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Bovine serum albumin with gallic acid: Molecular modeling and physicochemical profiling

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Abstract:
Introduction. Gallic acid is a biologically active natural compound with strong antioxidant properties. Gallic acid is highly soluble and stable. It is known to increase the thermal stability of protein. However, its bioavailability is low, but interaction with proteins can solve this problem. Bovine serum albumin can bind various ligands, including polyphenols. The resulting complex of gallic acid and bovine serum albumin can become a promising functional food additive. *Study objects and methods.* This research featured in silico molecular modeling of gallic acid and bovine serum albumin using the HyperChem program. The methods of infrared spectrometry, potentiometry, and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) made it possible to describe the physicochemical profile of the complex. *Results and discussion.* The molecular modeling confirmed that hydrophobic interactions were responsible for the chemical bond between gallic acid and bovine serum albumin. The SDS-PAGE test showed that the protein molecule remained intact. The reducing properties of the complex grew as the concentration of gallic acid increased. At 100 mg/L of gallic acid, the reducing properties were 7.8 ± 1.3 mg/L equivalent of gallic acid. At 200 and 300 mg/L, the values reached 15.90 ± 2.65 and 23.30 ± 5.05 mg/L, respectively. The IR spectrometry revealed a significant difference between the samples with different concentrations of gallic acid. *Conclusion.* The research managed to predict the properties of the complex of bovine serum albumin and gallic acid during its formation. The resulting complex had the highest reducing properties at 0.69 g of bovine serum albumin and 300 mg of gallic acid. The obtained parameters can be used in the food industry to develop new food additives.

Keywords: Serum proteins, SDS-PAGE, IR spectroscopy, amperometric detector, polyphenols

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INTRODUCTION

Bovine serum albumin is a small and stable protein. Its molecular weight is 66.4 kDa. This protein predominates in bovine blood, where it is responsible for maintaining blood oncotic pressure and transporting various substances [1]. In milk, the amount of serum proteins is about 0.7–1.3% of the total mass [2]. The food industry obtains bovine serum albumin from animal blood and uses it to control such technological aspects of production as foam formation and gelation, emulsification, and encapsulation [3–7]. Bovine serum albumin has a number of important functional properties, e.g. an extensive profile of essential amino acids [8]. It is also known to inhibit lipid peroxidation in in vivo studies. In addition, it can reversibly bind most ligands [9, 10]. When it binds to polyphenols, bovine serum albumin changes its thermal stability, light absorption in the UV and visible range, the number of free amino groups and thiol groups, as well as secondary structure [11, 12].

Polyphenols are secondary metabolites of plant origin. They are found in plant materials and serve as

quality markers of fruits and vegetables [13]. Phenolic compounds proved beneficial in human diet [14]. They prevent obesity and reduce the risk of diabetes and coronary heart disease [15–17]. Phenolic compounds inhibit oxidative stress and free radicals while increasing molecular thermal stability [18, 19]. When binding, polyphenol molecules form complexes with oppositely charged biopolymers [20]. This property can protect proteins from oxidation by occupying free amino acid residues in the protein, thus solving the problem of antioxidant delivery [21].

Gallic acid is a hydrolyzed tannin found in many fruits and vegetables. Green and black teas are especially rich in gallic acid, where it is represented by gallates, e.g. epigallocatechin-3-gallate, epicatechin-3-gallate, epigallocatechin, etc. Gallic acid has antimicrobial, anti-inflammatory, antitumor, and antioxidant properties [22, 23].

The final reaction product of gallic acid and bovine serum albumin can exhibit different properties of protein and polyphenol, which makes it a promising food additive in complex food systems. The food industry demonstrates a growing interest in this research area, but the interaction patterns between gallic acid and bovine serum albumin still remain understudied.

The research objective was to establish the interaction patterns between bovine serum albumin and gallic acid using molecular modeling, as well as to identify how gallic acid affects the protein.

STUDY OBJECTS AND METHODS

Molecular modeling. Molecular modeling (HyperChem) helped predict the chemical bonds and energy state of the complex of gallic acid and bovine serum albumin. It featured the conformational structures typical of proteins in polypeptide chain sequences and active centers. To study the properties of gallic acid, we applied the AMBER method, while the Polak-Ribière method was used to simulate the potential energy of the system [24, 25]. Bovine serum albumin was represented by a hydrated 4F5S molecule from the Protein Data Bank with pre-calculated minimum-energy conformer, and a three-dimensional electrostatic potential map [26]. The PockDrug open web server was used to detect the binding sites [27]. To predict the interaction pattern, the AutoDock Vina calculated the charges in the molecule by the Gasteiger method [28, 29].

Bovine serum albumin molecule depends on the pH of the medium and exists in several isomeric forms of conformers E, N, F, and B. The E-form (unfolded) is the main isomer at low pH (< 4.0). As pH reaches 4.0–4.5, the F-form (fast) of bovine serum albumin appears as a result of the rapid N→F transition. The transition occurs as domain III unfolds. It has leveled α-helical structures. B-isomer appears at pH = 7.0–9.0 and subsequently causes A-isomer to appear, which is an aggregated form of bovine serum albumin [30]. The experiment featured neutral pH (7.4) because it is in this environment that bovine serum albumin is found in blood plasma in its stable B-form [26].

Formation of the complex of bovine serum albumin and gallic acid. Gallic acid belongs to food phenolic compounds with antioxidant effects and has low toxicity [31]. The amount of gallic acid to be added was determined according to the recommended intake of food and biologically active substances: 100 mg/L is adequate for human consumption, 300 mg/L being the upper limit [32].

The experiment included three concentrations of gallic acid (100, 200, and 300 mg/L), bovine serum albumin (0.69 g/L), neutral pH, and room temperature. Commercial bovine serum albumin (Diaem) was lyophilized and had a purity of ≥ 99.0%, while anhydrous gallic acid (Diaem) had a purity of ≥ 98.0%. Solutions included deionized water (Smart2Pure, Thermo Scientific, USA). Tris-HCl buffer (pH 7.4) was used as a solvent for bovine serum albumin. All solutions were kept in a refrigerator at 4°C for 12 h before the experiment.

Physicochemical methods for studying the complex of bovine serum albumin and gallic acid. The experiment involved sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), liquid chromatography with amperometric detection, and Fourier-transform infrared spectroscopy (FTIR).

SDS-PAGE. The test was based on Laemmli disk electrophoresis technique with denaturing agents [32]. Polymerization of the acrylamide gel featured a Bio-Rad vertical electrophoresis chamber (glass size = 10×10 cm, gel thickness = 1 mm). The concentration of the separating gel was 12.7%. The protein content was measured spectrophotometrically using the calibration curve of standard solutions of bovine serum albumin.

The experiment involved a bromophenol blue solution, a separating buffer, a dissociating mix based on β-mercaptoethanol, and a sample with a protein content of 10 μg. They were poured into tubes in equal measures. The resulting mix was stirred and incubated in water bath for 10 min. The electrophoresis occurred at 8–24 mA until the band of the leading dye reached the bottom of the gel. After the electrophoresis, the gel was placed in 30% trichloroacetic acid to fix proteins, followed by staining in Coomassie G-250 solution. The electrophoregrams were processed using the Gel Doc EZ gel (Image Lab, BioRad).

Amperometric method. The experiment involved a TsvetYauza 01-AA amperometric detector (Russia) with chromatogram registration. A solution of gallic acid was used as standard to evaluate the ability of the complex to enter into an oxidation (reduction) reaction at the electrode [34]. Gallic acid has stronger reducing properties than bovine serum albumin. As a result, gallic acid equivalent was used to measure the gallic acid interaction with the protein and to define the total antioxidant activity of the complex compared to gallic acid. Another experiment was conducted to

separate the complex from unreacted gallic acid. It involved a laboratory dialysis bag with a cut-off mass of 12–14 kDa.

FTIR spectroscopy. The transformation of chemical bonds was carried out in liquid samples using a Nicolet iS50 FTIR spectrometer (Thermo Scientific, USA) in an attenuated total internal reflector Smart iTR ATR (ZnSe) (Thermo Scientific, USA). Infrared radiation between two substances with different refractive indices creates a damped wave, which gradually penetrates the sample with each subsequent internal reflection. This technique analyzes objects in different states of aggregation and gives access to the structure and composition of the sample.

IR spectrometry is based on the ability of substances to absorb infrared radiation. Absorption occurs at the wavelengths with the energy that corresponds to the excitation energies of vibrational motions in molecules. Absorption bands indicate certain bonds in the compound under analysis. Absorption bands of amide groups I and II indicate the main conformations of a molecule, which marks the noncovalent addition reaction [35].

Statistical data analysis employed STATISTICA 10 software (StatSoft, Inc.).

RESULTS AND DISCUSSION

Molecular modeling. Molecular modeling determined interaction patterns between bovine serum albumin and gallic acid, as well as their binding sites. The energy of the gallic acid molecule was 1.984 kcal/mol; the energy gradient was 0.0997 kcal/mol. Figure 1 shows a three-dimensional electrostatic density map of bovine serum albumin that was constructed to determine the binding sites.

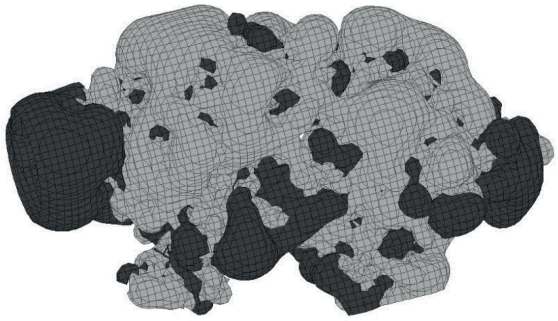

Figure 1 Electrostatic density map of bovine serum albumin: light gray – negatively charged area, dark gray – positively charged area

According to scientific publications, the molecule of gallic acid was to react along the negatively charged region, which was more favorable as far as energy was concerned. PockDrug data were used to confirm the hypothesis [27]. P23 appeared to be the suitable locus in bovine serum albumin. The ligand attached to a pocket of 530.57 angstroms. The hydrophobicity index was 2.28 on the Kyte-Dullittle scale, which means that the number of hydrophobic interactions was higher by the same number of times in this area, and the conformation was likely to stabilize [36]. The binding site was located deep inside the protein structure in a hydrophobic gap lined with the following amino acids: Lys 421, Glu 186, Asp 561, Gln 521, Asp 517, Arg 458, and Glu 424.

The binding energy was –5.6, –5.5, –5.4, –5.4, –5.3, –5.3, and –5.2 kcal/mol, respectively. The ligand was also located within this locus, thus stabilizing the conformation. The proposed interaction between gallic acid and bovine serum albumin was hydrophobic (Fig. 2). Such interactions increase the entropy of the system. As the temperature rises, their

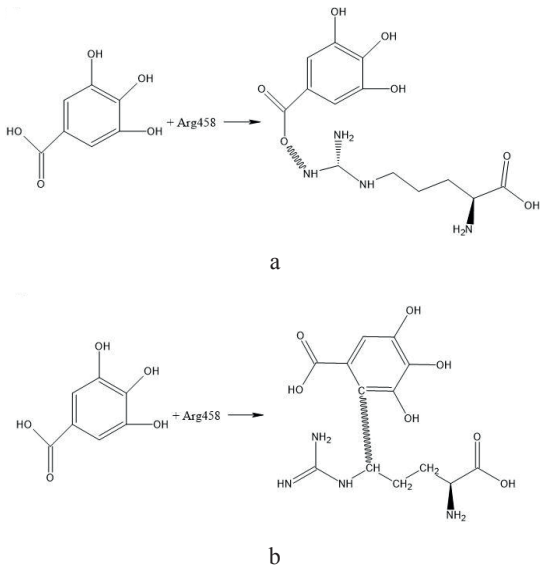

Figure 2 Modeled reaction between gallic acid and bovine serum albumin: (a) ionic reaction, (b) hydrophobic reaction

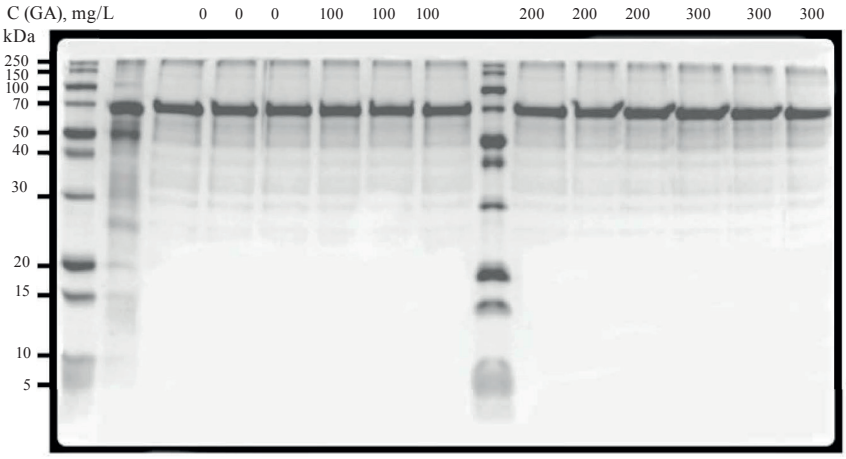

Figure 3 SDS-PAGE of the complex of bovine serum albumin and gallic acid in concentration of 100–300 mg/L (lanes 6–8, 10–12, and 13–15, respectively). Lanes 3–5 show bovine serum albumin standard (0.69 g/L)

number increases. In aromatic chains, such interactions reach their energy maximum already at 42°C, in aliphatic chains – at 60°C. Thus, temperature rise can lead to hydrophobic flocculation of the resulting complex and its subsequent precipitation. Therefore, the experiment was conducted at room temperature, and SDS-PAGE was used to monitor the state of the complex.

In protein, all carbon atoms not bound to polar atoms are hydrophobic, as are sulfur atoms in cysteine and methionine. This arrangement also opened up the possibility of other reactions because several ionic and polar residues were in the immediate vicinity of the binding site between gallic acid and bovine serum albumin.

The model confirmed the chemical bond between molecules of gallic acid and bovine serum albumin due to the polar regions of the protein with negatively charged regions and the hydrophobic regions of the protein. The bonds in the pocket can change the molecular bonds of the protein and reduce the reducing properties of gallic acid. The model showed changes in the secondary structure of the protein in the complex with gallic acid. Some gallic acid molecules react along the hydrophobic sites of bovine serum albumin, while others react along the polar regions. Therefore, gallic acid can be found in reaction solutions with a polar solvent, e.g. water.

Gallic acid is known to bind to bovine serum albumin in an amount of 45–65% of the ligand [37]. The bond formation of the polyphenol + bovine serum albumin complex depends on the size of the ligand: the larger the ligand, the more pronounced the binding to the protein, e.g. various catechins and bovine serum albumin [37]. Binding constant K increased in the series catechin-epigallocatechin-epigallocatechin-3-gallate up to 2.7×10^5, 2.9×10^5, and 3.2×10^5 mol/L, respectively. Based on these data, the reaction mix had an excess of gallic acid.

SDS-PAGE. Figure 3 shows the gel electrophoresis of samples denatured with sodium dodecyl sulfate. From left to right, the concentration of gallic acid in the complex increases. The first lane on the left illustrates the marker of protein molecular weight. The next lane shows the bovine serum albumin standard. The control solution of bovine serum albumin usually has a band of about 70 kDa and coincides with the band of the experimental solution without gallic acid. According to the gel electrophoresis, the gallic acid molecule did not affect the molecular weight of the protein and did not promote the formation of protein conglomerates.

The attachment of a gallic acid molecule to bovine serum albumin did not affect the charge and mass of the entire complex because the molecular mass of bovine serum albumin (68 kDa) significantly exceeded the mass of gallic acid (170 Da), and the charge was concentrated in the pocket of bovine serum albumin. An increase in the concentration of gallic acid resulted in no additional bands, which means no additional protein complexes with a different molecular weight and minor protein components. The samples with the complex showed no additional bands in the range of 68–5 kDa that usually designate the destruction of bovine serum albumin molecule. Thus, the SDS-PAGE analysis detected neither significant changes in the molecular weight nor violations of the integrity of the protein molecule.

Amperometric assessment method with gallic acid standard. According to the amperometric measurement of a standard bovine serum albumin solution by gallic acid equivalent, the indicator was 5 ± 1 μg/L of gallic acid, which corresponded to the low reducing properties of the protein in the amperometric detector (Fig. 4).

When the concentration of gallic acid was 100 mg/L, the bovine serum albumin + gallic acid values corresponded to 7.8 ± 1.3 mg/L in gallic acid equivalent. The result indicated a decrease in the reducing properties of gallic acid. When the concentration of gallic acid reached 200 and 300 mg/L, the reducing properties of the complex increased ($P < 0.05$) in terms

of the gallic acid equivalent and reached 15.9 ± 2.65 and 23.3 ± 5.05 mg/L gallic acid, respectively.

The recorded parameters in the experimental solution decreased in comparison with the level of gallic acid added to the reaction mix. This change indicated the reaction with bovine serum albumin. The chromatogram showed that the indicators of the complex changed proportionally as more gallic acid entered the reaction. Thus, the binding to bovine serum albumin increased as the concentration grew. The molecular modeling suggested a partial reaction between gallic acid and bovine serum albumin.

Gallic acid was removed from the reaction mix with a concentration of 300 mg/L using dialysis membranes with a cut-off mass of 12–14 kDa. The reducing properties of the concentrate of the complex were 63% of the initial level.

The most favorable thermodynamic conditions for the development of H-bonds were likely to form when a part of the unreacted gallic acid was in the solution. This result corresponded to the data obtained about the binding of bovine serum albumin to polyphenols, which was in t he range of 45–65% [37].

FTIR spectroscopy. Figure 5 shows the spectral data. The main parameters evaluated were the changes in the amide I region. During the formation of the complex, the concentration of gallic acid in the bovine serum albumin solution was gradually increased (from 100 to 300 mg/L).

FTIR spectroscopy made it possible to estimate the concentration of gallic acid that caused the greatest changes in the properties of the protein. The data can facilitate further practical studies. The molecular modeling and amperometric parameters showed that not all gallic acid bound to bovine serum albumin. Another experiment made it possible to determine the sample with the maximal change in the spectrum intensities.

The experiment evaluated the changes in the intensities and shifts of the amide I and amide II bands of the protein. Amide I had the following wavelengths:

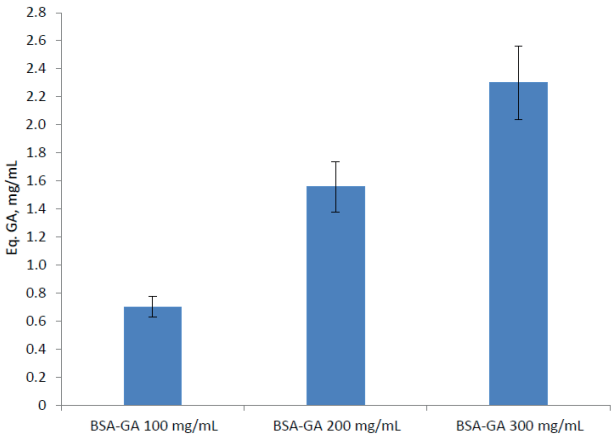

Figure 4 Changes in gallic acid equivalent during amperometric testing of the bovine serum albumin and gallic acid complex at 100, 200, and 300 mg/L of gallic acid

1652 (α-helix), 1618 (β-sheet), 1675 (the molecule unfolds), 1694 (β-antiparallel sheet), and 1633 cm^{-1} (random turns) [35]. These absorption bands are characteristic of all proteins, and they can be used to evaluate the changes in the secondary structure that occurs when gallic acid incorporates into the bovine serum albumin molecule. The ongoing reaction was expected to affect amide II in the region of 1546 cm^{-1} (stretching the C-N bond in combination with the N-H bond). It was also expected to change the spectral line at 1390 cm^{-1}, which is typical of bovine serum albumin and causes its protonation. Another characteristic band of gallic acid (1539 cm^{-1}), also showed an increase in the concentration of gallic acid in the solution.

Figure 5 shows changes in the absorption band at 1652–1653 cm^{-1}. They indicate a gradual decrease in the number of α-helices, which means that amino acids are losing the possibility of hydrogen bond formation when the concentration of gallic acid is high [37]. The number of hydrogen bonds might have decreased following the increase in the concentration of gallic acid involved. The characteristic band at 1539 cm^{-1} grew together with the concentration in the solution. The absorption band at 1652 cm^{-1} demonstrated a short-wave shift to the blue region by 1653.16 cm^{-1}, which also marked a certain change in peptide bonds in this region of the protein.

The initial IR spectra contained various broad peaks in the ranges in question, which complicated the analysis. In such cases, it is recommended to use the first- and second-order derivatives of the original spectra. This research involved the Savitzky-Golay method to perform differentiation in all three ranges [4].

To make the assessment more accurate, the second-order derivative of the original spectra was used in the regions of the bovine serum albumin peptide bond and the characteristic gallic acid band. The spectral line at 1546 cm^{-1} grew less intensive as the concentration of gallic acid decreased. Consequently, valence and deformation stretching of the C-N and N-H bonds occurred in the bovine serum albumin molecule, following the decrease in the number of peptide bonds. The spectral line at 1390 cm^{-1} grew more intensive as the concentration of gallic acid increased, which means that the number of protons in the system increased when gallic acid was added. These changes indicated that hydrogen bonds between bovine serum albumin and gallic acid could develop by attaching polyphenol hydroxyl residues to protein peptide bonds [38].

The spectral line at 1618 cm^{-1} showed changes in the β-sheet. These changes were opposite to the changes in the α-helix. In addition, the number of antiparallel structures increased at 1694 cm^{-1}. Bovine serum albumin unfolded at 1675 cm^{-1}, with a slight shift to the short-wavelength region. The unfolding might have been a consequence of the decreasing number of α-helices in the structure. It confirmed the changes in the region of 1652 cm^{-1}. The shape of random coils did not change when the gallic acid concentration changed (1633 cm^{-1}).

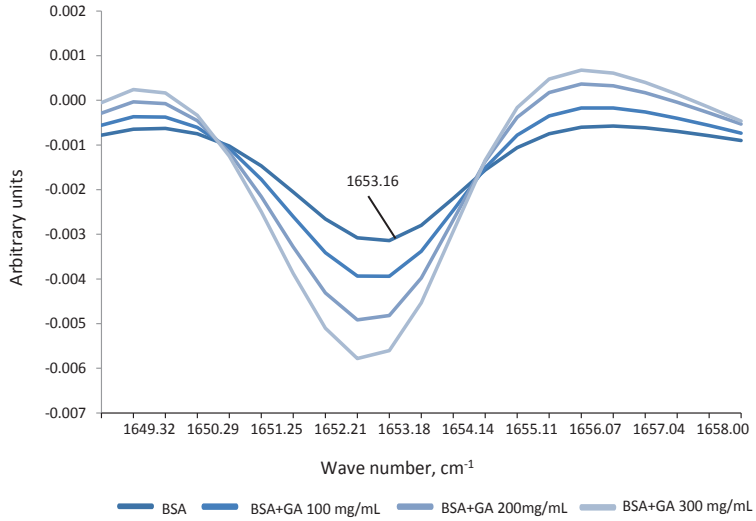

Figure 5 Derivatives of spectral lines. BSA – standard solution of bovine serum albumin. GA – gallic acid (in different concentrations)

The IR spectra of the bovine serum albumin molecule showed a slight unfolding. Protonation resulted from the decrease in the number of peptide bonds. The falling number of α-helices indicated the replacement of free hydrogen bonds. All these changes indirectly confirmed the formation of the bovine serum albumin + gallic acid complex. The sample with 300 mg/L of gallic acid had the strongest effect on bovine serum albumin. This ratio can be recommended to develop new food additives.

CONCLUSION

The present research featured molecular modeling of the reaction between bovine serum albumin and gallic acid. The experiment confirmed that chemical bonding developed between the molecules in the binding pocket of the protein. The bonding occurred mainly through hydrophobic interactions. It involved polar regions with negatively charged areas of the protein molecule. Presumably, some changes in chemical bonds affected the secondary structure of the protein. In a polar solvent, the bonds of gallic acid with the protein molecule might be partially unstable.

According to SDS-PAGE, electrophoresis of gallic acid did not violate the integrity of the protein molecule and did not produce minor proteins. When the concentration of gallic acid increased during the reaction with bovine serum albumin, it did not lead to the formation of protein complexes with a molecular weight higher than that of bovine serum albumin.

The chromatogram of the bovine serum albumin + gallic acid complex revealed amperometric changes that corresponded with the increase in the concentration of gallic acid introduced into the reaction. Therefore, free gallic acid in the solution provided the most favorable thermodynamic conditions for chemical binding. The obtained data confirmed the molecular modeling about the duality of gallic acid binding. When gallic acid was removed from the reaction, the reducing properties of the complex fell down to 63%, following the proportion of binding in the pocket with bovine serum albumin.

The FTIR data confirmed the molecular modeling. The number of peptide bonds in the bovine serum albumin molecule decreased due to valence and deformation stretching of the C-N and N-H bonds. As the amount of gallic acid grew, the number of protons in the system increased, as did the possibility of hydrogen bonding between bovine serum albumin and gallic acid. As a result, bovine serum albumin unfolded, and the secondary structure of the protein changed. The highest binding occurred when the concentration of gallic acid was 300 mg/L, which corresponded to the reaction mix with an excess of gallic acid. This concentration can be recommended for further use in the food industry as part of new food additives.

The present research was limited to a laboratory experiment and requires further study by thermodynamic calculations.

CONTRIBUTION

R.O. Budkevich designed and supervised the project. N.M. Fedortsov, E.V. Budkevich, I.A. Evdokimov, and S.A. Ryabtseva performed the experiments and discussed the results.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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