1032)	0.7477 (-0.2907)	0.6466 (-0.4359)	0.3761 (-0.9779)	-
	20	0.8154 (-0.2041)	0.5628 (-0.5748)	0.4182 (-0.8718)	0.1425 (-1.9485)	-
	30	0.7350 (-0.3073)	0.4739 (-0.7466)	0.2709 (-1.3059)	0.5339 (-2.9301)	-
	40	0.6650 (-0.4078)	0.3540 (-1.0373)	0.1764 (-1.7346)	0.0195 (-3.9373)	-
Rennet whey	5	-	0.978 (-0.0220)**	0.9339 (-0.0683)	0.8208 (-0.1975)	0.5553 (-0.5882)
	10	-	0.9542 (-0.0468)	0.8903 (-0.1162)	0.6890 (-0.3712)	0.3122 (-1.1639)
	20	-	0.9114 (-0.0927)	0.7801 (-0.2483)	0.4858 (-0.7219)	0.0873 (-2.4385)
	30	-	0.8660 (-0.1441)	0.6963 (-0.3619)	0.3609 (-1.0189)	0.0326 (-3.4208)
	40	-	0.8390 (-0.1748)	0.6052 (-0.5022)	0.2357 (-1.4451)	0.0080 (-4.8217)

** values in parentheses correspond to the ln (a/a_o)

contd..... Appendix II

Sample	Time of heating (min.)	Temperature (°C)			
		70	71	72	73
		Activity left (a/a_o)			
Acid whey (pH 6.8)	5	0.9926 (-0.0074)**	0.9584 (-0.0425)	0.8929 (-0.1132)	0.6625 (-0.4117)
	10	0.9844 (-0.0156)	0.9304 (-0.0720)	0.7845 (-0.2426)	0.4428 (-0.8145)
	20	0.9658 (-0.0347)	0.8479 (-0.1649)	0.6310 (-0.4604)	0.1815 (-1.7066)
	30	0.9558 (-0.0452)	0.7906 (-0.2349)	0.5070 (-0.6792)	0.0910 (-2.3965)
	40	0.9269 (-0.0759)	0.7200 (-0.3284)	0.3387 (-1.0825)	0.0342 (-3.3752)

Sample	Time of heating (min.)	Temperature (°C)			
		64	65	66	67
		Activity left (a/a_o)			
Lacto-peroxidase in acetate buffer (0.1 M, pH 6.4)	5	0.9276 (-0.0751)**	0.8301 (-0.1863)	0.7226 (-0.3248)	0.5915 (-0.5250)
	10	0.8573 (-0.1540)	0.7038 (-0.3512)	0.4910 (-0.7112)	0.3286 (-1.1130)
	20	0.7225 (-0.3250)	0.4939 (-0.7052)	0.3158 (-1.1527)	0.0940 (-2.3640)
	30	0.6368 (-0.4513)	0.3630 (-1.0132)	0.1581 (-1.8446)	0.0400 (-3.2170)
	40	0.5351 (-0.6252)	0.2528 (-1.3752)	0.0877 (-2.4332)	0.0116 (-4.4560)

** values in parentheses correspond to the ln (a/a_o)