Isolation and Characterization of a Novel Acidic Polysaccharide Containing Tartrate and Glyoxylate Residues from the Mineralized Scales of a Unicellular Coccolithophorid Alga *Pleurochrysis carterae*

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Mary E. Marsh
From the Dental Science Institute, The University of Texas Health Science Center, Houston, Texas 77225

Ding-Kwo Chang and Garry C. King
From the W. M. Keck Center for Computational Biology, Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251

The characterization of mineral-associated polyanions from the unicellular alga *Pleurochrysis carterae* is described. This species is useful for the study of mineralization, because it produces calcified scales known as coccoliths in homogeneous cell culture. Three acidic polysaccharides (PS-1, PS-2, and PS-3) were extracted from the coccoliths with EDTA and were separated and purified by differential precipitation with magnesium ions and chromatography on DEAE-cellulose. PS-1 and PS-3 are predominantly polymers of galacturonic acid containing lesser amounts of other monosaccharides. PS-2 has an unusual structure. Chemical, enzymatic, and two-dimensional NMR analyses demonstrate that the repeating unit of PS-2 is \( [\rightarrow 4 \text{D-gluconuronic acid(31-2)} \times \text{meso-tartrate(3-1)} \times \text{glyoxylate(1-1)}]. \) Thus PS-2 has a high density of negatively charged groups available for calcium ion binding, similar to the phosphoprotein polyanions of other species. Polysaccharides containing tartrate and/or glyoxylate have not been previously described; these residues may be introduced into PS-2 by a postpolymerization process involving oxidative cleavage of glucuronate or mannuronate residues.

Highly acidic macromolecules are postulated intermediates in tissue mineralization, because they sequester large numbers of calcium ions and occur in high concentrations at mineralizing foci in some tissues (1, 2). Two similar mineralization-associated polyanions have been described. The phosphophoryns are aspartic acid-rich, highly phosphorylated proteins associated with vertebrate dentin (3). The most acidic rat phosphophoryns are approximately 50% phosphoserine and 35% aspartic acid, giving them a net charge of \(-1.3\) electron units (eu) per residue (4). Phosphophoryns self-associate in the presence of calcium ions forming discrete 25-nm particles (5). Similar phosphoproteins accumulate at the shell-mineralization front in some bivalve mollusks (6, 7). The bivalve proteins contain 25-30% phosphoserine and 20-35% aspartic acid, producing a net charge of about \(-0.8\) eu per residue. These proteins occur as discrete 40-nm calcium-rich aggregates surrounding the growing CaCO₃ crystals at the mineralization front (7).

The mineralizing function of polyanions is poorly understood, largely because functional studies are hampered by the complexity of shell- and dentin-forming tissues. This paper describes the mineral-associated polyanions of another organism, *Pleurochrysis carterae*, which should be more amenable to detailed functional studies. *P. carterae* is a unicellular coccolithophorid alga easily grown in homogeneous cell culture. It is surrounded by an extracellular layer of mineralized scales called coccoliths (see Fig. 1) which are formed intracellularly in Golgi-derived vesicles (8). There are two probable sources of mineral-associated polyanions in this organism: the organic coat which surrounds the CaCO₃ crystals on mature coccoliths, and a collection of particles morphologically similar to the bivalve phosphoprotein aggregates. The algal particles contain carbohydrate material and have a high calcium content (9, 10). They surround the growing coccolith crystals intracellularly and are thought to contribute calcium ions to the developing mineral phase and organic matter to the crystal coat. This study describes the isolation and characterization of three coccolith-associated polyanions, one of which is a novel acidic polysaccharide with a repeating unit of glucuronate, tartrate, and glyoxylate residues.

**EXPERIMENTAL PROCEDURES**

Culture Conditions—The CCOII strain of *P. carterae* was obtained from the Bigelow Laboratory (West Boothbay Harbor, ME) and grown in F/2 medium (11) prepared with synthetic sea water (12). Small cultures of 1 liter or less were grown aerobically at 18 °C in conical glass flasks. These cultures were exposed to a photocycle of 18 h of illumination followed by 6 h of darkness and were aerated by manually swirling for a few seconds once a day. Large scale cultures, grown at room temperature in 60-liter rectangular glass aquaria, were exposed to constant illumination

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* The abbreviations used are: eu, electron units; PS-1, polysaccharide 1; PS-2, polysaccharide 2; PS-3, polysaccharide 3; HPLC, high-performance liquid chromatography; COSY, two-dimensional scalar correlated spectroscopy; NOESY, two-dimensional dipolar correlated spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy.

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Isolation of Mineral-associated Polysaccharides—In the method used in this study, 45.6 g of packed cells were sonicated in 125 ml of 50 mM Tris, 10 mM CaCl₂, pH 8.0, and then centrifuged 5 min at 600 × g. Nonmineral-associated material was extracted from the coccolith-coccosphere preparations using two 5 min, 100,000 × g pellets. This procedure disrupts the cells and larger organelles (chloroplasts and nuclei) but leaves coccoliths intact. In addition, coccoliths were sonicated in 200 ml of 0.5 M EDTA, pH 8.0. The EDTA extract was centrifuged at 100,000 × g for 1 h, and the supernatant fluid was equilibrated with water and concentrated to approximately 20 ml by distillation using an Amicon YM10 membrane. Polysaccharide 2 (PS-2) was precipitated by addition of 3.0 ml of 1.0 M MgCl₂, and the colloidal precipitate was pelleted by centrifugation at 100,000 × g for 1 h. Then polysaccharides 1 and 3 were coprecipitated from the supernatant fluid by addition of 3.0 ml of 1.0 M CaCl₂ and 0.25 ml of 3.0 M Tris, pH 8.8, and the pellet was collected by centrifugation at 7000 × g for 10 min. Both the calcium and magnesium precipitates were dissolved in 3 ml of 0.5 M EDTA, pH 8.0, and dialyzed versus 50 mM NaCl.

PS-1 and PS-3 from the calcium precipitate were resolved by chromatography on a 1.0 × 20-cm column of DEAE-cellulose with a 0.1–0.35 M NaCl gradient in 50 mM Tris, pH 8.0 (see Fig. 2). PS-1 was further purified by chromatography on a 1.6 × 200-cm column of Sephadryl S-300 in 50 mM NaCl, 10 mM Tris, pH 8.0. PS-3 was purified on the same Sephacryl column under identical conditions and then on a 1.0 × 7.0-cm column of DEAE-cellulose with a 0.05–0.70 mM sodium acetate gradient at pH 5.5. PS-3 eluted in about 0.4 M acetate. PS-2 from the magnesium precipitate was further purified on a 1.0 × 27-cm column of DEAE-cellulose with a 0.15–0.30 M NaCl gradient in 10 mM Tris, pH 8.0. PS-2 eluted in about 0.23 M NaCl.

In a simpler preparatory method, the mineral-associated polysaccharides were extracted by stirring the cells in 10 volumes of 5% trichloroacetic acid for 10 min. This procedure dissolves the coccolith crystals (CaCO₃) and polysaccharides and releases most of the carbonate as CO₂. The acid extract was centrifuged at 14,000 × g for 1 h, and the supernatant fluid was equilibrated with water and concentrated to approximately 20 ml by distillation using an Amicon YM10 membrane. Polysaccharide 2 (PS-2) was precipitated by addition of 3.0 ml of 1.0 M MgCl₂, and the colloidal precipitate was pelleted by centrifugation at 100,000 × g for 1 h. Then polysaccharides 1 and 3 were coprecipitated from the supernatant fluid by addition of 3.0 ml of 1.0 M CaCl₂ and 0.25 ml of 3.0 M Tris, pH 8.8, and the pellet was collected by centrifugation at 7000 × g for 10 min. Both the calcium and magnesium precipitates were dissolved in 3 ml of 0.5 M EDTA, pH 8.0, and dialyzed versus 50 mM NaCl.

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In a simpler preparatory method, the mineral-associated polysaccharides were extracted by stirring the cells in 10 volumes of 5% trichloroacetic acid for 10 min. This procedure dissolves the coccolith crystals (CaCO₃) and polysaccharides and releases most of the carbonate as CO₂. The acid extract was centrifuged at 14,000 × g for 10 min, and the calcium salts of PS-1, -2, and -3 were precipitated from the supernatant fluid by increasing the pH to about 8.0 with 3 M Tris, pH 8.8. The polysaccharides were resolved by magnesium ion fractionation and ion-exchange chromatography as described above.

Polysaccharide Analysis—Uronic acid residues were reduced to the corresponding hexose residues by the carbodiimide-NaBH₄ method of Taylor and Conrad (13), with no attempt to control the pH during the reduction step. After repeating the three procedures on each polysaccharide, 95% or more of the uronate residues were reduced as determined by the disappearance of the color of a 2,2,3,3-d₄-3-(trimethylsilyl)propionate.

Polysaccharides isolated from detergent- and guanidine-extracted coccoliths are acidic polysaccharides referred to as PS-1, PS-2, and PS-3, with the numbers indicating their elution order from DEAE columns at pH 8.0 (Fig. 2). However, PS-2 is generally separated from PS-1 and PS-3 before chromatography by precipitation with MgCl₂. Although all three polysaccharides are precipitable with calcium ions, only PS-2 aggregates in the presence of magnesium.

All experiments described in this report were performed on polysaccharides isolated from detergent- and guanidine-extracted coccoliths. However, the polysaccharides could be isolated more simply by extracting the cells with 6% trichloroacetic acid and then precipitating the polysaccharides as calcium salts by increasing the pH of the extract to 8.0. Addition of

RESULTS

P. carterae cells are surrounded by extracellular mineralized scales called coccoliths composed of organic oval-shaped bodies with a rim of CaCO₃ crystals (Fig. 1). In order to show that the polysaccharides described in this study are associated with the mineralized coccoliths, the scales were separated from other components by repeated sonications and recovery of the coccoliths in 600 × g pellets. This procedure disrupts the cells and larger organelles (chloroplasts and nuclei) but leaves coccoliths intact. In addition, coccoliths were sonicated in detergent and guanidine solutions in the presence of calcium ions to insure that all extractable polymers were removed except those associated with the mineral phase or otherwise maintained in insoluble calcium aggregates.

The three polysaccharides liberated from the coccoliths with EDTA are acidic polysaccharides referred to as PS-1, PS-2, and PS-3, with the numbers indicating their elution order from DEAE columns at pH 8.0 (Fig. 2). However, PS-2 is generally separated from PS-1 and PS-3 before chromatography by precipitation with MgCl₂. Although all three polysaccharides are precipitable with calcium ions, only PS-2 aggregates in the presence of magnesium.

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**FIG. 1.** Electron micrographs of *P. carterae* cells. The extracellular coccoliths are composed of organic oval-shaped bases with a rim of CaCO₃ crystals (*arrowhead*). X indicates a coccolith base which is apparently reversed with the rim crystals facing the cell surface, and the arrow indicates a coccolith viewed on edge showing both the organic base and the mineral rim. An organic coat surrounds the crystals but cannot be distinguished in scanning electron images. Cells were fixed 30 min in 1% OsO₄ in sea water, dehydrated in ethanol, dried from Peldri I₁ (Ted Pella, Inc.), and examined in a JEOL JSM 820 scanning electron microscope. Magnifications are 4500 X (a) and 11,000 X (b).

Calcium was unnecessary, since the extract already had a high calcium content derived from the acid-solubilized CaCO₃ crystals. Similar polyanion fractions with a similar degree of heterogeneity were obtained with both isolation schemes.

PS-1 and PS-3 migrate in relatively tight bands on polyacrylamide gels (Fig. 3), indicating that they have a rather narrow molecular weight range. However, PS-2 forms a ladder of discrete bands spanning the length of the gel, indicating that its degree of polymerization may range from less than 10 to over 1000. The full range of PS-2 lengths is apparent in Fig. 3a which shows the unfractionated polyanions. Purified PS-2 (Fig. 3c) is relatively depleted in the low molecular weight species. PS-2 is the most abundant coccolith polyanion; 343 mg were obtained from 45 g of cells. On a weight basis PS-1 and PS-2 represent about 22 and 77% of the polyanion fraction, respectively. PS-3 is a minor component constituting less than 2%.

**FIG. 2.** DEAE-cellulose chromatography of coccolith polyanions. PS-2 is generally separated from PS-1 and PS-3 before chromatography; however, the position at which PS-2 elutes in this buffer system is indicated with a dotted curve. Elution was with a 0.10-0.35 M NaCl gradient (dashed line) in 50 mM Tris, pH 8.0. Ten-ml fractions were collected at a flow rate of 60 ml per h. PS-1, PS-2, and PS-3 elute in approximately 0.15, 0.23, and 0.275 M NaCl, respectively.

**FIG. 3.** Polyacrylamide gels of the coccolith polyanions. EDTA extract of isolated coccoliths (a) and 25 μg each of purified PS-1 (b), PS-2 (c), and PS-3 (d). Gels were stained with Stains-All. No additional bands were observed with a sensitive silver stain (29).
PS-1 is a polyuronide with a glucuronic acid/galacturonic acid ratio of 1:3 and contains small amounts of uncharged carbohydrate residues (Table I). PS-3 is a mannos- and galacturonic acid-rich polysaccharide and was the only sulfated polyanion derived from the coccoliths.

Glucuronic and galacturonic acid in a mole ratio of 10:1 were the only carbohydrate residues identified in PS-2 by paper chromatography and HPLC. However, the uronic acid residues represented only about 50% of the PS-2 mass, and paper chromatograms of PS-2 hydrolysates displayed two bands which did not correspond to any monosaccharides tested. The $^{13}$C NMR spectrum of PS-2 (Fig. 4) contained 12 major carbon resonances of which six could be assigned to glucuronic acid. Of the six additional resonances, three appear in the carboxyl region (170–180 ppm), and one lies in the anomeric carbon region (100–110 ppm). This spectrum suggested that PS-2 might be a repeating diuronide in which 1 residue of the repeating unit was oxidatively cleaved in such a manner as to yield a dicarboxylic acid and a carboxylic acid aldehyde upon acid hydrolysis. With this clue from the NMR spectrum, it was easily established that PS-2 contains equal molar ratios of D-glucuronic acid, meso-tartaric acid, and glyoxylic acid (Table I). The absolute configuration of glucuronic acid was established by demonstrating that it could be reduced to D-glucose. The meso configuration of tartaric acid is coupled to glucuronate H1, indicating a glucuronate (pl+) residue. The remaining spin-coupled signals at 4.50 and 4.41 ppm were assigned to the C3 and C2 protons of tartrate, respectively (Table II).

Glucuronic acid was established by demonstrating that it could be reduced to D-glucose. The meso configuration of tartaric acid was assigned by cross-peak connectivities between all carbon-proton correlations in the HMQC spectrum of PS-2 (Fig. 6a) and one-dimensional $^1$H-$^1$H COSY (Fig. 5a). The interresidue correlations determine the sequence of the PS-2 constituents. Glucurionate C1 has a long-range correlation to tartrate H2, and tartrate C2 is coupled to glucurionate H1, indicating a glucurionate configuration (32).

Two-dimensional NMR spectra establish the connectivity of the PS-2 constituents. All $^1$H resonances can be assigned from the COSY spectrum (Fig. 5b). It is clear that the signal at 5.15 ppm is derived from the "anomeric" (acetal) proton of glyoxylate, since this is the only isolated resonance. The other anomeric proton signal at 4.64 ppm is from D-glucurionate, and its upfield chemical shift indicates that it has the $\beta$ configuration (32). Cross-peak connectivities beginning with the anomeric resonance yield all the glucurionate proton assignments. The vicinal coupling constants, approximately 6 Hz, are consistent with the all axial orientation of $\beta$-glucuronate protons. The remaining spin-coupled signals at 4.50 and 4.41 ppm were assigned to the C3 and C2 protons of tartrate, respectively (Table II).

The HMOC spectrum of PS-2 (Fig. 6a) shows resolved cross-peaks for all eight $^{13}$C-$^1$H single-bond correlations. Thus the noncarboxyl $^{13}$C resonances are readily assigned by correlation to their directly bonded protons. The HMOC spectrum (Fig. 6b) shows a number of correlations through two- and three-bond couplings, including those involving carboxyl signals (Fig. 6b, inset). The interresidue correlations determine the sequence of the PS-2 constituents. Glucuronate C1 has a long-range correlation to tartrate H2, and tartrate C2 is coupled to glucurionate H1, indicating a glucurionate configuration (32).
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2) tartrate linkage. The acetal carbon of glyoxylate has a long-range correlation to both tartrate H3 and glucuronate H4, and both glucuronate C4 and tartrate C3 are coupled to the glyoxylate acetal proton, indicating a tartrate(3-1)-glyoxylate(1-4)glucuronate linkage. Therefore the repeating structure of PS-2 is \((\beta(1\rightarrow4))\)D-glucuronate(\(\beta(1\rightarrow2)\))meso-tartrate(3-1)glyoxylate(1-), as shown in Fig. 7.

**DISCUSSION**

In contrast to the mineralizing phosphoproteins of shells and dentin, the mineral-associated polyanions in *P. carterae* coccoliths are polysaccharides. The major polyanion PS-2 is unique in that it is the first tartrate- and glyoxylate-containing polysaccharide to be described. The biosynthesis of PS-2 has not been examined, but it seems likely that tartrate and glyoxylate may be introduced into the polysaccharide in a postpolymerization or copolymerization process. Possible precursors of PS-2 are repeating disaccharides in which \(\beta(1\rightarrow4)\) linked D-glucuronic acid alternates with \((1\rightarrow4)\) linked glucuronic or mannuronic acid, since oxidative cleavage of the C2-C3 bond to the carboxylate level in either of the latter residues would yield \(\beta(1\rightarrow4)\)D-glucuronic acid alternating with meso-tartaric and glyoxylic acids as in PS-2. The anomic configuration of the putative tartrate-glyoxylate precursor is unknown since the configuration of the glyoxylate acetal has not been established; preliminary modeling based on NOESY data (not shown) is consistent with either an \(\alpha\) or \(\beta\) linkage. Of the possible precursors, poly(1\(\rightarrow4\))D-glucuronic acid with alternate \(\alpha\) and \(\beta\) linkages occurs in the teichuronopeptide of bacterial cell walls (33), and poly-D-glucuronic acid with undetermined linkages has been reported in fungal cell walls (34). Organisms capable of oxidizing C-C bonds in polysaccharides to yield tartrate residues have not been reported; however, tartrate is derived from ascorbic acid by cleavage of the C4-C5 bond in grapes and by cleavage of the C2-C3 bond in geraniums (35).

In addition to glucuronate, tartrate, and glyoxylate, PS-2 also contains lesser amounts of other residues. Galacturonic acid was detected by HPLC, and several small unidentified signals were observed in \(^1H\) and \(^{13}C\) NMR spectra. The distribution of minor components in PS-2 has not been determined. The glucuronate-tartrate-glyoxylate linkage was established with \(^1H\) detected HMBC spectroscopy (28), which has been used to sequence oligosaccharides (36-38) and polysaccharides (39, 40) more complex than the PS-2 repeating unit. Heteronuclear NMR experiments may also be useful in determining the distribution of minor components in PS-2; however, this study was limited to a determination of the glucuronate-tartrate-glyoxylate sequence.

PS-1 which represents about 22% of the coccolith polyanions has a more conventional composition than PS-2. It is

**Table II**

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<td></td>
<td>6</td>
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* Positions are numbered as in Fig. 7.

* Chemical shifts are in ppm relative to external sodium 2,2,3,3-d4-3(trimethylsilyl)propionate.

**Fig. 6.** Proton-carbon correlation spectra of PS-2. a, HMOC spectrum. G, T, and X refer to glucuronate, tartrate, and glyoxylate residues, respectively, and the numbering system is shown in Fig. 7. b, HMBC spectrum. Peaks are labeled in order of their correlated \(^{13}C\) and \(^{1}H\) atoms, respectively, e.g. G1T2 indicates the long-range correlation between glucuronate C1 and tartrate H2, while T2G1 indicates the correlation between tartrate C2 and glucuronate H1. s indicates incompletely suppressed \(^{1}H\)-\(^{13}C\) single bond correlations involving the glyoxylate acetal. *Inset*, carboxyl region. Cross-peak (r) from a minor component is indicated.
predominantly a polymer of galacturonic and glucuronic acids with lesser amounts of charged residues, and it is less acidic than PS-2. In PS-1 most residues are singly charged. In PS-2, residues are alternately singly charged (glucuronate) and triply charged (tartrate and glyoxylate), giving the polysaccharide a net charge of -2.0 e per residue.

There are interesting parallels between PS-1 and PS-2 and the rat dentin phosphophosphates. In rat dentin there is a very acidic phosphoprotein with an average charge of -1.3 e per residue and a less acidic phosphoprotein with an average charge of -0.75 e per residue (4, 5). In both coccoliths and dentin the very acidic polyanion is about three times more abundant than the less acidic polyanion. PS-1 and PS-2 coprecipitate in the presence of calcium ions as do the dentin phosphoproteins, but only the very acidic coccolith and dentin polyanions aggregate in the presence of magnesium (5). It is possible that calcium coprecipitates of very acidic and less acidic polyanions occur naturally in dentin and coccoliths and have a common mineralizing function. So, this would be an example of parallel evolutionary development since the polyanions are chemically different, and the organisms in which they occur are distantly related.

PS-3 is a minor coccolith component, representing less than 2% of the polyanion fraction, and is the only sulfated polysaccharide identified in this study. However, a preliminary report (41) from another group described two sulfated acidic polysaccharides isolated from P. carterae coccoliths which have no apparent relationship to the polysaccharides described here. One of the sulfated polysaccharides previously described was composed of galacturonic, glucuronic, and iduronic acids in a mole ratio of 2.4:1.1:1.0, respectively. The other sulfated polysaccharide was predominantly polysaccharic acid with lesser amounts of uncharged residues, but no glucuronic acid. Different strains of P. carterae were used in this and the previous study which may account for the differences observed in coccolith polyanions. Analysis of additional P. carterae strains will be needed to establish the degree of polysaccharide diversity within this species.

The coccoliths are formed intracellularly in large Golgi-derived sacules. The organic base plate is completed before mineralization begins. Small vesicles containing carbohydrate- and calcium-rich particles are produced from the Golgi system and pass through the cytoplasm to fuse with the coccolith sacule (8-10). Then CaCO₃ deposition begins on the rim of the base plate, and the particles surround the growing crystals until mineralization ceases. After this point the particles are no longer observed, but the crystals are surrounded by a carbohydrate-rich coat apparently derived from the particles (9). We speculate that the polyanions described in this report are components of the crystal coats, since they are solubilized from the isolated coccoliths when the mineral phase is dissolved. Antibera to PS-1 and PS-2 have been prepared for localizing the polyanions in coccoliths and intracellular pools. Speculation on the functional role of the polyanions is deferred until their distribution in situ has been established.

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