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Structure characterization of human serum proteins in solution and dry state

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Abstract: The present report describes application of advanced analytical methods to establish correlation between changes in human serum proteins of patients with coronary atherosclerosis (protein metabolism) before and after moderate beer consumption. Intrinsic fluorescence, circular dichroism (CD), differential scanning calorimetry and hydrophobicity (S_o) were used to study human serum proteins. Globulin and albumin from human serum (HSG and HSA, respectively) were denatured with 8 M urea as the maximal concentration. The results obtained provided evidence of differences in their secondary and tertiary structures. The thermal denaturation of HSA and HSG expressed in temperature of denaturation (T_d, °C), enthalpy (ΔH, kcal/mol) and entropy (ΔS kcal/mol K) showed qualitative changes in these protein fractions, which were characterized and compared with fluorescence and CD. Number of hydrogen bonds (*n*) ruptured during this process was calculated from these thermodynamic parameters and then used for determination of the degree of denaturation (%D). Unfolding of HSA and HSG fractions is a result of promoted interactions between exposed functional groups, which involve conformational changes of α-helix, β-sheet and aperiodic structure. Here evidence is provided that the loosening of the human serum protein structure takes place primarily in various concentrations of urea before and after beer consumption (BC). Differences in the fluorescence behavior of the proteins are attributed to disruption of the structure of proteins by denaturants as well as by the change in their compactability as a result of ethanol consumption. In summary, thermal denaturation parameters, fluorescence, S_o and the content of secondary structure have shown that HSG is more stable fraction than HSA.

Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; HSA, human serum albumin; HSG, human serum globulin; IF, intrinsic fluorescence; S_o, hydrophobicity.

Proteins have a specific intramolecular structure and amphoteric nature, containing the balanced portions of hydrophilic and hydrophobic groups. Moreover, in human metabolism the proteins can be subjected to action of different substances, such as detergents and acids. The stability of protein structure may be evaluated by its disruption, therefore, the protein stability can be determined by studying its denaturation and influence on human metabolism. The quantitation of changes occurring during beer consumption is important for understanding the mechanisms that can affect protein metabolism (1–3). Studies on protein denaturation and renaturation *in vitro* have great importance for understanding such mechanisms. It has been shown in recent studies that the denaturation of protein depends on its conformational state (4,5). Fluorescence permitted quantitative estimation of denaturation using fluorescence parameters, which are a linear measure of denaturation in aqueous solutions in a recent investigation (6,7). The function–structure relationships of human serum protein fractions have been widely studied (8–11) but, until now, no information of the changes in denaturation of human serum proteins in solid and liquid stages as a result of beer consumption has been reported. The importance of characterization of the changes in the proteins of patients is evident, because these proteins can be used as potential indicators of protein metabolism. Therefore, the relationships between structural and functional properties of human serum proteins (albumins and globulins) in patients with coronary atherosclerosis before and after beer consumption were studied using fluorescence, hydrophobicity, CD and DSC.

This work reports a new application of DSC in order to determine the conformational changes (percentage of denaturation) in dry-heated solid human serum proteins. IF and CD measurements confirmed the results obtained by DSC.

Experimental Procedures

Materials

Plasma proteins were separated into two fractions by 2 M ammonium sulfate (HSA) and 4 M ammonium sulfate (HSG) over 20 h at 4°C. Protein fractions were then purified using ion-exchange chromatography (albumin-HSA and γ-globulin-HSG), dialyzed against water for 72 h and lyophilized. Proteins were dissolved in 0.01 M phosphate buffer, pH 7.2. Human albumin (A1653) and γ-globulin (G4386), as well as all other chemicals, were purchased from Sigma Chemical Co. St. Louis.

Human studies

The study population was recruited from patients undergoing coronary bypass surgery owing to coronary artery disease in the Institute of Cardiology, University Medical Center, Rehovot, Israel. For 30 consecutive days the diet for 19 patients of the experimental groups was supplemented once a day by 330 mL Maccabee beer (about 20 g of alcohol). The 19 patients of the control group consumed 330 mL of mineral water Netivot during 30-day period. The experimental and control groups were examined before and after completion of the study period.

Protein determination

Protein assays were performed by Lowry method (12) and human serum albumin (HSA) was used as a standard.

Absorbance values were measured using Uvikon 930 UV spectrophotometer (Kontron AG Instruments, Zürich, Switzerland). Protein concentration corresponded to the absorbance less than 0.1 in a 1-cm path length to guarantee a linear increase in the relative fluorescence intensity.

Fluorescence measurements

Intrinsic fluorescence measurements of proteins were done using a Model FP-770 Jasco-Spectrofluorometer (Japan Spectroscopic Co., Ltd, Hachioji City, Japan). Fluorescence emission spectra were taken at excitation wavelength of 295 nm and recorded over the frequency range from the excitation wavelength to a wavelength of 500 nm (6,13). The temperature of the samples was maintained at 30°C using a thermostatically controlled cell holder. A series of emission spectra were recorded for protein solution at a concentration of approximately 0.015%. All chemicals used were reagent grade from Sigma. Deionized distilled water was used throughout. Treatment of serum proteins involved the addition of urea to the protein solutions in concentrations of 8 M as the maximal concentration for denaturation. Per cent of denaturation was determined after incubation of

protein with denaturants for 1 h. The magnitude of protein denaturation was calculated using the following equation:

$$\text{per cent denaturation (\%D)} = (I_0 - I_1)/I_0 \times 100$$

where I_0 and I_1 are fluorescence intensity of protein in the absence and presence of denaturants, respectively.

Hydrophobicity

S_0 was determined by 1-anilino-8-naphthalenesulfonate fluorescent probe measurements with 0.01 M phosphate buffer, pH 7.0 from 0.001 to 0.02% of protein concentration at $\lambda_{ex}=357$ nm and the fluorescence intensity was measured at 513 nm (14). The index of protein hydrophobicity was calculated as initial slope of fluorescence intensity vs. protein concentration (%) plot.

CD and DSC

CD spectra were measured with a Jasco J-600c spectropolarimeter (Japan Spectroscopic Co., Ltd), using a 0.05 cm quartz cell at room temperature under constant nitrogen purge. Solutions (0.01 mg/mL) of proteins were prepared by dissolving the lyophilized powder in 0.01 M phosphate buffer, pH 7.2. The absorbencies of all solutions were kept below 1.0 (4,15). Denaturation of proteins was performed with 8 M urea. CD spectra represent an average of eight scans collected in 0.2 nm steps at a rate of 20 nm/min over the wavelength range 180–250 nm of far-UV. CD spectra were baseline-corrected, and the data are presented as the mean residue ellipticities (θ). The molar ellipticity per residue at 222 nm (θ_{222}) was calculated and used as the index of protein denaturation. The CD spectra were evaluated with Contin software according to Provencher's algorithm, allowing the comparison of secondary structures from different proteins (16–18).

The extent of denaturation in solid proteins was estimated on a Perkin Elmer DSC System DSC Mettler using GraphWare TA-72 (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Portions (4–6 mg) of solid proteins were transferred into preweighed aluminum pans and sealed. Solid proteins were mixed with urea in a 1 : 1 proportion. An empty pan was used as the reference. The pans were heated in the calorimeter at 5°C/min over the range 30–140°C. All thermodynamic parameters were found. The denaturation temperature (Td) and changes in the enthalpy of denaturation (ΔH) were computed from the thermograms (19,20). To show the effect of proteins during denaturation standard human serum albumin and γ-globulin were examined by the same method. All data were determined in triplicate for all experimental conditions.

To verify the statistical significance of studied parameters means (M), their 95% confidence intervals of samples analyzed three times $\pm$ SD were defined. Differences between groups were established using 2-way ANOVA. The P-values of <0.05 were considered significant.

Results

Intrinsic fluorescence and hydrophobicity

Fluorescence spectra of native HSA and HSG and denatured with ethanol after beer consumption as well as with 8 M urea demonstrate a shift in the wavelength of emission from 339.5 nm to 363.5 nm, and a decrease in fluorescence intensity of denatured sample as a result of beer consumption (Fig. 1, curves 1–4; Table 1). The hydrophobicity of albumin and globulin fractions before beer consumption was, respectively, 9.98 and 21.74, and 14.12 and 23.20 after consumption (Table 1). The increase in surface hydrophobicity (an indicator of protein stability) was correlated with the decrease in protein compactability and can be explained by the altered, partly unfolded proteins denatured with 8 M urea (23.44 and 39.42 before beer consumption, and 26.04 and 40.12 after). The slight increase in the hydrophobicity of globulin compared with albumins showed that this plasma fraction is less stable after beer consumption. The surface hydrophobicity of plasma protein was increased by urea (Table 1). This suggests that the surface hydrophobicity of serum protein is easy to increase and denaturation occurs easily during the treatment with urea. Current hypotheses suggest that surface hydrophobicity is one of the characteristics of the protein most likely to define its surface behavior and, consequently, its emulsifying properties (14,21).

These results are in agreement with Tanaka *et al.* (22), who have shown that in the urea-induced denatured state of HSA, a red-shift in the wavelength of maximum fluorescence occurred over urea concentrations ranging from 4 M to 6 M. This shift indicated that a structural change in domain II occurred simultaneously with the unfolding of domain III in this concentration range. The maxima emission wavelengths (λ_{max}) of HSA and HSG before beer consumption were 337.0 and 336.0 nm, respectively, and after BC shifted to 334.0 and 335.0 nm. The surface hydrophobicity slightly increased after BC. In a solution of 8 M urea the λ_{max} of human serum albumin shifted to 342.5 and that of human serum globulin to 346.4 nm. In contrast, in the same

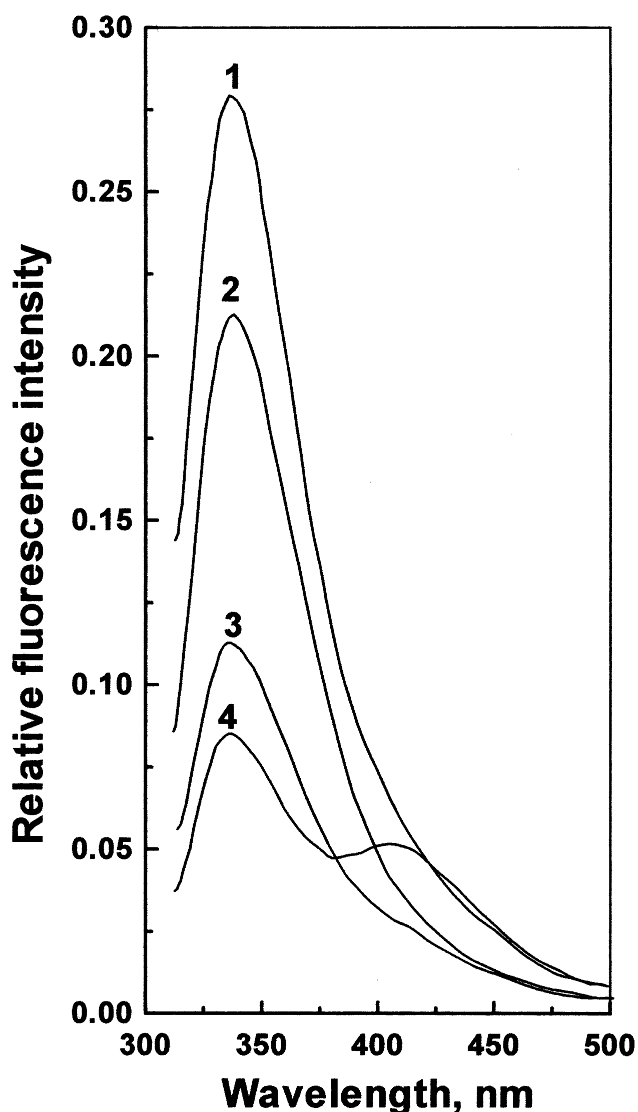

Figure 1. Effect of denaturation of human serum albumin (HSA) and globulin (HSG) before and after beer consumption, at λ_{exc} 295 nm: 1, native HSG before consumption; 2, native HSA before consumption; 3, HSG after consumption; 4, HSA after consumption. Samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration was 0.01 mg/mL at 30°C.

solution, but after beer consumption, the λ_{max} positions of HSA and HSG shifted to 357.7 nm and 355.9 nm, respectively. A decrease in fluorescence intensity, a shift in the maximum of emission and increase in surface hydrophobicity was observed, which reflected the unfolding of proteins.

CD studies

The values obtained for secondary structure contents for different states of protein fractions are reported in Table 1 and Fig. 2.

The far-UV CD spectra for different states of HSA and HSG (native and denatured with ethanol owing to

Table 1. **Structural properties of native and denatured human serum albumin (HSA) and human serum globulin (HSG)[a]**

Proteins	I_{295}	S_o	%D	α-helix	β-sheet	R
Before beer consumption						
HSA	0.29±0.03	9.98±1.0	0	67±6.3	4±0.4	29
HSA+8 M urea	0.12±0.01	23.44±2.3	61.1	40±3.9	9±1.0	51
HSG	0.21±0.02	21.74±2.2	0	8±0.8	56±5.3	36
HSG+8 M urea	0.08±0.01	39.42±4.1	57.4	4±0.3	58±5.6	38
After beer consumption						
HSA	0.11±0.01	14.12±1.5		64±6.2	7±0.8	29
HSA+8 M urea	0.14±0.01	26.04±2.5	84.0	32±3.1	11±1.1	57
HSG	0.09±0.01	23.20±2.3		6±0.5	64±6.2	30
HSG+8 M urea	0.04±0.01	40.12±3.9	81.4	3±0.2	76±7.3	21

a. Values are means±SD of triplicates. I_{295}, fluorescence intensity at 295 nm; S_o, hydrophobicity; %D, per cent denaturation; R, random coil.

beer consumption) are shown in Fig. 2. The unfolding of these protein fractions results from interactions promoted between exposed functional groups, which involve conformational changes of α-helix (native HSA=67%; native HSG=8%), β-sheet (native HSA=4%; native HSG=56%) and aperiodic structure (native HSA=29%; native HSG=36%).

Denaturation with 8 M urea resulted in an increase in β-sheet content (HSA=9%; HSG=58%), in sacrifice of α-helix (HSA=40%; HSG=4%). Albumin can withstand 8 M urea even at 44°C, with temporary loss of α-helix, but without irreversible change (23).

Polymerization and loss of helical structure occurs within 1 min at 65°C and pH 9.0. Refolding of the reduced molecule occurs spontaneously in the presence of reduced disulfide system (24).

Optical activity of α-helix in far-UV permits the use of CD studies for investigations of conformational changes in protein solutions (16). The CD of native HSA spectrum (Fig. 2, curve 1) shows a negative peak centered around 222 nm. This band demonstrates a significant α-helical content in protein. Another smaller band at 213 nm is characteristic for proteins with significant quantities of β-structure. Takeda *et al.* (10) and Moriyama *et al.* (25) reported a 66% α-helical, 3% β-structure and 31% random coil content for HSA. Similar data were obtained (Table 1) for HSA and HSG in these studies. A negative maximum around 202 nm is typical for a protein with a disordered random coil structure. Urea causes the protein to undergo

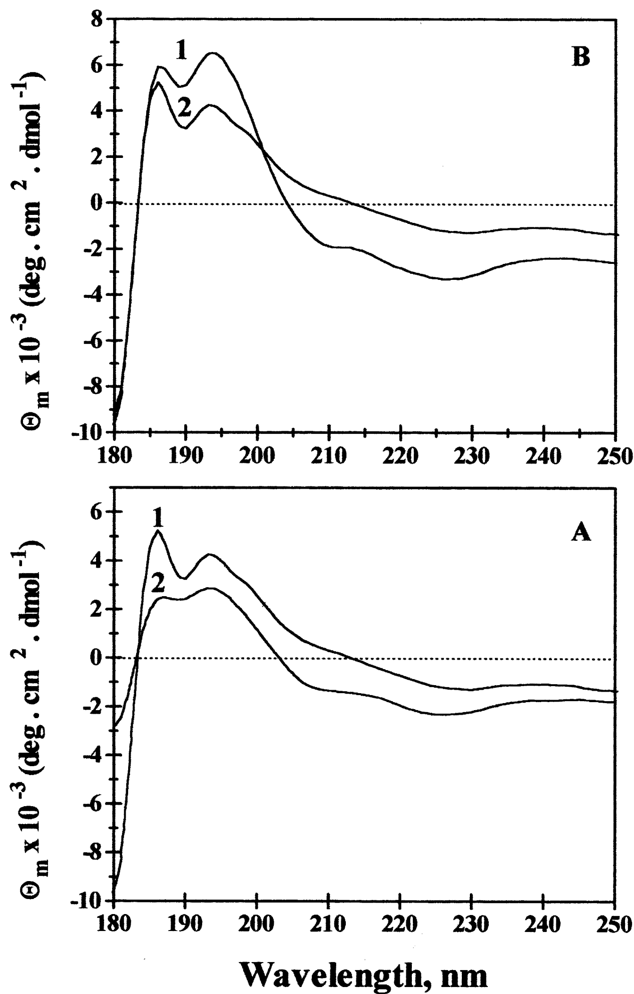

Figure 2. Circular dichroism spectra of human serum proteins (albumin, HSA; globulin, HSG). (A) After beer consumption: 1, HSA and 2, HSG. (B) Before beer consumption: 1, HSA; 2, HSG. Samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration was 0.01 mg/mL at 30°C. Denaturation was performed with 8 M urea. Each spectrum was run at room temperature.

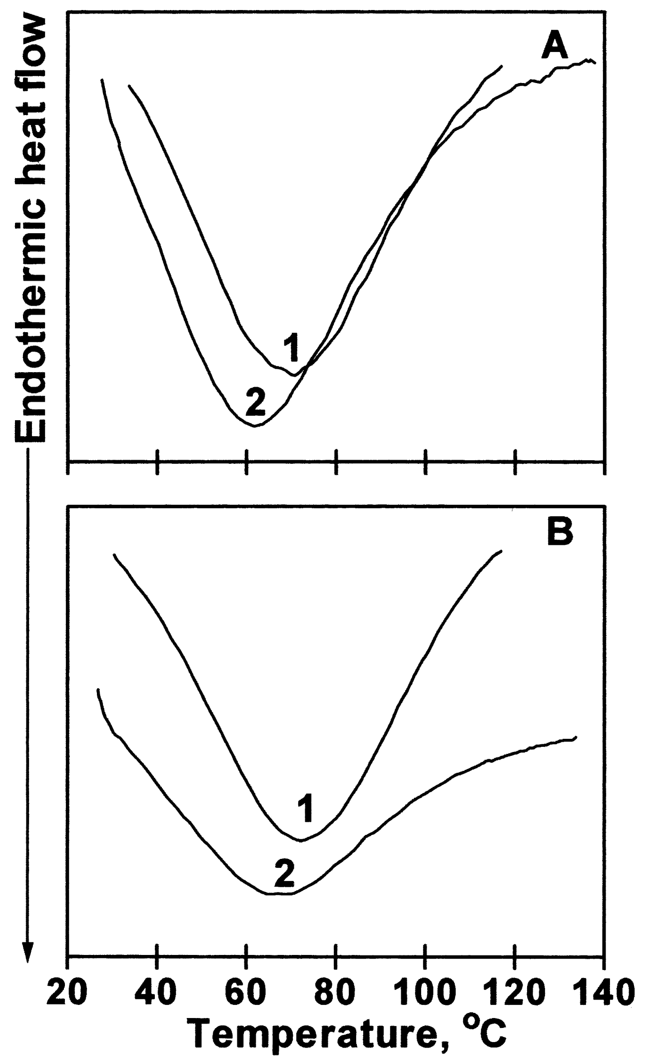

Figure 3. Differential scanning calorimetry thermograms of human serum proteins (albumin, HSA; globulin, HSG). (A) After beer consumption: 1, HSA, 2, HSG. (B) Before beer consumption: 1, HSA, 2, HSG. The protein samples were denatured in dry state by mixing samples with urea in a 1 : 1 ratio. Samples were dissolved in 0.01 M phosphate buffer at pH 7.2. The protein concentration was 0.01 mg/mL at 30°C.

disorganization of its secondary structure, involving a complete helix-to-coil transition. These data about the changes in α-helix, β-structure and random coil in HSA denatured with 8M area are in agreement with others (25). Urea denaturation of the protein fractions leads to a decrease in α-helical content (40% compared to 67% in the native state) (Table 1). The spectrum for HSA does not closely resemble that of the native state and demonstrates the much less pronounced negative maxima at 222 nm and 208 nm typical of α-helix. The far-UV spectrum of the ethanol-denatured protein is similar to the native protein. However, a decrease in α-helical content compared with native state was observed on denaturation with ethanol (Table 1). These data agree with results of Moriyama *et al.* (25), who described the changes in helicity of HSA–sodium dodecyl sulfate complex.

DSC thermograms and thermodynamic data

The DSC scans for native and denatured protein fractions are shown in Fig. 3. The native structure of all protein fractions was stable up to a critical temperature, and then disrupted with intense heat absorption.

Broadening of the peak, as well as decrease in ΔH indicates denaturation (Fig. 3). An increase in the temperature and time of heat treatment caused a decrease in enthalpy and slight shift in T_d to the lower value with the addition of denaturants. All sample proteins showed a gradual decrease in the extent of denaturation with increase in temperature. Thermal protein denaturation involves the rupture of

disulfide bonds (one bond contributes a ΔH=25 kcal/mol and a negligible S) and the rupture of n hydrogen bonds (ΔH= 4 kcal/mol, and ΔS=0.012 kcal/mol per protein molecule). The thermal stability depends mostly on the number of hydrogen bonds. So, the number of broken hydrogen bonds can be calculated as: $n=\Delta S/0.012$ and $n=(\Delta H-25)/4$, where n is the number of broken hydrogen bonds; ΔS is entropy and ΔH is enthalpy of denaturation (26). The number of hydrogen bonds ruptured during this process was used for determination of the degree of denaturation (%D).

Disordering of the system takes place upon heating. A considerable number of protein molecules shift to a state that contributes much less to the unfolding transition, causing a significant decrease in the calorimetric enthalpy. The enthalpy changes of the initial and remaining DSC endotherm were measured and used for calculation of per cent denatured protein fractions (Table 2). The entropy (S) values, which are associated with state transition and affirm disordering of protein structure, were also calculated (Table 2). Denaturation of around 50% was observed in all cases. Disruption of hydrogen bonds through heat denaturation is also reflected in a decrease in α-helix content of denatured protein, according to the findings of Kato *et al.* (27).

With the ΔH (kcal/mol) of native HSA (2303) and ethanol-denatured HSA (2102), one can approximately estimate that 558 and 508 of hydrogen bonds were broken during the conformational transition of native protein and that denatured with ethanol after beer consumption. These results are in harmony with others demonstrating that hydrogen bonding is the main stabilizing force in protein stability (28–30). In the case of HSA and HSG denatured with urea 30% and 10% of hydrogen bonds, respectively, were ruptured during treatment. Saito & Taira (19) also reported the influence of hydrogen bonds disruption on enthalpy changes in DSC. Such difference in results can be attributed to the fact that β-sheet is the main ordered structure of γ-globulin compared with other fractions.

The heat-induced gelation of serum proteins in general has been studied extensively (15,29,31). Preferential interaction parameters of multisubunit protein, α-globulin and monomeric protein human serum albumin were determined in different cosolvents (32).

***Table 2.* Thermal properties of native and denatured human serum albumin (HSA) and human serum globulin (HSG)[a]**

Proteins	T_d, °C	ΔH	ΔS	*n*
Before beer consumption				
HSA	71.4±7.0	2303±228	6.7	558
HSA+urea	57.0±5.6	1645±159	4.4	399
HSG	63.7±6.1	5302±529	15.7	1308
HSG+urea	60.0±5.8	2047±203	14.3	1189
After beer consumption				
HSA	70.7±6.9	2102±201	6.1	508
HSA+urea	52.0±5.1	1460±138	4.2	353
HSG	60.7±5.9	5238±523	15.7	1308
HSG+urea	59.6±5.7	4635±457	13.9	1158

a. Values are means±SD of triplicates. ΔH, enthalpy of denaturation, kcal/mol; *n*, number of ruptured hydrogen bonds; ΔS, entropy of denaturation, kcal/molK; T_d, temperature of denaturation; Proteins were denatured by mixing the sample with urea (1 : 1).

Discussion

Little if any work has been devoted to the use of DSC to study protein conformational studies before and after beer consumption. Denaturation and aggregation of plasma proteins were shown to commence at 55°C and heat-induced gelation of plasma protein was observed at 60–65°C. A marked increase in values relating to gel compressive strength occurred on increasing the heating temperature from 60 to 65°C. In general, these results clearly show that denaturation of plasma proteins precedes gel formation, which is in line with the two-stage mechanism (33). Similar biphasic features were observed earlier and confirmed the existence of a stable intermediate conformation. According to the two-state model, which applies to small globular proteins (34), after the rapid disappearance of ordered structures (mostly α-helical) there still remains large part of folded protein molecule. These molecules become unfolded during the subsequent denaturation (35). Uversky *et al.* (36) have shown that processes of HSA denaturation and unfolding are completely reversible. The release of ligands from HSA results only in a small decrease in stability but not in transformation into the molten globule state.

HSA unfolds by a pathway involving at least three distinct steps (37). The rate of gel formation is determined by the rate of molecular unfolding (step 1) below 57°C, while aggregation (step 2) is rate-determining above this temperature. This finding is consistent with the commonly held view that in the heat-set gelation of globular proteins, denaturation precedes aggregation and provides the driving force for protein–protein interactions. Gel strength continues to increase with increasing heating temperature up to 95°C. Simulations indicate that two-state conformational

transitions with enthalpy changes greater than approximately 30 kcal/mol should be observable by DSC.

Heating above the optimum gelation temperature causes an increased tendency towards random protein–protein interactions, loss of ordered structure and the formation of aggregates.

It has been postulated that hydrogen bonding is the major attractive force in gels formed at temperatures $<70^{\circ}C$. An increase in β-sheet formation occurs with increase of temperature, and this is directly involved in forming a hydrogen-bonded dimer. The involvement of hydrophobic interactions in the heat-induced association of proteins is suggested by negative effects of urea. The detergents and denaturants destabilize hydrophobic interactions. Disulfide bonds are predominant and both hydrogen and hydrophobic interactions also contribute to the formation of plasma protein gels. The gelling of plasma protein fraction is largely governed by the properties of albumin (31).

Results for standard human albumin and globulin were obtained and interpreted in order to compare with the proteins investigated. These results were found to be similar (not shown).

The thermal denaturation of HSA and HSG expressed in temperature of denaturation (T_d, °C), enthalpy (ΔH, kcal/mol) and entropy (ΔS, kcal/mol K) showed similar changes in protein fractions, which were also characterized by fluorescence (Table 1).

The addition of denaturants caused an induction of α-helical structure as evident from the mean residue ellipticity value at 222 nm. These results are in agreement with Muzammil *et al.* (8), who suggests that human serum proteins in the presence of denaturants exist in a partly folded state characterized by native-like secondary structure and tertiary folds. Our results are supported also by Chmelik (34). It was concluded that the disulfide-reduced state, with partly folded variable conformation, is involved in the reversible interconversion between the denatured reduced form and native disulfide-bonded form of HSA (38)

Conclusion

We have investigated the potential use of differential scanning calorimetry to characterize conformational changes in proteins, with emphasis on a conformational change in two separated fractions of human serum proteins, which may be related to the differences in the protein metabolism after the consumption of beer.

In our previous reports (6,7) intrinsic fluorescence was used to study the unfolding of serum protein fractions induced by denaturants and beer consumption in patients with high cholesterol levels. The effect of denaturant and beer consumption in patients with coronary artery disease is the displacement of the tryptophan residues to a more polar environment upon unfolding. Such displacement gives a shift to lower energy in the maximum of emission and quenching of fluorescence intensity. Increase in surface hydrophobicity was correlated with the increase and extent of protein denaturation and can be explained by the altered, partly unfolded protein fraction conformation induced by urea. Albumin fraction of plasma is less stable after beer consumption than globulin.

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