STUDIES ON PROTEINS OF SUNFLOWER SEED (*Helianthus annuus* L.) - STRUCTURAL STABILITY AND INTERACTION WITH POLYPHENOLS

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MAY 1997

(Dedicated to My Parents

DECLARATION

I hereby declare that the thesis entitled "STUDIES ON PROTEINS OF SUNFLOWER SEED *{Helianthus annum* L.) - STRUCTURAL STABILITY AND INTERACTION WITH POLYPHENOLS" which is submitted herewith for the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY of the UNIVERSITY OF MYSORE, MYSORE is the result of the work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance of Dr. V. Prakash during the period 1992-1997.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

P. Surya Prakash

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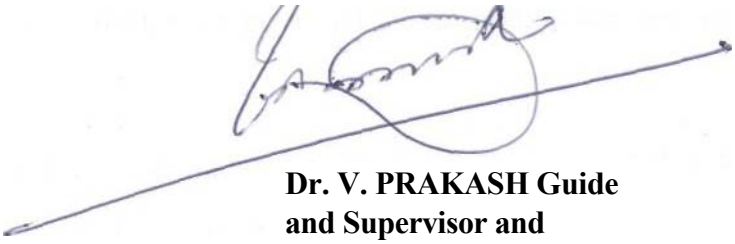
Place: Mysore, India

CERTIFICATE

I hereby certify that this Ph. D. thesis entitled "STUDIES ON PROTEINS OF SUNFLOWER SEED *(Helianthus annuus* L.) - STRUCTURAL STABILITY AND INTERACTION WITH POLYPHENOLS" submitted by Mr. P. SURYAPRAKASH for the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY of the UNIVERSITY OF MYSORE, MYSORE, is the result of research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under my guidance and supervision during the period 1992-1997. This has not been submitted either partially or fully to any other degree or fellowship earlier.

Date: 30 May, 1997
Place: Mysore, India



Dr. V. PRAKASH Guide
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P. Sunja Prakash

Date: 30 May, 1997
Place: Mysore, India.

CONTENTS

LIST OF ABBREVIATIONS

ΔA	change in absorbance
A	absorbance
ΔA_{max}	maximum change in absorbance
C_L°	total ligand concentration
C_{Lfree}	free ligand concentration
CGA	chlorogenic acid
CA	caffeic acid
dm	decimeter
$(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$	preferential interaction parameter on g/g basis
ξ_3	preferential interaction parameter on g/g basis
$(\delta g1/\delta g2)_{T,\mu 1,\mu 3}$	referential hydration parameter on g/g basis
$(\delta m_3/\delta m_2)_{T,\mu 1,\mu 3}$	preferential interaction parameter on mole/mole basi
ϵ	molar extinction coefficient
$\Delta\epsilon$	change in molar extinction coefficient
M	molarity
μM	micromolar
GuHCl	guanidine hydrochloride
GuHSCN	guanidine thiocyanate
μm	micrometer
nm	nanometer
$[\theta]$	observed ellipticity
$[\theta]_{mrw}$	mean residue molar ellipticity
mol	mole
kJ	kilo Joule
ΔG	change in Gibb's free energy

ΔC_p°	change in heat capacity
ΔH	change in enthalpy
ΔS	change in entropy
S	sedimentation coefficient
β	fractional binding
f_1	fraction of the total binding at class 1 binding sites
f_2	fraction of the total binding at class 2 binding sites
K_{a1}	association constant at class 1 binding sites
K_{a2}	association constant at class 2 binding sites
K_{ai}	association constant at class i^{th} binding sites
n	total number of binding sites
n_H	Hill coefficient ; index of cooperativity in binding
γ	number of moles of ligand bound per mole of protein
min	minute
K_a	reciprocal of dissociation constant
Σ	summation
eg.	example
η_{red}	reduced viscosity
$[\eta]$	intrinsic viscosity
t	flow time of the sample in viscosity measurements
t_o	flow time of buffer in viscosity measurements
g_3	bulk solvent composition
MW	molecular weight
M_3	molecular weight of cosolvent
M_2	molecular weight of protein
m_3	molality of cosolvent
ρ	density of sample

ρ_o	density of buffer
C	concentration in mg/mL
$\bar{V}$	partial specific volume
T	temperature
μ_1	chemical potential of water
μ_3	chemical potential of cosolvent
m_2	molarity of protein
m_3	molarity of cosolvent
P	pressure
ϕ_2^o	apparent partial specific volume of protein extrapolated to zero concentration in isomolal condition
$\phi_2^{\mu o}$	apparent partial specific volume of protein extrapolated to zero concentration in isopotential condition
TNBS	2, 4, 6, - trinitrobenzoic acid
NBS	N-bromosuccinimide
TEMED	N, N, N', N' - tetramethyl ethylene diamine
QA	quinic acid
TNP-lysine	trinitrophenyl-lysine
V_e	elution volume
V_o	void volume
$S_{20,w}$	sedimentation coefficient corrected for viscosity and temperature
pH	negative logarithm of hydrogen ion concentration
°C	degree centigrade
pK_a	negative logarithm of acid dissociation constant
tris	tris-hydroxy methyl amino methane
NaOH	sodium hydroxide

PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
ΔF	change in fluorescence intensity
ΔF_{max}	maximum change in fluorescence intensity
F_{corr}	fluorescence intensity corrected for inner filter effects
F_{obs}	observed fluorescence intensity
PB	phosphate buffer
ANS	1-aniline 8-naphthalene sulphonic acid
CD	circular dichroism
EDTA	ethylene diamine tetra acetate
Fig	figure
g	gram
mL	millilitre
rpm	revolutions per minute
mg	milligram
µL	microlitre
T_m	thermal transition temperature
UV	ultraviolet
w/v	weight by volume
$E^{1\%}$	extinction coefficient of 1% solution
%	percentage
nm	nanometre
NaCl	sodium chloride

LIST OF TABLES

LIST OF FIGURES

12 A. The plot of (β/l-β) versus CA for CA-11S protein fraction interaction obtained from uv-absorption spectral titration.

B. Scatchard plot of CA-11S protein fraction interaction obtained from uv-absorption spectral titration.

13 A. Binding isotherm of CA-11S protein fraction interaction obtained from equilibrium dialysis.

B. Scatchard plot of the CA-11S protein fraction interaction obtained from equilibrium dialysis.

14. Far-UV circular dichroic spectra of 1IS protein fraction in presence of CA.

15 A. Scatchard plot of QA-11S protein fraction interaction obtained from fluorescence titration data.

B. The plot of (1/1-β) versus (CL°/β) of QA-11S protein fraction interaction obtained from fluorescence titration data.

16. SDS-PAGE pattern of 2S protein fraction.

17 A. Native-PAGE pattern of 2S protein fraction.

B. Fluorescence emission spectrum of 2S protein fraction.

C. UV-absorption spectrum of 2S protein fraction.

18. Scatchard plot of CGA-2S protein fraction interaction at pH 3.0.

19 A. Scatchard plot of CGA-2S protein fraction interaction at pH 5.8.

B. Scatchard plot of CGA-2S protein fraction interaction at pH 6.6.

20. Scatchard plot of CA-2S protein fraction interaction at pH 5.8

21 A. Velocity sedimentation profile of 1 IS protein fraction in presence of GuHCl.

B. Percent composition of 11S, 7S and 2S protein fractions as a function of GuHCl concentration.

22. Reduced viscosity profile of 11S protein fraction as a function of GuHCl concentration.

23 A. UV-difference absorption spectrum of 11S protein fraction at different concentrations of GuHCl.

B. Change in Δε value of US protein fraction as a function of GuHCl concentration.

INTRODUCTION

INTRODUCTION

Proteins are biological macromolecules and have either a role in the structural organization or in a definite function of the cells. Proteins when ingested orally are digested by the human physiological system and the resulting amino acids will be absorbed, distributed and utilized by different kinds of cells for the synthesis of its required proteins. Only some of the 20 amino acids are synthesized by the cell's enzymatic machinery. Thus the essential amino acids such as arginine, lysine, leucine, valine, isoleucine, methionine, phenylalanine and tryptophan have to be supplemented to the human beings. Balanced diet should contain proteins having high amounts of these essential amino acids and the nitrogen source from amide containing amino acids.

Protein intake in underdeveloped countries is low due to the high cost of protein rich sources such as meat. It is for this reason the cheaper vegetable sources of protein are being looked into for an efficient usage and large scale catering of the population (Simpson, 1982). The rich sources of vegetable proteins are oilseeds, legumes and cereals. Though legumes, pulses contain many a times antinutritional factors such as proteinase inhibitors and cereals are low in lysine content, these are widely used in food supplements after processing. Efforts are made to understand the physico-chemical and functional properties of oilseed proteins as reviewed by Prakash and Rao (1986).

Oilseeds are predominantly grown for their edible oil content, however, are rich in quality protein. The major oilseeds grown worldwide are soybean, groundnut, sesame seed, sunflower seed, rape seed, mustard seed, cotton seed and safflower seed.

Sunflower *(Helianthus annuus* L.)

Its young leaves and developing heads display heliotropism. It belongs to the family compositae and subfamily helianthaceae. Its seed is called an achene, a small dry indehiscent one-seeded fruit. Achene contains majorly the outer pericarp or hull and inner edible kernel.

Based on the composition of the kernels, achenes are of two types. They are (1) high oil yielding smaller sized with black and thin hulls and (2) low oil yielding larger achenes, with black striped hulls (Clandinin, 1958).

Kernel contains the seed coat called testa, one to two layers of aleurone cells coalesced with the seed coat called as an endosperm and the inner embryo. The embryo has two cotyledons attached to a protruding growing point. Pericarp consists mainly the fibre. Oil and protein are present in the kernel. Several breeding programmes are made to get seeds of low percentage hull, high oil content and high protein content (Clandinin, 1958).

Chemical composition of the kernel

The composition of the seed depends upon variety. It contains 13-23% crude protein, 21-49% crude fat, 15-22% crude fibre, 3-4% of ash and 13% starch (Earle *etal,* 1968; Smith, 1971; Sosulski, 1984). Kernel contains 16-26% crude protein, 41-58% crude fat, 2-10% crude fibre and 3-6% ash (Smith and Johnson, 1948; Smith, 1971; Wan *et al,* 1979; Sosulski, 1984). The defatted meal usually contains 37-56% crude protein, 1-3% crude fat, 4-14% crude fibre and 6-9% ash (Smith, 1971; Robertson, 1975; Sosulski, 1984; Saeed and Cheryan, 1988).

The oil consists of oleic and linoleic acids comprising 85% and the remaining 15% is made of palmitic and stearic acids. Laurie, arachidonic, behemic, lignoceric and lindeinic acids are scarcely present (Robertson, 1975). Different kinds of sterols present are stigmasterol, beta- sitosterol, campesterol, 7-stigmasterol, 7-avenasterol and carotenoids such as xanthophyll and carotene.

Various sugars present in the seed are arabinose, fructose, galactose, glucose, rhamnose, xylose, galacturonate, hydrolyzed cellulose, raffinose, trehalose and sucrose.

The oil seed meal is richer in B-complex vitamins such as nicotinic acid, pantothenic acid, thiamine, riboflavin and niacin (Rector *et al,* 1946; Clandinin, 1958). The meal also

contains calcium, phosphorus and vitamin A (Brummett and Burns, 1972).

The nitrogen content is predominantly present as proteins (87-90%) and the remaining 10-13% is in the form of peptides and amino acids. There are mainly two groups of proteins present in the oilseeds. They are salt soluble globulins comprising about 50% of the total protein and water soluble albumins making up 25% of total protein (Schwenke and Raab, 1973; Rahma and Rao, 1979). Prolamines are present in scarce (Prakash and Rao, 1986). The solvent extracted seed flour is highly digestible (94%) and has a biological value or protein efficiency ratio of 64%, very much comparable to soybean oil meal (Clandinin, 1958). The net protein utilization, net protein ratio are 85 and 82 when compared to 100 for casein (Clandinin, 1958). The major constraint in the utilization of sunflower meal is the presence of phenolic compounds which impart colour to the proteins and decreases the bioavailability of certain amino acids. The major phenolic compounds present in the meal are chlorogenic acid (CGA) and caffeic acid (CA) which are present to an extent of 2-5% and 0.13%, respectively (Robertson, 1975; Sosulski, 1984). Several other phenolic acids present in the flour are trans-cinnamic acid, isomer of chlorogenic acid, p-coumaric acid, isoferulic acid, sinapic acid and hydroxy cinnamic acid-sugar ester (Sabir *et al,* 197'4a; Leung *et al,* 1981).

The total proteins of sunflower seed

The total proteins of sunflower seed are mainly characterized by the designation of 11S, 7S and 2S fractions, the percentage of which are 55%, 7% and 38%, respectively, as seen by the analytical ultracentrifugation pattern. These protein fractions occur with higher polymer with average sedimentation coefficient of 16S - 18S. The total protein is extractable both in water and salt solutions (Prakash and Rao, 1986). The interaction of total proteins has been reported (Sabir *et al,* 1974b).

However, to have a meaningful idea and meaningful interpretation of interaction of the polyphenols is desirable to study the interaction of ligand with pure protein fractions. In this thesis an attempt is made to consolidate biophysical and thermodynamic parameters of the interaction with ligands such as polyphenols (CGA, CA and QA), denaturants (GuHCl and GuHSCN), physical conditions (such as temperature) and interactions with cosolvents (glycerol, sorbitol, sucrose, and trehalose).

The high molecular weight protein (Helianthinin, IIS protein fraction)

The major protein fraction of Sunflower seed total protein is termed as 11S protein fraction or Helianthinin (Prakash and Rao, 1986). It is a multisubunit protein having a sedimentation coefficient of 1IS. It has been isolated by several workers (Joubert, 1955; Schwenke *et al,* 1974; Schwenke *et al,* 1975a, b; Baudet and Mosse, 1977; Schwenke *et al,* 1978; Schwenke *et al,* 1979; Rahma and Rao, 1981a; Dalgalarrondo *et al,* 1984; Durante *et al,* 1989; Sastry and Rao, 1990; Theerthaprasad, 1990a), however, most of the fractions contain the polyphenols associated with it. The physico-chemical characteristics of the protein are given in Table 1.

The low molecular weight fraction of sunflower seed protein (2S protein fraction)

The 2S protein fraction isolated and characterized in this laboratory (Venktesh and Prakash 1993) has a molecular weight of 15,000 and is fairly rich in α-helical content and has been shown to be rich in sulfur containing aminoacids and lysine. The protein has an hydrophobic index of 663 and intrinsic viscosity of 7.28 (Prakash, 1994). These properties of 2S protein fraction indicates two major difference as compared to 11S protein fraction namely, its monomeric nature and it is not the 2S protein fraction of the dissociated fraction of 11S protein fraction. The 2S protein fraction does not have an acidic or basic subunit. Therefore, it was interest to compare the properties of the structural stability of both US protein fraction and 2S protein fraction with different ligands to arrive at a conclusion on mechanism and thermodynamics of interaction.

Table 1 : Physico-chemical properties of 11S protein fraction

Property	Values
Molecular weight	2.4×10^5 - 3.5×10^5
Intrinsic viscosity	3.7 mL/g
Sedimentation coefficient	11.2 - 12.8 S
Extinction coefficient, $E^{1\%}_{1cm}$	8.2 - 8.8
Fluorescence emission maximum	325 - 330 nm
Carbohydrate content	0.31% (w/v)
Chlorogenic acid content	0.31% (w/v)
Number of subunits	6
Secondary structure	
a) Alpha helix	10%
b) Beta pleated	30%
c) Aperiodic	60%
Shape	Oblate ellipsoid
Size	10.4-11.8 x 10.4-11.8 x 7.08-8.8 nm
Axial ratio	0.85
Hydration	0.25 g solvent / g protein

(Compiled from Plietz *et al,* 1978; Schwenke *et al,* 1979; Prakash and Rao, 1986)

Quaternary structure of IIS protein fraction

Available literature clearly suggests that 11S protein fraction is a multisubunit protein. SDS-gel electrophoresis of the protein revealed two major pairs of disulphide linked polypeptide subunits (David and Franklin, 1982). Molecular weight of one of the pair is 59K with two subunits of molecular weights 37K and 22K. The other pair has a molecular weight of 51 K comprising of two subunits of molecular weights 29K and 22K. There are three pairs of acidic and basic subunits. Three pairs have equal concentration in SDS-gels without the reαucing agent. All these have molecular weights in the range 40-50K (Kortt and Caldwell, 1990). Durante *et al,* (1989) has reported molecular weights of 39K, 32K, 26K and 23K for the polypeptides in SDS-PAGE and the 50K bands are present in minor quantities suggesting that these are the precursor molecules and there exists an equilibrium between disulphide linked precursor subunit and the reduced acidic and basic subunits. Subunits have pi in the range 5.0-6.2. Both size and charge heterogeneity is found in subunits (Dalgalarrondo *et al,* 1985).

The six subunits are arranged as a trigonal antiprism as suggested by small angle X-ray scattering and electron microscopic studies. The shape is an oblate ellipsoid (Plietz *et al,* 1978; Plietz *et al,* 1983). Prakash and Rao (1984, 1986, 1988) have proposed a general model for US proteins of major oilseeds based on the available data which is shown in Fig. 1. According to this, the protein constists of six acidic subunits with homology between them and six basic subunits with homology amongst them. These acidic and basic subunits are linked by inter chain disulphide bonds. These six acidic-basic pairs associate noncovalently to form a hexagonal structure with two trimers present one above the other.

Association - dissociation phenomena of 11S protein fraction

The association-dissociation phenomena of this multisubunit protein is studied in fair detail. Effect of acidic and alkaline pH on the association and dissociation phenomena of the protein is studied by different biophysical techniques (Sripad, 1985). Below pH 4.2

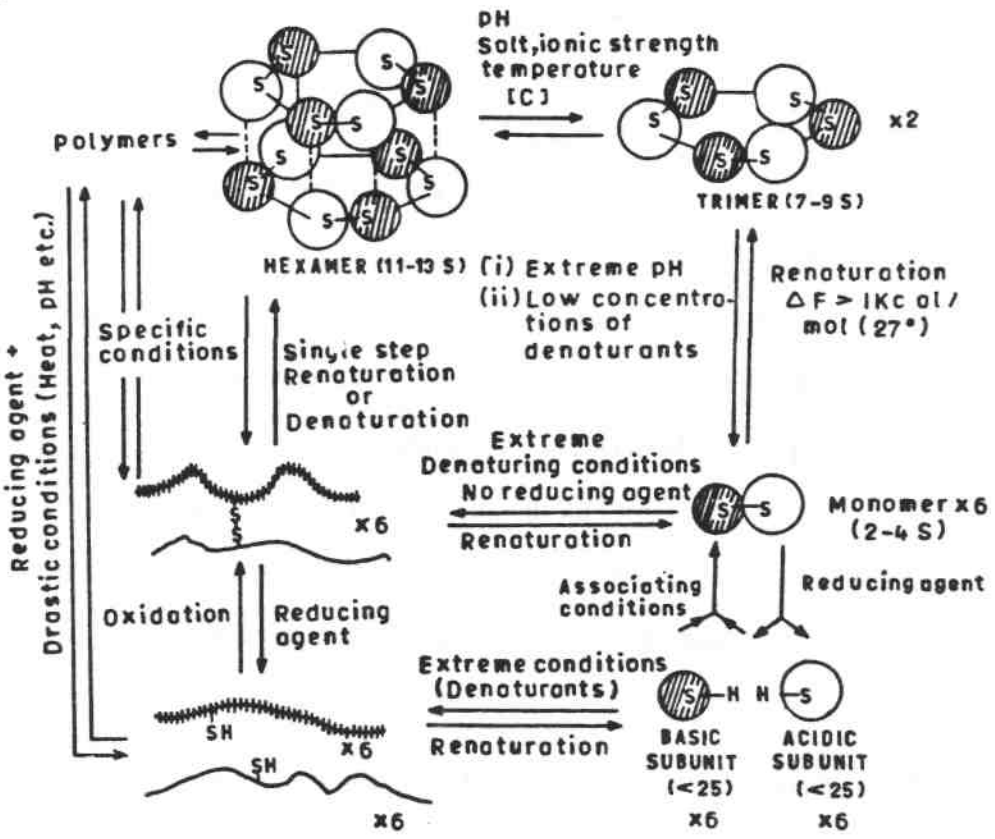

Fig. 1. Schematic model for the high molecular weight oilseed protein quaternary structure. Dashed lines indicate weak non-covalent interactions, solid lines indicate strong non-covalent interactions, S-S, disulphide bridges. (Prakash and Rao, 1986. Reproduced with permission of the author)

dissociation occurs. At pH 3.0 it is in 2S form. Below pH 2.5 reaggregation and refolding of the protein is noted. The protein is in dissociation equilibrium till pH of 10.5 without denaturation. Above this pH the protein is converted into 2S form and at pH 11.5 it is dissociated and denatured (Sripad, 1985).

In presence of 1M NaCl at pH 4.0 it is in 1IS form and at pH 7.6 it is in 1IS form (Sastry and Rao, 1990). Solutions of low ionic strength have dissociated the 1IS component to a 7S component (Schwenke *et al,* 1974). The protein is dissociated into 2S form upon acetylation or succinylation. The percent of 11S gradually decreased with increasing percentage of succinylation, with above 55% succinylation the protein is in 7S form, and above 70% of succinylation all the protein is in 2S form (Schwenke *et al,* 1985; Schwenke *et al,* 1986; Sastry and Rao, 1991). Above 70% of succinylation of the protein denaturation takes place. All these data has suggested the importance of ionic interactions in the maintenance of the quaternary structure.

Dissociation pattern of this protein in presence of urea, guanidine hydrochloride and sodium dodecyl sulphate is studied empirically (Rahma and Rao, 1981b). The 1IS component dissociates into 2S through 7S intermediate in urea and GuHCl while no intermediate is detected in SDS induced dissociation. However no detail information is available in terms of preferential interaction parameters in the presence of these denaturants. Circular dichroic data of the native protein yielded 10% alpha-helix, 30% beta structure and 60% aperiodic structure (Schwenke *et al,* 1975b; Prakash and Rao, 1986; Paredes-Lopez *et al,* 1994).

The cDNA encoding the acidic-basic subunit of 11S protein fraction is cloned and sequenced. The primary structure of the unit is deduced from cDNA SF2A1 (Wright, 1988). The genomic clone SGH3 is also obtained. The primary structure of the protein is obtained.

The acidic subunit of the genomic clone has 285 amino acids and basic subunit has 188 amino acids.

The low molecular weight protein fraction (conhelianthinin, 2S protein fraction)

The second major protein fraction of the total protein is water soluble albumin, having a sedimentation coefficient of 2S and is normally termed as 2S fraction or conhelianthinin (Prakash and Rao, 1986). Its isolation is done by several workers. Tannin and pectin are used to isolate albumin by their complexation ability (Schwenke *et al,* 1977). Schwenke and Raab have extracted albumins in water and fractionated on gel filtration column (Schwenke and Raab, 1973; Schwenke *et al,* 1973; Theerthaprasad, 1987; Theerthaprasad, 1990b). There are seven fractions obtained upon NaCl gradient on carboxy methyl sephadex column. All have distinct molecular weights. Selective pH precipitation is used to isolate the protein (Schwenke and Simon, 1974). The molecular weight of the albumins is found to be in the range of 14-16 K. All these are basic proteins and have distinct amino acid compositions. All the albumin components have high content of amido amino acids such as glutamine and asparagine. The pi of these proteins is >8.8 (Kortt and Caldwell, 1990). In the acidic pH of 4.3 they are separated on PAGE. The SDS- PAGE analysis in the absence and presence of beta- mercaptoethanol yielded a single subunit for each albumin component.

The protein fraction is isolated from acidic butanol treated flour and characterized (Venktesh and Prakash, 1993). It has an absorption maximum of 275 nm. It has a sedimentation coefficient of 1.82S which is independent of protein concentration. There are four separable fractions obtained through gel filtration on Sephadex G-75 gel column. The molecular weights of the three major bands obtained on SDS-PAGE are 32.5K, 24.5K and 19.5K. The fluorescence emission maximum of the protein is 333 nm. The secondary structures of the fraction are 27% alpha helix, 38% beta structure and 35% aperiodic structure. The secondary structures of individual proteins and tertiary structures of them is not known.

Polyphenols in sunflower seed

Polyphenols, especially chlorogenic acid (CGA) are implicated in the enzymatic browning of various fresh fruits and vegetables (Hulme, 1953; Weurman and Swain, 1953; Williams, 1957; Sakamura and Obata, 1963; Goldstein and Swain, 1963). Non enzymatic oxidation of CGA and CA also induce browning reactions (Cilliers and Singleton, 1989). Polyphenols decrease the action of various enzymes such as trypsin, alpha chymotrypsin, papain (Feeney, 1968; Sastry, 1985), lipoxygenase (Koshihara *et al,* 1984) and polygalacturonase (Barbeau and Kinsella, 1985).

Polyphenols are attributed to the plant defense mechanism by inhibiting the actions of several enzymes (White, 1957; Beart *et al,* 1985). CGA has been indicated in antiviral action against herpes virus-hominis type 1 and type 2 in vitro (Thiel *et al,* 1985). CGA and CA are shown as antioxidants (Iwahashi *et al,* 1990; Reddy and Lokesh, 1992; Cuvelier *et al,* 1992). Polyphenols form the basis of colour, flavour and astringency of beverages (Maga, 1978). Colouration of the sunflower protein isolates is due to the chlorogenic acid. Due to the wide variety of actions, polyphenol interaction with proteins is widely studied.

Protein-polyphenols interaction

Phenolic compound interaction by hydrogen bonding with N-substituted amides is reported (Loomis and Battaile, 1966). Interaction of hydrogen bond forming compounds with tannins is studied (Gustavson, 1966). Condensed tannins are found to bind to amide bonds below pH 7-8 and this binding is decreased above pH 8. Hydrolyzable tannins bind strongly at pH 3-4 and decreases above a pH of 5 indicating the importance of phenolic groups and unionized carboxylic group in the hydrogen bond formation (Loomis and Battaile, 1966; Loomis, 1974). Polyamide/Dowex-1-polyphenol interaction is due to the formation of hydrogen/ionic bonding (Gray, 1978). Aqueous organic solvents that are capable of hydrogen bonding such as alcohols, water and acetone are used to decrease the interaction of polyphenols with proteins (Hestrin *et al,* 1955; Pomenta and Burns, 1971; Sosulski *et al,*

1972; Sabir *et al,* 1973; Fan *et al,* 1976; Sodini and Canella, 1977; Taha and Nockrashy, 1981; Sripad *et al,* 1982; Sastry, 1985).

Polyphenols are envisaged to complex predominantly via hydrogen bonding with proteins (Haslam, 1974). They are also found to interact by hydrophobic interaction (Goldstein and Swain, 1965; Loomis, 1969, 1974; Oh *et al,* 1980). The binding is reported to increase with temperature and decrease with increase in ionic strength (Barbeau and Kinsella, 1983).

Quantitative measurements of binding of phenolic compounds such as resorcinol, catechol, pyrogallol and methy gallate with bovine serum albumin has been studied. The results of this study indicated hydrophobic interaction at acidic pH values. Gallic acid metabolites at pH 2.2 have exhibited an enthalpy- entropy compensation effect in binding with BSA. The results have also shown that size of the polyphenol is important, with above certain optimum size the binding decreases or unchanged (Mc Manus *et al,* 1985). Reduced conformational flexibility has reflected in reduced capacity to bind with BSA (Mc Manus *et al,* 1985). Tannic acid-gelatin complexation is found to be more at isoelectric pH of the protein (Van Buren and Robinson, 1969; Reed *et al,* 1975).

Polyphenol-BSA and polyphenol-lysozyme interaction led to precipitation of the complexes which is dependent on protein concentration at pH 4.0. At low protein concentration of 1×10^{-6} M, the number of binding sites are 120, but at a high protein concentration of 1×10^{-5} M, the number of binding sites are 60 (Van Buren and Robinson, 1969; Reed *et al,* 1975). However, the number of binding sites are 12-14 for polyphenols of gallic acid type with BSA (Beart *et al,* 1985). It is also proposed that polyphenol association at low protein concentration leads to the formation of monolayer of polyphenol around the surface of the protein making the surface more hydrophobic, and at high protein concentrations the multidentate polyphenol is found to crosslink the protein molecules leading to precipitate (Mc Manus *et al,* 1985). Both hydrogen and hydrophobic

bonding are envisaged to be present in the interaction of polyphenols with proteins (Mc Manus *et al,* 1985). Covalent interaction of the oxidized polyphenols, either by enzymatic or non enzymatic means, with the amino and thiol groups of proteins is reported (Mason, 1955a, b; Loomis and Battaile, 1966; Pierpoint, 1966, 1969 a,b; Vithayathil and Gupta, 1981). Haslam and Lilley have reviewed about the nature of astringency by polyphenols (Haslam and Lilley, 1988). Polyphenol interaction forms β-pleated like conformation (Haslam, 1974).

CGA - Sunflower protein interaction

Interaction of CGA with low molecular weight proteins of sunflower is studied mostly at an empirical level (Sabir *et al,* 1974b; Theerthaprasad, 1988). Interaction of CGA with a sunflower protein isolate at pH's of 3, 5, 7, 9 is studied earlier (Saeed and Cheryan, 1989). There are two to three classes of binding reported. Hydrogen and electrostatic interactions are proposed in the interaction. CGA interaction with a polyphenol free 1IS protein fraction is studied at different pH values (Sastry and Rao, 1990). However no detailed investigations are performed from a thermodynamic point of view to get an insight into the nature and energetics of interaction.

Effect of chemical denaturants on proteins

The native structure of globular proteins consist of well folded compact secondary plus tertiary structures. The broad structural change of the native protein from a globular compact form into a random coiled polypeptide chain is referred to as denaturation (Tanford, 1968). This change in structure of the protein occurs within a narrow range of denaturant concentrations and hence the process is cooperative. The denaturants such as GuHCl, GuHSCN and urea bind to the native protein by hydrogen bonding to the peptide groups and replaces the hydrogen bonding contacts that water make with the native protein. GuHCl and urea are shown to bind with a stoichiometry of 1 with each peptide group (Lee and Timasheff, 1974; Prakash *et* ***al,*** 1981). These denaturants also bind to the aromatic side chains with a stoichiometry of one (Lee and Timasheff, 1974; Prakash *et al,*

1981). Thus the denaturants bind to both polar and non polar groups of the protein. They are stated to increase the solubilities of both polar and non polar molecules in proportion to their accessible surface areas (Nozaki and Tanford, 1963). This non specific binding leads to the exposure of aromatic side chains to the bulk solvent which are otherwise buried in the compact hydrophobic interior of the protein structure. This binding is more to the denatured protein molecule than native protein in terms of number of binding sites due to their ability to bind with the exposed non polar amino acid side chains also (Pace, 1975; Tanford, 1968). This preferential binding is positive in magnitrie and can be obtained from the denaturant induced native to denatured equilibrium constant measurements. The denaturation transition is usally two-state and refers to the existence of only native and denatured conformations and at a concentration of denaturant the equilibrium number of molecules of these native and denatured forms will be altered leading to the change in equilibrium constant.

The existence of stable intermediate states of the protein apart from pure native state and pure denatured state is made for a number of proteins. This intermediate state usually does not have rigid tertiary structure contacts but the secondary structure is similar or even more pronounced than the native state. The intermediate formation leads to the exposure of some non polar groups and is compared with its binding with ANS through molten globule state (Ptitsyn, 1995).

For multisubunit proteins the dissociation of the oligomeric structure into its subunits is an extra step that will be induced by the denaturants. This dissociation either can occur prior to denaturation as a distinct step or both can be happening simultaneously. And this dissociation process is dependent on protein concentration if it is a distinct step. If both dissociation and denaturation occurs simultaneously the entire transition will be a single one and depends on protein concentration. Measurement of the conformational stability of the

reference native protein in a given particular conditions requires the knowledge of the detailed mechanism of dissociation, denaturation and the presence of stable thermodynamic intermediates. This conformational stability is calculated to be less and is in the range of 10-20 kcal/mole (Pace, 1975). This is due to the compensative contributions from both enthalpy and entropy to the change in free energy at a particular temperature (Tanford, 1968; Pace, 1975). In this study the interaction of guanidinium salts with the multimeric protein namely US protein fraction is studied in detail by using several biophysical techniques with a focus on preferential interaction parameters.

Interaction of cosolvents **on structural stability of proteins**

Structural integrity of protein is dependent on the presence of components other than protein in the solution. The ligand binding ability such as binding of a substrate with an enzyme molecule is determined by the native rigid three-dimensional tertiary structure of the protein. Structure stabilizing cosolvents such as glycerol are usually added to the protein in the past to maintain the structural integrity and thereby its activity (Jarabak *et al,* 1966; Timasheff and Arakawa, 1989). It is shown to be preferentially excluded from the surface of the protein molecule due to its balancing effect of the solvophobicity from the non polar surfaces of the molecule and favourable interaction with polar surfaces replacing water molecules (Gekko and Timasheff, 1981; Timasheff and Arakawa, 1989). This preferential exclusion is shown with a number of proteins (Gekko and Timasheff, 1981; Rajeshwara, 1994; Rajendran et al., 1995,). Sorbitol is also used frequently as a stabilizer of the protein structure. The preferential exclusion nature of sorbitol also is reported (Rajeshwara, 1994; Rajendran et al., 1995). Sucrose and trehalose are also used as structure stabilizers of the protein molecules and these are shown to be preferentially excluded from the protein surface due to their effect on the increase in surface tension of water (Lee and Timasheff, 1981; Kita *et al,* 1994; Lin and Timasheff, 1996). This preferential exclusion of the stabilizing cosolvent is compensated at the immediate surface of the protein molecule by preferential

binding of water molecules, the preferential hydration (Timasheff and Arakawa, 1989). Both the cosolvent molecules and water molecules compete for the same sites on the protein surface by hydrogen bonding. However, cosolvent molecules are also bound at some of the specific sites but overall balance is towards the preferential hydration of the protein by an indirect mechanism.

The extent of stabilization of the protein, therefore depends on the chemical surface nature of the protein molecule too and depends on the concentration of the cosolvent used. Therefore, a study is made of the effect of giycerol, sorbitol, sucrose and trehalose with 1IS protein and is presented in this report. The preferential binding parameters are calculated from the measurement of partial specific volumes of the protein in presence of these cosolvents by densitymetric technique. And this preferential exclusion of cosolvents as a result of their effect on increase in surface tension of water forms the basis of the increase in apparent T_m of the transitions of native protein to intermediate state (Lin and Timasheff, 1996). The thermodynamic stabilization is due to the denatured protein interacting even more unfavourably as the area of exclusion will be more as compared to the native state of the protein and thus the denaturation equilibrium is shifted towards stabilization (Timasheff and Arakawa, 1989; Arakawa *et al,* 1990 a,b; Lin and Timasheff, 1996).

Effect of temperature on proteins

Temperature is an important physical parameter that alters the hydrophobic and hydrogen bonding properties of the protein (Schellman, 1987). Hydrophobic interaction strength is predominantly changed with increase in temperature (Kauzmann, 1959; Schellman, 1987) and changes the structure of the protein since hydrophobic interaction contributes significantly to the maintenance of the three dimensional structure. The heat of the reaction, the ΔH and $\mathbf{T\Delta S}$ of the system are functions of temperature is suggested by the data obtained from differential scanning calorimetry. This dependence leads to an another parameter **which** is **constant** to **that** system and is the $\Delta C_P°$. As a result of this the ΔG of the

protein unfolding equilibrium is non-linearly dependent on temperature (Schellman, 1987). However, the detailed data of thermodynamic parameters in the absence of or in the presence of chemical additives is obtained only when the two-state native to denatured state transition is assumed. The literature on the presence of intermediates in the temperature induced unfolding pathway is scanty. And this intermediate state presence alters the conformational stability parameter and the other thermodynamic parameters.

Multisubunit proteins have association - dissociation step occuring simultaneously or sequentially with denaturation step. While the hydrophobic bonding is affected even at low temperatures the hydrogen bonding between the bulk solvent water molecules and that present in the maintenance of secondary structures such as alpha-helix and beta-pleated sheets of the protein are affected at much higher temperatures. This unfolding data at higher temperatures is usually difficult to obtain.

In view of the above literature available, the structural stability of sunflower protein fractions under different conditions such as GuHCl and GuHSCN, cosolvents along with the effect of polyphenols on binding and the thermal properties have been monitored by various biophysical techniques. Primarily the interaction of the polyphenols with the sunflower seed protein fractions have been monitored to understand the nature of interaction from an energetics point of view. The information generated in terms of the structural stability of these proteins would throw more light in general on ligand-protein interactions.

SCOPE AND OBJECTIVES

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Interaction of chlorogenic acid with polyphenol free 11S protein fraction would give an insight into the nature and extent of the binding. The understanding of the nature of functional groups of the protein and CGA involved in the interaction can be obtained from the alteration in the physical parameters such as pressure and temperature, and chemical parameters such as the pH of the solution and concentration of the ligand. Since CGA is a bidentate ligand having two parts CA and QA, interaction of the protein with CA and QA would further help in understanding of the mechanism of the interaction of the CGA. Interaction of these ligands with chemically modified proteins also give more detailed information on the nature of the interactive forces and amino acid residues involved in the interaction. Such a study will lead to the understanding of the nature of the interactive forces present in the binding and form a rationale for the general interaction mode of the polyphenol- protein systems and also help in understanding the functional role of the polyphenols in plant systems.

Interaction of GuHCl, GuHSCN, sucrose, trehalose, glycerol and sorbitol with protein may lead to the change in the stability of the protein molecule. The extent of the stability offered by the protein molecule is an intrinsic property of the protein and is dependent on the nature of the interaction of protein with these ligands. The mechanism of dissociation and denaturation of the protein whether sequential or simultaneous by chemical denaturants such as GuHCl, GuHSCN and the association-dissociation and denaturation of the protein in presence of the known stabilizing chemical additives, cosolvents can be useful in understanding the structural stability of the protein in more detail in various solution conditions and forms a rationale for the nature of the specific effects of cosolvents and denaturants. The parameters of either preferential interaction or preferential hydration of the protein in presence of these cosolvents can give us an insight into the extent of the interaction of these cosolvents. The data on the thermal stability of the protein in presence of all these

chemical denaturants and cosolvents would be useful in understanding the structural stability as a function of the temperature.

The objectives of the present study are

1) To isolate 11S and 2S protein fractions and to study the interaction of CG A, CA and QA with 11S and 2S protein fractions and also to study the interaction of these ligands with succinylated 11S protein fraction, tryptophan modified 11S protein fraction, succinylated and tryptophan modified 11S protein fraction, and effect of NaCl on such interaction. This study would enable to have an insight into the detailed mechanism of the nature and mechanism of the interaction of polyphenols with sunflower seed proteins.

2) To study the interaction of GuHCl, GuHSCN with 11S protein by using several biophysical techniques to obtain the mechanism of interaction including the presence of intermediate structures in the respective unfolding pathway.

3) To study the effect of temperature on the thermal stability of the 1IS protein fraction in various solution conditions of pH, salt concentrations, polyphenols, denaturants and in presence of sucrose, trehalose, glycerol and sorbitol.

4) To study the extent of preferential interaction of sucrose, trehalose, glycerol and sorbitol with 1IS protein fraction and understanding the rationale for the thermal stability of the protein in the presence of these cosolvents.

MATERIALS AND METHODS

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Materials

The chemicals used were as follows: Sepharose 6B, Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden); CGA, CA, QA, SDS [151-21-3], acrylamide [79-06-1], GuHCl [50-01-1], GuHSCN [593-84-0], xylose, glycerol, sucrose [57-50-1], trehalose, sorbitol, N,N-methylene bisacrylamide [110-26-9], N,N,N',N'-tetramethyl ethylene diamine (TEMED) [110-18-9], coomassie brilliant blue R-250 [6104-59-2], 2,4,6-trinitro benzene sulphonic acid (TNBS) [2508-19-2], N-bromosuccinimide (NBS) [128-08-5], lysine monohydrochloride [657-27-2], succinic anhydride [108-30-5], trizma base (tris, tris hydroxy methyl amino methane) [77-86-1], 2-mercaptoethanol [60-24-2], N-acetyl L- tryptophan ethyl ester, N-acetyl L-tyrosine ethyl ester (Sigma Chemical Company, StLouis, USA); sodium sulphite, (Ranbaxy chemicals Pvt. Ltd, S.A.S Nagar, India); sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen phosphate, ammonium sulphate, ammonium persulphate (SD Fine Chemicals, Boisar, India); potassium chloride, sodium hydroxide, EDTA disodium salt, hydrochloric acid, citric acid, acetic acid, boric acid, borax, sulphuric acid, diethyl ether (Qualigens Fine Chemicals, Bombay, India); potassium hydrogen phthalate, sodium bicarbonate (Sarabhai M Chemicals, Baroda, India); glycine, sodium acetate, sodium citrate (E. Merck (India) Ltd., Bombay, India); bromocresol green [76-60-8], methyl red [493-52-7], bromophenol blue [115-39-9] (Himedia Laboratory Pvt. Ltd., Bombay, India); dialysis tubing (Thomas Scientific Co., Philadelphia, USA).

Methods

Preparation of sunflower seed flour

Sunflower *(Helianthus annuus* L.) seeds of Morden variety are purchased from Karnataka State Seeds corporation. They are dehulled and the kernels are flaked to 0.1 mm thickness using Aktiebolaget Kwarnmaskiner Malmo type J machine. The flakes are defatted

in stainless steel percolators using n-hexane as the solvent until the fat content is less than 1% in the flakes. These flakes are airdried and powdered in a Brabender quadrumat senior automatic pilot mill (Guisberg, Germany).

Isolation of 11S protein fraction

The total protein is extracted from the flour using 0.02 M phosphate buffer (PB) containing IM NaCl at pH 5.8 using a flour to solvent ratio of 1:10. The slurry is stirred for one hour and then centrifuged to obtain the supernatant, at 6000 rpm in a Sorvall RC-5B refrigerated centrifuge. The supernatant is precipitated with 20% (w/v) ammonium sulphate. The precipitate is dissolved in and dialyzed versus buffer, and then fractionated on a Sepharose 6B-100 column. The fractions of 4 mL are collected using a pharmacia FRAC 300 fraction collector in conjunction with a pharmacia peristaltic pump P-l. The fractions are monitored for the absorbance at 280 nm and 325 nm to monitor protein and CGA content using a Shimadzu UV-150-02 Spectrophotometer. The elution pattern is represented by plotting the absorbance versus Ve/V_0 where V_e is the elution volume in mL and V_0 is the void volume in mL. The void volume of the gel is determined by passing blue dextran 2000 through the column and measuring its absorbance at 620 nm. The peak eluting at a Ve/V_0 of 2.0 is collected, dialyzed free of salts and then lyophilized and stored at -18°C.

Isolation of 2S protein fraction

2S protein fraction is isolated by size-exclusion HPLC method using Shimadzu LC-RA series chromatographic system with a SPD-6AV UV-detector. The total protein is extracted as mentioned above and the 20% (w/v) ammonium sulphate precipitate is loaded on a supelco column which was preequilibrated with buffer. The protein fraction corresponding to the molecular weight of 13,000 is collected and reprecipitated with 45% ammonium sulphate and then dissolved in water, dialyzed versus water to remove salts and lyophilized and stored at -18°C. Standard molecular weight protein markers such as RNase, Lysozyme, Chymotrypsinogen and human serum albumin are used to calibrate the column. The protein is characterized and confirmed by SDS-PAGE for its homogeneity and molecular weight.

Protein estimation

Nitrogen content of the protein is estimated using microkjeldahl method (AOAC, 1984). Sulphuric acid and catalyst mixture are added to the protein solution, boiled for digestion and subsequent conversion of nitrogen into ammonium sulphate using a Buchi 430 digestion apparatus. This is then cooled, distilled with 40% sodium hydroxide and the liberated ammonia is collected into boric acid containing an indicator. The indicator consists of 0.033% methyl red and 0.167% bromo cresol green in ethanol. Then this solution is ύtrated using N/140 HC1. The titre value of the sample is then compared with the value obtained for standard ammonium sulphate of analytical grade (Fluka) which is similarly distilled into the boric acid. The nitrogen content of the protein is multiplied with a factor of 6.25 to obtain the protein content.

Concentration of protein

The protein which is used for the microkjeldahl estimation of nitrogen is diluted appropriately and the absorbance of the solution is measured at 280 nm wavelength which is the absorption maximum. Then the obtained nitrogen content is converted to the protein. Then the absorbance of the protein and die protein content measured from microkjeldahl are correlated to obtain the $E^{1\%}$, $_{1cm}$, 280 nm of the protein. The $E^{1\%}$, $_{1cm}$ ^{at} 280 nm of the protein is 8.8 at pH 6.0 and the value is 6.09 at pH 8.6 after giving correction for scattering.

Polyacrylamide gel electrophoresis

The homogeneity of the isolated US and 2S protein fractions is tested by PAGE. Acrylamide of 9% (w/v) and N,N'-methylene bis acrylamide of 0.3% (w/v) are mixed in a suitable buffer of defined ionic strength and the contents are filtered. Then TEMED and ammonium persulphate are added to the acrylamide solution and then gels are casted in cylindrical glass tubes and water is added on the top of the gel. The protein samples mixed with bromophenol blue and 5% glycerol prepared in the same buffer are loaded on top of the

gels after gel formation and then subjected to gel electrophoresis using a constant current of 3 mA/tube after a prerun, before sample loading, is done at 1 mA/tube. After the bromophenol blue has reached the bottom of the gel the gels are removed from tubes and stained with 0.25% (w/v) coomassie brilliant blue, then destained using a mixture of 10% (v/v) acetic acid and 10% (v/v) ethanol.

SDS-PAGE

Polyacrylamide gels are casted in cylinderical glass tubes essentially according to the procedure described above except that the SDS of 0.1% (w/v) is added to the acrylamide solution before polymerization. Protein samples are prepared according to the procedure described by Weber and Osborn, 1969. The protein is mixed with 1% SDS and 1% beta-mercapto ethanol and then incubated at 37°C for 4 hours and then dialyzed versus buffer containing 0.1% (w/v) SDS and 0.1% (w/v) beta-mercaptoethanol. Then the samples are loaded on a 12% (w/v) acrylamide gel polymerized with 0.4% (w/v) bis- acrylamide. The staining and destaining procedures are similar as described above.

Velocity sedimentation

Total protein or 11S or 2S protein fraction in 1M NaCl at pH 6.0 are centrifuged at 59,780 rpm in a Beckman model E analytical ultracentrifuge equipped with phase plate Schlieren optics and RTIC unit. Photos are taken at regular time intervals. They are developed and the movement of Schlieren peak is analyzed to obtain sedimentation coefficient value by using standard procedure as described by Schachman (1959).

For association-dissociation study of 1IS protein fraction the protein in 0.5 M NaCl at pH 6.0 and a concentration of 10 mg/mL is used. The protein is mixed with differing concentrations of either GuHCl or GuHSCN and then dialyzed versus the denaturant solutions and, then centrifuged at 10,000 rpm and the supernatant obtained is subjected to analytical ultracentrifugation as described above. Photos are taken at regular intervals and

the s value is calculated for the different peaks individually. The areas of die individual peaks are calculated.

Sedimentation equilibrium

The 11S protein fraction at a concentration of 9.13 x 10^{-7} M is centrifuged to equilibrium at 13,000 φm and then the absorbance at 280 nm and 325 nm as a function of radial distance is monitored by Schlieren optics and the gradient is analyzed by curve-fitting to evaluate the molecular weight of the protein in absence and presence of CGA whose free concentration is 1.3 x 10^{-5} M. The procedure described by Yphantis (1964) is followed for all the calculation.

Amino acid composition

1IS protein is taken in an evacuated sealed tube in presence of 6N HC1 and digested at 110°C for 24 hours. Then the hydrolyzed protein is washed free of acid and a known quantity is fractionated on ion exchange column and the retention times of separated amino acids are compared with standard amino acids. The respective peak areas of the sample and standards are used to get the content of each amino acid present in the protein molecule.

Circular dichroic measurements

Far-UV circular dichroic spectra of helianthinin (1.65 x 10^{-6} M) in presence of 9.8 x 10^{-6} M and 8 x 10^{-5} M CGA, 1 x 10^{-5} M and 1 x 10^{-4} M CA, 1 x 10^{-5} M and 1 x 10^{-4} M QA are recorded in the range of 200-260 nm using a Jasco J-20C spectropolarimeter at 25°C with the temperature being maintained using a circulating water bath. A quartz cylindrical cell having 1 mm pathlength is used for die measurements. Similarly the CD spectra of buffer solutions is also recorded. The difference in die signal between protein solution and buffer solution is converted to die mean residue molar ellipticity using a mean residue molecular weight of 113 for die protein (calculated from amino acid composition). (Schmid, 1989).

$$[\theta]_{mrw} = \theta \times \frac{100 \times MRW}{c \times d} \quad (1)$$

The [θ]mrw at each wavelength is analyzed for the percentage of secondary structural contents using CDESTTMA programme (Courtesy: Prof. G.D. Fasman of Brandeis University, Waltham, MA, USA).

For the denaturation measurements the protein is mixed with GuHCl of specified concentrations and then their CD- spectra is measured using a similar procedure. The [θ]mrw as a function of wavelength is analyzed for secondary structural contents.

Absorption measurements

Absorption measurements of proteins are made either in Shimadzu UV-150-02 or Shimadzu UV-160A recording spectrophotometer using the buffer as the reference solution. The absorption spectra are taken in Shimadzu UV-160A recording spectrophotometer in the wavelength range of 200- 400 nm.

UV-DifFerence spectra

UV-difference spectra of protein in presence of varying concentrations of GuHCl, GuHSCN at pH 6.0 in presence of 0.5M NaCl is measured according to standard procedure (Herskovits, T.T, 1967). After performing the baseline correction with both the cuvettes containing the cosolvents, the spectra of the protein in presence of a particular concentration of cosolvent is recorded. Similarly the protein spectrum alone using the buffer as reference is recorded. Then the spectrum of the protein is subtracted from the sample spectrum (that containing cosolvent). From this the difference in spectral absorbance, ΔA at different wavelengúis such as 280 nm, 287 nm and 292 nm is calculated. These are then converted into molar difference in extinction coefficient values, Δε. The protein concentrations used are in the range of 2.5 x 10^{-6} M to 4.7 x 10^{-6} M depending upon the solvent.

In a similar manner the difference spectra are obtained for the protein in presence of glycerol, sorbitol, sucrose and trehalose at pH 8.6 with the buffer being 0.05M glycine-NaOH containing 0.8 M NaCl.

Precipitation kinetics

US protein fraction (4.7 x 10^{-6} M) with varying concentrations of GuHCl or the protein of 2.4 x 10^{-6} M concentration with varying concentrations of GuHSCN are used to record the absoφtion as a function of time at 660 nm Then the maximum concentration of the denaturant at which this turbidity is maximum is calculated by means of the pseudo-first order rate constants by Guggenheim plot of the data (Hiromi, 1979). In another set of experiment, at different denaturant concentrations the protein concentration dependence of the rate constants is followed.

Ligand binding by absorption spectroscopic titration

CGA, CA and QA prepared in the respective buffer solution at pH 6.0, are added in increments of 10 microliters to the protein solution taken in a quartz cuvette of 1 cm pathlength. The addition is continued upto a total dilution of the protein by 10% (v/v). After each addition the absoφtion of the protein is recorded. The difference in absoφtíon at 325 nm, 310 nm or 240 nm is calculated for 11S-CGA, 11S-CA and 11S-QA solutions, respectively using the protein absorbance alone used as a reference.

In some cases the titration is carried out using the tandem quartz cuvettes essentially according to the procedure described earlier (Kronman and Robbins, 1970). The protein and buffer are taken separately into the two cells of the tandem reference cuvette, and the buffer and ligand are added in 5 µL increments to the protein cell and buffer cell, respectively and mixed separately using syringe needles. For the sample cuvette the ligand is added to the protein compartment only and mixed well. Then the difference spectra are measured at each stage of the titration of the experiment.

In another experiment, CGA of 5.41 x 10-^{6} M is titrated with a concentrated stock solution of the 11S protein and the absorption spectra are recorded, and the ΔA at 280 and 325 nm is measured. This experiment is essentially done according to the procedure described earlier (Oberfelder and Lee, 1985). The ΔA at each titre point is analyzed by a plot of 1/ ΔAi versus $1/C_p°$ to evaluate the ΔA_{max} from which the molar difference in extinction coefficient is calculated for a stoichiometry of one.

Then using this ΔE for an average stoichiometry of one the bound ligand concentration on the protein is calculated utilizing the data of forward titration data of protein by the ligand. This bound ligand concentration is converted to moles of ligand bound per mole of protein, γ, and this is analyzed for the association constant of the protein-ligand complex formation by a Scatchard plot of analysis or Klotz plot of analysis. Since the Scatchard plot and Klotz plots are curved typical of positive cooperativity the following equation is used for analysis which includes the n_H (the Hill coefficient) the index of cooperativity (Cantor and Schimmel, 1980).

$$\gamma = \frac{n.K_a^{nH}.C_L^{nH}}{1+K_a^{nH}.C_L^{nH}} \qquad (2)$$

where γ is the moles of ligand bound per mole of protein, n is the total number of binding sites, K_a^{nH} is the intrinsic association constant and C_L^{nH} is the free ligand concentration which is the difference between total molar concentration of ligand and protein.

Ligand binding by fluorescence spectroscopic titration

The fluorescence spectra of the protein after each addition (10 μL) of either CGA or CA or QA is recorded at different temperatures. The maximum dilution of the protein did not exceed 10% (v/v). The fluorescence excitation maximum is 285 nm and the emission is recorded in the range 300-400 nm. The band widths of the excitation and emission are 5 and 10 nm, respectively.

From the spectra the ΔF is calculated for each addition of the ligand after giving correction for dilution and inner filter effects due to the absorbance of CGA and CA in the emission range of the intrinsic fluorophores of the protein. The inner filter effect correction is given according to the following equation (Birdsall, *et al.,* 1983):

$$F_{corr} = F_{obs} \frac{e^{-\varepsilon C_L^{\circ} d} - e^{-\varepsilon C_L^{\circ}}}{\varepsilon C_L^{\circ}(1-d)} \qquad (3)$$

where F_{corr} and F_{obs} are the corrected fluorescence intensity and observed fluorescence intensity values of the protein. The parameters ε and d are molar extinction coefficient at a particular wavelength and pathlength, respectively. C_L° is the total ligand concentration The correction is given both at excitation and emission wavelengths.

The ΔF data is transformed to the fractional saturation, β, and it is analyzed using Scatchard plot.

The K_a, reciprocal of the average association constant is calculated from the curve fitted values of f_i and K_{ai} by following equation (Dahlquist, 1978) which is approximated to the slope of the Scatchard plot when y tends to zero.

$$K_a = 1/K_{dav} = 1\Big/\left(\sum_{i=1}^{2}(f_i/K_{ai})\right) \qquad (4)$$

The number of the binding sites, n, of the multiple classes of binding are obtained from the fractional binding data from the x intercept of a plot of 1/1-β versus $C_L^{\ o/\beta}$ for the line with a slope approximately equivalent to $\mathbf{K_a}$.

Fluorescence spectroscopic tïtration for iigand binding by 2S protein fraction

Chlorogenic acid is added in increments to 2S protein fraction at pH 6.0 (0.02 M PB) and the intrinsic fluorescence spectra of the protein is recorded, corrected for inner filter effects and then the data is analyzed for the binding parameters by the Scatchard plot. The experiment is performed at pH 3.0, 5.8 and 6.6 and at different temperatures of 20°C, 28°C and 37°C at each pH.

Ligand binding by equilibrium dialysis

1IS protein fraction of 1 x 10^{-5} M in a volume of 0.5 mL is taken into Spectra-Por dialysis tubings and the protein is dialyzed versus different concentrations of CGA or CA for 12 hours at 31°C after which the dialysis tubings are removed. Then the ligand concentration present in the outside compartment after dialysis is measured for its absorbance at 325 nm for CGA and 311 nm for CA. Buffer of 0.5 mL is similarly dialyzed versus ligand and used as a control for dilution and membrane binding effects. The difference in the ligand concentration of sample and reference is the bound ligand concentration (Steinhardt and Reynolds, 1969). This data is converted to γ, the number of moles of ligand bound per mole of protein and then analyzed using equation 2, since cooperativity in binding is observed, to obtain the association constant.

Thermal transition measurements

1IS protein fraction of 0.4 absorbance at 280 nm is mixed with 1 x 10^{-5} M and 1 x 10^{-4} M CGA and the absorbance of these solutions at 287 nm is measured as a function of temperature with temperature increments of 1°C using a Gilford thermal response spectrophotometer, using the respective concentration of ligand solution used as the reference. The data is analyzed for apparent T_m of the transition of increasing absorbance according to the procedure described earlier (Pace *et al,* 1989). The transitions of increasing absorbance are analyzed by first derivative plots and the mid transition temperature is noted as the apparent T_m of the protein. Similar thermal transition measurements are made for the protein in presence of 1 x 10^{-6} M, 1 x 10^{-5} M and lx 10^{-4} CA.

1IS protein fraction of different concentrations both in presence and absence of 0.8 M NaCl are used to record their absorbance at 287 nm as a function of temperature using a similar procedure and analyzed for the apparent T_m values.

The effect of NaCl of different concentrations such as 0.1 M, 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1 M mixed with the protein of constant protein concentration (1.9 μM) at pH 8.6 is studied as a function of temperature and the analysis is made according to the procedure described above.

The effect of cosolvents such as sucrose, trehalose, glycerol and sorbitol on the thermal absorption transitions of the protein are measured by following the absorbance at 287 nm at pH 8.6 in presence of 0.8 M NaCl.

The effect of varying concentrations of GuHCl and GuHSCN on the thermal absorption transition of the protein is similarly measured and analyzed at pH 6.0 in 0.15 M PB, 0.5 M NaCl and analyzed for the apparent T_m values.

Viscosity measurements

11S protein fraction in presence of various concentrations of GuHCl are incubated for several hours and centrifuged to get clear solution and this is used for viscosity measurements. The viscosity is measured by the flow of the liquid through Ostwalds capillary viscometer that has a flow time of 192 sec for water at a temperature of 20°C. The temperature is maintained by immersing the viscometer in a water bath whose temperature is regulated by a Heto circulating water heater and a virtis water cooler operating simultaneously. A protein concentration of 10 mg/mL is used. The flow times of both protein-GuHCl, protein alone and GuHCl alone are measured and these are used to calculate the reduced viscosity using the following equation (Bradbury, 1970):

$$\eta_{red} = \frac{\eta_{sp}}{C} = \left[\frac{t - t_o / t_o}{C}\right] \qquad (5)$$

where C is the concentration of protein in mL/g, η_{red} is the reduced viscosity, η_{sp} is the specific viscosity, t_0 is the flow time of sample and t is flow time of buffer/guanidinium salt.

Preferential interaction measurements

The partial specific volume, v, of helianthinin in 0.05 M glycine, 0.8 M NaCl (pH 8.6) is determined by measuring its density. Protein is vacuum dried over phosphorus pentoxide and dissolved in either buffer or cosolvents such as glycerol, sorbitol, sucrose and trehalose and then the densities are measured in a DMA - 55 precision densitymeter. The v is calculated from the obtained density data using the following equation (Kielly and Harrington, 1960; Lee and Timasheff, 1974; Gekko and Timasheff, 1981; Prakash, 1982).

$$\bar{v}_{c\to o} = \frac{1}{\rho_0}\left(1-\left(\frac{\rho-\rho_0}{C}\right)\right) \qquad (6)$$

The v is the measured partial specific volume under concentration, C which is derived from the density of the protein solution p and the buffer solution p_0 in both isomolal and isopotential conditions and the extrapolated v values to zero protein concentration respectively are the $<j>2^0$ and $\Phi 2^0$ and these are used to calculate the preferential binding parameter, (δg3/δg2)T,μι,μ3 (Casassa and Eisenberg, 1964; Cohen and Eisenberg, 1983):

$$\xi_3 = \left(\frac{\delta g_3}{\delta g_2}\right)_{T,\mu_1,\mu_3} = \left(\frac{(\delta\rho/\delta g_2)_{T,P,\mu_3} - (\delta\rho/\delta g_2)_{T,P,m_3}}{(\delta\rho/\delta g_3)_{T,P,m_2}}\right) = \frac{1}{\rho_3}\left(\frac{\phi_2^0 - \phi_2'^0}{1-\bar{v}_3\rho_3}\right) \qquad (7)$$

where P_3 is density of third component (cosolvent) and V3 is the partial specific volume of the third component. The preferential hydration of the protein, (δgı/δg2)T, μ1,μ3 is calculated using the equation (Arakawa and Timasheff, 1982; Timasheff and Kronman, 1959):

$$\left(\frac{\delta g_1}{\delta g_2}\right)_{T,\mu_1,\mu_3} = -\frac{1}{g_3}\left(\frac{\delta g_3}{\delta g_2}\right)_{T,\mu_1,\mu_3} \qquad (8)$$

$$g_3 = M_3 m_3 / 1000 = \frac{\text{Concentration of component 3 in g / mL}}{(\rho_3 - \text{Concentration of component 3})} \quad (9)$$

where g3 is the bulk solvent composition of third component, m_3 is the molality of third component and M3 is *the* molecular weight of third component.

The preferential interaction parameter in mole/mole basis is calculated from the following eαuation (Arakawa and Timasheff. 1984):

$$\left(\frac{\delta m_3}{\delta m_2}\right)_{T,\mu_1,\mu_3} = \frac{M_2}{M_3}\left(\frac{\delta g_3}{\delta g_2}\right)_{T,\mu_1,\mu_3} \quad (10)$$

Where M2 is the molecular weight of the protein and M3 is the molecular weight of the cosolvent.

Modification of tryptophan residues

The exposed tryptophan residues of the protein are modified using N-bromosuccinimide according to the procedure described earlier (Spande and Witkop, 1967). The protein in 0.1M acetate buffer at pH 4.0 and 0.15 M PB at pH 6.0 is taken into a quartz cuvette and then titrated with NBS. The absorption at 280 nm is measured at each addition of NBS using a Shimadzu-uv-150-02 spectrophotometer. The titration is continued till the onset of the increase in absorbance. The ΔA_{max} of this titration is used to calculate the number of tryptophan residues modified using the following equation (Spande and Witkop, 1967).

$$n = \frac{\Delta A_{max} \times 1.31 \times MW}{A \times a.f. \times 5500} \quad (11)$$

where ΔA_{max} is the maximum change in absorbance duly corrected for dilution, A is the initial absorption of the protein at 280 nm, MW is the molecular weight of the protein (2,41,000), a.f. is the absorptivity factor to convert the absorbance at 280 nm of the protein to mg/mL (a.f. = 1.13 for 1IS protein fraction).

Similarly estimate of the number of tryptophan residues modified of the protein in presence of CGA, CA and QA are calculated, while taking care of the absorbance change due to the addition of NBS to the reference solution containing these ligands. The protein is dialyzed versus water after modification and lyophilized for further use. The extent of exposed tryptophan residue modification by NBS is calculalted at pH 4.0 and pH 6.0 in the absence and presence of ligands such as CGA, CA and QA. In the reference cuvette corresponding ligand is also titrated and the maximum AA is calculated from which the number of tryptophan residues modified are calculated (Spande and Witkop, 1967). From comparison of the data of the modification of native protein and protein-ligand mixture the number of tryptophan residues protected from modification are calculated.

Modification of lysine residues

The exposed lysine residues of the protein are modified by adding 100 mole excess of succinic anhydride in increments at pH 7.5 with the pH of the solution kept constant by addition of NaOH. The procedure followed is similar to that described earlier (Klotz, 1967). The solution is kept for nearly two hours and then the protein is dialyzed versus water and lyophilized for further use. The available lysine content of the modified and unmodified proteins is estimated by using 0.1% (w/v) of 2,4,6-trinitro benzene sulphonic acid at pH 10.5. (Hall et al., 1973).

For doing both the modifications the lysine residues of helianthinin are first modified by succinic anhydride essentially according to the procedure described above and is dialyzed free of salts and lyophilized. This protein is dissolved in buffer and then modified using N-bromosuccinimide to obtain the lysine and tryptophan modified helianthinin. This is dialyzed free of salts and then lyophilized for further use.

Estimation of available lysine

Available lysine content of 11S protein after modification of the lysine residues by succinylation is made using a reported method (Hall et al., 1973). The 2,4,6- trinitro benzene sulphonic acid (TNBS) is added to the protein solution in sodium bicarbonate to form the epsilon- trinitrophenyl lysine (ε-TNP lys). This ε-TNP-lysine is then released by hydrolysis with hydrochloric acid which is determined spectrophotometrically at 346 nm or 415 nm after removing the picric acid and alpha-TNP-lysine by extracting with diethyl ether. A sample blank by adding **HC1** to the protein prior to the addition of TNBS and a reagent blank consisting of water instead of the protein are treated similarly. The absorption at 346 nm or **415** nm is correlated to the amount of lysine by preparing a standard graph of the reaction of **TNBS** with lysine monohydrochloride. From the difference in the available lysine on native protein and succinylated protein the extent of succinylation is calculated as 86%.

Chapter 1

INTERACTION OF POLYPHENOLS WITH 11S AND 2S PROTEIN FRACTIONS

INTERACTION OF POLYPHENOLS WITH 11S AND 2S PROTEIN FRACTIONS

Isolation and characterization of 11S protein fraction

The total protein is extracted from the sunflower seeds according to the procedure described under Materials and Methods. In Fig. 2A (a) is shown the sedimentation velocity pattern of the total protein from sunflower seed. From the figure it is clear that the total protein consists of three protein fractions whose sedimentation coefficients are as 11S, 7S and 2S. The percent composition of these fractions are 55%, 7% and 38%, respectively and is in conformity with the reported values in literature (Prakash and Rao, 1986).

In order to isolate the 11S component fairly devoid of free polyphenols, the total protein was subjected to 20% (w/v) ammonium sulphate precipitation and eluted on a Sepharose 6B gel filtration column. The elution profile is shown in Fig. 2B. There are two major peaks as monitored by absorption at 280 nm for protein and a peak rich in poyphenols as indicated by absorption at 325 nm . The peak eluting at a Ve/Vo of 2.0 is identified as the 1IS protein fraction as indicated by earlier studies in this laboratory and is pooled and further purification carried out.

The homogeneity of the isolated protein is established by velocity sedimentation pattern and native PAGE. In Fig. 2A (b) is shown the velocity sedimentation profile of the purified US fraction, helianthinin. This peak has a sedimentation coefficient ($s2o_{,w}$) of 11.14S. In Fig. 3A is shown the electrophoretic pattern of this protein, which showed single band corresponding to the 11S protein fraction. In Fig. 3B is shown the electrophoretic pattern of this protein in presence of SDS and β-mercaptoethanol which showed multiple bands indicating that US protein is a multimeric protein. The molecular weights of these bands are in accordance with the values reported by other workers in the range of 23K-35K (Dalgalarrondo *et al,* 1985; Durante *et al,* 1989; Kortt and Caldwell, 1990). The US protein is hexameric with each subunit containing one acidic subunit and one basic subunit linked together by disulphide bonds.

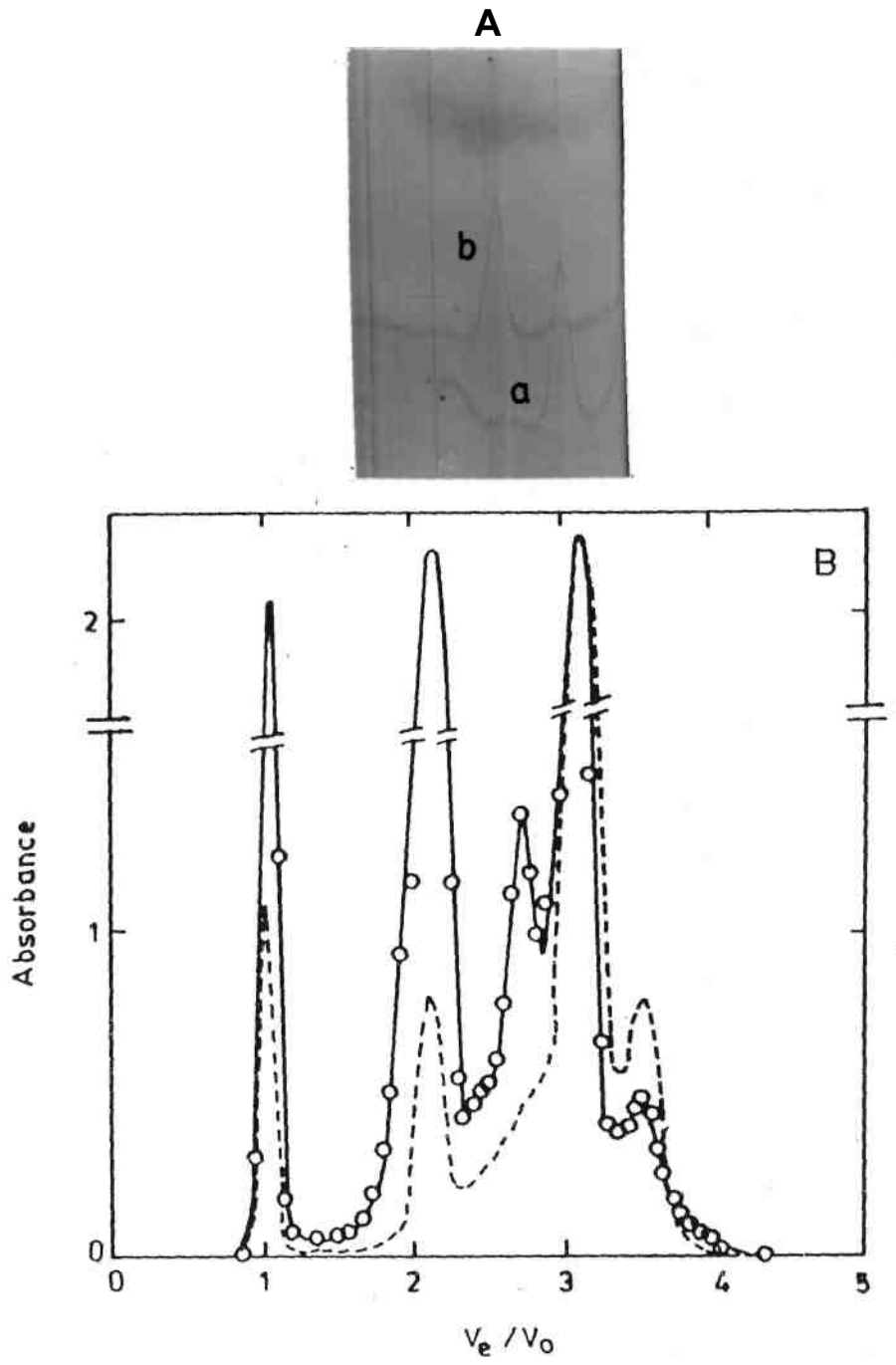

Fig. 2A(a). Sedimentation velocity pattern of the total protein and 11S protein C tO of sunflower seed. The protein concentration used for the experiment is 8.0 mg/mL for both fhe proteins. The photograph was taken at 36th minute after attaining 2/3 the maximum speed of 59,780 rpm.

B. Elution profile of the 20% (w/v) ammonium sulphate precipitate of the total protein of sunflower seed on Sepharose 6B-100 gel filtration column. The eluted sample is monitored at 280 nm (-o~) and 325 nm (----------) for protein and CGA, respectively in the eluted fractions.

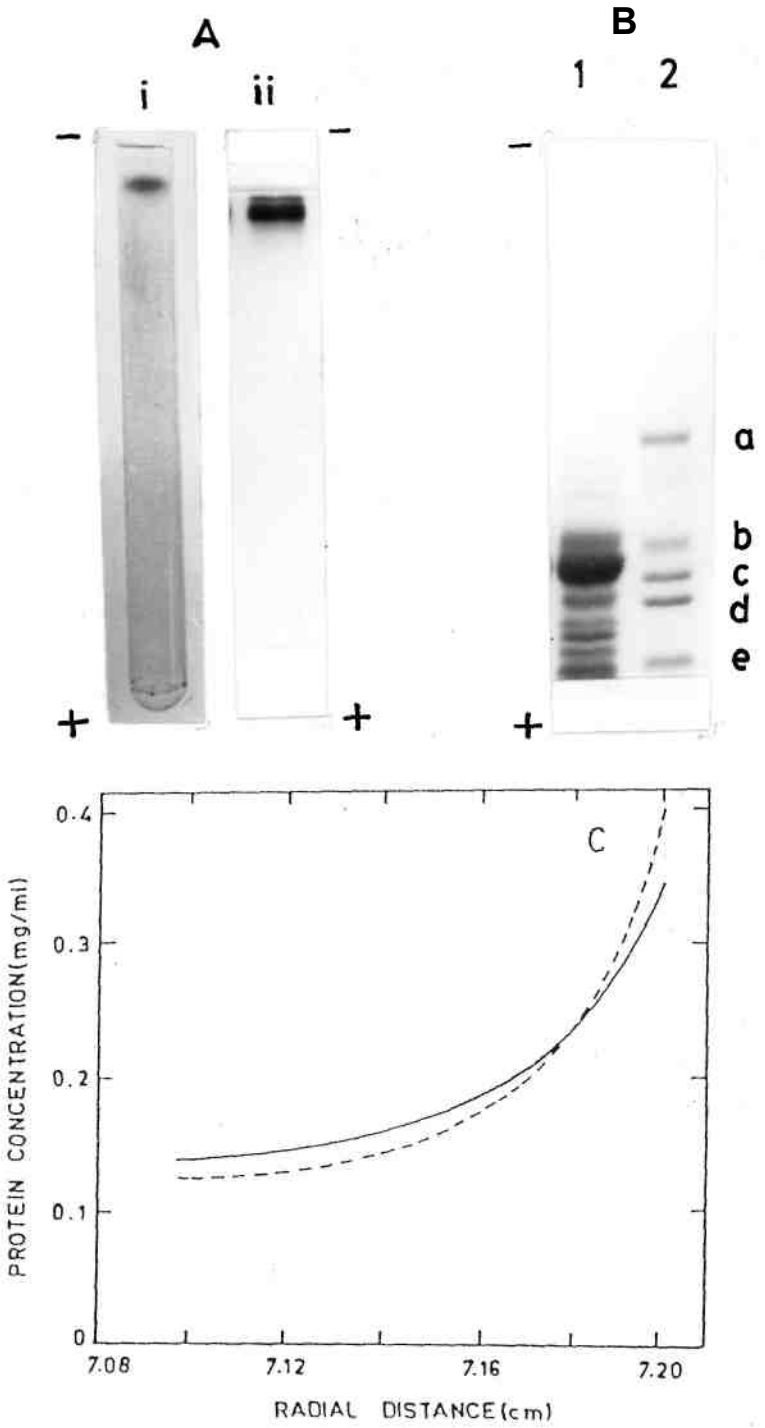

Fig. 3A. (i) Native PAGE pattern of 11S protein fraction in 0.15M phosphate buffer, pH 6.0. A protein concentration of 5 x 10' M was used in the experiment, (ii) Native-PAGE pattern of 11S protein fraction at pH 8.2 in 0.1 M

Tris glycine buffer, pH 8.2.

B. SDS-PAGE pattern of 11S protein fraction in 0.1 M Tris-glycine buffer, pH 8.2 (lane 1). In lane 2 is shown the electrophoretic pattern of the standard molecular weight markers protein representing (a)BSA, (b)ovalbumin, (c) glyceraldhyde 3-phosphote dehydrogenase, (d) carbonic anhydrase and (s) trypsinogen.

C. Distribution of the protein concentration of 11S protein fraction (—) and 11 S protein fraction in presence of CGA (—) in sedimentation equilibrium at 13000 rpm. The protein and CGA concentrations used (or the experiment are 9.13 x 1θ' M and $1.3x10^{-5}$M, respectively.

In Fig. 3C is shown the sedimentation equilibrium pattern of US protein. The molecular weight calculated by curve-fitting of this pattern is 2,41,000 ± 5000. The amino acid composition of the protein was evaluated and 99% correlation was obtained with the reported values (Prakash and Rao, 1986). The protein has more of aspartic acid and glutamic acid residues and is rich in amide content.

In Fig. 4 is shown the structure of CGA which has both hydrophobíc and hydrophilic functional groups. The hydrophobic part of CGA is the aromatic CA part which is primarily responsible for its absorption spectrum and fluorescence emission spectrum. The CGA molecule has UV-absorption spectrum with a peak in absoφtion intensity at 325 nm. The absoφtion maximum of the CA part of CGA is at 311nm. CGA has the fluorescence emission maximum at 420 nm. It does not contribute to the fluorescence emission intensity in the region of 330 nm where the US protein has its intrinsic fluorescence emission maximum.

The hydrophilic part of CGA is the presence of two hydroxyl groups on the aromatic ring of CA, and three hydroxyl groups and one carboxylate group on the cyclohexane ring of QA. The knowledge of the ionization behaviour of these hydrophilic groups at different pH values, net charge and isoelectric point of CGA are important in understanding of its interaction with proteins. The pK_a of the carboxylate group is reported as 3.9 (Sastry, 1985). The ionization of phenolic groups of the CGA was monitored by the titration following the absorbance at 295 nm, where the phenolate ion absorbs. There are two transitions, one of decreasing absorbance upto a pH 9.5 and another of increasing absorbance due to ionization and subsequent simultaneous formation of o-quinone. In the first derivative plot of this data there are two peaks corresponding to the ionization of first phenolic group and second phenolic group with the mid points of these peaks, the apparent pK_a, are at 8.6 and 10.8.

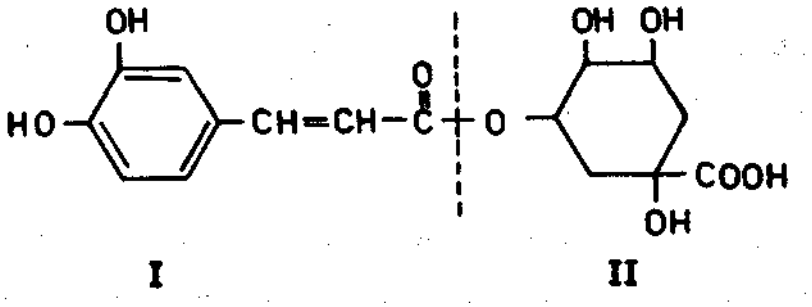

Fig. 4. Structure of 3'-0-caffeoyl D-quinic acid. It consists of two parts (I). Caffeic acid part and (II). Quinic acid part.

Interaction of polyphenols with 11S protein fraction

Interaction with CGA?

Effect of pH

The (11S-CGA) complex was prepared at a protein concentration of 2.35 μM and a total CGA concentration of lxl0-^{4} M and the mixture incubated for one hour at 30°C at different pH values. The incubated mixture was centifuged at 6000 φm for 30 min and the supernatant monitored at 325 rnn. In Fig. 5 is shown the percentage of the CGA content in the supernatant at different pH values which indicates two distinct pH zones where the interaction and subsequent precipitation of CGA occured. They are (i) pH 3-4 and (ii) pH 6-8. The percent CGA content is lesser in the supernatant at pH 6 as compared to other pH values. This indicates that at pH 6, the maximum interaction takes place which leads to precipitation of the protein-ligand complexes. However, in the supernatant even soluble 11S-CGA complexes will contribute to the absoφtion at 325 nm apart from the unbound CGA. However it gives a qualitative indicator of the pH dependent phenomena of 11S-CGA interaction.

The interaction of CGA with 11S protein is studied by titration of the intrinsic fluorescence intensity of protein by the addition of CGA at different temperatures. Addition of CGA to the protein quenches the intrinsic fluorescence intensity of the protein. In Fig. 6 is shown the representative fluorescence spectra of the protein at 28°C after giving corrections for inner filter effect in presence of various concentrations of CGA. There is an increase in the emission maximum of protein with increasing concentrations of CGA. There are two peaks in normalized quenching spectrum one at 310 nm and the other at 355 nm or 350 nm. This data suggests the differential quenching of the tyrosine and exposed as well as partly exposed tryptophan groups upon binding. As the concentration of ligand is increased the 355 nm peak is not present, instead a 350 nm peak is present. This is due to the conformational change of the protein exposing some of the partly buried tryptophan residues to the polar

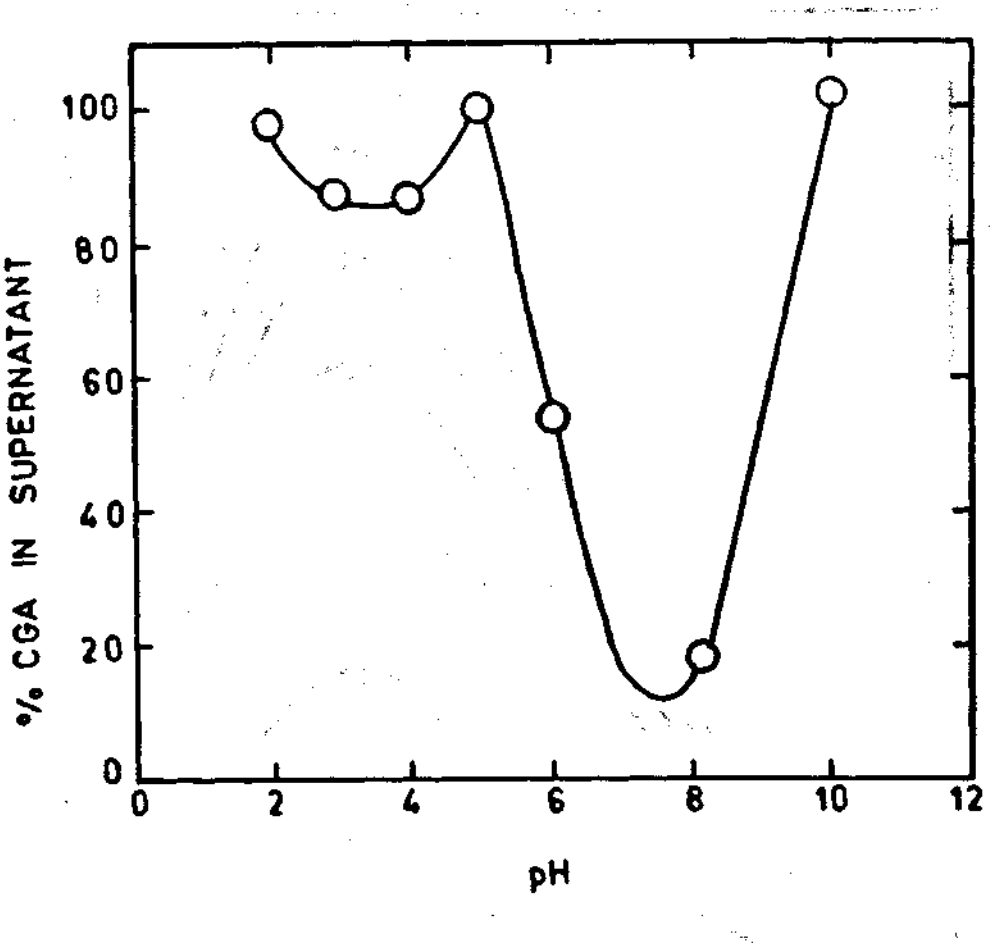

Fig. 5. Percent CGA in the supernatant of the 11 S protein fraction incubated with 1 x 10"^{4} M CGA for one hour at different pH values at 30°C. The supernatant is obtained after centrifugation at 6000 rpm for 30 minutes at 30°C. The protein concentration used for incubation is 2.4 x lO^M. CGA content is measured by the absorbance at 325 nm. Different buffer systems used are KCl-HCl for pH 2, citrate buffer for pH 3, acetate buffer for pH 4-5, phosphate buffer for 6-8 and carbonate buffer for pH 10.

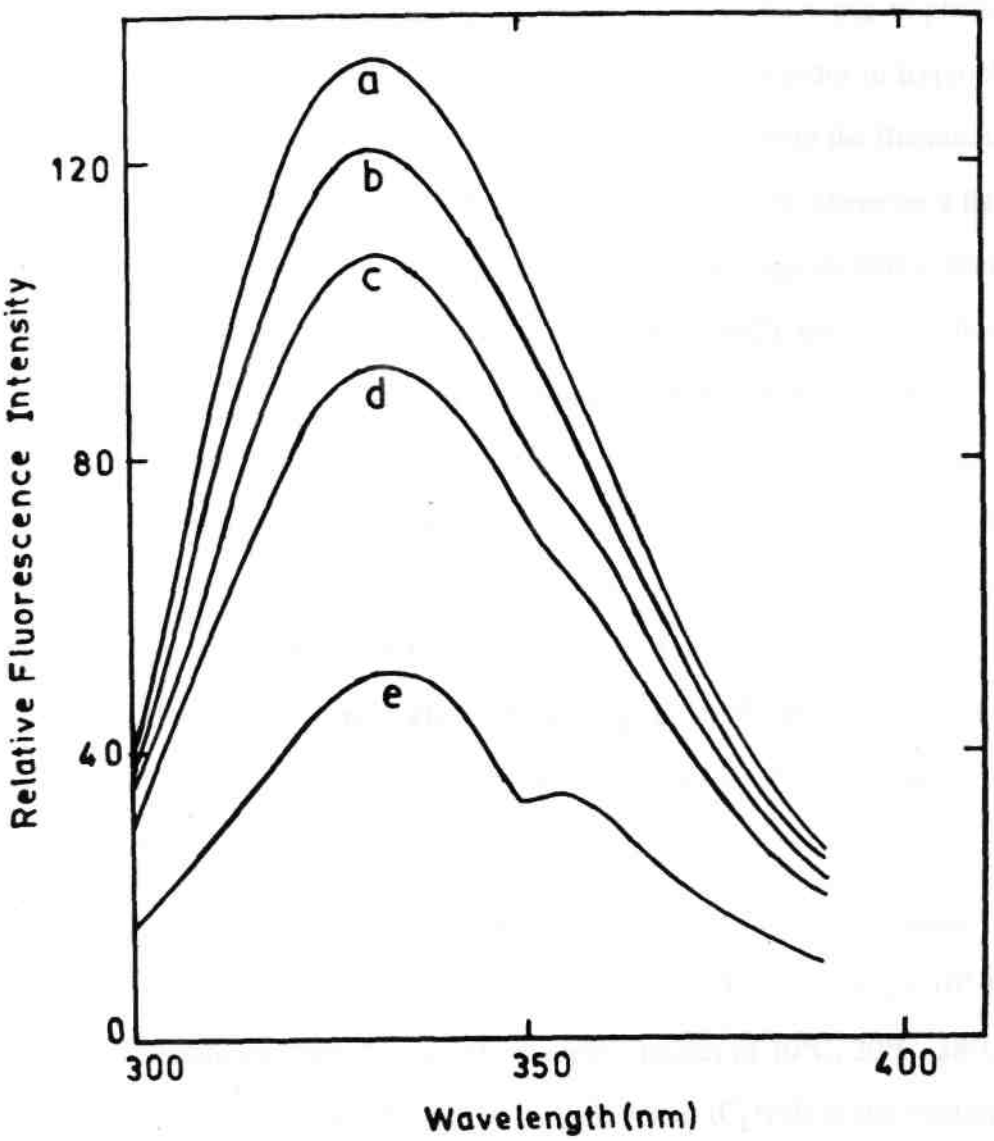

Fig. 6. Fluorescence emission spectra of the 11S protein fraction in 300 nm -400 nm wave length range in 0.15 M phosphate buffer, pH 6.0. The concentration of the protein is 4.7×10^{-4}M. (a) native 11S protein fraction. The 11S protein fraction incubated with (b) 4.68×10^{-6} M CGA , (c) 1.44×10^{-5} M CGA, (d) 3.03×10^{-5} M CGA and (e) 1.10×10^{-4} M CGA concentrations.

environment as will be clear from the CD data also. Usually in the native protein or ligand (of non absorbing nature) bound protein, the fluorescence intensity due to phenylalanine and tyrosine are not seen as peaks due to their resonance energy transfer to tryptophan residues. However in this case, the ligand absorption spectrum overlaps with the fluorescence emission spectrum of phenylalanine, tyrosine and tryptophan and therefore whenever a ligand is bound the energy of the emission is efficiently transferred to the ligand rather than to aromatic amino acids of protein. Therefore this transfer can individually takes place from tyrosine to CGA and from tryptophan to CGA. The binding of CGA to both tyrosine and tryptophan is further illustrated by the interaction of the ligand with model compounds, N-acetyl L-tyrosine ethyl ester and N-acetyl L-tryptophan ethyl ester. There are two classes of binding sites for CGA with tryptophan ethyl ester. The association constants of high and low affinity classes are 1.5×10^6 M^{-1} and 0.94×10^4 M^{-1}, respectively. The fraction of total number of binding sites for high and low affinity class of binding are 0.05 and 0.95, respectively. The association of CGA with N-acetyl L-tyrosine ethyl ester is of single class with association constant is being 6×10^3 M^{-1}.

In Fig. 7A is shown the Scatchard plot of the 11S-CGA interaction data at 28°C, which is biphasic indicating two classes of binding sites. The K_a is 1.2×10^4 M^{-1}. In Table 2 is given the binding parameters at different temperatures of 10°C, 20°C, 28°C, 35°C, 40°C and 45°C. In Fig. 7B is shown the plot of $(1/1- \beta)$ versus (C_L^0/ β) of the binding data at 28°C at higher concentrations of ligand to calculate n. The total number of binding sites are 150 ± 20 at 28°C. In Table 3 is given the data of association constants, K_a and the free energy changes of the interaction of CGA with protein at different temperatures.

As is clear from Table 3, the temperature dependent energetics are not linear. The enthalpy change and entropy changes are more positive with increase in temperature in the range of 10°C - 45 °C. This indicates the involvement of both hydrophobic and hydrogen bonding/ionic interactions in the ligand binding. The contribution of these individual

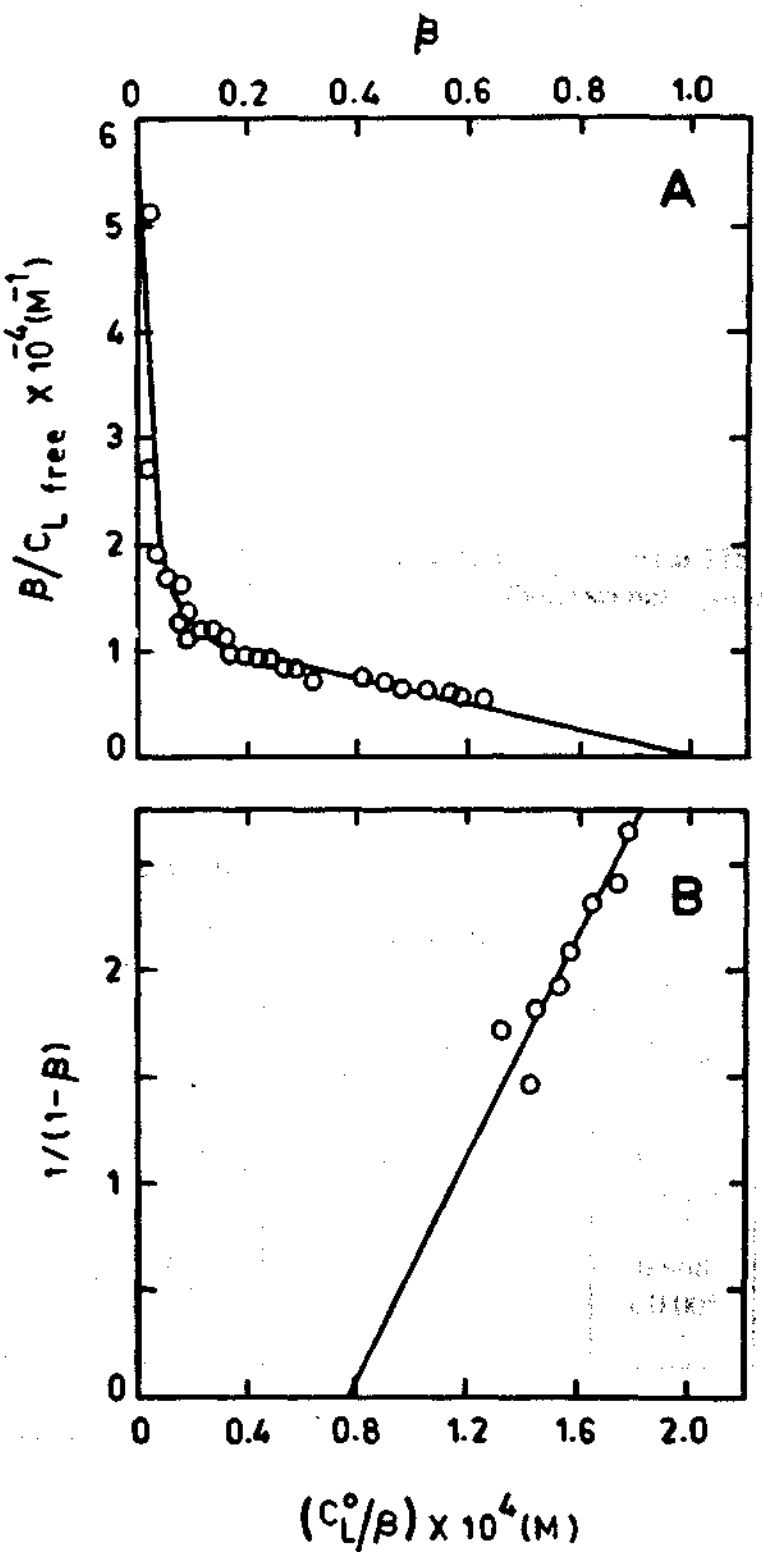

Fig. 7A. Scatchard plot of the binding data of CGA interaction with 11S protein fraction in 0.15 M phosphate buffer, pH 6.0. The data is obtained from fluorescence spectroscopic titration. Protein concentration of 4.7 x 10-^{7} M is used in the experiment. B. Plot of (1/1- β) versus C_L *o/* β to calculate the total number of binding sites for CGA interaction with 11S protein fraction. The data is derived from the fluorescence spectroscopic titration.

Table 2 : Binding parameters of the interaction of CGA with IIS protein fraction for different classes of binding sites obtained from fluorescence spectroscopic titration at pH 6.0

Temperature (°C)	f_1	$K_{a1} \times 10^{-6}$ (M^{-1})	f_2	$K_{a2} \times 10^{-4}$ (M^{-1})
10±1	0.060 ± 0.010	1.45 ± 0.10	0.940 ± 0.01	1.60 ± 0.10
20±1	0.025 ± 0.005	0.80 ± 0.05	0.975 ± 0.005	0.93 ± 0.05
28±1	0.028 ± 0.005	1.60 ± 0.10	0.972 ±0.005	1.17 ± 0.16
35±1	0.030 ± 0.005	2.00 ± 0.10	0.970 ± 0.005	0.87 ± 0.05
40±1	0.032 ± 0.005	3.50 ± 0.20	0.968 ± 0.005	1.20 ± 0.10
45±1	0.042 ± 0.004	3.80 ± 0.20	0.958 ± 0.004	1.25 ± 0.10

Table 3 : Association constants and the corresponding free energy changes for interaction of CGA with IIS protein fraction as a function of temperature at pH 6.0

Temperature (°C)	K_a x 10^{-4} (M^{-1})	ΔG (kJ/mol)
10±1	1.70 ± 0.12	-23 ± 2
20±1	0.95 ± 0.05	-22 ± 2
28±1	1.20 ± 0.11	-24 ± 2
35±1	0.89 ± 0.05	-23 ± 2
40±1	1.23 ± 0.10	-25 ± 2
45±1	1.32 ± 0.12	-25 ± 2

interactions at 28°C is calculated from the data of interaction of CGA with chemically modified proteins as described later.

As the nature of thermodynamic forces shown to be involved in the interaction are hydrophobic and hydrogen/ionic bonding, the following modifications of the protein are done to understand the alteration of respective thermodynamic strength of interaction.

Modification of the exposed tryptophan residues of 11S protein shifts the fluorescence emission maximum to 308 nm from 332 nm for the native protein. This is possibly due to the energy transfer to the modified tryptophan residues which absorb above a wavelength of 300 nm. Addition of CGA quenches the fluorescence intensity of the protein at 308 nm and this data is analyzed for interaction parameters. There are two classes of binding sites obtained from the analysis. The fraction of the total binding of class 1 and class 2 are 0.02 and 0.98, respectively. The association constants of class 1 and class 2 are $0.5xl0^6$ $M1^1$ and $0.56X10^4$ M^{-1}, respectively. The K_a value is 5.7×10^3 M^{-1}. The decrease in the average strength of binding by tryptophan modification is 1.86 kJ/mol.

In another set of experiments the ligand CGA is added to the protein at pH 6.0 to form the complex and then it is titrated with N-bromosuccinimide for tryptophan residues. The results suggest that there is protection of six tryptophan residues towards modification due to the direct binding of CGA with exposed tryptophan residues.

Lysine residues were modified to an extent of 86% of the total residues by succinylation which led to the shift in the fluorescence emission maximum of the protein from 332 nm to 340 nm. This could be due to the altered structure of the protein. This is in accordance with the higher intrinsic viscosity [η], at >76% succinylation of protein when compared to the [η] of native protein (Schwenke *et al,* 1985; Schwenke *et al,* 1986). The fluorescence emission maximum shows the dominance of fluorescence spectrum by tryptophan residues. Addition of CGA quenches the intrinsic fluorescence intensity of the succinylated US protein. The quenching data is analyzed for the binding parameters which

shows that there are two classes of binding sites. The binding parameters of the interaction, f1, f_2, K_{al} and K_{a2} are 0.02,0.98, 2.6 x 10^6 M-^{1} and 0.87 xl0^4 M-^{1}, respectively. The K_a of the binding is 8.9 x 10^3 M-^{1}. There is no decrease in the average first site association strength of the binding as a result of the succinylation. The average decrease in the strength of binding at the last site is 0.75 kJ/mol. Thus succinylation affected the strength of binding at low affinity class of sites but not at high affinity class.

The succinylated 11S protein is modified for its exposed tryptophan residues. This doubly modified protein has a fluorescence emission maximum of 308 nm which is due to the absorption of the energy associated with the fluorescence intensity efficiently by modified tryptophan residues. CGA addition decreases the intrinsic fluorescence intensity of this doubly modified protein. This double modification led to a single class of low affinity sites. The K_a of the binding is 2.0 ± 0.1 x 10^4 M-^{1}. Thus the tryptophan residues are present at the high affinity class of sites. The absence of the high affinity class completely on this doubly modified protein is probably due to a change in the structure of the protein molecule as succinylation alone could not change the strength at high affinity class of sites.

Non-covalent modification of the surface environment by 1 M NaCl is done. The fluorescence emission maximum of this protein is similar to that of native protein. Addition of CGA quenches the intrinisic fluorescence intensity of US protein precomplexed withlM NaCl. The binding is described by a single class of sites with an intrinsic K_a of 0.5 x 10^4 M-^{1}. There is positive cooperativity in the binding to this protein which is due to the facilitation of increase in multimeric structure by NaCl. The absence of high affinity class of sites is due to the following reason. Multimerisation of the structure of the protein leading to the burial of aromatic chromophores which are otherwise present at protein-protein interfaces. Oligomerisation of the protein in presence of 1M NaCl is a known phenomena for the high molecular weight proteins from various oilseeds (Prakash and Rao, 1986). This cooperativity in binding is also observed in the case of the data obtained from equilibrium

dialysis where higher protein concentration is used. Higher protein concentration also leads to the multimerisation of the protein at a particular temperature (Prakash and Rao, 1986). The decrease in the strength of the binding is little lesser to that of tryptophan modification.

Modification of the surface hydrophobic residues of the protein is done by adding 2.84 x 10^{-5} M of l-anilino-8- naphthalene sulphonic acid (ANS) that has quenched the intrinsic fluorescence intensity without changing the emission maximum. The spectrum has an additional peak corresponding to the binding of ANS to the exposed hydrophobic regions of the protein. The concentration of ANS used is that at the saturation of the increasing fluorescence intensity at the additional peak with addition of increasing concentrations of ANS. Addition of CGA to the US protein precomplexed with ANS leads to the decrease in the emission intensity after correcting for inner filter effects. The binding is of single class with a K_a of 1.3 ± 0.1 x 10^3 M^{-1}. As a result of this modification the decreased average strength of the interaction is 5.52 kJ/mol. Thus ANS, a hydrophobic probe, complexation has decreased the strength of the binding significantly and hence hydrophobic interaction is the predominant mode of binding of CGA.

Quinic acid of 9.32 x 10^{-5} M and 2.78 x 10^{-4} M are added to the protein and incubated to get the complexed protein. The emission maximum of the protein is not changed upon QA addition. The interaction of CGA with precomplexed US protein with QA (US protein + 9.32 x 10^{-5} M QA) has the binding parameters such as f_1, K_{a1}, f_2 and K_{a2} of 0.03, 0.1 x 10^6 M-l, 0.97 and 1.20 x 10^4 M^{-1}, respectively. The K_a of the binding is 1.23 x 10^4 M^{-1}. The data indicates that there is a significant inhibition of the high affinity class binding strength as a result of the complexation of QA at high affinity sites. This experiment suggests that both the CGA and QA bind to the similar sites of high affinity class. The decreased strength is an evaluation of the contribution of the QA part of CGA in binding.

When the QA of 2.78 x 10^{-4} M is added to the protein and allowed to complex, the interaction parameters of CGA with this complexed protein are 0.02, 0.1 x 10^6 M^{-1}, 0.98 and 1 x 10^4 M^{-1}, respectively. The K_a of the binding is 1.02 x 10^4 M^{-1} and as a result of this precomplextion there is a decrease in the strength of the binding of CGA by 0.42 kJ/mol when compared to native protein.

Interaction of CGA with 11S protein is studied by UV-absorption spectral titration. Fluorescence spectral intensity of a protein is more sensitive to the surrounding environment such as the binding of a ligand molecule, than absorption spectral intensity. These absorption changes also indicate whether there is any direct interaction of aromatic functional groups of the protein. Thus binding is also followed to know the extent of difference in binding that could be observed between these techniques. In Fig. 8A is shown the difference absorption spectra of CGA. There are two peaks with absorption maximum at 243 nm and 325 nm with a shoulder at 295 nm. This spectra resembles that of the CGA absorption spectra except at lower wavelengths suggesting that the aromatic moiety of the CGA is involved in the binding. The positive difference spectra is due to the facilitated absorption when the motion of the aromatic moiety is restricted as a result of the binding. This difference spectral data at 325 nm is analyzed for the binding.

In Fig. 8B is shown the binding isotherm of the interaction data. In the inset of Fig. 8B is shown the Klotz plot of the interaction. The K_a, n and n_H used for curve- fitting of the binding isotherm, Klotz plot, Scatchard plot of the data are 1 x 10^4 M^{-1}, 50 ±10 and 1.3, respectively. The data is not obtained above a free CGA concentration of 1 x 10^{-4} M due to higher absorptivity of CGA alone contributing to the absorbance of nearly 2.0. Hence the data obtained is curve-fitted for minimal number of binding sites and K_a.

The low number of binding sites obtained by difference spectral titration is due to the reason that the fluorescence intensity of protein is sensitive even when ligand binds to non-

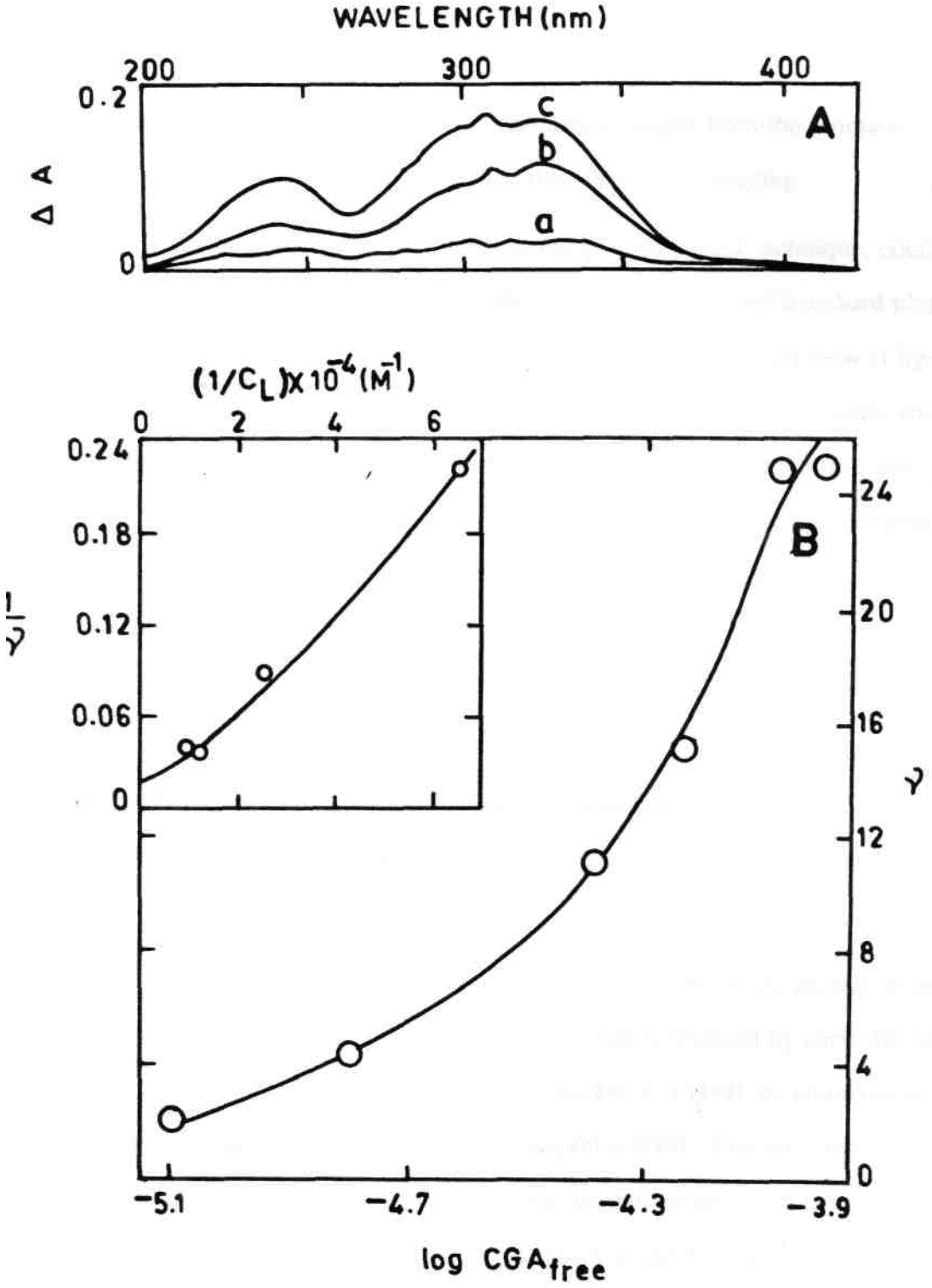

Fig. 8A. UV-difference spectra of 11S protein fraction in the presence of CGA at 10^{-5} 10^{-5} 10^{-4} (a) 2.94 x 10 M, (b) 5.95 x 10 M and (c) 1.89 x 10 M concentrations at pH 6.0. The protein concentration used for the experiment is 3.2 x lO^M. The baseline is obtained for protein versus protein without any ligand.

B. Binding isotherm of the CGA interaction with 11S protein fraction. The data is derived from the UV- difference spectral measurement at 325 nm. *Inset* Klotz plot of the interaction data obtained from the UV-difference spectra.

absorbing regions of the protein molecule due to energy transfer from the aromatic moieties to the CGA molecule, however, absorption is not that sensitive for binding.

The interaction is studied by the direct binding measurement technique, equilibrium dialysis at 31°C. In Fig. 9A and B are shown the binding isotherm and Scatchard plot of the data. The binding isotherm curve is not complete since at higher concentration of ligand the binding is not measurable that is due to favourable self association of protein and CGA molecules respectively. However, this precipitation is not seen when using low protein concentration. The Scatchard plot is typical of positive cooperativity in binding. The simultaneous curve-fitting of the binding isotherm with the Scatchard plot and Klotz plot for minimal number of binding sites and the association constant is performed for the data. The expermental data is curve fitted using K_a, n and n_H of 3.5 x 10^4 M^{-1}, 400 ± 100 and 3.67, respectively.

The higher association constant obtained by equilibrium dialysis may be due to the positive cooperativity observed where nearly 100 fold more protein is used,compared to fluorescence and uv-spectral measurements.

In Fig. 3C is shown the sedimentation equilibrium pattern of the protein in presence of ligand. The molecular weight of protein - ligand complex obtained by curve fitting using the oligomeric equilibrium of US protein is 2,68,000 ± 15,000 in comparison to the molecular weight of protein alone obtained is 2,41,000 ± 5000. This increase in molecular weight of the protein in presence of CGA is due to the binding of CGA. At this low concentration of protein an approximate calculation of possible total ligands bound to the protein purely based on weight average molecular weight is 80 ± 10.

Since the binding of CGA resulted in an increase in the fluorescence emission maximum significantly, any large conformational changes brought out by this ligand is monitored by circular dichroic measurements. In Fig. 10 is shown the far-UV CD spectra of the protein in presence of CGA. The alpha-helix, beta-sheet, beta-turn and aperiodic

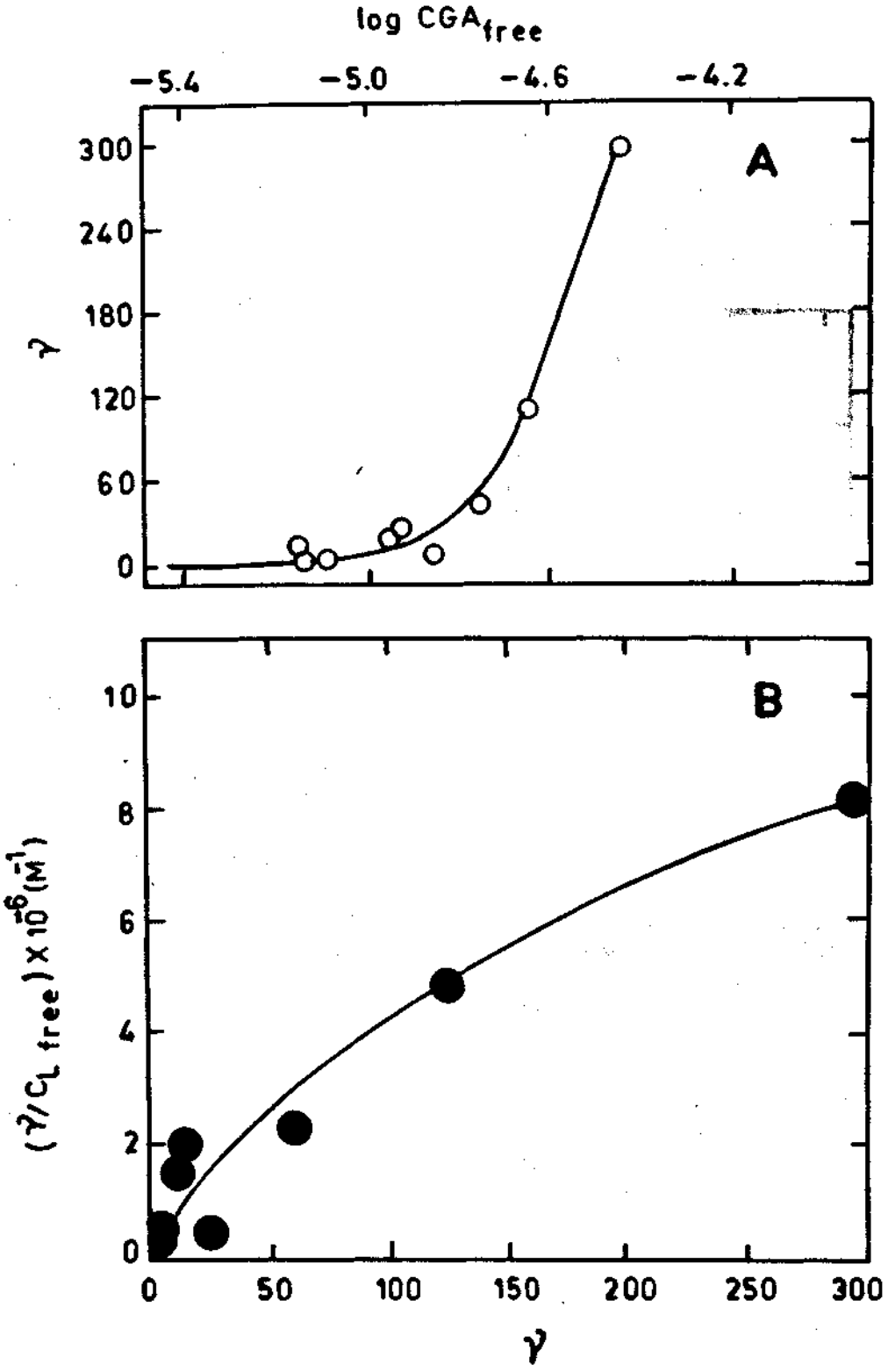

Fig. 9A. Binding isotherm of the CGA interaction with 11S protein fraction obtained from equilibrium dialysis. The experiment is in 0.15 M phosphate buffer, pH 6.0. The protein concentration used for the experiment is 1.04x10*^{5}M.

B. Scatchard plot of interaction of 11S protien fraction -CGA data derived from equillibrium dialysis experiments.

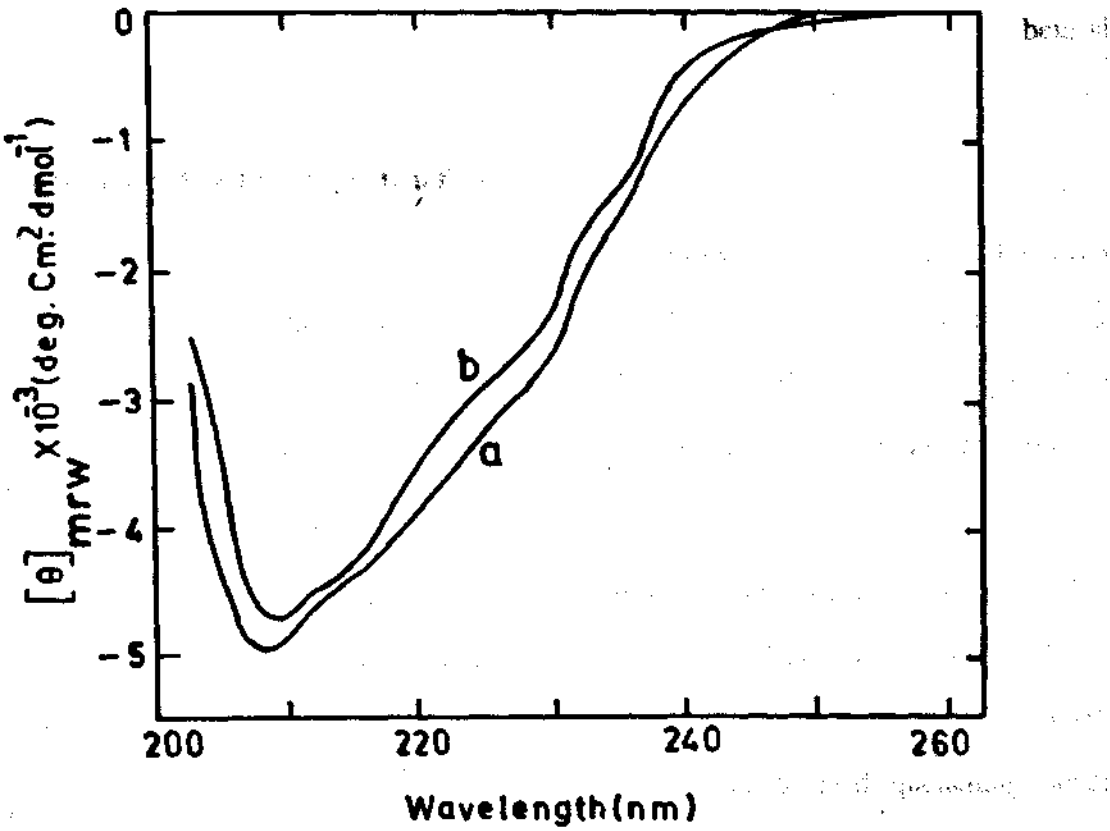

Fig. 10. Far-UV circular dichroic spectra of (a) native 11S protein fraction (b) 11 S protein fraction in presence of 1 x 10^{-5} M CGA. The protein concentration used is 1.9 x $l0^{-6}$M.

structure contents of the protein in presence of 9.8 x 10-^{6} M CGA are 7%, 25%, 26% and 42%, respectively, and in presence of 8 x 10-^{5} M CGA are 6%, 24%, 26% and 44%, respectively. The control protein has 9% alpha-helix, 20% beta-sheet, 28% beta-turn and 43% aperiodic structure as determined from CD spectra. This data indicates that there is marginal decrease in alpha helix and significant beta turn contents change while beta sheet content is increased.

Interaction of CA with 11S protein fraction

Caffeic acid is intrinsically present in the sunflower seeds. CA is the aromatic constituent of CGA. The interaction of CA with 11S protein will lead to the further understanding of the nature of interactions involved in the complexation of CA part of CGA. The interaction of CA with the protein is studied as a function of temperature by measuring the fluorescence emission spectra.

The fluorescence emission spectra of protein are measured in presence of CA and corrected for inner filter effects due to the absorption of CA. There is a shift in the fluorescence emission maximum towards higher wavelengths by nearly 8 nm at 5.4 x 10-^{5} M CA. This effect is similar to that of CGA. The normalized quenching spectrum ($\Delta F_{corr}/F_0$ versus λ) of fluorescence intensity has a predominant peak at 310 nm. The 310 nm peak is due to the efficient energy transfer of the emitted energy of exposed tyrosine(s) wavelength to the bound CA which has its maximum absorption coefficient at 311 nm. Quenching is also significant at other wavelengths, however, could be due to the long range, low efficient energy transfer. This data suggests that tyrosine is present at the CA binding site.

The above data is further supported by the interaction of CA with model compound N-acetyl L-tyrosine ethyl ester. The K_a of the binding is 2 x 10^4 M^{-1} with a stoichiometry of one. The CA molecule also interacts with N-acetyl L-tryptophan ethyl ester. There are two classes of binding with tryptophan but single class with tyrosine. This difference may be

due to the difference in the nature of aromatic ring of these two amino acids. The two classes of binding is also observed in the case of the interaction of CGA with tryptophan. The association constant of the binding site of CA with the model peptide N-acetyl-L-tryptophan ethyl ester is (1.2 ± 0.1) x 10^4 M^{-1}. However, if one analyses the data carefully there is less than 1% fraction of different class of binding with an association constant of (1 ± 0.1) x 10^6 M^{-1}, quite possibly due to the result of interaction at high affinity binding site. However, since it is not significant the main values are derived from a single class of binding with model peptide.

The fractional binding of CA with 11S protein is calculated from the intrinsic fluorescence quenching data of protein. This data as a function of the concentration of CA is analyzed for the binding parameters. In Fig. 11A is shown the binding data as Scatchard plot. From the figure it is clear that there are two classes of binding sites representing the biphasic nature of the plot. The K_a calculated at 28°C is 3.7 x 10^4 M^{-1}. The average association constant for the individual classes are calculated by curve-fitting of the biphasic plot. There is a single class of binding sites at 11 °C and 20°C and two classes at 28°C, 35°C and 45°C as analyzed by Scatchard plot. In Table 4 is given the f_1, f_2, K_{a1}, K_{a2} of this binding at different temperatures. In Table 5 are given the K_a and the corresponding change in free energy values. In Fig. 11B is shown the plot of 1/1- β versus C_L^0/ β for CA binding with 11S protein fraction. The number of binding sites at 28°C are estimated to be 60 ± 10.

It is clear from the data of Table 5 that the free energy changes of the interaction are non-linear suggesting the presence of both hydrophobic interaction and hydrogen bonding/ionic interaction. The $\Delta C_p°$ of the data is positive. The $\Delta C_p°$ is more positive in the case of the data evaluated from the K_{a1} than K_{a2}. Thus it is clear that the high affinity class sites have more of hydrophobic component involved in it. The separation of the

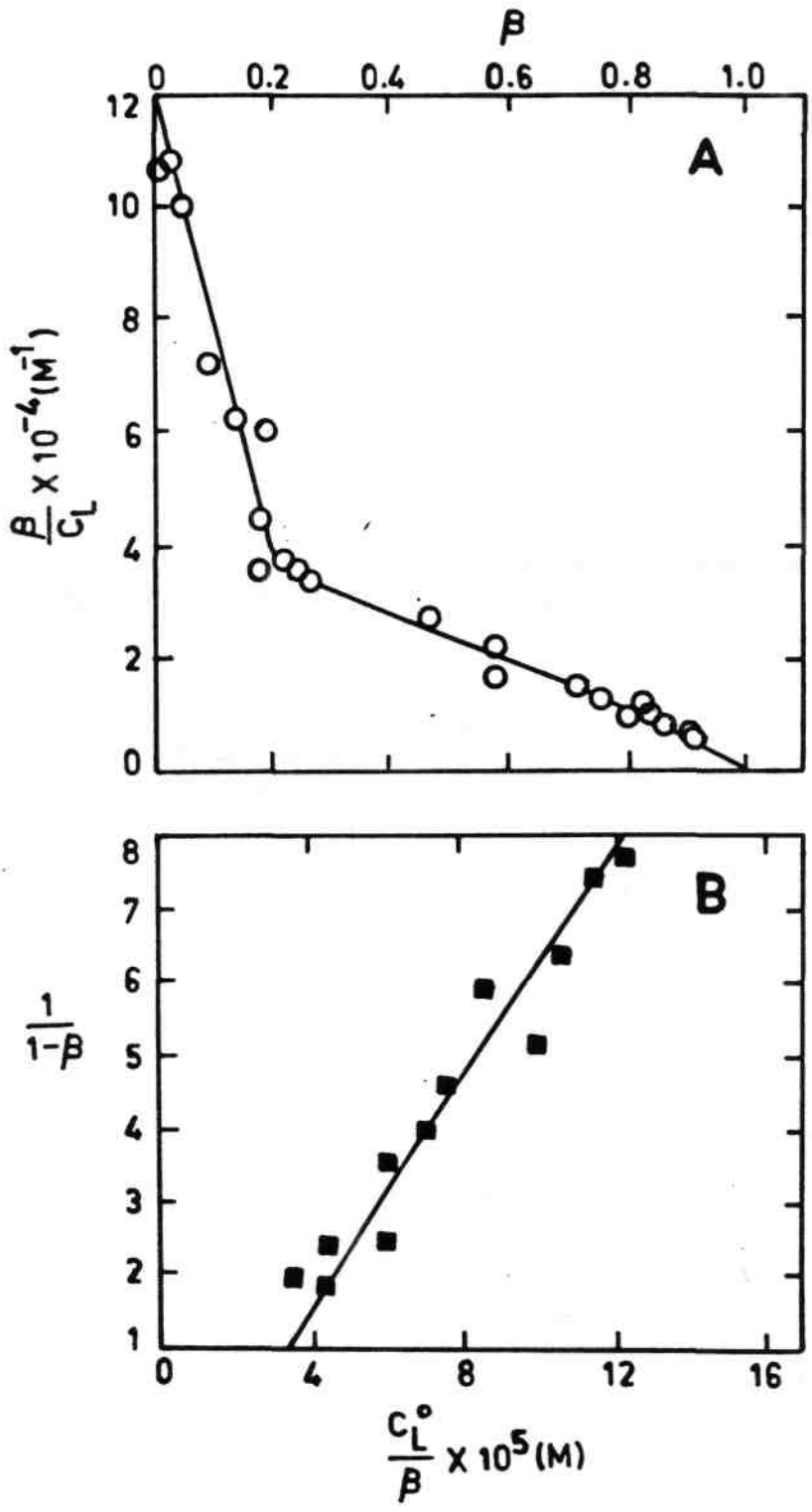

Fig. **11 A.** Scatchard plot of the interaction of CA with 11S protein fraction in 0.15 M phosphate buffer, pH 6.0. The protein concentration used is 4.7 x 10-^{7}M.

B. Plot of (1/1 —β) versus *(C_L o/* β) for the interaction of CA with 11S protein fraction. The data is derived from the fluorescence spectroscopic titration.

Table 4 : Binding parameters for the interaction of CA with US protein fraction at pH 6.0 obtained from fluorescence spectroscopic titration

Temperature (°C)	f_1	$K_{a1} \times 10^{-6}$ (M^{-1})	f_2	$K_{a2} \times 10^{-4}$ (M^{-1})
11±1	*	*	1.00	3.90±0.20
20±1	*	*	1.00	3.25±0.10
28±1	0.12±0.02	0.8±0.10	0.88±0.02	3.30±0.20
35±1	0.15±0.02	1.6±0.15	0.86±0.02	2.60±0.15
45±1	0.25±0.04	3.0±0.20	0.75±0.04	4.00±0.20

♦This class is not detected at these temperatures

Table 5 : Association constants and the corresponding free energy changes for interaction of CA with US protein fraction as a function of temperature at pH 6.0

Temperature (°C)	K_a x $10^{-4}(M^{-1})$	ΔG (kJ/mol)
11±1	3.85 ± 0.30	-25 ± 1.5
20±1	3.22 ± 0.11	-25 ± 1.5
28±1	3.70 ± 0.30	-26 ± 2.0
35±1	3.03 ± 0.22	-26 ± 2.0
45±1	5.26± 0.50	-29 ± 2.0

contributions of hydrophobic and ionic/hydrogen bonding components is tried only at 28°C by the interaction data of CA with modified proteins as described later.

Since the temperature dependent interaction energetics have suggested the presence of both hydrophobic and ionic bonding interactions in the binding of CA, the interaction is therefore carried out with the protein that has more net negative charge (succinylation), with the tryptophan modifed protein (tryptophan is a hydrophobic residue), and with the succinylated and tryptophan modified protein. The K_a of the interaction of CA with tryptophan modified protein is 1.5 x 10^4 M^{-1} and 2.2 x 10^4 M^{-1} with succinylated and tryptophan modified protein suggesting the dominance of hydrophobic interaction (the K_a with the native protein is 3.7 x 10^4 M^{-1}). The double modification of lysine and tryptophan residues has resulted in a single class of binding sites with a decreased strength of binding when compared to the low affinity class of the CA binding with native protein. These results suggest that both lysine and tryptophan are present at high affinity sites. The addition of 1M NaCl also decreased the strength of the interaction of the high and low affinity class sites comparable to that of tryptophan modified protein. The presence of high affinity class of sites on the tryptophan modified protein for CA is due to the binding with tyrosine residues.

In an other experiment the ligand CA is added to form the complex and then titrated with N-bromosuccinimide. The result showed that three residues are protected out of six exposed residues of 1IS protein, towards modification.

The interaction is followed by UV-difference absorption spectroscopy at 29°C. The data measured at 311 nm is analyzed for the binding parameters. In Fig. 12A is shown the fractional binding plot, (β/l-β) versus free concentration of CA. The plot is biphasic indicating two classes of binding sites. The K_a evaluated from the plot is 5.95 x 10^4 M^{-1}. In Fig. 12B is shown the Scatchard plot of **the** data. The curve represents two classes of binding profiles.

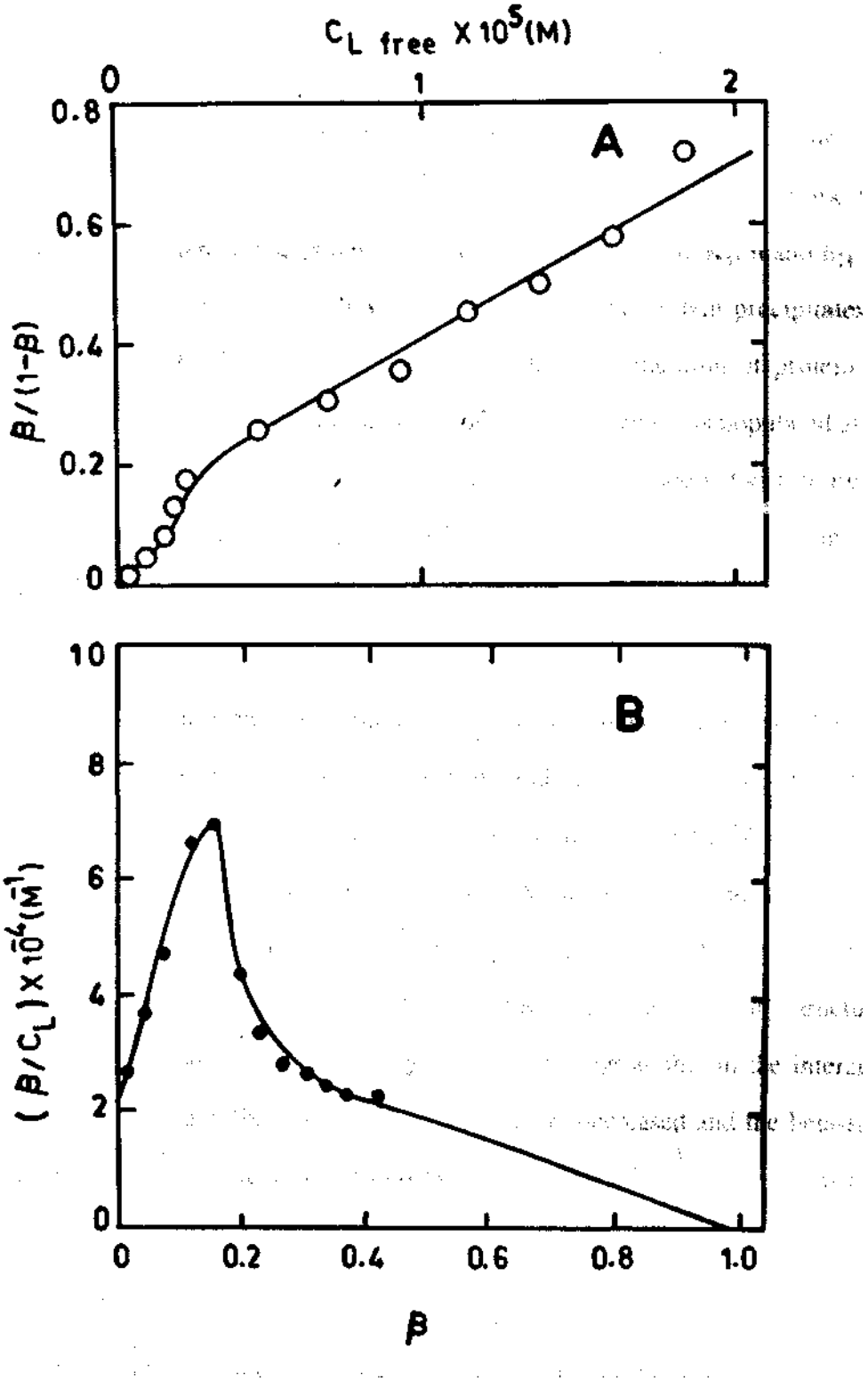

Fig. 12A. Plot of (β/1- β) versus free concentration of CA in the 11S protein - CA interaction data from UV-absorption spectroscopic titration in 0.15 M phosphate buffer, pH 6.0. The protein concentration used is 1.85 x $l0_{-6}$M.

B. Scatchard plot of the interaction data derived from the above titration experiments.

The interaction of CA with 11S protein is also followed by equilibrium dialysis. In Fig. 13A and B are shown the binding isotherm and Scatchard plot of the data, respectively. The obtained experimental data is best curve fitted by the intrinsic K_a, n and n_H of 6.4 x 10^4 M"[1], 60 and 1.93, respectively. Above 5 x 10-[5] M CA the protein precipitates and ligand depletion is zero. The cooperativity is due to higher concentration of protein used where hexamer concentration will be more than that of monomer (which is populated at low protein concentrations). The association constant obtained by this method is twice more than that of the data of fluorescence spectroscopy of CA binding at low affinity class of sites. And this is due to the cooperativity. The high affinity sites are not detected by this method, as also in the case of CGA.

The fluorescence emission maximum is increased in presence of CA. Does this change represents also the broad secondary structural changes is evaluated from the far-UV CD spectral analysis for the protein in presence of the ligand. In Fig. 14 is shown the far-UV CD spectra of the protein in presence of CA. The alpha-helix, beta-sheet, beta-turn and aperiodic structure contents of the protein in presence of 1 x 10^{-5} M CA are 7%, 24%, 26% and 43%, respectively, and in presence of 1 x 10-[4] M CA these secondary structural contents are 8%, 24%, 26% and 42%, respectively. This data suggests that in the interaction of CA with 11S protein alpha-helix and beta-turn contents are decreased and the beta-sheet content is increased to a similar extent to that in presence of comparable concentrations of CGA with protein.

Interaction of QA with 11S protein fraction

Quinic acid is the other constituent of CGA and it has hydroxyl groups and a carboxyl group. The interaction of QA with the protein could therefore possibly give an insight into the extent of hydrogen bonding and ionic interactions.

The interaction of QA with US protein is followed by fluorescence spectroscopic titration as a function of temperature. The addition of QA quenches the intrinsic fluorescence

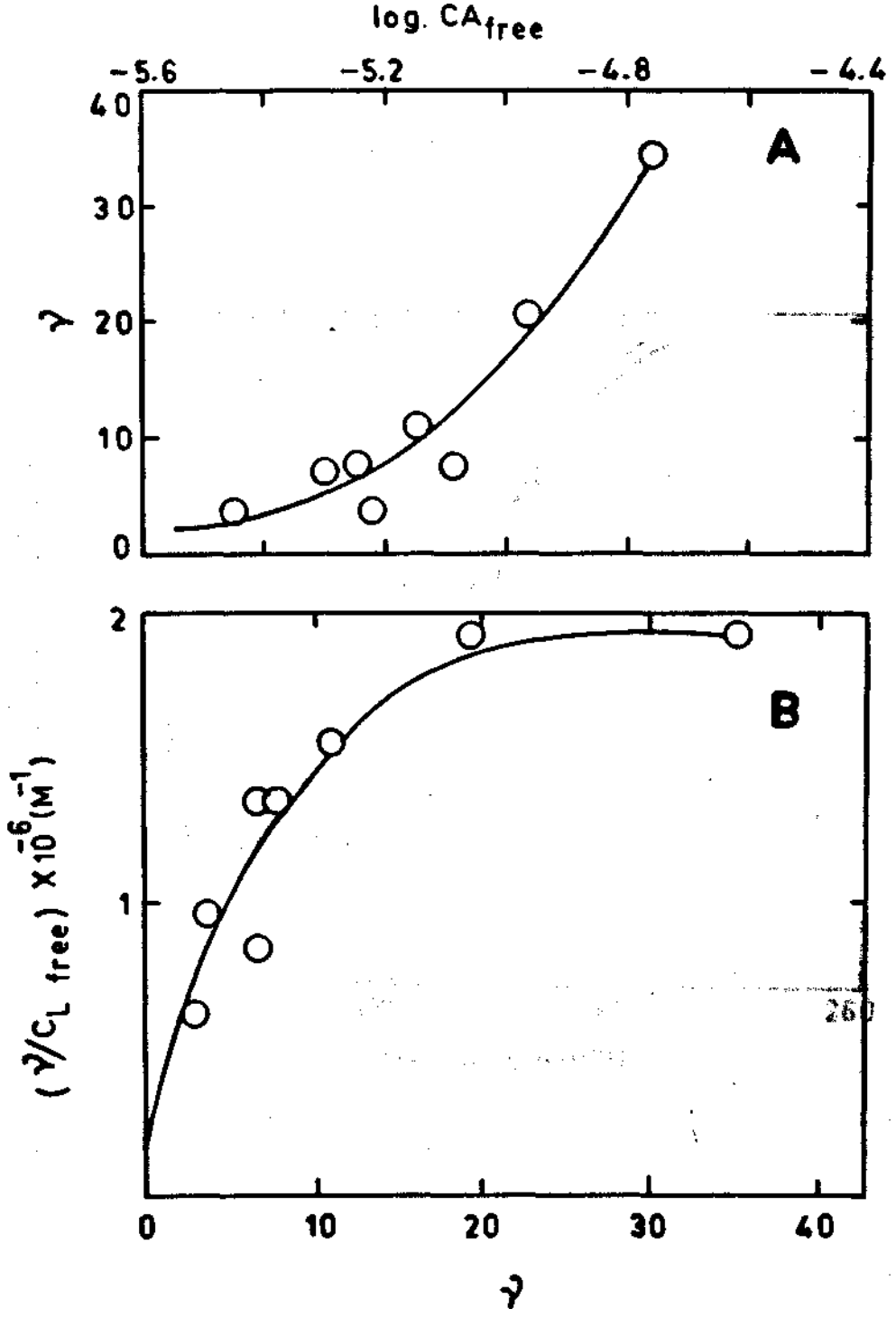

Fig. 13A. Binding isotherm of the CA interaction with 11S protein fraction in 0.15 M phosphate buffer, pH 6.0. The data is obtained from equilibrium dialysis experiment using a protein concentration of 1.03 x 10-^{5}M.

B. Scatchard plot of the CA interaction data with 11S protein fraction. The data is obtained from equilibrium dialysis.

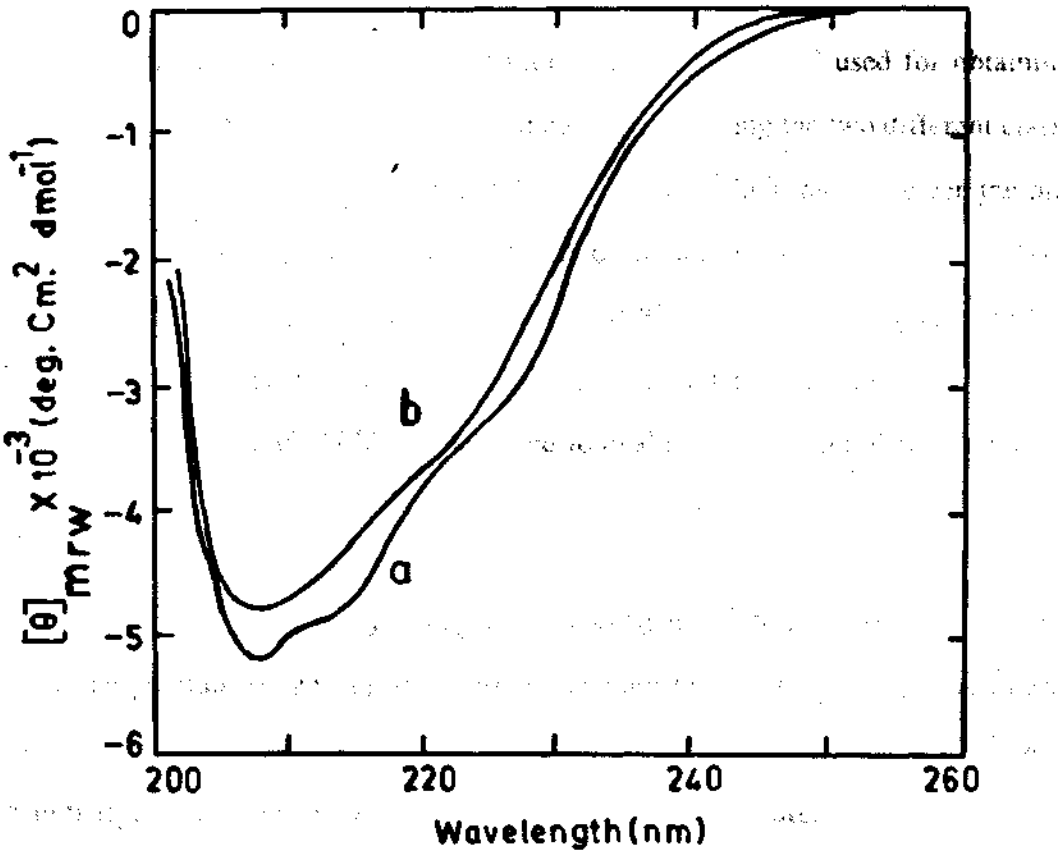

Fig. 14. Far-UV circular dichroic spectra of (a) native 11S protein fraction and (b) 11S protein fraction in presence of 1 x 10^{-5} M CA. The protein concentration used for the experiment is 1.86x10^{-6} M.

intensity of the protein similar to that of CA and CGA. However, the quenching is less when compared to CGA and CA, as QA is a non-absorbing ligand to overlap the quenching spectrum of complexation. There is no shift in fluorescence emission maximum of the protein upon binding.

In Fig. 15A is shown the Scatchard plot of the data at 28°C used for obtaining the interaction parameters K_a and fi. The plot is biphasic representing the two different classes of binding sites. The K_a value of the binding is 6.62 x 10^3 M^{-1}. In Table 6 is given the binding parameters such as fi and Kai for individual classes of binding sites. There are two classes of binding sites at 10°C, 15°C and 28°C, 35°C and 45°C. In Table 7 is given the binding parameters such as K_a, and the free energy change of the interaction. In Fig. 15B is shown the plot of (1/1- β) versus (C_L^0/ β) at 28°C used to evaluate n. The number of binding sites at 28°C is 64 ± 10.

As shown, the free energy changes from average binding constant values are not linear with temperature suggesting the presence of both hydrogen bonding/ionic interaction and hydrophobic interaction in QA binding. This non-linearity involves a positive $\Delta C_p°$ indicating that the binding is predominantly of hydrophobic in nature.

The interaction is carried out between QA and modified proteins at 28°C. The interaction is not at all present with succinylated protein. Therefore, this abolition of interaction probably is due to the change in a positive charge of lysine to negative magnitude upon succinylation.

The interaction is not detected with tryptophan modified protein also. Thus tryptophan moieties are involved in the interaction from this experiment and as indicated from the NBS protection experiments. However, the inability of QA to bind with the protein

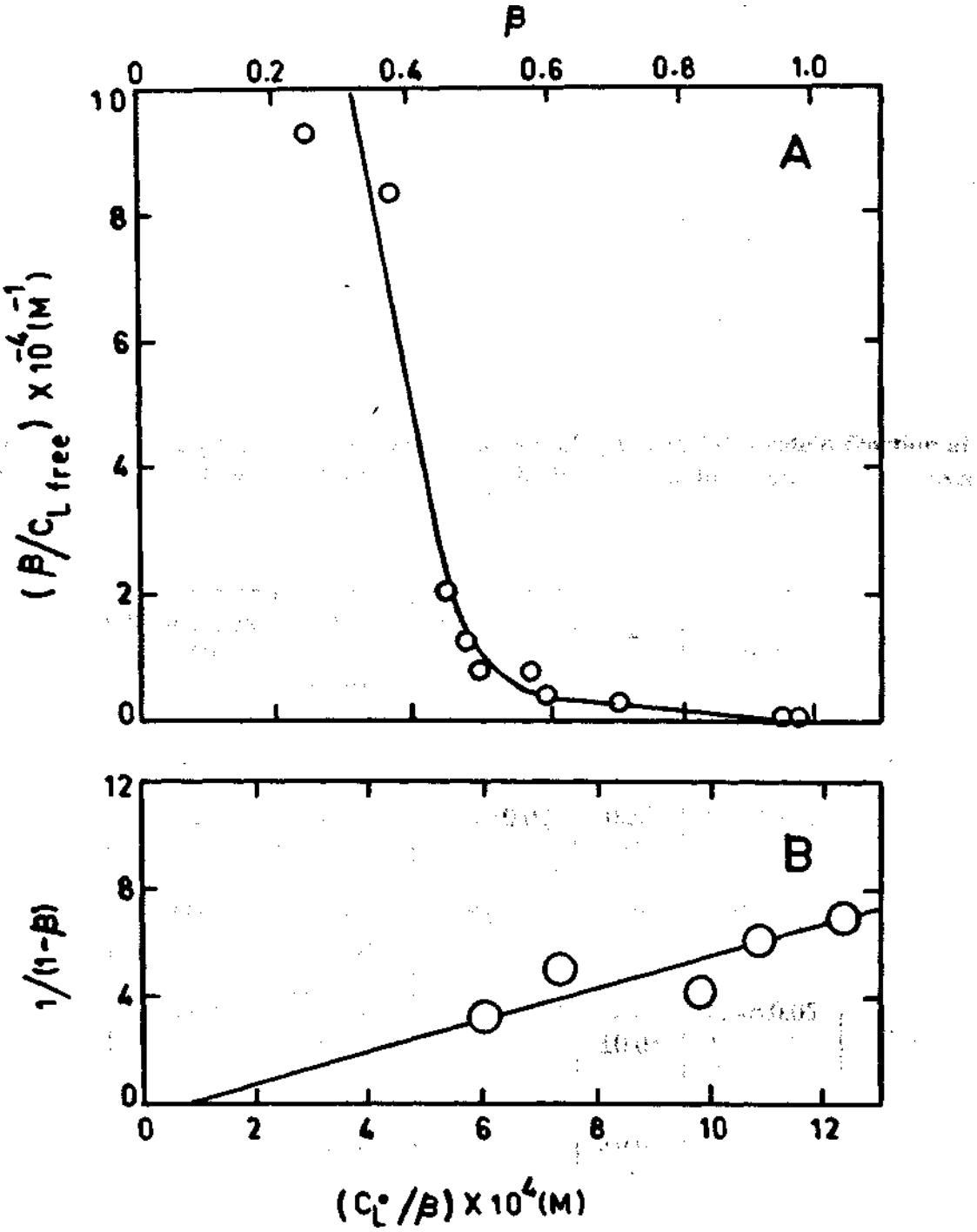

Fig. 15A. Scatchard plot of the QA interaction with 11S protein fraction at pH 6.0. The data is obtained by fluorescence spectroscopic titration.

B. The plot of (1/1- β) versus (C_Lo/ β) of the data obtained from the above titration experiment.

Table 6 : Binding parameters of the interaction of QA with US protein fraction at pH 6.0 for different classes of binding sites calculated from fluorescence spectroscopic titration

Temperature (°C)	f_1	$K_{a1} \times 10^{-5}$ (M^{-1})	f_2	$K_{a2} \times 10^{-4}$ (M^{-1})
10±1	0.50 ±0.05	0.30±0.05	0.50 ±0.05	0.10±0.01
15±1	0.45 ±0.05	0.40±0.02	0.55 ±0.05	0.10±0.01
28±1	0.40 ±0.05	6.00±0.50	0.60 ±0.05	0.40±0.02
35±1	0.50 ±0.05	3.50±0.45	0.50 ±0.05	0.60±0.05
45±1	0.50 ±0.05	30.00±1.0	0.50 ±0.02	3.20±0.40

Table 7: Association constants and the free energy changes for the interaction of QA with 11S protein fraction at pH 6.0 obtained from fluorescence spectroscopic titration

Temperature (°C)	$K_a \times 10^{-3}$ (M^{-1})	ΔG (kJ/mol)
10±1	2.00 ± 0.12	-18 ± 1.0
15±1	1.78 ± 0.10	-18 ± 1.0
28±1	6.62 ± 0.50	-22 ± 2.0
35±1	11.11 ± 2.60	-24 ± 2.0
45±1	64.00 ± 16.00	-30 ± 2.0

after tryptophan modification is due to the insensitiveness of the fluorescence spectroscopic signal due to binding at other sites. The important point is that unlike the CA and CGA which are absorbing in the region of intrinsic fluorescence spectrum of the protein the QA is non-absorbing therefore even it is binding to the non-aromatic moieties such as lysine as suggested it does not result in a change in fluorescence signal.

The results from the CD studies show that the control protein has 9% alpha-helix, 20% beta- sheet, 28% beta-turn and 43% aperiodic structure as estimated from CD spectra. In presence of 1 x 10^{-5} M QA the alpha- helix, beta-sheet, beta-turn and aperiodic structure contents of the protein are 10%, 20%, 28% and 42%, respectively, and 10%, 20%, 27% and 43%, respectively in presence of 1 x 10^{-4} M QA. Thus there are no significant changes in the secondary structure of the protein upon interaction with QA. It can be recalled that fluorescence emission maximum is also not changed by binding of QA.

Isolation and characterization of 2S protein fraction

The total protein is precipitated with 20% (w/v) ammonium sulphate and passed through a Sepharose 6B-100 gel filtration column. In Fig. 2B is shown the elution profile of this total protein. The peak eluting at a Ve/V_0 of 3.0 is collected and precipitated with 45% (w/v) ammonium sulphate. In Fig. 16 is shown the SDS-PAGE pattern of the precipitated protein which has a single diffused band. The molecular weight corresponding to this band is 13 K. The PAGE pattern and molecular weight very well correlates with earlier reports (Schwenke and Simon, 1974; Kortt and Caldwell, 1990). In Fig. 17A is shown the native PAGE pattern of the protein at pH 8.2. In Fig. 17B is shown the fluorescence emission spectrum of the protein. In Fig. 17C is shown the UV- absorption spectrum of the protein. The absorption maximum, fluorescence excitation maximum and fluorescence emission

Fig. 16. SDS-PAGE pattern of 2S protein fraction in Tris-glycine buffer, pH 8.2. The experiment is performed at a constant current of 3 mA/tube using 10% acrylamide gels with *0.4%* cross linking of bis acrylamide.

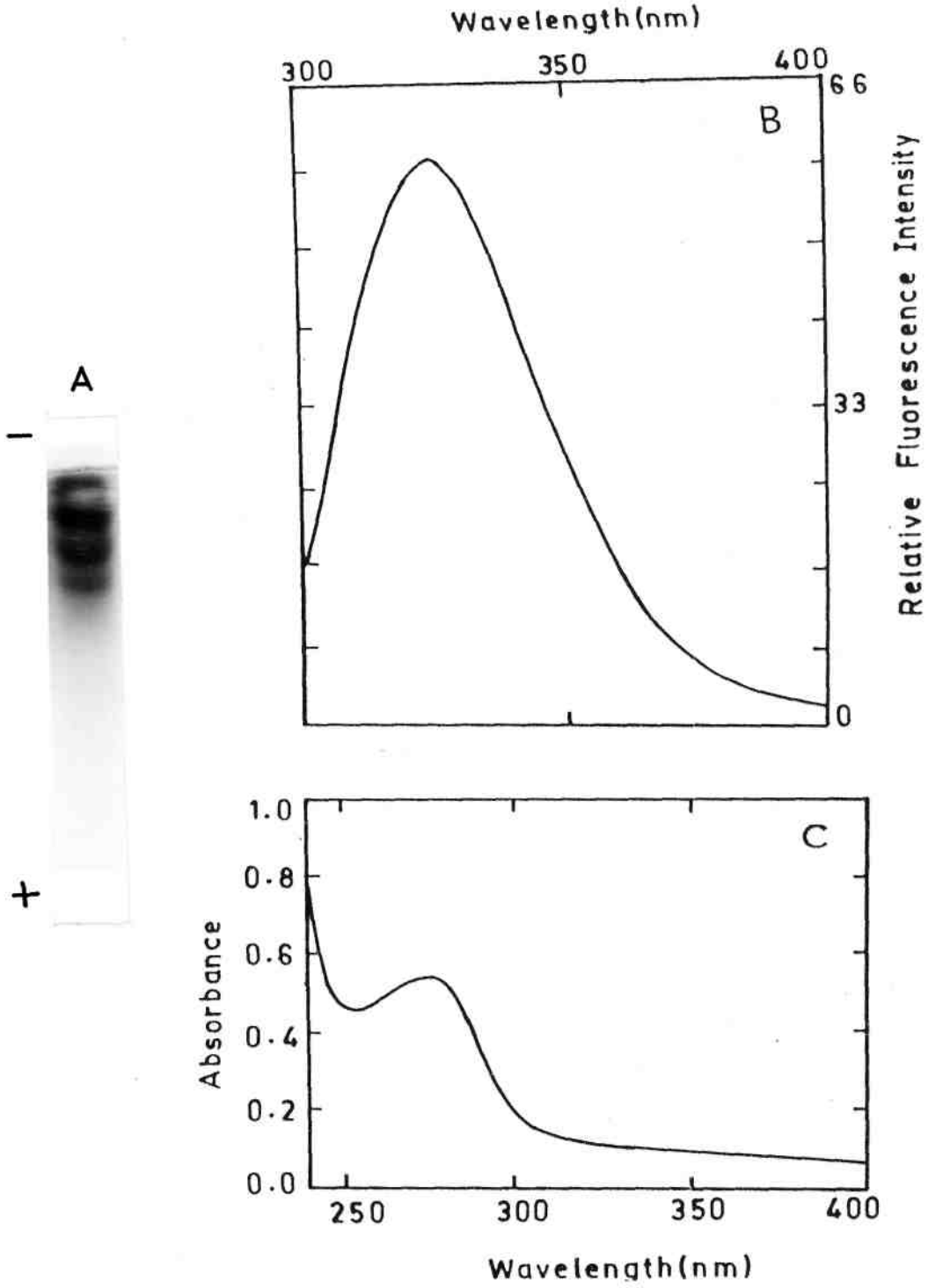

Fig. 17A. Native-PAGE pattern of 2S protein fraction at pH 8.2. The electrophoretic experiment is performed at a constant current of 3 mA/gel.

B. Fluorescence emission spectrum of 2S protein fraction in the wavelength regíon of 300-400 nm with an excitation maximum at 285 nm.

C. UV-absorption spectrum of 2S protein fraction.

maximum of the protein are 276 nm, 286 nm and 338 nm, respectively indicating the dominance of the spectral intensity by the buried tryptophan residues. This fraction is earlier shown to have a sedimentation coefficient of 1.82 S and is independent of protein concentration (Venktesh and Prakash, 1993).

Interaction of CGA with 2S protein fraction

The interaction of CGA with albumin fraction is carried out at pH 3.0, 5.8 and 6.6 at different temperatures of 20°C, 28°C and 37°C by fluorescence spectroscopic titration. CGA addition quenches the relative fluorescence intensity and this data is analyzed for the K_a from Scatchard plots. In Fig. 18 is shown the binding data at pH 3.0, 28°C, as Scatchard plot from which the K_a, fi and nH are evaluated by curve-fitting. The plot is biphasic indicating that there are two classes of binding sites. In Table 8 is given the various thermodynamic binding parameters of the data at pH 3.0 at different temperatures. In Table 9 is given the K_a and the interaction energetics. There is marginal increase in the ΔG with increase in temperature.

In Fig. 19A is shown the Scatchard plot of the binding data at pH 5.8, 28°C. It is clear from the figure that there are two classes of binding sites. In Table 8 is given the thermodynamic binding parameters for the individual classes of binding sites such as K_a, fi and n_H. There are two classes of binding sites at 20°C, 28°C and 37°C. In Table 9 is shown the K_a's as a function of pH and temperature. The ΔG at pH 3.0, 5.8 and 6.6 over the temperature range of 20 to 37°C remained constant in the range of -23 ± 2 kJ/mol.

In Fig. 19B is shown the Scatchard plot of the binding data at pH 6.6, 28°C. It is clear from the figure that there are two classes of binding sites. There are two classes of binding sites also at 20°C and 37°C at this pH. In Table 8 is given the thermodynamic binding

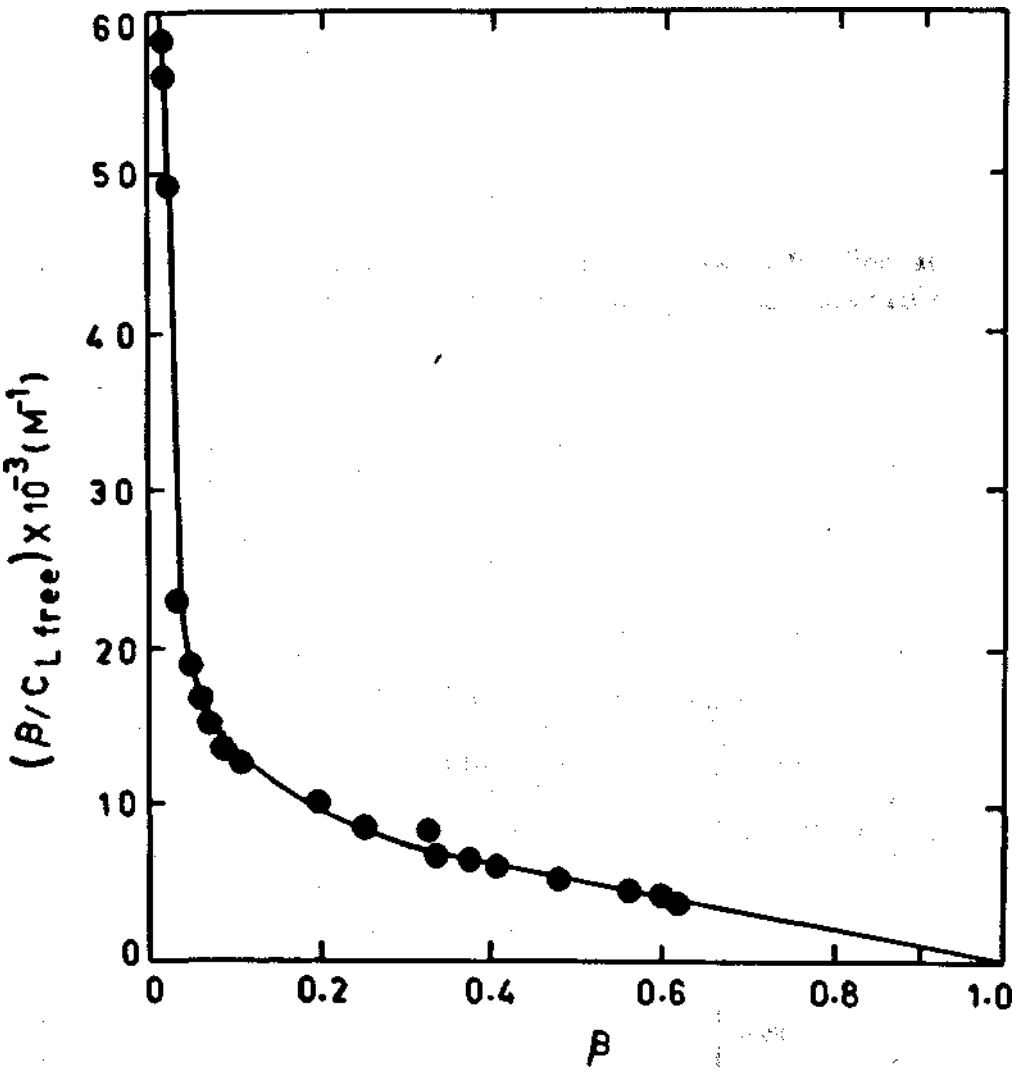

Fig. 18. Scatchard plot of the CGA interaction with 2S protein fraction at 0.02 M citrate-phosphate buffer, pH 3.0, at 28°C. The data is obtained by fluorescence spectroscopic titration. The biphasic curve indicates two classes of binding sites. Protein concentration used was 7.6 x 10 M.

Table 8: Binding parameters of CGA with 2S protein fraction at various pH and temperature values at different classes of binding sites as obtained from fluorescence spectroscopic titration

pH	Temperature (°C)	f_1	$K_{a1} \times 10^{-6}$ (M^{-1})	f_2	$K_{a2} \times 10^{-4}$ (M^{-1})
3.0	20±1	0.100±0.02	4.50±0.50	0.900±0.02	1.08±0.10
	28±1	0.022±0.005	5.50±0.50	0.978±0.005	1.00±0.10
	37±1	0.030±0.005	6.00±0.40	0.970±0.005	0.85±0.15
5.8	20±1	0.010±0.002	1.00±0.20	0.990±0.002	0.97±0.10
	28±1	0.010±0.002	2.00±0.10	0.990±0.002	0.74±0.10
	37±1	0.020±0.004	4.50±0.20	0.980±0.004	0.83±0.10
6.6	20±1	0.015±0.004	4.00±0.20	0.985±0.004	1.09±0.10
	28±1	0.015±0.004	1.50±0.20	0.985±0.004	0.85±0.10
	37±1	0.020±0.004	3.50±0.30	0.980±0.004	0.81±0.10

Table 9: Association constants and free energy changes of the binding of CGA with 2S protein fraction at different pH values and different temperatures

pH	Temperature (°C)	$K_a \times 10^{-4}$ (M^{-1})
3.0	20±1	1.20 ± 0.12
	28±1	1.02 ± 0.10
	37±1	0.88 ± 0.13
5.8	20±1	0.98 ± 0.10
	28±1	0.74 ± 0.11
	37±1	0.85 ± 0.10
6.6	20±1	1.11 ± 0.10
	28±1	0.86 ± 0.10
	37±1	0.83 ± 0.10

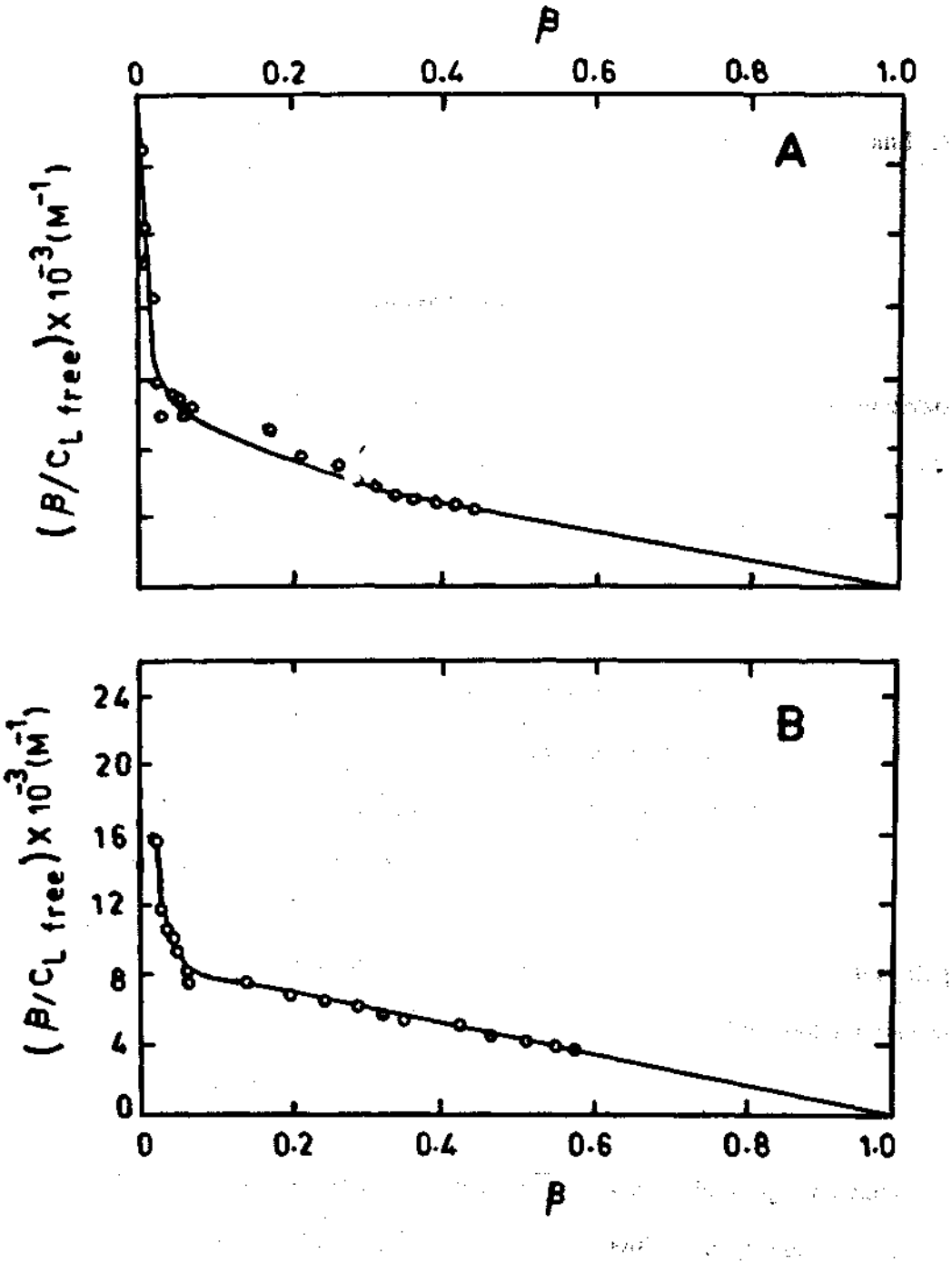

Fig. 19A. Scatchard plot of CGA interaction with 2S protein fraction in 0.02 M phosphate buffer, pH 5.8. The data is obtained by intrinsic fluorescence emission spectral titration of 2S protein fraction with **CGA.** A Protein concentration of 7.6 x 10-^{6} M was used.

B. Scatchard plot of **CGA** interaction with 2S protein fraction in 0.02 M phosphate buffer at pH 6.6. A Protein concentration 7.6 x l0^{-6}M is used in the experiment.

parameters for the individual classes of binding sites such as K_a, fi and n_H. In Table 9 is given the K_a and the interaction free energies. The ΔG is similar at 20°C and 28°C but marginally increased at 37°C.

Interaction of CA and QA with 2S protein fraction

The binding of CA with 2S protein fraction is studied at pH 5.8 to understand the contribution of the CA part in the interaction of CGA. In Fig. 20 is shown the scatchard plot of the binding data of CA with 2S protein fraction at 28°C. The plot is biphasic indicating two classes of binding sites. The fraction of the total binding at hight affinity class, f_1, is 0.01 ± 0.002 and fraction of total binding at low affinity class, f2 is 0.99 ± 0.002. The association constants at high affinity (K_{a1}) and low affinity (K_a2) class of sites is 8 ± 1 x 10^6 M^{-1} and 1.4 ± 0.2 x 10^4 M^{-1}, respectively. The K_{a1} and K_{a2} are higher in the case of CA binding when compared to the CGA binding. The K_a of the binding is 1.4 x 10^4 M^{-1}. The strength of the association of CA is higher than that of CGA binding.

The QA addition did not alter the intrinsic fluorescence intensity of the 2S protein fraction at 28°C, pH 6.0 with ionic strength of 0.02. Thus QA does not interact with 2S protein fraction is contrast to its interaction with 1IS protein fraction.

As shown, the association constants of high affinity class binding are nearly similar or little higher in the case of 2S protein fraction than with US protein. The marginal temperature dependence of the ΔG of binding of 2S protein fraction is similar to that with 11S protein.

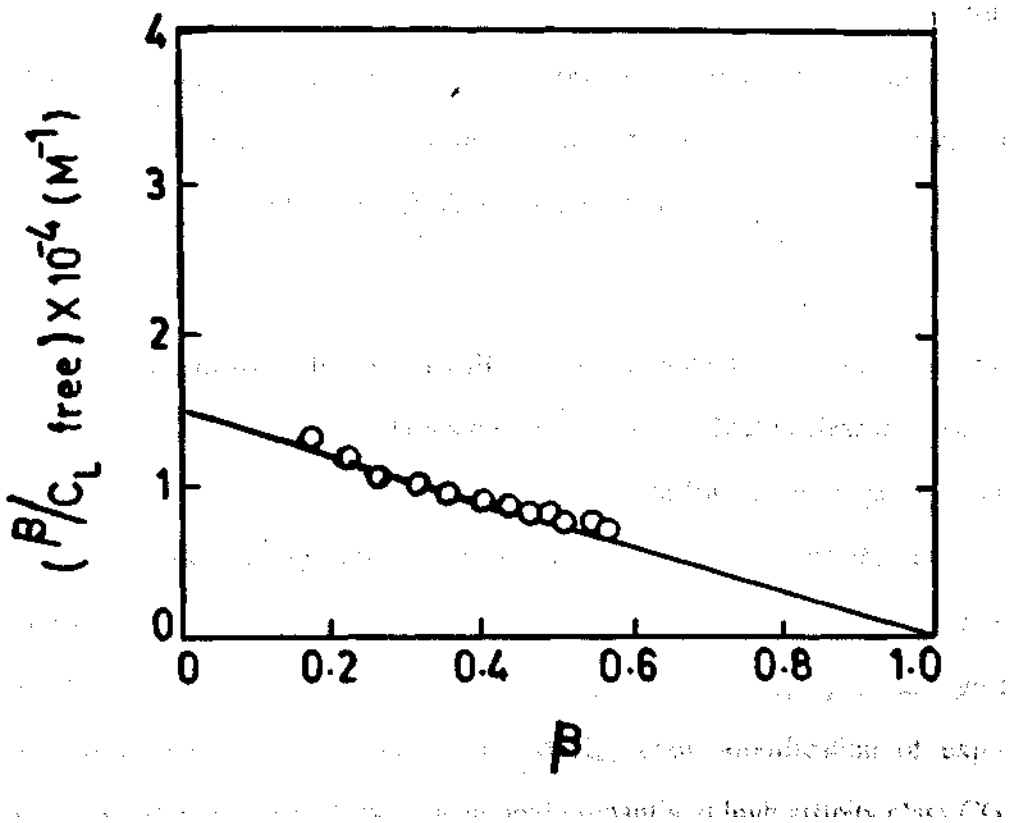

Fig. 20. Scatchard plot of the CA interaction with 2S protein fraction in 0.02 M phosphate buffer, pH 6.0.

Thus similar mechanism of the interaction of the protein is observed between 2S and 11S protein fractions which have completely different iso-electric pH values with 2S have an alkaline pI and 1IS has an acidic pI. What may be important therefore could be the positive net charge induced binding potential with a negatively charged ligand at pH 6.0 where the interaction is studied. In the case of 11S the protein is nearly neutral in charge potential while the 2S is positively charged and this difference can account for the increased interaction strength of the ligand with 2S protein fraction when compared to that with 1IS protein. In 11S protein, one of the subunit, the basic one is positively charged and is favourable to interact with the CGA molecules at this pH.

DISCUSSION

This following discussion will lead to the understanding of the nature of the binding from protein at high affinity class sites. From this study it is clear that there are two classes of binding sites for CA and CGA as measured from fluorescence spectroscopy. The number of binding sites of high affinity class for CA and CGA from this experiment ($n.f_1 = 6$) approximates to the number of tryptophan residues protected from NBS modification by complexation of CGA or CA and QA. The presence of tryptophan at high affinity class of sites is also indicated by the decreased K_{a1} upon modification of exposed tryptophan residues. The presence of tryptophan predominantly at high affinity class CGA sites are also indicated by the peak in normalized fluorescence quenching spectrum of the protein at 355 nm at ligand concentrations much lower than the reciprocal of K_a. The K_{a1} of CGA binding with protein or tryptophan is similar suggesting the presence of this amino acid side chain at the CGA binding site. As a result of CA complexation with protein at ligand concentrations lower than the reciprocal of K_a, there are three protected tryptophan residues from NBS modification.

The absence of high affinity class of sites of CGA with ANS bound protein suggests that predominantly hydrophobic residues are present in this class. This is the class where

interaction strengths are higher and thermodynamic parameters, ΔH and ΔS are increasingly become positive with increase in temperature for CA and CGA. The positive $\Delta C_p°$ is also indicative of the presence of hydrophobic bonding predominantly. Thus hydrophobic interaction involving the hydrophobic tryptophan residues is present in this class.

The presence of tyrosine at high affinity class is also indicated by the greater contribution of the fluorescence quenching at 310 nm than that at 332 nm or 355 nm at ligand concentrations lower than the reciprocal of K_a. The presence of a peak at 310 nm apart from that at 355 nm in the normalized quenching spectrum at low ligand concentrations also indicates that tyrosine is present at high affinity class of sites. However, tyrosine alone can not contribute to the total magnitude of the high affinity class site binding as is in the case of binding with tryptophan, since interaction of tyrosine alone with CGA has an association constant of 6×10^3 M^{-1} (from model compound study), however, the high affinity class K_a is 1.6×10^6 M^{-1} at 28°C.

The association constant of CA binding at high affinity class is similar to that of CGA binding at high affinity class. In contrast, the association constant and total number of binding sites of high affinity class of QA are completely different from that of CGA binding. Thus CA part of the CGA binds at the high affinity class of sites.

The other similarity in the binding of CA and CGA is that of the secondary structural changes measured by circular dichroism and the change in fluorescence emission maximum of the protein. In contrast there are no significant conformational changes for QA binding as detected by circular dichroism and fluorescence emission maximum. These secondary structural changes are saturated at low ligand concentrations. The β-pleated sheet content increase is probably due to the formation of such conformation by interaction of CGA with ketoimide groups of the protein which supports the earlier view of protein-polyphenol interaction (Haslam, 1974).

The decrease in the binding affinity of CGA at high affinity class of sites by addition of QA is due to the competitive nature of the binding of QA with QA part of CGA. Thus QA part of CGA is also involved in the binding at high affinity class of sites. This is also suggested by the result that three tryptophan residues are protected from NBS modification at ligand concentrations lesser than the K_a. Further evidence that QA binds to tryptophan is evident from its binding with N-acetyl L-tryptophan ethyl ester but not with N-acetyl L-tyrosine ethyl ester. ,

There is a comparative similarity in the binding of CGA with model compound tryptophan and with the protein in terms of number of binding classes. Even CA binds to the tryptophan with two classes of nearly identical association constants as CGA binds with. Thus the similarity of binding is due to the common aromatic group of CA and CGA. However, this could be .due to the two types of complexes of tryptophan-CGA or tryptophan-CA being formed, with the high affinity class complex type being less populated and low affinity site class complexes being highly populated.

In contrast the binding of CGA and CA with model compound tyrosine is of single class with its association constant more closely equivalent to that of low affinity class sites of protein. Thus, there is commonality in the interaction of either CA or CGA with respective to their binding with tyrosine with a single class, and two classes with tryptophan. This difference in the number of classes of binding of tyrosine and tryptophan with either CA or CGA is due to ther difference in the structural component of aromatic rings of the two amino acids.

From the above discussion it is clear that both tryptophan and tyrosine residues are involved in the high affinity class of sites. From the results of NBS protection experiments and fluorescence spectroscopy it is probable that three high affinity class binding sites consists of the binding of CA with tryptophan residues, and the other three binding sites of

high affinity class are formed by tyrosine at the CA binding subsite and tryptophan with QA binding subsite.

The free energy changes as calculated from the average dissociation constant values are non-linear and increasing ΔH and AS being obtained with increase in temperature for CA and CGA in the temperature range 10°C - 45°C, however, the ΔH and ΔS are decreasing with increase in temperature for QA in the temperature range of 15 - 45°C. The $\Delta C_p°$ is positive for CA, QA and CGA binding with US protein. These are indicative of predominant hydrophobic bonding in CA or QA or CGA binding with the protein.

As regarding the nature of the binding sites, the low affinity class of binding sites have predominantly hydrophobic interaction since the $\Delta C_p°$ as calculated from the average dissociation constant is positive for CGA, CA and QA binding with 1IS protein. At the binding sites of CA and as well as at the binding sites of QA, the CGA molecule binds. Thus the sum of the number of binding sites of CA and QA are equal to the total number of binding sites of CGA.

In the case of human serum albumin (Muralidhara and Prakash, 1997) the kinetics at 10°C and 40°C shows that the interaction is relatively a slow process and no secondary structural changes are observed in the native human serum albumin molecule at different temperatures used in their study. They have also shown that the stereochemical behaviour of individual ligand molecules such as CA, QA and CGA play a dominant role in the mechanism of the binding. In the case of human serum albumin, CA had a higher strength of binding as compared to CGA and QA.

Since in the present study this protein, a multimeric protein the contribution of the dissociation of the protein has a large role in binding initiated complex of the protein. Further, since this is an acidic protein with a large amount of hydrophobic amino acids, the hydrophillicity/hydrophobicity of **the 11**S protein fraction would be a major deciding factor

when compared to monomeric protein human serum albumin as has been reported by Muralidhara and Prakash, 1997.

Many proteins have been used in this kind of study to understand the mode of interaction of polyphenols with proteins (Loomis *et al,* 1974; Oh *et al,* 1980). Thus the balance between the stereochemistry of the ligand, the temperature of the interaction study as well as the free concentration of ligand would drive the complex towards the saturation point (For example at exceedingly large ίigand/protein ratio) and in this study the conformational changes induced as a result of binding of these ligands does not appear to alter the association-dissociation of the protein. Thus the binding of the polyphenols does not affect the subunit interactions as in 1IS protein fraction. The role played by indirect effect of water structure on the binding phenomena of polyphenols can not be ignored (Jencks, 1969).

At higher concentrations of the protein the cooperativity in binding is observed and this is due to the multimeric nature of this protein. At low temperatures of the study also this cooperativity is observed and this is due to the temperature dependent oligomerization of the protein with the oligomer dissociating at higher temperatures.

Chapter 2

INTERACTION OF GUANIDINIUM SALT WITH 11S PROTEIN FRACTION

INTERACTION OF GUANIDINIUM SALTS WITH 11S PROTEIN FRACTION

Understanding of how proteins function is related to the structure of protein and the energetics of its interaction with ligands. The structural stability is influenced by the interaction with ligands as described in the earlier chapter. The extent of change in structure brought out by the ligand interaction is dependent on the conformational stability of the protein. The pathway by which the protein unfolds and refolds is important including the presence of stable intermediates as they alter the conformational stability. Usually the protein unfolding involves a two state model defined by the reversible pathway N <-> U where N represents the native state and U represents the unfolded state of the protein.

In the case of multimeric proteins there will be an extra step of dissociation. The dissociation and unfolding of the protein occur either sequentially or simultaneously. In the simultaneous process the entire transition depends on many factors, among which an important parameter is protein concentration. If the dissociation is a distinct step, this step depends only on the protein concentration. In this study the interaction of guanidinium salts with 11S protein fraction is carried out to understand the nature of the dissociation and unfolding by measurements of several biophysical parameters by different techniques.

A. Interaction of GuHCl with 11S protein fraction

Interaction of GuHCl with 11S protein fraction is studied by the velocity sedimentation technique to monitor the association-dissociation pattern. In Fig. 21A is shown the velocity sedimentation profile of the protein in 0.5 M (lower pattern) and 1M GuHCl (upper pattern). From the figure it is clear that there are three peaks corresponding to the sedimentation coefficients of 11S, 7S and 2S. In Fig. 21 B is shown the percent composition of 11S, 7S and 2S protein fractions. The percent fraction of 7S component increases with increase in concentration of GuHCl upto 1.2 M above which it decreases. The percent fraction of 2S is gradually increases with the increase in the concentration of GuHCl,

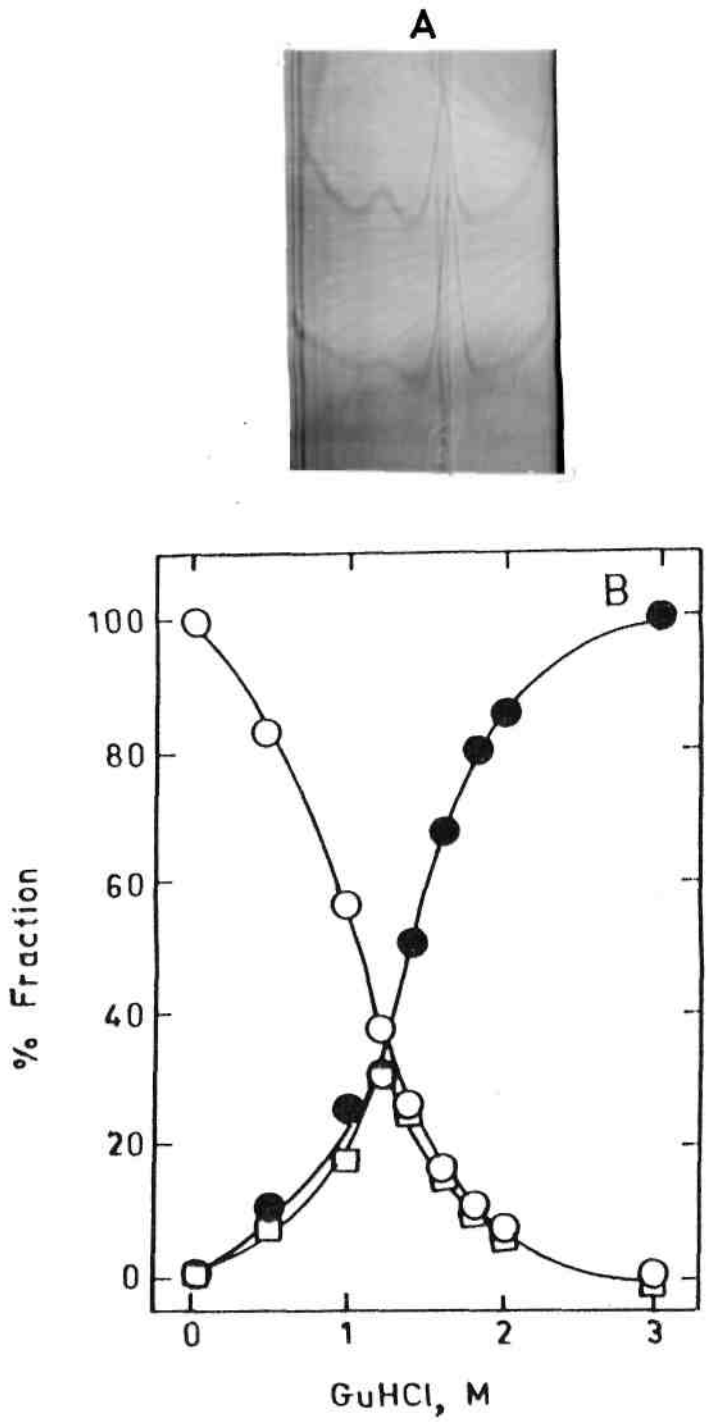

Fig. 21 A. Velocity sedimentation pattern of the 11S protein fraction in presence of 0.5 M GuHCl (lower), and 1M GuHCl (upper). The photograph was taken at 49th minute after reaching 2/3 maximum speed of 59780 rpm. Identical protein concentration of 10 mg/mL was used in both the experiments.

B. Effect of GuHCl concentration on the association-dissociation of 11S protein fraction. Percent fractions of 11S (-O), 7S (-α-) and 2S (-#-) protein fractions as a function of GuHCl concentration.

and in 3M GuHCl the protein is completely in 2S form. Thus this data indicates that the dissociation of 1IS occurs to 2S through an intermediate, 7S component.

The velocity sedimentation study suggests that the 1IS protein fraction is completely in 2S form in 3M GuHCl and this experiment is carried out at 10 mg/mL concentration of protein. The unfolding data obtained by techniques that are sensitive to the change in tertiary structure like fluorescence spectroscopy needs lesser protein concentration. At this low protein concentration the protein may or may not exist in a multimeric form to clearly know the nature of dissociation and denaturation. Hence measurements of the viscosny of the protein in presence of different concentrations of GuHCl is made at similar protein concentration as used in velocity sedimentation.

In Fig. 22 is shown the reduced viscosity profile of US protein fraction in various concentrations of GuHCl. The native protein has a reduced viscosity of 3.8 mL/g. The reduced viscosity increases to 28 mL/g in 4 M GuHCl and above which there is no further change. This value is in the range of the viscosity values obtained for completely random coiled structure of other proteins (Tanford, 1968). This higher concentration of GuHCl needed for completion of denaturation process as measured by viscosity (4 M) when compared to that needed for complete dissociation (3 M), while carrying out the experiments at same protein concentration, suggests that the dissociation and denaturation reactions probably are sequential. The data is not obtained between 0.7 M GuHCl and 2.5 M GuHCl due to precipitation of protein in this region.

The exposure of aromatic chromophores of the protein during unfolding is studied by difference absorption spectral measurements. In Fig. 23 A is shown the difference absorption spectra of 1 IS protein fraction in presence of 1 M, 2 M and 3 M GuHCl. It is clear from the figure that there are three peaks that have difference absorbance [(protein + GuHCl) - native protein] of negative magnitude in the range 260-320 nm. They are at 280 nm, 287 nm and 292 nm. These peak wavelengths indicate the exposure of both tyrosine and tryptophan

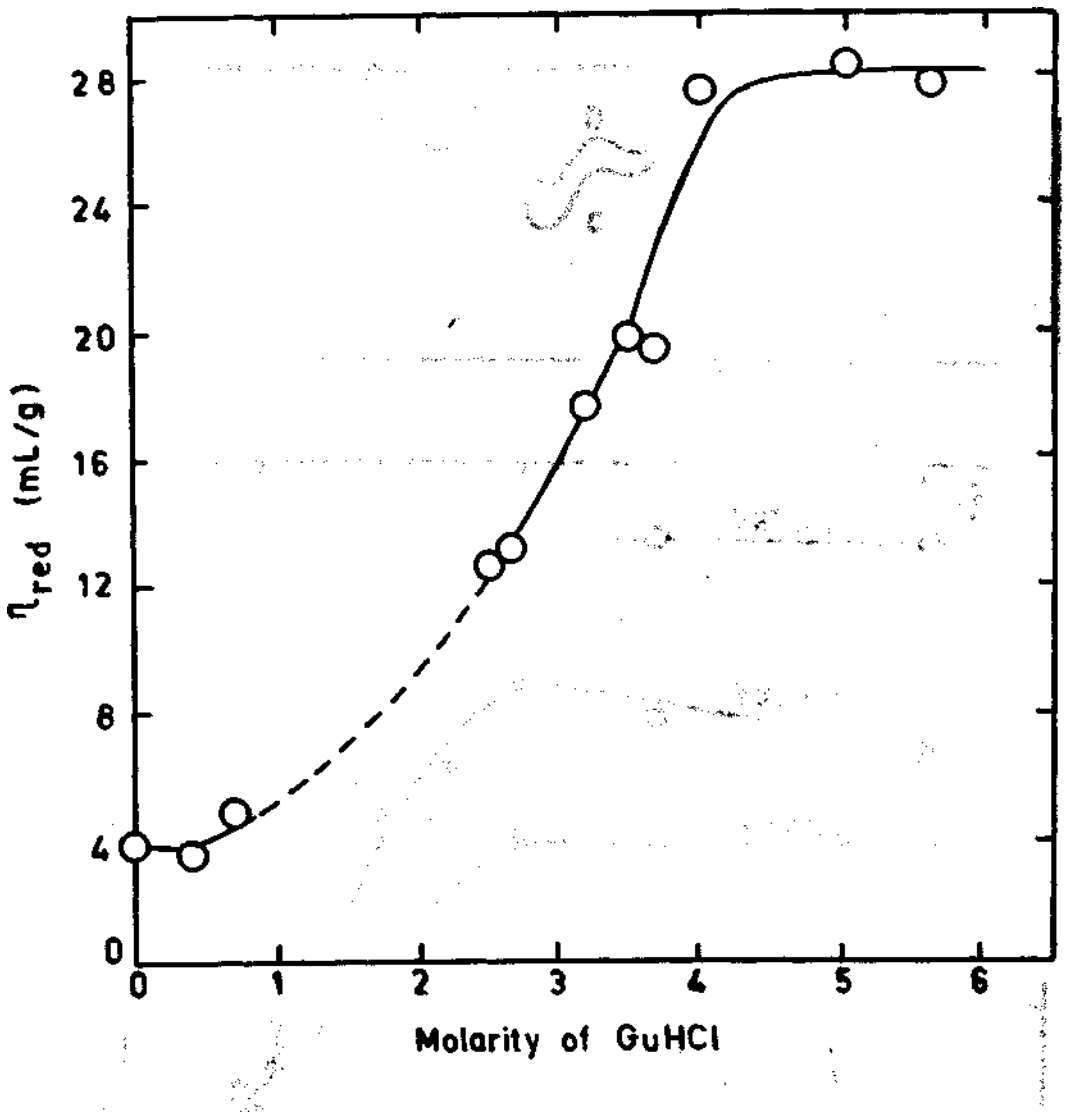

Fig. 22. Reduced viscosity profile of 11S protein fraction in various concentrations of GuHCl. A Protein concentration of 4.1 x 10^{-5} M is used. Dotted line on the curve indicates the non-availability of data due to protein precipitation.

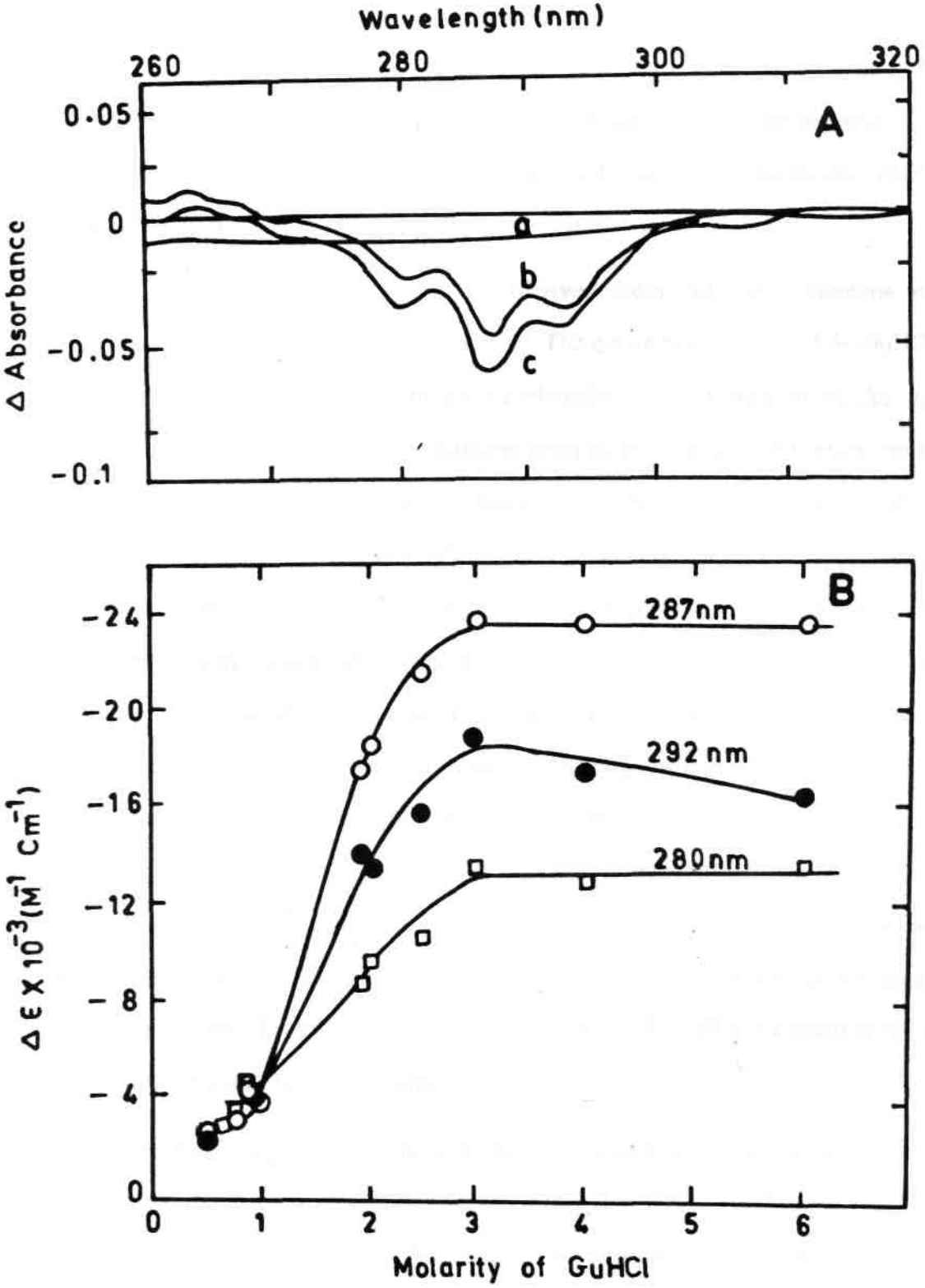

Fig. 23A. UV-difference absorption spectra of 11S protein in presence of (a) 1M GuHCl (b) 2M GuHCl and (c) 3M GuHCl concentration. Protein concentration of 2.5 x 10^{-6} M was used in all the experiments. The baseline is obtained for protein versus protein without any ligand.

B. Changes in Δe of 11S protein fraction as a function of GuHCl concentration at different wavelengths.

residues of the protein to the bulk polar solvent (Donovan, 1969). The negative magnitude is observed earlier for other proteins (Herskovits and Jaillet, 1969; Herskovits, 1967; Prakash and Nandi, 1977).

In Fig. 23 B is shown the Δe (derived from ΔA) as a function of GuHCl concentration at 280 nm, 287 nm and 292 nm. The Δe increases upto a 1 M GuHCl. Above 1 M GuHCl the Δ€ decrease in all the wavelengths. The increase in the Δe values as a function of GuHCl concentration at different wavelengths has got two major results. Up to concentration of 3 M GuHCl in all the wavelengths, the Δe value increases after which it plataeus in the case of 280 nm and 287 nm and 292 nm a decrease in absorbance is observed. This large change in Δe is due to the exposure of aromatic chromophores to the bulk solvent clearly above 3 M concentration as monitored in all the three wavelengths. The role played by the aromatic chromophores namely, tryptophan, tyrosine and phenylalanine is clearly evident. However, if one looks at the amino acid composition of the protein, the quantum yield in the Δe is a result of nearly 20 tryptophan residues and 50 tyrosine residues per mol protein as compared nearly 100 phenylalanine molecules which has less quantum yield (Shwenke *et al,* 1974). This is due to the exposure of some aromatic chromophores, upon binding of GuHCl, which are originally present at subunit-subunit contact regions. The increase in the Δe of negative magnitude from 1 M to 3M GuHCl is a result of the increasing number of molecules being denatured.

The unfolding of the 1IS protein fraction is followed by measurement of the intrinsic fluorescence intensity in presence of various concentrations of GuHCl. In Fig. 24 is shown the relative percentage of the fluorescence intensity of the protein, with the protein concentrations being 0.19 μM and 0.33 μM as a function of GuHCl. There is a transition

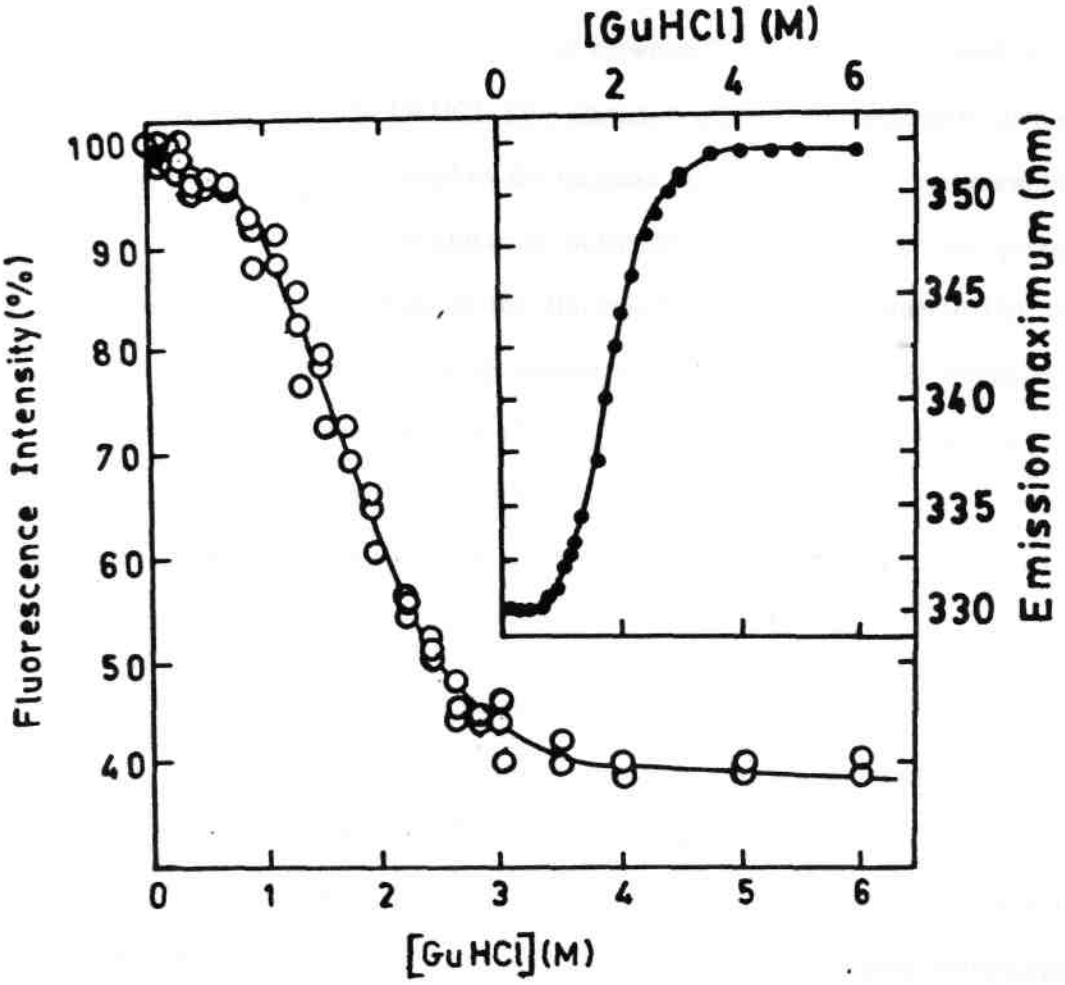

Fig. 24. Percent fluorescence emission intensity of the 11S protein fraction as a function of GuHCl concentration. The experimental points are obtained at a protein concentration of 1.9×10^{-7}M *Inset*: Fluorescence emission maximum of the protein as a function of GuHCl concentration.

of fluorescence intensity occuring between 0 -0.7 M GuHCl at protein concentrations of 0.19 μM and 0.33 μM. The concentration of GuHCl at the midpoint of this transition is 0.05 M.

The predominant transition is observed between 0.7 M and 3.5 M GuHCl. The mid point of this transition is 1.7 M GuHCl. This transition pattern is independent of the protein concentration and this is accompanied by the increase in the fluorescence emission maximum of the protein. The protein concentration independency suggests that the protein is in monomeric form and the decrease in the fluorescence intensity is due to the increasing number of molecules of the protein being denatured. The first transition of emission intensity does not represent the formation of a molten globule as indicated by the ANS binding experiment in presence of GuHCl. The ANS intensity is gradually decreased as a function of GuHCl after 0.2 M GuHCl suggesting that ANS binding to the exposed hydrophobic clusters is decreasing as a result of denaturation.

In Fig. 24 inset is shown the intrinsic fluorescence emission maximum of the protein as a function of the concentration of GuHCl. The emission maximum of the protein is increased from 330 nm for control to 352 nm in presence of 3.5 M GuHCl suggesting that the exposure of aromatic chromophores mainly tryptophan to the bulk solvent as a result of which the entire fluorescence emission spectrum shifts towards higher wavelengths with decreased intensity (Shifrin *et ah,* 1971; Teale, 1960; Teale and Weber, 1957). This fluorescence emission maximum data is reversible and the data is indicated in the figure.

The change in secondary structure of the protein due to its interaction with GuHCl is followed by measuring the far-UV CD spectra. In Fig. 25 is shown the far-UV CD spectra of the protein in presence of 2 M, 3M and 6 M GuHCl. As the concentration of GuHCl is increased the mean residue molar ellipticity, $[\theta]_{mrw}$, is predominantly changed in the wavelength region of 222 nm. In Table 10 is shown the $[\theta]_{mrw}$ at 222 mm for 1IS protein fraction in selected concentrations of GuHCl for the circular dichroic data. There is a

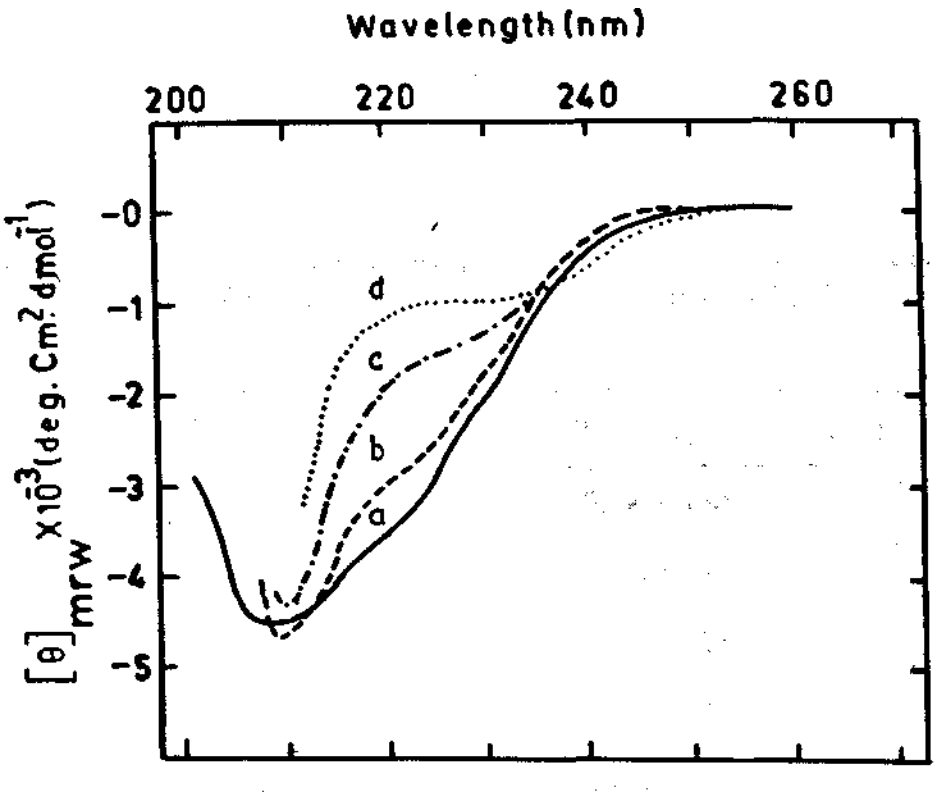

Fig. 25. Far UV-circular dichroic spectral pattern of the 11S protein fraction (a) In buffer. 11S protein fraction in presence of (b) 2M GuHCl, (c) 3M GuHCl and (d) 6M GuHCl. A Protein concentration of $2x10^{-6}$ M was used. A mean residue weight of 113 (calculated from the amino acid composition) is used.

Table 10 : Change in value of $[\theta]_{mrw}$ at 222nm of IIS protein fraction in presence of GuHCl at pH 6.0 calculated from circular dichroic data

Sample	$[\theta]_{mrw}$ at 222 nm (deg. cm^2. $dmol^{-1}$)
Native 11S	-3360 ± 150
11S + 1 M GuHCl	-3380 ± 150
11S + 2 M GuHCl	-2770 ± 125
11S + 3 M GuHCl	-1700 ± 125
11S + 4 M GuHCl	-1470 ± 100
11S + 6 M GuHCl	-1100 ± 100

progressive decrease in the $[\theta]_{mrw}$ at 222 nm as a function of increasing concentration of GuHCl suggesting the loss in secondary structures such as alpha helix and beta structure.

Since at the mid transition concentrations of GuHCl, the protein is precipitating at higher protein concentrations, the precipitation behaviour is studied at 25°C. The coagulation is not observed at low protein concentrations even after 24 hours in presence of any GuHCl concentration. However, at higher protein concentrations the coagulation is observed with the maximum precipitation is occuring at 1.6 M GuHCl. In Fig. 26 is shown the absorbance of the protein at 660 nm due to scattering by the aggregated protein molecules as a function of time. As the protein concentration is increased at a fixed denaturant concentration of 1.6 M GuHCl, the lag period for aggregation is decreased suggesting the requirement of a higher protein concentration. This precipitation is due to association of completely denatured molecules of the protein as indicated by the significant increase in emission maximum and significant decrease in fluorescence intensity at these GuHCl concentration.

B. Interaction of GuHSCN **with 11S protein fraction**

The nature of dissociation and denaturation reactions may be entirely dependent on the type of the denaturant used. Therefore the interaction of GuHSCN which belong to the same class of denaturant as that of GuHCl is studied with 1IS protein fraction to understand the nature of unfolding.

In Fig. 27 is shown the velocity sedimentation pattern of US protein fraction in presence of 0.25 M (upper pattern) and 0.5 M GuHSCN (lower pattern). It is clear from the figure that there are three peaks corresponding to the sedimentation constants of 1IS, 7S and 2S. In presence of 2 M GuHSCN the protein is completely in 2S form. Thus the dissociation occurs at much lower concentration of this denaturant as compared to GuHCl. The measurement of the percent compositions of different fractions as a function of GuHSCN is not possible due to instantaneous and higher precipitation of the protein at concentration

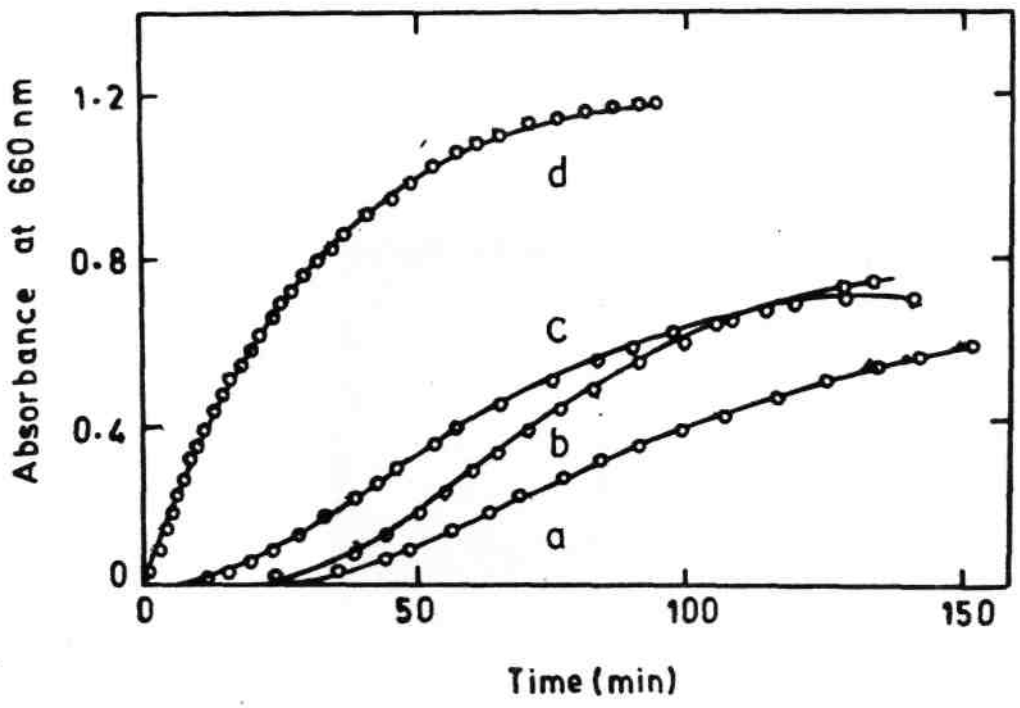

Fig. 26. Absorbance of 11S protein fraction in 1.6 M GuHCl at 660 nm as a function of time measured for different concentrations of the protein. (a) 8 mg/mL, (b) 10 mg/mL, (c) 12 mg/mL and (d) 14 mg/mL.

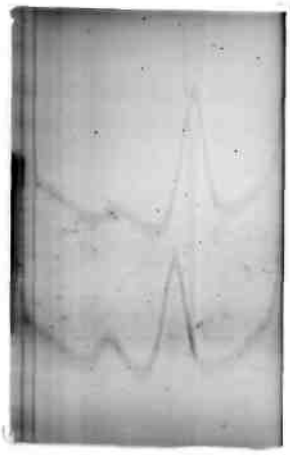

Fig. 27. Velocity Sedimentation pattern of 11S protein fraction in presence of 0.25 M (upper) and 0.5 M (lower) of GuHSCN concentrations. The photograph is taken at 35th minute after attaining 2/3 the maximum speed of 59780 rpm-

greater than 0.5 M GuHSCN. However, from the figure it is clear that the dissociation of 1IS to 2S occurs through an intermediate 7S component and this sequential dissociation is similar to that in the presence of GuHCl.

The UV-difference absorption spectra of the protein at varied concentrations of GuHSCN is studied. In Fig. 28A is shown the UV-difference spectra which has three peaks of negative magnitude with the peak wavelengths being 280 nm, 287 nm and 292 nm. In Fig. 28B is shown the Δe as a function of GuHSCN concentration. This pattern is similar to that of the GuHCl induced unfolding. The Δe at dissociated state (<0.25 M) is nearly similar to the data obtained from the interaction of GuHCl. Thus the molar Δe at low concentrations of denaturant is due to the presence of a stable thermodynamic state, i.e. the formation of 2S from 7S or US. The Δe is increased from $-4 \pm 1 \times 10^3$ M^{-1} cm^{-1} in presence of 0.2 M GuHSCN to $-19 \pm 2 \times 10^3$ M^{-1} cm^{-1} in presence of 2.5 M GuHSCN. The increase in this magnitude of Δe indicates gradual increase in the number of protein molecules being denatured as the denaturant concentration increases.

In Fig. 29A is shown the relative fluorescence intensity of protein as a function of GuHSCN concentration. The curve is biphasic. The extent of decrease in percentage of fluorescence intensity by GuHSCN at 2 M where it is nearly saturated is almost similar to that induced by GuHCl at 3.5 M concentration. The lesser molarity of GuHSCN to reach the similar structure of the protein (unfolded state) when compared to GuHCl is due to the efficiency of GuHSCN.

In Fig. 29B is shown the fluorescence emission maximum of the protein as a function of GuHSCN concentration. It is increased from 330 nm for native protein to 352 nm in presence of 2 M GuHSCN. This indicates that all the aromatic chromophores, especially tryptophans are exposed to the bulk solvent. The mid point of this transition is 0.7 M GuHSCN. These two similarities in intensity and emission maximum suggest that a similar

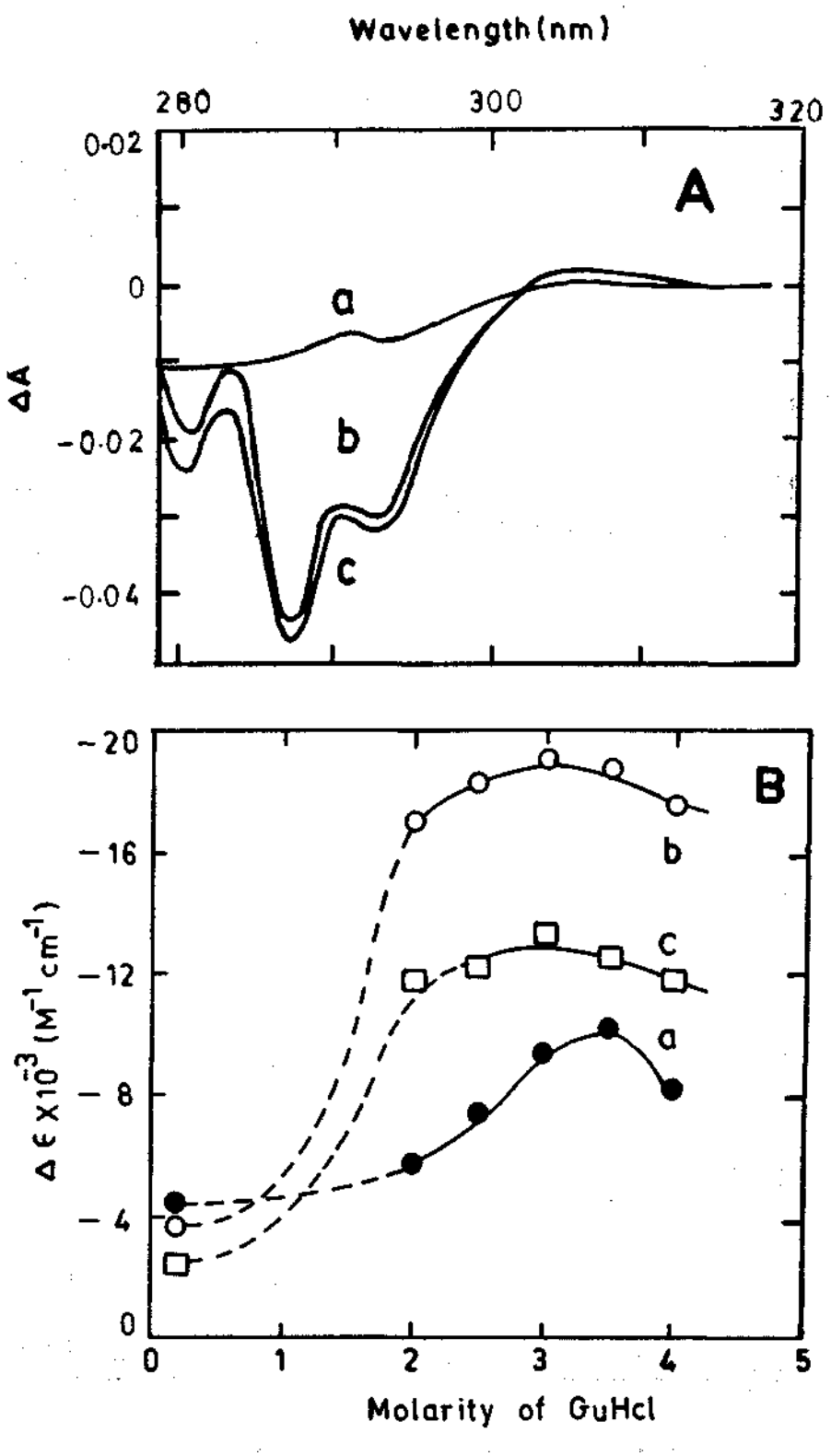

Fig. 28A. UV -difference spectra of 11S protein fraction in presence of (a) 0.2M, (b)2M and (c)4M GuHSCN concentrations in the wavelength range of 280 nm - 320 nm.

B. Differential molar absorption coefficient as a function of GuHCl concentration at (a) 280 nm, (b) 287 nm and (c) 292 nm. The data is derived from the UV difference spectral measurements.

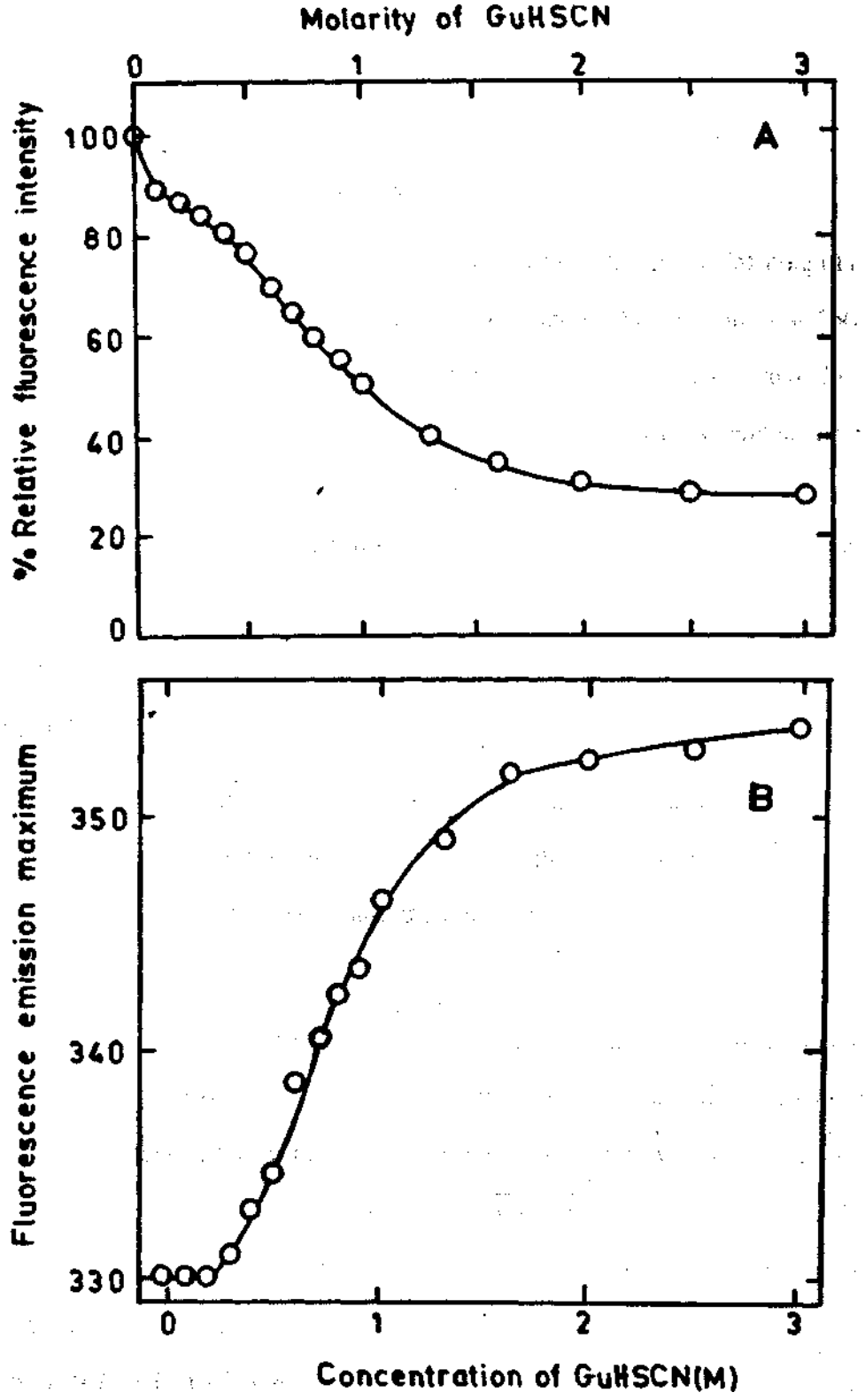

Fig. 29A. Relative fluorescence emission intensity of 11S protein fraction as a function of GuHSCN concentration. The protein was excited at 285 nm and a concentration of 2 x 10^{-7} M is used.

B. Fluorescence emission maximum of 11S protein fraction as a function of GuHSCN concentration.

thermodynamic state is reached at either 2M GuHSCN or 3.5 to 4 M GuHCl. The extent of absorption spectral alteration also favour this conclusion.

Since the precipitation of the protein is happening, the nature of coagulation of the protein at room temperature (25 °C) is studied by measuring the absorption at 660 nm of the protein at intermediate concentrations of GuHSCN. The data suggests that the lag period does not exist as in the case of GuHCl. And this precipitation is dependent on the protein concentration. The precipitation is-more at higher protein concentr ation. The precipitation of the protein is also studied as function of GuHSCN concentration. The precipitation is maximum at 1.2 M GuHSCN where the protein in denatured form is significant as is evident from the fluorescence data.

DISCUSSION

The dissociation of 11S to 2S occurs through an intermediate 7S component. Dissociation and denaturation are sequential. Dissociation results in change in an extinction coefficient of $-4 \pm 1 \times 10^3$ M^{-1} cm^{-1} and the denaturation results in a Δe value of $-23 \pm 4 \times 10^3$ M^{-1} cm^{-1} at 287nm.

There are two classes of low affinity binding sites that bring out the denaturation of the 2S form of 11S protein fraction. The first class being the binding to the charged, polar and non-polar residues of the surface of the native protein bringing out a partial loosening of the surface structure of the protein. This interaction is indicated by decrease in fluorescence intensity at 332 nm and Δe at 225 nm. The decrease in the fluorescence intensity of the protein but not its emission maximum upto a denaturant concentration of either 0.2 M GuHSCN or 0.7 M GuHCl is probably due to the formation of an intermediate state. This loosening involves the decrease in fluorescence intensity from 100% to 93 ± 2%. The unchanged fluorescence emission maximum at these denaturant concentrations is suggestive of the maintainance of the inner hydrophobic core intact. This is also suggested by the marginal or no change in the difference absorption upto 1 M GuHCl.

However, at higher concentrations than the above mentioned denaturant concentration, the hydrophobic solvation takes place. This is the major transition of denaturation involving increase in fluorescence emission maximum and a negative Δe at 280 nm, 287 nm and 292 nm. The fluorescence intensity decreases to 40 ± 5% in GuHCl or GuHSCN. This transition results in an increase in Δe from -4 ± 1 x 10^3 M^{-1} cm^{-1} to -23 ± 4 x 10^3 M^{-1} cm^{-1}. In this transition the interaction of guanidine salt with the exposed hydrophobic moieties of the intermediate state occurs with each binding site saturation leads to the exposure of a further hydrophobic site. The cooperativity is more for GuHSCN than for GuHCl. The mid point of this transition roughly equals the reciprocal of the binding constant of the guanidinium salt with the hydrophobic core moities of the protein.

The denaturation transition is two state between native structure and completely unfolded form is suggested by the following. (1) the decrease in fluorescence intensity and emission maximum as denaturant concentration is increased in a cooperative fashion and the coincidence of the predominant transitions measured from the absorption, fluorescence spectral measurements. (2) however, there is no change in the fluorescence emission maximum but decreased intensity as temperature is increased from room temperature to 60°C.

From the data it is clear that the nature of the dissociation and denaturation of the 11S protein fraction are sequential processes in both the denaturants studied with the GuHSCN inducing the unfolding at much earlier concentrations when compared to that of GuHCl. This result is similar to that as obtained earlier for the denaturation of wheat germ lipase and human serum albumin in presence of these denaturants (Rajeshwara and Prakash, 1994; Muralidhara and Prakash, 1997).

von Hippel and Wong (1965) has shown the decreased thermostability of number proteins in GuHCl and GuHSCN. We also know that the stabilization/destabilization of proteins depend upon protein solubility in denaturants, of course principally based structural

topology of proteins (Bull and Breese, 1976). The stability of fully folded or partially folded US protein fraction depends upon the solution conditions apart from the nature of the denaturant and concentration of protein. It is important to interprete these results with great caution towards the schematics of the interaction between denaturants and proteins. GuHCl has a less bulkier anion group as compared to GuHSCN. In the case of 1IS protein fraction the interaction of GuHCl and GuHSCN both suggest that there is an initial step of dissociation which may be rapid but firm step of denaturation exposing aromatic groups if they are in the contact area, to ultimately open up the molecule which is getting denatured at higher concentrations of denaturant. During this process of sequential dissociation-denaturation very similar results are observed in case of human serum albumin where molten globule state induced by new conformations in terms of stability as well as the structural changes that it accompanies in the protein (Muralidara and Prakash,1997).

Chapter 3

EFFECT OF TEMPERATURE ON 11S PROTEIN FRACTION

EFFECT OF TEMPERATURE ON 11S PROTEIN FRACTION

The oligomeric nature of the US protein fraction with the sandwiched model proposed by Prakash and Rao (1986) with the hexamer sliding over the trimer with the minor alteration in temperature and the trimer dissociation to monomer (an acidic and basic subunit) is highly dependent on the temperature. However, the bulk solvent and its properties play a major role in the properties of the dissociated subunits and also the parent molecules. Hence it is vital that one should understand the thermal stability of 11S protein fraction in denaturants and cosolvents which have a direct effect on the solvent that is water. This chapter addresses the stability of 11S protein fraction as a function of temperature in these specific solvents and denaturants under varied conditions to assess the stability of this molecule under conditions of extremes of temperature.

11S protein fraction

Temperature is an important physical parameter that influence the motion of the particles in three dimensions and alters many a times the bonding properties. The changes in bonding properties could be of weak and noncovalent intermolecular and/or intramolecular type. In proteins these can be the hydrophobic bonding and hydrogen/ionic bonding of the system (Creighton, 1993). The binding of small solutes with a protein molecule resulting in a different thermodynamically stable solute bound state of the protein, the binding of one protein molecule with another protein molecule or subunit-subunit interaction to form a thermodynamically stable oligomeric protein state involves these non-covalent interactive forces (Creighton, 1993; Kyte, 1995). Since temperature modulates these interactive forces that maintains the folded conformation of the protein molecules, it is important to study the effect of temperature on the macromolecular properties of the 1IS protein fraction.

The unfolding of a multisubunit protein has an extra step of dissociation. Thus temperature may affect the association-dissociation property of an oligomeric protein and unfolding of the folded protein molecule. The magnitude of the change brought out by the

temperature depends on the reference state of the protein including its ionization state and small molecular weight solute bound state. The unfolding is usually a two state phenomenon. However, several stable intermediates are possible in the unfolding. Characterization of such thermodynamically stable intermediate states as a function of temperature is important to understand the mechanism of unfolding in a better way (Tanford, 1968). These intermediate states need not be correlative with the chemical denaturant induced transitions. The unfolding brought out by chemical denaturants such as GuHCl, GuHSCN is described in the earlier chapter. The effect of temperature on the thermal transitions of 11S protein fraction in presence of polyols, disaccharides, guanidinium salts and polyphenols is studied in depth and presented in this chapter.

The protein structure is dependent on the properties of the water, small molecular weight solute and the other macromolecules present in it. One such intrinsic property is the pH of the solution which changes the ionization state of the protein depending on the isoelectric point of the protein. Different chemical structures which are intrinsically dependent on their amino acid constitution differ in their thermal transition profiles as the temperature is increased, due to the differences in entropy and enthalpy changes (Tanford, 1968). Similarly the different tertiary structures of the protein as occur due to changes in pH or electrolytes also show different effect on the heat changes and entropy changes with change in temperature. And the changes can be monitored by the direct calorimetric experiment or in an indirect way by monitoring a particular physical property of the system. Absorbance at 287 nm of the protein is an useful probe in this regard. This absorption, as a probe of protein structure is widely used in the earlier literature (Hermans and Scheraga, 1961; Brandts and Hunt, 1967; Lee and Timasheff, 1981; Arakawa *et al,* 1990b). In the present study the absorbance at 287 nm is used as a probe to monitor the structure of the protein at pH 6.0 and 8.6 in absence and presence of the NaCl. The mid point transition of these temperature curves are calculated by the method described earlier (Pace *et al,* 1989).

(a) pH and buffer ions

In Fig. 30 A, B and C is shown the first derivative plots of the absorbance at 287 nm as temperature is increased from 25°C to 95°C at pH 6.0, 7.9 in PB (A), at pH 7.9 and 8.6 (tris buffer) (B) and 8.6 (glycine -NaOH buffer) (C), respectively. There are two peaks at the above mentioned pH values whose peak temperatures are designated as apparent T_{m1} and apparent T_{m2} for low temperature transition and high temperature transitions, respectively. The apparent T_{m1} and apparent T_{m2} values are 76 ± 1°C and 90 ± 1°C, respectively at pH 6.0 in phosphate buffer; The apparent T_{m2} is 89 ± 1°C at pH 7.9 in phosphate buπer; apparent T_{ml} and apparent T_{m2} are 69 ± 1°C and 89 ± 1°C, respectively at pH 7.9 in tris -HC1 bufffer; They are 68 ± 1°C and 89 ± 1°C, respectively at pH 8.6 in tris-HCl buffer; They are 71 ± 1°C and 93 ± 1°C, respectively at pH 8.6 in glycine-NaOH buffer. Thus increasing the pH has a destabilizing effect on first transition T_{ml}. However,not much change is observed for the apparent T_{m2} as a function of pH from 6.0 to 8.6. The buffer ion also has an effect as shown by the data at pH 8.6 where in presence of glycine-NaOH buffer there is an increase of 3°C in apparent T_{m1} and apparent T_{m2} values compared to that in tris-HCl buffer. Thus change in pH significantly alters the thermal transition temperature of the protein that inturn alters the stability.

Effect of sodium chloride on apparent T_m of 11S protein fraction

In a similar fashion salts such as sodium chloride can also bind to the polar and charged groups of the protein molecule and can alter temperature dependent structural transition of the protein. In Fig. 31A is shown the first derivative plot of the thermal denaturation transition of protein at pH 6.0 in presence of 0.5 M NaCl. Upon comparision of the app T_m values of native protein with and without sodium chloride, the data suggest that there is not that much change. Thus concentration of salt has less effect on the structural stability of protein. In Fig. 3IB is shown the first derivative plot of the thermal denaturation transition of 11S protein fraction at pH 8.6 in presence of sodium chloride. The apparent

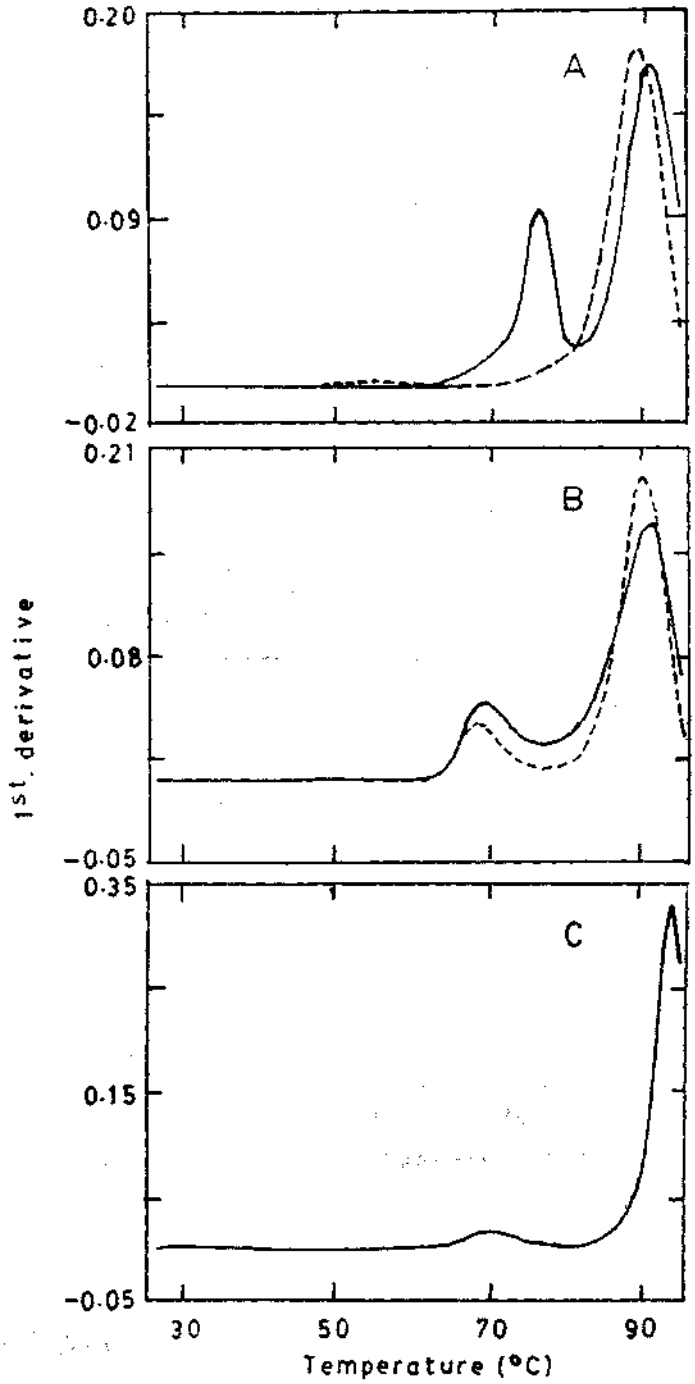

Fig. 30A. First derivative plot of the absorbance of the US protein fraction monitored at 287 nm, at pH 6.0 (-----) and pH 7.9 (—). The buffer used is 0.15 M phosphate buffer for both the pH values.

B. First derivative plot of the absorbance at 287 nm of the 1 IS protein fraction at pH 7.9 ( ) and pH 8.6 (—). For both the pH values the buffer used is tris - HC1,0.15 M.

C. First derivative plot of the absorbance at 287 nm of the 1 IS protein fraction at pH 8.6 with the buffer used being glycine - NaOH, 0.15 M.

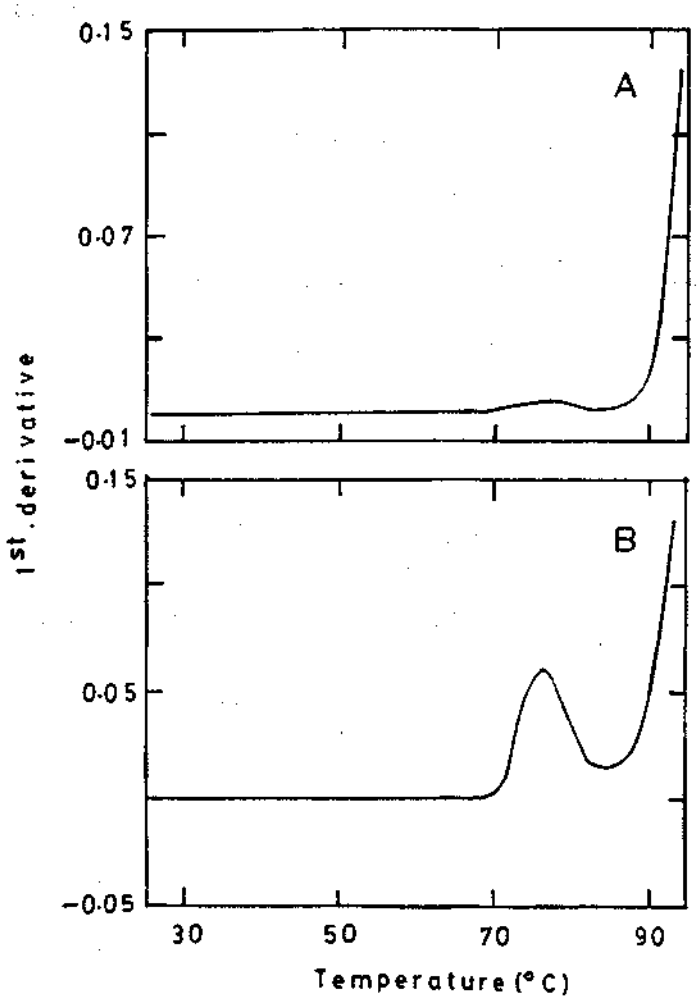

Fig. 31 A. First derivative plot of the absorbance at 287 nm of 1 IS protein fraction at pH 6.0 in presence of 0.5 M NaCl. The buffer used is phosphate buffer, 0.15 M.

B. First derivative plot of the absorbance at 287 nm of 1 IS protein fraction at pH 8.6 in presence of 0.8 M NaCl. The buffer used to maintain the pH is glycine - NaOH, 0.15 M.

T_{m1} values are as shown in the Table 11. In presence of 0.5 M sodium chloride at pH 6.0 the apparent T_{m1} value is 76 ± 2°C. At pH 8.6 when the concentration of sodium chloride is increased to 0.8 M soidum chloride, the apparent T_{m1} value increases from a control value of 71 ± 1°C to a value of 76 ± 1°C. These data indicates the role of electrostatic interaction and also the charge shielding on the protein (Jencks, 1969).

Thus the above data on the effect of sodium chloride on the thermal transition temperature of US protein fraction under neutral pH conditions suggests that at low concentration of sodium chloride, the protein is nearly in the native state with thermal transition temperature being 76 ± 1°C. These data give a basis for the selection of conditions under which apparent T_m of protein is increased in different cosolvents at various concentrations for partial specific volume measurement in the next chapter.

Effect of cosolvents on apparent T_m of 11S protein fraction

The US protein fraction is very sensitive to denaturants as a function of temperature. The role of the inter and intra subunit hydrophobic interaction as a stabilizing factor beyond 55°C (Jencks, 1969) would be the one of the factor that destabilizes the protein at higher temperature. If one looks at the role played by the cosolvents namely glycerol, sorbitol, sucrose and trehalose, the cosolvents in general had a stabilizing effect on macromolecules . The extent of stabilization depends on nature, concentration of cosolvents and nature of the protein also. The effect of cosolvents namely glycerol, sorbitol, sucrose and trehalose on the apparent T_m of 11S protein fraction is studied. There is no distinct second transition in presence of cosolvents as compared to in different pH or salt concentration and/or denaturants. Therefore, only the apparent Tml (first transition) is monitored as a probe for the structural stability of the protein in these conditions.

Since the protein is unstable at higher pH in the presence or absence of sodium chloride, the effect of the cosolvents on the stability of 1IS protein fraction is investigated at

Table 11 : Apparent T_{m1} of IIS protein fraction at pH 6.0 and pH 8.6 under different NaCI concentrations

pH	NaCl (M)	Apparent T_{m1} (± 1°C)
6.0	0.0	76
	0.5	76
8.6	0.0	71
	0.1	69
	0.2	70
	0.4	73
	0.8	76
	1.0	76

different concentrations of protein at higher pH. The presence of sodium chloride was essential for complete solubility of the protein and not to have any scattering effect of solution at low temperature to have stable base line. In Table 12 is shown apparentT_{m1} of the US protein fraction in presence of various cosolvents. From the data it can be seen that compared to control which is having apparent T_{m1} of 76 ± 1°C, as the concentration of glycerol increases from 2-30% the apparent T_{ml} also increases progressively from 79°C to >95 ± 2°C. In the case of sucrose the effect is less pronounced as compared to glycerol under similar concentration. As the concentration of sucrose increases the apparent T_{ml} also increases, at 25% sucrose the apparent T_{m1} is 86 ± 1°C. Trehalose appears to be less effective in bringing about thermal stability of protein under similar concentration of other cosolvent, say at 20%, the apparent T_{m1} is 95 ± 1°C in glycerol, 87 ± 1°C in case of trehalose , 88 ± 1°C in case of sorbitol and 86 ± 1°C in case of 25% sucrose (Table 12). Thus data indicates that glycerol has higher stabilizing effect on the protein at concentration range studied between 2-30%. Such a stability can be accompanied only through a water mediated indirect preferential hydration phenomenon.

Effect of denaturants on the apparent T_m of 11S protein fraction

Guanidine salts are known to affect the structural stability of protein in general. The nature of interaction of guanidine salts has a major role to play in terms of anion part namely Cl" and SCN- ions. The effect of GuHCl in concentration range of 0.1-1.3 M suggested that as the concentration of GuHCl increases the thermal stability of the protein also increase and beyond 1.3 M the protein does not have any transition, as monitored by absorbance at 287 nm. For example at 0.1 M GuHCl there is marginal increase of apparent T_{m1} by 1°C. At higher concentration such as 0.8 M GuHCl the protein is more unstable and apparent T_{m1} is 70±1°C.

Similar results are observed in case of GuHSCN as shown in Table 13 where the transition is much sharper even at 0.5 M GuHSCN, where the apparent T_{m1} reaches to a value of 62°C. In the case of GuHCl at 0.1 M there is a slight increase in apparent T_{ml} but is

Table 12 : Mid transition temperatures of 11S protein fraction in presence **of** various **cosolvents**

Cosolvent (w/v)	Apparent T_{m1} (± 1°C)
2% glycerol	79
4% glycerol	80
10% glycerol	85
20% glycerol	95
30% glycerol	>95
10% sorbitol	83
20% sorbitol	88
30% sorbitol	>95
2.5% sucrose	79
5.0% sucrose	79
15% sucrose	83
25% sucrose	86
5% trehalose	80
15% trehalose	83
20% trehalose	87
30% trehalose	89

* Apparent T_{m1} of 1IS protein fraction is 76 ± 1°C

Table 13 : Apparent T_{m1} of IIS protein fraction in presence of different concentrations of Guanidinium class of salts at pH 6.0

Additive	Concentration (M)	Apparent T_{m1} (± 1°C)
GuHCl	0.1	77
	0.4	75
	0.6	72
	0.85	70
	1	69
	1.3	No transition seen
GuHSCN	0.1	74
	0.3	69
	0.4	66
	0.5	62

* Apparent T_{m1} of 1IS protein fraction is 76 ± 1°C

not significant. The apparent T_{ml} value increases from 76 ± 1°C of control protein to 77 ± 1°C in presence of 0.1 M GuHCl, indicating marginal stability of protein at these concentration of denaturant. Prakash and Timasheff (1997) has explored the phenomenon of these unusual stabilization of protein at lower concentration of denaturant (Prakash and Timasheff, 1997), where preferential hydration dominates on preferential interaction. This has been shown in the case of ribonuclease and lysozyme. Muralidhara and Prakash (1997) has explored the phenomenon in the case of HSA, where they have proposed a molten globule state of protein at the lower concentration of denaturants. In the case of 1 IS protein fraction a similar molten globule preferentially hydrated state could exists at the lower concentration of denaturants. Since the technique *per se* has an error built into the system of 1°C, major emphasis is not laid in these small change in T_m in pursuing it to drive home the above point. However, the trend observed is explained on the available literature data as can be applied on 1IS protein fraction.

Effect of polyphenol on apparent T_m of 11S protein fraction

In Table 14 is shown the apparent T_{m1} of US protein fraction as a function of different polyphenol namely CGA, CA and QA at 1 x 10^{-5} M pH 6.0 without sodium chloride. Compared to the control value of 76 ± 1°C there is a major decrease of 2°C in apparent T_{m1} of protein indicating the destabilization of US protein fraction as the interaction between the polyphenol and the protein is less at these higher concentration of ligand as explained in chapter 1 in terms of the extent and data of interaction of the polyphenols. There is no major change between polyphenols and all of them decrease the stability by lesser extent (Table 14). However, if one compares the results of extent of interaction, one sees major changes in presence of both CGA and CA as compared to QA as explained **in** chapter 1. But these properties does not effect the apparent T_{m1} in presence of equimolar concentrations **of ligand** at pH 6.0. The selection of pH is to avoid quinone formation at the **concentration** of ligands at any other higher pH value above pH 6.0 which **would** interfere in absorbance of protein at 287 nm.

Table 14. Apparent T_{m1} of 11S protein fraction in presence of polyphenols at pH 6.0

Polyphenol	Concentration (M)	Apparent T_{m1} (± 1°C)
CGA	1×10^{-5}	74
CA	1×10^{-5}	74
QA	1×10^{-5}	74

* Apparent T_{m1} of 11S protein fraction is 76 ± 1°C

The above results suggest that the thermal stability of US protein molecule in presence of certain ligands such as hydrogen ions, sodium chloride, guanidinium salts such as GuHCl, GuHSCN,cosolvents such as glycerol, sucrose, sorbitol and trehalose as well as polyphenols such as CGA, CA and QA has a profound effect on the destabilization of the protein from the point of view of the structure as monitored by change in absorbance of die protein in presence of these ligands. These results indicates that the fragile structure of US protein molecule namely oligomeric structure of 1IS to 7S dissociation and its perturbation in presence of these ligands would effect the above property. One see that 11S protein molecule will be in a different state possible in much more compact state in the case sodium chloride to a certain extent and to a larger extent in the case of cosolvents. However, in the.case of guanidinium salts and polyphenols the protein molecule is probably much more open there by ΔG required to push native to denatured state (elevated temperature is much less in presence of these destabilizing ligands as compared to control protein without ligands in the system).

In the former case of cosolvents because of preferential hydration, discussed in next chapter probably the protein assumes molten globule state invoking a high thermal stability in presence of these cosolvents. Therefore, it is vital to address this question of stability of protein in presence of different cosolvents by quantifying and measurements of such interaction or be it preferential hydration, the extent and the nature of interaction is quantified through partial specific volume measurements and determination of related thermodynamic parameters as described in succeeding chapter 4.

Chapter 4

INTERACTION OF COSOLVENTS WITH 11S PROTEIN FRACTION

INTERACTION OF COSOLVENTS WITH 11S PROTEIN FRACTION

In the previous chapters the effect of temperature on the US protein fraction under the equilibrium concentration of several solvents such as denaturants, cosolvents and salt suggest the stabilization/destabilization of the molecule. However when one looks at the stabilization of the 1IS protein fraction the preferential interaction/hydration of the protein in these cosolvents is of immediate interest. The information on the preferential interaction and preferential hydration has to have a firm thermodynamic base and in the present chapter the effect of different cosolvents on the partial specific volume under isomolal and isopotential conditions both in different concentrations of protein and different concentrations of cosolvents is investigated and the results presented. The role played by water is very critical and crucial and the interpretation of the results are cautiously attempted keeping in the view of the role of water in this three component system. The nature and extent of interaction of cosolvent with the protein is also monitored by appropriately selecting the conditions of these cosolvents.

The measurement of partial specific volume of proteins in any particular solvent is dependent upon the nature of the solvent, the nature of the protein,the concentration of the protein and the temperature of partial specific volume measurements. The partial specific volume of proteins under isomolal and isopotential conditions in a particular solvent helps in understanding the extent of preferential interaction/preferential hydration as compared to its native condition. The extent of interaction of cosolvent with water also plays a major role for eg. in the case of 1IS protein fraction of sunflower seed the solvents used are glycerol, sorbitol, sucrose and trehalose which are shown to have stabilization effect as indicated in the earlier chapter in the thermal stability measurement of the protein. The data of fluorescence and UV-difference spectral measurements in these cosolvents shows the perturbation of aromatic chromophores to different extents as a result of changes in the micro environment of aromatic chromophores. These results

tries to consolidate the results of stabilization through net preferential interaction/net preferential hydration at different solution conditions to arrive at thermodynamic understanding of US protein fraction interactions with cosolvents. All these results are correlated to the structural features of the US protein fraction and the results are interpreted keeping in view of the chemical nature of the cosolvent and is correlated with the conformation status of the protein in these solvents.

Partial specific volume measurements

To quantitate the interaction of cosolvents with 1IS protein fraction it is essential to measure the partial specific volume of the protein both at constant molality and constant chemical potential conditions. The 1IS protein fraction in the buffer has a value of 0.763 + 0.002 and 0.776 + 0.003 ml/g both at isomolal and isopotential conditions. The results are shown in Fig. 32. The effect of different cosolvents on the partial specific volume of the protein is measured and the results are as follows.

(a) In Glycerol

The effect of glycerol on the multimeric 1IS protein fraction is studied by partial specific volume measurements. In Fig. 33A is shown the apparent partial specific volume values of the protein as a function of protein concentration in presence of 10% and 40% (w/v) concentrations of glycerol both at isomolal and isopotential conditions. The apparent partial specific volume values are extrapolated to zero protein concentration. From the difference in the partial specific volumes of the protein at isomolal and isopotential conditions the preferential interaction parameter (ξ_3) is calculated. Both isomolal and isopotential values are changed from a native value of 0.763 ml/g levels. In the case of monomeric protein, human serum albumin (HSA), the v_{app} measured under identical conditions, the isomolal values in different cosolvent concentration does not change. However, in the case of the 1IS protein fraction the isomolal values changes. This may have a role on the association-dissociation phenomenon of the multisubunit

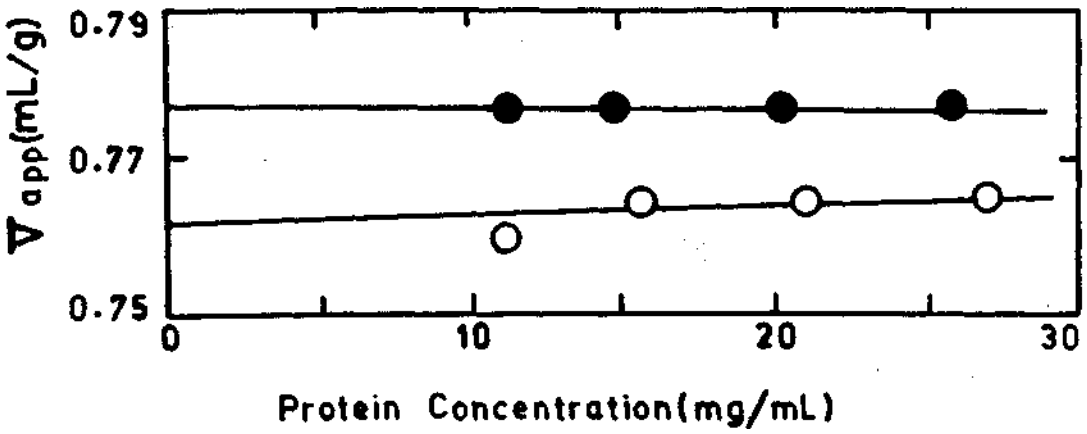

Fig. 32. Apparent partial specific volume of 11S protein fraction under isomolal (open circles) and isopotential conditions (filled circles).

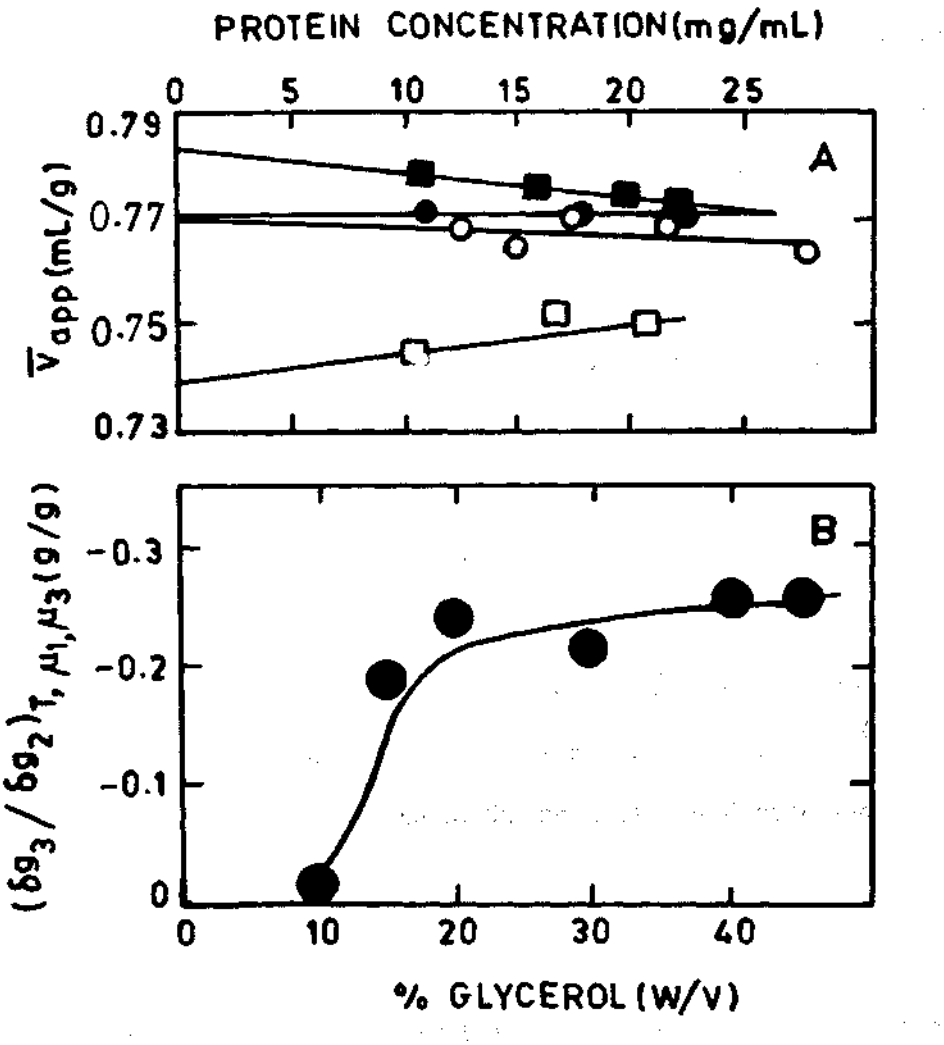

Fig. 33A. Representative plots of apparent partial specific volume of 11S protein fraction in 10% glycerol (circles) and 40% glycerol (squares). The isomolal (open symbols) and isopotential (filled symbols) values are shown in the figure.

B. Variation of preferential interaction parameter of 11S protein fraction in 10-45% concentration range of glycerol.

protein though dissociation of subunits not significantly seen. However, the isopotential values shows an increase compared to isomolar values at the corresponding solvent concentration. This is due to the preferential hydration of protein as observed in different protein system (Lee and Timasheff, 1974; Arakawa and Timasheff, 1984; Gekko and Morikawa, 1981a, b; Gekko and Timasheff, 1981; Rajeshwara and Prakash, 1996; Rejendran *et.al,* 1995; Muralidhara and Prakash, 1997). In Fig. 33B is shown the plot of ξ_3 values as a function of glycerol concentration. The ξ_3 value is negative in magnitude suggesting a preferential exclusion of the cosolvent molecules from the vicinity of the protein molecule. The ξ_3 values in case of HSA also increase following a sigmoidal pattern reaching a maximum value of -0.154 + 0.018 g/g at 40% glycerol concentration (Muralidhara and Prakash, 1997). At similar concentration the ξ_3 values for 1 IS protein fraction is -0.253 + 0.006 g/g. But as a mol to mol basis these values makes a large difference. The values being -133 ± 23 and -663 ± 105 mol/mol of additional molecules structured around HSA and US protein fraction, respectively. This indicates the contribution of protein surface area in the net preferential hydration process. The ξ_3 aslo increases sigmoidally and then saturates above 15% (w/v) glycerol in case of US protein fraction.

In Table 15 is shown the partial specific volume of the protein at isomolal ($\varphi_2°$) and isopotential ($\varphi_2°$) and various preferential interaction parameters calculated from the above basic data of partial specific volume at different concentrations of glycerol. The preferential hydration value is increased with glycerol concentration upto 15% (w/v) and then decreased reaching a constant value of 0.4 ± 0.05 g/g at 40% (w/v) cosolvent concentration. The preferential interaction parameter on mole to mole basis has a value of -625 ± 90 on mol/mol basis at 20% glycerol concentration and above this concentration there is no change in the preferential interaction value.

Table 15. Preferential interaction parameters of glycerol with 11S protein fraction

Interaction Parameter	Glycerol (%) (w/v)					
	10	15	20	30	40	45
ϕ_2^0 (mL/g)	0.769 ±0.002	0.731 ±0.002	0.710 ±0.002	0.736 ±0.003	0.739 ±0.003	0.734 ±0.003
$\phi_2'^0$ (mL/g)	0.772 ±0.002	0.770 ±0.002	0.757 ±0.003	0.776 ±0.003	0.783 ±0.004	0.775 ±0.005
$(\delta g_3/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.015 ±0.006	- 0.188 ±0.022	- 0.238 ±0.029	- 0.210 ±0.040	- 0.253 ±0.038	- 0.253 ±0.066
$(\delta g_1/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.142 ±0.057	1.147 ±0.134	1.044 ±0.127	0.561 ±0.038	0.456 ±0.049	0.382 ±0.029
$(\delta m_3/\delta m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 39 ±15	- 493 ±80	- 625 ±90	- 552 ±75	- 662 ±100	- 663 ±105

(b) In Sorbitol

The partial specific volume of 11S protein fraction is measured as a function of sorbitol concentration both under isomolal and isopotential conditions at different protein concentrations. In Fig. 34A is shown the apparent partial specific volume of the protein as a function of protein concentration at 10% and 40% (w/v) sorbitol concentration as a representative plot. The extrapolated partial specific volume values to zero protein concentration, is calculated for both isomolal and isopotential conditions. The preferential interaction parameter is calculated from the partial specific volume of isomolal and isopotential conditions according to the procedure described under materials and methods. The result is shown in Fig. 34B. The $\xi 3$ value is increased upto 30% sorbitol concentration above which it decreases. In Table 16 is given the preferential hydration values, preferential interaction parameter values both on g/g basis and mol/mol basis. In compensation for the preferential exclusion of sorbitol molecules the water molecules are preferentially present around the protein molecule. This preferential hydration value is increased to 0.988 g/g at 20% (w/v) sorbitol above which it decreases. The preferential interaction parameter on mole to mole basis is increased to -374 mol/mol basis at 30 % (w/v) sorbitol concentration above which it decreased.

(c) In **Sucrose**

The apparent partial specific volume values of the protein are measured at different protein concentrations in presence of different concentrations of sucrose. In Fig. 35A is shown the representative plot of apparent partial specific volume of the protein in presence of 10% and 40% (w/v) sucrose concentrations. The partial specific volume values are calculated upon extrapolation of V_{app} values to zero protein concentration. The preferential interaction parameter values calculated from partial specific volume at isomolal and isopotential conditions are shown as a plot of ξ_3 as a function of sucrose concentration on g/g basis in Fig. 35B. From the figure it is apparant that there are two

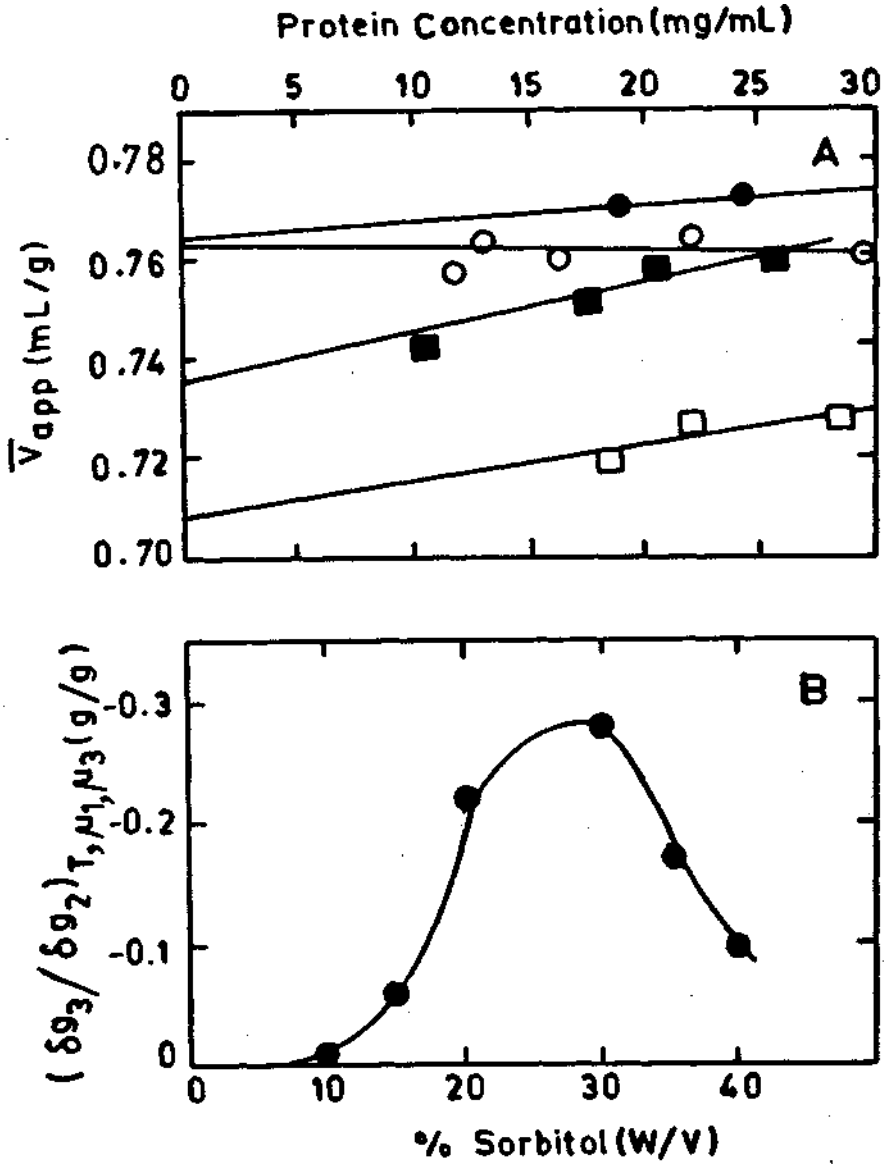

Fig. 34A. Representative plots of apparent partial specific volume of 11S protein fraction **in** 10% sorbitol (circles) and 40% sorbitol (squares). The isomolal (open symbols) and isopotentiai (filled symbols) values are shown in **the figure.**

B. **Variation** of **preferential interaction** parameter of **11S** protein fraction in **10-40% concentration range of sorbitol.**

Table 16. Preferential interaction parameters of sorbitol with 11S protein fraction

Interaction Parameter	Sorbitol (%) (w/v)					
	10	**15**	**20**	**30**	**35**	**40**
ϕ_2^0 (mL/g)	0.763 ±0.002	0.733 ±0.002	0.707 ±0.003	0.710 ±0.003	0.742 ±0.003	0.708 ±0.003
$\phi_2'^0$ (mL/g)	0.764 ±0.002	0.751 ±0.002	0.774 ±0.002	0.795 ±0.004	0.790 ±0.004	0.735 ±0.004
$(\delta g_3/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.003 ±0.002	- 0.057 ±0.016	- 0.220 ±0.017	- 0.283 ±0.023	- 0.166 ±0.036	- 0.095 ±0.026
$(\delta g_1/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.030 ±0.028	0.357 ±0.044	0.988 ±0.131	0.784 ±0.087	0.378 ±0.082	0.181 ±0.022
$(\delta m_3/\delta m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 4 ±2	- 76 ±12	- 292 ±20	- 374 ±17	- 220 ±48	- 125 ±14

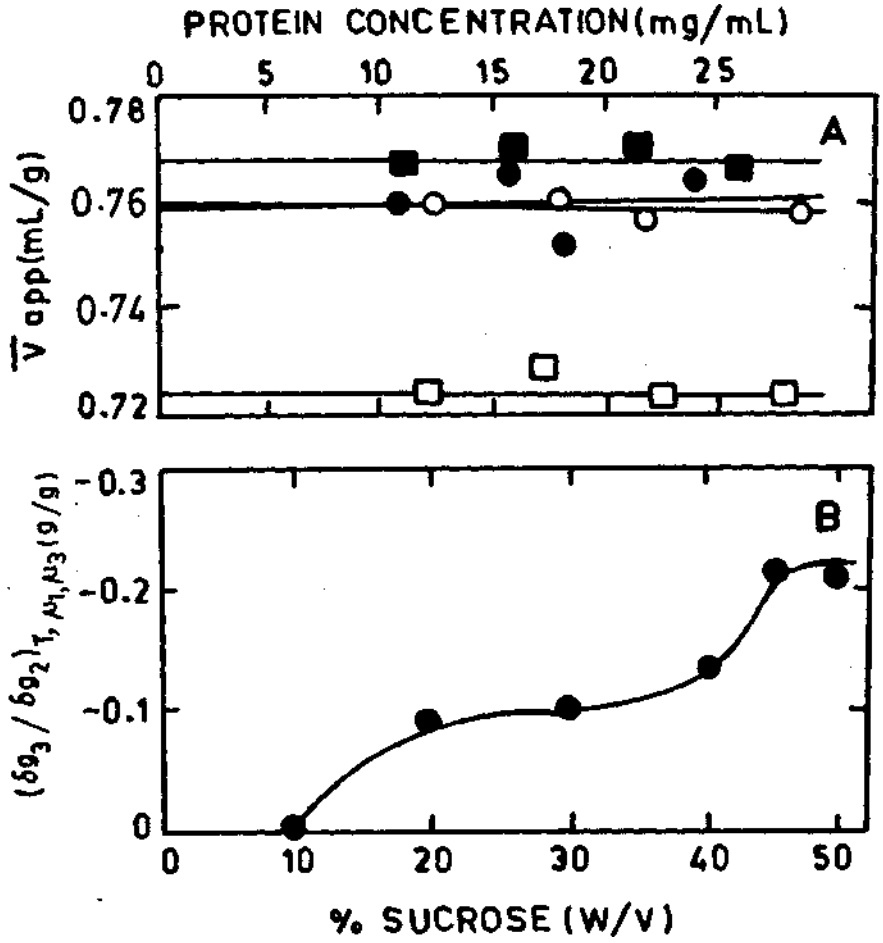

Fig. 35A. Representative plot of apparent partial specific volume of 11S protein fraction in 10% sucrose (circles) and 40% sucrose (squares). The isomolal (open symbols) and isopotential (filled symbols) values are shown in the figure.

B. Variation of preferential interaction parameter of 11S protein fraction in 10-50% concentration range of sucrose.

transitions of ξ3 values, namely, between 10% to 30% concentration and 30% to 40% concentration above which it reaches a plateau. These two transition regions might have induced microenvironment changes of the protein surface (fluorescence spectral measurements are explained in Fig. 39). The ξ_3 values are negative and increases upto 45 % (w/v) sucrose concentration above which it is saturated. In Table 17 is shown the ξ_3 values and the preferential interaction parameter on mole to mole basis, preferential hydration and partial specific volume at isomolal and isopotential conditions. If one compares the ξ_3 values from 10 to 50% concentrations, onj sees a progress increase of a ξ_3 values from 0 to -0.219 , g/g , as the peak. On a mol/mol basis this gives the value of -155 + 18 moles of cosolvent/mol of protein after which it reaches a plateau value at 45% sucrose concentration (w/v). These suggest a rather peculiar role played by sucrose compared to glycerol, sorbitol and trehalsoe. The results of trehalose are described below.

(d) In Trehalose

The preferential interaction parameter values of trehalose with 1IS protein fraction is calculated by measuring the partial specific volume of the protein in presence of different concentrations of trehalose. In Fig. 36A is shown the apparent partial specific volume of the protein as a function of protein concentration. The ξ_3 values are plotted versus the trehalose concentration in Fig. 36B. These ξ_3 values are negative which means that water molecules are predominantly present around the immediate domain of the protein molecule by exclusion of the cosolvent molecules. As the concentration of trehalose increases ξ_3 values are increased and the curve follows sigmoidal path till 40% (w/v) trehalose concentration. The preferential interaction parameter values and other related parameters are shown in Table 18. It is clear from the data that the preferential hydration value is not significantly changed from 0.3 ±0.1 g/g

Table 17. Preferential interaction parameters of sucrose with 11S protein fraction

Interaction Parameter	Sucrose (%) (w/v)					
	10	**20**	**30**	**40**	**45**	**50**
ϕ_2^0 (mL/g)	0.760 ±0.002	0.741 ±0.002	0.722 ±0.003	0.724 ±0.003	0.708 ±0.002	0.705 ±0.003
$\phi_2^{'0}$ (mL/g)	0.760 ±0.002	0.773 ±0.002	0.757 ±0.002	0.768 ±0.004	0.777 ±0.004	0.770 ±0.004
$(\delta g_3/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.0	- 0.092 ±0.006	- 0.099 ±0.006	- 0.131 ±0.006	- 0.219 ±0.006	- 0.207 ±0.006
$(\delta g_1/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.0	0.416 ±0.036	0.280 ±0.026	0.257 ±0.042	0.364 ±0.040	0.298 ±0.039
$(\delta m_3/\delta m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	0.0	- 65 ±10	- 70 ±14	- 93 ±16	- 155 ±18	- 146 ±22

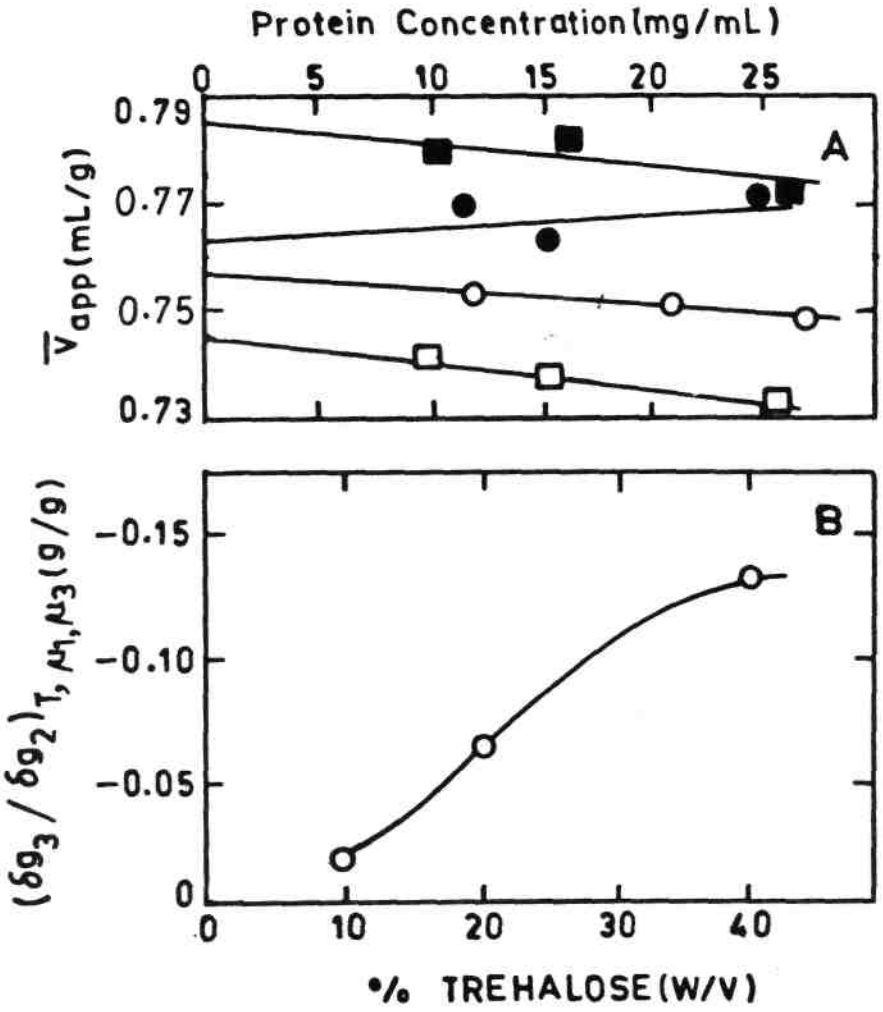

Fig. **36A.** Representative plots of apparent partial specific volume of 11S protein fraction in 10% trehalose (circles) and 40% trehalose (squares). The isomolal (open symbols) and isopotential (filled symbols) values are shown in the figure.

B. Variation of preferential interaction parameter of 11S protein fraction in **10-40%** concentration **range of trehalose.**

Table 18. Preferential interaction parameters of trehalose with US protein fraction

Interaction Parameter	Trehalose (%) (w/v)		
	10	20	40
ϕ_2^0 (mL/g)	0.757 ±0.002	0.752 ±0.003	0.745 ±0.004
$\phi_2'^0$ (mL/g)	0.763 ±0.003	0.774 ±0.003	0.785 ±0.004
$(\delta g_3/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	- 0.018 ±0.008	- 0.066 ±0.016	- 0.133 ±0.007
$(\delta g_1/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.175 ±0.058	0.299 ±0.037	0.256 ±0.033
$(\delta m_3/\delta m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	- 12 ±4	- 42 ±12	- 85 ±20

upon increasing the trehalose concentration. However, the preferential interaction parameter on mole to mole basis increases with increase in trehalose concentration.

In Fig. 37 (A-D) is shown the UV-difference spectra of 1IS protein fraction at 40% concentrations of glycerol, sorbitol, sucrose and trehalose in the wavelength range 260-320 nm namely FIG. 37A, B, C and D , respectively. In all the above solvents the perturbation of aromatic chromophores of 1IS protein fractions is observed with peaks of positive magnitude principally at 280 nm and 287 nm except in trehalose where only 280 nm peak is observed. This clearly indicates the microenvironment changes of exposed tyrosine and tryptophan residues in the protein with contribution of phenylalanine being minimum. This also indicates conformational changes in the protein molecule. However, the minor structural alterations in the protein are measured by fluroscence emission spectra.

In Fig. 38 is shown a representative plot of fluroscence emission intensity of 1IS protein fraction between 300 nm to 400 nm in different concentrations of glycerol. The fluorescence intensity of the protein increases progressively, as a function of glycerol concentration in the range 10%, 20% and 40% represented by spectral curves b), c) and d) respectively. In the case of sorbitol also the fluroscence intensity increases but to a lesser extent compared to glycerol. In the case of both sucrose and trehalose the fluroscence intensity decreases with the increase in solvent concentration. But the fluorescence emission maximum of the protein is not changed in any of the cosolvents mentioned above even at 40% level indicating the absence of conformational changes in the protein molecule in presence of these cosolvents. The results are shown in Fig. 39.

From Fig. 39 it can seen that there are two major results (i) Sorbitol and Glycerol increase the fluorescence intensity of protein as the result of microenvironmental changes around aromatic chromophores on the other hand in case of sucrose there is a marginal increase of about 10% suggesting no huge changes in the

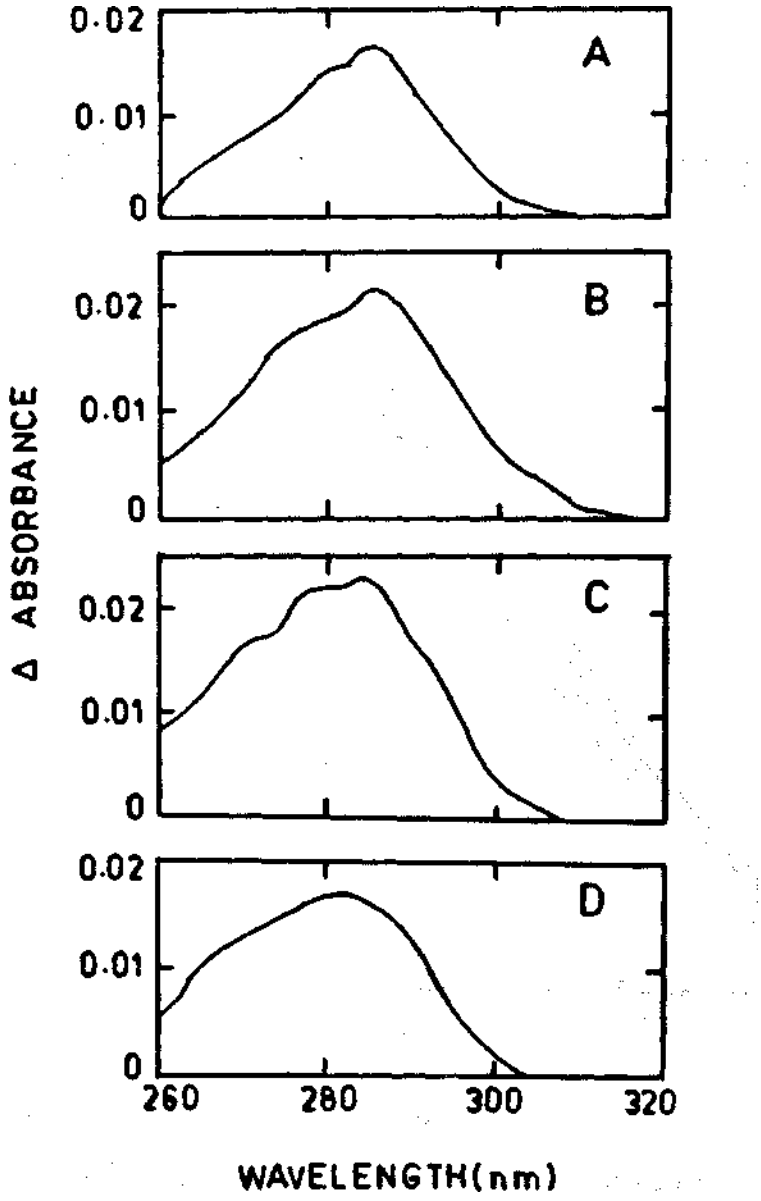

Fig. 37. UV-difference spectra of 11S protein fraction at 40% concentration of (A) Glycerol, (B) Sorbitol, (C) Sucrose and (D) Trehalsoe. The differential absorbance was monitored using the identical protein concentrations of 0.6 mg/mL.

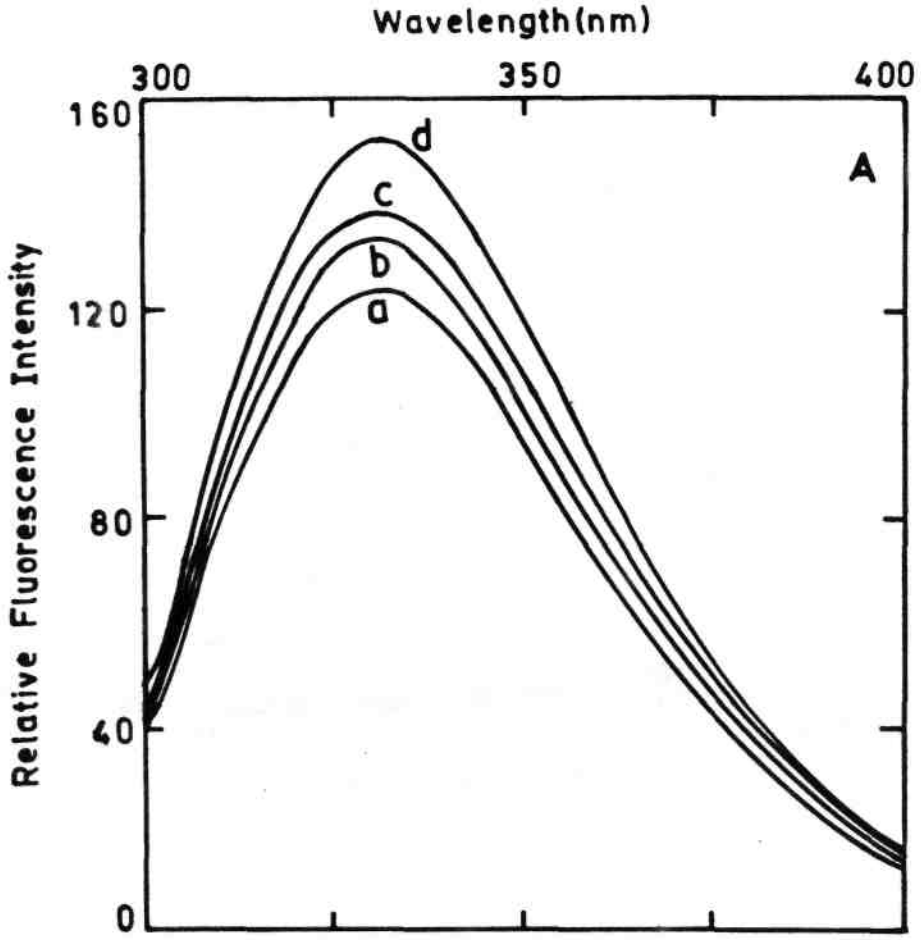

Fig. 38. Representative plots of cosolvent induced changes in the fluorescence emission intensity of 11S protein fraction in different concentration of glycerol (a) buffer only, (b) 10% glycerol, (c) 20% glycerol and (d) 40% glycerol concentration.

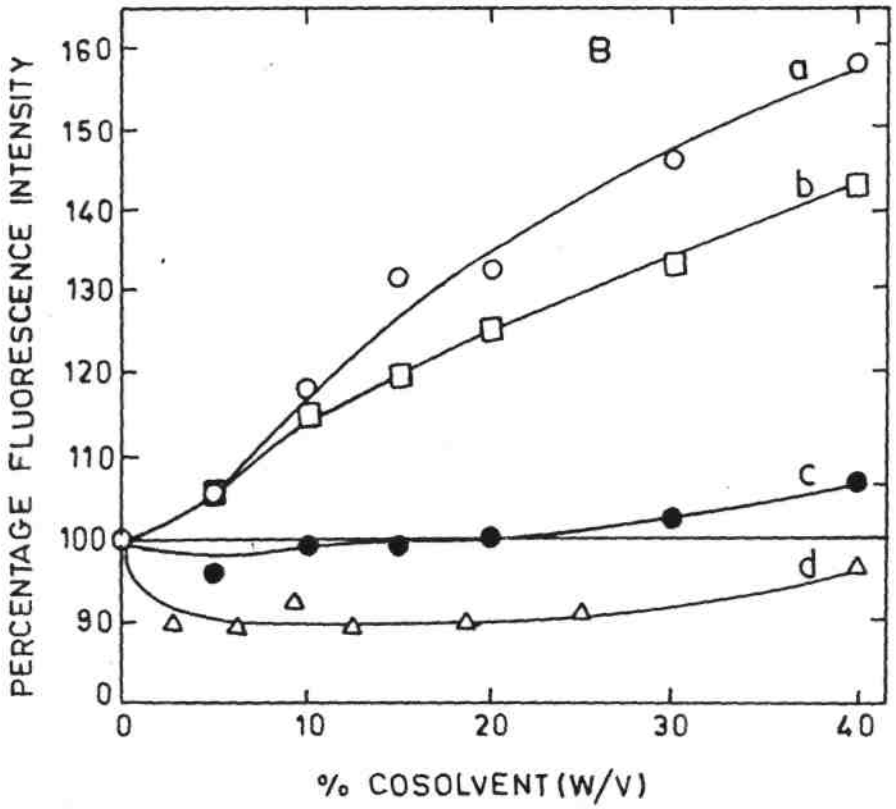

Fig. 39. Relative fluorescence intensity of 11S protein fraction at different cosolvent concentrations in (a) glycerol, (b) sorbitol, (c) sucrose and (d) trehaiose. The protein without any cosolvent is set for 100% fluorescence intensity in arbitrary units.

protein compared to native protein molecule. However, in the case of trehalose the changes are negative and the protein appears to be having less perturbed chromophores such as tryptophan as compared to native protein.

The cosolvent induced perturbation of aromatic chromophores of monomeric proteins is well documented in the literature (Herskovits. 1967; Villaneuva and Herskovits, 1971; Rajeshwara and Prakash, 1994, 1996; Rajendran *et al,* 1995; Muralidhara and Prakash, 1997). But in the case of a multimeric protein like 1IS protein fraction also the perturbation of chromophores occurs with minimal changes in the fluorescence emission maximum, which indicates the rigidity of the protein structure against cosolvent induced changes in the multimeric protein architecture. However, the interaction of cosolvents with proteins either monomeric or multimeric is dependent upon number of properties of both protein as well as cosolvent and their interaction between each other and with water as a solvent.

DISCUSSION

The cosolvent interaction with the 11S protein fraction of sunflower seed, the cosolvents being glycerol, sorbitol, sucrose, trehalose, at different concentrations indicates an increase in the stability of the protein as a function of the concentration of cosolvents. What does this mean ? From the preferential interaction and preferential hydration study it is clear from the above data that the cosolvent induces stabilization through water mediated marginal structural alterations. It is a well known fact that the role of water structure and interaction of water with the amino acid residues has a major role in the stabilization process of a protein. These are generally interlinked with enthalpy-entropy compensation and the thermodynamic state of a protein (Eftink *et al,* 1983).

The various parameters calculated from the above results of cosolvent interaction namely $(\delta g_3/\delta g_2)T,\mu 1, \mu 3$ and $(\delta m_3/\delta m_2)\ T,\mu 1, \mu 3$ suggests that the driving force for the

cosolvent mediated stability of the 1IS protein fraction is entropically driven preferential hydration in these cosolvents. The minor changes of the surface properties of the protein and alteration of equilibrium of association- dissociation of the hydrophobically stabilized 11S protein fraction are the main driving force towards that hydrated state resembling the molten globule state of the molecule.

In a typical three component system of the protein, cosolvent and water it is vital and important that we have to take into consideration the nature and extent of interaction between cosolvent and water, cosolvent and protein, and water and protein. The indirect stabilization process as a result of preferential hydration of the 11S protein fraction (Gekko and Morikawa, 1981a,b) can also bring about minimal physico-chemical changes in some proteins. The role of surface tension, the number of hydroxylic groups and the number of hydrophobic residues all determine the final stability of the multimeric protein in presence of cosolvents (Lin and Timasheff, 1996). The nature of the cosolvent and its precise balance between preferential hydration and preferential interaction determines to a large extent the ultimate process of minimizing the denaturation of the protein and thus imparting structural stability of the protein molecule.

Thus a delicate balance exists between surface hydrophobicity of the protein, nature of cosolvent, temperature of interaction between cosolvent and protein, the hydrophobicity / hydrophillicity index of protein, all of which directly or indirectly contribute to the stability of the multimeric, 1IS protein fraction of sunflower seed.

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SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Understanding of how proteins function is related to their structure, conformation and energetics of the interaction with small molecules and macromolecules in solution. Proteins are polyelectrolytes with their surface being occupied by charged, polar, neutral and nonpolar functional groups. The free energy of the protein molecules in solution is dependent on the summative of favorable and unfavorable noncovalent interactions with ligands and added cosolvent[c]. The strength and extent of interactions between protein and ligands is dependent on the chemical surface of the protein, chemical reactivity of ligands and the stereochemistry of acceptor and donor functional groups of both the molecules.

1IS protein fraction (helianthinin) is a major protein fraction from sunflower *(Helianthus annuus* L.) seeds. The seeds contain a 2S protein fraction which is the other protein fraction. Helianthinin has been isolated and characterized. Its subunit structure and association and dissociation phenomenon are studied in fair detail. 2S protein fraction has also been isolated and characterized. The seeds contain several phenolic constituents of which the polyphenols such as chlorogenic acid (CGA), caffeic acid (CA) and the non-phenolic quinic acid (QA) are present in significant amounts. CGA is implicated for the colouration of the seed proteins at alkaline pH. Interaction of CGA with the 11S and 2S protein fractions has been attempted. However, the conformational stability and energetics of both the protein fractions with individual ligands such as CGA, CA, QA or in combination is not attempted. Also the destabilization/stabilization of 1IS protein in presence of denaturants such as guanidine hydrochloride (GuHCl), guanidine

thiocyanate (GuHSCN), and cosolvents such as glycerol, sorbitol, sucrose and trehalose is not studied in detail from a thermodynamic point of view.

Based on the background of literature available, studies are carried out of the (i) interaction of CGA, CA and QA with 1IS and 2S protein fractions and (ii) interaction of guanidinium class denaturants with 1IS protein fraction (iii) effect of temperature on structural stability of 1IS protein fraction in presence of different ligands such as hydrogen ion, sodium chloride, polyphenols, guanidinium salts and cosolvents and (iv) interaction of cosolvents with 1IS protein fraction.

Introduction consists of a detailed review of the literature on polyphenols, sunflower seed proteins and the interaction between them. This is followed by description of the scope and objectives of the present study. Materials and methods consists of the materials used for the study, the methodology adopted in carrying the experiments and the analysis procedure of the data obtained by each technique.

Results are organized in four chapters. Chapter I constitutes isolation and characterization of 1IS and 2S protein fractions, and their interaction with CGA, CA and QA. Chapter II comprises the interaction of guanidinium class of denaturants with US protein fraction. Chapter III consists of the effect of temperature on the structural stability of 1IS protein fraction. Chapter IV consists of the data on interaction of glycerol, sorbitol, sucrose and trehalose with 1IS protein fraction.

The results on the isolation and characterization of US and 2S protein fractions and their interaction with CGA, CA and QA are presented in Chapter I.

The important results are :

1. Helianthinin is isolated to homogeneity by 20% (w/v) ammonium sulphate precipitation of total proteins followed by gel filtration and the protein eluted at Ve/Vo of 2.0 on a Sepharose 6B-100 column is pooled, dialyzed and freeze dried. The homogeniety of the protein is checked by established biophysical techniques.

2. The protein has a molecular weight of 2,41,000 + 5000 and its $S_{20,w}$ value is 11.14 + 0.05 S. Its amino acid composition correlates excellently with literature values for the amino acid composition of the 1IS protein fraction. Its absorption maximum, fluorescence excitation maximum and emission maximum are 279, 285 and 332 nm, respectively. Its reduced viscosity is 3.8 mL/g and partial specific volume is 0.763 + 0.003 mL/g.

3. 2S protein fraction is isolated by 20% (w/v) ammonium sulphate precipitation followed by gel fractionation through HPLC size fractionation. The protein eluting at a Ve/Vo of 2.8-3.1 is precipitated with 45% (w/v) ammonium sulphate. The precipitate is pooled and dialyzed versus water and freeze dried. The homogeniety of the protein is checked by established biophysical techniques. The sedimentation coefficient of the protein is determined to be 1.82 + 0.03 S.

4. Its molecular weight is 13000 ± 1000 and is a single band in SDS-PAGE. The absorption maximum, fluorescence excitation maximum and emission maximum are 276, 286 and 338 nm, respectively.

5. CGA binding alters the absoφtion and the fluorescence spectra of both 11S and 2S protein fractions. CGA interacts with 1IS and 2S protein fractions in a pH dependent manner. The maximum strength of the interaction is at pH 6.0. The association constant (Ka) values as evaluated from titration of pH of the protein suggests a stronger binding.

6. The alpha-helical content of 1IS protein is decreased from 9% for control to 6.0% in 8 ∧ 10^{-5} M CGA and 8% in 1 x 10^{-4} M CA as monitored by circular dichroic measurements indicating no significant changes in the secondary structure of the protein as a result of interaction with CA and CGA.

7. The US protein has multiple classes of binding sites for CGA, CA and QA and the number of binding sites at 28°C are 150 + 20, 60+10 and 64+10, respectively.

8. There are multiple classes of binding sites for CGA present on 2S protein fraction also.

9. Modification of tryptophan and lysine residues of 11S protein fraction and non-covalent modification of surface amino acids by 1M NaCl and 1-amino 8-naphthalene sulphonic acid decreases the binding strength of CGA suggesting the involvement of —* hydrophobic amino acid residues such as tryptophan in the binding process.

10. Based on the above data principally hydrophobic, hydrogen bonding and ionic interactions are envisaged in the interaction of CGA, CA and QA with both the proteins and the affinity of the three ligands and their strength clearly indicated in terms of CA > CGA > QA.

The Chapter Π consists of the results on the association-dissociation and denaturation phenomena of 11S protein fraction in presence of guanidinium salts at pH 6.0. The results are given below:

1. Interaction of GuHCl with 11S resulted in dissociation of 11S to 2S through an intermediate 7S (trimer). The dissociation of the protein is complete at 3M GuHCl with a maximum trimer concentration being present at 1.75 M GuHCl.
2. In intermediate concentrations of GuHCl the protein precipitates and is a protein concentration dependent phenomena.
3. Dissociation is complete by 3M GuHCl and denaturation is complete at 4M GuHCl suggesting that dissociation and denaturation are sequential and the data is supported by analytical ultracentrifugation and fluorescence measurements.
4. The fluorescence emission maximum is increased from 332 nm for the control protein to 352 nm in presence of 3.5 M GuHCl suggesting the exposure of tryptophan residues to bulk solvent.
5. The reduced viscosity of the protein is increased from 3.8 mL/g for the control protein to 28 mL/g in 4M GuHCl.
6. Interaction of GuHSCN with 1 IS protein fraction results in dissociation from 1 IS to 7S and subsequently to 2S in a sequential manner. The dissociation is complete at 2M GuHSCN and the denaturation of the protein also begins at this concentration as suggested by fluorescence spectroscopy.

7. The fluorescence emission maximum of the protein increases from 332 nm for the control to 352 nm in presence of 2M GuHSCN suggesting the exposure of the tryptophan residues to bulk solvent at nearly half the concentration of GuHCl.

The Chapter IΠ constitutes the study of thermal stability of 11S and 2S protein fractions. The results of this chapter are as follows.

1. Helianthinin at pH 6.0 and 8.6 has different apparent Tm. The protein has more stabilised structure at pH 6.0 compared to pH 8.6 with apparent T_{m1} of76+l°C and 71 + 1°C, respectively.
2. Effect of NaCl on the apparent Tm of 1IS protein fraction suggests that apparent Tm changes as a function of NaCl concentration which is pH dependent for e.g. at pH 6.0 in 0.5 NaCl there is no change in apparent T_{m1} on the other hand the apparent T_{m1} shifts to higher temperature from 0-1 M NaCl concentrations at pH 8.6.
3. The effect of cosolvent on the apparent Tm of 1 IS protein fraction suggests the more thermally stable protein. The apparent T_{m1} increases as a function of glycerol concentration with a value of 95 + 1°C at 15% glycerol concentrations.
4. In sorbitol the apparentT_{m1}increases by 7°C at 10% concentration whereas at 30% the value is greater than 95°C.
5. In the case of sucrose, in the concentration range 2.5 - 25%, does not induce high degree of thermal stability and the apparentT_{m1} value at 25% sucrose is 86 + 1 °C.

6. On the other hand in case of trehalsoe in the concentration range 5 - 30% only 9% increase in the apparent T_{m1} is observed with the apparent T_{m1} values being 80 + 1 and 89 + 1 at *5%* and 30% trehalose concentration, respectively.

7. Apparent Tm of 1 IS protein fraction in GuHCl and GuHSCN is highly concentration dependent and also the nature of guanidinium salts. For e.g. at 0.1 M the apparent T_{m1} is 77 + 1°C and 74 ± 1°C for GuHCl and GuHSCN, respectively. At 0.6M GuHCl the apparentT_{m1} is 72°C whereas even at 0.5 GuHSCN the apparent T_{m1} is already reached 62°C indicating the destabilization of the protein molecule in presence of such low concentration of denaturants.

8. In the case of polyphenols such as CGA, CA, QA the apparent T_{m1} of 1IS protein fraction does not change significantly at 1 x 10^{-5}M concentrations where all the three cases the apparentT_{m1} is between 74 + 1 °C as compared to the control protein value of 76 + 1°C. This indicates that even there is difference in the stereochemical property between these ligands the macroscopic properties of the protein are effected to the same extent.

The Chapter TV consists of the preferential interaction and association phenomena of 1IS protein fraction with cosolvents at pH 8.6. The results are as follows.

1. The partial specific volume (mL/g), of the control protein in isomolal and isopotential condition is 0.763 + 0.003 and 0.777 + 0.001.

2. The partial specific volume (mUg), values of the protein in isomolal condition are 0.769, 0.731, 0.710, 0.736, 0.739 and 0.734, respectively in presence of 10%, 15%, 20%, 30%, 40% and 45% glycerol, respectively. The isopotential v values are 0.772,

0.770, 0.757, 0.776, 0.783, 0.775, respectively in presence of 10%, 15%, 20%, 30%, 40% and 45% glycerol, respectively.

3. The partial specific volume (mL/g) values of the protein in isomolal condition are 0.763, 0.733, 0.707, 0.710, 0.733 and 0.708, respectively in presence of 10%, 15%, 20%, 30%, 35% and 40% sorbitol, respectively. The isopotential partial specific volume (mL/g) values are 0.764, 0.751, 0.774, 0.795, 0.751, 0.735 in presence of 10%, 15%, 20%, 30%, 35% and 40% sorbitol, respectively.

4. The partial specific volume (mL/g) values of the protein in isomolal condition are 0.760, 0.741, 0.722, 0.724, 0.708 and 0.705 in presence of 10%, 20%, 30%, 40%, 45% and 50% sucrose, respectively. The isopotential partial specific volume (mL/g) values are 0.760, 0.773, 0.757, 0.768, 0.777, 0.770, respectively in presence of 10%, 15%, 20%, 30%, 40%, 45% and 50% sucrose, respectively.

5. The partial specific volume (mL/g) values of the protein in isomolal condition are 0.757, 0.752, 0.745 in presence of 10%, 20% and 40% trehalose, respectively. The isopotential partial specific volume (mL/g) values are 0.763, 0.774, 0.785 in presence of 10%, 20% and 40% trehalose, respectively..

6. The preferential interaction parameter ($\xi 3$) values as calculated from the above "v data of both sucrose and trehalose with protein is -0.13 g/g at 40% (w/v) concentrations.

7. The $\xi 3$ values as calculated from the above data of glycerol and sorbitol at 40% concentrations with protein are -0.25 g/g and -0.095 g/g respectively.

8. Absoφtion coefficient, El%, 1cm at 280 nm is increased from 6.09 for control protein to 6.28, 6.32, 6.37 and 6.32 in presence of glycerol, sorbitol, sucrose and trehalose at 40% concentrations, respectively.

9. Fluorescence intensity of the protein increases significantly in presence of glycerol and sorbitol, while the effect is marginal in sucrose and trehalose suggesting the stabilization of the protein molecule.

Thus the focus of this thesis on the interaction of ligands with 11S protein fraction addresses the very vital issue of destabilization of protein structure in presence of above mentioned ligands and such destabilization has also the contribution of water structure to the molten globule structure of the 11S protein fraction. The results explain not only the stabilization-destabilization of IIS protein fraction but also the interaction mechanism of ligands with 11S protein and 2S protein fractions at multiple concentrations of ligand and protein. The data also opens up newer vistas in understanding the 1IS protein fraction from different angles of association-dissociation and denaturation and to a large extent conformational changes in presence of denaturants and ligands which contribute to the structural understanding of this protein from an energetics point of view.

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