

V. STUDIES ON CATARACT

1. CURCUMIN AND TURMERIC DELAY STREPTOZOTOCIN - INDUCED DIABETIC CATARACT IN RATS

Chronic hyperglycemia is a major determinant in the development of secondary complications of diabetes, including diabetic cataract. Studies indicate that diabetes increase the risk of development of cataract. Though the etiology of cataract is not fully understood, oxidative damage to the constituents of the eye lens is considered to be a major mechanism in the initiation and progression of various types of cataracts, including diabetic cataract. Diabetes causes increased oxidative stress in various tissues including lens.

On the other hand, a number of studies suggest that intake of antioxidant rich foods may slow the progression of cataract. Curcumin, the active principle of turmeric, has been shown to have significant antioxidant activity. Earlier, the effect of curcumin against galactose-induced cataract model using two levels of curcumin, 0.002 and 0.01% in the diet were studied. Interestingly, though curcumin delayed the onset of cataract at both the levels, maturation was delayed by 0.002% curcumin, but not by 0.01% (*Mol Vis* 9: 223-230, 2003). Since galactose-induced cataract does not mimic typical diabetic cataract of humans, the effects of curcumin was investigated in another model of diabetic cataract, streptozotocin (STZ) induced diabetic cataract model.

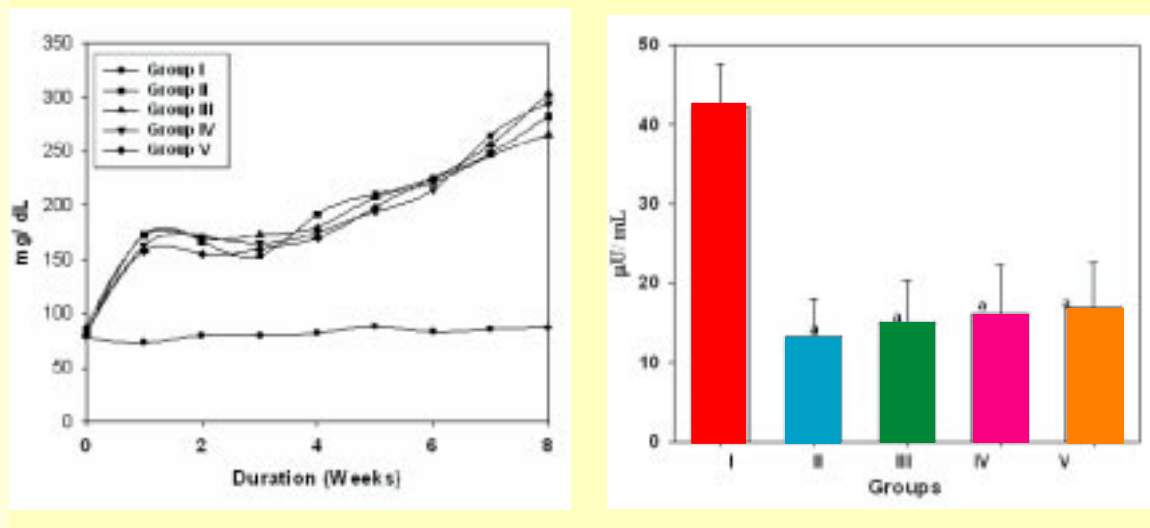
Methodology

WNIN rats were selected and diabetes was induced by streptozotocin (35 mg/kg body weight; IP) and divided into 4 groups (Group II-V). The control (Group I) rats received only vehicle. While Group I and Group II animals received AIN-93 diet, rats in Group III, Group IV and Group V received 0.002%, 0.01% curcumin and 0.5% turmeric in AIN-93 diet respectively, for a period of 8 weeks. Cataract progression due to hyperglycemia was monitored by slit lamp biomicroscope and classified into 4 stages. At the end of 8 weeks, the animals were sacrificed and the biochemical pathways involved in the pathogenesis of cataract such as oxidative stress, polyol pathway, alterations in protein content and crystallin profile in the lens were investigated to understand the possible mechanism of action of curcumin and turmeric. Blood glucose and insulin were also determined.

Results

1. Despite the increased food intake, the body weight of Group II animals was decreased (194g), when compared to the controls (385g). However, the decrease in body weight due to hyperglycemia was not ameliorated either by treatment with curcumin or turmeric.
2. Both curcumin and turmeric did not prevent the streptozotocin-induced hyperglycemia, as assessed by blood glucose and insulin levels indicating that curcumin and turmeric treatment had no effect in the state of STZ-induced hyperglycemia (Figure 18).

Figure 18: Effect of curcumin and turmeric on blood glucose (A) and insulin (B) levels in streptozotocin-induced diabetic rats



3. Interestingly, curcumin delayed the progression and maturation of STZ-induced diabetic cataract in a dose dependent manner. The effect was even more pronounced with turmeric treatment (Figure 19,20).

Figure 19: Effect of curcumin and turmeric on average stage of streptozotocin-induced cataract as a function of time

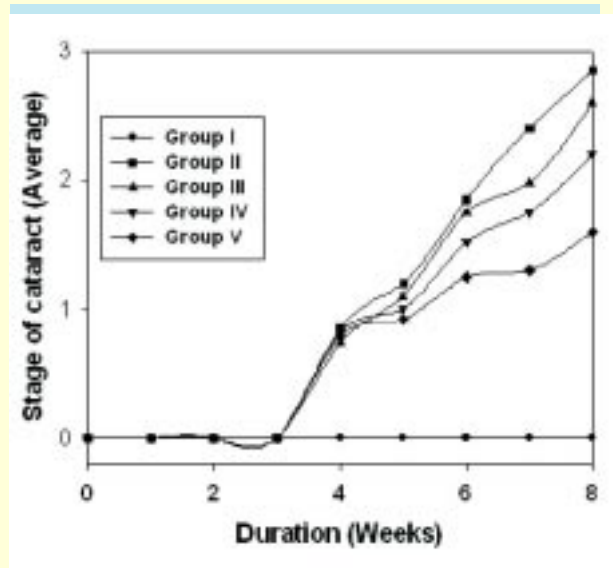
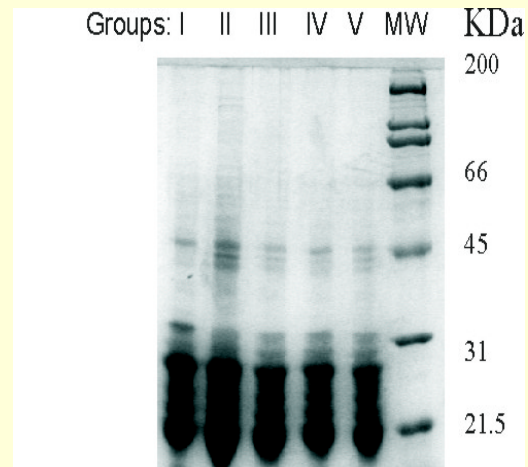


Figure 20: Effect of curcumin and turmeric on protein cross-linking in soluble fraction of lens. Soluble protein was loaded onto a polyacrylamide gel. Molecular weight standards in kDa are indicated alongside of the gel



4. Curcumin and turmeric treatment appear to have countered the hyperglycemia-induced oxidative stress, since there was a reversal of changes with respect to lipid peroxidation, reduced glutathione, protein carbonyl content and activities of antioxidant enzymes in a significant manner (Tables 12 & 13).

Table-12. Effect of curcumin/turmeric on lipid peroxidation, protein carbonyls and glutathione in rat lens

Parameter	(Group I)	(Group II)	(Group III)	(Group IV)	(Group V)
TBARS (n mol/g lens)	6.68±0.78	12.35±0.92 ^a	9.52±5.25	7.32±2.36 ^b	8.48±2.7 ^b
Carbonyls (μ mol/mg protein)	2.00±0.45	3.20±0.34 ^a	2.69±0.72	2.34±0.88	2.27±0.74
GSH ((g/g lens)	434±42.46	174±5.99a	198±12.71 a	209±6.84a	224±8.9ab

The data presented above are the mean ±SD (n=4). The superscript 'a' denotes that the data are significantly different from G I and the superscript 'b' denotes that the data are significantly different from G II (p<0.05).

Table 13. Effect of curcumin/ turmeric on activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glucose-6-phosphate dehydrogenase (G6PD) in rat lens

Enzyme	Group I	Group II	Group III	Group IV	Group V
SOD	39.6(3.54)	40.8(5.74)	39.7(5.62)	38.4(8.53)	40.3(9.08)
GPx	17.0(0.83)	23.4(1.62a)	21.1(1.31b)	19.9(2.21b)	20.8(1.01b)
G6PD	5.2±0.36	4.2±0.55 ^b	4.2±0.40	4.5±0.18	5.0±0.52 ^b

The data are the mean ± SD (n=4). The superscript 'a' denotes that the data are significantly different from G I and the superscript 'b' denotes that the data are significantly different from G II (p<0.05). While SOD activity was expressed as units/min/100 mg protein, activity of GPx and G6PD was expressed as mol of NADPH oxidized/h/100 mg protein and mol of NADP reduced/h/100 mg protein respectively.

- Also treatment with turmeric or curcumin appears to have minimized osmotic stress as assessed by polyol pathway enzymes (Table 14).

Table 14. Effect of curcumin/ turmeric on activities of polyol pathway enzymes, aldose reductase (AR) and sorbitol dehydrogenase (SDH), in rat lens

Enzyme	Group I	Group II	Group III	Group IV	Group V
AR	22.6±0.70	29.2±3.23 ^a	26.8±0.86 ^b	22.4±1.81 ^b	23.1±0.64 ^b
SDH	3.6±0.53	4.0±1.24	3.9±0.01	3.5±1.01	3.1±0.94

The data are the mean ±SD (n=4). The superscript 'a' denotes that the data are significantly different from G I and the superscript 'b' denotes that the data are significantly different from G II. AR activity was expressed as moles NADPH oxidised/h/100 mg protein and SDH activity as moles NADH oxidised/h/100 mg protein.

- Interestingly, feeding of curcumin and turmeric improved the altered total and soluble protein levels in diabetic lens (Table 15). The ability of curcumin/ turmeric to prevent the loss of soluble proteins of lens in STZ-treated rat was remarkable (Table 15).

Table 15: Effect of curcumin/ turmeric on lens protein content in rat lens

Parameter	Group I	Group II	Group III	Group IV	Group V
Total protein (mg/g lens)	495±37.3	385±27.8 ^a	398±53.1	472±48.3 ^b	468±17.5 ^b
Soluble protein (mg/g lens)	359±33.8	180±40.5 ^a	237±50.3	297±39.1 ^b	305±20.4 ^b
% Soluble protein	72.5	46.9	59.7	62.9	65.1

The data are the mean ± SD (n=4). The superscript 'a' denotes that the data are significantly different from G I and the superscript 'b' denotes that the data are significantly different from G II.

- Most importantly, curcumin and turmeric treatment not only prevented the decrease in protein content but also aggregation and insolubilization of lens proteins due to hyperglycemia as assessed by HPLC (Table 16) and SDS-PAGE (Figure 19).

Table 16. Distribution of crystallins in soluble protein fraction. Data are average of three HPLC runs for area under the curve

Peak	Group I	Group II	Group III	Group IV	Group V
HMW Peak	8631	24655	13853	10493	11172
α-Crystallin	215461	201370	202881	206158	202936
β-Crystallin	351479	277659	298147	308971	317596
γ-Crystallin	347580	294613	299900	318304	340523

Data are arbitrary units for absorbance at 280 nm.

Conclusions

The results indicate that turmeric and curcumin are effective against development of diabetic cataract in rats. Moreover, these results thus provide a clue, for the first time, that turmeric or curcumin may act downstream to glucose-mediated changes. Further, these results imply that the ingredients in our dietary sources, such as turmeric, may provide a viable food based, as well as pharmacological approach in the treatment of diabetic complications.

2. CHAPERONE ACTIVITY OF α-CRYSTALLIN UNDER DIABETIC CONDITIONS: MODULATION BY CURCUMIN

The α-Crystallin contributes to 30% of total lens protein and consists of two different subunits of each 20 kDa. These subunits self associate to form heterogeneous polydisperse complex of molecular mass of 800 kDa. α-Crystallin, being a member of small heat shock protein family acts as molecular chaperone and prevents the aggregation of other lenticular proteins/enzymes denatured by heat and other stress conditions. It has been established that chaperone function of α-crystallin is critical in maintenance of transparency of the lens *vis-à-vis* cataract formation. Diabetes is one of the major risk factors of cataract formation. In view of the prevailing and predicted epidemic of diabetes in developing countries like India, diabetic cataract may become the major leading cause of blindness along with senile cataract.

Chaperone function of α -crystallin in hyperglycemic conditions is of great concern with respect to lens transparency. It has been shown that oxidative stress can deteriorate the α -crystallin chaperone activity. Studies implicate that impaired chaperone function of α -crystallin could be involved in the formation of diabetic cataract. Therefore, it is essential to understand the role of chaperone function in diabetic conditions and the ways and means by which we can maintain and/ or modulate the chaperone potential of α -crystallin under diabetic conditions. The aim of the study was to investigate the effect of diabetes on chaperone activity of α -crystallin and to investigate modulatory effect, if any, of curcumin on chaperone activity of α -crystallin.

Methodology

Three month old male WNIN rats (b.w. 225 g) received 0.1 M citrate buffer pH 4.5 as vehicle (Group I; n=8), where as the experimental rats received a single intraperitoneal injection of streptozotocin (STZ; 35 mg/kg) in the same buffer. After 72 h, fasting blood glucose levels were monitored and animals having blood glucose levels less than 250 mg/dl were excluded from the experiment and rest were distributed into three groups. Experimental animals received either only AIN-93 diet (Group II; n=13) or received AIN-93 diet containing 0.002% (Group III; n=9) and 0.01% curcumin (Group IV; n=9). Animal care and protocols were in accordance with and approved by the Institutional Animal Ethics Committee. After 8 weeks of STZ injection rats from all the four groups were sacrificed by CO₂ asphyxiation and eye balls were enucleated. Lenses from three rats in each group were pooled for the studies. Water soluble proteins were analyzed on a Sephacryl S-300 gel filtration column and fractions corresponding to α H and α L-crystallins were pooled separately. Chaperone activity of α -crystallin (both α H and α L) was assessed by aggregation and enzyme inactivation assays. Far- and near-UV CD spectra of α H- and α L-crystallins were recorded. Intrinsic tryptophan fluorescence and fluorescence of 8-anilino-1-naphthalene-sulfonic acid (ANS) bound to α -crystallin was also measured.

Results

There was a marked difference in the relative distribution of crystallins between the groups. α H-crystallin peak has been elevated in diabetic rat lens (Group II) compared to control rat lens (Group I). Further there was a decrease in both β - and γ -crystallin fractions also in Group II (Figure 21).

- Feeding of curcumin (Group III and IV) reverted the altered crystallin profile in a dose dependent manner (Figure 21).
- α L-Crystallin from Group II rat lens showed 50% decrease in chaperone activity in suppressing the heat-induced aggregation of β L-crystallin when compared to the activity of α L-crystallin from Group I (Figure 22). Similar results were observed with α H-crystallin.
- Interestingly, the chaperone-like activity of α L from Group III and IV was improved than Group II rat lenses. Strikingly, aggregation kinetics of β L-crystallin displayed longer lag time in the presence of α L from Group III and IV compared to α L from Group II (Figure 22). Curcumin feeding also improved chaperone activity of α H-crystallin isolated from diabetic lenses.
- Similar to aggregation assays, the ability of α L-crystallin from Group II to prevent heat-induced inactivation of G6PD was declined as compared to Group I (Figure 23).
- Furthermore, in contrast to the marginal protection in aggregation assays, α L-from Group III and IV (curcumin treated) rat lens exhibited a remarkable protection against G6PD inactivation(Figure 23).
- α L from Group II showed lesser ANS binding when compared with α L from Group I (Figure 21), which correlated well with the decreased chaperone activity of α L-crystallin from Group II (Figure 22).

- Furthermore, improved chaperone activity of α L-crystallin from Group III and IV is also reflected in increased ANS binding when compared to that of α L-crystallin from Group I (Figure 24).
- Far-UV CD signal for α -crystallin isolated from diabetic rat lens is decreased compared to control rat lens, indicating altered secondary structure. Altered tryptophan fluorescence (Figure 25) and changes in near-UV CD spectra indicated altered tertiary structure of α -crystallin due to hyperglycemia.
- Although, curcumin treatment has not affected the altered secondary structure due to hyperglycemia in a significant manner, curcumin-mediated modulation of altered tertiary structural changes are quite noticeable (Figure 25).

Figure 21: Separation profile of total soluble lens protein on gel filtration. Protein (80 mg) from Group I, Group II, Group III and Group IV was loaded onto a Sephacryl S-300 HR column

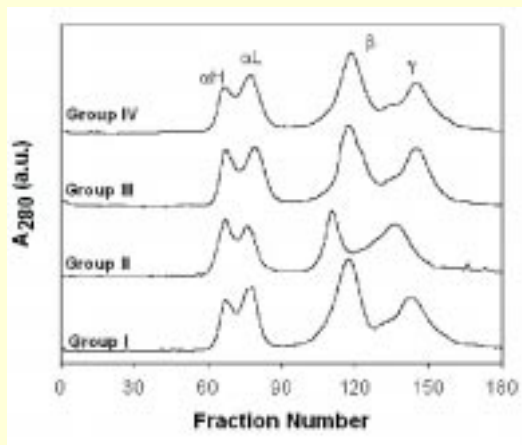


Figure 23: Chaperone activity of α L-crystallin in enzyme inactivation assays. Protection of heat-induced inactivation of glucose-6-phosphate dehydrogenase at 42°C by α L-crystallin. Bar 1-G6PD alone, Bars 2, 3, 4 and 5, are G6PD along with α L-crystallin from Group I, Group II, Group III and Group IV, respectively. Data were average of three chaperone assays

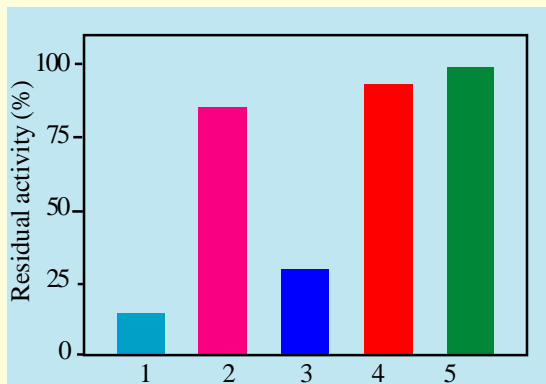


Figure 22: Chaperone activity of α L-crystallin as assessed by the suppression of heat-induced aggregation of β _L-crystallin. β _L-Crystallin was incubated at 60°C in the absence (trace 1) or in the presence of α L-crystallin (0.025 mg/ml) from Group I (trace 2), Group II (trace 3), Group III (trace 4) and Group IV (trace 5). Data were average of three chaperone assays

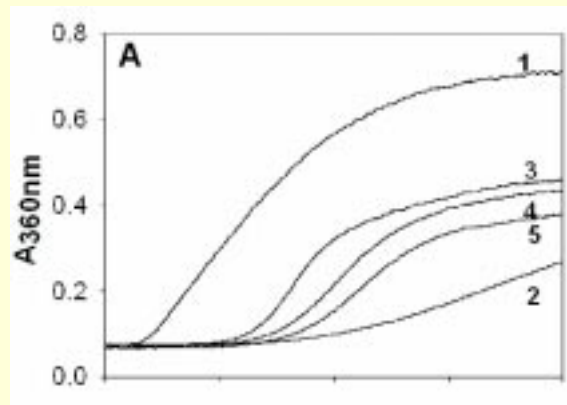


Figure 24: Hydrophobicity of α L-crystallin as assessed by ANS fluorescence. Traces 1-4 correspond to α L-crystallin from Group I, Group II, Group III and Group IV, respectively. Data were average of three assays.

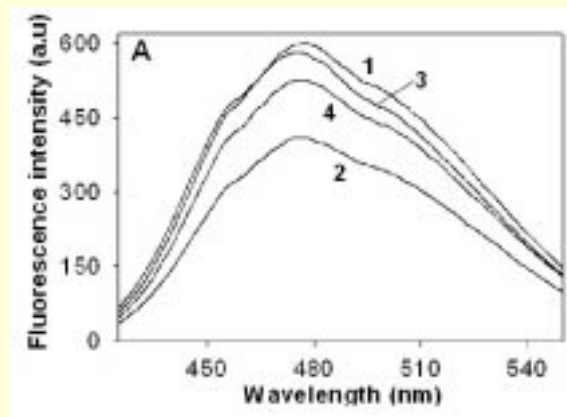
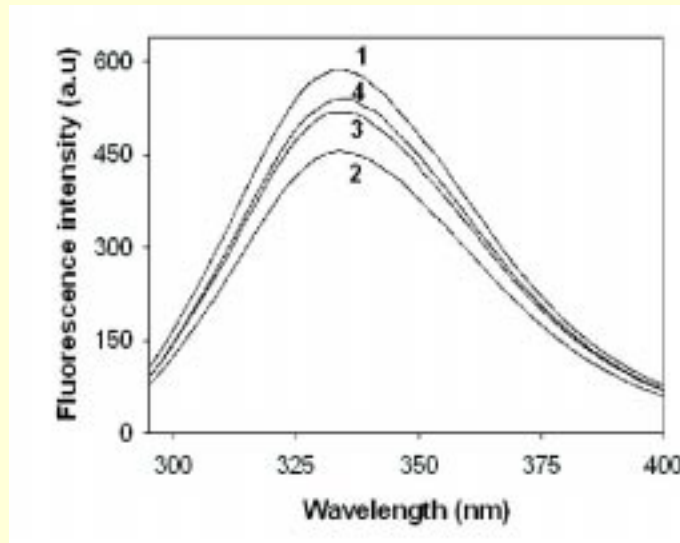


Figure 25: Tryptophan fluorescence spectra of α L-crystallin. Traces 1-4 correspond to α L-crystallin from Group I, Group II, Group III and Group IV, respectively. Data were average of three experiments



Conclusions

Present study demonstrated that in STZ-induced diabetic cataract α -crystallin exhibited diminished chaperone activity, which was positively modulated by dietary curcumin and delayed progression and maturation of cataract. Though, multiple actions of curcumin could be involved in delaying STZ-induced cataract in rats, we observed that antioxidant effect of curcumin was the predominant mechanism (Invest Ophthalmol Vis Sci, 2005). Thus, one of the possible explanations for the modulatory effect of curcumin on α -crystallin chaperone activity in diabetes could be decreased oxidative stress by curcumin in hyperglycemia.

3. INHIBITION OF PROTEIN GLYCATION BY DIETARY AGENTS

Diabetic complications is a major cause of morbidity and mortality in both the developed and developing nations, which are manifested in several organ systems in the form of vascular complications such as retinopathy, nephropathy, neuropathy and non-vascular complications such as cataract and glaucoma. Hyperglycemia is the primary factor that initiates and promotes the complications. Multiple molecular mechanisms have been proposed to explain the pathogenesis of long term complications of diabetes, one of them which is considered to be a prominent upstream phenomenon down the line that leads to various deleterious consequences is non enzymatic glycation. The high glucose levels in diabetes may cause tissue damage by the nonenzymatic glycation of proteins. Excess reducing sugars react non-enzymatically with the amino groups of proteins to form Schiff's base intermediates, which rearrange to more stable Amadori products via the Maillard reaction. These Amadori products further undergo chemical modification to form advanced glycation end products (AGE). It has been shown that formation of AGE in vivo contributes to several pathophysiologicals associated with ageing and diabetes mellitus, such as cataract.

Hence role of antiglycating agents delaying the onset or progression of diabetic complications has gained the considerable importance. Aminoguanidine and few other chemical compounds have been shown to be effective against protein glycation, but none of them are clinically successful. Therefore, the aim of the study was to investigate antiglycating potential of dietary agents for the prevention of secondary complications of diabetes.

Methodology

Briefly, total soluble lens proteins (TSP) from bovine eyes were used for in vitro glycation. Each 1 ml incubation mixture contained 10 mg of protein, 0.2 M PBS, glycating sugar (100 mM fructose), 50 µg of penicillin & streptomycin and 0.01% sodium azide. Various concentrations of aqueous extracts of dietary sources were added to the above mixture when used. Tubes were then sealed and incubated in dark at 37°C for 3 weeks. After incubation solutions were dialyzed extensively against buffer to remove unbound sugars and the extent of glycation was assessed by various complimentary methods;

- (i) Determination of protein cross linking on SDS-PAGE,
- (ii) Advanced glycation related fluorescence (ex: 370 nm and em: between 400-500 nm) and
- (iii) Protein carbonyls and tryptophan fluorescence as function of protein oxidation and conformational changes.

Results

Incubation of bovine lens total soluble protein (TSP) with fructose resulted in cross-linking of polypeptides in a dose dependent manner and these cross-links are similar to the cross-links observed in many types of cataractous lenses. Similarly, upon glycation there was an increase in non-tryptophan fluorescence due to formation of AGE. Glycation of lens proteins also leads to oxidative damage and altered conformational changes as shown by increased carbonyl content and decreased tryptophan fluorescence respectively. Since there is very little information on antiglycating agents, particularly from dietary sources, the dietary agents were coded that have shown promising antiglycating potential, to protect intellectual property rights and the code names are given in Table 17.

On the basis of the results obtained after analyzing the extent of inhibition of glycation, the extracts that are found to be promising are MB1, MB2, MAB1, MSB1, MYB1, MYB2, MYB3, MYB4, MYB5 and MYB6 (Table 17). They inhibited protein glycation in a dose dependent manner. Inhibition of glycation in comparison to control was assessed by different parameters. Order of effectiveness against glycation is given based on concentration of extracts required for inhibiting glycation by various methods

- The following agents in that order were effective in preventing the formation of cross-links of lens protein profile based on SDS-PAGE; MYB1 > MYB5 > MYB2 > MAB1 > MB1 > MSB1 > MB2 (Figure 26)
- Inhibition of AGE fluorescence was found to be in the following order; MYB1 > MB1 > MYB2 > MYB5 > MAB1 > MB2 > MSB1 (Figure 27)
- Reduction in the carbonyl content of the glycated protein by dietary extracts was observed in the following order; MYB1 > MB2 > MYB2 > MAB1 > MYB5 > MB1 > MSB1 (Figure 28)
- Recovery brought about by the extracts in the tryptophan fluorescence of protein found in the following order; MYB5 > MB2 (Figure 29)

Table 17: Code names of the dietary extracts screened for the inhibition of protein glycation.

S.No.	Code Name	Ranking*	S.No.	Code Name	Ranking*
1	MB1	3	6	MYB1	1
2	MB2	5	7	MYB2	2
3	MAB1	6	8	MYB3	4
4	MSB1	8	9	MYB4	9
5	MSB2	10	10	MYB5	7

* Ranking was assigned by averaging the potential of the extract in preventing protein glycation by all the three methods, cross-linking, AGE fluorescence and protein carbonyls methods. Results were reproduced 3 times for the agents with ranking 1-4.

Fig. 26: SDS-PAGE of lens protein upon *in vitro* glycation in the absence and presence of MYB1

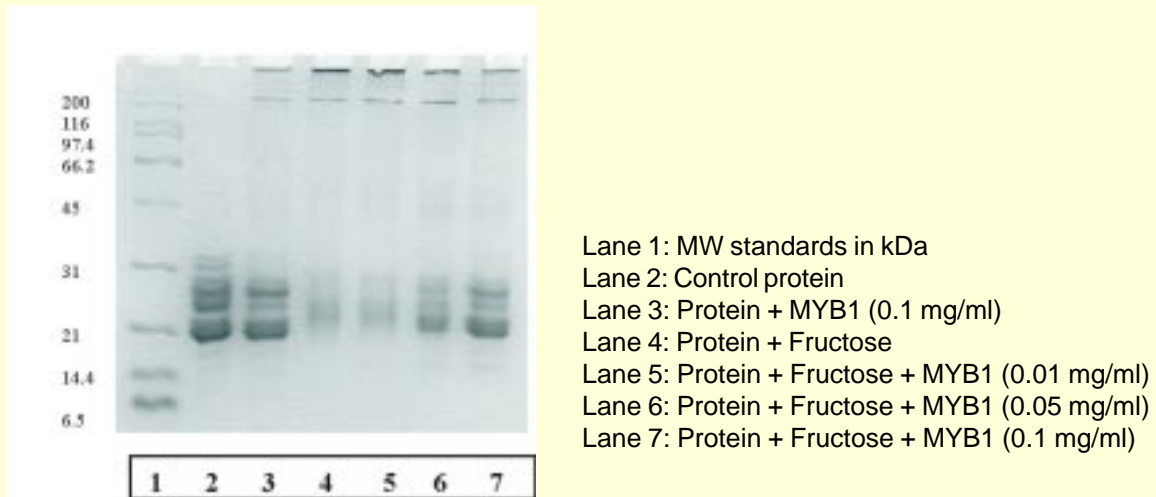


Fig. 27. AGE fluorescence on *in vitro* incubated protein with fructose in the absence and presence of MYB1

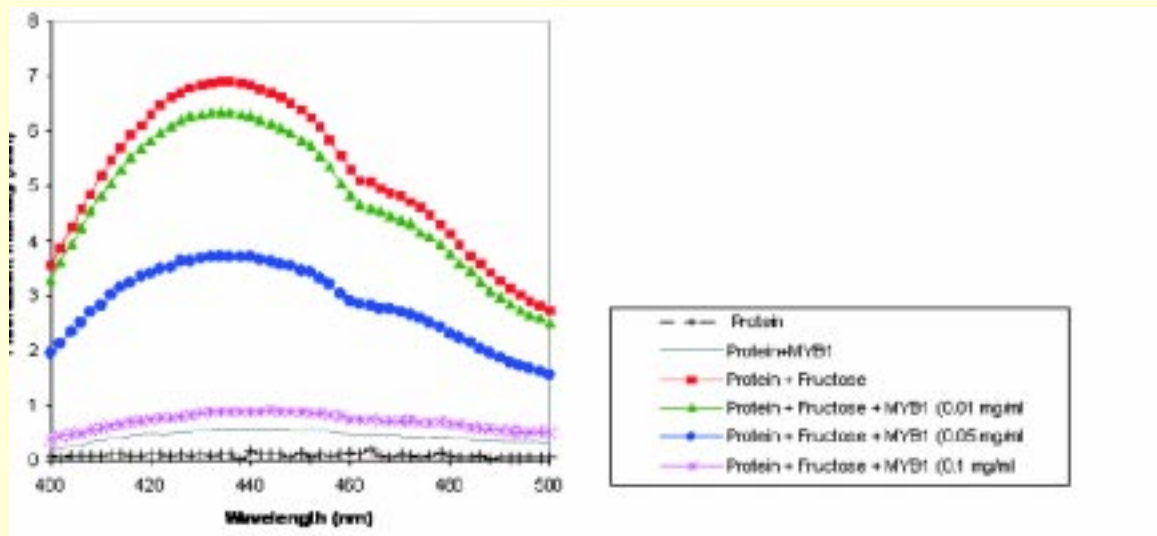


Fig. 28: Carbonyl content of lens soluble protein upon *in vitro* glycation in the presence and absence of MB2

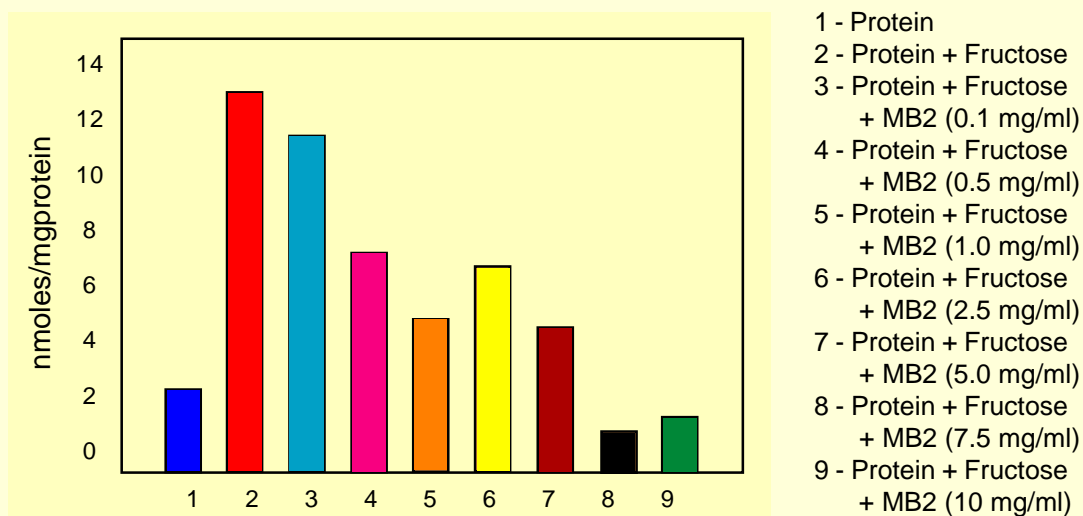
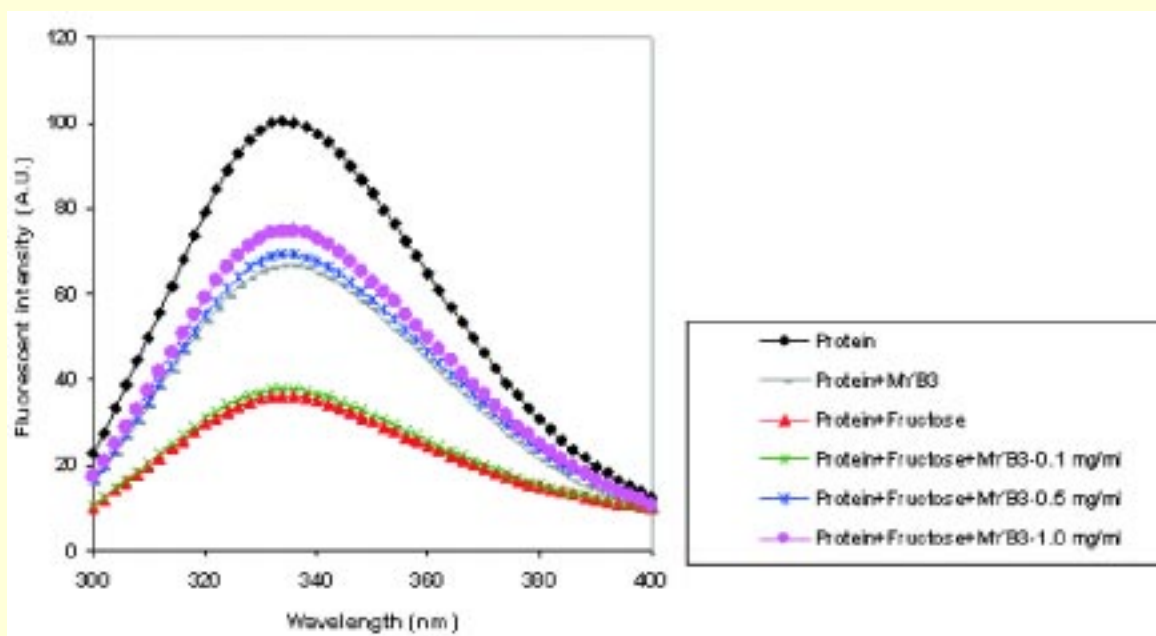


Fig. 29. Tryptophan fluorescence of TSP upon *in vitro* glycation in the presence and absence of MYB3



Conclusions

MYB1, MYB2, MB1 and MYB3 were found to be the effective inhibitors of protein glycation *in vitro*, MYB1 being the most potent. Hence, these agents may be exploited for their potential in the management of secondary complications of diabetes. Studies are underway to investigate the mechanism of antiglycation by the effective agents and their significance in the pathophysiology.

4. EFFECT OF METHYLGLYOXAL ON DEGRADATION AND STABILITY OF α -CRYSTALLIN

Several diabetic complications including cataract are thought to be result of accumulation of advanced glycation end products (AGE) generated from modification of proteins by different glycation agents. Methylglyoxal (MGO), a major dicarbonyl compound, is present in high concentrations in lens compared to plasma or any other tissue and its levels increase several folds during diabetes. Compared to other potential glycation agents, MGO has very high affinity for proteins and is known to react with Arg, Lys, His and Cys residues forming AGE. α -Crystallin, molecular chaperone of eye lens plays an important role in maintaining the transparency of eye lens by preventing the inactivation or aggregation of several enzymes/proteins and also as a key structural element. Being a long-lived protein and rich in basic amino acids, α -crystallin may be more susceptible to non-enzymatic browning by MGO. Earlier it was reported that MGO-modified α -crystallin exhibited enhanced chaperone-like activity in aggregation assays but it was less effective in preventing enzyme inactivation (Biochem J, 379, 1-10, 2004).

Further, modified α -crystallin has showed decreased hydrophobicity, altered secondary/ tertiary structure and increased oligomeric size (Biochem J, 379, 1-10, 2004). Although altered/damaged proteins are known to be more susceptible to degradation, effect of non-enzymatic glycation of protein on its susceptibility to degradation was not well studied. Eye lens contains high levels (3-5 mM) of ATP and its binding has been shown to protect α B crystallin from proteolytic digestion. Therefore, we have investigated the effect of MGO-modification on α -crystallin degradation and stability and the role of ATP mediated protection. Finally, physiological significance of MGO effects on α -crystallin *vis a vis* lens transparency was studied using lens organ culture system.

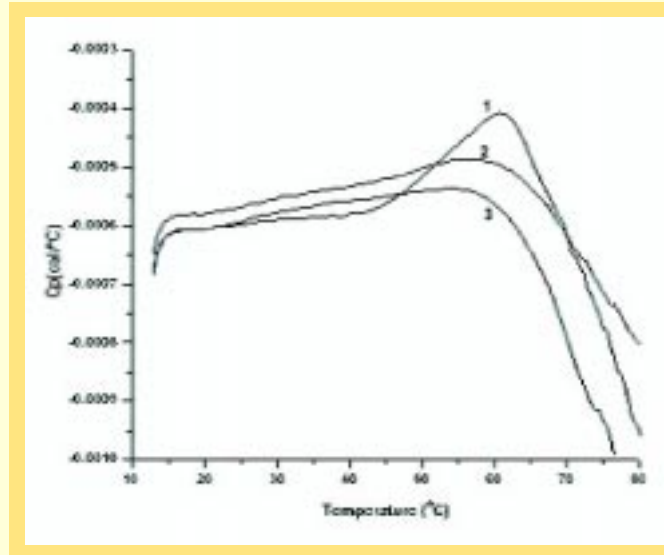
Methodology

The α -Crystallin was isolated from bovine lenses by gel filtration (Sephacryl S-300HR) and incubated with various concentration of methylglyoxal (MGO) for different time periods at 37°C under sterile conditions. Unbound MGO was removed by extensive dialysis. Extent of protein damage was analyzed by assessing formation of protein carbonyls by 2, 4-DNPH method. Effect of MGO-modification on stability was studied by differential scanning calorimetry (DSC) and denaturant induced unfolding. Susceptibility of MGO-modified α -crystallin to proteolytic digestion by trypsin and chymotrypsin was analyzed by SDS-PAGE. ATP binding to native and MGO-modified α -crystallin was studied using tryptophan fluorescence quenching. Bovine lenses were cultured in modified TC-199 medium with antibiotics at 37°C under 95% air and 5% CO₂. Lenses were incubated with and without 1 mM MGO for different time periods. After incubation, lenses were homogenized and soluble and insoluble fractions were prepared by centrifugation.

Results

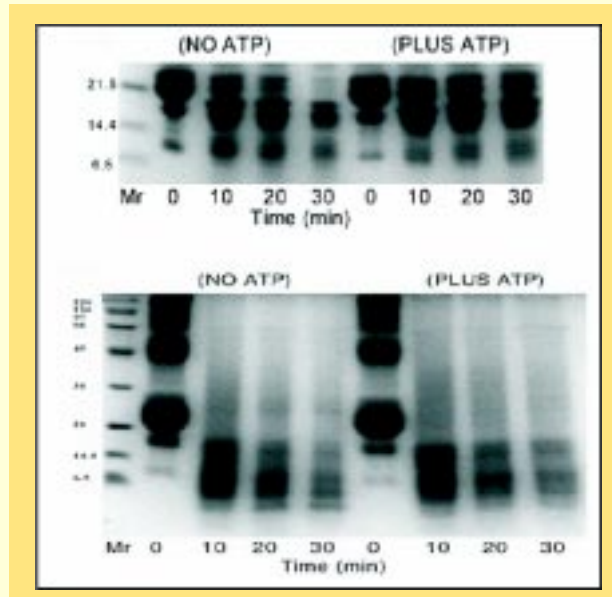
- MGO modification leads to severe oxidative damage as shown by increased protein carbonyl formation.
- Methylglyoxal modification of α -crystallin, reduced its stability in a concentration dependent manner as studied by differential scanning calorimetry, T_p was shifted to 56 and 53 from 65°C respectively for 10 and 100 mM MGO-modified α -crystallin (Figure 30).
- Denaturant induced unfolding studies show that MGO-modified α -crystallin unfolds at lower concentrations of denaturant compared to native α -crystallin. These results further support DSC data and suggest that methylglyoxal modification decreases the stability of α -crystallin.

Fig. 30. Tracings of typical differential scanning calorimeter thermograms (heat capacity versus temperature) of native and modified α -crystallin. Trace 1- native α -crystallin, trace 2 & 3 - 10 and 100 mM MGO modified α -crystallin.



- Proteolytic digestion studies of native and MGO-modified α -crystallin suggest that methylglyoxal modification increases the susceptibility to degradation by proteases such as trypsin and chymotrypsin.
- Binding of ATP could protect native α -crystallin against proteolytic digestion by trypsin but not MGO-modified α -crystallin (Figure 31)

Fig. 31. Trypsin digestion of native (A) and MGO-modified (B) α -crystallin in the absence & presence of ATP at 37°C as analyzed by SDS-PAGE. α -Crystallin was modified by incubating with 5 mM MGO for 24 h. The time course for the proteolysis was indicated at the bottom of the gel. Molecular weight standards in kDa are marked alongside.



- Stem-Volmer plot for the ATP binding to native and MGO-modified α -crystallin indicates decreased affinity for the binding of ATP to MGO-modified α -crystallin. Scatchard analysis indicates decreased binding sites for the ATP upon MGO-modification of α -crystallin (Table 18).

Table 18. Binding of ATP to native and MGO modified α -crystallin.

Group	Binding constant (K_d , nM)	Binding sites (N)
Native α -crystallin	3.130	7.9
Modified α -crystallin		
1 mM	1.611	4.5
5 mM	1.27	0.14
10 mM	2.296	0.10

Values are average of three experiments

- Lens organ culture studies indicate presence of MGO in the culture medium leads to the opacification of the calf lenses (Figure 32).
- Total soluble protein analyzed by gel filtration indicates MGO treated lenses having high amounts of high molecular weight α -crystallin (α_H) compared to control lenses.
- Furthermore, α -crystallin fractions isolated from MGO-treated lenses showed diminished chaperone activities.
- Western blot analysis of the soluble and insoluble fractions of MGO-treated and control lenses indicates that MGO-modification of α -crystallin leads to AGE formation and insolubilisation causing the scattering of light.

Fig 32. Methylglyoxal-induced opacification of lens in organ culture: Calf lenses were cultured in modified TC-199 in the presence of 1 mM MGO for '0' days (A), 2 days (B) and 4 days (C). Transparency of the lens that was cultured for 4 days in the absence of MGO was similar to '0' days lens

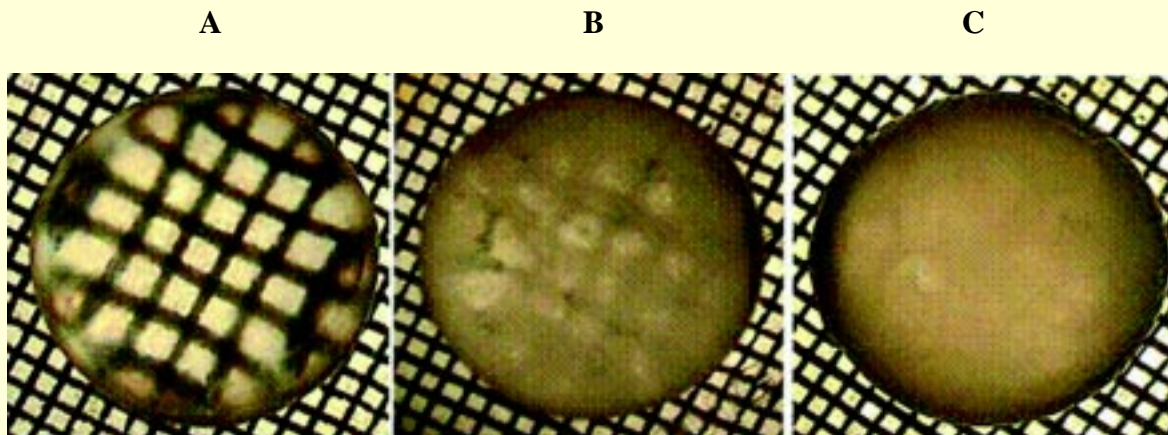
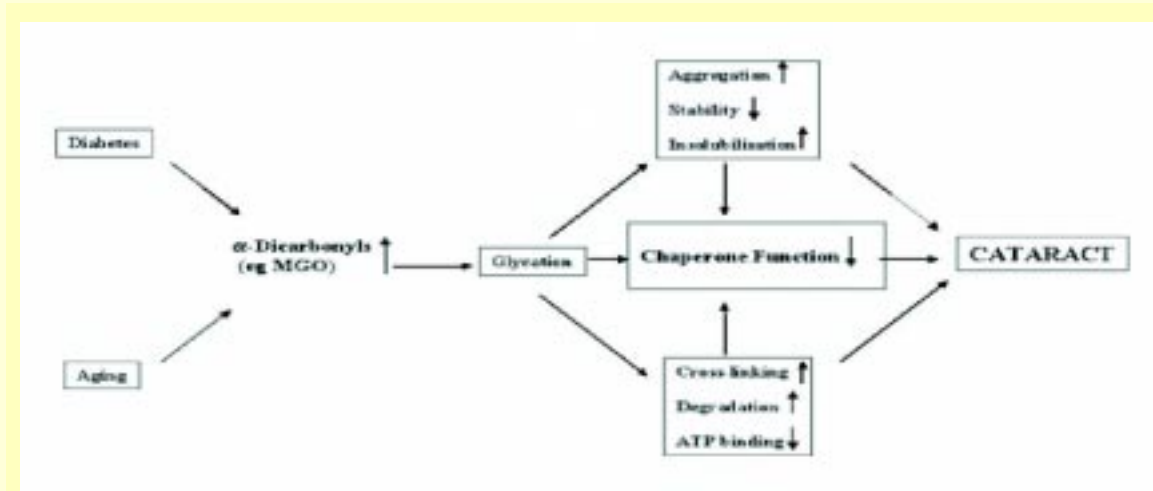


Fig. 33. Schematic representation of possible molecular effects of methylglyoxal on α -crystallin *Vis a vis* lens opacification



Conclusions

The studies show that nonenzymatic browning of α -crystallin by MGO leads to decrease in its stability and unfolding that in turn leads to the exposure of buried proteolytic sites causing enhanced proteolytic degradation. The ATP could not protect the glycated α -crystallin from proteolytic degradation as was observed with native α -crystallin. As depicted in figure 33, unfolding and conformational changes due to MGO modification increase susceptibility to degradation and subsequent light scattering due to cross-linking/ insolubilisation. Hence, it is quite possible that posttranslational modification imposed by dicarbonyls may have unfavorable effects on the ability of α -crystallin to inhibit protein aggregation/ enzyme inactivation *in vivo*. Results of the present study provide the basis for the role of non-enzymatic glycation on α -crystallin chaperone activity in age-related brunescence and diabetic cataracts.

5. INSIGHTS INTO THE HYDROPHOBICITY AND CHAPERONE FUNCTION OF α -CRYSTALLIN: ISOTHERMAL TITRATION CALORIMETRY STUDY

The α -Crystallin, a member of the sHSP group constitutes a major portion of the eye lens cytoplasm. Lenticular α -crystallin is a hetero-oligomer with two subunits, α A and α B, mostly present in a stoichiometry of 3:1. α A and α B are 20 kDa each and share ~60% sequence identity. Both homo and heteropolymers of α -crystallin exhibit chaperone-like activity by suppressing protein aggregation. It is clear that, in addition to providing refractive properties to the eye lens, α -crystallin is instrumental in maintaining transparency of the eye lens with its chaperone-like activity. Despite their high sequence homology, the relative importance of α A- and α B-crystallins is not completely understood. Although the mechanism of chaperone function is not understood completely, numerous studies implicate surface-exposed hydrophobic sites on α -crystallin and other sHSP in binding to partially unfolded proteins.

The finding that increased exposure of hydrophobic surfaces on structurally perturbed α -crystallin is associated with increased chaperone like function, substantiates the role of hydrophobicity in chaperone function of sHSP. However, the enhanced chaperone-like activity with increase in temperature was not

similarly associated with increased hydrophobicity of α A- and α B-crystallins. α A- and α B-Crystallins differ not only in their hydrophobic character with temperature but also in their secondary and tertiary structure, molecular size and other physicochemical properties. This makes an exact correlation between hydrophobicity and chaperone activity difficult to establish. In the present study isothermal titration calorimetry (ITC) was employed to determine the number of binding sites and the thermodynamics of 8-anilino-1-naphthalene sulfonic acid (ANS) binding to α A- and α B-crystallin. Hydrophobicity and chaperone activity of α A- and α B-crystallins were correlated at different temperatures to get greater insight into the role of hydrophobicity in the chaperone-like function of α -crystallin.

Methodology

Recombinant α A- and α B-crystallins were purified according to previously reported methods (*FEBS Lett*, 2002, 522, 59-64). The chaperone activity of α A- and α B-crystallin was studied by assessing their ability to suppress the aggregation of insulin B chain at 15 and 30°C (induced by DTT). Hydrophobicity was measured with ITC using ANS as a hydrophobic ligand. In brief, 4-8 μ l aliquots of ANS stock solution were added to α A or α B crystallin solution and heat changes accompanying these additions were recorded. The data so obtained were fitted using non-linear least squares minimization method for determining the binding stoichiometry (N), binding constant (K_b), and change in enthalpy (ΔH^b), using Origin software (Microcal Inc) and two site model provided the best fit for the data. The change in entropy (ΔS) was calculated from $\Delta G = \Delta H - T\Delta S$. For chaperone assays performed in the presence of ANS, α A and α B-crystallins were preincubated with saturating amounts of ANS at specified condition as described in figure legends and excess ANS was removed by dialysis.

Results

1. The quantification of the hydrophobic surfaces in α A- and α B-crystallins by ITC indicate presence of two binding sites, one with high affinity, low capacity and another with low affinity, higher capacity. At 30°C, α B showed higher number of ANS binding sites than α A (Table 19 & Figure 34).

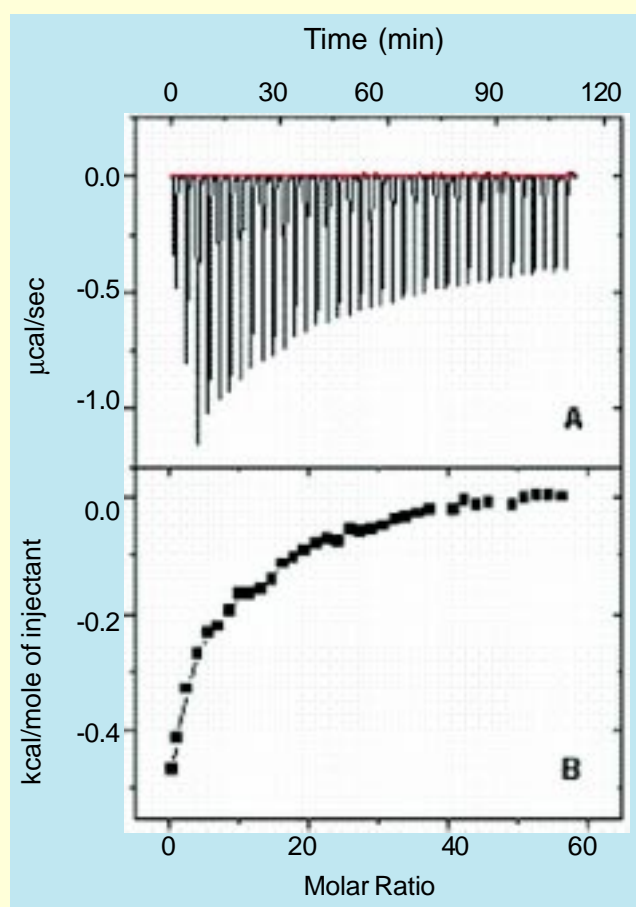
Table 19. Calorimetric data of ANS binding to α A- and α B-crystallins at 15°C and 30°C

Parameters	15°C		30°C	
	α A	α B	α A	α B
N1	3.5 \pm 0.47	3.1 \pm 0.32	0.98 \pm 0.06	5.9 \pm 0.26
K1 (M^{-1}) $\times 10^5$	2.4 \pm 0.16	4.6 \pm 0.42	7.8 \pm 0.66	2.1 \pm 0.42
ΔG_1 (kcal/mol)	-7.0 \pm 0.67	-7.4 \pm 0.61	-6.4 \pm 0.56	-7.0 \pm 0.89
ΔH_1 (kcal/mol)	-368 \pm 22.4	-612 \pm 29.1	-1075 \pm 93.3	-1262 \pm 196
ΔS_1 (cal/mol/K)	23.4 \pm 2.61	23.8 \pm 1.62	18.8 \pm 2.31	20.2 \pm 3.21
N2	12.2 \pm 1.40	11.9 \pm 1.57	18.0 \pm 2.32	27.1 \pm 3.05
K2 (M^{-1}) $\times 10^5$	1.9 \pm 0.09	0.42 \pm 0.037	0.73 \pm 0.079	4.1 \pm 0.60
ΔG_2 (kcal/mol)	-5.6 \pm 0.63	-4.7 \pm 0.62	-5.0 \pm 0.65	-10.6 \pm 1.20
ΔH_2 (kcal/mol)	-127 \pm 10.6	-506 \pm 39.2	-243 \pm 16.1	-56.9 \pm 5.70
ΔS_2 (cal/mol/K)	19.2 \pm 2.35	14.8 \pm 1.25	16.8 \pm 1.05	20.9 \pm 2.5

(N= Number of binding sites; K= binding constant; ΔG = Change in free energy; ΔH = Change in enthalpy; ΔS = Change in entropy; The numbers 1 and 2 indicate thermodynamic parameters associated with site 1 and site 2)
In each case the errors associated have been mentioned.

- In agreement with higher number of hydrophobic sites, α B-crystallin, demonstrated higher chaperone activity than A at 30°C.
- Thermodynamic analysis of ANS binding to α A- and α B-crystallins indicate that high affinity binding is driven by both enthalpy and entropy changes with entropy dominating the low affinity binding (Table 19).

Fig. 34. Calorimetric titration profile of the binding of ANS to native α A-crystallin at 30°C. Panel A: Exothermic heats associated with the injection of ANS into the sample cell containing α A-crystallin. Panel B: Binding isotherm corresponding to the data in panel A.

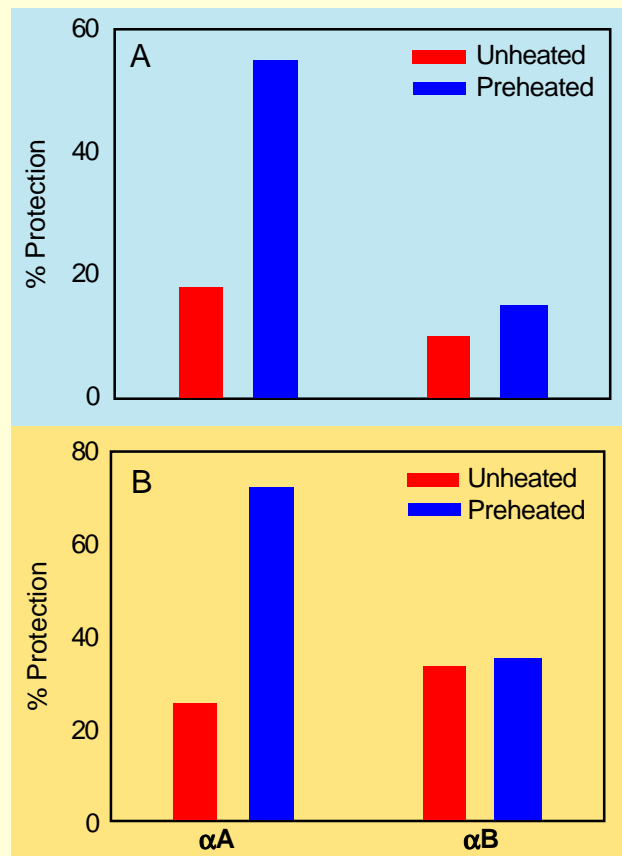


- Interestingly, although ANS binding sites were similar for α A and α B, at 15°C, A was more potent than α B in preventing aggregation of insulin B-chain.
- While, there was no change in high affinity sites of α A and α B for ANS upon preheating, there was an increase in low affinity sites for α A and α B.
- However, preheated α A, in contrast to α B, exhibited remarkably enhanced chaperone activity indicating no quantitative correlation to its surface hydrophobicity (Figure 35).
- Chaperone activity of α A and α B-crystallins studied both in the absence and presence of ANS suggests that there is no direct quantitative relation existing between hydrophobicity and chaperone like activity (Table 20).

Table 20. Percentage loss of chaperone activity of α A and α B crystallin (native and preheated) in the presence of ANS at 15 and 30°C. Data are average of three chaperone assays. The chaperone activity in the absence of ANS was considered as 100%

	α A		α B	
	15°C	30°C	15°C	30°C
Native	42	44	62	65
Preheated	60	54	55	63

Fig. 35. Chaperone activity of native and preheated α A and α B-crystallins studied at 15 (Panel A) and 30°C (Panel B). Aggregation of insulin with DTT in the absence of α -crystallin was considered as 100% to calculate the % protection observed in the presence of chaperone.



Conclusions

ITC data along with the data on chaperone activity suggest that although, hydrophobicity appears to be a factor in chaperone-like activity of α -crystallins, it does not correlate quantitatively to its chaperone function. Factors other than hydrophobicity could be involved in the chaperone activity of α -crystallin, which needs to be investigated. Further, this approach of studying the surface hydrophobicity using ITC may be employed to establish the role of hydrophobicity in chaperone activity of other sHSP.