

Intrinsic Tryptophan Fluorescence of Human Serum Proteins and Related Conformational Changes

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The unfolding of human serum proteins (HSP) was studied by measuring the intrinsic fluorescence intensity at a wavelength of excitation corresponding to tryptophan's or tyrosine's fluorescence and surface hydrophobicity. The maxima emission wavelengths (λ_{\max}) of human serum albumin (HSA) and human serum globulin (HSG) before beer consumption (BC) were 336.0 and 337.0 nm and after BC shifted to 335.0 and 334.0 nm, respectively. The surface hydrophobicity slightly increased after BC. In a solution of 8 M urea the λ_{\max} of BSA shifted to 346.4 and that of BSG to 342.5 nm. In contrast, in the same solution but after BC the λ_{\max} positions of HSA and HSG shifted to 355.9 and 357.7 nm, respectively. A decrease in fluorescence intensity, a shift in the maximum of emission, and an increase in surface hydrophobicity which reflected unfolding of proteins were observed. Here we provide evidence that the loosening of the HSP structure takes place primarily in various concentrations of urea before and after beer consumption. 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.

KEY WORDS: Human serum proteins; fluorescence; ethanol; denaturation.

1. INTRODUCTION

The serum proteins comprise a dynamic system with various biological functions and are generally considered together because of their common biosynthetic origin, participation in the same processes, and occurrence as major extracellular components of the circulatory system

(Tanford, 1969; Korsten *et al.*, 1991; Grattagliano *et al.*, 1996). Moreover, human protein metabolism can be subjected to the action of different substances, such as detergents and acids. Therefore it is interesting to study the conformational changes of proteins during denaturation. In addition, studies on protein denaturation and renaturation *in vitro* are important for understanding the mechanisms which affect protein structures. This problem has attracted the attention of many researchers (Miyazawa *et al.*, 1984; Gruen *et al.*, 1987; Walbridge *et al.*, 1987). Some epidemiological, experimental, and clinical studies show (Klatsky and Armstrong, 1993; Gorinstein *et al.*, 1997) that moderate alcohol consumption has a cardioprotective effect. Until now, red wine has been the cardioprotective beverage of choice (Klatsky and Armstrong, 1993). But recently published work points to a

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⁹ Abbreviations: BC, Beer consumption; HSA, human serum albumin; CAD, coronary artery disease; CG, control group; EG, experimental group; HSG, human serum globulin; HSP, human serum proteins; IF, intrinsic fluorescence; S_0 , hydrophobicity.

change in this attitude. It is suggested that because of its effectiveness, low cost, acceptable adverse-event profile, and availability in convenient single-dose dispensers, beer should replace red wine as the cardioprotective beverage of choice (Innes, 1998). It is common knowledge, however, that alcohol consumption has not only beneficial, but also adverse effects. It was reported that consumption of alcoholic beverages negatively influences protein metabolism (Preedy *et al.*, 1994; Reilly *et al.*, 1997). In our previous investigations no quantitative changes in serum proteins after moderate beer consumption were found (Gorinstein *et al.*, 1997, 1998), but possible qualitative changes could not be excluded. Therefore it was decided to investigate the influence of short-term moderate beer consumption on the structural and functional properties of serum proteins in patients with coronary artery disease (CAD)⁹ by intrinsic fluorescence (IF) and surface hydrophobicity (S_0). As far as we know there have been no prior investigations of this.

2. MATERIALS AND METHODS

The study population was recruited from patients undergoing coronary bypass surgery due to CAD in the Institute of Cardiology in the University Medical Center, Rehovot, Israel. Thirty-eight patients were randomly divided into two equal groups: 19 patients (experimental group, EG) were given an antiatherosclerotic diet supplemented daily with 330 ml of beer (about 20 g of alcohol) and 19 patients (control group, CG) instead drank mineral water (Netivot) for 30 consecutive days. In order to find possible qualitative changes after beer consumption, the human serum proteins were separated into two groups by 2 M ammonium sulfate (albumins-HSA) and 4 M ammonium sulfate (globulins-HSG) during 20 hr at 4°C. The suspension was extensively dialyzed against water for 48 hr. Then the protein fractions were purified using ion-exchange chromatography (albumin and γ -globulin) and lyophilized (Saito *et al.*, 1988). Protein assays were performed by the Lowry *et al.* (1951) method and human serum albumin (HSA) was used as a standard. Absorbance values were measured using a Uvikon 930 UV spectrophotometer (Kontron AG Instruments, Zürich, Switzerland). Protein concentration corresponded to an absorbance of less than 0.1 in a 1-cm pathlength to guarantee a linear increase in the relative fluorescence intensity.

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

wavelengths of 274 and 295 nm and recorded over the frequency range from the excitation wavelength to a wavelength of 500 nm (Arntfield *et al.*, 1987; Zemser *et al.*, 1994). All solutions were prepared in 0.01 M phosphate buffer, pH 7.2. The best dissolution was for proteins precipitated with 4 M ammonium sulfate. The temperature of the samples was maintained at 30°C using a thermostatically controlled cell holder. A series of emission spectra was recorded for protein solutions with a concentration of approximately 0.015%. The magnitude of protein denaturation was calculated using the following equation: percentage denaturation (%D) = $(I_0 - I_1)/I_0 \times 100$, where I_0 and I_1 are the fluorescence intensities of protein in the absence and presence of denaturants, respectively. All chemicals were reagent grade from Sigma Co., and were used without further purification. Deionized distilled water was used throughout. Treatment of serum proteins involved the addition of denaturants to the protein solutions in concentrations of, e.g., urea 0, 2, 4, 6, and 8 M. Percentage denaturation was determined after incubation of protein with denaturants for 1 hr. All data were determined in triplicate for all experimental conditions. Hydrophobicity (S_0) was determined by 1-anilino-8-naphthalenesulfonate (ANS)-fluorescent probe measurements with 0.01 M phosphate buffer, pH 7.0, from 0.001% to 0.02% of protein concentration at $\lambda_{\text{ex}} = 357$ nm and the fluorescence intensity was measured at 513 nm. The index of protein hydrophobicity was calculated as the initial slope of the plot of fluorescence intensity versus protein concentration (%) (Kato and Nakai, 1980).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) in 8% gels which were stained with 0.1% Coomassie brilliant blue.

2.1. Statistical Analysis

To verify the statistical significance of the means (M) of the parameters, the 95% confidence intervals of triplicate samples \pm SD were defined. Differences between three groups of serum proteins were established using two-way ANOVA. The p values of $<.05$ were considered significant.

3. RESULTS AND DISCUSSION

SDS-PAGE of HSA and HSG gave molecular weights of 66 and 150 kDa, respectively, in agreement with other results (Marshall, 1984). The results of electrophoresis are omitted.

The emission spectra of HSG are shown in Fig. 1. The protein emission spectra of CG patients after the experiment did not differ from those before the experiment. Therefore the data of the control group as well as the spectra are omitted in this report. The change in protein conformation, even in the presence of strong denaturants, might be the result of differences in serum protein-bound fatty acids that change as a result of EtOH consumption. Therefore the comparison below is done between the serum samples of patients before (without any influence of ethanol as a standard of native protein) and after beer consumption including denaturing studies.

The emission spectra of HSG from patients of the experimental group before beer consumption (BC), measured at excitation wavelengths of 295 and 274 nm, contained a single emission peak with maximum at 337.0 and 337.3 nm (Figs. 1A and 1B, curve 1), respectively, and those after BC showed a peak at 334.0 and 334.6 nm (Figs. 1A and 1B, curve 2), respectively. Considering the

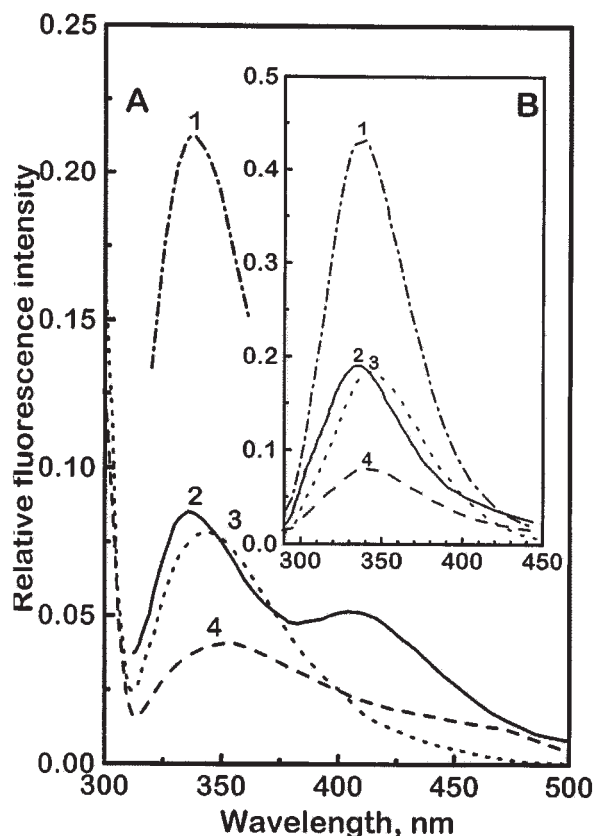


Fig. 1. Effect of denaturation with 8 M urea of human serum globulin (HSG) before and after beer consumption (BC) on fluorescence properties at (A) $\lambda_{exc} = 295$ nm and (B) $\lambda_{exc} = 274$ nm. (1) Native before BC; (2) influenced by ethanol after BC; (3) denatured before BC; (4) denatured after BC.

amounts of tyrosine and tryptophan, one could expect two emission peaks. But even one tryptophan residue gives a spectrum with a peak corresponding to tryptophan emission in the interval 331–342 nm at both excitation wavelengths 295 and 274 nm (Khan *et al.*, 1980; Gruen *et al.*, 1987). For an excitation of 295 nm tryptophan is the only aromatic amino acid to absorb light. For an excitation of 274 nm there is a transfer of energy from the excited tyrosine to tryptophan, which also corresponds to a single emission peak (Khan *et al.*, 1980; Permyakov and Burstein, 1984; Gryczynski *et al.*, 1988).

A lower level of relative fluorescence intensity was observed at $\lambda_{max} = 295$ nm (Table I). Tryptophan gives a strong emission that corresponds to a significant quantity of tryptophan residues in this fraction. At the same time, for an excitation wavelength of 274 nm a shoulder corresponding to tyrosine emission at 308 nm and in some samples two distinct peaks (at 305 and 336.5 nm) appeared. This means that the distance between tyrosine and tryptophan is great enough to give two emission peaks for tryptophan and tyrosine residues.

Comparison of the fraction before (Figs. 1A and 1B, curve 1) and after BC (Figs. 1A and 1B, curve 2) shows a small shift at 295 as well as at 274 nm to a more compact structure. There is a decrease in the fluorescence intensity for the samples after BC (Table I).

Table I. Fluorescence Properties of Native and Denatured HSG and HSA before and after Beer Consumption^a

Proteins	I_{274}	I_{295}
HSG + 0 U Before BC	0.430 ± 0.04	0.212 ± 0.02
HSG + 0 U After BC	0.190 ± 0.02	0.085 ± 0.01
HSG + 8 M U Before BC	0.183 ± 0.02	0.081 ± 0.01
HSG + 8 M U After BC	0.080 ± 0.01	0.041 ± 0.01
HSA + 0 U Before BC	0.643 ± 0.06	0.279 ± 0.03
HSA + 0 U After BC	0.250 ± 0.03	0.112 ± 0.01
HSA + 8 M U Before BC	0.252 ± 0.03	0.120 ± 0.01
HSA + 8 M U After BC	0.103 ± 0.01	0.143 ± 0.02

^a Mean values of triplicates ± standard deviation. I_{274} , Fluorescence intensity at excitation of 274 nm; I_{295} , fluorescence intensity at excitation of 295 nm; U, urea. BC, Beer Consumption.

Emission spectra of HSA from EG patients before BC, measured at excitation wavelengths of 295 and 274 nm, contained a single emission peak with maximum at 335.9 and 335.5 nm (Figs. 2A and 2B, curve 1), respectively, and those after BC showed a peak at 335.0 and 334.9 nm (Figs. 2A and 2B, curve 2), respectively. At $\lambda_{\text{exc}} = 295$ nm tyrosine was not shown. Our results, in agreement with others, show that HSA is composed of three homologous domains, each of which displays specific structural and functional characteristics. HSA is known to undergo different pH-dependent structural transitions, but we used a constant pH (Henkens *et al.*, 1982; Ward and Bokman, 1982; Chmelik *et al.*, 1988; Dockal *et al.*, 2000).

Denaturation of proteins with urea was examined at excitation wavelengths of 295 and 274 nm (Figs. 1 and 2). Increasing levels of urea led to a gradual decrease in fluorescence intensity at 274 nm up to a urea concentration

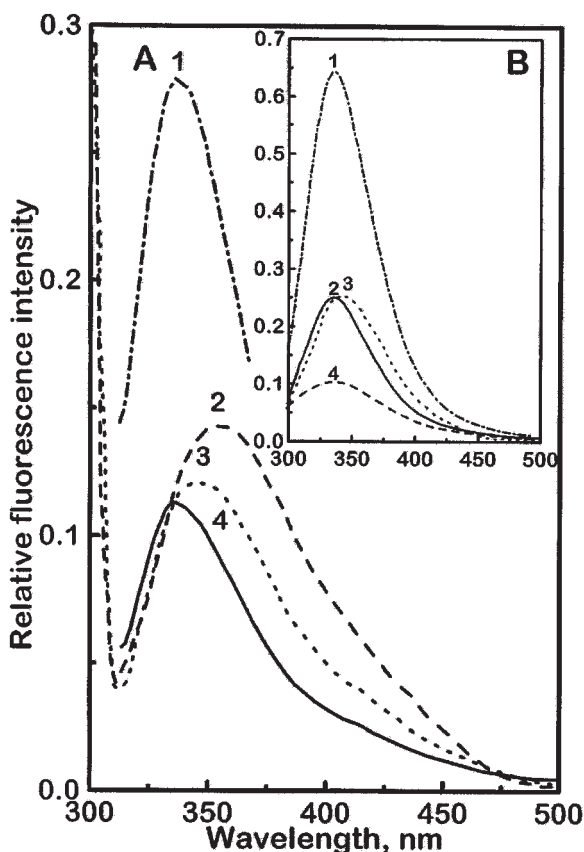


Fig. 2. Effect of denaturation with 8 M urea of human serum albumin (HSA) before and after beer consumption (BC) on fluorescence properties at (A) $\lambda_{\text{exc}} = 295$ nm and (B) $\lambda_{\text{exc}} = 274$ nm. (1) Native before BC, (2) influenced by ethanol after BC, (3) denatured before BC, (4) denatured after BC.

of 3 M, at which point a significant decrease in the fluorescence intensity and a small shift in the wavelength appeared. Above a concentration of 6 M urea a shoulder at approximately 307 nm appeared due to tyrosine fluorescence. Simultaneously, the emission peak (tryptophan fluorescence) shifted from 337.5 to 352.5 nm for all protein fractions (Figs. 1B and 2B, curves 3 and 4). These values continued to change up to the addition of 8 M urea, where they were maximal (emission maximum shifted to 358 nm). The appearance of a shoulder and a shift in the maximum of emission were the results of protein unfolding. As explained above, a distinct shoulder appears as a result of the cessation of energy transfer from excited tyrosine to tryptophan residues with unfolding, when the distance between the tryptophan and tyrosine residue increases. On the other hand, a shift of the tryptophan residues to a more polar environment upon unfolding causes a shift to the lower energy in the maximum of emission and a quenching of fluorescence intensity. The results following the increase of urea concentration are omitted and only the maximal denaturation with 8 M urea is shown in this report. Our results agree with others (Damodaran, 1987) in showing that low levels of urea promote the refolding of denatured and reduced serum proteins, while high levels of urea (>2 M) decrease the rate and extent of refolding. The effects of denaturants on the refolding behavior of serum proteins are attributed to subtle changes in the structure of the associated water (Damodaran, 1987). Our results are in agreement with others (Tanford, 1969) that HSA can withstand 8 M urea even at 44°C with temporary loss of α -helix, but without irreversible change (Gonzalez-Jimenez, 1994). Moreover, the fluorescence quenching depends on the excitation wavelength. Excitation at 278 nm, quenches 25% of the native fluorescence, but only 6% is quenched by excitation at 290 nm. From these spectroscopic studies our results are compatible with the possibility that the interaction could take place mainly on one of the subdomains. This explanation is in agreement with others who observed the same results studying the binding of chlorphenilamine to human serum albumin in comparison with other proteins (Gonzalez-Jimenez, 1994; Moriyama *et al.*, 1996).

Denaturation of HSA with 8 M urea after BC leads to changes in emission wavelength and an increase in fluorescence at 295 nm. In addition, the emission wavelength at 355.9 nm corresponded to unfolded protein structure where all tryptophan residues are situated in aqueous medium. Tryptophan emission was not quenched after unfolding in the presence of water. Our results agree with other work in indicating that urea interacts with proteins by electrostatic forces, yielding a randomly

coiled conformation in its unfolded state. The free energy change of unfolding versus concentration of urea suggests that the variation of the electrical charge of proteins influences the final state of the unfolded form of the protein and denaturation and unfolding are completely reversible (Gonzalez-Jimenez, 1994; Uversky *et al.*, 1997; Farruggia and Pico, 1999).

After BC the globulin fraction was more stable. The difference in compactability was small in albumin and globulin, but the highest stability was for globulin denatured with 8 M urea. Fluorescence measurements showed a decrease in fluorescence intensity and a shift in the maximum of emission, reflecting unfolding of these proteins with urea (Table I). The difference in the extent of denaturation between the protein fractions may be explained by the differences in amounts of amino acids and by the sulfur bridges existing in these proteins.

The hydrophobicity of globulin was 21.736 and 23.203 before and after BC, respectively. The increase with denaturation with 8 M urea corresponded to 39.415 and 40.118, respectively (Fig. 3A). Results for the albumin fraction were 9.9753 and 14.118 and with urea 23.438 and 26.044 before and after BC (Fig. 4A), respectively. Increase in surface hydrophobicity (an indicator of protein stability) was correlated with a decrease in protein compactability of globulins (Fig. 3B, 55.8%) and albumins (Fig. 4B, 61.1%) under the influence of ethanol. The results of denaturation of globulins (Fig. 3B, 57.4% and 81.4% before and after BC, respectively) and

albumins (Fig. 4B, 61.1% and 84.0% before and after BC, respectively) can be explained by the altered, partially unfolded proteins denatured with 8 M urea. A slight increase in the hydrophobicity of albumin in comparison with globulins showed that this fraction of serum is less stable after beer consumption. The more hydrophobic the protein, the greater is its ability to reduce interfacial tension (Kato and Nakai, 1980).

4. CONCLUSIONS

In this study intrinsic fluorescence was used to follow the denaturation of albumin and globulin. The present work describes the changes in functional properties of these proteins in solution using variable conditions of denaturation under the influence of ethanol. After beer consumption, native and denatured albumin and globulin showed a decrease in fluorescence intensity, a shift to a less polar environment, and an increase in surface hydrophobicity. Their stabilities differed slightly. Native and denatured HSG were the most stable, following by HSA. Before BC 8 M urea denatures the protein fractions to different extents. The λ_{max} in emission spectra is 346.4 nm for HSA and 342.5 nm for HSG. Although the nature and structure of HSA and HSG differ completely, the environment around the chromophores is similar. This behavior changes after BC, showing similar extents of denaturation for HSA and HSG. The maximum wavelengths were at 355.9 nm for HSA and 357.7 nm for HSG, corresponding to the structure with chro-

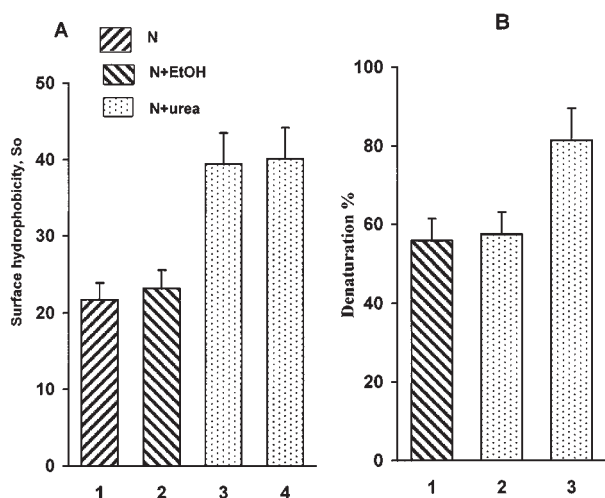


Fig. 3. Effect of denaturation with 8 M urea of human serum globulin (HSG) before and after beer consumption (BC) on (A) surface hydrophobicity and (B) extent (%) of denaturation. For panel A, (1) native before BC, (2) influenced by ethanol after BC, (3) denatured before BC, and (4) denatured after BC. For panel B, (1) after BC, (2) denatured before BC, and (3) denatured after BC.

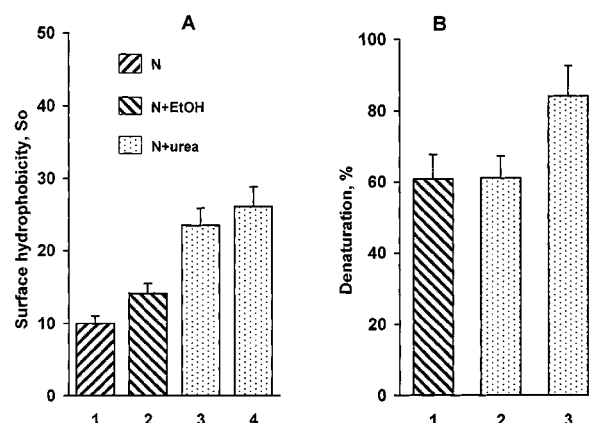


Fig. 4. Effect of denaturation with 8 M urea of human serum albumin (HSA) before and after beer consumption (BC) on (A) surface hydrophobicity and (B) extent (%) of denaturation. For panel A, (1) native before BC, (2) influenced by ethanol after BC, (3) denatured before BC, and (4) denatured after BC. For panel B, (1) after BC, (2) denatured before BC, and (3) denatured after BC.

mophores almost totally exposed to the medium. Ethanol seems to make the protein structure more labile and therefore the action of urea on albumin and globulin differ from that before BC. It is suggested that HSA and HSG fractions have the potential to be used as additional indices of protein metabolism during beer consumption. A short term of moderate beer consumption did not induce any quantitative changes in serum proteins, but in most patients (12 of 19) a tendency to a lower stability and minor structural deviations were detected.

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