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ABSTRACT

Graminaceous monocots generally contain low levels of hydroxyproline-rich glycoproteins (HRGPs). As HRGPs are often at the cell surface, we used the intact cell elution technique (100 millimolar AlCl₃) to isolate soluble surface proteins from Zea mays cell suspension cultures. Further fractionation of the chloroformic acid-soluble eluate on the cation exchangers phospho-cellulose and BioRex-70 gave several retarded, hence presumably basic fractions, which also contained hydroxyproline (Hyp). One of these fractions yielded a pure HRGP after a final purification step involving Superose-6 gel filtration. As this HRGP was unusually rich in threonine, (25 mole%) we designated it as a threonine-hydroxyproline-rich glycoprotein (THRG); it contained about 27% carbohydrate occurring exclusively as arabinosylated Hyp, predominantly as the monosaccharide (15%), and trisaccharide (25%) with 48% Hyp nonglycosylated—a characteristically graminaceous monocot profile. Amino acid analysis confirmed the basic character, and gave a low alanine content. Reaction with Yariv artificial antigen was negative. These character-

Current widespread interest in structural HRGPs stems from their localization in the extracellular matrix and their possible roles in the growth regulation (3, 7), stress response (5, 9, 17, 19, 22, 23) cell-cell recognition (6, 22) and reproductive physiology (6). Understandably, work has concentrated on the richest sources of HRGPs which are: dicotyledonous higher plants (14, 22) and volvocalean algae (21). Monocotyledonous plants, in particular the GMs, are generally hydroxyproline-poor (14) and their HRGPs have been virtually ignored, yet monocots represent a highly significant group of angiosperms, not least because they contain the cereals which sustain civilization.

We report here the first isolation and partial characterization of an HRGP from a graminaceous monocot, Zea mays. We also provide evidence that this HRGP may be an extensin, a class of HRGP well known as a major structural component of the dict primary cell wall. This HRGP is unusually rich in threonine, and hence we refer to it as a THRG.

MATERIALS AND METHODS

Suspension Cultures. We grew maize cell suspensions (variety 'Black Mexican', a gift from Dr. Tom Hodges, Purdue University), in 1 L flasks containing 500 ml Murashige and Skoog medium (20) (2 mg/L, 2,4-D) shaken at 120 rpm on a gyratory shaker at 27°C under 900 lux of constant fluorescent lighting, and subcultured every 11 d to an initial packed cell volume of 3%.

Cell Wall Preparation. We prepared maize cell walls by suspending the cells in distilled H₂O, cooling in an ice bath, and sonicating the cells 10 min followed by alternating H₂O washes and pelleting the walls by bench-top centrifugation until the walls were free of cell debris, judging by microscopic examination. Next, we boiled the walls 5 min in 5% SDS, removing the SDS via alternating acetone washes and pelleting in a bench-top centrifuge followed by water washes and lyophilization.

Hydroxyproline Assay. We determined hydroxyproline after hydrolysis (6 N HCl, 110°C, 18 h) by Kivirikko's method (12) which involves alkaline hypobromite oxidation and subsequent coupling with acidic Ehrlich's reagent and monitoring at 560 nm.

Hydroxyproline Arabinoside Profile. We determined Hyp arabinosides (16) after alkaline hydrolysis (0.44 N Ba(OH)₂; 18 h, 105°C) careful neutralization with concentrated H₂SO₄, followed by centrifugation and freeze-drying the supernatant fraction. We dissolved the lyophilate in distilled H₂O and applied 0.5 mL containing 100 to 200 µg hydroxyproline to a 75 × 0.6 cm column (H⁺ form) of Technicon Chromobeads C washed with water and eluted with a 0 to 0.5 N HCl gradient, monitoring the post-column hydroxyproline assay reaction at 560 nm.

Preparation of Crude HRGP by Intact Cell Elution. We prepared batches of crude THRG from 11-d cultures (575 ml/flask; 17 flasks/batch) harvested on a 2 L coarsely sintered funnel followed by a water wash and then gentle agitation of the cell pad (about 600 g fresh weight) in 1 L of 100 mM AlCl₃ (a nonplasmolyzing concentration) for 3 min before final suction. The eluate was reduced in volume to 100 ml at 32°C. After adding TCA (final concentration 10% w/v) to the concentrated eluate (18 h, 4°C), centrifugation (13,000g, 45 min, SS-34 rotor head) yielded a hydroxyproline-poor pellet (0.5% Hyp dw, discarded) and hydroxyproline-rich supernatant which was dialysed 36 h at 4°C and then freeze-dried. We designate this TCA-soluble fraction 'crude HRGP.'

Phospho-Cellulose Ion Exchange Chromatography. We dissolved crude HRGP (10 mg/ml) in 12 mM (pH 3.0) McIlvaine buffer (18), and applied a maximum of 30 mg to a Bio-Rad
Cellex-P phospho-cellulose column (8 mm i.d. × 100 mm) equilibrated with 12 mM McIlvaine buffer (pH 3.0). We eluted with a 3.0 to 6.8 pH gradient (in McIlvaine buffer) followed by a 0 to 1 M NaCl gradient in 15 mM (pH 6.8) McIlvaine buffer at a flow rate of 19 ml/h, monitoring the absorbancy at 220 nm.

BioRex-70 Ion Exchange Chromatography. We dissolved the Cellex-P Peak 2 in 2 ml 30 mM sodium phosphate buffer (pH 7.4) buffer and applied a maximum of 20 mg to a BioRex 70 (100–200 mesh) column (8 mm i.d. × 100 mm) equilibrated with 30 mM sodium phosphate buffer (pH 7.4), and eluted with a buffered 0 to 1 M NaCl gradient at a flow rate of 19 ml/h, monitoring the absorbancy at 220 nm.

Gel Permeation Chromatography. We injected 1 mg of semi-purified THRGP in 250 μl 200 mM (pH 7.0), 0.02% azide-sodium phosphate buffer onto a Pharmacia Superose-6 HPLC gel filtration column, and eluted at a flow rate of 14 ml/h, monitoring the absorbancy at 220 nm.

Amino Acid Analysis. We used a Pickering High Speed Na+ cation exchange column (3 mm i.d. × 150 mm) in series with a BX-8 cation exchange column (3.7 mm i.d. × 70 mm, Benson Co.), eluted by Pickering buffers A, B, and C. Postcolumn fluorometric detection involved NaOCl oxidation and OPA coupling which allowed Hyp and Pro detection (24). Data capture was by an IBM 9000 computer with IBM CAPS software.

Sugar Analyses. We analyzed sugars as their alditol acetates (1) by GC using a 6 foot × 2 mm i.d. PEG224 column programmed from 130 to 180 at 4°/min for neutral sugars and a 6 foot × 2 mm i.d. OV 275 column programmed from 130 to 230 at 2°/min for amino sugars using an SP4100 computing integrator for data capture.

SDS Gel Electrophoresis. (Based on Laemmli and Favre [13].) We loaded 2 to 30 μg of THRGP to the 'sepracomb' of commercially prepared Sepra-gel gradient (10–20% polyacrylamide, Separation Science, Inc.) We detected protein by silver staining (30).

TEM Sample Preparation. We prepared THRGP for TEM by following the methods of Tyler and Branton (27).

Precipitation with β-Glycosyl Yariv Antigen. We reacted 400 μg of THRGP with Yariv Antigen according to Jermy and Yeow (10).

THRGP Reaction with Polyclonal Antibodies Raised Against Tomato Extensin Precursors. We determined cross-reactivities of antibodies raised against tomato extensin precursors (11) with the THRGP by ELISA (based on Ref 8). We coated each test well of 96 well polystyrene plates (Nunc, Thomas Scientific) with 0.2 μg antigen (THRGP, P1, P2, dP1, dP2) in 200 μl (pH 9.6), 50 mM NaHCO3 buffer, for 15 h at 4°C, washed the plate once in H2O and briefly dried it before blocking all remaining protein binding sites by addition of 200 μl 1% BSA in PBS (final pH 7.5), for 30 min at 37°C, followed by washing twice with H2O and then drying.

**Fig. 2.** THRGP purification flow chart. Fractionation of the crude eluate via cation exchange chromatography and gel filtration yielded pure THRGP.

**Fig. 3.** Fractionation of the crude salt eluate from maize suspension cultured cells using cation exchange chromatography. A Cellex-P column was eluted with a citrate-phosphate pH gradient (pH 3.0–7.0) followed by a 0 to 1 M NaCl gradient in buffer. CP2 contains the THRGP.
We diluted the control (preimmune) and test sera as follows: ×200 for P1, dP1, and pre-immune control, and ×800 for P2, dP2, and preimmune control, in pH 7.5 PBS, and then added 25 µl of the diluted sera to the antigen-coated wells containing 25 µl 1% BSA-Tween-20 (1 µl/ml)/PBS at pH 7.5. After 1 h at 37°C we washed the plate twice in H_{2}O, added 50 µl diluted (×2000) goat-anti-rabbit serum coupled to peroxidase (Cappel Laboratories) in BSA/Tween-20/PBS to each well, incubated at 37°C for 30 min, washed the plate five times with H_{2}O, again briefly dried the plate, and then added 100 µl substrate to each well (11 mg ABTS and 15 µl 30% H_{2}O_{2} in 50 ml pH 4, 50 mM citrate buffer). After 30 min incubation at 23°C we added 100 µl NaF/EDTA stopping reagent (prepared by adding 50 µl 40% w/v tetrasodium EDTA to 50 ml 6 mM NaF in 2.5 mM HF) to each well, and then determined the absorbance at 405 nm.

**Assay of Agglutination.** We assayed the agglutinating effect of a serial dilution of THRGP (100–10 ng/ml) on 1% suspension of rabbit erythrocytes in phosphate-buffered saline according to the method of Allen and Neuberger (2).

**RESULTS**

**THRGP Elution from the Cell Surface.** The amount of elutable crude HRGPs rose as a function of culture age. After subculture, total soluble eluate fell to a minimum at 2 d and rose to level off at d 11 (Fig. 1). Thus, for bulk preparations, we used 11 d cells and 100 mM AlCl_{3}, the cells yielding 5.4 mg crude HRGP/g cells dw and fractionated further as shown in the flow sheet (Fig. 2). The crude HRGP was 1.7% Hyp on a dw basis (i.e. we recovered 92 µg soluble Hyp/g dw cells). The 100 mM AlCl_{3} did not plasmolyse the cells.

**Treatment of the Crude Eluate with 10% (w/v) TCA.** Overnight precipitation with 10% (w/v) TCA at 4°C followed by centrifugation precipitated 50% by weight of the crude eluate. The TCA pellet was 72% protein and 0.5% Hyp dw, but the THRGP remained soluble. The yield of TCA-soluble crude THRGP was 2.7 mg/g cells dw. The crude HRGP was 60% protein and 3.5% Hyp dw, representing a 60-fold Hyp enrichment over the whole cell Hyp content of 0.06%.

**Wall-Bound Protein and Hydroxyproline Content.** The maize cell wall is about 12% protein, determined by amino acid analysis, and 0.15% Hyp dw, determined by the method of Kivirikko and Liesma (12).

**Cation-Exchange Chromatography of the Dialysed TCA-Soluble Salt Eluate.** Chromatography on phosphocellulose yielded a void and three major protein peaks, designated CP1, CP2, and CP3, respectively (Fig. 3). The void and CP1 contained a trace of Hyp while CP2 contained 8.4% Hyp dw, and CP3 contained 2.7% Hyp dw. CP1 eluted at pH 3.8 in the pH gradient, CP2 at 200 mM NaCl, and CP3 at 450 mM NaCl in the salt gradient. Further chromatography of CP2 on Biorex-70 (Fig. 4) gave a Hyp-poor void (4% Hyp dw) and a Hyp-rich THRGP fraction (12% Hyp dw), designated BioRex Peak 1, which eluted at 200 mM NaCl (Fig. 4).

**Gel Filtration of BioRex Peak 1 THRGP.** Gel filtration on Superose-6 gave a major Hyp-rich (18% Hyp dw), threonine-rich peak at 2 × V_{o} and a minor Hyp-containing peak (3% Hyp dw) at 2.5 × V_{o} (Fig. 5).

**SDS Gel Electrophoresis of THRGP.** The THRGP migrated as a single fuzzy band with an apparent mol wt of 71.6 kD.

**Amino Acid Analysis of THRGP after Superose-6.** The THRGP contained 25 mol% threonine, 24 mol% Hyp, and was rich in proline, lysine, and serine (Table I).

**Neutral Sugar Analysis.** Alditol acetate derivatization of the THRGP showed arabinose as the only THRGP sugar substituent. The arabinose:Hyp molar ratio was 1.44:1. The arabinose accounted for 27% by weight of the THRGP.

**Hydroxyproline Arabinoside Profile.** A Hyp-arabinoside profile of the THRGP showed 48% nonglycosylated Hyp and Hyp arabinoside 3 as the major glycosylated component (Table I). The arabinose accounted for 35% by weight of THRGP. A Hyp-arabinoside profile of the maize cell wall showed 24% nonglycosylated Hyp and Hyp tri-arabinoside as the major glycosylated component (Table I).

**Electron Microscopy of THRGP.** TEM shadowed preparations of the THRGP showed rod-like monomers (see “Discussion”) averaging 70 ± 3 nm in length (Fig. 6).

**Precipitation with β-Glycosyl Yariv Antigen.** The THRGP did not react with Yariv Antigen, even at the relatively high level of 0.5 mg/ml where a standard AGP (0.5 mg/ml) (sycamore) gave
Table I. THRGP Composition

<table>
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* The average of amino acid analysis from three different THRGP preparations.

b Prepared from maize cell suspension cultures.

Fig. 6. TEM of the THRGP. TEM shadowed preparations of the maize THRGP appeared as rod-like monomers 70 nm long.

FIG. 7. Reactivity of THRGP with antibodies raised against tomato extensin precursors. Antibodies raised against glycosylated and deglycosylated tomato extensin precursor P1 (dP1) cross-reacted significantly with the THRGP. Antibodies raised against glycosylated and deglycosylated tomato extensin precursor P2 (dP2) also cross-reacted with the THRGP, but to a lesser extent.

precursors P1 and P2). Antibodies raised against glycosylated and deglycosylated tomato P2 (P2 and dP2) cross-reacted 28 and 12%, respectively, with glycosylated THRGP. Antibodies against P1 and deglycosylated P1 (P1 and dP1) cross-reacted 68 and 40%, respectively, with glycosylated THRGP. Control preimmune rabbit serum did not react with the THRGP.

Assay of Agglutinin. THRGP did not agglutinate rabbit erythrocytes.

DISCUSSION

Monocots are, by comparison with dicots, relatively poor in Hyp, although it has been clear for some time that monocot
Hyp-containing proteins do exist, both in the grasses (4; A. Bleeker, H. Kende, unpublished data) and other monocot families. Thus, van Etten (28) identified the seedcoat as a tissue often enriched in Hyp, while early work showed that cell walls prepared from coleoptiles contained detectable Hyp (14) some of it O-arabinosylated although to a much less extent than in the dicots (16).

Much of this work implicitly assumes that easily soluble HRGPs correspond to arabinogalactan proteins, while the insoluble HRGPs correspond to extensins. The latter hypothesis is difficult to test. However, a recent reinvestigation of our 'intact cell elution' technique (14) showed that under optimal conditions, we could ionically desorb soluble monomeric extensin precursors to wall-bound extensin directly from the cell surface of intact tomato cells grown in suspension culture (24). We and others have characterized soluble extensin monomers, chemically (5, 25, 29), immunologically (11) and electron microscopically (26, 29), as a small family of highly glycosylated, basic, hydroxyproline-rich, rod-like proteins of about 80 nm contour length and about 50 nm persistence length. Thus, we now have the tools to determine whether or not extensin occurs in graminaceous monocots. The question is relevant to current ideas about the control of cell extension (cf. oat coleoptiles) and a recently proposed model for the primary cell wall of dicots (15), which invokes an extensin 'weft' to mechanically couple the load-bearing microfibrillar polymer 'warp'-cellulose.

Our data here show successful application of the intact cell elution technique to maize cell suspension cultures. More than 30 proteins appeared in the crude eluate but only four or five occurred as major components, of which at least two were HRGPs, one of them being unusually rich in threonine. We purified the THRGP to constant composition; it migrated as a single band on SDS-PAGE; however, the apparent size of 71.6 kD is probably an overestimate judging by its contour length and glycosylation profile. Its status as a monomer is suggested by its behavior on Superose-6 gel filtration and SDS gel electrophoresis. Ten amino acids accounted for 98 mol% of the amino acid residues, being richest in threonine and Hyp, each accounting for 25 mol%, with a high proline, lysine, serine content, and lesser but significant amounts of tyrosine, histidine, glycine, glutamate, alanine, and valine. Such a biased composition is typical of HRGPs in general and extensins in particular, although the threonine-rich feature is novel; and, like extensin, the THRGP is highly basic. Furthermore the total Hyp plus proline content of 39 mol% implies a polyproline-II conformation similar to extensin (29), while the Hyp arabinoside profile is consistent with an extensin glycosylation pattern, and corroborates earlier work (16) which showed a high (about 50%) proportion of nonglycosylated Hyp residues in the monocots. However, the absence of galactose from the THRGP as well as the high threonine content distinguish it from dicot extensins.

To examine rotary-shaded material visualized THRGP molecules as flexible rods similar to those of dicot extensins (26, 29) although somewhat shorter, but consistent with their elution position on Superose-6 close to dicot extensin monomers.

Polyclonal antibodies raised against tomato extensins P1 and P2 cross-reacted significantly with the THRGP confirming the presence of common epitopes and proclaiming these THRGP molecules at least 'extensin-like.' The extent of true homology awaits further peptide mapping (in progress) and amino acid sequence determination of the major repeating peptides if the THRGP is highly periodic like other extensins.

The intriguing question of THRGP role also awaits future work, but the rapid 'disappearance' of ethalable THRGP within 48 h after subculture (Fig. 1), and its nondetectability in the growth medium, implies a covalent association with the primary cell wall, possibly as a network like the dicots, but as a very much looser weave.

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