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CHARACTERIZATION OF THE α-D-GLUCAN FROM THE PLASTIDS OF *Cecropia peltata* AS A GLYCOGEN-TYPE POLYSACCHARIDE

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ABSTRACT

A water-soluble polysaccharide extracted from the plastids of *Cecropia peltata*, a higher green plant, has been characterized by enzymic methods as a polysaccharide similar to phytoglycogen.

INTRODUCTION

The storage polysaccharides of plants and animals, *viz.* starch and glycogen, respectively, although related in structure, have properties sufficiently different to make them readily distinguishable in all but a few cases. Thus, most starches consist of two polysaccharides: amylose, an essentially linear α-(1→4)-D-glucan with a d.p. which may range from a few hundred to several thousand; and amylopectin, which consists of relatively short chains of (1→4)-linked α-D-glucose residues (average chain-length, $\overline{CL} = 20–25$). Glycogens, on the other hand, do not contain a linear component and have $\overline{CL}$ rather shorter than in amylopectin (10–14).

Although starch is generally a two-component system, this cannot be taken as a characteristic property since in some plants, *e.g.* waxy maize[1], only the branched component is present. Distinction between starch and glycogen-type polysaccharides is most conveniently made on the basis of the properties of the branched component. This publication illustrates how this may be performed in an unequivocal manner by the use of specific glycoside hydrolases of defined specificity and established purity.

Although the distribution of glycogen is generally considered to be restricted to animal tissues and micro-organisms, there is one well-authenticated example of its presence in plants, this being in the su_1 mutant of *Zea mays* L. (sweet corn)[2,3]. In this plant, glycogen is synthesized as the result of some, as yet undefined, enzymic abnormality and co-exists with a normal granular starch. Two other claims for the existence of glycogen-type polysaccharides in plant tissues, in wild apple (*Malus sylvestris*)[4] and *Clossostemon bruguierii*[5], have been disproved[6]. The polysaccharide from the former source is a degraded amylopectin; the latter is a normal two-component starch. The present work has, however, identified the glucan from the

Müllerian bodies of *Cecropia peltata* (for botanical details, see Discussion) as being a polysaccharide with the properties of glycogen. This had previously been indicated by ultrastructural and histochemical studies[7].

MATERIALS AND METHODS

Materials. — Müllerian bodies, collected using forceps from greenhouse-grown plants, were ground for 10 min at 0° in 10mM mercuric chloride solution in a Potter–Elvehjem homogenizer. After centrifugation (1000 *g*, 20 min), the polysaccharide was precipitated from the opalescent, supernatant solution with ethanol (2 vol.). The polysaccharide was purified by dissolution in water and precipitation with ethanol, this procedure being carried out 5 times. Finally, the polysaccharide solution was treated with mixed-bed ion-exchange resin (Bio-Rad Laboratories) to remove any residual mercuric chloride which would otherwise interfere with enzymic hydrolyses. The alcohol-precipitated polysaccharide was dried at 40°. In a typical preparation, 555 mg (fresh weight) of Müllerian bodies yielded 163 mg (dry weight) of polysaccharide.

alpha-Amylase from hog pancreas[8] was purchased from Worthington Biochemical Corporation. beta-Amylase was prepared from sweet potatoes by a modification[9] of the method of Nakayama and Amagase[10], followed by chromatography on DEAE-Sephadex A-50 to remove contaminating maltase[11]. *Cytophaga* isoamylase[12,13] was partially purified by using DEAE-cellulose as previously decribed[14], followed by molecular-sieve chromatography on a column of Biogel P-100. Crystalline pullulanase from *Aerobacter aerogenes*[15] was supplied by Dr. Christiane Mercier. *Aspergillus niger* glucoamylase was purified by Qureshi[16], using the method of Fleming and Stone[17].

Analytical methods. — Total carbohydrate was measured by using the phenol–sulphuric acid method[18]. Total (1→4)-linked α-D-glucose in polysaccharide solutions was measured by enzymic conversion into D-glucose, using a mixture of glucoamylase and alpha-amylase as described previously[19]. Reducing sugars were determined by a colorimetric method[20] employing the Somogyi alkaline copper reagents[21]. D-Glucose was determined specifically by the D-glucose oxidase method of Lloyd and Whelan[22]. Iodine spectra were recorded with a Coleman–Hitachi recording spectrophotometer.

The methods used for enzymic analysis of the polysaccharide were as follows. beta-Amylolysis of the polysaccharide (125 μg/ml) was carried out with sweet-potato beta-amylase (100 U/ml) in acetate buffer (200mM, pH 4.8), followed by determination of the liberated maltose. The beta-amylase was stable under the conditions used (*cf.* ref. 23). alpha-Amylolysis of the polysaccharide (50 μg/ml) was performed in sodium glycerophosphate buffer (40mM, pH 7.0), using hog-pancreatic alpha-amylase (100 U/ml). The reducing sugars liberated were estimated in terms of maltose equivalents. Debranching with pullulanase (1.25 U/ml) was carried out in acetate buffer (pH 5.5, 100mM), the substrate concentration being 2 mg/ml. Debranching with isoamylase was performed as described previously[14], the enzyme concentration used

being 0.1 U/ml and the substrate concentration 2 mg/ml. The extents of hydrolysis brought about by the two debranching enzymes were determined by measurement of the reducing chain-ends liberated. In each case, the digests were incubated for 24 h at 37°.

All enzyme concentrations are expressed as International Units; 1 Unit (U) is the amount that liberates 1 μmole of reducing power (as D-glucose equivalents for the debranching enzymes, and maltose equivalents for alpha- and beta-amylase) from the respective substrate per minute at 37°.

RESULTS

Nature of the water-soluble polysaccharide. — Degradation of the polysaccharide with a mixture of *Aspergillus niger* glucoamylase and alpha-amylase, under the conditions described previously[19], gave a conversion into D-glucose in good agreement with the total carbohydrate content as measured by the phenol–sulphuric acid method[18]. This was taken to show the poly-α-D-glucose nature of the polysaccharide.

Iodine spectrum of the glucan. — The polysaccharide stained feebly with iodine, the spectrum of the complex having λ_{max} 500 nm. Addition of an equal volume of saturated, aqueous ammonium sulphate caused a marked enhancement of the absorption of the iodine complex and a shift in λ_{max} to ~550 nm (Fig. 1).

Degradation with amylolytic enzymes. — The extents of degradation of the polysaccharide by alpha-amylase, beta-amylase, pullulanase, and isoamylase are shown in Table I. Since the products of isoamylase action were completely converted into maltose by subsequent action of beta-amylase, the extent of hydrolysis by this

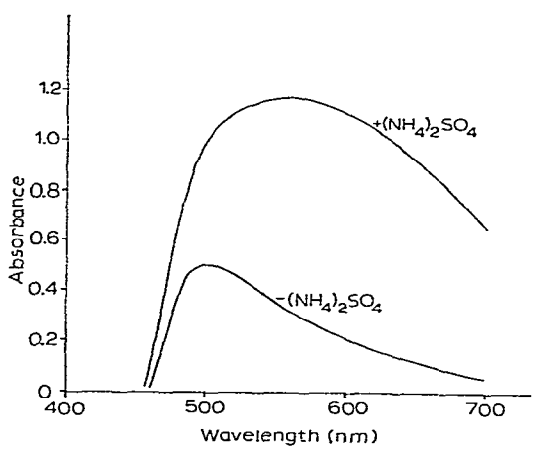

Fig. 1. Spectra of the iodine complex of *Cecropia peltata* polysaccharide in the presence and absence of ammonium sulphate. To polysaccharide solution (0.25 ml containing 133 μg of polyglucose) was added 0.25 ml of iodine reagent (0.2% of iodine in 2% aqueous potassium iodide) and 0.5 ml of saturated ammonium sulphate [+$(NH_4)_2SO_4$] or 0.5 ml of water [−$(NH_4)_2SO_4$], and the spectra were recorded in 1.0-cm cells against water.

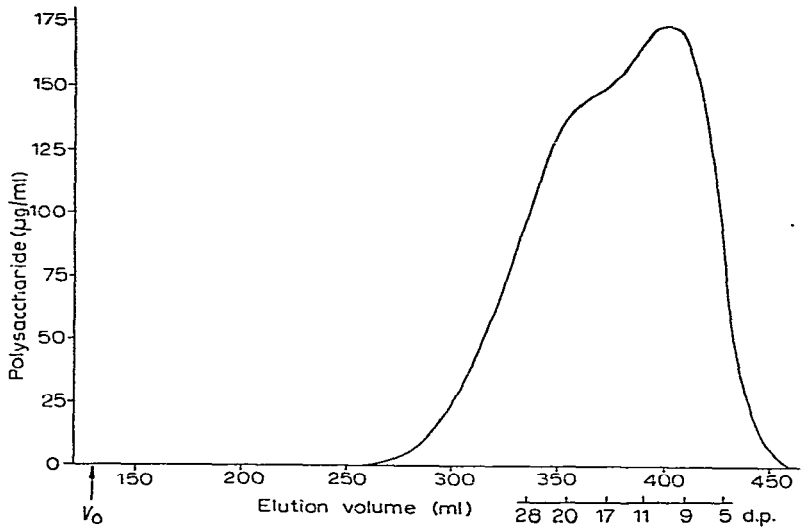

Fig. 2. Fractionation on Biogel P-10 of the products of debranching of *Cecropia peltata* polysaccharide with isoamylase. The polysaccharide was debranched and fractionated as described in the text. The void volume of the column is indicated by V_0. The degree of polymerization (d.p.) values shown were calculated from the carbohydrate contents and measured reducing powers.

TABLE I

ENZYMIC DEGRADATION OF *Cecropia peltata* POLYSACCHARIDE

Enzyme(s)	*Hydrolysis* (%)
Glucoamylase + alpha-amylase	100[a]
beta-Amylase	52.5[b]
alpha-Amylase	85.0[b]
Isoamylase	7.85[a] (100% debranching, corresponds to $\overline{CL}$ 12.7)
Pullulanase	1.05[a] (13.5% debranching)
Isoamylase + beta-amylase[c]	100[b]

[a]Sugars released determined as glucose equivalents. [b]Sugars released determined as maltose equivalents. [c]Used consecutively.

enzyme corresponds to complete debranching, thus enabling the extent of debranching by pullulanase also to be calculated.

Large-scale debranching and unit-chain profile of the polysaccharide. — Polysaccharide (10 mg) was incubated with isoamylase (0.4 unit) in acetate buffer (100mM, pH 5.5), the total volume of the digest being 5.0 ml. After incubation at 37° for 24 h, the enzyme was inactivated by heating at 100° for 5 min. The liberated unit chains were then fractionated on a column (95 × 2.5 cm) of Biogel P-10 by elution with 10mM phosphate buffer (pH 7.0) containing 1% of sodium chloride, fractions of 9.3 ml being collected automatically. Determination of the carbohydrate in the effluent[19] showed a distribution of unit chains as illustrated in Fig. 2.

DISCUSSION

Cecropia peltata is a small, neotropical tree of the family Moraceae. Species of this genus maintain a protective ant population which provides protection against overgrowth by other plants and decimation by herbivorous animals[24]. The ants live in the hollow stem internodes where they are provided with a food supply in the form of multicellular structures called Müllerian bodies. The Müllerian bodies are continuously differentiated from a pad of tissue on the abaxial surface of the leaf base and are harvested and eaten by the ants. These structures are formed by the plant alone and are not in any way dependent on an ant stimulation for their production. The present study was initiated after histochemical examination[7] indicated that glycogen rather than starch was stored in the plastids of the Müllerian body cells, and was aimed at putting the characterization on a rigorous biochemical footing.

The solubility of the polysaccharide, and the opalescent nature of the solution obtained, together with the weak iodine-staining power, which have been mentioned previously[7], are indicative of the polysaccharide's being a glycogen, but by themselves are insufficient to characterize it as such. For example, amylopectin may also be freely soluble, particularly after isolation by fractionation of starch or after slight degradation. An earlier characterization of a polysaccharide from wild apple as phytoglycogen[4] has been shown to be erroneous[6], the solubility being attributable to degradation during purification. In addition, polysaccharides other than those of the starch and glycogen class[25], as well as protein, substantial quantities of which are often present in polysaccharide preparations, may also interact with iodine to give the appearance of weak iodine-complex formation. However, the establishment of the poly-α-(1→4)(6)-D-glucose nature of the polysaccharide by enzymic conversion into D-glucose, taken together with its iodine-staining properties, does lend support to the suggestion that the polysaccharide is a glycogen. The wavelength of maximum absorption of the polysaccharide–iodine complex (λ_{max} 500 nm) is somewhat higher than that of phytoglycogen (450–480 nm[26]) but considerably less than that of amylopectin (~550 nm). The change in λ_{max} on addition of ammonium sulphate is in agreement with what would be expected of a glycogen; glycogens show large changes in both absorbance and λ_{max} (the latter changing by as much as 60 nm to values as high as 550 nm) on addition of ammonium sulphate[26–28]. Under the same conditions, there is little change in the iodine spectra of amylopectins. The extents of degradation by alpha-amylase (85%) and beta-amylase (52.5%) are also in agreement with the polysaccharide's being a glycogen since they are within the ranges (75–85% and 45–55%, respectively) expected for this type of polysaccharide, and rather less than what would be expected for amylopectin (90–100%; 55–60%). However, even these analytical measurements do not constitute an unequivocal characterization.

The best evidence comes from a consideration of the effect of the debranching enzymes pullulanase and isoamylase on the polysaccharide. The specificities of both these enzymes have been rigorously characterized[12,13,15,29]. Isoamylase will completely debranch both glycogen and amylopectin; action on the *Cecropia* poly-

saccharide caused 7.85% hydrolysis (Table I), equivalent to a $\overline{CL}$ of 12.7 for the polysaccharide[14]. Pullulanase action on amylopectin results in nearly complete debranching but the enzyme is without significant action on undegraded glycogens; carefully extracted rabbit-liver glycogen[15,30] and sweet-corn phytoglycogen[6], for example, are almost completely resistant to the action of this enzyme. Glycogens modified during extraction and purification, however, may be debranched to a certain extent (up to 50% in some cases), but never completely. Thus, comparison of the action of the two debranching enzymes makes it a simple matter to characterize a branched polysaccharide as glycogen or amylopectin. When the extent of debranching by isoamylase is greater than that by pullulanase, the polysaccharide is a glycogen. For amylopectin, the extents of debranching are almost identical. Thus, in the present case, the figures for the degrees of debranching by pullulanase and isoamylase (13.5% and 100%, the latter value indicating a chain length of 12.7), taken together with the other available evidence, make it clear that the polysaccharide from the *Cecropia* plastids is of the glycogen-type.

Our conclusion is confirmed by examination of the unit-chain profile of the isoamylase-debranched polysaccharide (Fig. 2), which is closely similar to that obtained on debranching of sweet-corn phytoglycogen[31] and distinct from that of amylopectin[31]. It is interesting to note that the similarity goes as far as the preponderance of unit chains on the high molecular weight side of the distribution. This is not observed with other (mammalian and bacterial) glycogens. Thus, *Cecropia* must take its place beside sweet corn as a higher plant synthesizing a glycogen-type storage polysaccharide.

We do not propose to speculate at the present time regarding the mechanism of biosynthesis of phytoglycogen, as compared with the biosynthesis of starch. For sweet corn, this has, in any case, been discussed in a recent review article[32]. However, it may well be the case that the Müllerian bodies of *Cecropia*, which, unlike sweet corn, do not synthesize a granular starch as well as the soluble polysaccharide, may provide an ideal system for examining the mechanism of biosynthesis of the soluble polysaccharide.

In conclusion, we point to the present work as an example of how simple the characterization of polysaccharides of the glycogen/amylopectin type becomes using purified, characterized glycoside hydrolases, in contrast to the classical chemical methods (*cf.* ref. 33).

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