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THE DISTRIBUTION OF D-GALACTOSYL GROUPS IN GUARAN AND LOCUST-BEAN GUM: NEW EVIDENCE FROM PERIODATE OXIDATION

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ABSTRACT

A detailed theoretical analysis of the kinetics of the periodate oxidation of guaran and locust-bean gum, together with the results of methylation analyses carried out by Lindberg's group on partially oxidised and borohydride-reduced samples of the galactomannans, has provided new evidence for the arrangement of the α-D-galactopyranosyl side-groups along the mannan chains. The results indicate that, in guaran, the D-galactosyl groups are arranged in small groups of mostly two to four units, attached to contiguous D-mannosyl residues in the chains. These are separated mostly by small groups of two, or occasionally three, contiguous, unsubstituted D-mannosyl residues. In locust-bean gum, there are long blocks of contiguous, unsubstituted D-mannosyl residues, long blocks in which every second D-mannosyl residue is substituted with D-galactose, and shorter blocks in which there is a high density of D-galactosyl groups.

INTRODUCTION

The new method is based upon the fact that the kinetics of periodate oxidation of (1→4)-linked polysaccharides are strongly influenced by the formation of hemiacetals between the aldehyde groups of oxidised residues and hydroxyl groups on unoxidised residues adjacent to them in the chains[1–3]. The formation of such an *inter*-residual hemiacetal may either inhibit or completely prevent oxidation of the unoxidised residue, depending upon the position of the equilibrium that is set up between the hemiacetal and other states of combination of the aldehyde group*. As expected, the position of the equilibrium is highly dependent upon the stereochemistry of both the oxidised residue and the unoxidised residues adjacent to it[3,4].

*These would include hydrates, hemialdals, and, when possible, *intra*-residual hemiacetals[1,3].

It is also strongly influenced by any unsubstituted hydroxyl groups remaining in the oxidised residue, because these would lead to the formation of *intra*-residual hemiacetals in competition with the *inter*-residual forms[1,3,5,6].

These findings suggest that, in principle, it should be possible to determine the sequence of different monosaccharidic units in a chain, simply by carrying out a sufficiently detailed theoretical analysis of the *shape* of the curve obtained by plotting periodate uptake against time. In practice, the scope of the method is limited by the large number of independent parameters**, the existence of experimental error, and the cost of computer running-time. For heteropolysaccharides with a non-regular structure†, it is realistic to try to determine only the nearest-neighbour†† and next-nearest-neighbour†† frequencies, and from these to generate representative sequences, either in the computer, or simply with a table of random numbers[7,8]. The significance of these frequencies is that they are the simplest possible way of explaining the kinetic data; the true structure may be more complex, but not less so. It should perhaps be noted that the same limitations are shared by ^{13}C-n.m.r. methods, which are also being developed for non-regular polysaccharides in these laboratories[12].

Full details of the theoretical principles of the method are given in recent papers[13,14] and in a thesis[15]. We now report on experimental results and conclusions for guaran and locust-bean gum, which were selected for the following reasons.

(*a*) Their high molecular weight made it possible to ignore end-group effects, including over-oxidation[16], which would also be blocked by the 6-*O*-substitution of D-mannosyl residues near to the reducing ends of the chains.

(*b*) Their structure can be formally regarded as a linear arrangement of (1→4)-linked, unsubstituted and 6-*O*-substituted β-D-mannosyl residues. The α-D-galactopyranosyl side-groups do not appear to enter into *inter*-residual hemiacetal formation of any kind, probably because the rings involved would be too large to be stable[5].

(*c*) The *inter*-residual hemiacetals formed between oxidised and unoxidised D-mannosyl residues in the main chain differ greatly in stability, depending upon whether or not HO-6 in the oxidised residue is unsubstituted[5]. When it is not, there is no possibility for the competitive formation of *intra*-residual hemiacetals, and the *inter*-residual hemiacetals formed with unoxidised neighbours are so stable that protection of the latter against subsequent oxidation is virtually complete[3,5]. When

**These include the ratio of the initial rates of oxidation of the different monomer units, and the positions of all the equilibria between the *inter*-residual hemiacetals and other forms.

†By this is meant polysaccharides that are not composed of a simple oligosaccharidic repeating-unit, and it implies that the biosynthetic enzymes do not show absolute specificity. It is only possible to describe the structure of such polysaccharides in terms of conditional probabilities that are an expression of the substrate specificities of the enzymes[9,10].

††These are the conditional probabilities that any given unit, or pair of units, respectively, will be followed by a unit of a particular identity. They are often expressed as "doublet" (or "diad") frequencies, and "triplet" (or "triad") frequencies, which are, respectively, the probabilities that any doublet or triplet, selected at random along the chain, will have a particular identity[10,11].

HO-6 is unsubstituted, only partial inhibition of the oxidation of adjacent residues is possible[3,5].

(*d*) Although no direct measurement of the positions of the equilibria set up by oxidised, unsubstituted D-mannosyl residues was possible (because of the insolubility of pure mannans), other data were available which compensated for this. The most important data were the methylation analyses performed by Hoffman *et al.* on samples of guaran[17] and locust-bean gum[18] that had been partially oxidised with periodate and then reduced with borohydride. In these analyses, unoxidised, 6-*O*-substituted and unsubstituted D-mannosyl residues were recovered as 2,3-di-*O*- and 2,3,6-tri-*O*-methyl-D-mannose, respectively. The molar ratio, D (2,3-Man/2,3,6-Man), of the yields of these two fragments had already provided information about the doublet frequencies in the two galactomannans[17,18], and is again used in this paper*.

The foregoing advantages had to be purchased at the price of correcting the periodate-oxidation curves for periodate consumed independently by the D-galactosyl groups.

RESULTS

Experimentally, the procedure consisted in careful measurement of the consumption of periodate by the two galactomannans as a function of time, and correction for the periodate consumed by the D-galactosyl groups, to give curves describing

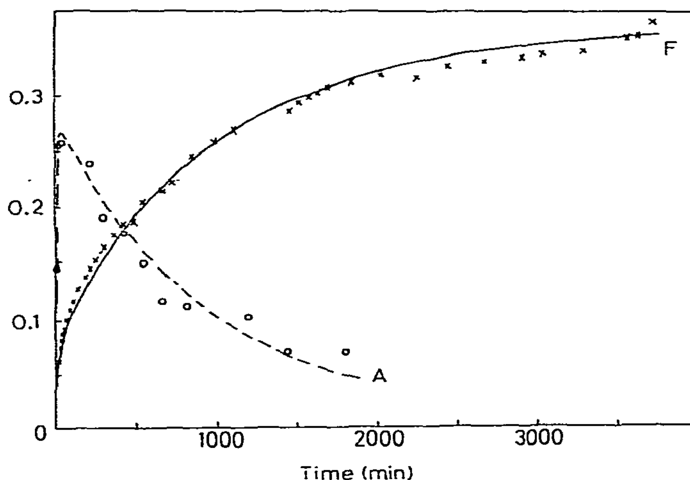

Fig. 1. Oxidation of guaran (4mM) in sodium metaperiodate (12.5mM) at 20°. F is formic acid liberated, and A is formaldehyde liberated by re-oxidation of partially oxidised samples after reduction with borohydride. Both quantities are expressed as mol per 162 g of guaran.

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*In the present work, the chromatographic peak-area ratios reported by Hoffman *et al.*[17,18] are corrected to molar ratios.

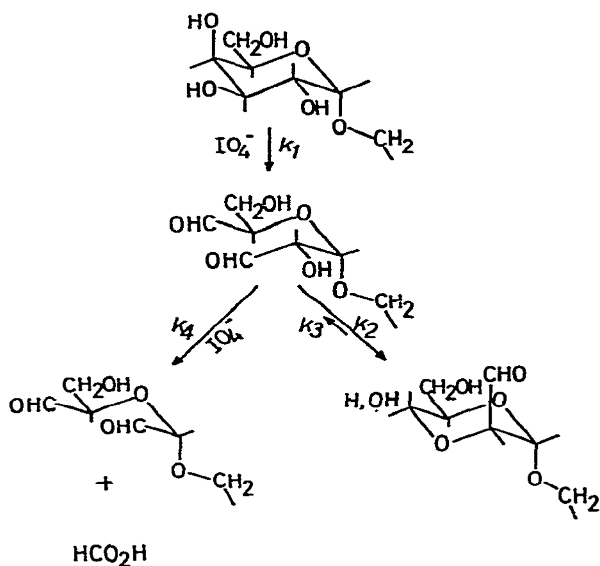

Fig. 2. Assumed reaction scheme for the oxidation of the D-galactosyl groups in guaran and locust-bean gum. Only the kinetically relevant steps are shown, and further cyclisation of the dialdehydes is likely. The curves in Fig. 1 are obtained by putting $k_1 = 10.7$ l.mol^{-1}.min^{-1}, $k_2 = 23$ min^{-1}, $k_3 = 0.007$ min^{-1}, and $k_4 = 51.2$ l.mol^{-1}.min^{-1}.

the oxidation of the mannan chains alone. For the interpretation, it was also necessary to measure the ratio (k_2/k_1) of the rates of oxidation of unsubstituted and substituted D-mannosyl residues, respectively.

Correction for periodate consumed by the D-*galactosyl groups.* — For guaran, this was achieved by making two additional measurements: namely, (*i*) the liberation of formic acid (F) as a function of time; and (*ii*) the formaldehyde (A) liberated by re-oxidation of twelve samples of guaran that had been oxidised for different times, and then reduced with sodium borohydride. Since both F and A could only have arisen from doubly- and singly-oxidised D-galactosyl groups, respectively, the amount of periodate consumed by these groups at any time is simply expressed as ($2F + A$).

The results for F and A are given in Fig. 1. The curves in Fig. 1 are theoretical[15], and conform to a mathematical expression of the reaction scheme shown in Fig. 2, which is known to be valid for methyl α-D-galactopyranoside[19,20].

For locust-bean gum, repetition of this laborious experimentation was obviated by using the same mathematical expression. The implicit assumption that the kinetics of oxidation of the D-galactosyl groups would be the same as in guaran was apparently justified by the close fit between theory and experiment that was achieved for the galactomannan as a whole.

Measurement of the ratio (k_2/k_1) *of the rates of oxidation of unbranched and branched* D-*mannosyl residues.* — The methylation data of Hoffman *et al.*[17] had already indicated that unsubstituted D-mannosyl residues were initially oxidised

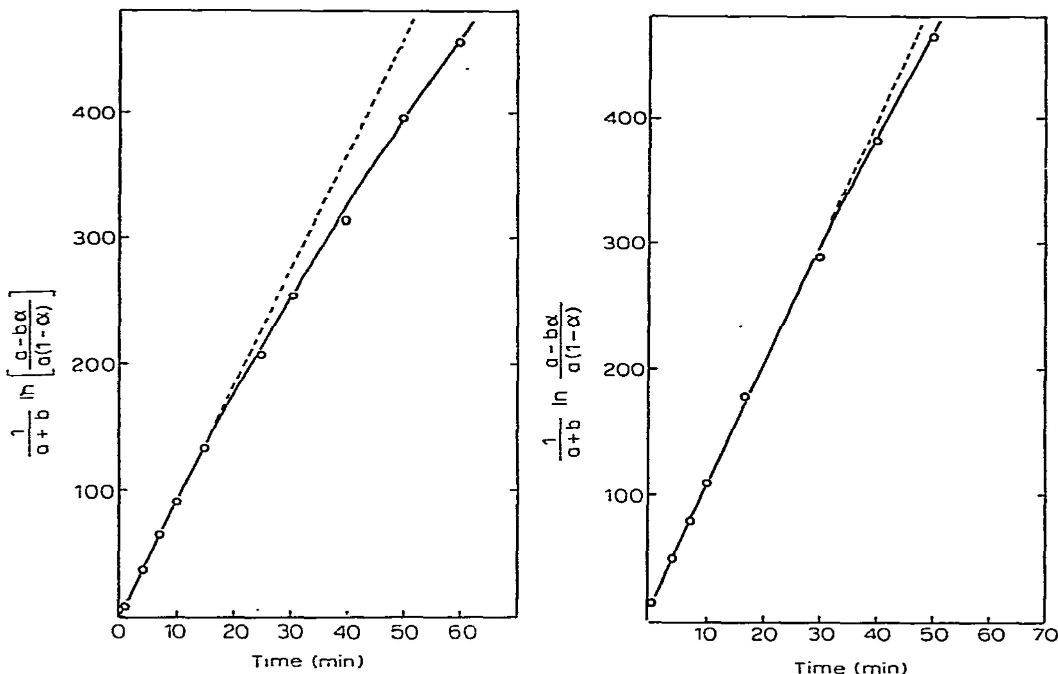

Fig. 3 (left). Second-order kinetic plot for re-oxidation of guaran after an initial oxidation for 170 h under the conditions of Figs. 1 and 5, followed by reduction with sodium borohydride. The initial concentrations of periodate and *vic*-diol groups were 6.25mM and 1.55mM, respectively. The temperature was 20°.

Fig. 4 (right). Second-order kinetic plot for re-oxidation of locust-bean gum after an initial oxidation for 48 h under the conditions of Fig. 6, followed by reduction with sodium borohydride. The initial concentrations of periodate and *vic*-diol groups were 6.25mM and 1.72mM, respectively, and the temperature was 20°. The small intercept on the ordinate axis is probably due to the presence of a small proportion of incompletely (singly) oxidised D-galactosyl groups, which are oxidised very rapidly.

somewhat faster than substituted ones. Because of the presence of the D-galactosyl groups, and the rapid changes in rate brought about by hemiacetal formation, it was not possible to determine the ratio accurately from the initial slopes of the periodate-uptake curves. Both galactomannans were therefore exposed to periodate until all the D-galactosyl groups had been fully oxidised; they were then reduced with sodium borohydride, and re-oxidised with careful measurement of the periodate uptake as a function of time.

In Figs. 3 and 4, the results are plotted according to second-order kinetics in the usual way, whereby obedience to the second-order rate-equation would give a straight line with a slope equal to the rate coefficient. The slight curvature actually observed is consistent* with the presence of two different kinds of D-mannosyl

*It is also consistent with the possible presence of a small fraction of contiguous, unsubstituted D-mannosyl residues.

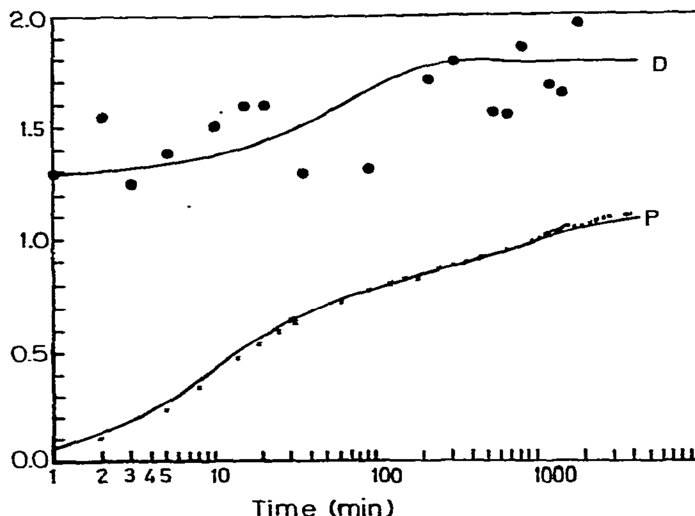

Fig. 5. Oxidation of guaran (4mM) in sodium metaperiodate (12.5mM) at 20°. P is periodate consumed, expressed as mol per 162 g of guaran, and D is the ratio of unoxidised, branched D-mannosyl residues to unoxidised, unbranched D-mannosyl residues, as determined by methylation analysis[17].

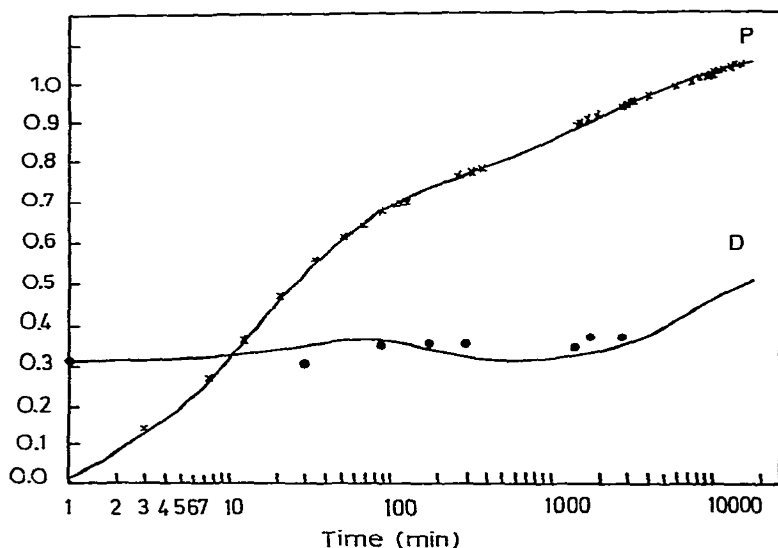

Fig. 6. Oxidation of locust-bean gum under the conditions of Fig. 5.

residues having slightly different reactivity. The initial slopes could, however, be measured with good accuracy, and indicated rates of 9.1 and 10.4 $l.mol^{-1}.min^{-1}$ for guaran and locust-bean gum, respectively. From these results, together with the methylation ratios (D) of 1.75 and 0.37 for the partially oxidised and reduced samples of guaran and locust-bean gum, respectively, it was calculated that the ratio, k_2/k_1, was 1.4 $\pm$0.1.

A significant, incidental finding in these experiments was that the sum of the periodate consumed in the first oxidation and that consumed in the second oxidation, after borohydride reduction, corresponded almost exactly to the expected Malapradian oxidation-limits of 1.36 and 1.21 mol. per hexosyl residue, for guaran and locust-bean gum, respectively.

Theoretical interpretation of the periodate-oxidation curves. — Full details of the specific mathematical techniques that were used are given in a thesis[15], and will also be published in a suitable journal of chemical physics. In principle, they consisted in varying the doublet and triplet frequencies until the predicted theoretical curves matched the experimental ones. This task was less onerous than it may seem, because the four doublet and eight triplet frequencies are not fully independent quantities; given the quantitative composition of the galactomannan, it was only necessary to determine three additional probabilities in order to be able to calculate them all[15].

Figs. 5 and 6 illustrate the agreement achieved for guaran and locust-bean gum, respectively, by showing experimental points for the periodate (P) consumed by the whole polysaccharides, and for the methylation ratios (D)*. The curves are those predicted theoretically by the doublet and triplet frequencies shown in Table I, after adding the contribution of the D-galactosyl groups (to P). The uncertainties in the values in Table I are rather variable. For the doublet frequencies, the uncertainty is $\sim\pm 0.015$, whereas for the triplet frequencies, and especially for F_{222}, it may be as high as ± 0.03.

An idea of what these probabilities mean in terms of structure is given by the

TABLE I

SEQUENTIAL STRUCTURE OF GUARAN AND LOCUST-BEAN GUM, IN TERMS OF DOUBLET AND TRIPLET FREQUENCIES[a]

Doublet frequencies	*Guaran*	*Locust-bean gum*
F_{11}	0.35	0.07
F_{12} ($= F_{21}$)	0.215	0.165
F_{22}	0.22	0.60
Triplet frequencies		
F_{111}	0.17	0.04
F_{211} ($= F_{112}$)	0.18	0.03
F_{122} ($= F_{221}$)	0.19	0.03
F_{222}	0.03	0.57
F_{121}	0.02	0.13
F_{212}	0.04	0.14

[a]The subscripts 1 and 2 refer to branched and unbranched D-mannosyl residues respectively.

*It will be appreciated that the values for D are less accurate than those for P, because the methylation analyses entailed five steps, whereas the periodate assays consisted of a simple titration.

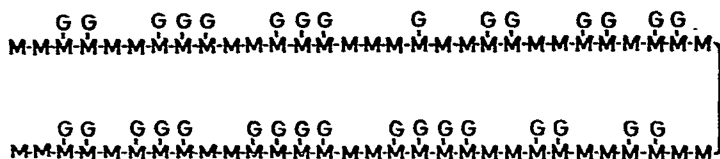

Fig. 7. Representative structure in guaran, obtained with a random-number generator in the computer.

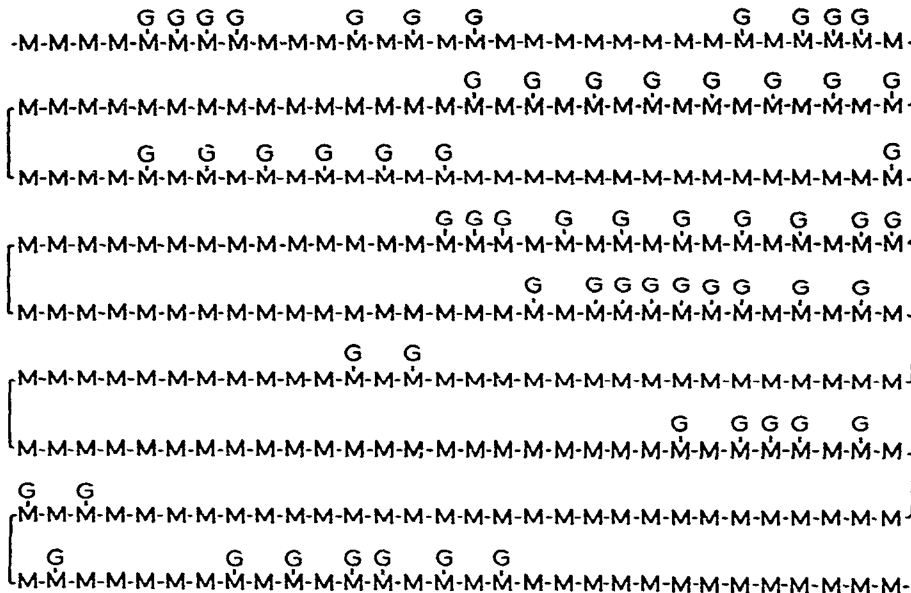

Fig. 8. Representative structure in locust-bean gum, obtained with a random-number generator in the computer. The doublet and triplet frequencies in this structure do not agree perfectly with those in Table I, because the chain shown is not sufficiently long in relation to the length of the blocks.

representative sequences shown in Figs. 7 and 8, which were printed out by the computer. Although the uncertainties in the smaller probabilities are, percentage-wise, large, any improvement in accuracy is unlikely to change the overall impression of small groups in guaran and blocks in locust-bean gum. The greatest uncertainty lies in the average length of the blocks in locust-bean gum.

DISCUSSION

In their pioneering work on guaran, Whistler and his co-workers[21,22] noted that the yields of oligosaccharidic fragments, as well as the physical properties of the polymer, could best be explained by assuming that the D-galactosyl groups were fairly uniformly distributed along the chains. X-Ray diffraction spectra of stretched films of guaran seemed to confirm this, and were interpreted by Palmer and Ballantyne as indicating an alternating arrangement of branched and unbranched D-mannosyl residues along the chain[23].

A major, *a priori* reason for questioning the idea of a "strictly alternating" structure for guaran is to be found in the composition of the polymer itself. Most samples, including the first to be described by Heyne and Whistler[24] and the one studied here[5,17], contain 36% of D-galactose. This apparently small excess of D-galactosyl groups has a surprising significance from the point of view of structure; if, for example, they were inserted at regular intervals into a strictly alternating arrangement, 40% of all the D-galactosyl groups in the molecule would become members of a triplet.

After a lapse of many years, Courtois and Le Dizet[25] re-opened the question by studying the action of a purified β-D-mannanase on locust-bean gum and several other galactomannans (which, however, did not include guaran). The results indicated that about a third of the D-galactosyl groups in locust-bean gum were concentrated in regions of high density ("blocks"), while the remainder occupied isolated (non-contiguous) positions on the chains.

Recently, Baker and Whistler[26] used alkaline degradation of a 6-sulphone derivative to cleave the galactomannan chains selectively at positions where the D-mannosyl residues were unsubstituted with D-galactosyl groups. The results seemed to confirm the alternating structure for guaran, whereas for locust-bean gum they differed markedly from the results of Courtois and Le Dizet[25], in indicating that the D-galactosyl groups were present exclusively in long blocks.

The present results (Figs. 7 and 8) are in reasonable agreement with the findings of Courtois and Le Dizet[25], but indicate the virtual absence of alternating sequences in guaran. Further work is needed to determine whether the existing conflicts are due to defects in the methods used, or to natural variations in structure from one sample of galactomannan to another.

EXPERIMENTAL

The materials and methods were essentially as described earlier[5,17,18], except that a special rapid-mixing procedure was used to start the oxidations, and, for measurements made during the first 20 min of reaction, to stop the reaction instantaneously at the desired time. These modifications were necessary to avoid errors due to the slow delivery times of pipettes, and are described in adequate detail in a paper dealing with amylose oxidation[27]. In some experiments, 1-propanol (2% v/v) was included in the reaction mixture as a precaution against free-radical depolymerisation[1,28,29], but it had no effect upon the results.

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