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HYDRATION OF BACTERIAL LECTIN IN NATIVE STATE AND AFTER IMMOBILIZATION ON SURFACE OF HYDROPHOBIC SILICA

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The aim of the work was to study the peculiarities of interaction of the surface of bacterial lectin of Bacillus subtilis IMB B-7724 in the native state and under different model conditions with water molecules by ^{1}H NMR; to create a composite system based on the studied lectin, in which the protein molecule is minimally affected by the surface of the carrier, because protein molecules are capable to bind a significant amount of water localized in the spaces between the polymer chains. A method of "dry" immobilization of bacterial lectin on the surface of hydrophobic silica has been developed.

Hydration of native lectin and lectin fixed on the surface of hydrophobic silica AM-1-175 was studied by low-temperature ^{1}H NMR spectroscopy. It has been shown that the immobilization of lectin on the surface of AM1 is accompanied by an increase in the interfacial energy γ_S from 4.1 to 5.2 J/g. This is due to an increase in the concentration of strongly bound water. Analysis of changes in the distributions of radii R of clusters of adsorbed water allows us to state that in water adsorbed by native lectin, there are two main maxima at R = 1 and 3 nm. In the immobilized state, the maximum at R = 1 nm is present in both types of water (of different order), but the second maximum is observed only for more ordered associates.

Chloroform medium slightly reduces the binding energy of water to native lectin molecules (from 4.3 to 4.1 J/g), but in the case of immobilized lectin in $CDCl_3$ medium, the value of $\Sigma\gamma_S$ increases from 5.2 to 7.4 J/g. That is, the weakly polar medium promotes to increase in the interaction of water with interfaces, which is manifested in a relative increase in the number of water clusters of smaller size (Fig. 4). It should be noted that weakly associated forms of water (signal 3) are also represented by several types of clusters that have a radius in the range R = 1–10 nm, and their size distribution changes significantly during immobilization of lectin on the surface of AM1. Probably, weakly associated types of water are formed both in cavities, between polymer chains of protein molecules, and on the surface of AM1, free of protein.

***Keywords**: bacterial lectin of Bacillus subtilis IMB B-7724, ^{1}H NMR spectroscopy, surface hydration, hydrophobic silica AM1-175, composite system, water clusters*

Lectins are proteins or glycoproteins that are capable to bind carbohydrates specifically on the surface of some cell types [1–3]. Due to this, in some cases there is agglutination of cells. In addition, lectins may have mitogenic activity (promote the process of division) with respect to blood cells [4]. Initially, lectins were isolated from plant seeds (beans, *Canavalia*), which made it possible to thoroughly study their properties. In particular, it has been found that concanavalin can bind specifically to certain groups of sugars, which allows it to interact with the receptor system of many cell types. Depending on the properties and distribution in the tissues, lectins can play an important physiological role. The characteristic property of lectins to clearly recognize other molecules makes it promising to research them, including purification, structural analysis and biotechnological applications in various fields, such as molecular and cell biology.

In expanding the scope of application of lectins, a significant step was the development of methods for obtaining of microbial lectins. It turned out that this type of lectins can act as substances with high anti-cancer activity [5]. In [6–8], methods have been described for obtaining of lectin using aerobic culture *Bacillus subtilis*, which is toxic to cancer cells.In this way, series of nutrient media have been used, such as Gauze's medium, Hottinger Broth with a number of corrective additives, such as lyophilized autolysate of liquid brewer's yeast, and various mineral salts.

Currently, the direction of creation of a new generation of anti-cancer drugs based on complex composite systems is being actively developed; their basis is magnetite nanoparticles, which can be directed to the tumor development zone by means of an external magnetic field. A multilayer coating is created on the outer surface of magnetite, which provides controlled delivery to the affected area of active substances such as anti-cancer antibiotics, monoclonal antibodies, lectins, cisplatin and others [9–12]. In particular, the processes of adsorption immobilization of cytotoxic bacterial lectin of *Bacillus subtilis* IMB B-7724 on the surface of magnetite and carbon-containing nanocomposite $Fe_3O_4/Al_2O_3/C$ at room temperature were studied in [12]. It has been found that the adsorption capacity of lectin on the surface of magnetite is 25.3 mg/g, and $Fe_3O_4/Al_2O_3/C$ NC – 36.3 mg/g (at initial lectin concentrations of 0.06–0.4 mg/mL). The extraction extent of lectin R (%) was 12–38 % for magnetite and 46–67 % for $Fe_3O_4/Al_2O_3/C$ NC. The dependence of the adsorption capacity on the exposure time in the lectin solution was studied. Nanobiocomposite based on MF and bacterial lectin was found to have a synergistic cytotoxic effect on MCF-7 cells, causing up to 40% cell death. The IC50 values for the nanobiocomposite and lectin for MCF-7 cells were 100 and 126 μg/mL, respectively. A promising way to use lectins as cytotoxic agents can be other composite systems, in which in the outer shell of magnetically carried nanoparticles they use an inert adsorption layer (*e.g.*, silica), capable of non-rigid fixation of lectin molecules, which weakly affects the conformational order of the protein molecule, and therefore retains its capability to selectively interact with cancer cell receptors.

It is known that most of protein molecules during their adsorption from aqueous solutions are irreversibly sorbed on the surface of silica adsorbents; this is due to the multicenter adsorption of polymer chains, that significantly change their conformation during adsorption in accordance with the minimization of free energy of the biopolymer-surface system [13–15]. And this applies to both hydrophilic and hydrophobized silica. To reduce the effect of the surface on the binding of lectin protein molecules, hydrophobic silica AM-1-175 was chosen, and lectin was fixed on the surface by "dry" immobilization, during which anhydrous mixtures of lectin and methyl silica powders were mechanochemically mixed.

The purpose of the work is to study features of the interaction of the surface of bacterial lectin of *Bacillus subtilis* IMB B-7724 in the native state and under different model conditions with water molecules by ^{1}H NMR; to create a composite system based on the lectin studied, in which the protein molecule is minimally affected by the surface of the carrier, because the protein molecules are capable to bind a significant amount of water localized in the spaces between the polymer chains.

EXPERIMENTAL PART

Materials. As methyl silica, hydrophobic silica AM-1 was used, with a specific surface area S_{BET} = 175 m^2/g, manufactured by the Kalush Research and Experimental Plant of Chuiko Institute of Surface Chemistry of National Academy of Sciences of Ukraine, obtained by chemical modification of the surface of original nanosilica A-200 with dimethyldichlorosilane. As a result of reactions, pairs of dimethylsilyl groups are formed, crosslinked with siloxane bridges $–Si(CH_3)_2–O–Si(CH_3)_2–$. The BET specific surface area of the investigated sample AM-1 according to nitrogen adsorption was S_{BET} = 175 m^2/g, and the total pore volume V_p = 0.8 cm^3/g.

To obtain hydrophobic nanosilicas, a method was used for chemical modification of the surface of hydrophilic silica by replacing surface hydroxyl groups with methyl-, di- and trimethylsilyl and other hydrophobic groups. AM-1-175 is an industrial grade of methyl silica (TU U 24.6-05540209-006-2006). The reaction of electrophilic substitution of protons of silanol groups is carried out according to the scheme:

$$\equiv SiO\text{-}H + ClSi(CH_3)_3 \rightarrow \equiv SiOSi(CH_3)_3 + HCl.$$

The adsorbents obtained in this way are chemically inert, non-toxic and are used in various fields of industry as thickeners for polymers, thixotropic agents and hydrophobic powders which are practically not wettable with water, having a high affinity to non-polar organic liquids.

Bacterial lectin of *Bacillus subtilis* IMB B-7724 was synthesized, its physicochemical and cytotoxic properties were studied at R.E. Kavetsky Institute of Experimental

Pathology, Oncology and Radiobiology of National Academy of Sciences of Ukraine [16]. It has been found that lectin is a glycoprotein (protein – 86.0 %, carbohydrates – 7.0 %) with a molecular weight of 18–20 kDa. Elemental composition: carbon – 34.00 %, hydrogen – 7.04 %, nitrogen – 16.61 %, oxygen – 42.35 %. The amino acid composition is dominated by leucine and aromatic amino acids (tyrosine and phenylalanine). The highest affinity of lectin of *Bacillus subtillis* IMB B-7724 was shown to N-acetylneuraminic and N-glycolylneuraminic acids (minimum inhibitory concentrations of carbohydrates – 0.3 mM). Lectin has high thermal stability, resistance to changes in pH, stable during storage for a long time [16].

The cytotoxic properties of lectin of *Bacillus subtilis* IMB B-7724 in the composition of magnetically sensitive nanocomposites and magnetic fluids were studied in [12].

^{1}H NMR spectroscopy. NMR spectra were measured using a high-resolution NMR spectrometer (Varian "Mercury") with the operating frequency of 400 MHz. Eight 60° probe impulses with a duration of 1 μs and a bandwidth of 20 kHz were used. The temperature in the sensor was regulated with an accuracy of ± 1 deg. Signal intensities were determined by measuring the area of the peaks using the procedure of decomposition of the signal into its components under the assumption of the Gaussian form of signal and optimization of the zero line and phase with an accuracy of ± 10 %. To prevent supercooling of water in the objects studied, the measurement of unfrozen water concentration was performed by heating samples pre-cooled to the temperature of 210 K. Temperature dependences of NMR signal intensity were measured in an automated cycle, when the sample was kept at constant temperature for 9 min, and measuring time was 1 min. NMR measurements were performed in air.

Since the concentration of water in the samples is known, from the intensities of the signal of water (I), values of the concentration of unfrozen water (C_{uw}) can be calculated at any temperature: $C_{uw} = I_T/I_{T>273}\ h$ (mg/g). The process of freezing (melting) of interfacial water, localized in a solid porous matrix, takes place in accordance with the changes in the free Gibbs energy due to the influence of a surface [17]. These changes are the smaller, the farther an investigated layer of water is from a surface. At $T = 273$ K, the water freezes, the properties of which do not differ from those of the bulk, and in decreasing of temperature (without taking into consideration the effect of supercooling) the layers of water freeze, closer to a surface, and for interfacial water a ratio is true:

$$\Delta G_{\text{ice}} = -0.036(273.15 - T), \qquad (1)$$

where the numerical coefficient is a parameter associated with the temperature coefficient of change of free Gibbs energy for ice. Then, according to the technique described in detail in [18–21], the amounts of strongly and weakly bound water (SBW and WBW, respectively) can be calculated, as well as the thermodynamic characteristics of these layers.

If in a colloidal system the total water content significantly exceeds the total volume of pores (interparticle gaps), then part of water may be in a free state when the water molecules do not feel the disturbing effect from the surface of the particles. It is difficult to determine accurately the amount of "bulk" water, so we will consider as bulk the part of water that corresponds to the ratio: $h > 1.5$ g/g.

Interfacial energy of solids or biopolymers was defined as the modulus of the total reduction of free energy of absorbed water due to the presence of the inner boundary of water-polymer phases by the formula:

$$\gamma_S = -K \int_0^{C_{\text{uw}}^{\max}} \Delta G(C_{\text{uw}})\, dC_{\text{uw}}\,, \qquad (2)$$

where $C_{\text{uw}}^{\max} = 1.5$ g/g.

To determine the geometric dimensions of the nanoscale fluid aggregates limited by a solid surface, the Gibbs-Thomson equation can be used [22, 23], which connects the radius of spherical or cylindrical pores (R) with the value of the freezing temperature depression:

$$\Delta T_m = T_m(R) - T_{m,\infty} = \frac{2\sigma_{sl} T_{m,\infty}}{\Delta H_f \rho R} \qquad (3)$$

where $T_{\text{m}}(R)$ is the melting temperature of ice localized in pores with radius R, $T_{\text{m},\infty}$ is the melting temperature of bulk ice, ρ is the density of solid phase, σ_{sl} is the energy of interaction of a solid with a liquid, and ΔH_{f} is the volumetric enthalpy of melting.

The value (ΔG), as well as the interfacial energy γ_S, were calculated according to the

equations (1, 2). As strongly bound water was considered the part of interfacial water for which the decrease in Gibbs free energy $\Delta G < 0.5$ kJ/mol.

RESULTS AND DISCUSSION

Fig. 1 shows ^{1}H NMR spectra of hydrated lectin, measured at different temperatures, as well as lectin immobilized in the dry state on the surface of methyl silica by mechanochemical activation. Both samples contained an equal amount of adsorbed water ($h = 0.1$ g/g). The mass ratio of methyl silica (AM-1-175) and lectin was 4/1.

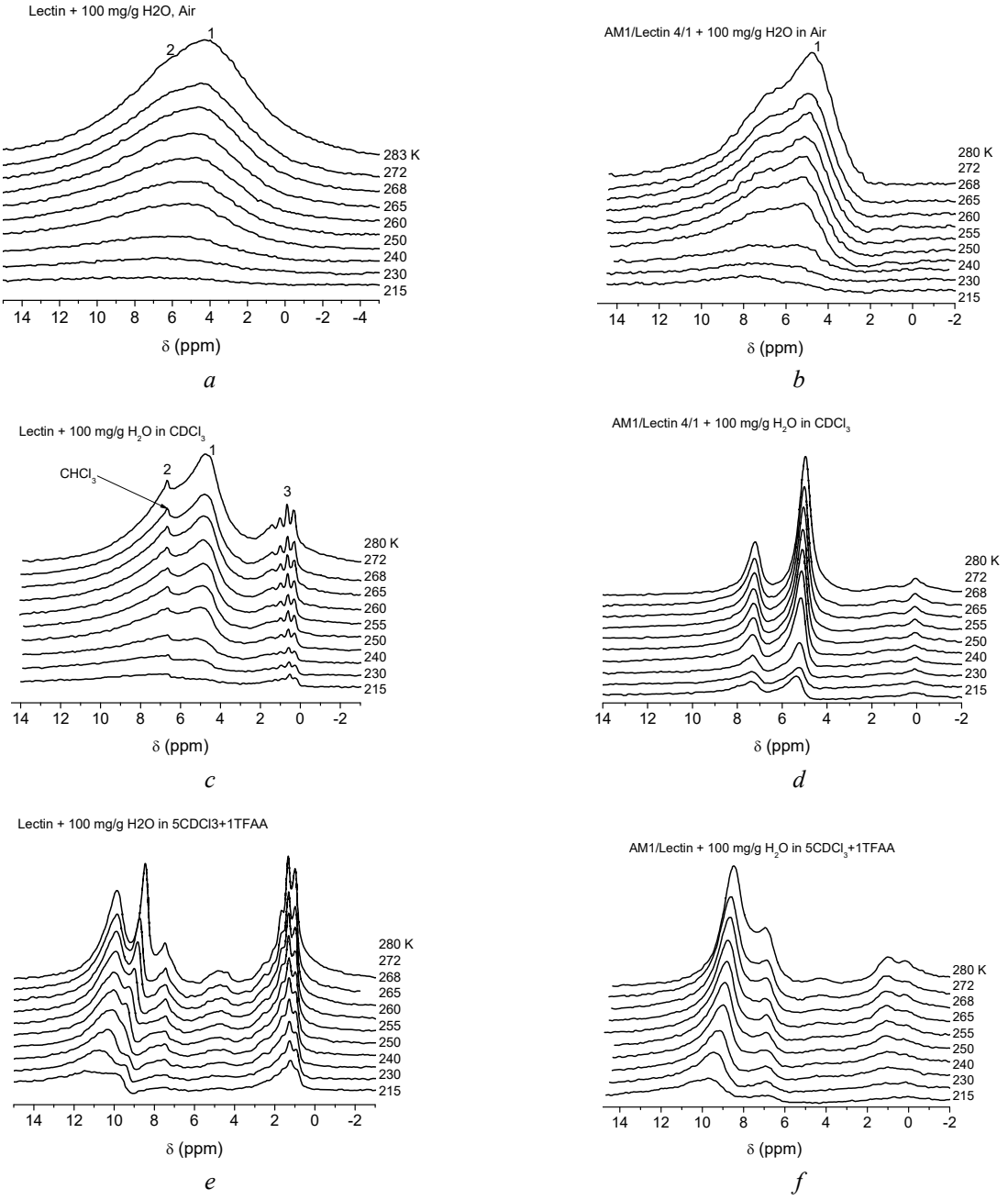


Fig. 1. ^{1}H NMR spectra, measured at different temperatures, of water adsorbed on the surface: native lectin (*a*, *c*, *e*); AM-1 (*b*, *d*, *f*) in air (*a*, *b*), in $CDCl_3$ medium (*c*, *d*) and in mixed medium $5CDCl_3$ + 1 TFAA (*e*, *f*)

In the spectrum of hydrated lectin, two signals of water are observed, the intensity of which decreases with lowering of temperature due to the freezing of certain amounts of water. The chemical shifts of the signals are $\delta_H = 4.5$ and 7 ppm for main and side signals, respectively. The presence of two signals (1 and 2) indicates the existence of at least two types of adsorbed water, which are characterized by different ordering of the hydrogen bond network. Since with increasing order of water molecules, its chemical shift increases (the signal shifts in the direction of weak magnetic fields), signal 2 should be attributed to water clusters, with an ordered structure similar to the structure of hexagonal ice. At the same time, signal 1 has a chemical shift close to the chemical shift of liquid water, in which each water molecule is involved in the formation of 2–3 hydrogen bonds [18–21].

During the adsorption of lectin on the surface of AM1, the type of spectra is close to the spectra of native lectin. Signals 1 and 2 are also observed in the spectra. And the ratio of their intensities is close to that obtained in pure lectin. This indicates not a very strong influence of the surface on the conformation of protein molecules and on the conformations of polymer chains. Thus, we can consider that our method of immobilization of protein molecules on the surface ensures the preservation of conformational stability of lectin molecules.

The environment of the non-polar organic solvent (chloroform) can significantly change the structure of the clusters of adsorbed water. Thus, if the clusters are adjacent to the hydrophobic surface of methyl silica or hydrophobic regions of the polymer chains of a protein molecule, it may be more energetically advantageous to replace water with chloroform. On the other hand, as shown in [18–21], on the border with solids of different nature, the layers of water may be formed as non-ordered by hydrogen bonds, similar to a concentrated solution of water in chloroform, which is stabilized by surface interactions.

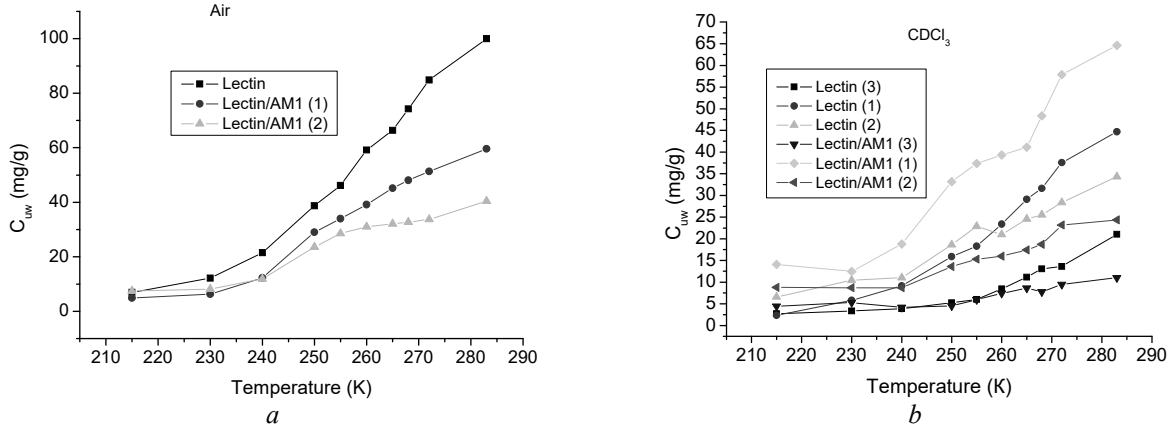
As follows from the data of Figs. 1 *c*, *d*, in air and organic media, in the spectra the signals appear in the range $\delta_H = 0$–2 ppm, corresponding to the weakly associated forms of interfacial water (signal 3). The ratio between intensities of signal 1 and 2 varies slightly. In the case of lectin immobilized on the surface of AM-1, the intensity of signal 1, caused by the highly ordered form of water, becomes slightly higher.

As shown in [23–27], an addition of a strong acid to the system can lead to the differentiation of water clusters according to their capability to dissolve the acid. Unlike bulk water, water in a clustered state dissolves polar substances, including acids, much worse. This is due to the significant rearrangement of water cluster when entering into that the molecules changing the structure of the network of hydrogen bonds. Indeed, when deuterated trifluoroacetic acid (TFAA) is added, several signals appear in the spectra, ranging from 12 to 0 ppm (Fig. 1 *e*, *f*). In this case, signals with large values of chemical shift correspond to clusters (or domains) with a high concentration of acid. The signal with $\delta_H = 5$ ppm and less corresponds to water that does not dissolve the acid. It should be noted that in the presence of strong acid, the intensity of the signals of disordered water increased significantly. For native protein, these signals become basic, even greater than the signals of water-acid solutions.

Fig. 2–4 show the temperature dependences of unfrozen water concentration, calculated on the basis of change in water signal intensity, as well as dependences of Gibbs free energy change on unfrozen water concentration, and distributions on the radii of adsorbed water clusters, calculated in accordance with equation (1–3) for native lectin and lectin, immobilized on the surface of hydrophobic silica AM1 at the weight ratio of 4/1 and hydration $h = 0.1$. Table 1 shows the characteristics of the layers of water, adsorbed in samples, corresponding to the different signals recorded in spectra. In this case, signal 3 corresponds to weakly associated water that does not participate in the formation of hydrogen-bound complexes with other molecules, and signals 1 and 2 – less and more ordered clusters of adsorbed water.

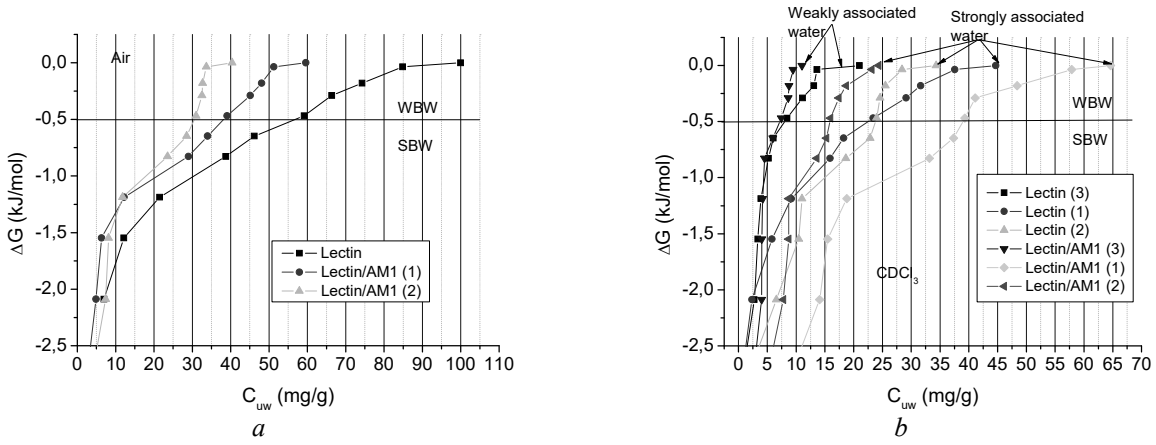
The amount of strongly and weakly bound water (C_{uw}^S and C_{uw}^W, respectively) was calculated on the basis of $\Delta G(C_{uw})$ dependences, and it was considered that if for water the change in Gibbs free energy caused by adsorption interactions $\Delta G < -0.5$ kJ/mol, the water is strongly bound [18–21]; the value of interfacial water-surface (protein or composite) energy – γ_S, that characterizes the total decrease in free energy of water due to the presence of an interface; the maximum decrease in Gibbs free

energy in the layer of strongly bound water $-\Delta G^S$, that characterizes the maximum influence of a surface on adjacent water. For each group of signals belonging to one system, Table 1 shows the total values of interfacial energy $\Sigma\gamma_S$, relating to all water, regardless of its type.



a *b*

Fig. 2. Temperature dependences of the concentration of unfrozen water of native lectin and lectin immobilized on the surface of AM1, containing 100 mg/g of adsorbed water in air (*a*) and in $CDCl_3$ (*b*) media



a *b*

Fig. 3. Dependences of the change of Gibbs free energy on the concentration of unfrozen water of native lectin and lectin immobilized on the surface of AM1, containing 100 mg/g of adsorbed water in air (*a*) and in $CDCl_3$ (*b*) media

Table. Characteristics of interfacial water in native hydrated lectin and lectin immobilized on the surface of AM1

Sample	Medium	Signal	C_{uw}^S, mg/g	C_{uw}^W, mg/g	ΔG^S, kJ/mol	γ_S, J/g
Native lectin	air		55	45	–3.5	4.3
	$CDCl_3$	3	8	14	–3.5	0.8
		1	23	22	–3	1.7
		2	23	12	–3	2
						Σ4.1
Lectin/AM1	air	1	38	22	–3.5	2.8
		2	31	9	–3.5	2.5
						Σ5.2
	$CDCl_3$	3	7	5	–5	0.9
		1	38	22	–5	4.4
		2	17	8	–5	2.1
						Σ7.4

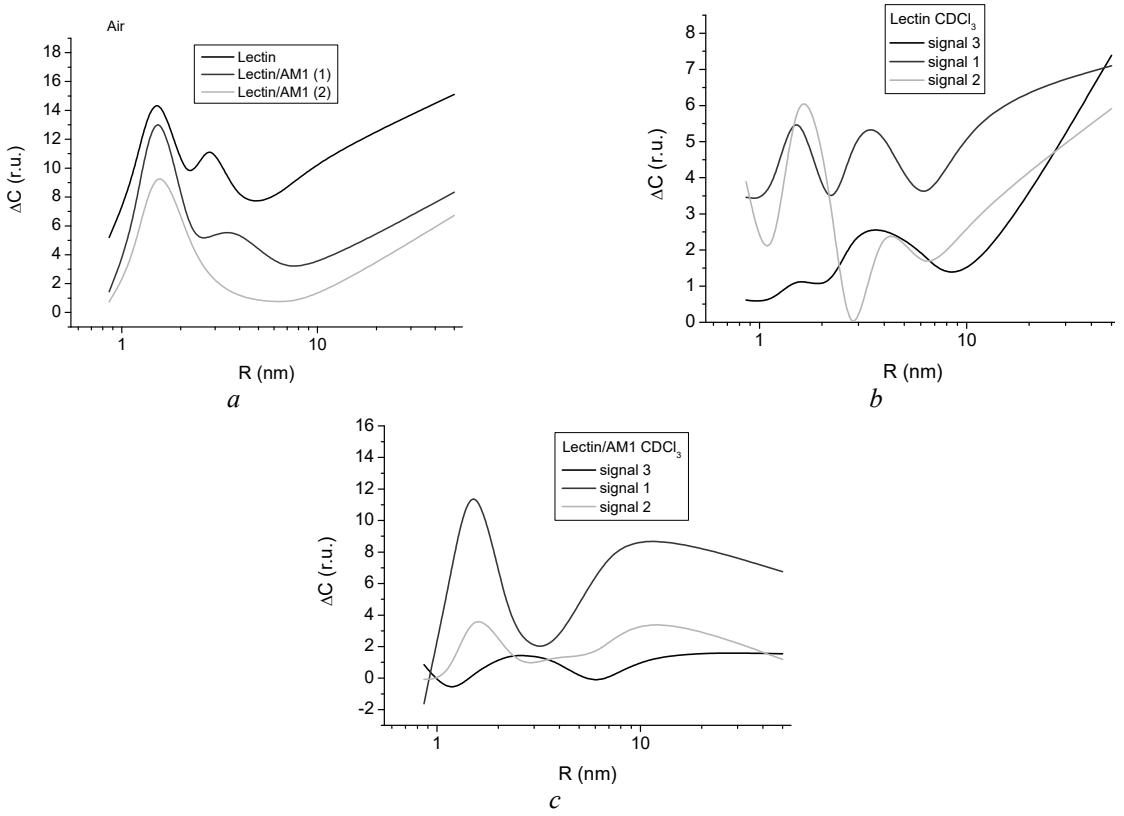


Fig. 4. Distributions by radii of the clusters of the adsorbed water of native lectin and lectin immobilized on the surface of AM1, containing 100 mg/g of adsorbed water in air (*a*) and in $CDCl_3$ (*b*, *c*) media

As can be seen from the above results, the immobilization of lectin on the surface of AM1 is accompanied by an increase in γ_S from 4.1 to 5.2 J/g. This is due to the increase in the concentration of strongly bound water (Table). If we analyze the changes in distributions of the radii of clusters of adsorbed water (Fig. 4 *a*), we can state that in the water adsorbed by native lectin molecules, there are two main maxima at $R = 1$ and 3 nm. In the immobilized state, the maximum at $R = 1$ nm is present in both types of water (of different order), but the second maximum is observed only for more ordered associates (signal 1).

Chloroform medium slightly reduces the binding energy of water to native lectin molecules (from 4.3 to 4.1 J/g), but in the case of immobilized lectin in $CDCl_3$ medium, the value of $\Sigma\gamma_S$ increases from 5.2 to 7.4 J/g. That is, the weakly polar medium promotes to an increase in the interaction of water with the interfaces, which is manifested in a relative increase in the number of water clusters of smaller size (Fig. 4). It should be noted that weakly associated forms of water (signal 3) are also represented by several types of clusters that have a radius in the range $R = 1$–10 nm, and their size distribution changes significantly during immobilization of lectin on the surface of AM1 (comparison of Fig. 4 *b* and *c*). Probably, weakly associated types of water are formed both in cavities, between polymer chains of protein molecules, and on the surface of AM1, free of protein.

CONCLUSIONS

The peculiarities of the interaction of the surface of bacterial lectin of *Bacillus subtilis* IMB B-7724 in the native state and under different model conditions with water molecules were studied by ^{1}H NMR methods. It has been found that water adsorbed by lectin molecules is in the form of two types of clusters, one of which is characterized by a network of hydrogen bonds similar to liquid water, and the other – by more ordered, close to the structure of hexagonal ice. The size of the adsorbed water clusters is 1–10 nm. The size distributions of water clusters depend on the conditions of lectin

immobilization on AM1 surface and the medium where the measurements are performed.

A method of "dry" immobilization of bacterial lectin of *Bacillus subtilis* IMB B-7724 on the surface of methylated silica has been developed, with a weak effect of surface on protein molecules.

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Гідратація бактеріального лектину в нативному та іммобілізованому на поверхні гідрофобного кремнезему станах

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Метою роботи було вивчення методами ^{1}H ЯМР особливостей взаємодії поверхні бактеріального лектину Bacillus subtilis IMB B-7724 в нативному стані та в різних модельних умовах з молекулами води; створення композитної системи на основі дослідженого лектину, в якій білкова молекула зазнає мінімального впливу з боку поверхні носія, оскільки білкові молекули здатні зв'язувати значну кількість води, локалізованої в проміжках між полімерними ланцюгами. Розроблено спосіб «сухої» іммобілізації бактеріального лектину на поверхні гідрофобного кремнезему.

Методом низькотемпературної ^{1}H ЯМР-спектроскопії вивчено гідратацію нативного лектину та лектину, закріпленого на поверхні гідрофобного кремнезему марки АМ-1-175. Показано, що іммобілізація лектину на поверхні АМ1 супроводжується збільшенням міжфазної енергії γ_S від 4.1 до 5.2 Дж/г. Це відбувається за рахунок збільшення концентрації сильнозв'язаної води. Аналіз змін в розподілах за радіусами R кластерів адсорбованої води дозволяє констатувати, що в воді, адсорбованій нативним лектином, присутні два основних максимуми при R = 1 та 3 нм. В іммобілізованому стані максимум при R = 1 нм присутній в обох типах води (різної впорядкованості), проте другий максимум спостерігається лише для більш впорядкованих асоціатів.

Середовище хлороформу дещо зменшує енергію зв'язування води з молекулами нативного лектину (від 4.3 до 4.1 Дж/г), проте у випадку іммобілізованого лектину в середовищі $CDCl_3$ величина $\Sigma\gamma_S$ збільшується від 5.2 до 7.4 Дж/г. Отже, слабкополярне середовище сприяє підвищенню взаємодії води з межами поділу фаз, що проявляється в відносному збільшенні кількості кластерів води меншого розміру (рис. 4). Слід звернути увагу, що слабоасоційовані форми води (сигнал 3) також представлені кількома типами кластерів, які мають радіус в діапазоні R = 1–10 нм, причому їхній розподіл за розмірами значно змінюється при іммобілізації лектину на поверхні АМ1. Ймовірно, слабоасоційовані форми води утворюються як в порожнинах, між полімерними ланцюгами білкових молекул, так і на поверхні АМ1, вільній від білка.

***Ключові слова**: бактеріальний лектин Bacillus subtilis IMB B-7724, ^{1}H ЯМР-спектроскопія, гідратація поверхні, гідрофобний кремнезем АМ1-175, композитна система, кластери води*

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