Carbohydrate Research 343 (2008) 2687–2691

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

Production of oligoglucuronans using a monolithic enzymatic microreactor

M. L. Tavernier [a], E. Petit [a], C. Delattre [b], B. Courtois [a], J. Courtois [a], A. Strancar [c], P. Michaud [b,*]

[a] LPMV, Université de Picardie Jules Verne, Avenue des facultés, Le Bailly, F-80025 Amiens Cedex 1, France
[b] LGCB, Université Blaise Pascal-Polytech Clermont Ferrand, 24 rue des Landais, F-63174 Aubière, France
[c] BIA separations d.o.o., Teslova 30, SI-1001 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 10 February 2008
Received in revised form 29 May 2008
Accepted 3 June 2008
Available online 8 June 2008

Keywords:
Monolithic enzymatic microreactor
Glucuronan lyase
Oligoglucuronan
CIM® disk

ABSTRACT

A glucuronan lyase (EC 4.2.2.14) was immobilized on a monolithic Convective Interaction Media (CIM®) disk. The immobilization yield was equal to 29% of the initial activity and 35% of the initial protein amount. Degradations of three glucuronans with various O-acetylation degrees were investigated and compared with degradations using free enzyme. The immobilized glucuronan lyase was inhibited by the O-acetylation degree like the free enzyme. ^{1}H NMR analyses were used to study the O-acetylation degree of oligoglucuronans and demonstrated that the average degrees of polymerization were inclusive between 4 and 13 after 24 h of degradation. This first immobilization of a glucuronan lyase constitutes a new tool to produce oligoglucuronans.

Degradations of anionic polysaccharides have been fully studied since acidic oligosaccharides were known as biological activators. Enzymatic degradation has been preferred to physical or chemical treatments which were generally difficult to carry out.[1–3] On the contrary, enzymatic degradation of anionic polysaccharides produced specific oligosaccharides with high production yields.[4]

Glucuronan is an anionic polysaccharide excreted by the *Sinorhizobium meliloti* M5N1CS mutant strain (NCIMB 40472). It is a (1,4)-β-D-polyglucuropyranosyluronic acid variably O-acetylated at C-3 and/or C-2 position depending on the Mg^{2+} concentration in the culture medium.[5] Oligoglucuronans were easily obtained by using the glucuronan lyase from *Trichoderma* sp. GL2.[6] This enzyme is acts on glucuronan by β-elimination, leading to the formation of a double bond (between C-4 and C-5) at the new formed non-reducing end. The Δ-(4,5)-unsaturated oligoglucuronic acids can be easily detectable by UV absorption at 235 nm.[7] These oligomers have shown immunostimulating properties (production of Il-1, Il-6 and TNF-α cytokines) and applications on elicitation of plant natural defences.[8,9] All these biological activities were generally due to specific degrees of polymerization (dp). Thus degradation processes are still investigated to produce specific dp and thereby to reduce purification steps. A way to improve these processes leads to immobilize the enzyme. Effectively, immobilization makes possible the reuse of the catalyst for different batches and permits an easy product recovery. This system can be applied to polysaccharide degradation in order to produce large amounts of oligosaccharides without the need for increasing the amount of enzyme necessary to achieve the reaction. Many immobilization techniques have been developed up to now such as support binding, entrapment or cross-linking using, for example, alginate beads, epoxy-activated microsphere of water insoluble (1,3)-α-D-glucan or polysaccharide–silica nanocomposites.[10–13]

Activated supports differ by their nature (synthetic resins, biopolymers or inorganic matrix) and/or by their couplings (epoxy, CNBr or NHS groups).[14] Recently, new monolithic materials were employed for enzyme immobilization. Convective Interaction Media (CIM®)-disk is a macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) support with a high binding capability.[15] Contrary to traditional columns made of porous beads, this kind of matrix has a good mass transfer controlled by convective effects. Then, CIM®-disks have been successfully employed for affinity chromatography after functionalization with ligands of high molecular mass.[16–18] In addition, CIM®epoxy disks have been used to immobilize many enzymes.[19–23]

In our case, the high porosity (1 μm) and low back pressure offered by CIM® disks are particularly adapted to the immobilization of polysaccharide lyases. These characteristics make possible to load on the disks such viscous substrates as polysaccharides. Recently, immobilization of a pectin lyase on a CIM® epoxy disk led to the production of large amounts of oligogalacturonans in a short time.[24] In this work, a CIM® epoxy disk was used to immobilize a glucuronan lyase from *Trichoderma* sp. GL2.[6] Then, different substrates were applied on this new enzymatic microreactor. The oligoglucuronans obtained were finally characterized.

The extracellular glucuronan lyase was produced by the strain *Trichoderma* sp. GL2 which was grown in a mineral medium using deacetylated glucuronan as single carbon source. After 4 days of

* Corresponding author.
E-mail address: philippe.michaud@univ-bpclermont.fr (P. Michaud).

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.06.002

Table 1
Purification balance of glucuronan lyase crude extract

Step	Total activity (U)	Total protein (mg)	SA[b] (U/mg)	Yield (%)	Purification (fold)
CE[a]	467	15.4	30.3	100	1
Anion-exchange chromatography	320	2.8	115.1	68.5	3.8
Size exclusion chromatography	181	0.59	306.8	38.7	10.1

[a] Crude extract.
[b] Specific activity.

cultivation the mycelium was removed. Extracellular proteins were concentrated by ultrafiltration and a partial purification of the glucuronan lyase was performed to eliminate poly- and oligosaccharide traces. After a step of anion-exchange chromatography followed by a step of size exclusion chromatography, 181 U (9 mL) of glucuronan lyase were recovered with a purification factor of 10 (Table 1).

A first immobilization of the enzyme was performed on the epoxy CIM® disk with 587 μg of proteins corresponding to 181 U of glucuronan lyase in a phosphate buffer (0.3 M, pH 8). Epoxy groups have been chosen because they form covalent linkages with nucleophilic groups such as primary and secondary amines, sulfhydryl groups or hydroxyl groups, depending on the pH of the immobilization step.[25] Alkaline conditions are necessary to have a good immobilization yield. In our case, immobilization was performed at pH 8. This pH is in the range of the glucuronan lyase stability.[26] After 24 h of dynamic immobilization, a loss of 32 U was measured in the glucuronan lyase solution. So, a second step of static immobilization was performed by plunging the disk into the glucuronan lyase solution. At the end of both immobilization steps, residual activity and amount of protein in the glucuronan lyase solution were evaluated to 128 U and 380 μg, respectively. A decrease of 29% of the glucuronan lyase activity was noticed in this solution and the total amount of protein diminished of 35%. In this way, it has been deduced that the theoretical immobilized activity was equal to 53 U.

A deacetylated glucuronan (DS = 0), a native glucuronan (DS = 1.2) and a highly acetylated one (DS = 2) were chosen to investigate their degradation by the immobilized glucuronan lyase. The degrees of substitution (DS) of glucuronans were evaluated by ^{1}H NMR spectroscopy. These three solutions of polysaccharide (2 g/L) were sent through the CIM®-disk in a closed circuit and the increase of absorbance at 235 nm was measured to evaluate the immobilized enzyme activity (Fig. 1). This demonstrated that the activity was inversely proportional to glucuronan substitution degree. The immobilized enzyme was then inhibited by acetates like the free enzyme. Pressure was recorded during the three degradations. The final system pressure for native glucuronan was about 0.38 MPa, whereas it was about 0.7 MPa and 0.8 MPa for highly acetylated and deacetylated glucuronans, respectively. However, these pressures fitted with the system working conditions.

Immobilized glucuronan lyase activity was calculated for deacetylated glucuronan considering the initial absorbance (Fig. 1). The activity was around 0.04 U, which is 1300 times less than the theoretical immobilized activity (53 U). Activity decreased 4 times (0.011 U) and 14 times (0.002 U) for native and highly acetylated glucuronans, respectively.

After a precipitation step to release salts (isopropanol 7 vol/vol), the final products of the three degradations (t = 24 h) were analyzed by ^{1}H NMR in order to identify and characterize oligoglucuronans produced in the different processes (Fig. 2). Unsaturated oligoglucuronans generated by β-elimination were responsible for specific signals in ^{1}H NMR analysis, such as a doublet at 5.8 ppm characteristic of the H-4 of an unsaturated unit (H-4Δ), and signals at 5.2 ppm, 4.4 ppm assigned to H-1 of an unsaturated residue (H-1Δ) and to H-1 of the repeating unit (H-1), respectively.[27] The ratio between H-1 signal integration of β Δ-(4,5)-glucuronic acid (H-1Δ) and all H-1 signal integrations (H-1Δ, H-1, H-1α and H-1β) allowed to access to the average polymerization degree. The acetylation degree of the three products could also be estimated, thanks to the integration of protons of acetates reported to the integration of all H-1 signal integrations (Table 2). The more the substrate was acetylated, the higher was the oligoglucuronan average dp. These results are in accordance with the inhibition of the glucuronan lyase by acetates.[26]

Similar 24 h degradations were carried out using 53 U of free enzyme. Final products were characterized through ^{1}H NMR analyses. After a 24 h degradation, smaller oligoglucuronans were recovered (Table 2).

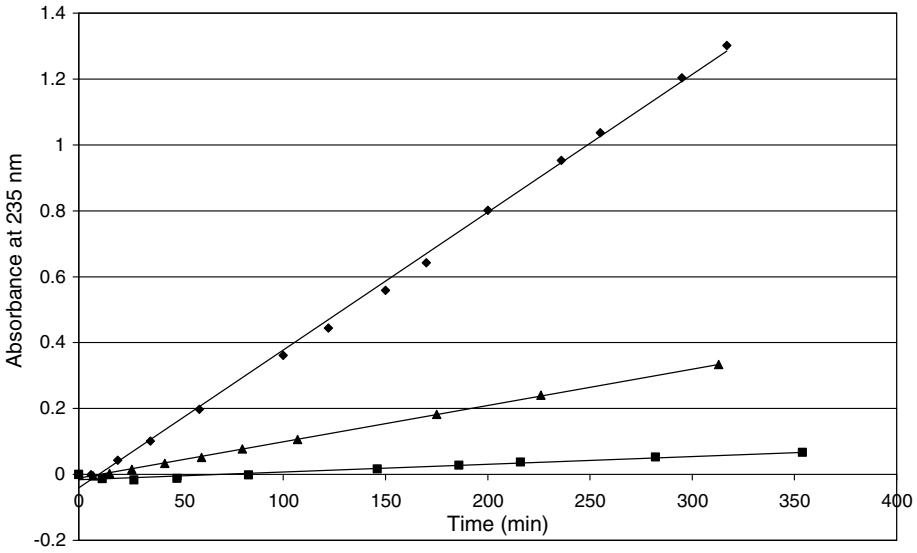

Figure 1. Evolution of absorbance at 235 nm for the degradations of glucuronans (♦: deacetylated glucuronan, ▲: native glucuronan, ■: highly acetylated glucuronan) at 2 g/L in potassium acetate 50 mM pH 5.5 using glucuronan lyase activity immobilized on a CIM® disk.

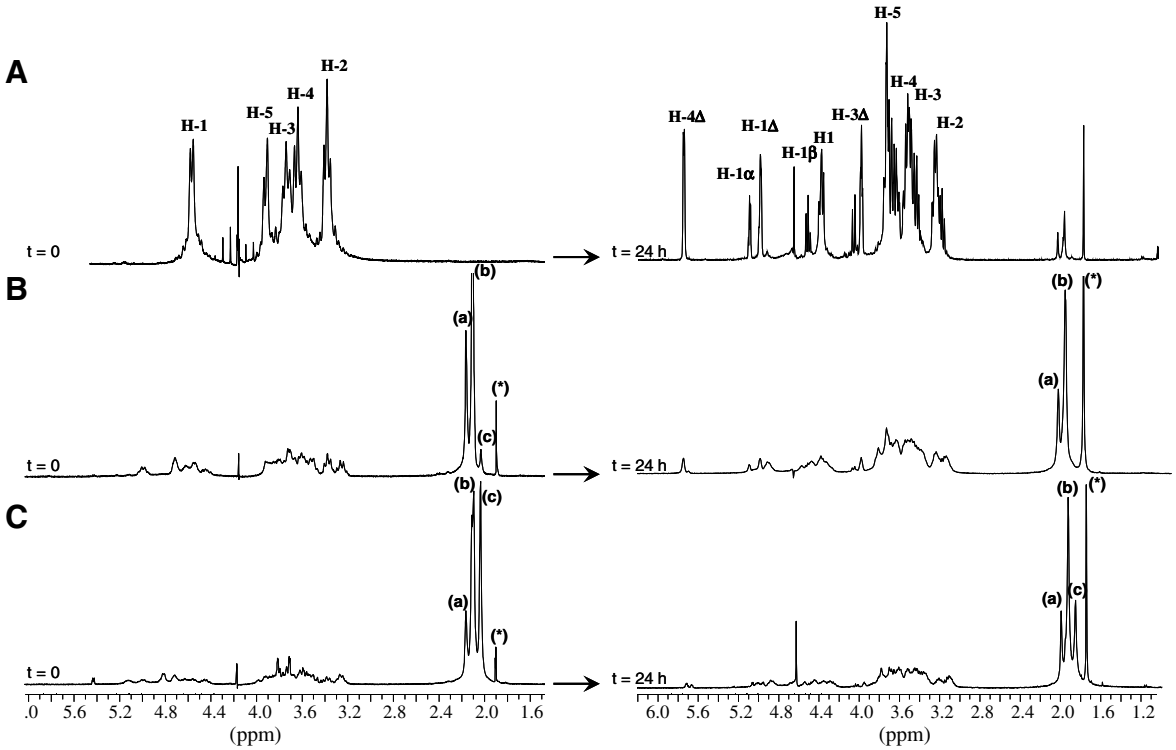

Figure 2. ^{1}H NMR spectra of substrate and product of degradation by immobilized glucuronan lyase on CIM® disk. (A) Degradation of deacetylated glucuronan, (B) degradation of native glucuronan, (C) degradation of highly acetylated glucuronan. H-4Δ: H-4 of an unsaturated unit corresponding to the non-reducing terminus unit of glucuronan; H-1Δ: H-1 of the unsaturated non-reducing terminus unit; H-1: H-1 of the non-terminal unit. (a) ^{1}H of the acetyl group at C-2 in the 2-O-acetylated residues; (b) ^{1}H of the acetyl group at C-3 in the 3-O-acetylated residues; (c) ^{1}H of the acetyl group at C-3 in the 2,3-di-O-acetylated residues and (*) free acetate.

Table 2
Degradation of three glucuronans with immobilized and free enzymes

		Deacetylated glucuronan	Acetylated glucuronan	Highly acetylated glucuronan
Immobilized enzyme	Average degree of polymerization	3.9	8.7	13.5
	Acetylation degree	0	0.7	1.4
Free enzyme	Average degree of polymerization	1.7	3.4	5
	Acetylation degree	0	0.4	1.3

Finally, we have an immobilized material that can be used again on several occasions. Oligoglucuronan pools with higher dp than those obtained with the free enzyme were produced. For example, oligoglucuronans up to dp 8 with a majority of dp 2–4 were recovered after a degradation of glucuronan with the same free enzyme.[6] Moreover, the addition of the free enzyme directly in the culture medium of *S. meliloti* M5N1CS after production of glucuronan led to the production of an unsaturated and non-acetylated oligoglucuronan with a degree of polymerization of 3.[28] Nevertheless, tedious purification steps were necessary to remove the culture medium. In conclusion, the use of the CIM disk immobilized glucuronan lyase will make it possible to increase the range of dp available for biological tests, in particular for dp higher than 5–6. Moreover, we have validated that monolithic matrices are efficient chromatographic supports for which mass transfer is much faster. Then monolithic matrices are available for the treatment by immobilized enzymes of very viscous solutions.

1. Experimental

1.1. Production of native, highly acetylated and deacetylated glucuronans

Native glucuronan was produced by the *S. meliloti* M5N1CS mutant strain which was grown at 30 °C in a 20 L bioreactor (SGI) with 15 L of *Rhizobium* Complete (RC) medium supplemented with sucrose 1% (w/v) (RCS medium).[29] Highly acetylated glucuronan was produced by cultivating the same strain on RCS medium supplemented with Mg^{2+} at the beginning of the culture (2.2 g/L instead of 0.2 g/L in standard conditions).[5] For each culture, inoculum was a 1.5 L of RCS medium inoculated with *S. meliloti* M5N1CS, and was incubated at 30 °C for 20 h on a rotary shaker (120 rpm).

After 72 h of incubation, broth was centrifuged at 33,900*g* and 20 °C for 40 min. Polysaccharides in the cell-free broth were precipitated by addition of 3 isopropanol volumes and collected by centrifugation (33,900*g*, 20 min, 20 °C). Pellets were then freeze-dried. The dry glucuronan was dissolved (4 g/L) in water and isopropanol precipitation step was repeated twice.

Deacetylated glucuronan was obtained after incubating native glucuronan during 12 h at pH 11.8 (addition of KOH 2 M) and at 50 °C. The deacetylated glucuronan was purified by a similar isopropanol precipitation.

1.2. Enzyme assay

GL activity was measured by recording the increase of absorbance at 235 nm using an UV-1700 spectrophotometer (Shimadzu). The reaction mixture was composed of 1 mL of 0.2% (w/v) glucuronan solution in potassium acetate buffer (50 mM; pH 5.5) and an appropriate volume (2–10 μL) of enzyme solution. The

amount of product released was assessed assuming that the molar absorption coefficient of Δ-(4,5) unsaturated oligoglucuronan (degree of polymerisation of 3) is 4931 M^{-1} cm^{-1}. One unit (U) of the enzyme activity corresponds to the release of 1 μmol of product per min.

1.3. Production and purification of *Trichoderma* sp. GL2 glucuronan lyase

Trichoderma sp. GL2 was cultivated on 4 L in a minimal medium supplemented with glucuronan (4 g/L) as single carbon source for 4 days as described previously.[6] The extracellular medium was then collected by a step of vacuum filtration (150 μm), followed by filtration on a Millistak+ Mini MC0CH cellulose ester capsule (Millipore, Bedford, USA). Afterwards, the extracellular medium was concentrated down to 525 mL (1325 U) using an ultrafiltration device (normal molecular weight cut off (NMWCO): 5 kDa, 0.1 m^2 from Sartorius, Goettingen, Germany).

The enzyme was partially purified at room temperature (20 °C) through a procedure using low-pressure liquid chromatography (Äkta Purifier System, Amersham Biosciences). In all steps, proteins were routinely monitored by measuring the absorbance at 280 nm with a UV-900 detector. First, 180 mL (9 injections of 20 mL) of the crude extract (CE) were applied on an anion-exchange column High Trap DEAE FF 1 mL (GE Healthcare, Chalfont St Giles, UK) equilibrated with a 20 mM Tris HCl buffer, pH 8.5 (buffer A) at a flow rate of 1 mL/min. The column was washed with equilibration buffer (35 mL). Bound proteins were eluted with 15 mL of a linear KCl gradient from 0 mM to 100 mM in buffer A followed by a 10 mL step at 100 mM of KCl. The column was washed with 500 mM and 1 M of KCl solution. Fractions of 0.5 mL were collected and characterized for enzymatic activity. Active fractions were pooled, washed and concentrated down to 2 mL on a stirred Amicon cell with a 1×10^4 NMWCO membrane. This active pool was loaded on Hiload 16/60 Superdex 75 prep grade (GE Healthcare, Chalfont St Giles, UK) first equilibrated with potassium acetate 50 mM, KCl 150 mM (pH 5.5) at a flow rate of 0.7 mL/min. Fractions of 0.5 mL were collected and monitored for glucuronan lyase activity. Active fractions were pooled, washed with phosphate buffer (0.3 M, pH 8), concentrated down to 9 mL and stored at −20 °C.

1.4. Immobilization of glucuronan lyase on CIM® disk epoxy

A CIM® epoxy disk was held on its support (CIM® housing, BIA Separations, Ljubljana Slovenia) and equilibrated with a phosphate buffer (0.3 M, pH 8). 9.5 mL of a glucuronan lyase solution (potassium phosphate buffer, 0.3 M, pH 8) were applied on the disk 24 h at 20 °C. Recirculation at 0.3 mL/min was performed using an Äkta Purifier system (GE Healthcare, USA). Dynamic immobilization step was followed by a static immobilization step. The disk was plunged into the glucuronan lyase solution for 24 h under gentle agitation. The immobilization was ended by washing the disk with 5 mL of phosphate buffer (0.3 M, pH 8) followed by 5 mL of phosphate buffer containing KCl 1 M. Finally, the disk was equilibrated with a solution of potassium acetate (50 mM, pH 5.5).

1.5. Immobilized enzyme activity assay

Three solutions of glucuronan (deacetylated, native and highly acetylated glucuronans) at 2 g/L in 50 mL of potassium acetate (50 mM pH 5.5) were applied to the CIM®-disk at the flow rate of 1 mL/min using an Äkta Purifier chromatography system (GE Healthcare, Chalfont St Giles, UK) in closed circuit for 24 h. Absorbance at 235 nm was measured using an UV-1700 spectrophotometer (Shimadzu, Duisburg, Germany) and aliquots were collected at different times for the quantification of the produced oligoglucuronans.

1.6. Protein assay

Protein amount was determined using Coomassie-protein assay (Bradford, 1976) as described in microassay Biorad instruction manual using bovine serum albumin as standard.

1.7. ^{1}H NMR analyses

^{1}H NMR analyses were achieved with a Bruker Avance 300 spectrometer of 300 MHz equipped with $^{13}C/^1H$ dual probe. Oligoglucuronan pools were analyzed at 25 °C, whereas polysaccharide spectra were achieved at 80 °C. The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 μs, a relaxation time of 1 s and a number of 128 scans. The HOD signal was presaturated by a presaturation sequence. All samples were previously dissolved in D_2O (99.9% D) and freeze-dried to replace exchangeable protons with deuterium. The freeze-dried samples were then dissolved in D_2O at a concentration between 10 and 30 g/L. The O-acetylesterification distribution and proportion for all glucuronans were analyzed by ^{1}H NMR spectroscopy with integration of the signals from downfield, upfield and acetyl regions according to previous studies.[27] They were expressed as the number of acetate per glucuronic acid residue.

Acknowledgement

This work was supported by the Conseil Régional de Picardie and the Fonds Social Européen.

References

1. Wasikiewicz, J. M.; Yoshii, F.; Nagasawa, N.; Wash, R. A.; Mitomo, H. *Radiat. Phys. Chem.* **2005**, *73*, 287–295.
2. Shimokawa, T.; Yoshida, S.; Takeuchi, T.; Murata, K.; Ishii, T.; Kusakabe, I. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1532–1534.
3. Heyraud, A.; Courtois, J.; Dantas, L.; Colin-Morel, P.; Courtois, B. *Carbohydr. Res.* **1993**, *240*, 71–78.
4. Delattre, C.; Michaud, P.; Courtois, B. *Minerva Biotechnol.* **2005**, *17*, 107–117.
5. Michaud, P.; Roblot, C.; Courtois, J.; Seguin, J. P.; Heyraud, A.; Barbotin, J. N.; Courtois, B. *Lett. Appl. Microbiol.* **1995**, *20*, 110–112.
6. Delattre, C.; Michaud, P.; Lion, J. M.; Courtois, B.; Courtois, J. *J. Biotechnol.* **2005**, *118*, 448–457.
7. Michaud, P.; Da Costa, A.; Courtois, B.; Courtois, J. *Crit. Rev. Biotechnol.* **2003**, *23*, 233–266.
8. Courtois-Sambourg, J.; Courtois, B.; Patent FR 2781673, 2000.
9. Lienart, Y.; Heyraud, A.; Sevenou, O.; Patent FR 2795289, 1999.
10. Schipunov, Y. A.; Burtseva, Y. V.; Karpenko, T. Y.; Shevchenko, N. M.; Zvyagintseva, T. N. *J. Mol. Catal. B* **2006**, *40*, 16–23.
11. Sheldon, R. A. *Adv. Synth. Catal.* **2007**, *349*, 1289–1307.
12. Busto, M. D.; Garcia-Tramontin, K. E.; Ortega, N.; Perez-Mateos, M. *Bioresour. Technol.* **2006**, *97*, 1477–1783.
13. Wang, T.; Hanxiang, L.; Kaili, N.; Tianwei, T. *Biosci. Biotechnol. Biochem.* **2006**, *12*, 2883–2888.
14. Temporini, C.; Calleri, E.; Campèse, D.; Cabrera, K.; Félix, G.; Massolini, G. *J. Sep. Sci.* **2007**, *30*, 3069–3076.
15. Mihelič, I.; Koloini, T.; Podgornik, A.; Štrancar, A. *J. High Resol. Chromatogr.* **2000**, *23*, 39–43.
16. Josić, D.; Schwinn, H.; Štrancar, A.; Podgornik, A.; Barut, M. *J. Chromatogr. A* **1998**, *803*, 61–71.
17. Champagne, J.; Delattre, C.; Shanthi, C.; Satheesh, B.; Duverneuil, L.; Vijayalakshmi, M. A. *Chromatographia* **2007**, *65*, 639–648.
18. Rucevic, M.; Clifton, J. G.; Huang, F.; Xuesong, L.; Callanan, H.; Hixson, D. C.; Josic, D. *J. Chromatogr. A* **2006**, *1123*, 199–204.
19. Benčina, M.; Benčina, K.; Štrancar, A.; Podgornik, A. *J. Chromatogr. A* **2005**, *1065*, 83–91.
20. Benčina, M.; Babič, J.; Podgornik, A. *J. Chromatogr. A* **2007**, *1144*, 135–142.
21. Vodopivec, M.; Podgornik, A.; Berovič, M.; Štrancar, A. *J. Chromatogr. B* **2003**, *795*, 105–113.
22. Bartolini, M.; Cavrini, V.; Andrisano, V. *J. Chromatogr. A* **2005**, *1065*, 134–135.
23. Vodopivec, M.; Berovič, M.; Jančar, J.; Podgornik, A.; Štrancar, A. *Anal. Chim. Acta* **2000**, *407*, 105–110.
24. Delattre, C.; Michaud, P.; Vijayalakshmi, M. A. *J. Chromatogr. B* **2008**, *861*, 203–208.

25. Wheatley, J. B.; Schmidt, D. E., Jr. *J. Chromatogr. A* **1999**, *849*, 1–12.
26. Delattre, C.; Michaud, P.; Keller, C.; Elboutachfaiti, R.; Beven, L.; Courtois, B.; Courtois, J. *Appl. Microbiol. Biotechnol.* **2005**, *70*, 437–443.
27. Dantas, L.; Courtois, J.; Courtois, B.; Seguin, J. P.; Gey, C.; Heyraud, A. *Carbohydr. Res.* **1994**, *265*, 303–310.
28. Delattre, C.; Michaud, P.; Keller, C.; Courtois, B.; Courtois, J. *Biotechnol. Prog.* **2005**, *21*, 1775–1781.
29. Courtois, B.; Hornez, J. P.; Courtois, J.; Derieux, J. C. *Anal. Biochem.* **1983**, *182*, 141–147.