CHARACTERIZATION AND INHIBITION STUDY OF BOVINE LENS ALDOSE REDUCTASE

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ABSTRACT

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Aldose Reductase E.C.1.1.1.21 is an NADPH dependent enzyme and it has been implicated in the formation of cataract , particularly in diabetics. Aldose reductase is a protein found in the human erytrocyte with Mr 37.000 and also found in the either lenses or in many tissues such as liver, brain, kidney of various species. Although many studies have been performed with aldose reductase, little is known about the physiological role of this enzyme in the lens, retina and nerve tissues. In the presence of NADPH mediator, aldose reductase converts glucose to sorbitol, which is further oxidized to fructose by the enzyme sorbitol dehydrogenase. Aldose reductase activity in the process of sugar cataract formation through *in vitro* studies revealed that the rate of cataract formation is directly dependent upon the levels of aldose reductase present in the lens.

In this study ,aldose reductase was isolated from bovine lens by differential centrifugation and ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE celluse. The conditions such as substrate concentration, cofactor concentration, effect of temperature and effect of pH on the enzyme for maximum activity towards substrate glyceraldehyde were optimized.

The bovine lens aldose reductase showed its maximum activity at pH values 6-6.5 and seemed to be saturated by glyceraldehyde at $5x10^{-5}$ M concentration. The V_{max} and K_m values of enzyme for glyceraldehyde as a substrate were also calculated as 1.695 nmol/min and 17.86 μM, respectively. The enzyme was saturated by its cofactor NADPH at a concentration of $1.75x\ 10^{-6}$ M . The Vmax and Km values of the enzyme for the cofactor was also calculated as 2 nmol/min and 1.69 μM respectively. The inhibition studies were carried out using partially purified aldose reductase on DE-52 cellulose ion exchanger. Aldose reductase was inhibited by inhibitors I_1 , I_2, and I_3 to various extends. The IC_{50} values were calculated as 165.3 μM, 499 μM, and 35.62 μM , respectively.

Keywords : Aldose Reductase , bovine lens , cataract

ÖZ

SIĞIR MERCEĞİ ALDOSE REDÜKTAZ ENZİMİNİN KARAKTERİZASYONU VE İNHİBİSYON ÇALIŞMASI

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Aldose redüktaz NADPH' a bağlı bir enzimdir ve diyabete bağlı katarakt oluşumuyla anılmaktadır. Aldose redüktaz insan eritrositi ile pek çok hayvan türünün merceğinde bulunan M_r 37.000 olan bir enzimdir. Hakkında pek çok çalışma yapılmış olmakla beraber, mercekte, retinada ve sinir hücrelerindeki aldose redüktazın fizyolojik rolü hakkında çok az bilgi mevcuttur. NADPH' in varlığında aldose redüktaz glikozu sorbitole çevirir. Sorbitol ise sorbitol dehidrojenaz ile fruktoza oksitlenir. Aldose reduktazın, hayvan dokularındaki çalışmalarındaki aktivitesi katarakt oluşumunun mercekteki aldose redüktaz seviyesi ile direk olarak ilgili olduğunu göstermiştir.

Bu çalışmada aldose redüktaz sığır merceğinden sentrifügasyon ve amonyum sülfat çöktürmesini izleyen DEAE selüloz da iyon değiştirme kromatografisi ile izole edildi. Substrat konsantrasyonu, sıcaklık ve pH gibi şartlar,substrat gliseraldehite karşı maksimum aktivite elde etmek için optimize edildi.

Aldoz redüktaz İnhibisyonu, DE-52 kolonunda, yarı saflaştırılmış enzimin üç yeni sentezlenmiş inhibitörünün, I_1, I_2, ve I_3, kullanılmasıyla çalışıldı. Buna göre IC_{50} değerleri bu üç inhibitör için sırasıyla 165.3 μM, 499 μM ve 35.62 μM olarak bulundu.

Sığır lensi aldose redüktazının en yüksek aktiviteye pH 6-6.5 arasında ulaştığı saptanmış ve enzimin $5x10^{-5}$ M gliseraldehit konsantrasyonunda doyuma ulaştığı görülmüştür. Bu çalışmada aldose redüktazın gliseraldehit için V_{max} ve K_m değerleri sırasıyla 1.695 nmol/da ve 17.86 μM olarak hesaplanmıştır. Aldose Reduktazın $1.75x10^{-6}$ M kofaktör NADPH konsantrasyonu için V_{max} ve K_m değerleri de sırasıyla 2 nmol/da ve 1.69 μM olarak hesaplanmıştır.

Anahtar kelimeler: Aldose redüktaz, sığır gözü, katarakt.

To My Family

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
NADP+	Nicotinamide adenine dinucletide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucletide phosphate (reduced)
BIS	N,N'-methylene bisacrylamide
DEAE	Diethylaminoethyl
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylenediamine
TRIS	Tris (hydroxymethyl) aminomethane

CHAPTER 1
INTRODUCTION

Advances in the treatment of Diabetes Mellitus have resulted in longer life expectancy of diabetic patients. This longer survival, however, had been accompanied by an increased probability for the development of complications secondary to chronic hyperglycemia often associated with Diabetes Mellitus. In patients having diabetes for 15-20 years, one or more of the diabetic complications such as cataractogenesis, retinopathy, epitheliopathy, neuropathy, and nephropathy have been observed. Although the biochemical mechanisms responsible for all these complications not clearly defined, increased sorbitol levels in the respective tissues have been implicated as one of the causative factors (1). The diabetic cataract is an example of this process. The studies, *in vivo* and *in vitro*, on eye lenses have confirmed that raised glucose concentrations lead to increased accumulation of sorbitol which is formed by reduction of glucose in the presence of NADPH and aldose reductase in this tissue (2). Therefore, aldose reductase is strongly associated with the pathogenesis of some symptoms of diabetes. The most reasonable evidence for the role of aldose reductase in sugar cataracts has also emerged from studies with aldose reductase inhibitors.

1.1 Aldose Reductase

Aldose reductase (alditol: $NADP^+$ oxidoreductase, EC 1.1.1.21) which is cytoplasmic and a non-metal containing monomeric enzyme, is an NADPH-dependent oxidoreductase with a broad substrate specificity catalyzing NADPH mediated reaction of aldo-sugars and aldehydes to their corresponding alcohols.

Aldose reductase has been detected and purified to homogeneity from the various mammalian tissues (3-14), such as lens (3-5,8,9), kidney (6,7), brain (12,13), muscle (11-13) and erythrocytes (9,13,14).

The presence of aldose reductase in the eye lens has been first reported by Van Heyningen (15) in 1959. Hayman and Kinoshita (16), in 1965, have described some of the properties of the partially purified bovine lens aldose reductase. Then, in 1976, the purification of the bovine lens aldose reductase to homogeneity has been first described by Sheaff and Doughty (4) who showed that the enzyme has a broad substrate specificity for the compounds having aldehyde function, exhibiting an optimum pH at 5.5. In this study, the enzyme has been purified 15,000 fold and shown to have a molecular weight of 37,000 which was suggested from both molecular sieve chromatography and SDS-PAGE with β-mercaptoethanol. The enzyme structure has been described as a single polypeptide chain behaving as an active monomer. The α-helical content of the enzyme has also been estimated from circular dichroism data as 5% and the pI of the enzyme as 4.85 (4).

It has been reported that the aldose reductase isolated from pig lens has exhibited the molecular weight in agreement with most of the earlier estimates of the molecular weight determined from various other species, such as rat (3), bovine (4), human (8), and rabbit (11) lens enzymes.

Several human ocular tissues including the cornea, conjunctiva, lens, retina and optic nerve have been immunohistochemically examined for the presence of aldose reductase (8). The enzyme localization has been described for cornea, in both the corneal epithelium and endothelium; for conjunctiva, in only a single layer of basal cells; for retina, in some Ganglion and Mueller's cells; for lens, in metabolically active epithelial cells and in lens fibers; and for optic nerve, in axons enclosed in myelin sheaths (8,10). These determinations of enzyme localization in human ocular tissues

have confirmed the earlier observations that have been performed with ocular tissues of various animals (17-20).

The physical and kinetic characterization studies (4,16,20) of aldose reductase suggest that the bovine lens enzyme follows an ordered addition of substrates with NADPH binding first and an ordered release of products with NADP being released last (21). The rate limiting step in the reaction catalyzed by the bovine lens enzyme has been shown to be the isomerization of E.NADP binary complex (21,22). The amino acid sequence of the enzyme has been deduced from the nucleotide sequence of cDNA (23), and also from direct amino acid sequencing (24). The x-ray structure of the enzyme as well as its binary complexes with ATP-ribose (25) and NADPH (26) has been published. In such studies it has been reported that according to the x-ray analysis of the enzyme structure, the active site of the enzyme is highly hydrophobic, and the majority of amino acids lining the cavity are nonpolar (25,26), and in the enzyme-NADPH binary complex, the C-4 of the nicotinamide of NADPH, the reactive end of the molecule, is in close proximity to three polar residues, Cys-298, Tyr-48, and His-110, and one of these residues may be the acid-base catalyst for the oxidation/reduction reactions (26).

In the kidney, the presence of aldose reductase has also been demonstrated (6) and the highest concentrations of enzyme have been found in the inner medulla and papilla, and lower concentrations in the cortex. The enzyme has been found as precisely located in the tubular epithelial cells of the thin limbs of the loop of henle, collecting tubules, the interstitial cells in the inner medulla, the glomerular epithelial cells, the mesengial cells, and the epithelial cells of distal convoluted tubules (27).

In recent years multiple forms of aldose reductase become of great interest from various view points (28-31) and isoforms of the enzyme have been purified from rabbit muscle (11), bovine kidney (32), bovine, rabbit and porcine lens (11,33-36), and also from human tissues (13).

Two structurally different forms of bovine lens aldose reductase have been identified (33) as unactivated form which is sensitive to inhibition by sorbinil and activated form, which is obtained in the presence of oxygen radical generating systems, insensitive to sorbinil. Chromatography of bovine kidney aldose reductase using Matrex Orange A affinity gel have resulted in separation of activated and unactivated enzyme forms (32). The activated and unactivated forms of bovine kidney aldose reductase display different physical and kinetic properties, but are indistinguishable by SDS-PAGE isoelectric focusing or immunodetection by Western blot (32). Das and Srivastava (10) have demonstrated that aldose reductase in human aorta, brain, lens and skeletal muscle exists in the activated and unactivated forms. The unactivated form can be activated in the presence of glucose, glucose-6-phosphate and NADPH. The activated form of aldose reductase exhibited lower K_m for glucose and glyceraldehyde as substrates and V_{max} increased as compared to native enzyme indicating that activated enzyme could more efficiently catalyze the reduction of these substrates (10). All the activated forms of aldose reductase are much less sensitive than the unactivated (native) forms for aldose reductase inhibitors. Ohta et al (37). have isolated two forms of aldose reductase also from dog kidney inner medulla. The high-K_m aldose reductase have exhibited lower isoelectric point, lower activity for aldo sugars and lower sensitivity for aldose reductase inhibitors when compared with aldose reductase. High -K_m aldose reductase has been easily converted into aldose reductase in the absence of dithiothreitol. The generated reductase as revealed immunochemical identity with aldose reductase as well as high-K_m aldose reductase .

The major function attributed to aldose reductase is its role as a promoter of the polyol pathway in which glucose is converted to fructose via the sorbitol formation. In recent years, interest has been generated on this enzyme because of its role in the formation of sorbitol. Since in the polyol pathway, aldose reductase is associated with the conversion of sugar to sugar alcohols, the enzyme also catalyzes the reduction of many hexoses other than glucose to their respective sugar alcohols via the oxidation

of reduced NADPH, such as the reduction of xylose to xylitol and galactose to galactitol (Figure 1).

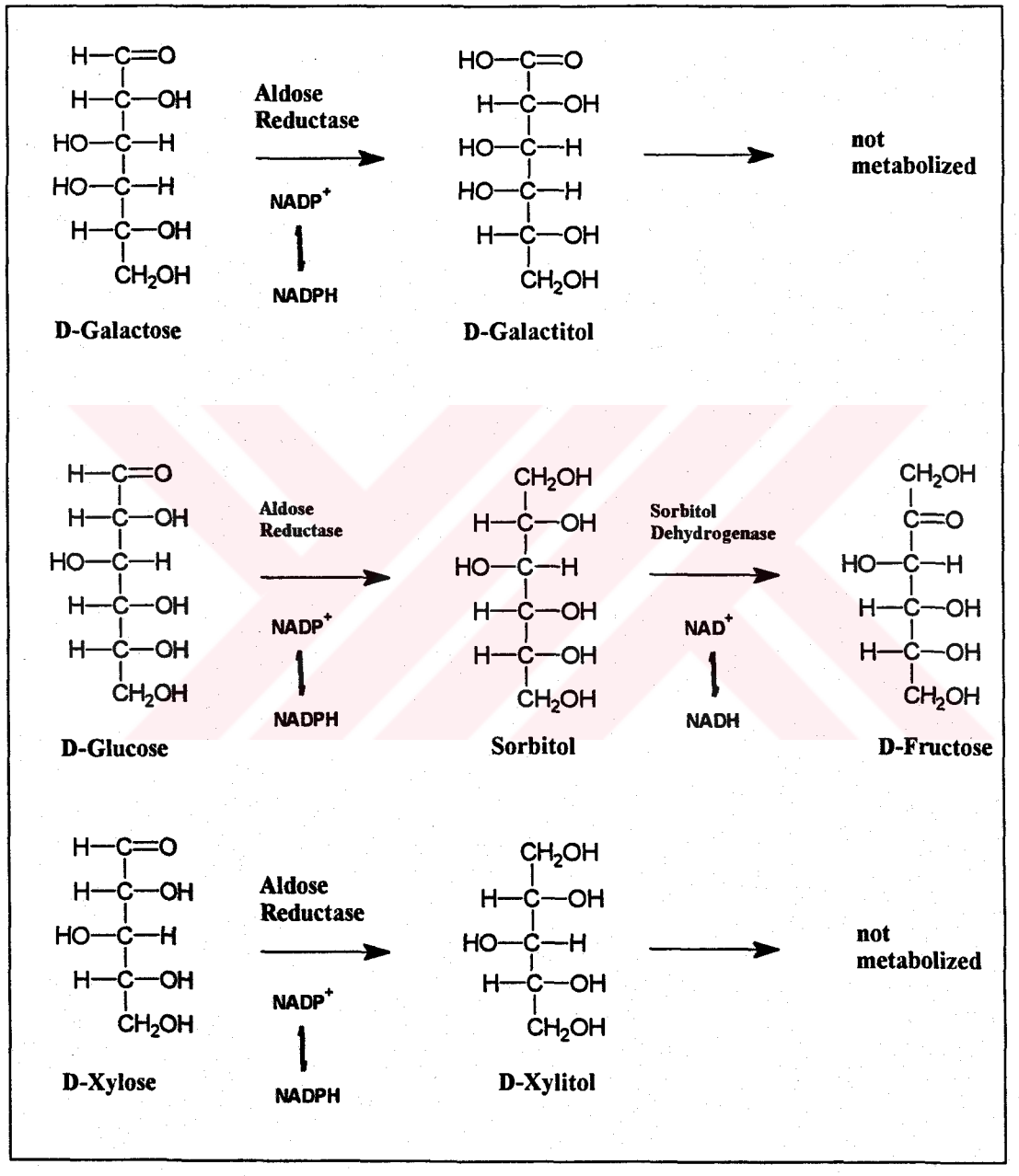


Figure 1. Polyol formation from intracellular monosaccharides by aldose reductase.

1.2 Polyol Accumulation and Diabetic Complications

Under normal conditions, the glucose entering into the cell is rapidly phosphorylated by the enzyme hexokinase and metabolized mainly via the glycolytic pathway to lactate and/or pyruvate providing the major energy source for the cells. A proportion of glucose is also metabolized via pentose phosphate pathway in the lens. However in diabetes and galactosemia, the concentration of glucose and galactose increases several folds. The hexokinase pathway is fully saturated even at normal glucose levels, so that if hyperglycemia occurs such as diabetes mellitus saturation of hexokinase becomes evident, and the excess glucose cannot be metabolized by Embden-Meyerhof (glycolysis) pathway (Figure 2). Therefore the aldose reductase becomes activated and gives rise to the conversion of glucose to sorbitol. Actually, aldose reductase has a much lower affinity for glucose than hexokinase, and therefore, glucose can enter the sorbitol (or polyol) pathway only when its levels are raised.

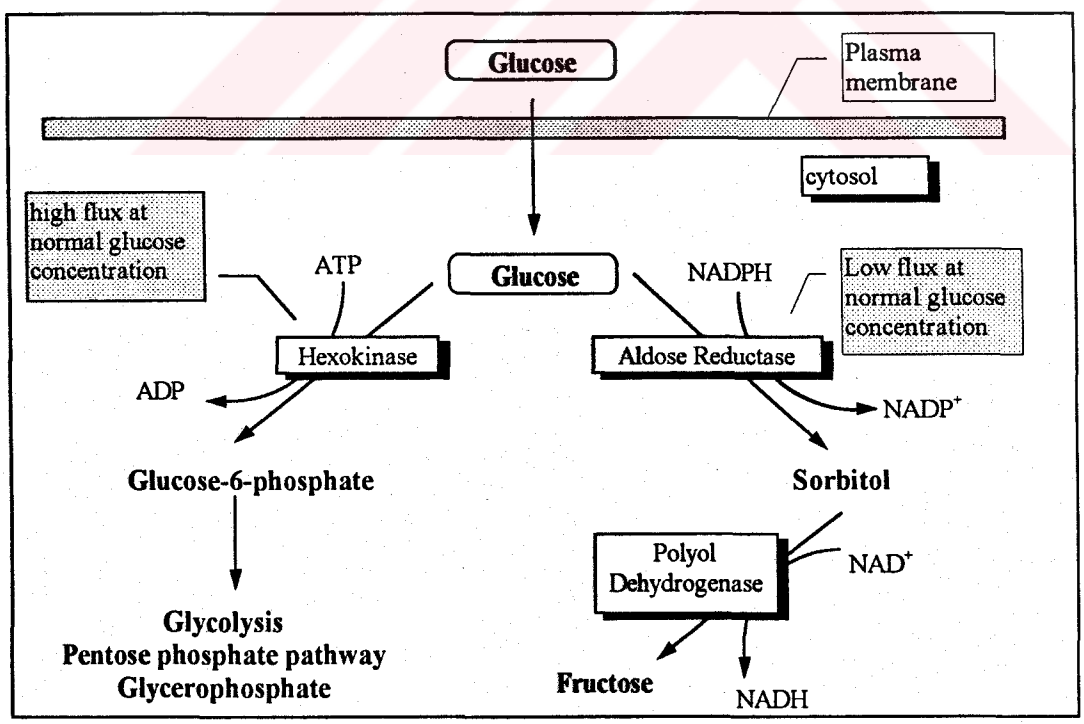


Figure 2. Polyol pathway in accordance with the Embden-Meyerhof (glycolysis) (39)

The reduction of glucose by aldose reductase (Figure 2) is the first and the rate limiting step in the polyol pathway (3). Via this pathway, called polyol, or sorbitol pathway, the sorbitol, is then further oxidized to fructose by the enzyme sorbitol dehydrogenase. Under normal conditions, during the normaglycemia, the polyol pathway constitutes a minor pathway of glucose utilization (approximately 3%) (14).

The physiological significance of this pathway is first described by Hers (1956), however, the aldose reductase-independent intermediate sorbitol in most tissues still remains unknown (33). In this pathway, the carbohydrate molecule of each carbon bearing an hydroxyl group converted to sugar alcohol which, in turn, is oxidized to ketose by the second enzyme of the path, NAD^+-dependent sorbitol dehydrogenase (polyol dehydrogenase, or i-iditol dehydrogenase; EC 1.1.1.14). (Figure 3). This enzyme has the ability to oxidize a variety of sugar alcohols including the sorbitol (Figure 1) but it is inefficient in oxidizing galactitol (dulcitol) thus leading to its accumulation in galactosemia (38).

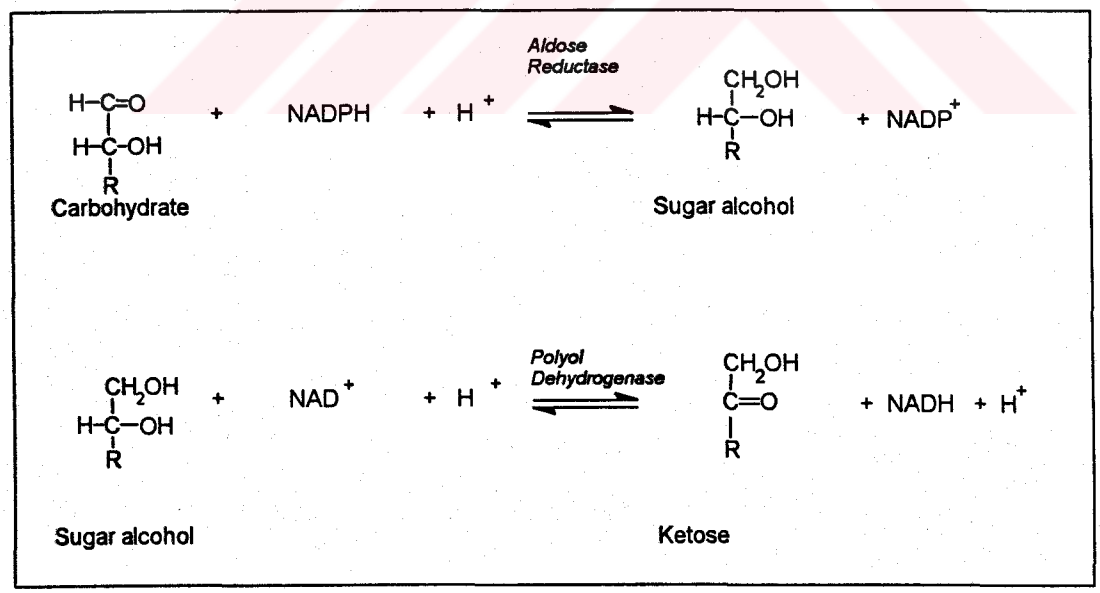


Figure 3. The reduction of aldose by the enzyme aldose reductase to the corresponding alcohol via the oxidation of NADPH and the oxidation of ketose intermediate by the polyol dehydrogenase via the reduction of NAD^+

As aldose reductase activity is increased several fold in response to raised glucose amount (14), the activity of the sorbitol dehydrogenase is not proportionately increased and sorbitol accumulates within the cell. Impermeability of sorbitol through most of the biologic membranes, finally, leads to the sorbitol accumulation that may cause osmotic stress by resulting in electrolyte imbalance, membrane stretching, and the cell death (38). The abnormal levels of sorbitol have been found in tissues from patients with Diabetes Mellitus where the saturation of hexokinase causes the accumulation of polyols. In the lens, therefore, high concentrations of sorbitol probably increase the osmotic pressure enough to cause cellular swelling with eventual disruption and cell death. This process, at the end, can result in lenticular opacity, which is termed as sugar cataract. The role of aldose reductase in the process of sugar cataract formation through *in vivo* animal studies has been first demonstrated by Kinoshita (16). In those studies, it has been revealed that the rate of cataract formation is directly dependent upon the levels of aldose reductase present in the lens (18). Although the etiology of ocular complications associated with diabetes remains unknown, it has been reported that aldose reductase increasingly being linked to these complications (2). The results of these reports (7) supported by the animal studies that reveals the dependence of the appearance of ocular complications associated with diabetes on the aldose reductase-initiated accumulation of sugar alcohols.

In 1984, Kador et al. reported that there are differences between human ocular diabetic complication (Table 1) and those manifests in animals due to the differences in distribution or levels of aldose reductase (8).

Table 1. Aldose reductase localization and related diabetic complications in eye (6,8,10,13).

Tissue	**Localization**	**Diabetic Complications**
Cornea	Epithelium	Keratopathy
Lens	Epithelium	Cataract
Retina	Pericytes of Retina Capillaries	Retinopathy
Optic nerve	Axons enclosed myelin sheaths	Neuropathy

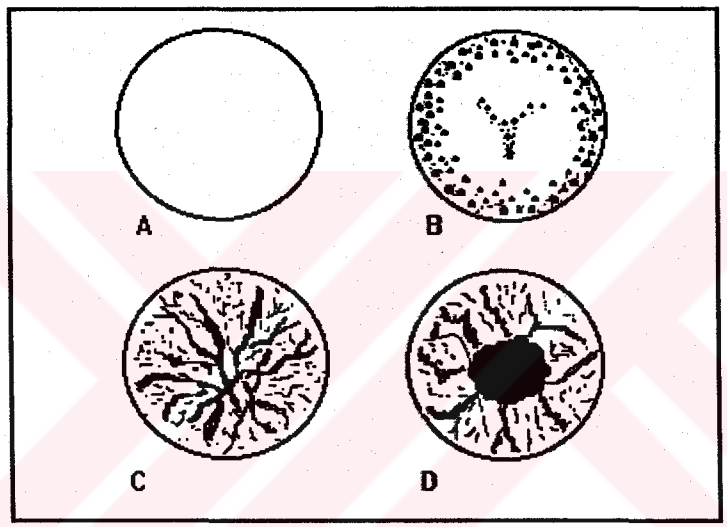
Such studies carried out to explain the diabetic complications of tissues associated with the aldose reductase has been mainly based on the immunohistochemical localization of the enzyme in both the rat and dog eye (40,41). In these studies, several human ocular tissues, including the cornea, conjunctiva, lens, retina and optic nerve have been examined by immunohistochemical assay for the presence of aldose reductase. In human cornea, aldose reductase localization has been found in both the corneal epithelium and endothelium. Although the degeneration of the basal cells of the corneal epithelium under diabetic or galactosemic conditions can be prevented with aldose reductase inhibitors, the physiologic importance of aldose reductase inhibitors in the corneal epithelium is still unknown. Generally, with the exception of the retinal pericytes and rat corneal epithelium, the animal studies reveal an association between the presence of aldose reductase and the appearance of reported diabetic pathology (8).

It is remarkable that the tissues, such as sural nerve, having the lowest sorbitol dehydrogenase and aldose reductase activities display the highest sorbitol accumulation both under basal and diabetic conditions, or vice versa (42). Possibly these are due to the presence of different isoenzymes of aldose reductase and sorbitol dehydrogenase in different tissues and thus the different sorbitol metabolism (43).

It has been suggested that the sorbitol pathway which functions in the production of sorbitol intermediates, contributes to the osmoregulation of certain tissues (1). Kinoshita and his research group has demonstrated that the onset of diabetic complications such as diabetic retinopathy, keratopathy, neuropathy, and nephropathy are also associated with adverse sorbitol production (7).

The formation of diabetic cataracts has been exclusively studied in terms of Aldose Reductase and polyol pathway. Since the polyol pathway is important by means of the development of diabetic complications, it is also important to consider the pathogenesis of diabetic cataracts (16,45-47).

The osmotic hypothesis or polyol theory of sugar cataract development that has been first pronounced by Kinoshita and coworkers in 1974, whom states that the aldose reductase-initiated accumulation of polyols produces hyperosmotic effect resulting the swelling in lens fiber and eventual cataract formation (48). This theory basically explains that the biochemical progression of cataract formation (Figure 4) due to the loss of cellular integrity in cells accumulating sugar alcohols that resulted from an intracellular increase of fluid in response to the aldose reductase-initiated sugar alcohol accumulation.



A, Normal Rat Lens

B, Vacuolar Stage Of Rat Lens

C, Cortical Opacity Of Rat Lens

D, Nuclear Opacity Of Rat Lens

Figure 4: Pictures Of Actual *In vitro* Cataract Formation That Correlate With The Proposed Biochemical Changes(7).

That is, the cataract formation has been related to an osmotic effect owing to the accumulation of polyols, such as sorbitol in diabetic rats or galactitol in galactose fed rats (49). The pictures of actual *in vitro* cataract formation that correlate with the proposed biochemical changes are shown in Figure 4 and Figure 5.

According to polyol theory, the increase in sorbitol and fructose concentration in the diabetic lens causes water to be drawn into the lens fibers, thus causing them to swell (Figure 5-B). Because, the avascular lens initially swells in response to the hyperosmotic effects of intracellular sugar alcohol accumulation (50). This swelling, in turn, leads to altered membrane permeability resulting in a decrease of the normally high potassium to sodium ratio, as sodium enters the cell by diffusion, and resulting a reduction in the lenticular levels of reduced glutathione, myoinositol, ATP and free amino acids (Figure 5-B). Because the entrance of sodium causes the potassium passage out of the cell, together with the chloride levels within the cell increase, the lens swells. In following stages, while the significant alterations of membrane permeability become apparent (50), the lenticular levels of sodium, then, exceed those of potassium and a selective shut-down of lens protein synthesis with loss of dry weight occurs (Figure 5-C). In this stage, those changes result in the swollen lens fibers that eventually rupture with the liquefaction of the fibers to form visible vacuoles initiating cataract formation (Figure 4-B). The progress then continues, as vacuole formation increase towards cortical opacification (Figure 4-C and Figure 5-C) due to the affected protein synthesis in the lens. Afterwards, the lens membrane becomes freely permeable to all constituents except for highly larger proteins, and so cataract progresses to the nuclear cataract stage (Figure 4-D and Figure 5-D). The nuclear cataract stage is the final stage of biochemical cataract progression where a complete loss of osmotic integrity occurs. Moreover, the complete loss of osmotic integrity with completely diffusible electrolytes and amino acids becomes evident as proteins readily penetrate the highly permeable lens (Figure 5-D).

Osmotic hypothesis that can explain the lens complications within the diabetic conditions due to the polyol accumulation can not explain the diabetic complications occurring in tissues other than the lens, although these complications are also due to

the polyol accumulation (Figure 4). This is because the concentrations of sorbitol in tissues are in significant to induce osmotic alterations, and its related changes caused by water accumulation in the cell.

In addition, *in vitro* studies postulating the validity of the osmotic hypothesis revealed that the diabetic lens complications, due to the loss of cellular integrity in cells, in response to the aldose reductase-initiated accumulation of sugar alcohols are more prominent than those complications in response to the self accumulation of sugar alcohols (51).

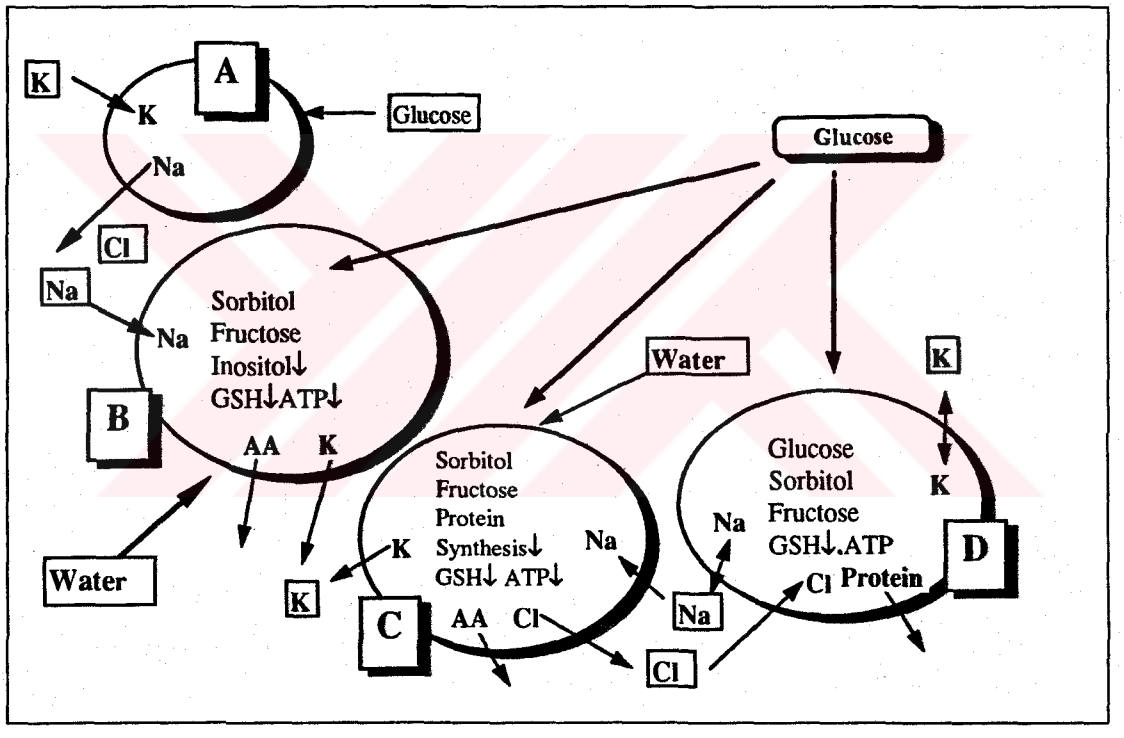


Figure 5: Biochemical changes in the rat lens during the development of sugar cataract.(45,51). (K, potassium ion; Na, sodium ion; AA, amino acid; Cl, chloride ion; GSH, reduced glutathione).

It has been demonstrated that (19) in young diabetic rats, if the blood glucose level is maintained at 2.5 mg/ml blood (12), the cataract of the lens inevitably develops as in the cases where the young rats have fed xylose or galactose. These three forms of cataract that resemble each other clinically are called as 'sugar cataracts' because

there is a raised level of monosaccharides in the blood in all those cases. Although cataracts develop in both diabetic and galactose-fed animals at a rate directly depending on the amount of Aldose Reductase present in the lens (19), in mice, where lens Aldose Reductase is extremely low, cataracts do not develop in either of these metabolic states.

1.3 Aldose Reductase Inhibitors And The Inhibition Of Aldose Reductase In Sugar Cataract

Under hyperglycemic conditions, aldose reductase is involved in the development of neuropathies, nephropathies, angiopathies and sugar cataracts in diabetics. Aldose reductase inhibitors, compounds that block the flux of glucose through polyol pathway, have been shown to attenuate, prevent, and in some cases to reverse the hyperglycemic tissue injury(2,45). There are many considerable studies supporting the effects of aldose reductase inhibitors on sugar cataracts. Many studies in diabetic animals have demonstrated that treatment with aldose reductase inhibitors normalizes tissue polyol levels (17,43,52) by which it has beneficial effects on development of cataracts. Recently, many aldose reductase inhibitors have been shown to affect erythrocyte sorbitol levels in diabetics (9,13,14,46). Therefore aldose reductase constitutes an important pharmacological target for the clinical management of secondary diabetic complications. Since aldose reductase inhibitors inhibit the pathogenesis of diabetic complications at a point after hyperglycemia, they do not pose the risk of hypoglycemia (53, 54). Additionally because these compounds have no effect on plasma glucose levels they can be used as an adjuvant to therapies that attempt to rigorously control blood glucose. As a result, great interest has been placed over the years on the development of potent aldose reductase inhibitors.

Aldose reductase inhibitors can be categorized in four groups: carboxylic acids, hydantoins, flavonoids and other compounds. The majority of inhibitors that have been used for human clinical trials are the members of the carboxylic acid category (Epalrestat, statil, tolrestat and ponalrestat) containing carbonyl or thiocarbonyl

groups. These reactive groups allow accepting a pair of electrons from the enzyme thus leading to its inhibition. There have been only few inhibitors, such as sorbinil, used in clinical trials belong to the hydantoin category (6). As reported, flavonoids, however, have not reached the clinical trials (54). Some of the inhibitor structures, used in the human clinical trials (55-60), shown in Figure 6.

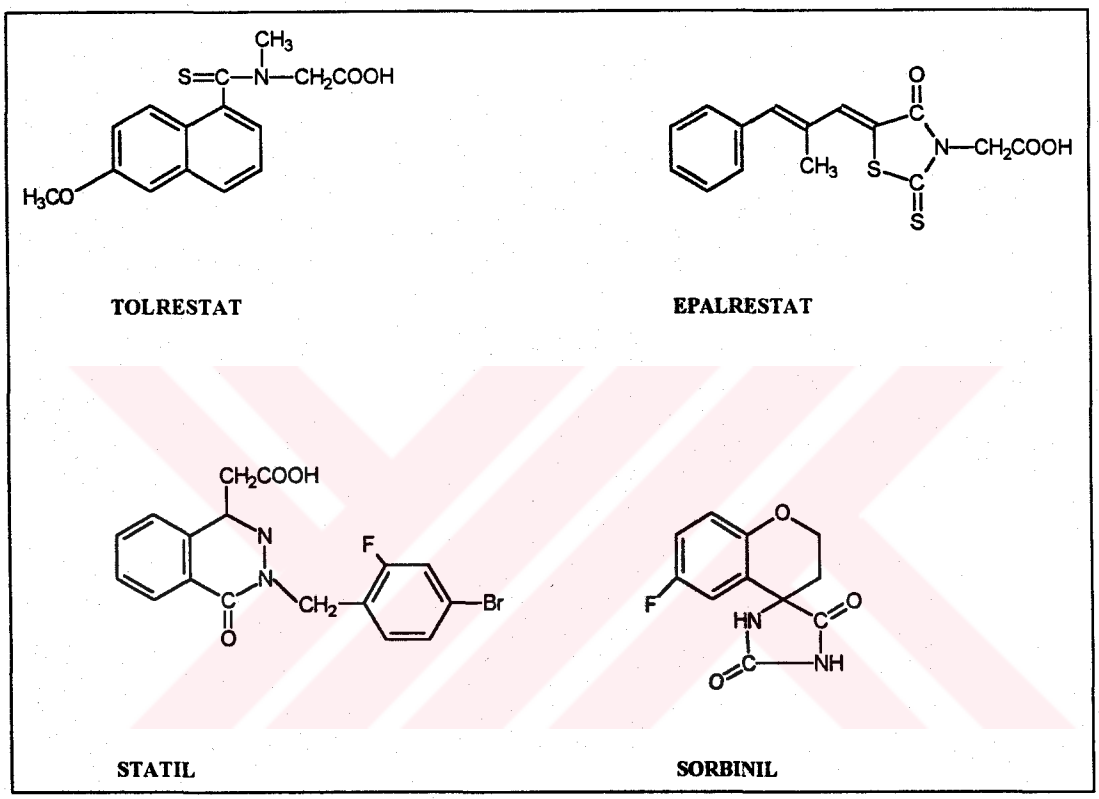


Figure 6. Some of the aldose reductase inhibitors used in human clinical trials (55-60)

The studies for the inhibition of aldose reductase that successfully prevent the onset of cataract, in the middle 1960's, began with the observation that long chain fatty acids inhibit aldose reductase in lens homogenates (16). Therefore, the long-chain fatty acids were the first known inhibitors of aldose reductase.

Octanoic acid, one of the aldose reductase inhibitors, is such a compound that substantially deficient of redox potential, was the first promising inhibitor to produce

59% inhibition at 0.1 mM against bovine lens aldose reductase (16). This led to the development of tetramethylene glutaric acid (TMG) and its analogues as inhibitors (61).

In 1968, it has been demonstrated that tetramethylene glutaric acid (TMG) could alter the cataractous process by modifying the activity of aldose reductase (44). Also it has been demonstrated that by reducing the synthesis and accumulation of galactitol in *in vitro* cultured lenses, TMG minimized increases in lens hydration and the appearance of vacuoles; however, its inability to penetrate the membranes made the compound ineffective *in vivo*. There are also many considerable studies supporting the effects of aldose reductase inhibitors on sugar cataract. Therefore, a number of more potent inhibitors have been developed with the ability to delay or prevent the cataract formation proportional to both their potency and ability to achieve appropriate concentrations in the lens (7).

These include the potent hydantoin sorbinil (S-6-fluoro-spirochroman-4-5′-imidazolidone-2′,4′-dione) has been utilized to demonstrate that induced sugar cataracts, either by diabetes or 50% dietary galactose feeding animals, could be prevented with Aldose Reductase inhibitors. Moreover, it has been also demonstrated that cataract formation could be reversed through treatment of sorbinil; however, the administration of these aldose reductase inhibitors required at the early stages of cataract formation (62,63,). Alrestatin, 3-dioxo-1-H-benz[de] isoquinoline-2(3H)-acetic acid, (AY-22284) has been reported as the first orally effective inhibitor to delay the appearance of the nuclear opacities in galactose fed rats (19).

In 1975, the inhibitory action of various flavanoids had tested and all the flavanoids tested had significant inhibitory activity, and in those study it has shown that quercetin, quercitrin and myricitrin were much more effective as aldose reductase inhibitors than either AY 22,284 (19) or TMG (17,44). Although it has known that flavanoids are non-toxic compounds, as we mentioned before it has not reached the clinical trials.

Another inhibitor reported is N-(-3-nitro-2-pyridyl)-3-methylanaline (AY-20263) that could delay the cataract formation (48), but the solubility complications of the compound necessitated its administration by intraocular injection as a dimethyl sulfoxide solution. Statil (ICI 128,436) which is a potent non-spyrohydantoin-related aldose reductase inhibitor has reduced the increased lens sorbitol and fructose levels in streotozotocin diabetic rats (43).

Clinical experience has been reported for a number of aldose reductase inhibitors, most notably tolrestat, sorbinil, ponalrestat, epalrestat, imirestat and ADN-138 (64-76). Tolrestat has been shown to improve nerve ultra structure and function after long term treatment and is currently approved for marketing in several countries (64-76). Epalrestat is currently approved for marketing in japan. The trials of sorbinil was first reported in 1983 (77). Although sorbinil penetrates well into human lens and erythrocytes and treatment with it improved both the structure and function of nerves in diabetic neuropathic patients, it was withdrawn from clinical trials because of toxicity.(66,71,78). Ponalrestat, imirestat, and ADN-138 have also been withdrawn clinical trials due to toxicity or lack of efficacy.

Orally active aldose reductase inhibitors are limited for the most part to two classes, the carboxylic acids and the cyclic imides (69-71,79,80). Even though both series have similar intrinsic activity for the isolated aldose reductase , their *in vivo* potencies are quite different. *In vitro,* both series reduce aldose reductase activity by 50 % at approximately 10^{-8} M concentrations (79,80). *In vivo,* in the 4-days galactosemic rat animal model, the carboxylic acids reduce polyol accumulation in the sciatic nerve by 50 % at doses ranging from 3.0 to 15.0 mg/kg/day, while the imides require only 0.1-0.5 mg/kg/ day for the same response (77). The increased potency of the imide type aldose reductase inhibitors is possibly due to better pharmacokinetic properties, including absorption and tissue penetration. Tolrestat was known as the most effective aldose reductase inhibitors within the carboxylic acid series.

The kinetics of most of the aldose reductase inhibitors has also studied by many researchers. Stribling et al (43) described the effects of ICI 128,436 (statil) a potent

inhibitor of aldose reductase *in vivo* and vitro. They showed that it inhibits enzymes isolated from rat, bovine and human sources. Studies have shown that ICI 128,436 is a noncompetitive inhibitor (inhibitor binds at a site other than the active site of the enzyme) with respect to aldehyde substrate.

In a study that examined kinetics of inhibitors to human kidney aldose reductase , the inhibitor sorbinil exhibited noncompetitive inhibition to both the aldehyde substrate and coenzyme NADPH (81). However, in that study two other inhibitors, tolrestat and statil, exhibited uncompetitive inhibition (inhibitor binds to the enzyme-substrate complex thereby affecting both the V_{max} and K_m) towards the aldehyde substrate but exhibited like the inhibitor sorbinil noncompetitive inhbition to the coenzyme NADPH (6).

It has been reported that using drugs which are the aldose reductase inhibitors, the onset of cataracts can be significantly delayed in the experimental animals, if the aldose reductase inhibitors is given at the onset of the disease (82). The cataractogenic process can only be reversed during the early stages prior to the onset of nuclear cataract (83).

It has been indicated that the inhibition of aldose reductase by some special chemicals is dependent on the degree of purification of the enzyme as well as on the substrate used to assay the activity (33). A wide variety of compounds have been shown to inhibit the aldose reductase reaction, as measured by the change in absorbance at 340 nm (oxidation of NADPH), when oxidizing monosaccharides are used as substrates. The aromatic hydrocarbonyl groups aid in the attachment of the inhibitor to the enzyme (2,51). In this case, the NADPH oxidation assay is invalid as a measure of aldose reductase, and so these compounds must have alternative modes of activation that relate to the free radical mechanism of the NADPH oxidation. In this respect, quercitrin, and sorbinil all inhibit *in vitro* free radical mediated-NADPH oxidation in the presence or absence of the NADPH-binding protein glutathione reductase (2).

The variation in sensibility to the inhibition, possibly be related to the loss of effectors stabilizing the 'inhibitor binding site' (33) during the purification as suggested by the effect of NADPH in the studies in which it maintains the sensibility to inhibition of the human placenta enzyme(84).The variable response of aldose reductase to inhibitors could also be attributed to the presence of multiple forms of the enzyme displaying in different sensitivity to inhibition. In this regard, two forms of aldose reductase in rabbit muscle and lens (11,36) have been described and the possibility of interconversion of two enzyme forms has been reported for Aldose Reductase of human erythrocyte (85).

Activation process leads to the generation of a form of aldose reductase which is insensitive to the inhibition by sorbinil. The activation process of aldose reductase occurs during incubation of bovine lens extracts with oxygen radical generating systems (86). It has been often observed that the activation processes of the enzyme causing the differences in sensitivity to inhibition of the activated and inactivated states of the enzyme which accounts for the reduction in the inhibitory effect of sorbinil during the purification of the aldose reductase, when conditions leading to enzyme activation occur (87,88). Since the sensitivity to the inhibition and the activation of enzyme appeared to be correlated, the sorbinil lose its inhibitory capacity before it reaches to its maximal activation. Accordingly, it has been suggested that the difference in the capacity of inhibition is due to the presence of multiple sites of oxidative modification, one of which probably affects inhibition by sorbinil while one or more yields activation (33).

1.4 Scope Of The Work

Better clinical management has resulted in the prolonged life span of diabetic subjects. In patients having diabetes for 15-20 years, one or more of the diabetic complications such as cataractogenesis, retinopathy, neuropathy, and nephrýpathy have been observed. Although the biochemical mechanisms responsible for all these complications is not clearly defined, increased sorbitol levels in the respective tissues

have been implicated as one of the causative factors. Sorbitol in tissues is formed by the reduction of glucose by aldose reductase.

The role of aldose reductase in diabetic and galactosemic cataractogenesis has been well established because aldose reductase inhibitors prevent or significantly delay cataractogenesis. Therefore, many inhibitors of aldose reductase have been intensively studied for possible use in the prevention of diabetic complications.

In this study, we aimed to establish the optimal conditions for the bovine lens aldose reductase and to find out the characteristics of aldose reductase. For this purpose , aldose reductase was isolated and partially purified by ammonium sulfate precipitation and DE-52 column chromatography.

A spectrophotometric assay was used for determination of aldose reductase activity by monitoring the reduction of NADPH to $NADP^+$ at 340 nm. The reaction conditions, such as enzyme amount, pH of the reaction mixture, NADPH concentration, substrate glyceraldehyde concentration, effect of temperature, on the reaction rate were optimized. Inhibition of the partially purified bovine lens aldose reductase by newly synthesized inhibitors which have the chemical structures given in the Figure 7 were studied.

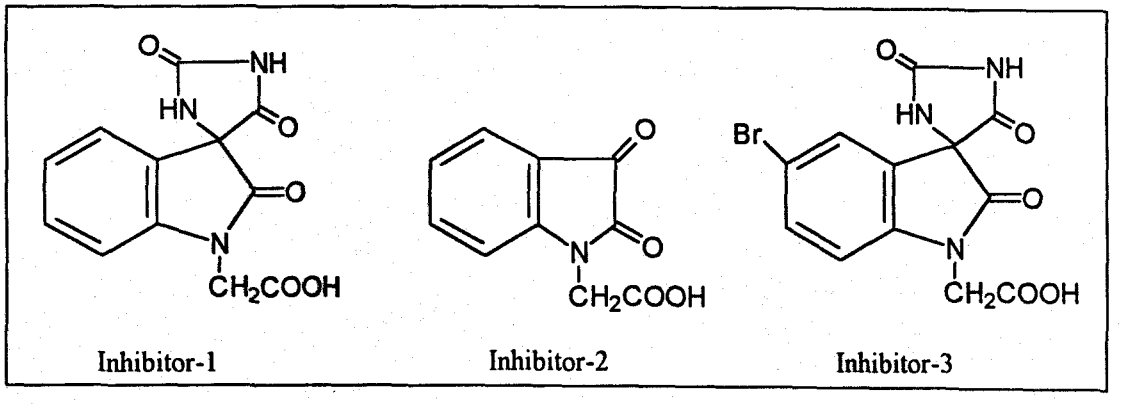


Figure 7. Chemical structures of the newly synthesized aldose reductase inhibitors.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Ammonium sulfate, DL-glyceraldehyde, bovine serum albumin (BSA), lithium sulfate, nicotinamide adenine dinucleotide phosphate (NADPH), sodium chloride (NaCl), molecular weight markers, ammonium persulfate, ß-mercaptoethanol, diethylaminoethyl-cellulose (DE-52), sucrose, N,N'-methylenebisacrylamide (BIS), hydroxymethyl aminomethane (TRIS), N,N,N',N'-tetramethylenediamine (TEMED) cellulose, acrylamide and membrane dialysis tubings were all purchased form Sigma Chemical Company, Saint Louis, Missouri, USA. The aldose reductase inhibitors used in this study were synthesized and kindly donated by Prof. Dr. Erdem Büyükbingöl from Faculty of Pharmacy, Ankara University.

2.2 Methods

2.2.1 Preparation of Aldose Reductase Crude Extract From Bovine Lens

The bovine eyes were obtained from local abattoir (Et ve Balik Kurumu-Ankara) immediately after slaughtering, and were placed in plastic bags packed in crushed ice. Upon reaching the laboratory the lenses were excised from the eyes and washed with distilled water. Lenses were weighed and homogenized in three volumes of cold distilled water in a Potter-Elvehjem homogenizer coupled with a motor (Black and Decker, V850 , multispeed drill)-driven Teflon pestle at 2400 rpm, and centrifuged

(Sorvall RC-2B Automatic Refrigerated Centrifuge, Ivan Sorvall Inc.,Newton, Connecticut 06740, USA) by using SS-34 rotor at 10,000x*g* for 30 minutes to remove insoluble material.

The supernatant was saturated with ammonium sulfate to 40% and saturated sample was centrifuged at 10,000x*g* for 25 minutes. Pellet was discarded and supernatant was brought to 50% ammonium sulfate saturation to remove additional proteins. The 50% ammonium sulfate saturated sample was centrifuged at 10,000x*g* for 25 minutes. Pellet was discarded, and the supernatant was further saturated to 75% and centrifuged at 10,000x*g* for 25 minutes. Supernatant was discarded and pellet was dissolved in minimum quantity of 0.05 M NaCl. The volume of the suspension was recorded and the sample was dialyzed overnight against 0.05 M NaCl (double replacement of the dialysis solution).

The volume of the sample was recorded after dialysis and the sample was stored in 0.5 ml aliquots in eppendorf tubes or in 15 ml fractions in storage bottles in deep freezer at –80°C for determination of protein and aldose reductase activity or for further purification.

2.2.2 Partial Purification of Bovine Lens Aldose Reductase by Ion-Exchange Chromatography

Bovine lens aldose reductase was partially purified using DEAE-cellulose column chromatography. The bovine lens (crude extract) was applied to DE-52 column with the dimension of 1.5 cm X 22 cm and the activity peaks recovered from the gradient elution were referred to as partially purified aldose reductase.

2.2.2.1 Preparation of The Chromatography Columns

A) Regeneration of DEAE-cellulose (DE-52) : The ion exchanger (approx. 50 ml) was soaked in 500 ml of 0.5 N HCl for 30 to 45 minutes stirring it gently from time

to time using a glass rod. Then the resin was filtered through a coarse filter paper and washed several times by soaking in deionized water until the pH was around 4.0. The same treatment was then repeated using 0.5 N NaOH until the pH was around 8.0. Finally, most of the supernatant liquid was removed and the DE-52 was soaked in 0.5 M phosphate buffer, pH 6.8, and stored in the refrigerator to be used later.

B) Equilibration of the Column : After the ion exchanger was regenerated and packed into the column (1.5 cm X 22 cm), it was equilibrated with about 1 liter of 0.01 M phosphate buffer, pH 6.8 , at a flow rate of 48 ml/hour, at 4°C.

2.2.2.2 Preparation and the Application of Samples

The 22 ml sample from the dialyzed bovine lens crude extract having about 8250 units of Aldose Reductase activity was applied into the DE-52 column at a flow rate of 40 ml/hour. The column was washed, using 500 ml of the equilibration buffer, 0.01 M phosphate buffer, pH 6.8, at a flow rate of 48 ml/hour. Around 5.5 ml were collected until no absorption at 280 nm was detected.

2.2.2.3 Gradient Elution

After adequate washing , the bovine lens anionic peaks were eluted from the DE-52 columns with a linear 400 ml phosphate buffer gradient of 10 mM to 200 mM pH 6.8. Fractions about 5.5 ml were collected during the gradient elution using a flow rate of 40 ml/hour.

2.2.2.4 Fraction Analysis

The protein concentrations of the collected fractions were determined spectrophotometrically by absorbance measurement at 280 nm. The aldose reductase activity was determined spectrophotometrically by monitoring the oxidation of NADP

at 340 nm as will be described later in the analytical procedures and expressed as nmoles NADPH oxidized / min/ml of effluent.

2.2.3 Analytical Procedures

2.2.3.1 Protein Determination

Protein concentration of samples were determined according to the method of Lowry et al (1951) by using the crystalline BSA as a standard. The aliquots of 0.1 to 0.5 ml of 1:500 diluted samples were taken into test tubes and were completed to the final volume of 0.5 ml with distilled water. Then they are mixed with 2.5 ml alkaline copper reagent which was prepared by mixing 2% copper sulfate, 2% sodium potassium-tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively.

The mixture then allowed to stand for 10 minutes at room temperature (25°C). 0.25 ml of 1 N Folin reagent was added. Each tube containing the mixture completely mixed within 8 second by vortex before addition of the Folin reagent to the next pair of tubes. After waiting 30 minutes at room temperature (25°C), the intensity of color developed in each tube were measured at 660 nm . The standard calibration curve of 0 to 0.2 mg BSA (Figure 7) was constructed and it was used in determining the protein concentration of the samples.

2.2.3.2 Determination of Aldose Reductase Enzyme Activity

The aldose reductase activity was measured spectrophotometrically according to the method Kinoshita et al (16). The method is based on the reduction of DL-gyceraldehyde as a substrate to glycerol by aldose reductase during which NADPH is oxidized to $NADP^+$. The oxidation of NADPH was followed spectrophotometrically at 340 nm using spectronic 21 spectrophotometer. In this study, the bovine lens crude

extract or partially purified aldose reductase, that were prepared as previously described, was used as the enzyme source.

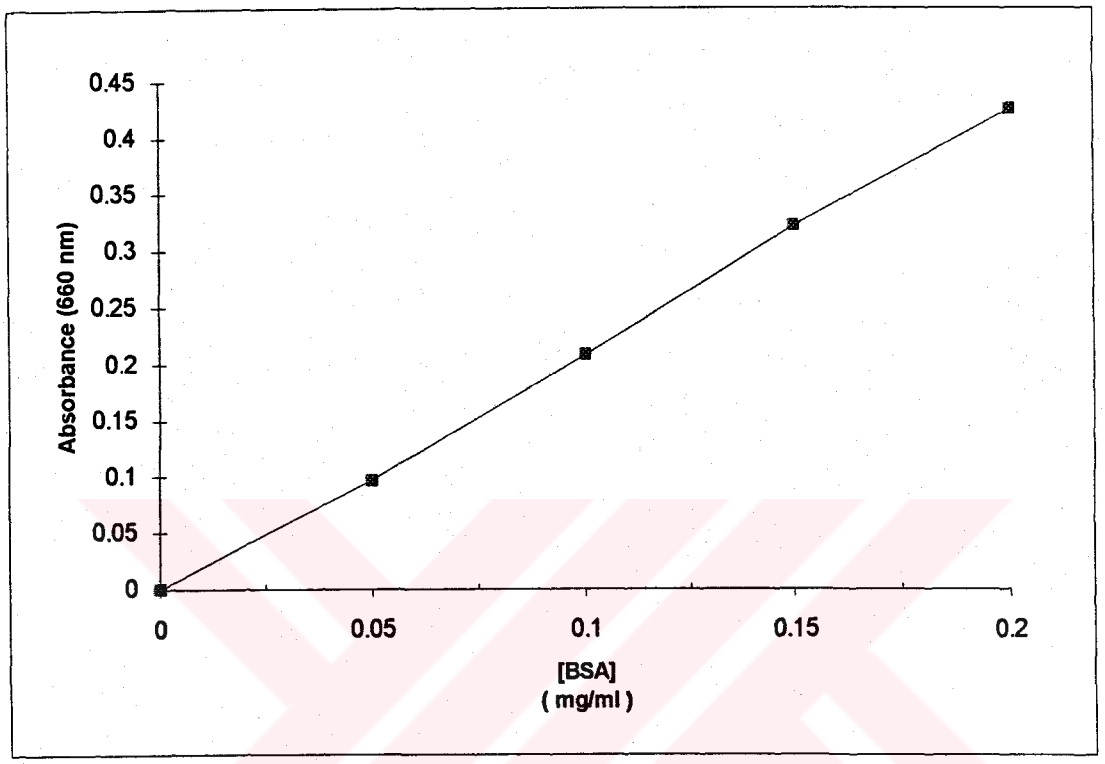


Figure 8: BSA Standard curve for protein determination.

As shown in Table 2, typical assay mixture contained $5x10^{-4}$ M freshly prepared DL-glyceraldehyde as substrate, approximately 2.15 mg partially purified aldose reductase, 0.4 M lithium sulfate , $5x10^{-5}$ M NADPH and 50 mM potassium phosphate buffer, pH 6.2 in a final volume of 1.0 ml.

All the ingredients of the reaction mixture, except DL-glyceraldehyde were pre-incubated for 3 minutes in the cuvette. The reaction was initiated by the addition of DL-glyceraldehyde and followed by monitoring the decrease in the absorbance at 340 for 5 minutes. The decrease in the absorbance in the reaction mixture without glyceraldehyde was also recorded under the same conditions as background (blank). The difference in slopes (ΔOD 340nm/min) of reaction and the blank measurements

and the extinction coefficient of 6220 mM^{-1} were used for the calculation of aldose reductase activity. The specific activity was expressed as nmoles NADPH oxidized per minute per mg protein. One unit of enzyme was defined as the amount of enzyme catalyzing the oxidation of 1 nmole NADPH per minute.

Table 2 : The Constituents of the Reaction Mixture for aldose reductase

Constituents	**Volume Added (µl)**	**Stock Soln**	**Final Conc.**
Glyceraldehyde	100	$5X10^{-3}$ M	$5X10^{-4}$ M
NADPH	35	$1.5X10^{-3}$ M	$5X10^{-5}$M
50 mM buffer pH 6.2	500	—	—
Li_2SO_4	200	2M	400mM
Aldose reductase	50	—	—
H_2O	to complete final volume to 1ml	—	—
Total Volume	1 ml.	—	—

2.2.4 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of anionic detergent, sodium dodecyl sulfate (SDS), was performed on the 4% stacking gel and 12% separating gel in a discontinuous buffer system as described by Laemmli (1970).

The five proteins given below were obtained as a lyophilized mixture from Sigma and they were used as molecular weight standards:

-Bovine Albumin (Mr 66000)
-Egg Albumin (Mr 45000)
-Carbonic Anhydrase (Mr 29,000)
-Trypsinogen (Mr 24,000)
-Trypsin Inhibitor (Mr 20,100)

2.2.4.1 Preparation of Reagents

A) Stock Separating Gel Buffer (1.5 M Tris-HCl, pH 8.8)

36.3 g Tris base were dissolved in about 100 ml distilled water and the pH was adjusted with 1 M HCl. Finally completed to 200 ml.

B) Stock Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8)

12.1 g Tris base were dissolved in about 100 ml distilled water and pH 6.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

C) Stock Gel Solution (Acrylamide-BIS, 20%A, 2.67% C)

60.0 g acrylamide were dissolved in about 175 ml distilled water and then 1.6 g bis-acrylamide was added and solution was filtered through course filter paper.

Note: %A represents acrylamide monomer percent concentration and %C indicates the cross-linking monomer concentration which were calculated as below:

% A =[(g acrylamide / total volume)] x 100
% C =[(g BIS) / (g acrylamide + g BIS)] x 100

D) 10% SDS Solution

10 g SDS were dissolved in water with gentle stirring and completed to a final volume of 100 ml.

E) Catalyst (10% Ammonium Persulfate)

Prepared freshly by dissolving 100 mg ammonium persulfate in a final volume of 1 ml distilled water.

Prepared freshly by dissolving 100 mg ammonium persulfate in a final volume of 1 ml distilled water.

F) Tracking Dye (0.05% Bromophenol)

Tracking dye solution was prepared by dissolving 5 mg solid bromophenol blue in a final volume of 10 ml.

G) 5 X Electrode (Running) Buffer (25 mM Tris , 192 mM Glycine, pH 8.3)

Stock buffer solution for the upper and the lower buffer reservoirs was prepared by dissolving and completing 15 g Tris base and 72 g glycine to 1 liter distilled water, which was designated as 5 X buffer. The pH of the buffer was not adjusted with acid or with base. This buffer was diluted 1:1 and 1 g solid SDS was added to 1 liter of buffer before use.

H) 4 X sample Dilution Buffer (SDS Reducing Buffer)

0.25 M Tris-HCl buffer, pH 6.8 containing 8% SDS, 40% glycerol, 20% ß-mercaptoethanol, 0.004% bromophenol blue. It was prepared by mixing the following volumes of given solutions:

2.5 ml	1 M Tris-HCl, pH 6.8
4.0 ml	Glycerol
2.0 ml	ß-mercaptoethanol
0.8 ml	Tracking Dye
0.8 ml	10% SDS
Distilled water to 10.0 ml	

2.2.4.2 Preparation of Electrophoresis Gels

The 12% separating gel polymerizing solution was prepared just before use by mixing the given volumes of stock solutions in the written order as below.

12.0 ml	Acrylamide-BIS Gel Solution
10.035 ml	Distilled water
7.5 ml	Separating Gel Buffer
0.3 ml	10% SDS
0.15 ml	Catalyst
0.015 ml	TEMED

The 4% stacking gel polymerizing solution was prepared just before use by combining the following volumes of stock solutions in order as indicated below.

1.3 ml	Acrylamide-BIS Gel Solution
6.1 ml	Distilled Water
2.5 ml	Stacking Gel Buffer
0.1 ml	10% SDS
0.05 ml	Catalyst
0.01 ml	TEMED

2.2.4.3 Procedure

Vertical slab gel electrophoresis was carried out using two glass plates (18 X 20 cm) forming a gel sandwich. This gel sandwich was assembled by laying down one of the glass plates on a clean and smooth surface , then spacers (1 mm) , followed by the second glass plate and finally fixing the two glass plates together with a pair of metal clamps form each side. Assembled gel sandwich was placed vertically in a small reservoir containing hot agar solution and left 30 minutes until the agar cooled down.

The separating gel polymerizing solution was poured into the gel sandwich from the edge of one of the spacers until the desired height of the solution (14-15 cm) in the sandwich was obtained. Using a syringe, the top of the gel polymerizing solution was covered with a layer of distilled water, approximately 2 mm thick, by gently squirting against the edge of one of the spacers to ensure the formation of a smooth gel surface. The gel was then allowed to polymerized at room temperature for 30 minutes. After polymerization, the layer of water was removed completely using filter paper without touching the gel surface.

The stacking gel polymerizing solution was prepared and pour into the gel sandwich over the polymerized separating gel along an edge of one of the spacers until the sandwich was filled completely. Then 1.00 mm Teflon comb with 15 well formers was inserted into the layer of stacking gel polymerizing solution without trapping air bubbles in the tooth edge of comb and the gel was then allowed to polymerize for 30 minutes.

2.2.4.4 Preparation of Samples and Molecular Weight Standards

Aliquots from the protein samples to be analyzed and from the standard mixtures were diluted 3:1 with the 4X sample buffer and water (1 part protein 1 part sample buffer and 2 parts water) to obtain 62.5 mM Tris-HCl buffer, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. Then the samples and the standards were put in a boiling water bath for 5 minutes.

2.2.4.5 Loading the Gel

The Teflon comb carefully removed without hurting the wells. Then, wells were filled with electrode buffer using a Pasteur pipette to remove any air bubbles if present. The assembled gel sandwich was cut carefully from the outer sides of the glass plates, and

it was then placed vertically in the lower buffer reservoir which was half filled with the electrode buffer.

Using a 25µl Hamilton syringe with a tipped needle, crude extracts of 5, 10, 15 µl and partially purified sample about 20 µl and molecular weight standards about 15 µl were loaded into one or more wells carefully as thin layer at the bottom of the wells. This was ensured because of the glycerol added to the 4X sample dilution buffer which made the protein solution density greater than the electrode buffer's density.

2.2.4.6 Running The Gel

After sample application , the top side of the gel was connected to the upper buffer reservoir, which was completely filled with the electrode buffer, with two layers of Whatman filter paper. Then the electrodes were fixed properly into the electrode buffer in the upper and the lower reservoirs and connected to the power supply (Shandon Southern V500/150) and was run at 10 mA constant current through the stacking gel. When tracking dye entered into separating gel, the current was increased to 20 mA. The power supply was turned off when the tracking dye reached to 8-9 cm form the beginning of the separating gel. The total running time was approximately 6 hours.

2.2.4.7 Silver Staining of The Gel

After electrophoresis was completed, the gel sandwich was taken and the clamps were removed. The gel sandwich was placed horizontally and the upper glass was removed carefully leaving the gel on the lower glass. The left-top corner of the gel was cut to indicate the order of wells and it was also cut from the dye track. Then the gel was removed carefully from the lower plate and subjected to silver staining according to Blum et al. (1987) , as explained in Table 3 .

2.2.4.8 Mobility Calculation

The relative mobility (R_f) of each protein was determined by dividing its migration distance from the top of the separating gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

The R_f values (abscissa) were plotted against the known molecular weights (ordinate) on semi-logarithmic paper, and standard line was drawn and used for calculating the molecular weight of proteins.

Table 3: Procedure for Silver Staining of Proteins in Polyacrylamide Gels.

Steps	Solutions[a]	Time of Treatment[b]
1) Fix	50% MeOH; 12% AcOH 0.5 ml 37% HCOH / l	≥ 1 h
2) Wash	50% EtOH	3 X 20 min
3) Pretreat	$Na_2S_2O_3.5H_2O$ (0.2 g/l)	1 min [c]
4) Rinse	H_2O	2 X20 sec[c]
5) Impregnate	$AgNO_3$ (2 g/l); 0.75 ml 37% HCOH / l	20 min
6) Rinse	H_2O	2 X 20 sec[c]
7) Develop	Na_2CO_3 (60g/l) 0.5 ml 37% HCOH/l; $Na_2S_2O_3.5H_2O$ (4 mg/l)	10 min
8) Wash	H_2O	2 X 2 min
9) Stop	50% MeOH ; !2% AcOH	10 min
10) Wash	50% MeOH	≥ 20 min[d]

[a] Solutions freshly prepared in a quantity that is 10 fold larger than the volume of the gel.

[b] Steps 1-10 were carried out on a shaker at room temperature (20-25^0 C) .

[c] The times indicated here should be observed exactly in order to ensure a reproducible image development.

[d] After step 10, gel is transferred to 4°C for storage.

2.2.5 Inhibition of Bovine Lens Aldose Reductase

Three different newly synthesized inhibitors were used for testing their inhibitory effects on aldose reductase enzyme activity. The stock inhibitor solutions were prepared as described below:

- I_1 solution (0.1 M): 27.5 mg inhibitor-1 was dissolved in 0.05% dimethylsulfoxide (DMSO).
- I_2 solution (0.1 M): 22 mg inhibitor.-2 was dissolved in 0.5% dimethylsulfoxide (DMSO).
- I_3 solution (0.1 M): 35 mg inhibitor.-3 was dissolved in 0.5% dimethylsulfoxide (DMSO).

The desired concentrations of inhibitors were prepared by serial dilution of the stock inhibitor solutions in 0.05% or 0.5% DMSO accordingly. In order to keep the DMSO content constant during the activity determinations, always 50 µl of inhibitor solution of varying concentrations was added into the standard assay mixture.

The enzyme activity determined in the standard reaction mixture containing DMSO (0.05% or 0.5%) was only considered as control activity. The presence of DMSO did not have any effect on blank measurements recorded in the absence of DL-gyceraldehyde.

I_1 was dissolved in 0.05% DMSO solution and , I_2 and I_3 in that of 0.5% The standard assay mixture was prepared as explained under "Methods".

Before testing Inhibitor effect, all of the ingredients except inhibitor with 0.05% and 0.5% DMSO for I_1 and I_2 , I_3 ,respectively, were used as a reference for that study.

The desired concentrations were reached by serial dilution of the inhibitors with DMSO. For each inhibitor its effect on activity were analyzed by using different concentration ranges.

The standard assay mixture was used in this study, all of the ingredients including 50 µl DMSO without any inhibitor was read as blank and again with the standard assay with different concentrations of 50 µl inhibitors except DMSO were read as sample.

CHAPTER 3
RESULTS

3.1 Bovine Lens Aldose Reductase Activity

The bovine lens aldose reductase activity was determined according to the method of Kinoshita (16) as described under "Methods". Crude extract prepared from bovine lens homogenate or partially purified fractions were used as enzyme source.

Aldose reductase activity was determined in the crude extracts prepared from 10 to 12 bovine lenses. Among the pooled lens extracts the lowest activity was measured as 0.7 nmole NADPH oxidized /min/mg protein and the highest activity was measured as 1.83 nmole NADPH oxidized /min/mg protein. The average aldose reductase activity calculated as 1.18 ± 0.18 (Mean ± SE, n=4) nmole NADPH oxidized/min/mg protein.

3.2 Partial Purification of Bovine Lens Aldose Reductase

Partial purification of aldose reductase from bovine lens was achieved by using DEAE-cellulose column chromatography as explained under "Methods". The bovine lens cytosolic fractions or crude extract were applied to DE-52 column to recover anionic peaks after the potassium phosphate gradient elution. The 22 ml of crude extract contained approximately 46,200 units of enzyme towards DL-gyceraldehyde before applying it into the column. During elution by the linear potassium phosphate buffer gradient of 200 ml, 10 mM and 200 ml, 200 mM of pH 6.8, three different protein peaks were obtained, as shown in Figure 9.

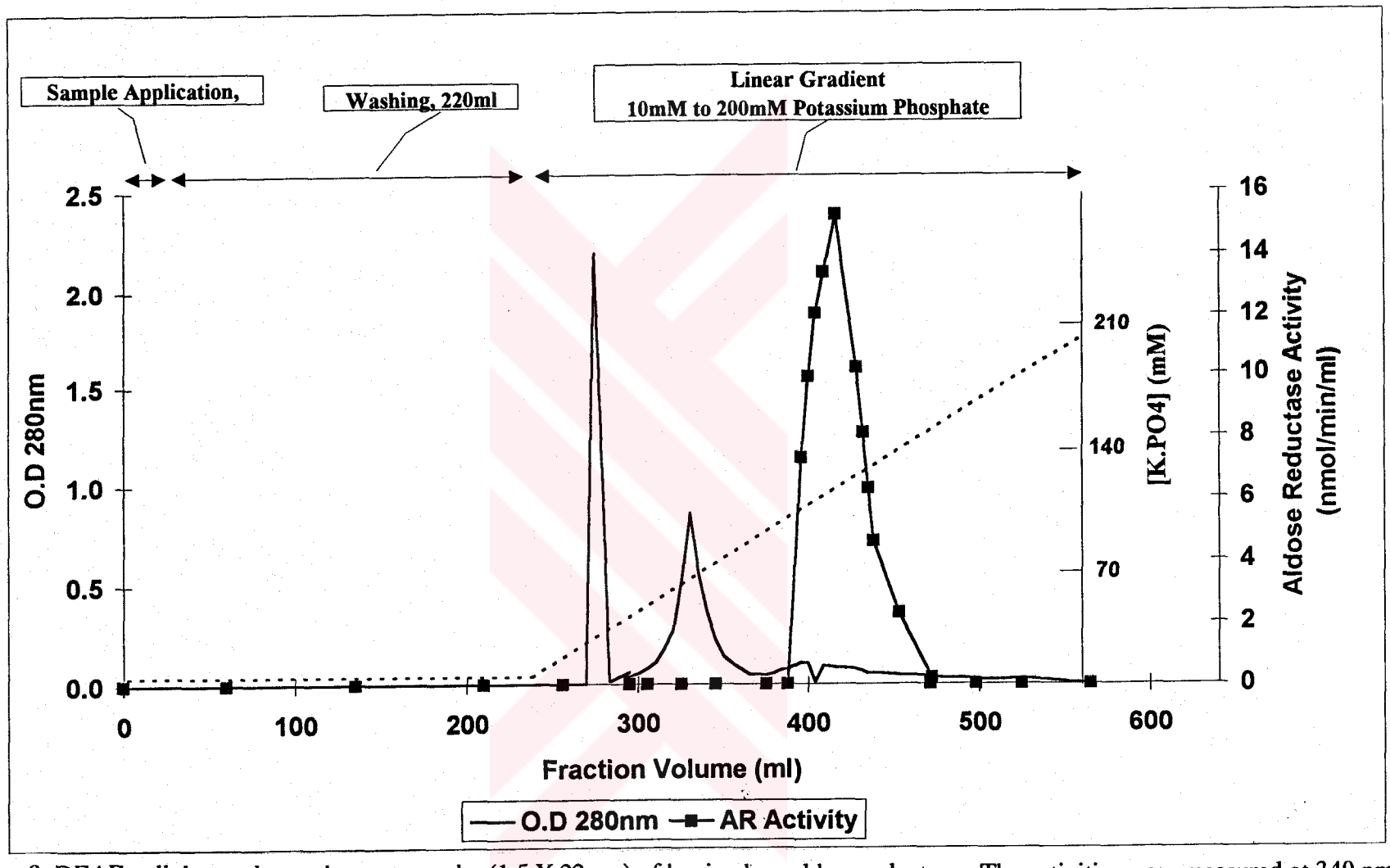


Figure.9: DEAE-cellulose column chromatography (1.5 X 22 cm) of bovine lens aldose reductase . The activities were measured at 340 nm as described under "Methods".

The tubes containing highest aldose reductase activity were pooled and the partially purified aldose reductase was stored at - 80oC in 5 ml aliquots for further studies.

3.3 Storage Stability of Aldose Reductase Activity

In order to determine the effect of storage on aldose reductase activity, the crude extracts prepared from bovine lenses were divided into small aliquots in eppendorf tubes and kept at -80 °C in deep freezer. At indicated days of storage one of the aliquots was thawed and the aldose reductase activity was determined as explained under "Methods". Figure 10 shows the days of stability of the enzyme.

Thawing the frozen crude extract once did not affect the aldose reductase activity considerably, but second thawing of the same sample resulted in the loss of activity approximately 12%. Therefore, the measurements of enzyme activity were always performed using the samples thawed once. As shown in Figure 10, the aldose reductase activity was stable only for 14 days. After 14 days the enzyme activity was decreased; at 20th day 66% and at 22nd day 50% of original activity was retained.

The stability problem can be seen in partially purified aldose reductase drastically, as the enzyme activity was lost in deep freezer without any thawing or refreezing of the enzyme. In Figure 11, the bar diagram shows the enzyme stability of the partially purified aldose reductase. First day measurements were taken immediately after polling the aldose reductase containing tubes eluted from DE-52 cellulose column, without being frozen. In 15 days, the enzyme lost the half of its original activity. Next 15 days, the activity did not change considerably, but in one month, the enzyme activity was lost.

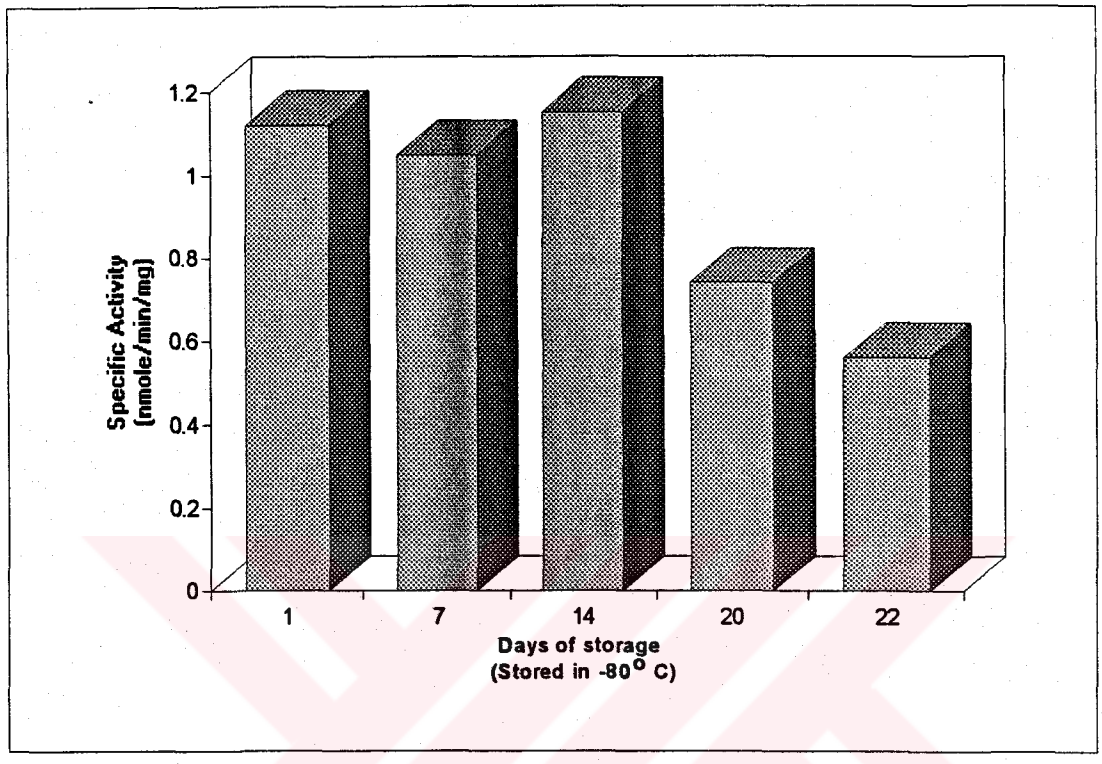


Figure 10. Stability of bovine lens aldose reductase crude extract. Crude extract was stored in small aliquots at - 80 °C.

The measurements were taken in the aliquots thawed once at the indicated time periods.The reactions were carried out at 25 °C as described under "Methods". The points were the means of duplicate determinations from at least two separate experiments.

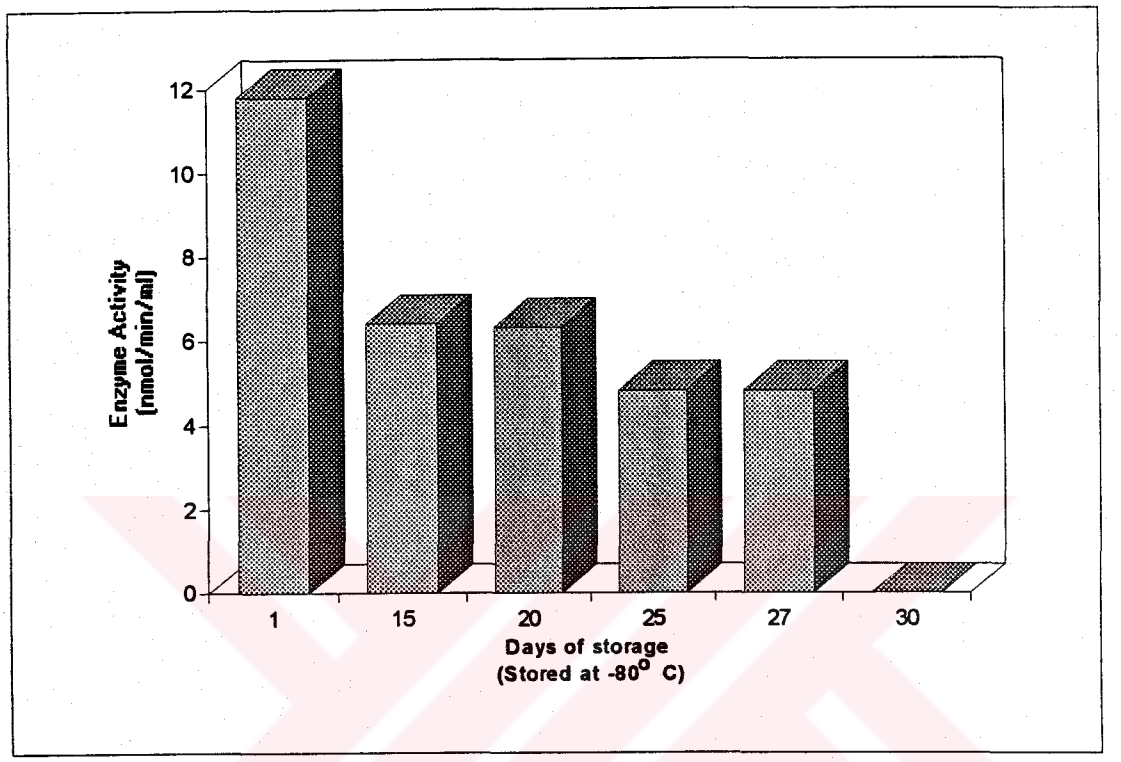


Figure 11. Stability of partially purified bovine lens aldose reductase. The partially purified sample was stored in small aliquots at - 80 °C.

The measurements were taken in the aliquots thawed once at indicated time periods.The reactions were carried out at 25 °C as described under "Methods". The points were the means of duplicate determinations from at least two separate experiments.

3.4 Characterization of Bovine Lens Aldose Reductase Activity

3.4.1 The Time Courses of Aldose Reductase Activity

The time course of a typical DL-gyceraldehyde reduction catalyzed by aldose reductase is illustrated in Figure 12. The reaction was linear with time up to at least 6 minutes. Therefore, the reactions were followed for 3 minutes and the change in OD in 1 minute in the absence of gyceraldehyde as a blank was used to calculate the aldose reductase activity as explained under "Method".

3.4.2 Effect of Protein Amount On Enzyme Activity

Figure 13 shows the effect of protein amount in the reduction of DL- gyceraldehyde by bovine lens aldose reductase . The protein amount in the reaction mixture was varied from 1.15 mg to 9.20 mg. The reaction rate was linear with enzyme amount up to approximately 7.0 mg crude extract protein per 1.0 ml incubation mixture.

In order to obtain sufficient quantity of products for spectrophotometric determination,protein concentration of 2.3 mg/ml of incubation mixture was routinely used throughout this study.

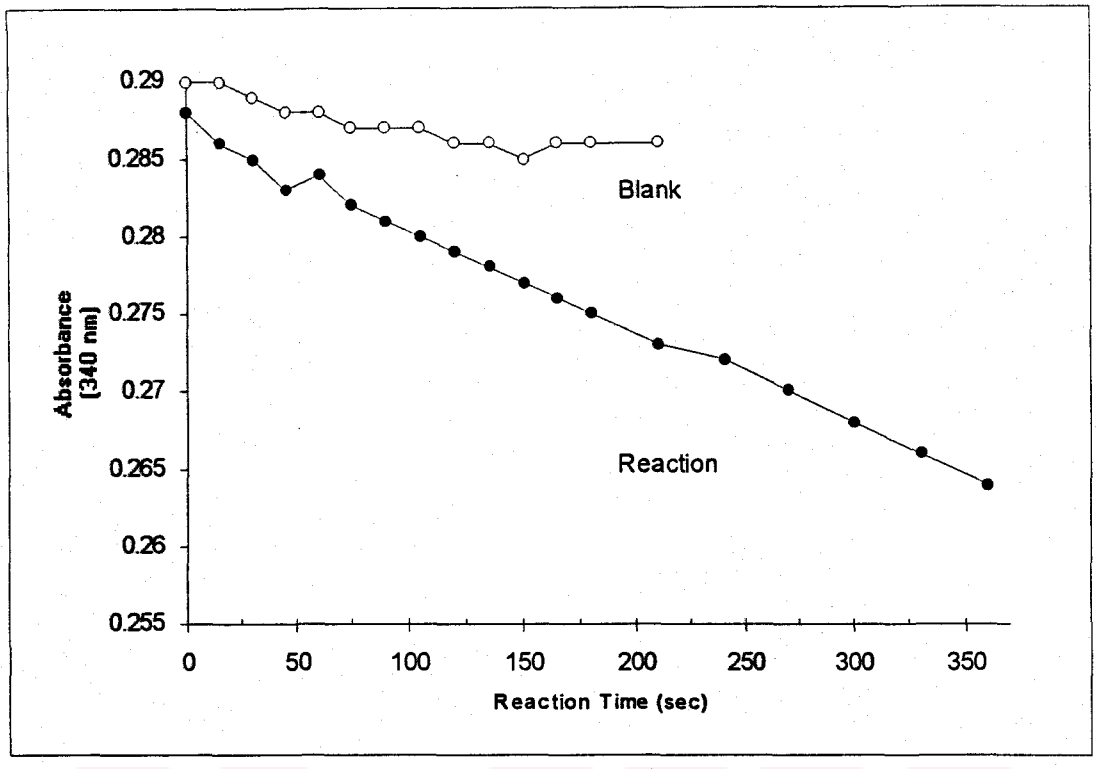


Figure 12. Time course of aldose reductase activity. The reactions were carried out at 25°C as described under "Methods".

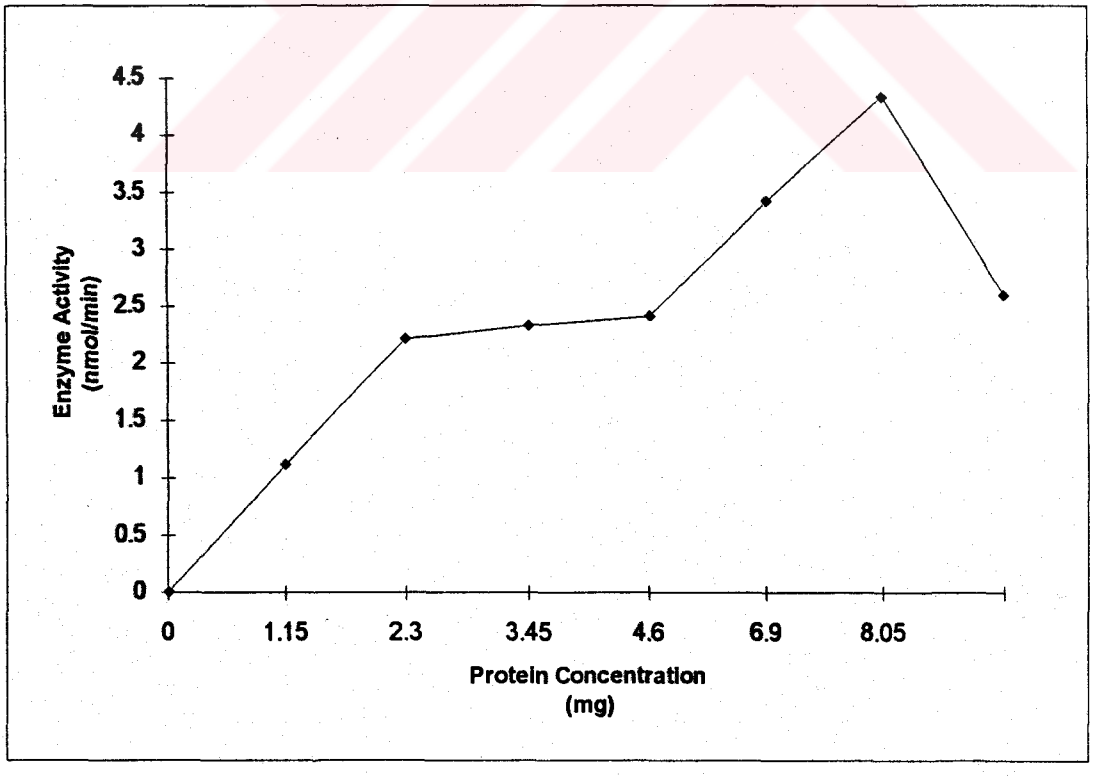


Figure 13 Effect of enzyme amount on the rate of bovine lens aldose reductase activity.

3.4.3 Effects of Li_2SO_4 Amount On Enzyme Activity

Table 4 shows the effect of Li_2SO_4 concentration on aldose reductase activity. Li_2SO_4 was used as an activator and added into the reaction mixture at varying concentrations. The maximum activity was obtained at 400 mM of Li_2SO_4. As shown in Table 3 ,when the aldose reductase activity was determined in the absence of Li_2SO_4 ,the specific activity was found as 0.33 nmole /min/mg protein. However, as Li_2SO_4 concentration was increased from 10 mM to 400mM the specific activity of the enzyme was increased from 0.56 to 1.63. Therefore, for the further measurement of aldose reductase activity, Li_2SO_4 concentration was chosen as 400 mM.

Table 4: Effect of Li_2SO_4 concentration on aldose reductase activity.

Li_2SO_4 Concentration (mM)	**Enzyme Activity[a] (nmole/min/mg)**
400	1. 63
200	1.16
40	0.75
20	0.6
10	0.55
0	0.33

[a] The reactions were carried out at 25 °C as described under "Methods". Blank measurements contained corresponding amounts of Li_2SO_4. The specific activity values are mean of duplicate measurements obtained from at least two separate experiments.

3.4.4 Effect of pH On Enzyme Activity

Figure 14 shows the effect of pH on bovine lens aldose reductase activity. The pH assays were carried by using 0.1M phosphate buffer with pH ranges varying from 6.0 to 8.0; 0.1 M acetate buffer with pH ranges varying from 4.0 to 6.0 and 0.1 M citrate buffer pH ranging from 5.0 to 7.5. The highest activity was observed between pH 6 to 6.5 when the phosphate buffer was used. However, when the reactions were carried out in citrate buffer, the enzyme activity was much lower than the specific activity of aldose reductase determined in phosphate buffer. Therefore phosphate buffer, pH 6.2, was used for determination the characterization of other properties of aldose reductase.

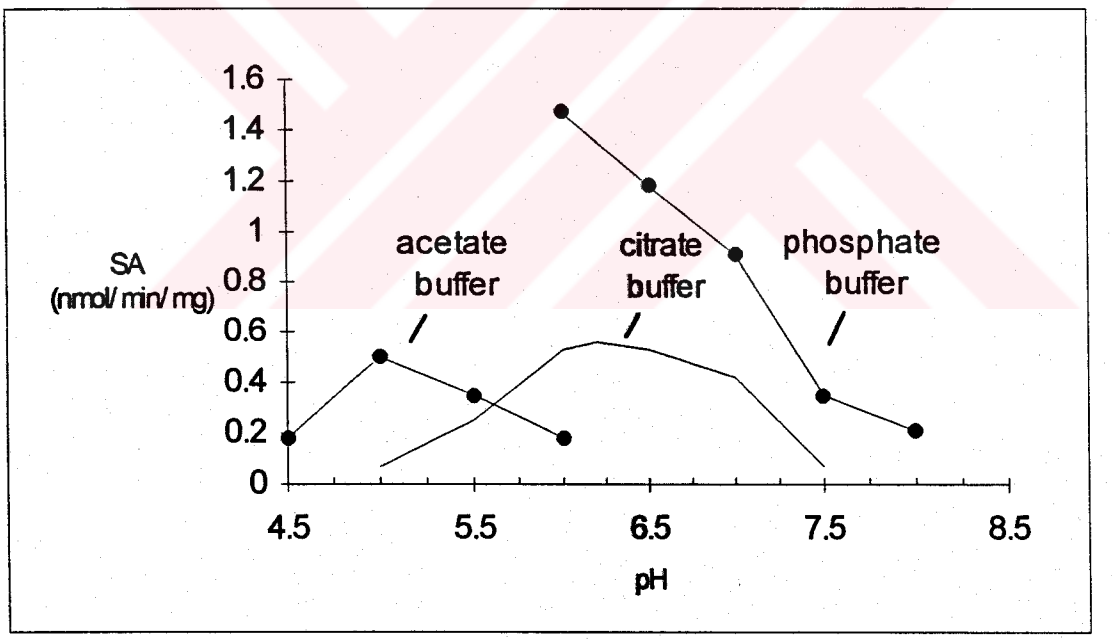


Figure 14. Effect of pH on bovine lens aldose reductase activity.

All the activity measurements were carried out at 25 °C in 0.1 M phosphate buffer as described under "Method". The points seen on the graph are the means of three different sets of experiment and each point is the mean of duplicate determinations

3.4.5 Effect of Glyceraldehyde Concentration

Figure 15 illustrates the substrate concentration curve for bovine lens aldose reductase. The optimal glyceraldehyde concentration for the enzyme activity determinations was found to be approximately $5x10^{-5}$ M. The enzyme seemed to be saturated by its substrate at around $5x10^{-5}$ M glyceraldehyde concentrations. As the substrate concentration was further increased, the enzyme activity did not change.

The Lineweaver-Burk plot (Figure 16) derived from glyceraldehyde saturation curve was linear, suggesting a simple Michealis-Menten kinetics. By use of this plot the V_{max} and K_m values were found as 1.695 nmol/min and 17.86 μ M respectively.

3.4.6 Effect of NADPH Concentration On Enzyme Activity

The effect of cofactor concentration on the bovine lens aldose reductase activity is shown in Figure 17. As shown in the figure the saturation is reached at 1.75 $x10^{-6}$ M concentration and the linear Lineveaver-Burk plot for NADPH suggesting a simple Michealis-Menten kinetics (Figure 18). The V_{max} and K_m values were found as 2 nmol/min and 1.69 μ M respectively.

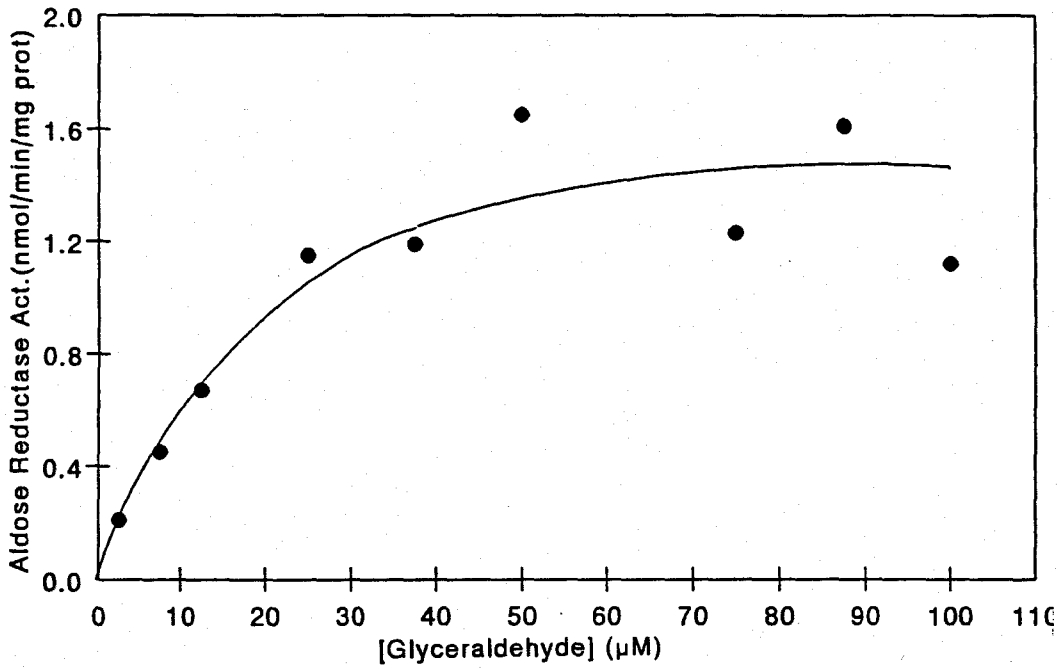


Figure 15. Effect of glyceraldehyde concentration on bovine lens aldose reductase activity. The reactions were carried out at 25 °C as described under "Methods". The points on the graph are the means of at least three different sets of experiment and each point is the mean of triplicate determinations.

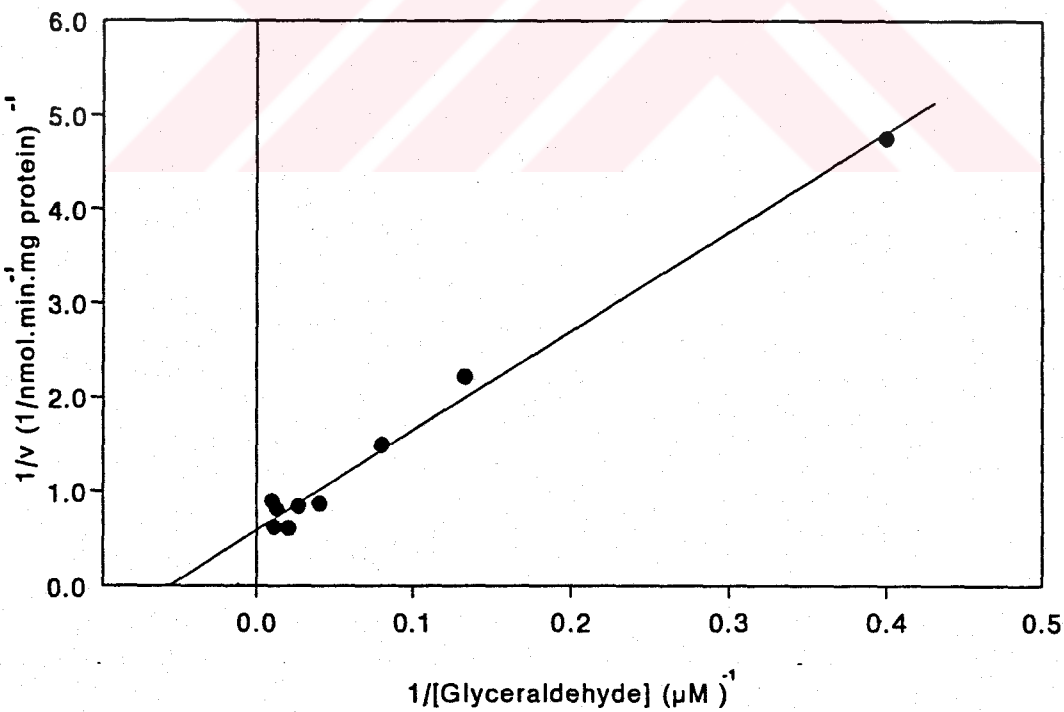


Figure.16: Lineweaver-Burk plot for glyceraldehyde saturation curve.

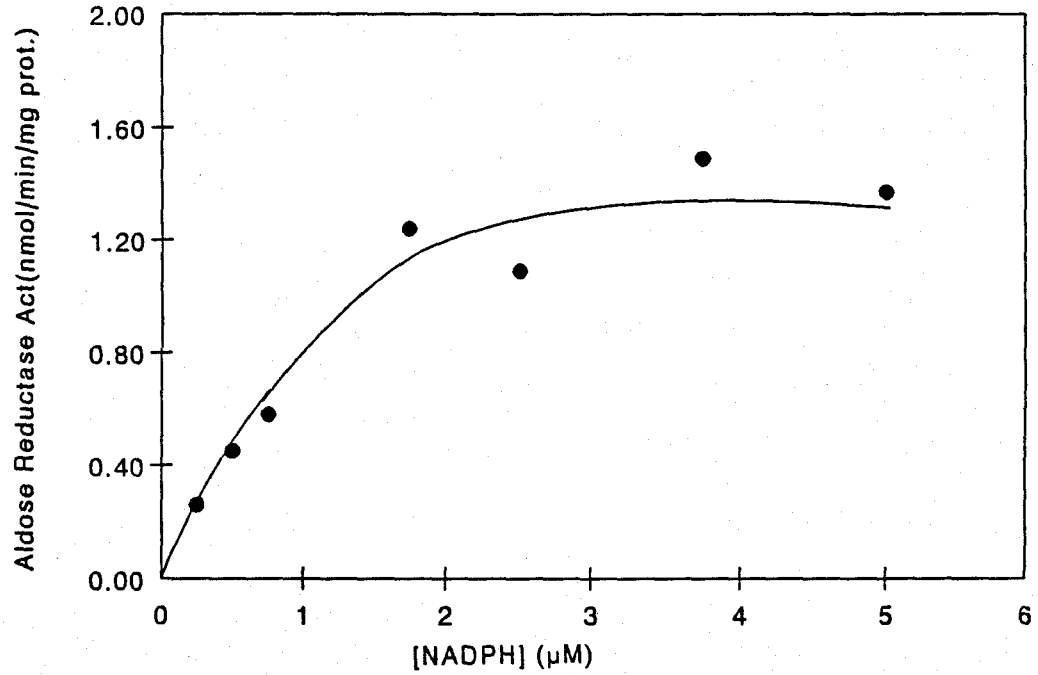


Figure 17. Effect of NADPH amount on bovine lens aldose reductase activity. The reactions were carried out at 25 °C as described under "Methods". The points on the graph are the means of at least three different sets of experiment and each point is the mean of triplicate determinations.

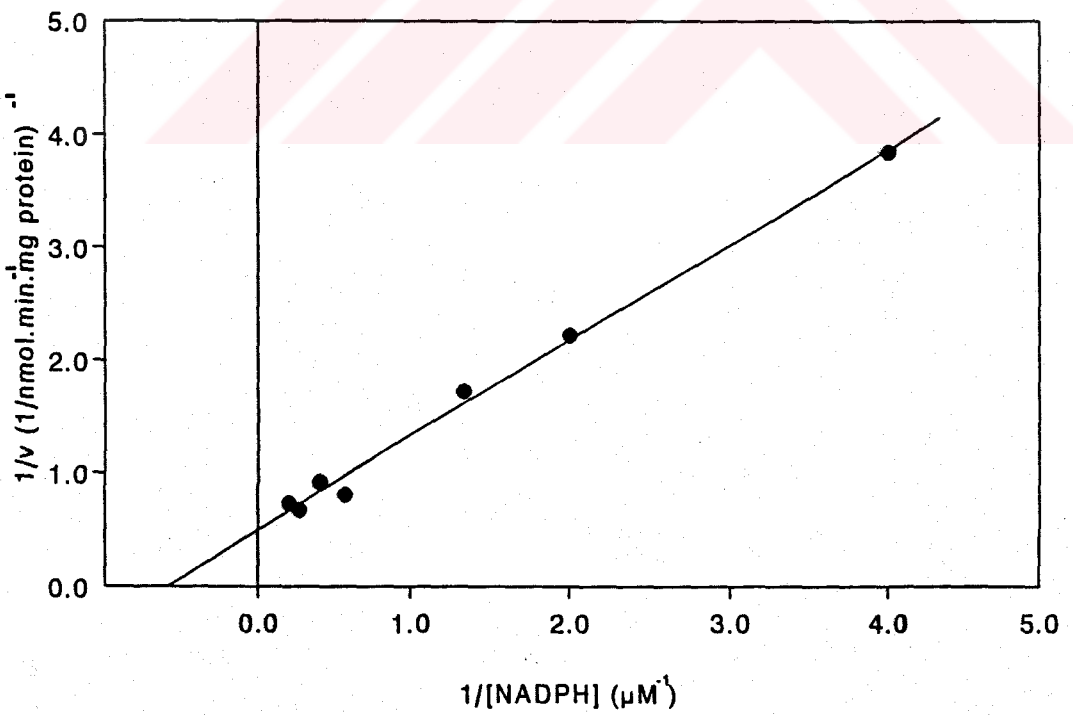


Figure 18. Lineweaver-Burk plot for NADPH saturation curve.

3.4.7 Effects of Temperature On Enzyme Activity

Figure 19 shows the effect of temperature on bovine lens cytosolic aldose reductase activities detected by incubating the reaction mixture constituents, without adding the enzyme source (bovine lens cytosolic fractions), at indicated temperatures for 3 minutes, then adding the cytosolic fraction and following the rate of reactions for 5 minutes. Eight different incubation temperatures namely, 10°C, 15°C, 20°C, 30°C, 37°C, 40°C, 45°C, 50°C and 60°C,were used in this experiment. The reaction mixture constituents were the same as described previously (Table.2) under the "Methods" .

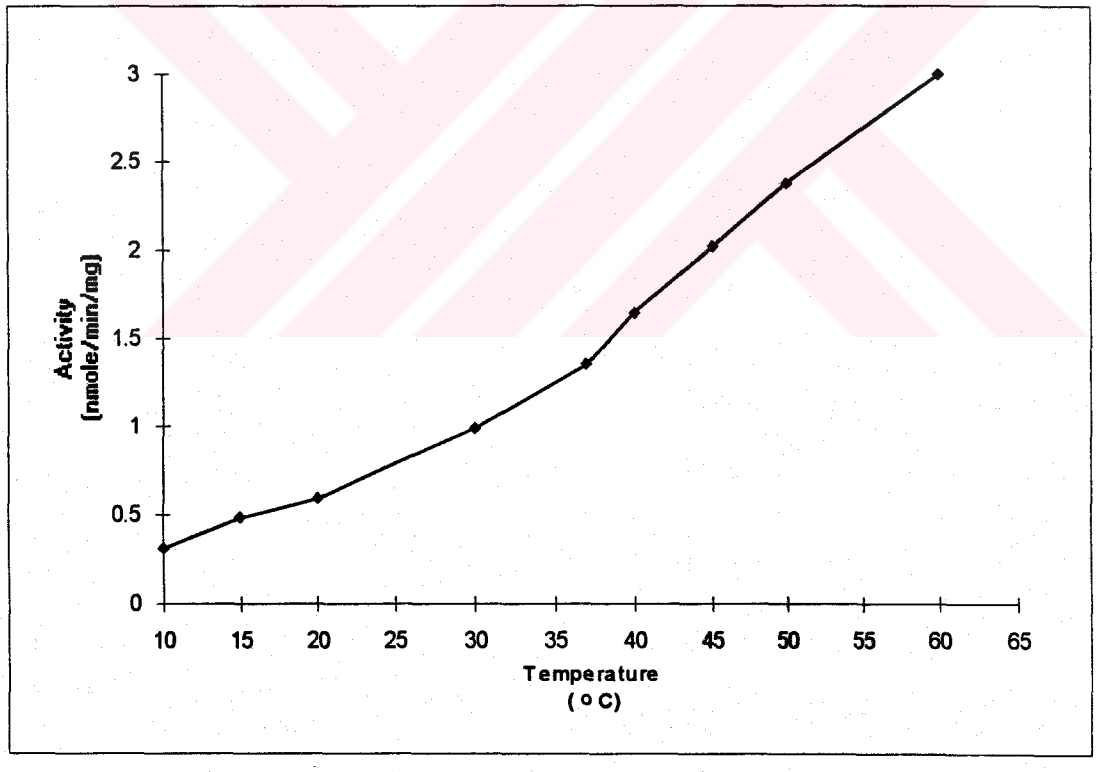


Figure.19: Temperature effect on aldose reductase enzyme activity.

3.5 Effect Of Inhibitors On Enzyme Activity

The inhibition studies were carried out using partially purified bovine lens aldose reductase. The aldose reductase inhibitors used in this study were synthesized by Prof. Dr. Erdem Buyukbingöl. Inhibitors are insoluble in water, therefore DMSO solution was used as solvent. Various concentrations of inhibitors were prepared by serial dilution in DMSO as explained under "Methods". The corresponding DMSO solution was added into reaction medium as control. DMSO alone did not affect the enzyme activity considerably.

Figure 20 shows the inhibition of bovine lens aldose reductase by I_1. As seen in the figure, inhibitor-1 was effective even at 5μM concentration, however the most dramatic decrease in activity was observed after 50 μM concentration. The inhibition pattern was also shown in Figure 21 as percent activity versus inhibiotr-1 concentration. The IC_{50} value was calculated as 165.3 μM.

The inhibition of bovine lens aldose reductase by inhibitor-2 was shown in Figure 22. As shown in figure after the concentration 37.5 μM the activity decreased and when the concentration reaches to 2500 μM almost no any activity was observed. This result was confirmed with that of given in Figure 23 in which the concentration of inhibitor versus percent activity was shown and the IC_{50} value of this inhibitor was calculated as 499 μM.

The inhibitory effect of I_3 on bovine lens aldose reductase was shown in Figure 24. In this bar diagram the inhibition started at 2.5 μM but the most effective concentration was the concentrations above the 25 μM. In Figure 25 this result was also explained by using the percent activity versus inhibitor concentration and again for this graph except for the 25 μM the activity decreases gradually up to the concentration of inhibitor reaches to the 250 μM here there was a sligth increase after that concentration . The IC_{50} was found as 35.62 μM.

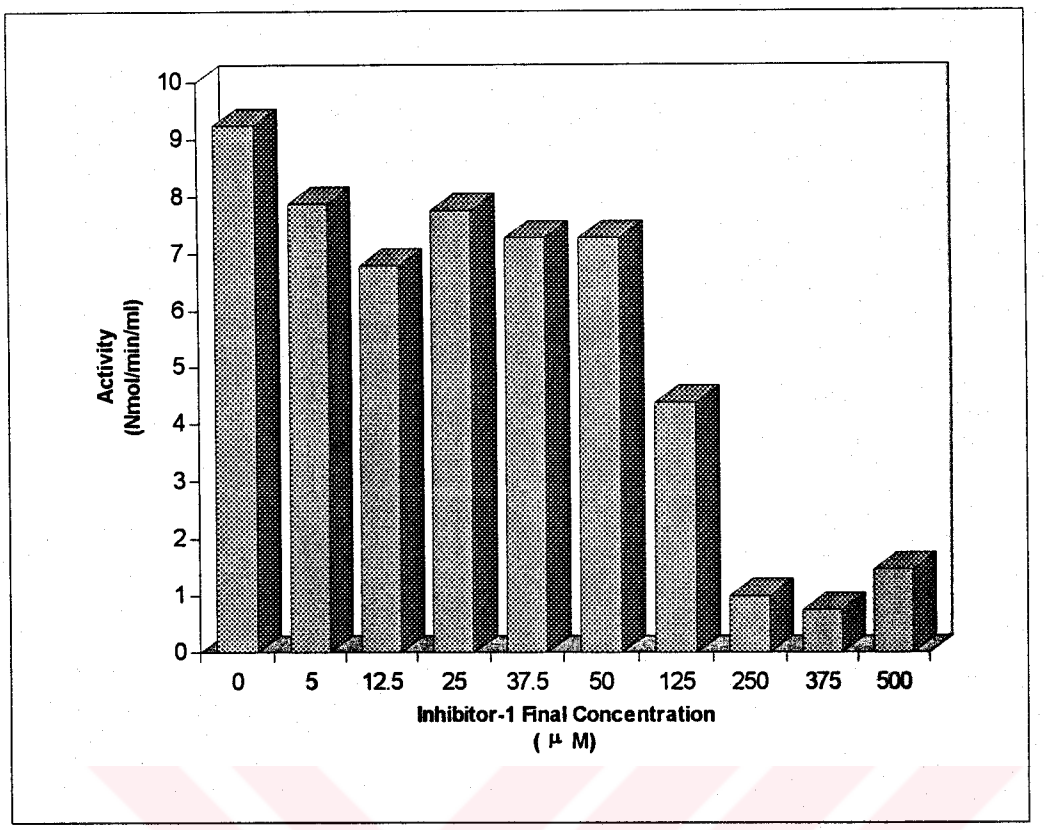


Figure 20. .Effect of inhibitor (I_1) concentration on the enzyme activity.

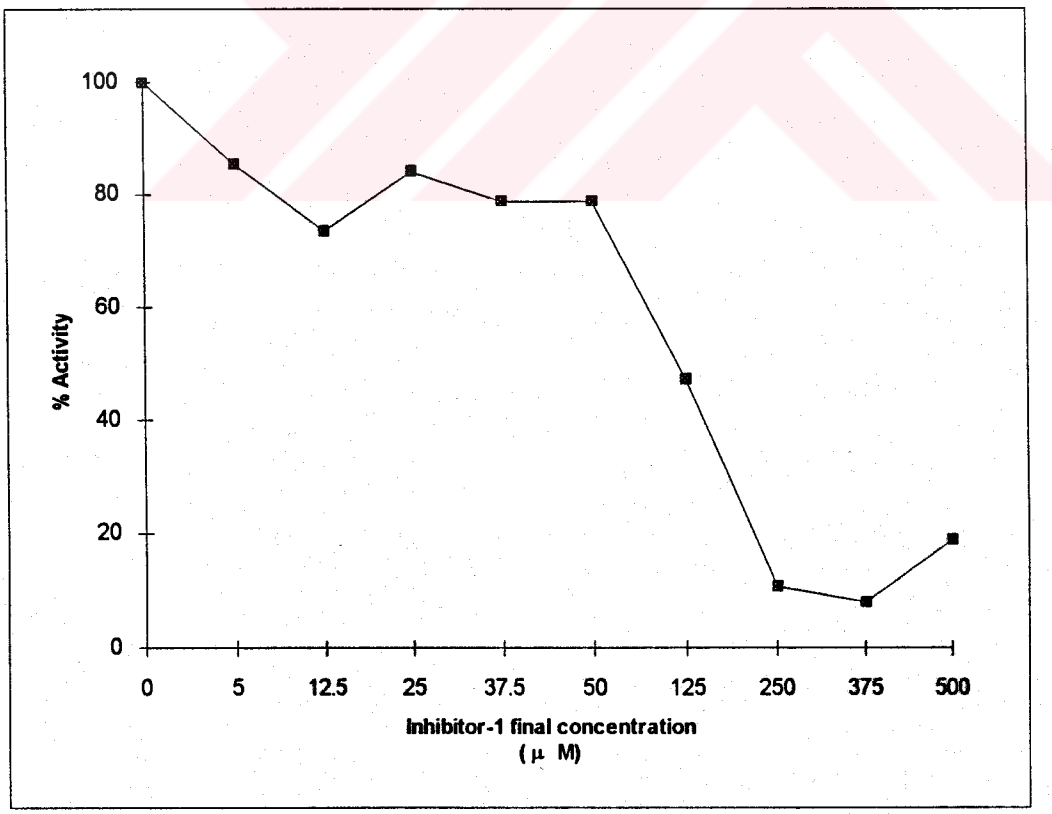


Figure 21. Percent activity of inhibitor-1 (I_1).

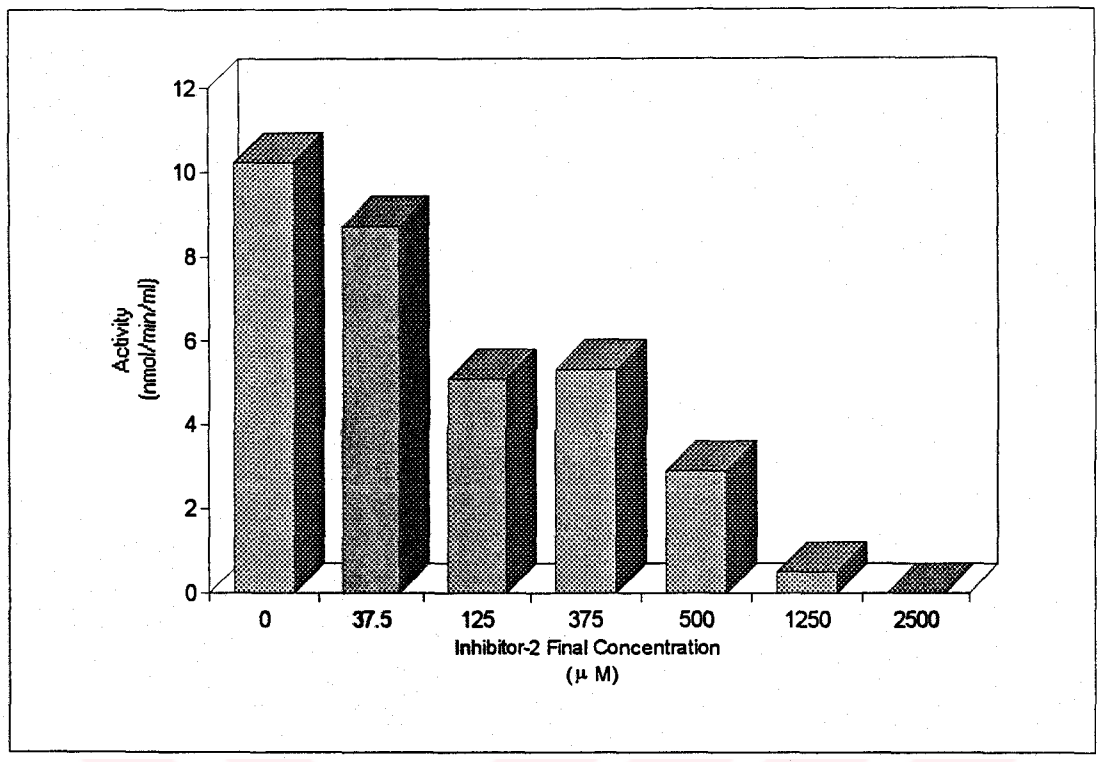


Figure 22. Effect of inhibitor (I_2) concentration on the enzyme activity.

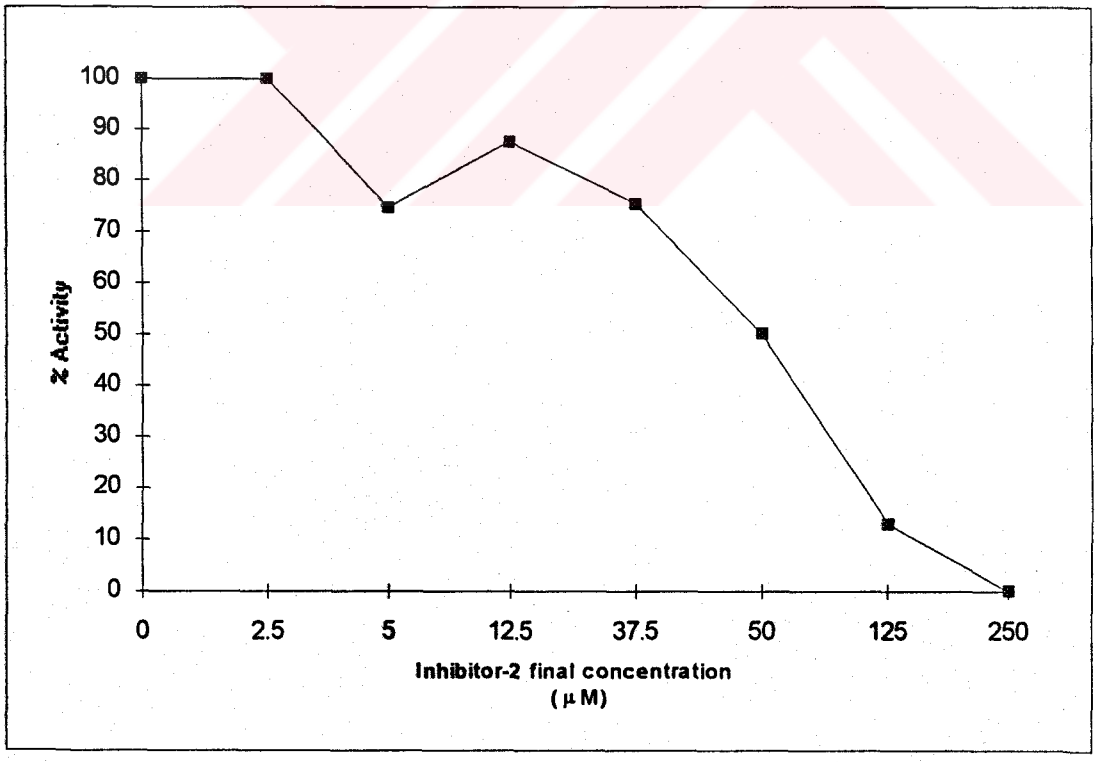


Figure 23. Percent activity of inhibitor-2 (I_2).

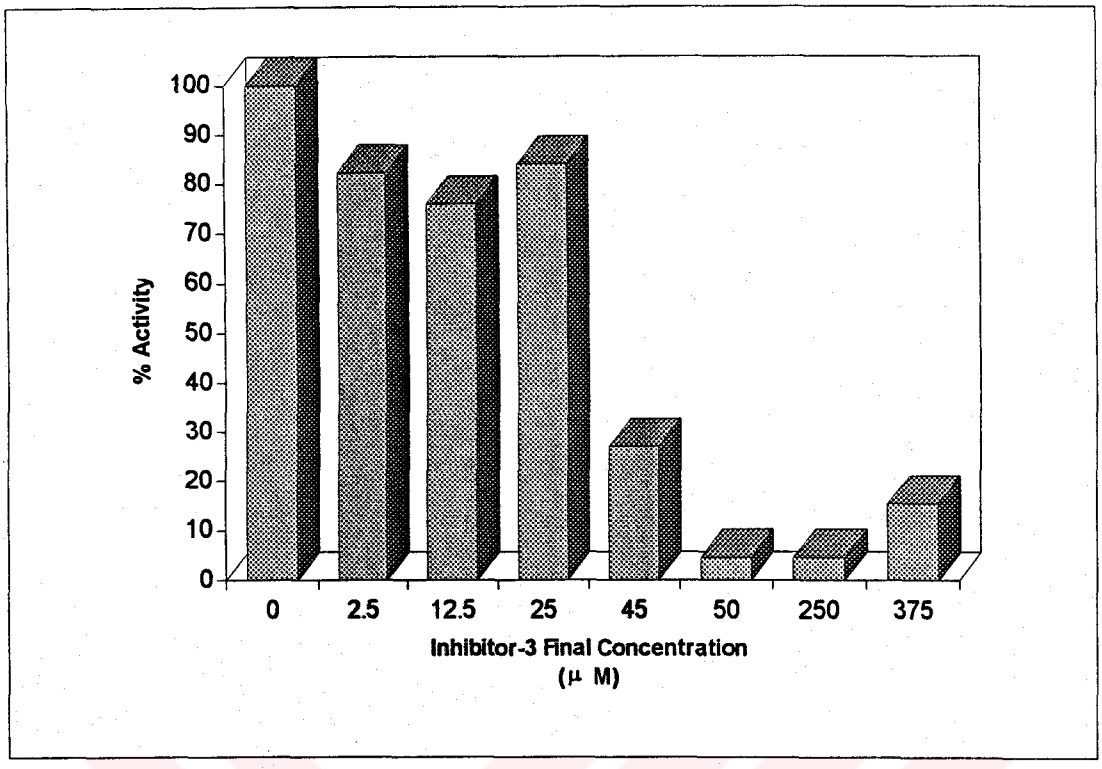


Figure 24. Effect of inhibitor (I_3) concentration on the enzyme activity.

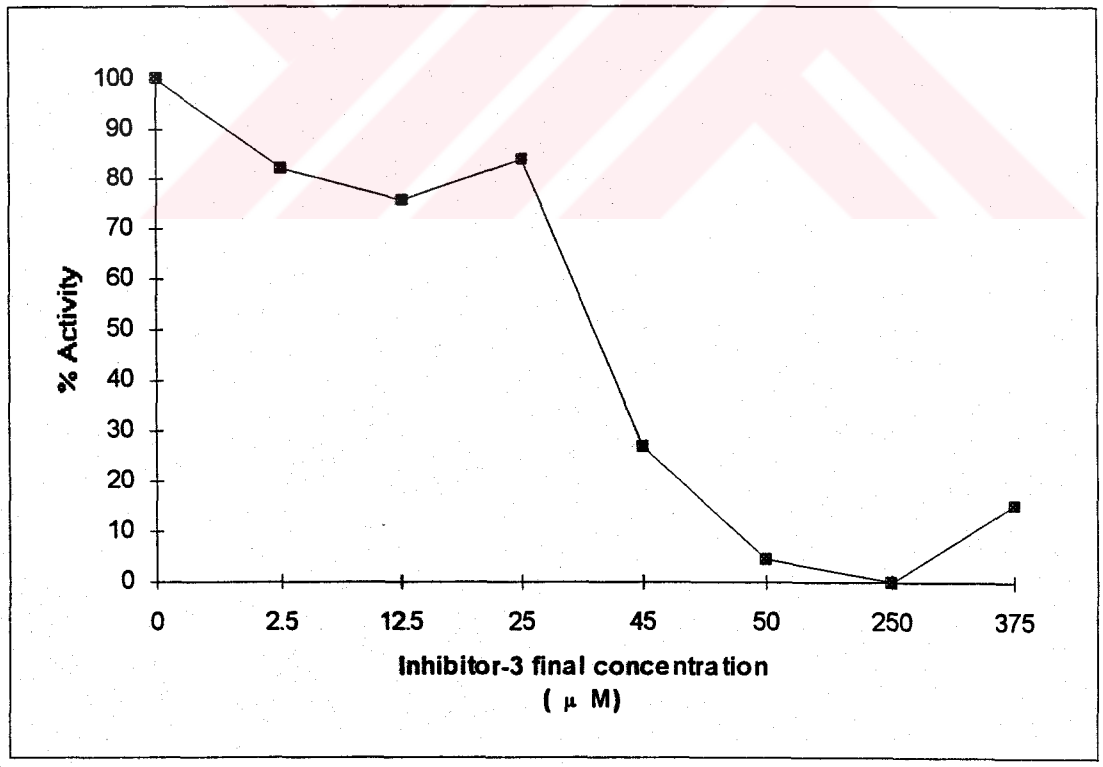


Figure 25. Percent Activity of inhibitor-3 (I_3).

3.6 SDS Polyacrylamide Gel Electrophoresis

Aldose reductase isolated from bovine lens, following column chromatography on ion exchangers, were analyzed by SDS polyacrylamide gel electrophoresis. The molecular weights of the subunits were calculated using the curve for molecular weight of standards.

Figure 26 shows the photograph of the one typical SDS-polyacrylamide gel after silver staining. The lanes contained molecular weight standard proteins, bovine lens crude extract and some of the fractions collected after DE-cellulose column chromatography. As it is seen in the photograph, it is easy to identify the individual subunits found in the eluted from the column.

Figure 27 demonstrates a typical molecular weight standard curve obtained for the following proteins; Bovine Serum Albumin (Mr 66.000 D), Egg Albumin (Mr 45.000 D), Carbonic Anhydrase (Mr 29.000 D), Trypsinogen (Mr 24.000 D), Trypsin Inhibitor (Mr 20.000 D).

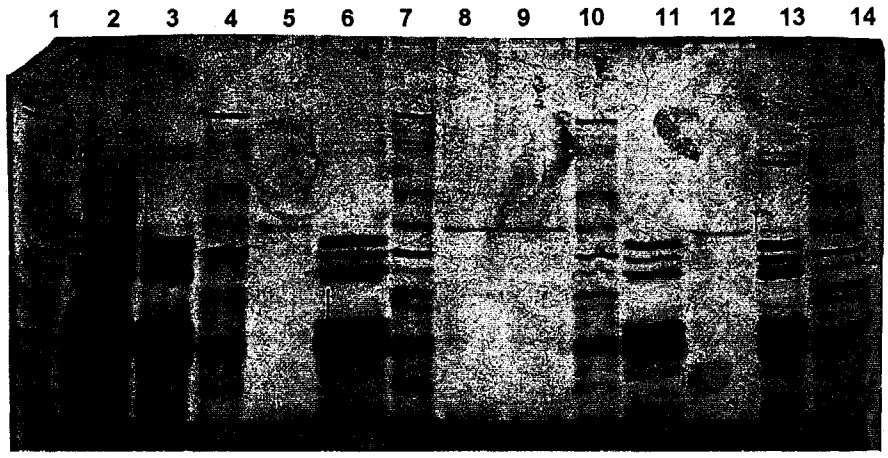


Figure.26: Typical SDS-polyacrylamide slab gel (12%) electrophoresis after silver staining.

-Lanes 5, 8, 9,12 contain partially purified bovine lens aldose reductase, (0.03 mg protein/ml for each),

-Lanes 2, 3, 13 contain the crude extract of bovine lens aldose reductase (7.6 mg protein/ml for each),

-Lanes 6,11 contain the crude extract of bovine lens aldose reductase (0.61 mg protein/ml for each),

- Lanes 1, 4, 7, 10, 14 : contain molecular weight markers:

-Bovine Albumin	(Mr 66.000 D)
-Egg Albumin	(Mr 45.000 D)
-Carbonic Anhydrase	(Mr 29.000 D)
-Trypsinogen	(Mr 24.000 D)
-Trypsin Inhibitor	(Mr 20.100 D)

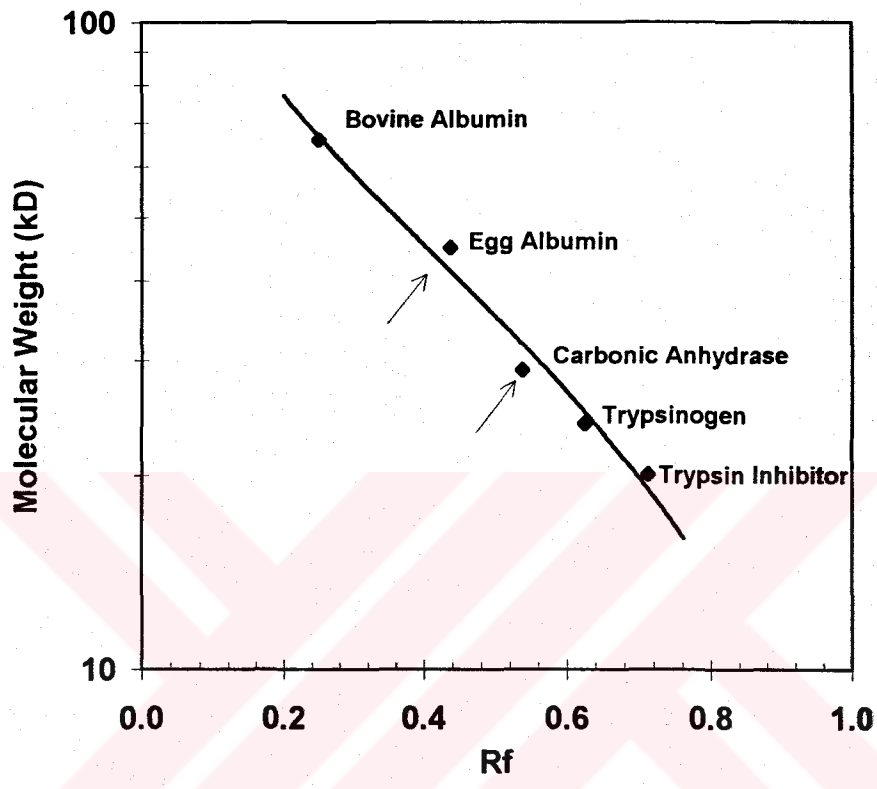


Figure.27: Typical molecular weight standard curve of molecular weights of protein standards versus relative mobility on 12 % SDS- polyacrylamide slab gel. The plot is drawn on a semi-logarithmic scale. The range for relative mobility of partially purified aldose reductase is within the arrows.

-Bovine Albumin	(Mr 66.000 D)
-Egg Albumin	(Mr 45.000 D)
-Carbonic Anhydrase	(Mr 29.000 D)
-Trypsinogen	(Mr 24.000 D)
-Trypsin Inhibitor	(Mr 20.100 D)

CHAPTER 4

DISCUSSION

Blindness in diabetics is largely due to retinopathy and/or cataract. Cataract is one of the three major causes of blindness worldwide. The majority of studies indicate that hyperglycemia and the duration of diabetes are major risk factors for the development of cataract as for the other diabetic complications (2). Therefore cataract in older diabetics is far more common than in childhood.

Animal studies have revealed an increasing number of associations between the aldose reductase initiated accumulation of sugar alcohols and the appearance of ocular complications associated with diabetes. The strongest evidence for this enzyme's affect comes from studies which reveal that inhibition of aldose reductase can significantly delay or prevent the appearance of many of these ocular diabetic complications. Moreover, the manifestation of many of these complications in galactosemia and their subsequent prevention with aldose reductase inhibitors clearly implicates the sorbitol pathway in these diabetic complications.

Sorbitol is known to accumulate in numerous tissues in diabetes, and this accumulation has been long associated with several late diabetic complications. The diabetic cataract is example of this process, and studies *in vivo* and *in vitro* on eye lenses have confirmed that raised glucose concentrations lead to increased accumulation of sorbitol in this tissue (42).

Aldose reductase which is responsible for the sorbitol formation is widely distributed in mammalian tissues. It has been reported that aldose reductase can be

activated in the presence of high glucose levels and that the enzyme under these circumstances displays different kinetic properties compared with the unactivated enzyme seen at low glucose levels (10). However, the results of Naeser et al (42) do not support this notion. The sural nerve which has the lowest aldose reductase activity displays the highest sorbitol accumulation both under basal and diabetic conditions. In contrast, the optic nerve of diabetic rats displays very low sorbitol content. This may indicate different isozymes of aldose reductase in the tissues and thus different sorbitol metabolism (42).

The aldose reductase levels and their K_m using partially purified bovine and human enzymes have been reported by Srivastava et al. (89) as 24 mM/g bovine lens and 188 mM/g human lens, respectively, and K_m for substrate glucose as 144 mM. However, as reported in work of Sheaff and Doughty (4) the bovine lens aldose reductase have not exhibited classical Michealis-Menten kinetics but rather have demonstrated two affinity constants for both substrate and cofactor. This has been manifested by a concave downward curvature in the Lineawaver-Burk double reciprocal plots. This type of kinetic behavior is commonly interpreted as being due to the presence of two related enzymes. However, other mechanisms to explain this behavior have also been postulated. The possibility that NADPH has been contaminated with $NADP^+$ could be considered as an explanation for the curvilinear kinetic behavior. In our studies, however, we have not observed any such curvilinear double reciprocal plots most probably due to the purity of our bovine lens preparation as seen in Figure 26.

The partial purification of bovine lens aldose reductase has been carried out on anion exchanger (DE-52 cellulose) column as described in 'Materials and Methods'. Figure 9 illustrates the elution profile of aldose reductase enzyme from the column. The bulk of the protein has been eluted from the column at lower phosphate buffer concentrations during the gradient. The aldose reductase activity has been detected from the column at the elution of a very low protein amount. When a sample from the highest activity peak was applied to SDS-PAGE , the enzyme fraction has been shown to be almost pure, although few bands have become visible slightly by silver nitrate staining of the polyacrylamide gel. Sheaff and Doughty (4) have also purified bovine

lens aldose reductase . Enzyme that had been purified through the final ammonium sulfate precipitation and dialyzed against 10 mM potassium phosphate buffer, pH 7.0, could be lyophilized and stored at -60 °C indefinitely. Some activity have been lost under these conditions, but it is restorable by incubation with sulfhydryl reagents. However, as soon as the enzyme has been purified through DEAE-cellulose column it becomes labile to freezing and thawing. With no protective agents added to the enzyme solution, freezing and thawing results in total loss of activity, as we have experienced during our studies.

The purity of the enzyme preparation has enabled us to study the kinetic parameters of bovine lens aldose reductase so that K_m has been determined as 17.86 μM when glyceraldehyde is used as substrate, exhibiting V_{max} of 1.7 nmol/min/mg protein. The affinity of enzyme towards its cofactor NADPH has also been determined as 1.69 μM and the V_{max} has been calculated as 2 nmol/min/mg protein using linear double reciprocal plots (16). Hayman and Kinoshita (16) have reported the K_m value of partially purified bovine lens as 30 μM when glyceraldehyde was used as a substrate. Our results are in quite good accordance with their findings.

The activation of the enzyme was established by following the NADPH oxidation as well as the sorbitol formation using glucose as substrate. The activated form of aldose reductase exhibited monophasic kinetics with glucose and glyceraldehyde whereas the unactivated form or native enzyme exhibited a biphasic kinetics with both the substrates (6.).

The substrate specificity of lens aldose reductase is broad, it does seem to be ascribable to a single enzymatic activity. The tendency for the K_m of an aldose to increase with chain length may be caused by requirement for a free aldehyde group in the substrate. The best aldose substrates have the hydroxyl on carbon atom 2 in the same configuration as that of D-xylose. These compounds in which this hydroxyl is either inverted or reduced are inferior substrates. Similarly, the K_m of D-glyceraldehyde is half as large as that of DL-glyceraldehyde (16).

The substrate specificity and other properties of lens aldose reductase have been quite similar to those of seminal vesicle enzyme (90).The two enzymes have comparable K_m values with different substrates (16). They are both stimulated to the same extend by ammonium sulfate. Addition of lithium sulfate resulted in doubling of enzyme activity obtained from bovine lens similar to the results in the literature.

Liu et al (91) has proposed a sequential mechanism for the reduction of aldehydes by aldose reductase when p-chlorobenzaldehyde was used as substrate. No substrate inhibition has been observed at either high p-chlorobenzaldehyde or high NADPH concentration. The maximum velocity has been found to be pH dependent and decreased at high pH. To avoid changes in the composition of the buffer and ionic strength of the reaction mixture on varying pH, a three buffer system have been chosen which consisted of acetate, citrate-phosphate and phosphate buffers. When phosphate buffer system has been used in activity determinations, the maximum activity for reduction of glyceraldehyde has been reached at pH range of 6.0 to 6.5. The optimum working pH is chosen as pH 6.2 throughout the study.

As mentioned before the diabetic complications are proposed to be due to accumulation of sorbitol in tissues. The most convincing evidence in support of this hypothesis comes from *in vivo* experiments involving the inhibitors of aldose reductase. In galactosemic rats, systemic administration of an aldose reductase inhibitor effectively delays the onset of cataractous process (18).

Most of the aldose reductase inhibitors share similar structural characteristics, such as presence of carbonyl or thiocarbonyl group. In a study that examined kinetics of inhibitors to human kidney aldose reductase , the inhibitor sorbinil exhibited noncompetitive inhibition. However, tolrestat and statil exhibited uncompetitive inhibition. The inhibition of enzyme activity varies from inhibitor to inhibitor (6). Tolrestat has a K_i of 0.033 μM as compared to sorbinil with Ki of 1.24 μM.
The inhibition study with three newly synthesized inhibitors namely I_1, I_2, I_3 the IC_{50} values have calculated as 165.3 μM, 499 μM, 35.62 μM respectively.

The activated enzyme was less susceptible to inhibition by aldose reductase inhibitors such as sorbinil, alrestatin, quercetrin as compared to unactivated enzyme. Partially purified aldose reductase from the normal human lens exhibited properties similar to the native enzyme of other tissues (6). Whereas the enzyme from clear lens obtained from diabetic subjects with severe hyperglycemia expressed properties similar to the *in vitro* activated enzyme of aorta brain and muscle (6).

Drug toxicity is another problem. Whatever the scientific rationale behind the inhibitors, at the very last they should not produce toxicity problems of their own if they are to be marketed successfully. Over the years, one by one they have fallen by the wayside. Sorbinil, which penetrates well into human lens and erythrocytes, and once thought to be a promising drug, was plagued by toxicity problems and its performance in trials for human neuropathy was poor even where it was well tolerated. Sorbinil is associated with the occurrence of fever and skin rash in around 10% of patients and several more serious hypersensitivity reactions have occurred (77). Alrestatin caused a frequent photosensitive skin rash and was quickly abandoned. Tolrestat caused a reversible elevation in hepatic transaminases in 2% of treated patients, and there was occasional dizziness (77).

Aldose reductase is widely distributed enzyme in mammalian tissues and it is difficult to see why it should be so if it genuinely has no physiological function in many of the sites in which it is found. However, no such physiological role has so far been demonstrated, so it is unlikely that long term inhibition of this enzyme would have detrimental effects.

Although the field of aldose reductase research has greatly expanded, major research efforts remain ahead. For example, the physiological role of this enzyme and the specific mechanisms by which aldose reductase initiates diabetic complications remain to be elucidated.

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