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EXTRACTION, PARTIAL CHARACTERIZATION, AND STUDIES OF SOME SPECTROSCOPIC PROPERTIES OF THE ANIONIC POLYSACCHARIDE FROM *COLOCASIA ANTIQUORUM*

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ABSTRACT

The natural anionic polysaccharide (Ps-A) isolated from arum (*Colocasia antiquorum*) tubers contains 56% neutral sugars as galactose, mannose, rhamnose, and arabinose, and 40% anionic components as galacturonic acid and mannuronic acid. The equivalent weight of Ps-A estimated by conductometric and spectrophotometric titrations is rather high, 1510 ± 5 , which on saponification is reduced to 479. This indicates that about two-thirds of the carboxylic groups of Ps-A exist in the esterified form. Ps-A and its saponified product (Ps-B) induce strong metachromasia in the dyes 1,9-dimethyl methylene blue and pinacyanol chloride (PCYN). Ps-A, with its lower charge density, induces relatively sharp and single banded metachromatic spectra, but the spectra induced by Ps-B, with its higher charge density, is broad and multiple banded. Ps-A induces weak dichroism in PCYN, indicating a bit of helical asymmetry in its conformation; Ps-B does not induce dichroism in this dye. The failure of Ps-B to induce dichroism presumably indicates that Ps-B has a nonhelical conformation.

INTRODUCTION

Colocasia is a small genus of about 13 species of perennial herbs distributed in the tropical parts of southeastern Asia, of which 5 or 6 species occur in India where they are cultivated for their edible tubers. There is some confusion regarding the nomenclature of the cultivated species; some consider *Colocasia antiquorum* as a species distinct from *Colocasia esculenta*, and other regard *Colocasia esculenta* as a variety of *Colocasia antiquorum*.

There are, however, hundreds of varieties [1, 2] differing in the color of leaf blades and petioles, and the size, shape, color, and nutritive value as well. Tubers of many of the varieties have marked acidity and are not consumed. Though the major constituents of the tubers besides moisture are carbohydrates, they contain a good percentage of proteins, calcium, phosphorus, iron, and vitamins B₁ and C [3]. Hence the tubers are nutritious—nearly one-and-one-half times more so than potato [2]. Moreover, the starch grains in the tubers are among the smallest in food plants, and hence they are digested easily and recommended as infant and invalid food. The starch of the tuber is also considered useful for sizing textiles where penetration, and not the coating power, is the main consideration [2]. The tuber mucilage is also used as a glue for making impermeable paper. The starch contains amylose [4] and finds use in the production of industrial alcohol [5] besides as a thickener of soups and gravies, and in making pancakes, biscuits, and cakes.

In spite of its general uses, it has never been completely characterized. This paper deals mainly with spectroscopic studies of the mucilage part, but we have made an attempt to partially characterize it, particularly the sugar content of the mucilage fraction. We avoided boiling of tubers or using strong alkali when extracting the anionic polysaccharide since such an extraction seems to solubilize some starch.

EXPERIMENTAL

Extraction and Purification of the Polysaccharide from the Tubers

The epidermal surface of 500 g of tubers, collected during autumn, was cleaned off, washed, homogenized in 600 mL of 5% potassium acetate solution, stirred for 2 hours, and filtered in a nylon cloth. The filtrate was centrifuged for 15 minutes at 7000 rpm. To the supernatant, 5.0 g sodium acetate and about 750 mL distilled ethanol were gradually added with gentle stirring. A copious white precipitate appeared. The precipitate was washed several times with ethanol and dried in vacuum. The yield was 1.5 g. 800 mg of the dry mass was dissolved in 50 mL distilled water, centrifuged for 1 hour at 15,000 rpm, and 9.0 mL of 5% cetyl pyridinium chloride (CpCl) solution was added to the clear supernatant. A fibrous white precipitate appeared. The precipitate was washed thoroughly with water to which 10.0 mL of 10% sodium acetate in methanol had been added, and the mixture was stirred in a magnetic stirrer. After prolonged stirring, the compound went into solution. The solution was filtered and centrifuged for 1 hour at 15,000 rpm. The polysaccharide in the supernatant was precipitated with ethanol (200 mL); 50 mg sodium acetate was added to facilitate the precipitation. The precipitate was redissolved in water and the steps beginning with the precipitation by CpCl solution

were repeated twice. Finally, it was washed with acetone and dried in vacuum. The yield was 200 mg. We called this Ps-A. The moisture content of the sample was estimated to be 3.2 wt%.

Saponification of Ps-A

Ps-A (100 mg) was dissolved in 10.0 mL of 0.1 M KOH, flushed with nitrogen, and stored overnight at laboratory temperature in the nitrogen atmosphere for the saponification of any ester group. The solution was neutralized to pH 8.0 by adding dilute acetic acid, and then dialyzed for 24 hours against water. The polysaccharide in the tube was then precipitated by adding 25 mg potassium acetate and 25.0 mL distilled ethanol. The product was washed several times with ethanol to free it from potassium acetate, and then dried in vacuum. This saponified product of Ps-A was labeled Ps-B. The yield was 65.0 mg.

Water-soluble anionic polysaccharides of plant origin usually contain some neutral sugars [6–8] besides uronic acids. The neutral sugars were estimated by the phenol sulfuric acid method [9] and the uronic acids by the carbazole-sulfuric acid method [10]. The neutral sugars were also identified and estimated by gas-liquid chromatography (glc) [11].

The sources of the dyes used, namely methylene blue, acridine orange, 1,9-dimethyl methylene blue, and pinacyanol chloride, have been described elsewhere [12, 13].

glc was performed with a glass column (6 mm × 1.83 m) containing 3% ECNSS-M on gas chrom Q (100–120 mesh) at 190°C for alditol acetates of neutral sugars using a Hewlett-Packard gas chromatograph (model 5730A) equipped with a flame-ionization detector and a 7127A recorder. Nitrogen was used as the carrier gas. Absorption and circular dichroic spectra were recorded with a Shimadzu spectrophotometer UV-150-02 and a spectropolarimeter Jasco-500-C using 1.0 cm quartz cuvettes. All experiments were carried out at laboratory temperature (25°C), and all evaporations were done below 40°C under reduced pressure.

RESULTS AND DISCUSSION

A quantitative estimation of neutral sugar was made colorimetrically by the phenol sulfuric acid method [9] from a standard curve of glucose. The average sugar content of Ps-A from a set of such data was 56.8%. The neutral sugar content of Ps-A was also estimated by glc. The Ps-A was hydrolyzed to the monomers, and the neutral sugars were separated from the uronic acid(s) by ion-exchange chromatography. They were eventually converted into alditol acetate derivatives [14] and extracted with chloroform. A few microliters of this chloroform solution was injected into the glc column and, by comparison with standard peaks, the neutral components were identified to be galactose, mannose, arabinose, and rhamnose. Their percentage (w/w) contents were estimated from chromatogram as 43.4, 6.6, 6.5, and 2.1, respectively. Thus, the total content of neutral sugars by glc was 58.6, which is a 2% deviation from the colorimetric method estimate.

To identify the hexouronic acid(s) of Ps-A, a glc run was made after converting the uronic acids to the corresponding neutral sugars and subsequently to their

alditol acetates. The chromatogram showed two peaks of almost equal intensity, corresponding to galacturonic and mannuronic acid. Based on the result of the quantitative estimation of total uronic acid (40%), the polysaccharide contains 20% each of galacturonic and mannuronic acids.

Taking the 40% content of hexuronic acid of Ps-A, the equivalent weight, i.e., the average weight containing one anionic charge, is 419 in its acid form, and obviously 457 as the potassium salt. Adding the 3.2% moisture content of the sample, this equivalent weight should be 472. Of the various methods of estimation available for determining the equivalent weight of an anionic polymer, we used spectrophotometric and conductometric titration methods. Figure 1(A) represents a spectrophotometric titration curve using purified acridine orange. The equivalent weight is 1520 ± 5 , which is obviously much higher than the value obtained from the hexouronic acid estimation. The estimation of equivalent weight was repeated by direct conductometric titration with standardized HCl; Fig. 1(B) represents one such titration curve which gave an equivalent weight of 1510 ± 3 , in close agreement with the spectrophotometric titration data. The wide difference between the results of titration and those calculated from hexouronic acid determination of the unsaponified polysaccharide Ps-A suggests that some carboxylic groups in native Ps-A exist in the ester form, as is common in plant polysaccharides [15]. To check this contention, the equivalent weight of the saponified product Ps-B (as the K salt) was determined to be 479, which is in fairly good agreement with the value obtained from hexouronic acid estimation. Comparison of the equivalent weights of Ps-A (1510) and Ps-B (479) shows that in the native polysaccharide Ps-A, approximately two out of every three carboxylic groups exist as an ester. These esterified groups

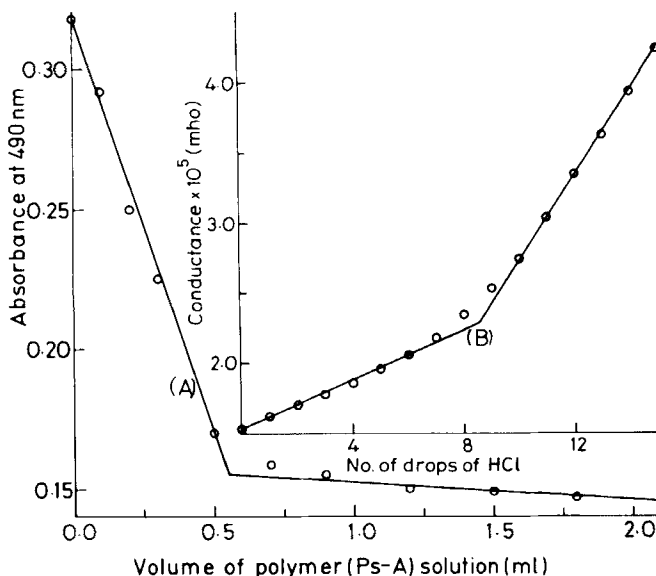


FIG. 1. (A) Spectrophotometric titration of 1.20×10^{-5} M AO by $164.7 \mu\text{g/mL}$ of Ps-A solution in H_2O . (B) Conductometric titration of 6.0 mg of Ps-A by 9.06×0.001 N HCl (1 drop of HCl = 0.051 mL).

will not respond to spectrophotometric or conductometric titrations, but they will be converted to the carboxylate group through hydrolysis during the colorimetric method of estimating hexouronic acid as well as during glc. This clearly explains the large discrepancy in the equivalent weight determination results by different methods.

If Ps-A had been a straight chain polymer, the carboxylate groups would be separated by a considerable distance, and the cationic dyes bound at the anionic carboxylate groups would be almost isolated and devoid of any dye-dye interaction. On the other hand, if Ps-A had a folded conformation, either random coil or helical, the dye cations bound at the available anionic sites would be placed relatively close and thus exhibit metachromasia. Figure 2 shows that Ps-A induced metachromasia in the strongly aggregating dye 1,9-dimethyl methylene blue (DMMB), with a distinct metachromatic band at 540 nm and a relatively small absorbance at the monomer band of the dye. However, the relatively weaker chromotropic character of Ps-A is reflected by the blue shift of 90 nm of this metachromatic peak as compared to the metachromatic peak in this dye blue-shifted by as much as 130 nm from its monomer band by the helical polysaccharides alginate [12] and pectate [13]. Though the induction of metachromasia in a dye by a polymer gives only a crude reflection of the aggregation of dyes on the polymer backbone, the picture becomes much clearer from a study of the induction of circular dichroism, yet the weak chromotropic property of Ps-A hints at a compact conformation that brings the anionic sites nearer to each other. Pinacyanol chloride (PCYN), a cyanine dye, exhibits metachromasia in the presence of a polyanion. It exhibits multiple banded metachromasia when it binds with a 1:1 stoichiometry, and sharp and single banded metachromasia when it binds the polyanion in a 1:2 stoichiometry (dye/polymer) [16]. The molarity of the polymer, in terms of its equivalent weight, is defined as the average weight containing one anionic site. The dye pinacyanol has

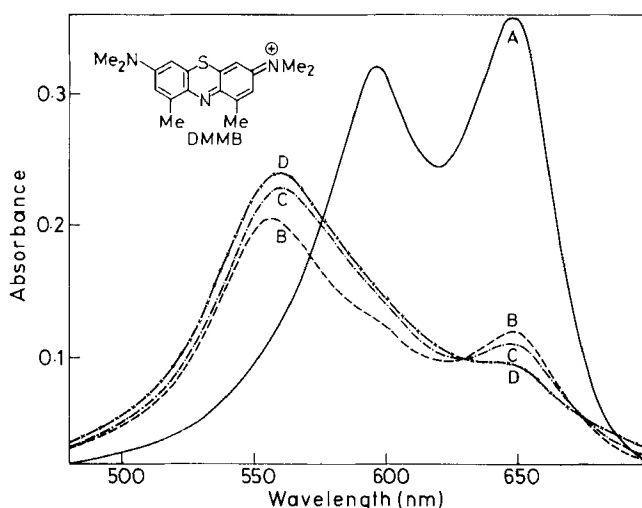


FIG. 2. Absorption spectra of 1.20×10^{-5} M DMMB in water (A) and that in the presence of Ps-A at P/D of 1.1 (B), 3.3 (C), and 9.9 (D). P/D is the polymer/dye molar ratio.

a stronger aggregating tendency because it is larger and hence is expected to be more hydrophobic. Its structure is relatively flexible and capable of undergoing *cis-trans* isomerization [17]. When large pinacyanol cations are bound to a polyanion with a 1:1 stoichiometry, there will possibly be overcrowding, which might cause steric effects in the bound dyes and result in irregular stacking and hence multiple-banded metachromasia. Therefore, for compact coiled polymers with a reasonably high charge density with which the dye could make a 1:1 stoichiometric compound, multiple-banded metachromasia is much more expected than is sharp, single-banded metachromasia. Figure 3 shows the metachromatic spectra of this dye in the presence of different concentrations of Ps-A. Obviously, the shape of all these spectra are sharp and single banded, and almost independent of the polymer concentration. Two-thirds of the carboxylic groups in Ps-A exist in ester forms, the aggregated dyes on the polymer backbone do not suffer overcrowding, and hence there is a prominent single-banded metachromasia with a sharp peak at 490 nm. Figure 4 shows the metachromatic spectra of PCYN in the presence of Ps-B, the saponified product of Ps-A. Here a multiple-banded metachromasia is observed. Ps-B has a higher charge density and yet binds dye cations at every anionic site, as is apparent from the equivalent weight estimation. The shapes of the metachromatic spectra of PCYN depend not only on the type of polymer but also on the conformation of the inducing polyanion. Pal and Ghosh [18] showed that poly(sodium methacrylate) induces single-banded metachromasia with the dye, but if the polymer solution is heated to 90°C followed by pipetting of the dye and cooling to room temperature, the metachromatic spectrum changes to a multiple-banded one.

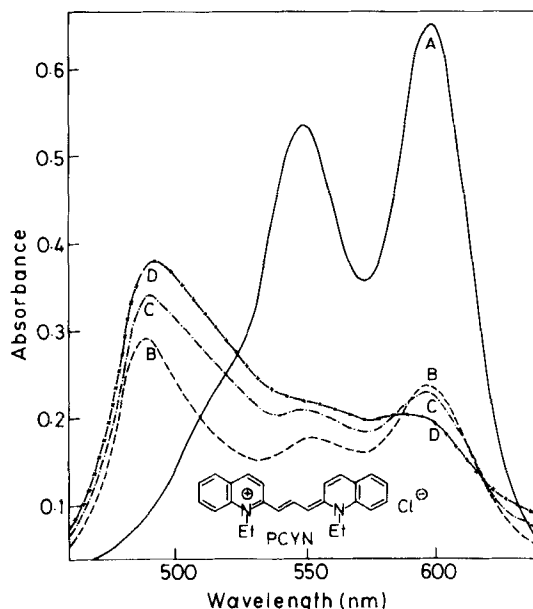


FIG. 3. Absorption spectra of 1.20×10^{-5} M PCYN in water (A) and that in the presence of Ps-A at P/D of 1.1 (B), 3.3 (C), and 9.9 (D).

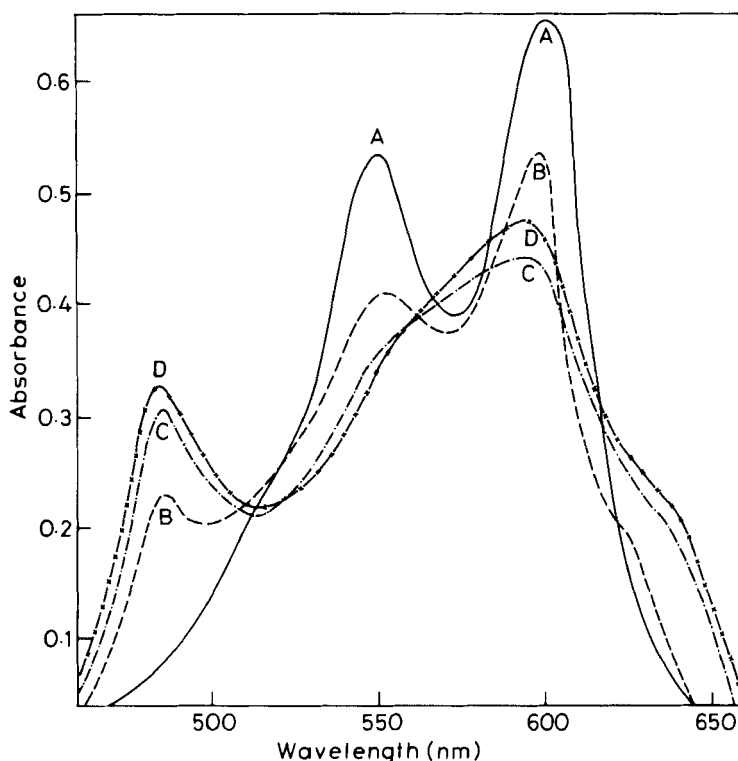


FIG. 4. Absorption spectra of 1.20×10^{-5} M PCYN in water (A) and that in the presence of Ps-B at P/D of 1.1 (B), 3.3 (C), and 9.9 (D).

The building units of Ps-A are the hexouronic acids, mannuronic and galacturonic, both of which are optically active. It is therefore expected that some chirality may be induced, even in achiral dyes bound at the carboxylic groups. If, in addition, the host polymer has a chiral helical conformation, the dye cations bound to it may be aggregated with a systematic twist to form a kind of superhelix around the helix of the polymer. The planar symmetric dyes have their magnetic and electric dipoles at right angles to each other in individual dyes and also in a stack of dyes. Thus, the electric and magnetic moments of the dyes cannot couple and give a rotational strength of zero values. If the dye molecules are made to aggregate helically (or at least with a systematic twist in one sense), the magnetic moment of a dye molecule in such a twisted aggregate may be coupled with the electric moment of a neighboring dye if not at a right angle to each other. This gives a nonzero value to the rotational strength. Such aggregates are expected to exhibit circular dichroism (CD) in the absorption region of the dye aggregate. However, the possibility of a nonzero value of the rotational strength obviously depends on the structures and dimensions of the dye molecules besides being primarily dependent on the polymer. Our experience with conformational studies of biopolymers using CD with extrinsic chromophores as the probe shows that even polymers with known helical conformations do not induce dichroism in all dyes they bind. We recorded the CD spectra of both Ps-A and Ps-B in the presence of the dyes acridinine orange, methylene blue,

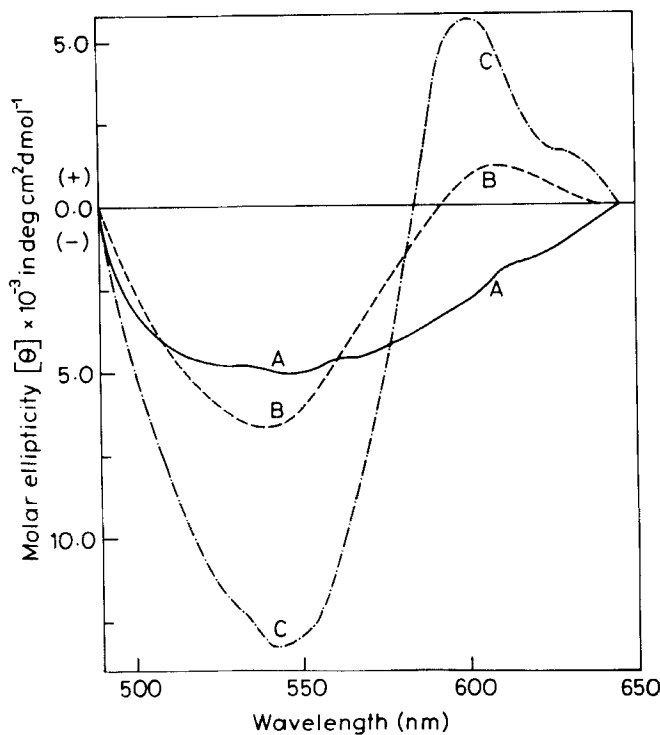


FIG. 5. Circular dichroism spectra of 1.20×10^{-5} M PCYN induced by Ps-A at P/D of 1.1 (A), 3.3 (B), and 9.9 (C).

DMMB, and PCYN. In Fig. 5 the molar ellipticity values $[\theta]$ of the recorded circular dichroic spectra of PCYN induced by Ps-A are plotted. The figure shows that Ps-A induces a negative biphasic CD spectrum at P/D = 9.9 with a trough at 540 nm and a peak at 590 nm. The crossover point is at 570 nm, that is, at the absorption region of the dye with $[\theta]$ of the order of 10^4 . The other dyes tried do not show any induced CD by Ps-A. The induction of CD in PCYN with its moderate $[\theta]$ value and the failure of induction in other dyes probably indicates a random coil conformation. The helical regions of the polymer backbone may be responsible for the induction of CD. Perfectly helical polymers usually induce CD with $[\theta]$ values of the order of 10^5 . This result is also in close agreement with the metachromatic behavior of dye-Ps-A systems. Our CD spectra of Ps-B-dye systems, including PCYN, show no induction. Thus, the small amount of conformational asymmetry observed in the case of Ps-A vanishes completely on saponification.

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