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## INVESTIGATION OF THE STRUCTURE OF WATER-SOLUBLE GLUCAN YEAST *SACCHAROMYCES CEREVISIAE*

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### Introduction. Formulation of the problem

The rapid increase in the pace of life and mental stress, extreme environmental factors have a negative impact on the modern human body and, first of all, cause a violation of the immune system, which underlie the pathogenesis of a large number of diseases.

One of the possible ways to increase the resistance of the human body is the additional inclusion in the diet of biologically active compounds and dietary supplements. In recent years, interest in immunocorrection of natural origin has significantly increased. Among them, an important role is played by beta-glucans (non-cellulose polysaccharides). They are present in a number of crops, algae, fungi, bacteria and yeast [1,2]. Beta-glucans obtained from various

**Abstract.** It is known that a well-functioning immune system is important for human health. There are many natural and synthetic preparation that are widely used as immunomodulators. One such natural preparat is  $\beta$ -glucan. Beta-glucans are a group of natural polysaccharides. They are recognized as an effective immunocorrector. Their use is advisable both for the prevention of immunodeficiency pathologies and for the complex treatment of many diseases from cardiovascular to oncological. The physiological activity of  $\beta$ -glucan depends on the type and configuration between monosaccharide residues, branching and conformation of macromolecules, solubility in water. One major source of  $\beta$ -glucan is the baker's yeast *Saccharomyces cerevisiae*. Much research has been carried out over the years examining cell wall glucans from *Saccharomyces cerevisiae*. This work is the development devoted to the characterization of water-soluble beta-glucan obtained as a result of controlled degradation with the enzyme Rovabio Excel AP of glucan cell walls of yeast *Saccharomyces cerevisiae*. In this study conditions were selected which allow to accumulate the maximum water-soluble fractions with a molecular mass of 1–30 kDa presumably as fractions with a high immunomodulatory effect. The results of the paper show that glucan can be isolated from *Saccharomyces cerevisiae* in very pure form by the method used in this study. Thus structural analysis gives reliable results. The structural characterization of pure product was performed using the common analytical procedures: enzymes hudyolyses and spectral analyses FTIR, NMR spectroscopy. On the basis of the obtained results it was concluded that investigated glucan is a (1 $\rightarrow$ 3)- $\beta$ -linked glucose polymer with (1 $\rightarrow$ 6)- $\beta$ -linked side chains with sparsely branched. Further work will concern the physiological effect of water-soluble glucan in comparison to the native glucan. The structural requirements for example for an immunomodulation in humans or animals are still under discussion.

**Keywords:** polysaccharide, baker's yeast *Saccharomyces cerevisiae*, water-soluble glucan, immunomodulator.

sources are distinguished by their structure. Cereal glucans are water-soluble polysaccharides. They have a linear structure of macromolecules, which include blocks from the residues of  $\beta$ -(1 $\rightarrow$ 4)-D-glucopyranose, separated by  $\beta$ -(1 $\rightarrow$ 3)-bonds. These block fragments are trimers or tetramers, the ratio of which varies. The main sources of such glucans are oat and barley [3].

### Analysis of recent research and publications

Branched  $\beta$ -(1 $\rightarrow$ 3)/(1 $\rightarrow$ 6)-D-glucans are found in some strains of yeast, a number of fungi and seaweed. Common features of their structure are: cortex, built from  $\beta$ -(1 $\rightarrow$ 3)-linked  $\beta$ -D-glucopyranose residues, the presence of lateral branches in 0-6 positions of glucose residues of the main chain (Fig. 1).

The degree of branching and the size of the side chains are variable and depend on the type of

feedstock. Representatives of these glucans (particular in  $\beta$ -glucan baking yeast) are used as multimodal immunomodulators of the biological activity of the human body in the treatment of oncological diseases, hypertension, to lower blood cholesterol, allergies, and the prevention of hyperglycemia in diabetes mellitus [4-6].

The mechanism of immunomodulatory action of  $\beta$ -glucan was found in 80-ies of the last century [7]. It has been established [8] that the surface of macrophages has a specific receptor dectin-1. This

receptor is a glycoprotein complex that can bind to a site of the  $\beta$ -glucan molecule, which consists of seven monosaccharide units and has a spiral configuration. The binding of this receptor to  $\beta$ -glucan is the starting link in the subsequent immune response.

One of the most perspective sources of  $\beta$ -(1 $\rightarrow$ 3)/(1 $\rightarrow$ 6)-D-glucan is baker's yeast *Saccharomyces cerevisiae*. It is localized in cell walls (Fig. 2.), insoluble in water and characterized by a complex structural organization [9-11].

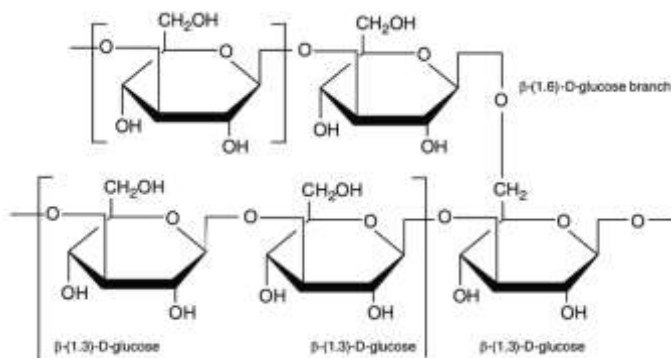


Fig. 1. The structure of yeast glucan

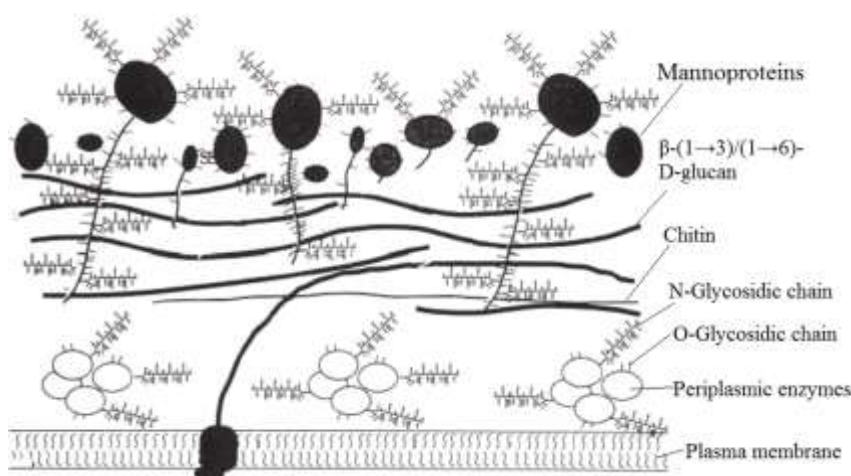


Fig. 2 Composition and structure of the yeast (*Saccharomyces cerevisiae*) cell wall

There are a number of ways to isolate the glucan cell walls of yeast. However, obtaining the desired product with a satisfactory degree of purity is difficult task [12]. The main problem is the removal of reinforcing biopolymers- mannan and protein, which are a tightly bound complex. It is solved both by extraction, using appropriate agents under rather harsh conditions, and by their hydrolytic degradation by appropriate enzyme preparations.

The physiological activity of  $\beta$ -glucan depends on the type and configuration between monosaccharide residues, branching and conformation of macromolecules, solubility in water [13-17]. However, one of the important characteristics of soluble polysaccharides, allowing to predict the level of their physiological activity, is

the value of molecular weight. It is established that the increase in the physiological activity of yeast glucan can be achieved by converting it into a water-soluble form, with the most effective effect being provided by a preparation with a molecular mass in the range of 1–30 kDa [18].

Earlier, we justified the advisability of using hydrogen peroxide to obtain  $\beta$ -glucan cell walls of yeast and the possibility of its transformation into a water-soluble form by enzymatic modification with the enzyme Rovabio Excel AP [19, 20].

This work is the development of these studies and is devoted to the characterization of water-soluble beta-glucan obtained as a result of controlled enzymatic degradation of glucan cell walls of yeast *Saccharomyces cerevisiae*.

## Research materials and methods

**Materials.** To carry out the experimental part of the work used: yeast *Saccharomyces cerevisiae*.

Enzyme preparations: Rovabio Excel AP Penicillium funiculosum, with xylanase (endo-1,4- $\beta$ -);  $\beta$ -glucanase (endo-1,3-(4)) activities (Aventis, France);  $\beta$ -glucanase Helix pomatia with  $\beta$ -(1 $\rightarrow$ 3)-glucanase activity (No. 67138 "Sigma Aldrich", Germany); B-glucanase with  $\beta$ -(1 $\rightarrow$ 6)-glucanase activity ("Sigma Aldrich", Germany);  $\alpha$ -amylase of animal origin ("Sigma", Germany).

Gel chromatography markers: dextran (Leuconostoc mesenteroides) with molecular weights of 1000, 9000–11000, 15000, 35000, 70,000 and 100,000 Da ("Sigma", Germany), dextran (Leuconostoc mesenteroides) with a molecular weight of 25,000 Da ("Fluka", Germany).

Sephadex: G-50, G-75, G-150 (Pharmacia, Sweden).

### Obtaining the Glucan of Cell Walls

Glucan preparations of cell walls were prepared according to the method of [19].

### Enzymatic methods

To confirm the belonging of the studied polysaccharides to the category of  $\beta$ -(1 $\rightarrow$ 3)/(1 $\rightarrow$ 6)-glucans, enzymatic methods were used.

Enzymatic hydrolysis of glucan cell walls of yeast (sample 100 mg) was carried out with  $\beta$ -glucanase with  $\beta$ -endo-1,3(4)-glucanase activity 1 unit/mg in 0.1 M phosphate-citrate buffer pH 4.6 at a temperature of  $37 \pm 0.2^\circ\text{C}$  for 20 minutes. Then the enzyme was inactivated. The precipitate was separated by centrifugation. Soluble carbohydrates were determined in the supernatant by the anthrone method.

To determine the possible presence impurities of glycogen in their preparations was treated with  $\alpha$ -amylase. To do this, a sample of 100 mg glucan of the cell wall was filled with 20 cm<sup>3</sup> of a 2%  $\alpha$ -amylase solution in 1 N acetate buffer pH = 4.7 and incubated at  $37 \pm 0.2^\circ\text{C}$  for 60 minutes. Then the enzyme was inactivated by boiling. The precipitate was separated by centrifugation. Soluble carbohydrates were determined in the supernatant by the anthrone method.

Water-soluble glucan was obtained by hydrolysis with the enzyme preparation Rovabio Excel AP glucan cell walls of yeast. Enzyme concentration in the solution (0.75–3.0 mg/cm<sup>3</sup>), the length of hydrolysis (24–72 hours) the ratio E:S (1:15 – 1:30) was varied at  $t = 50^\circ\text{C}$ ; pH = 4.5 and at  $t = 37^\circ\text{C}$  and pH = 6.0. After hydrolysis, the fermentolysates were boiled for 15 minutes to inactivate the enzyme, centrifuged and the carbohydrate content of the Anthrone method and protein determined in the supernatant by the Lowry method [21].

### Determination of molecular weight

The molecular weight of the water-soluble glucan was determined by gel-chromatography on Sephadex G-50, G-75 and G-150. Column dimensions are

H = 38 cm; D = 3.1 cm; V = 121 cm<sup>3</sup>. The elution rate was set constant using the pump. The column with Sephadex was calibrated using markers with known molecular weights. The column was introduced from 7 to 10 mg of a water-soluble glucan. Fractions were collected in 2 cm<sup>3</sup>. The content of carbohydrates in the fractions was controlled by the Anthrone method.

### Physical-chemical methods of investigation (Structural Analysis)

FTIR spectra were obtained using a FTIR Spectrometer Frontier (Perkin Elmer, USA) instrument in the 4000–500 cm<sup>-1</sup> region using KBr discs. Samples were tablets containing 4 mg of the glucan.

NMR spectroscopy was carried out on a Bruker Avance 400 wb spectrophotometer (Germany). The dried samples were placed in a water-soluble glucan standard ampoule (diameter 5 mm and height 178 mm). A microdoser was added a solvent (deuterium water). The contents of the ampoule were thoroughly mixed until a clear solution was obtained. Inclusion of insoluble particles was not allowed.

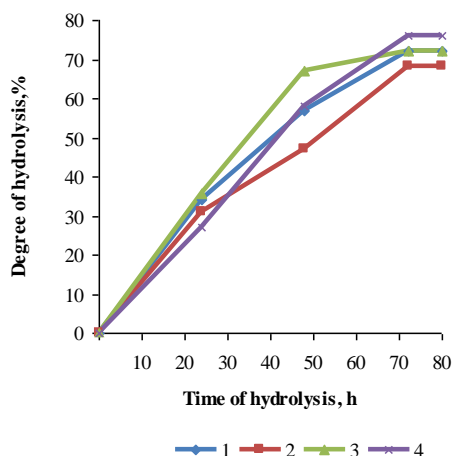
To obtain the proton spectrum (<sup>1</sup>H), the weight of the sample of the polysaccharide was about 5 mg. To obtain a spectrum on <sup>13</sup>C nuclei, the mass of the sample of the polysaccharide was 20–30 mg. The spectra were recorded at an operating frequency of 125.1 MHz. Deuteroacetone was used as an internal standard.

## Results of the research and their discussion

Obtained preparations of glucan cell walls give a negative reaction with iodine. This indicates the absence of glycogen in them, which is part of the yeast. Furthermore, in the processing of the samples  $\alpha$ -amylase, which cleaves glycogen, the formation of water soluble carbohydrates were detected. Conversely, these polysaccharides are cleaved by enzymatic preparations with  $\beta$ -(1 $\rightarrow$ 3)- and  $\beta$ -(1 $\rightarrow$ 6)-glucanase activity. To produce a water-soluble glucan, a preparation prepared using a 24% hydrogen peroxide solution was used. Since it contains no protein, carbohydrate content is 97.3%, yield is 8.1% for dry weight of yeast.

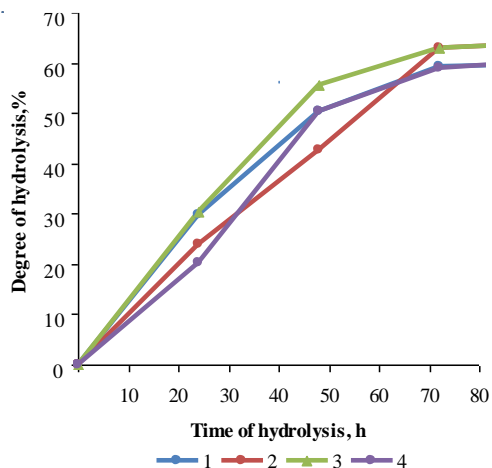
In Fig. 3 and 4 show the conditions for carrying out the enzymatic hydrolysis of glucan and the amount of products passing into the solution during a certain processing time. A comparison of the data shows the dynamics of the accumulation of water-soluble fractions under various conditions of enzymatic hydrolysis of glucan indicates a significant difference in the rate of fermentolysis. It is shown that the high yield (about 75% of the weight of the initial polysaccharide) of the water-soluble fraction occurs with the following parameters of fermentolysis: enzyme concentration 0.50 mg/cm<sup>3</sup>, ratio E:S = 1:30 and process duration 72 hours at  $t = 50^\circ\text{C}$ ; pH = 4.5.

As expected, at  $t = 37^\circ\text{C}$ , pH = 6.0, the degree of transition of water-soluble fragments to the solution is much smaller and their accumulation does not reach 65%.



**Fig. 3. Dynamics of accumulation of water-soluble products of glucan hydrolysis at  $t = 50^{\circ}\text{C}$ ;  $\text{pH} = 4.5$**

- 1 – E concentration  $0.25 \text{ mg/cm}^3$ , E: S = 1:15;
- 2 – E concentration  $0.25 \text{ mg/cm}^3$ , E: S = 1:30;
- 3 – E concentration  $0.50 \text{ mg/cm}^3$ , E: S = 1:15;
- 4 – E concentration  $0.50 \text{ mg/cm}^3$ , E: S = 1:30



**Fig. 4. Dynamics of accumulation of water-soluble products of glucan hydrolysis at  $t = 37^{\circ}\text{C}$ ,  $\text{pH} = 6.0$**

- 1 – E concentration  $0.50 \text{ mg/cm}^3$ , E: S = 1:30;
- 2 – E concentration  $0.25 \text{ mg/cm}^3$ , E: S = 1:15;
- 3 – E concentration  $0.50 \text{ mg/cm}^3$ , E: S = 1:15;
- 4 – E concentration  $0.25 \text{ mg/cm}^3$ , E: S = 1:30

The purpose of the next stage of the research was to determine the conditions for enzymeolysis in which the maximum accumulation of polysaccharide fractions having molecular weights in the range of 1–30 kDa [18]. For this purpose, the molecular weight distribution of the water-soluble fractions of each of the samples obtained at  $t = 50^{\circ}\text{C}$  was determined;  $\text{pH} = 4.5$ . Since under these conditions the greatest accumulation of water-soluble products takes place. The relevant data are presented in Table 1. (samples 1–6 – preparations obtained at an enzyme concentration of  $0.25 \text{ mg/cm}^3$ , 7–12 – preparations obtained at an enzyme concentration of  $0.50 \text{ mg/cm}^3$ ).

As can be seen from the presented data, each sample of glucan is a heterogeneous molecular weight mixture. It contains fragments with a degree of polymerization over a wide range of values. Investigation of the polymolecular composition of glucan hydrolysates showed that an increase in the fermentolysis time up to 72 hours and enzyme concentration to  $0.5 \text{ mg/cm}^3$  in the composition of the reaction mixture resulted in the accumulation of water-soluble hydrolysis products with a target molecular weight. However, a further increase in fermentolysis time will not only lead to a deterioration in the microbiological indices of the hydrolysates, but also to an increase in the costs of their production.

Thus, the conditions ensuring the maximum accumulation of water-soluble products of fermentolysis make it possible to obtain the highest yield (46.6% of the weight of the initial polysaccharide) of a water-soluble form of glucan with a molecular mass of 1–30 kDa.

The presence in the IR spectra of water-soluble glucan absorption bands typical of a  $\beta$ -(1 $\rightarrow$ 3)/(1 $\rightarrow$ 6)-glucan. The FTIR spectrum is shown in Fig. 5. The band at  $890 \text{ cm}^{-1}$  is a characteristic feature of polysaccharide having the beta-configuration i.e. ( $\text{C}_1\text{-H}$ ) deformation mode in addition to the bands that distinguish 1 $\rightarrow$ 3 linkages at 2920, 1370, 1250, and  $1200 \text{ cm}^{-1}$  absorption bands arising from the (CC) and (COC) stretching vibrations at 1160 and two partially overlapping bands at  $1078 \text{ cm}^{-1}$  and  $1048 \text{ cm}^{-1}$  attributable to ring and (C-OH) side group stretching [22].

**Table 1 – Molecular weight distribution of samples of water-soluble beta-glucans ( $t = 50^{\circ}\text{C}$ ,  $\text{pH} = 4.5$ ), %**

No	Time of hydrolysis, h	The relation E: S	Molecular weight, kDa			Yield soluble products having a molecular weight of 1–30 kDa, % dry weight of the starting glucan
			>30	1–30	<1	
1	24	1:15	18.8	75.7	5.5	22.5
2		1:30	20.2	71.3	9.5	17.1
3	48	1:15	20.6	70.4	9.0	35.3
4		1:30	27.5	58.2	14.3	24.9
5	72	1:15	34.7	49.2	16.1	29.1
6		1:30	30.0	43.1	26.6	27.3
7	24	1:15	36.9	63.1	no	12.8
8		1:30	36.6	60.2	3.2	18.3
9	48	1:15	29.6	66.0	4.4	30.0
10		1:30	28.4	64.3	7.3	38.7
11	72	1:15	30.8	56.7	9.5	35.7
12		1:30	19.6	62.0	19.4	<b>46.6</b>

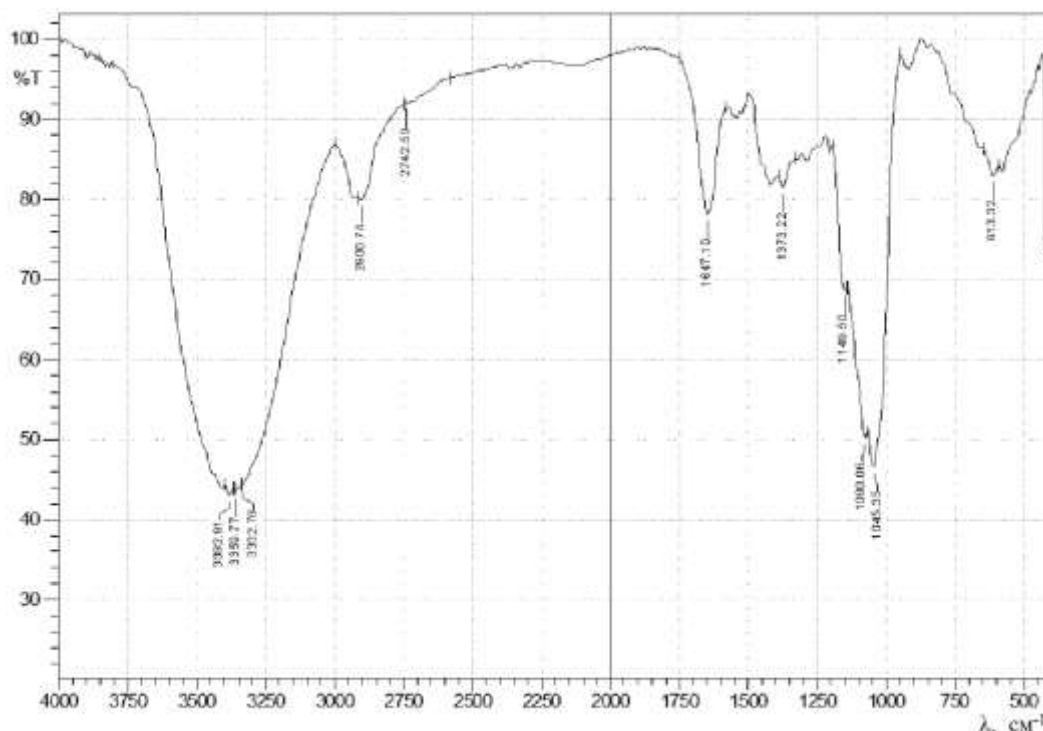


Fig. 5. FTIR spectrum of water-soluble yeast

The spectrum  $^1\text{H}$  NMR water-soluble glucan is shown in Fig. 6. An intense peak (4.5 ppm) is detected on the spectrum, which is identified with the presence of the  $\beta$ -anomeric form of the macromolecule. These data are consistent with the data of FTIR spectroscopy.

Chemical shifts of proton magnetic resonance at 3.00–4.50 ppm indicate that this polymer is a compound of carbohydrate nature. The presence of proton signals in the spectrum of anomeric carbon atoms at 4.47 ppm indicates the presence of  $\text{H}^1$   $\beta$ -1 $\rightarrow$ 3-linked glucopyranose and at 4.36- $\text{H}^1$   $\beta$ -1 $\rightarrow$ 6-linked glucopyranose.

Evidence supporting  $\beta$ -anomeric carbons in the glucan was confirmed by  $^{13}\text{C}$ -NMR data. The  $^{13}\text{C}$  NMR data did not reveal peaks corresponding to the  $\alpha$ -configuration of the anomeric carbon;  $\alpha$ -glucose anomeric carbons resonate at approx  $\delta$  100 ppm and  $\beta$ -glucose carbons slightly downfield at  $\delta$  104 ppm [23].

The spectrum  $^{13}\text{C}$  NMR water-soluble glucan is shown in Fig. 7. In the  $^{13}\text{C}$  NMR spectrum of the water-soluble glucan identified only a signal at 102.4 ppm. These data suggest the presence of only  $\beta$ -anomeric carbons, which agrees with the data  $^1\text{H}$  NMR and FTIR spectroscopy.

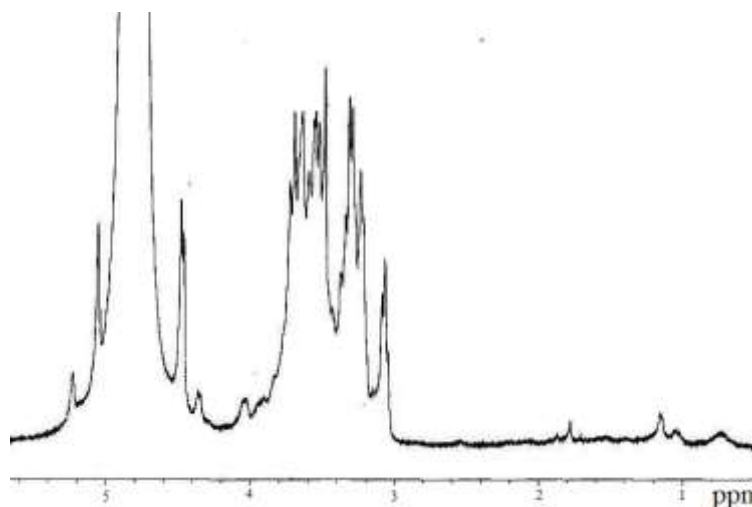


Fig. 6  $^1\text{H}$  NMR spectrum of water-soluble glucan yeast

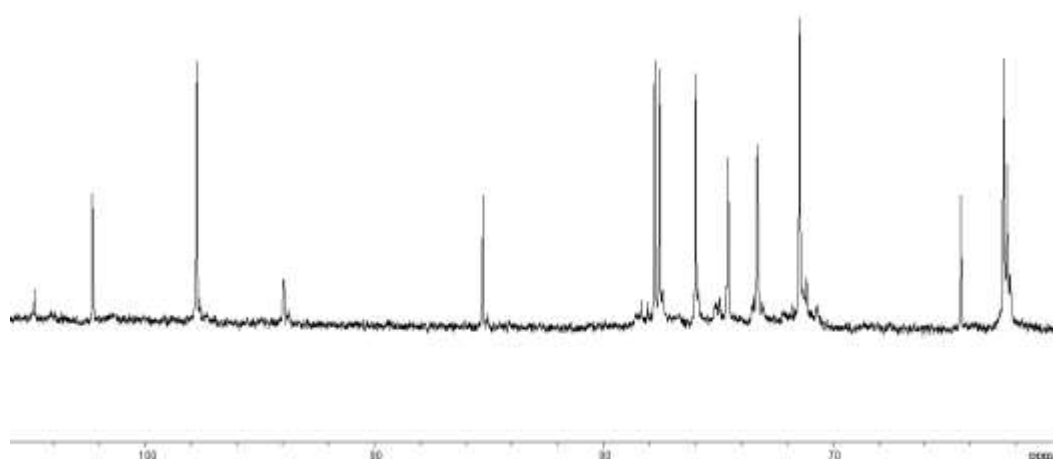


Fig. 7. <sup>13</sup>C NMR spectrum of water-soluble yeast glucan

The <sup>13</sup>C NMR spectrum of the test sample contains signals corresponding to carbon atoms of two types of glucans. The main peaks of 102.4 (C<sub>1</sub>), 72.9 (C<sub>2</sub>), 85.6 (C<sub>3</sub>), 71.5 (C<sub>4</sub>), 73.4 (C<sub>5</sub>) and 62.5 (C<sub>6</sub>) ppm are characterized by β-(1→3)-glucans. While, small

peaks at 104.6 (C<sub>1</sub>), 76.5 (C<sub>3</sub>), 75.8 (C<sub>5</sub>), 73.2 (C<sub>2</sub>), 64.4 (C<sub>4</sub>), and 63.1 (C<sub>6</sub>) indicate the presence of β-(1→6)-linked glucose residues in the form of branches attached to the main chain. The data obtained are similar to those described in the literature (Table 2).

Table 2 – Chemical shifts of signals in <sup>13</sup>C NMR spectra of glucan

Carbohydrate residues	Chemical shifts, ppm						Glucan Source
	C1	C2	C3	C4	C5	C6	
→3)-β-Glc-(1→	105.34	76.04	87.36	71.03	78.50	63.63	Laminaran [24]
→3)-β-Glc-(1→	102.49	72.46	85.76	68.18	76.02	60.67	Cell walls of yeast [25,26]
→6)-β-Glc-(1→	104.66	74.71	76.57	71.14	77.25	70.48	Mushrooms [27]
→3,6)-β-Glc-(1→	102.96	73.04	85.76	68.53	74.96	68.68	Mushrooms [28]
→3,4)-β-Glc-(1→	103.02	73.69	85.34	68.99	76.48	61.97	Barley [29]

Thus, from the data of IR, and also of <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy, it follows that the action of hydrogen peroxide and the action of the enzyme in no way affect the structure of the resulting water-soluble glucan. It is identical to the structure of other β-D-(1→3)/(1→6)-glucans. Its main chain is constructed from the residues of β-D-glucopyranose, which are interconnected (1→3)-glycoside bonds. To the positions of O-6 monosaccharide remains of the cortex, side branches are attached.

### Conclusion

The results of the above paper show that β-glucan can be isolated from *Saccharomyces cerevisiae* in very pure form by the method used in this study.

A method for biotransformation of structural glucan into a water-soluble form was developed by controlled destruction of the enzyme preparation Rovabio Excel AP. The maximum accumulation of water-soluble glucan hydrolysis products with a molecular mass of 1–30

kDa occurs under the following fermentolysis conditions: enzyme concentration 0.5 mg/cm<sup>3</sup>, ratio E:S = 1:30, duration 72 hours.

The structural characterization of pure product was performed using the common analytical procedures: enzymes hydrolysis and spectral analysis FTIR, NMR spectroscopy. On the basis of the obtained results, it was concluded that the macromolecule of water-soluble glucan has a branched structure, the basis of which consists of the residues of D-glucopyranose, which are connected by β-(1→3)-glycoside bonds. To the O-6 positions of monosaccharide residues, lateral branches, built from β-glucopyranose monomer units.

Further work will concern the physiological effect of water-soluble glucan in comparison to the native glucan. The structural requirements for example for an immunomodulation in humans or animals are still under discussion.

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## **ДОСЛІДЖЕННЯ СТРУКТУРИ ВОДОРОЗЧИНОГО ГЛЮКАНА ДРІЖДЖІВ *SACCHAROMYCES CEREVISIAE***

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**Анотація.** Відомо, що функціональна активність імунної системи має велике значення для здоров'я людини. Існує багато синтетичних та природних препаратів, які претендують на місце ефективних імуномодуляторів. Один з таких природних препаратів є  $\beta$ -глюкан. Бета-глюкани є ефективними імунокоректорами, використання яких доцільно як для профілактики імунодефіцитних патологій, так і для комплексного лікування багатьох захворювань від серцево-судинних до онкологічних. Фізіологічна активність  $\beta$ -глюкана залежить від багатьох факторів: типу та конфігурації

між залишками моносахаридів, розгалуження та конформації макромолекули, розчинності у воді. Одним з головних джерел  $\beta$ -глюкана є хлібопекарські дріжджі *Saccharomyces cerevisiae*. Протягом багатьох років було проведено багато досліджень з вивчення глюканів клітинної стінки дріжджів *Saccharomyces cerevisiae*. Дану роботу присвячено характеристиці водорозчинного  $\beta$ -глюкана, отриманого в результаті контрольованої ферментативної деградації (ферментним препаратом Rovabio Excel AP) глюкана клітинних стінок дріжджів *Saccharomyces cerevisiae*. У цьому дослідженні підібрано умови, які дозволяють максимально накопичувати водорозчинні фракції з молекулярною масою 1–30 кДа, які володіють високим імуномодулювальним ефектом. Показано, що за допомогою запропонованого методу з *Saccharomyces cerevisiae* можна виділити глюкан у чистому вигляді. Структурну характеристику чистого продукту проводили за допомогою загальних аналітичних процедур: ферментативного гідролізу та спектрального аналізу. На основі отриманих результатів, зроблено висновок, що досліджуваний глюкан – це (1 $\rightarrow$ 3)- $\beta$ -зв'язаний полімер D-глюкози з (1 $\rightarrow$ 6)- $\beta$ -зв'язаними бічними ланцюгами, він є з рідкорозгалуженим полісахаридом. У подальших дослідженнях планується вивчення фізіологічних властивостей водорозчинного глюкана, порівняно з нативним.

**Ключові слова:** полісахарид, пекарські дріжджі *Saccharomyces cerevisiae*, водорозчинний глюкан, імуномодулятор.

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