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Buckwheat Flour as a Matrix for Sorption of Plant Phenolics: Homology Modeling, Molecular Docking, and FTIR Study.

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ABSTRACT

This study aimed to determine the mode of interaction of bilberry leaves extract phenolics with the 13S buckwheat globulin by means of molecular docking study in the following three steps: 1) homology modeling of the protein, its refinement using molecular dynamics simulation and validation; 2) molecular docking of the bilberry leaves phenolic compounds with the developed model; 3) validation of the docking results by the FTIR spectroscopy analysis. We have shown that 13S globulin contains three main binding sites for phenolic compounds, including the one located within the 378-406 amino acid sequence - major buckwheat allergen. The highest binding affinity was predicted for CinchonainIb and Id (-10 kcal/mol). Docking results were confirmed by FTIR spectroscopy, which indicated the conversion of aperiodic structures and 310-helices to α-helices. These findings will substantiate further research in the field of development of new forms of biologically active substances.

Keywords: 13S buckwheat globulin, bilberry leaves extract, homology modeling, molecular docking, FTIR, plant phenolics.

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INTRODUCTION

Plant phenolic and polyphenolic compounds have a wide range of biological effects [1]. They have antioxidant, antiallergenic, anti-inflammatory, anti-microbial, anti-tumor, capillary-strengthening properties [2]. In this regard, phenolic compounds used as biologically active compounds particularly for the treatment of Type 2 Diabetes Mellitus [3].

One of the main problems significantly affecting the exerted biological activity of polyphenols is their low bioavailability [4]. The studies on the interaction of polyphenols and food matrices, such as proteins, carbohydrates, dietary fibers, and fat have been conducted to improve the bioavailability of phenolic compounds [5].

Recent studies have reported the potential of soybean (defatted flour, protein concentrate, protein isolate) and wheat flour to adsorb polyphenols from bilberry and cranberry juices. Sorption of polyphenols was performed by mechanical mixing of bilberry or cranberry juice in different concentrations with flour at room temperature, followed by centrifugation. It has been found that these flours effectively adsorb and stabilize bioactive polyphenols from the juices. In addition, this procedure resulted in the separation of sugars [6,7]. Similar studies on the sorption of polyphenols from bilberry and black currant juices on soy flour in an acidic medium (pH 3.7), followed by centrifugation, separation of the precipitate and freeze drying were presented in the study [8]. However, usage of these flours is limited by their significant role in the pathogenesis of common food allergies.

Buckwheat *(Fagopyrum esculentum)* flour can be used as an alternative matrix for people sensitive to wheat and soy proteins. Besides buckwheat is also recognized as food allergen and should be mandatorily labeled in local regulations, this type of food allergy has a low prevalence in the global population [9,10]. In a recent review, buckwheat was described as a functional food with several health benefits such as reduction of plasma cholesterol level, neuroprotection, anticancer, anti-inflammatory, antidiabetic and other effects [11].

Common buckwheat proteins are presented by 18-25% of albumins, 15-70% globulins, 0-5% prolamins and 4-23% glutelins[12]. The major buckwheat protein is 13S globulin – a legume-like protein that is expressed between 7 and 28 days after pollination only in immature seeds. It is a hexamer with subunits composed of an acidic (30 to 38 kDa) and a basic (23 to 25 kDa) chain derived from a single precursor and linked by a disulfide bond near 44-77 and 120-384 amino acid residues [13]. It has been assumed that these subunits might be thiamin binding sites [14]. The secondary structure of this protein is presented by 25% of α-helices, 30% of β-sheets and 45% of aperiodic structures [15]. To date, there is no information on the crystal structure of this protein.

Only one study examined the buckwheat flour as a matrix for adsorption of plant phenolics. Authors have demonstrated hypoglycemic action of a buckwheat flour complex with a bilberry (*Vaccinium myrtillus*) leaves phenolic extract on a fat male mice C57BL/6 diabetic model [16]. Bilberry leaves are a source of a wide range of phenolic compounds such as catechins, proanthocyanidins (and their condensation products), phenolic acids and flavonols[17].

The aim of this study is to determine the mode of interaction of bilberry leaves extract phenolics with the 13S buckwheat globulin.

MATERIALS AND METHODS

Materials

The amino acid sequence of the buckwheat 13S globulin (565 residues) for the homology modeling experiment was obtained from the UniProt database (UniProtKB: O23878).The list of ligands for molecular docking study composed of molecules from Table 1.

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Table 1: Compounds used for molecular docking study

CID	Common Name	IUPAC Name
List for preliminary docking study		
1130	Thiamine	2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethanol
412810	2'-Ethylthiamine	2-[3-[(4-amino-2-ethylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethanol
8682	Oxythiamine	5-[[5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium-3-yl]methyl]-2-methyl-1H-pyrimidin-6-one
4477692	6'-Methylthiamine	[2-[3-[(4-amino-2,6-dimethylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethoxy-hydroxyphosphoryl] hydrogen phosphate
12414318	DL-2-(1-Hydroxyethyl)thiamine	1-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium-2-yl]ethanol
150952	O-Benzoylthiamine	2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethyl benzoate
List of the phenolic compounds of the bilberry of leaves		
1203	Catechin	2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol
255538	Epicatechin	(3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol
689043	Caffeic acid	(E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid
1794425	cis-Chlorogenic acid	(1S,3R,4R,5R)-3-[(Z)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid
25210304	trans-Chlorogenic acid	3-[(Z)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid
5280459	Quercetin-3-rhamnoside	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one
5281643	Quercetin-3-galactoside	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one
72277	Epigallocatechin	(2R,3R)-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-trio
65084	Gallocatechin	(2R,3S)-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol
10456516	Cinchonain Ia	(2R,3R,10S)-2,10-bis(3,4-dihydroxyphenyl)-3,5-dihydroxy-3,4,9,10-tetrahydro-2H-pyrano[2,3-f]chromen-8-one
442675	Cinchonain Ib	(2R,3R,10R)-2,10-bis(3,4-dihydroxyphenyl)-3,5-dihydroxy-3,4,9,10-tetrahydro-2H-pyrano[2,3-f]chromen-8-one
21676383	Cinchonain Ic	(4R,8R,9R)-4,8-bis(3,4-dihydroxyphenyl)-5,9-dihydroxy-4,8,9,10-tetrahydro-3H-pyrano[2,3-h]chromen-2-one
21676382	Cinchonain Id	(4S,8R,9R)-4,8-bis(3,4-dihydroxyphenyl)-5,9-dihydroxy-4,8,9,10-tetrahydro-3H-pyrano[2,3-h]chromen-2-one
11765545	Cinchonain IIa	(2R,3R,4S,10R)-2,10-bis(3,4-dihydroxyphenyl)-4-[(2R,3R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-8-yl]-3,5-dihydroxy-3,4,9,10-tetrahydro-2H-pyrano[2,3-h]chromen-8-one

All structure files were downloaded from PubChem at National Center for Biotechnology Information (NCBI).

Bilberry leaf extract was obtained from Kharms LLC, Russia, St. Petersburg. Brown buckwheat flour was purchased from Khlebzerno product LLC, Russia, Taganrog. Their complex was obtained by mechanical

mixing of the components in an acidic medium (pH=3.6), followed by centrifugation and freeze-drying according to the method of [16].

The tertiary structure of the buckwheat 13S globulin was simulated using homology modeling with Phyre2 server (Protein Homology/analogy Recognition Engine V 2.0) in the intensive mode [18]. Modeled structure was then solvated in water box (tip3p model), minimized and equilibrated under NVT and NPT ensembles. Then, the 100 ns molecular dynamics (MD) simulations at 300 K, with a coupling of the model were carried out. MD simulations were performed using the GROMACS 5.1.3 package with the standard OPLS-AA force field [19].

The quality of the developed protein model was assessed using the standard protocol of the ProSA service [20]. *Molecular docking* was performed using the Autodock Tools environment and AutodockVina software [21]. The search space for all docking calculations included the entire surface of the protein. A high exhaustiveness, 50, was used in AutodockVina calculation because of the relatively wide search space. Preliminary molecular docking experiment was conducted in order to evaluate the validity of the developed protein model. In this experiment predicted affinities of thiamine (and its derivatives) complexes with buckwheat globulin were compared with experimental values of free energies for these complexes. Binding affinities of the best poses in the thiamin binding pocket was considered.

Experimental confirmation of the data obtained during the computational experiment was performed by Fourier Transform Infrared Spectroscopy (FTIR) on a Tenzor 27 spectrometer (Bruker Optic GmbH, Germany) coupled with attenuated total reflection (ATR) module. Scan parameters: specter for each sample was obtained from averaging of 64 scans in the range from 4000 to 800 cm^{-1}, followed by the ATR spectrum correction, water vapor and carbon dioxide compensation, the baseline correction, normalization, and calculation of the second derivative with 13-point smoothing arrays of Savitzky and Golay to aid in the visualization of overlapping absorptions. Samples were analyzed in 6 replicates. The proportion of individual protein structures was determined in accordance with the method [22].

Statistical methods*:*

All analyses were carried out using SPSS, version 20. The paired t-test was used to compare differences in spectral data. A P value <0.05 was considered significant.

RESULTS AND DISCUSSION

Protein structure modeling

Homology modeling of the buckwheat 13S globulin reviled that 11S globulins of amaranth (*Amaranthus hypochondriacus*), pumpkin (*Cucurbita maxima*), coconut (*Cocos nucifera*), almond (*Prunus dulcis*) and rapeseed (*Brassica napus*) have the highest identity to its amino acid sequence (Table 2).

Table 2: Proteins used as a template for modeling the tertiary structure of 13S buckwheat globulin

UniProtKB	PDB ID	% i.d.	Confidence, %	Alignment score	Description
Q38712	3QAC	50	100	363	A Chain, 11S amaranth *(Amaranthus hypochondriacus)* proglobulin
P13744	2E9Q	54	100	291	A Chain, 11S pumpkin (*Cucurbita maxima)* proglobulin
A0A222NNM9	5WPW	56	100	281	A Chain, 11S coconut *(Cocos nucifera)* globulin (cocosin)
Q43607	3EHK	55	100	270	C Chain, 11S almond *(Prunus dulcis)* globulin (amandine)
Q7XB53	3KGL	48	100	238	B Chain, 11S rapeseed *(Brassica napus)* globulin (procruciferin)

Note: % i.d. – percentage of identity

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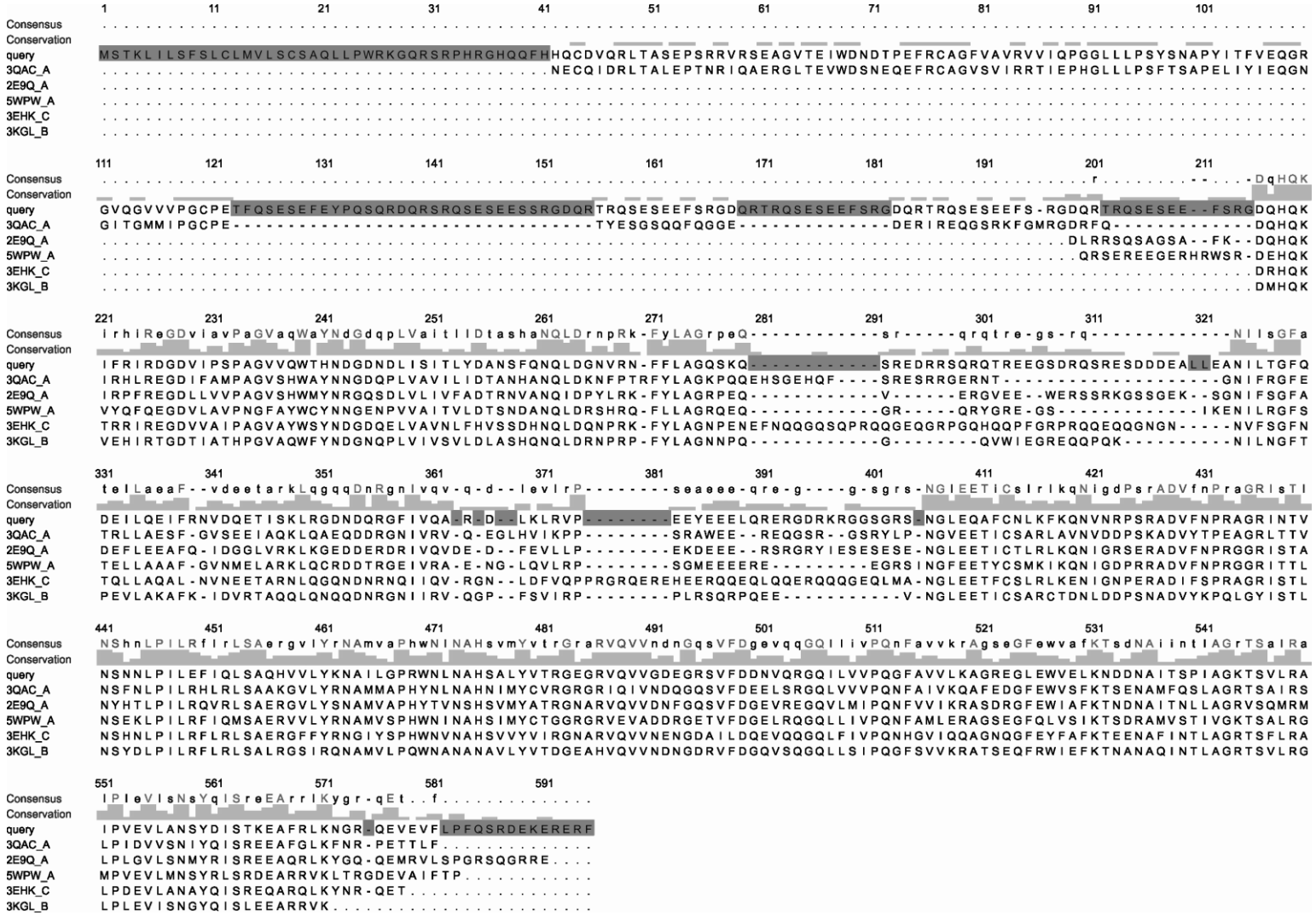

Fig 1: Structure-based sequence alignment of test globulins. 'query' – indicates 13S buckwheat globulin; the symbol '-' represents gaps and identical residues; lowercase and uppercase letters in the Consensus line indicate the low or high degree of consensus respectively; residues highlighted by dark gray are residues that have no appropriate templates.

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High levels of Confidence, % i.d. and Alignment scores indicate a true homology between 13S buckwheat globulin amino acid sequence and these templates. High structural homology indicates the relationship of these plant species. Therefore, it is not surprising that 11S amaranth proglobulin (pseudo-cereal of the same Caryophyllales Order) has the highest Alignment score to buckwheat globulin. High Confidence for other globulins is associated with highly conserved domains of two jelly-roll β-barrels and two extended α-helix domains of 11S globulins [23]. However, buckwheat 13S globulin has significant divergence from 11S globulins of templates (Figure 1).

As shown in Figure 1, most divergent sequences are located near the N-terminal domain. The longest sequence with no appropriate template includes 1-41 residues. In addition, there are three long sequences 123-155, 169-182, 202-215 and one sequence in the C-terminal domain from 582nd to 594th residues. These sequences were modeled automatically using low confident *ab initio* approach to folding simulation.

After 100 ns of MD simulation, the three-dimensional model built for the 13S globulin consisted of a typical cupin motif (Figure 2A). This result is in agreement with data obtained by [24] for 13S buckwheat globulin.

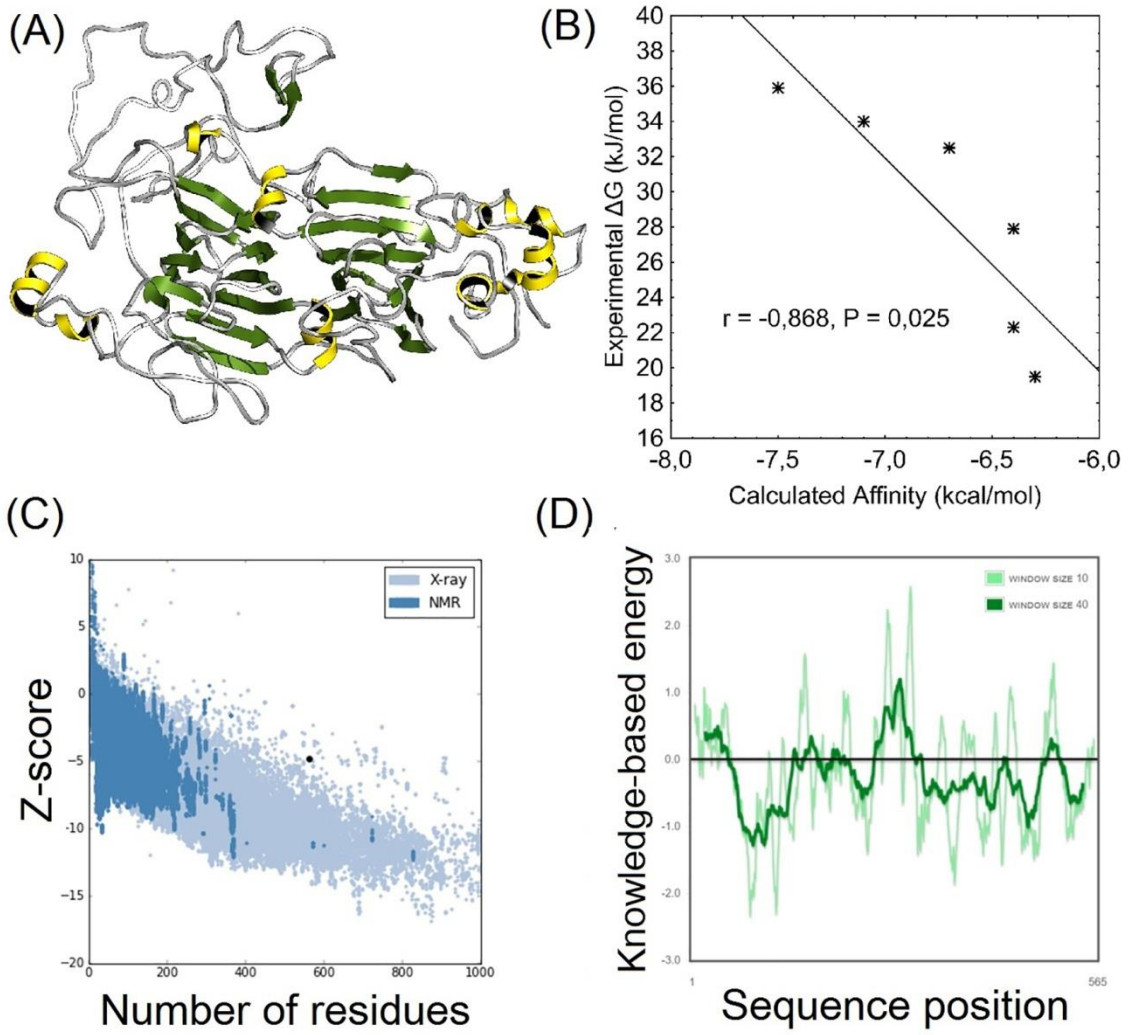

Fig 2: Results of the 13S globulin modelling (A – minimised and equilibrated structure of the protein, B – results of the correlation analysis, C – global quality plot (Z-score of the model is mentioned by black dot), D – local quality plot)

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The model is characterized (Figure 2C) by global quality within the range of scores typically found for native proteins of similar size (Z-score = -4.82). However, the plot of the local quality (Figure 2D) indicates the presence of positive energy values regions – the problematic parts of the model. These parts are corresponding to sequences with no appropriate template. In spite of this, the high correlation (Pearson r = -0.868, P=0.025) of predicted affinity with the experimental data ΔG is shown (Figure 2B).

Molecular docking study

The purpose of this part of the study was to explore the mode of interaction between bilberry leaves phenolics with 13S buckwheat globulin. Figure 3 presents a graphical summary of the best ligand poses from the docking experiment. Modes of the interaction of ligands may be classified depending on binding sites into three clusters: thiamine binding site, major allergen site (residues from 378 to 406), and external binding sites (clusters 1, 2 and 3 accordingly).

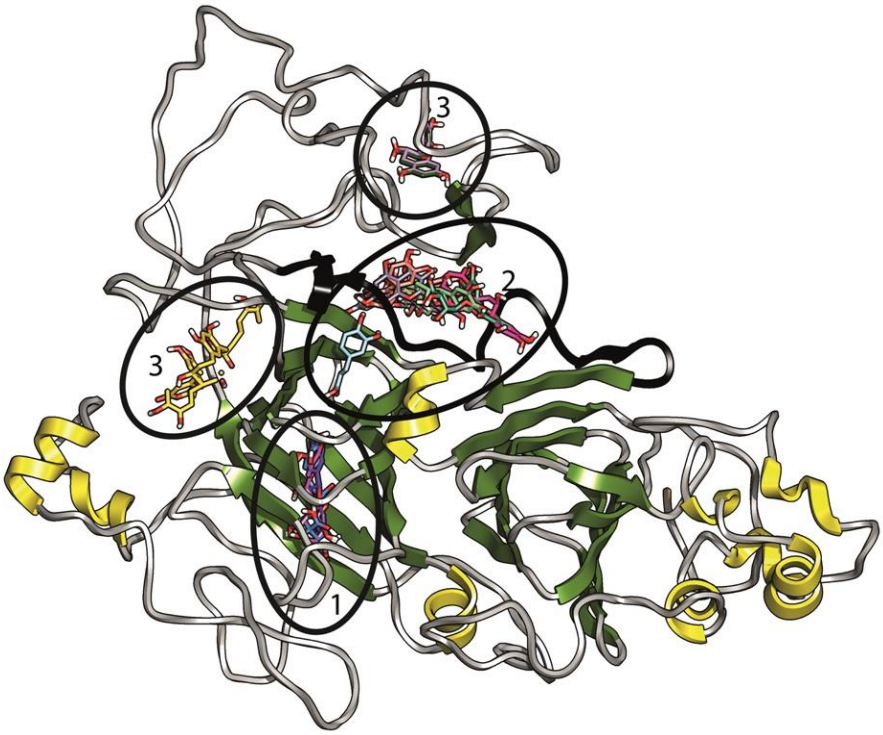

Fig 3: Graphical summary of the best ligand poses. Ovals are indicating positions of cluster by number; major allergen site is presented by black ribbon.

As can be seen from Table 3, cluster 1 includes cis- and-trans-chlorogenic acids, epicatechin, and epigallocatechin. Cis-chlorogenic acid showed the highest affinity to the binding site (-9 kcal/mol). Formation of hydrogen bonds with Tyr251 and Ser276 is common for all ligands in this cluster. Additionally, hydrophobic interaction with Val83 and Leu93 residues plays a significant role in binding affinity. That is why trans-chlorogenic acid (the only ligand without hydrophobic interactions) showed the lowest affinity (-8.6 kcal/mol) in this cluster.

Table 3: Predicted values of binding affinity and cluster distribution

Ligand	Predicted affinity, kcal/mol	Cluster No
Caffeic acid	-6.9	2
Catechin	-9.0	3
Cinchonain Ia	-9.8	2
Cinchonain Ib	-10.0	2

Cinchonain Ic	-9.9	2
Cinchonain Id	-10.0	2
Cinchonain IIa	-10.0	3
cis-Chlorogenic acid	-9.0	1
Epicatechin	-8,9	1
Epigallocatechin	-8,9	1
Gallocatechin	-8,8	3
Quercetin-3-galactoside	-9,5	2
Quercetin-3-rhamnoside	-8,9	2
trans-Chlorogenic acid	-8,6	1

The most interesting finding was that the greater part of bilberry leaves phenolics have high binding affinity to the major allergen of buckwheat (cluster 2). CinchonainIb and Id showed the highest affinity to the binding site (-10 kcal/mol). The key criteria, affecting the binding affinity is the possibility to form bonds (hydrogen or π-cat) with charged residues especially with Lys389, Arg397, Asp399. For instance, quercetin-3-rhamnoside (-8.9 kcal/mol) has lower binding affinity than quercetin-3-galactoside (-9.5 kcal/mol). The main difference between them is a binding mode to Lys389, Arg397. Quercetin-3-galactoside binds directly to the amino groups of the residues, whereas quercetin-3-rhamnoside binds to the carbonyl oxygen of the backbone chain. The same is true for the caffeic acid that has the lowest binding affinity (-6.9 kcal/mol), binding only to the Phe388, Lys389 thru the amino groups of backbone chain.

This finding is important considering the relevance of charged residues to the lg-E-binding region of 13S buckwheat globulin[24]. It is possible, therefore, that bilberry leaves extract may act as an antiallergenic agent that impairs the recognition of this reactive peptide by the antigen-presenting cells [25].

The 3-rd cluster includes catechin, cinhonainIIa, and gallocatechin. CinchonainIIa showed the highest affinity to the binding site (-10 kcal/mol). Affinity to external sites is non-specific in nature and characterized by the formation of hydrophobic interactions and hydrogen bonding with backbone chain atoms of the neutral amino acid residues.

FTIR spectroscopy study

The purpose of the third part of this research was to evaluate the changes in the protein secondary structure of the buckwheat flour after adsorption of phenolics from bilberry leaves extract.

Figure 4 presents the second derivative of FTIR absorbance spectra for native flour and its complex with bilberry leaves extract within the region 1500-1800 cm^{-1}. Peaks within this region are mostly associated with Amide I and II signals of protein.

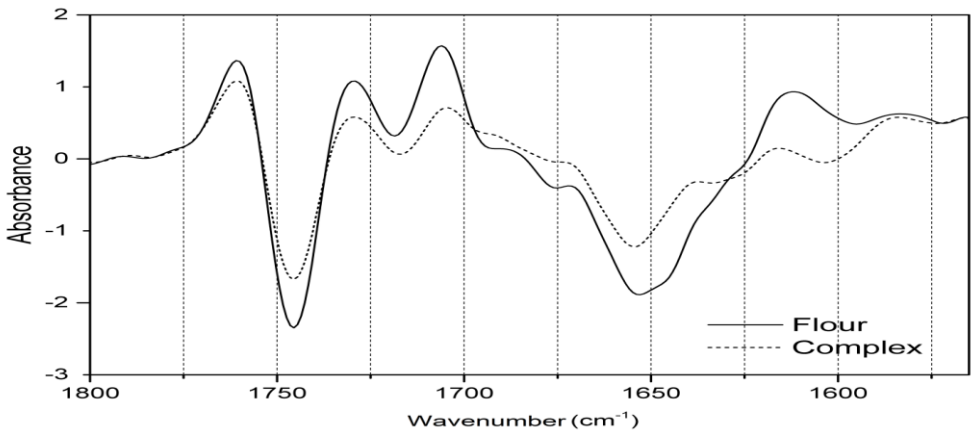

Fig 4: Representative second derivative FTIR spectra for the buckwheat flour (solid line) and its complex with phenolic compounds (dashed line).

The calculation of the relative peak areas (RPA) under the absorbance curves for flour that it is rich in α-helices (1651 cm^{-1} band, 34.11±4.78% RPA) and 3_{10}-helices (1696 cm^{-1} band, 27.69±5.39% RPA). The bands at 1679 cm^{-1} (15.08±0.29% RPA) and at 1629 cm^{-1} (15.35±0.67% RPA) indicate the occurrence of antiparallel β-sheets and β-Turns. Small band at 1641 cm^{-1} (7.77±0.82% RPA) reflects the presence of aperiodic structures.

The analysis of the FTIR spectra for a complex of flour with bilberry leaves extract revealed several significant changes in protein structure. The RPA of aperiodic structures decreased to 3.19±0.66% ($t=10.61$, $P<0.001$, $N=6$). Moreover the RPA of 3_{10}-helices decreased to 15.97±8.96% ($t=2.75$, $P=0.021$, $N=6$) At the same time the RPA for α-helices increased to 49.24±14.37% ($t=-2.45$, $P=0.034$, $N=6$). Any significant changes were not observed for other protein structures.

These findings illustrate that absorption of bilberry leaves extract phenolics leads to the formation of α-helices due to the conversion of aperiodic structures and 3_{10}-helices.

CONCLUSIONS

The main goal of the current study was to determine the mode of interaction of bilberry leaves extract phenolics with the 13S buckwheat globulin. For that purpose, we have developed and validated the three-dimensional model of this protein. By means of molecular docking study, we have shown 13S globulin contains 3 main binding sites for phenolic compounds. The most important binding site is located within the 378-406 amino acid sequence and represents the major buckwheat allergen. We have confirmed these results by FTIR spectroscopy experiment, which indicated the conversion of aperiodic structures and 3_{10}-helices to α-helices.

Taken together, these results suggest that buckwheat flour is a promising matrix for sorption of plant phenolics. Being limited to computational methods, this study lacks experimental data on binding affinities of selected ligands. Nevertheless, an implication of these findings will substantiate further research in the field of development of new forms of biologically active substances.

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