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Borohydride reactivity of cellulose reducing ends

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Abstract

A comparative study of the kinetics of sodium borohydride reduction of reducing ends on model celluloses and soluble cellooligosaccharides (COS) was conducted to better understand the nature of reducing ends encountered by reducing end-specific exo-acting cellobiohydrolases. Apparent second-order rate constants for the reduction of glucose, COS (degree of polymerization from two to five) and typical microcrystalline and amorphous cellulose substrates were determined. In general, rate constants for the reduction of the cellulose-associated borohydride-accessible reducing ends were similar to those describing the reduction of the COS. Thus, no significant differences were observed between the amorphous celluloses and COS. The reactivity of the microcrystalline cellulose preparation was found to be significantly ($p=0.0018$) lower ($\sim$20%) than that of the reference COS. All of the celluloses and COS had significantly lower rates of reaction than glucose ($p<0.0001$). The results indicate that the reagent-accessible reducing ends on typically employed amorphous cellulose substrates behave similar to those of cellooligosaccharides free in solution.

Keywords: Cellooligosaccharide; Cellulose; Reduction; Reducing ends; Kinetics; Sodium borohydride

1. Introduction

Cell-free fungal enzyme systems capable of efficiently catalyzing the saccharification of cellulose are typically comprised of a mixture of endo- and exo-acting cellulases. The predominant enzymes in these mixtures are usually the exo-cellulases. Exo-cellulase catalysis is dependent on an initial seating of an appropriate cellulose chain end in the enzyme's catalytic site. The appropriate end, be it either the reducing end or the non-reducing end of the cellulose molecule, is dependent on the nature of the enzyme (Barr, Hsieh, Ganem, & Wilson, 1996; Becker et al., 2001; Davies & Henrissat, 1995; Koivula et al., 2002; Nutt, Sild, Pettersson, & Johansson, 1998; Valjamae, Sild, Pettersson, & Johansson, 1998). Those exo-acting cellulases that preferentially act via the reducing end are presumably dependent on acquired mechanisms for interaction with such ends. Interactions of this type will of course be dependent on the complimentary chemistry of the enzyme and the substrate's reducing end. The present study was designed to provide information on the reactivity of such reducing ends in typically employed cellulose substrates. An important related question is whether or not the reducing ends of soluble cellooligosaccharides adequately represent the reducing ends in typically employed cellulose substrates, since both classes of substrates are used to study exo-cellulase activity (Mattinen, Linder, Teleman, & Annila, 1997; Medve, Karlsson, Lee, & Tjerneld, 1998; Sild, Nutt, Petterson, & Johansson, 1998; Vrsanska & Biely, 1992). The experimental approach was to compare the borohydride-reactivity of a series of cellooligosaccharides (COS) with that of celluloses typically employed in the study of exo-acting cellulases. 'Reactivity' is here based on each of the compounds', all of which have reducing end terminal glucosyl residues, susceptibility to reduction by sodium borohydride.

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2. Materials and methods

2.1. Substrates

Glucose was purchased from Sigma Chemical Co (St Louis, MO). Cellooligosaccharides were prepared by acid hydrolysis of the microcrystalline cellulose and subsequent fractionation using stearic acid-treated charcoal-based columns (Miller, Dean, & Blum, 1960). Microcrystalline cellulose (Avicel, PH 101) was from FMC (Philidelphia, PA). Amorphous cellulose was produced from MCC by the method of Isogai and Atalla (1991) using a SO_2-diethylamine-dimethylsulfoxide solvent system for cellulose dissolution. Phosphoric acid swollen cellulose (PSC) was prepared from the MCC starting material according to Ståhlberg, Johasson, and Petterson (1993). Amorphous and phosphoric acid-swollen celluloses were kept in water containing 0.02% sodium azide until used.

2.2. Hydrolysis of sodium borohydride

Rates of hydrolytic decomposition of sodium borohydride ($NaBH_4$) were determined as the change in total reducing power of the borohydride containing solution with time Reaction conditions were 25 mM sodium borohydride, 100 mM sodium phosphate, pH 8.0, and 22 °C. Total reducing power was quantified as the capacity for the cupric to cuprous ion conversion, as measured using the bicinchoninic acid reagent (BCA, Garcia, Johnston, Whitaker, & Shoemaker, 1993). The time course of the reaction was followed for 600 min and experiments were conducted in duplicate on separate occasions. Total reducing power was converted to sodium borohydride concentrations by the use of calibration curves prepared using standard sodium borohydride solutions.

2.3. Reduction of glucose, cellooligosaccharides, and celluloses

Reaction mixtures were designed to be 25 mM $NaBH_4$ and 0.033 mM reducing ends (total reaction volume of 1 ml) The molar concentration of reducing ends in the glucose and the cellooligosaccharide solutions was taken as the molar concentration of each compound itself. For the celluloses, the molar concentration of reagent-accessible ($NaBH_4$-accessible) reducing ends was taken as the difference in the total reducing ends per unit weight cellulose before and after exhaustive reduction by sodium borohydride under the given reaction conditions (Kongruang, Han, Breton, & Penner, 2004). Reductions were done in 100 mM sodium phosphate, pH 8.0, at 22 °C. Time courses for the reaction were determined by monitoring the decrease in reducing ends over a 90-min reaction period. At predetermined times (0, 5, 10, 15, 20, 25, 30, 40, 50, 60, and 90 min) the reaction was terminated by lowering the pH (w/20 μl HCl) to rapidly decompose the borohydride reducing agent. The number of reducing groups remaining in reaction mixtures was then determined by the copper-based bicinchoninic acid method (Garcia et al., 1993).

2.4. Rate of incorporation of sodium boro [^{3}H] hydride

Solid sodium boro [^{3}H] hydride, specific activity 370 mCi/mmol (13.7 GBq/mmol), was obtained from ICN Biomedicals (Irvine, CA) A 0.2505 M NaB^3H_4-working solution (specific activity 0.738 mCi/mmole), in 0.1 M NaOH, was prepared using the commercial NaB^3H_4 preparation combined with cold $NaBH_4$. Reductions were initiated by the addition of 100 μl NaB^3H_4-working solution to a 20 ml scintillation vial containing the reducing carbohydrate suspended in 0.88 ml 0.1 M sodium phosphate, pH 8. Initiated reactions were allowed to proceed at 22 °C for specified times and then terminated by the addition of 20 μl 37% HCl. Acidified reaction mixtures were allowed to stand open for a minimum of 30 min. Reaction mixtures were then evaporated to dryness on a hot plate at 50 °C (approximately 1 h). The evaporation step was necessary to drive off residual labeled $^3H_2$ gas (Conrad, Bamburg, Epley, & Kindt, 1966). The solid residue remaining was then resuspended in 1 mL phosphate buffer and to that suspension was added 10 ml scintillation cocktail (Scintisafe Gel, Fisher Scientific, Pittsburgh, PA). Samples were counted in a Beckman LS 6500 Scintillation System (Beckman Instrument, Inc., Fullerton, CA) at 20 min per vial. Time courses of isotope uptake were developed using reaction times of 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 120 min. Controls included the treatment of previously $NaBH_4$ reduced celluloses as described above to assess the extent of non-specific isotope incorporation. Experiments were done in triplicate and all reductions and subsequent treatments involving tritium, up to the scintillation counting, were done in a hood.

2.5. Curve fitting and statistical analyses

Progress curves of all reactions were analyzed using DYNAFIT, a kinetic simulation program (Kuzmič, 1996) Concentration changes, with time, were fitted assuming the borohydride/carbohydrate reaction obeys second order kinetics. Changes in borohydride composition due to hydrolytic degradation were modeled using first order kinetics. Thus, time courses were obtained by solving an initial value problem described by the following system of differential equations:

$$d[BH_4^-]/dt = -k_d[BH_4^-] - k_{app}[BH_4^-][G]$$

$$d[B(OH)_4]/dt = k_d[BH_4^-] + k_{app}[BH_4^-][G]$$

$$d[G]/dt = -k_{app}[BH_4^-][G]$$

$$d[GH]/dt = k_{app}[BH_4^-][G]$$

where BH_4^- is borohydride; $B(OH)_4$, borate; G, aldose form of carbohydrate; and GH, alditol form of carbohydrate. Parameter k_d (s^{-1}) is the first order rate constant describing borohydride decomposition and k_{app} ($M^{-1}\,s^{-1}$) the second order rate constant describing the reaction between borohydride and the aldose form of the carbohydrate. A modification of the Livermore solver of ordinary differential equations was used to numerically solve the above equations and compute progress curves. Progress curves, and thus the corresponding rate constants, were optimized by least-squares regression of the calculated and real data (Marquardt, 1993 and Reich, 1992). Statistical differences between best-fit second order rate constants obtained for the reduction of the different test compounds were determined using multiple regression analyses (extra-sum-of-squares F-test) by SAS software (SAS Institute, Inc., 1990), yielding two-sided '*p*-values'. Significant differences were defined as $p<0.002$. Cellotriose was used as the prototypical cellooligosaccharide for statistical comparison of rates of reduction among cellooligosaccharides and celluloses.

3. Results and discussion

Time courses for the reduction of the different test compounds are presented in Fig. 1a–h. Included in each figure is the corresponding simulated time course generated using the second order rate constants that 'best fit' (least-squares optimization, Table 1) the real data. The general agreement between the calculated and real data indicates the second order rate expressions are adequate for such analyses. Rate constants for the reduction of the different cellooligosaccharides were found to be similar, all of them being significantly below the corresponding value for glucose. The relative values of the rate constants for glucose and cellobiose are in general agreement with a previous report showing glucose to be the more reactive (Bragg & Hough, 1957); values for the other cellooligosaccharides are not in the available literature. It is important that no significant differences were detected among the rate constants for cellooligosaccharides of differing degree of polymerization (DP). This observation leads to the conclusion that rate constants describing the reduction of celluloses, i.e. those polymers with much higher DP, will also be in this general range provided the reaction is not hindered by factors associated with the insoluble nature of the celluloses.

Comparison of the rate constants obtained for the reduction of the different celluloses indicates a demonstrable difference between the reactivity of the reducing ends associated with amorphous versus microcrystalline celluloses. Rates of reduction of the reducing ends in the amorphous cellulose preparations were not significantly different from those obtained for the soluble cellooligosaccharides ($p=0.3129$). In contrast, the corresponding rate constant for the microcrystalline cellulose was significantly lower ($p=0.0018$). The cellulose-associated rate constants obviously reflect only the reactivity of the 'reagent-accessible' reducing ends (Kongruang et al., 2004); these ends presumably being on the surface of cellulose aggregates/fibers.

Included in Table 1 is a minimal value for the rate constant describing the time course of glyceraldehyde reduction. This minimal value reflects the fact that glyceraldehyde was essentially consumed in the reaction prior to the initial time point used to monitor the reduction of the other test compounds. The relatively rapid rate of reaction with glyceraldehyde, relative to the other compounds, indicates the importance of the chemistry of the carbohydrate moiety in determining the overall rate of borohydride reduction. A major difference between glyceraldehyde and the other compounds is its inability to form the hemiacetal ring structure. One may surmise that the slower reaction rates for glucose, the cellooligosaccharides, and the celluloses is largely attributable to their existing, predominantly, in the ring form - which is not reduced by sodium borohydride. In the acyclic form, the saccharides would be expected to react at rates approaching that of glyceraldehyde. This rationale for the difference in reaction rates for glyceraldehyde and glucose minimizes the importance of potential steric effects limiting borohydride-glucose interactions.

The pH chosen for this study, pH 8.0, was a compromise between that necessary for sodium borohydride ($NaBH_4$) stability and relevance to the enzymatic saccharification of cellulose. Sodium borohydride decomposition is acid catalyzed (Davis & Swain, 1960), thus the reagent is far more stable at highly alkaline pHs. Fungal cellulases typically have pH optima below 7, pHs at which borohydride rapidly hydrolyzes. Thus, pH 8.0 was chosen since it is within the technologically relevant range and yet the rate of borohydride decomposition at this pH is such that it can be accounted for (see Section 2). The actual time course of $NaBH_4$ hydrolysis under these experimental conditions is presented in Fig. 2. The simulated data was generated using a first order rate expression with $k=0.000291\ s^{-1}$ ($0.017451\ min^{-1}$).

To check the validity of the experimental approach used to generate the previously discussed data, an analogous set of experiments, focusing on glucose and the experimental celluloses, was done using tritiated sodium borohydride. In this case the extent of reduction was determined by isotope uptake at the C-1 position (Bhat, Hay, Claeyssens, & Wood, 1990; Chirico & Brown, 1985; Evans, Sheppard, Turner, & Warrell, 1974; Mclean, Werner, & Aminoff, 1973; Richards & Whelan, 1973), rather than by measuring the amount of reducing carbohydrates remaining, over the course of the reaction. Time courses illustrating the results from this set of experiments are presented in Fig. 3a–d. The corresponding 'best fit' rate constants are tabulated in Table 2. The general

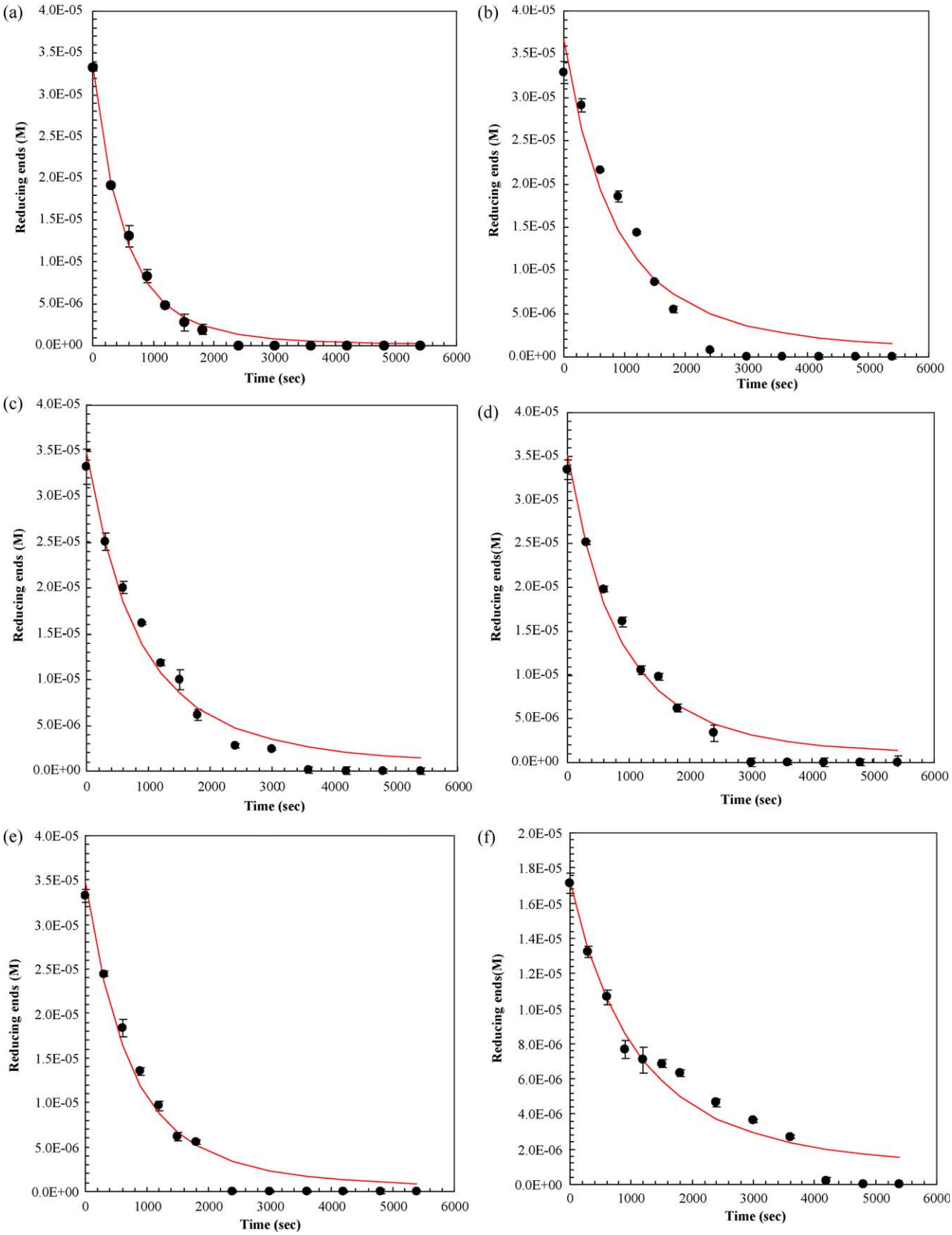

Fig. 1. Time courses, including experimental data (●) and fitted curves (–), for the reduction of (a) glucose, (b) cellobiose, (c) cellotriose, (d) cellotetraose, (e) cellopentaose, (f) microcrystalline cellulose, (g) amorphous cellulose, and (h) phosphoric acid- swollen cellulose by sodium borohydride. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Reaction conditions were 25 mM sodium borohydride, 0.033 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22 °C.

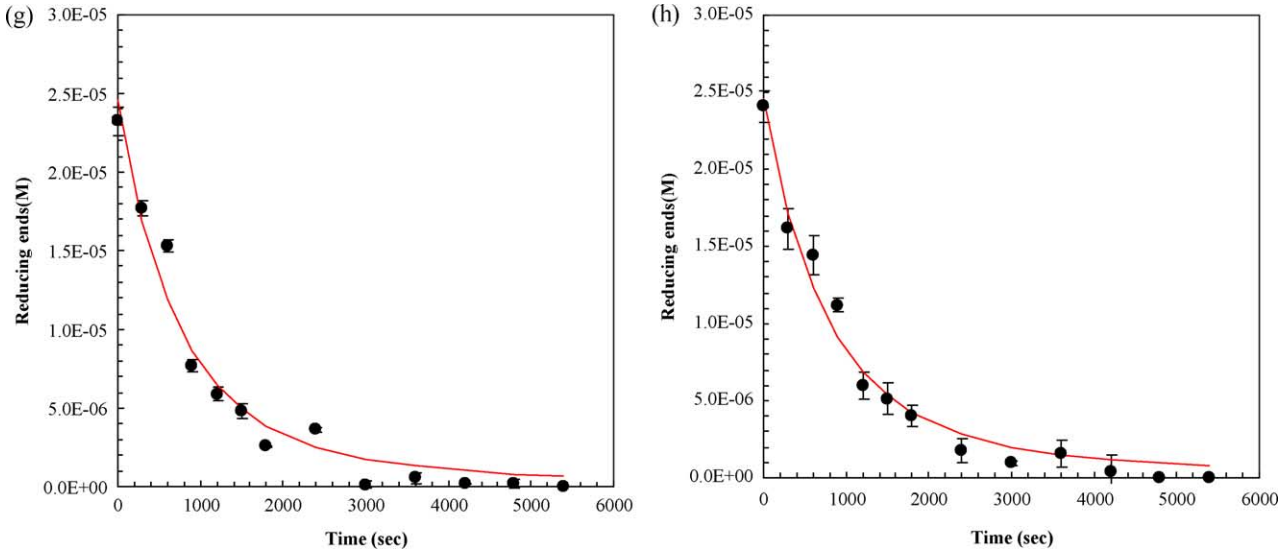

Fig. 1. (*Continued*)

agreement between the values presented in Tables 1 and 2 provide further credibility to the data presented herein.

The aim of this work was to generate information pertaining to the relative reactivity of the terminal glucosyl residues of cellooligosaccharides and those celluloses typically employed in saccharification studies. The premise is that the terminal anomeric carbon of each of these glucose-based oligosaccharides/polysaccharides will have similar reactivity. Support for this notion comes from the similar reactivities of the entire series of soluble cellooligosaccharides tested. The slower rate of reduction of the cellooligosaccharides, relative to glucose, is attributable to the substitution of the terminal glucosyl units at the 4th position (Rickborn & Wuesthoff, 1970). The number of glucosyl units appended at this position appears to have a relatively minor affect on borohydride reactivity - provided the molecule stays in solution. The implication being that the celluloses, having much greater DPs, will be reduced at rates similar to those of the soluble cellooligosaccharides provided the terminal glucosyl units behave in a manner analogous to that of terminal residues that remain in solution. If the terminal glucosyl units of the celluloses are found to have significantly lower reaction rates, then one may surmise that the reaction has been perturbed as a result of the nature of the insoluble substrate. Whether this perturbation is the result of an electronic inductive effect or some steric effect, such as may restrict $NaBH_4$ access to the terminal *aldehydo* group, is not distinguished in this study.

Table 1
Best-fit second order rate constants, with 95% confidence intervals, obtained from minimum least-square regression of time course data for sodium borohydride reduction of test compounds (Fig. 1a–h).

Test compound	Rate constant ($M^{-1}\,s^{-1}$)	Statistical analysis p-value[a]
Glyceraldehyde	$\gg 19.070 \pm 0.100$	–
Glucose	0.0781 ± 0.0250	<0.0001
Cellobiose	0.0466 ± 0.0052	0.5765
Cellotriose	0.0463 ± 0.0032	–
Cellotetraose	0.0480 ± 0.0035	0.8371
Cellopentaose	0.0465 ± 0.0038	0.2776
MCC[b]	0.0355 ± 0.0029	0.0018
AMCC[c]	0.0527 ± 0.0043	0.3129
PSC[d]	0.0502 ± 0.0033	0.7292

[a] Multiple regression (extra-sum-square F-test); $P<0.002$ indicates the associated rate constant is significant different from the reference compound (cellotriose).
[b] Microcrystalline cellulose.
[c] Amorphous cellulose.
[d] Phosphoric acid swollen-cellulose.

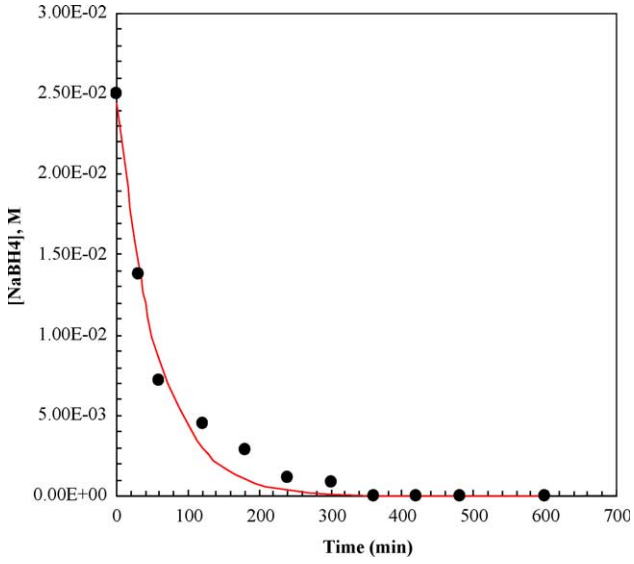

Fig. 2. Time course, including experimental data (●) and fitted curves (–), for hydrolytic degradation of sodium borohydride in 100 mM sodium phosphate buffer, pH 8.0, 22 °C.

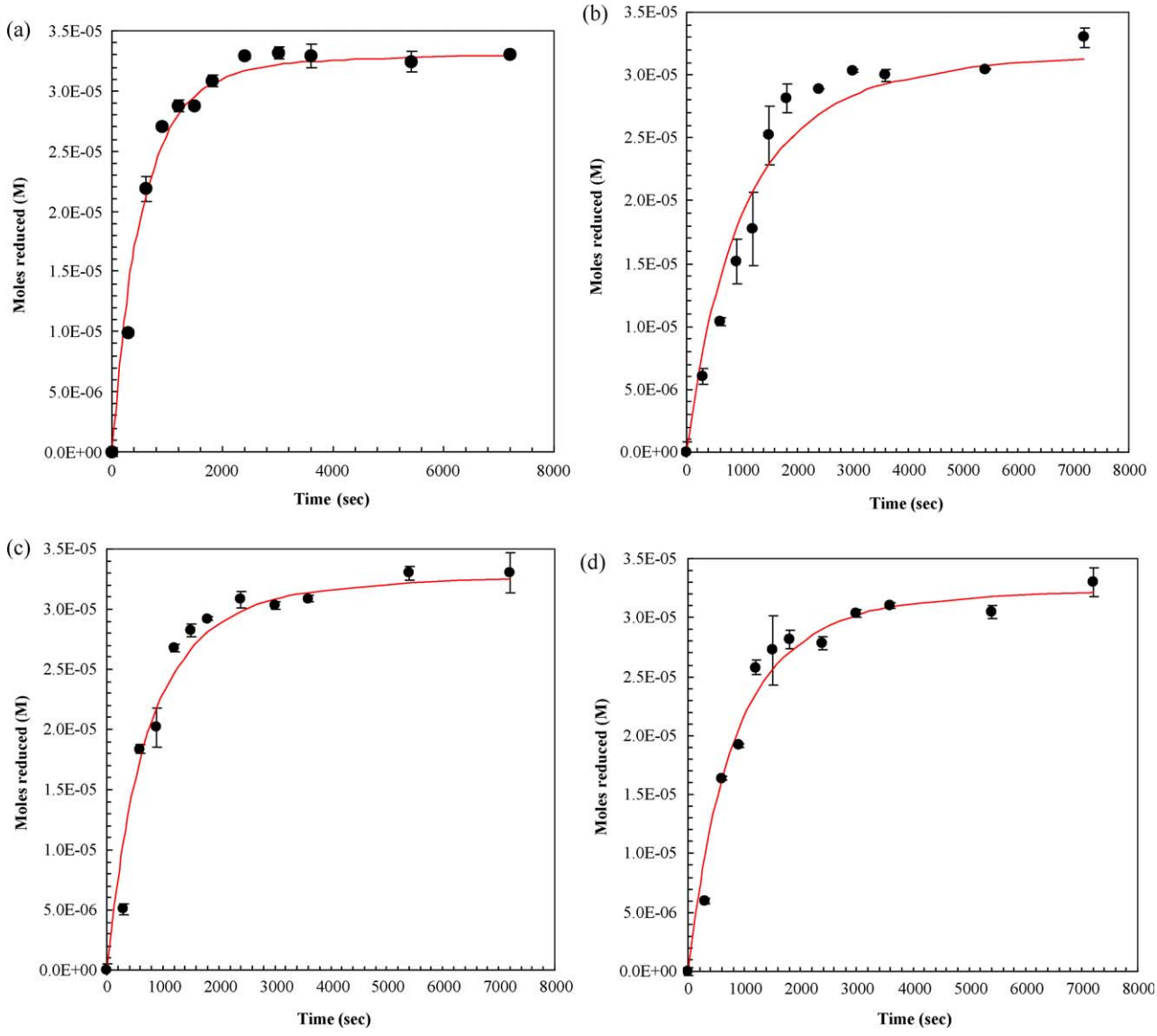

Fig. 3. Time course, including experimental data (●) and fitted curves (–), for the incorporation of tritium into (a) glucose, (b) microcrystalline cellulose, (c) amorphous cellulose and (d) phosphoric acid swollen cellulose resulting from treatment with ^{3}H-labeled sodium borohydride. Isotope incorporation is expressed in terms of moles reducing sugar (aldose) reduced. Reaction conditions were as in Fig. 1.

The relative reactivity of the different classes of test compounds (glucose, cellooligosaccharides, amorphous cellulose, and microcrystalline cellulose) are illustrated in Fig. 4a and b. The solvent-accessible reducing ends of the amorphous celluloses behave, with respect to $NaBH_4$ reduction, as though they are free in solution. This result was not a given in that amorphous celluloses are insoluble and are expected to have regions of partial ordering (Newman & Hemmingson, 1994). The terminal glucosyl units of the microcrystalline cellulose, however, are herein shown to be chemically distinct from those of their soluble cellooligosaccharide analogs. This finding seems reasonable in that a higher degree of order may be expected at the surface of microcrystalline cellulose preparations (Newman & Hemmingson).

Table 2
Best-fit second order rate constants, with 95% confidence intervals, obtained from minimum least-square regression of time course data for tritium incorporation resulting from ^{3}H-labelled sodium borohydride treatment of test compounds (Fig. 3a–d)

Test compound	Rate constant ($M^{-1}\,s^{-1}$)
Glucose	0.0744 ± 0.0042
MCC[a]	0.0392 ± 0.0029
AMCC[b]	0.0544 ± 0.0039
PSC[c]	0.0491 ± 0.0027

[a] Microcrystalline cellulose.
[b] Amorphous cellulose.
[c] Phosphoric acid swollen-cellulose.

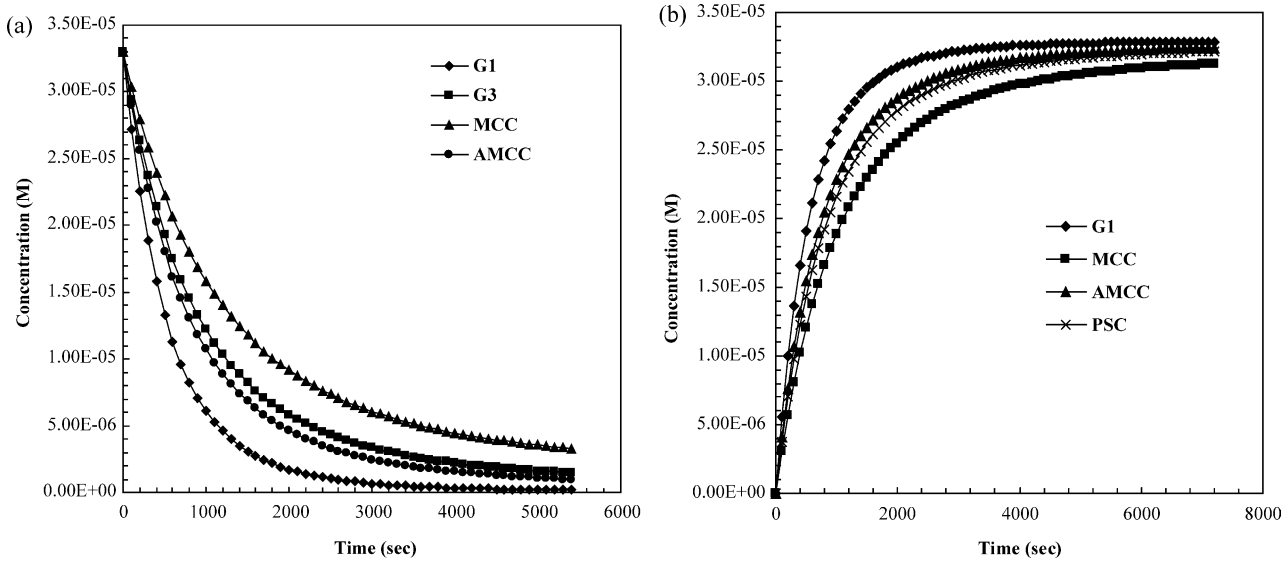

Fig. 4. Comparison of simulated time courses for the reaction of test compounds with (a) sodium borohydride and (b) ^{3}H-labelled sodium borohydride. Time courses were generated using the best-fit second order rate constants of Tables 1 and 2. Extents of reaction for glucose (G1), cellotriose (G3), microcrystalline cellulose (MCC), amorphous cellulose (AMCC) and phosphoric acid swollen cellulose (PSC) are expressed as described in Figs. 1 and 3.

4. Conclusion

These results give chemical evidence in support of the concept that the energetics of the initial interactions between exo-acting cellulases and the reducing ends of typical cellulose substrates will be at least partially dependent on the crystalline nature of those substrates. The implication is that the reactivity of the terminal residues of surface molecules in amorphous substrates, but not crystalline substrates, approaches that of the terminal residues of the soluble cellooligosaccharides.

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