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ОРИГИНАЛЬНЫЕ СТАТЬИ

Original article

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Isolation of protein-polysaccharide conjugates *from Solanum tuberosum L.* rootlets by extended reversed-phase flash chromatography

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Abstract. Polysaccharides and their conjugates with various biologically active compounds (proteins, nucleic acids, peptides etc.) are of great importance, they take a part in various biological processes. The one of the important feature of polysaccharides is possibility to form wide variety of branched structures with different molecular masses and, thus, they can form very complicated structural conjugates with wide range of biologically active compounds. Cells of tubers of cultivated potato (*Solanum tuberosum L.*) contain high amounts of polysaccharides conjugated with proteins and nucleotides. In this work we developed fast and efficient chromatographic procedure for the isolation of protein-polysaccharide conjugates from the rootlets of *Solanum tuberosum L.* It consisted of double-cartridge flash-chromatographic system, filled with the cross-linked macroporous styrene-divinylbenzene and C18 stationary phases. Isolated products were characterized by different analytical methods, including HPLC (reversed phase and size exclusion chromatography), gas chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (determination of proteins). Some structural features were determined by means of IR spectroscopy in the region characteristic for saccharides (1250-750 cm^{-1}). It could be concluded, that isolated conjugates consisted of arabinogalactane polysaccharide and protein with average MM 60 kDa. Analytical data obtained for both products, isolated using conventional precipitation method and flash-chromatographic procedure were similar.
Keywords: protein-polysaccharide conjugates, flash chromatography, high performance liquid chromatography.
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Научная статья

Выделение белково-полисахаридных коньюгатов из проростков *Solanum tuberosum L.* методом обращенно-фазовой флэш-хроматографии

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Аннотация. Полисахариды и их конъюгаты с различными биологически активными молекулами (белками, нуклеиновыми кислотами, пептидами и др.) имеют большое значение, поскольку принимают участие в различных биологических процессах. Одной из важных особенностей полисахаридов является способность образовывать разветвленные структуры с различной молекулярной массой, которые могут образовывать очень сложные структурные конъюгаты с широким спектром биологически активных

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соединений. Клетки проростков картофеля (*Solanum tuberosum L.*) содержат значительное количество полисахаридов, связанных с белками и олигонуклеотидами.

В данной работе разработана быстрая и эффективная хроматографическая методика выделения белково-полисахаридных конъюгатов из проростков *Solanum tuberosum L.*, состоящая из двухкартриджной флэш-хроматографической системы, заполненной стационарной фазой С18. Выделенные продукты были охарактеризованы комплексом аналитических методов, включая ВЭЖХ (обращенно-фазовая и эксклюзионная хроматография), газовая хроматография, электрофорез в полиакриламидном геле с додецилсульфатом натрия для определение белков. Структурные характеристики определяли методом ИК-спектроскопии в характеристичной для сахаридов области (1250-750 $см^{-1}$). Сделано заключение, что выделенные конъюгаты состоят из арабиногалактанового полисахарида и белка со средней ММ 60 кДа.

Аналитические данные, полученные для обоих продуктов, выделенных традиционным осадительным методом и при помощи флэш-хроматографии, позволяют сделать вывод, что белково-полисахаридные конъюгаты имеют близкий состав.

Ключевые слова: протеин-полисахаридные коньюгаты, флэш-хроматография, высокоэффективная жидкостная хроматография.

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Introduction

Polysaccharides constitute a structurally diverse class of polymers. Their molecules are composed of monosaccharide residues linked by glycosidic bonds. Since polysaccharides possess high structural variability (i.e., they can form a wide variety of branched structures with different molecular masses), they participate in numerous biological processes. Unlike nucleotides that form nucleic acids, and amino acids in proteins (which can interact with specific sites in a living organism only in a certain manner), polysaccharides and their conjugates can take part in various types of interactions. Polysaccharides and their derivatives are involved in signal recognition and cell–cell communication; they play key roles in the functioning of the immune system, fertilization, disease prevention, and blood clotting [1, 2].

Tubers of cultivated potato (*Solanum tuberosum L.*) contain high amounts of polysaccharides conjugated with proteins and nucleotides, as well as polyphenols, vitamins, phenolic acids, chlorogenic acid, cafeic acid, ferulic and *p*-coumaric acids, flavonoids (quercetin, kaempferol, rutin, and catechin), etc. These substances are important for the metabolic processes of human organism [3, 4]. It should be noted that the highest amounts of biologically active compounds are contained in potato rootlets.

The traditional procedure for the isolation of polysaccharides and their conjugates with proteins from the plant raw materials is rather complicated and involves liquid extraction and fine low-pressure chromatographic purification [5-7]. One of the simplest and most effective techniques for chromatographic separation and isolation of target compounds is flash chromatography, which provides a means for a fast efficient fractionation of biologically active compounds with similar properties [6].

The goal of this study was to develop a fast and efficient chromatographic procedure for isolation of protein-polysaccharide conjugates from the rootlets of *Solanum tuberosum L.* and to reveal the composition of the obtained polysaccharides.

Experimental

Materials. The rootlets were obtained from the “Novinka” white-skin potato variety (Novgorod, Russia). They were air-dried under permanent heat ventilation, and the resulting material was ground. The samples were stored at room temperature before use.

Extraction of water-soluble protein-polysaccharide conjugates and related compounds. Polysaccharides and the related water-soluble biologically active compounds

were extracted according to the modified protocol described in [8]. Dry rootlets (20 g) were mixed with 500 mL of distilled water, and the mixture was stirred vigorously for 1 h at 70°C. The suspension was filtered, and the solution was used in the experiments. The extraction procedure was performed twice to provide the maximal recovery of the water-soluble components. The combined filtrates were concentrated using a rotary evaporator at 50°C in order to increase the concentration by a factor of 20. The resulting solution was centrifuged at 15 000 rpm and freeze-dried.

Flash chromatography. The flash chromatographic unit (fig. 1) consisted of the self-packed cartridge for pre-concentration (10 ml of hyper cross-linked styrene-divinylbenzene phase MN-202, (pore volume 220A, pore volume 0.3 mg/cm^3, surface area 950 m^2/g, 80 μm, (Purolite, Great Britain)) with two polypropylene frits and the self-packed cartridge for fine purification (5 cm^3 C18 phase (10 μm) with two polypropylene frits). The flow rate of 0.2 M NaCl- 0.01% trifluoroacetic acid (pH 2.2) elution system was 1 cm^3/min; the flow was initiated with an LKB 12000 peristaltic pump (LKB, Sweden). A sample (50 mg) was dissolved in water (2 cm^3) and injected into the system with a syringe using a three-way tap. The UV absorption at 254 nm was monitored using an LKB 8300 UVCorp II UV detector (LKB, Sweden). The data were processed with the "MultiChrom" software (Ampersand, Russia). The fractions were collected with the aid of a FRAC-10 fraction collector (Pharmacia, Sweden). The isolated products were dialyzed against water and freeze-dried.

FTIR, HPLC, GC, and gel electrophoresis. FTIR spectra in the 4000-400 cm^{-1} range were acquired using an IR-8400 S Fourier transform infrared spectrophotometer (Shimadzu, Japan) in KBr pellets an using ATRP 8000A accessory for neat samples. Chromatographic analysis was performed with the use of a Smartline HPLC instrument (KNAUER, Germany) equipped with refractometric and spectrophotometric detectors. Chromatographic profiles, molecular masses and other parameters of the products were obtained using the ClarityChrom GPC/SEC software (Germany). A PSS Suprema Lux SEC column (dimensions: 7.8×300 mm) (Germany) with a pre-column (dimensions: 0.6×40 mm) was used to estimate molecular masses of protein-polysaccharide conjugates. Sodium chloride solution (0.2 M) was selected as an eluent; pullulans were used as standards for calibration. Tessek C18 columns (3×150 mm, 5 μm) (Czech Republic) were used in fraction analysis; elution was performed with a linear gradient in water–phosphate buffer system (pH 2.4) (0-100%, 15 min).

Gas chromatographic analysis was performed with the aid of a 7820A unit (Agilent Technologies, France) that included a quartz capillary column (dimensions: 30 m×

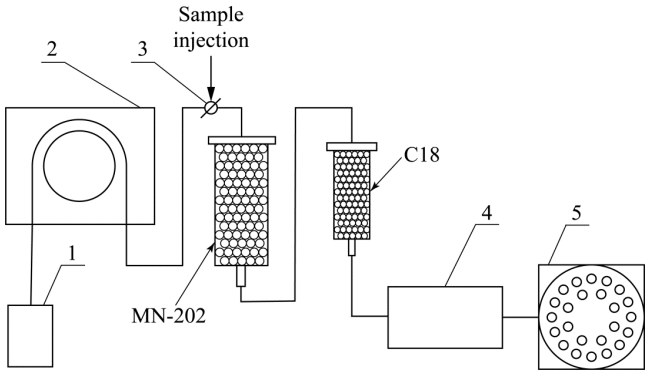

Fig. 1. Experimental setup of the protein-polysaccharide two-step isolation process: 1 – solvent vessel, 2 – peristaltic pump, 3 – tree way injection tap, 4 – UV detector, 5 – fraction collector.

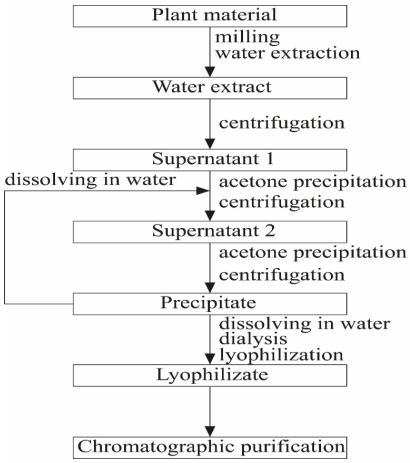

Fig. 2. Isolation of protein-polysaccharide conjugates from plant raw material.

0.32 mm) packed with the NR-20 stationary phase and a flame ionization detector ($T_{detection}$=300°C, $T_{injection}$=290°C, carrier gas: helium, flow rate: 40 cm^3/min). Conjugate was preliminary hydrolyzed with trifluoroacetic acid in a sealed ampoules and sialylated. Arabinose, inosite, glucose, galactose, and fructose were used as standards using sialylation protocol. Inositol was used as an internal standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli method [9] on the plates with the dimensions 10×10.5 cm coated with a minigel layer 1.5 mm thick. A Mighty Small electrophoresis unit (Mighty Small II, Hoefer Pharmacia Biotech Inc., San Francisco, CA) was used in SDS-PAGE analysis. The concentrations of polyacrylamide in the resolving gel were 7.5 and 10.0%; its concentration in the stacking gel was 4%. These gels were prepared with the use of 0.375 M Tris-Cl (pH 8.8) and 0.125 M Tris–Cl (pH 6.8) solutions, respectively. The electrophoresis buffer (pH 8.3) contained Tris base (0.02 M) and glycine (0.192 M). All buffers contained 0.1% of SDS. A typical separation process lasted for 4 h at 10°C with a current of 20 mA per gel.

Results and discussion

The key issue in isolation of polysaccharides and their conjugates from plant raw materials is separation of target products from the highly cross-linked matrix that is deposited on cellular wall during suberisation and consists of various lipid polymers, suberins, and lignin-like phenolics [10]. The conventional extraction methods involving subsequent re-precipitation of the target products are characterized by low reproducibility and low yields; as a rule, additional chromatographic purification of the products is required. Using a conventional procedure for the isolation of protein-polysaccharide conjugates (fig. 2) we isolated such conjugate in fairly low yield which was used as a reference in proposed separation process.

In this work, a simple and efficient low-pressure flash chromatographic unit was developed with the purpose of isolation of protein-polysaccharide conjugates from the aqueous extract that contained water-soluble components of potato rootlets. The setup includes two cartridges; the first cartridge filled with hypercrosslinked biporous styrene-divynilbenzene sorbent is intended for sorption of the cross-linked refuse matrix. The second cartridge serves for fine separation of the target fractions. A characteristic flash chromatography profile is presented in fig. 3 (A). The SEC, IR, and electrophoresis data indicate that the first fraction contains proteins with the average MM equal to 50-70 kDa; the second broad peak can be attributed to the protein-polysaccharide conjugate, and the third fraction contains unidentified washout.

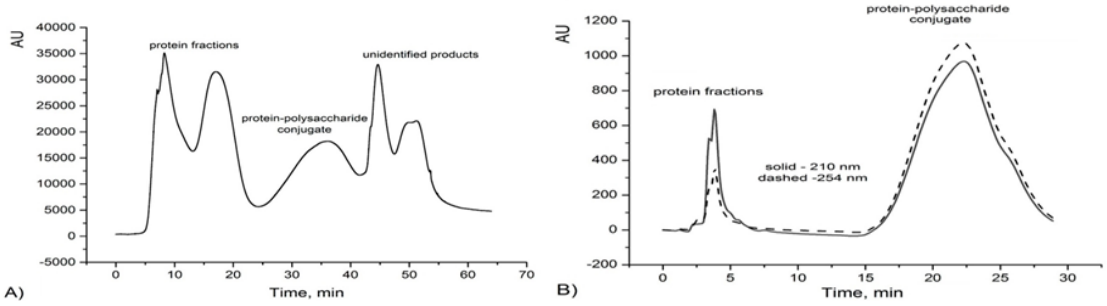

Fig. 2. Characteristic flash chromatography (A) and HPLC (B) profiles obtained using the C18 stationary phase. Linear gradient elution (eluent: water– phosphate buffer system (pH 2.4)).

The fractions were compared using reversed-phase HPLC performed under identical elution conditions (linear gradient, water – phosphate buffer system (pH 2.4)). A typical HPLC profile of the protein-polysaccharide conjugate is presented in fig. 3 (B). It is suggested that the protein and polysaccharide components are not linked by covalent bonds, which evidenced from HPLC data in different buffer elution systems (phosphate, citrate). Apparently, they are associated due to some non-specific interactions, because the ratio between intensities of the two main peaks in the HPLC profile depends on pH of the medium.

The results obtained by IR spectroscopy, gas chromatography (GC), size exclusion chromatography, and gel electrophoresis were used to suggest possible composition of the investigated protein-polysaccharide conjugate.

The most intensive bands in the IR spectra were used as “fingerprints”. The characteristic bands at 1725 and 1648 cm^{-1} are attributed to υ_sCOO and υ_sNHC(O) vibrations of proteins. The 1200-950 cm^{-1} region is characteristic of combined vibrations of saccharide ring (C-OH, C-O-C, C-C); the anomeric region (950-750 cm^{-1}) is attributed to the overlapped C-O and C-C stretching vibrations of glycoside and pyrane rings.

The complicated area of IR spectrum (1250 750 cm^{-1}) was interpreted using monosaccharide standards. Thus, the intense band at 979 cm^{-1} can be assigned to glucuronic acid; the bands at 1073 cm^{-1} and 883 cm^{-1} are related to galactose; the peak at 1136 cm^{-1} is attributed to glucose; the complex pattern near 1200 cm^{-1} is assigned to combined C-OH, C-O-C, C-C vibrations of various saccharide residues (arabinose, glucose, mannose) [11, 12].

The IR data agree well with the results of GC analysis. The GC data allowed us to evaluate percentages of monosaccharides (after hydrolysis of conjugate and sialylation of products) in the studied samples. The samples contain arabinose (17.4%), galactose (23.8%), glucuronic acid (13.1%), galacturonic acid (14.6%), xylose (2.5%), fructose (1.6%), and glucose (9.6%). Thereby, it may be concluded that the isolated polysaccharide could be assigned to the class of branched arabinogalactans.

The molecular mass of the isolated protein-polysaccharide conjugate was evaluated by SEC with the use of pullulan standards; the peak value was equal to 90 kDa. The molecular mass of the protein component was estimated by SDS-PAGE using the standard protein kit and was found to be 60 kDa. Analytical data obtained for both products, isolated using conventional precipitation method and flash-chromatographic procedure were similar.

Conclusions

A low-pressure flash chromatographic system was developed for the isolation of protein-polysaccharide conjugates from potato rootlets. The FTIR, HPLC, GC, and gel electrophoresis data were used to propose probable composition of the conjugates consisting of arabinogalactane polysaccharide and protein with average MM 60 kDa.

Конфликт интересов

Авторы заявляют, что у них нет известных финансовых конфликтов интересов или личных отношений, которые могли бы повлиять на работу, представленную в этой статье.

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