I.A.U
Karaj branch

Journal of
A p p l ied
C hemical
R esearch
jacr.kiau.ac.ir

Journal of Applied Chemical Research, 18, 4, 99-109 (2024)

Analysis of Disaccharides Interaction with Human Growth Hormone by Spectroscopy and Molecular Docking

Solmaz Kamrani[1], Parichehreh Yaghmaei[1], Maryam Nikkhah[2], Saman Hosseinkhani[3*]

[1]Department of biology, Science and Research branch, Islamic Azad University, Tehran, Iran

[2]Department of Nanobiotechnology, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran

[3]Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

(Received 10 Feb. 2024; Final revised received 18 May 2024)

Abstract

Sucrose and trehalose are disaccharides commonly used as stabilizer excipients in the formulation of therapeutic proteins. One of the essential therapeutic proteins that scientists focus on is the recombinant human growth hormone (rhGH). In the current study, we used UV spectroscopy and molecular docking simulation to investigate the effect of sucrose and trehalose on the stability of rhGH. The results showed that sucrose and trehalose could increase the thermal stability of rhGH. However, the aggregation of growth hormone in the presence of sucrose is less than in trehalose. Molecular docking studies showed that these differences could result from the interactions between growth hormone and the disaccharide. So, hGH has more regions that interact with sucrose than trehalose.

Keywords: Molecular Docking, Somatotropin, Stability, Sucrose, Trehalose.

***Corresponding author**: *Saman Hosseinkhani, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran. P. O. Box: 14115-175, E-Mail: saman_h@modares.ac.ir.*

Introduction

There are different excipients used in the protein formulations; like amino acids, osmolytes, salts, surfactants, proteins, polymers, chelators, anti-oxidants, specific ligands, and carbohydrates [1]. Disaccharides, especially sucrose, and trehalose, are considered a frequent group of excipients [2]. Sucrose consists of glucose and fructose molecules linked together with an α, β- 1, 2 bound and trehalose contains an α, α - 1, 1 bond that binds two glucose molecules (Fig.1). Both of these non-reducing disaccharides have anti-aggregate and stability effects on most of the therapeutic proteins [3].

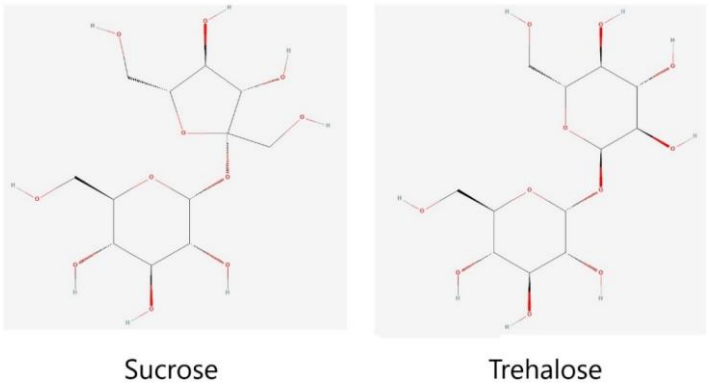

Figure 1. 2D Structure of sucrose and trehalose.

Recombinant human growth hormone (rhGH) is a therapeutic protein that can be stabilized by sucrose and trehalose using vitrification, water replacement, or a preferential exclusion mechanism [4]. The rhGH is a globular protein consists four alpha-helical structures connected with loops and strands. Its sequence is formed from 191 amino acid residues with two disulfide bonds linking its cysteine residues [5]. Its only tryptophan (Trp86) is buried in a relatively hydrophobic interior of the protein and interacts with Asp169 by hydrogen bound [6].

The stability of rhGH in lyophilized form in the presence of sucrose and trehalose was previously reported at different temperatures and various methods [7, 8]. Also, the stability effect of sucrose and trehalose on the rhGH was confirmed using fluorescence spectroscopy studies [9]. In the current report, we have studied the stability of rhGH in liquid form using UV spectroscopy, which is an accessible and low-cost technique, and sensitive to the chemical environment of proteins. As aromatic residues of proteins can absorb UV light, the alteration of protein conformation and structure can change their UV absorption [10].

In this study, we have studied the rhGH after expression and purification from the E. coli as reported earlier [11]. To investigate the thermal stability of the protein at different temperatures, its liquid state was used in the absence and presence of sucrose and trehalose. Also, we have performed molecular docking to monitor the interactions between hGH and the disaccharides.

Experimental

Material

Sucrose and trehalose were from Merck and Sigma Aldrich Co., respectively. Other materials used in this study were of analytical grade.

Expression of rhGH

The rhGH was synthesized using the *E. coli* expression system containing pET28a carrying the recombinant human growth hormone gene, as reported earlier [11]. Affinity chromatography was used to purify the His-tagged recombinant protein. After dialysis of the extracted protein, SDS-PAGE confirmed its purification, and its concentration was measured by Bradford assay.

The thermal stability study

Solutions of rhGH with and without 1.0 M sucrose or trehalose were prepared in Eppendorf tubes. The volume of each sample was 1.0 ml and the ratio of rhGH: disaccharide was 1:1. The samples were incubated in a bain-marie at different temperatures of 4, 25, 30, 40, 50, 60, and 70 ºC for 10 minutes. UV absorption of the incubated samples was recorded at the wavelength of 280 nm using a Biochrom WPA Biowave II UV/Visible spectrophotometer.

Next, to investigate aggregation of rhGH in the absence and presence of sucrose and trehalose, the samples were incubated at 37 °C for a month while continuous shaking at 180 rpm. Then, the absorbance of the samples was recorded at 280, 350, and 600 nm. Also, the aggregation index of the samples was calculated by equation 1 [12]:

$$AI = \left(\frac{OD\ 350\ nm}{OD\ 280\ nm - OD\ 350\ nm}\right) X\ 100$$

Equation 1. Aggregation Index.

Molecular docking

For molecular docking, the crystal structure of human growth hormone (PDB DOI: 10.2210/pdb1HGU/pdb), sucrose (PubChem DOI: 10.5517/cc584yh) [13], and trehalose (PubChem DOI: 10.5517/ccqf5y7) [14] were achieved from Protein Data Bank (PDB) and PubChem, respectively. Water molecules were removed from the single chain hGH and polar hydrogen atoms were added using Autodock Vina 1.2.0 [15] and Autodock tools (ADT). The whole of the protein surface was chosen as the grid box. According to the molecular docking results, one region of hGH can interact with both sucrose and trehalose. The region coordinate was 64.026, 27.491, and 52.062 as X, Y, and Z center. This region of the protein was set as the grid box with the

size of 12*12*12 along X, Y, and Z axes and 1.0 Å grid spacing for the second docking. After molecular docking, the nine best modes of ligands were introduced according to the docking score. Discover studio visualizer v21.1.0 was used to view and analyze the modes. Analyzing the amino acid residues involved in the interaction between hGH and disaccharides was also done using LIGPLOT v.2.2.7 software.

Results and Discussion

UV spectroscopy

To investigate the thermal stability of rhGH in the absence and presence of sucrose and trehalose, absorption spectra at different temperatures were obtained. The only Trp residue of rhGH is monitored as a chromophore that absorbs the UV light at 280 nm, which can be an indication of protein tertiary structure changes [10]. Figure 2 showed that the absorbance didn't change significantly up to 40 °C, indicating the relatively stable structure of the protein. After that, the UV absorption of Trp increased which could be a sign of Trp exposure as a result of protein denaturation. In contrast, decreased absorption could indicate the burying of Trp in the hydrophobic core of the protein, showing the folded state [10]. It could be suggested that the sucrose and especially trehalose partially prevent the changes of rhGH structure after 10 minutes of incubation at temperatures above 40 °C when starting the unfolding of proteins in the mentioned experimental conditions. It should be mentioned that the melting temperature (Tm) of rhGH was previously calculated at about 82 ℃ using CD spectroscopy and Differential Scanning calorimetry (DSC) after 1-minute incubation [16].

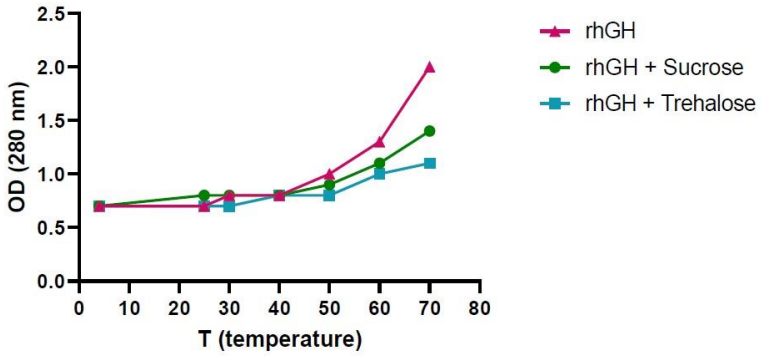

Figure 2. UV spectroscopy of rhGH in the absence and presence of 1.0 M sucrose and 1.0 M trehalose at different temperatures (4, 25, 30, 40, 50, 60, and 70 ℃).

To investigate the sucrose and trehalose effect on aggregation of rhGH, we incubated the samples at 37 °C for a month. Then, the spectroscopy was done at 600 nm to monitor the turbidity [14, 15] and at 280 and 350 nm to indicate the aggregation index of samples (eq.1). Figures 3 and 4 showed that

the turbidity and aggregation index increase in the presence of sucrose and trehalose after one month. The aggregations can be amorphous or regular structures [17]. The high-ordered aggregations like amyloids are more probable in the presence of sucrose [9].

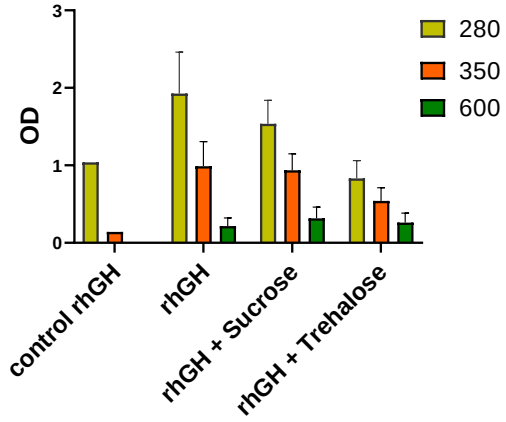

Figure 3. Absorption of rhGH in the absence and presence of sucrose and trehalose, after a month of incubation at 37 °C. The control rhGH was a fresh sample without incubation. The control rhGH is a clear sample and its turbidity (absorbance at 600 nm) is low and not detectable.

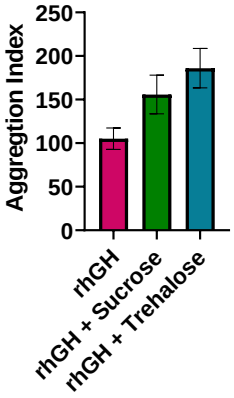

Figure 4. Aggregation index of rhGH in the absence and presence of sucrose and trehalose, after a month of incubation at 37°C.

Molecular docking

Intermolecular interactions were investigated using the molecular docking technique to better understand how sucrose and trehalose affect hGH structure and their possible differences. For this purpose, the x-ray structure of hGH; named 1HGU [15] was used. There is not an exact area on the protein to interact with the sucrose or trehalose, so we set the whole protein as a grid box at the first docking. Figure 5 showed the nine modes of sucrose (Fig. 5A) and trehalose (Fig. 5B) in different colors. It could be seen that most of the sucrose modes are at the region of the end of helix 5 and turn 2 and 3; between helix 1 and 2 (marked with a yellow circle in figure 5A) indicating more affinity of sucrose to this region. However, trehalose has the most affinity to growth hormone at a different region (between helix 3, 4, 5, and strand 3) as was shown in figure 5B by a yellow circle. Analysis of whole protein molecular docking showed that sucrose can have chemical interactions with hGH in 5 regions but trehalose in 2 regions of that. These results could show the better-closed coverage of sucrose in comparison to trehalose for hGH which can be a reason for the relatively lower value of the aggregation index of sucrose in comparison to trehalose in long times (Fig 3 and 4). However, since the area marked in Figure 5A was common for both disaccharides, this critical region was selected as a grid box at the second docking to study the binding mode of sucrose and trehalose to hGH.

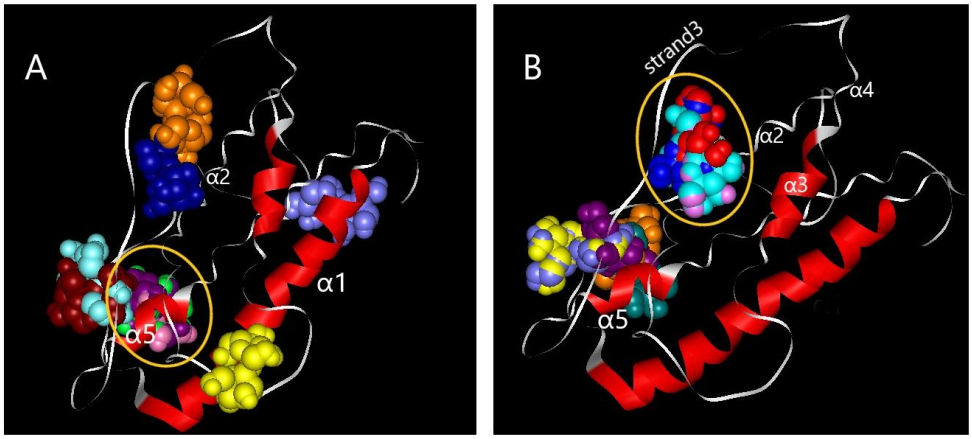

Figure 5. First docking. Viewer-Lite representation of sucrose (A) and trehalose (B) docked to hGH. The whole of the protein was selected as the grid box. Different colors show different modes of sugars in cartoon shapes. The best modes were indicated by yellow circles.

The second docking resulted in nine possible conformations of disaccharides with different affinities (table 1), and the best one of each sugar mode was selected based on free energy changes (ΔG). These results showed that sucrose and trehalose can bind to the hydrophilic surfaces of hGH with a high probability (Fig. 6 and 7).

Table 1. Docking scoring.

Mode	Docking of sucrose			Docking of trehalose		
	Affinity (kcal/mol)	Dist from best mode		Affinity (kcal/mol)	Dist from best mode	
		Rmsd l.b.	Rmsd u.b.		Rmsd l.b.	Rmsd u.b.
1	-6.0	0.000	0.000	-5.3	0.000	0.000
2	-6.0	1.741	5.909	-5.3	0.009	6.374
3	-5.4	1.486	1.709	-5.3	2.658	4.631
4	-5.3	1.838	5.893	-5.2	2.552	5.851
5	-5.3	1.861	3.633	-5.2	2.165	3.028
6	-5.3	1.360	2.082	-5.2	2.638	5.506
7	-5.1	1.781	5.875	-5.1	1.856	3.906
8	-5.0	2.541	4.776	-5.1	2.425	4.970
9	-5.0	2.936	6.227	-5.0	1.828	6.201

Furthermore, figures 6 and 7 depicted the hydrophobic and hydrogen intermolecular bonds that stabilize the complex of protein-disaccharide [4]. Data analysis with Ligplot+ showed hydrogen bonds could be formed between sucrose and Lys41, Leu45, Gln46, Gln49, Sre51 (residues between helix 1 and 2), Tyr 160, and tyr164 (residues near the helix 5) of hGH. The sucrose may also have some hydrophobic interactions with Gly161 and Cys165 (Fig. 7). On the other hand, the trehalose could forme some other hydrogen bonds with Lys41, Gln46, Gln49 (residues between helix 1 and 2), Tyr 160, and tyr164 (residues near the helix 5) of hGH. The residue involved in hydrophobic interaction with trehalose was Cys53. The binding energies of hGH complexes with sucrose and trehalose that were calculated by Autodock Vina were -6 and -5.3 kcal/mol, respectively. It could be suggested that the best conformation of sucrose and trehalose in a similar region of hGH showed eight hydrogen bonds between the sugars and the protein with completely different patterns and

relatively different ΔG (Fig. 6). Previous investigation on the insulin was indicated the effect of different polyphenols structure on their interactions with protein without inducing significant conformational change of the protein [18]. However, the data of small-angle neutron scattering (SANS) supported our data that sucrose and trehalose have anti-aggregation effects on myoglobin [3]. The investigation of sucrose and trehalose protecting effect on lysozyme also suggested the stronger affinity of trehalose to water [2]. The previous thermodynamic studies on the other proteins also showed the protective effect of sucrose so that it can increase the activation energy of the unfolding process [19]. The molecular dynamics simulations of hGH in the presence of disaccharides also showed the difference in sucrose and trehalose interaction with hGH so that the sucrose increases protein stability by preferential exclusion and the trehalose using vitrification [20]. As the data shown (Fig. 5), since the disaccharides do not attach to the hGH at the exact same position, on the other hand, the interactions of sucrose and trehalose with hGH don't induce significant conformation changes as stabilizers [21]. Also, based on previous studies, stabilizers do not exert their effect only through specific interactions [20]. Therefore, it probably appears impossible to observe significant and reproducible changes with competitive tests.

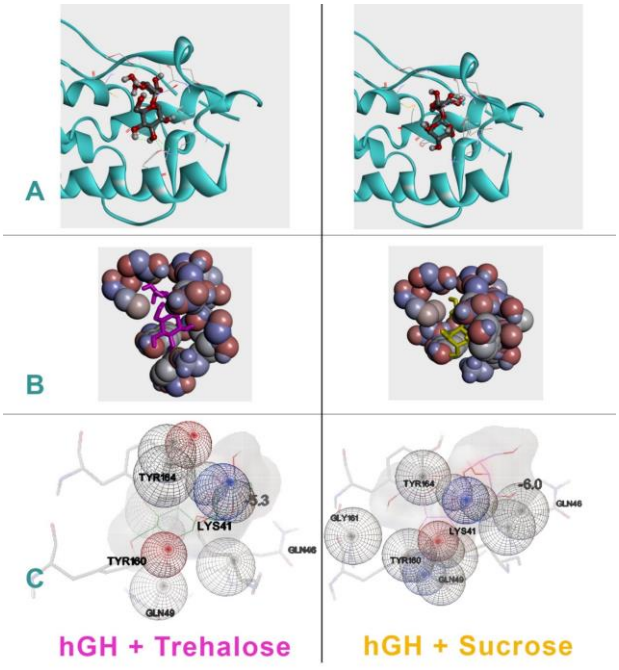

Figure 6. The best conformations of trehalose (left) and sucrose (right) docked to hGH. A; the ribbon style of hGH and ball and stick style of ligands. B; cartoon representation of ligands docked to hGH. C; amino acids in close contact with sugars.

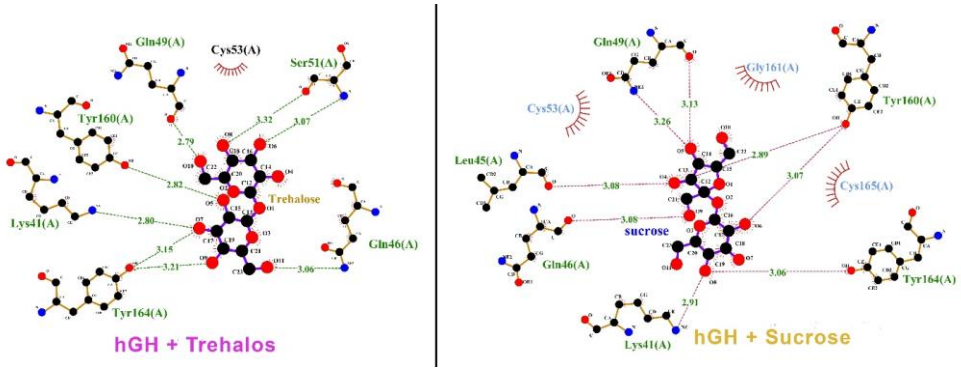

Figure 7. Lig plot representation of H-binding and hydrophobic interactions between trehalose (left) and sucrose (right) with hGH.

Conclusion

In summary, this study investigates the effect of sucrose and trehalose on the growth hormone by absorption spectroscopy and molecular docking simulation software. The effects of thermal stabilization of growth hormone can be observed by solutions containing sucrose or trehalose at temperatures above 40 ℃. It seems that the presence of disaccharides can cause the long-term thermal stability of rhGH, although the samples with sugars show more aggregation indexes. It can be considered that the formation of rhGH regular aggregate types is facilitated in the presence of disaccharides that need more documents to confirm. Docking results also show the different nature of the interactions of sucrose and trehalose with hGH, which can indicate the possible differences in their stabilizing mechanisms.

This study can be useful in understanding the probable interactions between growth hormones and disaccharides. The protein structure was investigated using an accessible and sensitive technique; UV spectroscopy. The limitation of this study is that it is restricted to two common stabilizer sugars which our main laboratory research was previously on [9], but it can be modeled by docking and molecular dynamics with other excipients to select the best condition of its formulation. It is also possible to investigate the effect of sucrose and trehalose or other excipient simultaneously on the rhGH.

Acknowledgment

Financial support of this work was provided by Nooragene Pishro Company (www. nooragen.com).

References

1. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein–excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development. Advanced drug delivery reviews. 2011;63(13):1118-59.

2. Starciuc T, Malfait B, Danede F, Paccou L, Guinet Y, Correia NT, et al. Trehalose or sucrose: which of the two should be used for stabilizing proteins in the solid state? A dilemma investigated by in situ micro-raman and dielectric relaxation spectroscopies during and after freeze-drying. Journal of Pharmaceutical Sciences. 2020;109(1):496-504.

3. Olsson C, Swenson J. The role of disaccharides for protein–protein interactions–a SANS study. Molecular Physics. 2019;117(22):3408-16.

4. Arsiccio A, Pisano R. The preservation of lyophilized human growth hormone activity: how do buffers and sugars interact? Pharmaceutical Research. 2018;35(7):1-13.

5. Ranke MB, Wit JM. Growth hormone—past, present and future. Nature Reviews Endocrinology. 2018;14(5):285-300.

6. Buijs J, Hlady V. Adsorption kinetics, conformation, and mobility of the growth hormone and lysozyme on solid surfaces, studied with TIRF. Journal of colloid and interface science. 1997;190(1):171-81.

7. Xu Y, Carpenter JF, Cicerone MT, Randolph TW. Contributions of local mobility and degree of retention of native secondary structure to the stability of recombinant human growth hormone (rhGH) in glassy lyophilized formulations. Soft Matter. 2013;9(32):7855-65.

8. Arsiccio A, Pisano R. Stability of proteins in carbohydrates and other additives during freezing: the human growth hormone as a case study. The Journal of Physical Chemistry B. 2017;121(37):8652-60.

9. Kamrani S, Yaghmaei P, Nikkhah M, Hosseinkhani S. Spectroscopic analysis of recombinant human growth hormone in the presence of sucrose and trehalose. Biotechnology and Applied Biochemistry. 2023;70(4):1543-53.

10. Biter AB, Pollet J, Chen W-H, Strych U, Hotez PJ, Bottazzi ME. A method to probe protein structure from UV absorbance spectra. Analytical biochemistry. 2019;587:113450.

11. Ghasemi R, Abdollahi M, Emamgholi Zadeh E, Khodabakhshi K, Badeli A, Bagheri H, et al. mPEG-PLA and PLA-PEG-PLA nanoparticles as new carriers for delivery of recombinant human Growth Hormone (rhGH). Scientific Reports. 2018;8(1):9854.

12. Hawe A, Kasper JC, Friess W, Jiskoot W. Structural properties of monoclonal antibody aggregates induced by freeze–thawing and thermal stress. European journal of pharmaceutical sciences. 2009;38(2):79-87.

13. PubChem Compound Summary for CID 5988, Sucrose [Internet]. 2022 [cited 2022,11,14]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Sucrose.

14. PubChem Compound Summary for CID 7427, Trehalose [Internet]. 2022 [cited 2022,11,14]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Trehalose.

15. Eberhardt J, Santos-Martins D, Tillack AF, Forli S. AutoDock Vina 1.2. 0: New docking methods, expanded force field, and python bindings. Journal of Chemical Information and Modeling. 2021;61(8):3891-8.

16. Gomez-Orellana I, Varinano B, Miura-Fraboni J, Milstein S, Paton DR. Thermodynamic characterization of an intermediate state of human growth hormone. Protein science. 1998;7(6):1352-8.

17. Pignataro MF, Herrera MG, Dodero VI. Evaluation of peptide/protein self-assembly and aggregation by spectroscopic methods. Molecules. 2020;25(20):4854.

18. Skrt M, Benedik E, Podlipnik Č, Ulrih NP. Interactions of different polyphenols with bovine serum albumin using fluorescence quenching and molecular docking. Food chemistry. 2012;135(4):2418-24.

19. Lee JC, Timasheff SN. The stabilization of proteins by sucrose. Journal of Biological Chemistry. 1981;256(14):7193-201.

20. Arsiccio A, Pisano R. The preservation of lyophilized human growth hormone activity: how do buffers and sugars interact? Pharmaceutical Research. 2018;35:1-13.

21. Salnikova MS, Middaugh CR, Rytting JH. Stability of lyophilized human growth hormone. International journal of pharmaceutics. 2008;358(1-2):108-13.