Carbohydrate Research, 140 (1985) 61–68
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

NEW GLUCURONOGLUCANS OBTAINED BY OXIDATION OF AMYLOSE AT POSITION 6

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(Received June 26th, 1984; accepted for publication, December 20th, 1984)

ABSTRACT

By adding finely powdered sodium nitrite to solutions of amylose in 85% (w/w) orthophosphoric acid, a series of D-glucurono-D-glucans was prepared, having number-average molecular weights of $\sim 10^4$ and GlcA/Glc ratios varying from 0.5 to 3.0. The products were soluble in water, both as sodium salts and free acids, formed complexes with iodine having λ_{max} in the range of 508–574 nm, and exhibited strongly pH-dependent chiroptical properties.

INTRODUCTION

It has been known for many years that cellulose can be oxidised at position 6 with liquid N_2O_4, to give a (1→4)-linked β-D-glucuronan (C-6-oxycellulose)[1]. Possibly because of the heterogeneous reaction conditions, which lead to excessive exposure of the more accessible chains to the reagent before the others can react, depolymerisation is severe, and the products, which are yellow to brown in colour, contain nitrogen[2].

The oxidation of cellulose by addition of finely powdered sodium nitrite to a homogeneous solution of the polymer in aqueous 85% orthophosphoric acid has been described[2]. Because of the high viscosity of the solution, the liberated N_2O_3 generated a foam which was stable for many hours before the bubbles coalesced and escaped. The high surface area within the foam, which expanded to 20 times the volume of the original solution, and the excess pressure within the bubbles appeared to be the major factors responsible for the smooth and rapid oxidation observed.

Oxidation by this method was not absolutely specific for position 6. Under the conditions required for complete oxidation of the primary alcohol groups, ~8% of the units were also oxidised at secondary positions, but these were readily reconverted into hexosyluronic acid residues by treatment with aqueous sodium

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0008-6215/85/$ 03.30

borohydride[2]. In agreement with other reports[3], this reduction occurred with considerable stereoselectivity, and it was not possible to detect any hexuronic acids other than D-glucuronic acid in acid-hydrolysates of the product.

We now describe attempts to apply the same method to amylose. The quest for suitable reaction conditions was less straightforward because higher concentrations of amylose were needed to give solutions of the required viscosity, and depolymerisation, apparently resulting from non-specific oxidation, was more severe. However, four partially oxidised samples were obtained, which should be of interest as model substrates for various enzymes, and in connection with studies of the iodine-binding reaction of amylose.

EXPERIMENTAL

Materials. — The amylose was a commercial sample ("Superlose", Stein, Hall & Co., Inc., New York). The number-average molecular weight, determined[4] on a water-soluble derivative (carboxymethylamylose), was 2×10^5. The iodine-binding capacity was 17.5%, corresponding to the presence of ~5% of amylopectin. It contained no nitrogen. Anhydrous sodium nitrite was ground to a fine powder and dried *in vacuo* over anhydrous calcium chloride at 40°. The amylose was dried under the same conditions. Standard sodium hydroxide solutions were prepared using triply-distilled water.

General methods. — All physical measurements were made at 25 ±0.05°, on solutions in 0.05M sodium perchlorate. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter, c.d. spectra with a Jasco J500A spectropolarimeter, osmotic pressures with a Melabs CSM-1 recording membrane osmometer, and intrinsic viscosities with a Schott-Geräte AVS/G automated capillary viscosity-measuring system. Potentiometric titrations were carried out with a Radiometer Type PHM52 digital pH meter. Volumes were measured with Hamilton precision microsyringes.

The D-glucose content of the samples was measured by heating portions (10 mg) in sealed tubes with 0.5M hydrochloric acid (3 mL) at 98° for 3, 6, 9, 12, 18, and 24 h. After cooling, portions (2.5 mL) of each hydrolysate were withdrawn, mixed with 0.2M sodium acetate (3 mL), and neutralised to pH 6 with 0.5M sodium hydroxide (2.4 mL). These solutions were then diluted appropriately for analysis by the D-glucose oxidase method[5]. The yields of D-glucose were plotted against time of hydrolysis, and the content of D-glucose in the sample was taken as the point of intersection of the initial and final slopes of the curve.

D-Glucuronic acid was assayed for the unhydrolysed samples (Na^+ salts) by the carbazole method[6], with C-6-oxycellulose as the standard[2]. The equivalent weights were measured by passing ~0.5% solutions (50 mL) through a column (8 × 4 cm) of Dowex 50 (H^+) resin to obtain the free-acid forms. A portion (5 mL) of each effluent was titrated potentiometrically with 0.1M sodium hydroxide, and another portion (10 mL) was freeze-dried and the residue was weighed.

Preparation of C-6-oxyamylose. — Amylose (10 g) was ground at ~22° with aqueous 85% (w/w) orthophosphoric acid (50 mL) until a clear, visco-elastic solution was obtained (~1 h). Sodium nitrite (10 g) was added portionwise, with vigorous grinding, during 5 min, and the mixture was stored at room temperature for 24 h. The foam was then broken up, releasing oxides of nitrogen. The rubbery, cream-coloured residue was ground with ether (3 × 200 mL), followed by decantation, and then with ethanol–water (2:1, 3 × 400 mL). The residual, granular solid was dispersed in ice-cold water (1 L), and solid sodium carbonate was added, with vigorous stirring, to pH 7. Sodium borohydride (20 g) was then added portionwise during 5 min, and the solution was stirred overnight at room temperature, then brought to pH 6 by the addition of glacial acetic acid, centrifuged for 1 h at 30,000*g*, dialysed exhaustively against distilled water, concentrated to 300 mL, and freeze-dried to yield a white solid (A1).

Sample A2 was prepared in the same way, and sample A3 was prepared from amylose (5 g), orthophosphoric acid (25 mL), and sodium nitrite (10 g). Sample A4 was prepared by further oxidation of sample A1 (Na^+ salt, 5 g) in orthophosphoric acid (10 mL) with sodium nitrite (5 g).

RESULTS

Conditions of oxidation. — The procedure described was arrived at by trial and error. The preparation and maintenance of a foam that was stable for many hours was crucial. In general, if the foam had not collapsed completely after 24 h, the oxidation was successful. This requirement could be met by adjusting the concentration of amylose in the initial solution to give a high viscosity, and by adding very finely powdered sodium nitrite, so as to generate very small bubbles. Attempts were also made to increase the viscosity by lowering the temperature, but then the rate of oxidation also decreased markedly; accordingly, all oxidations were performed at room temperature (22°).

There was a limit to the proportion of sodium nitrite that could be added successfully to a given volume of amylose solution due, in part, to the limited volume of N_2O_3 that the foam could contain, but also because the sodium phosphate formed in the early stages of the reaction made the foams more brittle.

Conditions of work-up. — After breaking down the residual foam and allowing all the oxides of nitrogen to escape, the excess of phosphoric acid was extracted with ether. Ethanol–water (2:1) may be used instead of ether, but this additionally extracts sodium phosphate and some of the more-degraded oxyamylose. To minimise the risk of an explosion, it has been recommended[2] that unreacted N_2O_3 should be reduced with formic acid, but this step is unnecessary provided that an excess of phosphoric acid has been used and sufficient time allowed for any unreacted N_2O_3 to escape.

Non-selective oxidation. — The initial products contained ketone groups, since they reacted with aqueous phenylhydrazine. Aqueous solutions also turned

TABLE I

CHARACTERISTICS OF C-6-OXYAMYLOSES[a]

Sample	*Yield (%)*	*Equiv. wt* [b]	*GlcA*		M_n ($\times 10^{-3}$)	$[\eta]$[e] (*dL g*$^{-1}$)	$[\alpha]_D^{20}$ (*degrees*)	$I_B\lambda_{max}$ (*nm*)
			c	*d*				
A1	—	495	0.38	0.34	10.3 ±0.3	0.098	185.8	560
A2	93	460	0.40	0.45	8.7 ±0.3	0.072	196.8	530
A3	86	360	0.52	0.56	11.1 ±0.4	0.157	202 6	574
A4[f]	66	255	0.75	0.67	10 2 ±0 3	0 058	173.8	508

[a]Na^+ salts. [b]Free-acid form. [c]Calculated as molar fraction from the equivalent weight. [d]Calculated as molar fraction from the sugar analysis. [e]In 0.78M NaCl at 25°. [f]Obtained by further oxidation of A1, the yield refers to the original weight of amylose.

yellow, and decreased in viscosity when made alkaline, evidently because of β-elimination. Reduction with sodium borohydride proceeded smoothly, rapidly, and with considerable stereoselectivity, since only D-glucuronic acid could be detected chromatographically among the acidic products of acid-hydrolysis.

There were indications that depolymerisation can occur as a result of non-

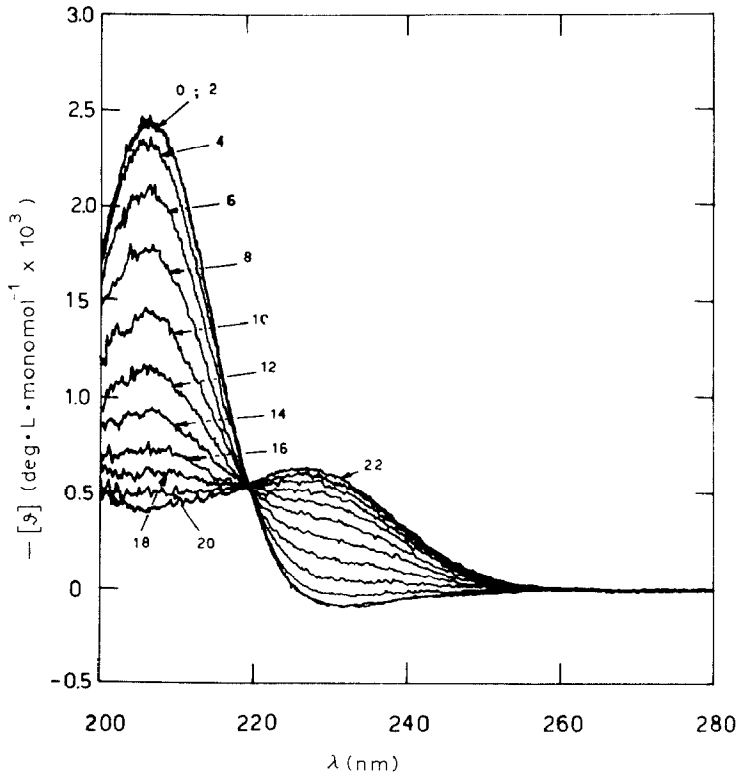

Fig. 1. C.d. spectra of C-6-oxidised amylose (A1) in 0.05M $NaClO_4$ at 25° as a function of 1.23M $HClO_4$ (μL) added to polymer (Na^+ form) solution (2 mL) (concentration, 10^{-2} equiv.L^{-1}).

specific oxidation. For example, when sample A1 was re-oxidised without prior reduction with borohydride, the product was so highly degraded that most of it passed through the dialysis membrane. On the other hand, re-oxidation after borohydride reduction afforded sample A4 in comparatively good yield (Table I).

Characterisation of the products. — Products A1–A4 were freely soluble in water, as either sodium salts or free-acid forms. Otherwise, they were typical glycuronoglycans, giving insoluble salts with Ca^{2+}, Sr^{2+}, Ba^{2+}, and heavy-metal cations, and with cetylpyridinium and cetyltrimethylammonium ions.

Intrinsic viscosities, $[\eta]$, and number-average molecular weights, $\bar{M}_n$, were determined in 0.78M NaCl at 25° in order to allow comparison with similar data for other amylose derivatives[4], and to eliminate the polyelectrolytic effect for samples of different charge density. The results (Table I) show that, whereas substantial depolymerisation occurs during the reaction, it does not appear to be related to the degree of oxidation. A molecular weight of $\sim 10^4$ consistently resulted from the starting value of 2×10^5 for the parent amylose sample[4], despite the fact that sample A4 was obtained by repeating the reaction on sample A1. Sample A4 appears to deviate from the trends exhibited by samples A1–A3, *i.e.*, with the sequences A3 > A1 > A2 for the values of $\bar{M}_n$, $[\eta]$, and $I_B\lambda_{max}$, and A3 > A2 > A1 for the values of GlcA and $[\alpha]_D^{20}$. These two correlations were expected, since $I_B\lambda_{max}$ and $[\eta]$ depend on $\bar{M}_n$, whereas $[\alpha]_D^{20}$ must depend mainly on the content of glucuronic acid. Because of the different procedure of preparation, sample A4 is

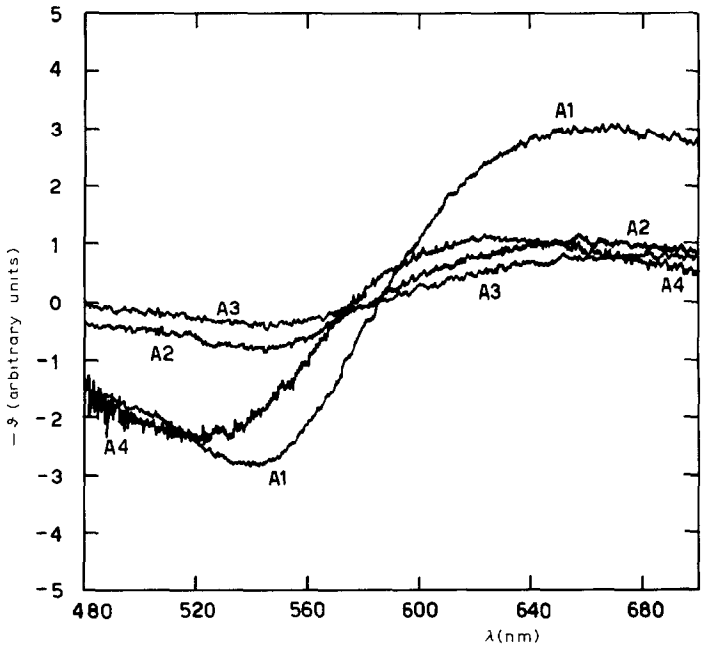

Fig. 2. C.d. spectra of the iodine complex of amylose derivatives in 0.01M KI at 25°. Polymer concentration, 5×10^{-3} equiv.L^{-1}; iodine concentration, 5×10^{-4}M.

probably not a homologue of A1–A3 and will not be considered further unless stated otherwise.

Chiroptical measurements were carried out on both the acid and the sodium-salt forms of samples A1–A4 (Table I). From the optical rotations of maltose and of samples A1–A3, an $[\alpha]_D^{20}$ value of +280° can be obtained for the 4-substituted α-D-glucuronate residue. This assumes additivity of the optical rotation on an average molar basis, with the absence of perturbations due to nearest-neighbor interactions. New bands appeared in the c.d. spectra in the region 190–250 nm, associated with the $n \rightarrow \pi^*$ and the $\pi \rightarrow \pi^*$ transitions of the carboxylate groups. These bands underwent a dramatic change in intensity, with a clear isodichroic point, when the pH of the solution was changed from neutral to acidic (Fig. 1).

The formation of a blue complex by the glucuronans with tri-iodide and iodine was studied by u.v.–visible absorption and c.d. spectroscopy. In all samples, a large shift of $I_B\lambda_{max}$ to smaller values with respect to that of amylose ($I_B\lambda_{max}$ 640 nm) was brought about by depolymerisation (Table I). The c.d. spectra are shown in Fig. 2. The retention of the chirality of the c.d. bands suggests that the left-handed helical conformation is still the most stable one, despite the change of neutral groups to carboxylate form.

DISCUSSION

Although proof is lacking, the conditions so far established as optimal for oxidation strongly suggest that the initial step is the formation of a nitrite ester, and that there is reduction of N_2O_3 to NO. Dilute aqueous nitrous acid does not oxidise carbohydrates and the N_2O_3 formed initially must have a finite but limited solubility in phosphoric acid, promoted by the foam, which allows rapid transfer between the gaseous and liquid phases.

Although the reaction conditions are strongly acidic, the rate of hydrolysis is surprisingly low. In earlier, kinetic studies[7,8] with simple glycopyranosides, cellobiose, and maltose, it was found that the slopes of the Zucker–Hammett plots were exceptionally low for phosphoric acid. The extent of hydrolysis during 24 h appears to be ~0.2% for cellulose and ~0.5% for amylose. The likely buffering action of the sodium phosphate formed in the early stages of the oxidation has not been investigated.

Severe depolymerisation appears to arise from non-specific oxidation, and it could be controlled by carrying out the oxidation in steps, with borohydride reduction after each. Sample A4 (Table I) was the product of a two-step oxidation of this kind, but it had physico-chemical properties which were markedly different from those of the samples obtained in a single oxidation step.

Under the alkaline conditions of borohydride treatment, depolymerisation by β-elimination could compete with reduction. During borohydride reduction of periodate-oxidised alginates, the rate of β-elimination could be greatly decreased relative to that of reduction, simply by increasing the concentration of reductant[9],

since the rate of reduction is proportional to, but the pH is independent of, the concentration of borohydride. In the present work, 2% borohydride was used, but this could profitably be increased to 20%.

The λ_{max} of the blue complex of amylose with iodine is a function of the d.p. of the amylose chains[10]. On the other hand, the stability of the iodine complex of carboxymethylamylose (CMA)[11,12] decreased markedly with increasing degree of substitution (d.s.). There was a marked decrease in λ_{max} and ε_{max} with increasing d.s. in samples of almost constant molecular weight[10,13]. In the present work, samples A3, A1, and A2 showed, in that order, a decrease in λ_{max} which parallels the decrease of $\bar{M}_n$ and of $[\eta]$, but not the trend of the degree of oxidation (see Table I). Also, for a given degree of modification (*i.e.*, either oxidation or carboxymethylation), λ_{max} and ε_{max} of the iodine complex are much larger for the C-6-oxyamylose than for CMA, possibly because the $-CH_2-O-CH_2-COO^-$ group is more bulky than the the COO^- group and/or because of the higher regioselectivity of the present reaction for position 6. Indeed, even minor amounts of carboxymethylation at positions 2 or 3 in CMA might prevent the formation of the helical structure. In contrast, all the optical data reported here for the C-6 oxyderivative indicate that the main conformational features required for the formation of helical channels in amylose are still present. Therefore, oxidation of the primary alcohol group to a carboxyl group is a controllable perturbation of many physico-chemical properties. Despite the extensive hydrolysis, the oxidised samples are excellent models for studying conformational properties of unmodified (slightly soluble) amylose in aqueous solution.

Another feature of the amylose derivatives described above is that the carboxyl function mimics natural glycuronates. Work is in progress on the physico-chemical characterisation of the present samples and of samples of C-6-oxidised cellulose, and to improve the oxidation procedure since, in its present form, it will not give products that are both highly oxidised and high in molecular weight.

ACKNOWLEDGMENTS

The authors thank the Consiglio Nazionale delle Ricerche (Rome), Progetto Finalizzato Chimica Fine e Secondaria, for financial support, and Kari-Anne Holsen for the sugar analyses.

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