α-(1→4)-D-GLUCANS

PART XXI[1]. THE MOLECULAR STRUCTURE OF STARCH-TYPE POLYSACCHARIDES FROM *Haematococcus pluvialis* AND *Tetraselmis carteriiformis**

SIR EDMUND HIRST, D. J. MANNERS**, AND I. R. PENNIE

Department of Chemistry, University of Edinburgh, and Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh (Great Britain)

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ABSTRACT

Polysaccharides isolated from the unicellular organisms *Haematococcus pluvialis* and *Tetraselmis carteriiformis* have been examined by chemical and enzymic methods, and characterised as having starch-type structures. After dispersion in alkali, the *H. pluvialis* starch was fractionated into amylose and amylopectin components. Fractionation of the *T. carteriiformis* starch was not completely successful, although an amylopectin component was obtained. The starches and components from both organisms were very similar in composition and structure to those from higher plants.

INTRODUCTION

Although the synthesis of starch is generally regarded as a characteristic of higher terrestrial plants, it is now clear that a similar polysaccharide is produced by some unicellular organisms. Examples include *Polytomella coeca*[2], *Polytoma uvella*[3], *Chilomonas parameciu*m[4], and *Nitella translucens*[5]. We now describe a chemical and enzymic investigation of the polysaccharide from two additional organisms.

Haematococcus pluvialis is a green-pigmented member of the Chlorophyceae (order, Volvocales), a group that characteristically stores both carbohydrate and fat, and has a cellulose-containing cell wall; *Polytoma uvella* is a non-photosynthetic member of this group[3]. *Tetraselmis carteriiformis* is a photosynthetic species of unicellular, marine-planktonic algae, and was selected as a representative of the class Prasinophyceae (order, Pyramimonadales). Members of this class contain chlorophyll *b* and were formerly included in the Chlorophyceae. The absence of a cellulose wall and the presence of scales on the surface of the cells, and of scales and hairs on the flagella, together with other features revealed by electron microscopy, have led to the establishment of a separate class of *Tetraselmis* and related algae[6]. A preliminary account of these results has been published elsewhere[7].

*Dedicated to Professor M. Stacey, C.B.E., F.R.S., in honour of his 65th birthday.
**To whom enquiries should be addressed.

MATERIALS AND METHODS

Analytical methods. — Glucose was determined by using a phenol–sulphuric acid reagent[8]; maltose was determined by the volumetric procedure of Somogyi[9], or the colorimetric Nelson–Somogyi method[10]. Descending paper chromatograms were prepared by using ethyl acetate–pyridine–water (10:4:3 v/v) as solvent, and visualized with alkaline silver nitrate[11]. The blue value (b.v.) of starch, amylopectin, and amylose was determined by the method of Bourne *et al.*[12]. For starch and amylose, λ_{max} of the iodine complex was determined by using the same b.v. solutions. For amylopectin, the λ_{max} was measured by using a solution containing ten times the concentration of polysaccharide and iodine. Potentiometric iodine titrations were carried out by the method of Bates *et al.*[13], on a scale reduced by a factor of twenty, so that only *ca.* 2 mg of starch or *ca.* 0.5 mg of amylose were required. The beta-amylolysis limits of polysaccharides were measured with either (*a*) a Wallerstein Laboratories (New York) preparation of barley beta-amylase in digests containing 50 units of enzyme per mg of polysaccharide at pH 4.6 and 37°, or (*b*) a crystalline, sweet-potato preparation from the Worthington Biochemical Corporation. The Wallerstein preparation contained a minute trace of alpha-amylase which was sufficient to affect the beta-amylolysis limit of amylose, but not that of starch or amylopectin (compare ref. 14). Debranching-enzyme preparations (isoamylase and pullulanase) were prepared from yeast and *Aerobacter aerogenes*, as described previously[15,16]. The extent of degradation by maltase-free, salivary alpha-amylase, prepared essentially by the method of Fischer and Stein[17], was measured in small-scale digests containing polysaccharide (0.5 mg) and alpha-amylase preparation (0.5 mg) in a total volume of 5 ml, which were incubated at 37° for 24 h. The relationship between the apparent percentage conversion into maltose (P_M) and the proportion of α-(1→6)-D-glucosidic inter-chain linkages (P_R) was investigated by using amylose, amylopectin, and glycogens of known chain-length, and found to be $P_R = 23{\cdot}5 - 0{\cdot}211(P_M)$. This equation agrees closely with previous results obtained with different preparations of salivary alpha-amylase[18].

EXPERIMENTAL

Haematococcus pluvialis. — The organism was grown and harvested by Dr. J. F. Ryley. Stock cultures were maintained on agar slopes (containing 0·1% of Oxoid peptone, 0.02% of potassium nitrate, 0.002% of magnesium sulphate, and 1% of agar) in daylight at room temperature (15–25°). For bulk growth, the peptone–acetate liquid medium previously described[3] in studies on *Polytoma uvella* was used. 2-Litre conical flasks, in batches of 24, each containing 1·5 litres of medium, were inoculated with 10 ml of a 6-day culture grown in the same medium. After growth for 8–12 days in daylight at room temperature, the cultures were harvested by using a Sharples supercentrifuge. Under these culture conditions, *ca.* 50% of the cells were motile, the rest being mainly non-motile palmellae with a few haematocysts. The cells were washed and stored in methanol, which removed a considerable amount of

green pigment. A total of 667 litres of culture medium was utilised in these studies.

The cells were very resistant to most physical and chemical methods of disintegration. They were unaffected by repeated freezing and thawing, by treatment with *N*,*N*-dimethylformamide, trichloroacetic acid, saponins, and sodium lauryl sulphate. A small amount of disruption occurred on exposure to ultrasonic vibrations at 0° for periods of up to 1 h, and on prolonged, rapid shaking with very small glass beads in a Mickle disintegrator. Cells which had been dried with ethanol, ether, and *in vacuo* over phosphorus pentoxide could be disrupted by grinding with clean carborundum in a chilled mortar. After extracting the ground material with hot water, and filtering to remove carborundum, the extract gave a blue stain with iodine, typical of starch. However, attempts to separate whole starch-granules from the abrasive particles by differential centrifugation were not successful.

Disintegration was finally achieved by using a Teflon homogeniser. A solid Teflon piston was rapidly rotated by a high-speed mechanical stirrer, in a closely fitting, hard-glass tube. An aqueous suspension of cells at 0° required homogenisation for 3–4 h before a high proportion of cell breakdown was observed. The resulting suspension was centrifuged at 1500 *g* for 15 min, and the precipitate washed with methanol and acetone until no further green pigment was removed. The homogenisation of whole cells (total wet weight, 15 g), in 10 batches, yielded a solid residue (extract *A*; wet weight, 6 g) which still retained some green colour, despite extensive washing with organic solvents. On total hydrolysis (M sulphuric acid, 100°, 2·25 h), extract *A* yielded glucose as the sole monosaccharide.

Attempts to free extract *A* from protein impurities were unsuccessful, since the available preparations of trypsin contained significant amounts of alpha-amylase. Starch was therefore isolated from extract *A* either by using methyl sulphoxide[19] or by the Sevag method[20].

A sample (0·144 g) of extract *A*, which had been washed with ethanol, acetone, and ether, and dried *in vacuo* over phosphorus pentoxide, was extracted for 6 days with methyl sulphoxide (15 ml) in an atmosphere of nitrogen. Undissolved material was removed by centrifugation; this contained very little carbohydrate, as shown by the phenol–sulphuric acid reagent[8]. The solution was heated to 65°, and 1-butanol (44 ml) was added with vigorous stirring. On slow cooling to room temperature, a precipitate formed which was collected by centrifugation, and dried by washing with ethanol, acetone, and ether, and then *in vacuo* over phosphorus pentoxide. The product (70 mg) was whitish-gray in colour, and gave a deep-blue stain with iodine. A portion (10·1 mg) of the product was dissolved in 2M sodium hydroxide, and the solution was neutralised and diluted to 10 ml. Aliquot portions of this solution were then analysed: the polysaccharide had b.v. 0·392, the iodine complex had a λ_{max} of 590–595 nm, an iodine affinity of 4·15%, and a beta-amylolysis limit of 61%. These results show the general properties to be similar to those of typical plant-starches.

Starch was also obtained by the Sevag method[20]. An aqueous suspension of extract *A* (*ca.* 75 ml) was shaken vigorously for 30 min with chloroform (15 ml) and 1-butanol (3 ml). After centrifugation at 1,000 *g* for 15 min, the solid material had

settled as two distinct layers — a thick, green layer covered by a thin, white layer (starch) — at the interface of the two liquid phases. The mixture was resuspended and centrifuged; the upper aqueous phase, containing the starch in suspension, was separated and subjected to three further Sevag treatments. The final suspension was collected by centrifugation, and the pure, white material washed and dried in the usual way; yield 0·5 g.

The purified starch had a D-glucose content of 95% and a b.v. of 0·404, and the iodine complex had a λ_{max} of 595 nm, *i.e.* the iodine-staining properties were very similar to those of the methyl sulphoxide extract.

The starch (250 mg) was dispersed in 2M sodium hydroxide (20 ml) at 40° for 4 h in an atmosphere of nitrogen, and fractionated with thymol as described previously[21]. The amylose–thymol complex was reprecipitated four times from 1-butanol-saturated water, and the final amylose–1-butanol complex was stored in 1-butanol-saturated water. The supernatant solution from thymol complexing was dialysed against running water, extracted with ether to remove thymol, and freeze-dried.

The resulting amylopectin (yield, 160 mg) had a D-glucose content of 98% and a b.v. of 0·12; the iodine complex had λ_{max} 550 nm. The beta-amylolysis limit was 57%; when incubated successively with isoamylase[15] and then beta-amylase, the percentage conversion into maltose was 69. When the amylopectin was incubated with a mixture of beta-amylase and isoamylase, there was 102% conversion into maltose. On incubation with salivary alpha-amylase, the percentage conversion into apparent maltose (P_M) was 88, indicating a P_R value of 4·9% which corresponds to an average chain-length of 20.4 D-glucose residues. Paper-chromatographic analysis confirmed the liberation of maltose, with smaller amounts of glucose and higher oligosaccharides.

The amylose–1-butanol complex was analysed to give the following results: b.v., 1·43; λ_{max} 665 nm; iodine affinity, 19.0%; beta-amylolysis limit, 88% with the crystalline, Worthington enzyme-preparation, and 103% with the amorphous, Wallerstein preparation.

Tetraselmis carteriiformis. — This organism was originally isolated by Dr. M. R. Droop of the Scottish Marine Biological Association Laboratory, Millport, and stock cultures were maintained by Mrs. K. M. G. Adam, Department of Zoology, University of Edinburgh. The organism was grown in shallow culture flasks (10 × 1 litre), which were autoclaved containing 100 ml of Droop's E.6 liquid medium[22]. To each flask, 1 ml of 10% aqueous D-glucose and 100 ml of sea water, both of which had been previously sterilised, were added aseptically. An inoculum (1 ml) was added from a stock culture of the alga, and the flasks were incubated under bright light for 18 days at room temperature (15–25°). The cells were harvested by centrifugation at 8,000 *g* for 30 min and stored in a concentrated, aqueous slurry at −20°. About 1.6 g of wet cells were obtained from a total of 2 litres of medium. Before freezing, the small, green cells were examined microscopically and shown to be very mobile due to the possession of flagella; after staining with iodine, blue particles were observed inside the cells.

The cells were disintegrated by ultrasonic treatment of an aqueous suspension

at 0–2° for several periods of 2 min. The suspension was then centrifuged at 38,000 *g*, and the supernatant solution was shown to be free of carbohydrate. The solid residue was washed once with water, and then several times with methanol to remove fats. Complete, acid hydrolysis of the grey-white residue gave glucose as the only monosaccharide. The residue was then deproteinised by the Sevag method[20], a suspension (50 ml) being shaken for 30 min with chloroform (10 ml) and 1-butanol (2 ml). After centrifugation at 1,000 *g*, a small amount of protein material had settled on the surface of the lower chloroform layer; the upper aqueous layer contained a white, granular suspension which was collected by centrifugation at a higher speed. The grey-white solid was washed with ethanol and ether, and then dried *in vacuo* over phosphorus pentoxide; yield 100 mg.

A solution of the polysaccharide (10 mg) was prepared by dispersion for 60 min in de-oxygenated, boiling water (*ca.* 9 ml), a stream of nitrogen gas being passed through the stirred suspension. Any undissolved material was removed by centrifugation, and the resulting solution analysed. The polysaccharide had a b.v. of 0·412, and the iodine complex had a λ_{max} of 585 nm and an iodine affinity of 4·05%. A partial, acid hydrolysate contained D-glucose, maltose, and higher maltosaccharides, and on incubation with beta-amylase the percentage conversion into maltose was 60.

A small-scale fractionation of the starch (50 mg) was attempted, under anaerobic conditions, by the thymol method[21]. A small amount of insoluble complex was collected by centrifugation and dispersed in boiling water, 1-butanol was added with stirring, and the dispersion was allowed to stand overnight. An amylose–1-butanol complex was not obtained, but centrifugation at 38,000 *g* gave a fine suspension which showed iodine-staining properties similar to the original starch. The supernatant solution from the thymol precipitation was washed with ether to remove residual thymol, traces of ether were removed by rotary evaporation, and the solution was freeze-dried to give a white powder (8 mg) which stained purple with iodine, indicating an amylopectin-type structure.

The amylopectin had a b.v. of 0·11, and the iodine complex a λ_{max} of 540 nm. The beta-amylolysis limit was 55%, and on incubation with a mixture of beta-amylase and pullulanase, there was 100% conversion into maltose. The alpha-amylolysis limit was 86%, corresponding to a P_R value of 5·4% and an average chain-length of 18·5 D-glucose residues.

DISCUSSION

The present investigations show that *H. pluvialis* and *T. carteriiformis* can now be added to the list of those organisms in the lower orders of the plant kingdom which synthesise a two-component starch. Studies of such organisms are often hampered by the difficulty in obtaining adequate amounts of cells, which also makes the subsequent purification of the polysaccharide more difficult. In addition, the work on *H. pluvialis* was hindered by our inability to effect disintegration of the cells by normal methods. This arose partly from the nature of the cell wall, and also from dehydration of the cells caused by storage in methanol. Since only small quantities of purified starch

were isolated, it was not possible to carry out fractionation experiments on the usual scale to give amylose and amylopectin components, to carry out a methylation analysis of these components, or to determine their molecular size by physico-chemical methods. Nevertheless, the interaction of the polysaccharides with iodine and their behaviour on partial hydrolysis with acid and on incubation with various specific amylolytic enzymes enable them to be characterised as starches.

The starch isolated from *H. pluvialis* by extraction with methyl sulphoxide had an iodine affinity of 4·15%; since the purified amylose had an affinity of 19·0%, this result shows that the amylose content of the starch was 22%. Attempts to fractionate starch purified by the Sevag method after dispersion in boiling water were unsuccessful. Similar difficulties have been reported in related studies on the starches from *Polytomella coeca*[2] and *Polytoma uvella*[3]. It was therefore necessary to disperse the starch in alkali, using anaerobic conditions to minimise any inadvertant, alkaline degradation of the components. The resulting amylose showed the expected high affinity for iodine; it was completely hydrolysed by the amorphous beta-amylase preparation, but with the crystalline enzyme, the beta-amylolysis limit was 88%. This result probably indicates the presence of a very small proportion of α-(1→6)-D-glucosidic inter-chain linkages, as has been demonstrated in amyloses from higher plant starches[23,24]. The amylopectin component was very similar to potato amylopectin; the highly branched structure was shown by the extent of alpha- and beta-amylolysis, and since the inter-chain linkages are hydrolysed by isoamylase, they are characterised as α-(1→6)-D-glucosidic in type.

TABLE I

COMPARISON OF THE PROPERTIES OF STARCH-TYPE POLYSACCHARIDES

Property	*Source*			
	Haematococcus pluvialis	Tetraselmis carteriiformis	*Potato var. Red Star*[a]	*Oats*[b]
Starch				
λ_{max}, nm	595	585	600	605
B.v.	0.404	0.412	0.42	0.40
Iodine affinity	4.15	4.05	4.02	4.10
Amylose content, %	22	20–21	20–21	22
beta-Amylolysis limit, %	61	60	65	66
Amylopectin				
λ_{max}, nm	550	540	555	540
B.v.	0.12	0.11	0.14	0.08
alpha-Amylolysis limit, %	88	86	89	88
Average chain-length	20	19	21	20
beta-Amylolysis limit, %	57	55	55	56
Exterior chain-length[c]	13–14	12–13	13–14	13
Interior chain-length[c]	5–6	5–6	6–7	6

[a]Data from ref. 7. [b]Data from ref. 1. [c]Calculated using the equations: average chain-length = exterior chain-length + interior chain-length + 1, where exterior chain-length = average chain-length × beta-amylolysis limit + 2·0.

Since the classification of *Tetraselmis* sp. has now been changed, it was of interest to determine whether there were any significant differences in the reserve carbohydrate from that in other members of the Chlorophyceae. The cells, which had been grown in pure culture, produced small quantities of an iodophilic polysaccharide having the analytical properties of a typical starch (Table I). It was unfortunate that attempts to fractionate the starch failed to give an amylose component; nevertheless, the major amylopectin component which was isolated was very similar to that from *H. pluvialis* and the other plants. It is therefore evident that the structure of the reserve polysaccharide is not a feature which can be used to distinguish this member of the class Prasinophyceae from other algae.

From a biochemical point of view, the present results imply a remarkable constancy in certain carbohydrate-metabolising enzyme systems in a wide range of organisms. In particular, the relative activity of starch synthetase and Q-enzyme would appear to be uniform, since these enzymes play a major role in controlling the relative amount of amylose and amylopectin in a given starch sample, and the degree of branching in the amylopectin component.

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