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FEATURES OF THE HEMICELLULOSE STRUCTURE OF SOME SPECIES OF REGIONAL RAW MATERIALS AND PRODUCTS OF THEIR ENZYMATIC HYDROLYSIS

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Abstract. Nowadays, it is recognized that a lot of polysaccharides are biologically active. It is well known that these biomolecules show the highest level of their activity if they are water-soluble preparations, their molecular weight being 15–25 kDa, and if they preserve the supramolecular structure of carbohydrates. Basing on the fact that β-glucans of mushrooms are characterized by the antitumor, anticoagulant, anti-inflammatory, and immunomodulatory activities, it is important to determine whether regional raw material contains polysaccharides of a similar structure, and to define the conditions for their fragmentation to obtain products with a given molecular weight.

The purpose of the work was to characterize the features of the structure of the hemicellulose complex of the *Agaricus bisporous* and *Pleurotus ostreatus* and products of their limited enzymatic hydrolysis.

To determine the primary structure of hemicellulose polysaccharides, the ^{1}H-NMR spectra of the samples were registered. It has been shown that β-D-(1→3)/β-(1→6)-glucan dominates in the hemicellulose of *Pleurotus ostreatus*. Among the hemicelluloses in the *Agaricus bisporis*, the main polysaccharide was galactoglucan. Its main chain consisted of β-D-glucopyranose residues interconnected with (1→3)-glucosidic bonds. The positions of O-6 monosaccharide are joined by the side branches in the form of β-D-glucopyranoses and the terminal residues of β-D-galactopyranoses. The hemicelluloses of *Pleurotus ostreatus* also contain manogalactan. Complexes of hemicelluloses of both types of mushrooms contain linear α-(1→3)-glucan in small quantities.

It has been studied how the molecular-weight distribution of products of limited hydrolysis of hemicelluloses depends on the conditions of their treatment with the enzyme with β-(1→3)-glucanase activity. The maximum accumulation of a fraction with a given molecular weight of 15–25 kDa was observed at a ratio of E:S = 1:45 and treatment time of 21 hours. A specific reaction with congo red has proved there is a triple helical conformation of the main chain of the polysaccharide for this fraction of carbohydrates, so the supramolecular structure of the molecule is preserved.

Key words: glucan, conformation, mushroom, enzymatic hydrolysis.

ОСОБЛИВОСТІ БУДОВИ ГЕМІЦЕЛЮЛОЗ ДЕЯКИХ ВИДІВ РЕГІОНАЛЬНОЇ СИРОВИНИ ТА ПРОДУКТІВ ЇХНЬОГО ФЕРМЕНТАТИВНОГО ГІДРОЛІЗУ

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Анотація. Встановлено, що переважаючими компонентами комплексів геміцелюлоз печериці двоспорової та гливи звичайної є відповідно β-(1→3)/(1→6)-глюкан і галактоглюкан. Кор останнього полісахариду побудовано з залишків β-D-глюкопіраноз, з'єднаних між собою (1→3)-глікозидними зв'язками. Досліджено залежність молекулярно-масового розподілу продуктів обмеженого гідролізу геміцелюлоз від умов обробки їх ферментом з β-(1→3)-глюканазною активністю. Доведено наявність скрученої конформації головного ланцюга для фракції вуглеводів гідролізату із молекулярною масою 15–25 кДа.

Ключові слова: глюкан, конформація, гриби, ферментативний гідроліз.

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Introduction. Formulation of the problem

For a long time, polysaccharides have been used in food industry as preservatives, thickeners, stabilizers, foaming agents in the production of a wide range of products. Such a considerable spectrum of their functional and technological properties cannot but attract attention of scientists of other branches of knowledge [1]. So, many studies have found that in the human body, these compounds are involved in a number of physiological processes, namely: recognition of molecules by means of receptors, cell adhesion, formation of a protective barrier. Besides, these polysaccharides exhibit antitumor, anticoagulant, anti-

inflammatory, and immunomodulatory activity. These qualities, as well as their stability *in vitro*, biocompatibility, biodegradability, and non-toxicity even in high doses, make these compounds ideal carriers of biological activity while creating dietary supplements with different physiological properties [2-3].

However, the analysis of the composition of drugs existing on the market shows that manufacturers use bioactive polysaccharides but to a limited extent. The reason is their low biological activity in the human body due to the peculiarities of the functioning of membrane transport, limited solubility of biopolymers in physiological fluids. It was found that insoluble polysaccharides are not absorbed in the gastrointestinal tract, and, therefore, are cannot enter the bloodstream, where they must act on the body. Soluble compounds characterized by high molecular weight do not exhibit their activity in full range either because they cannot penetrate into the cell due to the presence of membrane barriers. At the same time, low molecular weight carbohydrates, which are easily transported through the membrane, do not exhibit the physiological activity due to the lack of supramolecular structure in them. It was found that the degree of manifestation of the properties of polysaccharides in the human body is determined by such factors as solubility, molecular weight, and supramolecular structure [2-4].

Thus, the relevance of the work in this area lies in the fact that to ensure the maximum degree of manifestation of biological activity of polysaccharides in the human body, ways should be developed of modifying the polysaccharides that will make them able to dissolve in body fluids and lower their molecular weight, with supramolecular structures retained.

Analysis of recent research and publications

Nowadays, there are three main ways of modifying the structure and properties of biopolymers: chemical, physical, and biological [5-13].

The chemical method involves the introduction of new functional groups in the polysaccharides, thus increasing the solubility of the starting compounds. Depending on the nature of the substituent introduced, the following methods are distinguished: acetylation, alkylation, phosphorylation, carboxymethylation [5-8]. As a result of this modification, derivatives of soluble polysaccharides are obtained. At the same time, they have new functional properties. The main disadvantages of the chemical modification of polysaccharides include the change in their macromolecules' conformation, which leads to a partial or complete loss of the initial physiological activity of biopolymers, and the accumulation of undesirable synthesis products [7-8].

The chemical method also includes the hydrolysis of polysaccharides with alkaline and acids solutions. But this process is characterized by low selectivity, which prevents obtaining stable results [9].

Another way that has been widely used recently due to the development of new types of equipment is the physical one [10–12]. It consists in reducing the molecular weight of a polysaccharide as a result of the breakdown of glycoside bonds under the influence of physical factors. For this purpose, a polysaccharide is treated by ultrasound, radiation, and microwaves. But though these methods allow obtaining soluble polysaccharides with a certain molecular weight, they cause disruption of the supramolecular structure of the molecules. This, in turn, leads to a decrease in the biological activity of polysaccharides.

The basis of the biological method of modifying polysaccharides is their destruction by breaking certain types of bonds under the action of enzymes-hydrolases. Besides such advantages as high specificity, high efficiency, absence of by-products, enzymatic hydrolysis does not lead to disruption of the supramolecular structure of polysaccharides [13]. The latter factor is decisive in the physiological activity of such effective immunomodulators as β-(1→3)/(1→6)-glucans [14].

Many studies have found that to manifest their immunomodulatory activity, β-(1→3)/(1→6)-glucans should have a molecular weight within 15–25 kDa and a spatial structure in the form of triple helixes, since these particular spiral conformations are determining for their connection with glucan-binding receptors in a living organism [15].

In Ukraine, there are a number of regional raw materials that can be viewed as a source of such glucans. They include cultivated mushrooms, namely *Agaricus bisporus* and *Pleurotus ostreatus*. Earlier, in the work [16], a method of obtaining high molecular weight polysaccharides from this raw material was developed. However, the literature does not provide data on the peculiarities of the primary and supramolecular structure of glucans present in the raw material, nor information about their directed depolymerization to obtain carbohydrates with a certain molecular weight. This prevents the development of domestic technologies for the production of dietary supplements based on these polysaccharides.

To expand the spectrum of dietary supplements with immunomodulatory activity, a topical task of modern science is to determine the conditions for obtaining polysaccharides with a given molecular weight, preserving the native conformation of macromolecules.

The aim of this research was to characterize the features of the structure of the hemicellulose complex in *Agaricus bisporus* and *Pleurotus ostreatus* and products of their limited enzymatic hydrolysis.

Tasks of the research.

1. To characterize the composition and the primary structure of hemicelluloses in *Agaricus bisporus* and *Pleurotus ostreatus*;
2. To determine the conditions for enzymatic hydrolysis of hemicelluloses that allow obtaining products with a given molecular weight;
3. To investigate the supramolecular structure of hydrolysis products.

Research materials and methods

To obtain the complex of hemicelluloses, mushrooms were treated with 70% ethanol at 60°C for 40 minutes. The solid residue was separated by centrifugation, and then it was treated with a 3% NaOH solution at 98°C for 4 hours. The liquid phase was separated and acidified to pH=2 with phosphate acid. The resulting precipitate was separated by centrifugation. The obtained supernatants were neutralized with 1M NaOH solution, and ethanol was added in a volume ratio 1:4. The resulting precipitate was separated by centrifugation and dissolved in water.

The content of carbohydrates in the samples was determined by the anthrone method [17] using glucose (Sigma-Aldrich, USA) as a standard, that of protein by the Lowry method [18], melanins with a UV-2401 PC UV-Vis spectrophotometer (Japan) [19], using melanin (Sigma-Aldrich, USA) as a standard. To determine the monosaccharide composition of hemicelluloses, the samples were hydrolyzed with solutions of mineral acids [20], and then the hydrolysates were studied with a Hewlett-Packard 5890 chromatograph (Germany) [21].

To determine the structure of hemicelluloses, the sample was purified from the impurities of melanins and protein by treating them with a 10% H_2O_2 solution in ammonia (pH=10.0) at 90°C for 15 min. The solution was lyophilically dried, dissolved in deuterium water, and transferred to a standard ampoule (5 mm in diameter, 178 mm high) for further investigation. The spectra were recorded with a working frequency of 125.1 MHz. Deuteroacetone was used as an internal standard. The ratio of β-1→3- and β-1→6-bonds in the test sample was calculated from the ratio of the integral intensities of the carbon atoms signals of β-1→3 and β-1→6-bonds [22]. ^{1}H NMR spectra were recorded on a Bruker Avance 400 wb spectrophotometer (Germany).

Sephadex G-150 was used for gel chromatography, with column dimensions H=38 cm; D=3.1 cm; V=121 cm^3. The constant elution rate was set with a pump. The column with sephadex was calibrated with markers the molecular weights of which were already known.

The hemicelluloses obtained were hydrolysed with a 0.025% solution of the purified multienzyme preparation *Rovabio Excel AP* with endo-1,3(4)-β-glucanase activity. During this process, the enzyme-substrate ratio was varied taking the values 1:100, 1:45, 1:30, and the duration of hydrolysis ranged 12–72 h [23]. The hydromodule was 150. After the hydrolysis, the fermentolysate was boiled for 15 minutes to inactivate the enzyme, then centrifuged, and the supernatant was fractionated on a Sephadex G-50 column.

To confirm the presence of glucan in the form of a triple helix conformation, fractions of the hemicelluloses with certain molecular weights obtained after the elution of the hydrolysis products on the column G-50 were collected and lyophilically dried. Then 2 mg of the sample was dissolved in 2 cm^3 of a 0.15 M NaOH solution, after which 60 µl of a 0.01 M solution of congo red (Sigma-Aldrich, USA) in 96 % ethanol was added. After 10 minutes, the type of the absorption spectra of the solutions obtained was defined. The starch was used as a control sample [24].

Results of the research and their discussion

In the samples of mushroom hemicelluloses, carbohydrate component dominates, its mass fraction being higher in *Pleurotus ostreatus* (Table 1). In the hydrolysates of mushroom hemicelluloses (Table 2), galactose and glucose are found. Along with the mentioned monosaccharides, hydrolysates of *Pleurotus ostreatus* hemicelluloses also contain mannose.

Table 1 – Composition of the mushroom hemicelluloses (n = 5, P = 0.95)

Raw material	Content of components (% on the dry matter)		
	Carbohydrates	Proteins	Melanins
Pleurotus ostreatus	92.90±3.72	3.50±0.14	1.10±0.04
Agaricus bisporus	86.80±3.47	3.80±0.15	0.70±0.03

Table 2 – Monosaccharide composition of hemicellulose hydrolyzates, % ratio (n = 5, P = 0.95)

Raw material	Galactose	Glucose	Mannose
Pleurotus ostreatus	12.30±0.49	82.30±3.29	5.40±0.22
Agaricus bisporus	15.00±0.60	85.00±3.40	tr.

After additional purification in the preparation of the hemicelluloses obtained from mushrooms, the content of carbohydrates in *Pleurotus ostreatus* reached 96.2%, *Agaricus bisporus* – 92.1%. The carbohydrates were used to determine the primary structure of hemicellulose polysaccharides. For this purpose, the ^{1}H NMR spectra of the samples were recorded. The detected signals on the spectra were interpreted basing on the analysis of literary data.

On the ^{1}H-NMR spectra of polysaccharides (Fig. 1,2), signals were observed in the region of 3.0–5.4 ppm, which proved the carbohydrate nature of the samples.

The presence of two types of signals in the anomeric region of polysaccharides suggests the presence of monosaccharides with glycoside centres of the α- and β-configurations (Table 3). In the spectra, signals were identified corresponding to β-(1→3)-, β-(1→6)-bonded glucose residues. Signals of 3-substituted α-glucose residues are also found on the ^{1}H -NMR spectra of polysaccharides of both mushrooms. Besides, signals were identified corresponding to β-(1→6)- and β-(1→2)-bound galactose and mannose residues, respectively.

Thus, in the hemicelluloses of *Pleurotus ostreatus*, β-D-(1→3)/β-(1→6)-glucan dominates. The chain of the molecule consists of glucopyranose units, which are linked by β-(1→3)-glycosidic linkages. The positions of O-6 monosaccharide residues of the main chain are joined by lateral branches. They are represented by several monosaccharide residues. There are about 3 monosaccharide units of the core per one branch. It corresponds to the degree of branching 0.38.

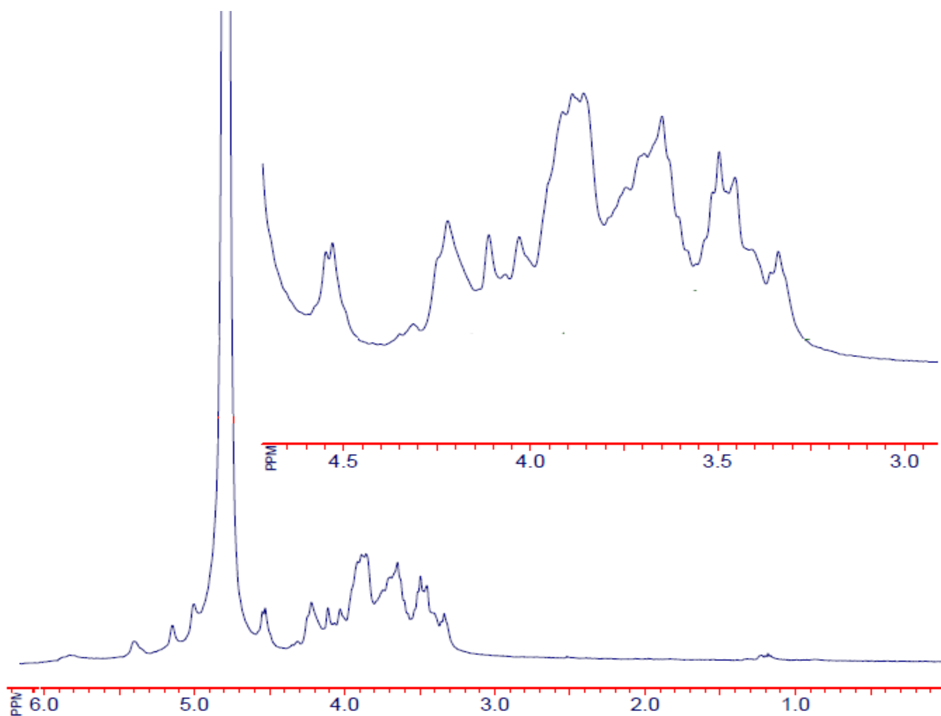

Fig. 1. ^{1}H NMR spectra of the hemicelluloses of *Pleurotus ostreatus*

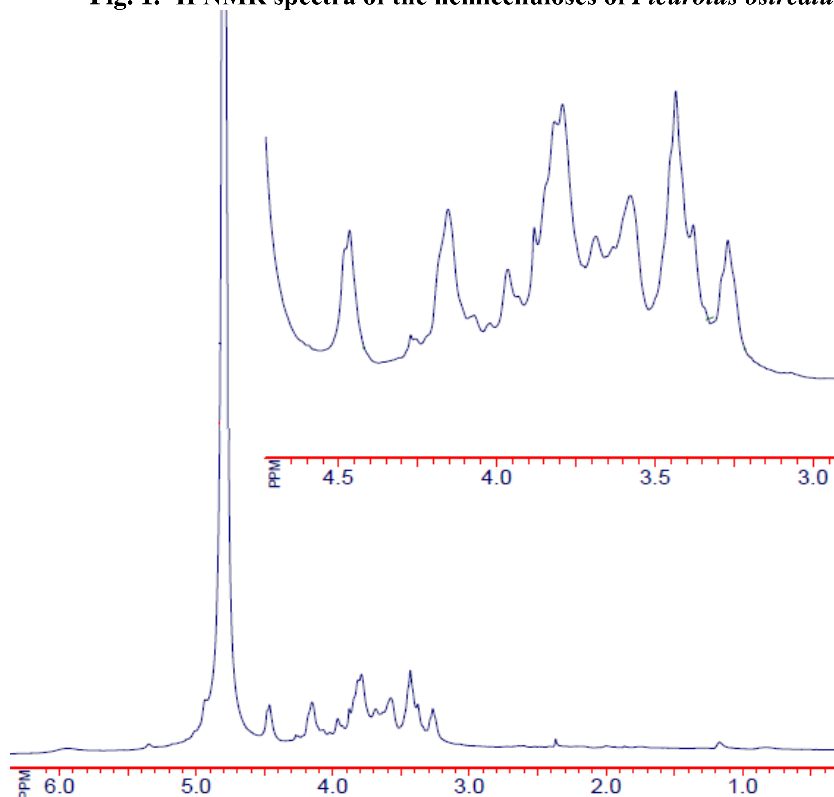

Fig. 2. ^{1}H NMR spectra of the hemicelluloses of *Agaricus bisporus*

Table 3 – Chemical shift of signals in ^{1}H NMR spectra of mushroom hemicelluloses

Carbohydrate moieties	Chemical shift, ppm					
	H1	H2	H3	H4	H5	H6
The hemicelluloses of *Pleurotus ostreatus*						
→3)-β-Glc-(1→	4.80	3.41	3.74	3.60	3.85	3.88
→6)-β-Glc-(1→	4.53	3.34	3.65	3.51	3.74	4.22
→3,6)-β-Glc-(1→	4.55	3.40	3.70	3.60	3.45	3.91
→3)-α-Glc-(1→	5.39	3.65	3.89	3.56	4.03	3.70
→6)-β-Gal-(1→	4.48	3.60	3.70	3.92	3.92	4.11
β-Man-(1→2	5.00	4.22	3.70	3.60	3.45	3.91
The hemicelluloses of *Agaricus bisporus*						
→3)-β-Glc-(1→	4.80	3.43	3.69	3.57	3.82	3.88
→6)-β-Glc-(1→	4.48	3.38	3.63	3.51	3.79	4.15
→3,6)-β-Glc-(1→	4.48	3.38	3.69	3.57	3.43	3.96
→3)-α-Glc-(1→	5.35	3.63	3.88	3.57	4.02	3.88
β-Gal-(1→6	4.46	3.58	3.69	3.96	3.79	3.82

The gemicelluloses of *Agaricus bisporus* contain galactoglucan. Its main chain consists of the residues of β-D-glucopyranose connected by (1→3)-glucosidic linkages. The positions of O-6 monosaccharide residues of the core are joined by the lateral branches. They are built from β-D-glucopyranose residues connected with each other by (1→6) -glucosidic bonds. Some of these branches contain terminal β-D-galactopyranose residues. By the ratio of the intensity of the signals due to the presence of β-(1→3)- and β-(1→6)-containing glucose residues, the degree of branching of the polysaccharide was determined as 0.3.

In the hemicelluloses of *Pleurotus ostreatus*, there is mangolactan. The main chain of this heteropolysaccharide consists of β-(1→6)-bound galactose residues, to which, by the O-2 position, the terminal residues of mannose are joined.

The hemicelluloses of both mushroom species also contain linear α-(1→3)-glucan.

To be capable of high immunomodulatory activity, polysaccharides should have a molecular weight of about 15–25 kDa. However, as we can see from Fig. 3, the samples are heterogeneous as to their molecular weight. None of them contains fractions with a specified molecular weight. Thus, the hemicelluloses of *Pleurotus ostreatus* contain fractions with molecular weight values ranging 70–90 kDa and 35–45 kDa. The hemicelluloses of *Agaricus bisporus* have the fractions with the molecular mass 90–100 kDa (the first fraction) and 35–45 kDa (the second fraction).

This makes fragmentation of samples necessary, so as to obtain β-glucans with a lower molecular weight.

Among the known methods of degradation of polysaccharides, enzymatic hydrolysis is the best method because it allows receiving products with specified characteristics by employing soft treatment modes.

The reason for choosing an enzyme preparation is the presence of certain enzymatic activity and its level. The backbone of the main polysaccharides of both mushroom types consists of β-(1→3)-connected glucose residues, that is why the enzyme preparation should have β-(1→3)-glucanase activity. As highly purified enzyme preparations are quite costly, it makes sense to use multienzyme preparations for the hydrolysis of glucans.

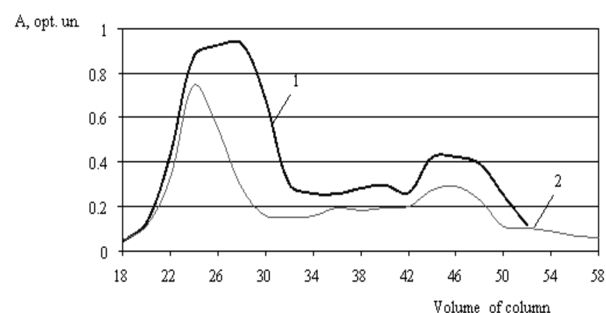

Fig. 3. Elution curves of *Pleurotus ostreatus* (1) and *Agaricus bisporus* (2) hemicelluloses on Sephadex G-150

The activity of several of such preparations was determined in [23]. The highest glucanase activity was observed in the multienzymes preparation *Rovabio Excel AP*. That is why this preparation was chosen to carry out limited depolymerisation of mushroom β-glucans.

Table 4 shows the data on the molecular weight distribution of enzymatic degradation products of mushroom hemicelluloses.

Analysis of the data shows that the maximum accumulation of a fraction with a given molecular weight (15–25 kDa) can be obtained at E:S=1:45 for 21 hours. A further increase in the fermentolysis time leads to a smaller content of the target product due to its degradation to low molecular fragments. However, the mass part of the fraction with a molecular weight over 25 kDa practically does not change.

Increasing the ratio of E:S to 1:30 allowed obtaining the maximum yield of products with a given molecular weight only 3 hours earlier than with E:S=1:45.

So it is unreasonable to use a large amount of the enzyme to obtain fragmented β-glucans.

Reducing the amount of the enzyme in the composition of the reaction mixture does not allow getting a large mass fraction of the target product even after 72 hours of hydrolysis. A further increase in the treatment time will not only reduce the safety profile of the drugs, but also raise the cost of enzymatic destruction of mushroom polysaccharides.

Table 4 – Molecular mass distribution of fermentolysis products of mushroom hemicelluloses, % ratio (n = 5, P = 0.95)

№	Ratio E : S	Duration of hydrolysis, h	Molecular weight, kDa		
			>25	15–25	<15
Hemicelluloses of *Pleurotus ostreatus*					
1	1 : 45	12	72.4±3.6	15.8±0.8	11.8±0.6
2		15	58.0±2.9	18.6±0.9	23.4±1.2
3		18	42.4±2.1	22.3±1.1	35.3±1.8
4		21	26.9±1.3	29.8±1.5	43.3±2.2
5		24	22.6±1.1	21.6±1.1	55.8±2.8
6		36	19.9±1.0	16.4±0.8	63.7±3.2
7	1 : 100	24	88.6±4.4	5.3±0.3	6.1±0.3
8		72	75.6±3.8	10.1±0.5	14.3±0.7
9	1 : 30	15	53.3±2.7	18.4±0.9	28.3±1.4
10		18	37.0±1.9	26.7±1.3	36.3±1.8
11		21	21.4±1.1	23.8±1.2	54.8±2.7
Hemicelluloses of *Agaricus bisporus*					
12	1 : 45	12	54.5±2.7	17.2±0.9	28.3±1.4
13		15	49.8±2.5	20.4±1.0	29.8±1.5
14		18	36.2±1.8	24.7±1.2	39.1±2.0
15		21	23.3±1.2	28.6±1.4	48.1±2.4
16		24	20.9±1.0	24.1±1.2	55.0±2.7
17		36	19.8±1.0	22.8±1.1	57.4±2.9
18	1 : 100	24	85.3±4.3	6.7±0.3	8.0±0.4
19		72	71.6±3.6	14.0±0.7	14.4±0.7
20	1 : 30	15	38.1±1.9	23.5±1.2	38.4±1.9
21		18	28.4±1.4	29.8±1.5	41.8±2.1
22		21	21.6±1.1	25.1±1.3	53.3±2.7

It should be pointed out that polysaccharide of *Pleurotus ostreatus* is hydrolysed more slowly than that of *Agaricus bisporus*.

A peculiarity of the spatial structure of β-(1→3)-glucans is the presence of a so-called 'triple helix' conformation. To spot its presence, the interaction with congo red is used, based on the formation of glucan-congo red complexes in an alkaline medium. At the same time, the maximum absorption of the colourant in the visible region shifts to the long-wave region.

The absorption spectra of congo red in the presence of hydrolysis products of mushroom hemicelluloses with a molecular weight of 15–25 kDa have been analysed and compared to that of starch (Figure 4).

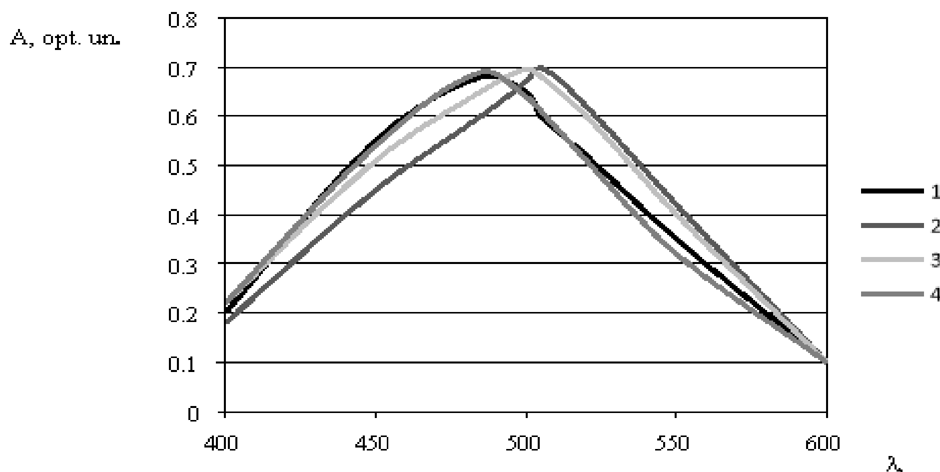

Fig. 4. Absorption spectra of congo red (1) and its complex with *Pleurotus ostreatus* (2), *Agaricus bisporus* (3) and starch (4)

It has been found that products of enzymatic hydrolysis of mushroom hemicelluloses cause a multichrome shift. This is characteristic of a series of β-(1→3)-glucans and confirms the presence of a triple helix conformation of the main chain in the structure of the β-(1→3)-glucans of the studied samples [24].

For further recommendations about the use of the obtained products of limited enzymatic hydrolysis of mushroom glucans, it is necessary to carry out their medical and biological research.

Conclusions

1. In the hemicelluloses of *Pleurotus ostreatus* β-D-(1→3)/β-(1→6)-glucan dominats, and linear α-(1→3)-glucan, manogalactan, has also been identified. The hemicelluloses of *Agaricus bisporus* contain galactoglucan. Its main chain consists of β-D-glucopyranose residues connected by (1→3)-glucosidic linkages. The lateral branches join the positions of O-6 monosaccharide residues of the core. They are built from residues of β-D-glucopyranose and β-D-galactopyranose, and connected with each other by (1→6)-glucosidic bonds. Besides, there is a linear α-(1→3)-glucan structure.

2. While carrying out a limited enzymatic hydrolysis of mushroom hemicelluloses, the maximum accumulation of a fraction with a given molecular weight (15–25 kDa) was observed at E: S = 1: 45, the duration of the process being 21 hours.

3. The triple helix conformation of the main chain of β-(1→3)-glucan has been found in the structure of products of limited enzymatic hydrolysis of mushroom hemicelluloses.

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