

## Influence of freezing down to 77.15 K on structure and antioxidant power of some proteins

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The purpose of the present work was to investigate influence of different freeze-thawing protocols on structure and antioxidant properties of isolated proteins. In our experiments we have studied human serum albumin, human hemoglobin and cytochrome C derived from equine heart frozen down to 77.15 K with 1–2 deg/min and 300 deg/min rate with following thawing on a water bath at 293.15 K. Native proteins were assumed as a control. Influence of freeze-thawing protocols on protein structure was investigated using spectrophotometric and fluorescent assays. Antioxidant activities of isolated proteins were estimated by their ability to reduce ABTS<sup>+</sup> radical. It has been established that unfolding derived from freeze-thawing exposure leads to protein antioxidant activity increasing while decreasing of such an activity may be connected with macromolecule aggregation. Character of freeze-thawing influence on antioxidant activity of proteins depends on molecule structure peculiarities and freezing protocols.

PACS: **33.20.-t** Molecular spectra;  
33.20.Lg Ultraviolet spectra;  
33.50.Dq Fluorescence and phosphorescence spectra.

Keywords: albumin, hemoglobin, cytochrome C, freeze-thawing, antioxidant activity.

### Introduction

For a long time proteins have been considered as a major target for detrimental action of free radicals. However there are new data supporting proteins antioxidant properties. Thus the recent studies of Bourdon *et al.* and Roche *et al.* allow considering serum albumin as the major antioxidant in blood plasma. Herewith its antioxidant capabilities are closely linked to molecule structure and may be also present in many other proteins. These facts may contribute to application of protein and its compositions in clinical practice to treat oxidation-induced diseases including atherosclerosis, aging, inflammation, and certain nervous system disorders [1,2]. Studying the mechanisms of protein nonenzymatic antioxidant activity has revealed that it is caused by the accessible to the solvent amino acids. According to the efficiency the studied amino acids were placed in the following order: cysteine >> tryptophan > tyrosine > histidine > cystine. A significant role can be attributed to methionine and phenylalanine as well [3].

One of the most perspective and proper ways to preserve antioxidant properties appears to be cryopreservation. Thus low temperatures are known to be widely used

in clinical practice in order to increase a drug shelf life and to preserve its potency. However freeze-thawing is known to lead in most cases to protein conformational changes, appearing in molecule loosening and increasing accessibility of protein active sites as well as in reversible or irreversible protein aggregation, degradation and severe loss of their activity [4,5]. Up to now there are no data about freezing influence on nonenzymatic antioxidant activity of proteins. Herewith the previously reported findings have revealed that protein unfolding derived from thermal structural changes and caused with other stressor factors leads to increasing of their antioxidant activity [6]. Thus we assume the cold denaturation, resulted from freeze-thawing to lead to protein antioxidant activity alterations. In most cases an antioxidant activity of proteins plays a leading role for their application in clinical practice. Increasing of this protein activity under several conditions will contribute to their effectiveness. Herewith such an increase is not expedient because of changing of protein native conformations and as a result molecules faster oxidation.

Hence the purpose of the present work was to investigate the influence of different freezing protocols on structure and antioxidant properties of isolated proteins.

## Materials and methods

In our experiments we have investigated crystalline cytochrome C (Cyt C) derived from equine heart (Serva, Germany); human hemoglobin (Hb) isolated from donor whole blood erythrocytes; human serum albumin (HSA), separated from donor whole blood plasma using methanol [7] and further purified using a 27×1 cm gel-chromatography column (Sigma Chemical Company) with Sephadex G-75 (Loba Feinchemie, Austria). Proteins were diluted with 50 mM Na-phosphate-buffer (pH 7.4) to obtain the 1 mg/ml concentration. Then the samples were frozen down to 77.15 K with 1 deg/min and 300 deg/min cooling rate. Samples were thawed in a water bath at 293.15 K. Native proteins were taken as a control.

### *ABTS cation radical decolorization assay*

In order to evaluate total proteins nonenzymatic antioxidant power and estimate contribution of different antioxidant centres the method based on ABTS<sup>+</sup> (2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical cation reduction was used [8]. ABTS<sup>+</sup> cation radical was produced by the reaction between 7 mM ABTS<sup>+</sup> (Sigma-Aldrich Chemical, Steinheim, Germany) in H<sub>2</sub>O and 2.45 mM ammonium persulfate (Sigma-Aldrich Chemical, St. Louis, MO, USA), stored for 12 h in the darkness at room temperature. Before usage, the ABTS<sup>+</sup> solution was diluted to get an absorbance of (0.700 ± 0.025) at 734 nm with a distilled water. Experimental sample (0.1 ml) was added to the obtained radical solution (3 ml) and the kinetics of ABTS<sup>+</sup> radical decolorization was recorded. Results were presented as percentage changes in absorbance of initial ABTS<sup>+</sup> solution.

### *Protein structure assay*

To assess the protein conformation changes their absorption spectra were analyzed. All spectrophotometrical measurements were carried out using spectrophotometers Pye Unicam SP800 (UK) and Perkin Elmer (UK).

Fluorescence emission spectra were monitored at the wavelength of maximum intensity with a Cary Eclipse fluorescence spectrophotometer (Varian, Australia) using 5 nm entrance and exit slits and excitation at 296 nm for registering tryptophan residues fluorescence [9].

### *Statistical analysis*

Statistical analysis was carried out using Statgraphics Plus version 2.1 for Windows (Manugistic, Rockville, MD, USA). All the analyses on antioxidant activity for each sample were done in triplicate set. The data were presented as mean ± standard deviation. The data were analyzed by *t*-test to find out difference between sample means. Values with *p* < 0.05 were considered significant (*p* — statistical significance level).

## Results

Proteins with a diverse structure were chosen in order to assess freeze-thawing influence on antioxidant properties. Hemoglobin is tetramer with a molecular weight of 64 kDa, the freezing of which may also lead to macromolecule aggregation. Albumin is a globular subunit protein with domain structure (63 kDa molecular mass). Freezing of albumin solution also results in molecule aggregation [10]. Equine heart Cyt C is a small globular protein with 12 kDa molecular mass when being in oxidized form. Influence of freeze-thawing on its structure has not been fully investigated yet. As for antioxidant properties they have been studied only for albumin [1,11,12].

The method based on ability to inhibit ABTS<sup>+</sup> radical is widely used in order to assess antioxidant properties of diverse biological fluids. During this reaction the blue ABTS<sup>+</sup> radical cation is converted back to its colorless neutral form. The profiles obtained employing kinetics of ABTS<sup>+</sup> decolorization by all the experimental samples indicated the occurrence of fast decay (reaction time less than 10 s) and the slow one observed after a longer time period. Taking into account that in all the cases the kinetics of ABTS<sup>+</sup> radical reduction included 2 phases, the activity of both rapid and slow scavenging antioxidant centers was assessed. Activity of antioxidants responsible for rapid reduction was evaluated by decolorization during first 10 s; the activity of antioxidants responsible for the slow one was estimated by decay during next 390 s.

The obtained results have revealed that a slow freezing down to 77.15 K (1 deg/min) leads to decreasing of albumin and hemoglobin total antiradical activity due to a decrease in activity of both rapid and slow reducing centers. For Cyt C a total antiradical activity does not change significantly. However the lowering of activity of rapid reducing centers and the increasing of activity of slow one have been shown. Rapid freezing down to 77.15 K (300 deg/min) leads to the increase in Cyt C antiradical activity, occurring due to slow reducing centers. The freezing of hemoglobin and albumin results in the protein antiradical activity lowering. However in the albumin case such a lowering occurs only due to a rapid phase, whereas in hemoglobin it is associated with both rapid and slow reducing centers (Table 1).

It is assumed that protein activity of rapid reducing centers is primarily associated with cysteine (Cys) SH-group which is not involved into the formation of disulfide bounds [13]. Albumin molecule has one free Cys however the analysis of first derivatives of protein absorption spectra testifies against increasing of protein chromophore accessibility to the solvent (Fig. 1). Slight spectra alteration to the long-wave region and increasing of light scattering level, which is evident due to absorbance increasing at 325 nm (Fig. 2), testify to molecule aggregation [14]. These alterations in albumin structure were the most manifested after slow freezing down to 77.15 K. Previously it has

Table 1. Freeze-thawing influence on protein antioxidant activity

Sample		Rapid reducing centers activity, %	Slow reducing centers activity, %	Total antioxidant activity (for 400 s), %
HAS	Native	7.6±0.29	12.5±0.6	20.1±0.58
	Slow freezing	4.3±0.21*	9.1±0.40*	13.4±0.41*
	Rapid freezing	5.4±0.19*	13.4±0.61*	18.8±0.6*
Hb	Native	16.4±0.12	13.7±1.03	30.1±1.06
	Slow freezing	3.9±0.24*	6.1±1.37*	10.0±1.70*
	Rapid freezing	2.0±0.14*	9.5±1.28*	11.5±1.40*
Cyt C	Native	7.2±0.17	21.0±0.35	28.2±0.35
	Slow freezing	4.6±0.15*	23.7±0.20*	28.3±0.21
	Rapid freezing	5.7±0.20*	25.4±0.25*	31.1±0.20*

Comment: \* — significant difference ( $p < 0.05$ ).

been established that albumin aggregation occurs due to Cys residues [15]. The findings match with the results of fluorescence assay. There was no evidence of significant

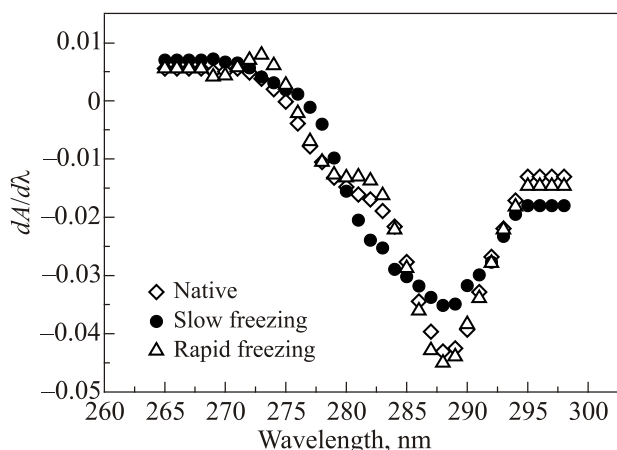


Fig. 1. Freeze-thawing influence on first derivatives of albumin absorption spectra.

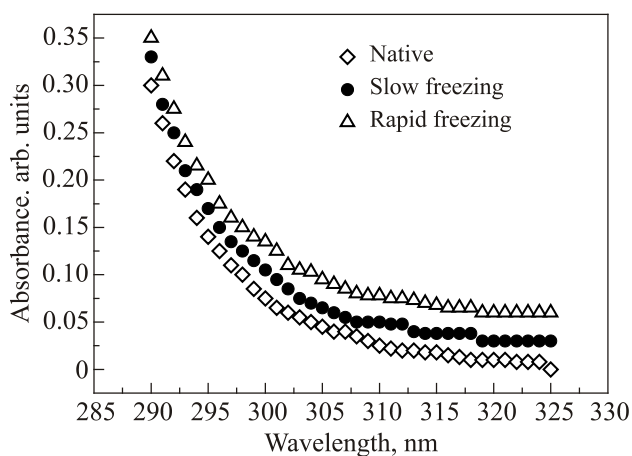


Fig. 2. Freeze-thawing influence on albumin absorption spectra.

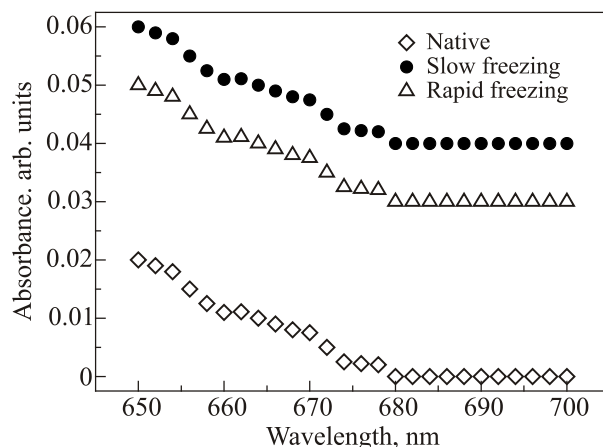


Fig. 3. Freeze-thawing influence on hemoglobin absorption spectra.

alterations of fluorescence parameters after freeze-thawing. Our findings may be explained by crystallization process and by time increasing when the protein remains in concentrated solution, resulting in higher extent of albumin aggregation, leading to a decrease in protein antioxidant activity.

Hemoglobin molecule has also free SH-groups of Cys [16]. Experimental data have shown the increasing of the level of hemoglobin light scattering, being the most pronounced after rapid freezing (Fig. 3). These findings agreed the data obtained for antioxidant activity. Thus all above mentioned facts may be an evidence of macromolecule aggregation due to free SH-groups. Herewith no significant changes in the protein hydrophobic regions were observed, as no alteration of negative maximum at 293 nm was detected. The shift of the negative maximum at 285 nm towards the short wavelengths region testifies to tyrosine increasing accessibility to the solvent (Fig. 4). This fact suggests the freezing to lead to loosening of the polar regions of the protein molecule. Such a loosening in its turn may result in the protein slow reducing centers activity increase.

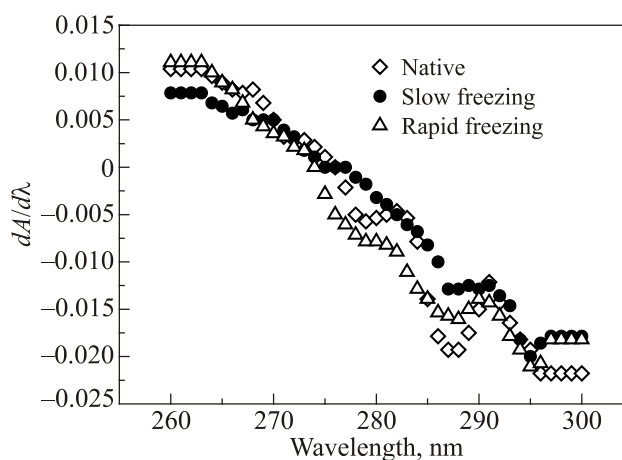


Fig. 4. Freeze-thawing influence on first derivatives of hemoglobin absorption spectra.

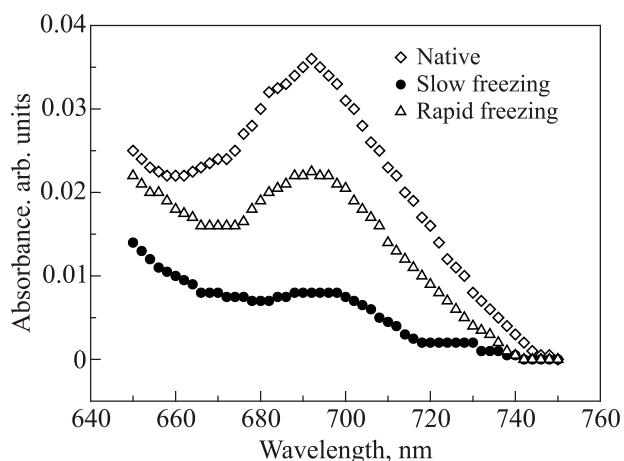


Fig. 5. Freeze-thawing influence on Cyt C absorption spectra.

Among several model proteins available for biophysical studies the Cyt C is the unique one because it provides optical probes to measure both global and local unfolding. Cyt C has a single triptophan residue (Trp-59) and its fluorescence measures the loose of compactness or the global unfolding of the entire protein. This is due to Trp-59 fluorescence is quenched in native state due to resonance energy transfer to the covalently linked heme. For the fluorescence to be seen Trp-59 has to be significantly displaced away from, that is only possible with a complete unfolding of the protein [17]. We have not observed the fluorescence of Cyt C after freeze-thawing. Thus we assume it to have a native-like compact structure. Other parameter for Cyt C conformation investigation is an absorption band at 695 nm which measures the stability of a local protein region in Cyt C. This absorption band corresponds to the charge transfer from the side chain sulfur of the methionine residue (Met-80) to the heme ferric iron. Molecular loosening in Met-80 region leads to the absorption band absence due to lack of ligation of the amino acid to the heme [18]. Cyt C freezing leads to decreasing of absorbance band intensity (Fig. 5), testifying to protein molecular loosening, being more considerable during slow freezing.

### Conclusions

It has been established that unfolding resulted from freeze-thawing exposure leads to protein antioxidant activity increasing while decreasing of the activity may be

associated with macromolecule aggregation. Character of freeze-thawing influence on proteins depends on molecule structure peculiarities and freezing protocols. Moreover protein ABTS<sup>+</sup> reducing activity may be considered as a parameter of their conformational stability when selecting cooling protocols.

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