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# POLYSACCHARIDES OF PLANT TISSUE CULTURES.

## III. ENZYMATIC HYDROLYSIS OF INDUSTRIAL WASTES OF THE BIOMASS OF A GINSENG TISSUE CULTURE

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UDC 581.143.6:547.917

Questions of the enzymatic hydrolysis of industrial wastes of the biomass of a ginseng tissue culture by Pektofoetidin P10× (I), Tsellyulaza P10× (II), and Tsellokoningin P10× (III) are discussed. The optimum conditions permitting the hydrolysis of the wastes by the following respective percentages have been selected: (I) 62; (II) 43; (III) 54. Subsequent treatment of the wastes with mineral acids raises the total degree of hydrolysis to 84-88%. The monocarbohydrate compositions of the hydrolysates have been studied by chromatographic methods.

The development of waste-free industrial processes requires the search for methods of rationally utilizing large-tonnage secondary resources of nonfood character. These are, in the first place, cotton lint, grape vines, prunings of fruit trees, the tops and stems of vegetable crops, sawdust, straw, and wastes from the processing of aromatic and medicinal plants [1-4], and also a new type of waste – the biomass of ginseng, rose-root stonecrop, and yam, the industrial cultivation of which has been developed intensively in the last few years [5-8].

*Student I. B. Smirnova took part in the work.

M. V. Frunze Simferopol' State University. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 492-499, July-August, 1988. Original article submitted July 21, 1987; revision submitted November 11, 1987.

0009-3130/88/2404-0415 $12.50 

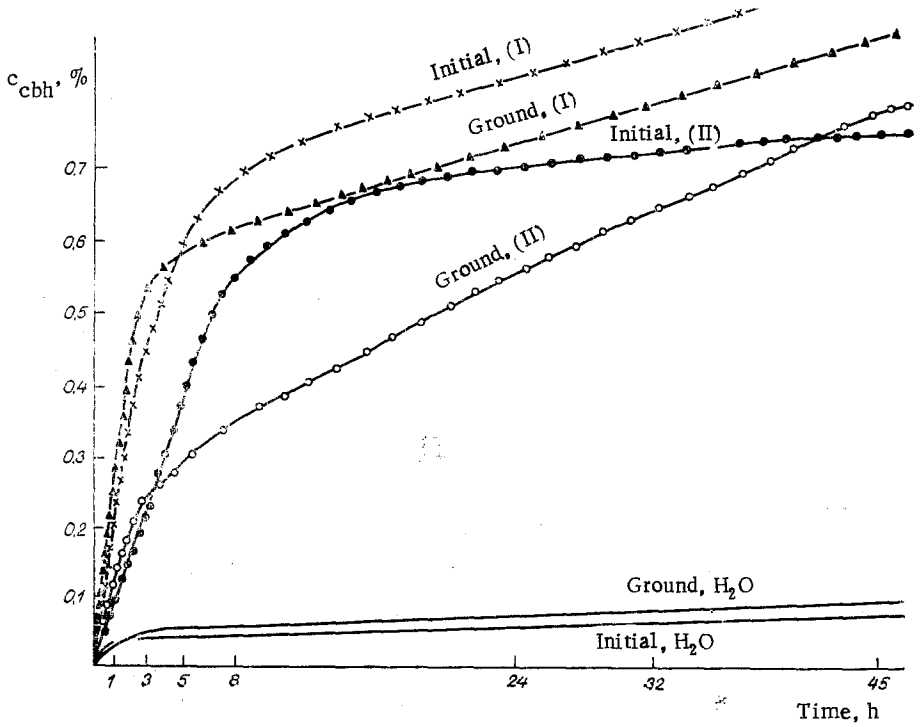


Fig. 1

TABLE 1. Dependence of the Efficiency of the Hydrolysis of the Biomass on the pH of the Medium (45°C, 5 h; biomass:enzyme: water = 1.0:0.5:50)

| Enzyme | Concentrations of carbohydrates (%) in the hydrolysates at the following pH values of the medium | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4,00 | 4,25 | 4,50 | 4,75 | 5.00 | 5,25 | 5,50 |
| I | 0,42 | 0,47 | 0,50 | 0,50 | 0,48 | 0,47 | 0,44 |
| II | 0,15 | 0,26 | 0,29 | 0,28 | 0,26 | 0,25 | 0,20 |
| III | 0,23 | 0,27 | 0,30 | 0,38 | 0,40 | 0,37 | 0,30 |

With the aid of acid hydrolysis it is possible to obtain from the biomass of a ginseng tissue culture a whole series of carbohydrates or to isolate a polysaccharide fraction [9, 10]. Interest is also presented in the study of the possibility of its enzymatic hydrolysis.

We have investigated the following enzymes: Pektofoetidin P10× (I), Tsellyulaza P10× (II), and Tsellokoningin P10× (III), the properties of which are described in the Experimental section. Enzymatic hydrolysis took place most effectively at a biomass:enzyme:water ratio of 1.0:0.1:50 at 45°C for 24 h. Longer incubation did not raise the degree of hydrolysis appreciably, obviously because of the suppressing action of the degradation products accumulating in the solution [11, 12], while a decrease in the degree of saturation of the enzymes by the substrate was accompanied by a higher consumption of the latter per unit of biomass. Increasing the liquor ratio led to a dilution of the hydrolysate and to the necessity for its subsequent evaporation.

We studied the influence of the pH of the medium on the rate of hydrolysis; the dependence of the efficiency of hydrolysis on the dispersity of the biomass; and the carbohydrate compositions of the hydrolysates obtained both by enzymatic treatment and by subsequent acid hydrolysis of the undecomposed residues. The choice of optimum pH values, particularly for new substrates, is of great importance. We have established optima in the 5-h incubation of the biomass for enzymes (I)-(III). In this process, as a rule, the accumulation of carbohydrates in the hydrolysates has a linear nature, i.e., their concentration apparently does not become a suppressing factor for the enzymes. The optimum pH for (I) and (II) is 4.5-4.7, and that for (III) is 5.0 (Table 1).

Another important factor is the dispersity of the initial wastes when they are sparingly soluble in water. In the opinion of A. A. Klesov [13] and a number of other workers [14], in the

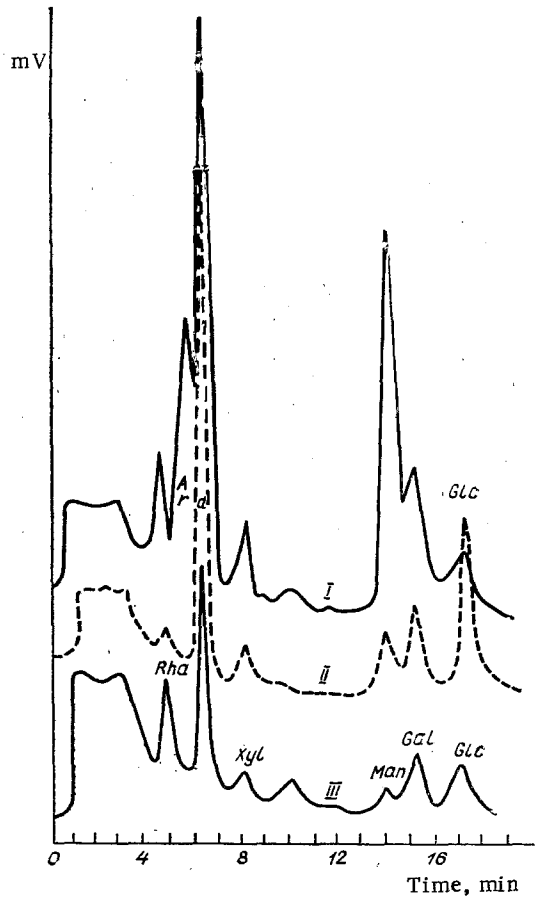


Fig. 2

enzymatic degradation of heteropolysaccharides an important role is played by the processes of adsorption of the depolymerases on the surface of the substrate and of the soluble products of enzymatic degradation by the surface layer of the initial substrate, which prevents the passage of the monosaccharides (MSCs) into solution until the reaction has proceeded for a definite time.

In view of this, we have studied the dependence of the efficiency of hydrolysis on the degree of dispersity of the biomass:

| Enzyme | Degree of hydrolysis of the biomass, % ground | unground |
|---|---|---|
| I | 46 (46—16=30) | 62 (62—11=51) |
| II | 34 (34—16=18) | 43 (43—11=32) |
| III | 37 (37—16=21) | 54 (54—11=43) |
| Control ($H_2O$, pH 4,5) | 16 | 11 |

The degree of hydrolysis is the ratio of the weight of substances that have passed into solution to the initial weight of biomass in 1 g. The values of the true enzymatic degree of hydrolysis are given in parentheses.

The comparative dynamics of the passage of carbohydrates into solution on the hydrolysis of the initial and ground biomasses are shown in Fig. 1. In the case of (I), the yield of carbohydrates was somewhat lower for the ground biomass, while in the cases of (II) and $H_2O$ it rose somewhat. Consequently, the preliminary mechanical grinding of the biomass lowers the efficiency of enzymatic cleavage. Thus, the enzymatic degree of hydrolysis of the unground biomass on treatment with (I) was 1.7 times greater than for the ground biomass, and on treatment with (II) and (III) 1.8 and 2.0 times higher, respectively. At the same time, the ratio of the enzymatic degrees of hydrolysis changed insignificantly. In the case of the ground biomass it was 1.7:1.0:1.2, and for the unground biomass 1.6:1.0:1.3 [the degree of hydrolysis for enzyme (II) was taken as unity]. On this basis, it may be concluded that the general kinetic laws of the degradation of the biomass by enzyme preparations do not

TABLE 2. Total Amount of Carbohydrates and Ratio of MSCs and PSCs in the Hydrolysates (weight of the biomass 1 g in 50 ml of medium)

| Hydrolysate after treatment with the enzymes | Total conc. of carbohydrates, % | Amount of carbohydrate, g | Including, % | |
|---|---|---|---|---|
| | | | MSCs | PSCs |
| I | 0.860 | 0.430 | 95.5 | 4.5 |
| II | 0.600 | 0.300 | 75.0 | 25.0 |
| III | 0.640 | 0.320 | 75.2 | 24.8 |
| Control: $H_2O$ (pH 4.5) | 0.055 | 0.027 | 80.0 | 20.0 |

change although its rate falls considerably. One of the reasons for this may be considered to be the competitive inhibition caused by the substrate itself, since when the biomass is ground the number of accessible bonds and groups with which the enzymes can form ineffective complexes rises considerably. In addition to this, the accessibility for the enzymes of compounds that are inhibitors – i.e., substances not hydrolyzed by the given enzymes but forming stable complexes with them – increases.

Thus, in the enzymatic hydrolysis of the biomass of a plant tissue culture, no preliminary grinding is required, as it is in the case of other plant wastes. This is no unimportant factor in the technical respect: The hydrolysates produced, which contain monosaccharides, amino acids, micro- and macroelements, etc., can be used as nutrient media in the microbiological industry.

As can be seen from Table 2, the amounts of MSCs was fairly high in the case of (I) (0.86%). According to GLC (Fig. 2), these hydrolysates contained only a small amount of glucose and their use as nutrient media requires preliminary enrichment with glucose to 1-2%. Hydrolysate (II) [enzyme (II)] was richer in glucose than (I) and (III). This is explained, on the one hand, by the smaller amount of cellulases in the case of enzymes (I) and (III) and, on the other hand, by the possible suppressing action on the cellulases of the galactose appearing in the hydrolysates as a result of the action of hemicellulases. Some decrease in the amount of carbohydrates in hydrolysates (II) and (III) is explained by the absence from these enzymes of proteases and pectolytic enzymes. Therefore, not only are they incapable of hydrolyzing pectin substances and structural glycoproteins but they hydrolyze typical substrates – cellulose and hemicelluloses – with difficulty since the cell wall forms an intricate complex of the biopolymers mentioned. A confirmation of this is given by the results on the amounts of MSCs and polysaccharides (PSCs) in the hydrolysates (Table 2).

On the whole, for the enzymatic treatment of the biomass the concentration of water-soluble carbohydrates in the hydrolysates as compared with a control aqueous extract (pH 4.5) increased by factors of 15.6, 10.9, and 11.6 for (I), (II), and (III), respectively. Under these conditions, 1 g of biomass gave 0.430, 0.300, and 0.320 g of water-soluble carbohydrates as compared with 0.27 g for $H_2O$, and the amounts of water-soluble PSCs were 4.5, 25.0, and 24.8%. The absence of uronic acids from the last two hydrolysates enables us to regard these PSCs as pectin substances, which is also explained by the absence of pectolytic enzymes in preparations (II) and (III).

When the biomass was treated with the enzymes, unhydrolyzable residues remained (%): (I) 38, (II) 57, (III) 46. Subsequent treatment with 0.5 N $H_2SO_4$ at 115°C of the residues not hydrolyzed by the enzymes permitted an additional hydrolysis of the residues by 69% for (I), 72% for (II), and 69% for (III). In the hydrolysates, galactose, glucose, mannose, arabinose, xylose, and rhamnose were detected chromatographically.

Subsequent hydrolysis by Pektofoetidin P10× and sulfuric acid enabled the degree of degradation of the wastes to be raised to 88%, the corresponding levels for (II) and (III) being 84 and 85%, respectively.

More profound investigations of the repeated action of the enzymes mentioned on the unhydrolyzed residues or their combinations with other preparations will permit a more complete cleavage of the biomass of ginseng and other tissue cultures.

## EXPERIMENTAL

Initial Raw Material. The wastes from a ginseng tissue culture after the industrial extraction of the desired product (panaxosides) consists of a light brownish porous air-dry mass readily undergoing mechanical grinding – the biomass.

Enzymes: (I) — Pektofoetidin P10×; (II) Tsellyulaza P10×; (III) — Tsellokoningin P10×. Enzyme (I) is a multicomponent preparation containing as its main components an endoglucanase (3.2.1.4) and an exoglucanase (3.2.1.74). It also contains a number of hemicellulases and other enzymes. The preparation is capable of hydrolyzing cellulose-containing substrates to glucose, mannose, xylose, cellobiose, and other MSCs. Enzyme (II) consists of a protopectinase, a polygalacturonase (3.2.1.15), a pectin esterase (3.1.1.11), and a group of proteases (3.4) and cellulases (endo- and exoglucanases). Enzyme (III) has as its main components an endoglucanase (3.2.1.4) and an exoglucanase (3.2.1.74), and also a number of hemicellulases.

Method of Hydrolysis. The biomass (1 g) was treated with 45 ml of distilled water. Then the necessary pH of the media (4.0-5.5) was created by the dropwise addition with stirring of 0.2 N NaOH or 0.1 N HCl. After this, 5 ml of a 2% aqueous solution of the appropriate enzyme was added and, where necessary, the pH was readjusted to the necessary value. The control was 1 g of biomass in 50 ml of $H_2O$ at the same pH.

Hydrolysis was carried out with constant thermostating (45°C) and periodic stirring. The pH was monitored and strictly maintained throughout an experiment. After predetermined intervals of time, three parallel samples (0.25 ml) were taken from the hydrolysate to determine the dynamics of hydrolysis (from the passage of carbohydrates into solution). The samples were diluted fourfold with water and then 0.5 ml was taken from each and color was developed by the orcinol/sulfuric acid method. The concentration of carbohydrates was determined by photocolorimetry (the optical densities of the parallel determinations were averaged). After the end of hydrolysis the insoluble part was separated off by filtration, and the filtrate was kept for 1 h in the boiling water bath (for the inactivation of the enzyme) and was centrifuged at 6000 rpm for 30 min. The supernatant was stored in the refrigerator. The precipitate on the filter was washed with 100-150 ml of $H_2O$ and was dried at 55-60°C.

Procedure for Determining the Degree of Hydrolysis of the Biomass. The residue of unhydrolyzed biomass remaining on a previously weighed filter after washing (until MSCs ceased to appear in the wash-waters) was dried at 55-60°C to constant weight. Simultaneously, under the same conditions, five control filters previously wetted with water were dried. The weight of dry residue was determined by difference (averaged value, $p < 0.001$). The degree of hydrolysis was determined as the ratio of the weight of the substances that had passed into solution on hydrolysis to the weight of the initial sample (1 g). The weight of substance that passed into solution through the action of the enzymes on the biomass was determined as the difference between the total weight of the substances in the hydrolysate and the weight of the soluble substances in the control aqueous extract.

The total concentration of carbohydrates in the hydrolsates was determined by the orcinol/sulfuric acid method [15]. After fourfold dilution of a 0.25-ml sample of a hydrolysate (three samples in parallel), a 0.5-ml portion was taken and to this was added 5 ml of a 2% aqueous solution of orcinol and 3.6 ml of 60% (by volume) $H_2SO_4$. The mixture was heated at 100-105°C for 1 h. The intensity of coloration was determined on a photoelectric colorimeter at $\lambda$ 520 nm. The values of the intensities were averaged ($p < 0.05$). The concentration of carbohydrates was determined from a calibration curve (for glucose) on the basis of the average values of the intensity of coloration of the samples. The concentration of carbohydrates that had passed into solution through the action of the enzymes in the biomass was determined as the difference between the total concentration of carbohydrates in the hydrolysate and the total concentration of carbohydrates in the control (aqueous extracts).

The calibration curve was plotted on the basis of three independent dilutions of glucose (from 1.5 to 0.01%). The intensity of the coloration was determined in the way described above.

Procedure for Chromatographic Analysis. A. Fractionation of the Hydrolysates on Sephadex G-50. A glass column (20 × 600 mm) filled with Sephadex was charged with 5 ml of hydrolysate or the control aqueous extract. Elution was performed with a buffer [pyridine: $CH_3COOH$:$H_2O$ (4:10:986)]. The fractions were obtained in a collector (with a 2.15-ml siphon) after 25 ml of buffer (the free volume) had been discarded. The substances were detected by the orcinol/sulfuric acid method. Graphs were plotted from the intensities of the coloration (not less than three independent determinations). The ratio of MSCs, oligosaccharides (OSCs), and PSCs was determined from the averaged values of the areas of the peaks ($p < 0.05$).

B. Descending Paper Chromatography. The MSCs from the hydrolysates were chromatographed with markers (glucose, galactose, mannose, rhamnose, xylose, sucrose, cellobiose, and glucuronic and galacturonic acids) on FN-8 paper in the ethyl acetate–pyridine–water (8:2:1) system [16]. The chromatograms were stained with aniline phthalate followed by thermostating at 105-110°C (15-25 min). The amount of hydrolysates deposited was 0.04 ml and the $R_f$ values were calculated for the MSCs determined in relation to glucose, which gave good agreement with literature figures [16]. The determination of the MSCs was only roughly quantitative and bore a preliminary nature.

C. Gas–Liquid Chromatography. The MSCs were analyzed in the form of polyol acetates [17-19], details of the method of obtaining which we have described previously [20], on a Chrom-5 chromatograph (Czechoslovakia)–FID; glass columns (3 × 2500 mm) filled with Chromaton NAW-DMCS (0.125-0.160 mm) with 5% of SE-60; temperature of the detector and the evaporator 260°C and of the column thermostat 218°C; rate of flow of carrier gas (nitrogen) 30 ml/min, and of air 300 ml/min. Acetates of the polyols of mannose, rhamnose, glucose, etc., prepared by the procedure indicated above, were used as markers.

Exhaustive Acid Hydrolysis of the Biomass Residues. The residue (0.5 g) of the biomass of a ginseng tissue culture after treatment with the corresponding enzymes was treated with 20 ml of 0.5 N $H_2SO_4$ and the mixture was thermostated at 115°C with stirring for 6-9 h. After the end of hydrolysis, the reaction mixture was neutralized with NaOH. The unhydrolyzed part was carefully washed (20-fold volume of water), filtered off and, after drying, weighed. The degree of acid hydrolysis was determined by difference.

## CONCLUSIONS

1. The optimum conditions for the hydrolysis of industrial wastes of the biomass from a ginseng tissue culture using complex enzyme preparations have been selected.

2. The carbohydrate compositions of the hydrolysates have been studied by chromatographic methods.

3. It has been shown that the treatment of the wastes with Pektofoetidin P10× (I) permits them to be hydrolyzed by 62%, with Tsellyulaza P10× (II) by 43%, with Tsellokoningin P10× (III) by 54%. The subsequent treatment of the residues with mineral acids permits the total degree of hydrolysis to be raised to 84-88%.

4. Preliminary grinding of the biomass does not lead to an increase in the degree of hydrolysis.

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# SYNTHESIS AND ISOLATION OF MONOESTERS OF SUCROSE AND ARACHIDONIC ACID

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UDC 547.396:658.567

Monoesters of sucrose and arachidonic acid have been synthesized by the transesterification of ethyl arachidonate with unprotected sucrose, and the structural isomers – 6-O-arachidonoylsucrose, 6'-O-arachidonoylsucrose, and 3-O-arachidonoylsucrose – have been separated chromatographically. The positions of the ester bonds in these compounds were determined by the $^{13}C$ NMR method.

The esters of sucrose and higher fatty acids are finding use in pharmacy, the food industry, cosmetics, and other sectors of the national economy as nonionic surface-active agents [1-4]. The use of these compounds in medicine may also prove to be promising due to their antimicrobial and antitumoral activity [5-8]. The preparation of esters of sucrose and saturated or slightly unsaturated fatty acids or mixtures of higher fatty polyenoic acids has been described [1, 4, 9]. However, it is known that with an increase in the degree of unsaturation of the hydrocarbon moiety the solubility of esters of sucrose in water rises [1], which is important for their use in medicine. Furthermore, interest in the preparation of esters of sucrose and polyunsaturated fatty acids is also due to the fact that these compounds may prove to be more active because of the high biological activity of the free polyunsaturated fatty acids [10].

We have synthesized monoesters of sucrose and arachidonic acid with the subsequent isolation of the individual monoesters.

Arachidonic acid obtained from the lipids forming waste products from the production of insulin [10] was esterified with absolute ethanol in the presence of a catalytic amount of thionyl chloride [11]. To obtain monoesters of sucrose and arachidonic acid, which possess a higher solubility in water than di- and polyesters of sucrose, we used the transesterification of ethyl arachidonate with an eightfold excess of unprotected sucrose in N,N-dimethylformamide in the presence of an alkaline catalyst. Here the different reactivities of the hydroxy groups in the sucrose were taken into account. To separate the resulting monoesters from the unchanged sucrose after the elimination of the dimethylformamide we used extraction with ethyl acetate. The use of ethyl acetate was due to the good solubility of the monoesters in it and also to its rapid separation from the aqueous phase. To free the monoesters from unchanged ethyl arachidonate, the sodium arachidonate formed, and dimethylformamide residues we used adsorption chromatography on silica gel.

The purified reaction product had a band of vibrations at 1730 $cm^{-1}$ in the IR spectrum, which corresponds to an ester bond, and was an individual substance according to TLC on Silu-

All-Union Scientific-Research Institute of the Technology of Blood Substitutes and Hormone Preparations, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 499-502, July-August, 1988. Original article submitted November 9, 1987.