Carbohydrate Polymers 87 (2012) 1828–1836

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

A transcriptomic approach to predict the impact of β-(1,3)-polyglucuronic acid sodium salt and derivatives in the main biological processes

C. Delattre[a,*], P. Michaud[b], L. Chaisemartin[a], J.Y. Berthon[a], L. Rios[a,c]

[a] GREENTECH Biotechnologies, Biopôle Clermont Limagne, 63360 Saint Beauzire, France
[b] Laboratoire de Génie Chimique et Biochimique, Université Blaise Pascal Polytech Clermont Ferrand, 24 avenue des Landais, 63174 Aubière Cedex, France
[c] ALTINAT, Biopôle Clermont Limagne, 63360 Saint Beauzire, France

ARTICLE INFO

Article history:
Received 5 October 2010
Received in revised form 10 August 2011
Accepted 4 October 2011
Available online 8 October 2011

Keywords:
β-(1,3)-Polyglucuronic acid sodium salt
Biological processes
Curdlan
DNA-array
Regioselective oxidation
Transcriptomic analysis

ABSTRACT

β-(1,3)-Polyglucuronic acid sodium salt was produced by the regioselective oxidation of curdlan using 2,2,6,6,-tetramethylpiperidine-1-oxyl radical (TEMPO)/NaBr/NaClO systems. This β-(1,3)-polyuronic acid sodium salt was depolymerized, *O*-sulphated and *O*-acetylated. All these molecules have been biologically screened for transcriptomic analysis using DNA array. The transcriptomical responses were studied after statistical gene ontology in order to evaluate biological responses disturbed by the individual treatments of human fibroblast by all these β-(1,3)-polyglucuronic acid sodium salt derivatives.

1. Introduction

For a long time, *O*-sulphated and *O*-acetylated polysaccharides either natural or chemically synthesized and their oligosaccharides derivatives have been largely described for their anticoagulant, antitumour, anti-inflammatory, antiviral and antithrombotical activities (Chevolot, Foucault, & Chaubet, 1999; Delattre, Michaud, Courtois, & Courtois, 2005; Delattre and Vijayalaksmi. 2009; Delattre et al., 2009; Feldman, Reynaldi, Stortz, Cerezo, & Damonte, 1999; Guan, Lin, & Ding, 2001; Huimin et al., 2005; Millet, Jouault, & Mauray, 1999; Nishino, Aizu, & Nagumo, 1991; Qi et al., 2006; Shi, Xu, & Li, 2000; Yuan et al., 2005). It was well-established that the sugar composition, the degree of substitution (by acetates, sulphates, phosphate), the specific position of substitution, the molecular conformation, the molecular weight distribution and the glycosidic branching of polysaccharides could modulate the biological activities (Melo, Feitosa, Freitas, & De Paula, 2002). It has already been shown that chemical modification of polysaccharides by using *O*-sulphation or *O*-acetylation with or without a reduction of the molecular weight could control and increase the biological activities of native polysaccharides (Chen & Wang, 1997; Nishino & Nagumo, 1992; Sun, He, Liang, Zhou, & Niu, 2009). Bio-active β-glucans in particular curdlan have been extensively studied (Delattre, Laroche, & Michaud, 2008). This linear and unbranched β-D-glucan is produced by *Agrobacterium* biovar 1 (*Alcaligenes faecalis var. myxogenes* strain 10C3) (Delattre et al., 2008; Lee, 2002; McIntosh, Stone, and Stanisich, 2005). Curdlan is made up of 12,000 D-glucose monomers linked by β-glucosidic bonds between C-1 and C-3. Even if curdlan is hardly used at all in foods (Harada & Sato, 1978), this polysaccharide can provide health benefits (Harada, Terasaki, & Harada, 1993). β-Glucan polysaccharides, curdlan and its derivatives have been applied in pharmacology as antithrombotic, anticoagulant, and anti-HIV agents (Delattre et al., 2008; Lee, 2002). Nevertheless, curdlan is totally insoluble in water and therefore its biological applications are low in the native form. Chemical modifications of curdlan have been investigated to generate active glycan derivatives for example carboxymethylated (Honda, Sugino, Asano, & Kakinuma, 1986) phosphorylated (Koumoto et al., 2004) aminated (Seljelid, 1986) and sulphated (Chaidedgumjorn et al., 2002) β-(1,3)-glucan. The biological activity of these derivatives has been usually related to their uronic acid, sulphate, or acetyl contents. More recently, the synthesis and characterization of a new β-(1,3)-polyglucuronic acid from curdlan has been described particularly with respect to applications in the pharmaceutical and cosmetic fields (Delattre et al., 2009).

The objective of this study was to produce *O*-sulphated and *O*-acetylated β-(1,3)-polyglucuronic acid derivatives (polysaccharides and oligosaccharides) in order to modulate biological

* Corresponding author. Tel.: +33 04 73 33 99 00; fax: +33 04 73 33 91 32.
E-mail address: Cedric_delattre@hotmail.com (C. Delattre).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.10.010

activities. Human fibroblast cell cultures have been incubated with these different β-(1,3)-polyglucuronic acid salt derivatives and the DNA array method was used to screen their biological activities.

2. Experimental

2.1. Production of curdlan

Curdlan was produced as previously described (Delattre et al., 2009) by the *Agrobacterium* sp. strain (ATCC 31750) in Yeast Broth media (30 °C, 600 rpm) in a 7.5 L bioreactor (Bioflo® 110 Newbrunswick Scientific). At the end of the growth phase, the culture pH was changed from pH 7.0 to 5.5 by addition of HCl (5 M). After 96 h of cultivation, curdlan was recovered from cultures by dissolution in 1 M NaOH followed by centrifugation to remove bacteria (15000 × *g* for 20 min at 4 °C). Finally, curdlan in supernatant was precipitated by neutralization with HCl (5 M), collected by centrifugation (15000 × *g* for 30 min at 4 °C), abundantly washed with distilled water to remove salts, and finally freeze-drying for 48 h.

2.2. Production of β-(1,3)-polyglucuronic acid sodium salt

β-(1,3)-Polyglucuronic acid sodium salt was prepared according to the oxidation procedure described by Delattre et al. (2009). Briefly, curdlan (10 g) was dissolved at room temperature in distilled water (1 L) for 60 min at pH 11.5 by adding NaOH (4 M). The solution was kept below 4 °C in an ice bath during oxidation step. TEMPO (86.6 mg), NaBr (1.92 g) and NaOCl (100 mL at 9.6%) were added to start oxidation. The pH was kept at 11 by addition of NaOH (1 M). The reaction was quenched by adding ethanol (50 mL) after 1 h and neutralized with HCl (5 M). After concentration (1/3) by evaporation under vaccum, β-(1,3)-polyglucuronic acid sodium salt was precipitated with cold isopropanol (3 volumes). The precipitate was washed with isopropanol, dissolved in distilled water, dialyzed against water during 24 h at 10,000 Da cut off dialysis membranes and freeze-dried.

2.3. Depolymerisation of β-(1,3)-polyglucuronic acid sodium salt

2.3.1. Radical depolymerisation

β-(1,3)-Polyglucuronic acid sodium salt was depolymerized according to Elboutachfaiti, Delattre, Michaud, Courtois, and Courtois (2008). Typically, β-(1,3)-polyglucuronic acid sodium salt (2 g) was dissolved in water (100 mL), and 40 mL of 1.6×10^{-3} M cupric acetate mono-hydrate were added. The temperature was kept at 60 °C. A 1% (w/w) hydrogen peroxide solution was added at a flow rate of 2 mL min^{-1} during 1 h. The reaction was then stopped, and the contaminating copper ions were removed from the product by using Chelex 100 resin. The solution was concentrated under vacuum (1/10), precipitated at 4 °C by isopropanol (7 volumes) and the mixture was centrifuged (15,000 × *g* for 20 min at 4 °C). The precipitate was washed with isopropanol, dissolved in distilled water and precipitated with isopropanol. Finally, the precipitate was dissolved in distilled water and freeze-dried to give low molecular weight β-(1,3)-polyglucuronic acid sodium salt (LMW1-PGU).

2.3.2. Thermal depolymerisation

β-(1,3)-Polyglucuronic acid sodium salt was depolymerized according to Mellal et al. (2006). Typically, β-(1,3)-polyglucuronic acid sodium salt solution at pH 4.8 in water (10 g/L) is first autoclaved at 120 °C for 40 min. After cooling in an ice bath, the solution was centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was then adjusted to pH 2 by addition of HCl (5 M) to precipitate the high molecular weight β-(1,3)-polyglucuronic acid sodium salt. After a second centrifugation step (14,000 *g* for 10 min at 4 °C), the supernatant was collected and adjusted to pH 7 by addition of NaOH (5 M). The solution was concentrated under vacuum (1/10), precipitated at 4 °C by isopropanol (7 volumes) and the mixture was centrifuged (15,000 × *g* for 20 min at 4 °C). The precipitate was washed with isopropanol, dissolved in distilled water and precipitated with isopropanol. Finally, the precipitate was dissolved in distilled water and freeze-dried to give low molecular weight β-(1,3)-polyglucuronic acid sodium salt (LMW2-PGU).

2.4. Sulphation of low molecular weight β-(1,3)-polyglucuronic acid sodium salt

The sulphation step of low molecular weight β-(1,3)-polyglucuronic acid sodium salt (LMW-PGU) was realized according to Yuan et al. (2005). Briefly, the sulphation reagent (SO_3-DMF) was prepared by dropping very slowly 40 mL of chlorosulphonic acid ($HClSO_3$) into 200 mL of DMF in an ice-water bath.

Dry LMW-PGU (2 g) was added to 80 mL DMF, and the mixture was vigorously stirred at 50 °C for 60 min to disperse LMW-PGU. Then 20 mL of SO_3-DMF reagent was slowly added. After 3 h at 50 °C, the mixture was cooled at room temperature and filtered to remove insoluble. The filtrate was then concentrated (1/10) under reduce pressure and precipitated with isopropanol (7 volumes) at 4 °C overnight. The precipitate was filtered off, dissolved in distilled water at the concentration of 20 g/L and neutralized with NaOH (5 M). The solution was precipitated with isopropanol (7 volumes) at 4 °C and filtered off. Finally, the sulphated LMW-PGU was dissolved in distilled water (50 mL) and freeze-dried.

2.5. Acetylation of low molecular weight β-(1,3)-polyglucuronic acid sodium salt

The acetylation of low molecular weight β-(1,3)-polyglucuronic acid sodium salt (LMW-PGU) was realized according to Yuan et al. (2005).

LMW-PGU (2 g) was dispersed in FA (50 mL), and the mixture was stirred at 80 °C for 20 min to disperse the carbohydrates. Then, 20 mL of Ac_2O/pyridine (1/1) solution was added. After 24 h under continuous stirring at room temperature, 200 mL of distilled water were added to react with the Ac_2O excess and the solution was concentrated (1/10) under reduce pressure and precipitated with isopropanol (7 volumes) at 4 °C overnight. The precipitate was filtered off, dissolved in distilled water at the concentration of 20 g/L and neutralized with NaOH (5 M). The solution was precipitated with isopropanol (7 volumes) at 4 °C and filtered off. Finally, the acetylated LMW-PGU was dissolved in distilled water (50 mL) and freeze-dried.

2.6. SEC MALLS analysis of β-(1,3)-polyglucuronic acid sodium salt derivatives

Average molecular weights and molecular weight distributions were determined by high performance size exclusion chromatography (HPSEC) with on line multi-angle laser light scattering (MALLS) and differential refractive index (DRI) detectors. The MALLS apparatus is the EOS from Wyatt Technology (CA, USA) filled with a K5 cell and a Ga–As laser (λ = 690 nm). The DRI detector is the ERC7515A from Erma Cr., Inc. (Japan). Columns [OHPAK SB-G guard column, OHPAK SB804 and 806 HQ columns (Shodex)] were eluted with $LiNO_3$ 0.1 M at 0.6 mL min^{-1}. Solvent was filtered through 0.1 μm filter unit (Millipore), degassed (ERC-413) and filtered through a 0.45 μm filter upstream column. The sample, filtered on 0.45 μm unit filter (Millipore) was injected through a 100 μL full loop.

For β-(1,3)-polyglucuronic acid sodium salt and oligosaccharides derivatives, the calculated d*n*/d*c* (refractive index increment) was estimated in 0.1 M at 0.150 mL/g and 0.115 mL/g respectively.

The collected data were analyzed using the Astra V-4-81-05 software package from Wyatt Technology (CA, USA).

2.7. Sugar assays

Glucuronic acid and glucose contents of β-(1,3)-polyglucuronic acid sodium salt was assayed with *meta*-hydroxyldiphenyl (Van Den Hoogen et al., 1998) and resorcinol (Monsigny, Petit, & Roche, 1988) in order to quantify the oxidation level. D-Glucose (Sigma) and D-glucuronic acid (Sigma) were used as standards. Quantification of neutral sugars was done according to the corrective formula described by Spick and Montreuil (1964). The glucuronic acid ratio (GA %) of polysaccharides obtained was expressed as the amount of glucuronic acid compared with this of all sugars (glucose and glucuronic acid).

2.8. NMR analysis

NMR analyses were performed at 30 °C with a Bruker Avance 400 spectrometer of 400 MHz equipped with $^{13}C/^{1}H$ dual probe. 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 1500 scans. Oxidized polysaccharides were dissolved in D_2O at a 50 g/L concentration. Curdlan was dissolved in NaOD (1 M) at a 50 g/L concentration.

2.9. IR analysis

Dried polysaccharides and LMW samples (1 mg) were dispersed in 100 mg of anhydrous KBr and pressed. The IR spectra were recorded at room temperature in the wavenumber range of 500–2000 cm^{-1} and referenced against air with a Nicolet 380 FT-IR instrument (Thermoelectron Corporation).

2.10. Sulphate content

For sulphate analysis, the sulphated β-(1,3)-polyglucuronic acid sodium salt derivatives fractions were quantitatively assayed by turbidity measurement after hydrolysis with HCl (4 M) and addition of gelatin–barium chloride ($BaCl_2$) (Dodgson & Price, 1962).

2.11. Acetate content

For acetate analysis, the acetylated β-(1,3)-polyglucuronic acid sodium salt derivatives fractions (10 g/L in water) were quantitatively determined by colorimetric assay using acetylcholine chloride as standard. For this, solution A (hydroxylamine hydrochloride (2 M) in NaOH (3.5 M)) and solution B ($FeCl_3$, $6H_2O$ (0.4 M) in HCl (0.1 M)) were prepared just before assay.

Briefly, mix acetylated β-(1,3)-polyglucuronic acid sodium salt derivatives solution (50 μL) with solution A (100 μL). After 2 min at room temperature, add HCl 4 M (50 μL) and solution B (50 μL). Mix the solution during 2 min at room temperature and absorbance read at 540 nm.

2.12. Screening activities of β-(1,3)-polyglucuronic acid sodium salt derivatives by transcriptomic analysis

DNA array method has been used to screen the biological activities of the β-(1,3)-polyglucuronic acid sodium salt and derivatives. Firstly, the cytotoxicity of the β-(1,3)-polyglucuronic acid sodium salt and derivatives on human fibroblast cell cultures has been evaluated. In a second step, we incubated human fibroblast cell cultures with 5 mg/mL of the β-(1,3)-polyglucuronic acid sodium salt and derivatives (with a coating method, until the cell confluence). RNA were extracted and analyzed with the DNA array method. The biological responses disturbed by the individual treatments of human fibroblast by all these β-(1,3)-polyglucuronic acid sodium salt derivatives has been investigated and correlated by genes ontology and analyzed using GeneSpring GX software (Agilent Technologies) and FatiGO softwares (http://fatigo.bioinfo.cipf.es/).

2.13. HPAEC analysis

The oligosaccharides composition produced by thermal/radical depolymerisation was determined using high performance anion exchange chromatography (HPAEC), on a Carbopac PA-1 analytical column (4 mm × 250 mm). Detection was performed with a pulsed amperometric ED50 detector (Dionex Corp., Sunnyvale, CA). Twenty-five microliters of sample were injected. The elution has been achieved by a gradient of 160 mM NaOH (solvent A) and 1 M ammonium acetate in 160 mM NaOH (solvent B) was applied at a flow rate of 1 m/min. The gradient contained four steps (expressed in percent B in A): 0% during 10 min; 0–100% from 10 to 40 min; 100% from 40 to 45 min; 100–0% from 45 to 50 min.

3. Results and discussion

3.1. Production of β-(1,3)-polyglucuronic acid sodium salt by oxidation of curdlan mediated by TEMPO–NaBr–NaOCl system

β-(1,3)-Polyglucuronic acid sodium salt derivatives have been easily produced using the TEMPO biochemistry as proposed in Fig. 1. β-(1,3)-Glucan (curdlan) was oxidized using TEMPO/NaOCl/NaBr system in order to produce β-(1,3)-polyuronides. We obtained a pure soluble β-(1,3)-polyglucuronic acid sodium salt in a good yield higher than 80% after 1 h of process as already described by Delattre et al. (2009). This pure β-(1,3)-polyglucuronic acid sodium salt was depolymerized in order to obtain different classes of low molecular weight β-(1,3)-poly- and oligoglucuronic acid sodium salt. SEC MALLS analysis was performed by coupling on-line a size exclusion chromatography (SEC), a multi-angle laser light scattering (MALLS) and a differential refractive index detector (DRI) in order to estimate the molecular weight of β-(1,3)-polyglucuronic acid sodium salt derivatives. As we can observe in Table 1 the average molecular weight of β-(1,3)-polyglucuronic acid sodium salt and LMW were estimated to be: 500 kDa, 9500 Da (LMW-1 from radical depolymerisation) and 8400 Da (LMW-2 from thermal depolymerisation) respectively.

It is appropriate to mention that the LMWs produced are composed of oligosaccharides with degree of polymerization (dp) up to 25 as confirmed by HPAEC analysis (Fig. 2) and Electrospray-QTOF-Mass analysis (data not shown). These results are comparable with literature of oligosaccharides obtained by thermal depolymerisation (Mellal et al., 2006) where oligogalacturonides (OGAs) with dp up to 25 were produced in around 30% yield.

Once produced, the LMWs fractions were *O*-acetylated and *O*-sulphated in good yield (80–90%) as described in material and method part. Therefore, we produced *O*-acetylated and *O*-sulphated LMW-polyglucuronic acid sodium salt with degree of sulphation (DS) and degree of acetylation (Dac) of 45% and 85% respectively (Table 1).

3.2. FT-IR spectroscopy analyzes

Curdlan and its oxidized derivatives have been analyzed by using FT-IR. As observed in these FT-IR analyses, the structural modification due to oxidation can be confirmed as summarized in Table 2. In these spectra, we have easily observed the specific characteristic vibrations for carboxylates as already demonstrated

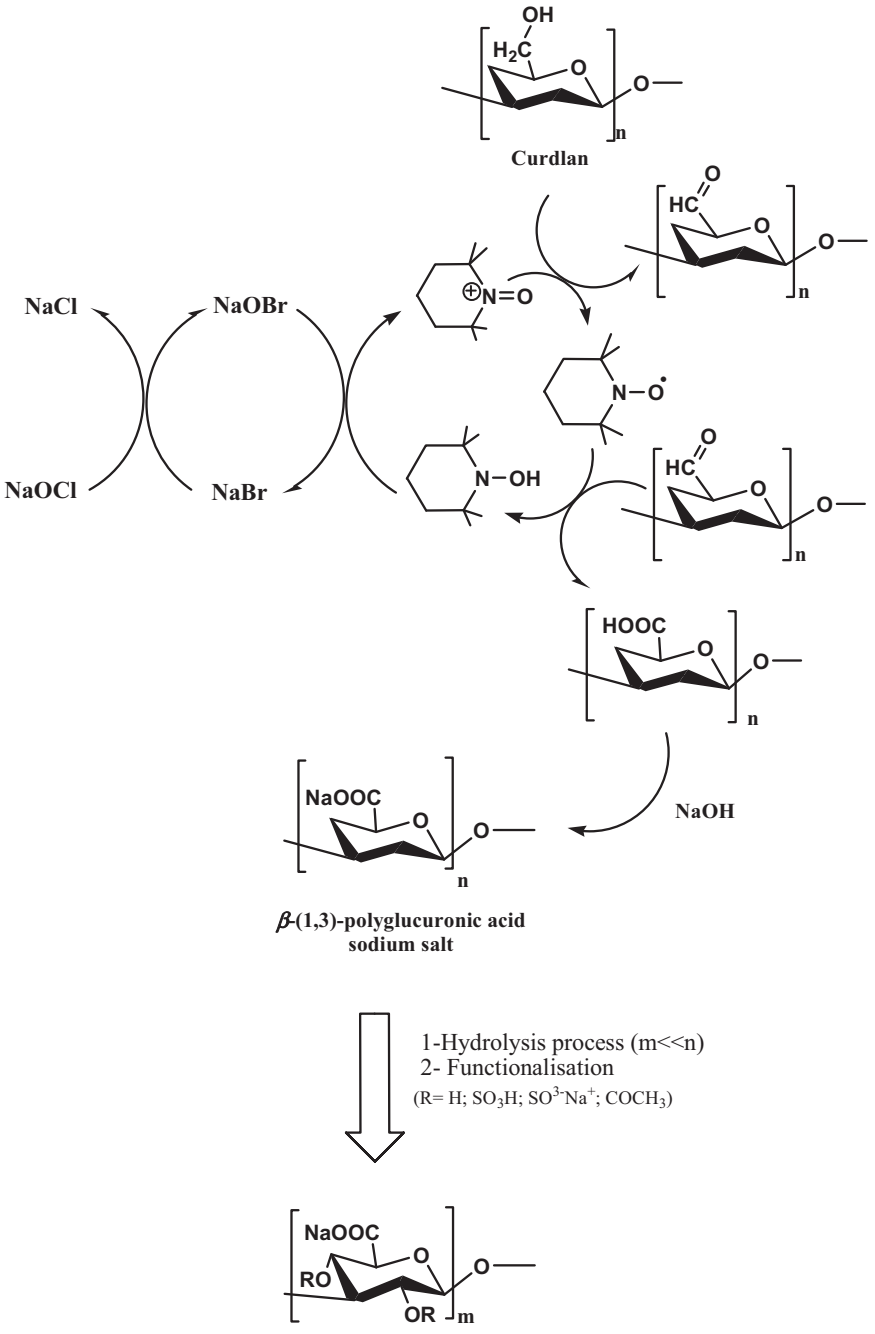


Fig. 1. Biotechnological strategies to produce β-(1,3)-polyglucuronic acid sodium salt derivatives.

Table 1
Characterization of β-(1,3)-polyglucuronic acid sodium salt and its derivatives.

Derivatives	Glc (%)	UA (%)	DS (%)	Dac (%)	Mw (kDa)
β-(1,3)-Polyglucuronic acid sodium salt	0	100	0	0	500
LMW-β-(1,3)-GlcA	0	100	0	0	<10
Sulphated LMW-β-(1,3)-GlcA	0	100	45	0	<10
Acetylated LMW-β-(1,3)-GlcA	0	100	0	85	<10

UA: uronic acid (glucuronic acid) estimated by colorimetric assay (Van Den Hoogen et al., 1998); DS: degree of sulphation estimated by turbidimetry assay (Dodgson & Price, 1962); Dac: degree of acetylation estimated by colorometric assay; Glc: glucose; MW: molecular weight.

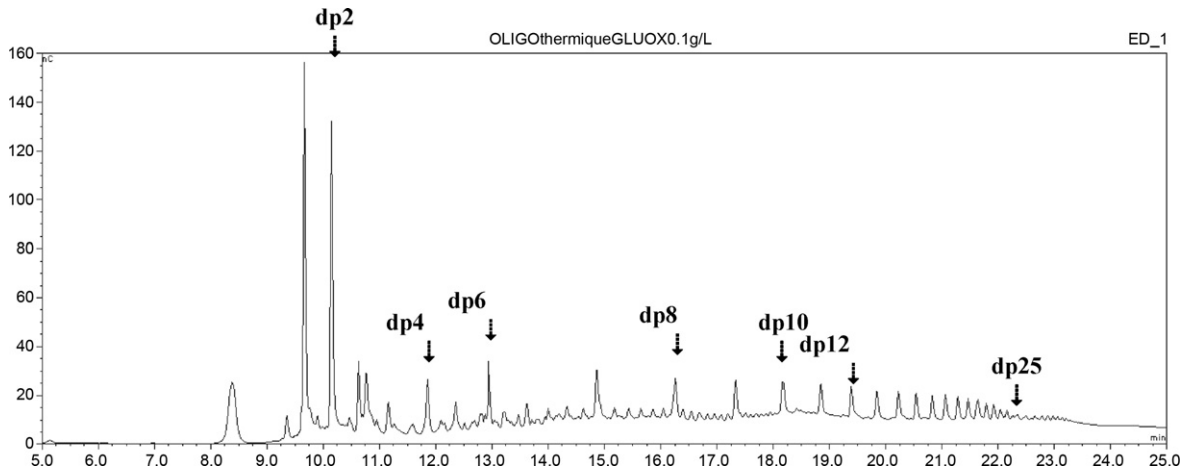


Fig. 2. General HPAEC analysis (DIONEX) of β-(1,3)-oligoglucuronic acid generated by chemical depolymerisation of β-(1,3)-polyglucuronic acid sodium salt generated by regioselective oxidation of curdlan using TEMPO/NaOCl/NaBr system (dp referred to degree of polymerization (estimated by ESI-QTOF-MS)).

Table 2
FT-IR analysis (wavenumber range 500–2000 cm^{-1}) of native curdlan ([→3)-β-D-Glc-(1→]) and β-(1,3)-polyglucuronic acid sodium salt.

Wave number (cm^{-1})	Fragments
1645	H–O–H
1610[a]	H–O–H
1420[a]	C=O[a]
1075	C–O

[a] The characteristic signal of oxidized curdlan.

by Delattre et al. (2009). In fact, in the region 1800–1200 cm^{-1} of which included the deformation vibrations of groups having a local symmetry, 2 signals were observed for β-(1,3)-polyglucuronic acid sodium salt. An intense absorption band at 1600 cm^{-1} was assigned to the asymmetrical COO^- stretching vibration, whereas the band at 1415 cm^{-1} was assigned to the symmetrical COO^- stretching vibration. These bands were not observed for unmodified curdlan.

3.3. ^{13}C NMR analyze

β-(1,3)-Polyglucuronic acid sodium salt produced during the oxidation step was analyzed by ^{13}C NMR as shown in Fig. 3. Six resonance peaks (Table 3) were characteristics of the β-(1,3)-polyglucuronic acid sodium salt and of the native β-(1,3)-glucan. The ^{13}C NMR analysis confirmed, based on the comparison with literature (Delattre et al., 2009), the absence of the C-6 resonance in the range 60–65 ppm after 60 min of oxidation, indicating the total oxidation of the primary hydroxyl group C-6 (Fig. 3). The spectrum of β-(1,3)-polyglucuronic acid sodium salt shows a new signal due to the carboxyl group around 175 ppm as evidence of carboxyl in agreement with the above mentioned FT-IR results. The presence of signals at 101.87 ppm (C-1), 70.05 ppm (C-4), 75.63 ppm (C-5), 82.80 ppm (C-3), 73.27 ppm (C-2) and more especially 175.47 ppm (COO–) validated the fact that we have obtained the pure form of this fully oxidized β-(1,3)-glucan (curdlan) as previously described by Delattre et al. (2009).

3.4. Transcriptomic analysis

3.4.1. General observations and descriptions

DNA microarray technology is currently used to propose the comprehensive profiling of genes that are up- or down-regulated in response to environmental stimuli (Bogard, Ameziane, & Lamoril, 2008). In our study, we used the microarray technology to analyze the gene expression of human fibroblast cells treated with β-(1,3)-polyglucuronic acid sodium salt and derivatives, and then to predict their impacts in the main biological processes (Fig. 4).

After decoding and filtering of the microarray data using GeneSpring GX software (Agilent Technologies) and FatiGO softwares (http://fatigo.bioinfo.cipf.es/), the expression data were normalized using the gcRMA method. Then, for each experiment, we have calculated the fold-changes in transcript levels as compared to the control (without β-(1,3)-polyglucuronic acid sodium salt derivatives) and we have selected genes whose transcript levels were either 2-fold higher or 1/2-fold lower than the control (without β-(1,3)-polyglucuronic acid sodium salt derivatives). Consequently, we identified lot of genes whose transcription levels were significantly expressed (up- or down-expressed) after the individual treatments of human fibroblast by all these β-(1,3)-polyglucuronic acid sodium salt derivatives. Among the expressing genes, the expression of: 48 genes (39 up-expressed genes and 9 down-expressed genes), 16 genes (13 up-expressed

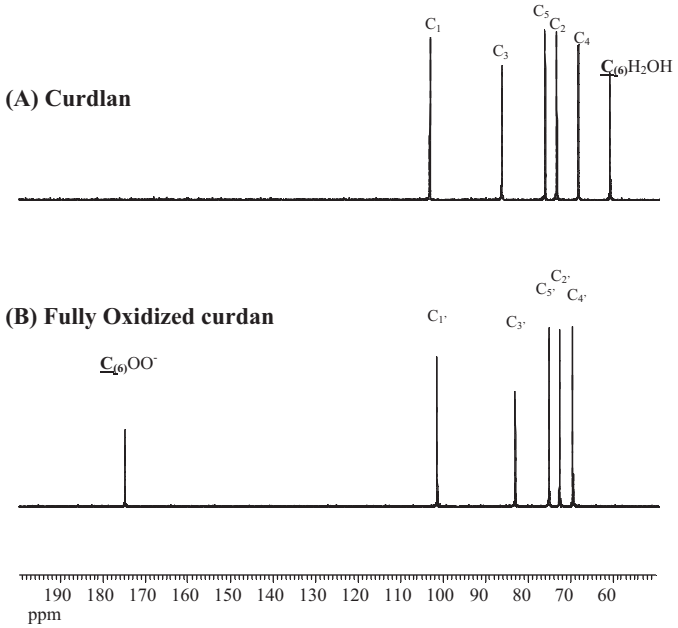


Fig. 3. ^{13}C analysis of (A) β-(1,3)-glucan (curdlan) and (B) β-(1,3)-polyglucuronic acid sodium salt generated by regioselective oxidation of curdlan using TEMPO/NaOCl/NaBr system.

Table 3
Chemical shifts (ppm) of ^{13}C (80 °C) for pure curdlan (in NaOD 0.1 M) and oxidized curdlan (in D_2O).

	Chemical shifts (δ ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Curdlan: [→3)-β-D-Glc-(1→]	103.61	73.93	86.74	68.86	76.63	61.45
Oxidized curdlan: [→3)-β-D-GlcA-(1→]	101.92	73.31	82.85	70.16	75.63	175.52

genes and 3 down-expressed genes), 48 genes (30 up-expressed genes and 18 down-expressed genes), and 9 genes (4 up-expressed genes and 5 down-expressed genes) were altered after the treatment of human fibroblast by β-(1,3)-polyglucuronic acid sodium salt, LMW2-β-(1,3)-oligoglucuronic acid sodium salt, acetylated LMW2-β-(1,3)-oligoglucuronic acid sodium salt and, sulphated LMW2-β-(1,3)-oligoglucuronic acid sodium salt respectively.

Therefore, in order to elucidate the physiological functions and the molecular functions that are affected by stimulation of human fibroblasts with β-(1,3)-polyglucuronic acid sodium salt and derivatives, we determined the percentage of down and up-regulated genes within specific categories of the gene ontology (GO) classification system (Fig. 5). Consequently, in this current study we categorized each biological process increased by fibroblast treatment with β-(1,3)-polyglucuronic acid sodium salt and derivatives. These transcriptomic analysis have shown that firstly, β-(1,3)-polyglucuronic acid sodium salt and derivatives did not induce the down-regulation of essential genes or the up-regulation of oncogenes. Secondly, lot of biological impact have been related according to the chemical modifications of β-(1,3)-polyglucuronic acid salt and derivatives. In fact, as observed in Fig. 5, it has been clearly related that β-(1,3)-polyglucuronic acid sodium salt and derivatives could induce some of the mains biological pathways *via* the specific stimulation in the expression of important genes according to biological process categorization or by molecular function categorization (Fig. 5). All these results clearly show that β-(1,3)-polyglucuronic acid sodium salt and derivatives implied a change in fibroblast's biological function. For example, some of metabolism-related genes, such as protein metabolism, lipid metabolism, or cytoskeleton organization were down-regulated significantly by stimulation of fibroblast with β-(1,3)-polyglucuronic acid sodium salt and derivatives (Fig. 5). It is important to mention that all these β-(1,3)-polyglucuronic acid sodium salt families increase the regulation of biological process. Comparing β-(1,3)-polyglucuronic acid sodium salt families, we observed a marked increase in the modulation of biological process between the polysaccharide form (63.16%) and the oligosaccharide form (75%). This reflects a greater cellular interaction for oligosaccharides as widely mentioned with the close relationship structure/function oligosaccharides in cell interaction (Delattre and Vijayalaksmi, 2009; Delattre et al., 2005, 2009; Varki, 1993). This interaction is slightly increased with the sulphated form (69.23%) but this interaction is especially important with the highly acetylated β-(1,3)-oligoglucuronic acid sodium salt where we get a modulation of biological processes up to 81.25% (Fig. 5). Structurally speaking, these acetylated β-(1,3)-oligoglucuronic acid sodium salts are oligo hyaluronan-like which are widely described for their biological functions. Then, these transcriptome analyses allow correlating the acetylated oligosaccharide with the higher modulation of biological processes.

In terms of biological activity, cell communication/cell interaction/or other cell regulator class genes were highly modulated and expressed by stimulation of human fibroblasts with β-(1,3)-polyglucuronic acid sodium salt and derivatives. For example, we estimated the modulation of cell communication/multicellular organismal development scored at: 31.6%/36.8%, 50.0%/50%, 42.3%/42.3% and 56.3%/62.5 for β-(1,3)-polyglucuronic acid sodium salt, LMW2-β-(1,3)-oligoglucuronic acid sodium salt, sulphated LMW2-β-(1,3)-oligoglucuronic acid sodium salt and acetylated LMW2-β-(1,3)-oligoglucuronic acid sodium salt respectively (Fig. 5).

These results indicated that metabolic activities were highly increased during responses. According to categorization by biological process (Fig. 5), receptor activity and signal transducer activity class genes were highly affected by stimulation. This result implied a change in human fibroblast's ability to perceive signalling upon β-(1,3)-poly- and oligoglucuronic acid sodium salt stimulation.

There are several lines of evidence that maintain an indispensable role of oligosaccharides in cellular communication (Delattre and Vijayalaksmi, 2009; Delattre et al., 2005; Varki, 1993) and this study confirm the impact of oligoglucuronic acid in the modulation

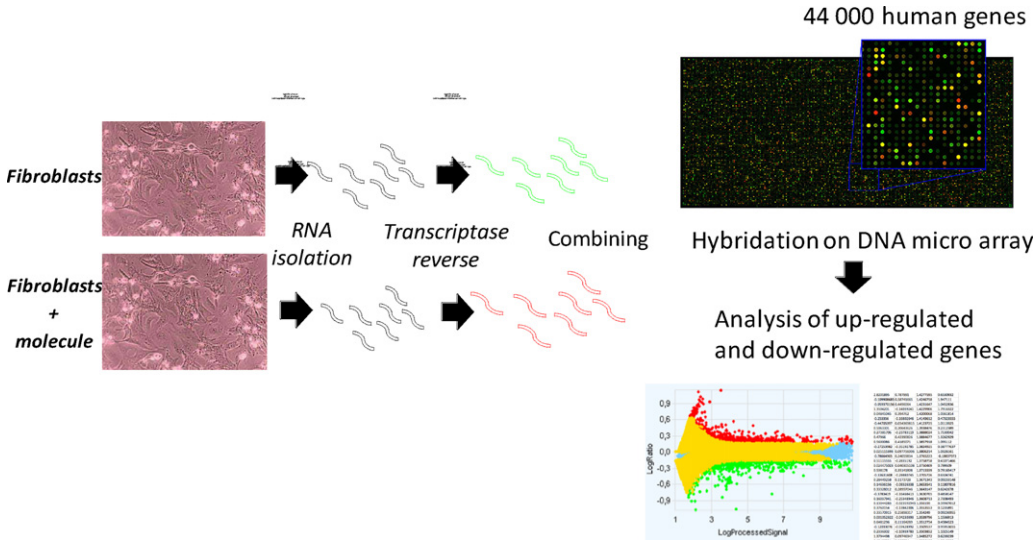
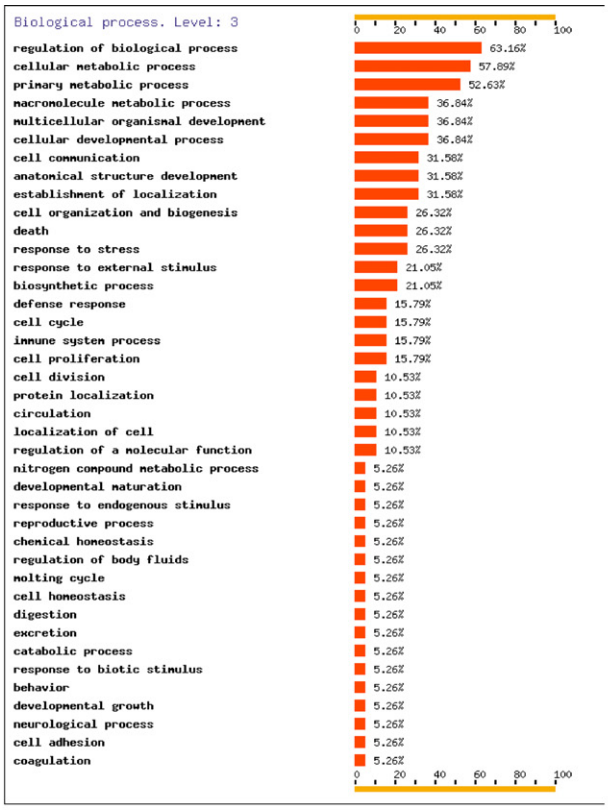
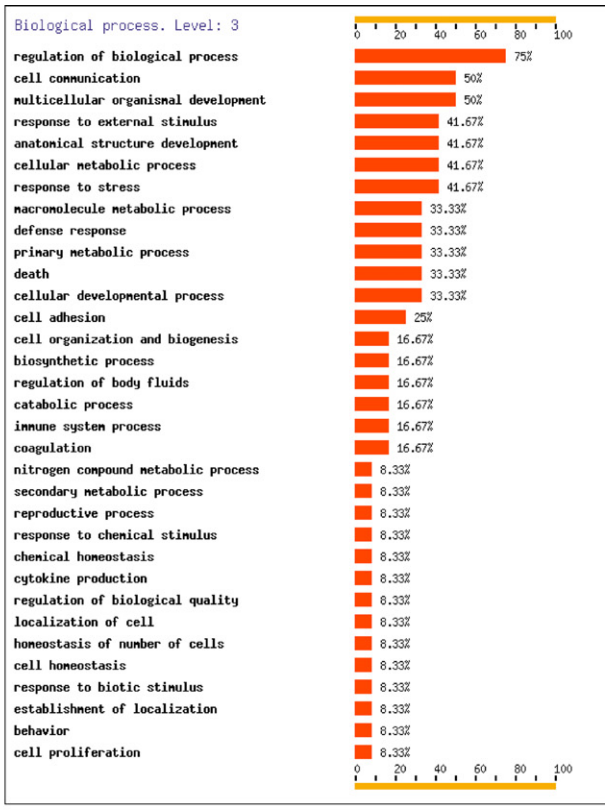


Fig. 4. Transcriptomic approach to screen the putative biological activities of β-(1,3)-polyglucuronic acid sodium salt derivatives.

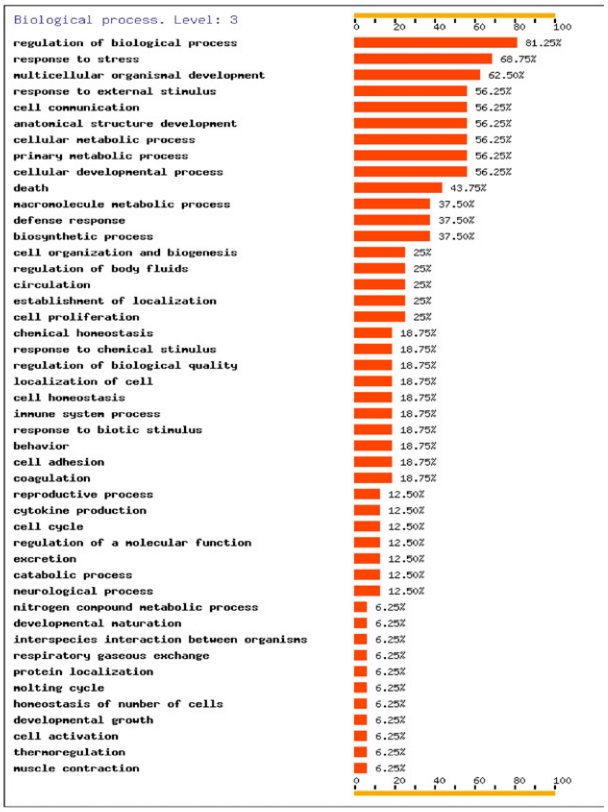


β-(1,3)-polyglucuronic Acid sodium salt



LMW2-β-(1,3)-oligoglucuronic acid sodium salt

Sulfated LMW2-β-(1,3)-oligoglucuronic acid sodium salt



Acetylated LMW2-β-(1,3)- oligoglucuronic acid sodium salt

Fig. 5. Screening of biological activities of β-(1,3)-polyglucuronic acid sodium salt and low molecular weight β-(1,3)-oligoglucuronic acid sodium salt derivatives (acetylated and sulphated) by transcriptomic method using DNA-array. The effect of hydrostimulation on genes expression in human fibroblasts was classified by gene ontology. The data represents the percentage of responsive genes in biological process and molecular function.

of cell communication and more especially with acetylated β-(1,3)-oligoglucuronic acid sodium salt as specific oligo-hyaluronan-like marker. This current study gives a first transcriptomic approach using β-(1,3)-polyglucuronic acid sodium salt and its derivatives whose primary purpose is to guide future research into the *in vitro* and *in vivo* biological test. This study gives a kind of "transcriptomic card" of the biological potential of β-(1,3)-polyglucuronic acid sodium salt and its derivatives. The analysis of the biological activities of these genes highlights their implication in the principal biological functions as the cell communication, the cellular development process, the cell proliferation, and the cell division (Fig. 5). This transcriptomic analysis could be the perfect tool to propose the "biological activities printing" of carbohydrate and derivatives.

3.4.2. Proposed biological impact of β-(1,3)-polyglucuronic acid sodium salt

If we studied in greater detail the genes classes expressed and its associated biological processes, this transcriptomic study demonstrated that the β-(1,3)-polyglucuronic acid sodium salt could be implicated in the process of healing and the metabolism of the lipids. In fact, the results of the transcriptomic analysis show that the oxidized curdlan activates genes implicated in the three phases of the healing process: the preparatory phase (formation of the blood clot in the event of wound and inflammatory reaction), the phase of proliferation (angiogenesis and cellular migration) and the phase of remodelling (cellular proliferation and synthesis of the extracellular matrix) (Fig. 5). Indeed, the oxidized curdlan activates genes CCL26, EGR1, PTGFD, PTGS2 and SECTM1 which are known to stimulate the inflammatory reaction, a constitutive step of the preparatory phase of the healing biological process. Activation of these genes allows to the recruitment of inflammatory cells (granulocytes, macrophages, lymphocytes) on the injured site and the production of cytokines and growth factors (IL-1, IL-6, IL-8, EGF, TGF-α, HB-EGF). These compounds constitute the earliest signals to activate the local fibroblasts and keratinocytes which are implicated in the proliferative and remodelling phases of the healing process. This β-(1,3)-polyglucuronic acid sodium salt also directly activates the proliferative phase of the healing process by inducing: (i) the over-expression of genes EDNRB, NPTX1, PTGFD and PTGS2 involved in the activation of the angiogenesis necessary to the installation of the neovascularisation on the site to be healed, (ii) the genetic over-expression of MMP1, MMP10 and PDGFD (which stimulates the expression of MMP1 and MMP9) implicated in the degradation of the extracellular matrix which is an essential phenomenon for the cellular migration (blood cells, vascular fibroblasts, endothelial and epithelial cells) towards the site injured but so essential for the elimination of the remains of the extracellular matrix injured by the wound.

In the same way, this β-(1,3)-polyglucuronic acid sodium salt also directly activates the phase of remodelling of the healing process by controlling negatively the activity of the matrix metalloproteinase (MMP), by the over-expression of the modulating gene TFPI2, by supporting the synthesis of types I and II collagen (by inducing the over-expression of gene EGR1, COL1A and WNT11).

It is important to mention that the three successive phases of the healing process (preparatory, proliferative and remodelling phases) are controlled in time. The oxidized curdlan takes part in the installation but also in the control of these three phases. Indeed, the oxidized curdlan can take part in the control of the induced inflammatory reaction during preparatory phase by over-expression of genes implied in anti-inflammatory processes (NPTX1, APOE). We observed that β-(1,3)-polyglucuronic acid sodium salt can regulate the proliferative phase by over-expression of genes having anti-angiogenic (EGR1, TFPI2, SPON1) or matrix anti-metalloproteinases activities (TFPI2). Moreover, this β-(1,3)-polyglucuronic acid sodium salt can control the phase of remodelling by over-expression, in the same time, of genes implicated in the synthesis of collagen (ECR1, WNT11) but also of those involved in its degradation (MMP1, MMP10, PDGFD).

The second biological process, on which acts this β-(1,3)-polyglucuronic acid sodium salt is the lipidic metabolism, as it activates the gene ANGPTL4 which codes for the adipokine protein. This adipokine synthesized by adipocytes induce a reduction of their masses and volumes (Mandard et al., 2006). Effectively, ANGPTL4 decreases the entry of fatty acids in the subcutaneous adipocyte by inhibiting LPL enzyme (lipoprotein lipase) (Sukonina, Lookene, & Olivecrona, 2006) and favour their liberation (lipolysis), stimulating the activity of the adipose triglyceride lipase (Mandard et al., 2006). The over-expression of ANGPTL4 gene could thus at the same time decrease the lipogenesis and increase the lipolysis locally (topical uses) on the subcutaneous adipocyte. This locally action of ANGPTL4 on subcutaneous adipocytes could lead to a locally increase of their sizes. So, the topical application of the β-(1,3)-polyglucuronic acid sodium salt on the subcutaneous adipocytes does not induce a general increase of ANGPTL4 in the body and does not modify the general metabolism of lipoproteins.

4. Conclusion

This transcriptomic analysis constitutes an interesting approach to predict the real impact of carbohydrates on biological processes. In fact, DNA microarray analysis could easily revealed comprehensive changes in transcript levels upon human fibroblast stimulations with carbohydrates. In this present study, curdlan has been regioselective carboxylated mediated by the TEMPO/NaOCl/NaBr system. This β-(1,3)-polyglucuronic acid sodium salt appears structurally as a glycosaminoglycans mimetic and due to the permanent worldwide boost in their use in pharmacology, the natural sources will not be enough for the biotechnological need in future. An important field of investigation is the development of successful alternatives to substitute glycosaminoglycans by using β-(1,3)-polyglucuronic acid sodium salt and all their derivatives from chemical substitution such as sulphation and acetylation. This study demonstrates that the biological processes disrupted by β-(1,3)-polyglucuronic acid sodium salt derivatives involving different modes of action can be identified by transcriptomic analysis. These preliminary results have shown the efficiency of the chemical modification of β-(1,3)-D-glucan (oxidation) and their non-toxicity on human fibroblasts cultures. A transcriptomic study about mRNA expressed by these cells using DNA array methods have identified in all the human genome the levels of gene expression induced by anionic β-(1,3)-D-glucans and its acetylated and sulphated derivatives. So, these results open the way to larger investigations using panel of acidic β-(1,3)-D-glucan with various sizes and chemical modifications. To confirm and reinforce all these preliminary transcriptomic results, works are in progress to experiment by *in vitro* and *in vivo* tests all these β-(1,3)-polyglucuronic acid sodium salt derivatives.

References

Bogard, M., Ameziane, N. & Lamoril, J. (2008). DNA microarray and genes expression patterns. Part I. Design and use. *Immuno-analyse et biologie spécialisée*, *23*, 71–88.

Chaidedgumjorn, A., Toyoda, H., Woo, E. R., Lee, K. B., Kim, Y. S., Toida, T., et al. (2002). Effect of (1 → 3)- and (1 → 4)-linkages of fully sulfated polysaccharides on their anticoagulant activity. *Carbohydrate Research*, *337*(10), 925–933.

Chen, H. & Wang, K. (1997). *Function and structure of glycoconjugates*. Shanghai, China: Shanghai Medical University Press., pp. 324–341 (in Chinese)

Chevolot, L., Foucault, A. & Chaubet, F. (1999). Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity. *Carbohydrate Research*, *319*, 154–165.

Delattre, C. & Vijayalaksmi, M. A. (2009). Monolith enzymatic microreactor at the frontier of glycomic toward a new route for the production of bioactives oligosaccharides. *Journal of Molecular Catalysis B: Enzymatic*, *60*(3), 97–105.

Delattre, C., Michaud, P., Courtois, B. & Courtois, J. (2005). Oligosaccharides engineering from plants and algae—Applications in biotechnology and therapeutic. *Minerva Biotecnologica*, *17*, 107–117.

Delattre, C., Laroche, C. & Michaud, P. (2008). *Bacterial and fungal polysaccharides produced by fermentation—An overview. Advances in fermentation Technology* (pp. 483–522). New Delhi: Asiatech Publishers Inc.

Delattre, C., Rios, L., Laroche, C., Le, N. H. T., Lecerf, D., Picton, L., et al. (2009). Production and characterization of new families of polyglucuronic acids from TEMPO–NaOCl oxidation of curdlan. *International Journal of Biological Macromolecules*, *45*, 458–462.

Dodgson, K. S. & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*, 106–110.

Elboutachfaiti, R., Delattre, C., Michaud, P., Courtois, B. & Courtois, J. (2008). Oligogalacturonans production by free radical depolymerization of polygalacturonan. *International Journal of Biological Macromolecules*, *43*, 257–261.

Feldman, S. C., Reynaldi, S., Stortz, C. A., Cerezo, A. S. & Damonte, E. B. (1999). Antiviral properties of fucoidan fractions from *Leathesia difformis*. *Phytomedicine*, *6*(5), 335–340.

Guan, M., Lin, W. & Ding, Y. (2001). Marine drugs-studies on the Chinese pharmacology newly in the great demand at the 21 century. *Chinese Journal of Marine Drugs*, *20*, 1–5.

Harada, T. & Sato, S. (1978). Production, properties and application of curdlan. *Hakko to Kogyo*, *36*, 86–97.

Harada, T., Terasaki, M. & Harada, A. (1993). In R. L. Whistler, & J. N. BeMiller (Eds.), *Industrial gums* (pp. 427–445). New York: Academic Press.

Honda, S., Sugino, T., Asano, S. & Kakinuma, A. (1986). Activation of the alternative pathway of complement by an antitumor (1,3)-beta-D-glucan from *Alcaligenes faecalis* var. myxogenes IFO 13140, and its lower molecular weight and carboxymethylated derivatives. *Immunopharmacology*, *1*, 29–37.

Huimin, Q., Tingting, Z., Quanbin, Z., Zhien, L., Zengqin, Z. & Ronge, X. (2005). Antioxidant activity of different molecular weight sulfated polysaccharides from Ulva pertusa Kjellm (Chlorophyta). *Journal of Applied Phycology*, *17*(6), 527–534.

Koumoto, K., Umeda, M., Numata, M., Matsumoto, T., Sakurai, K., Kunitake, T., et al. (2004). Low MW sulphated curdlan with improved water solubility forms macromolecular complexes with polycytidylic acid. *Carbohydrate Research*, *339*, 161–167.

Lee, I. Y. (2002). E. J. Vandamme, S. De Baets, & A. 1. teinbüchel (Eds.), *Polysaccharides I: Polysaccharides from prokaryotes. Biopolymers* (pp. 135–158). London: Wiley.

Mandard, S., Zandbergen, F., Va Straten, E., Wahli, W., Muller, M. & Kersten, S. (2006). The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma levels and adiposity. *The Journal of Biological Chemistry*, *281*(2), 934–944.

McIntosh, M., Stone, B. A. & Stanisich, V. A. (2005). Curdlan and other bacterial (1,3)-beta-D-glucans. *Applied Microbiology and Biotechnology*, *68*, 163–173.

Mellal, M., Ding, L. H., Jaffrin, M. Y., Delattre, C., Michaud, P. & Courtois, J. (2006). Separation and fractionation of oligouronides by shear-enhanced filtration. *Separation Science and Technology*, *42*, 1–13.

Melo, M. R. S., Feitosa, J. P. A., Freitas, A. L. P. & De Paula, R. C. M. (2002). Isolation and characterization of soluble sulfated polysaccharide from the red seaweed *Gracilaria cornea*. *Carbohydrate Polymers*, *49*(4), 491–498.

Millet, J., Jouault, C. S. & Mauray, S. (1999). Antithrombotic and anticoagulant activities of a low molecular weight fucoidan by the subcutaneous route. *Thrombosis And haemostasis*, *81*, 391–395.

Monsigny, M., Petit, C. & Roche, A. C. (1988). Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Analytical Biochemistry*, *257*, 107–111.

Nishino, T. & Nagumo, T. (1992). Anticoagulant and antithrombin activities of oversulfated fucans. *Carbohydrate Research*, *229*(2), 355–362.

Nishino, T., Aizu, Y. & Nagumo, T. (1991). The influence of sulfate content and molecular weight of a fucan sulfate from the brown seaweed *Ecklonia kurome* on its antithrombin activity. *Thrombosis Research*, *64*, 723–731.

Qi, H., Zhang, Q., Zhao, T., Hu, R., Zhang, K. & Li, Z. (2006). In vitro antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). *Bioorganic & Medicinal Chemistry Letters*, *16*(9), 2441–2445.

Seljelid, R. (1986). A water-soluble aminated beta 1-3D-glucan derivative causes regression of solid tumors in mice. *Bioscience Reports*, *6*, 845–851.

Shi, R. X., Xu, Z. X. & Li, Z. E. (2000). Antitumor activity of degraded *Chondrus ocellatus* polysaccharide. *Journal of Oceanologia et Limnologia Sinica*, *31*, 653–656.

Spick, G. & Montreuil, J. (1964). 2 causes of error in colorimetric determinations of total neutral sugar. *Bulletin de la Société de chimie biologique*, *46*, 739–749.

Sukonina, V., Lookene, A. & Olivecrona, G. (2006). Angiopoetin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. *Proceedings of the National Academy of Sciences*, *103*(46), 17450–17455.

Sun, Z., He, Y., Liang, Z., Zhou, W. & Niu, T. (2009). Sulfation of (1,3)-β-D-glucan from the fruiting bodies of *Russula virescens* and antitumor activities of the modifiers. *Carbohydrate Polymers*, *77*, 628–633.

Van Den Hoogen, B. M., Van Weeren, P. R., Lopes-Cardozo, M., Van Golde, L. M. G., Barnveld, A. & Van de Lest, C. H. A. (1998). A microtiter assay for the determination of uronic acids. *Analytical Biochemistry*, *257*, 107–111.

Varki, A. (1993). Biological roles of oligosaccharides: All the theories are correct. *Glycobiology*, *2*, 97–130.

Yuan, H., Zhang, W., Li, X., Lu, X., Li, N. & Gao, X. (2005). Preparation and in vitro antioxidant activity of [kappa]-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. *Carbohydrate Research*, *340*(4), 685–692.