STUDIES ON PROTEIN GLYCATION

SUMMARY
OF THE
THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE OF
Doctor of Philosophy
IN
BIOCHEMISTRY

BY
SHAMILA FATIMA

THESIS

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

2008

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SUMMARY

The term glycation is used to describe non-enzymatic sequence of reactions in which the nucleophillic groups of proteins, nucleic acids and lipids react with reducing sugars and result in the formation of complex adducts including advanced glycation endproducts (AGEs). Glycation is also accompanied by the formation of reactive oxygen species (ROS) and causes oxidation, degradation and other alterations in biomolecules including inactivation of enzymes, both *in vitro* and *in vivo*. Glycation is a slow process but has been implicated in a variety of disorders including diabetes, atherosclerosis and various micropathies. An attempt has been made in this thesis to evaluate simple and reliable strategies for the assay of protein glycation and screening of various inhibitors of protein glycation.

Glycated human serum albumin (HSA) is known to be involved in pathogenesis of several diabetic complications. The alterations resulting from incubation of HSA with various sugars was studied. HSA was incubated for upto 8 days with glucose, fructose or ribose at 37°C under aerobic conditions. The extent of glycation of HSA as well as accompanying structural alterations were studied using SDS-PAGE, fluorescence, hyperchromicity, circular dichroism (CD) and colorimetric techniques. There was a consistent decrease in tryptophan fluorescence and increase in fluorescence as ascribed to the formation of AGEs. The structural alterations shown by CD spectra were suggestive of increase in the beta structure and random coil at the expense of alpha helix. HSA incubated with the sugars revealed an increase in carbonyl content with increase in time of incubation. The alterations produced by fructose and ribose, measured by most parameters were more pronounced than those caused by glucose with the exception of ketoamine and periodate assays that indicated low reactivity of fructose. The protective role of various compounds, drugs and flavonoids against the sugar-induced alterations were also studied. Among the flavonoids, rutin and

quercetin were potent inhibitors of fructose-induced alterations in HSA, while naringin and myrecetin were moderately protective.

Incubation with various reducing sugars at 37° C also results in remarkable inactivation of bovine pancreatic RNase A and among those investigated, ribose was most reactive followed by fructose and glucose. The loss of RNase A activity was accompanied by parallel conformational and other changes in the enzyme as revealed by increase in new fluorescence, quenching of intrinsic fluorescence, hyperchromicity and loss in tertiary structure. SDS-PAGE of RNase A incubated with the sugars showed cross linking and/or fragmentation. Protective role of various compounds and some flavonoids against glycation induced enzyme inactivation and other changes were also studied. As observed earlier with HSA the order of protection provided by flavonoids was- rutin > quercetin> naringin> myricetin > catechin.

Taking into consideration the remarkable thermostablity of RNase A and high reactivity of ribose as well as sensitivity of RNase A activity to glycation, a novel assay procedure for the screening of inhibitors of glycation was developed. Incubation of RNase A with ribose at 60 °C resulted in rapid inactivation of the enzyme in two days with a parallel decrease in tyrosine fluorescence, enhancement in new fluorescence and hyperchromicity in the UV-region. No such alterations were observed when the incubation was carried out in absence of the sugar. Several drugs, flavonoids and other compounds that are known to act as inhibitors of glycation reactions also restricted the ribose-induced inactivation of RNase A. RNase A immobilized on CNBr-activated Sepharose was more stable but equally sensitive to incubation with ribose. The immobilized RNase A was superior for the screening of the inhibitors of glycation from natural sources which contain substances that interfered with the assay of enzyme. Immobilized RNase A could be readily removed from the reaction mixture, washed and the activity determined in the absence of the interfering substances. The immobilized preparation was also suitable for the assay of post Amadori inhibitors of glycation since it facilitated the ready separation of the enzyme from the reaction mixture after initial reaction with sugars.

An earlier study from this laboratory has shown that anti Cu, Zn-SOD antibodies protect the enzyme against inactivation induced by reducing sugars and dicarbonyls. RNase A bound to Sepharose supports precoupled with antiRNase A antibodies raised in rabbits were similarly more resistant to inactivation induced by some reducing sugars as compared to the soluble enzyme or that bound non-covalently to CM cellulose. The spectral properties (hyperchromicity, quenching of tyrosine fluorescence and new fluorescence) of the RNase A exposed to the sugars in the immobilized state showed that binding on the antibody support provides remarkable protection against the glycation-induced alterations, CM cellulose support was only moderately protective against sugar induced alterations.

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Certificate

This is to certify that the work embodied in the thesis entitled ***"Studies on protein glycation"*** has been carried out by Ms. Shamila Fatima under my supervision. It is original in nature and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

M. Saleemuddin 17/9/08
(Supervisor)

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(Shamila Fatima)

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Abstract

ABSTRACT

The term glycation is used to describe non-enzymatic sequence of reactions in which the nucleophillic groups of proteins, nucleic acids and lipids react with reducing sugars and result in the formation of complex adducts including advanced glycation endproducts (AGEs). Glycation is also accompanied by the formation of reactive oxygen species (ROS) and causes oxidation, degradation and other alterations in biomolecules including inactivation of enzymes, both *in vitro* and *in vivo*. Glycation is a slow process but has been implicated in a variety of disorders including diabetes, atherosclerosis and various micropathies. An attempt has been made in this thesis to evaluate simple and reliable strategies for the assay of protein glycation and screening of various inhibitors of protein glycation.

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Taking into consideration the remarkable thermostablity of RNase A and high reactivity of ribose as well as sensitivity of RNase A activity to glycation, a novel assay procedure for the screening of inhibitors of glycation was developed. Incubation of RNase A with ribose at 60 °C resulted in rapid inactivation of the enzyme in two days with a parallel decrease in tyrosine fluorescence, enhancement in new fluorescence and hyperchromicity in the UV-region. No such alterations were observed when the incubation was carried out in absence of the sugar. Several drugs, flavonoids and other compounds that are known to act as inhibitors of glycation reactions also restricted the ribose-induced inactivation of RNase A. RNase A immobilized on CNBr-activated Sepharose was more stable but equally sensitive to incubation with ribose. The immobilized RNase A was superior for the screening of the inhibitors of glycation from natural sources which contain substances that interfered with the assay of enzyme. Immobilized RNase A could be readily removed from the reaction mixture, washed and the activity determined in the absence of the interfering substances. The immobilized preparation was also suitable for the assay of post Amadori inhibitors of glycation since it facilitated the ready separation of the enzyme from the reaction mixture after initial reaction with sugars.

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List of Abbreviations

AGEs	Advanced glycation end products
BSA	Bovine serum albumin
CD	Circular dichroism
CM	Carboxymethyl
CML	N-(Carboxymethyl) lysine
DEAE	Diethyl ethyl aminoethyl cellulose
DETAPAC	Diethylenetriaminepentaacetic acid
3DG	3-deoxyglucosone
DNPH	Dinitrophenylhydrazine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSA	Human serum albumin
IgG	Immunoglobulin G
IR	Infrared
MALDI	Matrix-assiated laser desorption/ionization
MG	Methylglyoxal
MS	Mass spectrometry
NBT	Nitroblue tetrazolium
NEG	Non enzymatic glycosylation
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
RAGE	Receptor of AGE
RNase A	Pancreatic ribonuclease A
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SELDI-TOF	Surface enhanced laser desorption/ionization time of flight

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Introduction

1.0 INTRODUCTION

1.1 Protein glycation

Glycation is an omnipotent, non-enzymatic process, occurring slowly but continuously in cells of all living organisms. This process involves chemical binding of sugar to amino groups of proteins followed by a series of chemical transformations (Singh *et al.,* 2001). The nonenzymatic reaction of the amino groups of amino acids, peptides and proteins with reducing sugars, resulting in the formation of complex brown pigments and protein-protein crosslink, was first studied by L.C. Maillard in the early 1900s (Maillard and Gautier, 1912). The reactions therefore came to be known as the Maillard reactions. Food chemists however soon recognized the practical importance of the Maillard reaction in explaining the brown color formation and loss of protein quality in cooked and stored foods. Realization of the importance of Maillard reactions *in vivo* began in the mid-1970s with studies on hemoglobin A_{1c} (HbA_{1c}), naturally occurring minor human hemoglobin that is elevated in diabetics (Bookchin and Gallop, 1968). HbA_{1c} was known to be the post-translational adduct of glucose, linked nonenzymatically to the N-terminal valine amino group of the β chain of hemoglobin in humans (Rahbar *et al.,* 1969) and in animals (Koenig and Cerami, 1975). Flückigar and Winterhalter (1976) induced the formation of HbA_{1c} *in vitro* by incubating hemoglobin with glucose. Later on, the significance of the complex, late stage Maillard processes was recognized as mediators of several complications in diabetes (Bunn *et al.*, 1978) and aging (Monnier and Cerami, 1981).

Proteins bearing Amadori products have come to be referred to as glycated proteins (distinguishing them from enzymatically glycosylated proteins) and the process of Amadori product formation is termed glycation. The complex pigments and cross-links formed from glycated protein during the *in vivo* Maillard reaction are described as advanced glycation end-products, or AGEs (Bucala *et al.,* 1992).

Wolffenbuttel *et al.* (1996) suggested that modification of hemoglobin by advanced glycosylation end products (Hb-AGEs) would be a better index for long term glycemia in diabetic patients. While glycation can be detected in physiologic conditions like aging, the reactions are considerably faster and more intensive in the pathophysiologic conditions like the uncontrolled diabetes mellitus associated with persistently elevated blood glucose concentration.

Complex defence mechanisms restrict the glycation reaction *in vivo*, but small quantities of adducts of the proteins, nucleotides and basic phospholipids do occur under physiological states (Thornalley, 2003). The protection apparently goes astray in several disorders or when challenged with excess of reactive sugars over a prolonged duration. In addition to the multiple pathologies mediated by the *in vivo* generated AGEs, exogenous sources such as diet and smoking may also add significantly to the damage caused by those generated in the body (Kochinsky *et al.*, 1997).

1.1.1. Chemistry of glycation

Glycation involves a complex series of parallel and sequential reactions between reducing sugars and nucleophilic groups of proteins and other molecules leading to the formation of a variety of adducts, some of which are fluorescent and/or colored (Cerami and Ulrich, 2001). While the impact of glycation is countered in cells by high turnover and short half-life of many cellular proteins, long lived proteins accumulate glycation adducts with age (Sell *et al.*, 1996). Although all these adducts are removed by degradation of the glycated protein, removal of extracellular protein adducts requires specific recognition receptors, internalization and proteolytic processing (Horiuchi *et al.*, 1996; Gugliucci and Allard, 1996).

Glycation can be subdivided into three main stages: early, intermediate, and late. In the early stage, glucose or other reducing sugars react with nucleophilic groups like amino groups of proteins, nucleic acids and lipids to form unstable aldimine compounds (Schiff bases). Through rearrangement, the Schiff base gives rise to the stable ketoamine (Amadori product). The rate of non-enzymatic reaction *in vivo* depends on the concentration of the sugar, reactivity of the free amino groups of the biomolecules and also on the half-life of the biomolecules. Under *in vivo* conditions, the Amadori products reach equilibrium after approximately 15-20 days and accumulate both on short-lived and long-lived proteins (Thornalley, 1996). The Amadori rearrangement of lysine-glucose Schiff base is thought to be facilitated if a histadine side chain or another lysine group is present within about 5Å from the amino group on which the Schiff base has formed (Acosta *et al.,* 2000). In the intermediate stage the Amadori product is degraded into a variety of carbonyl compounds, such as glyoxal, methylglyoxal (MG) and deoxyglucosones which in turn act as propagators of the reaction (Thornalley *et al.,* 1999; Glomb and Monnier, 1995). In the late stage, the propagators further react with other free amino groups and through irreversible oxidation, dehydration and cyclization reactions form yellow-brown, heterogeneous AGEs (Fig.1).

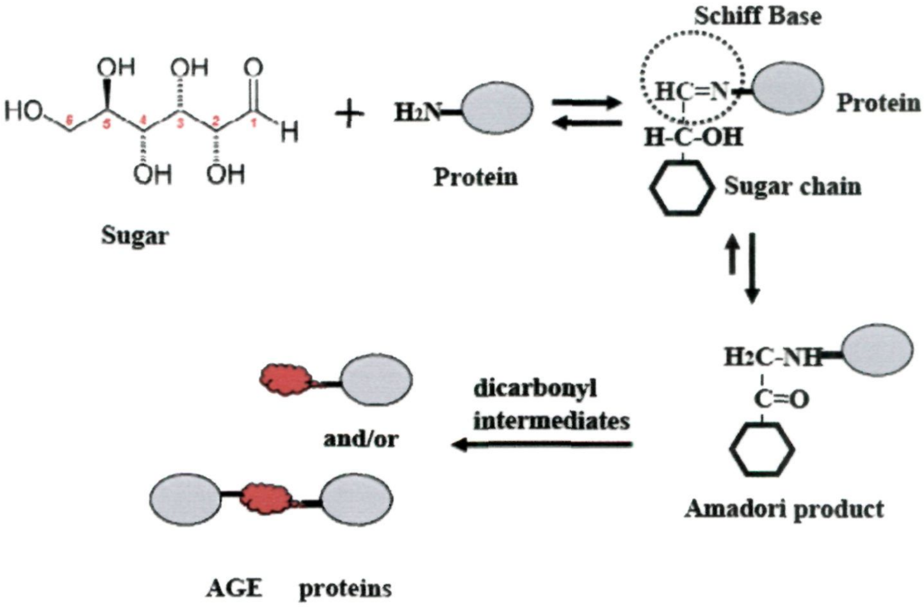

Fig. 1. Schematic representation of formation of AGEs (Lin, 2006)

Several AGEs exhibit characteristic fluorescence and have reactive groups that may cross-link proteins, form insoluble aggregates and cause serious disturbance in their physiological functions (Ahmed *et al.,* 2005).

In recent years, it has become increasingly clear that dicarbonyl compounds are the key intermediates in the formation of AGEs. The Amadori product can break down via its enol form to reactive, free dicarbonyl glyoxal compounds such as 3-DG, MG, and glyoxal (Thornalley *et al.,* 1999). These dicarbonyl compounds are even more reactive towards amino groups of proteins than the parent sugars and rapidly lead to the formation of crosslinks and AGEs (Fig. 2).

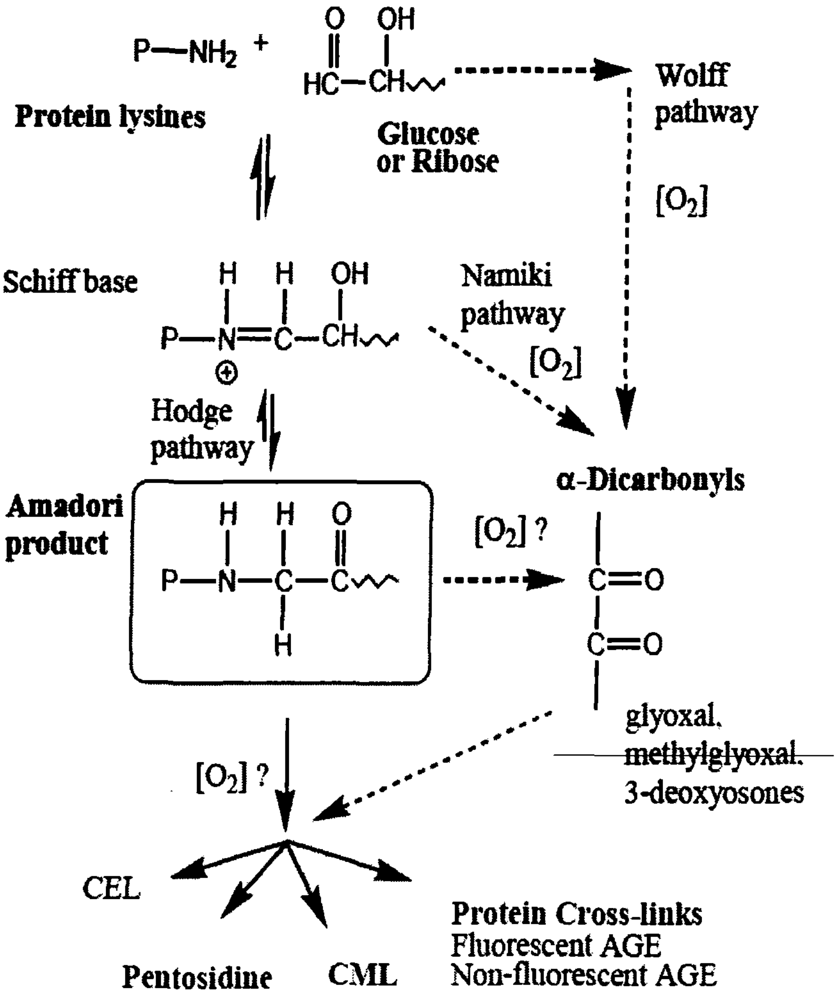
P—NH2 +
Protein lysines
HC—CH
Glucose
or Ribose
Wolff
pathway
[O2]
Schiff base
P—N=C—CH
Namiki
pathway
[O2]
Hodge
pathway
Amadori
product
P—N—C—C
[O2] ?
α-Dicarbonyls
C=O
C=O
glyoxal,
~~methylglyoxal,~~
3-deoxyosones
[O2] ?
CEL
Pentosidine
CML
Protein Cross-links
Fluorescent AGE
Non-fluorescent AGE

Fig. 2. Schematic presentation of Maillard reaction pathway (Booth *et al.*, 1997)

The increased levels of MG in diabetics and the identification of a number of AGEs that are derived from MG, make a strong case for the importance of this reactive α-oxoaldehyde in the development of diabetic complications (Bourajjaj *et al.,* 2003; Beisswenger *et al.,* 2005). Increased MG formation is the consequence of increased availability of MG precursors such as plasma glucose, consumption of ethanol, threonine or accumulation of fat (Wu, 2005). Overconsumption of food containing carbohydrates and fat with high levels of ethanol provide precursors for MG generation and eventually cause an overproduction of MG in vivo (Wu, 2006). MG initially reversibly reacts with arginine, lysine, or cysteine residues of proteins, and the subsequently irreversible reaction produces AGEs. MG is believed to be the most important source of AGEs (Shinohara *et al.,* 1996; Bourajjaj *et al.,* 2003).

Glyoxal, another reactive α-oxoaldehyde and a physiologic metabolite, is formed by the oxidative degradation of glucose, lipid peroxidation, ascorbate autoxidation and degradation of glycated proteins (Wells-Knecht et al., 1995; Mlakar *et al.,* 1996; Schwarzenbolz *et al.,* 2008). It can modify the side chains of various amino acids in proteins to give several glycation end products (Lederer and Klaiber, 1999; Brock *et al.,* 2003; Thornalley, 2002). Furthermore, glyoxal also reacts with free thiol groups on amino acids, peptides and proteins to form thiol- aldehyde adduct (Zeng and Davies, 2005).

Increased levels of MG/ glyoxal have also been reported in the blood from diabetic patients and in lens of streptozotosin-induced diabetic rats (Phillips, 1993; Mclellan, 1994; Lapolla *et al.,* 2003). Accumulation of glyoxal, MG and other oxoaldehyde in cells lead to the modification of proteins that may lead to protein degradation, enzyme inhibition and a cytokine-mediated immune response (Vaca *et al.,* 1994; Papuolis *et al.,* 1995; Brinkmann *et al.,* 1998; Westwood *et al.,* 1997). Since α-oxoaldehydes are more reactive than glucose in glycation reactions, these intermediates potentiate the rate of protein glycation. Because the formation of α-oxoaldehydes can also occur due to fragmentation of Schiff' base (Namiki pathway) as shown in Fig. 2, AGE may be formed without the delay required for the fructosamine formation via the Amadori rearrangement (Thornalley *et al.*, 1999).

1.1.2. Advanced glycation end products (AGEs) and their receptors

AGEs are the class of complex compounds formed as end products of glycation reaction (Wautier and Guillausseau, 2001). A number of AGEs are formed *in vitro* under conditions simulating the physiological state and some of these have also been isolated from human tissues affected by disorders like diabetes. Most of the AGE intermediates are highly unstable and reactive, some of which are difficult to analyze. The term melanoidins initially proposed by Maillard is presumably related with brown colour of the AGEs. Along with brown color, fluorescence is one of the qualitative properties used to characterize the AGE formation. The first fluorescent AGE crosslink pentosidine was first isolated and identified from dura mater collagen (Sell *et al.*, 1991).

Pentosidine and CML are produced by the oxidation reaction at the advanced stage while pyralline is produced by non oxidative reaction from α-oxoaldehydes such as 3DG (Frye *et al.*, 1998) Other important AGEs include hydroimidazolones derived from deoxyglucosone, N_ε-carboxymethyl lysine, N_ε-(1-carboxyethyl)-lysine, pyrraline, several bis (lysyl) imidazolium derivatives, pentosidine, argpyrimidine and arginine derived N_δ-(4-carboxy-4, 6-dimethyl-5,6dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-ornithine (Ahmad *et al.*, 2002; Kilhovd *et al.*, 2003). Based on the chemical structure, three types of AGE may be distinguished:

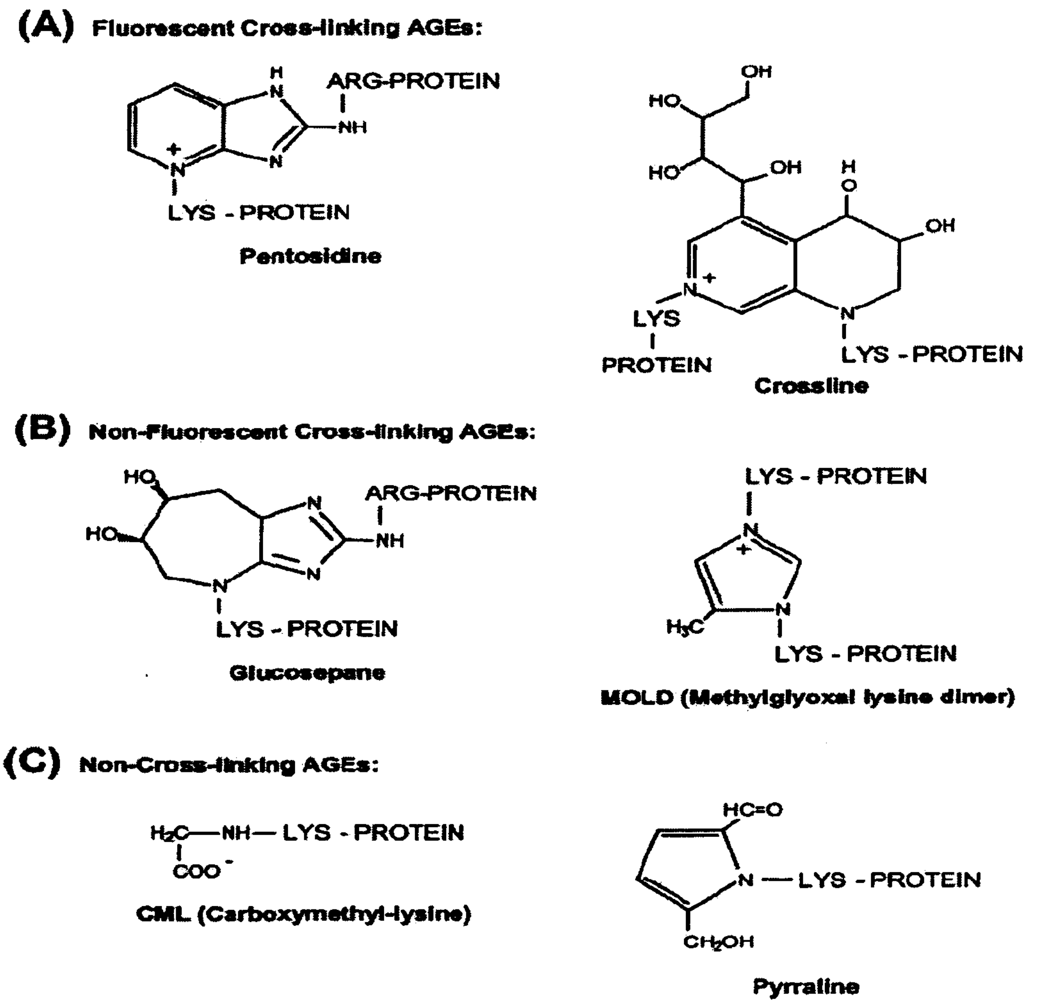
(A) Fluorescent Cross-linking AGEs:
ARG-PROTEIN
NH
LYS - PROTEIN
Pentosidine
HO
OH
LYS
PROTEIN
LYS - PROTEIN
Crossline
(B) Non-Fluorescent Cross-linking AGEs:
ARG-PROTEIN
LYS - PROTEIN
Glucosepane
LYS - PROTEIN
LYS - PROTEIN
MOLD (Methylglyoxal lysine dimer)
(C) Non-Cross-linking AGEs:
H₂C—NH— LYS - PROTEIN
COO⁻
CML (Carboxymethyl-lysine)
HC=O
N — LYS - PROTEIN
CH₂OH
Pyrraline

Fig. 3. Various types of AGEs

A number of AGE receptors (RAGE) have been identified in macrophages, endothelial and several other types of cells (Skolnik *et al.,* 1991). Phagocytic cells expressing RAGE internalize and digest AGE modified proteins and therefore these receptors are implicated in protein turnover, tissue remodeling and inflammation (Schmidt *et al.,* 2001; Vlassara, 2001). Interaction of AGE with its RAGE generates intracellular oxidative stress resulting in the activation of the transcription factor NF-*kappa B* and subsequent gene expression which is relevant in diabetic complications (Zill *et al.,* 2001). Circulating AGEs are modified by various enzymes to highly reactive glycotoxins that enters blood circulation where they generate new AGEs by reacting with plasma or tissue components. At this stage glycation accelerates the progress of deterioration (Jakus, 2000)

1.2. Glycation of proteins with various sugars

Majority of the innumerable studies carried out on glycation reactions as discussed earlier focused on those mediated by glucose (glucation), since glucose is the most abundant sugar in blood and tissues. It is now well recognized that glucose and the level of the sugar is elevated remarkably in diabetes. Other reducing sugars have also been shown to cause glycation. These include galactose (Urbanowsi *et al.,* 1982), mannose and fucose (Zaman *et al.,* 1981), glucose-6-phosphate (Hanney *et al.,* 2003), ribose (Khalifah *et al.,* 1996], glyceraldehydes (Acharya *et al.,* 1992), sialic acid (McKinney *et al.,* 2002), dihydroxyacetone (Peterson *et al.,* 2004) and lactose (Scaloni *et al.,* 2002). The extent of modification taking place *in vivo* have been minimal (1±2 mol of glycating agent attached per mol of protein) to high (30±40 mol per mol of protein) (Westwood and Thornalley, 1995; Thornalley *et al.,* 2000). Excellent reviews are available on various aspects of glycation reactions, mainly by glucose (Ulrich and Cerami, 2001; Stitt, 2003). A more recent review on the fructose mediated glycation has also appeared (Schalkwijk *et al.,* 2004).

1.2.1. Glycation by fructose

Recent years have witnessed a remarkable interest the *in vivo* and *in vitro* glycation reactions mediated by fructose (fructation) for a variety of reasons. Fructose, also described as the fruit sugar, is a ketohexose and the sweetest among the naturally occurring sugars. A recent study has shown that high intake of fructose by the vegetarians may contribute towards increase in AGE levels and presumably to accelerated ageing (Krajcovicova-Kudlacova *et al.,* 2002).

Fructose levels *in vivo* rarely increase beyond those of glucose but the high reactivity of the former makes it a serious contributor to the glycation reactions.

Since it is the open chain forms of various sugars that are reactive towards the amino groups, those with more stable open chains are more reactive. In solution the open chain form of fructose constitutes over 0.7% of the total sugar as compared to the 0.002% of glucose- a difference of over 350-fold (Bunn and Higgins, 1981).

Fructose is mainly generated through the polyol pathway in the body (Gabbay, 1975). While *in vivo* fructose levels are normally quite low as compared to those of glucose in majority of tissues, the ketosugar can accumulate in high concentrations in cells that operate the sorbitol pathway (Kinoshita *et al.,* 1979). For instance, in the human ocular lens, fructose concentration may rise up to 23-fold during diabetes, far exceeding the concentration of glucose (Tomlinson *et al.,* 1994). Similar local increases in fructose concentrations have also been demonstrated in conditions like diabetes in peripheral nerves, blood vessels, erythrocytes (Poulsom *et al.,* 1983 Tomblinson, 1985) and testis (Fakuola *et al.,* 1989) that operate the active polyol pathway. In addition, fructation appears to differ from glucation in its rapid second transformation i.e. rearrangement of the Amadori product in Heyns kind of rearrangement presumably due to the high reactivity of the resulting Amadori product (Suarez *et al.,* 1989).

Bunn and Higgins (1981) in an earlier study have shown that the rate of Schiff base formation between hemoglobin and fructose was 7.5 times faster than that with glucose. Suarez *et al.,* (1995) reported in a more recent study that fructated BSA is clearly more resistant to degradation by an ATP-dependent proteolytic system than the glucated protein due to the formation of higher amount of Maillard fluorophore. Since fructose is more active in glycation reactions and ε-aminogroup of lysine and α-amino groups, which are modified by glycation, have been implicated in ubiquitination and ATP dependent proteolysis (Hershko *et al.,* 1984), the observation was not unanticipated. Sakai *et al.,* (2002) observed that fructose is more readily degraded to dicarbonyl compounds in solution as compared to glucose.

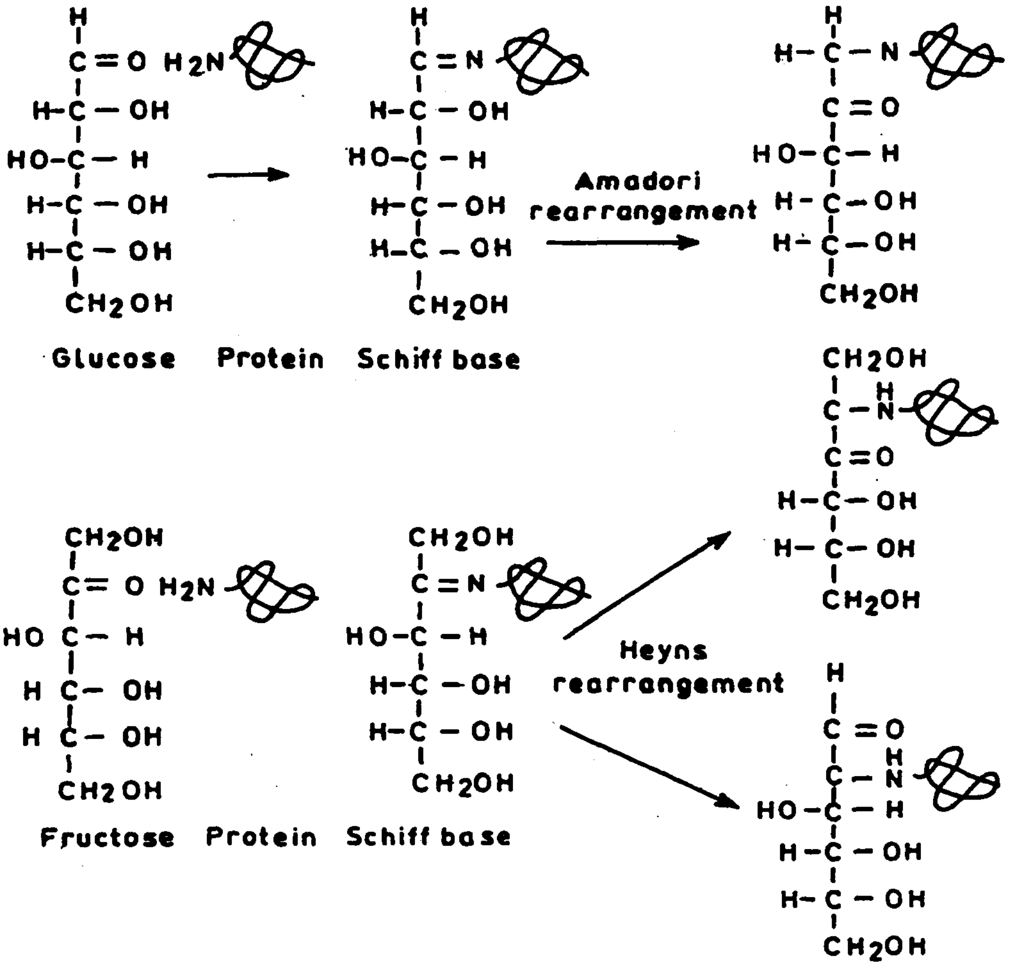
Glucose
Protein
Schiff base
Amadori rearrangement
Fructose
Protein
Schiff base
Heyns rearrangement

Fig. 4. The early reactions of proteins with glucose and fructose

Evidently, the post Amadori products of fructose, like those of glucose, are highly heterogeneous and complex and not yet fully characterized. They however seem to differ significantly from Amadori products generated during glucation (Suarez et al., 1989; McPhereson et al., 1998)

1.2.2. Glycation by ribose

As compared to glucose and fructose the pentose sugar ribose was found most potent glycating agent (Khalifah *et al.,* 1996; Drasta *et al.,* 2002). Ribose is a naturally occurring sugar synthesized in the body from glucose and is an essential component of ATP. Ribose also occurs in RNA, one of the main information-carriers of living organisms. Because ATP is rapidly used by muscles in high-intensity workouts and because RNA is important in protein synthesis, ribose supplements and energy drinks containing ribose are being promoted for energy enhancement and better exercise performance. Ribose supplements haven't been extensively studied, but emerging evidence does suggest that they benefit patients with congestive heart failure, a serious condition in which the heart cannot pump sufficient blood to meet the body's circulatory needs. In a recent study Omran (2007) reported ribose appears to improve heart function and quality of life among these patients by increasing levels and availability of ATP. Preliminary evidence also indicates that ribose may ease the pain and fatigue of patients with fibromyalgia and chronic fatigue syndrome. According to the conventional dogma, a pentose sugar, such as ribose, reacts first with a lysine residue on proteins to form an Amadori product, which then reacts with an arginine residue to form pentosidine (Sell and Monnier, 1989). Ribose levels are expected to rise in cells of diabetics where the pentose phosphate pathway is enhanced. A likely intramolecular source of reducing sugar is ADP ribose, a recognized histone glycation and glycoxidation agent *in vitro* (Lauren *et al.,* 1996). Syrovy (1994) compared glycation of rat skeletal muscle myofibrils with various sugars and the maximum decrease in the myofibrillar ATPase activity was observed by glycation with ribose. Incubation of link proteins and proteoglycans with ribose results in crosslinking both *in vivo* and *in vitro* (Pokharana and Pottenger, 2000). Janual *et al.,* (2003) showed that covalent coupling of reduced glutathione with ribose might contribute to the decrease in cell GSH levels and glutathione peroxidase activity.

Another enzyme aspartate aminotransferase has also been shown to be inhibited by glycation with ribose (Drasta *et al.,* 2002). Ribose also produces AGE ligands that have very high binding affinity for RAGEs (Valencia *et al.,* 2004).

1.3 Glycation of various biomolecules

As compared to glycation reactions involving molecules like nucleic acid and lipids, protein glycation has been studied extensively showing numerous structural alterations including exposure of thiols, protein compaction, cross linking, fragmentation and susceptibility to proteolysis (Seidler, 2005). Glycation by various sugars of a limited number of amino groups in proteins like hemoglobin, albumin and low density lipoproteins induce number of alterations in proteins and loss of biological activity (Turk, 2001). These include conformational alterations, exposure of hydrophobic residues and thiols, loss in allosteric sensitivity (Bunn *et al.,* 1970), ligand binding (McDonald *et al.,* 1979) and receptor recognition (Lorenzi *et al.,* 1994). Several enzymes undergo glycation induced loss of biological activity including malate dehydrogenase, glucose 6 phosphate dehydrogenase, glutathione reductase, glyceraldehydes-3-phoshate dehydrogenase, catalase and superoxide dismutase (Heath et al., 1996; Seidler, 2005; Jabeen et al., 2006).

AGE formation can result in the modification of the functional properties of the key extracellular matrix molecules. For example, in collagen, the most abundant protein in the body, AGEs induce intermolecular bonds (Monnier *et al.,* 1996). Formation of AGEs on laminin, another extracellular protein, causes reduction in polymer self-assembly and decreased binding of other major components like type IV collegen and heparin sulfate proteoglycan (Makino *et al.,* 1995; Haitoglou *et al.,* 1992). In diabetes mellitus, protein glycation and glucose auto-oxidation may generate free radicals, which in turn catalyze lipid peroxidation (Baynes, 1991).

Crystallins are synthesiszed during lens development and retained without turnover and at least some of these are as old as the organism itself (Hoenders and

Bloemendal, 1983). They are therefore among the most susceptible to glycation and numerous studies suggest that the glycation of lens crystallins in diabetes and aging results in their unfolding, aggregation and crosslinking. Yan and Harding (2003) have shown that γ-crystalline undergo glycation most readily and the primary target of glucose is γ-IIIb-crystallins, while that of fructose is γ-IIIa-crystallin. Glycation of α-crystallin caused crosslinking and high molecular mass aggregation along with alterations in secondary and tertiary structures (Kumar *et al.,* 2004). High susceptibility of the γ-crystallins to glycation has also been reported in earlier studies (Swamy and Abraham, 1991; Smith *et al.,* 1996). A report on the glycation of α-crystallins as related to their chaperone role is also available (Blakytyn and Harding, 1995). Yan and Harding (2006) observed that the glycation results in inactivation of its chaperonic function.

As in case of proteins, DNA bases also contain amino groups that offer potential sites for glycation and generation of AGEs, but deoxyguanosine appears to be most reactive under physiological conditions (Thornalley, 2003). Several nucleotides adducts from glycated DNA have been identified (Mistry *et al.,* 2003). The presence of AGE on DNA causes unusual transpositional rearrangements (Lee and Cerami, 1990; Bucala *et al.,* 1993). In addition to the damage caused to DNA by direct reaction with sugars, protein AGEs may exert remarkable genotoxic effects. Wondrak *et al.,* (2002) have shown that glycated proteins can serve as photosensitizers of DNA damage. Stopper *et al.,* (2003) have shown that AGE-BSA caused marked DNA damage as revealed by the comet-assay.

1.3.1. Glycation of HSA

HSA is the most abundant protein in plasma that comprises about 60% of the total protein found in blood (Shaklai *et al.,* 1984). HSA is important for maintaining osmotic pressure in the circulatory system (Peters, 1996) and also plays protective role as antioxidant (Coussons *et al.,* 1997). HSA has highly flexible structure and can bind various molecules for transportation throughout the body.

Primary, secondary and tertiary structure of HSA is well characterized. HSA comprises 585 amino acids with a molecular mass of 66,438 Da. It has 28 α-helical segments that comprise 67% of the secondary structure. Rest of the secondary structure consists of 10% β-turns and 23% extended peptide chain. The tertiary structure of HSA is arranged in 80x80x30Å heart shape in three homologous domains I, II, III (Fig. 5A). It contains only one tryptophan located in domain II (Peters, 1996; Coussons *et al.,* 1997; Weber, 1975).

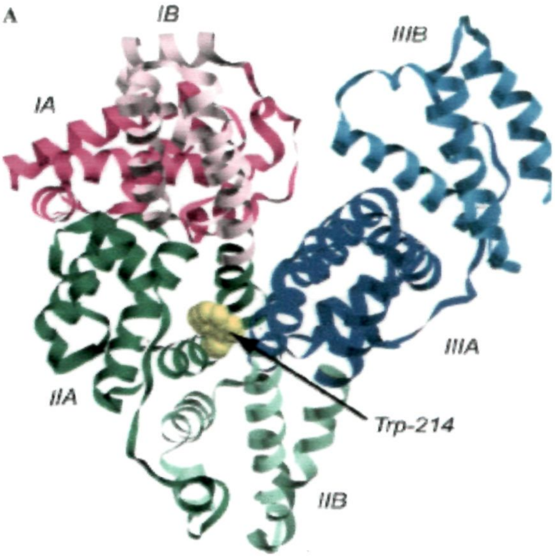

Fig. 5A. Ribbon diagram of three dimensional structure of HSA (Mendez *et al.,* 2005)

Glycation of albumin occurs at multiple sites. Glucose can be attached to Lys^{199}, Lys^{281}, Lys^{439}, and Lys^{525} as well as at some other lysine and arginine residues, and also at the N-54 terminal residues of polypeptides (Iberg and Fluckiger, 1986). Lysine-199 is one of the major glycation sites of HSA (Iberg and Fluckinger, 1986), plays a key role in the binding of anti-inflammatory drug (VanBoekel *et al.,* 1992) and such binding may result in a decrease in glycation of HSA with glucose 6 phosphate *in vitro* (VanBoekel *et al.,* 1992) presumably by competitive binding to a favored site of glycation (Fig. 5B).

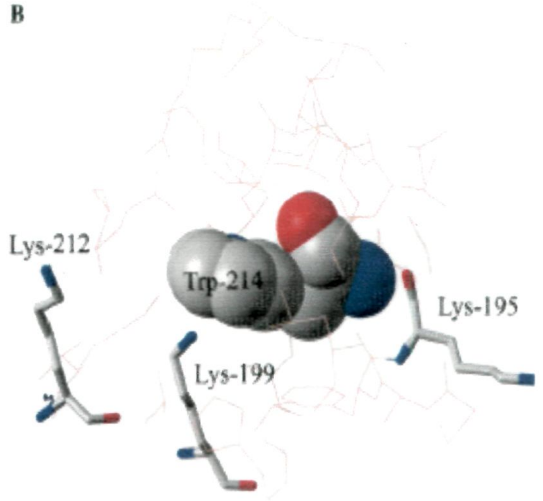

Fig. 5B. Structure of HSA around Trp with three closest lysine residues indicated (Mendez *et al.,* 2005)

In diabetes, the level of glycated HSA may rise from 6-10% to 20-30% and hence serves as the indicator of glycation (Nakajou *et al.,* 2003). HSA can potentially undergo glycation on some of its 58 lysine residues (Guthrow *et al.,* 1979). As a result, glycated HSA has been proposed as a useful marker for short term monitering of the diabetes patients. HSA also represents the major and predominant circulating antioxidant in plasma known to be exposed to continuous oxidative stress (Soriani *et al.,* 1994). Glucose and free radicals were found to impair the antioxidant properties of the serum albumin (Bourdon *et al.,* 1999). HSA possesses esterase activity due to the close proximity of Arg-410 and Tyr-411 residues (Watanabe *et al.,* 2000). According to Nakajou *et al.*, (2003) *in vitro* glycation of HSA with glucose causes inhibition of its esterase activity. Glycated and AGE modified albumin are reported to have different functional (Vlassara *et al.,* 1992) and immunological properties (Makita *et al.,* 1992) as compared to non-glycated counterparts. Sattarahmady et al., (2007) reported the formation of molten globule state in HSA after prolonged glycation. Also glycation may results in the formation of thermodynamically more stable high molecular weight aggregates than its unglycated forms (Khan et al., 2007)

1.3.2. Glycation of various enzymes

A large of number enzymes undergoes remarkable inactivation in response to glycation and the mechanism of loss of biological activity has received considerable attention. Diverse enzymes are modified by glycation. Some enzymes are inactivated due to modification in their active sites. Glyceraldehyde inactivates pancreatic glucokinase and substrate prevents inactivation (Murata *et al.,* 1993) suggesting active site modification. Fructose inactivates GADPH (Zhao *et al.,* 2000) suggesting that the glycolytic pathway in hexokinase containing cells may be at the risk of inhibition. The primary site of glycation is near the active site and glycation may change the microenvironment around the crucial C149 residue of the enzyme (He *et al.,* 1995). Yan and Harding (1997) studied the inactivation of two important antioxidant enzymes catalase and superoxide dismutase resulting from incubation with various sugars. Ribose and fructose inactivated the enzymes along with their antigenicity more rapidly than glucose and glucose-6-phosophate. Cu,Zn-SOD is inactivated initially by glycation at Lys-122 and Lys-128, followed by specific as well as non-specific fragmentation (Arai *et al.,* 1987)

The process of glycation *in vitro* is slow necessitating long incubations and high and usually unphysiological concentration of the sugars. Derham and Harding (2002) have however shown that entrapment of enzymes inside the resealed human erythrocyte ghosts that provides enzymes with a more physiological environment, promotes glycation. Using the system they demonstrated that incubation with fructose leads to a remarkable inactivation of catalase, malate dehydrogenase, glutathione reductase, fumerase, aldehyde dehydrogenase, lactate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase and superoxide dismutase. Glycation and crosslinking glycation products cause a decrease in chaperon function of α-crystallin (VanBoekel *et al.,* 1996; Hook and Harding, 2002). Fructation also inhibits the human erythrocyte membrane associated Na/K-ATPase and Na/K pump but α-crystalline entrapped in the resealed ghosts offers remarkable protection against the inactivation (Derham *et al.,* 2003)

Bousova *et al.* (2005) have demonstrated that fructose glycates aspartate aminotransferase more readily than glucose and during glycation a good correlation was observed between enzyme modification and catalytic activity as compared to that with pentosidine AGE formation. Fructose and several other hexoses and pentoses have been shown to inactivate δ-aminolevulinic acid dehydratase an enzyme involved in heme biosynthesis (Cabellero *et al.,* 1998). Since the activity of the enzyme is low along with the other heme enzymes porphobilinogen deaminase and uroporphyrinogen decarboxylase in diabetics, glycation of δ-aminolevulinic acid dehydratase may result in lowered synthesis of hemoglobin (Cabellaro *et al.,* 1998)

A number of other enzymes have also been shown to undergo inactivation by various sugars but more efficiently by fructose. These include glutathione reductase (Blaytyn and Harding, 1995), glucose-6-phosphate dehydrogenase (Ganea and Harding, 2005), malate dehydrogenase (Heath *et al.,* 1996), esterase (Yan and Harding, 2003), isocitrate dehydrogenase (Kil *et al.,* 2004), alanine transferase (Barenek *et al.,* 2001) and Ca^{2+} ATPase (Gonzalez *et al.,* 1993). Sorbitol dehydrogenase occurs both in glycated and non-glycated forms and the fraction of later is increased diabetic liver. Fructose was remarkably more active in bringing about glycation and inactivation of the enzyme in vitro (Hoshi *et al.,* 1996). Fructose has been to inactivate glyceraldehyde-3-phosphate dehydrogenase that indicates potential inhibitory effects on the glycolytic pathway (Zhao *et al.,* 2000; Hook and Harding, 1992). The inhibition is significant since inactivation of the enzyme results in the accumulation of glyceraldehydes-3-phosphate, which is highly reactive in glycation reactions.

Extensive glycation of aspartate aminotransferase causes decreased flexibility as evidenced by a decrease in 1-anilinonahthalene-8-sulfonate (ANS) binding (Seidler & Seibel, 2000). Using the same model enzyme and glyceraldehyde it was shown that glycation causes an increased Tm suggesting a glycation induced rigidification of the enzyme (Yeargans *et al.,* 2003). Loss of enzyme activity correlate with increased Tm when a concentration range of glycating agent was tested (Seidler *et al.,* 2000) suggesting that enzyme inhibition is due to decrease flexibility of the protein. The increase in the rigidity of the 3-dimentional structure of the glycated proteins may be in part attributed both to intra- and inter-molecular crosslinking. Free amino groups from N-terminus or from lysine residues are required for protein crosslinking as evidenced by incubation of α-dicabonyls (methylglyoxal, glyoxal) with lysine free peptides (that contain arginine) and arginine free peptides (that contain lysine) (Miller *et al.,* 2003). Protein crosslinking typically accompanies the glycation-induced loss of enzyme activity (Morgan *et al.,* 2002; Southwell *et al.,* 2002) Surface of the enzyme contains large number of hydrophilic residues to facilitate maintainance of the native structure of the enzyme. AGEs are hydrophobic hence they promote a condensation of substructures that result in local compaction within the protein (Yeargans *et al.,* 2003).

1.3.2.1 Glycation of RNase A

Pancreatic RNase A is an extensively studied highly thermostable enzyme (Raines, 1998). It is the first enzyme and third protein to be sequenced (Hirs *et al.*, 1960; Smyth *et al.,* 1963). RNase A is a small protein comprising of 124 amino acid residues with a molecular mass of 13,690 daltons (Pace *et al.,* 1995). It contains all the natural amino acids except tryptophan. The number of tyrosines in the enzyme is six and the protein is crosslinked by four disulphides. The secondary structure consists of long four stranded antiparallel β-pleated sheets and three short α-helices. The overall shape of the enzyme resembles that of a kidney (Fig. 6).

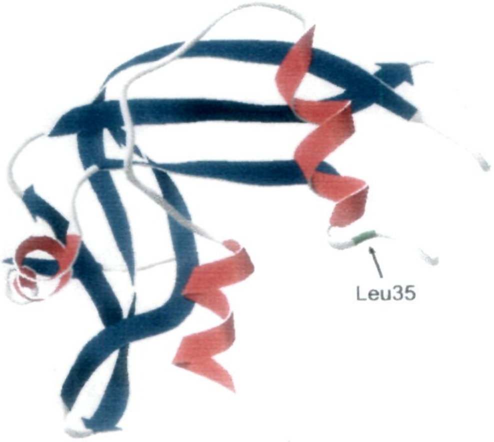

Fig. 6. Ribbon diagram of tertiary structure of RNase A (Brookhaven protein data bank and drawn with RASMOL)

RNase A contains 10 lysine residues including the amino terminal residue (Blackburn, 1976). The two amino groups have low pKa values Lys-1 (pKa ≈7.8) and Lys-41 (pKa ≈ 8.8) making them potentially more reactive in formation of the Schiff base, while the remaining lysine residues have pKa ≈10.5 and are less reactive. Three lysine (1, 7 and 41) are near or in the active site and modification of these three has been shown to decrease specific activity of the enzyme (Walter and Wold 1976). Earlier studies have described several reagents that are known to react preferentially with the active site lysine residues and inactivate enzyme at a rate much faster than overall rate of lysine modification (Richards and Wyekoff, 1971).

Brock *et al.,* (2003) identified Lys-41 in the active site of RNase A as the primary site for glycation and carboxymethylation. Glycation of RNase has been subjected to detailed investigation using glucose as model sugar but several reports on the reaction with other sugars are also available (Booth *et al.,* 1997). McPherson *et al.,* (1988) observed that while the *in vitro* rate of reaction with glucose and fructose with RNase were comparable, covalent non-disulphide crosslinking was induced

10 times more rapidly with fructose. In a previous study Arg-39 and Arg-85 were identified as the major sites of modification of RNase by α-dicarbonyls (Blackburn and Moore, 1982). Methylglyoxal, glyoxal and diacetyl also causes impairment of the RNase A activity (Miller and Gerrard, 2005). Irradiation of supercoiled phi X 174 DNA with solar simulated light in the presence of AGE modified RNase induced DNA single strand break (Wondrak *et al.,* 2002). The metal catalysed oxidation of phospholipids in the presence of RNase led to the loss of amino groups in RNase and incorporation of phosphate, hexanoate, entanedioate and palmitate into protein (Januszewsi *et al.,* 2005)

1.4. Protein oxidation in diabetes

Oxidative stress is suggested to be a potential contributor for the development of complications in diabetes (Baynes, 1991). Glycation has been considered as a fixative of free radical damage while glycated proteins act as free radical generators. Increased free radical production and reduced antioxidant defense responses, encountered in the diabetic state may give rise to increased oxidative stress (Halliwell & Gutteridge, 1990). Consequences of oxidative stress are adaptation or cell injury, i.e. damage to DNA, proteins and lipids, disruption in cellular homeostasis and accumulation of damaged biomolecules (Jakus, 2000). Under conditions of severe oxidative stress, free radical generation leads to protein modification. Proteins may be damaged directly by specific interactions of oxidants or free radicals with particularly susceptible amino acid residues. They may also be modified indirectly, with reactive carbonyl compounds formed by the auto-oxidation of carbohydrates and lipids, with eventual formation of advanced glycation/lipoxidation end products (Gumieniczek, 2005).

Diabetic patients exhibit elevated levels of intracellular iron and copper ions which in the presence of glycated proteins, have been shown to enhance the generation of free radicals *in vitro* (Dean *et al.,* 1991). These highly reactive species in turn are able to induce oxidative degradation of protein *in vitro* (Pacifici and Davies, 1991). Interestingly, metal-catalysed glycoxidative pathways were described to involve hexoses as possible precursors, in addition the to pentoses (Chace *et al.*, 1991; Dyer *et al.*, 1991). Under *in vivo* conditions, Cu^{2+} and Fe^{3+} are the most important metal-ion catalysts of the oxidative reactions. In blood plasma and in cellular cytoplasm virtually all of these ions are sequestered in specific metal transporters and other metalloproteins (Evans *et al.*, 1989; Rae *et al.*, 1999). However, copper metalloproteins such as ceruloplasmin and Cu- Zn superoxide dismutase and the iron metalloproteins such as hemoglobin and myoglobin have been shown to undergo significant conformational change and even fragmentation during glycation reactions, causing the release of bound metal (Takata *et al.*, 1996; Cussimanio *et al.*, 2003).

1.5. Pathogenecity of glycation and AGEs

Glycation cytotoxicity is the result of: (a) inhibition of specific functions of proteins; (b) cross-linking, aggregation, and precipitation of proteins; and (c) production of reactive oxygen species. AGEs have been shown to be intimately involved in the pathology of many diseases, including diabetes,(Metz *et al.*, 2003), inflammatory diseases, (Anderson and Heinecke, 2003), atherosclerosis and heart failure, (Zieman and Kass, 2004), macular degeneration (Howes *et al.*, 2004), osteoarthritis (DeGroot *et al.*, 2004), rheumatoid arthritis (Drinda *et al.*, 2004), Alzheimer's disease (Choei *et al.*, 2004 and Lueth *et al.*, 2004), poor bone healing (Santana et al., 2003), cataracts (Nagariaji *et al.*, 2002) and kidney disease (Alderson *et al.*, 2003). AGEs are especially accumulate on long-lived proteins such as collagen, the most abundant protein in the body, which makes up a significant part of skin, bones, cartilage, tendons, teeth and the cardiovascular system. AGEs cross-link various proteins, making them stiffer and less elastic. They damage normal three-dimensional protein structure, inhibit the physiologic function of proteins, and trigger inflammatory reactions. These stable compounds (AGEs) accumulate slowly throughout the lifespan and contribute to structural and physiologic changes in the cardiovascular system such as increased vascular and myocardial stiffness, endothelial dysfunction, altered vascular injury responses, and atherosclerotic plaque formation (Zieman and Kass, 2004). An overview of the role of protein glycation in the development of diabetic complications is presented below.

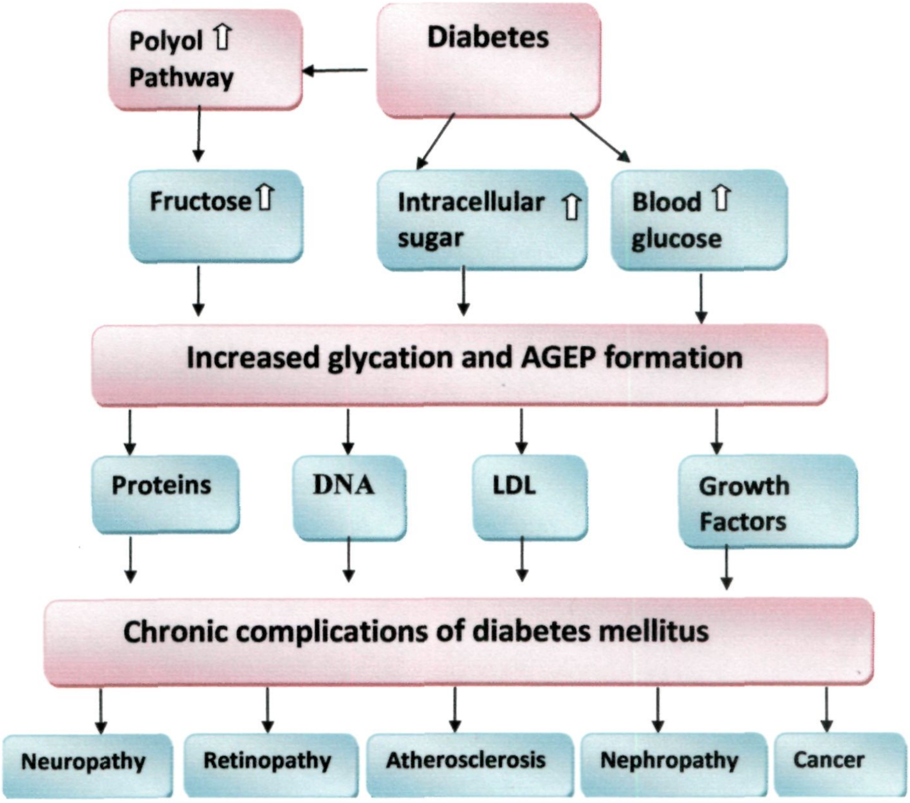
Polyol ⇧ Pathway
Diabetes
Fructose ⇧
Intracellular ⇧ sugar
Blood ⇧ glucose
Increased glycation and AGEP formation
Proteins
DNA
LDL
Growth Factors
Chronic complications of diabetes mellitus
Neuropathy
Retinopathy
Atherosclerosis
Nephropathy
Cancer

Fig. 7. Effect of enhanced AGE formation in diabetic complications (Ahmad and Ahmed, 2006)

AGEs are a major causal contributor to aging and the diseases of aging but unfortunately, there are few enzymes in the body that effectively break down the AGEs and their pathologic and unwanted cross-links in proteins. Glycation of proteins, lipids, and nucleic acids may play an important role in the natural processes of aging. Protein glycation has been associated with beta-amyloid deposits and formation of neurofibrillary tangles in Alzheimer disease, and possibly other neurodegenerative diseases involving amyloidosis (Colaco and

Harrington, 1994). Glycated proteins have also been shown to be toxic, antigenic, and capable of triggering cellular injury responses after uptake by specific cellular receptors (Vlassara *et al.,* 1994, Daniels & Hauser, 1992, Brownlee, 1994, Cohen *et al.,* 1994, Brett *et al.,* 1993 and Yan *et al.,* 1994). The group of glycation products consists of many different structures. They induce many cellular responses by binding to a specific receptor for the AGEs. AGE-HSA inhibits the proliferation of vascular endothelial cells and induces apoptosis in dose and time dependent manner (Lin *et al.,* 2003).

1.6. Assays of glycation

Considering the important role of glycation in causing damage to various tissues and organs, assays of glycation have received remarkable attention. In clinical practice the AGE measurement offers direct means of monitoring its accumulation in various tissues for use as prognostic marker and in evaluating the efficacy of various therapies. The development of methods based on high pressure liquid chromatography (HPLC) allows the detection of glycation. Mc Pherson *et al.,* (1988) used a procedure based on HPLC analysis of phenylthiocarbamoyl derivative of glycated proteins followed by calculation of position of C-1 and C-2 of hexose covalently bound to proteins. Analysis of glycated amino acids showed that 85% of the attached hexose is connected to ε-amino groups of lysyl residues via carbon 2(C-2) of the hexose chain and the remainder being connected via C-1. Ahmad *et al.,* (1986) identified CML in human lens and tissue collagen by HPLC which was confirmed by gas chromatography mass spectrometry. They observed that the oxidative degradation of Amadori adducts with proteins occur *in vivo* that leads to the formation and excretion of CML and lactic acid. However this procedure is complicated for routine analysis especially for *in situ* detections. Earlier studies employed strategies based on various chromatographic methods using the specific interaction of boronate ligands with the cis-diols of glycated proteins and these continued to be widely used for measuring glycated proteins (Furth, 1988).

Another approach for the quantification of AGE is an immunological assay (Makita *et al.,* 1992). Mitsuashi *et al.* (1997) had shown the immunological recognition of AGE by competitive ELISA and also suggested that *in vitro* modified proteins might not be appropriate standards for AGE that occur naturally *in vivo.* The lack of available certified glycation standards hamper standardization of various methods discussed. Miyazawa *et al.* (1998) isolated a novel antibody that exclusively recognizes fructated proteins but not adducts formed from other sugars. This method is simpler and more convenient than other quantitative detections of fructated proteins as there is no specific reliable method to detect glycation with fructose. Some of the literature results are based on the use of polyclonal and monoclonal AGE antibodies (Nagai and Horiuchi, 2003).

Radioimmunoassay (RIA) (Sheikh and Robb, 1993) and enzyme linked boronate immunoassay (ELBIA) (Ikeda *et al.,* 1998) based methods are also available to evaluate the glycation levels of intact proteins and their hydrolysis products. For *in vivo* detection of AGEs, immunohistochemical methods have been employed (Hayashi *et al.,* 2002). However all the existing procedures are time consuming requiring several hours to obtain results.

Some spectroscopic methods for detection of glycation levels in the various substrates of interest are also available (Odetti *et al.,* 1990). The glycation level of plasma proteins have been evaluated by measuring fructosamine levels with the help of a colorimetric assay based on the ability of ketoamine to reduce the nitro blue tetrazolium dye (Johnson *et al.,* 1983). Kobayashi *et al.* (1993) measured glycated albumin by a method based on colorimetry of ketoglucose released from glycated protein (ketoamine) on heating with hydrazine. Another procedure that uses thiobarbituric acid (TBA) is based on the estimation of a complex formed by the reaction of TBA with 5-hydroxymethyl furfural which is released by the dehydration of sugar in boiling oxalic acid (Ney *et al.,* 1981). Sensi *et al.,* (1988) developed a procedure in which the glycated proteins are treated with sodium nitrite and sulfanilic acid followed by the addition of sodium hydroxide to facilitate the formation of a chromophoric diazonium salt that absorbs maximally at 492 nm.

Among the existing protein glycation assays the quantification of Amadori product via periodate remains most convenient. This method was first proposed by Gallop *et al.,* (1981) for the quantification the glycation in hemoglobin and later on adapted using a microplate reader for sensitivity enhancement. Another important improvement was achieved by reducing proteins with sodium borohydride before periodate oxidation to decrease the interference by serum glycoproteins. The terminal reducing sugars of glycoproteins cause excessive background noise in the assay (Ahmad and Furth, 1991). Ahmed and Furth (1992) observed that in spite of the high fluorescence exhibited by the fructated proteins, the enhancement was reflected neither in the fructosamine nor phenylboronate affinity glycation assays. The underestimations were striking with the fructosamine assays that suggested only about 5% formation of fructosamine of that induced by glucose, while the phenylboronate affinity assay showed the glycation by fructose to be only 25%. The thiobarbutyric acid and periodate based assays were also shown to greatly underestimate the glycation caused by fructose (Ahmed and Furth, 1992). Syrovy (1994) compared various colorimetric methods and found the extent of glycation by various sugars by one method does not correspond well with the results obtained by another one.

Advanced glycation endproducts are also determined by 'total AGE fluorescence'. Avendano *et al.,* (1999) characterized AGE specific fluorescence in collagen by measuring emission at 440 nm following excitation at 370 nm. Increase in brown pigment formation and fluorescence was observed with the advanced glycation of long lived tissue proteins such as lens crystallin, collagen and myelin (Avendano *et al.,* 1999; Sajithlal *et al.,* 1998). Fluorimetric methods are compromised due to several analytical problems, as the major AGEs in biological systems are non fluorescent and therefore not detectable. The estimation of total AGE fluorescence is currently used as representative of qualitative measure of damage by glycation adduct fluorophores. Now a days spectroscopy is widely employed in conjugation with analytical methods like HPLC (Wilker *et al.,* 2001). Other commonly used spectroscopic techniques for the detection of glycation induced changes either in protein or sugar structure are CD (Bouma *et al.,* 2003), IR spectroscopy (Khajehour *et al.,* 2006) or NMR (Howard and Smales, 2005). Presence of sugar complicates the determination of protein structure by conventional technique like X-ray crystallography. NMR studies have been successfully used for small glycated peptides, however solving the structure of glycated protein by NMR is however problematic as the increase in rotational diffusion causes a line broadening effect (Howard and Smales, 2005). Khajehpour *et al.,* (2006) used IR spectroscopy to moniter the alterations in the sugar moiety in glycated proteins.

Mass spectrometry has been effectively employed in studies on protein glycation and the high quality of result makes its use of particular interest (Lapolla *et al.,* 2001). In addition to *in vitro* glycation determination, MALDI can also be used for *in vivo* glycated samples (Lapolla *et al.,* 2006). Stefanowicz *et al.,* (2001) evaluated high temperature glycation of proteins and peptides by electrospray ionization mass spectrometry. Wa *et al.,* (2007) studied the drug binding sites that are modified in albumin by glycation by MALDI time of flight mass spectrometry. The technique is based on a unique sugar moiety neutral loss that was observed in the fragmentation spectra of glycated peptides on Q-Tof type mass spectrometers. All three sugar moieties displayed characteristic fragmentation patterns accompanying the parent and the fragment ions, which could be explained by consecutive losses of water and formaldehyde. A reversed phase liquid chromatography followed by neutral loss scan mass spectrometric methods is developed by Gadgil *et al.,* (2007) for the screening of glycation in proteins. Gadgil *et al.,* (2007) identified 31 lysine glycated residues from total 59 lysine residues present in HSA.

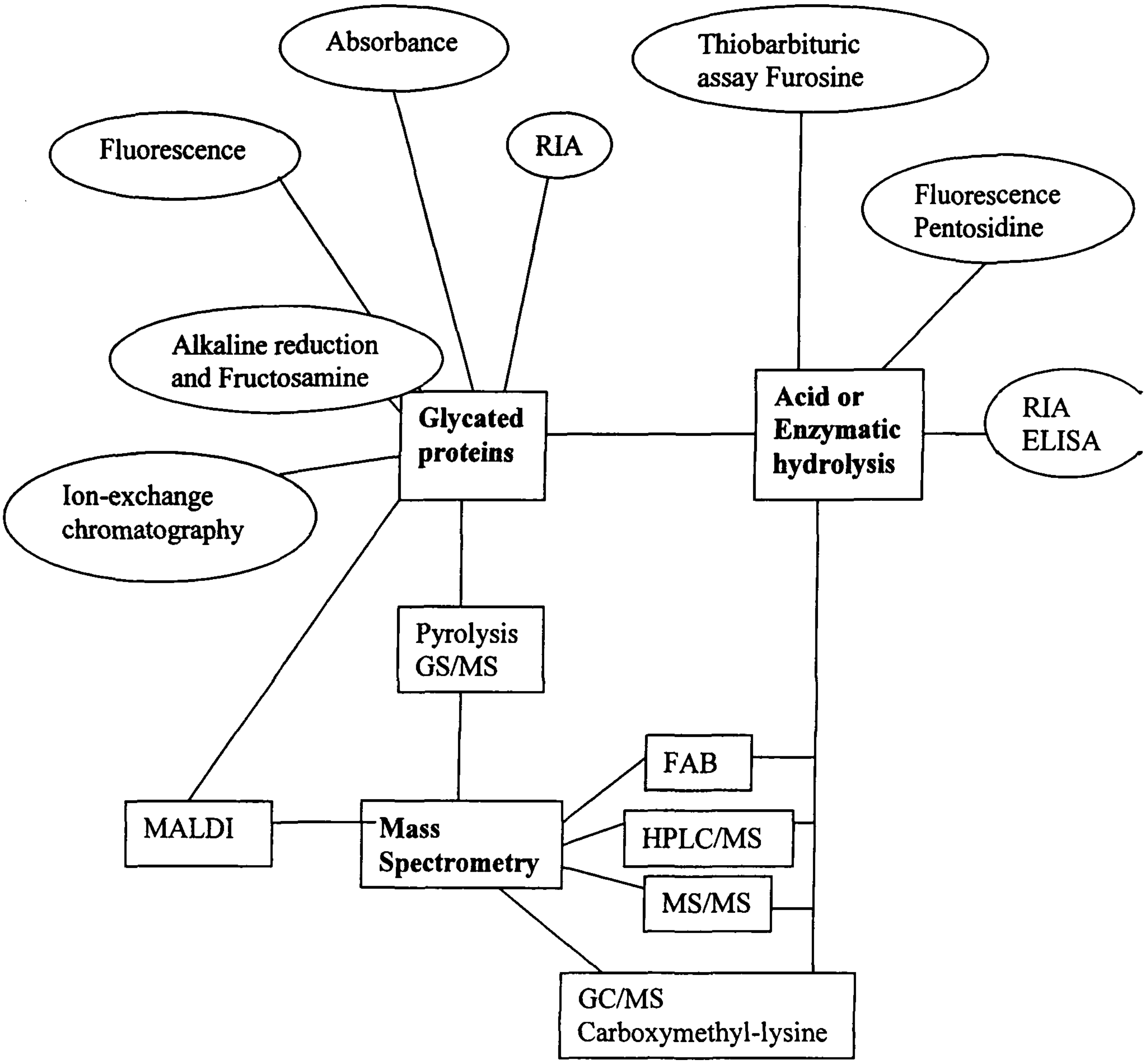
Absorbance
Thiobarbituric assay Furosine
Fluorescence
RIA
Fluorescence Pentosidine
Alkaline reduction and Fructosamine
Glycated proteins
Acid or Enzymatic hydrolysis
RIA ELISA
Ion-exchange chromatography
Pyrolysis GS/MS
FAB
MALDI
Mass Spectrometry
HPLC/MS
MS/MS
GC/MS Carboxymethyl-lysine

Fig. 8. Analytical approaches for the quantification of glycated proteins (Lapolla *et al.,* 2000)

1.7. Prevention of glycation

1.7.1 Natural defence

Body has several humoral and cellular defence mechanisms to protect tissues from deleterious effects of glycation reaction and AGE accumulation. These include the glyoxalase systems (I and II) that catalyses the deglycation of methylglyoxal (MG) to D-lactate (Thornalley, 1990; Thornalley, 1998). Other enzymes involved in the regulation of AGE formation are oxoaldehyde reductase and aldose reductase that detoxify reactive dicarbonyl intermediates (Boel *et al.,* 1995; VanderJagt *et al.,* 2001).

Additionally, a novel class of enzymes called as amadoriases were found that reverses the initial stage of the Maillard reaction between glucose and primary amines. The discovery of deglycating enzymes has implications for the repair of protein damage by fructose (Monnier, 2005). Another enzyme identified in erythrocytes, Fructosamine-3-kinase, catalyses the phosphorylation of protein bound fructosamine on their third carbon and leads to its destabilization and removal from protein (Collard *et al.,* 2004).

Receptors present on macrophages enable them to recognize and remove harmful AGE-proteins by endocytosis (Vlassara *et al.,* 1985). A variety of plasma amines may react with sugar and Amadori carbonyl groups to decrease the formation of AGEs. Antioxidants can protect biomolecules against the free radicals generated by glycation whereas transport proteins, for example, ceruloplasmin can bind transition metals such as cupric ions, preventing them from participating in autoxidative glycation or glyoxidation reactions.

1.7.2 Synthetic and natural inhibitors

Discovery and design of inhibitors for the glycation reaction should offer a promising therapeutic approach for the prevention of diabetes or other pathogenic complications. Currently several strategies are employed to control protein glycation. Most of the inhibitors have several sites of action. Some drugs have been specifically developed as AGE inhibitors or AGE breakers, RAGE and receptor signaling blockers. A number of therapeutic compounds are found to possess AGE inhibitory activity (Fig. 9).

The first compound that has been extensively studied *in vitro* and *in vivo* to be a powerful inhibitor of AGE formation is aminoguanidine (AG) (Brownlee *et al.,* 1986). AG prevents the formation of fluorescent AGEs and glucose derived collagen crosslinking. The mechanism of inhibition of AGE formation by AG involves trapping of reactive dicarbonyl intermediates such as MG, glyoxal and 3-DG (Thornalley *et al.,* 2000; Thornalley, 2003). In addition to chelating or antioxidant activity, AG also acts as true scavenger of carbonyl compounds (Thornalley *et al.,* 2000). OPB-9195, a hydrazine derivative similar to AG also inhibits pentosidine generation from diabetic plasma (Miyata *et al.,* 1998). Unfortunately, the clinical trials of this compound were hampered due to side effects related to the trapping of pyradoxal which results in vitamin B6 deficiency syndrome (Miyata *et al.,* 2005). Pyradoxamine, a form of vitamin B6, found to inhibit CML formation *in vitro* but it does not interact directly with Amadori intermediates but interfere with the post Amadori oxidative reactions by binding catalytic metal ions (Voziyan *et al.,* 2003; Chatyrkin *et al.,* 2008). It also traps reactive carbonyl compounds derived either from the sugars or lipids (Onarato *et al.,* 2000; Voziyan *et al.,* 2002). A lipophillic derivative of thiamine (vitamin B1), benfotiamine was also found to prevent vascular accumulation of AGE. Its proposed mechanism of action involves shunting of triose glycolytic intermediates towards the reductive pentose pathway (Babai-jadidi *et al.,* 2003). It also reduces aldose reductase mRNA expression and intracellular glucose and sorbitol levels (Berrone *et al.,* 2006).

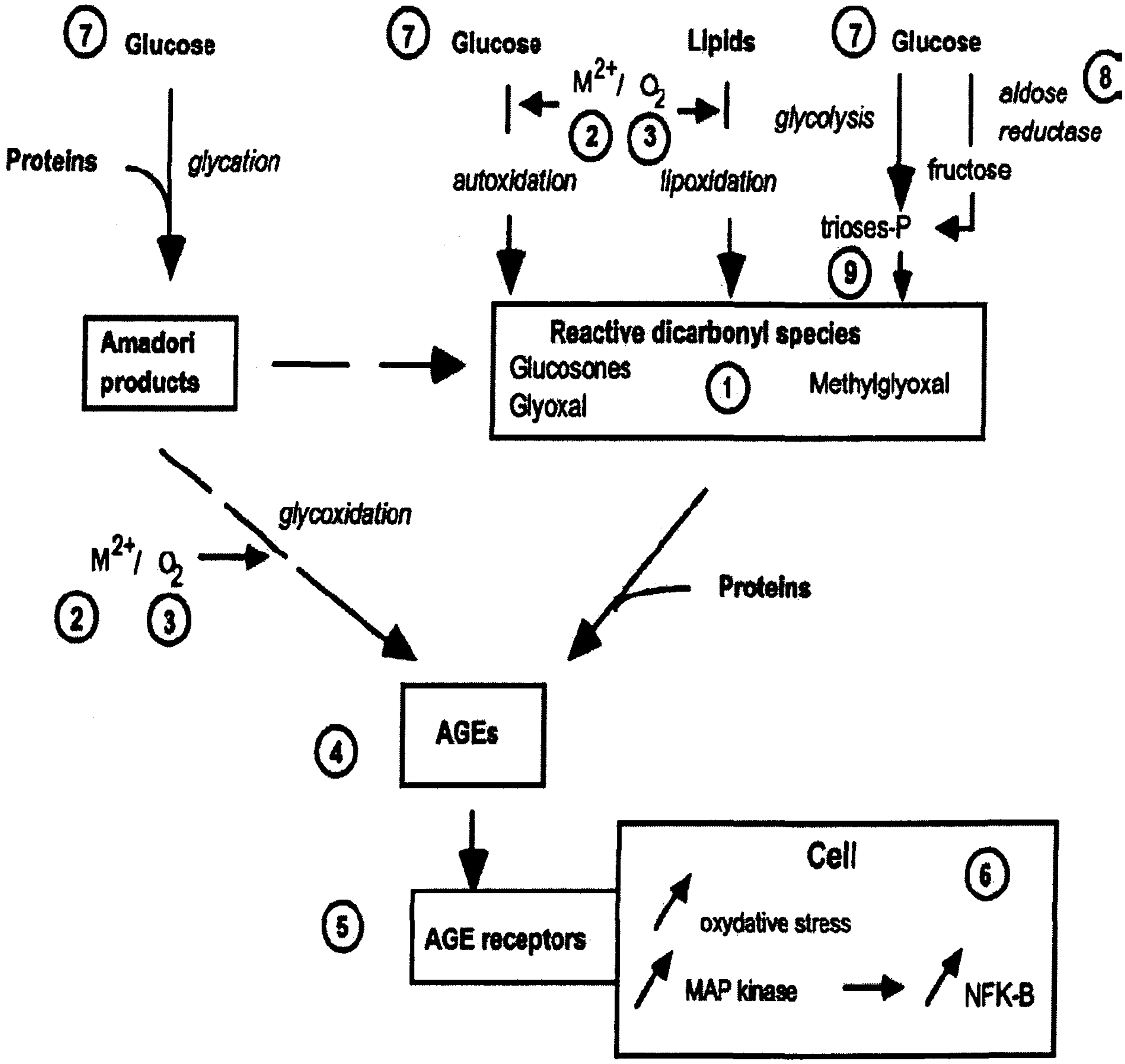
⑦ Glucose
Proteins
glycation
Amadori products
⑦ Glucose
M^{2+}/O_2
② ③
autoxidation
Lipids
lipoxidation
⑦ Glucose
glycolysis
aldose reductase
⑧
fructose
trioses-P
⑨
Reactive dicarbonyl species
Glucosones
Glyoxal
①
Methylglyoxal
glycoxidation
M^{2+}/O_2
② ③
Proteins
④
AGEs
⑤
AGE receptors
Cell
⑥
oxydative stress
MAP kinase
NFK-B

Fig. 9. Potential sites of inhibition of AGE formation and AGE-mediated damage (Peyrous and Sternberg, 2006) (1) trapping of reactive dicarbonyl species; (2) antioxidant activity by transition metal (M2+) chelation; (3) other antioxidant activity including free radical scavenging; (4) AGE cross-link cleavage (by AGE breakers); (5) AGE receptor (RAGE) blocking; (6) AGE receptor (RAGE) signaling blocking; (7) glycemia reduction by anti-diabetic therapy; (8) aldose reductase inhibition; (9) shunting of trioses-P towards the pentose-P pathway by transketolase activation.

The first compound that has been extensively studied *in vitro* and *in vivo* to be a powerful inhibitor of AGE formation is aminoguanidine (AG) (Brownlee *et al.,* 1986). AG prevents the formation of fluorescent AGEs and glucose derived collagen crosslinking. The mechanism of inhibition of AGE formation by AG involves trapping of reactive dicarbonyl intermediates such as MG, glyoxal and 3-DG (Thornalley *et al.,* 2000; Thornalley, 2003). In addition to chelating or antioxidant activity, AG also acts as true scavenger of carbonyl compounds (Thornalley *et al.,* 2000). OPB-9195, a hydrazine derivative similar to AG also inhibits pentosidine generation from diabetic plasma (Miyata *et al.,* 1998). Unfortunately, the clinical trials of this compound were hampered due to side effects related to the trapping of pyradoxal which results in vitamin B6 deficiency syndrome (Miyata *et al.,* 2005). Pyradoxamine, a form of vitamin B6, found to inhibit CML formation *in vitro* but it does not interact directly with Amadori intermediates but interfere with the post Amadori oxidative reactions by binding catalytic metal ions (Voziyan *et al.,* 2003; Chatyrkin *et al.,* 2008). It also traps reactive carbonyl compounds derived either from the sugars or lipids (Onarato *et al.,* 2000; Voziyan *et al.,* 2002). A lipophillic derivative of thiamine (vitamin B1), benfotiamine was also found to prevent vascular accumulation of AGE. Its proposed mechanism of action involves shunting of triose glycolytic intermediates towards the reductive pentose pathway (Babai-jadidi *et al.,* 2003). It also reduces aldose reductase mRNA expression and intracellular glucose and sorbitol levels (Berrone *et al.,* 2006).

Recently two new classes of aromatic compounds, derivatives of aryl (and heterocyclic) ureido and aryl (and heterocyclic) carboxamido-phenoxy-isobutyric acids and benzoic acids have been reported to be potent inhibitors of glycation and AGE formation (Rahbar and Figarola, 2003). *In vitro* studies showed that they could directly interact with several reactive dicarbonyls such as glyoxal and methyl glyoxal. They were also found to be potent chelators of Cu^{2+} and therefore can suppress hydroxyl radical production during sugar autoxidation and glycation reactions (Rahbar and Figarola, 2003).

Many studies suggested that metal catalyzed oxidation plays a critical role in glucose induced modification in collagen. Transition metals like Cu^{2+} ions can catalyze both glycation and glycoxidation in concentration dependent manner (Sajithlal *et al.,* 1999). In earlier experiments Wells-Knecht *et al.,* (1995) demonstrated that DETAPAC and EDTA act as metal ion chelators thereby sequestering free metal ions *in vivo.*

Carnosine appears to possess antiglycating, antioxidant and free radical scavenging activity. Carnosine inhibits inactivation and crosslinking of enzymes including superoxide dismutase glycation (Ukeda *et al.,* 2002) and oxidation (Stvolinskii *et al.,* 2003). It was found recently the imidazolium group of histadine on carnosine stabilizes the adduct formation at the primary amino group and hence it may play an important role for an anti crosslinking agent (Hobart *et al.,* 2004). Other compounds having AGE inhibitory activity are D-pencillamine and desferoxamine related to their antioxidant properties (Keita *et al.,* 1992; Jakus *et al.,* 1999). Some anti-inflammatory compounds such as acetylsalicylic acid, ibuprofen indomethacin were also reported to inhibit glycation by preventing the oxidative stress associated with the formation of AGE (Sobal and Menzel, 2000; Shastri *et al.,* 1998; Caballero *et al.,* 2000). Aspirin was also found to inhibit pentosidine formation (Fu *et al.,* 1994; Urios *et al.,* 2005) while Diclofenac, a non steroidal anti-inflammatory drug was shown *in vitro* to be an inhibitor of AGE formation, presumably by a non covalent interaction of the drug with serum protein (Van Boekel *et al.,* 1992).

A large number of other compounds have been designed and synthesized as AGE inhibitors. Some antidiabetic drugs metformin and progiatazone were also reported to be powerful AGE inhibitors (Rahbar, 2000). AL T -711 analog Nphenacyl thiazolium chloride claimed to catalytically break AGE crosslinks between proteins (Mentink, 2002).

Other approaches for the prevention of AGE mediated damage include (i) trapping of circulating AGE before they bind to their receptors (ii) inhibition of interaction of AGE with its receptor (iii) inhibition of signal transduction mediated by the activation of AGE receptor (Kim *et al.,* 2005). The prototype drug for trapping AGE ligands is soluble RAGE, which is the modified form of RAGE that constitute extracellular ligand binding domain (Wendt *et al.,* 2003). Another endogenous system for trapping AGE is lysozyme that has been reported to accelerate renal AGE clearance (Zheng *et al.,* 2001).

Yet other class of inhibitors includes aldose reductase inhibitors (ARI). Under hyperglycemic conditions excess glucose is metabolized into sorbitol by AR pathway. The fructose formation in this pathway leads to 3-deoxyglucozone and methyl glyoxal (Fig. 9), that may accelerate formation of AGEs. Sorbinil an ARI were found to decrease AGE related fluorescence in skin collagen of diabetic rats (Suarez *et al.,* 1988).

More recently, researchers have been investigating molecular chaperones for protection against glycation-induced inactivation (Ganea and Harding, 1995; Yan and Harding, 2003). Molecular chaperones not only assist during protein folding but also stabilize proteins and prevent aggregation (Hendrick and Hartl, 1993; Becker and Craig *et al.,* 1994).α-Crystallin, a molecular chaperone and lens structural protein, exhibit protection against glycation induced inactivation of enzymes like glucose-6-phosphate dehydrogenase, malate dehydrogenase and esterase (Ganea and Harding, 1995; Heath *et al.,* 1996; Yan and Harding, 2003). When incorporated into red cell ghosts α-crystallin protects membrane enzyme Na/K-ATPase against glycation and preserves its activity upon exposure to MG, fructose and H_2O_2. It is believed that α-crystallin may act through dynamic interactions, such that the chaperone may prevent the further unfolding but not bind to the target protein (Derham *et al.*, 2003).

Recent studies have highlighted the possible benefits of using plant extracts for decreasing glycation over the currently used drugs (Rates, 2001). Flavonoids represent the most common and widely distributed group of plant phenolics (Harborne, 1986) and are abundant in foods. They show important antioxidant and AGE inhibitory properties according to their structure (Odetti *et al.*, 1990; Farrar *et al.*, 2007). Quercetin and rutin are the most abundantly consumed flavonoids. Rutin with its free radical scavenging capability reduced the formation of glycated haemoglobin and increased haemoglobin levels in diabetic rats (Elgawish *et al.*, 1996). Nagasawa *et al.*, (2003) have shown that rutin and G-rutin (a water soluble rutin analogue) suppressed the formation of initial as well as advanced stages of Maillard reaction in tissues. Rousselot (2004) stated that improved antioxidant status is one mechanism by which dietary antioxidant treatment contributes to the prevention and reduction of diabetic complications. Quercetin has been shown to attenuate diabetic nephropathy in streptozotocin-diabetic rats (Anjaneyulu and Chopra, 2004). Numerous other studies indicat that dietary supplementation with antioxidant nutrients may be a safe and simple compliment to traditional therapies for preventing and treating diabetic complications (Ruhe and McDonald, 2001; Jung *et al.*, 2008).

Some herbal extracts and compounds isolated there form have been investigated for their potential against secondary complications of diabetes (Halaer *et al.*, 2003; Suryanarayana *et al.*, 2004). Green tea, a popular drink worldwide is found to contain large amount of tannins which are known for their antioxidant properties. Vincen and Zhang (2005) have shown that the extract can also delay diabetic complications. Another rich polyphenol containing extract from plant *Ilex paraguariensis* have been shown to inhibit *in vitro* formation of AGEs (Lunceford and Gugliucci, 2005). Garcinol, isolated from *Garcinia indica* fruit rind has been shown to possess antiglycating property *in vitro* along with antioxidant and metal chelating properties (Yamaguchi *et al.,* 2000). Mizutani *et al.,* (2000) isolated resveratrol, a natural phytoestrogen found in grapes which is found to inhibit AGE induced proliferation and collagen synthesis in vascular smooth muscle. Studies revealed that a natural compound curcumin isolated from *Curcuma longa* was shown to be a potant inhibitor of AGE formation and crosslinking of collagen in diabetic rats. A new compound puerariafuran isolated from the roots of *Pueraria lobata* was found to be active in causing inhibition of glycation (Jang *et al.,* 2006). *Cyperus rotundus* suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation (Ardestani *et al.,* 2007).

1.8. Objective of the study

Glycation, the sequence of of non-enzymatic reactions taking place between reducing sugars and the nucleophilic groups of proteins and other biomolecules, is ubiquitous and occurs in the cells of all living organisms, albeit at a very slow rate. The rates of glycation however increase remarkably during hyperglycemia, in diabetes and related disorders. Glycation is also accompanied by the formation of highly reactive and damaging reactive oxygen species (ROS). A number of proteins have been shown to undergo structural modifications and loss of biological function as a result of glycation. Several enzymes including those involved in antioxidant defense are also inactivated leading to serious disturbance in metabolism and exacerbate the damaging effect of glycation.

Available evidence suggests that agents that interfere with glycation reactions may be beneficial in restricting the complications accompanying diabetes and related disorders. Restriction/prevention of glycation and oxidative stress is therefore mooted as an effective strategy for alleviation of complications associated with hyperglycemia.

A number of drugs including aminoguanidine and benfiotamine are being investigated as potential antiglycation compound. Molecular chaperones and antibodies have also been investigated for their possible protective role against glycation. However no single compound seems to emerge as the perfect drug for combating glycation. Several plant extracts and compounds derived from plants have shown remarkable promise in this regard. Screening of a number of plants for inhibitors of glycation has therefore been undertaken by a number of investigators. While a large number of procedures are available for assay of glycation, majority of these are time consuming, necessitate specialized and expensive reagents and equipment. The objective of the work was to evaluate convenient, inexpensive assay procedures for the rapid screening of inhibitors of glycation from various crude preparations. Possibility of using HSA and RNase A as model proteins was investigated with glucose, fructose and ribose as the reactive sugars. High thermostability of RNase A facilitated carrying out of the assay at 60^0 C together with the highly reactive ribose, cut down of the duration of the activity based assay. The assay could be further improved by using immobilized RNase A. Also anti-RNase A antibody supports were shown in the study to protect the enzyme against inactivation caused by glucose, fructose or ribose.

Materials

2.0. MATERIALS

Chemicals and reagents used in the present studies were obtained from the sources as detailed below. Glass distilled water was used in all the experiments.

Sigma Chemical Co., U.S.A.	Acetyl acetone, Acrylamide, Aminoguanidine, Bovine pancreatic RNase A, Bovine serum albumin (BSA), Catechin, Coomassie brilliant blue R-250, CM-cellulose, Diethylenetriamine acetic acid (DETAPAC), Guanidinum HCl, Human serum albumin (HSA), Myricetin, Naringin, Nitroblue tetrazolium (NBT), Quercetin, Rutin, Tris (hydroxymethyl amino-methane).
Sisco Research Lab., India	Agarose, Ammonium per sulphate, Ammonium sulphate, Bromophenol blue, Cyanogen bromide, DEAE-cellulose, Ethylene diamine tetraacetic acid (EDTA), Glucose, Folin's Ciocalteu's phenol reagent, Formaldehyde, Fructose, Hydrogen peroxide, Lanthanum chloride, o-diansidine hydrochloride, Perchloric acid, Peroxidase, Ribose, Sepharose 4B, Sodium borohydride, Sodium periodate, Trichloroacetic acid (TCA), TEMED, Yeast RNA.
Qualigens Fine Chemicals, India	Acetic acid, Acetone, Acetonitrile, Copper sulphate, Diethyl ether, Di-hydrogen sodium phosphate, Di-sodium hydrogen phosphate, Ethanol, Glycerol, Hydrochloric acid, Magnesium chloride, β-mercaptoethanol, Potassium dichromate, Silver nitrate, Sodium acetate, Sodium bicarbonate, Sodium carbonate, Sodium chloride, Sodium hydroxide, Sodium metaperiodate, Sodium potassium tartarate, Sodium lauryl sulphate, Sulphuric acid, Tetra hydrofuran, Trichloroacetic acid, Tween-20, Zinc chloride.
Difco Laboratories Detroit, USA	Freund's complete adjuvant, Freund's incomplete adjuvant.
E. Merck, India	Ammonium sulphate, Glycine, Methanol.
Genei Pvt Ltd, Bangalore	Goat anti-rabbit immuno-globulin, horse raddish peroxidase.

Methods

THESIS

3.0 METHODS

Commercial HSA and RNase A that gave single band in SDS-PAGE were used without purification. Lyophilized HSA and RNase A were reconstituted with 20mM phosphate buffer pH 7.2 to make a stock solution of 10 mg/ ml and stored at -20°C.

3.1 Glycation reactions

3.1.1 *In vitro* glycation with various sugars

In order to induce glycation, the protocol used by Miyazawa *et al.* (1998) was adopted with slight modifications. HSA and RNase A were subjected to glycation with fructose, glucose or ribose. The proteins concentration was taken as (1 mg/ml) in 20 mM sodium phosphate buffer, pH 7.2 with respective sugar at the final concentration of 100 mM. The reaction mixtures were prefiltered through a nitrocellulose filter 0.2 µm pore-sizes and incubated in preautoclaved tubes in order to maintain sterile conditions during incubations. The incubations were carried out in the shaking water bath at 37°C for the indicated durations. Samples incubated without sugar similarly served as control. Each sample was extensively dialyzed against the buffer in order to remove excess of sugars before further analysis.

3.1.2 *In vitro* glycation of HSA and RNase A with fructose along with various compounds/drugs

HSA and RNase A (1 mg/ml) was incubated with 25 mM DETAPAC, EDTA, aminoguanidine, aspirin, paracetamol, pencillamine, ibuprofen and ascorbic acid in the presence of 100 mM fructose. The incubations were carried out in the shaking water bath at 37°C for 8 days. Samples incubated without sugar under similar conditions served as control. Before further analysis each sample was extensively dialyzed against the buffer in order to remove excess of sugars.

3.1.3. *In vitro* glycation of HSA and RNase with fructose along with various flavonoids

HSA and RNase A (1 mg/ml) was incubated with 10 μM quercetin, naringin, rutin, catechin and myrecetin in the presence of 100 mM fructose. The incubations were carried out in the shaking waterbath at 37°C for 8 days. Samples incubated without sugar under the similar conditions served as control. Before further analysis each sample was extensively dialyzed against the buffer in order to remove excess of sugars.

3.1.4. Incubation of soluble and immobilized RNase A with sugars

In order to induce glycation, soluble RNase A or that immobilized on antiRNase support or CM-Cellulose support was incubated along with 500 mM glucose, fructose or ribose in 20 mM phosphate buffer pH 7.2 containing 5 μM gentamycin to prevent any microbial growth. The reaction was carried out in a shaking water bath at 37°C for 8 days. After completion of incubation RNase A immobilized on the antibody-support or CM cellulose was eluted with 100 mM glycine/HCl buffer pH 3.0 and 100 mM NaCl followed by dialysis against phosphate buffer pH 7.2.

3.1.5. Glycation of RNase A at 60 ° C

RNase A (1mg/ml) was incubated with 500 mM glucose, fructose or ribose in 20 mM phosphate buffer pH 7.2 at 60 ° C for a period of 1, 2 and 4 days under sterile conditions. The incubations carried out under sterile condition but also contained 5 μM gentamycin. Where indicated RNase A was incubated with the inhibitors (25 mM) or plant extracts in the presence of 500 mM ribose. Control samples were incubated under similar conditions but without ribose or inhibitors. Similarly, RNase A was incubated with some plant extracts along with 500 mM ribose and incubated for 2 days under similar conditions. The plant extracts were prepared by homogenizing garlic, ginger, grapes, bitter gourd, turmeric and tomato in 20 mM phosphate buffer pH 7.2 followed by centrifugation. Green tea extract was prepared by stirring 1g of green tea with 20 ml of 50% (v/v) ethanol for 3 h. The mixture was centrifuged at 1500g for 15 min and the supernatant was used as a source of inhibitor (Zhang *et al.*, 2006). All the extracts were adjusted to assay pH. While using immobilized RNase A, the preparation was separated for the reaction mixture by centrifugation and washed thoroughly, after incubation with the sugar and extracts prior to the enzyme activity measurements.

3.2. Colorimetric/Spectrophotometric analysis

3.2.1. Determination of protein concentration

Protein was estimated by the method of Lowry *et al.* (1951). Aliquots of protein solution were taken in a set of tubes and final volume made up to 1 ml with 0.01 M sodium phosphate buffer, pH 8.0. Five ml of alkaline copper reagent (containing one part of 0.5% (w/v) copper sulphate, 1% (w/v) sodium potassium tartarte and fifty parts of 2% (w/v) sodium hydroxide) was added, followed after 10 minutes of incubation at room temperature with 0.5 ml of 1.0 N Folin-Ciocalteu's phenol reagent. The tubes were instantly vortexed. The colour developed was read at 660 nm after 30 minutes against the reagent blank. A standard curve was prepared using BSA as standard.

3.2.2. Determination of protein bound carbonyl groups

Protein -bound carbonyl groups were estimated according to the protocol of Levine et al., (1990); briefly 200 µl aliquot containing 0.1 mg of protein was mixed with 400 µl of 7 mM DNPH in 2 M HCl. The mixtures were run in duplicate and the control protein samples were devoid of DNPH. After incubation for 1 hour at room temperature the DNPH-hydrazones were precipitated by adding 500 µl of TCA (4% w/v), centrifuged for 5 mins at 14,000 x g and the pellet dispersed in ethanol-ethylacetate (1:1 v/v), in order to remove unreacted DNPH. After 4 such washes, the pellet was resuspended in 0.6 ml of 6 M guanidinium HCl solution in 20 mM phosphate buffer, already adjusting to pH 2.3 with trifluoroacetic acid. The hydrazones were dissolved completely only by freezing overnight at –20°C and thawing. From the solution, 200 µl aliquot was taken into a microplate and read at 379 nm. The results were expressed as the number of moles of carbonyl per mole of sample protein using a $\varepsilon_{379\,\mathrm{nm}} = 22000\ \mathrm{M^{-1}cm^{-1}}$.

3.2.3. Borohydride-periodate assay of glycated proteins

A previously described borohydride-periodate assay (Kennedy *et al.,* 1993) was used with few modifications. 100 µl of the samples were incubated for 1 hour at 37°C with 20 µl of 0.2 M sodium borohydride in ice cold 0.01 mM NaOH. The reaction was stopped by adding 20 µl of 0.2 M HCl. After this step, samples were incubated with 20 µl of 0.1 M sodium periodate for 30 min at 37°C followed by addition of 40 µl of ice cold 0.7 M NaOH and 15% zinc sulfate water solution. Precipitate was removed by centrifugation at 12,000 rpm. From each sample 200 µl supernatant were taken and mixed with 100 µl of color reagent (92 µl acetylacetone in 10 ml of 6.6 M ammonium acetate) quantification was accompalished at 405 nm in microplate reader.

3.2.4. Determination of protein ketoamine in glycated HSA

The glycation of HSA was quantified by a published colorimetric procedure using NBT (Mashiba *et al.,* 1992) with slight modification. BSA (10 mg/ml) was incubated with 500 mM glucose for 15 days at under sterile conditions 37°C in 20 mM phosphate buffer pH 7.2 which results in modification of protein with subsequent formation of ketoamines. Native and glycated HSA samples (50 µl) were added to the wells of the 96-well microliters plates in duplicate. One hundred microlitres of NBT reagent (250 µl in 100 mM carbonate buffer, pH 10.3) was added to each well and incubated in dark at 37°C for 2 hours. The plate was read in a microplate reader at 550 nm and amount of glycated HSA was calculated using the standard curve constructed with glycated BSA.

3.2.5. Assay of RNase A

RNase A activity was determined spectrophotometrically using yeast RNA as substrate. The standard reaction mixture in a total volume of 2.5 ml contained 2 mg of RNA and appropriate amount of RNase A in 0.1 M sodium acetate buffer, pH 5.0. The reaction was arrested after 10 minute incubation at 37°C by using stopping reagent (22 M lanthanum chloride in 1M perchloric acid), the reaction mixture was left on ice for 10 min and the precipitate formed removed by centrifugation. Subsequently 0.2 ml of the supernatant was diluted with distilled water and the absorption of acid soluble ribonucleotides measured at 260nm (Bergmeyer, 1984)

3.2.5.1. Effect of temperature

Both soluble and immobilized RNase A preparations were preincubated for 10 min at various temperatures ranging between 37-80°C in 0.1 M sodium acetate buffer, pH 5.0. The reaction was initiated by the addition of RNA followed by incubation at each temperature for 5 min and arrested by the addition of stopping reagent (22 M lanthanum chloride in 1M perchloric acid).

3.2.5.2. Effect of pH

To 250 µl of RNA (8 mg/ml) in 0.1 M NaH_2PO_4 were added increasing volume of 0.5 M NaOH and the volume made up to 450 µl with water. The volume of 0.5 M NaOH was increased in such a manner that the resulting pH of the solution ranged from 5-10. The reaction was initiated by adding 50 µl of RNase A (1.1 units) in distilled water followed by incubation at 37°C for 5 min. The reaction was terminated by 500 µl of stopping reagent. The mixture was left on ice for 10 min and precipitate removed by centrifugation (5000 x g, 10 min). Subsequently, 0.2 ml of supernatant was diluted with 0.8 ml distilled water and the absorbance of the acid soluble nucleotides measured at 260 nm. Similarly, the effect of pH on the activity of RNase bound to CNBr activated Sepharose was studied using 50 µl of the immobilized RNase A preparations (activity wise similar) instead of soluble RNase A.

3.3. Spectral Analysis

3.3.1. UV absorption spectroscopy

The UV absorption spectroscopy of native and sugar treated samples were obtained by measuring the absorption between 200 – 400 nm in a Shimadzu spectrophotometer using a cuvette of 1 cm pathlength. Three hundred micrograms of the samples in a total volume of 1 ml were taken for spectral analysis.

3.2.2. Flourescence spectroscopy

Samples (100 µg protein/ml) were analyzed by measuring intrinsic fluorescence at 25±0.2°C in a Hitachi F2000 spectrofluorometer (Tokyo, Japan). The samples were excited at 285 nm and emission range was taken at 310-460 nm. Fluorescence measurements were also made at 370 nm excitation and an emission range of 400-500 nm. Appropriate controls containing the substances used for the treatment were run and corrections were made wherever necessary.

3.2.3. Circular Dichroism

All the Circular Dichroism (CD) measurements were carried out at 25°C on a Jasco spectropolarimeter Model J-720 using a SEKONIC XY Plotter (Model SPl-430A), with a thermostatically controlled cell holder attached to a NESLAB water bath Model RTE 110 with an accuracy of ±0.10 °C. The instrument was equipped with a microcomputer and recalibrated with (+)-10-camphersulfonic acid. The spectrum was recorded with a scan speed of 20 nm/ min and with a response time of 20 nm/min. Each spectrum was recorded as an average of two scans. Far-UV CD spectra were taken at a protein concentration of 20 µM with a 1 and 10 mm path length cells, respectively. CD spectra were recorded in the wavelength ranges of 190-250 nm for far-UV.

3.4 Immunological methods

3.4.1. Immunization of rabbits

Male rabbits weighing 2.2 kg were subcutaneously injected with 150 µg of RNase A in 0.5 ml of 20mM phosphate buffer mixed (1:1) with Freund's complete adjuvant. The animals were rested for 15 days and booster doses, prepared by mixing 100 µg of RNase A/0.5 ml solution with equal volumes of Freund's incomplete adjuvant, were subcutaneously given at weekly intervals for 4 weeks. Blood was withdrawn through an ear vein and clot formation allowed to take place at room temperature for 6 hrs. Serum was collected by centrifugation and stored at 0°C.

3.4.2. Ouchterlony double diffusion

The procedure was described by Ouchterlony (1949) was used. Agar plates were prepared by pouring 25.0 ml of 1% (w/v) agarose solution in normal saline. The gel was allowed to solidify and wells punched. RNase A (about 30 µg) was loaded in the central well and 5-20 µl of adequately diluted antisera were added in the peripheral wells. The antigen was loaded after 4 hours at room temperature and subsequently overnight in a refrigerator.

3.4.3. Direct binding enzyme-linked immunosorbent assay (ELISA)

The titre of antigen specific antibodies was measured in the sera of immunized rabbits by ELISA. The 96 well microtitre plates (immunol 2HB, dynex, USA) were coated with 100 µl of RNase A at a concentration of 5 µg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6 for 2 hours at room temperature and then overnight at 4°C. They were washed 3 times with TBS-tween (0.14 M NaCl, 2.7mM KCl and 20 mM Tris pH 7.4 with 500 µl/l tween) to remove the unbound antigen. Unoccupied sites were blocked with 150 µl of 1.5% (w/v) BSA in TBS for 3 hours at room temperature. The plates were washed with TBS-tween and serially diluted antiserum samples (100 µl/well) in TBS to be tested were added to each well (1:100→1:10,00,000). The plates were incubated for 2 hours at room temperature. After the usual washing steps, the peroxidase reaction was initiated by the addition of the substrate tetramethyl benzidine/ H_2O_2, the reaction arrested by the addition of 4.0 N H_2SO_4 and absorbance at 450 nm measured in an ELISA reader. Each sample was coated in duplicate, and the results were expressed as mean of A test-A control. (A=absorbance at 450 nm).

3.4.4. Isolation and purification of Immunoglobulin G

IgG was isolated by a slight modification of the method of Fahey and Terry (1979). Fifty ml of antiserum was mixed with equal volume of 20 mM sodium phosphate buffer, pH 7.2 and 11.4 gms of solid ammonium sulphate was added. The mixture was stirred to dissolve ammonium sulphate and to make it 20% saturated. After 6 hours the sample was centrifuged at 2,000 x g for 20 minutes and the supernatant made 40% saturated with respect to ammonium sulphate by adding additional 12.3 gms of the solid. Precipitation was allowed to proceed for 12 hours at 4°C. The precipitate obtained was collected by centrifugation at 2,000 x g for 20 minutes. Most of the gamma globulin was present only in the 20–40% pellet which was dissolved in minimum volume of 20 mM sodium phosphate buffer, pH 7.2 and extensively dialyzed against the same buffer with frequent changes and stored at 4°C.

Ten grams of DEAE-cellulose was suspended in 0.5 N HCl for one hour and washed in a Buechner funnel with distilled water till the pH of the filterate was neutral. The exchanger was then treated with 0.5 N NaOH for 1 hour and again washed with distilled water till the pH of the filterate was neutral. DEAE-cellulose was then resuspended in 20 mM sodium phosphate buffer, pH 7.2 to obtain homogeneous slurry. Fine particles were removed by decantation and the slurry mixed with dialyzed ammonium sulphate fraction and stirred for 2 hours. The slurry was then centrifuged at 4,000 x g for 10 minutes to settle the particles and the supernatant thus obtained containing purified IgG was collected and analysed for the protein content. The homogeneity of purified protein was assessed by PAGE.

3.5. Immobilization techniques

3.5.1. Immobilization of antiRNase on cynogen bromide activated Sepharose

The antiRNase IgG was coupled to activated Sepharose-4B (Porath et al, 1967). Briefly, 1.0 g Sepharose-4B was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 2.0 ml of distilled water and 1.0 ml of 2.0 M sodium bicarbonate (Na_2CO_3). The slurry was stirred thoroughly at room temperature by placing it on a magnetic stirrer. Then 0.2 gm of cynogen bromide dissolved in 0.2 ml of acetonitrile was added to the beaker containing Sepharose gel and mixed thoroughly at 4°C for 15 minutes. The whole mass was then transferred to a glass sintered funnel and washed extensively with 0.1 M bicarbonate buffer, pH 8.5, distilled water and then with phosphate buffer. After thorough washings, the activated Sepharose was dried and suspended in 0.1 M sodium bicarbonate buffer, pH 8.5.

RNase A was immobilized on the antibody support by incubating 5500 units of the enzyme with 10 mg of Sepharose linked antiRNase in 0.1 M acetate buffer, pH 5.0 for 12 h at 4°C. The matrix was separated from the unbound enzyme by centrifugation and washed with the buffer. Amount of the enzyme immobilized were determined by subtracting from the amount of RNase added that present in the supernatant and washings by activity measurements.

3.5.2. Immobilization of RNase A on CNBr activated Sepharose

Immobilization of RNase A was carried out by activating Sepharose 4B as described in the above section. RNase A (10 mg) was added to the activated Sepharose in 0.1M sodium bicarbonate buffer pH 8.5 under stirring for 24 h at 4°C. Amounts of the enzyme immobilized were determined by subtracting from the amount added those of the supernatant and washing by activity measurement.

3.5.3. Immobilization of RNase A on CM-cellulose

Ten grams of CM-cellulose was suspended in 0.5 M NaOH for one hour and washed in a Buechner funnel with distilled water till the pH of the filterate was neutral. The exchanger was then treated with 0.5 N HCl for 1 hour and again washed with distilled water till the pH of the filterate was neutral. Ten mg of RNase A mixed with 1 g of regenerated CM-Cellulose was stirred at 4º C overnight. Amount of RNase A immobilized on support was calculated by subtracting the quantity of protein in the supernatant and washings from that added to the regenerated CM Cellulose.

3.5.4. Effectiveness factor

The effectiveness factor (η) of the immobilized preparation represents the ratio of actual to theoretical catalytic activity of the immobilized enzyme (Jafri and Saleemuddin, 1997). Actual activity value of the enzyme was determined by assaying an appropriate aliquot of the immobilized preparation. Theoretical activities of enzyme preparations were calculated by subtracting the soluble enzyme units remaining (after immobilization) from that added for immobilization. η value of an immobilized enzyme is a measure of internal diffusion effect and reflects the efficiency of immobilization procedure in retaining the enzyme in active form.

3.6. Slab gel electrophoresis

3.6.1. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed essentially according to the method of Laemmli (1970) using the slab gel apparatus manufactured by Biotech, India. A stock solution of 30% acrylamide containing 0.8% bisacrylamide was mixed in appropriate proportion to give the desired percentage of gel. It was then poured into the mould formed by two glass plates (8.5 x 10 cm) separated by 1.5 mm thick spacers. Bubbles and leaks were avoided. A comb providing a template for seven wells was quickly inserted into the gel film and polymerization allowed to occur. After 15–20 minutes, the comb was removed and the wells were cleaned, overlaid with running buffer. Samples containing 15–35 μg protein mixed with equal volume of sample buffer (containing 10% (v/v) glycerol, 0.06 M tris HCl, pH 6.8 and traces of bromophenol blue as tracking dye) were applied to the wells. Electrophoresis was performed at 100 V in the electrophoresis buffer containing 0.025 M Tris and 0.2 glycine until the tracking dye reached the bottom of the gel.

3.6.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE)

Sodium dodecyl sulphate (SDS) PAGE was performed by the tris-glycine system of Laemmli (1970) using slab gel electrophoresis apparatus, manufactured by Biotech, India. Concentrated stock solution of 30% acrylamide containing 0.8% bisacrylamide, 1.0 M tris (pH 6.8 and 8.8) and 10% SDS were prepared and mixed in appropriate proportion to give the final required percentage. It was poured in the mould formed by two glass plates (8.5 x 10 cm) separated by 1.5 mm thick spacers avoiding leaks and bubbles. A comb providing a template for seven wells was quickly inserted into the gel before the polymerization began. The comb was removed once the polymerization was complete and wells were overlaid with the running buffer. Protein samples were prepared in the sample buffer containing 1% (w/v) SDS, 10% (v/v) glycerol, 0.0625 M tris HCl, pH 6.8 and traces of bromophenol blue as tracking dye along with 5% β-mercaptoethanol. The samples were boiled at 100°C for five minutes. Electrophoresis was performed in electrophoresis buffer containing 0.025 M tris and 0.2 M glycine at 100 v till the tracking dye reached the bottom of the gel.

3.7. Staining procedures

After the electrophoresis was complete the gels were removed and the protein bands were visualized by staining.

3.7.1. Coomassie brilliant blue staining

Protein bands were detected by staining with 0.1% coomassie brilliant blue R-250 in 40% isopropanol and 10% acetic acid. The staining was carried out with 10% glacial acetic acid.

3.7.2. Silver nitrate staining

The procedure described by Merril *et al.* (1982) was followed. After electrophoresis the protein bands were fixed by rapidly immersing in a mixture of 40% (v/v) methanol and 10% (v/v) acetic acid for one hour with constant shaking. The gel was washed with 10% methanol and 5% (v/v) acetic acid twice, each time for 15 minutes to allow the gel to swell to normal size. This was followed by incubation in 3.4 mM potassium dichromate solution containing 3.2 mM nitric acid for 15 minutes and then thoroughly washed with distilled water. The washed gel was then immersed in 12 mM silver nitrate solution for 20 minutes and the again washed with distilled water and transferred to 280 mM solution of sodium carbonate containing 0.5% formaldehyde to make the gel alkaline. The reaction was stopped after 10 minutes by transferring the gel to 3% acetic acid solution for 5 minutes. The gels were washed 4 to 5 times with distilled water and finally stored in distilled water.

Results

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4.0 RESULTS

4.1 Studies on glycation of HSA

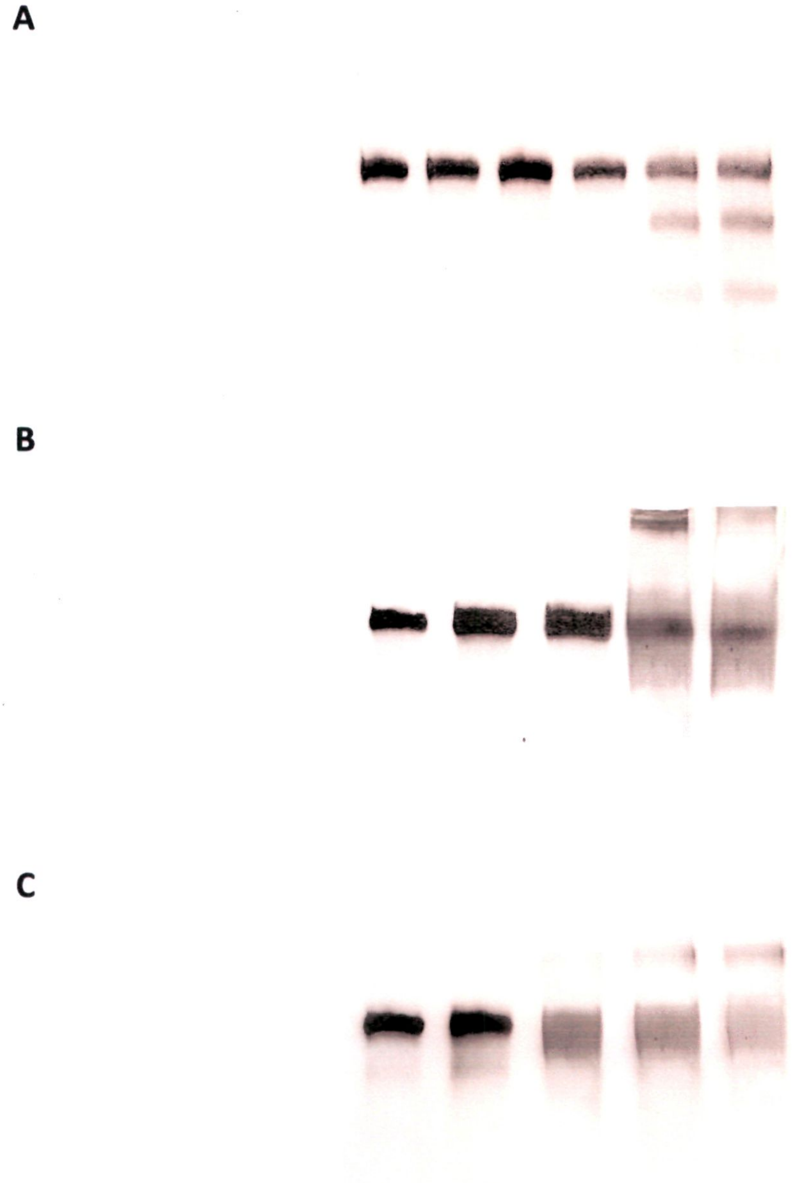
HSA is the most abundant protein in plasma and comprises about 60 percent of the total proteins found in blood plasma (Shaklai *et al.,* 1984). In normal adults, nearly 10 percent of the HSA is modified by glycation which increases 2-3 folds under hyperglycemic conditions such as diabetes (Guthrow *et al.,* 1979). As a result glycated albumin has been proposed as a useful marker for short term monitoring of diabetic patients and the levels of glycated albumin might also be used as an indicator of the degree of hyperglycemia (Nakajou *et al.,* 2003). Glycation studies on HSA mainly focused on the reaction with glucose although some information with other sugars is also available. This part of thesis presents data on comparison of the effect of various sugars on HSA and the comparative protective effect of some compounds/ drugs on glycation induced alterations in the molecule.

4.1.1 Effect of incubation with reducing sugars on the properties of HSA

4.1.1.1 SDS-PAGE

HSA was incubated with glucose, fructose or ribose for 8 days and analyzed by SDS-PAGE in the presence of the thiol reductant β-mercaptoethanol, as detailed in the text. HSA not exposed to the sugars migrated as a single band but on incubation with the sugars, clear alterations in the electrophoretic behavior were evident. These included broadening of the protein band and decrease in the staining intensity. Two other effects notable in the SDS-PAGE are the presence of oligomeric population in samples treated with ribose and fructose suggesting the formation of aggregates (Fig. 10 B, C) and presence of faster migrating peptides (Fig. 10 A) in the glucose treated protein. As shown in Fig. 10 the alterations induced by ribose and fructose were more obvious than those induced by glucose.

Fig. 10. SDS-PAGE of HSA incubated with reducing sugars. HSA was incubated with 100 mM glucose (A), fructose (B) or ribose (C) separately upto 8 days in 20 mM phosphate buffer, pH 7.2 at 37°C and subjected to electrophoresis. Lanes 1 contained HSA incubated without sugar, whereas Lanes 2-5 contained HSA incubated with sugars for 2, 4, 6 and 8 days respectively. Ten microgram of sample was loaded on each lane and subjected to SDS-PAGE in the presence of β-mercaptoethanol. The gels were silver stained for visualization of protein bands.


A
B
C
1
2
3
4
5

4.1.1.2. Protein bound carbonyl groups

HSA incubated with various sugars up to 8 days was analyzed for carbonyl content by reaction with DNPH (Fig. 11). The carbonyl content was considerably higher in the ribose treated sample followed by those treated with fructose and glucose. HSA incubated without sugar contained very small amount of carbonyl groups. Carbonyl content is considered a reliable indicator of glycation and is the most commonly used marker of protein oxidation and glycation, but the extent of carbonyl group formation varies from protein to protein, depending on the number and location of free amino groups present in the respective protein (Berlett and Stadtman, 1997; Beal, 2002). The relative reactivities of ribose, fructose and glucose in generating carbonyls substantiate the effects described in Fig. 10. The order of reactivity in the glycation reaction was ribose> fructose> glucose.

4.1.1.3. Colorimetric determination of glycation

Fig. 12 shows the progress of albumin glycation with the sugars, measured by borohydride-periodate assay. The amount of formaldehyde released at 405 nm was measured which is directly proportional to the extent of glycation (Kennedy *et al.,* 1993). The observations were supported by analysis of the ketoamine moieties formed by glycation of HSA (Fig. 13). Ribose was most active in generating ketoamine groups on HSA followed by glucose. HSA not incubated with the sugar and that treated with fructose contains negligible ketoamine groups. The relative reactivities of sugars as evident from Fig. 10 & 11 however appeared different with fructose showing higher reactivity. Thus, while fructose was more reactive than glucose in inducing alterations in electrophoretic behavior as well as generation of protein bound carbonyl (Fig. 10 & 11), it was found less effective in liberating formaldehyde as well as inducing the formation of ketoamine groups.

Fig. 11. Carbonylation of HSA incubated with various reducing sugars. HSA was incubated with 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 up to 8 days. The samples were derivatized with DNPH and assayed for protein bound carbonyl groups. Control sample (O) was incubated under identical conditions but without any sugar. Each value represent the mean± S.D. for three experiments performed in duplicates.

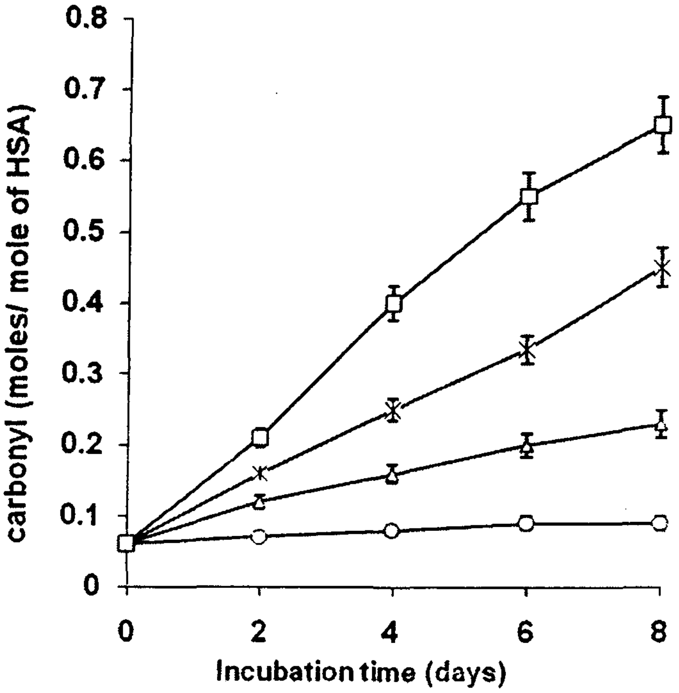
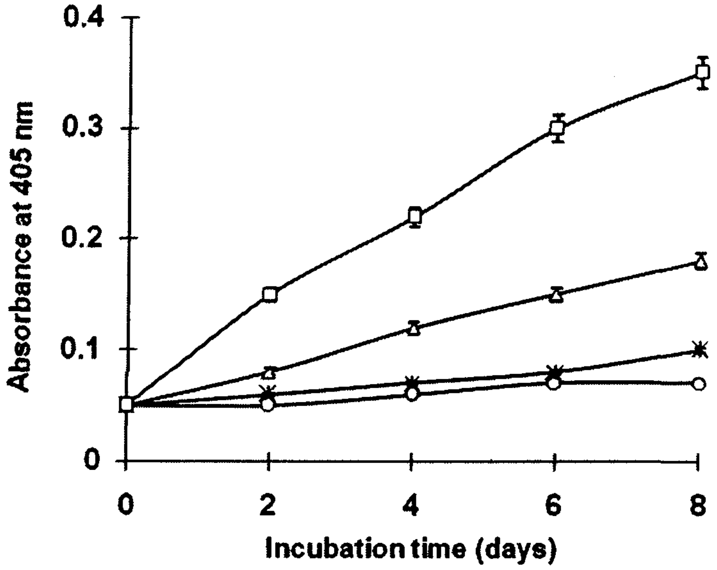
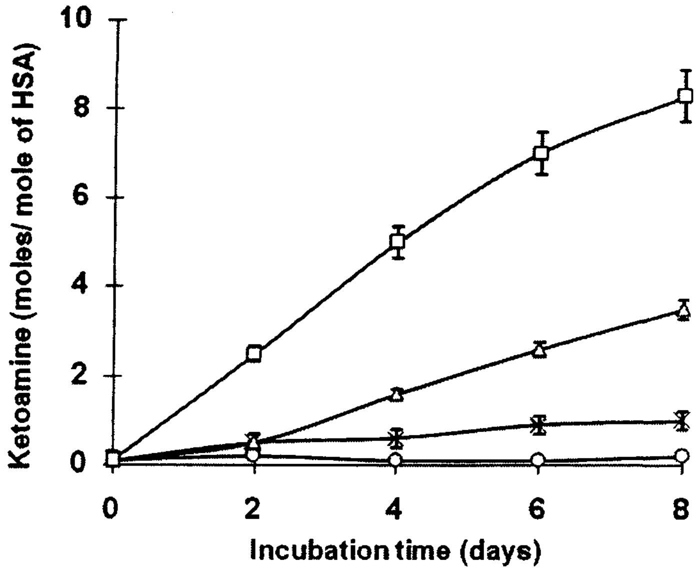

carbonyl (moles/ mole of HSA)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
Incubation time (days)

Fig. 12. Effect of various sugars on borohydride reduction in periodate assay. HSA was incubated with 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 up to 8 days. The samples were treated with borohydride and periodate to measure the amount of formaldehyde released by glycated and unglycated HSA at 405 nm. Control sample (O) was incubated under identical conditions but without any sugar.

Fig. 13. Effect of various sugars on ketoamine formation in HSA. HSA was incubated with 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 up to 8 days. The samples were treated with NBT and assayed for protein bound ketoamines. Control sample (O) was incubated under identical conditions but without any sugar.


0.4
0.3
0.2
0.1
0
Absorbance at 405 nm
0
2
4
6
8
Incubation time (days)


10
8
6
4
2
0
Ketoamine (moles/ mole of HSA)
0
2
4
6
8
Incubation time (days)

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4.1.1.4. Spectral properties of HSA

It is well recognized that the reaction of reducing sugars with protein can cause marked alterations in protein conformation. The glycation induced alterations in HSA were further examined with respect to their chromophoric and fluorophoric properties. Incubation with reducing sugars at 37°C for upto 8 days resulted in time dependent modification of HSA, as evident from the observed alterations in intrinsic fluorescence (Fig. 14), new fluorescence (Fig. 15) and absorbance at 280 nm (Fig. 16). As shown in the figures, HSA incubated with the sugars revealed quenching in the intrinsic fluorescence, while a marked increase was observed in new fluorescence and absorbance at 280 nm. Several investigators have shown that reaction of sugars and aldehyde with proteins leads to the formation of groups like pentosidine which are considered characteristic biomarker of glycation and accompanying autoxidation. These adduct show strong emission between 400-500 nm when excited at a wavelength of 370 nm (Schmidt, 1990; Traverso *et al.,* 1997).

To evaluate the changes in the secondary structure, the CD spectra in the range of 190– 250 nm were measured for the control and samples treated with glucose, fructose or ribose for 8 days. The spectrum of HSA revealed two minima, one at 208 nm and the other at 222 nm, which are indicative of the helical content of the protein (Fig. 17). It is evident that incubation with sugars lead to marked alterations in secondary structure. Glucose causes very little change in the secondary structure while maximum alteration was observed with ribose.

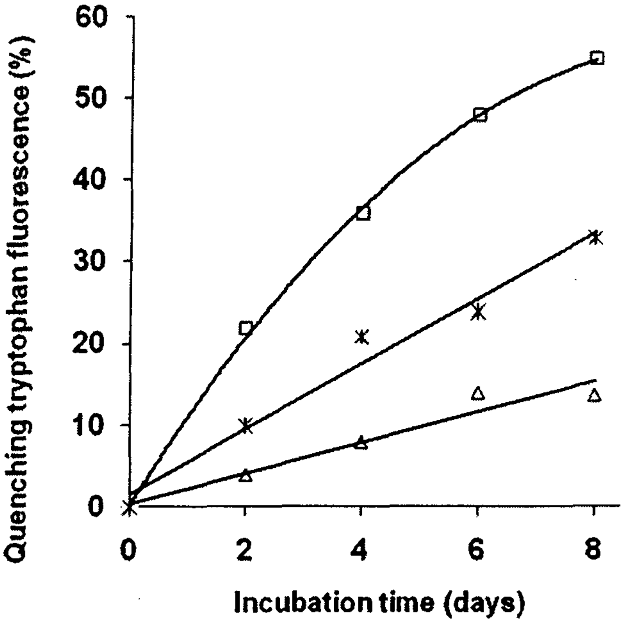
4.1.1.5. Effect of various compounds on glycation with fructose

Glycation reactions mediated by fructose (fructation) gained interest for variety of reasons. In tissues such as eyes and peripheral nerves polyol pathway is active and the concentration of fructose reaches that of glucose. The resultant fructose is known to be a strong glycating agent due the presence of high concentrations of the reactive open chain structures that react with protein amino groups at the rate

Fig. 14 A. **Effect of incubation with various sugars on tryptophan fluorescence of HSA.** HSA was incubated in the absence (O) or presence of 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 for upto 8 days at 37°C. Aliquots were withdrawn at 0, 2, 4, 6 and 8 days and dialyzed. Equivalent quantities of protein were withdrawn and the quenching was measured with respect to the HSA not incubated with either sugar.

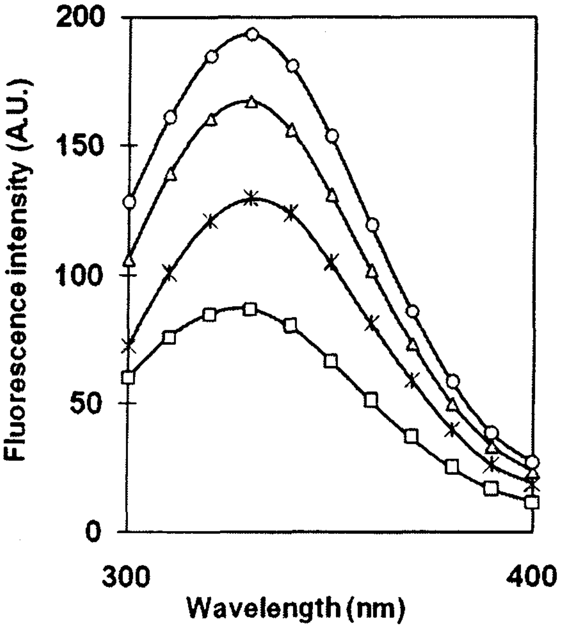
Fig. 14 B. **Tryptophan fluorescence spectra of HSA incubated with various sugars.** HSA was incubated in the absence (O) or presence of 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 for 8 days at 37°C. Samples were dialyzed prior to fluorescence measurement. The spectra were taken after excitation at 280 nm and emission range 300-400 nm.

A



60
50
40
30
20
10
0
Quenching tryptophan fluorescence (%)
0
2
4
6
8
Incubation time (days)

B

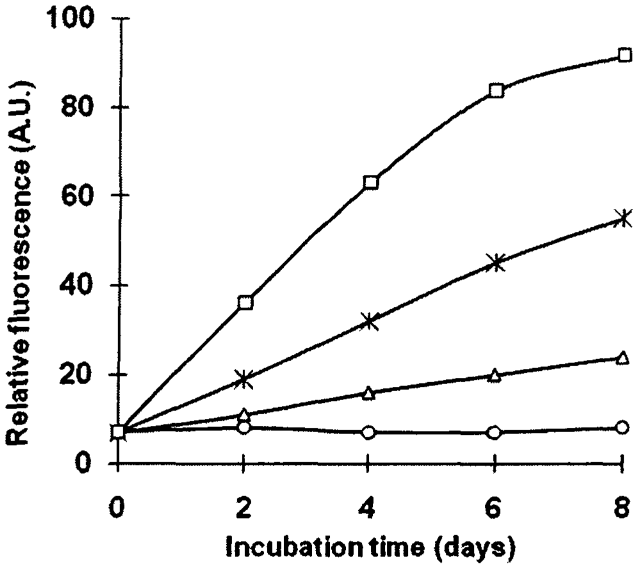


200
150
100
50
0
Fluorescence intensity (A.U.)
300
400
Wavelength (nm)

Fig. 15 A. Effect of incubation with various sugars on new fluorescence of HSA. HSA was incubated in the absence (O) or presence of 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 for upto 8 days at 37°C. Aliquots were withdrawn at 0, 2, 4, 6 and 8 days and dialyzed. Equivalent quantities of protein were withdrawn and the new fluorescence was measured with respect to the HSA not incubated with either sugar.

Fig. 15 B. New fluorescence spectra of HSA incubated with various sugars. HSA was incubated in the absence (O) or presence of 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 for 8 days at 37°C. Samples were dialysed prior to fluorescence measurement. The spectra were taken after excitation at 370 nm and emission range 400-500 nm.

A


100
80
60
40
20
0
Relative fluorescence (A.U.)
0
2
4
6
8
Incubation time (days)

B

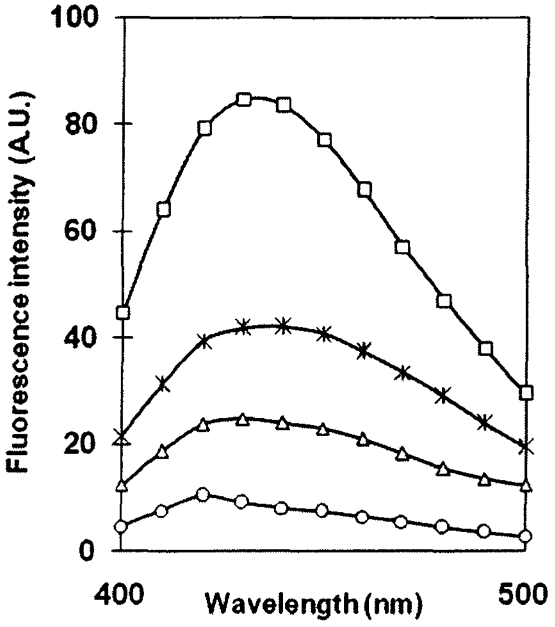
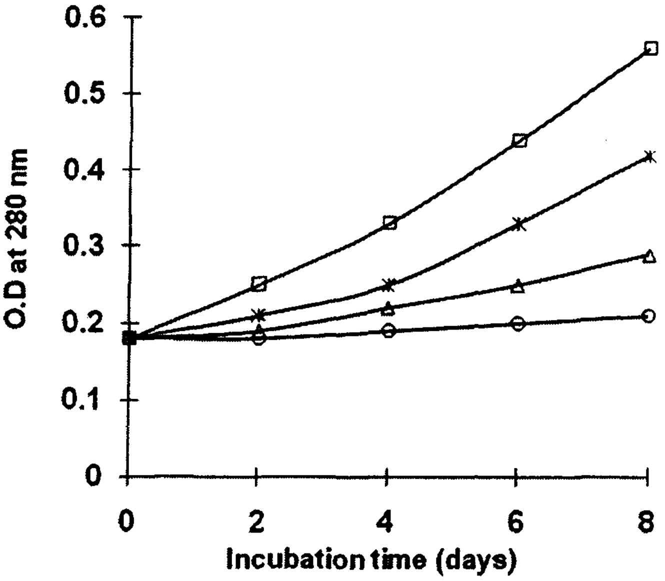

100
80
60
40
20
0
Fluorescence intensity (A.U.)
400
Wavelength (nm)
500

Fig. 16 A. Effect of incubation with various sugars on hyperchromicity of HSA. HSA was incubated in the absence (O) or presence of 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 for upto 8 days at 37°C. Aliquots were withdrawn at 0, 2, 4, 6 and 8 days and dialyzed. Equivalent quantities of protein were withdrawn and the absorbance at 280 nm was measured with respect to the HSA not incubated with either sugar.

Fig. 16 B. Absorption spectra of HSA incubated with various sugars. HSA was incubated in the absence (O) or presence of 100 mM glucose (Δ), fructose (X) or ribose (□) in 20 mM phosphate buffer, pH 7.2 for 8 days at 37°C. Samples were dialysed prior to measurement of absorbance at 280 nm.

A


0.6
0.5
0.4
0.3
0.2
0.1
0
O.D at 280 nm
0
2
4
6
8
Incubation time (days)

B

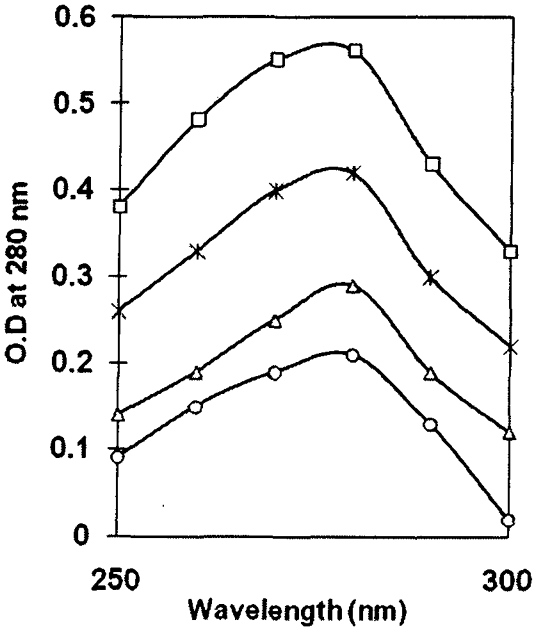
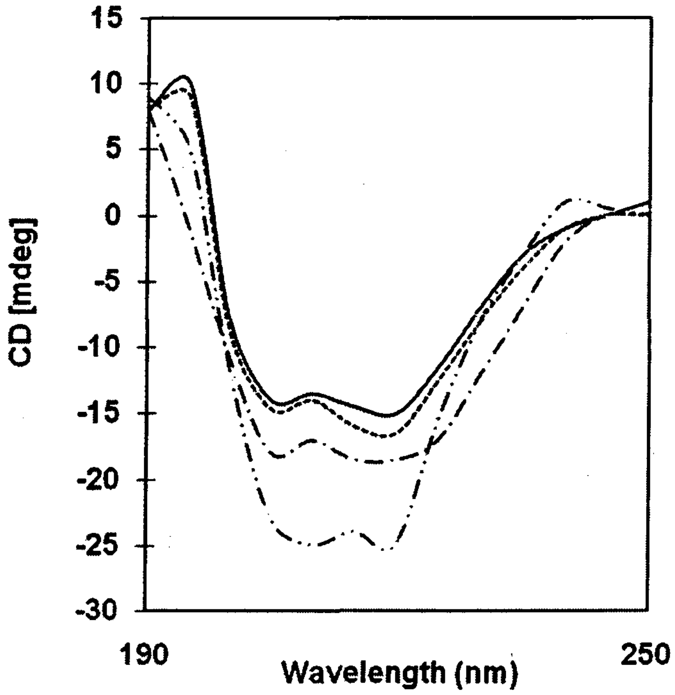

0.6
0.5
0.4
0.3
0.2
0.1
0
O.D at 280 nm
250
300
Wavelength (nm)

Fig. 17. Far UV CD spectra of HSA incubated with various sugars. HSA was incubated in the absence (—) or presence of 100 mM solutions of glucose (- - -), fructose (- · -) or ribose (- ·· -) in 20 mM phosphate buffer, pH 7.2 for 8 days at 37°C. Samples were dialyzed prior to the CD measurement.


15
10
5
0
-5
-10
-15
-20
-25
-30
CD [mdeg]
190
250
Wavelength (nm)

approx 10-folds higher as compared to that of glucose (Bunn and Higgins.1981). Fructose-mediated glycation and the protection provided by various compounds/ drugs were therefore investigated. The protective effect of some metal ion chelators, antioxidants, analgesics and other drugs have been investigated by measuring increase in new fluorescence, quenching of tryptophan fluorescence and absorbance at 280 nm (Fig. 18). Each bar represents the percent change of the control resulting from the inclusion of specific compound. The result obtained showed that DETAPAC, EDTA and aminoguanidine have strong protective effect while aspirin, ibuprofen, paracetamol and pencillamine exert only moderate protection. Ascorbic acid showed only marginal protective effect.

For further characterization of the protection provided by the compounds, HSA samples incubated with fructose and inhibitors were subjected to SDS-PAGE. The fructose treated samples showed characteristic variation already shown in Fig. 10, while the HSA samples incubated with various compounds along with fructose showed varying degrees of decrease in sugar-induced alterations in electrophoretic behavior. Aminoguanidine, EDTA, paracetamol were more effective compared to other compounds in this regard (Fig. 19, 20)

Fig. 21 showed SDS-PAGE of HSA incubated with fructose along with Cu^{2+} ions in the absence or presence of DETAPAC. The metal ions were found to markedly enhance the fragmentation of HSA as revealed by the disappearance of the protein band while some aggregated material remained at the top of the gel. DETAPAC was remarkably effective in reducing the combined effect of sugar and Cu^{2+} ions.

4.1.1.6. Effect of some flavonoids on glycation with fructose

The protection against fructose-mediated HSA glycation by flavonoids was determined by electrophoresis (Fig. 22) and alterations in spectral parameters (Fig. 23). *The result showed that some flavonoids were quite effective in restricting the* sugar induced alterations in the electrophoretic behavior of HSA. Quercetin and

Fig. 18. Protective effect of various compounds/ drugs on fructose-induced alterations in HSA. HSA was incubated with 100 mM fructose for 8 days in the presence of various compounds at a concentration of 25 mM. Incubated samples were dialyzed and equal quantities of protein were taken for new fluorescence (A), tryptophan fluorescence (B) and hyperchromicity (C). Relative values were calculated taking the value of HSA incubated without fructose as 100. All the values are mean± S.D for three experiments performed in duplicates.

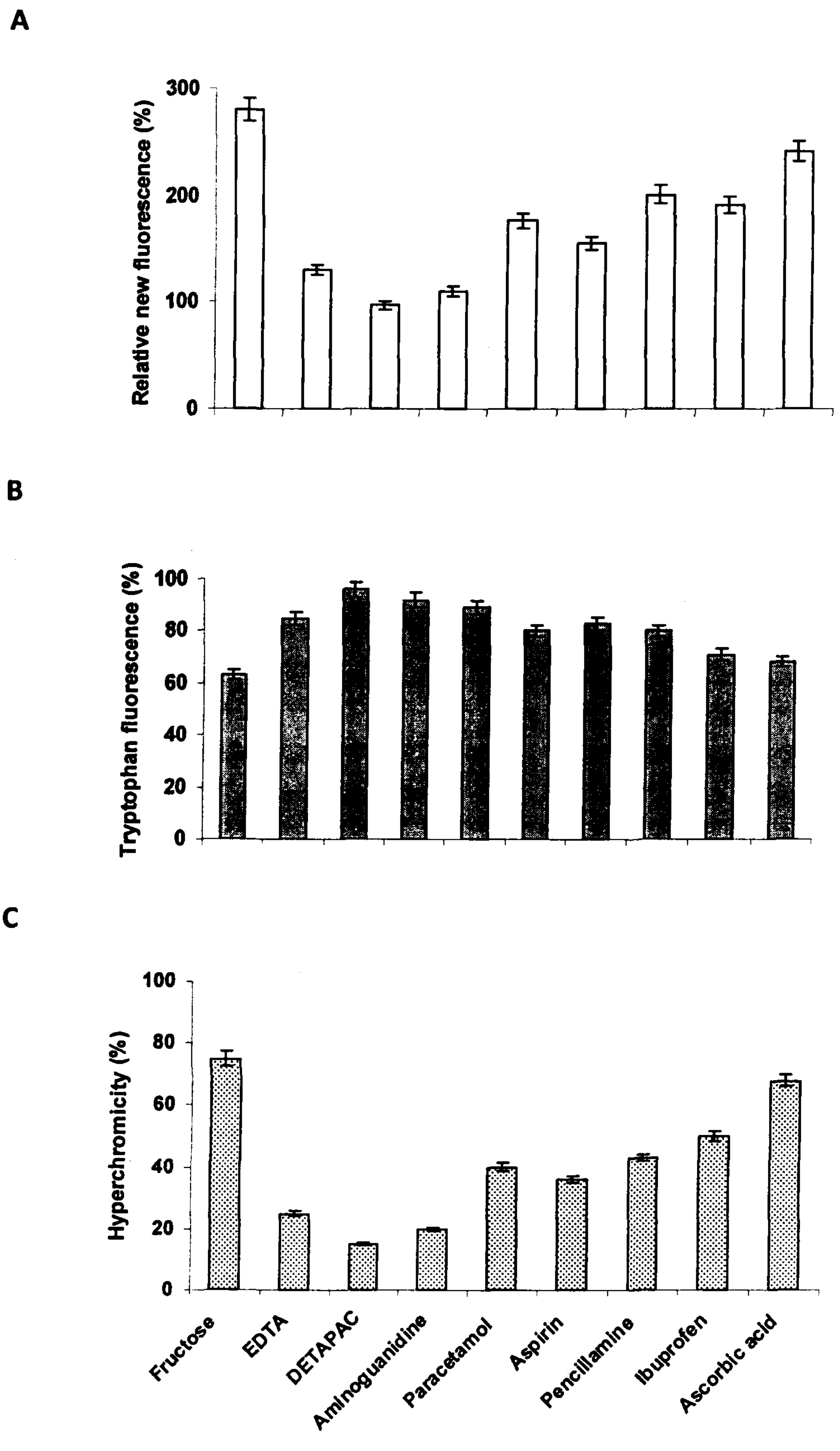
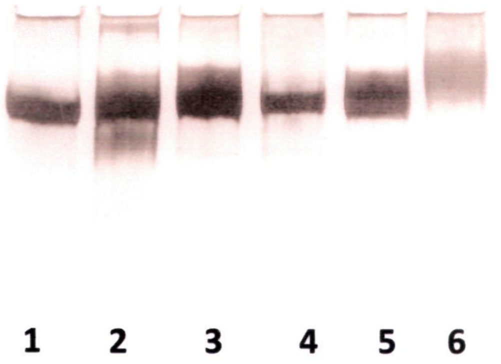

A
Relative new fluorescence (%)
300
200
100
0
B
Tryptophan fluorescence (%)
100
80
60
40
20
0
C
Hyperchromicity (%)
100
80
60
40
20
0
Fructose
EDTA
DETAPAC
Aminoguanidine
Paracetamol
Aspirin
Pencillamine
Ibuprofen
Ascorbic acid

Fig. 19. SDS-PAGE of HSA incubated with fructose in the presence of some inhibitors of glycation. HSA was incubated with 100 mM fructose in 20 mM phosphate buffer, pH 7.2 at 37°C for 8 days in the absence or presence of various compounds/ drugs at a concentration of 25 mM. Lane 1 contained HSA; Lane 2 contained HSA incubated with fructose; Lanes 3, 4, 5 and 6 contained HSA incubated with fructose along with EDTA, ibuprofen, pencillamine and ascorbic acid respectively.

Fig. 20. SDS-PAGE of HSA incubated with fructose in the presence of some inhibitors of glycation. HSA was incubated with 100 mM fructose in 20 mM phosphate buffer, pH 7.2 at 37°C for 8 days in the absence or presence of various compounds/ drugs at a concentration of 25 mM. Lane 1 contained HSA; Lane 2 contained HSA incubated with fructose; Lanes 3, 4 and 5 contained HSA incubated with fructose along with paracetamol, aminoguanidine and aspirin respectively.


1
2
3
4
5
6

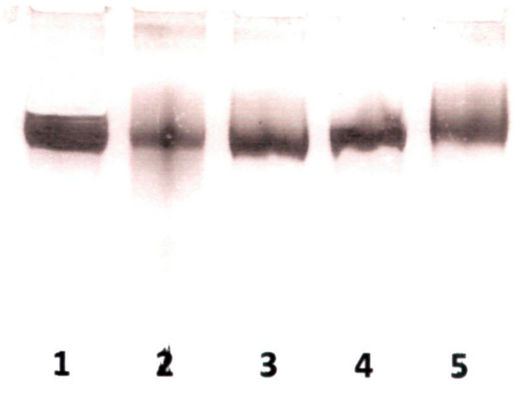

1
2
3
4
5

Fig. 21. SDS-PAGE of HSA incubated with fructose along with metal ions in the presence of DETAPAC. HSA was incubated with 100 mM fructose in 20 mM phosphate buffer, pH 7.2 at 37°C for 8 days in the presence of 0.1 mM Cu^{2+} ions. Lane 1 contained HSA; Lane 2 contained HSA incubated with fructose; Lane 3 contained HSA incubated with fructose and Cu^{2+} ions, Lane 4 contained HSA incubated with fructose and DETAPAC and lane 5 contained HSA incubated with fructose Cu^{2+} ions and DETAPAC respectively.

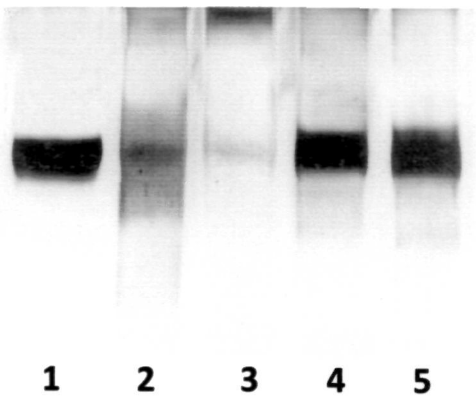

1
2
3
4
5

Fig. 22. **Protective effect of various flavonoids on fructose-induced alterations in HSA.** HSA was incubated with 100 mM fructose for 8 days in the presence of various flavonoids at a concentration of 10 μM. Incubated samples were dialysed and equal quantities of protein were taken for measuring new fluorescence (A), tryptophan fluorescence (B) and ultraviolet spectroscopy (C). Relative values were calculated taking value of HSA incubated without fructose as 100. All the values are mean± S.D for three experiments performed in duplicates.

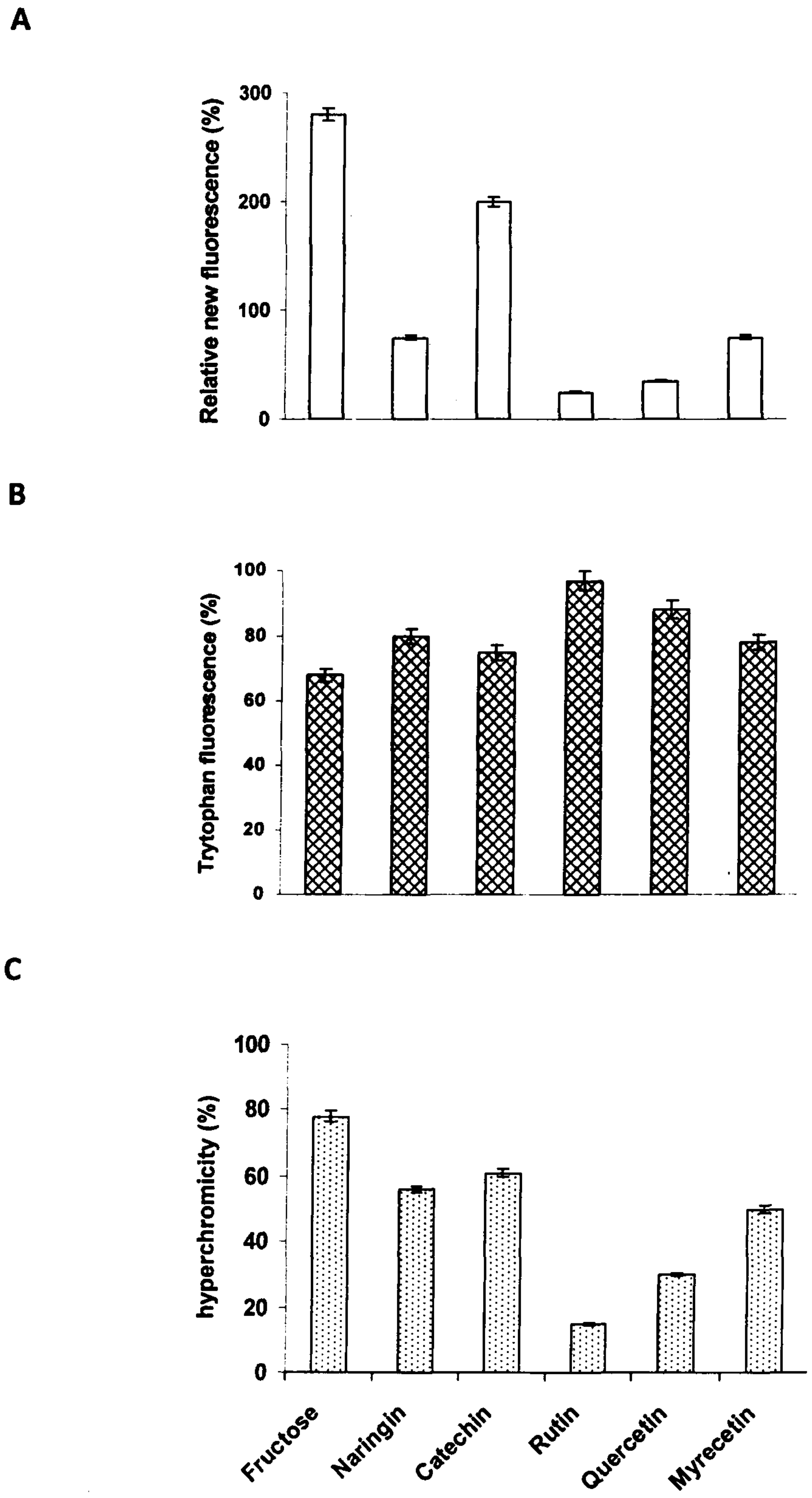
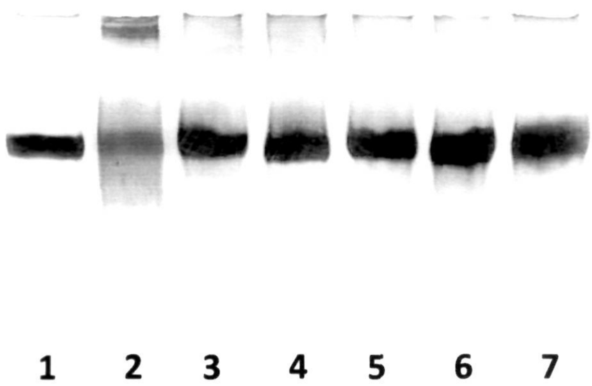

A
Relative new fluorescence (%)
300
200
100
0
B
Trytophan fluorescence (%)
100
80
60
40
20
0
C
hyperchromicity (%)
100
80
60
40
20
0
Fructose
Naringin
Catechin
Rutin
Quercetin
Myrecetin

Fig. 23. SDS-PAGE of HSA incubated with fructose in the presence of some flavonoids. HSA was incubated with 100 mM fructose in 20mM phosphate buffer, pH 7.2 at 37°C for 8 days in the absence or presence of flavonoids at a concentration of 10 μM. Lane 1 contained HSA; Lane 2 contained HSA incubated with fructose; Lanes 3, 4, 5, 6, and 7 contained HSA incubated with fructose along with quercetin, rutin, naringin, myricetin and catechin respectively.


1
2
3
4
5
6
7

rutin were more protective compared to naringin and myricetin, while catechin was not protective. New fluorescence, tryptophan fluorescence and hyperchromicity also suggest the protective effect of flavonoids to be in the order rutin> quercetin> naringin> myricetin> catechin.

4.2 Studies on RNase A

4.2.1. Effect of reducing sugars on RNase A

Earlier studies have shown that RNase A incubated with sugars undergoes structural modification and loss in catalytic activity like several other enzymes (Seidler, 2005). Use of RNase A therefore offers catalytic activity measurement as an additional parameter of glycation reaction. It is also well recognized that the enzyme is highly stable against various forms of inactivation. As shown in Fig. 24, glucose, fructose and ribose cause marked inactivation of RNase at 37^0 C, but the loss of catalytic activity was most rapid in the presence of ribose. Incubation with glucose, fructose or ribose resulted in loss of 36, 62 and 84 percent activity respectively after 8 days incubation at 37^0 C. RNase did not lose significant activity when incubated in absence of the sugars for upto eight days.

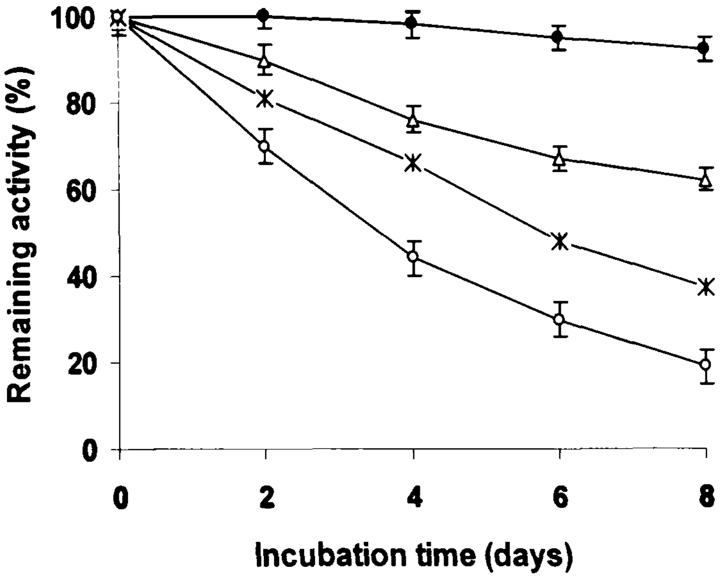
Fig. 25 illustrates SDS-PAGE of RNase exposed to the sugars revealed broadening of the RNase band, decrease in staining intensity as well as appearance of additional large and small molecular weight bands. The loss of band intensity also appeared maximum with ribose followed by fructose and ribose.

4.2.2 Effect of incubation with sugars on spectral properties of RNase A

The sugar induced alterations on RNase were also examined with respect to their chromophoric and fluorophoric properties. As shown in Fig. 26, a marked increase in new fluorescence was also observed with the sugars following the anticipated order i.e. ribose > fructose >glucose.

Fig. 24. Effect of reducing sugars on the activity of RNase A. RNase A (1 mg/ ml) was incubated with 100 mM glucose (Δ), fructose (x) or ribose (□) separately for 2, 4, 6 and 8 days in 20 mM phosphate buffer, pH 7.2 under sterile conditions at 37°C. Control sample contained RNase A (●) not incubated with any sugar up to 8 days. Aliquots were removed at appropriate intervals and the enzyme activity measured under standard assay conditions. Each value represents the average for three independent experiments performed in duplicate.

Fig. 25. SDS-PAGE of RNase A incubated with reducing sugars. RNase A was incubated with 100 mM glucose, fructose or ribose separately for upto 8 days in 20 mM phosphate buffer, pH 7.2 at 37°C and subjected to electrophoresis. Lanes 1 contained RNase A incubated in the absence of sugar whereas Lanes 2-4 contained RNase A incubated with glucose, fructose or ribose respectively. Ten microgram of sample was loaded on each lane and the gels were silver stained for visualization of protein bands.


Remaining activity (%)
100
80
60
40
20
0
0
2
4
6
8
Incubation time (days)

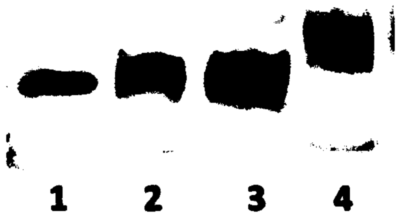
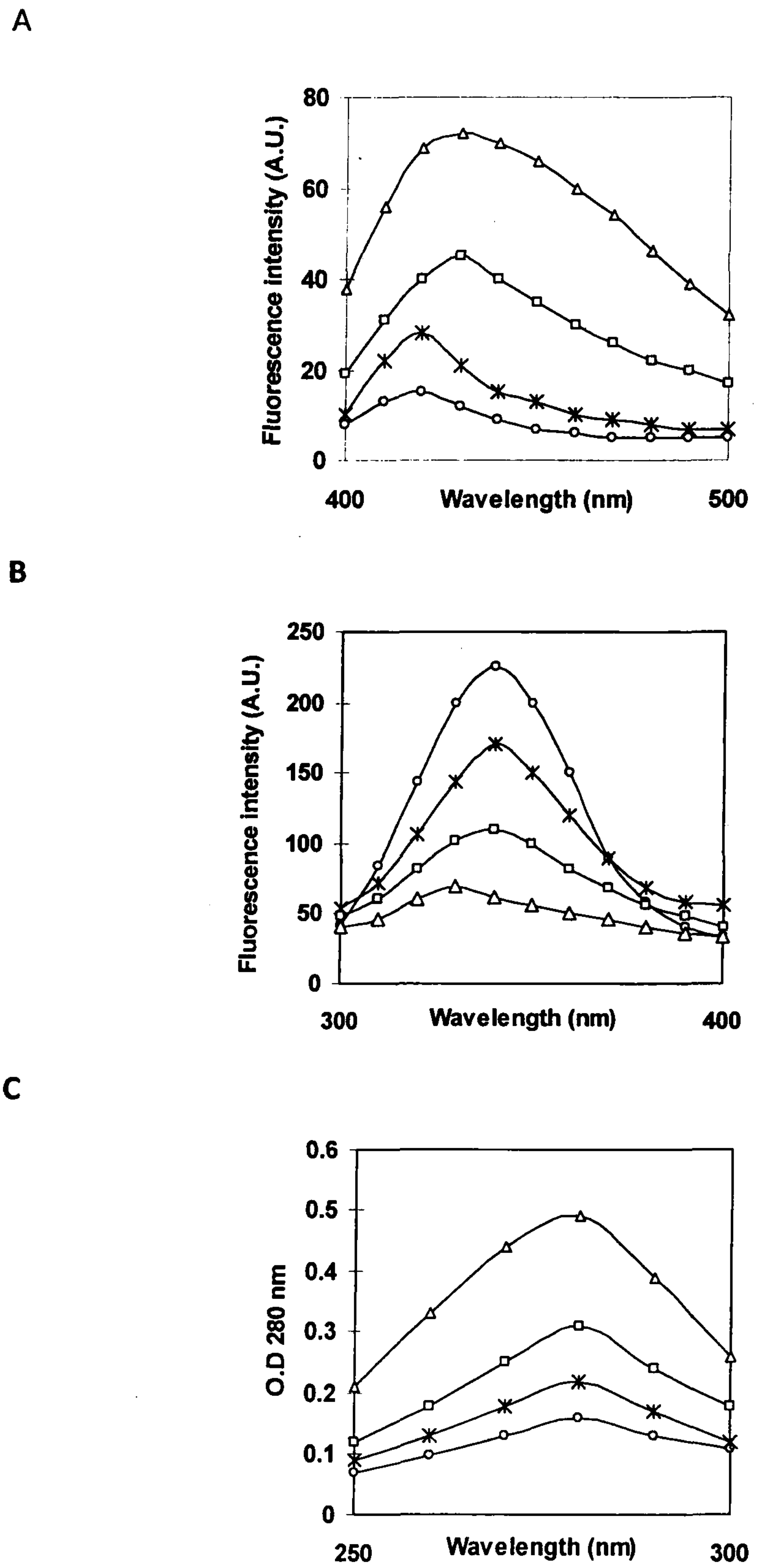

1
2
3
4

Fig. 26. Effect of sugars on the spectral properties of RNase A. RNase A (1mg/ml) was incubated with 100 mM glucose (X), fructose (□) or ribose (Δ) separately at 37°C for 8 days. Control sample contained RNase A (O) not incubated with any sugar up to 8 days.Samples were dialyzed for the analysis of new fluorescence (A), tyrosine fluorescence (B) and hyperchromicity (C). RNase A incubated without sugar (o) under similar conditions were taken as control.


A
80
60
40
20
0
Fluorescence intensity (A.U.)
400
Wavelength (nm)
500
B
250
200
150
100
50
0
Fluorescence intensity (A.U.)
300
Wavelength (nm)
400
C
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D 280 nm
250
Wavelength (nm)
300

Quenching of tyrosine fluorescence of RNase A was also measured as an additional index of alteration in structure/conformation. As observed with new fluorescence the magnitude of quenching also varied markedly and maximum quenching was observed in case of ribose-treated RNase, while enzyme treated with glucose revealed minimum quenching.

The UV spectra of RNase incubated with the sugars exhibited marked increase in absorbance at 280 nm as compared to the control sample incubated in the absence of sugar. However the magnitude of hyperchromicity induced by sugars varied markedly and among various sugars studied, maximum hyperchromicity was observed in ribose-treated RNase followed by that incubated with fructose and glucose.

Fig. 27 depicts the far UV-CD spectra between 200-250 nm of control and the sugar treated RNase A incubated for 8 days at 37°C. As can be seen from the figure a remarkable change in the spectrum of RNase A incubated in the presence of sugar was observed. Inclusion of ribose in the reaction mixture resulted in maximum alterations suggestive of marked loss of secondary structure.

4.2.3. Effect of various compounds on the fructose induced alterations in RNase A

The protective effect of some metal ion chelators, antioxidants, analgesics and other drugs that have been shown to interfere with glycation has been investigated. The protective effect of the compounds was assayed using various parameters such as effect on enzyme activity (Fig. 28 A), hyperchromicity (Fig. 28 B), generation of new florescence (Fig. 29 A) as well as quenching of tyrosine fluorescence (Fig. 29 B). Each bar represents the percent inhibition of glycation as compared to control. SDS-PAGE of RNase A incubated with fructose along with the compounds also revealed decrease in cross-linking as compared to RNase A incubated with fructose alone (Fig. 30). Among the compounds investigated aminoguanidine, DETAPAC and EDTA were found to be most protective, while

Fig. 27. Far UV CD spectra of RNase A incubated with various sugars. RNase A was incubated in the absence (—) or presence of 0.5 M solutions of glucose (- - -), fructose (- · -) or ribose (-···-) for 8 days at 37°C. Samples were dialyzed prior to the CD measurement.

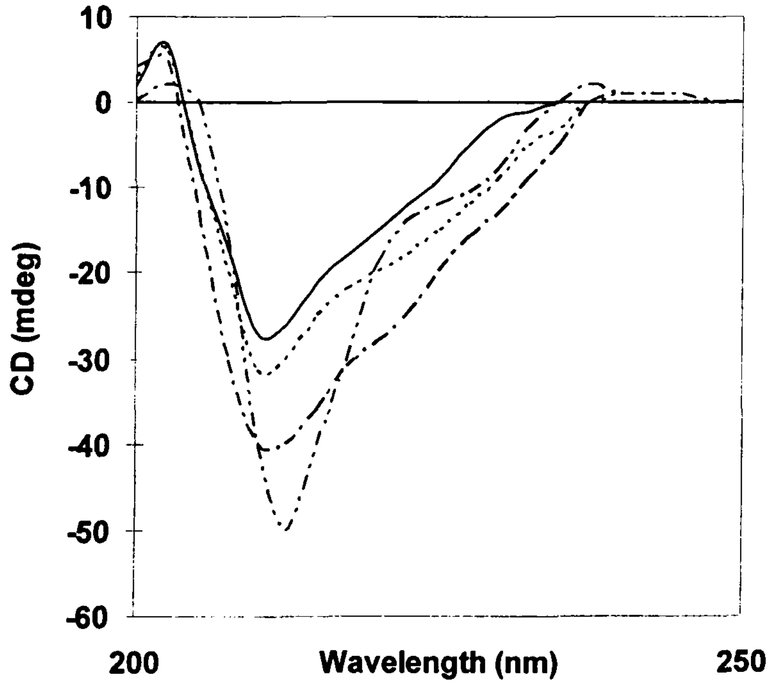
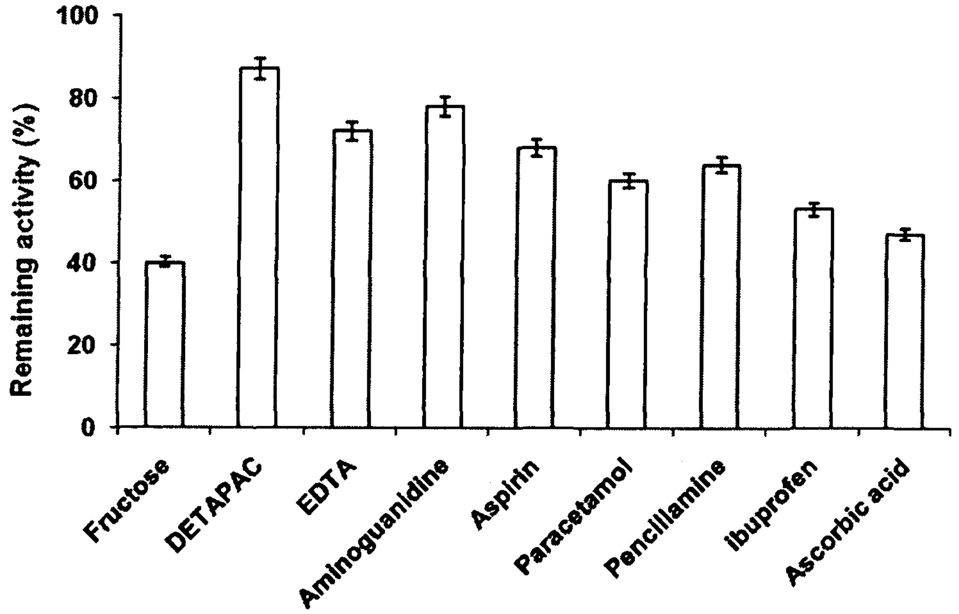

10
0
-10
-20
-30
-40
-50
-60
CD (mdeg)
200
Wavelength (nm)
250

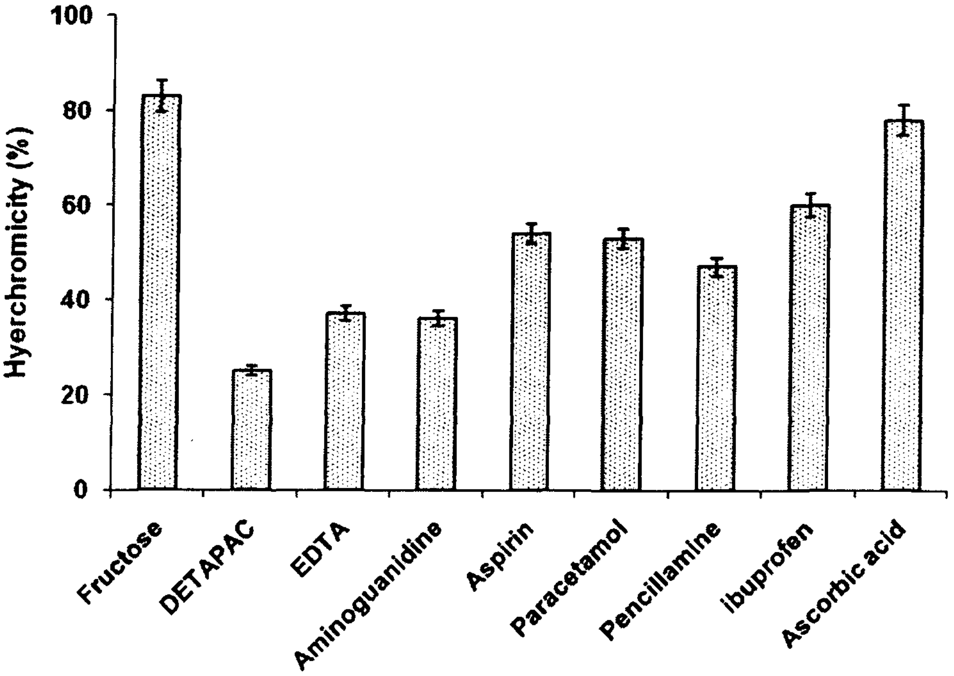
Fig. 28. Protective effect of various compounds/ drugs on fructose-induced alterations in RNase A. RNase A was incubated with 100 mM fructose for 8 days in the presence of various compounds at a concentration of 25 mM. Incubated samples were dialyzed and equivalent quantities of protein were taken for enzyme activity (A) and hyperchromicity (B). Percent remaining activity and hyperchromicity was calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.

A



Remaining activity (%)
100
80
60
40
20
0
Fructose
DETAPAC
EDTA
Aminoguanidine
Aspirin
Paracetamol
Pencillamine
ibuprofen
Ascorbic acid

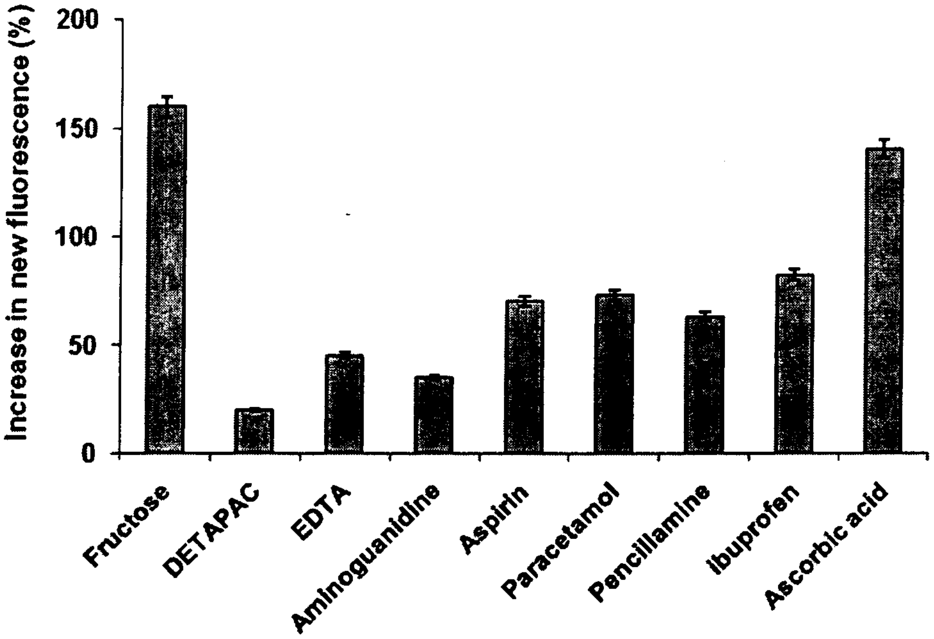
B



Hyerchromicity (%)
100
80
60
40
20
0
Fructose
DETAPAC
EDTA
Aminoguanidine
Aspirin
Paracetamol
Pencillamine
ibuprofen
Ascorbic acid

Fig. 29. Protective effect of various compounds/ drugs on fructose-induced alterations in RNase A. RNase A was incubated with 100 mM fructose for 8 days in the presence of various compounds at a concentration of 25 mM. Incubated samples were dialyzed and equivalent quantities of protein were taken for new fluorescence (A) and tyrosine fluorescence (B). Increase in new fluorescence and quenching in tyrosine fluorescence was calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.

A


Increase in new fluorescence (%)
200
150
100
50
0
Fructose
DETAPAC
EDTA
Aminoguanidine
Aspirin
Paracetamol
Pencillamine
ibuprofen
Ascorbic acid

B

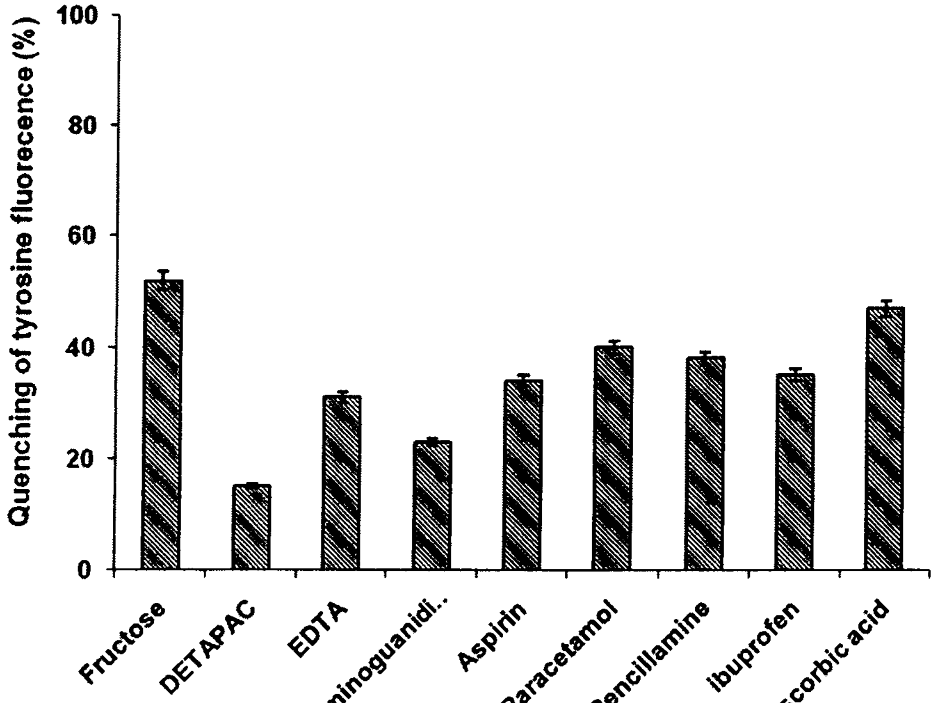
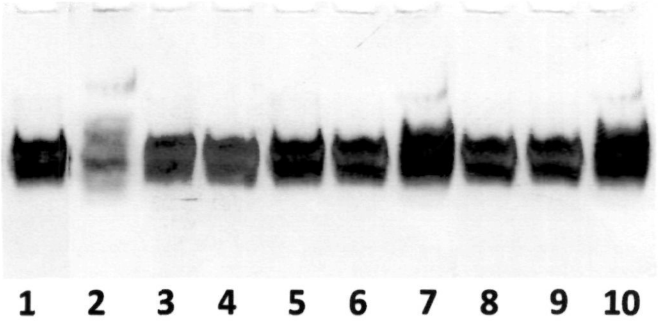

Quenching of tyrosine fluorecence (%)
100
80
60
40
20
0
Fructose
DETAPAC
EDTA
Aminoguanidi..
Aspirin
Paracetamol
Pencillamine
ibuprofen
Ascorbic acid

Fig. 30. SDS-PAGE of RNase A incubated with fructose in the presence of various compounds/ drugs. RNase A was incubated with 100 mM fructose in 20 mM phosphate buffer, pH 7.2 at 37°C for 8 days in the absence or presence of various compounds/ drugs at a concentration of 25 mM. Lane 1 contained RNase; Lane 2 contained RNase A incubated with fructose; Lanes 3, 4, 5, 6, 7, 8, 9 and 10 contained RNase A incubated with fructose and EDTA, DETAPAC, aminoguanidine, paracetamol, ibuprofen, aspirin, pencillamine and ascorbic acid respectively.


1
2
3
4
5
6
7
8
9
10

pencillamine, aspirin and iburofen were moderatly protective. Ascorbic acid was found to lower the quenching of intrinsic fluorescence as well as generation of new fluorescence to a certain extent but it neither reduced the cross-linking of RNase A nor provided significant protection against enzyme inactivation.

4.2.4 Effect of flavonoids on the fructose induced alterations in RNase A

The inhibition of fructose-mediated RNase A glycation by various flavonoids was also investigated by SDS-PAGE (Fig. 31), effect on enzyme activity (Fig. 32 A) and hyperchromicity (Fig. 32 B), generation of new fluorescence (Fig. 33 A) quenching of tyrosine fluorescence (Fig. 33 B). Fructose readily reacts with RNase A to produce high molecular weight aggregates as revealed by SDS-PAGE. As shown in the Fig. 31, RNase A incubated with fructose migrated as diffuse band as compared to native enzyme. Some flavonoids, especially rutin and quercetin protected RNase A against inactivation as well as formation of high molecular weight aggregates. The protective effect of naringin and myricetin were however moderate while catechin showed no significant inhibitory effect. Investigation of new fluorescence also supported the protective effect of rutin and quercetin but not catechin.

4.3. Evaluation of immobilized RNase A based assay for glycation

Taking under consideration, the potential of using loss of RNase A activity as parameter for screening of glycation inhibitors the possibility of using immobilized RNase A with enhanced stability and easy separability from the reaction mixture was investigated. For this purpose RNase A immobilized on CNBr activated Sepharose is used.

4.3.1 Stability studies of immobilized RNase A

4.3.1.1. Effect of temperature

The temperature activity profile of soluble and immobilized RNase A preparations

Fig. 31. SDS-PAGE of RNase A incubated with fructose in the presence of flavonoids. RNase A was incubated with 100 mM fructose 20 mM phosphate buffer, pH 7.2 at 37°C for 8 days in the absence or presence of flavonoids at a concentration of 10 μM. Lane 1 contained RNase A; Lane 2 contained RNase A incubated with fructose; Lanes 3, 4, 5, 6 and 7 contained RNase A incubated with fructose and rutin, quercetin, naringin, myricetin and catechin respectively.

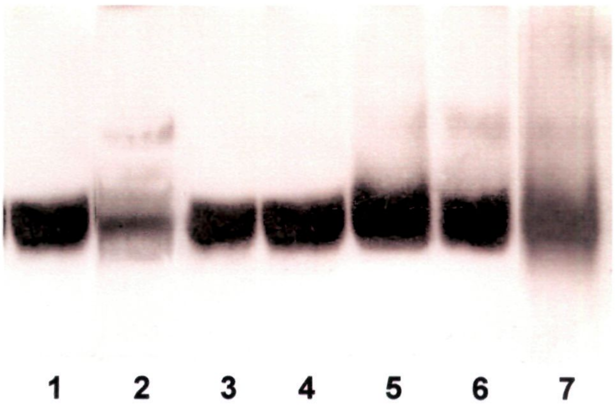
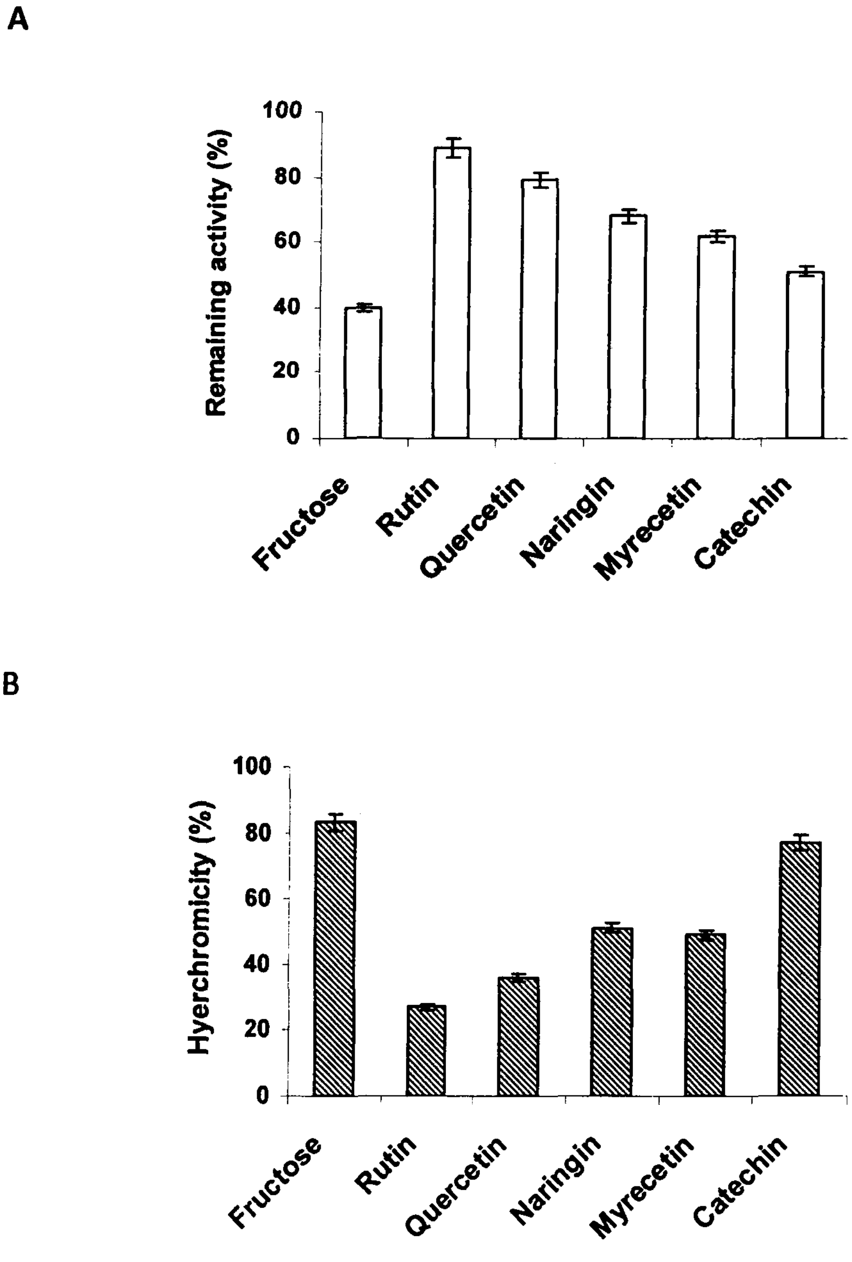

1
2
3
4
5
6
7

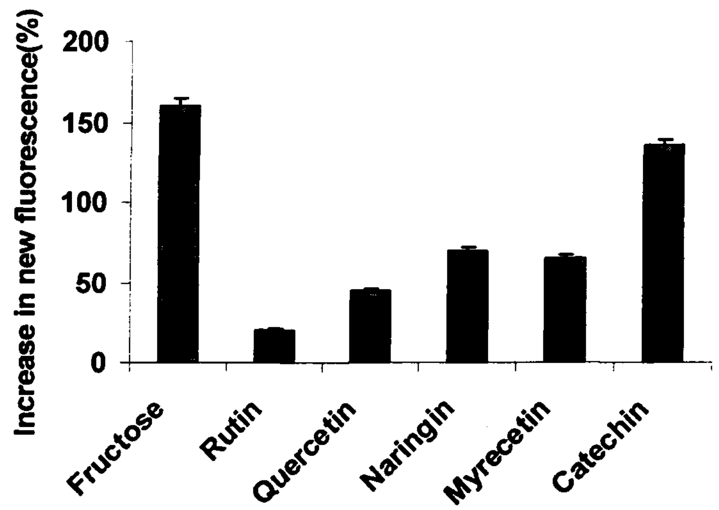
Fig 32. Protective effect of flavonoids on fructose-induced alterations in RNase A. RNase A was incubated with 100 mM fructose for 8 days at 37°C in the presence of various flavonoids at a concentration of 10 μM. The samples were dialyzed and equal quantities of protein were taken for increase in enzyme activity (A), and hyperchromicity (B). Relative values were calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.


A
Remaining activity (%)
100
80
60
40
20
0
Fructose
Rutin
Quercetin
Naringin
Myrecetin
Catechin
B
Hyerchromicity (%)
100
80
60
40
20
0
Fructose
Rutin
Quercetin
Naringin
Myrecetin
Catechin

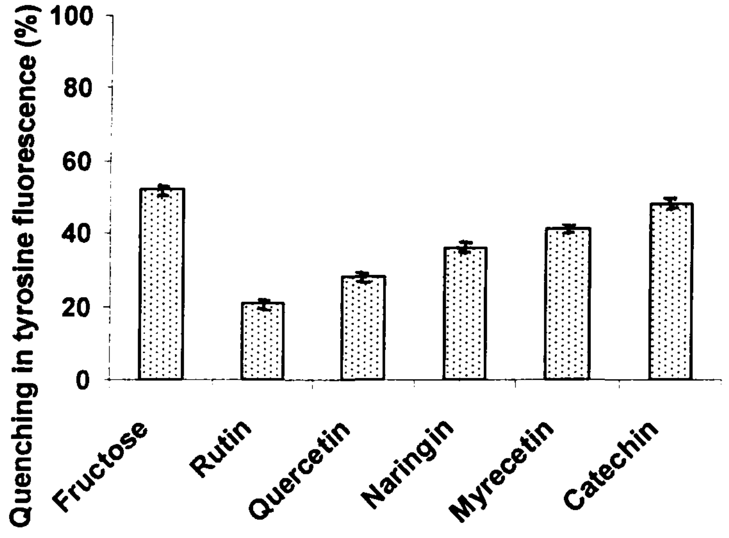
T-7069

Fig 33. Protective effect of flavonoids on fructose-induced alterations in RNase A. RNase A was incubated with 100 mM fructose for 8 days at 37°C in the presence of various flavonoids at a concentration of 10 μM. The samples were dialyzed and equal quantities of protein were taken for increase in new fluorescence (A) and quenching of tyrosine fluorescence (B) Relative values were calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.

A


Increase in new fluorescence(%)
200
150
100
50
0
Fructose
Rutin
Quercetin
Naringin
Myrecetin
Catechin

B


Quenching in tyrosine fluorescence (%)
100
80
60
40
20
0
Fructose
Rutin
Quercetin
Naringin
Myrecetin
Catechin

are shown in Fig. 34. While the immobilization did not alter the optimum temperature (60°C) of RNase A, the immobilized preparation retained a far greater fraction of activity at higher temperature as compared to the soluble enzyme. At 70°C, RNase A immobilized on Sepharose supports retained over 85% of initial activity while the soluble enzyme retained less than 70%. The immobilized preparations also appeared significantly more stable at 80°C.

4.3.1.2. Effect of pH

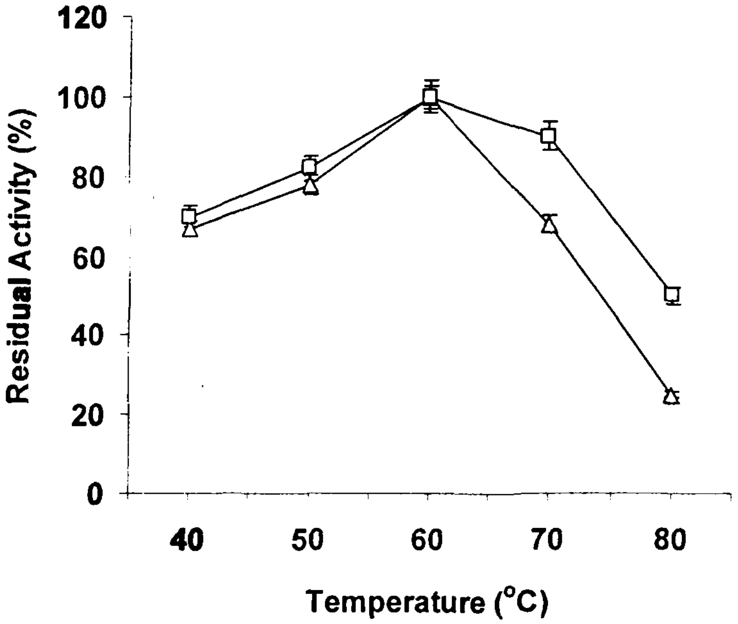
The pH activity profile of soluble and immobilized RNase A preparations are shown in Fig. 35. Immobilization on Sepharose support did not alter the optimum pH (pH 8.0) of the enzyme. The immobilized preparation however showed the moderate broadening of the pH-activity profile and retained greated fraction of activity both in acidic and alkaline range as compared to the soluble enzyme.

4.3.2. Effect of incubation with sugars on RNase activity

As has been reported earlier (Raines, 1998) bovine pancreatic RNase was thermostable and retained nearly complete enzyme activity after incubation at 60°C for two days (Fig. 36). Extension of the duration of incubation to four days resulted only in a loss of about 10 percent enzyme activity. Incubation of RNase with reducing sugars at 60°C however resulted in rapid inactivation of the enzyme. Among the sugars investigated, ribose was most active causing the loss of about 80 percent RNase activity in two days and the enzyme inactivation increased to 90 percent after four days of incubation. Glucose and fructose also inactivated RNase but to a smaller extent and a two day incubation at 60°C resulted in 20 and 60 percent loss of the enzyme activity respectively. RNase coupled to the CNBr-activated Sepharose was also susceptible to inhibition by the reducing sugars at 60°C and the pattern of inhibition resulting from the incubation with sugars with immobilized RNase was comparable with that observed with the soluble enzyme.

Fig. 34. Effect of temperature on the activity of soluble and immobilized RNase A. Appropriate quantities of soluble and immobilized RNase A preparation were incubated with 2 mg of RNA in a total volume of 0.5 ml at the indicated temperatures for 5 min in 0.1 M sodium acetate buffer, pH 5.0. The preparation of RNase A investigated were: soluble (Δ) and immobilized on Sepharose-4B (□). Each point represents the mean of three experiments.

Fig. 35. Effect of pH on the activity of soluble and immobilized RNase A. Activity of soluble and immobilized RNase A preparation were determined between pH 5-10 as described in the methods section , 3.2.5.2. The preparation of RNase A investigated were: soluble (Δ) and immobilized on Sepharose-4B (□).Each point represents the mean of three experiments


120
100
80
60
40
20
0
Residual Activity (%)
40
50
60
70
80
Temperature (°C)

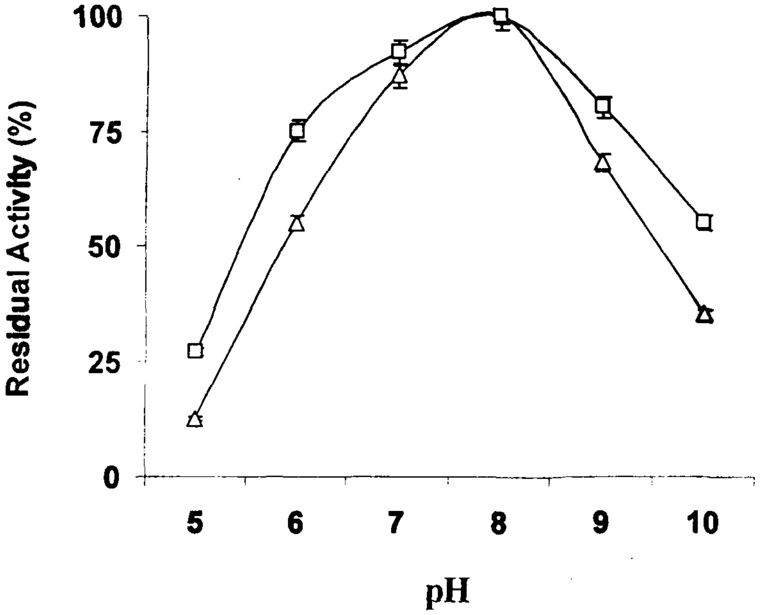
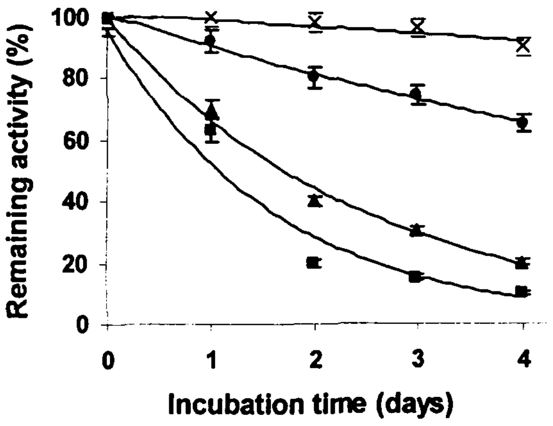
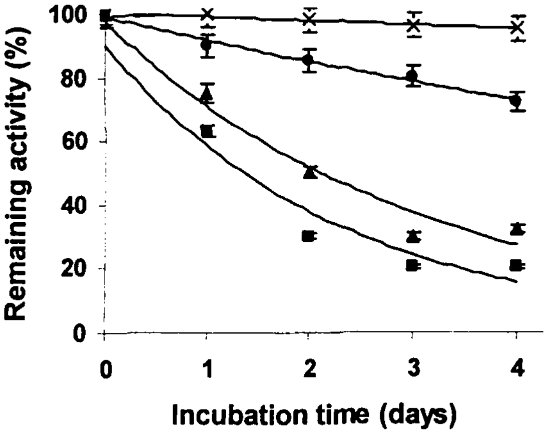

100
75
50
25
0
Residual Activity (%)
5
6
7
8
9
10
pH

Fig. 36. Effect of reducing sugars on the activity of soluble (A) and immobilized (B) RNase A. RNase was incubated with either 500 mM glucose (●), fructose (▲), ribose (■) for upto 4 days under sterile conditions at 60°C. Aliquots were removed at appropriate intervals and the enzyme activity was measured under standard assay conditions. Control sample contained RNase (X) not incubated with any sugar up to 8 days. Each value represents the average for three independent experiments performed in duplicate.

A


100
80
60
40
20
0
Remaining activity (%)
0
1
2
3
4
Incubation time (days)

B


100
80
60
40
20
0
Remaining activity (%)
0
1
2
3
4
Incubation time (days)

4.3.3. Effect of incubation with sugars at 60 °C in the spectral properties of RNase A

It is now well recognized that reaction of reducing sugars with protein can cause marked alterations in protein conformation (Ahmad, 2005). Incubation with reducing sugars at 60°C resulted in modification of soluble RNase A as evident from the observed alteration in intrinsic fluorescence (Fig. 37), new fluorescence (Fig. 38) and absorbance at 280 nm (Fig. 39). As shown in the figure RNase A incubated with the reducing sugars revealed a marked increase in new fluorescence and absorbance at 280 nm, while the quenching was observed in the intrinsic tyrosine fluorescence. The pattern of inhibition resulting from the incubation with sugars with immobilized RNase A was comparable with that observed with the soluble enzyme incubated with sugars for upto 8 days at 37°C. Clearly, ribose was most reactive in causing alterations followed by fructose and glucose. Quenching in the tyrosine fluorescence is attributed to the exposure of the phenolic groups to a less hydrophobic environment (Shaklai *et al.,* 1984). Similarly increase in absorbance at 280 nm is attributed to protein unfolding and exposure of the chromophoric groups (Traverso *et al.,* 1997).

Fig. 40 depicts the far UV-CD spectra of control and ribose treated RNase A incubated for 1, 2 and 4 days at 60°C between 205-250 nm. As can be seen from the figure, no remarkable change in the spectrum of RNase A incubated in the absence of sugar was observed while inclusion of ribose in the reaction mixture resulted in a time dependent alterations suggestive of marked loss of secondary structure. Seidler (2005) suggested that changes in secondary structure could lead to localized compaction of the protein.

4.3.4. SDS-PAGE of RNase A incubated with sugars

RNase A exposed to the various reducing sugars at 60°C was subjected to SDS-PAGE in presence of the thiol reductant β-mercaptoethanol. Fig. 41 showed that

Fig. 37. Tyrosine fluorescence spectra of RNase A. RNase A was incubated in the absence (o) or presence of 500 mM solutions of glucose (x), fructose (□) or ribose (Δ) for 2 days at 60°C. Samples were dialyzed prior to fluorescence measurement. The spectra were taken after excitation at 290 nm. Fresh RNase A (●) not incubated with either sugar was also taken along with RNase incubated at 60°C for 2 days.

Fig. 38. New fluorescence spectra of RNase A. RNase A was incubated in the absence (o) or presence of 500 mM solutions of glucose (x), fructose (□) or ribose (Δ) for 2 days at 60°C. Samples were dialyzed prior to fluorescence measurement. The spectra were taken after excitation at 370 nm. Fresh RNase A (●) not incubated with either sugar was also taken along with RNase incubated at 60°C for 2 days

Fig. 39. Absorption spectra at 280 nm of RNase A. RNase A was incubated in the absence (o) or presence of 500 mM solutions of glucose (x), fructose (□) or ribose (Δ) for 2 days at 60°C. Samples were dialysed prior to fluorescence measurement. The spectra were taken between 250-300 nm. Fresh RNase A (●) not incubated with either sugar was also taken along with RNase incubated at 60°C for 2 days.

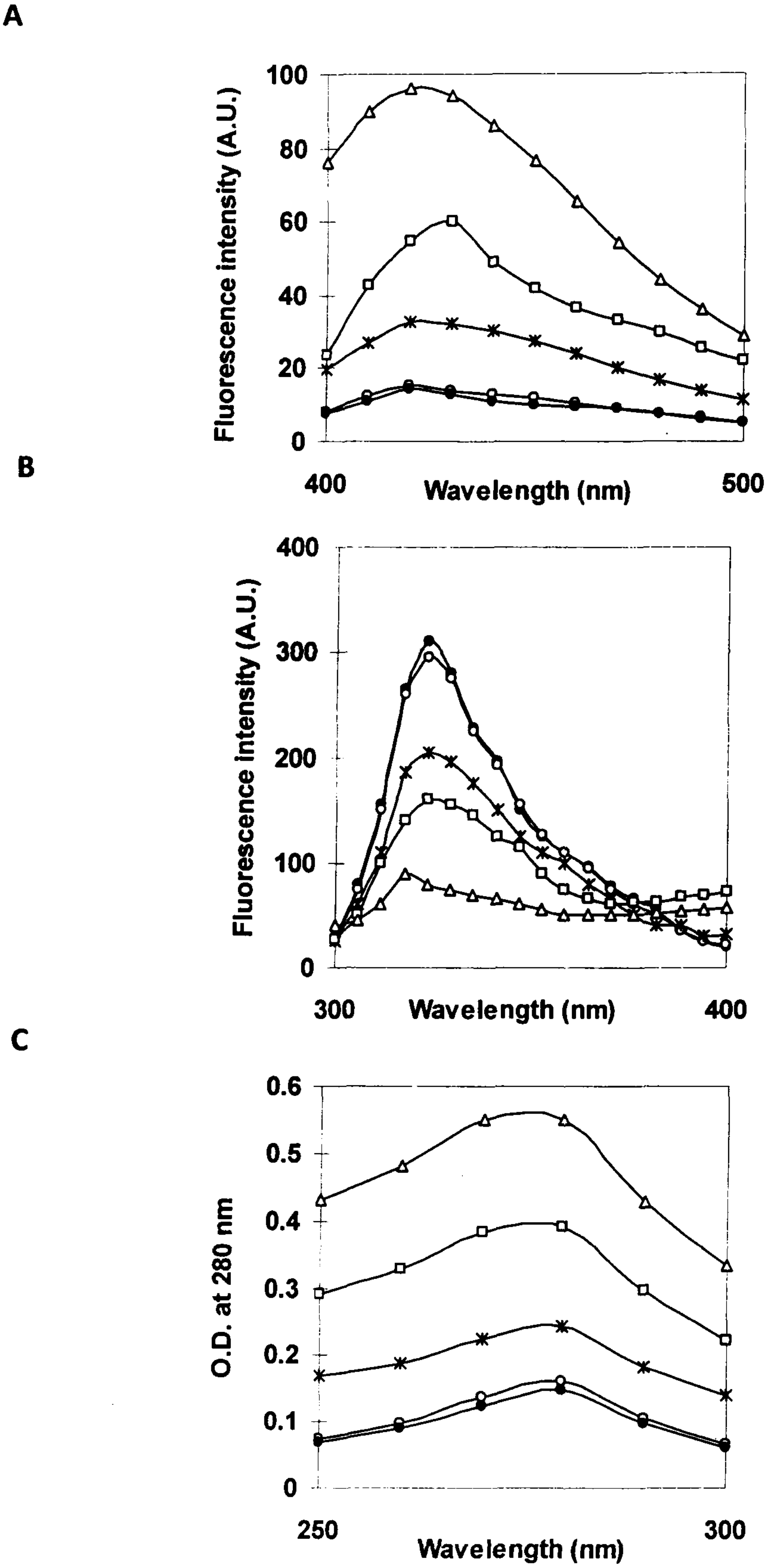
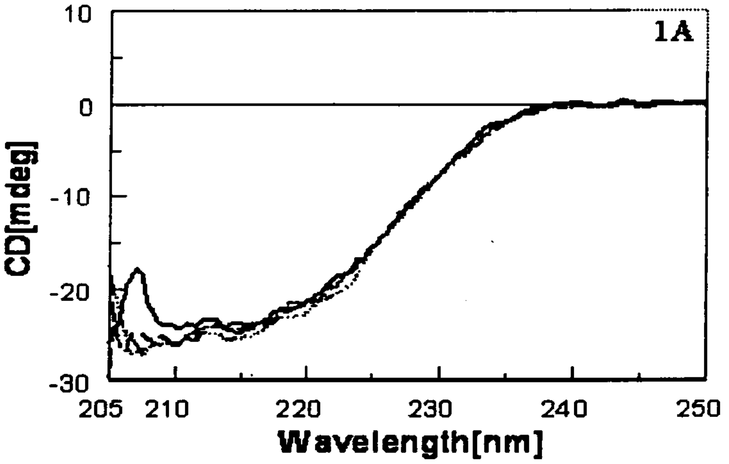

A
Fluorescence intensity (A.U.)
100
80
60
40
20
0
400
Wavelength (nm)
500
B
Fluorescence intensity (A.U.)
400
300
200
100
0
300
Wavelength (nm)
400
C
O.D. at 280 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
250
Wavelength (nm)
300

Fig. 40. Far UV CD spectra of RNase A incubated with ribose. RNase A was incubated in the absence (A) or presence of 500 mM solutions of ribose (B) for 1, 2 and 4 days at 60°C. Samples were dialyzed prior to CD measurement and 250 µl of sample was used for the analysis.


1A
CD[mdeg]
10
0
-10
-20
-30
205
210
220
230
240
250
Wavelength[nm]

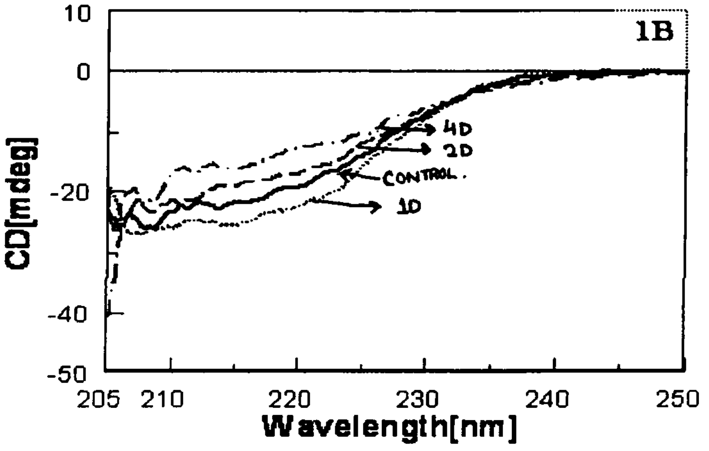
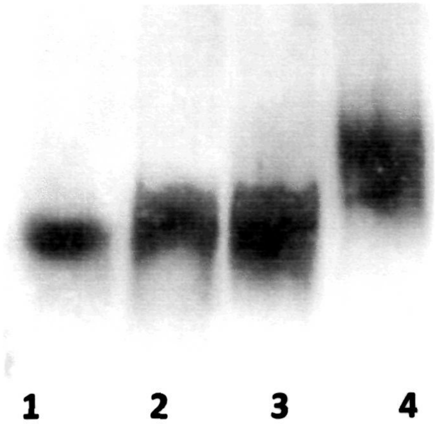

1B
CD[mdeg]
10
0
-20
-40
-50
205
210
220
230
240
250
Wavelength[nm]
4D
2D
CONTROL.
1D

Fig. 41. SDS-PAGE of RNase A incubated with various sugars. RNase A was incubated with 500 mM glucose, fructose or ribose in 20 mM phosphate buffer pH 7.2 at 60°C for two days under sterile conditions and subjected to electrophoresis. Lane 1 contained RNase A incubated in the absence of sugar; Lane 2-4 contained RNase A incubated with glucose, fructose and ribose respectively.


1
2
3
4

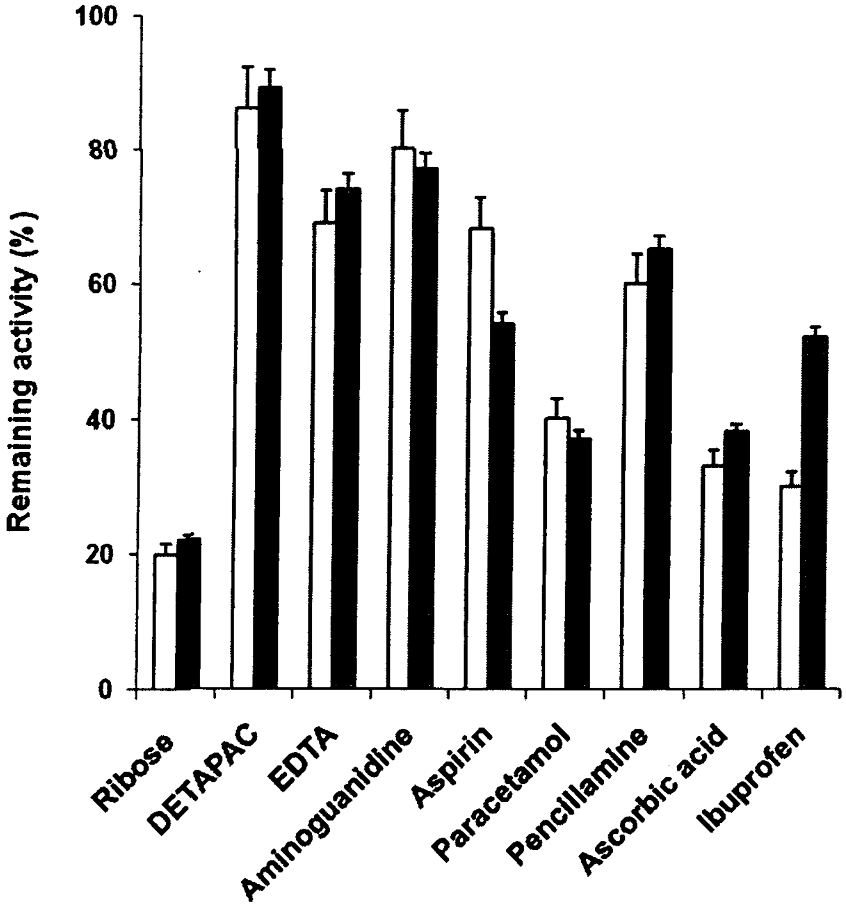
incubation of RNase with the reducing sugars resulted in marked broadening of the bands and/or decrease in electrophoretic mobility suggesting non disulphide crosslinking. The alteration in electrophoretic behavior was most prominent in the RNase incubated with ribose followed by those with fructose and glucose.

4.3.5. Effect of some glycation inhibitors

4.3.5.1. Effect of chemical compounds

The protective effect of some metal ion chelators, antioxidants, analgesics and other drugs that have been shown to interfere with glycation, on the activity of soluble and immobilized RNase A incubated with ribose at 60°C was also investigated. DETAPAC was most effective in protecting the enzyme activity, followed by aminoguanidine, EDTA, pencillamine, aspirin and paracetamol. Ascorbic acid and ibuprofen were less inhibitory. As evident from Fig. 42, a good correlation was observed between the extents of protection provided by several compounds when either soluble or immobilized RNase A was used. An excellent agreement in the protection offered by DETAPAC, EDTA, aminoguanidine, paracetamol and ascorbic acid was observed when either soluble or immobilized RNase A was used. In case of aspirin and ibuprofen however the protection appeared more marked when the immobilized enzyme was used. The difference may be attributed to interference by the drugs in the assay of soluble RNase A. Since the activity of immobilized RNase A was measured after centrifugation and washing of the immobilized preparation, the possible interference by the drugs in the assay is apparently eliminated. Aspirin is known to act by acetylation of free amino groups of proteins (Swamy and Abraham, 1989) and hence may contribute additionally towards the inactivation of RNase A. While similar action can be envisaged in case of the immobilized enzyme, it is not unlikely that some of the crucial side chain amino groups are less accessible due to immobilization of the enzyme.

Fig. 42. Protective effect of various compounds/ drugs on activity of RNase A treated with ribose. RNase A was incubated with 500 mM ribose for 2 days at 60°C in the presence of plant extracts at a concentration of 25 mM. Activity of soluble (□) and immobilized RNase A (■) incubated in the absence of ribose was taken as 100 for the calculation of percent remaining activity in various samples. All the values are mean± S.D for three experiments performed in duplicates


100
80
60
40
20
0
Remaining activity (%)
Ribose
DETAPAC
EDTA
Aminoguanidine
Aspirin
Paracetamol
Pencillamine
Ascorbic acid
Ibuprofen

4.3.5.2. Effect of some plant extracts

Ability of some plant-derived tissue extracts to protect RNase A against inactivation induced by ribose was also measured at 60°C. As shown in Fig. 43, extracts of green tea, ginger, garlic, grape, tomato and bitter gourd were significantly protective against the inactivation of both soluble and immobilized RNase A. The observed protection by ginger and bitter gourd extract was comparable when either soluble or immobilized RNase A was employed. There was however observed variation in the magnitude of protection in case of the intensely colored extracts derived from tomato, grapes and turmeric and the extent of protection appeared more marked in case of the soluble enzyme. Thus, while incubation of soluble RNase A with green tea extract resulted in the retention of 34 percent of control activity that observed with immobilized enzyme was 70 percent. Similarly, the observed retention in the RNase A activity incubated with the extracts of grape, tomato and turmeric extracts were respectively 33%, 31% and 42% while using soluble enzyme and 59%, 60%, and 64% when immobilized enzyme preparation was used.

4.3.5.3. Effect of some flavonoids

Fig. 44 showed the protective effect of various flavonoids on glycation induced inactivation of soluble and immobilized RNase A by ribose at 60°C at a concentration of 10 μM. The result indicated that quercetin and rutin showed maximum protection followed by myricetin and naringin. However catechin exhibited a little protection indicating that only some flavonoids are effective in the prevention of sugar induced alterations. There was however observed variation in the magnitude of protection in case of the intensely colored flavonoids such as quercetin and catechin and the extent of protection appeared more marked in case of the immobilized enzyme.

Fig. 43. Protective effect of some plant extracts on activity of RNase A treated with ribose. RNase A was incubated with 500 mM ribose for 2 days at 60°C in the presence of plant extracts at a concentration of 25 mM. Activity of soluble (□) and immobilized RNase A (■) incubated in the absence of ribose was taken as 100 for the calculation of percent remaining activity in various samples. All the values are mean± S.D for three experiments performed in duplicates.

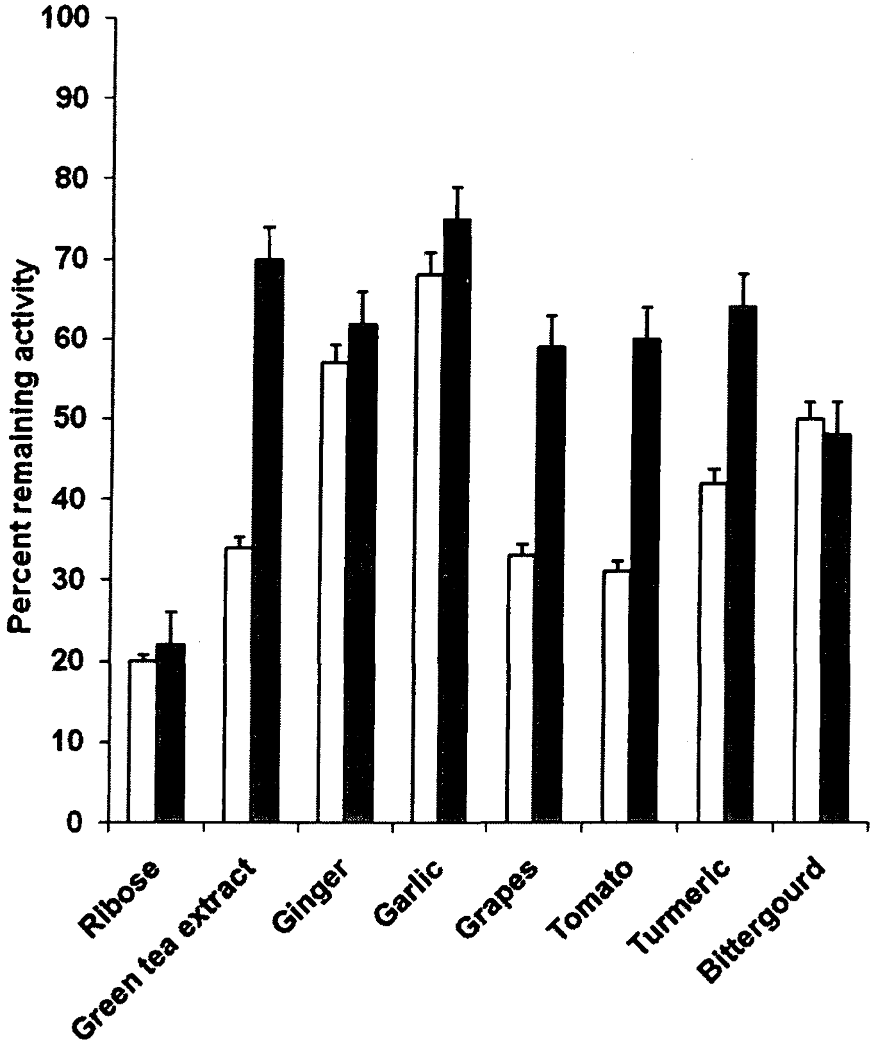
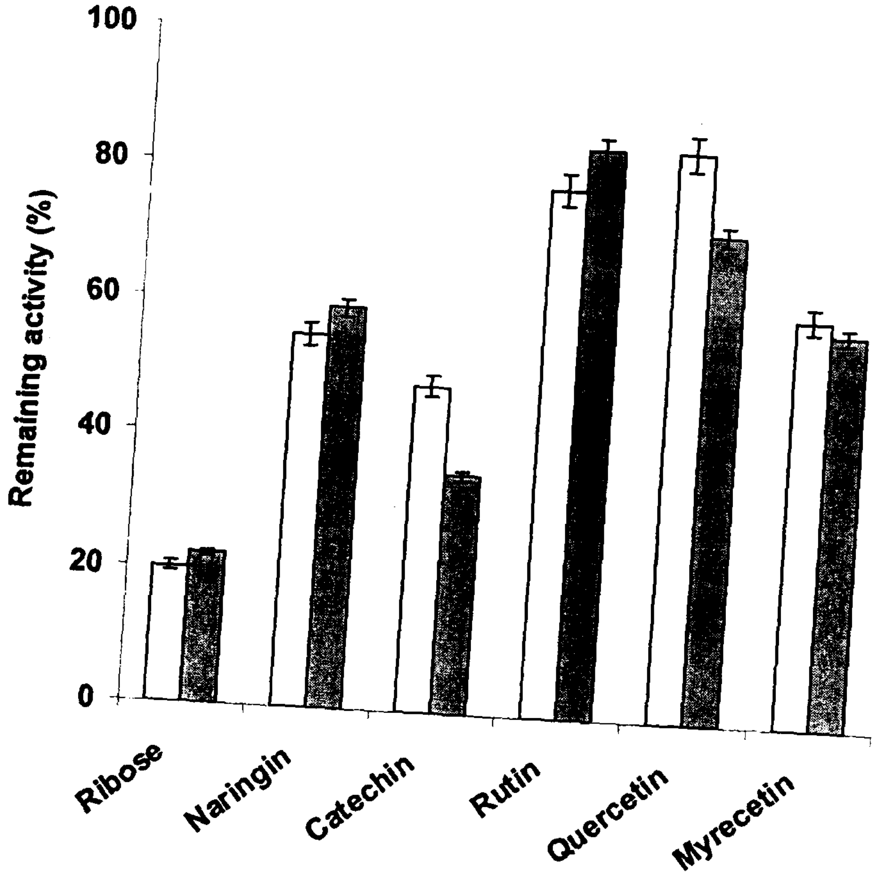

Percent remaining activity
100
90
80
70
60
50
40
30
20
10
0
Ribose
Green tea extract
Ginger
Garlic
Grapes
Tomato
Turmeric
Bittergourd

Fig. 44. Protective effect of various flavonoids on activity of RNase A treated with ribose. RNase A was incubated with 500 mM ribose for 2 days at 60°C in the presence of flavonoids at a concentration of 10 μM. Activity of soluble (□) and immobilized RNase A (■) incubated in the absence of ribose was taken as 100 for the calculation of percent remaining activity in various samples. All the values are mean± S.D for three experiments performed in duplicates.


Remaining activity (%)
100
80
60
40
20
0
Ribose
Naringin
Catechin
Rutin
Quercetin
Myrecetin

4.4. Use of antibody support for protection against glycation

4.4.1. Immunization of rabbits and purification of IgG

RNase A proved to be a good antigen, despite of its relatively small size. Fig. 45 shows the results of Ouchterlony double diffusion and ELISA suggesting the presence of antiRNase antibodies in the sera of immunized rabbits. The antiRNase IgG was purified to homogeneity on a protein A Sepharose column (Fig. 46) and the purity of the isolated IgG ascertained by SDS-AGE (Fig. 47 A). Two bands were visible on SDS-PAGE of IgG and their molecular weight as determined by the semilogarithmic plot of the molecular masses against the distance of migration correspond to those of heavy (50 kd) and light chain (25 kd) of IgG (Fig. 47 B).

4.4.2. Immobilization of RNase A on immunoaffinity and CM cellulose supports

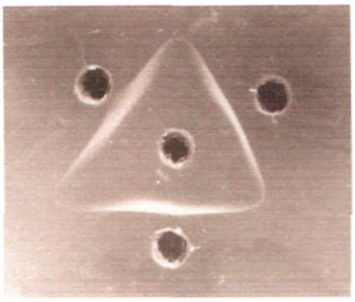
The IgG isolated from the sera of rabbits immunized with the native RNase A when coupled to CNBr activated Sepharose were effective in binding RNase A from the solution. The RNase A also bound effectively to CM-cellulose support at pH 7.2. As shown in the table 1, both the supports used contain comparable quantities of bound RNase A, but the immobilized preparations differed with regard to the effectiveness factor (Iqbal & Saleemuddin, 1983). The η values for RNase bound to antiRNase IgG and CM-cellulose support were 0.89 and 0.65.

4.4.3. Effect of various sugars on soluble and immobilized preparation of RNase A

The activity of soluble RNase A and that bound on to CM-cellulose during incubation with various sugars for eight days was markedly. As evident from Fig. 48, the inactivation was maximum with ribose and minimum with glucose. The remaining activity of RNase A immobilized on the ion exchanger incubated for eight days was only moderately higher than that of soluble enzyme incubated under the same conditions. Enzyme bound to the antibody support however retained markedly higher activity as compared to the soluble or CM-cellulose

Fig. 45. Detection of the antiRNase A antibodies in the antiserum. Ouchterlony double immunodiffusion (A) and ELISA (B) were performed to demonstrate the presence of antiRNase A antibodies. Details are given under methods (section 3.4.2 and 3.4.3)

A



B

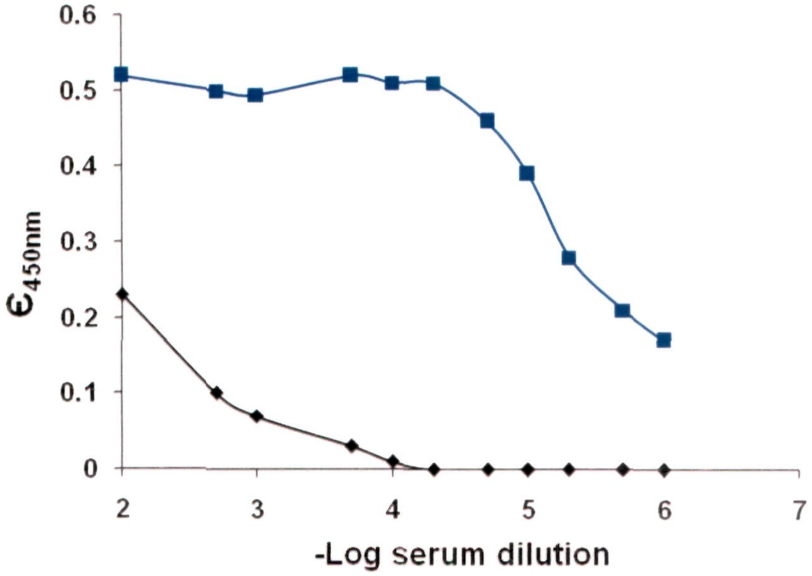


Fig. 46. Elution profile of antiRNase A IgG from a Protein A-Sepharose column. 2 ml of immune serum was loaded on (5 x 1 cm) column and washing performed with 0.1 M Tris/HCl buffer, pH 8.9, containing 3 M NaCl. The bound IgGs were eluted with 0.1 M glycine/HCl buffer, pH 3.0, and the eluate immediately neutralized with the 1 M Tris/HCl buffer, pH 8.9. Fractions of 0.5 ml were collected.

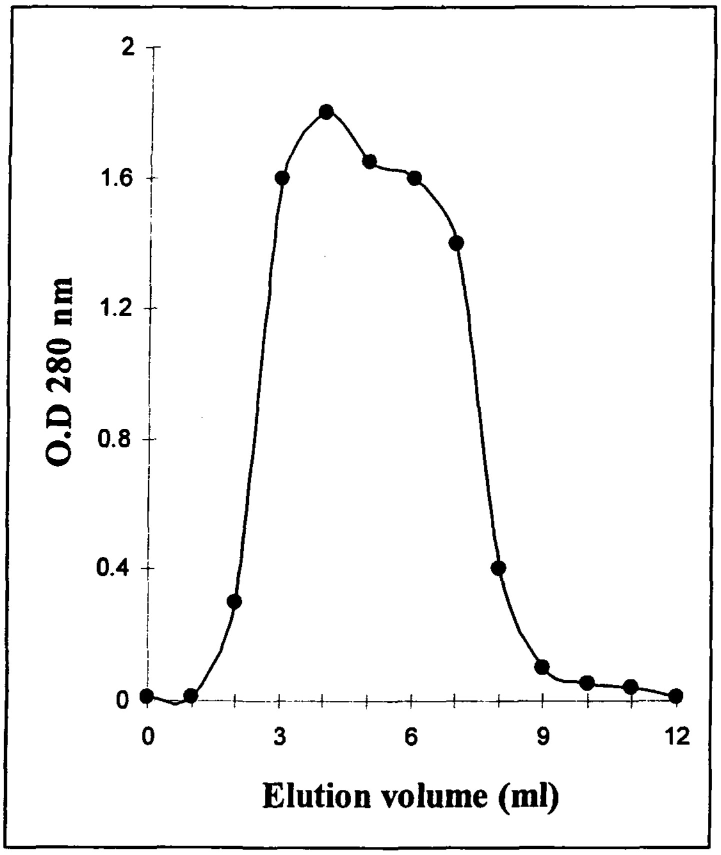
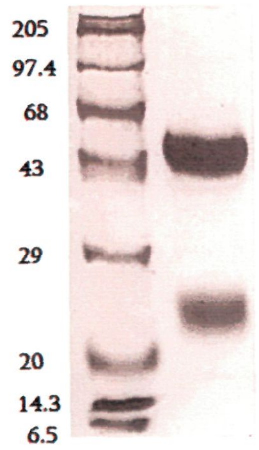

2
1.6
1.2
0.8
0.4
0
O.D 280 nm
0
3
6
9
12
Elution volume (ml)

Fig. 47. SDS-PAGE of IgG and determination of molecular weight of the IgG. The IgG purified from the sere of rabbits immunized with RNase A using a Protein A-Sepharose column was subjected to SDS-PAGE as described in the methods, section 3.3.9. Panel A Lane 1 contained 10μg standard marker protein mixture; myosin (205 kd), phosphorylase b (97.4 kd), BSA (68 kd), ovalbumin (43 kd), carbonic anhydrase (29 kd), soyabean trypsin inhibitor (20 kd), lysozyme (14.3 kd) and aprotinin (6.5 kd). Lane 2 contained 10μg of antiRNase A IgG. Panel B shows the semi logarithmic plot of the molecular masses against the distance of migration of the standard marker proteins. Arrow 1 and 2 indicate the position of the small and large molecular weight peptides, respectively.

A


205
97.4
68
43
29
20
14.3
6.5

B

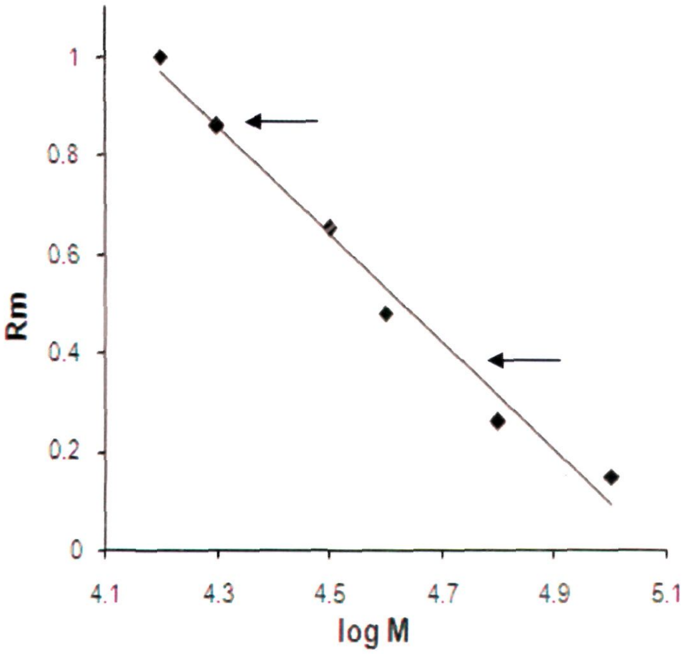
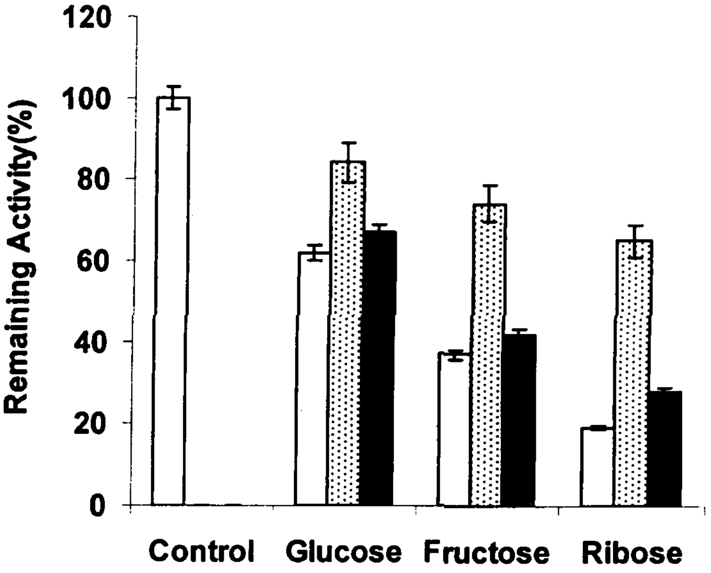


Table 1: Immobilization of RNase A on CNBr activated and antiRNase A coupled Sepharose supports.

Support	RNase A bound (U/ml)		Effectiveness factor η (b/a)
	Theoretical (a)	Actual (b)	
CM-cellulose	228.0	148.0	0.65
AntiRNase A-Sepharose	232.0	208.0	0.89

Each value represents the mean of three independent determinations. Maximum variation did not exceed ± 5.5 units.

Fig. 48. Effect of incubation with sugars on the activity of soluble (□) and that bound to CM-cellulose (⊡) or antiRNase antibody support (■). RNase A was incubated with 500 mM either sugar for 8 days under sterile conditions at 37°C. The RNase A bound to CM-cellulose support or antibody support was eluted and enzyme activity measured. Activity of RNase A incubated in the absence of sugar was taken as 100. Each value represents the average for three independent experiments performed in duplicate.


120
100
80
60
40
20
0
Remaining Activity(%)
Control
Glucose
Fructose
Ribose

bound enzyme. The difference was most remarkable in samples incubated with ribose, with the enzyme retaining over 65 percent activity as compared to the 20 and 30 percent activities respectively retained by soluble and CM-cellulose bound RNase A.

4.4.4. SDS-PAGE of RNase A exposed to the sugar in soluble and immobilized state

Fig. 49 shows the SDS-PAGE profiles of soluble RNase A incubated with the sugars as well as those of the enzyme exposed to the sugars while remaining bound to either CM-cellulose or the antibody support. The later were eluted and subjected to electrophoresis as described in the text. Soluble enzyme exposed to the sugars revealed marked broadening of the band, decrease in staining intensity as well as appearance of additional large and small molecular weight bands. In addition, staining observed at the top of the gel indicated the formation of very large molecular weight aggregates. The alterations were also more marked in samples incubated with ribose followed by fructose and glucose. RNase A immobilized on the antibody support was however clearly resistant to the sugar-induced alterations and showed minimum alteration in the electrophoretic behavior.

4.4.5. Effect of immobilization on sugar induced alterations in the conformation of RNase A

Spectroscopic studies of the soluble RNase A and that incubated with the sugars while remaining bound to the CM-cellulose or antibody Sepharose further revealed the protective role of the antibodies. Glucose induced a moderate increase in absorbance of the soluble enzyme and enzyme bound to CM-cellulose in eight days, while the alterations in the antibody bound RNase A preparation were minimal. The differences were more marked when the RNase A preparations were incubated with fructose or ribose. As evident from the Fig. 50, the

Fig. 49. SDS-PAGE of RNase A preparation incubated with reducing sugars. RNase A was incubated with 500 mM glucose (A), fructose (B) or ribose (C) separately up to 8 days in 20 mM phosphate buffer, pH 7.2 at 37ºC. Lane 1 contained RNase A incubated without any sugar. Lane 2 contained soluble RNase A, Lane 3 & 4 contained RNase A exposed to the sugars while remaining bound to antiRNase A antibody or CM-cellulose support.

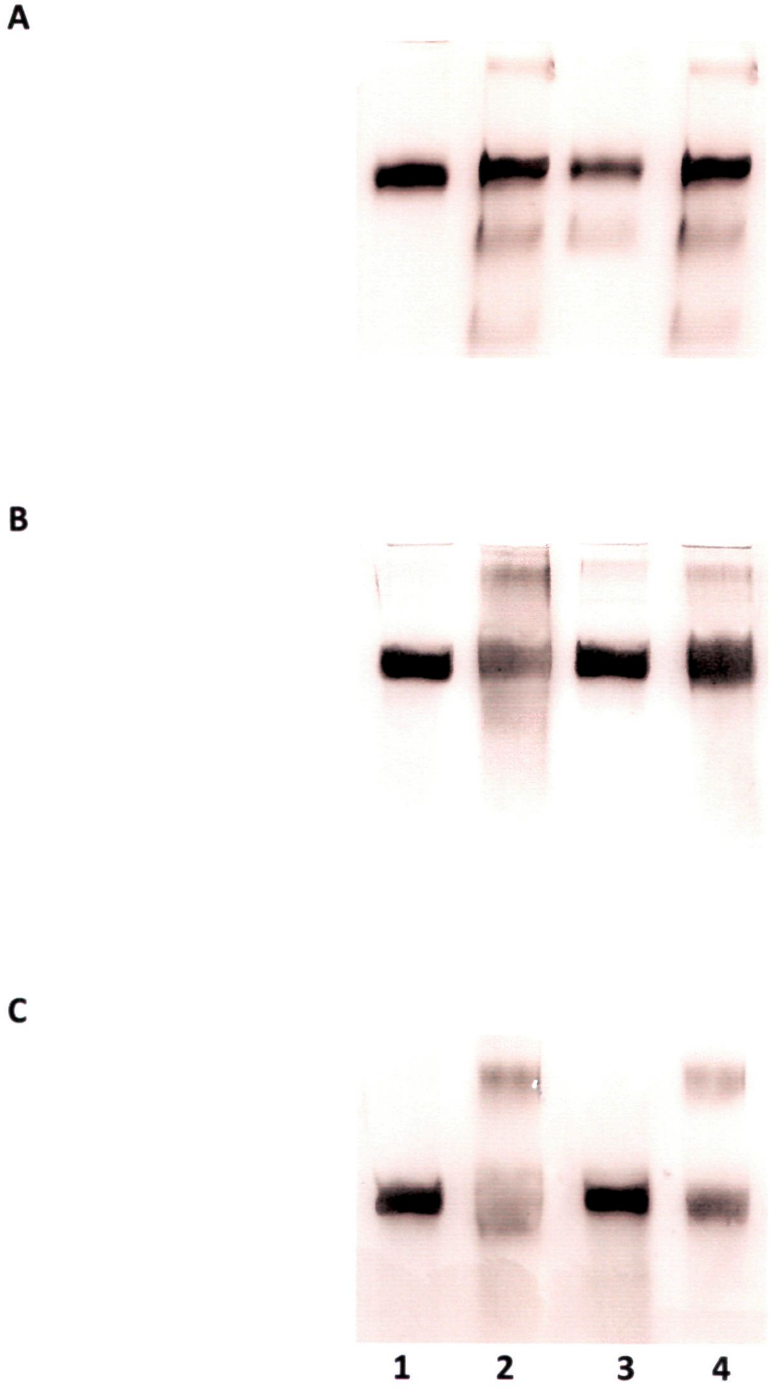
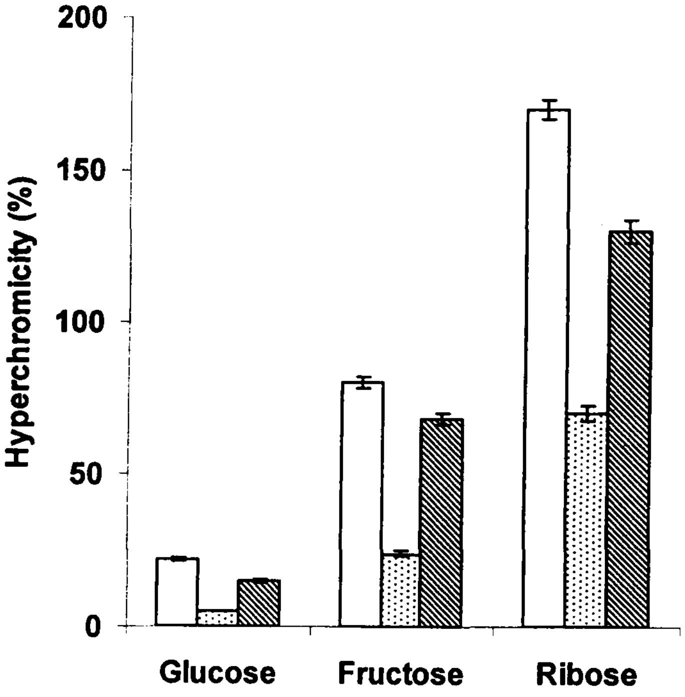

A
B
C
1
2
3
4

Fig. 50. Effect of various sugars on hyerchromicity of soluble RNase A (□), RNase A bound to antiRNase A Sepharose (▩) and CM-cellulose support (■). RNase A incubated with 0.5 M glucose, fructose, or ribose at 37°C for 8 days followed by dialysis in soluble RNase A and elution of the enzyme from immobilized preparation as described in the method section. Hyperchromicity was calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.

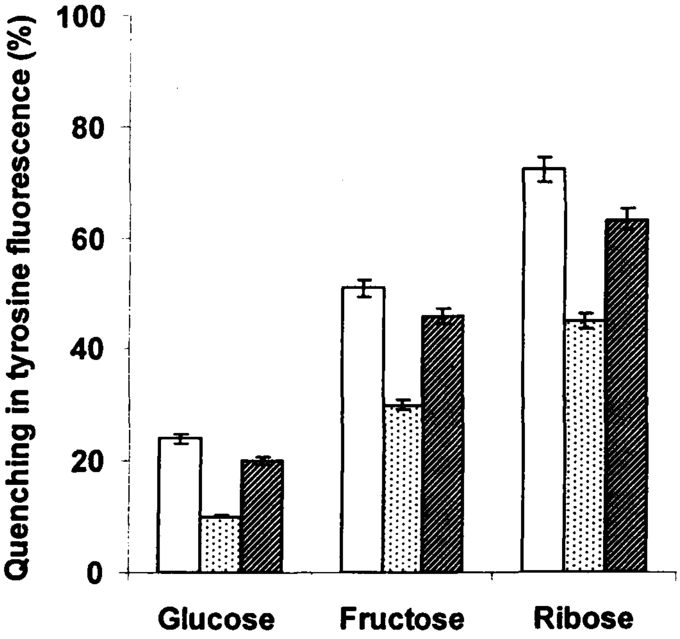

200
150
100
50
0
Hyperchromicity (%)
Glucose
Fructose
Ribose

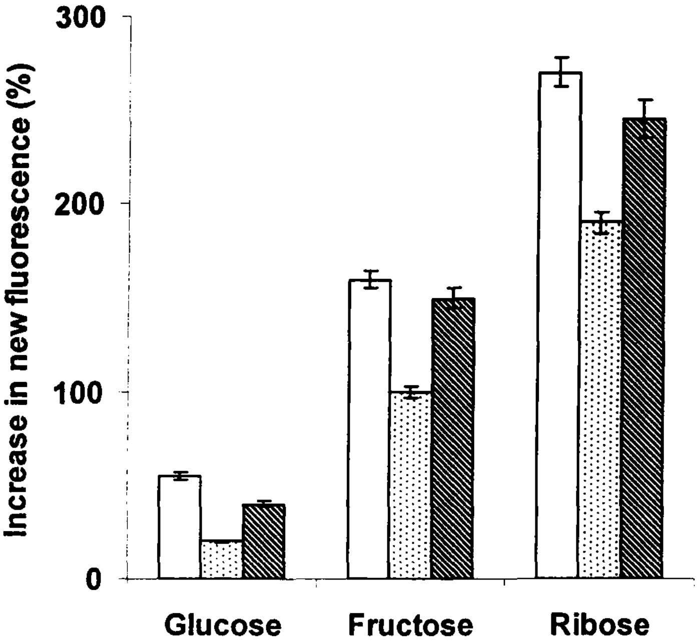
hyperchromicity of the CM-cellulose bound preparation was slightly and that of the antibody bound preparation remarkably lower as compared to that of the soluble enzyme incubated with the sugars. Conformational alterations resulting from the exposure to sugars is also evident from the measurement of fluorescence quenching determined after excitation at 280 nm and emission between 300-400 nm. Fig. 51 showed that maximum quenching was observed in case of the soluble preparation while RNase A that remained bound to the antibody support during exposure with the sugars showed minimum quenching. As observed in experiment with hyperchromicity, exposure to ribose caused maximum quenching.

Results with various sugars also show that ribose was most active in increasing new fluorescence and as compared to the soluble enzyme and that bound to CM-cellulose, RNase A immobilized on antibody support exhibited minimum new fluorescence (Fig. 52).

Fig. 51. Effect of various sugars on tyrosine fluorescence of soluble RNase A (□), RNase bound to antiRNase A Sepharose (⊡) and CM-cellulose support (▨). RNase A incubated with 500 mM glucose, fructose, or ribose at 37°C for 8 days followed by dialysis in soluble RNase A and elution of the enzyme from immobilized preparation as described in the method section. Quenching in tyrosine fluorescence was calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.

Fig. 52. Effect of various sugars on new fluorescence of soluble RNase A (□), RNase A bound to antiRNase A Sepharose (⊡) and CM-cellulose support (▨). RNase A incubated with 500 mM glucose, fructose, or ribose at 37°C for 8 days followed by dialysis in soluble RNase A and elution of the enzyme from immobilized preparation as described in the method section. Increase in new fluorescence was calculated taking the value of sample incubated without sugar as 100. All values are mean ± S.D for three experiments performed in duplicates.


Quenching in tyrosine fluorescence (%)
100
80
60
40
20
0
Glucose
Fructose
Ribose


Increase in new fluorescence (%)
300
200
100
0
Glucose
Fructose
Ribose

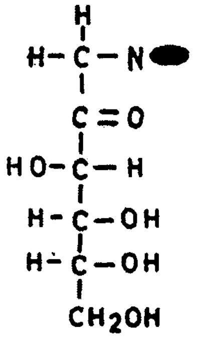
Discussion

5.0. DISCUSSION

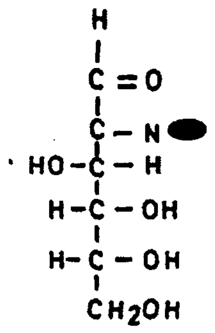
In an effort to evaluate simple and useful assay strategies for the screening of substances that interferes with glycation, two proteins HSA and RNase A were selected. Three sugars glucose, fructose and ribose with known differences in reactivity were used and a comparison was made of changes in the proteins induced by the sugars. The alterations studied included behavior in SDS-PAGE, intrinsic fluorescence, hyperchromicity and new fluorescence as well as adduct formation using ketoamine and borohydride-periodate assays. In case of RNase A, decrease in the ability to hydrolyze yeast RNA was also taken as a measure of glycation.

HSA is the most abundant protein in blood constituting about 40 mg/ml plasma (Shaklai *et al.,* 1984). HSA function as a carrier of wide variety of substances including amino acids, metals, hormones, metabolites like bilirubin, hemin and fatty acids and many therapeutic compounds. HSA plays a protective role *in vivo* as an antioxidant (Bourdon *et al.,* 1999). In normal individuals, nearly 10 % of the HSA is modified by glycation and the modification increases 2-3 folds under hyperglycemic condition (Guthrow *et al.,* 1979). Glycated albumin is therefore considered a useful marker for short term monitoring (2-4 weeks) of diabetic patients. The level of glycated albumin reflects the degree of hyperglycemia in diabetes (Nakajou *et al.,* 2002).

Glycation of protein has been shown to result in protein degradation and/ or crosslinking (Singh *et al.,* 2001; Rosca *et al.,* 2005) and the observed alterations in the migration behavior of HSA incubated with the sugars (Fig.10) apparently results from such effect. Glucose was least reactive but additional small molecular weight bands appeared in the gel after 8 days of incubation (Fig. 10A). Interestingly, samples incubated with fructose and ribose migrated as highly diffuse bands with the increase in incubation time and showed the presence of aggregates rather than fragments (Fig. 10B). High reactivity of the Amadori and other intermediate generated by ribose and fructose (ref) may contribute towards aggregate formation. Incubation of HSA with sugars also resulted in a time-dependent induction of carbonyl groups in the molecule which increased more rapidly in the samples incubated with ribose (Fig. 11). It is well recognized that carbonyl groups are introduced in the proteins as a consequence of glycation, and protein carbonyl content is considered as a reliable measure of glycation (Beal, 2002). Similarly, new fluorophore formation, quenching of intrinsic fluorescence, hyperchromicity at 280 nm as well as loss of tertiary structure, as evident from CD measurement were most prominent in HSA incubated with ribose. The difference in observed rates of inactivation resulting from the incubation with various sugars is attributable to their relative reactivities in glycation reactions (Seidler, 2005), which in turn dependent upon the concentration of the respective open chain form at equilibrium (Kaneto, 1994). Ketoamine and periodate assays however were contradictory showing fructose to be least reactive among the three sugars (Fig. 12, 13). The failure of these colorimetric assays to detect the Amadori product formed by the fructose may explain the observation. The Amadori product (AP) formed by fructose is an aldehyde with a carbonyl at C-1 which is unable to cyclize and hence cannot react with redox dyes such as NBT in the fructosamine assay and it also does not liberate formaldehyde in periodate assay from which the chromophores are generated.



Glucose-AP



Fructose-AP

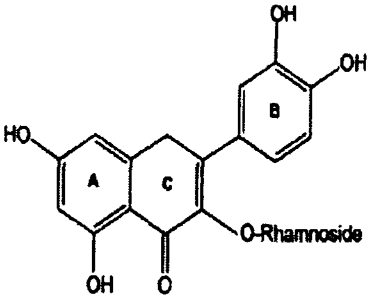
It is now well recognized that reaction of proteins with reducing sugars can cause marked alterations in their structure and conformation (Ahmed, 2005). The resulting covalent crosslinking may induce stress in the molecule, triggering the overall structural changes as revealed by quenching in intrinsic fluorescence, hyperchromicity, generation of new florescence and alterations in the CD spectra (Fig. 14, 15 and 16). Loss of intrinsic fluorescence is attributed to the destruction of the tryptophan and/or modification of the tryptophan microenvironment (Davies *et al.,* 1987). With increase in time of incubation with sugars there was a consistent increase in the peak value of emission in range 400-500 nm that suggests the formation of glycation adducts and AGEs. Increase in absorbance at 280 nm (Fig. 16) is attributed to protein unfolding and exposure of the buried chromophoric groups to the solvent (Traverso *et al.,* 1997). CD spectra (Fig. 17) also showed alterations in the helical behaviour of HSA. The structural alterations suggest by an increase of beta structure and random coil at the expense of alpha helix. The incubation of HSA with sugars appears to promote conformational changes leading to instability in the secondary structure. A comparison of intrinsic fluorescence with far CD in a recent study confirmed that glycated HSA lost tertiary structure before losing secondary structure (Mendez *et al.,* 2005). While the alterations in behavior of protein reported here are common to most proteins, the magnitude of glycation-induced changes may vary. It was shown earlier that while amino groups are primary targets, the extent of glycation does not directly depend on the number of glycation-prone residues but on the solvent accessible surface area of such residue (Moulick *et al.,* 2007). These studies however suggested that fructose and ribose behave very similar to glucose but react faster with HSA leading to more rapid modification of the protein.

A number of natural or synthetic compounds and drugs as AGE inhibitors have been proposed and identified. We have studied the protective effect of some metal ion chelators, antioxidants, analgesics and other drugs that have been shown to interfere with glycation by restricting structural alterations, protein crosslinking etc. For this study fructose was selected as a model sugar because of its high reactivity as compared to glucose and the fact that atleast in some tissues *in vivo,* glycation with the sugar is significant under specific conditions (Kinoshita *et al.,* 1979; Tomblinson, 1985). The inhibition of crosslinking and conformational alterations by radical scavengers and metal ion chelators DETAPAC and EDTA strongly suggest the role of free radicals in glycation. DETAPAC and EDTA are believed to act by preventing metal catalyzed oxidation of sugars (Wolff and Dean, 1988, Well-Knecht *et al.,* 1995). Aminoguanidine on the other hand blocks the formation of sugar carbonyl and ketoamine or their derivatives by competing with nucleophillic groups on proteins (Thornalley, 2003). Aspirin protect proteins against glycation by acetylating the free amino groups (Swamy and Abraham, 1989) while analgesics like ibuprofen and paracetamol act by reducing the oxidative stress (Malik, 1996). Our experiments (Fig.18, 19) suggested that ascorbic acid was least effective of all the inhibitors studied in preventing structural changes associated with glycation. Ascorbic acid is structurally similar to sugars therefore it is possible that it competes with fructose for sites of glycation. The protective property of ascorbic acid is also attributed to its ability to scavenge free radicals that play an important role in glycation reactions (Malik, 1996). The exact mechanism of action of these compounds needs to be further elucidated. The present study however indicates that these are powerful inhibitors which may act at multiple steps of glycation and AGE formation. This study also shows that fructose can be conveniently used as a model sugar for study of glycation inhibitors except while using the periodate and ketoamine assays.

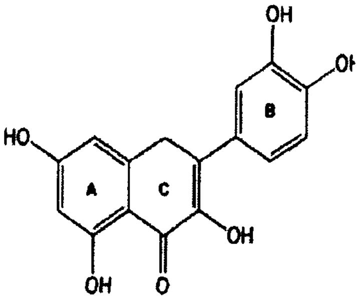
There are reports of several plant products that show remarkable AGE inhibitory activity. These include resveratrol, a natural estrogen present in grapes (Mizutani *et al.,* 2000), curcumin found in turmeric (Sajithlal *et al.,* 1998), S-allylcysteine a constituent of garlic (Ahmad and Ahmed, 2006), rutin that occurs in tomatoes

(Kiho *et al.,* 2004) and polyphenolic compounds in *Zea mays* (Farsi *et al.,* 2008). Green tea contains tannins (flavonoids) having significant antiglycation properties (Nakagawa *et al.,* 2002). Dearlove *et al.* (2008) showed polyphenolic substances in extracts of various herbs and spices inhibit fructose-mediated protein glycation.

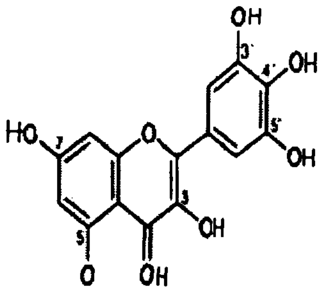
Several flavonoids are also known to act as antioxidants (Wu and Yen, 2005). Since some antioxidants also act as inhibitors of glycation (Urios *et al.,* 2007), we studied the effect of some flavonoid on HSA glycation by fructose. Some bioflavonoids have also been shown to reduce formation of glycated hemoglobin (Manuel *et al.,* 1999). According to Matsuda *et al.* (2003), the principal structural requirement of flavonoid for the inhibition of AGE formation, is the presence of vicinyl dihydroxyl group at 3'-, 4'-, 5 and 7-position. All the flavonoids used in our study fulfil this requirement, although the magnitude of their inhibition on the glycation reaction varied remarkedly.


OH
OH
HO
A
B
C
O-Rhamnoside
OH
O

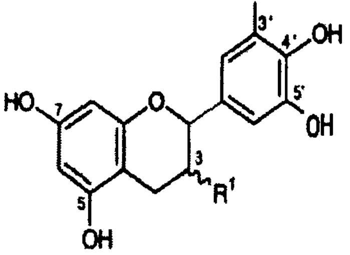
Rutin


OH
OH
HO
A
B
C
OH
OH
O

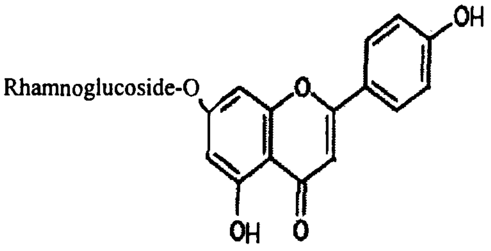
Quercetin


OH
3'
4'
OH
5'
OH
HO
7
O
3
OH
5
O
OH

Myrecetin


OH
3'
4'
OH
5'
OH
HO
7
O
3
R1
5
OH

Catechin


OH
Rhamnoglucoside-O
O
OH
O

Naringin

Our finding herein showed the reduced formation of glycation adducts in the presence of rutin and quercetin which is comparable with the protection provided by DETAPAC, EDTA and aminoguanidine. However 10 μM rutin and quercetin produced inhibitory effect with that of 25 mM aminoguanidine or DETAPAC, which is remarkable. The chelators used in the experiment were present in excess to ensure maximum inhibition. Inhibition of free radical generation derived from protein glycation and subsequent inhibition of protein modification is one of the mechanisms of prevention of glycation (Farrar *et al.,* 2007). The results suggest the usefulness of the assays in screening of the inhibitors of glycation and potential of some flavonoids in reducing the glycation-induced protein damage.

The data however suggest that inhibition of the formation of fluorescent adducts and AGEs presumably occurs by trapping of the ROS and carbonyl intermediates. Another parameter used for the study of protein glycation is the formation of protein crosslinks. Restriction of the crosslink formation by flavonoids suggests that their inhibitory activity is not only due to their antioxidant properties but also to some additional mechanism. In a recent study polyphenol rich extracts from the plant *Ilex paraguariensis* have been shown to inhibit the formation of AGEs (Lunceford and Gugliucci, 2005). In addition, green tea extract containing polyphenolic compounds have also shown to delay diabetic complications (Vincen and Zhang, 2005).

Our data suggest that micromolar concentration of flavonoids effectively reduce the sugar-induced protein damage. Consumption of the plant products in diet may therefore contribute to the restriction in protein glycation. Further characterization of flavonoid metabolites and metabolite structure-function relationship may lead to a better understanding of the role of these compounds in delaying progression of complications related to hyperglycemia. The study also suggests that fructose-induced glycation of HSA can be conveniently followed by the measurement of hyperchromicity, tryptophan fluorescence, new fluorescence as well as electrophoretic behavior of protein in the presence of SDS.

Earlier studies have shown that RNase A incubated with sugars undergoes structural modification and loss in catalytic activity (Khalifah *et al.,* 1996). As shown in Fig. 24, glucose, fructose or ribose cause marked inhibition of RNase A activity at 37^0 C and the loss of catalytic activity was most rapid in the presence of ribose. Incubation with glucose, fructose or ribose resulted in loss of 35, 62 and 84 percent activity respectively in eight days. The difference in observed rates of inactivation resulting from the incubation with various sugars as discussed earlier is attributable to relative reactivities of the sugars in glycation reactions (Seidler, 2005). The remarkably high reactivity of ribose towards RNase A may also be related to the structural similarity of the sugar with the enzyme substrate.

Pancreatic RNase A is an extensively studied and highly thermostable enzyme (Raines, 1998). RNase A undergoes remarkable alteration in structure and catalytic activity when incubated with glucose and other reducing sugars as discussed earlier. Lysine is a constituent of the active site of RNase A (Heinrickson, 1966), which is apparently highly reactive towards sugars and incubation with sugars resulted in a rapid inactivation of the enzyme. Since ribose is closely related to the enzymatic substrate of RNase and Lys-41 of the active site most easily form Amadori products (Watkins *et al.,* 1985) and AGEs (Brock *et al.,* 2003). Fig. 36 A shows that the catalytic activity of the enzyme is barely affected by incubation in the absence of sugars at 60°C for two days while the activity loss was remarkable in presence of the sugars especially ribose under the same conditions. This facilitated the assay of glycation at 60°C at which reactivity of sugar is very high which reduced the assay duration to two days. Most of the glycation assays used currently require weeks and even months. In an earlier study Matsuura *et al.,* (2002) have used BSA at 60°C to increase the rate of glycation however albumin is known to undergo unfolding at such temperature (Takedo *et al.,* 1993).

The loss in activity of RNase A resulting from the incubation with various sugars was comparable when either soluble or immobilized RNase A was used. This suggested that immobilization does not markedly alter the reactivity of sugars towards enzyme. It is interesting that RNase incubated in absence of sugars for two days at 60°C exhibited only minor alterations in hyperchromicity, quenching of intrinsic fluorescence and CD spectral behavior, suggesting the retention of three dimensional structure of enzyme (Fig.37, 38, 39 and 40). While it is true that ribose is not the principle sugar responsible for *in vivo* glycation reactions, structural alterations induced by ribose in proteins are similar to those caused by glucose. This include the cross reactivity of AGEs generated by the sugar with anti AGE antibodies raised against protein Glycated using glucose (Nagai and Horiuchi, 2003). In addition, ADP-ribose may actually cause glycation of proteins *in vivo,* although to a smaller degree (Cerventes-Lauren *et al.,* 1996). We therefore felt that a combination of RNase as model protein and ribose as model sugar provides a remarkable advantage in cutting down the assay duration and facilitates the rapid screening of inhibitors of glycation.

The comparable sensitivity of immobilized RNase A with that of its soluble counterpart towards glycation induced enzyme inactivation points out to the possibility of using the later with added advantage for screening of the anti glycation principles from natural source. Immobilized RNase A, in addition to being more stable (Younus *et al.,* 2001), offers the possibility of its rapid removal from the reaction mixture and hence elimination of components of the crude extracts that may interfere with the enzyme assay. As shown in Fig. 42, an excellent agreement between protections offered by DETAPAC, EDTA, aminoguanidine, paracetamol and ascorbic acid was observed when either soluble or immobilized RNase A was used.

Similarly, among the plant extracts that exhibited significant protective effects against ribose induced inactivation of RNase A, excellent agreement was observed in case of ginger, garlic and bitter gourd extracts both with soluble or immobilized enzyme. The extracts like those of green tea, grapes and tomato however appeared more protective when immobilized RNase A was used as discussed earlier, due to interference in the assay by components of the coloured extracts (Fig. 43). Since the concentration of ribose used in the assay is remarkably high, interference by the amino group modifying molecules present in the extract may not be significant unless they are highly reactive.

These results suggest the usefulness of RNase-ribose assay for the rapid screening of extracts for inhibitors of glycation. Since only measurement of catalytic activity of RNase A are required, a simple spectrophotometer is adequate for the analysis. Also use of immobilized RNase A provides additional advantage of high storage stability (Younus *et al.,* 2001) and possibility of distinguishing between early and posts Amadori inhibitors of glycation. For the study of such inhibitors, rapid removal of the glycating sugar after initial reaction with protein is required. This is normally accomplished by dilution or dialysis (Matsuura *et al.,* 2002). Immobilized RNase A on the other hand can be readily separated from the reaction mixture instantaneously by centrifugation and/ or filtration and freed of the sugar by washing. The immobilized enzyme will however not be reusable since part of the activity will be irreversibly lost during each assay.

A number of enzymes have been shown to undergo glycation induced loss in catalytic activity both *in vivo* and *in vitro.* Cabellero *et al.* (1998) and Bousova *et al.* (2005) suggested that the loss of catalytic activity of δ-aminolevulinic acid dehydratase and aspartate aminotransferase may be use as a measure of glycation. RNase A offers additional advantage being remarkably more stable and facilitating reaction at high temperature and thus cutting down the assay duration. Since the assay involves measurement of protection of RNase A activity, we believe it will measure only those substances that restrict the glycation reaction. Natural Inhibitor of RNase A present in the extract may contribute additionally towards the inhibition but can be taken care of by using an appropriate control in which enzyme may be preincubated with extract for short durations. Similarly possible by activators of the enzyme and/or RNase A present in the extract may corrected by running parallel appropriate controls.

Result shown in section 4.4 shows that antiRNase A antibody support protect the bound enzyme against inactivation and other alterations induced on incubation with reducing sugars. As shown in Fig. 48 the remaining activity of RNase A immobilized on CM- cellulose and incubated for eight days with sugars was only slightly higher than that of soluble enzyme incubated under the conditions. RNase A bound to the antiRNase A antibody support however retained markedly higher activity as compared to the soluble and CM-cellulose-bound enzyme. Apparently immobilization *per se* is not responsible for the observed protection provided by the antibody support, since CM- cellulose bound enzyme was only moderately protected (Fig. 48). It is interesting to note that RNase A is expected to bind to CM cellulose via side chain amino groups which are potential target of glycation reaction (Ganea and Harding, 2005), yet the observed protection against sugars was small. RNase A immobilized on the antibody support was however clearly more resistant to the sugar-induced alterations and also showed minimum alteration in the electrophoretic behavior (Fig. 49).

Spectroscopic studies of the soluble RNase A, and that incubated with the sugars while remaining bound to the antibody Sepharose further revealed the protective role of the antibodies (Fig. 50, 51 and 52). Considerable evidence suggests that binding of antienzyme antibodies to enzymes improves their resistance to inactivation induced by heat and other agents (Saleemuddin, 1999). Our more recent studies have also shown that inactivation of Cu,Zn-SOD by sugars (Jabeen and Saleemuddin, 2006a), glyoxal (Jabeen *et al.,* 2007) and methylglyoxal (Jabeen *et al.,* 2006b) can be restricted by complexing the enzyme with specific antibodies. In addition binding with antibody/antibody fragment resulted in restriction of enzyme aggregation as revealed by SELDI-TOF spectroscopy. Since both monomeric Fab and Fab_2 were protective, binding *per se* rather than crosslinking induced by the bifunctional antibodies fragment appears to be responsible for the observed protection (Jabeen *et al.,* 2007). The observation that enzyme bound to the antibody support is also resistant to glycation support this argument, since crosslinking of the antibody is less likely on the antibody support.

Binding of antibodies to protein antigen results in marked lowering of the free energy and stabilization of the protein antigens because of the high association constant. Hydrogen exchange rate investigation on lysozyme and cytochrome c amide group suggested that in the complex, the exchange rates were remarkably low not only at the point of contact between the antigen and the antibody but also in the regions far removed from the epitope recognized by the antibody (Rizzo *et al.,* 1992; Williams *et al.,* 1996). Several enzymes complexed with monoclonal/polyclonal antibodies exhibit enhanced stability against various forms of inactivation (Saleemuddin, 1999). Our earlier studies have shown that antibodies raised against the labile region of RNase were more protective than those recognizing the N-terminal peptide (Younus *et al.,* 2001). The labile region-specific antibodies also improved the thermal stability of a mutant RNase in which the labile region contained an altered cysteine (Younus *et al.,* 2002). Studies with anti- lysozyme antibodies however suggest that binding of the monoclonal antibody raised against the native enzyme improves the thermal stability of the enzyme even when it do not recognize epitopes located close to the mutation site. Thus D67H mutation of the human lysozyme related to systemic amylodosis undergoes partial unfolding and aggregation with a melting temperature 10^0 C lower than that of the native enzyme (Canet *et al.,* 2002). Binding of c-Ab-HUL6 increased the melting temperature of the enzyme by 15^0C, although the residue whose mutation leads to the destabilization and aggregation did not belong to the epitope and does not make any contact with the antibody (Dumoulin, *et al.,* 2003).

It may not be therefore essential to identify the specific epitope(s) of the target protein for raising protective antibodies, although such a selection may improve the effectiveness of the antibody. Further studies are however needed to establish that nature of protection against glycation is also similar.

Several advantages of immobilization of enzyme on antibody supports have been identified (Saleemuddin, 1999) and work described in this paper suggests enzymes immobilized thus may also be resistant to glycation induced alterations. A number of enzymes that catalyze transformations of reducing sugars or those that generates reducing sugars are employed in immobilized state in biosensors and reactors for continuous operation. Such enzymes may be exposed to reducing sugars continuously over long duration. Binding to antibody support may help extend the life of such reactors and sensors.

The mechanism by which antibodies protect enzyme against glycation needs further study. Binding of the antibodies may increase the conformational rigidity of the protein antigen and restrict unfolding. The antibodies may therefore decrease the possibility of sugar reacting with additional susceptible groups of the enzyme (Jabeen *et al.,* 2007). Some chaperones may protect the enzyme against glycation using similar mechanism (Yan and Harding, 2006). The observation that RNase A exposed to the sugars while remaining bound to the antibody support undergo fewer structural alterations supports this view.

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6.0. REFERENCES

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6. Participated in the National Symposium on "Recent advances in biochemistry and allied sciences" under the auspices of UGC-DRS II Programme of the department of biochemistry, F/O Life Sciences, Aligarh Muslim University, Aligarh during March 25, 2008.

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A procedure for the rapid screening of Maillard reaction inhibitors

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Abstract

A procedure for the rapid screening of inhibitors of glycation reaction, based on their ability to protect RNase against sugar induced inactivation of the enzyme is described. Glycation is implicated in variety of disorders including diabetes, atherosclerosis various micropathies yet is a slow process both in vivo and in vitro. In order to speed up glycation, the reaction was carried out at 60 °C using a thermostable protein RNase and ribose, a sugar that is known to react rapidly than glucose in the glycation reaction. It was observed that incubation of RNase with ribose at 60 °C in rapid inactivation of the enzyme with a parallel decrease in tyrosine fluorescence, enhancement in new fluorescence and hyperchromicity in the UV-region. No such alterations in the enzyme activity were observed when the incubation was carried out in absence of the sugar. Compounds and drugs that are known to act as inhibitors of glycation reaction restricted the ribose-induced inactivation of RNase. RNase immobilized on CNBr-activated Sepharose was also sensitive to exposure to ribose and appeared a better system to screen inhibitors of glycation from natural sources that contain substances that interfere with the assay of enzyme as well as in the study of post Amadori inhibitors of glycation.

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Keywords: Glycation inhibitors; RNase; Maillard reaction; Ribose

1. Introduction

During Maillard reaction reducing sugars react with amino groups of biomolecules including proteins, lipids, nucleic acids to form Schiff base which in turn undergoes transformation to a variety of advanced glycation end products (AGEs) [1,2]. The in vivo significance of Maillard reaction was first recognized with the detection of elevated levels of hemoglobin in the diabetic blood [3]. Subsequent evidence suggested that the AGE may play an important role in the etiology of variety of diabetic complications [4] and aging [5,6]. AGEs have also been implicated in a variety of disorders including inflammation [7], atherosclerosis [8], neurodegenerative disorders [9] and cancer [10]. Glycated protein may lose biological activity, turn toxic, develop new antigenic determinants and damage cell in a variety of ways after uptake mediated by specific receptors present on the surface of several cells [11,12].

Several lines of evidence suggest that agents that restrict AGE formation result in reduction of various diabetic complications including retinopathy [13,14] and can be considered as diabetic complication drugs. While several other strategies also appear promising [15–17], current emphasis is on the substances that act inhibitors of AGE formation. Among the compounds evaluated as AGE inhibitors, aminoguanidine (AG) [18], aspirin [19], vitamin B_6 [20] taurine [21] and quercitin [22] are important. Among these AG is in late stages of clinical trials, yet the effective dose of aminoguanidine is quite high with accompanying risks of side effects. In search of alternative drugs, screening of natural sources for potential glycation inhibitors has also been undertaken [22]. The standard procedures of assay of glycation require sophisticated equipment

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Abbreviations: AGE, advanced Glycation end products; DETAPAC, diethylenetriaminepenta acetic acid; EDTA, ethylenediaminetetraacetic acid; AG, aminoguanidine; RNA, ribonucleic acid; PAGE, polyacrylamide gel electrophoresis; CNBr, cynogen bromide; RNase A, ribonuclease A; SDS, sodium dodecyl sulphate.

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[23], expensive reagents like antibodies [24] and are time consuming requiring days and weeks because of the slow reaction of sugars with protein. Some attempts have been made to cut down assays duration by replacing glucose with reactive sugars like ribose [25] and by increasing the incubation temperature [26]. Matsuura et al. [26] described an assay in which the rate of reaction of glucose with BSA was accelerated by performing the incubation at 60 °C. AGE formation was detected by measuring quenching of intrinsic fluorescence and generation of the new fluorophore (Ex. 370. Em. 440 nm). While the authors claimed that incubation did not alter the native conformation of the protein. earlier studies suggest greater susceptibility of the BSA denaturation induced at such temperature [27]. A simple strategy for the screening of glycation inhibitors based on their ability to protect the catalytic activity of an enzyme sensitive to glycation is described in the manuscript. Bovine pancreatic ribonuclease (RNase) has been selected in view of its well known thermo stability [28] and the glycation reaction was carried out using a highly reactive sugar ribose at 60 °C [29]. The problem of interference by in the assay by substances present in the plant extract could be better addressed using an immobilized preparation of RNase.

2. Materials and methods

2.1. Materials

Bovine pancreatic RNase A, DETAPAC, aminoguanidine, yeast RNA, cynogen bromide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents for electrophoresis, glucose, fructose, ribose, lanthanum chloride and Sepharose 4B were obtained from SRL Chemicals (Bombay, India). All other chemicals and reagents were of analytical grade.

2.2. Glycation reaction

RNase (1 mg/ml) was incubated with 0.5 M glucose, fructose or ribose in 0.02 M phosphate buffer pH 7.2 at 60 °C for a period of 1, 2 and 4 days under sterile conditions. The incubation was carried out under sterile condition but also contained 5 μM gentamycin as an additional precaution. Where indicated RNase was incubated with the chemical inhibitors (25 mM) or plant extracts in the presence of 0.5 M ribose. Control samples were incubated under similar conditions but without ribose and inhibitors. Similarly RNase was incubated with some plant extracts along with 0.5 M ribose and incubated for 2 days under similar conditions. The plant extracts were prepared by homogenizing garlic, ginger, grapes, bitter gourd, turmeric and tomato in 0.02 M phosphate buffer pH 7.2 followed by centrifugation and 25 μl of the supernatant was added to the reaction mixture. Green tea extract was prepared by stirring 1 g of green tea with 20 ml of 50% (v/v) ethanol for 3 h. The mixture was centrifuged at 1500 ×g for 15 min and the supernatant was used as green tea extract [30]. All the extracts were adjusted to assay pH. While using immobilized RNase the preparation was washed thoroughly after incubation with the sugar and extracts prior to the enzyme activity measurements.

2.3. Measurements of RNase activity

RNase activity was determined spectrophotometrically using yeast RNA as substrate. The standard reaction mixture in a total volume of 2.5 ml contained 2 mg of RNA and appropriate amount of RNase A in 0.1 M sodium acetate buffer, pH 5.0. The reaction was arrested after 10 min incubation at 37 °C by using stopping reagent (22 M lanthanum chloride in 1 M perchloric acid), the reaction was left on ice for 10 min and the precipitate removed by centrifugation. Subsequently 0.2 ml of the supernatant was diluted with distilled water and the absorption of acid soluble ribonucleotides measured at 260 nm [31].

2.4. Spectroscopic analysis

The UV absorption measurements of RNase incubated without sugar (control) and that incubated with glucose, fructose and ribose were obtained by measuring the absorption between 200 and 350 nm in a Shimadzu spectrophotometer using a cuvette of 1.0 cm path length. Similarly, control and RNase incubated with sugars were studied by measuring intrinsic fluorescence at 25±0.2 °C in a Hitachi F 2000 spectrofluorimeter (Tokyo, Japan). The samples were excited at 285 nm and emission range was taken at 310–400 nm. New fluorescence measurements were also made at 370 nm and an emission range of 400–500 nm.

2.5. Electrophoresis

The electrophoretic behavior of control RNase samples and those treated with the sugars was analyzed by SDS-PAGE [32]. A resolving gel of 10% (w/v) acrylamide in tris HCl buffer, pH 8.8 with 0.1% SDS was employed. Silver staining of the gels was performed for the visualization of protein bands [33].

2.6. Circular dichroism

All the Circular dichroism (CD) measurements were carried out at 25 °C on a Jasco spectropolarimeter Model J-720 using a SEKONIC XY Plotter (Model SPI-430A), with a thermostatically controlled cell holder attached to a NESLAB water bath Model RTE 110 with an accuracy of ±0.10 °C. The instrument was equipped with a microcomputer and recalibrated with (+)-10-camphersulfonic acid. The spectrum was recorded with a scan speed of 20 nm/min and with a response time of 20 nm/min. Each spectrum was recorded as an average of two scans. Far-UV CD spectra and near-UV CD spectra were taken at a protein concentration of 20 μM with a 1 and 10 mm path length cells, respectively. CD spectra were recorded in the wavelength ranges of 190–250 nm for far-UV and 250–310 nm for near-UV.

2.7. Immobilization of RNase on CNBr activated Sepharose

Immobilization of RNase was carried out as described earlier [34]. Sepharose 4B washed thoroughly with distilled water in a sintered glass funnel, sucked dry and suspended in 1 ml of 2.0 M Na_2CO_3. Two hundred mg of CNBr in 0.2 ml of acetonitrile added to 1 g of Sepharose and the suspension was placed at 4 °C for 10 min with continuous stirring. The whole mass then washed thoroughly with 0.1 M bicarbonate buffer pH 8.5 and water. RNase (10 mg) was added to the activated Sepharose in 0.1 M sodium bicarbonate buffer pH 8.5 under stirring for 24 h at 4 °C. Amounts of the enzyme immobilized were determined by subtracting from the amount added those of the supernatant and washing by activity measurement. Under the conditions used 8.3 mg of RNase/gram of Sepharose was coupled to the matrix.

3. Results

3.1. *Effect of incubation with sugars on RNase activity*

As has been reported earlier [28] bovine pancreatic RNase was thermostable and retained nearly complete enzyme activity after incubation at 60 °C for two days (Fig. 1A). Extension of the duration of incubation to four days resulted only in loss of about 10% RNase activity. Incubation of RNase with reducing sugars at 60 °C however resulted in rapid inactivation of the enzyme. Among the sugars investigated, ribose was most active causing the loss of about 80% RNase activity in two days and the enzyme inactivation increased to 90% after four days of incubation. Glucose and fructose also inactivated RNase but to a smaller extent and a two day incubation at 60 °C resulted in 20 and 60% loss of the enzyme activity respectively. RNase coupled to the CNBr-activated Sepharose was also susceptible to inhibition by reducing sugars at 60 °C and the pattern of inhibition resulting from the incubation with sugars with immobilized RNase was comparable with that observed with the soluble enzyme.

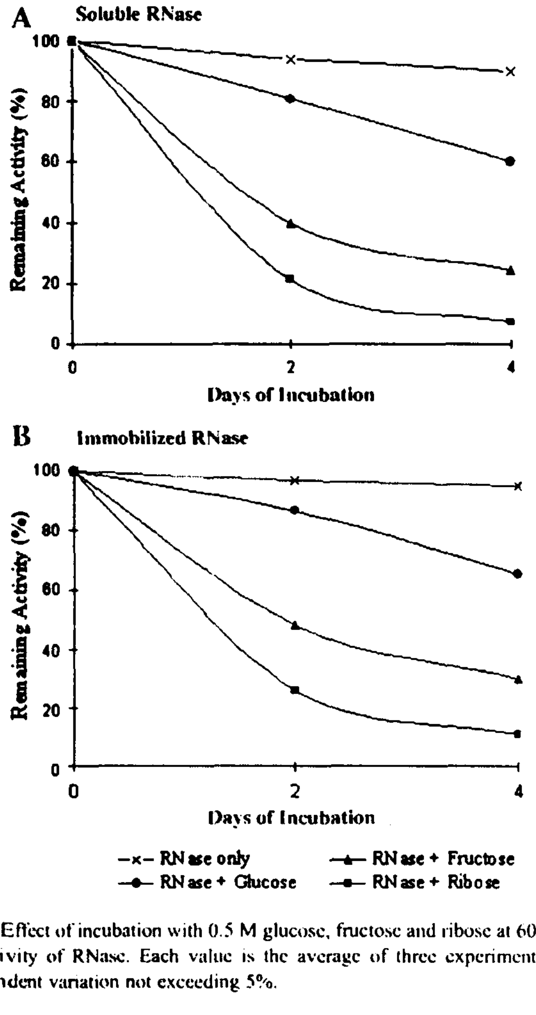

Fig. 1. Effect of incubation with 0.5 M glucose, fructose and ribose at 60 °C on the activity of RNase. Each value is the average of three experiments with independent variation not exceeding 5%.

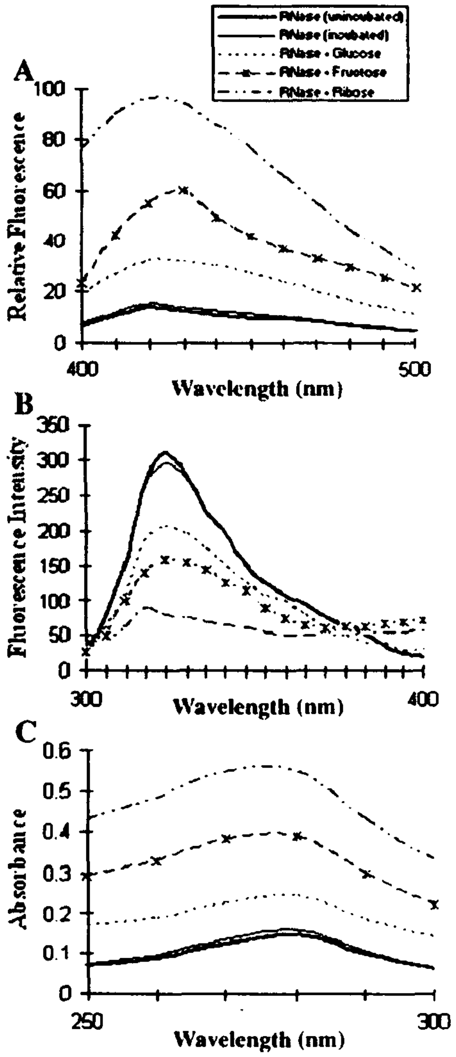

Fig. 2. Effect of sugars on the spectral properties of RNase. RNase (1 mg/ml) incubated with 0.5 M glucose, fructose, or ribose at 60 °C for 2 days followed by dialysis to remove excess sugar. Solutions were diluted to a concentration of 150 μg/ml for analysis of new fluorescence (A), tyrosine fluorescence (B), and hyperchromicity (C).

3.2. *Effect of incubation with sugars at 60 °C in the spectral properties of RNase*

It is now well recognized that reaction of reducing sugars with protein can cause marked alterations in protein conformation [35,36]. Incubation with reducing sugars at 60 °C resulted in modification in soluble RNase as evident from the observed alteration in intrinsic fluorescence, new fluorescence and absorbance at 280 nm (Fig. 2). As shown in the figure RNase incubated with the reducing sugars revealed a marked increase in absorbance at 280 nm and new fluorescence while the quenching was observed in the intrinsic tyrosine fluorescence. Ribose was most reactive in causing alterations followed by fructose and glucose. Several investigators have shown that reaction of sugars and dialdehydes with protein can also lead to the formation of groups that show strong emission between 400 and 500 nm when excited at a wavelength of 370 nm [37,38]. Incubation of soluble RNase with reducing sugars also resulted in marked decrease in intrinsic fluorescence and increase in absorbance at 280 nm and the magnitude of the alterations was maximum with ribose and least with glucose. Quenching in the tyrosine fluorescence is attributed to the exposure of the phenolic groups to a less hydrophobic environment [39]. Similarly, increase in absorbance at 280 nm is attributed to protein unfolding and exposure of the chromophoric groups [40]. A comparison of the alteration in RNase was summarized in Fig. 3. As evident, among the parameters studied, alterations in enzyme activity were most prominent.

Fig. 4 depicts the far UV-CD spectra of control and ribose treated RNase incubated for 1, 2 and 4 days at 60 °C between 205 and 250 nm. As can be seen from the figure no remarkable change in the spectrum of RNase incubated in the absence of sugar was observed while inclusion of ribose in the reaction mixture resulted in a time dependent alterations suggestive of marked loss of secondary structure. Seilder [41] suggested that changes in secondary structure could lead to localized compaction of the protein.

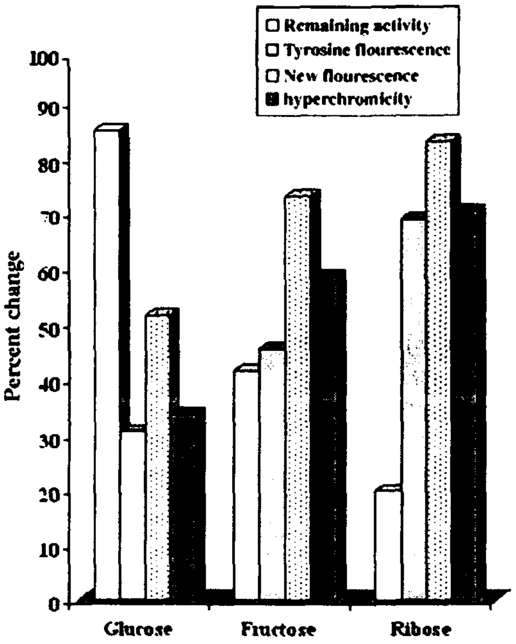

Fig. 3. Overall comparison of the alterations in RNase incubated with various sugars for two days at 60 °C by four particular parameters considering control as 100% activity. The values on vertical axis correspond to values given in Figs. 1 and 2.

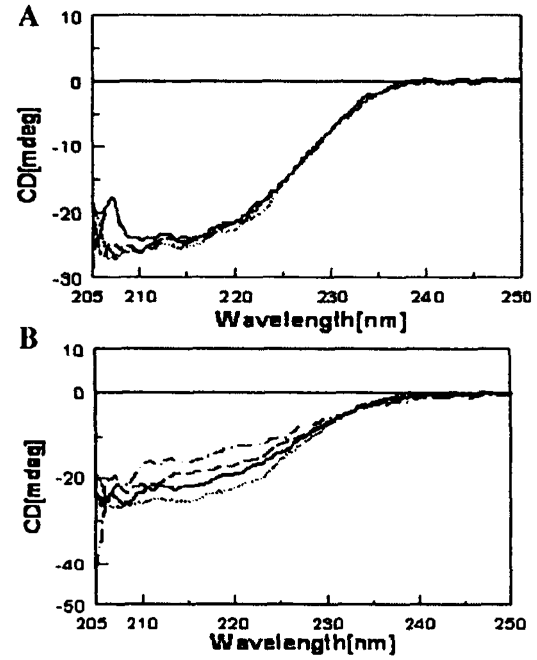

Fig. 4. Circular dichroic spectra of RNase incubated in the absence (A) or presence (B) of ribose for 1, 2 and 4 days at 60 °C in far UV region. Solutions were diluted to a concentration of 250 µg/ml for the spectral analysis.

3.3. SDS-PAGE of RNase incubated with sugars

RNase exposed to the various reducing sugars at 60 °C was subjected to SDS-PAGE in presence of the thiol reductant β-mercaptoethanol. Fig. 4 showed that incubation of RNase with the reducing sugars with the enzyme resulted in marked broadening of the bands and/or decrease in electrophoretic mobility suggesting crosslinking. Elbe et al. [42] have shown earlier that comparable alterations occur in RNase incubated with glucose at moderate temperature but up to 14 days. The alteration in electrophoretic behavior was most prominent in RNase incubated with ribose followed by those with fructose and glucose.

3.4. Effect of some glycation inhibitors

The protective effect of some metal ion chelators, antioxidants, analgesics and other drugs that have been shown to interfere with glycation, on the activity of some soluble and immobilized RNase incubated with ribose at 60 °C was also investigated. DETAPAC was most effective in protecting the enzyme activity, followed by aminoguanidine, EDTA and pencillamine, aspirin and paracetamol. Ascorbic acid and ibuprofen were less inhibitory. As evident from Fig. 5, a good correlation was observed between the extents of protection provided by several compounds when either soluble or immobilized RNase was used. An excellent agreement in the protection offered by DETAPAC, EDTA, aminoguanidine, paracetamol and ascorbic acid was observed when either soluble or immobilized RNase was used. In case of aspirin and

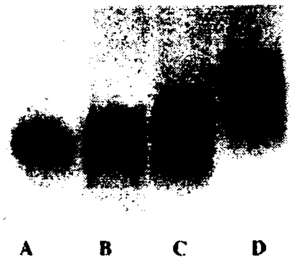

Fig. 5. SDS-PAGE analysis of the effect of incubation with various sugars for two days at 60 °C on RNase. RNase was incubated in the absence of sugar (Lane A) or presence of 0.5 M glucose (Lane B), 0.5 M fructose (Lane C) or 0.5 M ribose (Lane D).

ibuprofen however the protection appeared more marked when the immobilized enzyme was used. The difference may be attributed to interference by the drugs in the determination of soluble RNase activity. Since the activity of immobilized RNase was measured after centrifugation and washing of the immobilized enzyme, the interference by the drugs in the assay is apparently eliminated. Aspirin is known to act by acetylation of free amino groups of proteins [43] and hence may contribute additionally towards the inhibition of RNase. While similar action can be envisaged in case of the immobilized enzyme, it is not unlikely that some of the crucial side chain amino groups are less accessible in case of the immobilized RNase. Paracetamol is however, not known to lead to such modification.

Ability of some plant-derived extracts to protect RNase against inactivation induced by ribose was also measured at 60 °C. As shown in Fig. 6, green tea extract, ginger, garlic, grape, tomato and bitter gourd were markedly protective against the inactivation of both soluble and immobilized RNase. The observed protection by ginger and bitter gourd extract was comparable when either soluble or immobilized RNase was employed. There was however observed variation in the magnitude of protection in case of the intensely colored extracts derived from tomato, grapes and turmeric and the extent of protection appeared more marked in case of the soluble enzyme. Thus, while incubation of soluble RNase with green tea extract resulted in the retention of 34% of control activity that observed with immobilized enzyme was 70%. Similarly, the observed retention in of the RNase activity incubated with the extracts of grape, tomato and turmeric extracts were respectively 33%, 31% and 42% while using soluble enzyme and 59%, 60%, and 64% when immobilized enzyme preparation was used (Fig. 7).

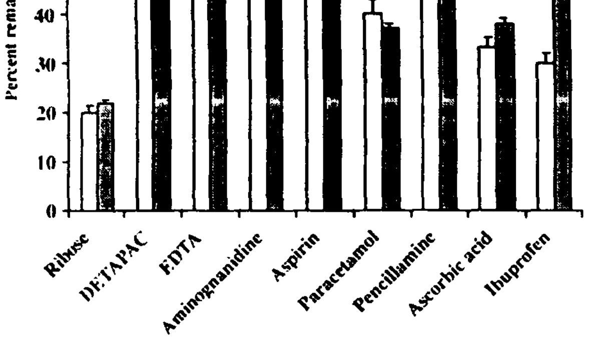

Fig. 6. Effect of various compounds at a concentration of 25 mM on the activity of RNase incubated with 0.5 M ribose at 60 °C for 2 days. Activity of RNase incubated in absence of ribose was taken as 100% for calculation of percent activity in treated samples.

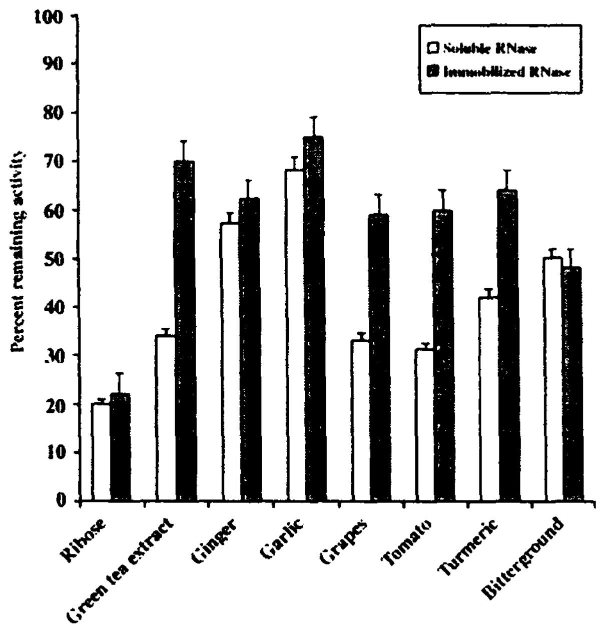

Fig. 7. Effect of some plant extracts on activity of RNase treated with 0.5 M ribose at 60 °C for 2 days Activity of RNase incubated in absence of ribose was taken as 100% for calculation of percent activity in various samples.

4. Discussion

Pancreatic RNase is an extensively studied thermostable enzyme [28]. RNase undergoes remarkable alteration in structure and catalytic activity when incubated with glucose and other reducing sugars [44]. Lysine is a constituent of the active site of RNase [45], which is apparently highly reactive towards sugars resulting in a rapid inactivation of the enzyme. Since ribose is closely related to the enzymatic substrate of RNase and Lys-41 of the active site most easily form Amadori products [44]. Fig. 1 shows that the catalytic activity of the enzyme is barely affected on incubation in the absence of sugars at 60 °C for two days, the activity loss was remarkable on incubation with the sugars under the same conditions, with ribose being most reactive. The high reactivity of ribose in glycation reaction has been ascribed to the stability of the open chain for the sugar [46]. The loss in activity resulting from the incubation with various sugars was comparable when soluble or immobilized RNase was used. This suggested that immobilization does not markedly alter the reactivity of enzyme towards sugars. It is interesting that RNase incubated in absence of sugars for two days at 60 °C exhibited only minor alterations in hyperchromicity and quenching of intrinsic fluorescence, CD spectral behavior suggesting the retention of three dimensional structure of enzyme (Fig. 2). While it is true that ribose is not the principle sugar responsible for in vivo glycation reactions, structural alterations induced by ribose in proteins are similar to those caused by glucose including the recognition of AGEs by anti-AGE antibodies [47]. In addition, ribose in form of ADP-ribose may actually cause glycation of proteins in vivo [48]. We therefore felt that use of RNase as model protein and ribose as model sugar provide remarkable advantage.

The comparable sensitivity of immobilized RNase with that of its soluble counterpart towards glycation induced enzyme inactivation points out to the possibility of using the later for screening of the antiglycation principles from natural source. Immobilized RNase in addition to being more stable [34] offers the possibility of its rapid removal from the reaction mixture and hence elimination of component of the extract that may interfere with the enzyme assay. As shown in Fig. 5, an excellent agreement between protections offered by DETAPAC, EDTA, aminoguanidine, paracetamol and ascorbic acid was observed when either soluble or immobilized RNase was used. Aspirin and ibuprofen however appeared more protective when immobilized RNase was used.

DETAPAC and EDTA are believed to act by preventing metal catalyzed oxidation of sugars [49]. Aminoguanidine on the other hand blocks sugar carbonyl and ketamine or their derivatives [50]. Aspirin protects the amino groups by acetylation [43,51] while analgesics like ibuprofen and paracetamol act by reducing the oxidative stress [52]. The protective property of ascorbic acid is attributed to its ability to scavenge free radicals that lay an important role in glycation reactions [53]. Thus, the protection from glycation induced inactivation by compounds with remarkable difference in their mechanism of action could be detected by the assay system.

Similarly, among the plant extracts that exhibited significant protection effects against ribose induced inactivation of RNase, excellent agreement was observed in case of ginger, garlic and bitter gourd extracts when soluble or immobilized enzyme was used. The extracts like green tea, grapes and tomato extracts however appeared more protective when immobilized RNase was used as discussed earlier. Since the concentration of ribose used in the assay is remarkably high, interference by the molecules present in the extract, that modify amino groups of enzyme is not very high unless they are highly reactive.

There are reports of several natural products in plants with AGE inhibitory effect. These include resveratrol, a natural estrogen in grapes [54], curcumin in turmeric [55], S-allylcysteine in garlic [56] and rutin in tomato fraction [57].Green tea contain tannins (flavonoids) having antiglycation properties [58]. This clearly makes a strong case for the screening of plants for potential inhibitors of glycation.

Immobilized RNase may be more useful for screening of inhibitors that act on post Amadori reactions [26]. For the study of such inhibitors, rapid removal of the glycating sugar after initial reaction with protein is required. This is normally accomplished by dilution or dialysis. Immobilized RNase on the other hand can be readily separated from the reaction mixture instantaneously by centrifugation and freed of the sugar by washing. Also the immobilized preparation may offer the advantage of additional storage stability [34].

A number of enzymes have been shown to undergo glycation induced loss in catalytic activity both in vivo and in vitro Cabellero et al. [59] and Bonsova et al. [60] suggested measurement of loss of enzyme activity of δ-aminolevulinic acid dehydratase and aspartate aminotransferase as a measure of glycation. In our opinion RNase offers additional advantage being remarkably stable and facilitating reaction at high temperature and thus cutting down the assay duration. Since the assay involves measurement of protection of RNase activity, we believe it will measure only those substances that restrict the glycation reaction. Natural Inhibitor of RNase present in the extract may contribute additionally towards the inhibition but can be taken care of by using an appropriate control. To conclude we believe that the assay described can be useful in the rapid detection of inhibitors of glycation, including those that inhibit post Amadori reactions from natural sources.

5. Description of the method and its applications

Here we described a simple strategy for the screening of various glycation inhibitors. Immobilized and soluble RNase was incubated with sugar along with inhibitors at desired conditions. After completion of incubation the immobilized preparation was washed thoroughly and activity measurement of RNase was performed. The problem of interfering substances present in the extract could be better addressed using immobilized preparation. In conclusion, the in vitro model described can be used to study and measure the formation of AGE in RNase and might help in determining the effect of various AGE inhibitors. Furthermore in this technique we removed all the possible substances that might interfere in the assay of RNase so that it helps in the accurate measurement without involving expensive reagents and sophiscated equipments.

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