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EXTRACELLULAR HYDROXYPROLINE-RICH GLYCOPROTEIN OF SUSPENSION-CULTURED TOBACCO CELLS

HIDETAKA HORI*† and SITIRO SATO‡

†Department of Botany, Faculty of Science, Hokkaido University, Sapporo 060, Japan;
‡Department of Botany, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

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Abstract—Hydroxyproline-rich glycoprotein was isolated from the used media of tobacco XD-6 cells cultured in suspension and purified by repeated ion exchange- and gel filtration-chromatography. The preparation was judged to be homogeneous from ultracentrifugation and isoelectric focusing. The dry wt of the glycoprotein was composed of 94% polysaccharide and 6% protein. Hydroxyproline accounted for 23% of the amino acids in the protein moiety. The polysaccharide moiety consisted of 44% galactose, 30% arabinose, 5% rhamnose, and 21% uronic acid. About 10% of the uronic acid residues were present as methyl esters.

INTRODUCTION

It is known that in higher plants cell walls have hydroxyproline containing glycoproteins. The glycoproteins are the main component of noncellulosic macromolecules in the cell wall along with polysaccharides. Researchers in many botanical fields have investigated the glycoproteins [1–7].

It is also well known that hydroxyproline-containing macromolecules accumulate in the media when plant cells are cultured in suspension [8–10]. Although knowledge of the glycoproteins has increased, types of molecules constituting the glycoproteins have not yet been made clear, nor is the relation of both known. Difficulties may occur in purifying the cell wall components, since chemical and enzymic treatment used in the extraction result in the destruction of the chemical structure of the components.

Separation of the glycoproteins from the used media is comparatively easy and this is a favourable method for studying not only glycoprotein accumulating in media but also those situated in cell walls. In order to determine which kinds of glycoproteins accumulated in the media, we purified these using ion exchange- and gel filtration-chromatography. In this paper we present the results of such analyses.

RESULTS AND DISCUSSION

The crude extract from the culture medium was applied to a DEAE-cellulose column and the macromolecules separated into 3 fractions. The first was eluted at a non-absorbing region, a second with 70 mM NaCl, and a third with 250 mM NaCl. The second fraction contained hydroxyproline, pentose, hexose and uronic acid, neither of the other two fractions contained hydroxyproline. Since it was rich in uronic acid, the third fraction seemed to a pectic substance. The second fraction was concentrated by evaporation and applied to Sephadex G-200. The hydroxyproline-containing macromolecules (hyp-macromolecules) appeared at the void volume as a single peak. Pentose, hexose, and uronic acid were also detected in this region. The hydroxyproline-containing eluate was concentrated and applied to a Sepharose 4B column. The hyp-macromolecules appeared again at the void volume as a single peak together with pentose, hexose, and uronic acid. In order to check whether the hyp-macromolecules which passed through the Sepharose 4B column were in fact a single substance or not, they were examined again on a DEAE-cellulose column. The preparation produced a single peak and eluted at 70 mM NaCl. In this fraction, pentose, hexose, and uronic acid were also detected. The protein peak (A at 280 nm) coincided in position with the hydroxyproline peak, (thus, this preparation is thought to be a hydroxyproline-containing glycoprotein 'hyp-glycoprotein'). The eluate was dialyzed against water for 24 hr. The dialysate designated as the purified extract was examined using isoelectric focusing chromatography in 100 ml of 0 to 50% discontinuous sucrose gradient with 2.4% carrier ampholytes (LKB, 40% pH 3.5–10). The hyp-glycoprotein was eluted as a single symmetrical peak at pH 2.5. The purified extract solution was altered to 0.15M NaCl solution by addition of solid NaCl and then centrifuged at 59 800 rpm at 19.4° with Spinco model E (Beckman). A single symmetrical peak is observed. The $S_{20,w}$ value was calculated as 5.8×10^{-13}/sec. Results indicate that this hyp-glycoprotein may be homogeneous in regard to molecular size. The MW of the hyp-glycoprotein was estimated to be larger than 5×10^6 from the Sepharose 4B-gelfiltration. However it is difficult to determine the MW from the elution point of a gel filtration, since the elution point varies erratically according to the molecular

*Present address: Department of Biological Science, University of Tsukuba, Sakura-Mura, Ibaraki 300-31, Japan.

Table 1. Protein and sugar content of the purified hyp-glycoprotein

Component	Amount of component (μg)	Recovery of component (%)	Relative recovery of component (%)
Original material	7270	(100)	—
Protein	436	6	6
Galactose	2850	39	41
Arabinose	1940	26	28
Rhamnose	307	4	5
Uronic acid	1370	19	20
Total recovery	6900	95	100

shape. Assuming that the glycoprotein has a rod like shape, the MW is estimated to be *ca* 600 000 from the S value.

The purified hyp-glycoprotein was analyzed for protein and sugar composition (Table 1). The preparation was composed of 94% polysaccharide substances and 6% protein. The sugar moiety was composed mainly of galactose, arabinose and a small amount of rhamnose. Uronic acid (as galacturonic acid) accounted for *ca* 20% of the total amount of sugars. About 10% of the uronic acid residues were present as Me esters.

Some researchers have reported that the sugar moiety of the macromolecules containing hydroxyproline extracted from culture media of plant cells is composed of galactose, arabinose, xylose, mannose and a small amount of fucose, rhamnose, and uronic acid [8, 10]. However our purified hyp-glycoprotein was composed only of galactose, arabinose, rhamnose, and uronic acid. Extracellular macromolecules which accumulated in culture medium contain such molecules as extracellular enzyme, pectic substances, xyloglucan, glycomannan, and others. Consequently, to clarify the composition of the hyp-glycoproteins it is essential to purify them using 'non severe' techniques.

Results of amino acid analysis are given in Table 2. Hydroxyproline accounted for 24% of the total number of amino acids. Serine, which plays the role of binding-site to sugar side-chains [5], and threonine, which is thought to be have the same function [2], accounted for 15% and 9% respectively. A high content of alanine (21%) is one of the characteristics of the hyp-glycoprotein. Our results agree with those of amino acid analysis, and protein- and sugar-content with the exception of proline, reported by Lamport [2] on hydroxyproline-rich protein-polysaccharides, extracted from TCA-soluble cytoplasmic fractions. One of the authors also obtained hydroxyproline-containing macromolecules from ethanol-insoluble cytoplasmic fractions of cultured tobacco cells. These macromolecules are also similar to the purified hyp-glycoprotein in sugar and protein content (unpublished data). The relation between extracellular and intracellular hyp-glycoproteins is not known.

Table 2. Amino acid-composition of the purified hyp-glycoprotein. Amino acids are expressed as number of residues per 1000 recovered amino acids

Amino acid	Residues/1000
Aspartic acid	33
Threonine	93
Serine	148
Glutamic acid	53
Hydroxyproline	238
Proline	53
Glycine	58
Alanine	213
Cystein	not detected
Valine	55
Methionine	5
Isoleucine	9
Leucine	19
Tyrosine	trace
Phenylalanine	not detected
Lysine	21
Histidine	trace
Arginine	not detected

Recently, Jermyn and May Yeow [11] studied the lectins extracted from the seeds of both angiosperms and gymnosperms. Some of these lectins closely agree with extracellular hyp-glycoprotein in regard to amino acids composition, carbohydrate: protein ratio and sugar composition. Kauss and Bowles [12] speculated that a lectin plays an important role in the assembly of cell wall carbohydrates and that a decrease of lectin activity brings a loosening of the cell wall.

EXPERIMENTAL

Cell culture and extraction procedure. Tobacco XD-6 cells were cultured in 500 ml flasks each containing 120 ml of modified Murashige and Skoog's medium for 7 to 10 days as previously described [13]. The media used (2.5 l.) was separated from the cells by filtration. An equal vol. of cold EtOH was added to the filtrate and the mixture allowed to stand 18 hr at 2°. The resulting ppt. was collected by centrifugation and suspended in 30 ml H_2O. This suspension was concd to 6 ml at 37° and resuspended in 50 ml of M NaCl using a homogenizer. Insoluble residues were sedimented by centrifugation and discarded. NaCl extraction was repeated ×3. The extract was concd to *ca* 40 ml at 37°, dialyzed against 5 l. of 10 mM Tris–HCl (pH 8.5) for 30 hr at 4° during which the buffer was changed ×3; the dialysate was designated as the crude extract.

Column chromatography. DEAE-cellulose was equilibrated with 10 mM Tris–HCl (pH 8.5) and eluted using a linear gradient of NaCl in the same buffer (0–0.8 M, 400 ml or 0–0.5 M, 200 ml). Sephadex G-200 and Sepharose 4B were equilibrated with the same buffer, and eluted at a flow rate of 10 ml/hr.

Chemical analysis. Hydroxyproline was determined by the method of ref. [14] after hydrolysis of the macromolecules according to ref. [15]. Pentose was determined by the method of ref. [16], using arabinose as standard. Hexose was determined by the method of ref. [17] using glucose as standard. Uronic acid was determined by the method of ref. [18], using galacturonic acid as a standard. The degree of esterification of uronic acid was determined by a method of ref. [19]. Protein was determined by the method of ref. [20], using BSA as standard.

GLC. For determination of neutral sugar content, the sample was hydrolyzed with N H_2SO_4 in a sealed tube at 110° for 4 hr. The hydrolysate was neutralized with satd $Ba(OH)_2$ and centrifuged to sediment insoluble $BaSO_4$. The supernatant was passed through a Dowex 50 WX4 (H^+) column. Neutral sugars in the eluate were determined by GLC.

Determination of amino acids. The same sample was hydrolyzed with 6N HCl in a sealed tube at 110° for 20 hr. The hydrolysate was dried, dissolved in H_2O, and passed through a Dowex 50 WX4 (H^+) column. Amino acids were applied to an amino acid analyzer.

Ultracentrifugation analysis. Sedimentation velocity expts were conducted using a Beckman-Spinco Model E.

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