ELSEVIER

Carbohydrate Research 268 (1995) 151–158

CARBOHYDRATE RESEARCH

Note

Analysis of *endo*-(1 → 5)-α-L-arabinanase degradation patterns of linear (1 → 5)-α-L-arabino-oligosaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection

Matthias P.H. Dunkel, Renato Amadò *

Swiss Federal Institute of Technology, Department of Food Science, ETH-Zentrum, CH-8092 Zurich, Switzerland

Received 28 March 1994; accepted in revised form 22 September 1994

Keywords: *Endo*-(1 → 5)-α-L-arabinanase (EC 3.2.1.99); (1 → 5)-α-L-arabino-oligosaccharides; High-performance anion-exchange chromatography (HPAEC); Pulsed amperometric detection (PAD); Enzymatic hydrolysis

Endo-(1 → 5)-α-L-arabinanases [*endo*-ABA (EC 3.2.1.99)] have proven to be essential analytical tools for the structural study of plant cell wall polysaccharides [1]. Reciprocally, the analysis of the reaction products released after enzymatic treatment has provided valuable information on the substrate specificity and the mode of action of *endo*-ABA [2–4]. Traditional separation methods for the analysis of carbohydrates, such as paper [5], gel filtration [6,7], and ion-exchange high-performance chromatography [8] have often exhibited poor resolution, selectivity, and sensitivity. In contrast, high-performance anion-exchange chromatography (HPAEC), used in combination with pulsed amperometric detection (PAD), has proved to be particularly appropriate for the determination of oligomeric carbohydrates [3,4].

As *endo*-ABA degradation patterns of polysaccharides (especially arabinans) have already been successfully examined with HPAEC–PAD [3,4], we took advantage of this technique to investigate the hydrolysis patterns of linear (1 → 5)-α-L-arabino-oligosaccharides [degree of polymerization 2–8 (DP_{2-8})] by a highly purified *Aspergillus*

* Corresponding author. Swiss Federal Institute of Technology, Department of Food Science, ETH-Zentrum, CH-8092 Zurich (Switzerland) Phone: +41 1 632 32 91 Fax: +41 1 632 11 23

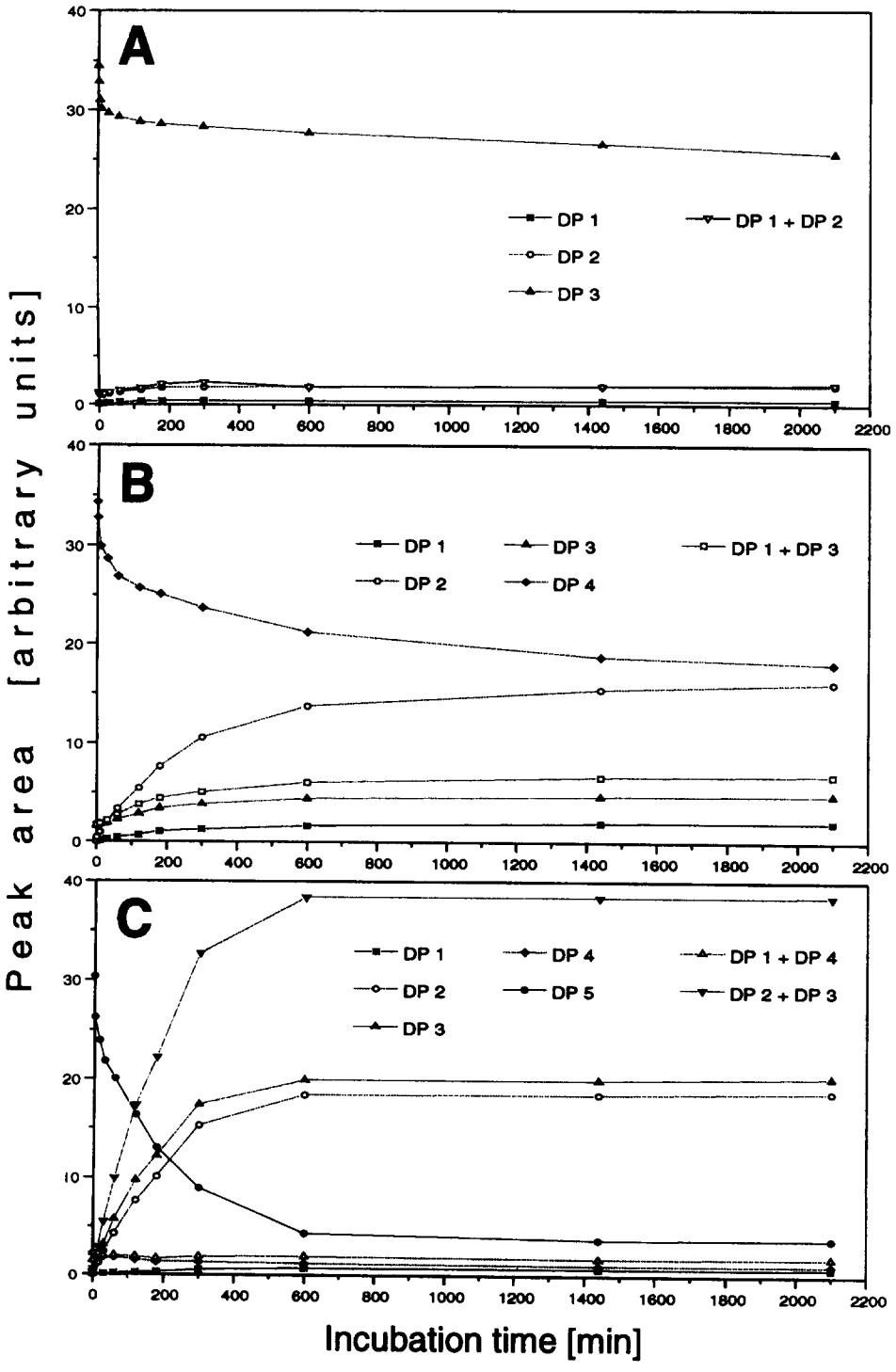
SSDI 0008-6215(94)00305-X

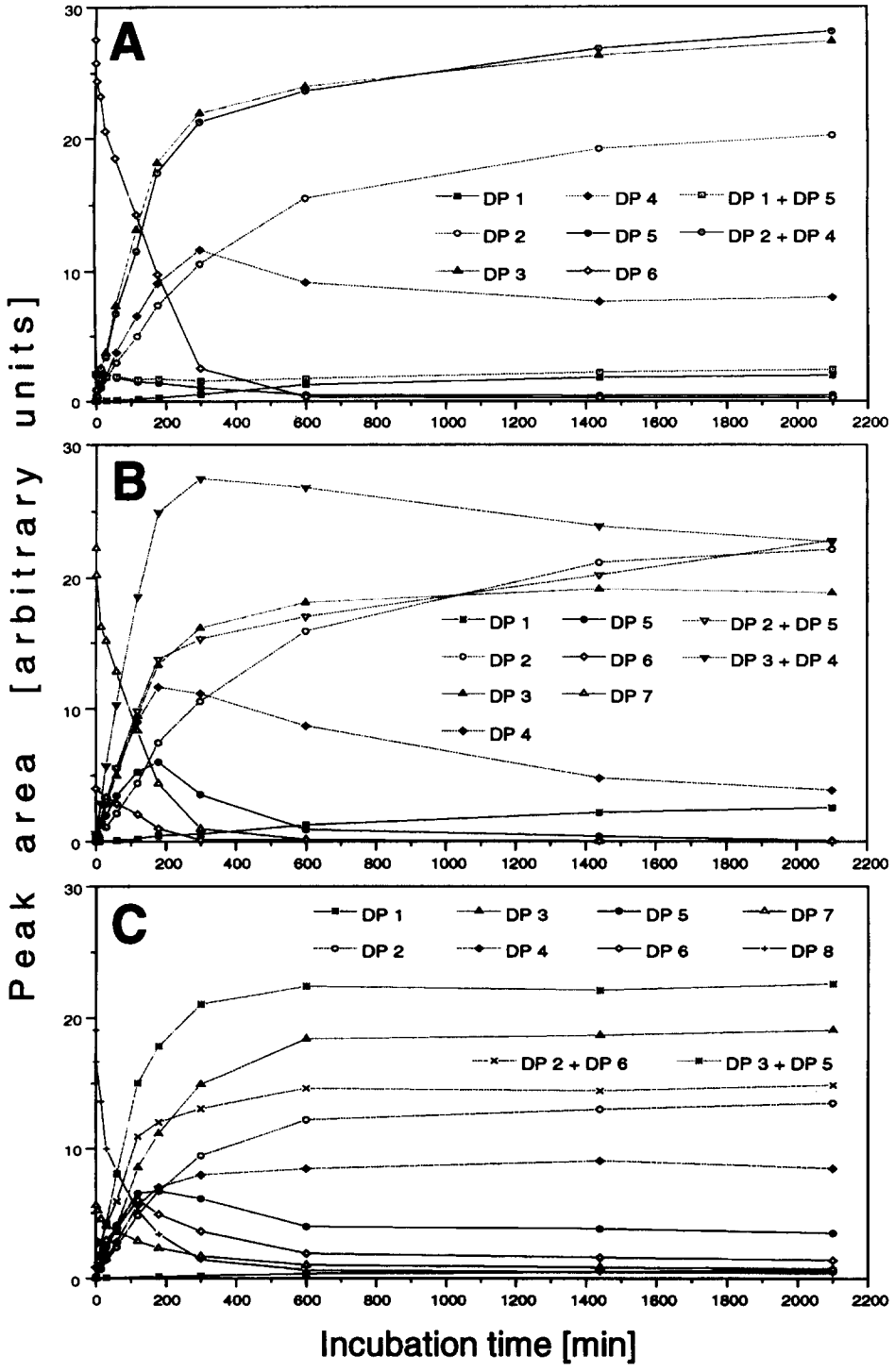
niger (*A. niger*) *endo*-ABA [9]. Two series of experiments were performed in order to detect (1) the intermediate hydrolysis products and (*2*) the end-products of the degradation of linear oligomers by *endo*-ABA. The structure of the molecules released by enzymatic hydrolysis was identified by comparison with chromatograms of the individual linear (1 → 5)-α-L-arabino-oligosaccharides.

Determination of intermediate enzymatic hydrolysis products.—In order to identify the intermediate products released by the mild hydrolysis of linear (1 → 5)-α-L-arabino-oligosaccharides by *endo*-ABA (Figs. 1 and 2), a low *endo*-ABA/substrate ratio and incubation times of 0 − 35 h were used (see Experimental section for details). The time course of incomplete *endo*-ABA hydrolysis of (1 → 5)-α-L-arabinotriose is presented in Fig. 1A; no significant degradation of the trimer into di- and mono-mers was observed. The incomplete enzymatic hydrolysis of (1 → 5)-α-L-arabinotetraose (Fig. 1B) showed that the degradation pattern $DP_4 \rightarrow DP_2 + DP_2$ was about twice as frequent as the $DP_4 \rightarrow DP_3 + DP_1$ pattern. The pentamer (Fig. 1C) was almost exclusively degraded into $DP_2 + DP_3$, as only a very limited amount of monomer had been released. HPAEC–PAD analysis of the hexamer degradation (Fig. 2A) revealed that DP_6 was degraded into almost equal amounts of $DP_2 + DP_4$ and $DP_3 + DP_3$. Hydrolysis to $DP_5 + DP_1$ was very limited. As the longer oligomers (DP_7 and DP_8) were found to be only partly pure (see Experimental section), their specific hydrolysis patterns could only be assumed. Nevertheless, it may be stated that the heptamer (Fig. 2B) was predominantly split into $DP_3 + DP_4$ rather than into $DP_2 + DP_5$; no significant $DP_6 + DP_1$ release was observed. The octamer (Fig. 2C) was primarily degraded into $DP_3 + DP_5$, followed by $DP_2 + DP_6$ and $DP_4 + DP_4$; the release of monomer was again not significant. These results suggest that: (*1*) the rate of the *endo*-ABA degradation of oligomers decreases progressively with decreasing length of the substrate ($DP_8 > DP_7 > DP_6 > DP_5 > > DP_4 > > > DP_3$); (*2*) arabinose is not a hydrolysis product under the relatively mild incubation conditions used; and (*3*) whenever a trimer can be generated, this mode of cleavage is preferred over any other (e.g., $DP_3 + DP_5 > DP_2 + DP_6$; $DP_3 + DP_4 > DP_2 + DP_5$, etc.; except for $DP_2 + DP_2 > DP_3 + DP_1$).

Determination of hydrolysis end-products after exhaustive enzymatic degradation.—In order to identify the end-products released by exhaustive degradation of linear (1 → 5)-α-L-arabino-oligosaccharides by *endo*-ABA (Figs. 3 and 4), a high *endo*-ABA/substrate ratio and incubation times of 36 and 72 h were used (see Experimental section for details). The only products detected consisted of arabinose and arabinobiose; (1 → 5)-α-L-arabinobiose was degraded at an extremely slow rate (Fig. 3). As identical end-products were obtained for all oligosaccharides (DP_{2-8}), only the degradation patterns of arabino-biose and -triose are illustrated in Figs. 3 and 4. These results were attributed to a low *endo*-ABA activity towards these molecules rather than to a significant inactivation

Fig. 1. Evolution of the amount of intermediately released (1 → 5)-α-L-arabino-oligosaccharides from the oligomeric substrates: A, (1 → 5)-α-L-arabinotriose (DP_3); B, (1 → 5)-α-L-arabinotetraose (DP_4); and C, (1 → 5)-α-L-arabinopentaose (DP_5), after incubation with *endo*-(1 → 5)-α-L-arabinanase (*A. niger*) for up to 35 h. Besides the time courses of the amount of individual oligomers, the relevant sum of oligomeric products resulting from specific enzymatic cleavage reactions is also displayed. DP $X = 1–5$ indicates the degree of polymerization of the respective (1 → 5)-α-L-arabino-oligosaccharides.


A
DP 1
DP 1 + DP 2
DP 2
DP 3
B
DP 1
DP 3
DP 1 + DP 3
DP 2
DP 4
C
DP 1
DP 4
DP 1 + DP 4
DP 2
DP 5
DP 2 + DP 3
DP 3
Peak area [arbitrary units]
Incubation time [min]


A
DP 1
DP 2
DP 3
DP 4
DP 5
DP 6
DP 1 + DP 5
DP 2 + DP 4
B
DP 1
DP 2
DP 3
DP 4
DP 5
DP 6
DP 7
DP 2 + DP 5
DP 3 + DP 4
C
DP 1
DP 2
DP 3
DP 4
DP 5
DP 6
DP 7
DP 8
DP 2 + DP 6
DP 3 + DP 5
Peak area [arbitrary units]
Incubation time [min]
0
10
20
30
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200

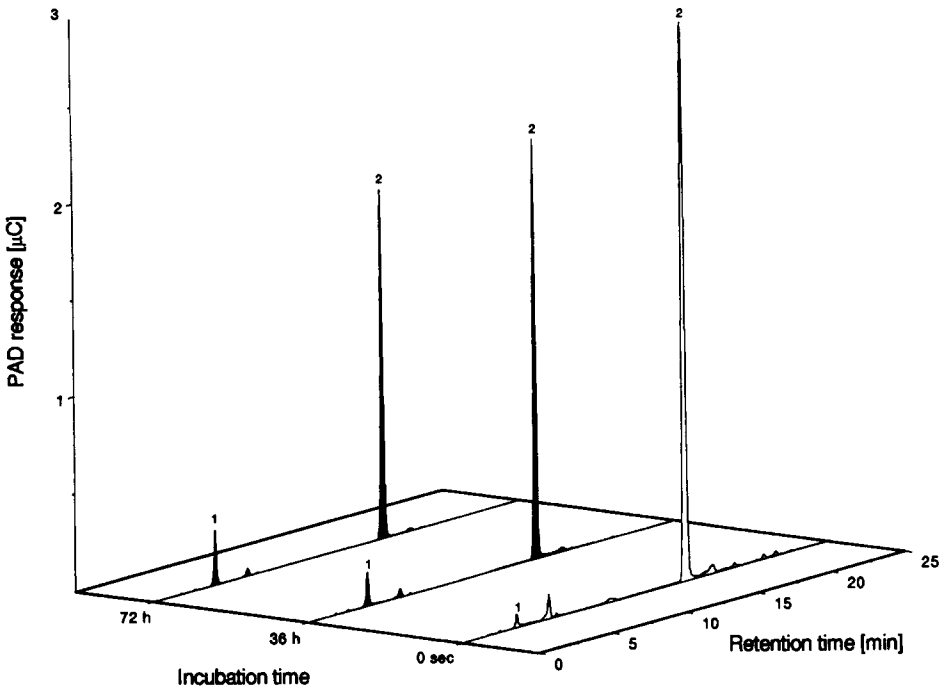


Fig. 3. HPAEC–PAD profile of hydrolysis end-products from (1 → 5)-α-L-arabinobiose (DP_2), after exhaustive degradation by *endo*-(1 → 5)-α-L-arabinanase (*A. niger*) for selected incubation times. Numbers on the top of some peaks indicate the degree of polymerization of the respective (1 → 5)-α-L-arabino-oligosaccharides (1, arabinose; 2, arabinobiose; 3, arabinotriose; etc.).

of the enzyme itself (data not shown) and were in contradiction to several previously reported results, in which (1 → 5)-α-L-arabino-biose and -triose were listed as the only hydrolysis end-products [3,4,7,8,10,11].

The data presented herein allow us to draw the following conclusions on the substrate specificity and the mode of action of *A. niger endo*-ABA: (*1*) as already observed by Beldman et al. [3], Lerouge et al. [4], and Schöpplein [7], *A. niger endo*-ABA shows extremely low degradation rates toward DP_2 or DP_3 (1 → 5)-α-L-arabino-oligomers; (2) as degradation patterns of the type $DP_1 + DP_X$ are generally not observed, the *endo*-ABA is active on glycosydic bonds situated in the inner part of the oligomeric substrate; (3)

Fig. 2. Evolution of the amount of intermediately released (1 → 5)-α-L-arabino-oligosaccharides from the oligomeric substrates: A, (1 → 5)-α-L-arabinohexaose (DP_6); B, (1 → 5)-α-L-arabinoheptaose (DP_7); and C, (1 → 5)-α-L-arabinooctaose (DP_8), after incubation with *endo*-(1 → 5)-α-L-arabinanase (*A. niger*) for up to 35 h. Besides the time courses of the amount of individual oligomers, the relevant sum of oligomeric products resulting from specific enzymatic cleavage reactions is also displayed. DP X = 1–8 indicates the degree of polymerization of the respective (1 → 5)-α-L-arabino-oligosaccharides.

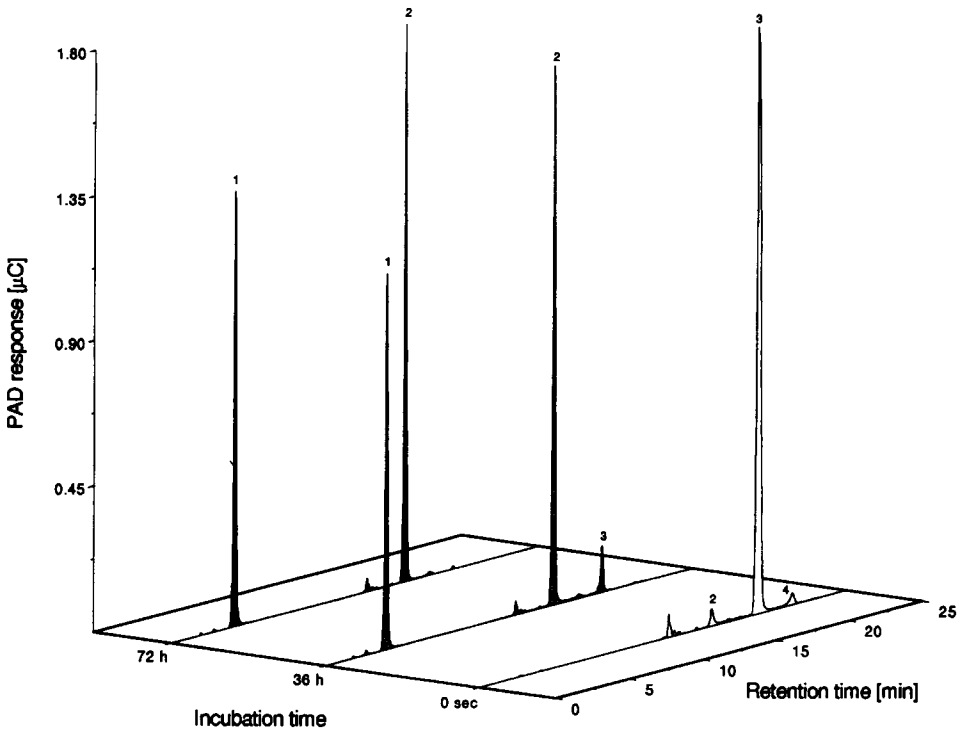


Fig. 4. HPAEC–PAD profile of hydrolysis end-products from (1 → 5)-α-L-arabinotriose (DP_3), after exhaustive degradation by *endo*-(1 → 5)-α-L-arabinanase (*A. niger*) for selected incubation times. Peak numbering as in Fig. 3.

cleavages involving the formation of the trimer (1 → 5)-α-L-arabinotriose play an important role in the hydrolytic process; and (4) the composition of end-products depends largely on the substrate and enzyme concentrations, as well as on the incubation times used. However, a more complete characterization of the catalytic properties of the *A. niger endo*-ABA would necessitate the detailed comparison of the hydrolysis kinetics of pure oligosaccharides of various chain length. In the scope of further studies, hydrolysis experiments should also be performed over a wide range of substrate concentrations; they could indeed lead to the evaluation of bond cleavage frequencies and to the assessment of possible transglycosylation effects.

1. Experimental

Enzyme and substrates.—Endo-ABA from *A. niger* was purified from the pectinase preparation Pectinex AR KPG 027 (Novo Nordisk Ferment AG, Dittingen, Switzerland) as reported by Dunkel and Amadò [9]. Linear (1 → 5)-α-L-arabino-oligosaccharides

(DP_{2-8}) were purchased from MegaZyme Pty. Ltd. (Sydney, NSW, Australia). Unless explicitly specified, all chemicals used were of analytical grade and supplied by Merck AG (Darmstadt, Germany) or Fluka Chemie AG (Buchs, Switzerland).

Enzymatic degradation of (1 → 5)-α-L-arabino-oligosaccharides.—*Endo*-ABA activities were expressed in nkat and determined as described previously [12].

Determination of intermediate enzymatic hydrolysis products.—(1 → 5)-α-L-Arabino-oligosaccharides were dissolved separately in 5 mL 50 mM NaOAc buffer (pH 4.8) and incubated at 40°C with 75 μL purified *endo*-ABA under slight stirring [final concentrations: 0.1% (w/v) oligosaccharide, 0.62 nkat *endo*-ABA]. After various incubation times, ranging from 0 s to 35 h (incubation time 0 s = substrate solution without enzyme), aliquots of 300 μL were withdrawn and inactivated at 100°C for 20 min.

Determination of hydrolysis end-products after exhaustive enzymatic degradation.—(1 → 5)-α-L-Arabino-oligosaccharides were dissolved separately in 1 mL NaOAc buffer as above and incubated at 40°C with 150 μL *endo*-ABA for 36 and 72 h [final concentrations: 0.05% (w/v) oligosaccharide, 1.24 nkat *endo*-ABA]. Aliquots of the samples were treated as described above.

The DP of the hydrolysis products was determined by HPAEC–PAD (see below), after having calibrated the column with L-arabinose and (1 → 5)-α-L-arabino-oligomers (DP_{2-8}). Substrate autohydrolysis was systematically checked by appropriate blanks (substrate solutions without enzyme, incubated and boiled under identical conditions to the samples). The purity of the respective oligomers (expressed in % of the total amount of sugars in the sample) was examined by applying them separately onto the column and eluting them under standard gradient conditions (dimer – hexamer, purity ⩾ 75%; heptamer, ~ 66%; octamer, ~ 52%).

HPAEC–PAD analyses.—HPAEC–PAD analyses of enzymatic hydrolysis products were performed on a Dionex Bio-LC DX-300 system (AGP pump, PED-2 detector with a gold working electrode and an Ag/AgCl reference electrode) interfaced to an AI-450 workstation (Dionex Corporation, Sunnyvale CA, USA). The chromatographic system was connected to an AS 3500 autosampler (SpectraPhysics, Fremont CA, USA). All eluents were degassed by flushing them with helium and pressurized with the Dionex eluent degas module. Separations were performed at room temperature on a CarboPac PA-100 column (4 × 250 mm) connected to a CarboPac PA-100 guard column (Dionex). A combination of three eluents was used for effective oligomer separations up to DP_{25}. Eluent *A* (Barnstead NANOpure water, Skan AG, Basle, Switzerland), eluent *B* [1.0 M NaOH, prepared from a 50% (w/v) NaOH solution (Baker, Deventer, The Netherlands)], and eluent *C* (1.0 M NaOAc) were mixed to form the following linear NaOAc gradient profile in 100 mM NaOH, 0 – 5 min, 0 M; 5 – 40 min, 0 – 0.5 M; 40 – 40.5 min, 0.5 – 0.9 M. The column was subsequently washed for 4.5 min with 0.9 M NaOAc and re-equilibrated for 15 min with 100 mM NaOH. Samples (20 μL) were injected via the autosampler and separations were performed at 1 mL/min. In order to optimize baseline stability and detector sensitivity, 0.5 M NaOH was added to the column effluent at 0.5 mL/min using a reagent delivery module (Dionex). Time and voltage parameters on the PAD detector were set as follows: E_1, E_2, and E_3 were, respectively +0.05 V, +0.75 V, and −0.15 V with the assigned pulse durations t_1, 0 – 0.4 s; t_2, 0.41 – 0.6 s; and t_3, 0.61 – 1.00 s. Integration of the signal occurred between 0.2 and 0.4 s.

Acknowledgements

The authors thank Novo Nordisk Ferment AG, Dittingen, Switzerland (Dr. Kurt Dörreich) for generous financial support and the gift of crude enzyme preparation.

References

[1] C. Brett and K. Waldron, in M. Black and J. Chapmann (Eds.), *Topics in Plant Physiology 2: – Physiology and Biochemistry of Plant Cell Walls*, Unwin Hyman, London, 1990, pp 1 – 57.
[2] R.F.H. Dekker, in J.M.V. Blanshard and J.R. Mitchell (Eds.), *Polysaccharides in Food*, Butterworths, London, 1979, pp 93–108.
[3] G. Beldman, M.J.F. Searle-van Leeuwen, G.A. De Ruiter, H.A. Siliha, and A.G.J. Voragen, *Carbohydr. Polym.*, 20 (1993) 159–168.
[4] P. Lerouge, M.A. O'Neill, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 243 (1993) 373–378.
[5] O. Yoshihara and A. Kaji, *Agric. Biol. Chem.*, 47 (1983) 1935-940.
[6] L. Weinstein and P. Albersheim, *Plant Physiol.*, 63 (1979) 425–432.
[7] E. Schöpplein, *Biochemische und chromatographische Charakterisierung von Fruchtsaft-Polysacchariden unter Verwendung hochgereinigter Glycanasen*, Dissertation, Universität Giessen, Geisenheim, 1989.
[8] A.G.J. Voragen, H.A. Schols, M.J.F. Searle-van Leeuwen, G. Beldman, and F.M. Rombouts, *J. Chromatogr.*, 370 (1986) 113–120.
[9] M.P.H. Dunkel and R. Amadò, *Carbohydr. Polym.*, (1994) in press.
[10] A.G.J. Voragen, F.M. Rombouts, M.F. Searle-van Leeuwen, H.A. Schols, and W. Pilnik, *Food Hydrocolloids*, 1 (1987) 423–437.
[11] F.M. Rombouts, A.G.J. Voragen, M.F. Searle-van Leeuwen, C.C.J.M. Geraeds, H.A. Schols, and W. Pilnik, *Carbohydr. Polym.*, 9 (1988) 25–47.
[12] M.P.H. Dunkel and R. Amadò, *Carbohydr. Res.*, 260 (1994) 131–135.