STUDIES ON β-GALACTOSIDASE FROM THE GOAT EPIDIDYMIS

A THESIS

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By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON β-GALACTOSIDASE FROM THE GOAT EPIDIDYMIS"** in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the Department of **Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from November, 1987 to August, 1993 under the supervision of **Dr. Ben M.J. Pereira.**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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Dedicated To My Parents

Abstract

Sperm produced in the testis spend varying periods of time in the epididymis during which they undergo significant changes collectively termed 'maturation'. Remodelling of sperm surface is one of the most prominent features observed. The sperm membrane like most other membranes is made up of a lipid bilayer with several glycoproteins tucked in. In the past few years, the role of glycoproteins in cell-cell interaction has been repeatedly emphasized. This concept has been extended to the interaction of egg and sperm too. The epididymis supports several glycosylating events such as addition, deletion and modification of carbohydrate moieties on the sperm surface. Several types of enzymes like glycosyltransferases and exoglycosidases responsible for these events are abundant in the epididymis. The action of these enzymes lead to a reorganized sperm surface which is reflected in the enhanced ability of sperm for forward progressive motility and interaction with ova.

In animals like goat which have been used in the present study, reproduction is seasonal and so is epididymal sperm maturation. Roorkee experiences five distinct seasons: spring, summer, monsoon, autumn and winter. Several environmental cues like termperature, light, humidity, rainfall and photoperiod ensure that breeding is restricted to the monsoon months of July, August and September. The excellent linear correlation between the weight of testis and epididymis together with information gathered from animal breeders in Roorkee confirmed that the monsoon season was the period of high sexual activity. In terms of monitoring the functional status of the epididymal

microenvironment, the activity of ß-galactosidase was measured in the various segments of the epididymis at all seasons. Epididymal ß-galactosidase is an exoglycosidase that influences sperm maturation by deletion of galactose moieties from sperm surface molecules. A spurt in ß-galactosidase activity in epididymal segments observed during the breeding season gave an indication that this enzyme may have a definite role to play in sperm maturation.

Interest on this enzyme thus grew and an attempt was made to purify the enzyme from the goat epididymis. Purification was done using the techniques of acid precipitation, salt precipitation, dialysis, cation exchange chromatography, gel filtration and preparative electrophoresis. This resulted in a 137 fold purification of ß-galactosidase. The purified preparation migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel subjected to electrophoresis. Discontinuous cathodic polyacrylamide gel electrophoresis followed by enzyme specific staining helped to prove that the homogeneous preparation was ß-galactosidase. Antibodies raised against purified ß-galactosidase gave a single precipitin line following double immunodiffusion on agarose gel, which stained for ß-galactosidase activity with 6-bromo-2-naphthyl ß-D-galactopyranoside. Thus, the homogeneity was also established immunologically.

Once the enzyme was purified in sufficient amounts an attempt was made to study its properties. Molecular weight of the purified preparation was determined and found to be 64K by gel filtration

and 63K by SDS-PAGE. The purified enzyme was quite stable at acidic pH (pH 4.2-5.0). Temperatures of upto 40°C did not substantially affect the ß-galactosidase activity. At higher temperatures significant loss in activity was observed within a few minutes. The enzyme was otherwise very stable at low temperatures. Storage at 4°, 0° and -70° C did not have any impact on the activity of ß-galactosidase during the entire period of three months for which the enzyme activity was checked.

Of the two specific substrates of ß-galactosidase tried, p-nitrophenyl ß-D-galactopyranoside was hydrolyzed 3.5 times faster (K_m = 0.102 mM; V_{max} = 1.084 μM/min/unit of enzyme) than o-nitrophenyl ß-D-galactopyranoside (K_m = 0.8mM; V_{max} = 0.32 μM/min/unit of enzyme). In addition, the preparation was to a limited extent able to hydrolyze p-nitrophenyl N-acetyl-ß-D-glucosaminide and p-nitrophenyl α-D-galactopyranoside indicating that it possesed N-acetyl-ß-D-glucosaminidase and α-D-galactosidase activities as well. The preparation did not however hydrolyze the substrates of other glycosidases namely, the p-nitrophenyl derivatives of α-D-mannoside, and α-D-and ß-D-glucoside.

γ-D-Galactonolactone competitively inhibited the hydrolysis of p-nitrophenyl ß-D-galactopyranoside with a K_i value of 0.231 mM. Enzyme activity was also strongly inhibited by Tris and D-galactose. The inhibitory action of synthetic sugars methyl α-D-glucopyranoside and methyl α-D-mannopyranoside was very small.

Ions are known to influence sperm maturation in a variety of ways including regulation of the catalytic activity of many enzymes. In order to know which of the ions are important, a survey was made of the ions present in the luminal fluid at various sites of the epididymal duct. Luminal fluid was collected and the composition determined using the techniques of Inductively Coupled Plasma Emission Spectroscopy and Flame Photometry. Cu^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and Zn^{2+} were detected in the samples, of the luminal fluid. In addition, trace levels of Ni^{2+}, Cr^{2+} and Ti^{2+} were also detected. However, Mn^{2+}, Cd^{2+} and Co^{2+} were not found. An attempt was then made to ascertain which of the ions from the luminal fluid influenced the activity of ß-galactosidase. In recent years, industrialization has led to pollution of the environment particularly with heavy metals. Heavy metal toxicity is of immense concern to animal species. The particular impact on the reproductive processes is even more important since the quantity and quality of the offspring are critically affected. With this in mind, the activity of the purified ß-galactosidase was measured in the presence and absence of specific ions. It was found that the enzyme activity was strongly inhibited by Cu^{2+}, Ni^{2+}, Pb^{2+}, Ag^{+} and Hg^{2+}.

The matter embodied in the thesis is presented in four parts. In the first part seasonal changes in epididymal ß-galactosidase activity have been shown implicating the importance of the enzyme in male reproduction. In the second part, strategies used to purify this enzyme have been described. The third part highlights the properties of the purified preparation. The last

part is concerned with the ionic regulation of the enzyme. Thus the work carried out represents a detailed study of one epididymal exoglycosidase, ß-galactosidase, which is known to play a role in sperm maturation.

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And now, the time has come for me, when I can sit, breathe deeply and look back. It was a long hike, strenuous times, when I did not have dreams. But today, when it is a total recall, I remember everyperson, near or far, who made a difference and provided me courage and help. I must apologize if some names remain unsung, it's not deliberate.

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Meenakshi Gaur

(MEENAKSHI GAUR)

Introduction

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A lot of attention has been focused on the events related to the maturation of sperm in several mammalian species. While this process is more or less continuous in some species, in others it is restricted to certain months, particularly during the season of breeding. At least in the case of discontinuous breeders surface and intracellular changes in sperm become obviously distinct in breeding months. Thus, for studies of this kind the choice is often in favour of using seasonal breeders like goat as the animal model.

Sperm present in the testicular lumen do not possess all the qualifying characters generally required for successful interaction with the egg. In fact, sperm undergo extensive modifications morphologically, biochemically and physiologically as they are transported through the epididymis. The secretory and absorptive nature of the epididymal epithelium is responsible for providing an altogether new microenvironment by transforming the composition of the luminal fluid in which the sperm are bathed. In the proximal region, the epididymis makes sperm maturation possible while at the distal end sperm are preserved in a dormant yet viable form until ejaculation. The active participation of the epididymis in these events is now fairly well established.

The epididymis synthesizes and secretes a whole lot of proteins in addition to several other substances. These proteins find

their way into the lumen where there is ample scope for interaction with sperm. Several proteins of epididymal origin are known to selectively adhere and incorporate into the sperm surface. The selective degradation and resorption of other sperm surface proteins by the action of proteolytic enzymes in the epididymis is also known. As a result sperm acquire several traits like change in antigenicity, surface charge and forward progressive motility. Thus, the changing protein profiles on the sperm surface are of immediate concern not only in the epididymis but also in events like capacitation and fertilization that follow.

The epididymal microenvironment provides an excellent opportunity for post-translational modification of pre-existing sperm membrane proteins. Many mechanisms such as methylation/ demethylation, phosphorylation/dephosphorylation, glycosylation/ deglycosylation and transglutamine crosslinking are operational, which have a functional significance in epididymal sperm maturation. But those involving glycosylation/deglycosylation have received much attention over the past few years. Recent concepts implicating carbohydrate moieties of proteins in cell-cell interactions have been the primary cause for the extra emphasis. The enzymes involved in glycosylation and deglycosylation are abundant in the epididymis. Their origin from the cells lining the epididymal epithelium has been established beyond doubt. On one hand galactosyltransferase,

fucosyltranferase and sialyltransferases are believed to be responsible for the transfer of glycosyl units on to proteins. On the other hand several lysosomal exoglycosidases such as α-fucosidase, α-mannosidase, α-glucosidase, ß-glucuronidase, N-acetylglucosaminidase, hexosaminidase and ß-galactosidase are involved in the trimming and processing of carbohydrates on existing glycoproteins. There is increasing evidence to suggest that glycoproteins on sperm surface, modified in this fashion during epididymal transit improve considerably the fertilizing ability of sperm.

In recent times, several attempts have been made to isolate, purify and characterise the glycosidases from different sources. It has been observed that properties of the enzymes isolated from different tissues within the same animal are not identical. More often than not, the properties of the enzymes suit the conditions prevalent in the tissues. The present work attempts to isolate, purify and study the properties of ß-galactosidase with the intention of understanding its function in the epididymis.

Literature Review

The main event that takes place in the life span of any animal species is `Reproduction'. It is this process that is responsible for the perpetuation of all animal species. Several environmental cues have their role in influencing reproduction and the animals respond by making the necessary physiological adjustments. Initially, when conditions were favourable reproduction occurred spontaneously and almost continuously all the year round. But, with the rapid depletion and seasonal availability of essential food supplies, it was necessary for animals to breed in a way that could ensure survival of maximum numbers of their progeny. Since reproduction is very demanding in terms of energy, restricting the period of breeding to a particular season, most favourable for the survival of the offspring, was one mechanism nature chose to keep the species from extinction. Thus, seasonal breeding also became a way of life. In studies of reproduction, it is more convenient to use seasonally breeding animals, than those breeding continuously because the special course of events that occur in the reproductive tract become prominent during a specific season and can easily be identified.

Both sexes synchronize their activities, physically and physiologically so that their reproductive efforts turn out to be successful. In the past several years reproductive biologists working on fertility control in mammals were more interested in studying the female reproductive system. The relatively fewer number of female gametes produced, their cyclical release and organized development spread over a sufficiently long period made

it an easier target for study. On the other hand, studies on the male reproductive system were neglected due to the large number of sperm produced and the highly complex mechanism by which maturation was achieved. If the number of journals that have sprung up recently exclusively on andrology are an indication, we may infer that reproductive biologists are trying to improve their understanding of the male reproductive system as well.

2.1 CURRENT CONCEPTS IN MALE REPRODUCTIVE PHYSIOLOGY: A SYNTHESIS

It is now clear that spermatogenesis takes place in the testis and sperm maturation in the epididymis (Orgebin-Crist, 1969; Bedford, 1975). The functional development of the sperm is a result of morphological, biochemical and physiological changes that are effected in the sperm during epididymal transit (Bedford, 1979; Amann, 1987; Robaire and Hermo, 1988). The epididymis is not merely a passive conduit for sperm between the testis and vas deferens but actively provides the right ingredients and microenvironment for sperm maturation (Howards et al., 1979). Thus, the sperm achieves forward progressive motility and enhanced fertilizing ability after sojourn through the epididymis.

Analysis of the fluid from the testis and epididymis has shown tremendous differences in composition. Electron microscopic studies attributed these variations to the absorption of testicular fluid in the initial segment and the secretory activity of the epithelium lining the epididymis (Hamilton, 1975). Receptor mediated endocytosis and pinocytosis are believed to be the mechanisms by which absorption occurs (Morales and

Hermo, 1983; Robaire and Hermo, 1988). Besides absorption, the epididymis also synthesizes several substances including sialic acid, glycerylphosphorylcholine, carnitine and proteins (Jones, 1974; Flickinger, 1979; Hinton et al., 1979; Setty et al., 1979; Brooks, 1981b; Casillas et al., 1984). These substances together with a variety of ions and small organic molecules are concentrated and secreted into the lumen for action on sperm (Cooper, 1986). Thus, the absorptive and secretory nature of the epididymis transforms the non-conducive testicular fluid into one that is conducive for sperm maturation and storage in the epididymis. It is relevant to mention here that while the proximal regions of the epididymis are concerned with sperm maturation, it is in the caudal segment that sperm are stored in a viable form, until ejaculation. The regional specialization of the epididymis to influence different functions is partly due to the presence of apical, basal, principal, clear and halo cells known to exist in this organ (Nicander, 1957; Yeung and Cooper, 1982). Each of these cells is associated with unique functions and its distribution along the duct is also not uniform (Abe et al., 1983; Goyal and Williams, 1991). Moreover, there is also variation in the response of these cells to different stimuli, particularly hormones (Goyal, 1983; 1985). Thus, in a way these cells influence the rates of absorption and secretion in different segments of the epididymis. As a consequence, it is not surprising to find variations in the composition of the fluid along the length of the duct.

2.2 EVENTS CONCERNED WITH SPERM MATURATION

The special milieu provided by the epididymal fluid is conducive for a ripening process that makes the epididymal sperm quite different from the testicular sperm. The prominent changes in sperm structure during sperm passage through the epididymis relate to the cytoplasmic droplet and the acrosome. The cytoplasmic droplet is envisaged as a repository of enzymes that are generally found in lysosome (Dott and Dingle, 1968). In addition, they are thought to be concerned with the nutrition of sperm since they are rich in metabolizable endogenous substrates (Voglmayr, 1975). This droplet to begin with arises in the middle piece of testicular sperm but gradually moves towards the tail and is ultimately lost as the sperm passes through the epididymis (Kaplan et al., 1984; Clermont and Hermo, 1985).

Another specialized structure is the acrosome that lies as a cap over the anterior portion of the sperm nucleus and is involved in the penetration of ova (Eddy, 1988). Both light and electron microscopic studies have shown that the fine structure, shape and size of sperm acrosome undergo dramatic changes in the epididymis (Fawcett and Phillips, 1969; Eddy, 1988; Lakoski et al., 1989) and also during capacitation and and acrosome reaction (Bawa et al., 1993). However, the precise significance of these changes is not entirely clear at the present time.

Several outstanding biochemical changes in sperm particularly those concerning metabolic pattern have been described and reviewed (Voglmayr, 1975; Brooks, 1981a; Inskeep and Hammerstedt,

1982). Differences in oxygen uptake and type of substrate utilization have been reported in sperm collected from various regions of the male reproductive tract (Brooks, 1979). Another striking observation concerns the reduction of phospholipid in sperm as they advance through the epididymal duct (Poulos et al., 1973, 1975). The resulting physiological implications with respect to changes in heat resistance (Bishop, 1961; Voglmayr et al., 1967), response to cold shock (Quinn and White, 1968a,b; Nikolopoulau et al., 1985) and harmful alkaloids (Tulsiani et al., 1990b) have also been investigated. The most spectacular feat achieved by sperm in the epididymis is the ability to fertilize ova (Orgebin-Crist and Olson, 1984; Fournier-Delpech and Courot, 1987; Williams et al., 1991, Harayama et al., 1993). Since this involves an interaction of the sperm and egg surfaces, a lot of effort has gone into studying the molecules that comprise the sperm membranes (Miller and Ax, 1990; Naz 1990).

2.3 MOLECULAR STRUCTURE OF SPERM MEMBRANES

Like all other membranes, sperm membranes possess a bilayered arrangement of neutral and charged lipids into which are inserted protein and glycoprotein molecules. Based on the manner in which these proteinaceous molecules are positioned in the lipid bilayers, they are referred to as transmembranal, integral and peripheral components. Atleast five principal domains with distinctive composition have been identified in sperm membranes (Friend, 1982; Holt, 1984; Peterson and Russell, 1985; Saxena et al., 1986). Spatial reorganisation of sperm membranes between the domains has not been found with respect to most membrane

proteins (Bearer and Friend, 1990). The mechanisms by which selective partitioning of antigens occur are never the less coming to light (Cowan et al., 1987). However, dynamic changes in the composition and characteristic of sperm membranes during maturation in the epididymis have been repeatedly shown (Bearer and Friend, 1982; Wolf and Voglmayr, 1984; Wolf et al., 1986; Wolf et al., 1990).

2.4 REMODELLING OF SPERM PLASMA MEMBRANE DURING EPIDIDYMAL TRANSIT

Several surface changes have been reported in the sperm of almost every species studied, during passage through the epididymis. In the sperm of male goats and ram changes in surface charge (Hammerstedt et al., 1979, 1982; Holt, 1984) intramembranous particle distribution (Mckinnon et al. 1991), lipid content (Parks and Hammerstedt, 1985), protein composition (Voglmayr and Sawyer, 1986; Voglmayr, 1987; Veeramachaneni et al., 1990) and lectin binding sites (Magargee et al., 1988; Sarkar et al., 1991) have been reported. The acquisition of a more negative charge involving methylation, phosphorylation and changes in sialic acid have been visualized as mechanism(s) for bringing together egg and sperm (Holt, 1980; Noland et al., 1984). It is believed that intramembranous particles represent the glycocalyx and therefore any change in their distribution on sperm surface might further influence egg-sperm interactions (Suzuki, 1981). Several properties like permeability, fluidity and rigidity are controlled by the lipid component of the sperm membrane (Wolf and Voglmayr, 1984; Nikolopoulau et al., 1985). Changes in the lipid profiles may thus affect functional

attributes like resistance to cold shock (Scott et al., 1967; Hammerstedt et al., 1979).

Many types of proteins have been identified in the plasma membrane of sperm (Brooks, 1981b; Eddy, 1988). Since sperm have little or no synthetic ability (Witkin and Bendich, 1977), it is likely that proteins are generally synthesized by the testis and packaged into the sperm membranes, where they are produced (Abraham and Bhargava, 1963a, b). Three possible explanations have been offered for the differences in the electrophoretic pattern of proteins obtained in sperm collected from various sites along the epididymis. Firstly, new proteins synthesized by the epididymis could be secreted into the lumen and incorporated into sperm (Moore, 1980; Gonzales et al., 1982; Tezon et al., 1985a,b; Ueda et al., 1990; Rankin et al., 1992). Secondly, proteins could be selectively eliminated from the sperm surface (Jones et al., 1981; Brown et al., 1983; Dacheux et al., 1989). Thirdly, the pre-existing sperm proteins could be modified by the action of epididymal enzymes (Hamilton and Gould, 1982; Cossu and Boitani, 1984; Skudlarek and Orgebin-Crist, 1986).

2.4.1 Remodelling by Addition of New Membrane Components

The synthetic ability of the epididymis is known for a long time (Barker and Amann, 1971; Huang and Johnson, 1975; Koskimies and Kormano, 1975; Dacheux and Voglmayr, 1983; Cooper, 1986). Over the past few years, an increasing number of proteins have been reported to be present in the epididymis. The use of radioisotopes, fluorescent dyes and electrophoretic techniques

have clearly established that the epididymal epithelium is responsible for the synthesis of several proteins (Kopecny and Peck, 1977; Kohane et al., 1980; Flickinger, 1985). Studies with cultures of cells isolated from the epididymal epithelium have also substantiated this view (Killian and Chapman, 1980; White et al., 1982). Klinefelter and Hamilton, 1985; Some proteins have been localized to more than one region of the epididymis, while others are solely confined to one restricted area (Bennett et al., 1974; Fain-Maurel et al., 1981, 1983; Flickinger, 1985). More details have come from electron microscopic studies which reveal movements of proteins from rough endoplasmic reticulum to Golgi to the luminal brush borders and luminal contents (Flickinger, 1983; Flickinger et al., 1984; Kopecny et al., 1984). In addition, several investigators have by different approaches demonstrated that proteins synthesized by the epididymis are actually secreted into its lumen (Flickinger et al., 1984). Further, the adherence, or at times even incorporation of these secreted proteins on sperm membranes has been noticed (Lea et al., 1978; Cornwall et al., 1990).

2.4.2 Remodelling by Loss of Membrane Components

There is accumulating evidence to show that certain proteins which once were part of the sperm are actually lost during epididymal transit (Voglmayr et al., 1982; Brooks and Tiver, 1984; Olson et al., 1987). For instance, coating of spermatozoa with an antigen secreted in the proximal region of the epididymis may be followed by its removal at a later stage of maturation (Fraser, 1984; Myles and Primakoff, 1985). Based on intensive

experimentation several explanations have been offered. One possibility is that proteins are selectively degraded in the epididymal lumen by the action of proteolytic enzymes (Jones, 1978; Mann and Lutwak-Mann, 1981). Another interesting prospect is that secreted epididymal proteins may initially bind to sperm membranes and then may have a direct influence on intracellular organelles in maturing spermatozoa by internalization (Jones, 1989b). Perhaps this is one of the mechanisms by which Forward Motility Protein (FMP), the protein kinases and phospho-diesterases of sperm are activated (Acott and Hoskins, 1978). Alternatively proteins are leached from the sperm membranes and taken up by the epididymal epithelium by special mechanisms involving change in pH and ionic strength (Harrison et al., 1982) There is substantial evidence to show that proteins introduced in the luminal fluid of the epididymis are taken up by the epithelial cells (Byers et al., 1985; Gerard et al., 1988). In addition to the principal cells present in the epithelium, clear cells are also believed to play a major role in this process (Bayard et al., 1981; White et al., 1982; Kopecny et al., 1984). Both fluid phased endocytosis and receptor mediated mechanisms have been implicated in the resorption of proteins that occurs in the epididymis of mammalian species (Veeramachanni et al., 1990, Veeramachaneni and Amann, 1991).

2.4.3 Remodelling Caused by Modification of Pre-existing Membrane Components

Many mechanisms have been considered for modifying sperm membranes during maturation in the epididymis. These include post-translational modifications of existing

proteins by methylation, transglutaminase-mediated crosslinking, phosphorylation and glycosylation (Jones, 1989b).

Protein carboxymethylation and demethylation are an important type of post-translational modification that are relevant for sperm maturation (Paik and Kim, 1980). Two enzymes namely protein carboxymethylase (PCM) and protein methylesterase (PME) are primarily implicated in this process. While PCM modifies free carboxyl groups of glutamate and aspartate residues, PME reverses this reaction (Gagnon et al., 1984). Indirect evidences provided by Gagnon et al. (1980) and Purvis et al. (1982) suggest that these enzymes participate in epididymal sperm maturation. A higher proportion of proteins with methyl ester groups have been detected in the caput and relatively low levels in caudal sperm (Gagnon et al., 1984). Methylated proteins in general are less soluble in aqueous media and tend to adhere to membranes spontaneously (Jones, 1989b). In accordance with this, PCM activity has been localized to sperm flagellum where it is believed to regulate motility (Bouchard et al., 1980).

It is known for quite sometime now that phosphorylation modifies the tertiary conformation of proteins (Tash et al., 1984). Both cAMP dependent and cAMP independent proteinkinases mediate this phosphorylation (Noland et al., 1984). Infact, phosphorylation of proteins has been reported on sperm membranes (Huacuja et al., 1977) as well as at intracellular sites (Chulavatnatol et al., 1982). It is proposed that phosphorylation/dephosphorylation

might be responsible for regulating the functional activity of sperm proteins.

Changes in antigenicity have been reported in sperm collected from various locations along the epididymal duct (Topfer-Petersen et al., 1990a,b; Mckinnon et al., 1991). Initially, it was thought that the cross-linking of proteins through disulfide bonds was responsible for such an event (Bedford, 1975). Since then, transglutaminase has also been implicated in bridging proteins by their glutamine and lysine residues (Nieto et al., 1977; Manjunath et al., 1984). Several investigators have also suggested that decreased antigenicity on account of bridging of sperm proteins in the epididymis may be necessary for protecting sperm from destruction by the immune system (Witkin et al., 1983; Esponda and Bedford, 1985).

The addition and deletion of carbohydrate moieties to proteins or lipids has received much attention (Jones, 1989a). There is good evidence that glycosylating and deglycosylating reactions are involved (Cossu and Boitani, 1984; Wenstrom and Hamilton, 1980). Several **in vitro** experiments have shown addition of sugars to sperm membrane proteins (Benoff et al., 1993). The presence of galactosyltransferase (Hamilton and Gould, 1982; Quasba et al., 1983), fucosyltransferase (Cossu and Boitani, 1984) and sialyl-transferase (Bernal et al., 1980) in the epididymis strongly suggests that such a process could operate in the organ. A wide variety of exoglycosidases have also been detected in the epididymal plasma (Conchie and Mann, 1957; Conchie et al., 1959a,

b; Mann and Lutwak-Mann, 1981). In fact, of all the tissues known, the epididymis is one of the richest sources of this class of enzymes (Conchie et al., 1959b). In addition, differences have been observed in the carbohydrate moiety of immunologically indentical sperm proteins isolated from different segments of the epididymis (Nicolson et al.,1977). Therefore, it is likely that the epididymal environment permits trimming and processing of carbohydrate chains in sperm membrane glycoproteins.

2.5 ROLE OF PROTEINS SECRETED FROM THE EPIDIDYMIS

The luminal proteins of epididymal origin may have several roles to play in the maturation of sperm. From literature it can be deduced that they interact with sperm in more than one way. Those proteins that bind to specific regions on the sperm may restrict the motion of integral proteins on sperm membranes and thereby contribute to the rigidity of sperm membrane (Gingell, 1976; Barber, 1982). In one study, Chaudhury and Majumder (1983) showed that motility of goat sperm is promoted by epididymal proteins. Alternatively, proteins together with lipids and sterols could alter the permeability of sperm membranes to ions (Zimmerman et al., 1979; Rufo et al., 1982, 1984). In addition, some proteins might aid directly in ion transport, which in turn might modulate sperm maturational events.

Proteins might also be responsible for storage of sperm in a viable form. In this regard, the identification of sperm survival factor is worth mentioning (Morton, 1979a,b). Epididymal luminal fluid is also known to contain a proteinaceous acrosin inhibitor that prevents the premature acrosome reaction thus

maintaining sperm in a decapacitated stage (Jonakova and Cechova, 1985). Protection against toxicity is also provided by the property of some proteins to bind to heavy metals (Emery, 1980). Further, proteins that prevent oxidation in tissues are also known to exist (Papkoff, 1966; Al-Timini and D'Ormandy, 1977). In ram the epididymal fluid contains a protein antagglutinin which prevents dilution induced agglutination of sperm (Dacheux et al., 1983). The influence of luminal proteins on immunosuppression and even antagonism to complement mediated haemolysis **in vitro** has also been reported (Anderson and Tartar, 1982; Tartar and Alexander, 1984; Esponda and Bedford, 1985).

2.6 ROLE OF CELL SURFACE CARBOHYDRATES

There is enough evidence to suggest that carbohydrate moieties on cell surface are responsible for the specificity of cell recognition and adhesion phenomena. The extension of this concept to gamete interaction has been extensively reviewed (Yanagimachi, 1981; Macek and Shur, 1988; Yanagimachi, 1988). The general concensus is that fertilization is a species specific event which is initiated by a series of specific interaction between ligands on the surface of gametes (O'Rand et al., 1988; Peterson and Hunt, 1989; Sacco et al., 1989, Williams and Jones 1993). The participation of fucose moieties in fertilization of sea urchins (Vacquier, 1986) and the ability of sulfated sugars to block fertilization (Ahuja, 1982; Huang and Yanagimachi, 1984; Peterson et al., 1984; Shalgi et al., 1986) clearly prove beyond doubt that carbohydrate moieties are actively involved in egg sperm interaction. It is possible to imagine that

carbohydrates could participate in other events too. For example, desialylation may be the key factor for selective elimination of abnormal sperm in the male reproductive tract (Czuppon 1984; Tashimori et al., 1991). This view stems from the work of Morell et al. 1968 on ceruloplasmin which revealed a rapid clearance of desialylated proteins while sialylated versions remain untouched.

2.7 ENZYMES THAT MODIFY GLYCOPROTEINS

The carbohydrate structures found on the surface of sperm are found to be associated both with proteins and lipids. The enzymatic machinery for the process of protein glycosylation is active in the epididymis of most mammalian species (Hamilton, 1980). A number of glycosyltransferases and mannosyltransferases that are responsible for the addition of sugars to sperm membrane have also been identified in the epididymis (Iusem et al., 1984). In addition, exoglycosidases necessary for removal of specific sugars from existing glycoproteins are also abundant in this organ (Conchie et al., 1959a,b; Guerin et al., 1990). Thus, a variety of cell surface glycosylation events have been demonstrated to be operational. At this stage it would be relevent to mention that clustering of monosaccharides on a protein backbone is more important than constituent sugars (Gallagher, 1984).

Biochemical, histochemical and immunological data have convincingly shown addition of sugars to sperm surface molecules

(Hamilton and Gould, 1982). Glycosyltransferases that generally catalyze these types of reactions have been extensively studied. Given below in Table 1 is the list of glycosyltransferases purified from diverse mammalian sources for which cDNAs have been obtained (Paulson and Colley, 1989).

TABLE - 1

Glycosyltransferase	Donor Substrate	Sequence Formed
Galactosyltransferases		
GlcNAcß1, 4-GT (EC 2.4.1.38)	UDP-Gal	Galß1,4GlcNAc-R
Galα1,3-GT (EC 2.4.1.151)	UDP-Gal	Galα1,3Galß1,4GlcNAc-R
Sialyltransferase		
Galα2,6-ST (EC 2.4.99.1)	CMP-NeuAc	NeuAcα2,6Galß1,4GlcNAc-R
Fucosyltransferases		
GlcNAcα1,3-FT (EC 2.4.1.65)	GDP-Fuc	Fucα1,3 Galß1,4 GlcNAc-R
		Fucα1,4 Galß1,3 GlcNAc-R
Galα1,2-FT (EC 2.4.1.69)	GDP-Fuc	Fucα1,2Galß1,4GlcNAc-R
		Fucα1,2Galß1,3GalNAc-R

N-Acetylgalactosaminyl-transferase		
Galα1,3-GalNAcT (Blood group A transferase)	UDP-GalNAc	GalNAcα1,3 \ Gal-R / Fucα1.2

Individual enzymes like galactosyltransferases, fucosyltransferases, sialyltransferases and mannosyltransferases have been identified in the epididymis too. The function of this category of enzymes is to transform the surface of sperm in the epididymis so that a successful fertilization of ova is ensured. It has also been suggested that these enzymes could be carried on sperm surface for action on ova during penetration (Durr et al., 1977; Bernal et al., 1980; Shur and Hall, 1982; Lopez et al., 1985; Ram et al., 1989; Tulsiani et al., 1989; Fayrer-Hosken et al., 1991). The presence of same enzymes at various sites along the male reproductive tract suggests that the donor substrates and corresponding acceptors are not always the same.

2.7.1 Glycosidases in the Male Reproductive Tract

In recent years, glycosidases are a class of enzymes that have drawn tremendous attention. The unusually high levels of these enzymes in the epididymis is not totally on account of their synthesis in the epididymis, but there are indications that the testis and sperm could also be important sources (Garbers et al., 1970; Hamilton, 1981; Guerin, 1987; Jauhiainen and Vanha-Perttula, 1986; Tulsiani et al., 1990a). Irrespective of their origin, it is fairly clear that their presence in the epididymis has a functional significance in the maturation of

sperm (Yeung et al., 1990). The activity of these enzymes increases with the age and androgen level of the animal (Conchie and Mann, 1957; Conchie and Findlay, 1959; Jones, 1974). It is believed that in the epididymis they effect sperm maturation by selectively deleting specific carbohydrate moieties from sperm surface glycoproteins (Olson and Hamilton, 1978; Brooks, 1981b; Jones et al., 1981; Orgebin-Crist et al., 1981; Chapman and Killian, 1984; Hall and Killian, 1987). Alternatively, they are absorbed or at times even incorporated in sperm plasma membranes for participating in later events such as cumulus dispersion or zona attachment or penetration (Allison and Hartree, 1970; Farooqui and Srivastava, 1980; Rodger and Young, 1981; Shur and Hall, 1982; Ben-Ayed et al., 1989; Tulsiani et al., 1989; Nikolajczyk and O'Rand, 1992).

Interest in biochemistry of epididymal glycosidases has grown because of their potential association with spermatozoa. In the past decade attempts have been made to understand in depth the functioning of enzymes like α-mannosidase, N-acetyl ß-glucosaminidase, α-and ß-fucosidases, α-glucosidase, ß-glucuronidase and ß-galactosidase (Hamilton, 1980; Jones and Brown, 1982; Skudlarek and Orgebin-Crist, 1986; Hall and Killian, 1987). Some of these enzymes are known to exist as multiple molecular forms in the epididymis (Dutta and Majumder, 1984, 1986). However, most of these isozymes were found to be active only at acidic or neutral pH (Dutta and Majumder, 1984). The importance of glycosidases could be easily realized from their wide distribution in the male reproductive tract **(Table-2)**.

TABLE - 2

ENZYME	SOURCE	REFERENCES
1	2	3
α-L-fucosidase	Rat epididymis	Leray et al., 1986.
N-acetyl ß-D-Glucosaminidase	Ram testis	Caygill et al., 1966
	Sperm acrosome	Allison and Hartree, 1970; Khar and Anand, 1977.
	Ram and Boar epididymis	Bullock and Winchester, 1973.
	Human seminal plasma	Parkes et al.,1984; Kapur and Gupta, 1986.
α-Mannosidase	Rat epididymis	Conchie and Hay, 1959; Snaith, 1977; Dutta and Majumder, 1984, 1986.
α-Glucosidase	Seminal plasma and Epididymis	Chapman and Killian, 1984; Paquin et al., 1984; Besancon et al., 1985; Hall and Killian 1987; Cooper et al., 1990; Viljoen et al., 1990; Fourie et al., 1991;
ß-Galactosidase	Rat epididymis Epididymal principal cells	Conchie and Hay, 1959 Skudlarek and Orgebin-Crist, 1986
	Rat epididymal fluid	Sosa et al., 1987; 1991
	Bovine testis	Distler and Jourdian, 1978; Verheijen et al., 1982.
	Ram testis	Caygill et al., 1966
	Rabbit testis	Nikolajczk and O'Rand, 1992
	Porcine testis	Yamamoto and Nishimura, 1987

1	2	3
Glycosidases	Human semen and male genital organs	Bostrom and Ockerman, 1971
	Bovine seminal plasma	Jauhianen and Vanha-Pertulla, 1987
	Human sperm	Tulsiani et al., 1990a.

2.7.2 Epididymal ß-Galactosidase

Of all the glycosidases, ß-galactosidase has caught the attention of several investigators. Histochemical studies have clearly indicated that the epididymis actively synthesizes this enzyme. (Cohen et al., 1952). Further, this enzyme is described as a glycoprotein secreted by the principal cells lining the epididymal epithelium (Kemp and Killian, 1978; Skudlarek and Orgebin-Crist, 1986). Although the activity of this enzyme has been suggested to modify the size and structure of glycoproteins on sperm membranes the exact components of this mechanism are obscure.

ß-galactosidase is abundant in the epididymis of most mammals. This was also confirmed in the present studies using goat as the animal model. With the onset of breeding which is seasonal in goats, a sudden rise in the epididymal ß-galactosidase activity was noticed. This trend continued to persist throughout the breeding period which led to the suspicion that ß-galactosidase is somehow associated with sperm maturation that occurs in the epididymis.

In order to precisely identify different molecules with which ß-galactosidase interacts, the first logical step would be to isolate and obtain this enzyme in a reasonably pure form.

Therefore, in the present study an attempt has been made to purify this enzyme from the epididymis of goat. An effort has also been made to study its properties with the intention of understanding its role in the physiology of the epididymis.

CHAPTER 1

Seasonal Changes in the activity of goat epididymal β-galactosidase

3.1 INTRODUCTION

Breeding literally refers to events leading to the production of offsprings. Natural selection ensures that this occurs in harmony with environmental conditions. Accordingly, continuous breeding is observed in mammals which inhabit locations with favourable environments all the year round. On the other hand, there are discontinuous breeders, who face a favourable environment only during a restricted time period each year (Bronson, 1985). Goats belong to the category of discontinuous breeders (Sadlier, 1969; Lincoln, 1976a). The precise timing of breeding is made possible year after year by a diverse array of environmental signals that are perceived. This leads to neuroendocrine adjustments and ultimately triggers the onset of breeding at the appropriate time (Pelletier and Ortavant, 1975a,b; Lincoln, 1976b).

Seasonal regulation of breeding in mammals is a complex process and involves at one level or another a proper interaction of appropriate ecological and physiological principles (Thibault et al., 1966;Gilmore and Cook, 1981). In the case of goat, the reproductive behaviour is known to be governed by two types of factors. The ones categorized as "ultimate factors" are actually long term signals such as food, rainfall, humidity, temperature and daylength that ensure the survival of the young ones. The second type termed as "proximate factors" are short term signals which include neuroendocrine and other stimuli like visual, auditory and olfactory signals that modulate reproduction (Baker, 1938). It is believed that ultimate factors enable the animals to

carryout the coarse adjustments as far as reproduction is concerned, while the fine tuning is done by proximate factors (Baker, 1938). The pathways by which both proximate and ultimate factors influence breeding have been extensively reviewed (Bronson, 1988). It is generally observed that the same species of goats inhabiting tropical and temperate zones breed at different times in the year. Obviously, the geographical locations and the climatic conditions prevalent in these regions have a profound role to play in the timing of events leading to reproduction.

Just as in other mammals, in goats too, sperm maturation in the epididymis is an important prelude to successful mating. Glycosylation/deglycosylation is one of the several mechanisms responsible for this process (Jones, 1989b). The extensive remodelling of sperm surface glycoproteins observed during epididymal transit is made possible by the action of several enzymes (Mann and Lutwak Mann, 1981; Eddy, 1988). ß-Galactosidase is one such enzyme that is responsible for processing glycoproteins on the sperm surface (Skudlarek and Orgebin-Crist, 1986). The objective of this study was to first identify the breeding season of goats at Roorkee and then establish if epididymal ß-galactosidase is in some way connected with this event.

3.2 MATERIALS AND METHODS

3.2.1 Location

The present studies were carried out over a period of one year i.e. from March 1990 to February 1991 at Roorkee, which is

located at 29.52° N and 77.53° E. The exact daily information regarding rainfall, relative humidity, temperature and the time of sunrise and sunset were collected from the local meteorological station. Based on the climatic conditions prevalent here, five distinct seasons could easily be distinguished annually viz. winter (December, January, February) spring (March, April), summer (May, June), monsoon (July, August, September) and autumn (October, November).

3.2.2 Animals

The present investigations were conducted on locally available domestic male goats of all age groups reared at the local breeding station under natural environmental conditions. The animals were group housed together, outdoors, under roofed shelter and were fed on the seasonally available plants and grasses. The animals were also allowed to graze in the open during the daytime and water was available at all times.

3.2.3 Collection of Tissue

The male reproductive tissues were collected throughout the period of investigations from the local abattoir as and when the animals were slaughtered for meat consumption. The tissues were brought to the laboratory in ice within one hour of slaughter. The testis and epididymis were cleared of the adhering adipose and connective tissue and weighed. While the testis was discarded, the epididymis was saved for biochemical studies. The epididymis was divided into twelve serial segments (Fig.1) as per

Besancon et al. (1985). The activity of ß-galactosidase and the protein content in each segment was determined.

3.2.4 Enzyme Studies

3.2.4.1 Extraction

Each epididymal segment was homogenized in 0.1M sodium acetate buffer pH 4.8 (100 mg/ml) using a mortar and pestle. The extract obtained was centrifuged at 12,000 xg for 20 minutes at 4°C in a Beckman J2-21 Centrifuge. The resulting supernatant was saved for assaying ß-galactosidase activity and total protein.

3.2.4.2 Assay of ß-Galactosidase

ß-Galactosidase was assayed according to the procedure of Conchie et al. (1959b) with slight modifications. Under optimal assay conditions, ß-galactosidase reacts with p-nitrophenyl ß-D-galactopyranoside to liberate p-nitrophenol (p-NP). The released p-nitrophenol produces a yellow color at alkaline pH, which can be measured spectrophotometrically at 400 nm.

Reagents

0.1M sodium acetate buffer pH 4.8: prepared by mixing 0.2M sodium acetate solution and 0.2M acetic acid.

A) 0.2M sodium acetate solutions: 8.16g sodium acetate trihydrate was dissolved in 300ml distilled water.

B) 0.2M acetic acid : 2.31ml of acetic acid was made upto 200ml with distilled water. 300ml of solution A and 200ml of solution B were mixed to get a final pH 4.8 and the volume

was made upto 1000 ml to give a final concentration of 0.1M.

Substrate solution : 1.25mM p-nitrophenyl ß-D galactopyranoside. 37.66mg of p-nitrophenyl ß-D galactopyranoside in 100ml of 0.1M sodium acetate buffer pH 4.8.

1.25mM standard p-nitrophenol: 13.911mg p-nitrophenol was dissolved in 10ml distilled water to give a final concentration of 10mM. 0.18ml of this solution was diluted with distilled water to a final volume of 12ml to make a working stock of 150 μM.

0.2M sodium carbonate solution : 21.198g sodium carbonate was dissolved in 1000ml distilled water.

Procedure

A suitable aliquot of enzyme extract was made upto 0.2ml with 0.1M sodium acetate buffer pH 4.8 and preincubated at 37°C for 15 minutes. To this 0.6ml of substrate solution was added. Incubation was carried out for further 15 minutes at 37°C. The reaction was terminated by the addition of 2ml of 0.2M Na_2CO_3. The yellow color developed was read at 400 nm spectrophotometrically against a blank. The blank was processed in a similar manner except that the enzyme extract was added after terminating the reaction with 0.2M Na_2CO_3.

The total enzyme activity is expressed as units, where one unit is defined as the quantity of enzyme capable of liberating 1

μmole of p-nitrophenol per minute at 37°C. The specific activity of the enzyme is expressed as units of enzyme per milligram of protein present in the extract.

3.2.5 Protein Estimation

Protein was estimated by the method of Lowry et al. (1951). The final color developed is a result of:

(i) Biuret reaction of protein with copper ions in alkali.

ii) Reduction of the phosphomolybdic phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

Reagents

Reagent A - 2% Na_2CO_3 in 0.1 N NaOH

Reagent B - 0.5% $CuSO_4 . 5H_2O$ in 1% potassium sodium tartrate. Freshly prepared.

Reagent C - Alkaline copper solution prepared by mixing 50ml reagent A and 1ml reagent B

Reagent D - Diluted Folin Ciocalteau reagent (1N).

Protein standard : Working standard contained 20-200 μg Bovine serum albumin (BSA)/ml from a stock solution of 1mg BSA/ml.

Procedure

A suitable aliquot of extract was taken in a test tube and the volume made upto 1ml with 0.1N NaOH. To this 1ml of reagent C was added and mixed. After standing at room temperature for 15 minutes, 0.1ml of reagent D was added and once again mixed. Forty minutes later the intensity of the color developed was

measured spectrophotometrically at 690 nm on a Beckman DU-6 spectrophotometer against a reagent blank which did not contain the extract.

3.2.6 Statistical Analysis

Regression analysis of the data on the weight of tissue obtained at different seasons of the year was performed using User Friendly Software (Lotus 123) on a personal computer.

3.3 RESULTS

The present study highlights the effect of various environmental factors on the breeding period of goats in Roorkee over a period of one year from March 1990 to February 1991. Depending on the climatic conditions experienced in this region, five distinct seasons have been recognized annually viz. spring (March, April), summer (May, June), monsoon (July, August, September), autumn (October, November) and winter (December, January, February).

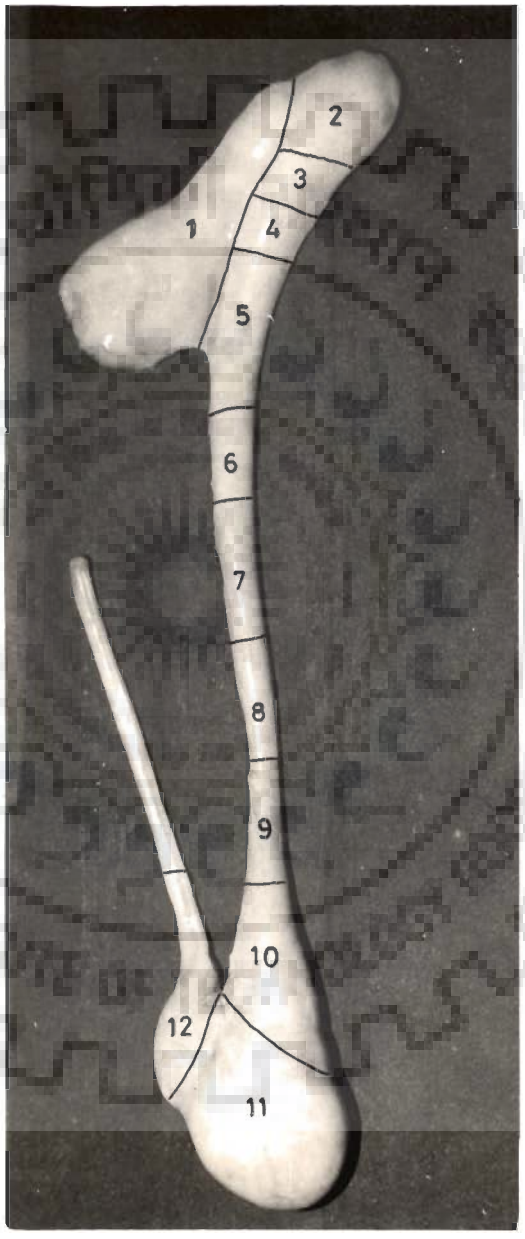
Mating in goats was predominantly confined to the monsoon months although stray incidents of mating were observed round the year. Animal breeders of this region also report very same pattern in animal behaviour. The analysis of data on goat epididymis and testis collected from the local abattoir during the five seasons also give some clue regarding mating in goats. Scatter plots of testicular and epididymal weights (Figs. 2-6) and subsequent regression analysis show a very good correlation between the two parameters in the monsoon months ($Y = 6.60X + 5.077$, $r = 0.934$) followed by spring ($Y = 8.59X + 9.178$, $r = 0.724$). No correlation was observed during other seasons i.e. summer ($Y = 5.00X + 11.31$,

r = 0.244), winter (Y = 3.43X + 28.71, r = 0.497) and autumn (Y = 3.12X + 27.50, r = 0.538).

Various environmental cues are known to signal the onset of mating in animals. Hence the present study has attempted to monitor the environmental factors like rainfall, temperature, humidity and photoperiod. The amount of rainfall recorded over the period of study is depicted in Fig.7. The total rainfall experienced seasonwise was spring (35.3mm), summer (89.2mm), monsoon (933.5mm), autumn (14.8mm) and winter (154.8mm). The rainfall recorded during the monsoon months was the highest (933.5mm).

The data recorded for minimum and maximum temperature on every day during the five seasons of the year is presented in Fig.8. The range of minimum and maximum temperature observed were spring (15.52 ± 0.40; 30.69 ± 0.74), summer (25.33 ± 0.30; 37.80 ±0.34), monsoon (25.44 ± 0.14; 32.90 ±0.23), autumn (15.0 ± 0.50; 30.29 ± 0.30) and winter (8.15 ± 0.28; 23.04 ±0.34). From the figure it is also obvious that the temperature is relatively more stable during monsoon as the variation in minimum and maximum temperature was found to be the least. Percent relative humidity was another environmental factor that varied during the five seasons: spring (62.37 ± 1.76), summer (59.80 ± 1.79), monsoon (85.49 ± 0.71), autumn (72.18 ± 0.76) and winter (80.2 ± 0.93). Most days during monsoon months showed uniformly high relative humidity which was rather steady and consistent during this entire season (Fig.9).

Fig. 1. Epididymal Segments. Goat epididymis was divided in 12 segments according to Besancon et al., 1985.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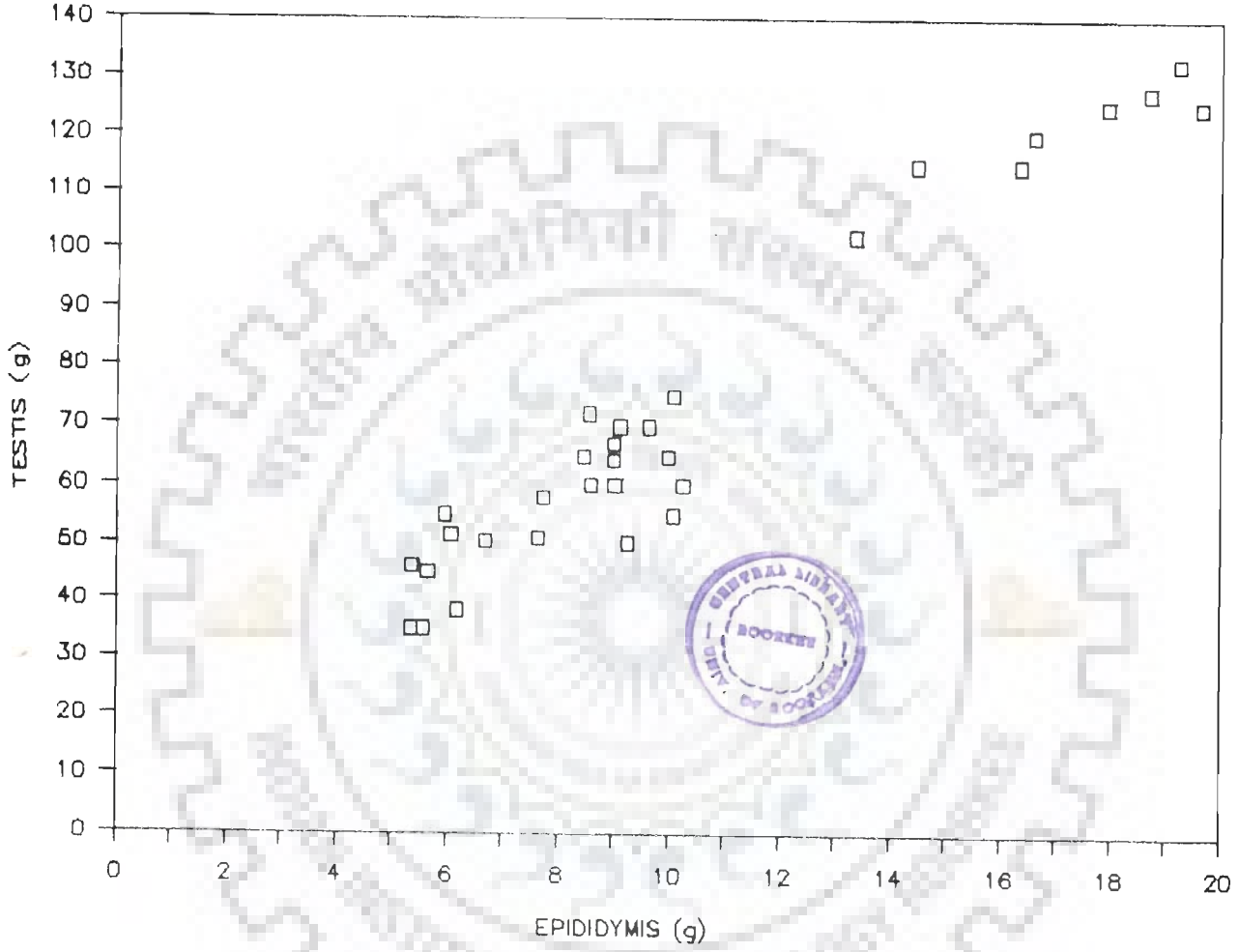


Fig. 2. Regression Analysis of Epididymal and Testicular Weights during Monsoon. Regression analysis of epididymal and testicular weights in a random population of male goats(n = 36) during monsoon, (Y=6.60X + 5.077; r=0.934).

Fig. 3. Regression Analysis of Epididymal and Testicular Weights during Spring. Regression analysis of epididymal and testicular weights in a random population of male goats (n=32) during spring, (Y=8.59X + 9.178; r=0.724).

Fig. 4. Regression Analysis of Epididymal and Testicular Weights during Summer. Regression analysis of epididymal and testicular weights in a random population of male goats (n=32) during Summer, (Y=5.00X + 11.31; r=0.244).

Fig. 5. Regression Analysis of Epididymal and Testicular Weights during Winter. Regression analysis of epididymal and testicular weights in a random population of male goats (n=36) during winter, (Y=3.43X + 28.71; r=0.497).

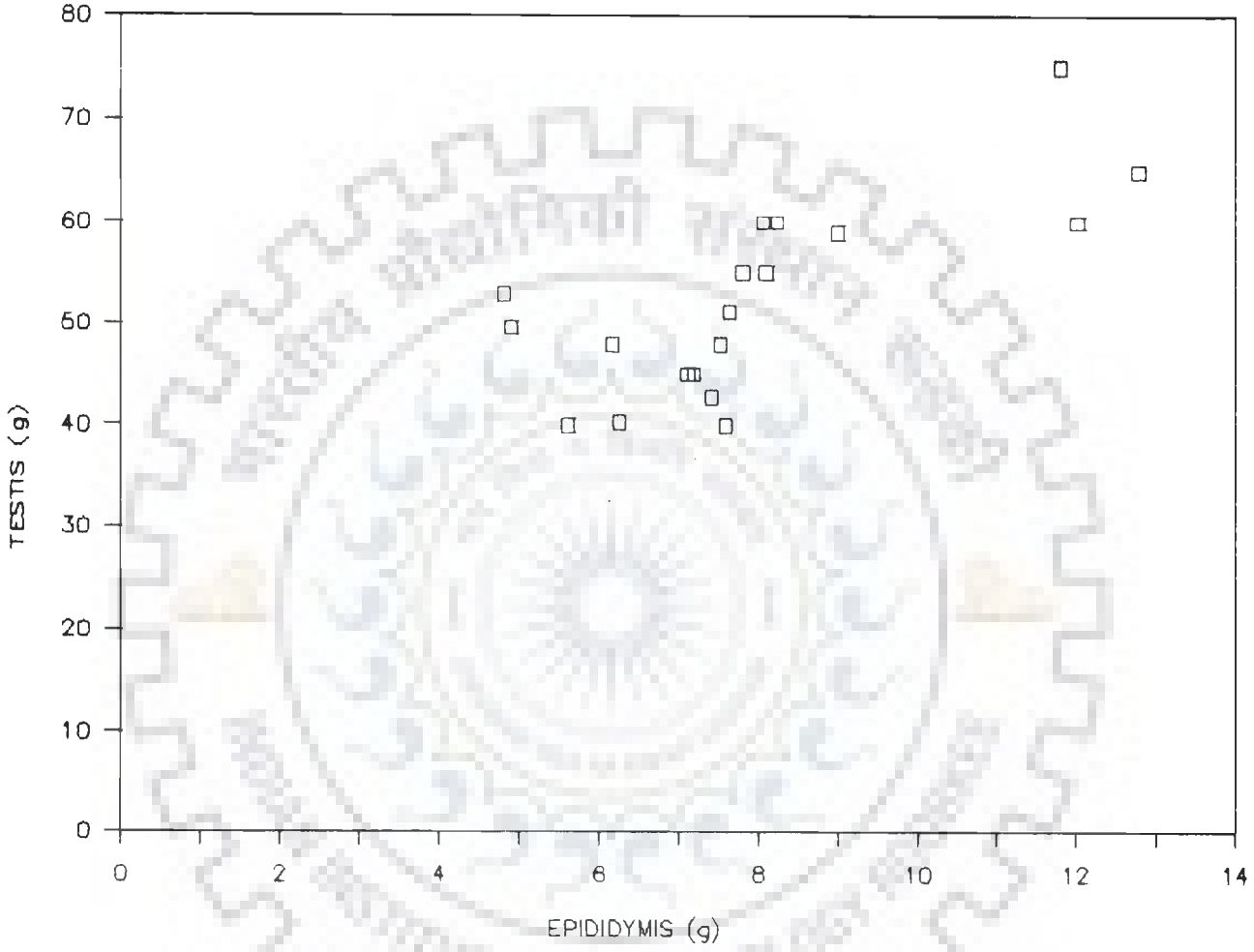


Fig. 6. Regression Analysis of Epididymal and Testicular Weights during Autumn. Regression analysis of epididymal and testicular weights in a random population of male goats (n=28) during autumn, (Y=3.12X + 27.50; r=0.538).

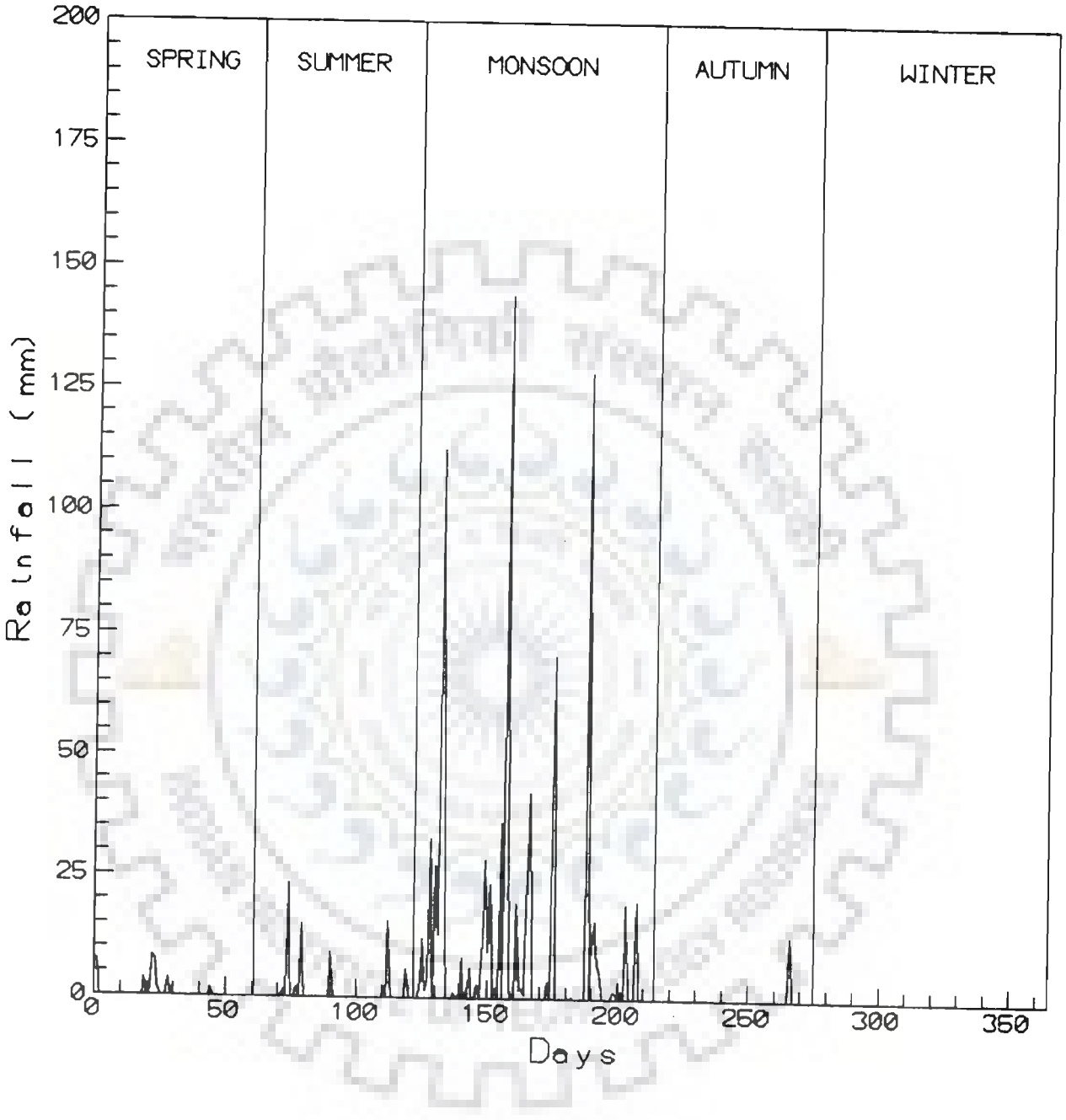


Fig. 7. Annual Pattern of Rainfall Received. Profile showing daily rainfall (mm) received during the five seasons over the period of study from March 1990 - February 1991 at Roorkee (29.52°N and 77.53°E).

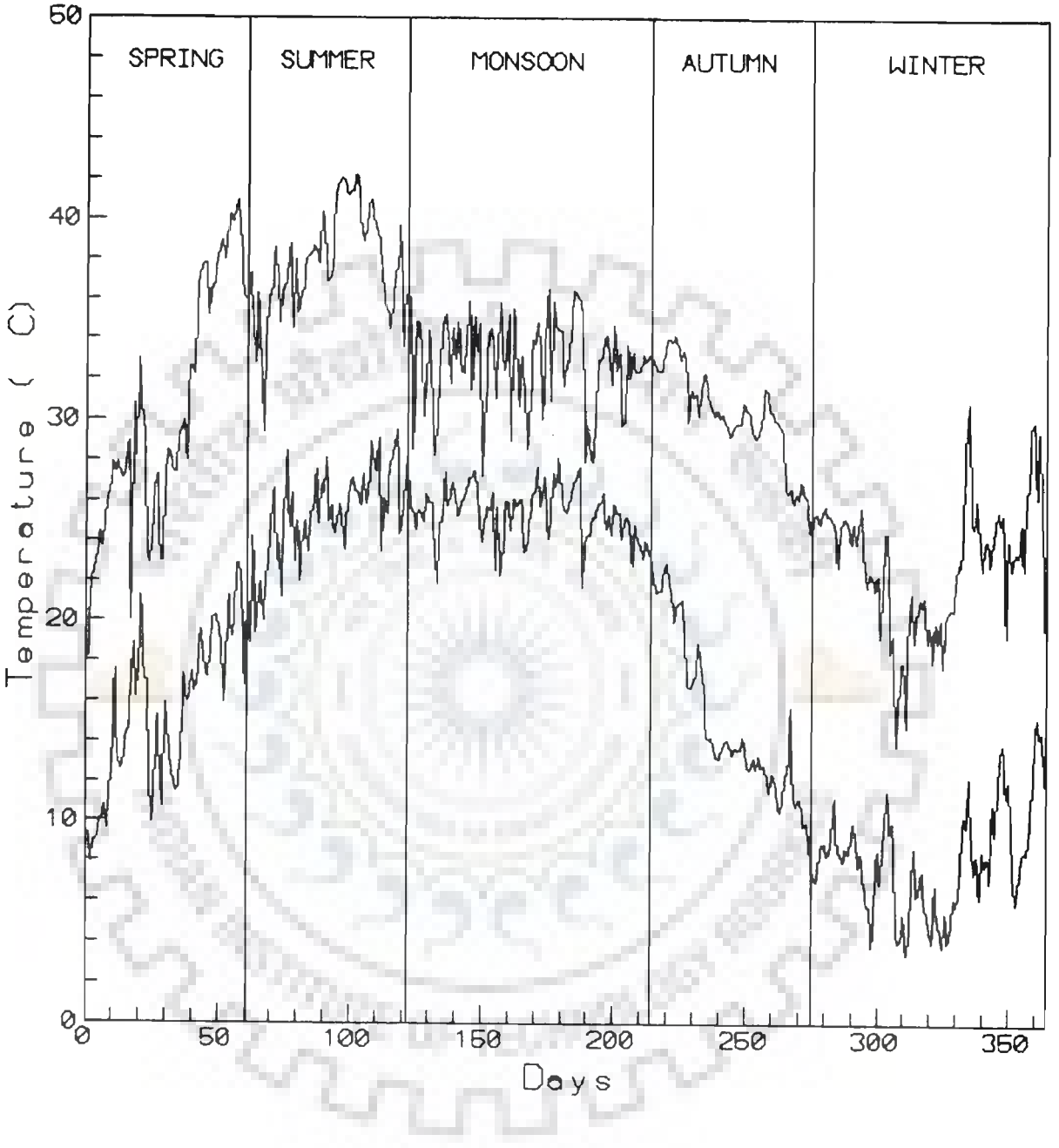


Fig. 8. Annual Pattern of Variations in the Minimum and Maximum Temperature. Daily variations in the minimum and maximum temperature recorded during the five seasons over the period of study from March 1990-February 1991 at Roorkee (29.52°N and 77.53°E).

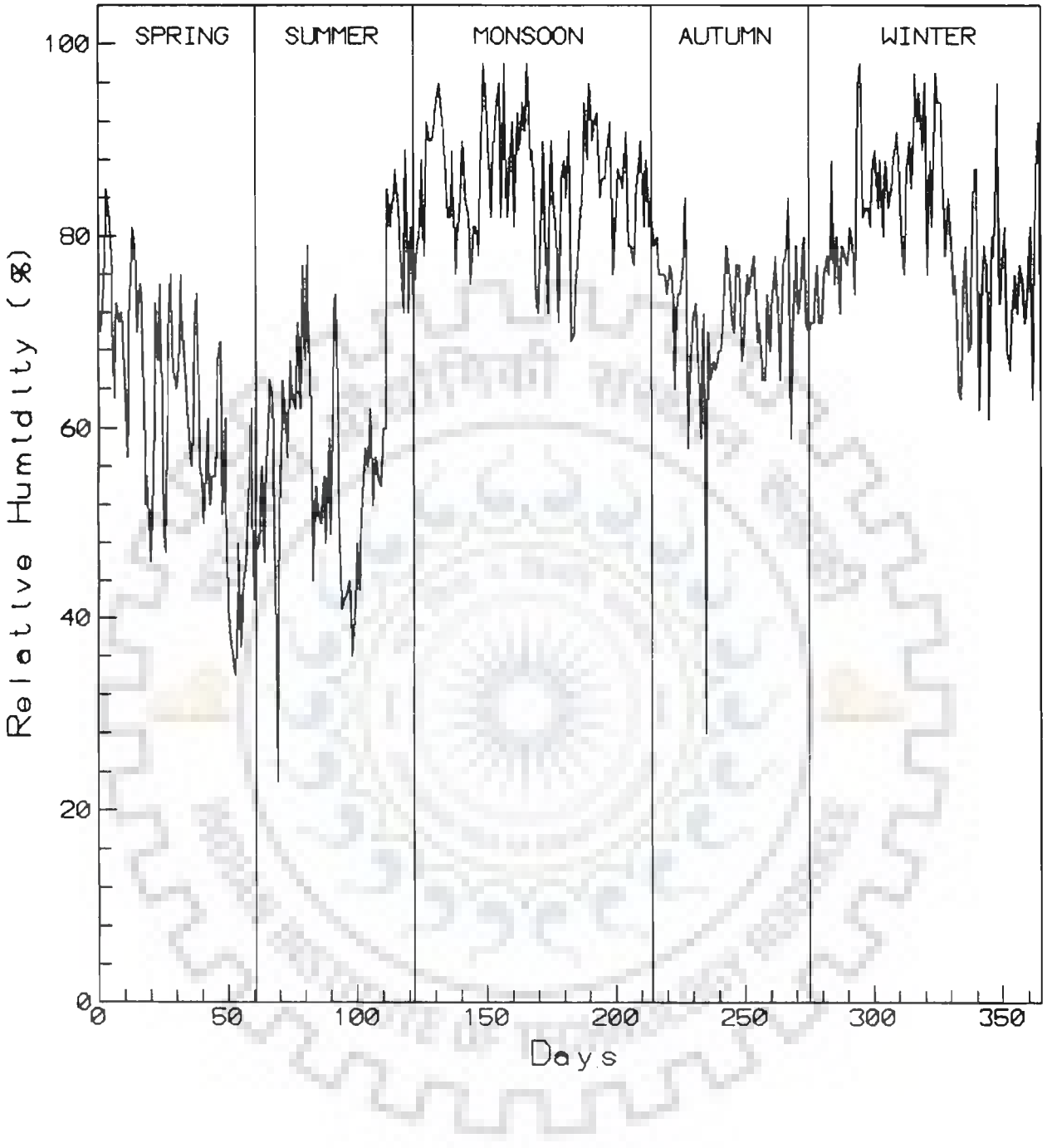


Fig. 9. Annual Pattern of Relative Humidity. Day to day variations in the relative humidity, expressed as percentage, during the five seasons over the period of study from March 1990-February 1991 at Roorkee (29.52°N and 77.53°E).

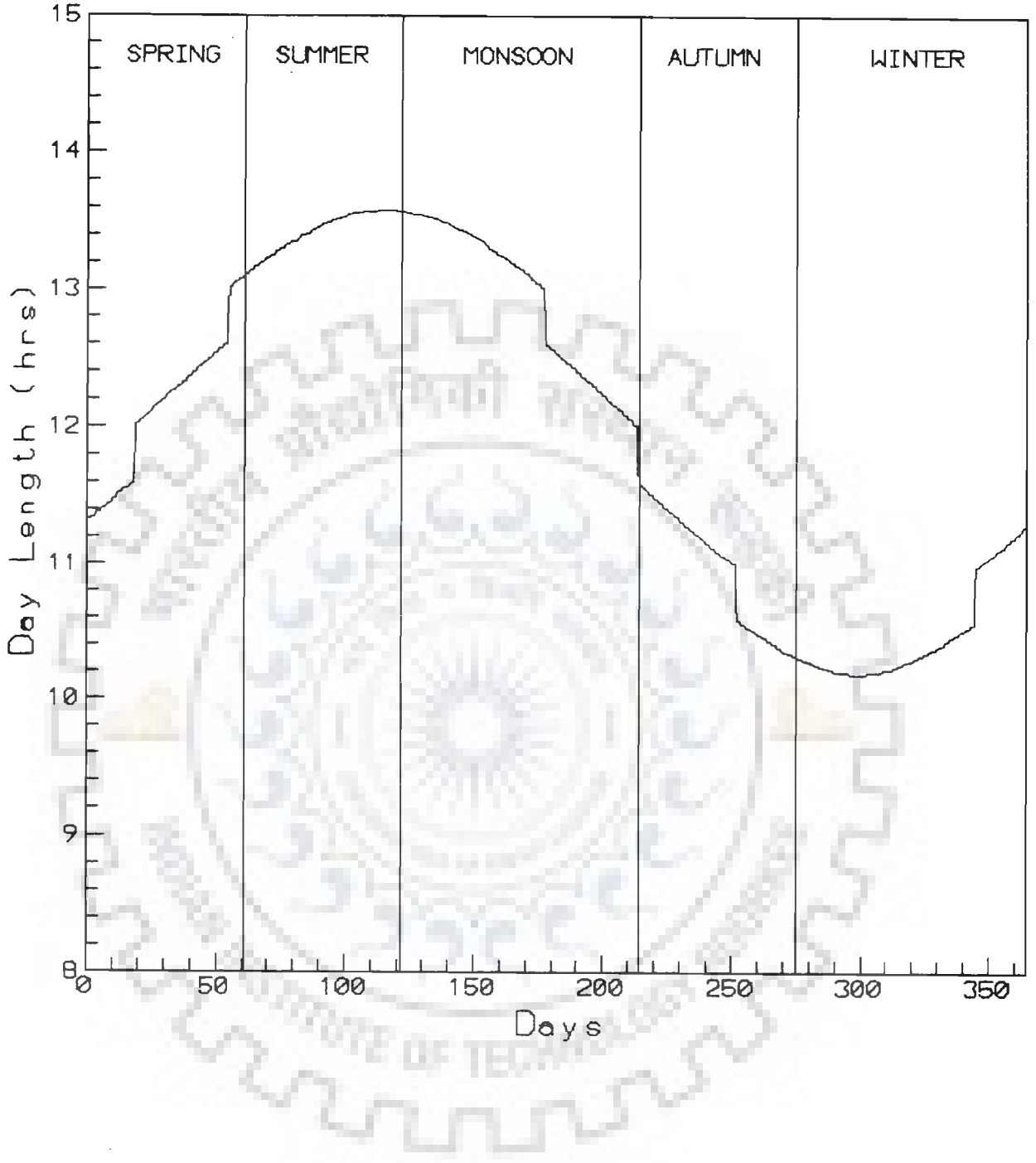


Fig. 10. Annual Pattern of Photoperiod. Day to day variations in photoperiod recorded during the five seasons over the period of study from March 1990 - February 1991 at Roorkee (29.52°N and 77.53°E). Photoperiod was calculated from the daily recorded data on the time of sunrise and sunset provided by the local Meterological Station.

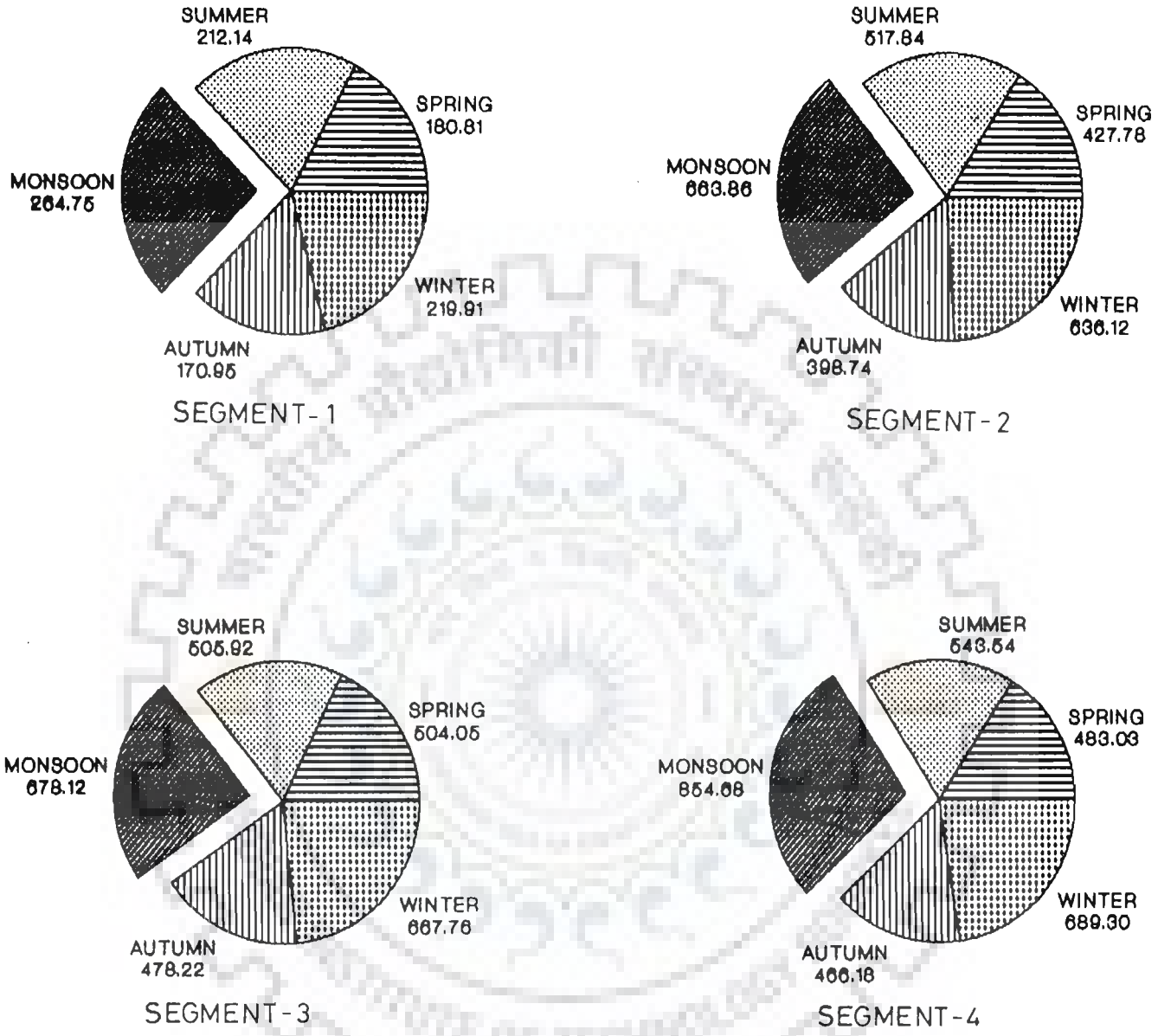


Fig. 11a. Annual Variations in the Total Activity of ß-Galactosidase in Goat Epididymal Segments (1-4) during the Five Seasons. The total activity is expressed as μmoles of p-nitrophenol liberated from p-nitrophenyl ß-D-galactopyranoside per min per g tissue. Each value denotes the mean enzyme activity (spring n=32; summer n=32; monsoon n=36; autumn n=28 and winter n=36).

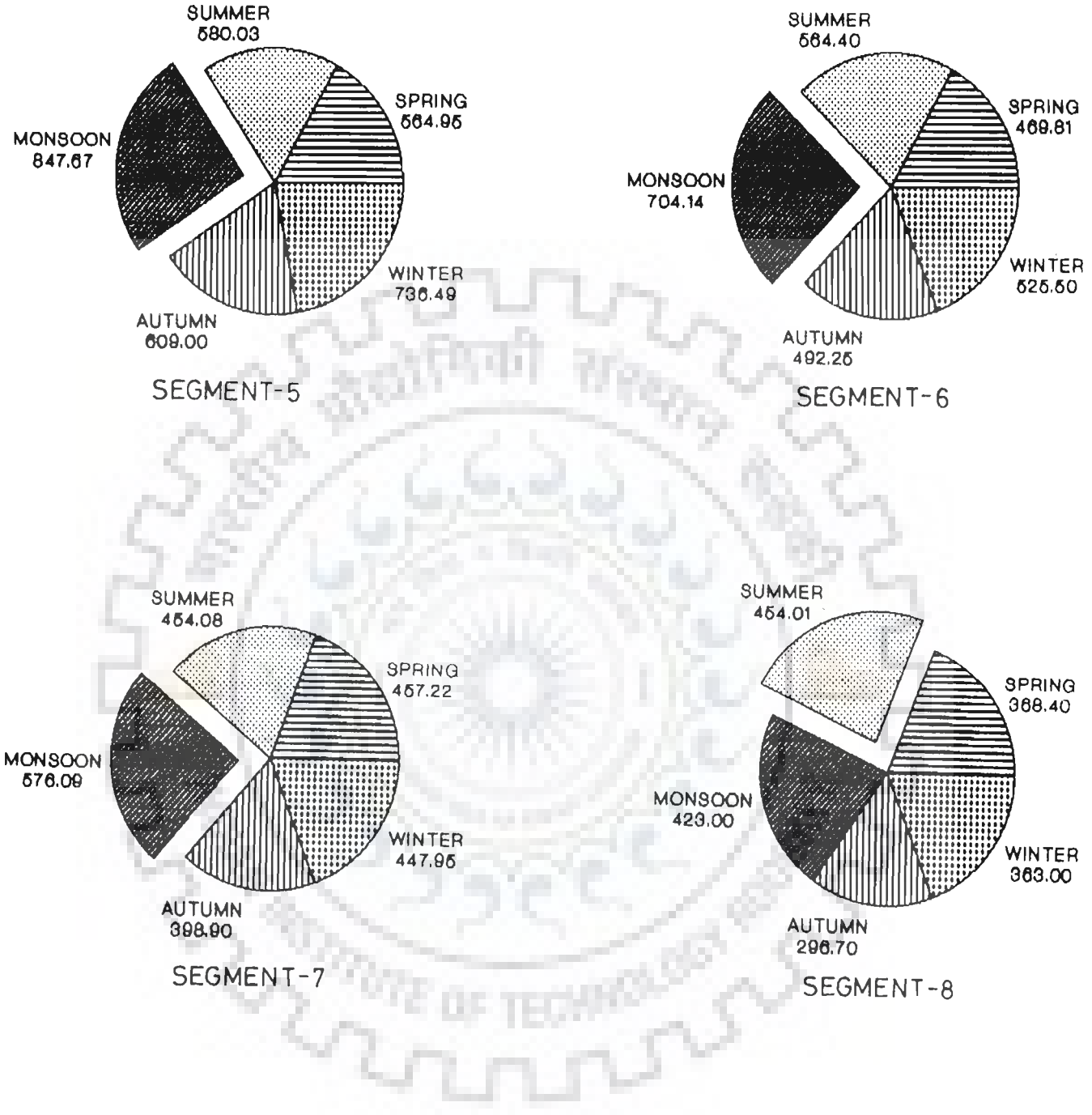


Fig. 11b. Annual Variations in the Total Activity of ß-Galactosidase in Goat Epididymal Segments (5-8) during the Five Seasons. The total activity is expressed as μmoles of p-nitrophenol liberated from p-nitrophenyl ß-D-galactopyranoside per min per g tissue. Each value denotes the mean enzyme activity (spring n=32; summer n=32; monsoon n=36; autumn n=28 and winter n=36).

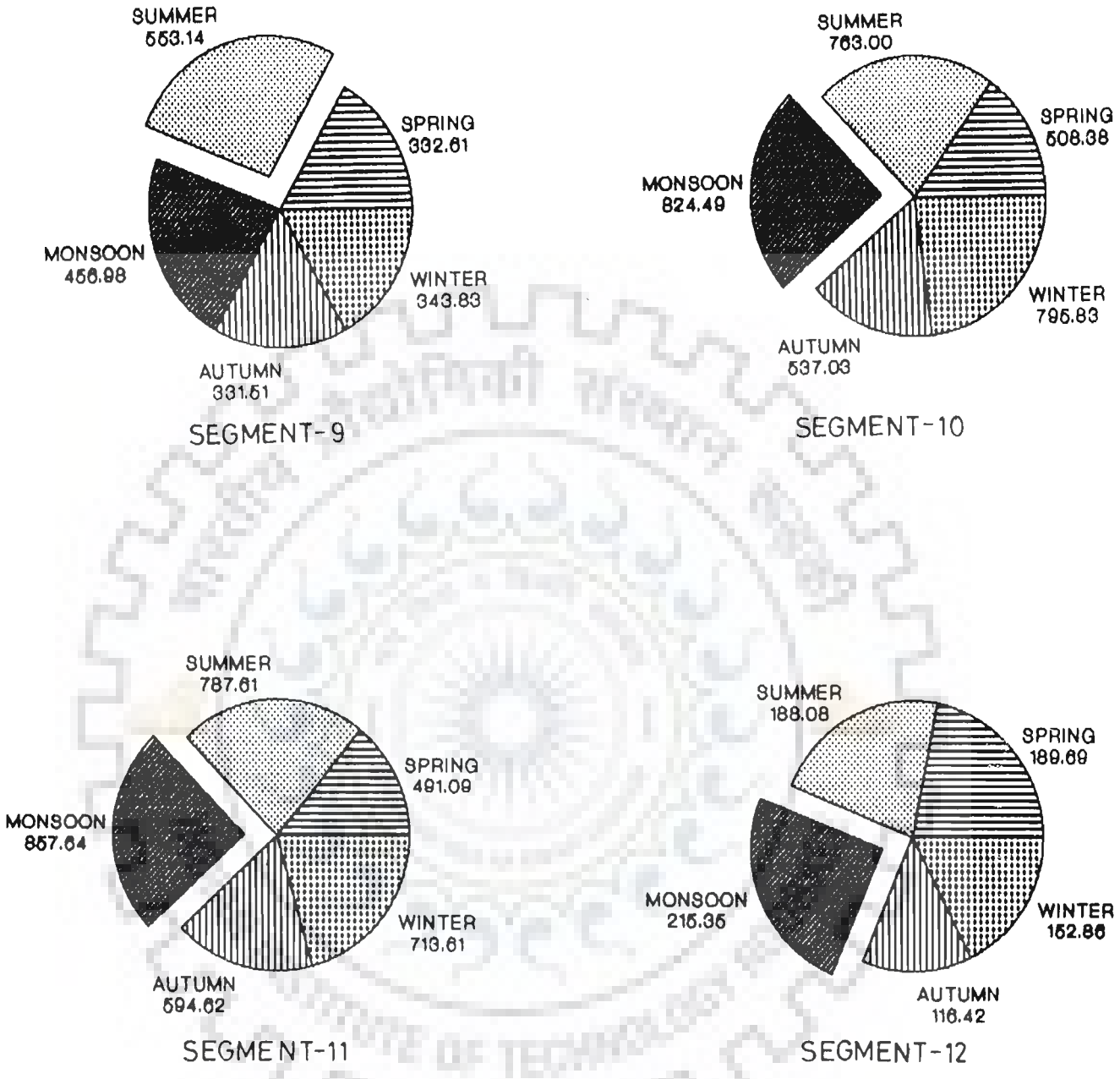


Fig. 11c. Annual Variations in the Total Activity of ß-Galactosidase in Goat Epididymal Segments (9-12) during the Five Seasons. The Total Activity is expressed as μmoles of p-nitrophenol liberated from p-nitrophenyl ß-D-galactopyranoside per min per g tissue. Each value denotes the mean enzyme activity (spring n=32; summer n=32; monsoon n=36; autumn n=28 and winter n=36).

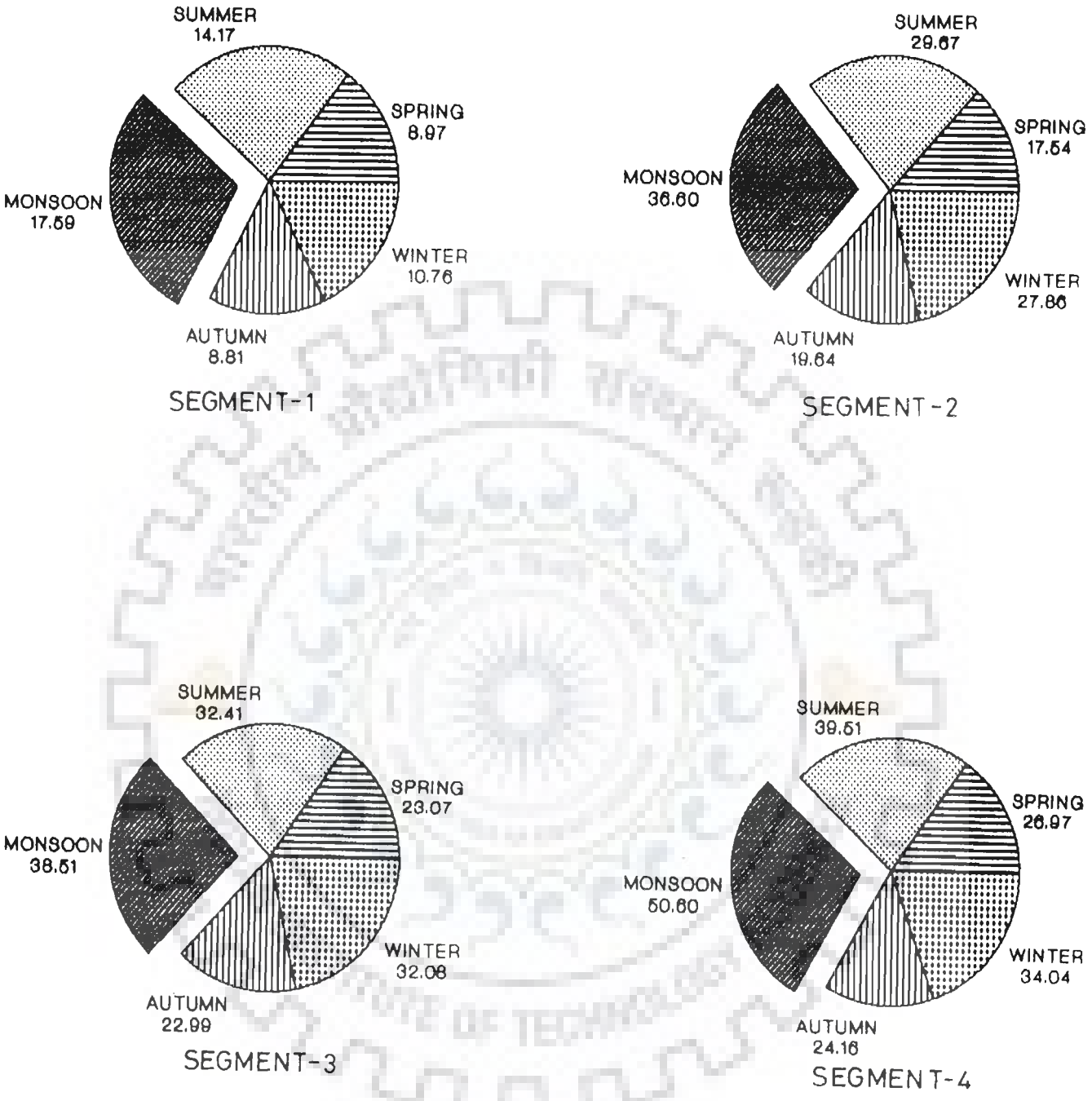


Fig. 12a. Annual Variations in the Specific Activity of ß-Galactosidase in the Epididymal Segments (1-4) of Goat during the Five Seasons. Specific activity is expressed as μmoles p-nitrophenol liberated per min per mg protein. Each value denotes the mean specific activity (spring n=32; summer n=32; monsoon n=36; autumn n=28 and winter n=36).

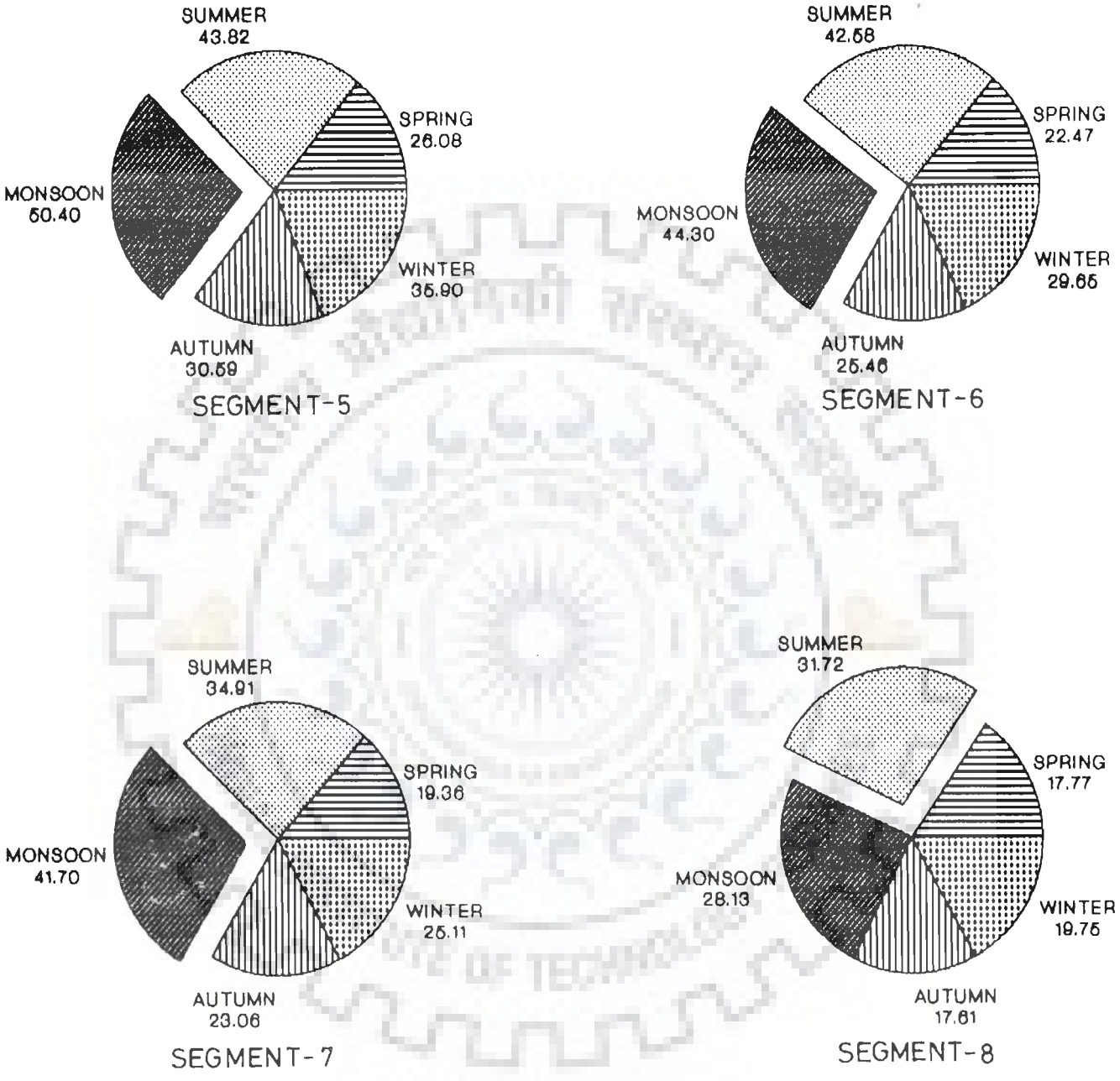


Fig. 12b. Annual Variations in the Specific Activity of ß-Galactosidase in the Epididymal Segments (5-8) of Goat Epididymis during the Five Seasons. Specific activity is expressed as μmoles p-nitrophenol liberated per min per mg protein. Each value denotes the mean specific activity (spring n=32; summer n=32; monsoon n=36; autumn n=28 and winter n=36).

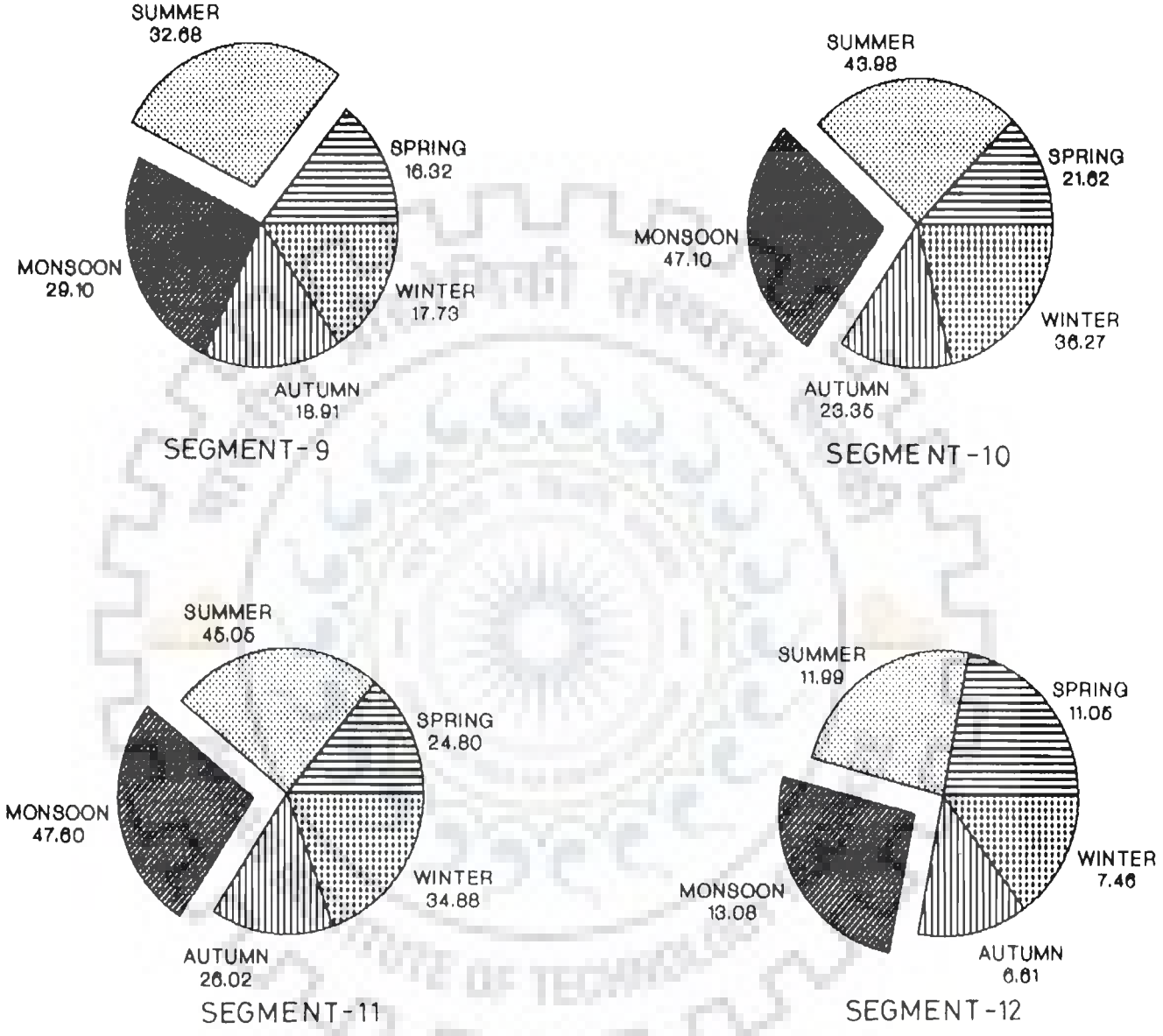


Fig. 12c. Annual Variations in the Specific Activity of ß-Galactosidase in the Epididymal Segments (9-12) of Goat during the Five Seasons. Specific activity is expressed as μmoles p-nitrophenol liberated per min per mg protein. Each value denotes the mean specific activity (spring n=32; summer n=32; monsoon n=36; autumn n=28 and winter n=36).

The daily variation in the photoperiod based on the time of sunrise and sunset which was collected from the local meteorological station is presented in Fig.10. Daylength was observed to increase until the third week of June after which it started decreasing. The long photoperiod just prior to the monsoon months may be of relevance to the onset of breeding cycles of goat.

The effects of environmental signals was also found to be reflected in the biochemical changes that occurred in reproductive tissues. Post-translational modifications of sperm glycoproteins that lead to sperm maturation in the epididymis are affected by the activity of glycosidases and ß-galactosidase belongs to this class of enzymes. The total and specific activity of ß-galactosidase in the epididymal segments measured in different seasons in the year are presented in Figs.11(a,b,c) and 12(a,b,c). The results clearly demonstrate that (i) the activity of ß-galactosidase varies among the 12 segments and (ii) the enzyme activity increase many fold in all 12 segments of the epididymis during the mating season which incidentally happens to be the rainy days of July, August and September.

3.4 DISCUSSION

A majority of seasonally breeding mammals show circannual peak in testicular spermatogenic activity and the desired endocrine profiles much needed for the initiation and maintenance of their breeding cycles (Fuentes et al., 1991). The annual resumption of the reproductive activity in the seasonally breeding goat perhaps

enables it to prepare metabolically for the favourable periods of time, when the chances of survival of its off-spring are maximum. These mammals infact are known to perceive and respond to several environmental signals and at times actually anticipate the upcoming seasons by making the necessary physiological adjustments (Lincoln, 1976a,b; Dacheux et al., 1981; Fuentes et al., 1993).

It is well known that spermatogenesis takes place in the testis while sperm maturation and storage occurs in the epididymis (Bedford, 1975; Orgebin-Crist, 1975; Robaire and Hermo, 1988). When the weight of the testis and the corresponding epididymis collected over a one year period was analysed season-wise, an interesting linear correlation emerged, which was particularly impressive for the monsoon season. In the past, several authors have reported that the daily sperm production in the testis and the rate of flow through the rete testis in discontinuous breeders vary with the season (Dacheux et al., 1981). Obviously the rate of sperm production in the testis and removal from the male reproductive tract is more balanced in the sexually active male goats during the breeding season than at other seasons. This view is further supported by the information gathered from several animal breeders who confirm that maximum sexual activity in goats occurs during the rainy months of July, August and September.

Since goats maintained under natural conditions have been used as the animal model for the present study, one could expect several

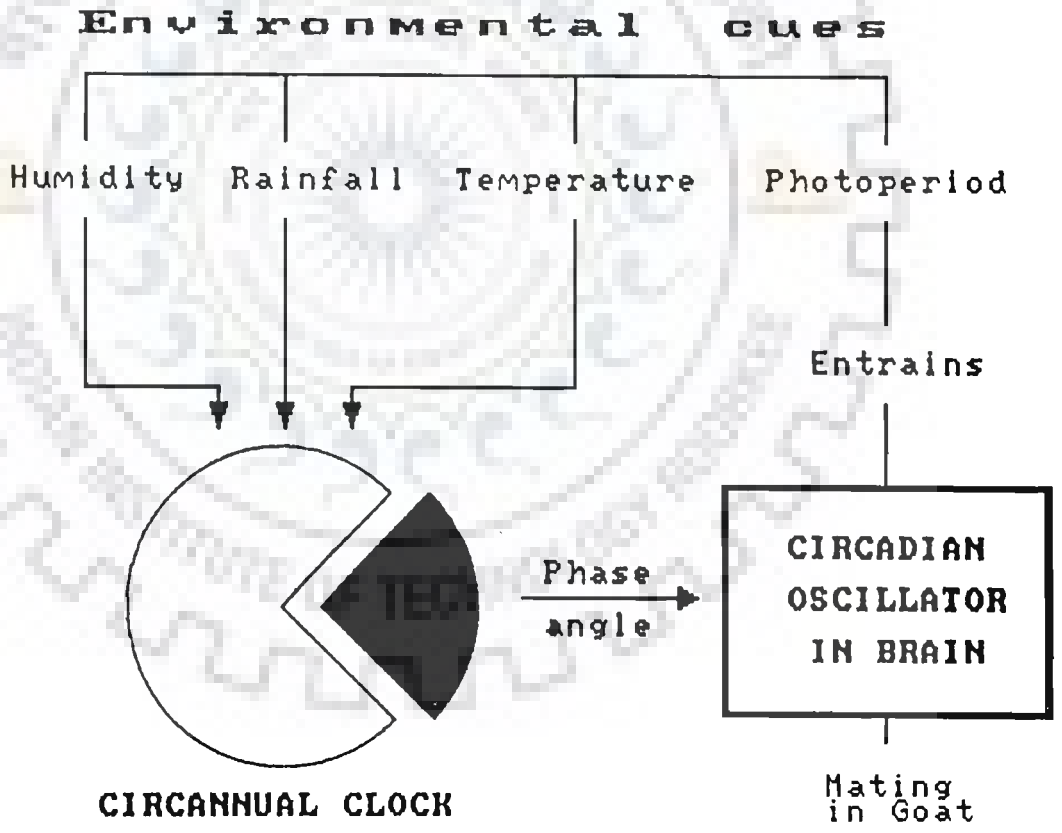
environmental cues to serve as signals for seasonal reproduction. In this context, rainfall was found to be an important factor that governs breeding. Although no direct evidence can be provided to pinpoint its precise role in the regulation of reproduction, rainfall perhaps acts as a stimulant, by promising surplus food. In addition, since goats are herbivorous it is possible that certain plant compounds particularly those secondary metabolites produced during sprouting may act as dietary signals for the onset of breeding, one such metabolite 6-methoxybenzoxazolinone (6-MBOA) has been reported in literature (Sanders et al., 1981). Similarly offset of breeding cycles has been attributed to consumption of certain plant compounds such as cinnamic acids which are abundant in degenerating plant parts (Berger et al., 1977). Thus, the influence of rainfall is visualized as indirect and might be yet another classical case of environment influencing the synthesis of certain factors in primary producers of the food chain which in turn affect primary consumers.

Although rainfall is experienced in two seasons, spring and monsoon, peak sexual activity is distinctly seen in monsoon months. This shows that rainfall may not be the only signal required for sexual activation of male goats in Roorkee. It is reasonable to expect that the temperature and humidity must be comfortable for breeding to occur. Further, reproduction requires tremendous nutrients and energy and in order to channelize the energy available properly, nature has perhaps choosen a season that is least demanding in terms of energy

required for thermoregulation. Thus, the high temperatures prevalent in summer and extremely low temperatures of winter months do not favour reproduction. On the other hand optimal temperatures combined with high relative humidity makes the monsoon season most suited for sexual performance. In this season, there is least variation in the daily maximal and minimal temperatures from the optimal levels. Thus, a major portion of the energy is saved that could have otherwise been spent in thermoregulation.

In many species light acts as a timer of the breeding season, either by acting as a signal that synchronizes an endogenous circannual rhythm or by acting as a stimulus for the neuroendocrine gonadal axis (Turek and Campbell, 1979). Among mammals that respond reproductively to photoperiods, two groups can be distinguished: one group that breeds with the onset of long days and another group that breeds with the onset of short days (Dark and Zucker, 1984; Karsch et al., 1984). From the data collected in the present study, it appears that goats belong to the second group: "the short day breeders". The switch over from the long photoperiod of summer to the relatively short photoperiod experienced during the monsoon seems to stimulate breeding in goats at Roorkee. This observation is in good agreement with previous reports on goats and rams inhabiting temperate regions (Pelletier and Ortavant, 1975a, b; Howles et al., 1980; Lincoln, 1984). The perception of shift in photoperiod is believed to involve the pineal gland and eyes, culminating in changed hormone levels (Thibault et al., 1966;

Pelletier and Ortavant, 1970; Muduuli et al., 1979; Reiter, 1980; Wood et al., 1991). Based on these endocrine profiles it has been suggested that the decreasing light photoperiod has two effects (i) stimulation of gonadotropin release, particularly LH and (ii) lowering the negative feedback of testicular androgens (Lincoln, 1976(a); Olster and Foster, 1988; Wood and Foster, 1992). The resulting rise in testosterone levels bring about the physiological adjustment in the target tissues and thus favour successful breeding.



HYPOTHETICAL SCHEME FOR ENVIRONMENTAL CONTROL OF MATING IN GOAT

It may now be reasonable to conclude that regulation of reproduction in male goats is a synchronized cascade of response to seasonal changes in rainfall, temperature, humidity and photoperiod. ß-Galactosidase is a glycosidic enzyme believed to be involved in processing of glycoproteins (Skudlarek and Orgebin-Crist, 1986). Although ß-galactosidase is known to occur in the testis, several studies have shown convincingly that the principal cells lining the epididymal epithelium are also capable of synthesizing this enzyme (Kemp and Killian, 1978; Skudlarek and Orgebin-Crist, 1986; Sosa et al., 1987). It is now clear that the ß-galactosidase of the epididymal origin is secreted into its lumen where the carbohydrate composition of sperm surface glycoproteins is being modified (Hall and Killian, 1987; Sosa et al., 1991). The fertilizing ability of sperm is thus believed to be enhanced. The present studies have shown the prevalence of high enzyme activity in specific segments of the goat epididymis which may suggest that the enzyme is synthesized in the epididymis and indeed should be involved in events that take place here. This view also stems from the higher enzyme activity measured in the distal segments of the epididymis than in the proximal region.

Another interesting feature is that although the segmental distribution pattern of ß-galactosidase remains more or less similar all the year round, the activity of this enzyme is enhanced manyfold during late summer and monsoon months. Since breeding also occurs during the monsoon season, it is tempting to speculate that the enzyme is involved in processing sperm surface

glycoproteins and thereby sperm maturation. It seems that sperm is prepared in this way for successful mating. It may be recalled that ß-galactosidase in the epididymis is an androgen dependent enzyme (Ronchie and Findlay, 1959). The rise in the testosterone levels caused by the onset of short photoperiod during the mating season (Lincoln, 1976a), may therefore be the reason for enhancement in the activity of epididymal ß-galactosidase. The similar pattern in the total and specific activity of ß-galactosidase during the breeding season excludes the possibility that the increase in enzyme activity is due to seasonal changes in water content in the epididymal tissue. Thus, it is most likely that the increase in enzyme activity is a true reflection of androgen stimulation during the breeding season.

3.5 CONCLUDING REMARKS

1. Based on the climatic conditions prevalent in Roorkee, five distinct seasons have been identified annually: spring; summer, monsoon, autumn and winter.

2. A good linear correlation between the weight of the testis and epididymis observed only in monsoon months is a reflection of balance in the rate of daily sperm production in the testis and the rate of sperm removal from the male reproductive tract.

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3. The rainy days of July, August and September were identified to be the breeding season for goats. Rainfall, temperature, humidity and daylength characteristic of the monsoon season

were found to be important environmental cues that regulate breeding in males.

a) Optimal temperature and high humidity were perhaps needed for comfort.

b) Rainfall provided the necessary nutrients and dietary inputs by increasing grasslands.

c) Change in photoperiod signalled the onset of physiological adjustments needed for breeding.

4. The abundance of ß-galactosidase in specific segments of the epididymis together with high activity during breeding season indicates that this enzyme is involved in sperm maturational events believed to occur in this organ.

5. The increase in epididymal ß-galactosidase during the breeding season could be visualized as the tissue response to the rising testosterone levels. This may be one among several physiological adjustments needed for successful breeding.

CHAPTER 2

Purification of β-galactosidase from the goat epididymis

4.1 INTRODUCTION

ß-Galactosidase (EC 3.2.1.23) is an exoglycosidase which participates in post- translational processing of glycoproteins by removal of galactose units. ß-galactosidase from bovine testis splits terminal galactose from Galß1-->3GlcNAc, Galß1-->4GlcNAc and Galß1--> 3GalNAc (Distler and Jourdian, 1978). The significance of such a transformation in glycoprotein structure has often been known to be reflected in the function of the molecule. It must be emphasized that accurate structural and functional studies are possible only with purified preparations of the molecules involved. With this intention many investigators attempted to purify ß-galactosidase from a number of plant and animal sources with varying degrees of success (Agrawal and Bahl, 1968; Bahl and Agrawal, 1969; Verheijen et al., 1982; Cecilia et al., 1991; Nikolaczyk and O'Rand 1992). From the reports it is apparent that although the reaction catalyzed by the enzyme is more or less identical the amino acid composition and other structural characteristics of the enzyme do not always remain the same. This implies that no universal procedure can be applied for the purification of ß-galactosidase, if the source happens to be different.

The purification of an enzyme is not always simple. Generally physical, chemical and immunological properties are utilized for the successful separation of molecules. It is only logical then that different strategies have been employed to isolate and purify ß-galactosidase from varied sources including male reproductive tissues. Each method of purification exploits an

inherent property of the enzyme. Several such characteristics of the enzyme can thus be of advantage in planning purification schemes. Inspite of the vast range of techniques available many a times it has not been possible to substantially purify the enzyme. This is primarily because some methods employ harsh conditions that lead to a loss in enzyme activity due to denaturation. The extent of damage and reversibility are some of the criteria that limit the choice of techniques that can be utilized. Another important point to be noted is that ß-galactosidase of certain tissue origin are tightly associated with other enzymes forming multienzyme complexes. Thus some researchers have only been able to copurify ß-galactosidase with other exoglycosidases (Verheijen et al., 1982; Yamamoto and Nishimura, 1987).

The catalytic activity of ß-galactosidase is now recognised to be important for sperm maturation in the epididymis. Histochemical and biochemical studies have indicated that the epididymal epithelium actively synthesizes and secretes this enzyme into the lumen for action on sperm (Skudlarek and Orgebin-Crist, 1986). In order to get a better insight into the mode of action and the possible regulatory mechanisms involved in the operation of this enzyme **in vivo** it is desirable to first isolate and purify this enzyme. Therefore in this chapter, the sequential strategy employed for purification of ß-galactosidase from goat epididymis is presented.

4.2 MATERIALS AND METHODS

4.2.1 Source of Enzyme

Adult goat epididymis were collected from the local abattoir immediately after the animal was slaughtered and brought to the laboratory in ice within one hour of slaughter. The epididymis were then cleared of adhering adipose and connective tissue and used as the starting material for purifying ß-galactosidase.

4.2.2 Extraction of Enzyme

The epididymis totalling a weight of approximately 100 g were cut into small pieces and homogenized in 75ml of prechilled distilled water using mortar and pestle. The homogenate was centrifuged at 10,000 xg for 20 minutes at 4°C in a Kuboto refrigerated centrifuge (Japan). After centri- fugation, the supernatant obtained was decanted and stored at 4°C. The pellet obtained was homogenized once again in 75ml distilled water and the extraction procedure was repeated. The supernatant after second extraction was pooled with the one obtained earlier so as to give a final ratio of 650 mg tissue/ml. The pooled supernatant was filtered through a glasswool pad to eliminate lipids and suspended particles, if any, and then subjected to acid precipitation.

4.2.3 Acid Precipitation

The precipitation of selective proteins at extremes of pH serves to eliminate some proteins from solutions thereby resulting in purification of desired proteins. This principle was used for

purifying ß-galactosidase by selectively precipitating the acidic proteins from the crude enzyme preparation.

Reagents

0.2M Sodium acetate buffer pH 3.6 : Prepared by mixing solutions A and B.

Solution A: 0.2M solution of acetic acid : 5.775ml acetic acid in 500ml distilled water.

Solution B: 0.2M solution of sodium acetate : 2.72g of $C_2H_3O_2Na.3H_2O$ in 100ml distilled water.

463ml of solution A and 37ml solution B were mixed to obtain 0.2M sodium acetate buffer pH 3.6.

To 150ml supernatant resulting from initial extraction with distilled water an equal volume of 0.2M sodium acetate buffer pH 3.6 was gradually added with continuous stirring and left for 2 hours at 4°C. The resulting precipitate was removed by centrifugation at 8,000 xg at 4°C in a Kuboto centrifuge and the clear supernatant obtained was further subjected to ammonium sulfate salt precipitation.

4.2.4 Ammonium Sulfate Salt Precipitation

Salting out is one of the well known techniques that has been routinely used in most purification studies. The added advantage with this technique is that besides being a purification step, it also simultaneously concentrates the sample. In preliminary experiments, in order to workout the salt concentration that

could selectively precipitate ß-galactosidase from the crude enzyme preparation in distilled water (400 mg tissue/ml), solid ammonium sulfate was added and gradually mixed using a magnetic stirrer. The precise amount of salt needed to obtain 0-90 percent saturation was worked out from the chart given in Methods in Enzymology 1, 76 (1955). At each level of saturation the precipitating proteins were collected by centrifugation at 10,000 xg for 20 min. in a Beckman J2.21 centrifuge. The volume of the supernatant was measured and appropriate amounts of salt were added to achieve the next higher percentage of saturation. The precipitates obtained at each step were resuspended individually in 5.0ml of 0.1M sodium acetate buffer pH 3.6 and then checked for ß-galactosidase activity and protein content.

Assay for monitoring the degree of enzyme purification

ß-Galactosidase activity was measured employing the method of Conchie et al., (1959b) as described earlier (Section 3.2.4.2). The total activity was calculated. Protein was also estimated simultaneously in the samples by Lowry's method (1951) (Section 3.2.5) from which the specific activity of enzyme was calculated.

4.2.5 Heterogeneity of ß-galactosidase

To check if ß-galactosidase exists as acidic and neutral form after ammonium sulfate salt precipitation the activity of the enzyme precipitated was determined at varying pH values using three different buffer systems over a wide pH range. Since appreciable amounts of enzyme were precipitated at 40, 50 and 60% salt concentration, the enzyme activity of these precipitates

alone were determined at varying pH. The three buffer systems used were sodium acetate buffer (pH 3.6-5.6), phosphate buffer (pH 5.8-8.0) and glycine-NaOH buffer (pH 8.6 - 10.6).

Reagents

0.1M Sodium acetate buffer (pH 3.6-5.6): Stock solutions of 0.2M acetic acid and 0.2M sodium acetate were prepared and mixed in the proportions indicated in the table to get the desired pH values.

Solution A: 0.2M acetic acid: 11.55ml glacial acetic acid in 1000ml distilled water.

Solution B : 0.2M sodium acetate solution : 27.2g $C_2H_3O_2Na.3H_2O$ in 1000ml distilled water.

X ml of A + Y ml of B were mixed and diluted to 100 ml to give a final concentration of 0.1M.

X	Y	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

Phosphate buffer (pH 5.8 - 6.0) : 0.2M stock solutions of monobasic and dibasic sodium phosphate were prepared.

Solution A: 0.2M Monobasic sodium phosphate solution : 2.78g in 100ml.

Solution B: 0.2M Dibasic sodium phosphate solution : 7.17g of $Na_2HPO_4.12H_2O$ in 100ml.

X ml of A + Y ml of B were mixed and diluted to a total volume 20ml.

X	Y	pH
9.20	0.80	5.8
8.77	1.23	6.0
8.15	1.85	6.2
7.35	2.65	6.4
6.25	3.75	6.6
5.10	4.90	6.8
3.90	6.10	7.0
2.80	7.20	7.2
1.90	8.10	7.4
1.30	8.70	7.6
0.85	9.15	7.8
0.53	9.47	8.0

Glycine-NaOH buffer (pH 8.6-10.6) : 0.2M stock solutions of A and B were prepared and mixed

Solution A: 0.2M Glycine solution : 15.01 g glycine in 1000ml distilled water.

Solution B: 0.2M NaOH : 8g NaOH in 1000ml distilled water.

50ml of A + x ml of B were mixed and diluted to a total volume of 200ml.

X	pH
4.0	8.6
6.0	8.8
8.8	9.0
12.0	9.2
16.8	9.4
22.4	9.6
27.2	9.8
32.0	10.0
38.6	10.4
45.5	10.6

Procedure: In separate tubes 0.1ml of either the crude extract (200 mg tissue/5ml of distilled water) or resuspended precipitate after 40-60% salt saturation was mixed with 0.1ml of the buffer of desired pH and preincubated for 15 minutes at 37°C. The incubations were further carried out for 15 minutes at 37°C after the addition of 1.25 mM substrate prepared in the same buffer. The reaction was terminated by the addition of 2.0ml of 0.2M Na_2CO_3. The absorbance, was read at 400 nm in a Beckman DU-6 spectrophotometer against a corresponding blank in which the substrate was added after the addition of Na_2CO_3.

4.2.6 Dialysis

Since the precipitates obtained between 40-60% ammonium sulfate salt precipitation contained maximum reextractable ß-galactosidase activity, the supernatant obtained after acid precipitation step was brought to 30% salt saturation. The sample was now centrifuged at 8,000 xg at 4°C for 20 minutes in a Kuboto centrifuge. The precipitate which pelleted out was discarded. The supernatant was saved and solid ammonium sulfate was added to bring the salt concentration to 60% . The mixture was left at 4°C for 4 hours and once again centrifuged at 10,000 xg for 30 minutes. This time the supernatant was discarded and the precipitate rich in ß-galactosidase was resolubilized to a final volume of 15 ml with 0.01M sodium acetate buffer pH 4.8 and dialysed extensively against several changes of the same buffer for 6-8 hours. The dialysis membrane whenever used was pretreated to remove any contaminants.

4.2.6.1 Treatment of Dialysis Membrane

Reagent: 5mM Ethylenediamine tetramine salt (EDTA) and 200mM sodium bicarbonate solution : 1.861g of EDTA and 16.8g $NaHCO_3$ were mixed and dissolved in 1000 ml distilled water.

Procedure: The dialysis tubing (Sigma Chemical Company) was cut to desired lengths (10-30cm) and placed in 500ml of 5mM EDTA, 200mM $NaHCO_3$ solution. It was boiled for 5 minutes following which the EDTA/$NaHCO_3$ solution was poured out. The tubing was rinsed briefly with distilled water. It was again placed in 500ml of fresh EDTA/$NaHCO_3$ solution and the procedure was

repeated. After the second rinse, the dialysis tubing was autoclaved for 10 minutes and was stored in distilled water containing 0.02% sodium azide at 4°C.

After the dialysis was complete, there appeared a precipitate in the dialysis bag. The dialysate was therefore centrifuged. The precipitate was discarded since no enzyme activity was detected. The supernatant which contained nearly 100 units of enzyme/mg protein was then subjected to ion exchange chromatography. Both cation and anion exchange resins were tried but the cation exchange resin CM-cellulose was found to be most useful in terms of yield, specific activity and degree of purification.

4.2.7 Cation Exchange Chromatography

4.2.7.1 Washing the resin

25g carboxymethyl cellulose (CM cellulose, medium mesh : Sigma) was suspended in 500ml distilled water overnight for swelling. Later, the fine particles were removed to prevent the clogging of the column by repeatedly washing the resin with distilled water and decanting the water overlay containing floating fine particles until it was completely free of all fines.

4.2.7.2 Charging the Resin

Reagents

Solution A: 0.5M NaCl : 0.5M NaOH solution: 14.61g NaCl and 10g NaOH were dissolved and made upto 500ml with distilled water.

Solution B: 0.5N HCl : 20.833ml HCl was diluted to 500ml with distilled water.

After removing the excess water, the swollen CM cellulose was transferred to solution A and left for 30 minutes with intermittent mixing. After completion of alkali treatment the resin was washed extensively with distilled water to wash away all traces of alkali. The washing was considered to be complete when the pH of the water obtained after washing was similar to the one freshly added. Once washing was complete, the resin was transferred to solution B and left for another 30 minutes. Washing was repeated till the resin was free of all traces of HCl. The charged resin was then packed into a glass column (1.2 x 20 cm).

4.2.7.3 Equilibration and Sample application

After the column was packed, the resin was equilibrated for 8-10 hours with 10mM sodium acetate buffer pH 4.8. Complete equilibration was ensured once again by checking the pH of the eluant and comparing it with the one added at the top of the column. 18.5ml of clear supernatant obtained after dialysis and containing 119.4 mg protein was applied in small volumes allowing the first volume to get into the resin bed and subsequently adding fresh sample. Finally the sides of the column were rinsed with 2ml of buffer to wash the adhering protein into the column bed.

4.2.7.4 Column washing and elution

The unbound proteins in the column were washed with 40ml of the equilibration buffer. The bound proteins were then eluted using a continuous linear gradient formed by mixing 70ml equilibrating

buffer and 70ml of the same buffer containing 500mM NaCl using a gradient mixer.

The flow rate was maintained at 20 ml/hr using a peristaltic pump and fractions of 1ml were collected. 0.02ml from every alternate fraction was checked for both enzyme activity and protein separately.

4.2.8 Concanavalin A Sepharose chromatography

Buffer : 0.1M Sodium acetate buffer pH 6.0 containing 0.1M NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$ and 1mM $MnCl_2$

4.2.8.1 Packing and Equilibration

Con A Sepharose 4B from Jack bean (Sigma) was packed in a glass syringe (1 x 5 cm). Con A Sepharose interacts with high affinity with N-linked oligosaccharides in which at least 2 outer mannose residues are either unsubstituted or are substituted only at position C-2 by another sugar. The column was equilibrated with 0.1M sodium acetate buffer pH 6.0 containing 0.1M NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$ and 1mM $MnCl_2$.

4.2.8.2 Sample application and Elution

The sample subjected to acid precipitation, ammonium salt sulfate precipitation and cation exchange chromatography was dialysed and applied to the Con A Sepharose column at a flow rate of 5 ml/hr. The non-specifically bound proteins were eluted using 2 volumes of the equilibration buffer supplemented with 1M NaCl. Specific elution of the bound glycoproteins was carried out by washing the column with 2 volumes of the equilibrating buffer containing 1M

NaCl and 500 mM α-methyl D-mannoside. Fractions of 1ml were collected at a flow rate of 6 ml/hr.

Protein was estimated in all the fractions by Lowry's method (1951) and the fractions rich in protein content were pooled and dialysed against 50mM sodium acetate buffer pH 4.8 for 6 hours. After dialysis ß-galactosidase activity was monitored.
Since there was substantial loss in ß-galactosidase activity after Con A Sepharose chromatography in the purification schedule it became necessary to skip this affinity column chromatography procedure and directly proceed with gel filtration after cation exchange chromatography.

4.2.9 Gel Filtration on Seralose 6B

Gel filtration separation that helps in the purification of biological molecules based on molecular size. The special advantages of using this technique could in a nutshell be summarised as follows:

(i) Because the chromatographic behaviour of almost all substances in gels is independent of temperature, pH, ionic strength and buffer composition, separation can be carried out under virtually all conditions.

(ii) Because adsorption is negligible very labile substances are not affected by the chromatography.

(iii) There is less zone spreading than with other chromatographic techniques.

(iv) The elution volume is related in a simple manner to molecular weight.

100ml of Seralose 6B (SRL India) supplied in preswollen form was packed in a glass column (1.4 x 80 cm) The column once packed was equilibrated with 50 mM sodium acetate buffer pH 4.8 containing 0.3M NaCl, for 5-6 hours. The concentrated pooled fractions after CM cellulose chromatography were applied in a volume of 2ml containing 9.44mg protein. The column was eluted with equilibrating buffer and fractions of 1ml were collected at a flow rate of 8 ml/hr. ß-galactosidase activity and protein content were measured in alternate fractions. The fractions rich in ß-galactosidase activity were pooled and dialysed against 1:10 dilution in distilled water of Tris- chloride buffer stock solution pH 6.7. The dialysate was then concentrated by lyophilization.

4.2.10 Preparative Electrophoresis

Electrophoresis was carried out by following the method of Davis (1964) with slight modification to facilitate large sample application.

Reagents

Tris-Glycine electrode buffer stock solution (pH 8.3): 6g Tris, 28.8g glycine were dissolved in 900ml of distilled water, the pH was adjusted to 8.3. Final volume was made upto 1000ml with distilled water. This stock was diluted in 1:9 ratio with distilled water before use.

Tris-chloride buffer stock solution (pH 8.9): 48ml of 1N HCl, 86.6g Tris and 0.23ml TEMED were dissolved in 90ml distilled

water. The pH was adjusted to 8.9. Final volume was made upto 100ml.

Tris-chloride buffer stock solution (pH 6.7): 48ml of 1N HCl, 5.98g Tris and 0.46ml TEMED were mixed in a final volume of 90ml with distilled water. The pH was adjusted to 6.7 with 1N HCl. Final volume was made upto 100ml with distilled water.

Resolving gel acrylamide stock solution: 28g acrylamide, 0.74g NN' Methylenebisacrylamide were mixed and made upto 100ml with distilled water. The solution was filtered and stored in a dark bottle at 4°C.

Ammonium persulfate solution: 0.1g ammonium persulfate was dissolved in 1ml distilled water.

Bromophenol Blue Solution : 25 mg bromophenol blue was dissolved in 10ml of Tris-chloride buffer solution (pH 6.7).

Sample buffer : 50ml Tris-chloride buffer stock solution (pH 6.7) and 40ml glycerol were mixed. Final volume was made 100ml with distilled water.

Resolving gel solution

Tris-chloride buffer stock pH 8.9	5.0ml
Resolving gel acrylamide solution	10.0ml
Distilled water	25.0ml
Ammonium persulfate solution	0.3ml

Stacking gel solution

Tris-chloride buffer stock solution pH 6.7	2.5ml
Resolving gel acrylamide solution	3.0ml
Distilled water	14.1ml
Ammonium persulfate solution	0.3ml

Resolving gel: The glass plates 18 x 16 cm were fixed with 1.5 mm spacers. The resolving gel solutions were mixed, deaerated and poured to a height of 12cm. Overlaying was done by careful addition of distilled water with a pasteur pipette and the gel was left to polymerise for 1 hour. After polymerization was complete the water overlay was decanted.

Stacking gel: The stacking gel solutions were mixed thoroughly and deaerated. After pouring out the overlying water, the resolving gel surface was rinsed with stacking gel solution. The rinsing solution was poured out and stacking gel solution was added to a height of 3.5cm. A blank comb with 1.5mm thickness with one well at one extreme was inserted and gel was left to polymerize at room temperature for 30 minutes.

Sample preparation: The lyophilized pooled fractions were mixed 1:1 with sample buffer. Prior to sample application 25 μl of bromophenol blue was added using micropipettes.

The sample was applied and electrophoresis was carried out at 4°C at 30mAmp, constant current mode using Hoeffer Electrophoresis system. Once the bromophenol blue migrated to about 9.5cm in the resolving gel, electrophoresis was stopped and gel was taken out. Negative staining of the side lane was done after separating the

extreme lane from remaining gel by a sharp paper knife. The remaining gel was stored at 4°C till the band was visible on the stained gel strip.

4.2.10.1 Negative staining by Copper chloride stain

The negative staining of the excised gel strip was done by the method of Lee et al. (1987) using 0.3M $CuCl_3$.

Procedure: The gel strip was washed briefly in several changes of deionized water and then transferred to 0.3M $CuCl_3$ solution placed in a trough. Gentle shaking was done for 3-5 minutes till the white bands started appearing. After completion of staining the bands were viewed against a dark background, and the distance of the band of interest was marked. The remaining gel was excised at the distance of 2mm above and 2mm below that position to ensure complete recovery of the protein band.

4.2.10.2 Electroelution

Gel tubes were used for this purpose. The bottom of gel tubes were sealed with stacking gel and the macerated pieces of the excised gel strip containing the enzyme obtained in the preparation procedure were packed into the tubes. The enzyme contained in the gel tube thus prepared was electrophoresed into a dialysis bag attached to the bottom of the gel tube. After electrophoresis was complete the dialysis bag was detached from the gel tube. The free end of the dialysis bag was tied and the contents were dialyzed against 0.1M sodium acetate buffer pH 4.8. The dialysate was then concentrated by lyophilization and both enzyme activity and protein content were measured.

4.2.11 Check for Homogeneity

4.2.11.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of the purified ß-galactosidase preparation was carried out following the standard procedure of Laemmli (1970).

Stock Solutions

Acrylamide: bisacrylamide (30:0.8): 30% W/V acrylamide containing 0.8% W/V bisacrylamide (N,N' methylene bisacrylamide): 30g acrylamide and 0.8g bisacrylamide were mixed in 50ml distilled water and the final volume was made upto 100ml filtered and stored at 4°C in a dark bottle.

TEMED (NNN'N'-Tetramethylethylene diamine): Used as supplied.

Ammonium persulfate (1.5% W/V): 0.15g of ammonium persulfate was dissolved in 10ml distilled water. This was prepared fresh each time before use.

Sodium dodecyl sulfate (10% W/V): 5g SDS was dissolved in water to a final volume of 50ml stored at room temperature.

Gel Buffer Stock solutions

Stacking gel buffer (0.5M Tris HCl pH 6.8): 6g Tris dissolved in 40ml distilled water was titrated to pH 6.8 with 1M HCl and brought to a final volume of 100 ml. It was filtered and stored at 4°C.

Resolving gel buffer (3.0M Tris HCl pH 8.8): 36.3g Tris and 48ml 1M HCl were mixed and brought to 100ml final volume with distilled water. The solution was filtered and stored at 4°C.

Reservoir buffer stock (0.25M Tris, 1.92M glycine, 1% SDS pH 8.3): 30.3g Tris, 144g glycine and 10g SDS were dissolved in distilled water and made upto 1L.

These stock solutions were mixed as indicated below to make 10% gels

Recipe for 10% Resolving Gel

Acrylamide: bisacrylamide	10.0ml
Resolving gel buffer stock	3.75ml
Ammonium per sulfate	1.5ml
TEMED	0.015ml
Water	14.45ml
SDS solution	0.30ml

Recipe for 3.75% Stacking Gel

Acrylamide: bisacrylamide	2.5ml
Stacking gel buffer stock	5.0ml
Ammonium per sulfate	1.0ml
TEMED	0.015ml
Water	11.30ml
SDS solution	0.2ml

Casting the Gel : 18 x 16 cm glass plates were fixed with 1.5mm spacers and the deaerated and resolving gel solution was poured to a height of 10cm. Once the gel solution was poured, overlaying was done with distilled water using a pasteur pipette and the gel was left to polymerize at room temperature for one hour. After the polymerization of the resolving gel was complete, the water overlay was poured out and the mixture of

stacking gel solutions was prepared. The resolving gel surface was rinsed by pouring little of this mixture before adding TEMED. After the gel surface is rinsed TEMED is added and the stacking gel mixture was added to a height of 3cm. The comb was inserted which provided 14 wells. Each well had a volume capacity of 100μl. The gel polymerization was complete after another 30 minutes.

Sample buffer (0.125M Tris HCl pH 6.8, 4% SDS and 40% Glycerol): The sample buffer was prepared by mixing 40ml glycerol, 4g SDS and 25ml Tris HCl buffer pH 6.8 (0.5M). The final volume was made upto 100ml with distilled water.

Sample preparation: Dialysed protein sample after preparative electrophoresis was concentrated by lyophilization and mixed in sample buffer and diluted 1:1 with water. Bromophenol blue solution containing 20% glycerol was added in the wells using micropipettes following which 40 μl of the sample was added.

Reservoir buffer was diluted with distilled water in 1:9 ratio and electrophoresis was conducted at constant current measuring 30mAmp. Time taken by bromophenol blue for completion of run was around 5 hours. After the run, the gel was removed and the distance migrated by bromophenol was measured and the gel was transferred to the staining solution.

4.2.11.1.1 Staining for Protein

Staining Solution

0.1% Coomassie Brilliant Blue R-250 in methanol: acetic acid : H_2O (40:10:50 V/V/V).

0.1g Coomassie Brilliant Blue was dissolved in 40.0 ml methanol. After complete solubilization, 10ml glacial acetic acid and 50ml distilled water were added to make the final volume 100ml. The staining solution was filtered through Whatman filter paper before use.

Staining was carried out at room temperature overnight by immersing the gel in the staining solution.

Destaining Solution

Methanol: acetic acid: Water (5:7:88 V/V/V): The destaining solution was made by mixing 20ml methanol, 28 ml acetic acid and 353ml distilled water so as to give a final volume of 400ml.

Destaining was carried out by transferring the gel into the destaining solution. The destaining solution was changed after 2 hours initially and after every 4 - 6 hours till the background was clear and the fine bands were clearly resolved.

4.2.11.2 Cathodic Discontinuous Polyacrylamide Gel Electrophoresis

It was not possible to specifically detect ß-D-galactosidase on gels after SDS PAGE by Laemmli system due to loss in enzyme activity caused by the denaturing conditions. Discontinuous PAGE of the purified preparation was therefore tried in acidic buffer system as described by Hames (1981), on completion of

electrophoresis, the gel was incubated with 6-bromo-2-naphthyl ß-D-galactopyranoside, a specific substrate used to identify ß-galactosidase activity (Knipple & MacIntyre, 1984).

Stock solutions

Acrylamide: bisacrylamide (30:0.8): 30g acrylamide and 0.8g bisacrylamide were dissolved in 50 ml distilled water and the final volume was made upto 100ml. The solution was filtered and stored at 4°C in a dark bottle.

TEMED: Used as supplied.

Ammonium per sulfate(1.5% W/V): 0.15g ammonium per sulfate was dissolved in 10ml distilled water. This solution was freshly prepared before use.

Fuchsin Tracking dye: 0.05g basic Fuchsin was dissolved in 10ml distilled water.

Gel Buffer stock solutions

Stacking Gel buffer (Acetic acid-KOH pH 6.8): 48ml 1M KOH and 2.9ml glacial acetic acid were mixed. Final volume was made 100ml with distilled water.

Resolving Gel Buffer (Acetic acid-KOH pH 4.3): 48ml 1M KOH and 17.2ml glacial acetic acid were mixed and water added to make the final volume 100ml.

Reservoir Buffer (Acetic acid-ß-Alanine pH 4.5): 31.2g ß-Alanine and 80ml glacial acetic acid were dissolved in and made to 1L with distilled water.

Sample Buffer: 25ml stacking gel buffer solution (pH 6.7) and 10ml glycerol were mixed. Final volume was made 100ml with distilled water.

Recipe for preparation of 5% Resolving gel

Acrylamide: bisacrylamide (30:0.8)	5.0ml
Resolving gel buffer stock	3.75ml
Ammonium per sulfate (1.5%)	1.5ml
TEMED	0.15ml
Water	19.6ml

Recipe for preparation of 2.5% Stacking gel

Acrylamide: bisacrylamide (30:0.8)	2.5ml
Stacking gel buffer stock	5.0ml
Ammonium per sulfate (1.5%)	1.5ml
TEMED	0.15ml
Water	11.35ml

Casting the gel: Eight absolutely clean glass gel tubes were marked at 7.5cm length with a glass marker and the opposite end of the tube was capped tightly. The resolving gel mixture was prepared and degassed. It was then carefully poured into the vertically arranged gel tubes to the marked heights using a pasteur pipette. The gel solution was overlayed with distilled water and left for 45 minutes to polymerize at room temperature. Once the polymerization was complete, the overlay was decanted and the stacking gel mixture was poured to a height of 1cm above the resolving gel. This gel was once again overlaid with a thin layer of distilled H_2O and left to polymerize for another 30

minutes. The overlying water in the tubes was decanted and the gel surface was rinsed with sample buffer. The tubes were then fixed into the electrophoretic assembly for sample application.

Sample preparation and application

Prior to sample application 10 μl of the tracking dye was added into each gel tube. The lyophilized protein was mixed with the sample buffer and 30-50 μg protein per tube was added using micropipettes.

Once the sample was applied the reservoir buffer was added and the electrophoretic apparatus was connected to the Powerpack. The polarity of the electrodes was changed i.e. cathode was connected to the reservoir and anode connected to the upper tank buffer so as to affect migration of the applied proteins towards cathode. Electrophoresis was carried out at constant current mode (3mAmp per tube) at 4°C in a refrigerator.

On completion of run (3 hours) the gels were recovered from the gel tubes by rimming the sides of the gel tubes forcing a jet of water with the help of a syringe. The gels were stained for protein and ß-galactosidase activity.

4.2.11.2.1 Staining for Protein

The rod gels were stained for protein with Coomassie Brilliant blue and destained as described earlier (Section 4.2.11.1.1).

4.2.11.2.2 Staining for ß-Galactosidase

Specific staining for ß-galactosidase was carried out by the method of Knipple and MacIntyre (1984) with slight modifications

using 6-Bromo-2-naphthyl ß-D-galactopyranoside (6-BNG). The staining is based on the principle that ß-galactosidase reacts with 6-bromo-2-naphthyl ß-D-galactopyranoside and the product formed when incubated with Fast blue tetrazolium salt forms a burgundy colored complex at the site of reaction.

Staining solution: 30 mg 6-Bromo-2-naphthyl ß-D-galactopyranoside (Sigma) was dissolved in 10ml methanol. To this 90ml of 0.1M sodium acetate buffer (pH 4.8) was added. Finally 100mg of o-Dianisidine, tetrazotized (Sigma) was added to give a final concentration of 1 mg/ml.

The gels recovered after cathodic discontinuous PAGE, were transferred into the staining solution and incubated at 37°C for 6-8 hours till a burgundy colored band indicating the site of active ß-galactosidase was visible.

After the staining was complete gels were placed in 7% acetic acid and stored in the same.

4.2.11.3 Immunological Studies

Immunological studies were carried out as described in Antibodies: A laboratory Manual (Harlow and Lane 1988).

Production of Antibodies: Polyclonal polyspecific antibodies were generated using white female rabbits of New Zealand strain. Four to six months old animals were purchased from Indian Drugs and Pharmaceuticals Ltd. Rishikesh. They were acclimatized to the conditions in the laboratory for 15 days, prior to immunization.

4.2.11.3.1 Collection of Preimmunization Serum

Prior to immunizations, the rabbit was bled by incising the outer marginal ear vein giving a small cut perpendicular to the vein. The blood (approximately 10ml) was collected in tubes and kept inclined at 37°C for 1 hour. The clot was then detached from the sides of the tubes using a pasteur pipette and stored overnight at 4°C for complete separation of serum from the clot. The following morning, serum was decanted and centrifuged at 800 xg for 25 minutes at 4°C. The straw colored serum thus obtained was stored at -20°C in small vials in aliquots of 0.2ml.

4.2.11.3.2 Immunization Schedule

One week after the preimmuniization bleeding the first dose of purified enzyme preparation mixed with Freunds' Complete Adjuvant was injected subcutaneously at multiple sites on the back of the animal. Fifteen days later the first booster dose was injected. The second booster was given after a gap of another fifteen days. The animals were bled from the marginal ear vein one week after the second booster and serum was separated and stored in small vials.

Reagents

Phosphate Buffered Saline (PBS) pH 7.2: Prepared by dissolving 8g NaCl, 0.2g KCl, 0.24g KH_2PO_4 and 1.44g Na_2HPO_4 in 800 ml distilled water. The pH was adjusted to 7.2 and the final volume was made 1000ml with distilled water.

Preparation of Injection

250μg of lyophilized enzyme obtained after preparative electrophoresis was suspended in 0.5ml PBS. This was then mixed with an equal volume of Freunds' Complete Adjuvant (Difco Laboratories, Detroit Michigan, USA) and an emulsion was generated by vigorous and prolonged mixing. To 0.5ml Freunds Complete Adjuvant Contained in 1.5ml eppendorf tube, 0.5ml protein solution in PBS was forcefully injected by piercing the needle tip into the tube through its lid. Vigorous mixing was done by repeatedly taking up the mixture in the syringe and then forcefully releasing it by pressing the plunger. This procedure was repeated till a thick white colored emulsion was generated. Satisfactory preparation of emulsion was checked by releasing a minute drop of emulsion on PBS. Once the intact drop was formed which did not disperse in the aqueous phase, it was taken as an indication that the protein was completely emulsified.

Method of Injections

The fur on a small area near the back of the animal was shaved off to mark sites for subcutaneous injections. Afterwards the first dose was injected at five marked sites with each injection having a volume of 0.2ml. The antigen was injected by carefully lifting the skin of the animal and releasing the antigen under the skin. The booster doses containing 100μg protein in 0.3ml PBS were prepared in a similar manner and injected as per schedule at the same sites.

4.2.11.3.3 Double Immunodiffusion

This technique popularly known as Oucterlony Technique (Ouchterlony, 1962) is widely used to analyse complex mixture of antigens. It is carried out on the principle that when the antigen and the corresponding antibody are allowed to diffuse in agarose, they form a precipitin band at the point of equivalence for the specific concentration of antigen and antibody.

Reagents

PBS prepared as described in section

1% Agarose: 1g agarose was suspended in 100 ml PBS pH 7.2 and placed in a boiling water bath till the agarose dissolved completely and a homogeneous clear solution resulted.

Method

The 1% agarose solution was brought to 60°C and poured between 2 glass plates (13.0 x 9.5 cm) fixed with 2mm spacers. The agarose solution was allowed to polymerize at room temperature for 20-30 minutes. When the polymerization was complete the glass plate from one side and the spacers were carefully removed. On each plate thus obtained 2 sets of wells were punched each set comprised of four peripheral wells punched at equidistance from each other as well as from the central well.

All the wells had a capacity of 20 μl. The central well was loaded with 20μl antigen, whereas the immunized serum was added to wells a and c. 20 μl of the immunized serum was added to a while 10μl of serum diluted 1:1 with PBS was loaded in well c. Wells b and d contained 20 μl of preimmunization serum and 20μl

PBS respectively. The gel was then placed in a closed sandwich box on a layer of moistened filter papers to maintain humidity and incubations were carried out at 37°C for 24 hours.

Washing the gel: Once incubations were complete the gel was slit to separate the two identical sets of wells and each set was washed for 48 hours in PBS with change in the washing solution at 6-7 hours intervals. Washing was done to ensure complete removal of the unprecipitated proteins prior to staining.

4.2.11.3.4 Staining the Gel

Although the precipitin lines were visible clearly, staining was used to facilitate photography of the pattern obtained. In this regard, Coomassie Brilliant Blue R-250 was used to enhance the resolution. To verify if the antibody was generated against ß-galactosidase the specific staining of this enzyme was used to stain the precipitin lines.

4.2.11.3.4.1 Coomassie Brilliant Blue Staining

Staining solution: 0.05g Coomassie Brilliant Blue was dissolved in 40ml methanol. To this, 10ml of glacial acetic acid was added and the final volume was made upto 100 ml by addition of 50ml distilled water.

The gel was immersed in the staining solution for 4-6 hours and then destained by transferring it to the destaining solution.

Destaining

Destaining solution: 10% V/V acetic acid, 5% V/V methanol: 10ml acetic acid and 5ml methanol were mixed and made upto 100ml with distilled water.

Destaining was carried out with repeatedly changing the destaining solution till the blue background disappeared leaving the clearly visible precipitin bands indicating the sites of antigen-antibody interaction.

4.2.11.3.4.2 Staining for ß-Galactosidase

Specific staining of the identically run agarose gel following immunodiffusion was carried out using 6-Bromo-2-naphthyl ß-D-galactopyranoside as described earlier (Section 4.2.11.2.2). Briefly, the gel was washed in 0.1M sodium acetate buffer pH 4.8 at $4^{o}C$ for 1 hour. The agarose gel was then immersed in staining solution and incubated at 37°C for 6-8 hours till burgundy colored band was visible between the wells containing antigen and antibody. The gels were then transferred to 7% acetic acid solution for termination of reaction and stored in the same.

4.3 RESULTS

Goat epididymal tissue has been used as a source for purifying ß-galactosidase. The enzyme was initially extracted in water and further purification was done using conventional techniques namely, acid precipitation, salt precipitation, dialysis, cation exchange chromatography, gel filtration and preparative electrophoresis.

Selective precipitation of proteins in the crude epididymal extract following exposure to the acidic pH resulted in 3 fold purification of ß-galactosidase with 60% recovery. The pattern of protein precipitation by sequential increase in ammonium sulfate salt concentration is presented in Fig. 13. Although most epididymal proteins precipitated between 20-80% salt saturation, the reextractable ß-galactosidase activity could be obtained along with proteins that precipitated between 40-60% salt saturation. Thus, this step resulted in a 6.5 fold purification over the crude enzyme preparation with 45% recovery of the enzyme protein.

The activity of ß-galactosidase in the crude enzyme preparation over the entire range of acidic, neutral and basic pH is presented in Fig. 14. High enzyme activity was observed only at acidic pH particularly between pH 4.4 - 6.0.

The enzyme activity was also checked in enzyme rich precipitates obtained after salt precipitation at various pH (Fig. 15). It was seen that even in these precipitates epididymal ß-galactosidase showed activity only at acidic pH. In fact no activity of ß-galactosidase was observed when the enzyme assays were carried out with buffers of pH higher than 6.0.

When ß-galactosidase rich precipitate obtained after 40-60% ammonium sulfate salt precipitation was resuspended in sodium acetate buffer pH 4.8 and dialysed against the same buffer, there appeared a precipitate in the dialysis bag. The contents of the dialysis bag were centrifuged to isolate this precipitate. The

precipitate was found to be largely devoid of ß-galactosidase activity and hence discarded. The supernatant, however, was rich in enzyme and showed a 7.6 fold purification over the crude preparation.

The elution profile of the concentrated protein applied on to CM-cellulose column is presented in Fig. 16. The applied protein eluted mainly as 3 peaks - the first one was due to the washing of the unbound proteins. The second peak which emerged with the start of NaCl gradient was of the loosely bound proteins which were eluted at lower salt concentration. The third peak which denoted the major peak was obtained due to the elution of the strongly bound basic proteins at higher NaCl concentration. When checked for the presence of ß-galactosidase activity, it was observed that the enzyme eluted as a sharp peak in fractions 129 - 139 at around 250 mM salt concentration in the elution buffer. The enzyme in these pooled fractions was found to have a 55 fold purification over the crude preparation with a specific activity of 72.51 units/mg protein.

Fig. 17 presents the elution profile of concentrated pooled fractions applied on Concanavalin - A Sepharose 4B column. The applied protein eluted as 2 peaks. The first peak which emerged as the major peak denoted the elution of non-specific proteins. Second peak which emerged following elution with 0.5M methyl α-D-mannopyranoside added to the equilibrating buffer was of the enzyme. When the enzyme activity and protein content were monitored in the fraction collected, about 60% loss in the enzyme activity was observed. Since the purification fold achieved was

also not very significant, this step was removed from the purification scheme.

The elution profile of the protein applied on Seralose-6B column is depicted in Fig. 18. The applied protein eluted as 2 major peaks between fractions 51 - 96. ß-galactosidase activity was recovered in fractions 63 - 69 of the first protein peak. These pooled fractions had a specific activity of 104 units/mg protein and thus gave 79 fold purification.

Preparative electrophoresis following gel filtration resulted in a substantial purification of ß-galactosidase. Selective excision of the protein band containing the enzyme followed by electroelution into a dialysis tubing was able to bring about 137 fold purification over the crude preparation with an activity of 179.66 units/mg protein.

Thus the sequential steps used in the purification of ß-galactosidase from goat epididymal tissue involved acid precipitation, salt precipitation, dialysis, CM-cellulose chromatography, gel filtration and finally preparative electrophoresis. The data on enzyme purification at each stage of purification is consolidated in the Table 3.

The electrophoretic protein pattern of the purified enzyme preparation as obtained by discontinuous SDS - PAGE is shown in electrophoretogram (Fig. 19). The slab gel was stained for protein with Coomassie brilliant blue and a single band was seen indicating that the preparation was electrophoretically

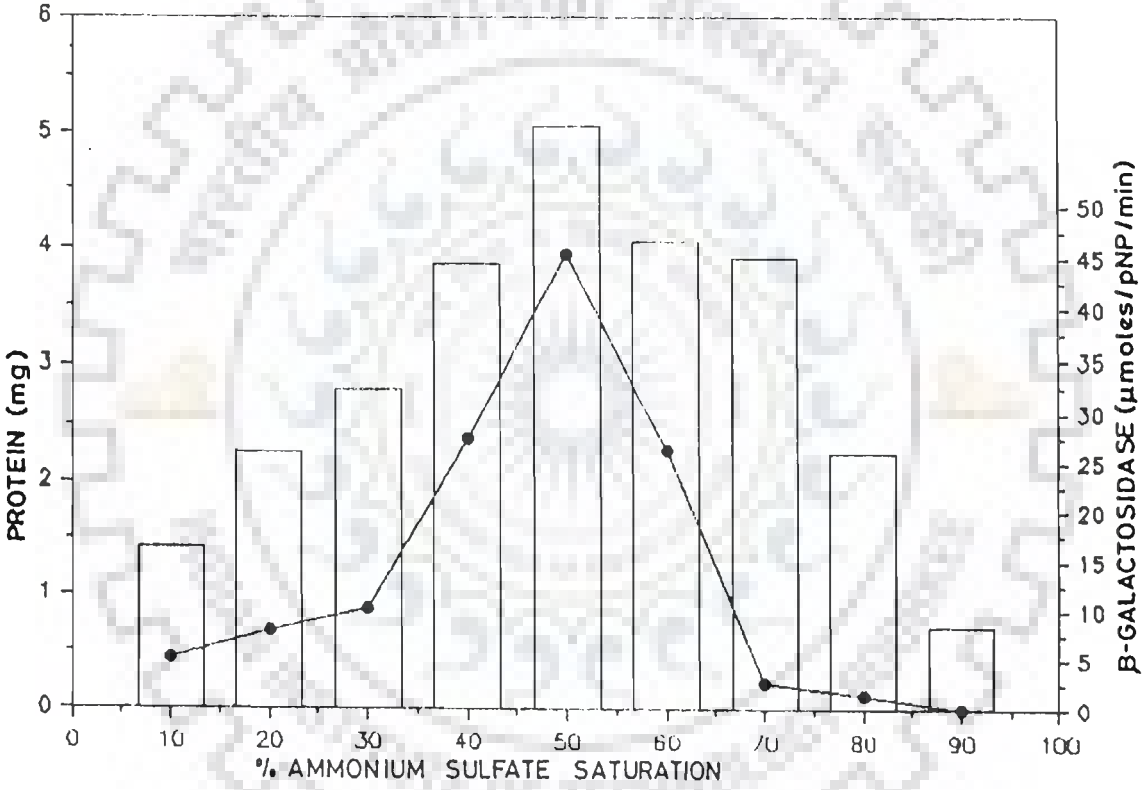


Fig. 13. Ammonium Sulfate Salt Precipitation of Proteins in the Crude Epididymal Extract Following Sequential Increase in Salt Saturation. The precipitates obtained at each step were resuspended in 0.1M Sodium acetate buffer pH 3.6 and estimated for (bars) protein and (-●-) ß-galactosidase activity.

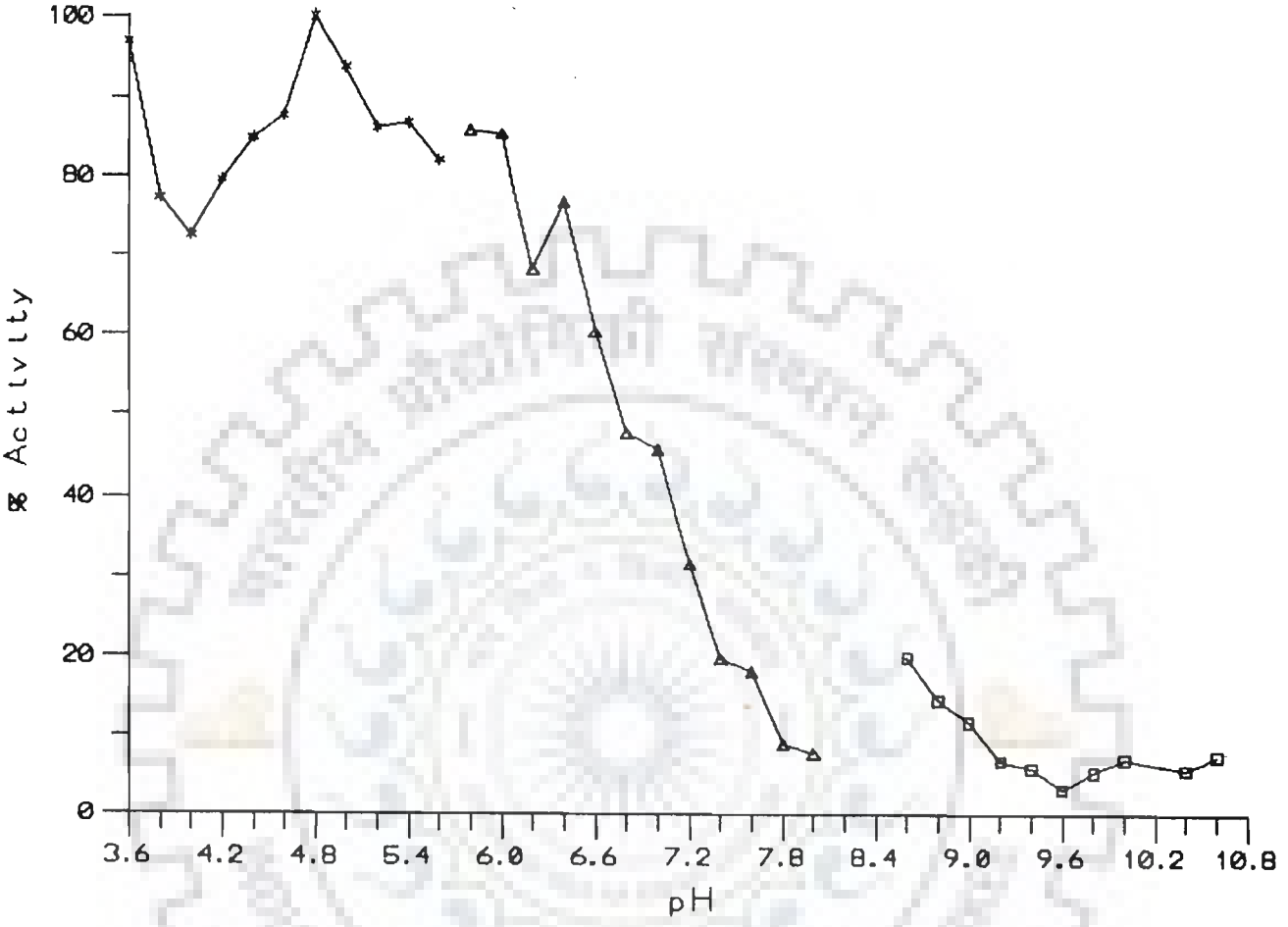


Fig. 14. pH Dependence of Crude Preparation of ß-Galactosidase From the Epididymis of Goat. The enzyme was incubated at 37° with 1.25mM p-nitrophenyl ß-D-galactopyranoside in: (-*-) 0.1M sodium acetate buffer, pH 3.6-5.6; (-▵-) 0.1M phosphate buffer, pH 5.8-8.0 and (—□—) 0.1M glycine-NaOH buffer, pH 8.6-10.6. p-Nitrophenol liberated was estimated according to the standard assay procedure.

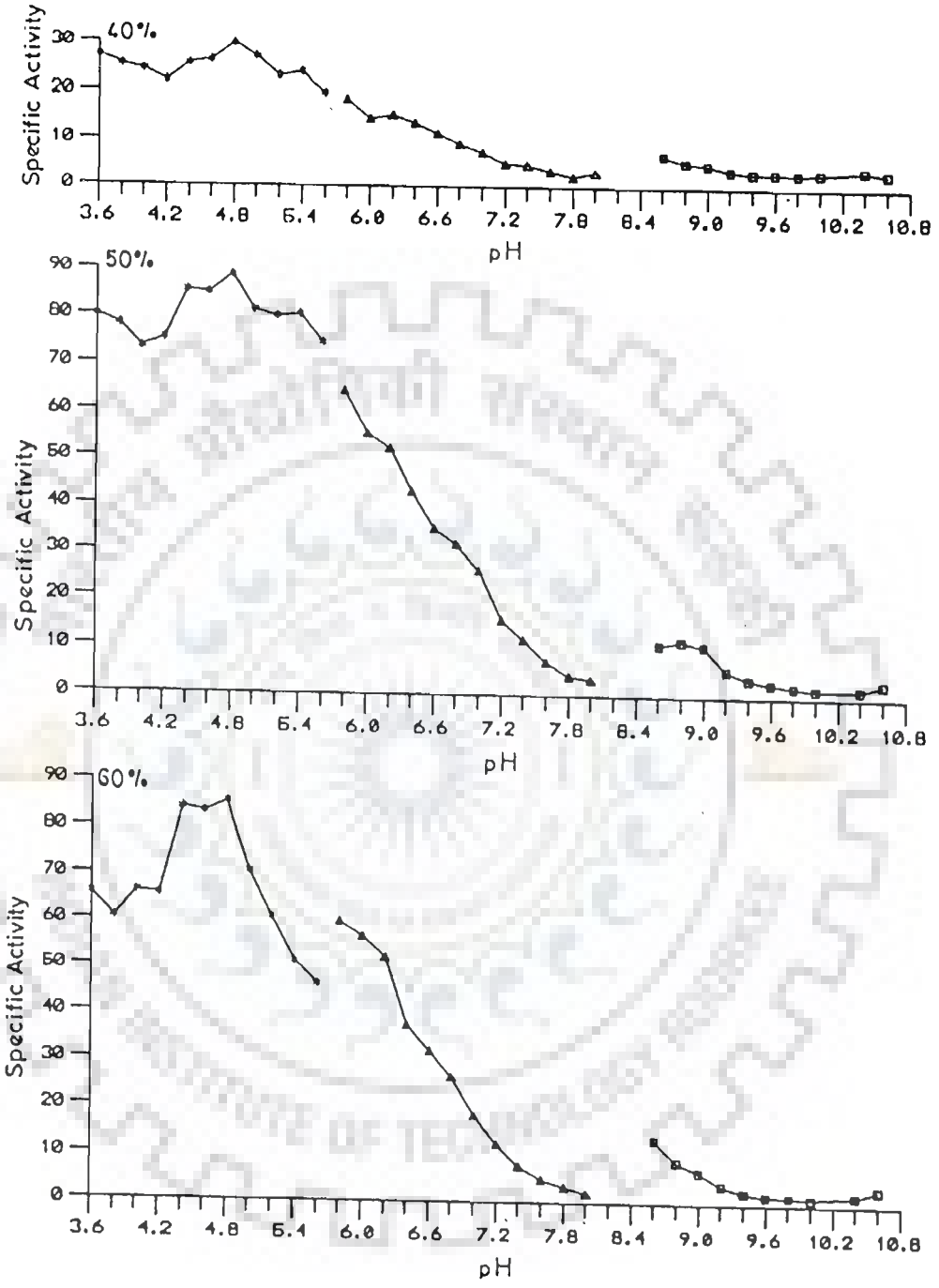


Fig. 15. pH Dependence of the Semipurified Preparation of ß-Galactosidase from the Epididymis of Goat. Equal aliquots of the resuspended preciptates obtained after (a) 40%, (b) 50% and (c) 60% ammonium sulfate salt precipitation were incubated at 37°C with 1.25 mM p-nitrophenyl ß-D-galactopyranoside in (-*-) 0.1M sodium acetate buffer, pH 3.6-5.6, (-Δ-) 0.1M phosphate buffer, pH 5.8 - 8.0 and (-□-) 0.1M glycine-NaOH buffer pH 8.6 - 10.6 for estimation of enzyme activity. Specific activity is expressed as enzyme units per mg protein estimated in the same precipitates.

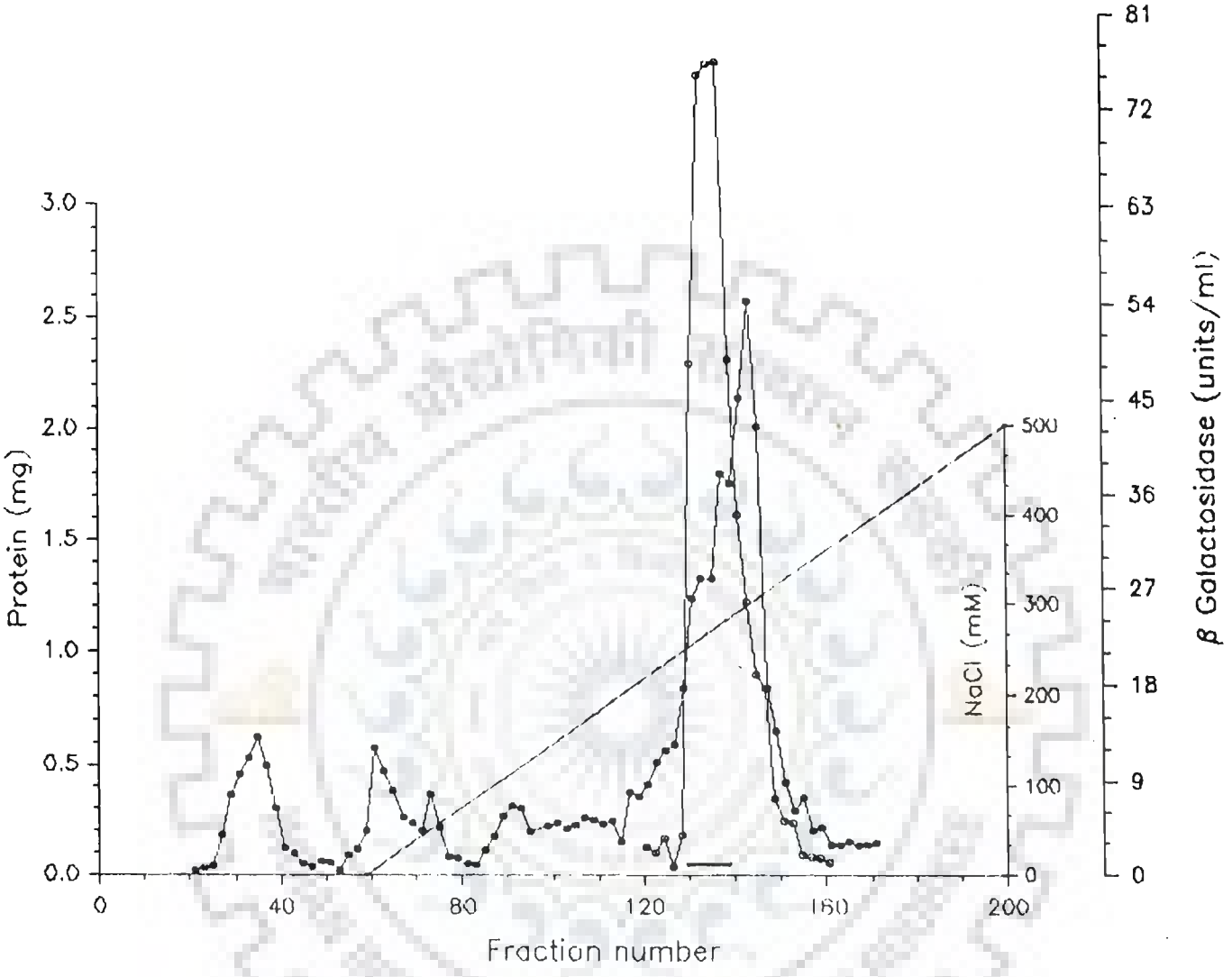


Fig. 16. Elution Profile of ß-Galactosidase Rich Supernatant Obtained after Dialysis on CM-Cellulose Column. 19.5ml enzyme solution (119.4mg protein) was applied on a CM-cellulose column (1.2 x 20cm) and eluted with a continuous linear gradient of 70 ml of 0.01M sodium acetate buffer pH 4.8 and 70ml of 0.01M sodium acetate buffer containing 0.5M NaCl. (-●-) protein; (—o—) ß-galactosidase; (----) NaCl gradient and (——) fractions pooled.

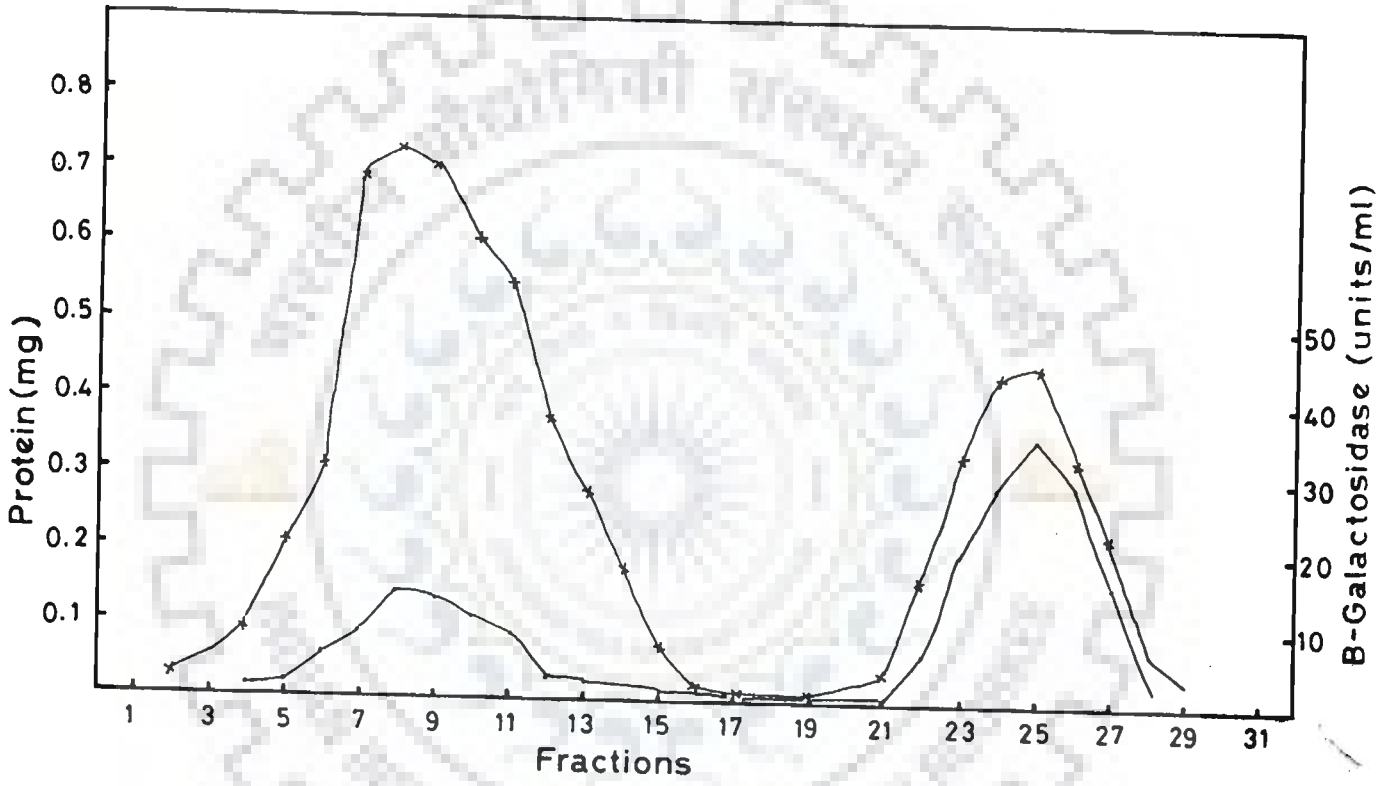


Fig. 17. **Concanavalin A Sepharose 4B Affinity Chromatography of Pooled Fractions From CM-Cellulose Column.** The pooled fractions from CM-cellulose (9.44mg protein) were applied on a Con A-Sepharose column (1 x 5cm). Elution was done using the equilibrating buffer containing 1M NaCl and 0.5M methyl α-D-mannopyranoside. (-*-) protein and (-·-) enzyme activity.

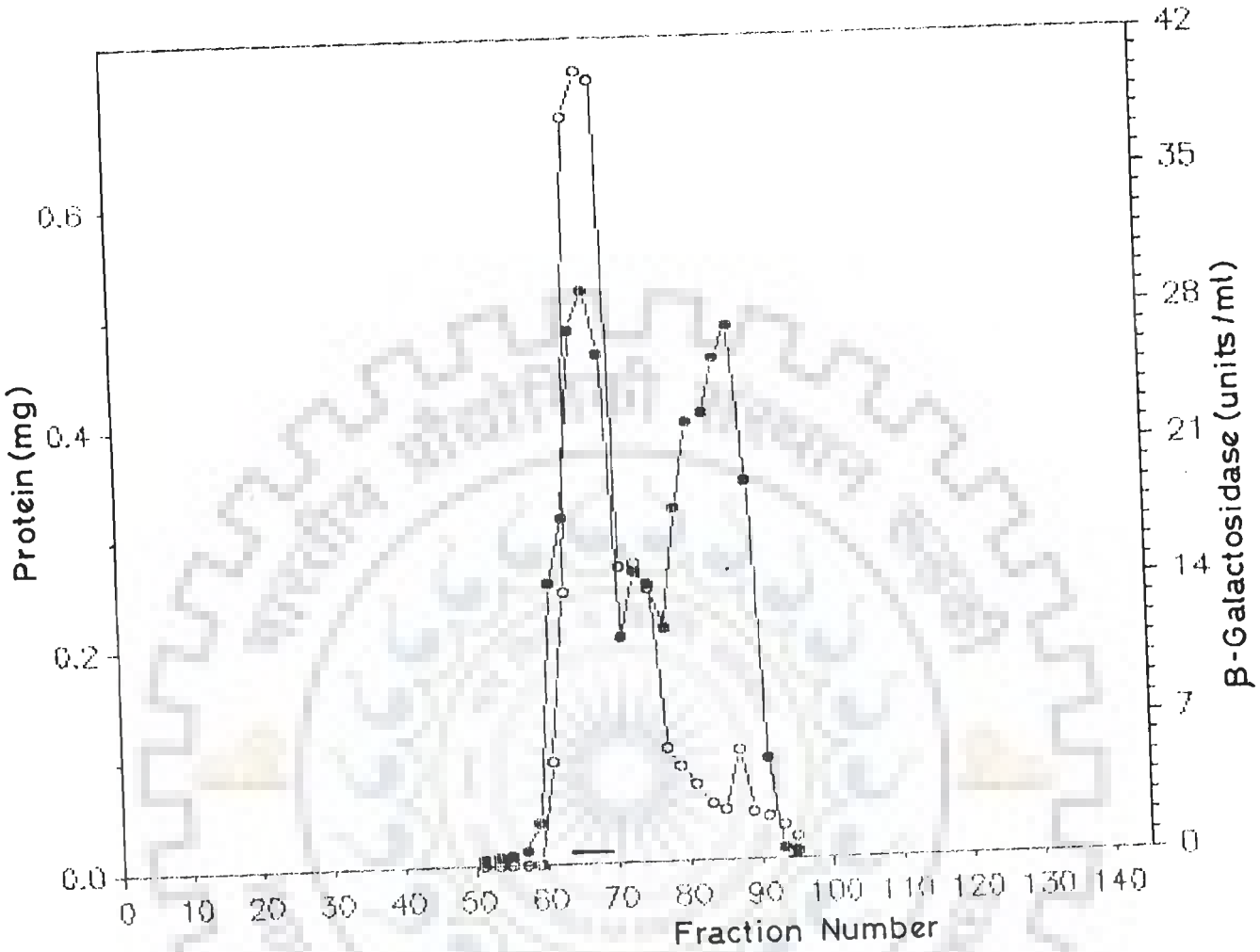
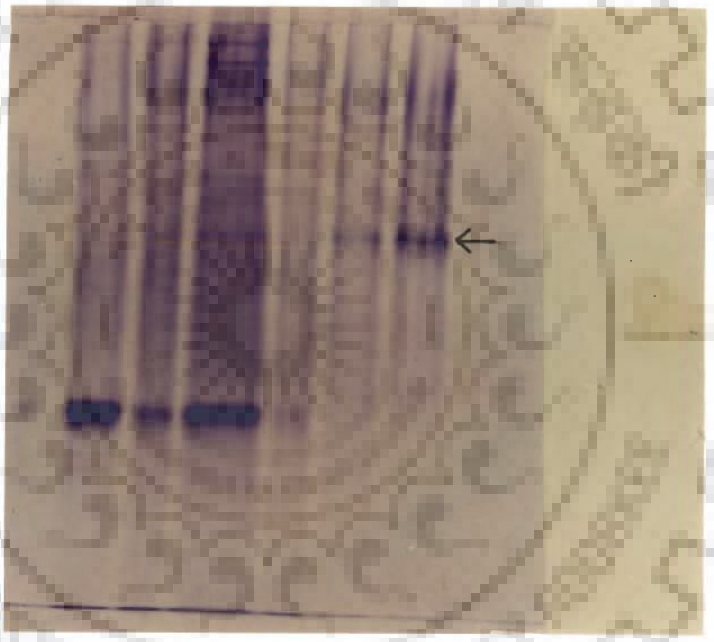


Fig. 18. Elution Profile of Pooled Fractions obtained after CM-Cellulose Chromatography on Seralose 6B. The pooled fractions(129-139) were dialysed against 0.05M sodium acetate buffer pH 4.8 containing 0.3M NaCl, concentrated and applied (9.44mg protein) on Seralose 6B column (1.4 x 80cm). The column was eluted with the same buffer. Fractions of 1ml/6min were collected and monitored for (– ● –) protein and (– o –) ß-galactosidase activity. (——) indicates fractions pooled for Preparative Electrophoresis.

Fig. 19. Ten Percent SDS-PAGE of Goat Epididymal ß-Galactosidase Following Various Purification Steps. SDS-PAGE was carried out on 10% gel under non-reducing condition. Lane 1, 100µg crude epididymal extract; Lane 2, 100µg acid precipitation fraction; Lane 3, 100µg dialysed supernatant following salt precipitation; Lane 4, 20µg precipitate after dialysis (discarded fraction) Lane 5 and 6, 20 and 40µg purified ß-galactosidase respectively, after preparative PAGE.


1 2 3 4 5 6

. 20. **Disc Gel Electrophoresis of Purified ß-Galactosidase on Cathodic Discontinuous Polyacrylamide Gel.** 50µg of the purified ß-galactosidase preparation obtained after preparative electrophoresis was applied on 5% rod gels. **Gel A:** stained with Coomassie Brilliant Blue R-250 **Gel B:** stained with 6-bromo-2-napthyl ß-D-galactopyranoside.

TABLE 3

PURIFICATION OF ß-GALACTOSIDASE FROM THE GOAT EPIDIDYMIS

	Volume (ml)	Total Protein (mg)	Enzyme Units	Specific Activity	Fold	Percent Recovery
Crude Extract	158.0	3157.5	4133.57	1.309	1.00	100
Acid precipitation Supernatant	300.0	593.4	2500.23	4.213	3.21	60.48
40-60% Ammonium sulfate salt precipitation	15.0	218.55	1858.50	8.503	6.49	44.96
Dialysed Supernatant	119.5	119.41	1191.84	9.981	7.62	28.83
CM - cellulose chromatography	7.5	9.44	684.57	72.518	55.39	16.56
Gel filtration	4.8	3.58	373.33	104.282	79.65	9.03
Preparative Electrophoresis	1.5	1.2	215.64	179.660	137.23	5.23

homogeneous. When an identically run gel was stained with 6-bromo-2-napthyl-ß-D-galactopyranoside, a specific substrate for ß-galactosidase, no activity was observed.

The purified preparation subjected to Cathodic discontinuous PAGE on tube gels gave a single band when stained for protein with Coomassie brilliant blue. A gel run under identical conditions stained with 6-bromo-2-napthyl-ß-D-galactopyranoside, to specifically identify ß-galactosidase activity also gave a single band at exactly the same location (Fig. 20).

The enzyme preparation obtained after preparative electrophoresis was pure enough to generate a polyspecific antisera to ß-galactosidase in rabbits. The double immunodiffusion pattern by Ouchterlony's procedure is presented in Figs. 21a,b. The gels were stained with Coomassie brilliant blue to enhance the resolution for photographic purposes. Clear precipitin lines are seen at both the dilution of the rabbit antisera tested. When an identical immunodiffusion was carried out and stained for enzyme activity with 6-bromo-2-naphthyl-ß-D galactopyranoside instead of staining with Coomassie brilliant blue, colour development was seen once again at the same site of the precipitin line. The positive reaction is visible prominently only when high concentrations of the antisera were loaded into the well.

4.4 DISCUSSION

In the present studies purification of ß-galactosidase from goat epididymis has been carried out. The final purified enzyme was electrophoretically homogeneous as seen on SDS-PAGE. Earlier

ß-galactosidase has been purified from a number of microbial, plant and animal sources (Gatt and Baker, 1970; Cheetham and Dance, 1976; Miyagawa, 1979; Cecilia et al., 1991) and has been shown to exist as monomeric as well as multimeric forms depending upon the sources. Among reproductive tissues, it is known that epididymis is the richest source of all glycosidases including ß-galactosidase. Many a times it has been proposed that this could be a good source for enzyme preparation (Conchie and Hay, 1959). But most purification procedures have used testis as the enzyme source (Distler and Jourdian, 1978; Yamamoto and Nishimura, 1987). The larger size of the testis may perhaps be one reason for preferring the testis to the epididymis. But when testis has been used as a source it has often been found that ß-galactosidase is co-purified with other enzymes. (Verheijen et al., 1982; Yamamoto and Nishimura, 1987). In the present study an attempt has been made to obtain a homogeneous preparation of ß-galactosidase from goat epdidymis. Previous reports and experience at this laboratary have shown that ß-galactosidase is a highly stable enzyme irrespective of the source (Conchie and Hay, 1959; Yamamoto and Nishimura, 1987, 1991). Therefore extraction was done in distilled water followed by selective elimination of a large number of contaminating proteins by acid precipitation. Recently this technique has been adopted as a routine procedure in the preliminary purification of ß-galactosidase from testis (Yamamoto and Nishimura, 1987; Nikolajczk and O'Rand, 1992). The activity of epididymal ß-galactosidase in the present study was found to be unaffected by exposure to acidic pH but it must be pointed out that at times

a sudden change in pH results in substantial loss of enzyme activity. Therefore, caution has to be exercised by changing the pH gradually.

In many enzyme purification procedures ammonium sulfate precipitation invariably finds a place. Previous studies have shown that ß-galactosidase is stable in the presence of ammonium sulfate and can be stored in its presence without loss in activity for long periods (Conchie and Hay, 1959). The results obtained in the present study also indicate that epididymal ß-galactosidase could be reasonably purified by this procedure. Infact the enzyme was found to largely precipitate at 40, 50 and 60 percent salt concentration. Earlier workers have also obtained similar results with testicular ß-galactosidase. While the work of Caygill et al. (1966) shows precipitation of the enzyme at 40% salt concentration with 63% enzyme activity, Yamamoto and Nishimura (1987) have obtained 80% recovery of enzyme at the 60% salt concentration.

An interesting feature of goat epididymal ß-galactosidase is that it is active only at acidic pH. The pH optima at which peak in enzyme activity was observed in the present study exactly coincided with those reported by Caygill et al. (1966) for ram testicular ß-galactosidase who used the same sodium acetate buffer system. Other investigators have also reported similar results but the pH optima are slightly different (Conchie and Hay, 1959; Distler and Jourdian, 1978; Nikolajczk and O'Rand, 1992). The difference in values may be due to differences in the

buffer systems (Caygill et al., 1966; Conchie and Hay, 1959), assay methods (Conchie and Hay, 1959), and species of animals used (Distler and Jourdian, 1978; Nikolajczk and O'Rand, 1992).

In non-reproductive tissues ß-galactosidase is known to occur as acidic and neutral form (Bullock and Winchester, 1973; Chinchetru et al., 1983). In reproductive tissues other glycosidases also exhibit such polymorphism (Snaith, 1977; Dutta and Majumder, 1984). Sometimes it has been reported that in crude extracts, acidic and neutral forms cannot be identified but can be detected only after further steps of purification (Snaith, 1977).

In the present study, however, only acidic ß-galactosidase could be detected in both crude and partially purified epididymal tissue extract.

It may be argued that since enzyme activity was observed at near neutral pH, neutral forms may exist. But it is unlikely that this is the case since prolonged exposure of both crude and partially purified preparations to neutral pH resulted in substantial lowering of enzyme activity. However when the samples exposed to neutral pH were acidified by addition of acetic acid or by dialysis against sodium acetate buffer pH 4.8, the enzyme activity was restored. It seems possible that the slight activity observed at near neutral pH may represent the tailing end of the activity of the acidic form of enzyme.

Dialysis of the pooled 40-60% salt precipitate fraction against a buffer of pH 4.8 resulted in precipitation of few other contaminating proteins. The precipitation of proteins at their

isoelectric point has been known for a long time. In addition, some investigators have also suggested that protein bound to mucosubstances are released and precipitate (Lusis et al., 1977). Thus precipitation of proteins at their isoelectric point and release of proteins from mucosubstances may be the cause for the precipitate obtained. The proteinaceous nature of the precipitate could be confirmed since removal of the precipitate following dialysis increased the specific activity of the enzyme.

The enzyme thus purified was subjected to ion exchange chromatography. The use of anion exchange resins was avoided since they need to be used at higher pH and test studies conducted in this laboratory revealed that frequent changes in pH of the system leads to substantial loss in enzyme activity. Thus cation exchange resins like SP-Sephadex C-50, CM-cellulose and Seralite WRC were tried for the purification of goat epididymal ß-galactosidase. Among these resins, CM-cellulose proved to be most efficient both in terms of the purification achieved and the recovery of enzyme. On previous occasions ß-galactosidase has been successfully purified using the same resin and eluting with pH gradient or salt gradients (Caygill et al., 1966; Watanabe et al., 1979). The purification reported by these authors is substantially lower than that obtained in the present study. This may be due to the difference in the pretreatment of samples prior to CM-cellulose chromatography. It may be relevant to mention that while Caygill et al., (1966) used a pH gradient for elution, in the present study a salt gradient has been used. The variation in the extent of purification could thus be attributed

to the use of pH or salt gradient for elution from the column.

It is well known that ß-galactosidase is a glycoprotein enzyme and its ability to bind lectins has often been exploited for purification schemes. Concanavalin-A is one such lectin that has been widely used for the purification of ß - galactosidase from several sources (Verheijen et al., 1982; Rodriguez-Berrocal, 1988; Chuang et al., 1991). Studies conducted at this laboratory using Concanavalin-A Sepharose 4B, showed that this affinity procedure was not useful since a loss in enzyme activity was observed. Inhibition of enzyme activity by methyl α-D-manno-pyranoside was ruled out since the removal of this synthetic sugar did not restore enzyme activity. Some investigators have changed the temperature of the column prior to elution with success, but a similar change in the temperature of the column did not prove useful in present studies. The precise reason for the loss in recovery and activity of ß-galactosidase from Concanavalin-A Sepharose is not known at the present time. This technique therefore was not used in the purification scheme.

Since the lectin affinity step was skipped, the pooled fractions after CM-cellulose chromatography were concentrated and subjected to gel filtration on Seralose-6B column. At the end of this procedure a 79 fold purification of ß-galactosidase was obtained over the crude extracts. In one study dealing with purification of ß-galactosidase from testis, gel filtration was found to be effective in separating ß-galactosidase from other glycosidases (Yamamoto and Nishimura, 1987). Since separation of molecules by

gel filtration is based on differences in molecular size, it is likely that the molecules whose sizes are greater than ß-galactosidase have been eliminated in this step.

A number of standard protocols have been used for studying ß-galactosidase electrophoretically in its native form (Chuang et al., 1991). The method of Davis, (1964) was found to give better resolution for carrying out native PAGE on preparative scale. Electroelution following preparative PAGE resulted in the recovery of ß-galactosidase which was found to be substantially purified. Preparative electrophoresis followed by electroelution has been used before to purify ß-galactosidase from a number of other sources. In the present study a 137 fold purification was achieved at the end of this procedure.

The enzyme obtained after preparative electrophoresis was apparently homogeneous since a single band was obtained when the preparation was run on a SDS-PAGE and stained with Coomassie Brilliant Blue R-250. But the enzyme activity could not be localized on the gels because of the denaturing conditions.

In order to stain identically run gels for both protein and enzyme activity it was necessary to use another system which was non-denaturing. Davis method (1964) was one option but here once again the gels stained for protein but not enzyme. This may be due to the use of Tris and a higher pH in this system which might have affected enzyme activity. Some workers could successfully localize the enzyme by preincubating the gels in acidic buffer before staining. Adoption of the same procedure in our

laboratory did not result in staining of the gel for the enzyme. An alternative procedure of electrophoresis, the Cathodic discontinuous PAGE was tried and the tube gels took up the stain for both protein and enzyme activity. Single bands were obtained in both cases confirming the homogeneity of the enzyme preparation.

The electrophoretically homogeneous preparation was injected into rabbits to produce polyspecific antisera. The polyspecific antisera was then reacted with purified enzyme on agarose gels by the diffusion method. It must be pointed out that antigen-antibody reaction proceeds on these gels to form precipitin lines. If multiple antigens were used in an immunization, then several antibodies could present in the antisera. Reaction of these multiple antigens and antisera would now result in multiple precipitin lines if tested by double immunodiffusion on gels. But in the present study a single precipitin line was obained showing immunologically also that the preparation was a homogeneous protein.

The next step was to prove that the homogeneous preparation was infact ß-galactosidase. The specific staining of the precipitin lines by 6-bromo-2-naphthyl ß-D-galactopyranoside provided the evidence to show that the antigen present at the precipitin line was ß-galactosidase. Another important fact that emerged from this experiment was that ß-galactosidase retains its activity following immunoprecipitation. Obviously the site at which ß-galactosidase binds to the antibody is different from that of its active site. In addition, it also follows that there is no

steric hinderance to enzyme activity by the antigen-antibody interaction.

4.5 CONCLUDING REMARKS

1. ß-Galactosidase has been purified from the epididymis of goat.

2. A 137 fold purification has been finally obtained following acid precipitation, salt precipitation, dialysis, CM-cellulose chromatography, gel filtration and preparative electrophoresis.

3. ß-Galactosidase was found to exist only in the acidic form.

4. The final purified product was found to be electrophoretically homogeneous by SDS-PAGE.

5. The homogeneous preparation was found to have ß-galactosidase activity in rod gels subjected to cathodic discontinuous PAGE.

6. The homogeneity was also confirmed immunologically in agarose gels by immunodiffusion techniques.

CHAPTER 3

Properties of purified β-galactosidase from the goat epididymis

5.1 INTRODUCTION

Due to the specialized nature of each tissue in an animal species it is not uncommon to see tissue specific differences in enzyme behaviour. The conditions prevalent in tissues are not always the same and so are the the factors that govern the activity of enzymes. To obtain a better understanding of enzyme behaviour in tissues it is essential to study the physico-chemical properties of an enzyme in its pure form. Many investigators in the past have attempted to do so and such studies have provided a lot of information based on which new physiological concepts have emerged.

ß-Galactosidase (EC 3.2.1.23) is known to specifically hydrolyze terminal galactose residues from glycoproteins, glycolipids (Aronson 1967; Rodger and Young, 1981; Swallow et al., 1984) and carbohydrates (Asp and Dahlquist, 1972). Much interest has been shown over the past several years to study its properties from a myriad of sources (Cheetham and Dance, 1976; Chao and Wells, 1978; Rodriguez-Berrocal, 1988). It has been widely seen that this enzyme exhibits tremendous variation in characteristics depending on the source (Ho and O'Brien, 1971; Suzuki and Kushida, 1973; Needleman and Koenig, 1974; Miyagawa, 1979; Cecilia et al., 1991). Besides, interesting features like aggregation and seggregation have been observed for ß-galactosidase within tissues (Lusis et al., 1977). Such types of molecular interactions are strictly dependent on pH and ionic makeup of the immediate surroundings to which the enzyme is exposed (Heyworth et al., 1981; Heyworth and Wynn, 1982, Hiraiwa

et al., 1986). As a result of this, investigators working on the enzyme from the same source have reported difference in molecular weight (Chytil, 1965, Cheetham and Dance, 1976, Yamamoto et al., 1982). At times, the occurrence of multiple forms of ß-galactosidase have also been demonstrated in some tissues (Furth and Robinson, 1965, Rodriguez-Berrocal et al., 1988; Martinez-Zorzano et al., 1989). Thus, it appears that physical and chemical properties of an enzyme are tailored to ensure that the biological functions in tissues are carried out smoothly and effectively.

It is known that ß-galactosidase is a glycoprotein and controls the processing of sperm surface glycoproteins in the epididymal duct. The precise conditions and regulatory mechanisms can only be understood if the properties of the enzyme are known in detail. In this chapter, are presented, some of the characteristics that have been determined for the enzyme, ß-galactosidase of epididymal origin.

5.2 MATERIALS AND METHODS

In the preceeding chapter the enzyme ß-galactosidase has been purified substantially and shown to be homogeneous by electrophoretic and immunological methods. This preparation of epididymal ß-galactosidase was used to study the properties of the enzyme.

5.2.1 Molecular Weight Determination

Molecular weight of the purified enzyme was determined at neutral

pH by gel filtration on Sephacryl S-200 and by discontinuous SDS PAGE under reducing conditions (Laemmli, 1970).

5.2.1.1 Gel Filtration on Sephacryl S-200

Buffer: 0.1M Citrate phosphate buffer (pH 7.0) containing 0.1M NaCl.

Solution A: 0.1M citric acid : 19.21 g citric acid in 1000ml distilled water.

Solution B: 0.2M dibasic sodium phosphate: 53.65g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000ml distilled water.

65ml of solution A and 436ml of solution B were mixed and 5.844g NaCl was dissolved. After adjusting the pH to 7.0 volume was made upto 1000ml in distilled water.

5.2.1.1.1 Column Packing and Equilibration

Swollen Sephacryl S-200 (Superfine:Sigma) was degassed and packed in a glass column (1.2 x 80 cm). The column was equilibrated for 6-8 hours with 0.1M citrate phosphate buffer pH 7.0 containing 0.1M NaCl. The void volume of the column was determined using Blue Dextran ($2x10^6$) (Sigma) at a concentration of 2mg/ml in equilibrating buffer.

5.2.1.1.2 Sample Application and Elution

The enzyme preparation obtained after preparative electrophoresis was dialysed overnight extensively against 2L of equilibrating buffer and concentrated to a volume of 1ml by lyophilization. This concentrated purified enzyme preparation was applied on the

column using a pasteur pipette. Once the sample got into the resin bed, the sides of the column were washed with 2ml equilibrating buffer and the column was connected to the reservoir containing equilibrating buffer. Flow rate of 6 ml/hr was maintained using a peristaltic pump (Pharmacia) and fractions of 1ml were collected in a fraction collector (SISCO, India). The optical density of the eluting fractions was recorded at 280 nm.

The column was calibrated using the Molecular weight standard kit supplied by Boehringer Mannheim Company, Germany (18,000 - 300,000 weight range). The Molecular weight markers used were cytochrome C (12,500), chymotrypsin (25,000), ovalbumin (45,000), albumin (68,000), aldolase (158,000), and ferritin (450,000). A calibration curve was prepared by plotting elution volume: void volume (Ve/Vo) ratio against log Molecular weight of known standards. Using the value Ve/Vo ratio obtained for ß-galactosidase on the same column, the molecular weight was read from the calibration curve.

5.2.1.2 Molecular Weight Determination by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis:

Molecular weight determination and subunit analysis was carried out by conducting SDS electrophoresis on slab gel containing 10% polyacrylamide gel following the procedure given by Laemmli (1970) as described earlier in Section 4.2.11.1.

The lyophilized purified preparation was resuspended in 0.0625M Tris-HCl (pH 6.8) and the prepared sample had a final

concentration of 2% SDS, 5% 2-mercaptoethanol and 10%, glycerol. The sample was boiled for 3 minutes, cooled to room temperature and loaded. Prior to sample application 10 μl of Bromophenol blue (0.002% in sample buffer) was added into the wells.

Standard molecular weight markers (Sigma) were processed in an identical manner and run simultaneously. The molecular weight markers used were in the weight range of 30,000 - 200,000 with carbonic anhydrase (29,000), ovalbumin (45,000), BSA (66,000), phosphorylase b (97,400), ß-galactosidase (116,000) and myosin (205,000).

Molecular weight of purified ß-galactosidase was determined from the calibration curve of molecular weight vs Relative mobility (R_f).

5.2.2 Studies on Stability

The pH stability, thermal stability and the storage stability of the purified ß-galactosidase were ascertained.

5.2.2.1 pH stability

Due to the acidic optimal pH exhibited by the crude and semipurified enzyme preparations the pH stability of the purified enzyme was determined in the acidic range only.

Reagents : All the reagents were prepared as described earlier (Section 3.2.4.2).

0.1M sodium acetate buffer pH 3.6-5.6.

1.25mM p-Nitrophenyl ß-D-galactopyranoside

0.2M Na_2CO_3

Procedure

10μg purified enzyme preparation was resuspended in distilled water and incubated at 37°C for 30 minutes in equal volumes of 0.1M sodium acetate buffer pH 3.6-5.6. To this, 0.6ml of 1.25mM p-Nitrophenyl ß-D-galactopyranoside, prepared in the respective pH buffer was added and further incubated for 30 minutes. The reaction was terminated by addition of 2ml of 0.2M Na_2CO_3. Absorbance was read spectrophotometrically at 400 nm. The percent activity was calculated at each pH value, taking the maximum activity exhibited as 100.

5.2.2.2 Thermal stability:

Reagents :

0.1M sodium acetate buffer pH 4.8

1.25mM p-Nitrophenyl ß-D-galactopyranoside

0.2M Na_2CO_3

Procedure

Aliquots containing 10 μg purified enzyme preparation in 0.2ml of 0.1M sodium acetate buffer pH 4.8 were incubated at various temperatures ranging from 35-60°C. The period of exposure to these temperatures were varied from 5-30 minutes. After the desired exposures at various temperatures, the tubes were cooled rapidly to 0°C by keeping them in ice so as to avoid further denaturation. The remaining activity for each incubation time was assessed by monitoring ß-galactosidase activity and expressed as percent residual activity taking the enzyme activity of the control containing unheated enzyme preparation as 100.

5.2.2.3 Storage Stability

Reagents

0.1 M sodium acetate buffer pH 4.8

1.25mM p-Nitrophenyl ß-D-galactopyranoside

0.2 M Na_2CO_3

Procedure: Storage stability of the purified ß-galactosidase was determined by storing 10 μg purified enzyme protein in 0.1M sodium acetate buffer pH 4.8. Separate vials containing enzymes were stored at 4°, 0° and -70°C. Initially, the enzyme activity was checked daily for each storage temperature till one week. Afterwards, it was monitored once in 72 hours upto a period of 3 months.

5.2.3 Substrate Specificity

Hydrolysis of various substrates by 10 units of the purified ß-galactosidase was carried out and compared with that of p-Nitrophenyl ß-D-galactopyranoside under identical assay conditions.

Reagents

All the substrates used in the present experiment were purchased from (Sigma Chemical Co). Stock solution of 5mM concentration for each substrate was prepared in 0.1M sodium acetate buffer pH 4.8.

p-Nitrophenyl ß-D-galactopyranoside : 15.065mg p-nitrophenyl ß-D-galactopyranoside was dissolved in 10ml buffer.

o-Nitrophenyl ß-D-galactopyranoside: 15.065mg o-nitrophenyl ß-D-galactopyranoside was dissolved in 10ml buffer.

p-Nitrophenyl ß-D-glucopyranoside : 15.065mg p-nitrophenyl ß-D-glucopyranoside was dissolved in 10ml buffer.

p-Nitrophenyl α-D-glucopyranoside : 15.065mg p-nitrophenyl α-D-glucopyranoside was dissolved in 10ml buffer.

p-Nitrophenyl α-D-galactopyranoside : 15.065mg p-nitrophenyl α-D-galactopyranoside was dissolved in 10ml buffer.

p-Nitrophenyl α-D-mannopyranoside: 15.065mg p-nitrophenyl α-D mannopyranoside was dissolved in 10ml buffer.

p-Nitrophenyl N-acetyl ß-D-glucosaminide: 171.115mg p-nitrophenyl N-acetyl ß-D-glucosaminide in 10ml buffer.

Procedure

0.2ml aliquots containing 10 μg purified ß-galactosidase in 100mM sodium acetate buffer pH 4.8 were preincubated at 37°C for 15 minutes. To these, 0.6ml of various substrate solutions were added separately and incubation was extended for 15 minutes. The reaction was terminated by the addition of 2ml of 0.2M Na_2CO_3. Absorbance of p-nitrophenyl released was read at 400 nm spectrophotometrically.

5.2.4 Kinetic Studies

The Michaelis constants (K_m) and maximal velocity (V_{max}) were determined using both o-nitrophenyl ß-D-galactopyranoside and

p-nitrophenyl ß-D-galactopyranoside as substrates as described by Lineweaver and Burk (1934).

5.2.4.1 Determination of K_m and V_{max} Using o-Nitrophenyl ß-D-Galactopyranoside as Substrate

Reagents

0.1M sodium acetate buffer pH 4.8

0.2M Na_2CO_3

o-Nitrophenyl ß-D-galactopyranoside solution : 40.17mg of o-nitrophenyl ß-D-galactopyranoside (Sigma) was dissolved in 10ml of 0.1M sodium acetate buffer pH 4.8 to give a final concentration of 13.33mM. Serial dilutions were then made using the same buffer to obtain final substrate concentration range of 0.625-10mM in the final assay volume.

Procedure

To 0.2ml aliquots containing 10 units of enzyme in 0.1M sodium acetate buffer pH 4.8, 0.6ml of variously diluted substrate solution was added to give a final substrate concentration range over 0.625-10mM. Incubation with substrate were carried at 37°C for 15 minutes and the reaction was terminated by the addition of 2ml of 0.2 M Na_2CO_3. Absorbance was read at 400 nm in a Beckman DU-6 spectrophotometer. The enzyme activity was calculated from the standard which was run simutaneously. K_m and V_{max} values were determined from the reciprocal plots 1/V Vs 1/[S] as described by Lineweaver and Burk (1934).

5.2.4.2 Determination of K_m and V_{max} Using p-Nitrophenyl ß-D-Galactopyranoside as Substrate

Reagents

0.1M sodium acetate buffer pH 4.8

0.2M Na_2CO_3

p-Nitrophenyl ß-D-galactopyranoside solution: 40.17mg p-nitrophenyl ß-D-galactopyranoside were dissolved in 10ml of 0.1M sodium acetate buffer pH 4.8 to get a stock of 13.33mM concentration. Serial dilutions were then made using the same buffer so as to obtain substrate concentrations ranging from 0.156 - 5mM in the assay mixture.

Procedure: 0.2ml aliquots containing 10 units of enzyme in 0.1M sodium acetate buffer pH 4.8 were incubated at 37°C with 0.6ml of the variously diluted substrate solution to give a final coancentration ranging from 0.156 - 5mM. Incubation was carried out for 15 minutes following which the reaction was stopped by addition of 2ml of 0.2M Na_2CO_3. Absorbance of the final product was read at 400 nm and activity was calculated. K_m and V_{max} values were calculated from the reciprocal plot 1/V Vs 1/[S] as described by Lineweaver and Burk (1934).

5.2.5 Inhibitor Studies

Determination of K_i: The inhibitor constant (K_i) was determined as described by Dixon (1953) using ϒ-D- galactonolactone, a competitive inhibitor of ß-galactosidase.

Reagents

0.1M sodium acetate buffer pH 4.8

0.2M Na_2CO_3

Y-D-galactonolactone: 71.24mg of Y -D-galactonolactone (Sigma) were dissolved in 10ml of 0.1M sodium acetate buffer pH 4.8 to obtain a final concentration of 40mM stock. Serial dilutions were carried out in the same buffer to give a final concentration ranging from 0.3125-5mM in the assay mixture.

p-Nitrophenyl ß-D-galactopyranoside: 40.17mg p-nitrophenyl ß-D-galactopyranoside was dissolved in 10ml of 0.1M sodium acetate buffer and serial dilutions were done using the same buffer to give a final concentration range of 0.625-5mM in the reaction mixture.

Procedure: To 0.2ml aliquots containing enzyme (10 units) and Y-D-galactonolactone over the concentrations ranging from 0.3125-5mM. 0.6ml the substrate solution was added. The effect of inhibition was determined at several substrate concentrations (final concentration of 0.625, 1.25, 2.5 and 5mM). Incubations were carried out at 37°C for 15 minutes. After terminating the reaction with 2ml of 0.2M Na_2CO_3, absorbance was read at 400 nm spectrophotometrically. The enzyme activity was calculated from the p-nitrophenol standard run simultaneously.

5.2.5.2 Inihibition by Tris and Sugars

During the present studies, the activity of ß-galactosidase was found to be inhibited in presence of Tris, D-galactose, methyl α-D-glucopyranoside and methyl α-D-mannopyranoside. Hence, the

precise effect of these compounds on purified ß-galactosidase was ascertained.

Reagents

0.1 M sodium acetate buffer pH 4.8

0.2M Na_2CO_3

Tris (Hydroxymethyl) aminoethane : 1.01g Tris (Boehringer Mannheim Co) was dissolved in 10ml sodium acetate buffer pH 4.8 to give a stock concentration of 800mM. Serial dilutions were made using the same buffer to obtain final concentration of 10, 25, 50 and 100mM in the assay mixture.

D-Galactose: 72.06mg D-galactose was dissolved in 10ml of 0.1M sodium acetate buffer pH 4.8 to give a stock concentration of 40mM. Serial dilutions were done using the same buffer to get a final concentration of 1.25, 2.5, and 5mM in the assay mixture.

Methyl α-D-glucopyranoside: 155.36mg methyl α-D-glucopyranoside (Sigma) were dissolved in 10ml of 0.1M sodium acetate buffer pH 4.8 to give a stock concentration of 80mM. Serial dilutions were made using the same bufer to achieve final concentrations of 2.5, 5 and 10mM in the reaction mixture.

Methyl α-D-mannopyranoside : 155.36mg of methyl α-D- manno-pyranoside (Sigma) was dissolved in 10ml of 0.1M sodium acetate buffer pH 4.8 to give a concentration of 80mM. From this stock, serial dilutions were done using the same buffer to give a final concentration of 2.5, 5.0 and 10mM in the assay volume.

Procedure : The effect of these compounds on the purified enzyme was assessed by estimating the change in enzyme activity following their additions during the assay. The assay mixture contained 10 units of enzyme, 1.25mM p-nitrophenyl ß-D-galactopyranoside and varying concentrations of inhibitors in 100mM sodium acetate buffer pH 4.8. Incubations were carried out at 37°C for 15 minutes following which the reaction was terminated by the addition of 2ml of 0.2M Na_2CO_3. A control was also included in the experiment to which no inhibitor was added but which was otherwise processed identically. The activity of this control was taken as 100 and accordingly the change in the enzyme activty following the addition of these compounds was assessed.

5.3 RESULTS

Fig. 22 presents the calibration curve obtained and used for determination of molecular weight of the purified ß-galactosidase following gel filtration on Sephacryl S-200. The enzyme eluted as a sharp peak showing a Ve/Vo ratio of 4.806 thereby indicating a molecular weight of approximately 64K. Following electrophoresis on 10% discontinuous SDS-PAGE under denaturing conditions on Laemmli system, ß-galactosidase migrated as a single band (Fig. 23). Comparison with appropriate standards run simultaneously indicate that the purified preparation had a molecular weight of 63K (Fig. 24).

The hydrolysis of p-Nitrophenyl ß-D-galactopyranoside by the purified ß-galactosidase in sodium acetate buffer at varying pH

is shown in Fig. 25. A broad peak of activity was obtained between pH 3.8 - 4.8 with slightly higher activity at pH 4.8. The enzyme activity declined sharply with rise in pH values above 5.0.

Purified epididymal ß-galactosidase was very stable at 35°C. At 40° and 45°C the enzyme was stable for 20 minutes beyond which there was a sudden drop in activity. However, at 50°C there was a 25% drop in the enzyme activity within 5 minutes and 50% drop after 10 minutes. The enzyme activity continued to be substantially inhibited, greater than 50% with further increase in incubation time. At temperatures of 55°and 60°C the inhibtion in enzyme activity was 70% and 90% respectively within 5 minutes (Fig. 26).

When the storage stability of ß-galactosidase was monitored, it was found that the enzyme could be stored at 4°, 0° and -70°C for three months without any significant loss in activity. Although it was observed that sudden thawing of the frozen samples resulted in a loss of enzyme activity which could amount to as much as 15%.

The rate of hydrolysis of various p-nitrophenyl substrates studied using purified epididymal ß-galactosidase is presented in Table 4. Higher rate of hydrolysis was observed for p-nitrophenyl ß-D-galactopyranoside as compared to o-nitrophenyl ß-D-galactopyranoside which was nearly 3.5 times more for p-nitrophenyl substrate. The enzyme was also able to hydrolyze p-nitrophenyl α-D-galactopyranoside and p-nitrophenyl ß-D-gluc-

osaminide but to a very limited extent. On the other hand, it was found that purified ß-galactosidase did not hydrolyze the p-nitrophenyl derivatives of α-and ß-D-glucopyranoside and α-D-mannopyranoside.

Fig. 27 presents the double reciprocal plot for the hydrolysis of the synthetic substrates o-nitrophenyl ß-D-galactopyranoside. The assay mixture contained varying concentrations of the substrate so as to provide partially to fully saturated conditions during the assay. The Michaelis constant (K_m) and the maximal velocity (V_{max}) were found to be 0.8mM and 0.32 μM/min/unit of enzyme respectively.

The double reciprocal plot 1/V versus 1/[S] for the hydrolysis of synthetic substrate p-nitrophenyl ß-D-galactopyranoside is presented in Fig. 28 . The Michaelis constant K_m and the maximal velocity (V_{max}) were found to be 0.102 mM and 1.084 μM/min/unit of enzyme respectively. Fig. 29 shows the Lineweaver-Burk plot 1/V versus 1/[S] both in absence and in presence of a fixed concentration of inhibitor γ-D-galactonolactone. The results obtained clearly indicate a competitive inhibition as the intercept of the plot of 1/V versus 1/[S] is the same in the presence and absence of inhibitor, although the slope is different. This reflects the fact that V_{max} is not altered.

Fig. 30 shows the same results plotted differently (1/V versus inhibitor concentration [I] at a fixed substrate concentration) as described by Dixon (1953). The rate of reaction varied with the concentration of substrate as is seen by the values obtained

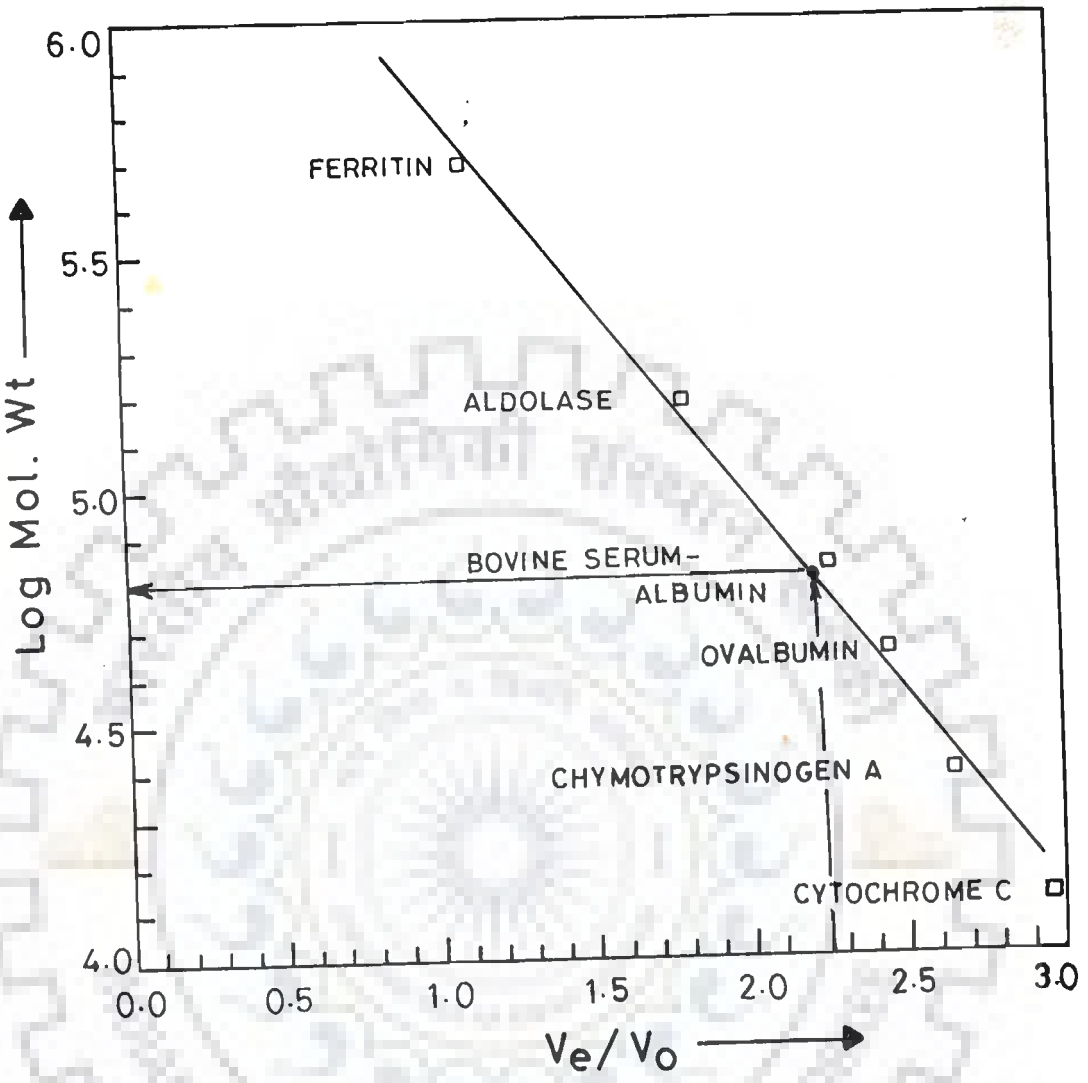
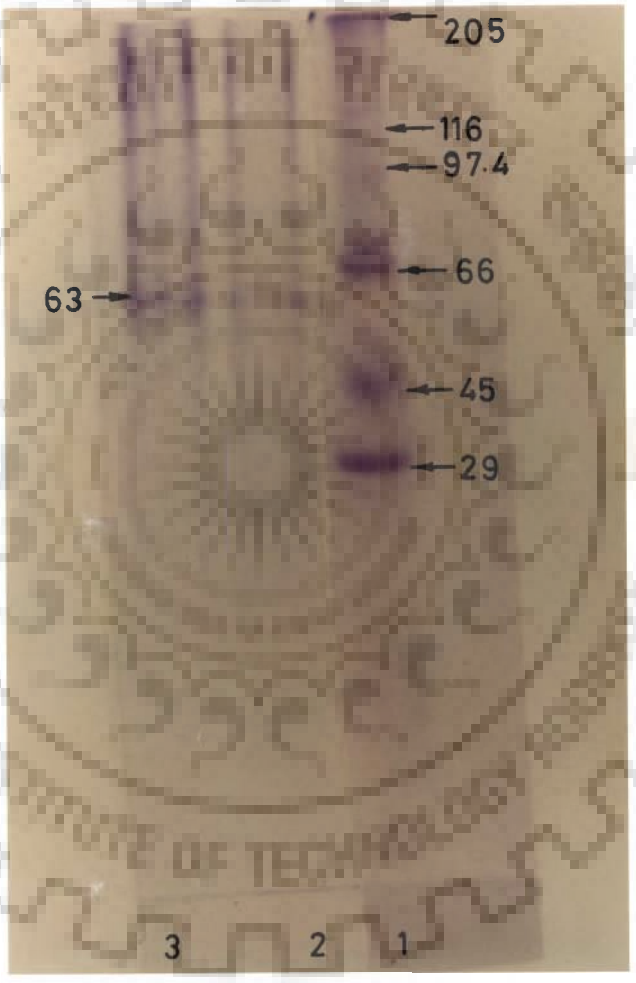


Fig. 22. Molecular Weight Determination of Goat Epididymal ß-Galactosidase by Sephacryl S-200 Chromatography. The void volume (V_o) was determined using Blue Dextran (2000K). The column was calibrated with ferritin (450K), aldolase (158K), bovine serum albumin (68K), ovalbumin (45K), chymotrypsinogen A (25K) and cytochrome C (125K). Ve/Vo ratio of the purified epididymal ß-galactosidase corresponds to log 4.806 thereby indicating an approximate molecular weight of 64K.

Fig. 23. SDS-PAGE of Purified ß-Galactosidase. SDS PAGE of completely reduced and denatured ß-galactosidase preparation obtained after preparative electrophoresis was performed on a 10% gel. Lane 1, Molecular weight markers; The standard protein and their molecular were as follows: myosin (205K), ß-galactosidase (116K) phosphorylase b (97.4K), BSA (66K), ovalbumin (45K) and carbonic anhydrase (29K); Lanes 2 & 3 10 and 20μg purified ß-galactosidase respectively.


205
116
97.4
66
63
45
29
3
2
1

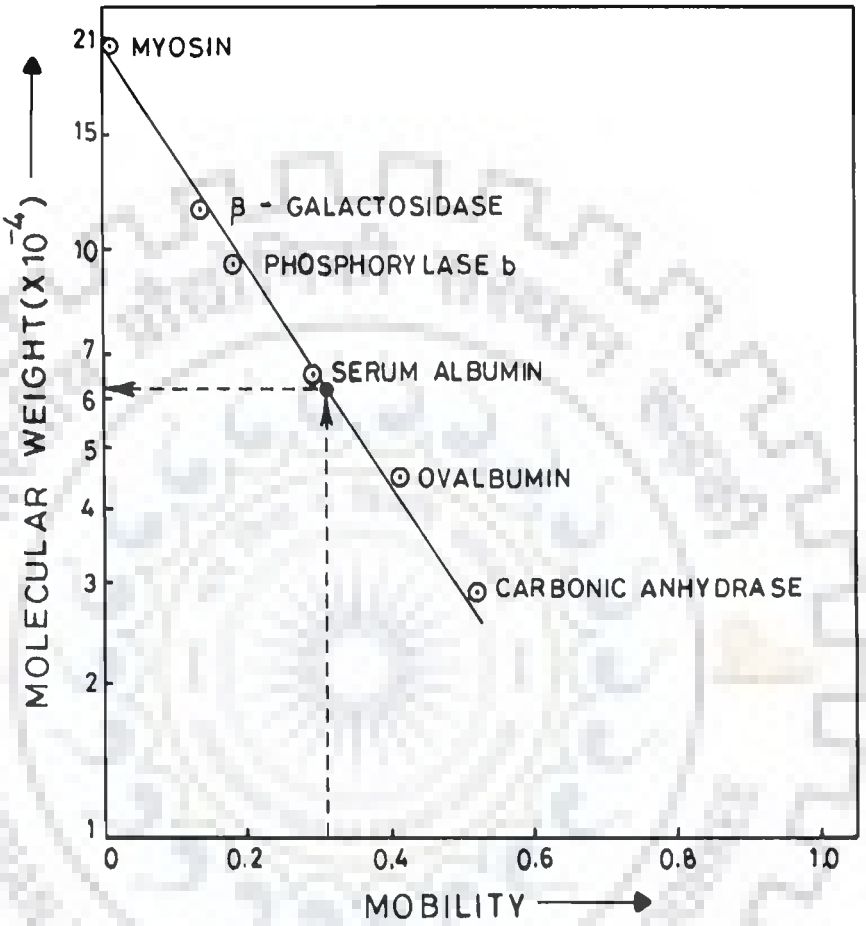


Fig. 24. Calibration Curve of Polypeptide Molecular Weight Versus Relative Mobility Following 10% SDS-PAGE Under Reducing Conditions. The molecular weight markers used were : myosin (205K), ß-galactosidase (116K), phosphorylase b (97.4K), bovine serum albumin (66K), ovalbumin (45K) and carbonic anhydrase (29K). Rf value of the purified ß-galactosidase corresponds to 0.315 thereby indicating an approximate molecular weight of 63K.

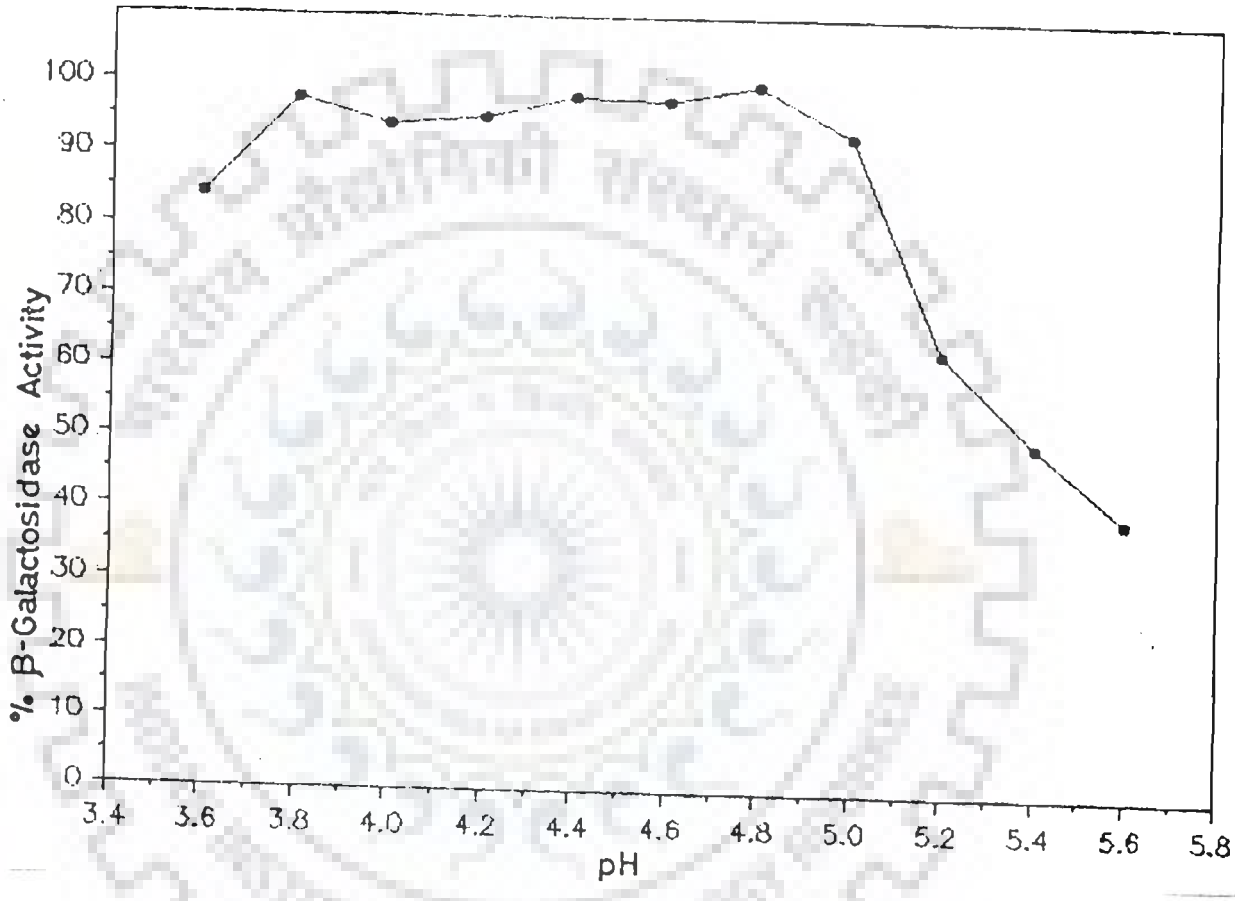


Fig. 25. Stability of Epididymal ß-Galactosidase at Different pH Values. The purified enzyme was preincubated with 0.1M sodium acetate buffer of pH 3.6-5.6 for 30 minutes. Further incubations with 1.25mM p-nitrophenyl ß-D-galactopyranoside prepared in the respective pH buffer were carried out for 15 minutes. Results are plotted as percentage of the maximum activity exhibited.

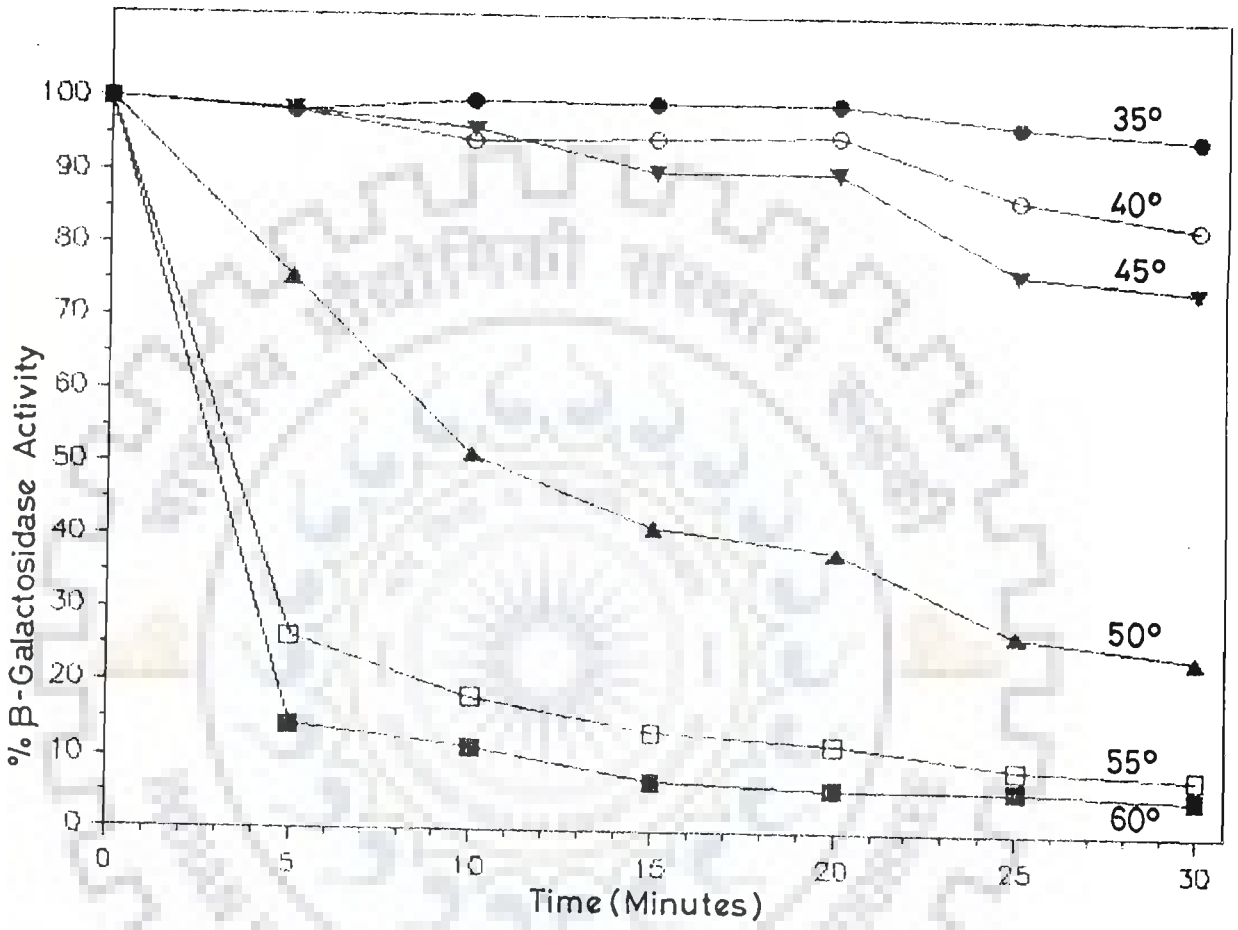


Fig. 26. Stability of Epididymal ß-Galactosidase at Different Temperatures. The purified enzyme preparation in 0.1M sodium acetate buffer was preheated at 35°, 40°,45°,50°, 55° and 60°C for varying periods of time upto 30 minutes. After each exposure, the enzyme preparations was cooled and assayed using the routine assay method. Results are plotted as percentage residual activity considering the activity of the unheated enzyme preparation as 100.

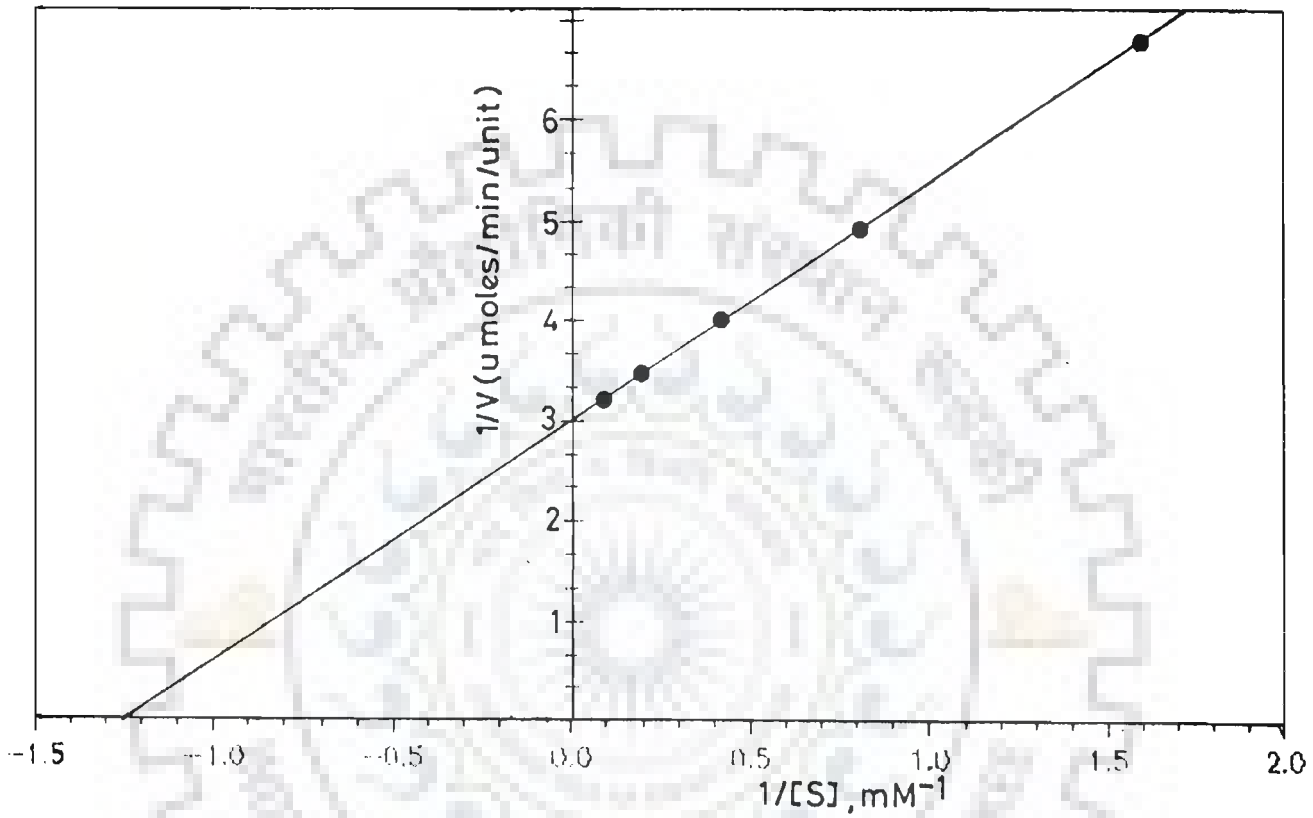


Fig. 27. Lineweaver-Burk Plot of Epididymal ß-Galactosidase using o-Nitrophenyl ß-D-Galactopyranoside as Substrate. The enzyme (10 units) was incubated for 15 minutes with varying concentrations (0.625-10mM) of o-nitrophenyl ß-D-galactopyranoside in sodium acetate buffer pH 4.8. Y intercept gives $1/V_{Max}$ value whereas the extrapolation of the line on X-axis gives $-1/K_m$ value.

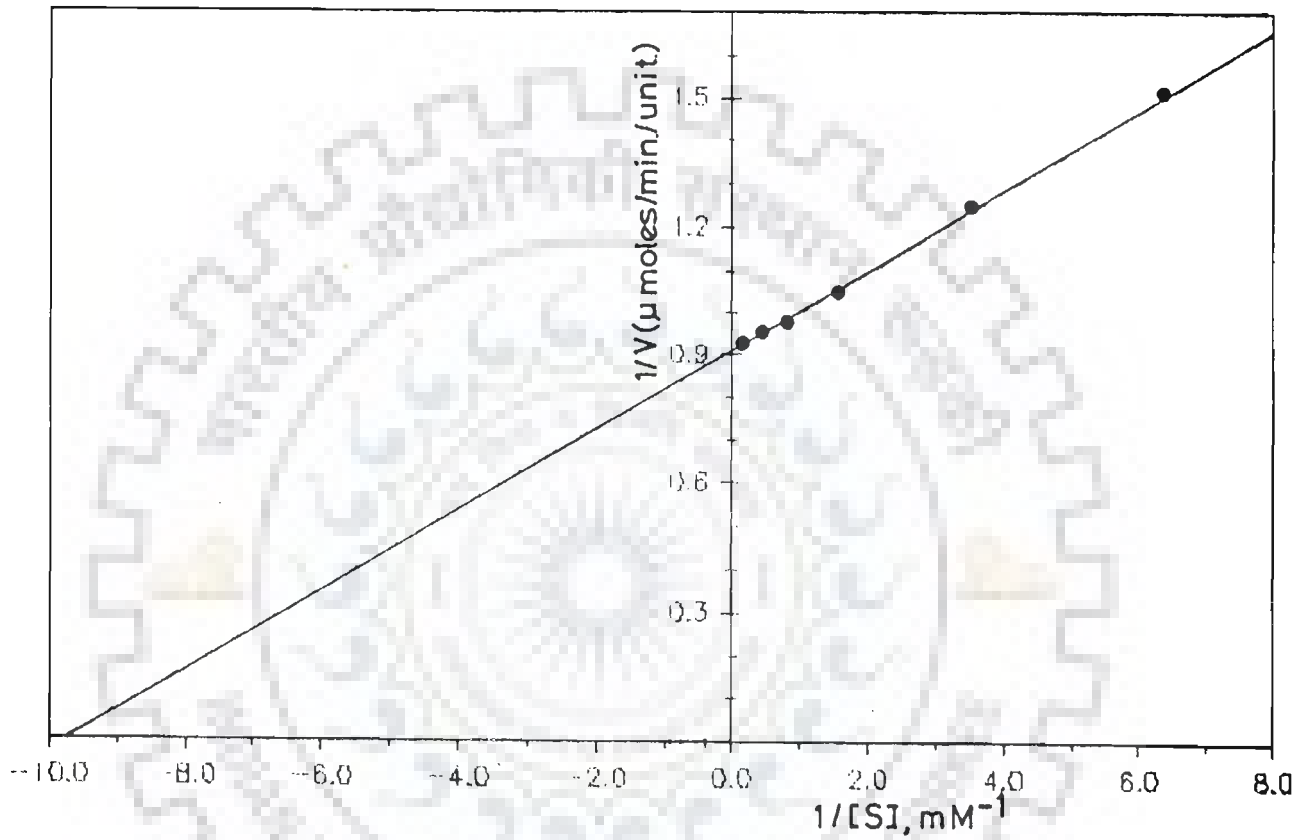


Fig. 28. Lineweaver-Burk Plot of Epididymal ß-Galactosidase using p-Nitrophenyl ß-D-Galactopyranoside as Substrate. The enzyme (10 units) was incubated at 37°C for 15 minutes with varying concentrations (0.156-5mM) of p-nitrophenyl ß-D-galactopyranoside in 0.1M sodium acetate buffer pH 4.8. Y intercept gives $1/V_{max}$ and the extrapolation of line on X-axis gives $-1/K_m$.

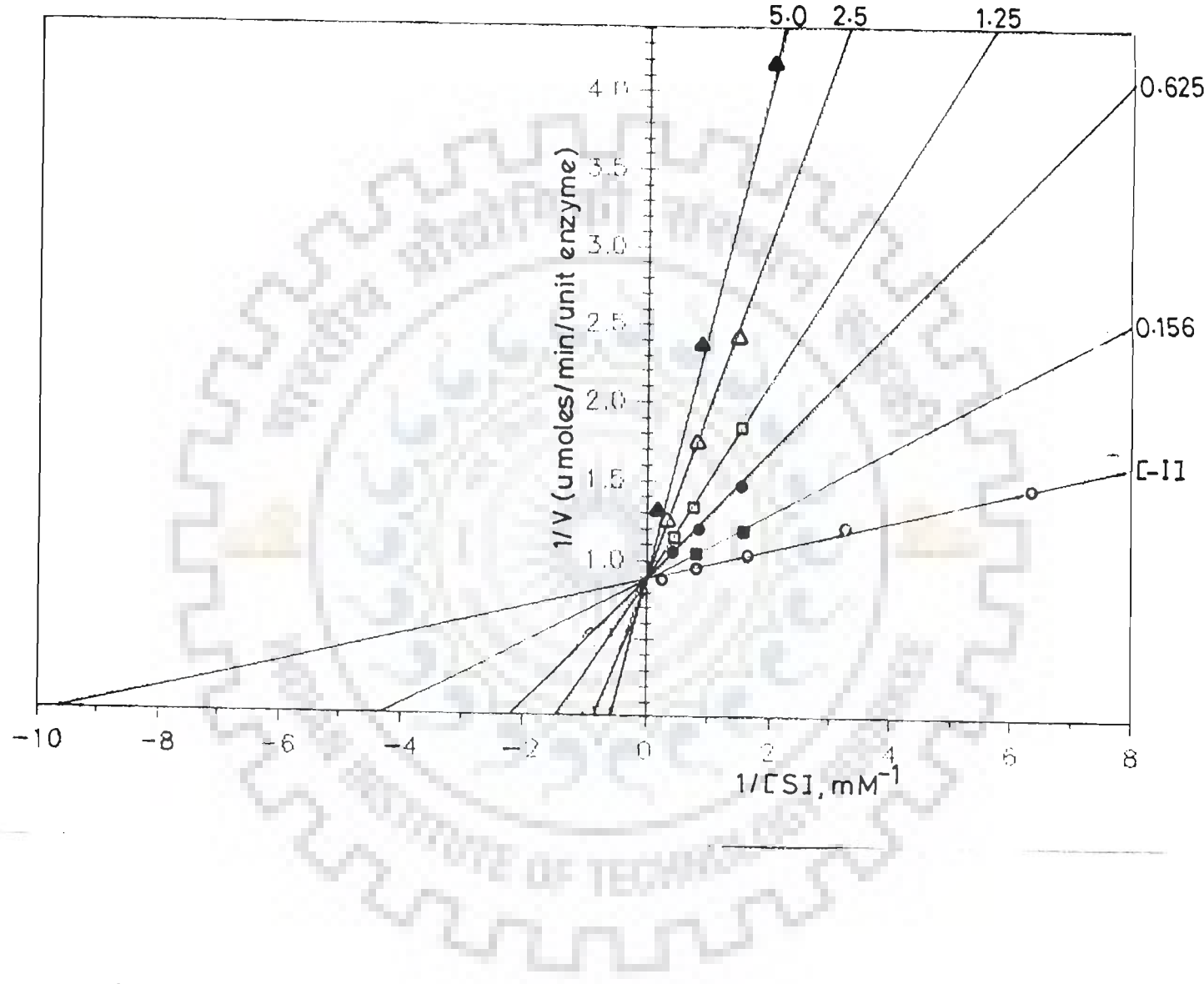


Fig. 29. Lineweaver-Burk Plot of Epididymal ß-Galactosidase showing Competitive Inhibition in the Presence of γ-D-galactonolactone. ß-Galactosidase activity was measured at fixed inhibitor concentration in the absence and presence of γ-D-galactonolactone (0.3125-5mM) at varying p-NP ß-D-galactopyranoside concentration.

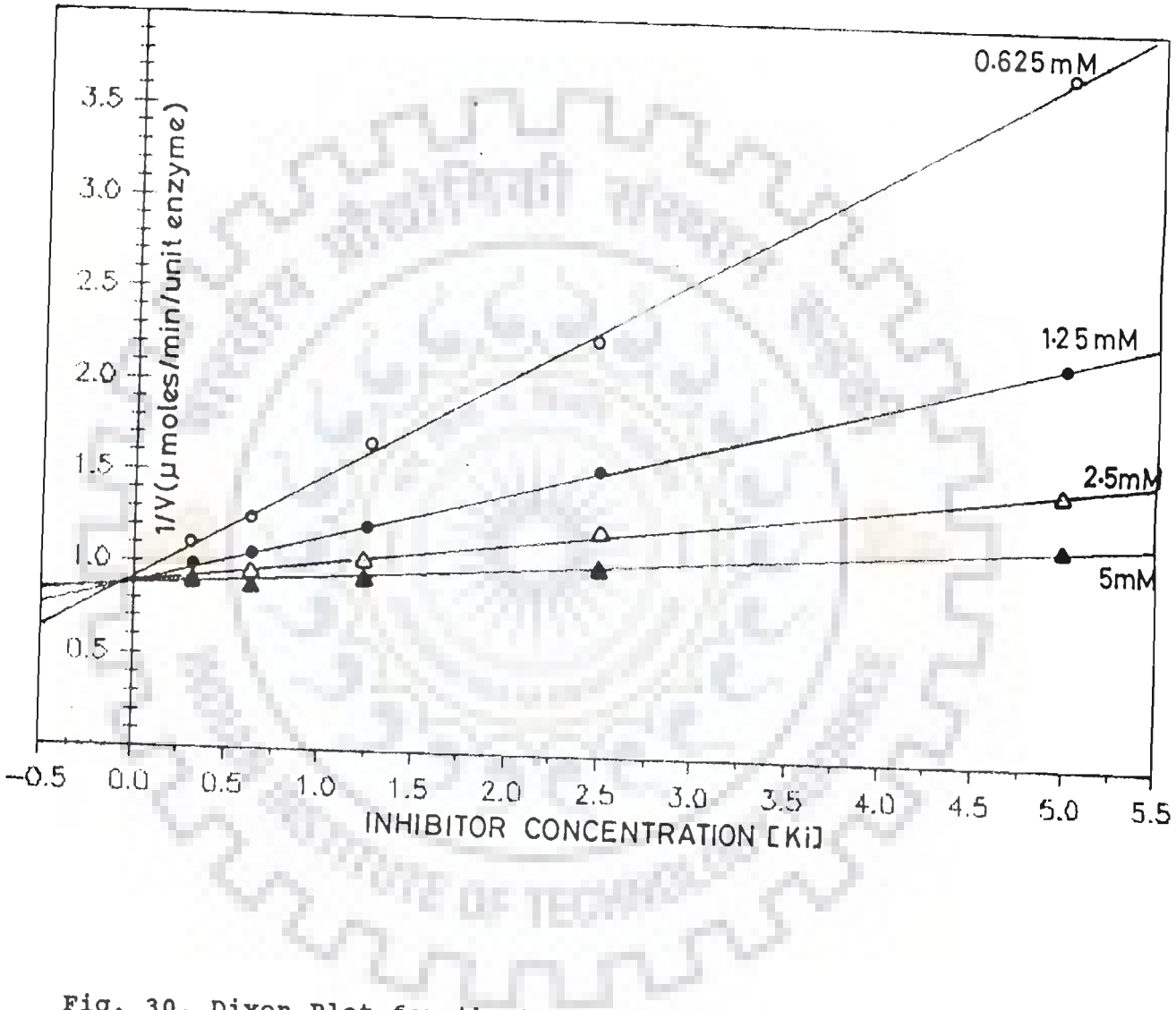


Fig. 30. Dixon Plot for the Determination of K_i using Y-D-Galactonolactone. ß-Galactosidase activity was measured at 5, 2.5,1.25 and 0.625mM p-NP ß-D-galactopyranoside concentration in the presence of varying concentration of Y-D-galactonolactone.

TABLE 4

HYDROLYSIS OF VARIOUS NITROPHENYL SUBSTRATES BY GOAT EPIDIDYMAL ß-GALACTOSIDASE :

Assays were performed in the pressence of 5mM concentration of each substrate.

Substrate	% Hydrolysis
p-Nitrophenyl ß-D-galactopyranoside	100.00
o-Nitrophenyl ß-D-galactopyranoside	28.96
p-Nitrophenyl α-D-galactopyranoside	2.97
p-Nitrophenyl N-acetyl ß-D-glucosaminide	3.25
p-Nitrophenyl α-D-mannopyranoside	0.00
p-Nitrophenyl α-D-glucopyranoside	0.00
p-Nitrophenyl ß-D-glucopyranoside	0.00

TABLE 5

EFFECT OF TRIS AND SOME SUGARS ON THE ACTIVITY OF GOAT EPIDIDYMAL ß-GALACTOSIDASE

Compound	Concentration (mM)	% Inhibition
Tris	100	98.14
	50	97.52
	25	90.25
	10	81.33
D-Galactose	5	50.73
	2.5	47.55
	1.25	28.42
α-Methyl D-Mannopyranoside	10	3.75
	5	2.08
	2.5	1.04
α-methyl D-Gluco-pyranoside	10	5.81
	5	4.15
	2.5	3.74

on Y-intercepts. Extrapolation of all these lines resulted in convergence at a single point. The value on the horizontal axis corresponding to this point gave the K_i value of 0.231mM.

The effect of Tris, D-galactose, methyl α-D-glucopyranoside and methyl α-D-mannopyranoside is presented in Table 5. The enzyme activity was inhibited in the presence of these compounds and the extent of inhibition varied depending on the concentration of these compounds in the reaction mixture. The extent of inhibition in enzyme activity brought about by Tris and D-galactose was very high. Very low concentration of Tris present in the assay could result in substantial inhibition of ß-galactosidase (81%). Similarly D-galactose caused more than 50% inhibition in enzyme activity at 5mM concentrations. The inhibition was further enhanced at higher concentration of both these inhibitors. Although the presence of two synthetic sugars methyl α-D-glucopyranoside and methyl α-D-mannopyranoside inhibited the enzyme activity, the degree of inhibition was relatively low (< 5%).

5.4 DISCUSSION

On a number of previous occasions the molecular weight of ß-galactosidase obtained from a variety of sources has been determined (Distler and Jourdian, 1978; Holmes and O'Brien, 1979; Yamamoto and Nishimura, 1987; Chuang et al., 1991). In the present study, the molecular weight of purified epididymal ß-galactosidase was found to be 63K by SDS-PAGE and 64K by gel filtration. This small variation could be attributed to the differences in techniques employed in the determination of

molecular weight. In most tissues associated with the male reproductive tract it has been repeatedly shown that the catalytic activity of ß-galactosidase resides in a protein whose molecular weight ranges between 63-68 K (Distler and Jourdian, 1978; Verheijen et al., 1982; Yamamoto and Nishimura, 1987). In one recent study, antisera to bovine ß-galactosidase was shown to recognise several proteins in rabbit testis homogenates (64K 58K, 89K and 98K). The authors concluded that several isoforms of ß-galactosidase exist in the testis (Nikolajczk and O'Rand, 1992). The significance of these results could be gathered from the pulse-chase experiments carried out by Skudlarek and Orgebin-Crist (1986), who have amply demonstrated that the cultured principal cells from rat epididymis synthesize and secrete high molecular weight precursors of the enzyme ß-galactosidase (84K, 87K, 90K and 100K). These precursors were reported to be activated when they were processed to the mature enzyme (Mr = 63K). There appears to be a similarity in the molecular weight of this mature enzyme from rat epididymal cells and the molecular weight of ß-galactosidase purified from the goat epididymis. Thus, it is likely that the ß-galactosidase purified in the present study from goat epididymis represents the mature form of the enzyme.

It must be mentioned that in the present study SDS-PAGE was carried out under reducing conditions and the purified preparation of ß-galactosidase migrated as a single band. Therefore, it seems highly unlikely that this homogeneous preparation was made up of multiple subunits.

A phenomenon of aggregation of ß-galactosidase at acidic pH and disaggregation of the same at near neutral pH has been reported earlier (Yamamoto and Nishimura, 1982; Hiraiwa et al., 1986). The authenticity of these findings could not be confirmed in the present studies since both SDS-PAGE and gel filtration were carried out at higher pH. However, it is worth mentioning that if such a phenomenon was true, then the molecular weight of 63-64K obtained represents the value of the disaggregated monomer.

Tests conducted on the preparation of ß-galactosidase purified from the goat epididymis showed that the enzyme is very stable at acidic pH. This is understandably so since ß-galactosidase is considered to be a lysosomal enzyme which are generally active at acidic pH. The stability of ß-galactosidase at acidic pH has also been reported in other male reproductive tissues (Conchie and Hay, 1959; Caygill et al., 1966; Distler and Jourdian, 1978; Yamamoto and Nishimura, 1987) showing that the goat epididymal enzyme is not different as far as this trait is concerned. The activity of enzyme was stable after prolonged exposure to temperatures of upto 35°C. However, with temperatures of 40° and 45°C the stability lasted only for about 20 minutes. Exposure to higher temperatures arrested the enzyme activity within 5 minutes. Thus, the results show that the enzymatic reaction declines rapidly since heat denatures the enzyme and the rate of denaturation is known to increase with increasing temperature (Creighton, 1990). Similar findings have also been reported for

ß-galactosidase from other sources (Conchie and Hay, 1959; Watanabe et al., 1979). Low temperatures of 4°, 0°and -70°C were suitable for storage of the enzyme preparation since no loss in activity was observed for periods of upto three months.

The epididymal enzyme preparation vigorously reacted only with substrates of ß-galactosidase. The specificity of the enzyme is clearly established from the inability of the enzyme preparation to hydrolyze the substrates of other glycosidases. The only substrates that reacted were p-nitrophenyl α-D-galactopyranoside and p-nitrophenyl-N-acetyl ß-D-glucosaminide. This interaction could be considered as non-specific, since less than 4% of substrates were hydrolyzed as compared to p-nitrophenyl ß-D-galactopyranoside. It is relevant to mention here that previous investigators have often ended up in co-purification of ß-galactosidase with other glycosidases (Verheijen et al., 1982; Yamamoto and Nishimura, 1987). But in the present study such a co-purification is ruled out since the extent of interaction with the substrates of other glycosidases, if any, was negligible. In addition, as shown in the previous chapter the enzyme preparation was homogeneous both electrophoretically and immunologically.

The kinetic studies using the p-nitrophenyl and o-nitrophenyl derivatives of ß-D-galactopyranoside as substrates have revealed vital characteristics of the purified ß-galactosidase preparation. It is generally known that K_m is an index of the affinity of enzyme for substrate and the V_{max} gives information on the turnover number. Based on these two parametrs it may be

concluded that p-nitrophenyl ß-D-galactopyranoside is a much better substrate than o-nitrophenyl ß-D-galactopyranoside as far as the purified preparation of ß-galactosidase from goat epididymis is concerned. In one previous study (Distler and Jourdian, 1978; Yamamoto and Nishimura, 1987) p-nitrophenyl ß-D-galactopyranoside was used as substrate to determine the K_m and V_{max} of testicular ß-galactosidase. Their results are in good agreement with the values obtained in the present study suggesting that epididymal and testicular ß-galactosidase may have similar kinetic properties (Distler and Jourdian, 1978).

In general most glycosidases are known to be competitively inhibited by aldonolactones. A review has indicated that the K_i values range between 3 - 50 μm (Lalegerie et al., 1982). But at times higher values of K_i have also been reported (Conchie and Hay, 1959). In the present studies γ-D-galactonolactone has been shown to be a competitive inhibitor for ß-galactosidase when p-nitrophenyl ß-D-galactopyanoside was used as the substrate. The competitive inhibition is due to the isosteric structure of the pyranose ring in both these compounds. The value of K_i was also found to be on the higher side (0.231mM). The differences between the K_i values observed in the present study and others reported in literature may be due to differences in the type of substrates used in the inhibition studies.

Tris was also found to inhibit the activity of ß-galactosidase prepared from goat epididymis. This inhibition may be due to the reaction of the amino group of Tris (substituted ammonium ion)

with the carboxyl group of the enzyme molecule which may lead to alteration in the confirmation required for the maintenance of enzyme activity as suggested by other investigators (Hartley and Vedamuthu, 1975). Such an inhibitory action of Tris on acidic ß-galactosidase activity has been also previously demonstrated (Cecilia et al. 1991).

The inhibition in epididymal ß-galactosidase activity brought about by D-galactose is interesting since this may be one of the mechanisms that regulates enzyme activity **in vivo**. It must be pointed out that the concentration of free D-galactose in the epididymis is very low under physiological conditions (Mann and Lutwak-Mann, 1981) and any slight rise in the concentration of this sugar could be of functional significance in sperm maturation by changes in enzyme activity. It is also known that ß-galactosidase secreted by the epididymis is absorbed on the sperm surface for participation in later events like cumulus dispersion and egg penetration (Hall and Killian, 1987). The precise interaction between ß-galactosidase carried on the sperm surface and the galactose present during these events needs to be investigated further.

Data obtained in the present study shows that the inhibition in enzyme activity brought about by the two synthetic sugars methyl α-D-mannopyranoside and methyl α-D-glucopyranoside was negligible. It is important to point out that both methyl α-D-mannopyranoside and methyl α-D-glucopyranoside have glycosidic linkage at the C-1 α position. Obviously ß-galactosidase is unable to interact with such sugars because

its active site perhaps can interact with ß-anomers only. Thus, the lack of inhibition by these two synthetic sugars may be due to the high specificity of ß-galactosidase for its substrates depending on the C-1 position of anomers.

5.5 CONCLUDING REMARKS

1. The properties of the purified preparation of ß-galactosidase from goat epididymis have been investigated.
2. The molecular weight of ß-galactosidase was found to be 63K by SDS-PAGE and 64K by gel filtration.
3. The enzyme was very stable at acidic pH and temperature of upto 35°C. Temperatures above 50°C, however, rapidly destroyed the activity of enzyme. Aqueous preparations of the enzyme could be stored at low temperature (4°, 0° and -70°C) without loss in activity for periods upto 3 months.
4. The epididymal preparation of enzyme did not react with the substrates of other glycosidases and was rather specific in its reaction with substrate of ß-galactosidase.
5. Kinetic studies demonstrated that p-nitrophenyl ß-D-galactopyanoside was a better substrate than the corresponding o-nitrophenyl derivative.
6. Y-D-galactonolactone competitively inhibited the activity of the enzyme preparation.
7. Tris and D-galactose were also found to substantially inhibit the activity of purified epididymal ß-galactosidase while methyl α-D-glucopyranoside and methyl α-D-mannopyranoside had little effect.

CHAPTER 4

Influence of metal ions on purified β-galactosidase from the goat epididymis

6.1 INTRODUCTION

It is well established that the epididymis controls the composition of the luminal fluid in which the sperm are bathed. So delicate is this mechanism that the luminal fluid at each site along the duct differs dramatically in different respects. Among the several characteristics that change, pH and osmolarity are of immense concern (Levine and Marsh, 1971; Howards et al., 1979; Acott and Carr, 1984; Carr et al., 1985). The ionic composition of the epididymal fluid influences these parameters significantly (Jones and Glover, 1975; Mann and Lutwak-Mann, 1981). The epididymal epithelium regulates the entry of ions into its lumen by both passive and energy dependent processes (Wong and Yeung, 1977; Jones, 1978). It is now clear that the epididymal events are to a large extent controlled by alterations in the concentration of ions. Of late, the role of ions in molecular cell signalling has also been well recognized. In addition, the ions are instrumental in stabilizing and maintaining the activity of several enzymes in the epididymis (Snaith, 1977; Dutta and Majumder, 1984). Therefore, it would be interesting to know which of these ions are predominantly found in the epididymal luminal fluid. The proximal regions of the epididymis support sperm maturation while distal segments serve as a repository for sperm (Orgebin-Crist et al., 1981). It would be relevant then to study the fluctuations in the levels of these ions along the length of the epididymis as well.

In a preceeding chapter it was shown that epididymal ß-galactosidase activity comes to prominence during the breeding season of goats. This observation gave room for suspicion of its involvement in epididymal sperm maturation. Detailed studies on ß-galactosidase by several other investigators have led them to the same conclusion (Hall and Killian, 1987). Since this enzyme was reasonably purified in this laboratory, it was thought worthwhile to see if any of the ions found naturally in the epididymal fluid had any regulatory role in the functioning of the enzyme.

In this era of industrialization, animal species are exposed to many hazards of heavy metal ions which are industrial wastes and environmental pollutants. Heavy metal toxicity has been the subject of intense study for well over forty years. More recently, reproductive dysfunctions have been reported to be associated with exposure to some metal ions (Clarkson et al., 1985; Jockenhovel et al., 1990). In males, testicular damage, inhibition of testicular steroidogenesis, inhibition of luminal acidification, necrosis in the caput epididymis and defective sperm maturation have been described (Gunn et al., 1970; Saksena et al., 1977; Saksena and Lau, 1979; Waalkes and Poirier, 1985). Thus, it would be also relevant to study the influence of potential environmental heavy metal ion pollutants on the activity of ß-galactosidase.

The present study addresses three issues:

i) Identification of ions in epididymal fluids and their regional distribution.

ii) Impact of ions found in the epididymal luminal fluid on purified ß-galactosidase.

iii) Impact of ions found as environmental pollutants on purified ß-galactosidase.

6.2 MATERIALS AND METHODS

6.2.1 Studies on Goat Epididymal Luminal Fluid

Goat epididymis were collected from the local abattoir and brought to the laboratory in ice within one hour of slaughter. After clearing the adhering adipose and connective tissue, each pair of epididymis (belonging to the same animal) was weighed. Six pairs of epididymis (each pair weighing between 20-28 g) were used in the present study.

6.2.1.1 Collection of luminal fluid

A pair of epididymis was cut into twelve segments according to the scheme of Besancon et al. (1985) as given in Fig. 1, Section 3.2.3. The tissue of each segment was taken in a small container and subjected to vacuum. The luminal fluid which oozed out from the site of the cut was aspirated into fine capillaries and transferred to miniature eppendorf tubes. The samples were then centrifuged at 12,000 xg for 10 minutes in a refrigerated centrifuge (Kuboto 1300, Japan). The sperm packed into a tight pellet and were discarded. The supernatant which essentially comprised the luminal fluid was saved and the elemental

composition determined after appropriate dilutions with deionized water.

6.2.1.2 Detection of Elemental Composition of Luminal Fluid

The elements present in liquid samples can be accurately detected quantitatively by using the Inductively Coupled Plasma (ICP) technique. Because of the higher temperature and chemically inert environment, ICP is considered superior to flame spectroscopy methods. Further, since simultaneous detection is possible for a number of elements, only small amounts of sample are required. Briefly, the principle involves imparting energy to the sample via a sufficiently high temperature environment which converts the elements in the sample to excited free atoms and ions. As they return to the ground state, they emit radiations at characteristic wavelengths from which their concentrations may be determined.

In the present studies, the polychromatic system of Labtam (Plasma Lab 8440, Australia) was used, which permits simultaneous determination of upto 48 elements at wavelengths ranging from 170-820nm. However, only biologically relevant elements were analysed.

Initially standard stock solutions of 1000 μg/ml (or 1000 ppm) were prepared as indicated below :

Calcium

2.497g $CaCO_3$ was dissolved in 50ml deionized water. HCl (approximately 10ml) to effect complete solubilization of $CaCO_3$

was added and diluted the resulting solution to a volume of 1L. Final concentration : 1000 μg/ml Ca.

Cobalt

1g metallic cobalt was dissolved in 50ml 1:1 HCl and diluted quantitatively to a volume of 1L. Final concentration : 1000 μg/ml Co.

Copper

1g metallic copper was dissolved in 50ml 1:1 HNO_3 and diluted quantitatively to a volume of 1L. Final concentration : 1000 μg/ml Cu.

Chromium

1g metallic chromium was dissolved in 50ml 1:1 HCl with gentle heating. This was cooled and diluted quantitatively to a volume of 1L. Final concentration : 1000 μg/ml Cr.

Cadmium

1g metallic cadmium was dissolved in 50ml 1:1 HCl and diluted quantitatively to a volume of 1L. Final concentration : 1000 μg/ml Cd.

Iron

1g metallic iron was dissolved in 50ml 1:1 HCl and diluted quantitatively to 1L. Final concentration : 1000 μg/ml Fe.

Lead

1g metallic lead was dissolved in 50ml 1:1 HNO_3 and diluted quantitatively to a volume of 1L. Final concentration: 1000 μg/ml Pb.

Magnesium

1g magnesium ribbon was dissolved in 50ml 1:1 HCl and diluted to 1L. Final concentration : 1000 μg/ml Mg.

Manganese

1g metallic manganese was dissolved in 50ml 1:1 HNO_3 and diluted quantitatively to a volume of 1L. Final concentration: 1000 μg/ml Mn.

Nickel

1g metallic nickel was dissolved in 50ml 1:1 HNO_3 and diluted to 1L. Final concentration : 1000 μg/ml Ni.

Titanium

1g metallic titanium was dissolved in 100ml 1:1 HCl and diluted quantitatively to a volume of 1L with 10% V/V HCl. Final concentration : 1000 μg/ml Ti. All further dilutions were made with 10% V/V HCl to prevent precipitation as titanium oxychloride.

Zinc

1g metallic zinc was dissolved in 50ml 1:1 HCl and diluted to 1L. Final concentration: 1000 μg/ml Zn.

At the time of analysis, the instrument was calibrated using working standards of 1 μg/ml and 5 μg/ml. The quantity of the elements detected in the luminal fluid samples were directly read as parts per million (ppm). The dilution was taken into consideration while compiling the results. In order to

facilitate comparison with other previous investigations the values obtained as ppm were converted and expressed as millimolar concentration.

As far as sodium and potassium are concerned it is generally recognized that Flame Photometric methods are superior. Therefore, samples of luminal fluid from each of the twelve epididymal segments were analysed by flame photometry (Evans Electroselenium Ltd., England). Commercial standards supplied by Mallinckrodt, Australia were used. The working standards were 1, 5 and 10 ppm for both sodium and potassium.

After determining the concentration of various elements in the luminal fluid collected along the epididymis it was thought useful to examine if these ions influenced epididymal ß-galactosidase activity.

6.2.2 Treatment with Ethylenediamine Tetracetate (EDTA).

In studies dealing with ionic regulation of enzyme activity it is often desirable to first determine the activity in the absence of ions. EDTA comes in handy in such investigations since it is known to selectively bind divalent cations in solutions. Most previous investigators have first eliminated the ions present in enzyme preparations using EDTA and then supplemented the system with fixed concentration of individual ions. From the changes in enzyme activity under these conditions, the ionic dependence of enzymes were established. Preliminary investigations of this kind not only give important clues regarding ionic dependence but

also help in understanding the mechanism of enzyme regulation **in vivo.**

In the present study the same approach has been used to pinpoint the effect of individual ions on purified epididymal ß-galactosidase. Equal aliquots of purified ß-galactosidase were exposed to three different concentrations (0.1mM, 0.5mM and 1mM) of EDTA. Further the time of exposure to each concentration of EDTA was varied (5 minutes, 30 minutes and 60 minutes) to make the analysis complete. After the respective preincubations, the activity of ß-galactosidase was assayed using the same colorimetric procedure as described in Section 3.2.4.2. These values were then compared with the activity of enzyme in a control that did not contain EDTA.

6.2.3 Metal Ions and ß-Galactosidase Activity

Once the pre-existing divalent cations were mopped up using EDTA, the effect of metal ions on ß-galactosidase preparation was ascertained. In the present studies the effect of two types of metal ions has been worked out: Firstly those that are naturally found in the epididymal luminal fluid and secondly those that are common environmental pollutants.

The procedure employed uses EDTA (1mM) treated purified ß-galactosidase preparations. Equal aliquots were exposed to various metal ions which were prepared by dissolving the appropriate salts in 0.1M sodium acetate buffer pH 4.8. The effect of each metal ion was determined basically at two concentrations : one

low (1mM) and one high (5mM). In some cases when inhibition was substantial, the studies were carried out at reduced concentrations as indicated.

Reagents

Initially stock solutions of all metal ions which were detected in the luminal fluid and others which are common environmental pollutants were prepared by dissolving their respective salts in 0.1M sodium acetate buffer pH 4.8 in a final volume of 10ml.

Calcium : 0.01470g $CaCl_2 . 2H_2O$ was dissolved to give a 10mM stock solution

Copper : 0.02496g $CuSO_4 . 5H_2O$ was dissolved to give a 10mM stock solution

Iron : 0.01622g $FeCl_3$ was dissolved to give a 10mM stock solution

Magnesium: 0.02033g $MgCl_2 . 6H_2O$ was dissolved to give a 10mM stock solution

Nickel : 0.02808g $Ni(SO_4)_2$ was dissolved to give a 10mM stock solution

Sodium : 0.05844g NaCl was dissolved to give a 100mM stock solution

Potassium: 0.07456g KCl was dissolved to give a 100mM solution stock

Zinc : 0.01362g $ZnCl_2$ was dissolved to give a 10mM stock solution

Lead : 0.03310g $Pb(NO_3)_2$ was dissolved to give a 10mM stock solution

Silver : 0.01690g $AgNO_3$ was dissolved to give a 10mM stock solution

Mercury : 0.02715g $HgCl_2$ was dissolved to give a 10mM stock solution

Manganese: 0.01979g $MnCl_2$ $4H_2O$ was dissolved to give 10mM stock solution

Cadmium : 0.02665g $Cd(CH_3COO)_2$ was dissolved to give a 10mM stock solution

Cobalt : 0.02379g $CoCl_2.6H_2O$ was dissolved to give a 10mM stock solution

Appropriate dilutions were carried out using 0.1M sodium acetate buffer pH 4.8 to give the desired low and high ion concentrations in the reaction mixture. The effect of most of the metal ions viz calcium, iron, magnesium, nickel, zinc, lead, manganese, cadmium and cobalt on ß-galactosidase activity **in vitro** was assessed at 1mM and 5mM concentration. Owing to higher inhibitions of the enzyme activity, lower concentration of copper, silver and mercury were used. Briefly, their concentrations were copper 0.25mM and 0.5mM, silver 0.0625mM and 0.125mM and mercury 0.0125mM and 0.025mM.

The concentrations of sodium and potassium are known to be high in the luminal fluid, so their effect on ß-galactosidase activity **in vitro** was analysed at higher concentration of 5mM and 25mM.

To determine if the effect of metal ions varied with time the enzyme activity was measured in samples exposed to the metal ions for 5 min, 30 min and 60 min. For comparison purposes a control was included which was neither exposed to EDTA nor any metal ion. All incubations were carried out at 37°C in a final volume of 0.2ml. At the end of the respective incubation periods, 0.6ml of 1.25mM p-nitrophenyl ß-D-galactopyranoside, also prepared in 0.1M sodium acetate buffer pH 4.8, was added to initiate the reaction. After 15 minutes of incubation at 37°C, the reaction was stopped by the addition of 2ml of 0.2M Na_2CO_3 solution. Separate blanks were run for each metal ion concentration which were processed in a similar manner except that the substrate solution was added after stopping the reaction with Na_2CO_3. The absorbance of the p-nitrophenol released was read at 400nm spectrophotometrically and the enzyme activity was determined.

6.3 RESULTS

The concentration profiles of various elements as obtained by Inductively Coupled Plasma analysis of epididymal luminal fluid collected from various segments of the goat epididymal duct are shown in Fig. 31. Cadmium, cobalt, lead and manganese were not found in the samples of epididymal luminal fluid. Other elements viz copper (0.0639-0.3079mM), calcium (0.0280-0.9750mM), nickel (0-0.6427mM), iron (0.1046-0.6744mM), magnesium (0.1811-

0.7395mM), chromium (0-0.2346mM), titanium (0-0.1948mM) and zinc (0.0168-0.2786mM) were found to be present and their levels fluctuated in the luminal fluid at different sites along the length of the epididymis.

The data on sodium and potassium concentration collected by flame photometric analysis of epididymal luminal fluid is depicted in the Fig. 32. In the proximal segments the values for sodium were significantly higher as compared to that of potassium. Another prominent observation was that Na^+/K^+ ratio was higher in the luminal fluid from the proximal segments of the epididymis and gradually decreased towards the distal end.

The effect of the metal ions detected in the luminal fluid on ß-galactosidase activity in vitro are depicted in Figs. 33a,b,c. Five minutes preincubation of the enzyme preparation with 1 and 5mM ion concentrations at 37°C had no significant effect on the enzyme activity as far as calcium, iron, magnesium, sodium, potassium and zinc were concerned. However, nickel and copper were found to inhibit ß-galactosidase activity. The percentage inhibition was more or less identical when 1 and 5mM concentration of nickel were used. On the other hand, the enzyme activity was more sensitive to copper since a higher percentage of inhibition was obtained with relatively low concentrations of copper in the incubation mixture. Extending the period of preincubation of enzyme with metal ions from 5 to 30 minutes or 60 minutes also yield similar results (Figs. 33b,c). The enzyme

activity remained unaffected by calcium, iron, magnesium, sodium, potassium and zinc at both high and low molar concentrations. Once again nickel and copper were the only two ions that inhibited the activity of ß-galactosidase. A careful glance through the data obtained at 5, 30 and 60 minutes preincubation of enzyme preparation with ions reveals that the percentage inhibition brought about by copper increased with time. The percentage inhibition in ß-galactosidase activity brought about by nickel at both the concentrations and the three time intervals studied did not vary much.

The effect of some elements associated with toxicity in animal tissues on epididymal ß-galactosidase is presented in Figs. 34a,b,c. The percentage residual activity of purified ß-galactosidase after preincubation with low and high concentrations of six metal ions was studied independently. All these metal ions except cobalt and manganese had an inhibitory effect on ß-galactosidase activity. The strongest inhibition was brought about by mercury followed by silver since inhibition was observed at much lower concentration than the other metal ions. Another interesting feature is that at both low and high ion concentrations of lead, silver, mercury and cadmium, the percentage inhibition in ß-galactosidase activity was found to increase with the period of preincubation. Manganese had no effect on ß-galactosidase activity but cobalt was slightly stimulating.

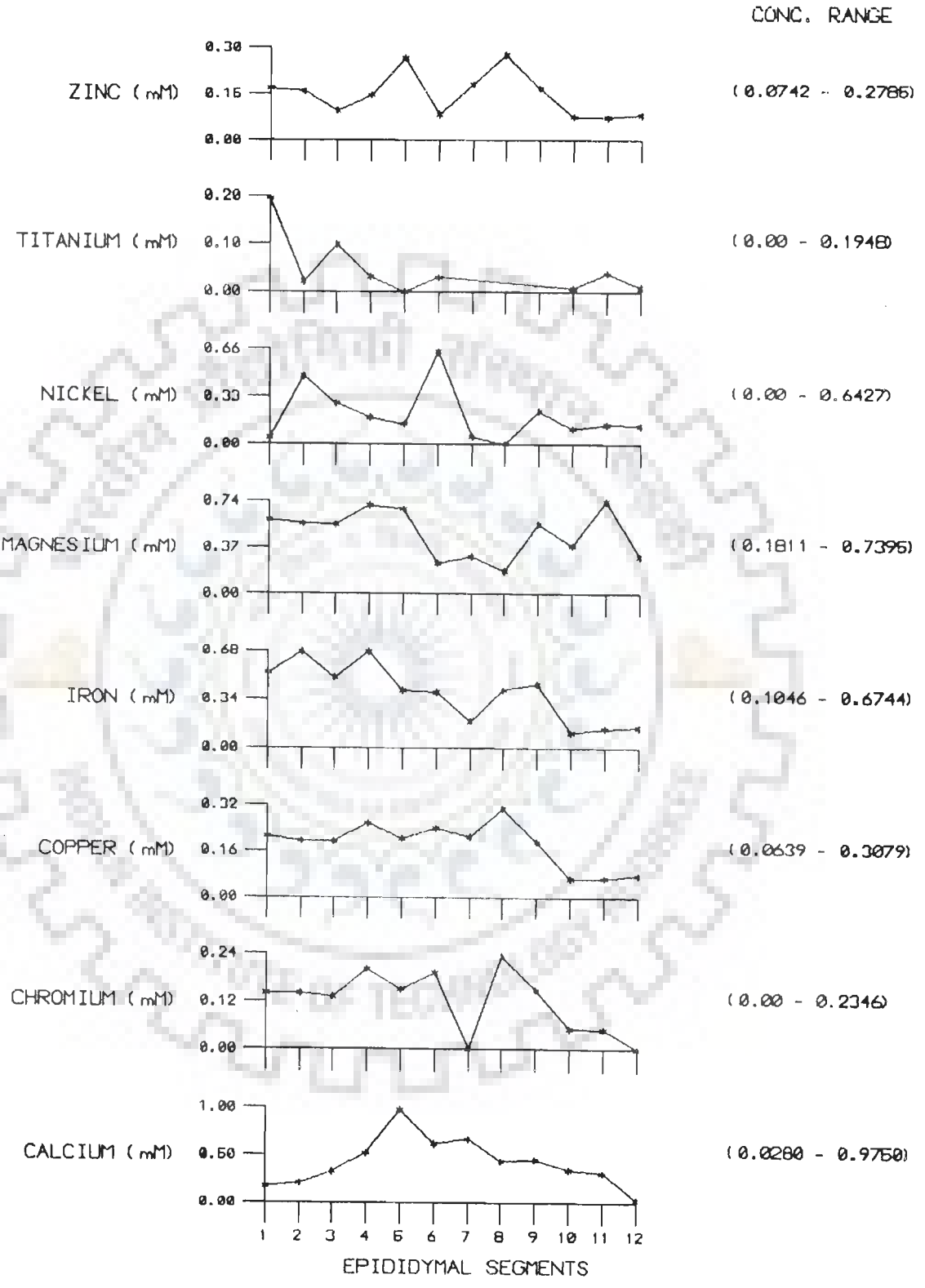
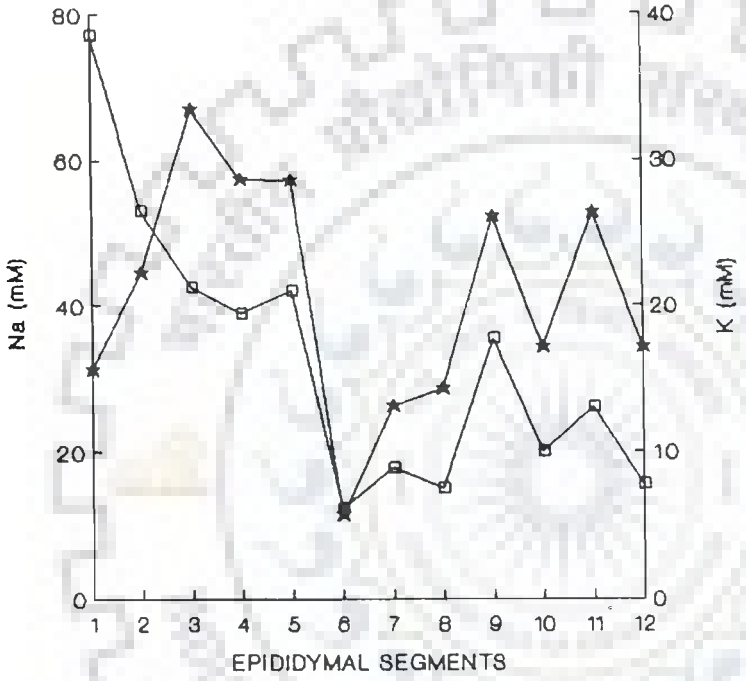


Fig. 31. Concentration (mM) of Various Elements in the Luminal Fluid Collected from 12 Segments of the Goat Epididymis. The epididymis was divided into 12 segments according to the scheme given by Besancon et al. (1985) and the concentrations of the various elements were analysed by Inductively Coupled Plasma (ICP) Technique.



SEGMENTS	Na/K
1	4.95
2	2.38
3	1.27
4	1.35
5	1.46
6	2.10
7	1.36
8	1.05
9	1.36
10	1.17
11	0.99
12	0.90

Fig. 32. (- □ -) Sodium (mM) and (- ★ -) Potassium (mM) Concentration in the Luminal Fluid Collected from 12 Segments of Goat Epididymis. The analysis was done by Flame Photometry. The table presents sodium; potassium ratio within the 12 epididymal segments.

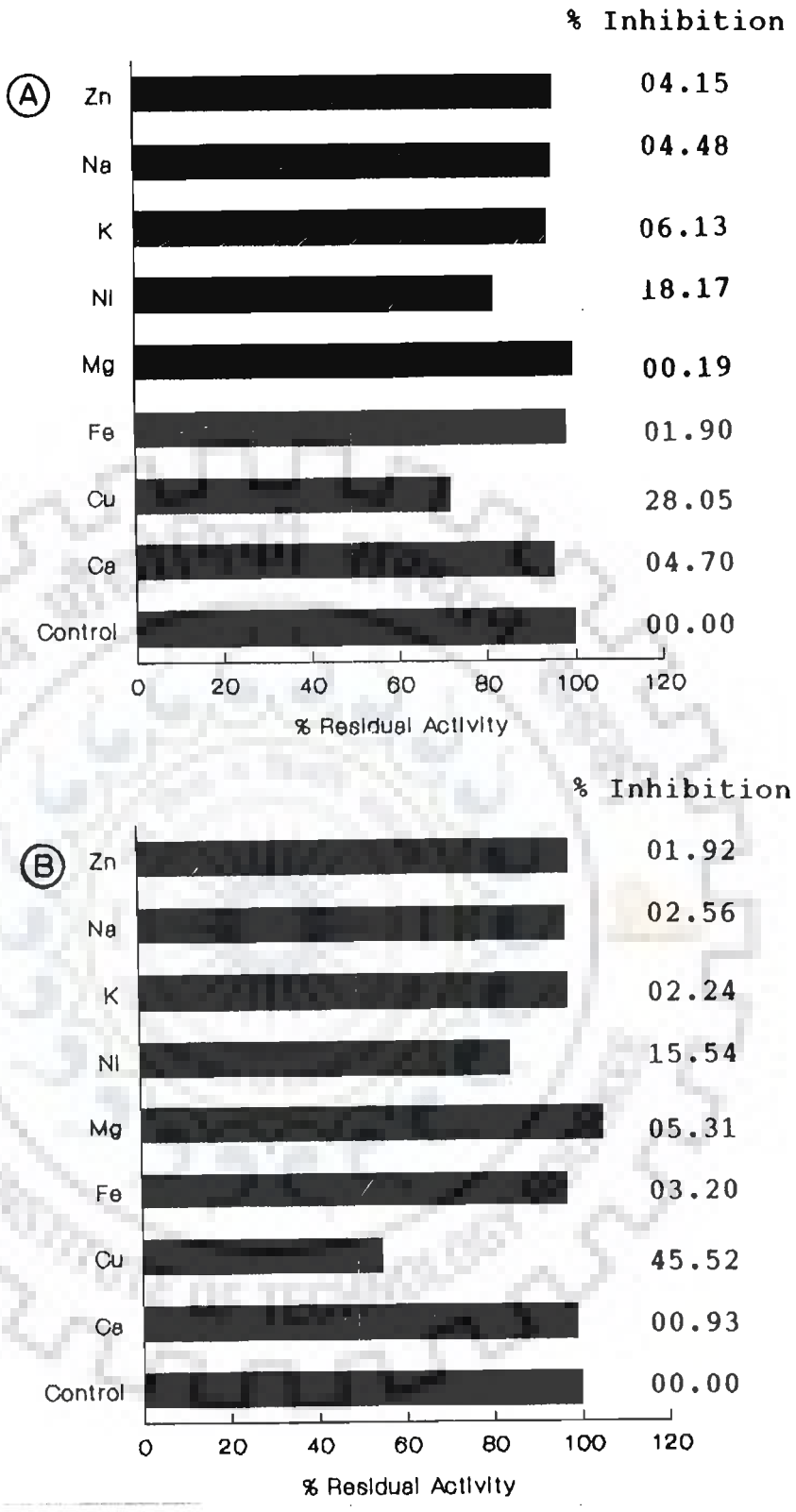


Fig. 33a. Effect of Metal Ions Detected in the Epididymal Luminal Fluid on the Activity of ß-Galactosidase. After 5 minutes preincubation with the ion at

(A) Low ion concentration; Ca^{2+} (1mM), Cu^{2+} (0.25mM) Fe^{2+} (1mM), Mg^{2+} (1mM), Ni^{2+} (1mM), K^{+} (5mM), Na^{+} (5mM) and Zn^{2+} (1mM).

(B) High ion concentration; Ca^{2+} (5mM), Cu^{2+} (0.5mM) Fe^{2+} (5mM), Mg^{2+} (5mM), Ni^{2+} (5mM), K^{+} (25mM), Na^{+} (25mM) and Zn^{2+} (5mM).

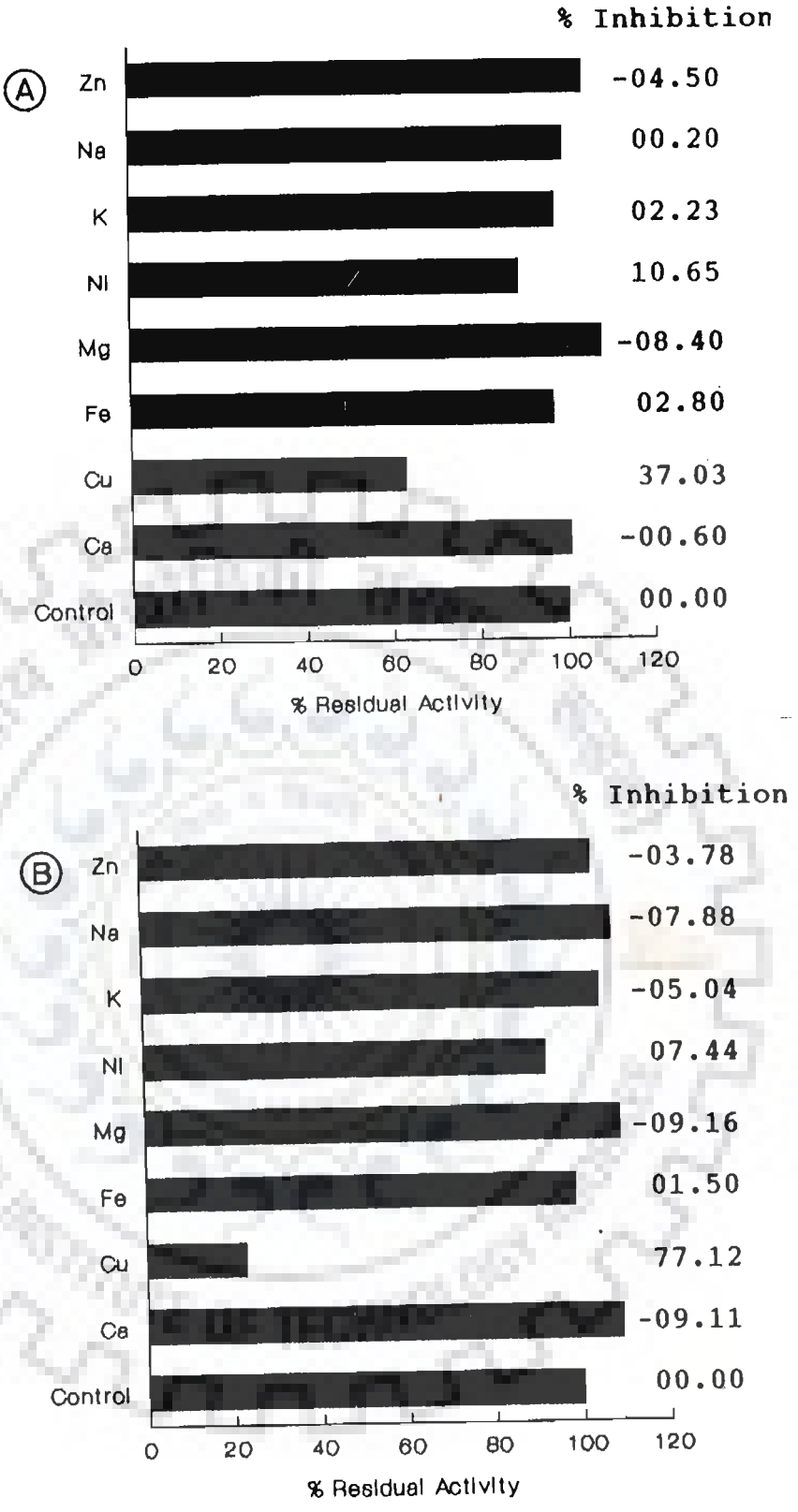


Fig. 33b. Effect of Metal Ions Detected in the Epididymal Luminal Fluid on the Activity of ß-Galactosidase. After 30 minutes preincubation with the ion at

(A) Low ion concentration; Ca^{2+} (1mM), Cu^{2+} (0.25mM) Fe^{2+} (1mM), Mg^{2+} (1mM), Ni^{2+} (1mM), K^{+} (5mM), Na^{+} (5mM) and Zn^{2+} (1mM).

(B) High ion concentration; Ca^{2+} (5mM), Cu^{2+} (0.5mM) Fe^{2+} (5mM), Mg^{2+} (5mM), Ni^{2+} (5mM), K^{+} (25mM), Na^{+} (25mM) and Zn^{2+} (5mM).

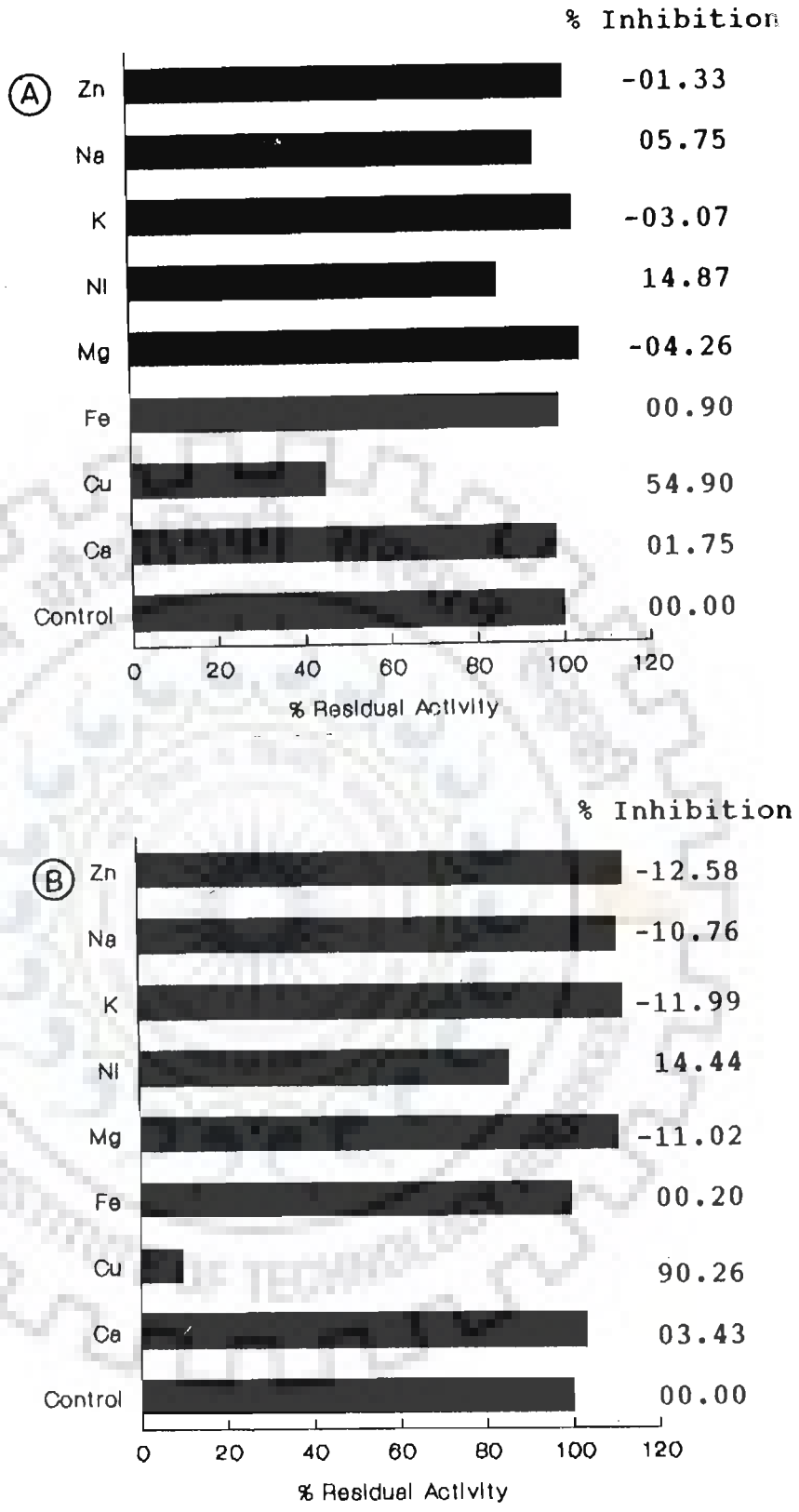
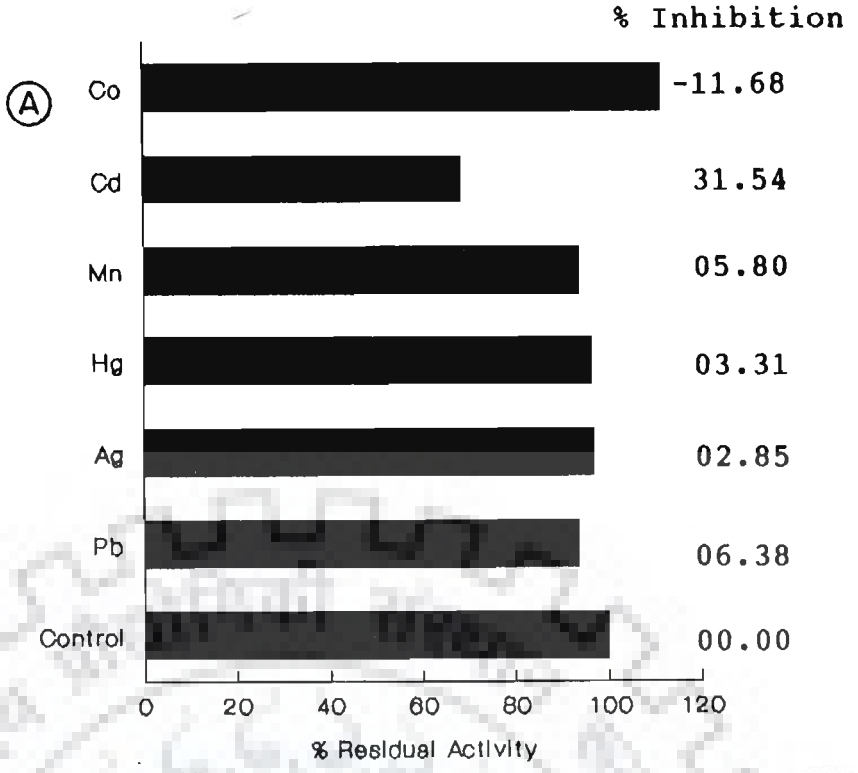


Fig. 33c. Effect of Metal Ions Detected in the Epididymal Luminal Fluid on the Activity of ß-Galactosidase. After 60 minutes preincubation with the ion at

(A) Low ion concentration; Ca^{2+} (1mM), Cu^{2+} (0.25mM) Fe^{2+} (1mM), Mg^{2+} (1mM), Ni^{2+} (1mM), K^{+} (5mM), Na^{+} (5mM) and Zn^{2+} (1mM).

(B) High ion concentration; Ca^{2+} (5mM), Cu^{2+} (0.5mM) Fe^{2+} (5mM), Mg^{2+} (5mM), Ni^{2+} (5mM), K^{+} (25mM), Na^{+} (25mM) and Zn^{2+} (5mM)



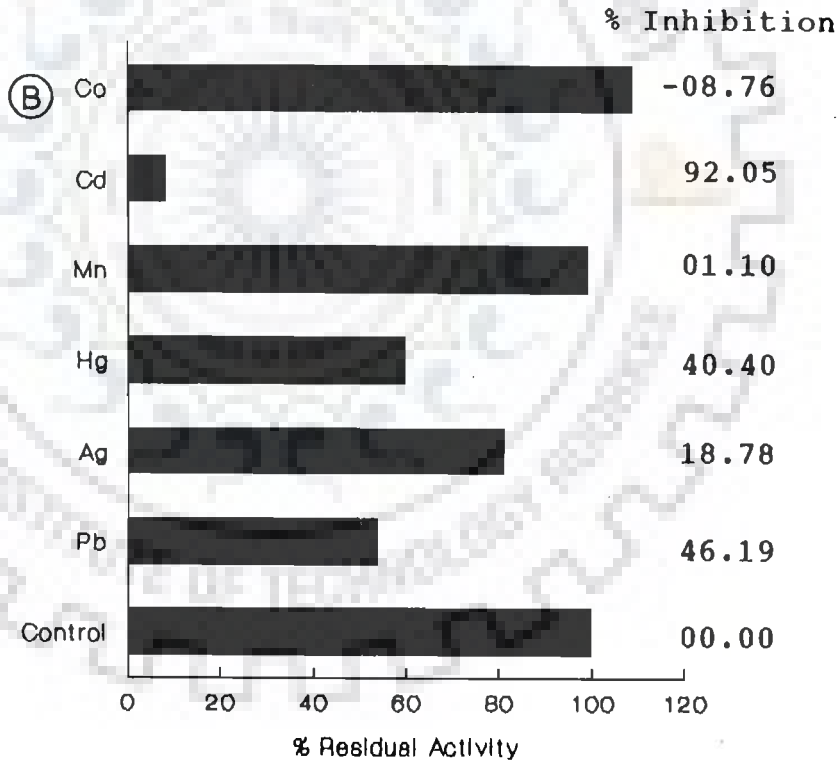


Fig. 34a. Effect of Metal Ions Found as Environmental Pollutants on the Activity of ß-Galactosidase. After 5 minutes preincubation with the ion at

(A) Low ion concentration: Pb^{2+} (1mM), Ag^{+} (0.0625mM), Hg^{2+} (0.0125mM), Mn^{2+} (1mM), Cd^{2+} (1mM) and Co^{2+} (1mM).

(B) High ion concentration: Pb^{2+} (5mM), Ag^{+} (0.125mM), Hg^{2+} (0.025mM), Mn^{2+} (5mM), Cd^{2+} (5mM) and Co^{2+} (5mM).

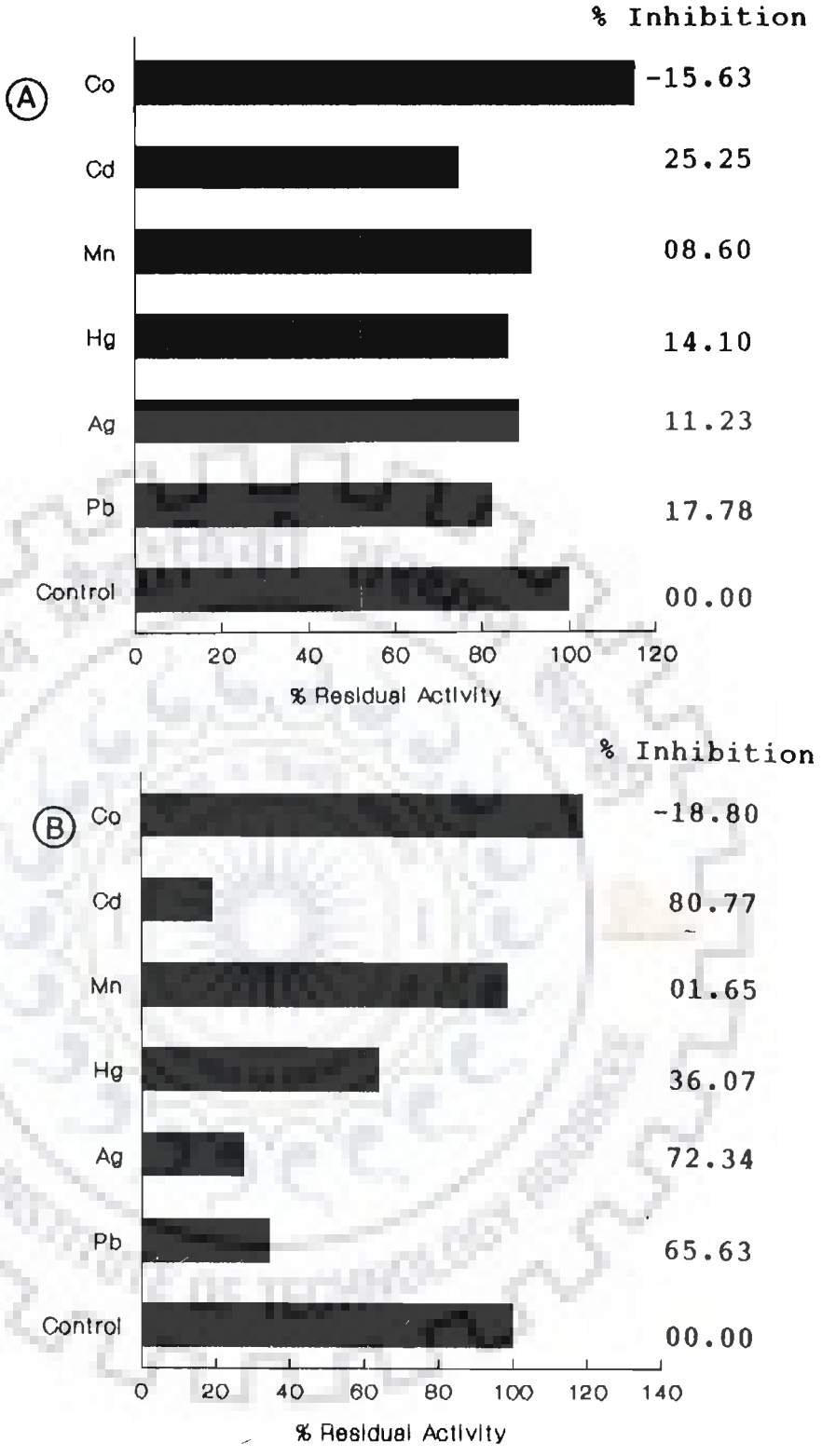


Fig. 34b. Effect of Metal Ions Found as Environmental Pollutants on the Activity of ß-Galactosidase. After 30 minutes preincubation with the ion at

(A) Low ion concentration: Pb^{2+} (1mM), Ag^{+} (0.0625mM), Hg^{2+} (0.0125mM), Mn^{2+} (1mM), Cd^{2+} (1mM) and Co^{2+} (1mM).

(B) High ion concentration: Pb^{2+} (5mM), Ag^{+} (0.125mM), Hg^{2+} (0.025mM), Mn^{2+} (5mM), Cd^{2+} (5mM) and Co^{2+} (5mM).

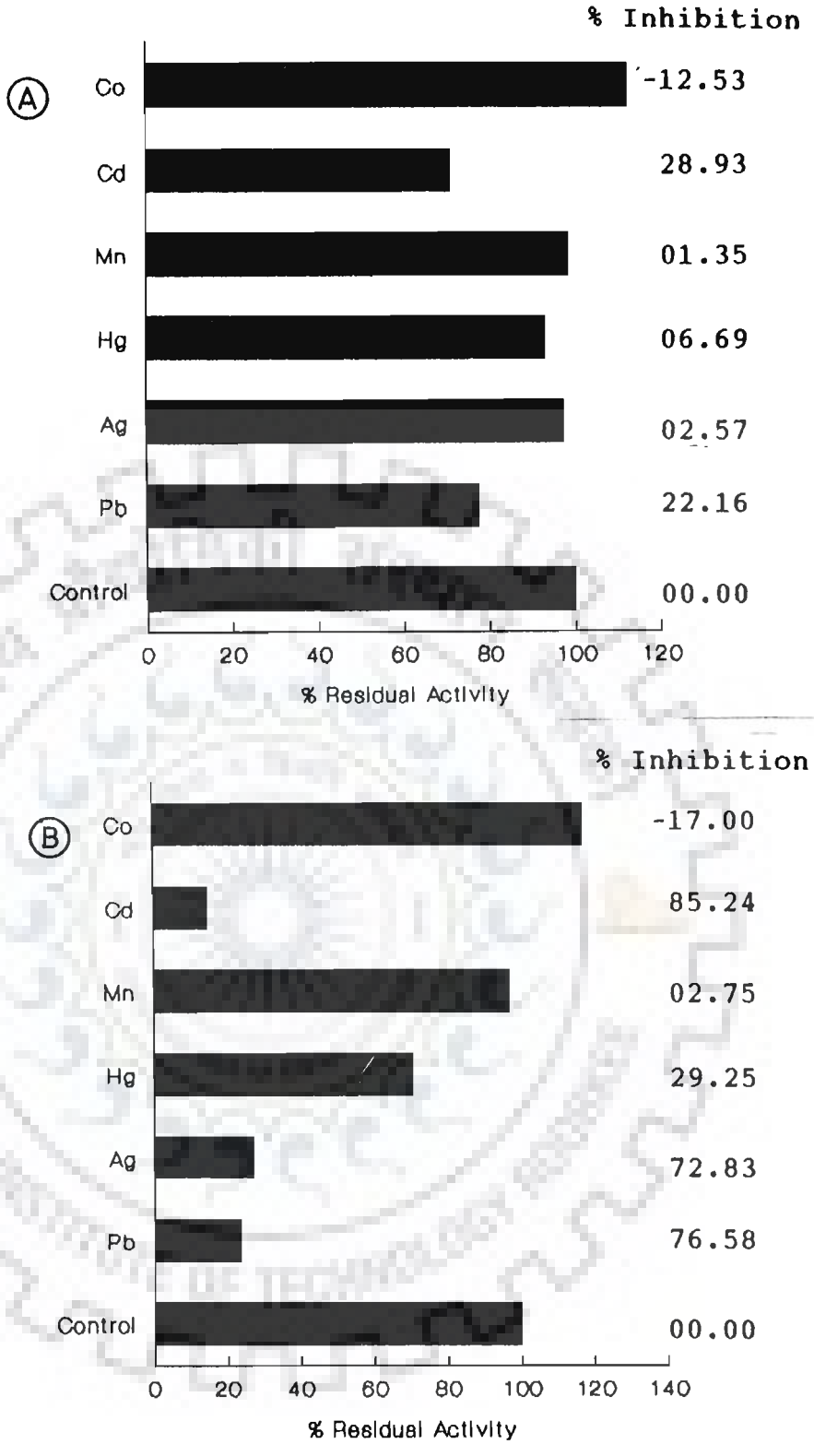


Fig. 34c. Effect of Metal Ions Found as Environmental Pollutants on the Activity of ß-Galactosidase. After 60 minutes preincubation with the ion at

(A) Low ion concentration: Pb^{2+} (1mM), Ag^{+} (0.0625mM), Hg^{2+} (0.0125mM), Mn^{2+} (1mM), Cd^{2+} (1mM) and Co^{2+} (1mM).

(B) High ion concentration: Pb^{2+} (5mM), Ag^{+} (0.125mM), Hg^{2+} (0.025mM), Mn^{2+} (5mM), Cd^{2+} (5mM) and Co^{2+} (5mM).

6.4 DISCUSSION

It has been fairly well established that the epididymal microenvironment provided by the luminal fluid nurture the sperm. The role of ions in this process is now increasingly being realized (Howards et al., 1979; Cooper, 1986). This implies that the ionic composition of the luminal fluid along the length of the epididymis constantly changes. It is not surprising therefore that in the present studies the concentration of ions in the luminal fluid collected from various sites of the epididymal duct did not remain the same.

Although many investigators in the past have determined the concentration of ions in epididymal luminal fluid using various techniques for collection of fluid and analysis methods, they have restricted their studies to select regions of the epididymis (Levine and Marsh, 1971; Back et al., 1974; Howards et al., 1975; Hinton and Howards, 1982). This has been mainly due to the tedious procedure involved in the collection of luminal fluid. Moreover, a majority of researchers have carried out their studies on rats and rabbits. Since the shape and size of the epididymis in different species are not same it would not be apt to make any direct segment to segment comparison between these and the present studies. Thus for comparative purposes the discussion is mainly restricted to larger mammals like goat, ram and bull. Even while doing so caution has been excercised since the levels of ions in the epididymal fluid could vary as a result of its interaction with sperm and epididymal epithelium.

In the present studies the values obtained for calcium, magnesium and zinc are comparable to those previously reported for ram (Jones, 1978). The presence of these cations are believed to be vital for sperm maturation (Arver, 1982; Breitbart et al., 1985); But there are contradictory reports about their functional significance in the epididymal fluid. According to some reports calcium may or may not be required for the initiation of motility (Turner and Howards, 1978; Serres et al., 1991) but only during acrosome reaction and capacitation (Fraser, 1987; Yanagimachi, 1988; Fraser and McIntyre, 1989). Others suggest that in species where the concentration of calcium is low in cauda epididymis exogenous calcium is required to initiate motility in contrast to the species which have high levels of calcium within the lumen of cauda epididymis. Besides, epididymal spermatozoa are known to accumulate large amounts of calcium from exogenous sources (Babcock et al., 1979; Hoskins et al., 1983) and the net uptake is dependent upon mitochondrial pyridine nucleotides (Vijayraghavan et al., 1989; Bretbart et al., 1990). Calcium may also be required by calcium binding proteins which bind to sperm membrane during the epididymal maturation (Lubac et al., 1976). The functional importance of magnesium is not entirely clear. Nevertheless, it has been shown on a number of previous occasions that several enzymes in the epididymis are dependent on magnesium for their activity (Cooper, 1986). In mouse, magnesium is known to substitute for the absence of calcium in bringing about sperm motility (Heffner and Storey, 1981). Zinc is believed to affect sperm motility (Lindholmer, 1974), sperm metabolism (Eliasson and Lindholmer,

1971; Huacuja et al., 1973), sperm membrane integrity (Silvestroni et al., 1989) and fertility (Suescun et al., 1981; Srivastava et al., 1982). Zinc is also required for regulating the activity of some epididymal enzymes like α-mannosidase (Snaith, 1977; Dutta and Majumder, 1984). In addition it has been suggested that zinc plays a role in sperm maturation by protecting thiol groups in sperm proteins from excessive oxidation (Kvist et al., 1987; Silvestroni et al., 1989). Owing to high nuclear zinc levels in ejaculated human sperm as compared to the epididymal sperm, it is speculated that male gametes take up zinc upon ejaculation (Kvist et al., 1985). Just like zinc, copper is known to react with thiol groups in sperm with great affinity and its biological relevance has been studied extensively (Makler and Zinder, 1980; Jockenhovel et al., 1990). Most of the copper is present tightly bound to proteins. Iron is also bound to proteins viz. transferrin, lactoferrin etc. which are known to be present in various male reproductive tissues (Wichmann et al., 1989; Ber et al., 1990). It has been reported that deficiency of iron in epididymal fluid is associated with infertility in humans (Wong and Lee, 1983, 1985). Thus it is clear that the presence of calcium, magnesium, zinc, copper and iron in the epididymal luminal fluid is of great functional significance.

There are no previous reports indicating the presence of nickel, chromium and titanium in the epididymal luminal fluid of animals under normal conditions. The present study is perhaps the first that reports the presence of these three elements in the goat

epididymal luminal fluid. It must be mentioned that Roorkee is situated at the foothills of Himalayas and the pastures used for grazing the animals are rich in mineral deposits. It is therefore likely that these elements have found their way into the male reproductive tract through the diet, a phenomenon referred in ecological terms as 'biological concentration'. To what extent the presence of these elements in the epididymal luminal fluid regulates the epididymal function remains to be established.

The present study is unique in the sense no previous investigations have analysed the sequential changes in the epididymal luminal fluid with respect to ionic composition. Nevertheless, the values obtained for sodium and potassium are comparable to those reported by Jones (1978), who has analysed the ionic composition of luminal fluid from the distal segments of ram. However, the values obtained are lower than those previously reported by Scott et al. (1963) for goats. The reason for this discrepancy may be attributed to the difference in the method of collection of fluid and the methods used in the analysis of various ions. Besides, species variation in the concentration of different elements in the epididymal luminal fluid cannot be ruled out.

From the present study it is pretty obvious that the sodium: potassium ratio decreases from initial to the distal segments. Earlier studies on the bull by Crabo (1965) report similar findings where this ratio decreased starting from the rete testis to corpus and declined further in cauda. The change in this

ratio may be due to the resorptive nature of epididymal epithelium and could be of functional significance. It would be relevant to mention here that the luminal environment in initial segments of the epididymis supports sperm maturation while distally sperm are maintained and stored in a quiescent yet viable form. The ratio of sodium to potassium is of importance to bring about sperm maturation and higher concentrations of potassium in cauda may be responsible to maintain the spermatozoa in a quiescent state. Very high concentration of potassium in the epididymis is known to have harmful effects on spermatozoa (Wong and Lee, 1983, 1985). Sperm motility is also dependent on precise sodium to potassium ratio (Scott et al., 1963; Quinn et al., 1965, 1966). Under altered conditions of sodium, the normal ion transport activities of the epididymis are affected (Cooper, 1986). Altered concentrations of sodium and potassium have been reported to bring about epididymal dysfunctioning in bulls (Gustaffsson et al., 1972). In the present studies the values obtained for potassium and sodium are low in cauda as compared to caput. In the earlier studies, similar findings have been reported in hamster (Jessee and Howards, 1976), ram and bull where the concentration of potassium is known to exhibit a decrease in cauda. In the case of rat, however, the findings are contradictory and the concentrations of potassium are reported to rise in cauda (Levine and Marsh, 1971; Jenkins et al., 1980). It appears that the factors responsible for such a discrepancy are many and complex since sperm of different species spend varying period of time in the epididymis. In addition, it must be

pointed out that laboratory rats are continuous breeders while breeding in ram, bull and goat is seasonal.

Most of the elements detected in the epididymal fluid had no influence on ß-galactosidase. This is evident from the activity of ß-galactosidase which remained unchanged after preincubation with the concerned elements **in vitro.** Further, addition of EDTA to the enzyme preparation did not alter the activity of ß-galactosidase. Nickel and copper were found to bring about an inhibition of ß-galactosidase which increased with increase in both time of exposure and concentration of ion. It is known that ß-galactosidase activity is susceptible to inhibition by compounds disrupting sulfhydryl groups (Hartley and Vedamuthu, 1975; Distler and Jourdian, 1978; Cecilia et al., 1991). It has also been separately established that copper and nickel interact with sulfhydryl groups. Earlier reports carried out on other testicular enzymes have indicated similar findings with copper where the extent of inhibition could be reversed partially after addition of chelating agent. Thus it is suggested that copper and nickel bring about inhibition of ß-galactosidase by interfering with sulfyhydryl groups that are vital for enzyme activity.

The promotion of several chemical industries have led to the extensive use of elements like mercury, silver, cadmium, lead cobalt and manganese as catalysts. The spent catalyst are potential sources of environment pollution and have adverse effects on biological systems (Mahaffey, 1985; Chawdhury et al.,

1989). The ones relating to the reproductive system assume great importance because they are directly involved in the perpetuation of species. (Holland et al., 1976; Pleban and De-Shen 1983; Roy Chaudhury and Vachhrajani 1987). The intake of these elements through diet finally results in their accumulation in the reproductive tract and cause fertility disorders (Thomas and Brogan, 1983; Clarkson et al., 1985; Barratt et al., 1989). The results of the present investigation show that mercury, silver, cadmium and lead inhibit the activity of ß-galactosidase drastically. Mercury is known to bind to sulfhydryl groups of cysteine residues in proteins. It has also been reported that mercury can bind to tryptophan and alter its absorptive spectrum. Mercury also quenches its flourescence and phosphorescence. Thus mercury possibly inhibits ß-galactosidase activity by changing the tertiary conformation of this enzyme. The other heavy metal ions viz. lead, manganese and cadmium are also known to have toxic effects (Hilderbrand et al., 1973; Margoes and Webb 1980; Sokol et al., 1985; Jockenhovel, 1990). On a number of previous occasions it has been experimentally shown that when salts containing these elements are administered to animals, changes in reproductive tissues occur for example, administration of cadmium induces alkalinization of luminal fluid in segments of the epididymis, inhibition of testicular steroidogenesis and infertility (Nagy, 1985; Caflisch et al., 1991). Since mercury, cadmium, silver and lead were found to inhibit the activity of epididymal ß- galactosidase in the present studies, it implies defects in post- translational

modifications of sperm proteins may result should they enter the epididymal lumen.

6.5 CONCLUDING REMARKS

1. The elemental composition of luminal fluid from various segments of goat epididymis has been studied by Inductively Coupled Plasma and Flame Photometric analysis.

2. Copper, calcium, iron, magnesium and zinc were detected in the samples of epididymal luminal fluid while manganese, cadmium and cobalt were absent.

3. The presence of nickel, chromium and titanium in the epididymal luminal fluid is reported for the first time. It is likely that these elements may have entered the epididymal lumen from dietary sources. The impact of these elements on male reproduction remains to be established.

4. The sodium/potassium ratio in the luminal fluid was found to decrease from the initial segments to the distal segments of the epididymis. This is possibly related to the storage of sperm in a quiescent yet viable form in the distal segments.

5. Of all the elements detected in the epididymal luminal fluid, copper and nickel strongly inhibited the activity of ß-galactosidase suggesting a regulatory role for these elements.

6. Lead, silver, mercury and cadmium also inhibited ß-galactosidase activity. Since these elements are extensively used as catalysts in chemical industries, their entry into the epididymal lumen through the food chain could impair the epididymal function. The inhibition of ß-galactosidase activity by these elements suggests that defects in remodelling of sperm surface proteins crucial for sperm maturation could occur.

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