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Characterization of cellulase-based enzyme reactors for the high-performance liquid chromatographic determination of β-D-glucan oligosaccharides

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ABSTRACT

Post-column enzyme reactors were used in series with an electrochemical detector for the selective high-performance liquid chromatographic (HPLC) determination of β-D-glucan oligo- and polysaccharides (degree of polymerization up to 30). Immobilized cellulase converted the eluting oligomers to β-D-glucose, which was oxidized by immobilized glucose oxidase. The production of hydrogen peroxide was measured with an electrochemical detector. The functioning of this system was verified for a whole range of glucosaccharides varying in both structure (positional isomers) and degree of polymerization. Fractional conversions and molar response factors were determined for all the compounds under study. Rate constants are discussed for the reactor system used applying a first-order kinetics model. Efficient HPLC separations were obtained for these oligo- and polysaccharides on a reversed-phase column using gradient elution. Detection limits were of the order of a few nanograms. The reactors were stable for several months.

INTRODUCTION

Interest in complex carbohydrates is growing very rapidly, especially in the field of biotechnology. Specific oligosaccharides have been reported as biologically active molecules in plant biochemistry (the so-called "oligosaccharins" [1]). Some of these compounds have a β-glucan structure [2]. β-Glucans are small polysaccharides consisting of only β-D-glucose units in the six-membered pyranose ring form, and all are linked from the glycosidic carbon 1 of the first glucose unit to carbon 2, 3, 4 or 6 of the next glucose unit. Complex structures with branched arrangements and mixed glycosidic linkages can occur. β-Glucan oligo- and polysaccharides also occur in food and beverage products. Their presence in biological samples is masked by the more abundant digestible α-glucans. In this laboratory, a method was needed for the

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quantitation of trace amounts of β-glucans in the presence of other types of oligo- and polysaccharides. A very selective and sensitive method was therefore required.

Enzyme reactors have been used successfully as post-column reactors in the high-performance liquid chromatographic (HPLC) determination of several biochemically interesting compounds [3] and in flow-injection analysis (FIA). In the latter field, selective enzymes are used for the measurement of mono- and disaccharides [4] and α-glucans [5,6]. For such measurements, oligomer hydrolysing reactors are used in combination with some system which detect the monosaccharides produced. Immobilized cellulase has been used in the bioengineering sciences for the large-scale saccharification of cellulose [7]. As far as is known, this enzyme complex has not yet been studied for analytical purposes. Little is known about its action on compounds other than β-(1$\rightarrow$4)-linked glucans. This paper reports an investigation of the ability of cellulase-based post-column enzyme reactors for the selective and sensitive HPLC determination of β-glucan oligo- and polysaccharides in general.

As enzyme reactors require water-rich eluents, reversed-phase (RP) columns were chosen for the separation of the oligo- and polysaccharides. Separations of carbohydrates on RP columns have been described previously (see refs. 8 and 9 for recent reviews and refs. 10–12 especially for RP separations). Until now the practical use of RP columns for the separation of carbohydrates had limited success because no sensitive and gradient-compatible detectors were available for this purpose. Recently, the combination of novel column materials with pulsed electrochemical detection (PED) highly improved the existing HPLC separation and detection capabilities for these compounds [13]. However, the non-selectivity of PED detectors is disadvantageous when specific carbohydrate molecules have to be determined in the very complex carbohydrate mixtures that are characteristic of biological extracts. Also, the technique requires highly basic eluents, which restricts the choice of chromatographic conditions and the recovery of separated compounds. The wide possibilities of the combination RP columns and enzyme reactors with electrochemical detection (ED) in carbohydrate analysis are reported here.

EXPERIMENTAL

HPLC determination

For gradient elutions, an SP8700 solvent delivery system (Spectra-Physics, San Jose, CA, USA) was equipped with an extra 1-ml mixing chamber (Ultrograd gradient mixer, LKB, Bromma, Sweden) at the low pressure side to eliminate gradient disturbances. Samples were injected with a Valco EC10U injector (VICI, Houston, TX, USA). Analytical separations were performed on a RoSiL C_{18} column (Bio-Rad RSL, Eke, Belgium), 150 $\times$ 4.6 mm I.D., 5 μm particle size. The detector consisted of a laboratory-made large-volume wall-jet electrochemical cell controlled by a three-electrode potentiostat (see ref. 14 for a detailed description). The working electrode was a 3 mm diameter platinum disc, operated at +700 mV *versus* the saturated calomel electrode. The chromatograms were recorded with an SP4100 computing integrator (Spectra-Physics). Enzyme reactors were placed between the analytical column and the electrochemical detector. The analytical column was omitted in FIA measurements. Since oxygen must be present for the regeneration of the flavin-adenine dinucleotide (FAD) co-enzyme (glucose oxidase), the eluents should not be thoroughly degassed.

Enzyme reactors

Glucose oxidase and cellulase were immobilized on aminopropyl-derivatized glass beads using glutaraldehyde as described by Weetall [15]. A 100-mg mass of aminopropyl glass, 50 nm pore size, 200–400 mesh (Sigma, St. Louis, MO, USA) was first activated in 3 ml of a 2.5% (v/v) solution of glutaraldehyde (grade 1, Sigma) in 0.1 *M* phosphate buffer (pH 7) for 90 min under vacuum. The derivatized glass beads were rinsed with water and added to a solution of either 5 mg of glucose oxidase [EC 1.1.3.4, from *Aspergillus niger*, 283 U mg^{-1} (Serva, Heidelberg, Germany)] or 50 mg of cellulase [EC 3.2.1.4, from *Penicillium funiculosum*, 5.9 U mg^{-1} (Sigma)] in 3 ml of 0.05 *M* acetate buffer (pH 5.0). Vacuum was applied for 15 min, after which the reaction proceeded for 3 h at 4°C with occasional shaking (vortex-mixing). After filtration, the glass beads with the immobilized enzyme were slurry-packed (10 MPa) into 2.1 mm I.D. stainless-steel columns of either 60 or 50 mm length. The reactors were stored at 4°C with buffer solution when not in use. All experiments with the reactors were run at ambient temperature at pH 5. This pH was found to be a good compromise for the optimum functioning of the enzyme reactors and the electrochemical detector.

Carbohydrates

Glucose (Merck, Darmstadt, Germany), cellobiose (Nutritional Chemical, Cleveland, OH, USA), laminaribiose (Sigma), gentiobiose (Sigma), cellulose (microcrystalline, Merck), curdlan (Serva) and laminarin (Sigma) were commercially available. Cellodextrins were obtained through the partial hydrolysis of cellulose by hydrochloric acid; 100 mg of cellulose were added to 2 ml of 37% hydrochloric acid (p.a., UCB, Leuven, Belgium). After 2 h of stirring, 6 ml of water were added. The acid was evaporated under reduced pressure and 6 ml of water were added to the residue. The remaining acid was neutralized with sodium carbonate. Insoluble material was removed by centrifugation at 10 000 *g* for 30 min.

Laminaridextrins were prepared from curdlan [16]. A 10-mg mass of curdlan was subjected to formolysis in 5 ml of 90% formic acid for 60 min at 100°C. The acid was removed under reduced pressure and 5 ml of a 0.1 *M* solution of trifluoroacetic acid were added to the residue. Hydrolysis occurred at 100°C for 60 min. The resulting hydrolysate was neutralized over Dowex MWA-1 (OH^- form, Serva) and centrifuged.

A 10-mg mass of laminarin was hydrolysed as described for curdlan, except that formolysis was omitted and the hydrolysis time was reduced to 30 min.

For the reduction of oligosaccharides with sodium borohydride (Sigma), 10-ml solutions of these hydrolysates were adjusted to pH 12 using concentrated sodium hydroxide, and 200 mg of sodium borohydride were added. After standing overnight at ambient temperature, the solutions were adjusted to pH 5 with acetic acid. These solutions were injected without further purification.

Semi-preparative chromatography

Cellodextrins (cellotriose to cellohexaose). A 10-g mass of cellulose was hydrolysed as described earlier and the volume was reduced to 40–50 ml. In a first chromatographic step, the higher oligomers of the hydrolysate were separated on a semi-preparative octylsilica column (RSiL C_8, 250 × 10 mm I.D., 10 μm particle size, Bio-Rad/RSL). Water was used as an eluent at a flow-rate of 5 ml min^{-1} (HP-1084A

liquid chromatograph, Hewlett-Packard, Avondale, PA, USA), with refractive index detection (R404, Waters, Milford, MA, USA). A first fraction contained cellobi-, -tri- and -tetraose. Cellopentaose and cellohexaose were collected in a second and a third fraction. Ninety 200-μl injections were performed. In a second step, the first fraction was injected on an analytical octadecylsilica column (RoSiL C_{18} column, 150 × 4.6 mm I.D., 5 μm particle size, Bio-Rad/RSL), eluted with water at 0.8 ml min^{-1}. Cellotri- and -tetraose were collected using fifty 200-μl injections. The amounts of collected oligomers ranged from 23 to 90 mg with a purity >90% for cellotriose and >95% for cellotetra-, -penta- and -hexaose.

Oligosaccharides from curdlan hydrolysates were separated by gradient preparative-scale HPLC on an octylsilica column. Injections (6 ml) containing 80 mg of hydrolysed curdlan were performed on a 250 × 10 mm I.D. RSiL C_8 column, 10 μm particle size (Bio-Rad/RSL). The column was eluted with a water (A)–acetonitrile (B) gradient (4 ml min^{-1}, from 0% B to 5.5% B in 55 min). The column eluent was split into a detection and a collection line using a flow splitter (Valco) and a metering valve (Hoke, Creskill, NY, USA) to obtain a split-ratio of 7 (collection line) over 1 (detection line). The detection line was mixed with a 0.05 M acetate buffer (pH 5). Detection was performed using the cellulase–glucose oxidase–ED system. Ten repetitive injections yielded 27 oligomers in amounts ranging from 22.6 mg [degree of polymerization (DP) 3] to 1.2 mg (DP30).

RESULTS AND DISCUSSION

Detection principle

β-Glucans, eluting from the chromatographic column, are hydrolysed to glucose monomers through the action of immobilized cellulase in a first enzyme reactor. The produced β-D-glucose is oxidized in a second enzyme reactor containing immobilized glucose oxidase. The oxidation of glucose is coupled to the reduction of oxygen to hydrogen peroxide. The hydrogen peroxide produced is detected electrochemically. (The glucose oxidase reactor has been studied for many FIA and HPLC applications [17].)

Commercial cellulase contains a mixture of enzymatic activities. Three major activities have been defined and can be found in all cellulase preparations [18]: *endo*-1,4-β-D-glucanase, *exo*-1,4-β-D-glucanase and β-D-glucosidase. Their action converts β-(1→4)-linked D-glucosaccharides (cellodextrins) to β-D-glucose. The cellulase under study (from *P. funiculosum*) is also known to act on β-(1→3)-D-(laminarioligomers), β-(1→6)-D-(gentiooligomers) and β-(1→2)-D-glucosidic bonds (sophorooligomers). Hydrolysis yields β-D-glucose (same configuration of the anomeric carbon) [19]. Michaelis constants (K) and relative velocities can be found for the hydrolysis of DP2 to DP6 cellodextrins only [20]. Information on the relative rates of hydrolysis of other than cellodextrin oligosaccharides by cellulase preparations is unfortunately very scarce. The following section therefore gives quantitative information on the relative rates of hydrolysis of different glucosaccharides obtained with immobilized cellulase reactors.

Quantitative evaluation of the enzyme reactors: theory

Under the conditions used in this work (low substrate concentrations) and in

analytical applications of enzyme reactors in general, first-order kinetics can be assumed (see also the discussion by Goldstein [21]) (see eqn. 1). This is particularly true under these conditions of strict detector linearity. For applications of enzyme reactors in fields where high productivities are required, this is not so, and other descriptions of reactor behaviour need to be found.

$$v = \frac{dS}{dt} = -K_{ps}^{(app)}S \quad (1)$$

Here v is the rate of substrate conversion in the enzyme reactor, S is the substrate concentration, t is time and $K_{ps}^{(app)}$ the apparent pseudo first-order rate constant, equal to $V_{max}/K_M^{(app)}$ (V_{max} is the maximum rate of substrate conversion and $K_M^{(app)}$ is the apparent Michaelis constant) [22]. In enzyme reactors, the rate of substrate conversion is affected by mass transport limitation phenomena. Therefore, the $K_{ps}^{(app)}$ values depend on the flow-rate, on the carrier particle characteristics (see Johansson *et al.* [22] for a detailed discussion) and on the reactor dimensions. This work was restricted to measuring $K_{ps}^{(app)}$ values at flow-rates of 1 ml min^{-1}, keeping the type of matrix particles and reactor dimensions constant.

Integration of eqn. 1 yields eqn. 2, which can be rearranged to eqn. 3:

$$\ln\left(\frac{S_{t=\tau}}{S_{t=0}}\right) = \ln(1 - X) = -K_{ps}^{(app)}\tau \quad (2)$$

$$X = 1 - e^{-K_{ps}^{(app)}\tau} \quad (3)$$

wherein $S_{t=\tau}$ is the amount of substrate at the outlet of the enzyme reactor and $S_{t=0}$ is the amount of substrate at the inlet of the enzyme reactor, τ and X are, respectively, the residence time of the substrate in the reactor and the fractional conversion of the substrate.

The effectiveness of an enzyme reactor can be expressed quantitatively using $K_{ps}^{(app)}$ values, fractional conversions (X), or $\tau_{1/2}$ values (the residence time required to convert 50% of the substrate). Still more practical than half-life values may be the use of a half-length $L_{1/2}$, *i.e.*, the reactor length required to convert 50% of the substrate: $L_{1/2} = \tau_{1/2}F^{-1}$ when no retention occurs during transit through the enzyme reactor (F being the linear velocity of the eluent in the reactor). As mentioned earlier, $K_{ps}^{(app)}$ and therefore also $\tau_{1/2}$ and $L_{1/2}$ are dependent on parameters which influence mass transport.

Characteristics of the cellulase reactor

Tables I and II show the fractional conversions (X) and the molar response factors (MRFs) obtained with the cellulase reactor for fifteen different oligomers. These measurements were performed by injecting known concentrations of carbohydrates in an FIA set-up (no column) consisting of a pump, an injector, the cellulase and glucose oxidase reactor and the electrochemical detector. Fractional conversions were defined as the ratio of the amount of β-D-glucose liberated by the enzyme reactor, divided by the maximum amount of β-D-glucose that could theoretically be liberated

TABLE I

MRFs AND FRACTIONAL CONVERSIONS (X) FOR CELLODEXTRINS [β-(1→4) GLUCO-OLIGOMERS] FOR DIFFERENT DP VALUES

Obtained with a cellulase reactor operated at a flow-rate of 1 ml min^{-1} using 0.05 M acetate buffer of pH 5 as an eluent. Reactor length × I.D. are given as column heading. The third series of values was obtained with sodium borohydride-reduced cellodextrins; the fourth series was obtained using 10% acetonitrile as the eluent.

DP	Column dimensions (mm)							
	50 × 2.1		100 × 2.1		50 × 2.1 (reduced)		50 × 2.1 (10% acetonitrile)	
	MRF	X (%)	MRF	X (%)	MRF	X (%)	MRF	X (%)
2	1.2	62	1.3	63	0.7	34	1.0	50
3	2.0	67	2.0	66	1.0	33	1.5	50
4	2.3	57	2.5	63	1.9	46	1.8	44
5	2.9	57	3.2	64	2.4	47	2.7	53
6	3.6	61	3.5	59	3.9	66	3.3	55

(100% hydrolysis). To determine the amount of glucose freed from the oligosaccharides by the cellulase reactor, known concentrations of β-glucans ($2 \cdot 10^{-10}$ mol) containing standard additions of glucose ($4 \cdot 10^{-10}$, $8 \cdot 10^{-10}$ and $16 \cdot 10^{-10}$ mol) were injected (a correction factor is applied to deal with the α/β anomerisation, as the glucose oxidase reactor only detects the β-anomer, which is present at 65% (25°C) for a glucose solution in equilibrium [23]). At least three standard additions were made for each data point and each sample was injected three times. The relative errors for these

TABLE II

MRFs AND FRACTIONAL CONVERSIONS (X) OBTAINED WITH A CELLULASE REACTOR AT DIFFERENT FLOW-RATES

Samples were β-(1→4) (cellobiose and cellopentaose), β-(1→6) (gentiobiose) and β-(1→3)-linked gluco-oligomers (laminaribiose and curdlan DP5–30). Column dimensions 50 × 2.1 mm.

Carbohydrate	Flow-rate (ml min^{-1})					
	0.5		1		4.5	
	MRF	X (%)	MRF	X (%)	MRF	X (%)
Cellobiose	0.57	28.5	0.54	27	0.38	19
Gentiobiose	0.64	32.2	0.79	40	0.60	30
Laminaribiose	0.44	22.0	0.42	21	1.4	68
Cellopentaose	3.57	71.4	3.6	72	2.6	52
Curdlan DP5	1.43	28.7	1.0	20	1.6	31
Curdlan DP10	1.61	16.1	1.1	11	0.96	9.6
Curdlan DP15	0.93	6.3	0.95	6.4	0.64	4.3
Curdlan DP20	0.99	4.9	1.0	5.2	0.45	2.2
Curdlan DP25	0.87	3.5	0.83	3.3	0.55	2.2
Curdlan DP30	0.82	2.7	0.55	1.8	0.57	1.9

data are up to 10% (linear regression error variance analysis). The MRFs are defined as the response (peak area, or number of Coulombs for an amperometric detector) provoked by the β-glucan, divided by the response provoked by an equimolar amount of β-D-glucose.

The fractional conversions are highest, around 60%, for the cellodextrins [β-(1→4)-glucans] (Table I). There is little variation in the fractional conversion within the series for degrees of polymerization from 2 to 6. This may indicate that for these substances, the reactor works at its maximum conversion capacity. This is in accordance with the fact that doubling the reactor length has no effect on the fractional conversion. Table I also shows that MRFs increase with increasing DP. The ratio MRF/(DP − 0.35) (0.35 is a correction factor, taking into account the α/β anomerisation of the end-glucose) is reasonably constant within each series of experiments. For the sodium borohydride reduced cellodextrins, this constancy is found in the ratio MRF/(DP − 1) as one of the glucose units is chemically transformed, and therefore not available for oxidation by glucose oxidase. After reduction pretreatment, the MRFs and fractional conversion are smaller, especially for molecules with low DP values. However, reducing the end-glucose unit of a β-glucan chain does not drastically alter the detection capability of the system for these compounds. This can be ascribed to the reaction route of the β-glucanases in which the terminal glucose units at the non-reducing end are hydrolysed first. The addition of 10% acetonitrile to the eluent also reduces the MRF and fractionation values, with 16 and 18%, respectively, as mean values for the five compounds.

Table II shows the MRF and fractionation values, measured for three disaccharides, cellopentaose and for six linear β-(1→3)-linked oligosaccharides at different flow-rates. It is clear from these data that the immobilized cellulase also catalyses the hydrolysis of β-(1→6)-linked compounds (gentiobiose) and β-(1→3)-linked oligomers from DP2 (laminaribiose) up to DP30. There is a tendency of decreasing MRFs and fractional conversions towards β-(1→3)-linked oligomers, as compard to the β-(1→4)-linked cellodextrins (Table I). These results suggest a lower activity of the immbilized enzyme complex towards other than β-(1→4)-linked glucans. However, the compounds are still easily detectable with the described system, as the MRFs are comparable to that of β-D-glucose. There are no significant differences between the results for flow-rates from 0.5 to 4.5 ml. This suggests that the enzyme reactor works at its maximum fractional conversion, *i.e.*, that the reactor length is much higher than $L_{1/2}$ (this is confirmed by results discussed for Table III later). For β-(1→3) oligomers, the MRFs decrease with increasing degrees of polymerization. This trend is shown in Fig. 1. There is no straightforward explanation as to why higher oligomers of this type have lower fractional conversions while the reactor works at its maximum efficiency. Substrate hydrolysis of 100% does not seem to be realizable, no matter how long the reactor is. A better understanding of the way in which the enzymes catalyse complex β-glucans is needed to improve the reactor efficiency, which leads to higher MRFs and lower detection limits. Little quantitative data are known for the MRFs for saccharides in PED applications. However, it is clear that the latter technique can give drastically reduced MRFs for higher oligomers, as Larew and Johnson [24] used enzyme reactors to improve the PED molar responses for such compounds.

Fig. 2 shows the fractional conversions as a function of the reactor length for

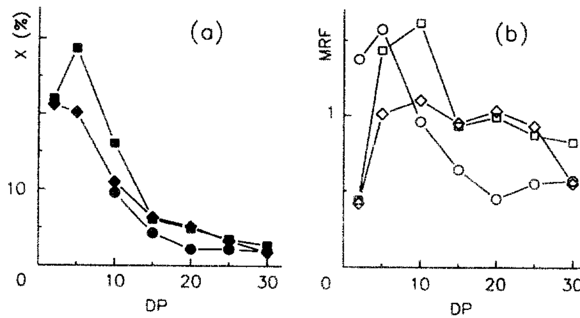

Fig. 1. (a) Dependence of fractional conversion (X) on DP value for β-(1→3)-linked glucooligomers of DP2, -5, -10, -15, -20, -25 and -30 at flow-rates of (■) 0.5 ml min^{-1}, (◆) 1 ml min^{-1} and (●) 4.5 ml min^{-1}, obtained with the immobilized cellulase reactor (50 × 2.1 mm I.D.). (b) Dependence of MRF on DP value for β-(1→3)-linked glucooligomers of DP2, -5, -10, -15, -20, -25 and -30 at flow-rates of (□) 0.5 ml min^{-1}, (◇) 1 ml min^{-1} and (○) 4.5 ml min^{-1} for the immobilized cellulase reactor (50 × 2.1 mm I.D.).

β-(1→3) oligomers. The experimental data were obtained using standard additions as described earlier (nine measurements for each data point). A decreased fractional conversion with increasing DP can be seen from this figure. Conversion rates, $K_{ps}^{(app)}$ (or half-length $L_{1/2}$), remain more or less constant for the different oligomers: the six lines shown in Fig. 2 reach a plateau value for approximately the same reactor lengths. The experimental data from Fig. 2 were used in a non-linear least-squares curve-fitting algorithm (the program was based on simplex iterations and run on a PC/AT 286 computer). The data were fitted in a function of the form $X = X_{max}[1 - \exp(-K_{ps}^{(app)}\tau)]$, which is theoretically predicted to describe the reactor behaviour (see eqn. 3) if first-order kinetics are used. Using the G-test $[G = \Sigma(X_{i,exp} - X_{i,cal})^2/\sigma_i^2]$ of a χ^2 distribution, all the calculated plots described the experimental data at the 95% confidence level, except for DP30. These results suggest that the simple first-order kinetics model, although probably oversimplified, gives a fairly practical description of the reactor behaviour. From the theoretically fitted curves, $K_{ps}^{(app)}$, X_{max} and $L_{1/2}$ values were obtained (except for DP30) (Table III). It is clear that the $K_{ps}^{(app)}$ and $L_{1/2}$ values are reasonably independent of DP. The measured $L_{1/2}$ values confirm that

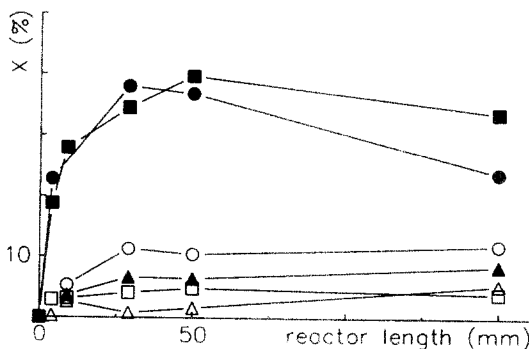

Fig. 2. Fractional conversion (X) of curdlan oligomers [β-(1→3) glucooligomers] as a function of reactor length (L) for an immobilized cellulase reactor (2.1 mm I.D.) at a flow-rate of 1 ml min^{-1}. (■) DP5; (●) DP10; (▲) DP15; (□) DP20; (○) DP25; and (△) DP30.

TABLE III

APPARENT PSEUDO FIRST-ORDER RATE CONSTANT, $K_{ps}^{(app)}$, MAXIMUM FRACTIONAL CONVERSION, X_{max}, AND HALF-LENGTH, $L_{1/2}$, OBTAINED FOR SOME CURDLAN OLIGOMERS WITH AN IMMOBILIZED CELLULASE REACTOR SYSTEM

Column, 2.1 mm I.D.; flow-rate, 1 ml min^{-1}. Curdlan oligomers [β-(1→3) glucooligomers] with known DP value. The values were calculated from the experimental data in Fig. 2 (simplex iterative curve fitting) (see text).

DP	$K_{ps}^{(app)}$ (s^{-1})	X_{max} (%)	$L_{1/2}$ (mm)
5	0.68	37	4.3
10	0.60	37	4.9
15	0.70	6.3	4.1
20	0.55	4.3	5.2
25	0.32	12	9.1
30	–	–	–

maximum reactor efficiencies were obtained, as the $L_{1/2}$ values are about ten times smaller than the reactor length.

The selectivity for β-glucans was evaluated by injecting a mixture which contained α-glucans (starch hydrolysate) at a concentration 50 times higher than the working range for β-glucans. Except for a large glucose peak, no major baseline disturbances could be detected at high sensitivity (10 nA). Some commercial glucose oxidase preparations were found to contain α-glucan hydrolysing enzymes. This causes the immobilized glucose oxidase reactor to behave as an α-glucan detector. Such contaminations were absent in the glucose oxidase preparation used in this study.

Characteristics of the glucose oxidase reactor

The quantitative determination of the fractional conversions as a function of reactor length (residence time) for the immobilized glucose oxidase reactor was rather troublesome. Similar problems were experienced in evaluating the hydrogen peroxide concentrations as those discussed by Tyrefors and Carlsson [25]. In Fig. 3 the integrator counts are plotted against the glucose oxidase reactor length (I.D. 2.1 mm) for a flow-rate of 1 ml min^{-1}. The same curve-fitting algorithm as described earlier was applied to the data in Fig. 3. The $K_{ps}^{(app)}$ value calculated from the resulting curve was 0.76 s^{-1}. From this value, $L_{1/2}$ was determined to be 3.8 mm and $\tau_{1/2}$ = 0.9 s. Therefore, an immobilized glucose oxidase reactor with an I.D. of 2.1 mm and a length of 60 mm has a fractional conversion >99.99%.

For both reactors, no major loss of activity could be detected for at least 3 months. The detection system was linear for glucose in the range 10^{-6} (1.8 ng injected) to $5 \cdot 10^{-3}$ M (9 μg injected). Linear calibration graphs were also obtained for cellodextrins of DP2 and DP6, and for laminaridextrins of DP2, -6 and -28 in the range $5 \cdot 10^{-6}$–$5 \cdot 10^{-4}$ M. Extra-column band broadening caused by the reactors was determined by a method described by Verzele and Dewaele [26]. A σ_{ec} value of 31 μl was obtained for a reactor of 50 mm length and 2.1 mm I.D.

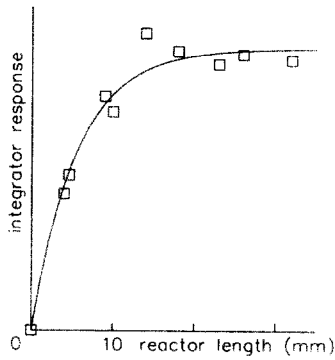

Fig. 3. Conversion of glucose (or production of hydrogen peroxide) with increasing reactor length by an immobilized glucose oxidase reactor (2.1 mm I.D., flow-rate 1 ml min^{-1}, 10^{-9} mol glucose injections).

Chromatographic separations

One of the problems in the RP chromatography of reducing carbohydrates is the doublet formation or band broadening due to α/β anomeric equilibria. Previous attempts to avoid this phenomenon by speeding up mutarotation were rather unsatisfactory [12]. Reduction of the terminal aldehyde group with sodium borohydride eliminates the anomerization problem, leading to less complicated chromatograms. Fig. 4a shows an isocratic mode separation of sodium borohydride-reduced cellodextrins [β-(1→4)-linked linear glucose chains] on an octadecylsilica column; Fig. 4b shows a gradient mode separation of non-reduced cellodextrins using

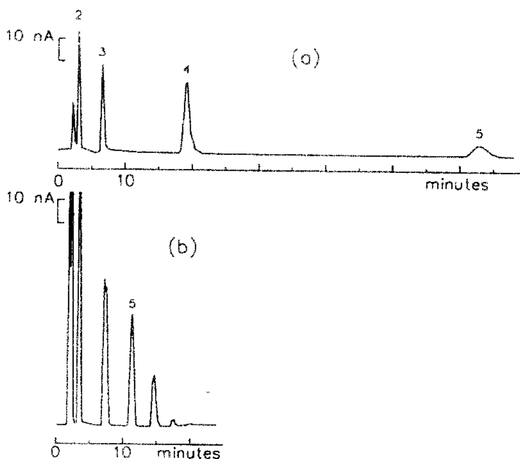

Fig. 4. (a) Chromatogram of sodium borohydride-reduced cellodextrins (reduced partial hydrolysate of cellulose) run under isocratic conditions using an immobilized cellulase (50 × 2.1 mm I.D.) and glucose oxidase (60 × 2.1 mm I.D.) reactor. Peak numbers refer to the DP value of the eluting compound. Chromatographic conditions: column, RP C_{18}; eluent, 0.05 *M* acetate buffer (pH 5); flow-rate, 1 ml min^{-1}; detector, platinum electrode at +700 mV *versus* saturated calomel electrode. (b) Chromatogram of cellodextrins (not reduced) using the immobilized enzyme reactors (cellulase and glucose oxidase) for detection run under gradient conditions. Eluent: A = 0.05 *M* acetate (pH 5), B = acetonitrile. Gradient programme from 0 to 5% B in 15 min; other conditions as in (a).

acetonitrile as the organic modifier. Peak broadening and splitting due to the separation of α/β anomers can be seen, but the overall separation efficiency is still sufficiently high. Laminaridextrins, obtained through the partial hydrolysis of curdlan [a mainly β-(1→3)-linked linear glucose chain], are also separated using the gradient system (Fig. 5). A simpler chromatographic pattern is obtained with sodium borohydride reduction pretreatment (Fig. 5b) than without this pretreatment (Fig. 5a). Sodium borohydride reduction is not strictly necessary for baseline separation of the oligosaccharides. More complex, branched oligosaccharides were obtained through the partial hydrolysis of laminarin. Water-soluble laminarin itself is a branched β-D-glucan containing about 30 glucopyranosyl units. The backbone is made up by (1→3) and (1→6) bonds while branching points consist of (1→6) bonds. The hydrolysate shows a complex chromatographic peak pattern (Fig. 6). Figs. 4–6 show that the immobilized cellulase can be used effectively for the detection of β-glucans after RP separations, for DP values as high as 30. The retention of the glucosaccharides increases with decreasing water solubility. Highly water-soluble carbohydrates such as the lower amylose [α-(1→4) linked] oligomers up to DP5 could not be retained on the C_{18} RP phases which were used in this study. A highly derivatized RP C_{18} phase, provided by Bio-Rad/RSL, could achieve this. Detection of the carbohydrates was very sensitive. For cellotetraose [capacity factor (k') $\approx$ 10] a detection limit was estimated to be 6 ng (10^{-11} mol injected, peak height about $10\sigma_{noise}$).

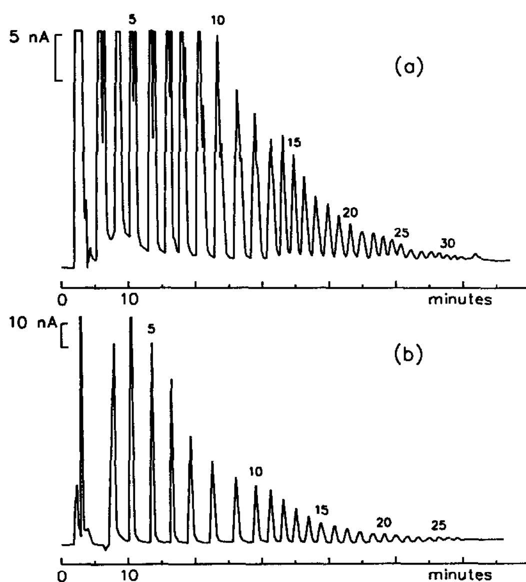

Fig. 5. (a) Chromatogram of partial hydrolysate of curdlan [laminaridextrins, linear β-(1→3)-linked glucooligomers], run under gradient conditions. Eluent: A = 0.05 M acetate (pH 5); B = acetonitrile. Gradient programme from 0 to 2.5% B in 3 min, 5% B at 12 min, 6.7% B at 30 min, 8.3% B at 60 min, 10% B at 65 min. Other conditions as in Fig. 4a. (b) Chromatogram of sodium borohydride-reduced partial hydrolysate of curdlan, run under gradient conditions. Eluent and gradient pogramme as in (a). Other conditions as in Fig. 4a.

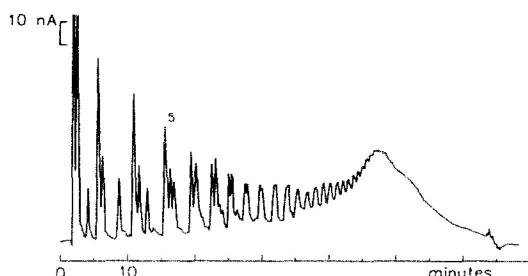

Fig. 6. Chromatogram of partial hydrolysate of laminarin (complex mixture of branched glucooligomers with mixed linkages, see text). Eluent: A = 0.05 *M* acetate (pH 5); B = acetonitrile. Gradient programme from 0 to 5% B in 20 min, 10% B at 60 min. Other conditions as in Fig. 4a.

Both the enzyme reactors and the ED system behaved well in water–acetonitrile gradient systems. There are indications that the large-volume wall-jet detector has better characteristics for gradient elution than thin-layer designs [27]. At high detector sensitivities (10 nA full scale), baseline problems were experienced during gradient elution when methanol was used as an organic modifier. Working electrode currents increased significantly with increasing methanol concentrations, resulting in a shifting baseline (probably due to the oxidation of methanol on the platinum electrode). Replacing methanol by acetonitrile solved this problem. It is known that enzymes can often exert their catalytic activity even in the presence of organic solvents, and that the stability of enzymes is also frequently enhanced after immobilization. No significant loss of activity of the reactor couple was observed for at least fifty gradient elution analyses comparable to that shown in Fig. 5.

CONCLUSIONS

In combination with gradient RP-HPLC and ED, immobilized cellulase reactors provide a powerful means for the selective and sensitive determination of β-glucans in complex carbohydrate mixtures. β-(1→4)-Linked glucosaccharides and various other structurally isomeric glucosaccharides up to at least DP30 are rapidly broken down by the immobilized enzyme complex. The reactors can be used in a gradient system, which highly improves and facilitates the separation of oligosaccharides on RP columns. The stability of the reactor is very good. The cellulase reactor can be improved to yield higher MRFs, especially for compounds other than β-(1→4)-linked glucans. Therefore, a search for enzymes with a higher hydrolytic activity towards such β-glucans and a better understanding of their mode of action is necessary. Future work in this laboratory will also be directed to the use of selective hydrolases in multiple enzyme reactor systems to obtain qualitative (structural) information on the chromatographically separated carbohydrates.

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