

ON THE POSSIBILITY OF INDIRECT DETERMINATION OF THE GLASS TRANSITION TEMPERATURE OF PROTEINS FROM VISCOSITY MEASUREMENTS AND AVRAMOV'S MODEL

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The paper presents the results of viscosity determinations on aqueous solutions of hen egg-white lysozyme, bovine β -lactoglobulin, human and porcine immunoglobulin IgG at a wide range of concentrations and at temperatures ranging from 5°C to 55°C. Viscosity-temperature dependence of the proteins solutions is analyzed based on a formula resulting from the Avramov's model. One of the parameters in the Avramov's equation is the glass transition temperature T_g . It turns out that for all studied proteins, the T_g of the solution increases with increasing concentration. To determine the glass transition temperature of the dry protein $T_{g,p}$, a modified form of the Gordon-Taylor equation is used. This equation gives the relationship between T_g and the concentration of the solution, and $T_{g,p}$ and a parameter dependent on the strength of protein-solvent interaction are fitting parameters. Thus determined the glass transition temperature for the studied dry proteins is in the range from 227.3 K (for bovine β -lactoglobulin) to 260.6 K (for hen egg-white lysozyme).

INTRODUCTION

The proteins in the solutions may occur in native form only in a specific pH range, and relatively narrow temperature range. Freezing point of the solution and the temperature of protein denaturation effectively define boundaries from the side of low and high temperatures, respectively. In this temperature range, the temperature dependence of solution viscosity can be based on several models of viscous flow of liquid. To the most important can include free volume model (Vinogradov & Malkin, 1980), a modified Arrhenius model (Monkos 1996) and the Avramov's model (Avramov, 1998). These models allow to obtain different types of information on the test system, because they consider the mechanism of flow in different way. In the present paper, only Avramov's model is taken into consideration. Based on this model, one can obtain three-parameter equation binding viscosity with temperature. One of the parameters in this equation is the glass transition temperature T_g .

As a result of the cooling process, liquids crystallize. However, if the liquid is supercooled below the melting point, it then solidify forming a non-crystalline state which is usually called the glass. This process occurs at a characteristic temperature called the glass transition temperature (Martinez & Angell, 2001). For temperatures above T_g , anharmonic motions of bonded and nonbonded groups of atoms in proteins dominate, as

in the liquid state. At temperatures below T_g , in turn, harmonic motions predominate, as in the solid state. In other words, the glass transition temperature of the protein is the temperature in which its properties change from liquid-like to solid-like. In the literature of recent years can be seen increasing interest to glass transition phenomenon of biopolymers such as proteins or polysaccharides (Grasmeijer *et al.*, 2013; Marsh *et al.*, 2013; Khatkar *et al.*, 2013; Roughton *et al.*, 2012; García *et al.*, 2012; Panagopoulou *et al.*, 2011; Rodríguez Furlán *et al.*, 2011; Hernández *et al.*, 2011; Khodadadi *et al.*, 2010; Jansson & Swenson, 2010). The glass transition temperature of proteins is determined primarily using calorimetric and rheological measurements and dielectric spectroscopy. In the present paper T_g for dry hen egg-white lysozyme (HEWL), bovine β -lactoglobulin (BLG), human immunoglobulin IgG (HIgG) and porcine immunoglobulin IgG (PIgG) has been obtained on the basis of viscosity measurements of aqueous solutions of these proteins, from Avramov's model and modified Gordon-Taylor equation.

HEWL is an enzyme that acts as a glycoside hydrolase. It is a small globular protein with a molecular mass $M = 14.32$ kDa (Squire & Himmel, 1979) and well known structure (Smith *et al.*, 1993). In solution it can be treated as prolate ellipsoid of revolution with the main axes 4.5 nm and 3 nm (Monkos, 1997). In addition, infrared spectra analysis of HEWL in solution

and crystalline form showed that in both cases its structure is very similar (Hadden *et al*, 1995). HEWL is used as a model protein in various biophysical and biochemical studies. Its structure, dynamics, and - in particular - the hydrodynamic properties have been examined by different experimental techniques (Monkos, 2011 and references therein).

BLG is the major whey protein of the cow's milk. It is also small, globular protein consisting of 162 amino acid residues and having a molecular mass of 18.155 kDa (Oreccini *et al*, 2001). It is able to bind a wide range of ligands, aromatic molecules, alkanone species and is thought to be a member of the lipocalin family of hydrophobic carrier molecules (Kuwajima *et al*, 1996). At physiological pH BLG forms dimers, in which the monomers are noncovalently linked (Aymard *et al*, 1996). BLG is used as a model protein in different studies of folding, stability and self-association.

The immunoglobulins can be divided into five major classes: IgG, IgA, IgM, IgD and IgE. They constitute about 20% of the total plasma proteins. IgG immunoglobulins, in turn, constitute approximately 75% of them (Goodman, 1991). IgG molecules with a molecular mass of 156 kDa, consist of four polypeptide chains – two identical light chains and two identical heavy chains. They are folded in three globular structures – two F_{ab} fragments and one F_c fragment (Al-Lazikani *et al*, 1997). As a result, the entire molecule looks like the letter T or Y. This means that the immunoglobulins IgG are non-globular proteins.

MATERIALS

Highly purified HEWL, BLG and PIgG were purchased from Sigma Chemical Co. and HIgG from Polish Chemical Reagents factories and were used without further purification for all the measurements. From crystalline form, the proteins were dissolved in distilled water and the solutions thus obtained were filtered through a paper filter to remove possible undissolved dust particles. These solutions were stored in a refrigerator until just prior to viscometry measurements, when they were warmed from 5°C to 55°C. The pH values of such prepared samples were measured by using pH meter. The pH values of such solutions of immunoglobulins – in the whole range of measured concentrations - were close to their isoelectric points and were as follows: 5.6 for HIgG and 5.9 for PIgG. The isoelectric point for HIgG is in the range (5.8 – 6.6) (Young, 1963). The pH values of HEWL and BLG solutions were, in turn, outside of their isoelectric points, and were as follows: 7.0 for HEWL and 7.2 for BLG. The isoelectric point for HEWL is in the range (11- 11.2) (Young, 1963) and is equal to 5.6 for BLG (Roth *et al*, 2000). The pH values for different

concentrations differed slightly from each other, and the values given above are the average values.

VISCOMETRY

The viscosity measurements of aqueous solutions of test proteins were carried out using an Ubbelohde-type capillary microviscometer with a flow time for water of 28.5 s at 25°C. Microviscometer was placed in a water bath whose temperature was stabilized with an accuracy of $\pm 0.1^\circ\text{C}$. All measurements were performed using the same viscometer. Measurements began after a few minutes of delay that the system may reach a state of equilibrium. Flow times were measured with an accuracy of ± 0.1 s. For most concentrations the viscosity measurements were conducted from 5°C to 55°C by steps of 5°C. Limitation of viscosity measurements to the temperature of 55°C results from the fact that above this temperature the solutions flow times increase with increasing temperature, which means that the proteins are gradually denatured.

The densities of solutions were measured by using weighing method, while the proteins concentrations were determined by a dry weight method in which solutions were dried at high temperatures for several hours. Viscosity measurements were made for individual proteins in the following ranges: from 17 kg/m³ to 285 kg/m³ for HIgG, from 28 kg/m³ to 223 kg/m³ for PIgG, from 25 kg/m³ to 343 kg/m³ for HEWL. Viscosity measurements of BLG solutions were made for concentrations in the range from 20 kg/m³ to 444 kg/m³. However - in the further analysis - viscosity data were used only for the solutions above the concentration of 119 kg/m³, since only above this concentration and the temperature range used herein, BLG molecules are present in the form of dimers (Aymard *et al*, 1996).

RESULTS AND DISCUSSION

According to the Avramov's model molecules in a flowing liquid jump from one equilibrium state to the other. During the jumps molecule has to overcome some energy barrier (or activation energy), which may be different for different jumps. It is further assumed that the frequency of these jumps is different for different molecules and it is subject to the Poisson distribution. This allows calculate the average frequency of these jumps. Finally, Avramov's model assumes that the viscosity of the liquid is inversely proportional to the mean frequency of these jumps. On the basis of these assumptions, one can get the relation between the viscosity and temperature of the liquid. For solutions, where viscosity is dependent on both temperature and concentration, this dependence has the following form:

$$\eta(c, T) = \eta_{\infty}(c) \exp \left[\frac{\Theta(c)}{T} \right]^{\alpha(c)}, \quad (1)$$

where $\eta_{\infty}(c)$, $\Theta(c)$ and $\alpha(c)$ are parameters dependent on the concentration.

To adjust the viscosity - obtained from Avramov's relation - to the experimental values of viscosity numerical values of the parameters $\eta_{\infty}(c)$, $\Theta(c)$ and $\alpha(c)$ are required. The relevant calculations were performed using a non-linear regression procedure in the

computational statistical program. Figure 1 shows the viscosity measurement results for all the proteins tested here, but - for each protein - only for a single selected concentration. Curves exhibit fit to the experimental points in accordance with relation (1), with parameters values obtained from the above-mentioned method. As seen this relation then gives a perfect fit over the whole range of measured temperatures. Equally good fit is obtained for all the concentrations of the studied proteins.

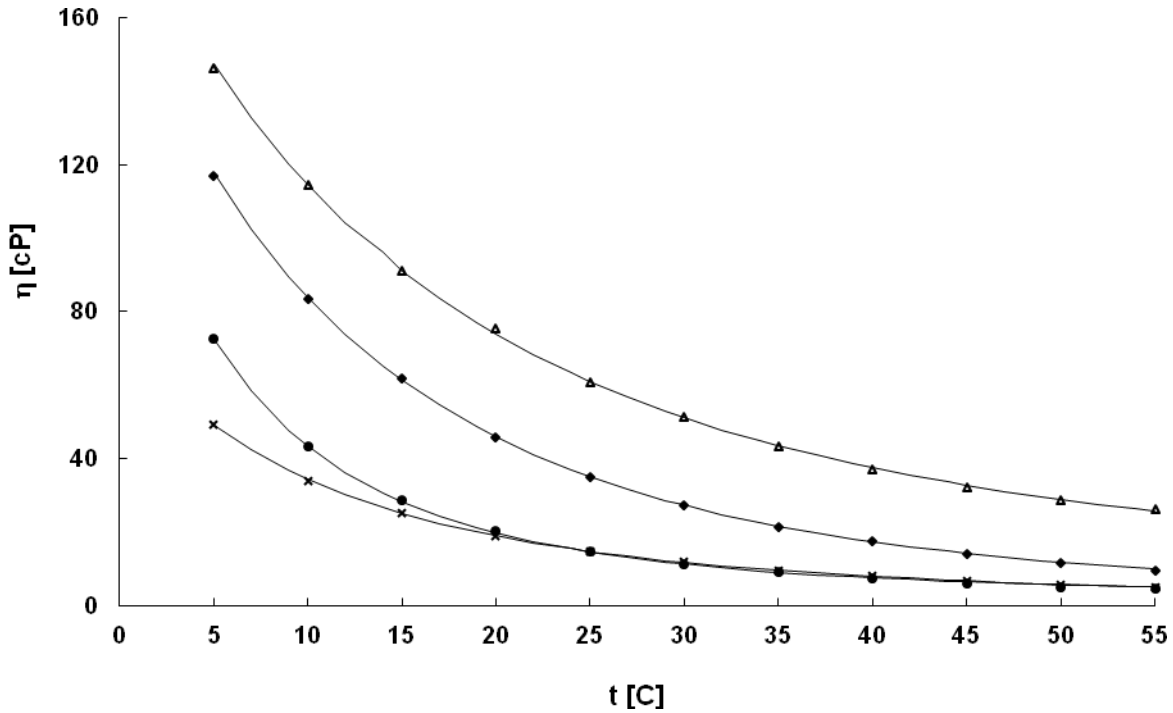


Fig. 1. Temperature dependence of the viscosity of BLGL (Δ), HIgG (\blacklozenge), HEWL (\bullet), and PIgG (\times) aqueous solutions for concentrations: $c = 430, 433, 343$ and 210 kg/m^3 , respectively. The curves show the fit obtained by using equation (1) with the parameters given in Table 1.

Table 1. The parameters of equation (1) for the selected concentrations of the studied proteins solutions.

Protein	$\eta_{\infty}(c)$ [cP]	$\Theta(c)$ [K]	$\alpha(c)$
HIgG ($c = 433 \text{ kg/m}^3$)	0.398	461.3	3.437
PIgG ($c = 210 \text{ kg/m}^3$)	1.192	351.8	5.591
HEWL ($c = 343 \text{ kg/m}^3$)	2.437	317.9	9.157
BLGL ($c = 430 \text{ kg/m}^3$)	4.637	372.7	4.241

From the point of view of purposes of this work, the most interesting parameter is the $\Theta(c)$. According to the Avramov's model $\Theta(c) = T_g(c)\varepsilon^{1/\alpha(c)}$, where $T_g(c)$ is the glass transition temperature of the solution and the quantity ε means the ratio of the activation energy corresponding to its value at the maximum of the probability distribution function to a dispersity of the

activation energy. The glass transition temperature of the solution depends on the glass transition temperature of the individual components. The glass transition temperature of binary mixtures is considered in a classical thermodynamic approach by Gordon and Taylor (Gordon & Taylor, 1952). According to this approach T_g of the mixture depends on the T_g of

components, and the strength of the interaction between them. For a mixture of protein and water, Gordon-Taylor formula can be written in the following way:

$$T_g = \frac{w_p T_{g,p} + k w_w T_{g,w}}{w_p + k w_w}, \quad (2)$$

in which w_p and w_w denote weight fractions of the protein and water, and $T_{g,p}$ and $T_{g,w}$ are glass- transition temperature of the dry protein and water, respectively. The parameter k is related to the strength of protein-water interaction. Couchmann showed that this parameter is equivalent to the ratio of the heat capacity changes of the pure components at T_g (Couchmann, 1978).

To be able to apply the Gordon-Taylor equation for solutions, the weight fractions should be expressed by the concentration of the solution. According to the definition: $w_p = m_p/(m_p + m_w)$, $w_w = m_w/(m_p + m_w)$ and m_p and m_w are masses of the dissolved protein and water in a solution, respectively. The mole numbers of the dissolved protein and water, in turn, are defined as: $N_p = m_p/M_p$, $N_w = m_w/M_w$ and M_p and M_w are their molecular masses. It follows that $w_p = N_p M_p / (N_p M_p + N_w M_w)$ and $w_w = N_w M_w / (N_p M_p + N_w M_w)$. Given the above, and using the definition of the molar fractions of the dissolved protein and water: $X_p = N_p / (N_p + N_w)$ and $X_w = N_w / (N_p + N_w)$ one can transform equation (2) to the following form:

$$T_g = \frac{X_p M_p T_{g,p} + k X_w M_w T_{g,w}}{X_p M_p + k X_w M_w}. \quad (3)$$

The molar fraction of the dissolved protein can be expressed by the concentration of the solution in the following way (Monkos, 1996):

$$X_p = \frac{c}{\rho_w \frac{M_p}{M_w} - c \left(\rho_w \frac{M_p}{M_w} \xi - 1 \right)}, \quad (4)$$

where ρ_w and ξ denote the water density and the effective specific volume of a protein, respectively, and

c is the solution concentration in kg/m^3 . The effective specific volume is a coefficient of proportionality between the effective molar volume and the molar mass of a macrolute. Since the sum of the molar fractions of the individual components of the solution is equal to 1, so $X_w = 1 - X_p$. Taking this into account - after substituting equation (4) into the formula (3) - we get Gordon-Taylor equation in the form convenient for use in the case of solutions:

$$T_g(c) = \frac{c (T_{g,p} - k \rho_w \xi T_{g,w}) + k \rho_w T_{g,w}}{c(1 - k \rho_w \xi) + k \rho_w}. \quad (5)$$

The effective specific volume of all studied proteins has been experimentally determined earlier and it is equal to: $2.6 \times 10^{-3} \text{ m}^3/\text{kg}$ for HEWL (Monkos, 1997), $1.27 \times 10^{-3} \text{ m}^3/\text{kg}$ for BLG (Monkos, 2006), $2.25 \times 10^{-3} \text{ m}^3/\text{kg}$ for HIgG (Monkos, 2009), $2.71 \times 10^{-3} \text{ m}^3/\text{kg}$ for PIgG (Monkos, 2009).

The glass transition temperature of the bulk water has been determined by a number of authors. The values obtained are slightly different from each other and the most frequently cited value is $T_{g,w} = 136 \text{ K}$ (Sartor *et al.*, 1994; Johari *et al.*, 1987; Hallbrucker *et al.*, 1989; Teeter *et al.*, 2001; Katkov & Levine, 2004).

As mentioned above, the glass transition temperature of the solution $T_g(c)$ is related to the parameters of Avramov's model $\Theta(c)$ and $\alpha(c)$ by the relation: $\Theta(c) = T_g(c) \varepsilon^{1/\alpha(c)}$. For the calculation of $T_g(c)$ - at a given concentration - in addition to the values of $\Theta(c)$ and $\alpha(c)$ the value of the parameter ε is needed. It turns out that - according to equation (5) - $T_g(c)$ increases monotonically with increasing concentration. The value of the parameter ε should be chosen so that in the limit of zero concentration $T_g(c)$ was equal to the $T_{g,w}$. In this way determined the values of parameter ε are given in Table 2. In the Avramov's work value of the parameter ε was determined for a number of inorganic liquids. The author received the values of ε in the range (25.1 - 35.1) with the average value (30.5±2). As can be seen from Table 2 the values of ε obtained for HEWL and BLG are very close to the lower limit of the range.

Table 2. The glass transition temperature $T_{g,p}$ and the parameters ε and k of the Avramov's model for the studied proteins.

Protein	ε	$T_{g,p}$ [K]	k
HIgG	20.2	241.7 ± 5.6	0.4603 ± 0.061
PIgG	20.85	240.9 ± 4.5	0.2592 ± 0.032
HEWL	23.6	260.6 ± 5.6	1.237 ± 0.14
BLGL	24.25	227.3 ± 13	1.250 ± 0.36

From the relation $\Theta(c) = T_g(c)\varepsilon^{1/\alpha(c)}$ - with known values of the parameters ε , $\Theta(c)$ and $\alpha(c)$ - one can calculate the $T_g(c)$ values for each concentration of the studied proteins. This way calculated values of $T_g(c)$ have been presented in Figures 2 and 3. Since the effective specific volume of the proteins and the glass transition temperature of water are known, the only unknown parameters in the modified Gordon-Taylor equation (5) are the glass transition temperature of the dry protein and the parameter k .

The function of equation (5) can be fit to the experimental points if $T_{g,p}$ and k are treated as the fitting parameters. Such calculations were performed using non-linear regression procedure in the computational statistical program. Thus calculated $T_{g,p}$ and k are given in Table 2. As shown in Figure 2 and 3 the function of modified Gordon-Taylor equation gives a good fit to the $T_g(c)$ values obtained in the above-described manner.

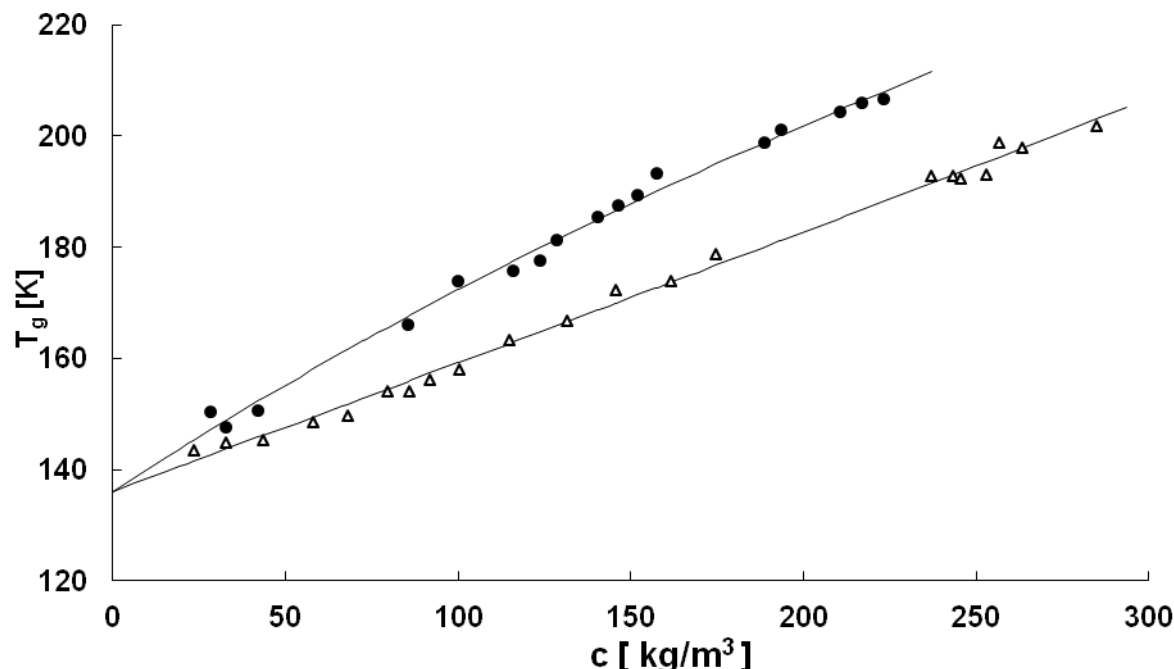


Fig. 2. Plot of the glass-transition temperature $T_g(c)$ of the PIgG (●) and HIgG (△) aqueous solutions versus concentration. The curves show the fit according to relation (5) with $T_{g,w} = 136$ K and with the parameters shown in Table 2 and in the text above.

To fulfill its physiological functions biological macromolecules, and in particular, globular and non-globular proteins, must demonstrate a structural flexibility. This may be demonstrated among others by examining the proteins in solution by viscometry method (Monkos, 2000, 2004, 2013). This flexibility of proteins causes that all interactions cause a slight displacements of atoms, resulting in the change of protein conformation. Change of the protein conformation, in turn, causes that slightly changes also its energy. This can be described by the conformational energy landscape like in glasses. It is now widely accepted view that the protein molecule may be present in one of the many isoenergetic conformational substates, corresponding to the valleys in the protein energy landscape (Frauenfelder *et al.*, 1991). As a result of all kinds of interactions, protein energy may vary due

to transitions between the conformational substates. As the lowering of the temperature, these transitions become more slower, and in certain temperature the protein is frozen in a specific substate. This temperature is called the glass transition temperature of the protein $T_{g,p}$. In the vicinity of the glass transition temperature a strong changes in temperature dependence of various physical quantities, such as heat capacity, density and elastic modulus are observed.

There are not too many literature data on the value of the glass transition temperature of proteins. For example, for small DNA oligonucleotide duplex d(CGCGCG)₂ in aqueous solution - using molecular dynamic simulations method - a glass transition temperature in the range (223 – 234) K was obtained (Ringe & Petsko, 2003). The results of some works indicate that the glass transition process of proteins does

not occur at one temperature but in a wide temperature range (Morozov & Gevorkian, 1985; Sartor *et al*, 1994). For example, calorimetric studies of lysozyme, myoglobine and albumin suggest the existence of a glass transition in a wide temperature range from 130 to 240 K (Morozov & Gevorkian, 1985). The glass transition process in hydrated hemoglobin, in turn, extends from about 150 K up to temperature of denaturation (Sartor *et al*, 1994). Furthermore, the glass transition temperature for a specific protein, obtained by different methods may differ significantly from each other. For example, the

glass transition temperature of myoglobin obtained by dielectric spectroscopy and differential scanning calorimetry method is in the range (190 - 210) K (Jansson & Swenson, 2010), and as determined by the molecular dynamics simulation is 220 K (Steinbach & Brooks, 1993). The above examples show that to the strict determination of the glass transition temperature of proteins would be desirable a confrontation the results from different methods.

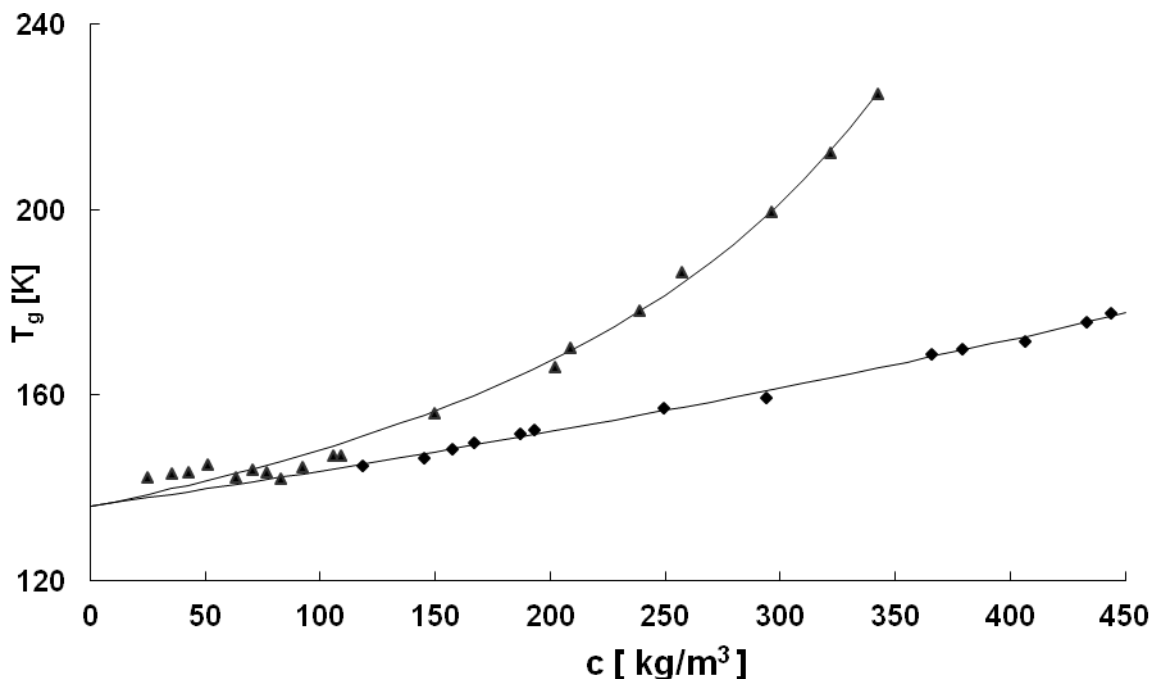


Fig. 3. Plot of the glass-transition temperature $T_g(c)$ of the HEWL (\blacktriangle) and BLGL (\blacklozenge) aqueous solutions versus concentration. The curves show the fit according to relation (5) with $T_{g,w} = 136$ K and with the parameters shown in Table 2 and in the text above.

The glass transition temperatures for studied proteins solutions obtained in this work (Figures 2 and 3), are above the glass transition temperature of water (136 K) up to 202 K for HIgG, 212 K for PIgG, 225 K for HEWL and 178 K for BLGL. As can be seen, they lie in the range of the glass transition temperatures for the hydrated proteins reported in the literature. Moreover, comparison of the above values with the results given in Table 2 shows that the glass transition temperature of a given protein in a dry state is always higher than the glass transition temperature of this protein solutions. Unfortunately, as far as I know, there is no appropriate data in the literature concerning the glass transition temperature of proteins in the dry state.

CONCLUSIONS

Functional dependence of viscosity on temperature, resulting from Avramov's model can be used to analyze the results of viscosity measurements of globular and non-globular proteins solutions in the temperature range from 5°C to 55°C. One of the three parameters found in the Avramov's equation is glass transition temperature. It depends on the concentration of the solution. For each of the studied protein, increase in concentration of solution causes a non-linear increase of T_g . Functional dependence of the glass transition temperature of the solution on the concentration can be given on the basis of a modified form of the Gordon-Taylor equation. The glass transition temperature of the dissolved protein is

one of the parameters of this equation. Numerical values of $T_{g,p}$ obtained for the studied proteins are in the range from 227.3 K (for bovine β -lactoglobulin) to 260.6 K (for hen egg-white lysozyme).

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