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Yanna André, Emeline Richard, Mélanie Leroux, Isabelle Jeacomine, Eric Bayma, et al.. Production of unsulfated chondroitin and associated chondro-oligosaccharides in recombinant *Escherichia coli*. *Carbohydrate Research*, 2024, 544, pp.109243. 10.1016/j.carres.2024.109243 . hal-04882048

HAL Id: hal-04882048

<https://hal.science/hal-04882048v1>

Submitted on 13 Jan 2025

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Production of unsulfated chondroitin and associated chondro-oligosaccharides in recombinant *Escherichia coli*

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ABSTRACT

We designed metabolically engineered non-pathogenic strains of *Escherichia coli* to produce unsulfated chondroitin with and without chondroitin lyase to produce the chondroitin polymer or its related oligosaccharides. Chondroitin was synthesized using chondroitin synthase KfoC and chondroitin was degraded using P135, a chondroitin lyase from *Pedobacter heparinus*. P135 behaved as a true endo-enzyme generating a large panel of oligosaccharides ranging from trimers to 18-mers instead of the di- and tetramers obtained with most chondroitin lyases. Two series of oligosaccharides were characterized, sharing an unsaturated uronic acid (4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid, Δ UA) residue at their non-reducing end. The major “even-numbered” series was characterized by a terminal reducing *N*-acetylgalactosaminyl residue. The minor “odd-numbered” series oligosaccharides carried a terminal reducing glucuronic acid residue instead.

Cultures were conducted in fed-batch conditions, and led to the production of up to 10 g L⁻¹ chondroitin or chondroitin oligosaccharides.

All products were purified and fully characterized using NMR and mass spectrometry analyses.

This is the first report of the microbial production of large chondro-oligosaccharides.

1. Introduction

Glycosaminoglycans (GAGs) are important components of vertebrate extracellular matrices. Among them, chondroitins are characterized by a polysaccharide skeleton of repeating disaccharide β 4glucuronic acid (β 4GlcUA)- β 3*N*-acetylgalactosamine (β 3GalNAc) units. Chondroitin is usually sulfated, thus defining the family of chondroitin sulfates, although unsulfated chondroitin can be found in some bacteria [1,2]. The pathogenic *Escherichia coli* K4 strain produces a capsular fructosylated chondroitin, called K4 polysaccharide, synthesized by chondroitin synthase KfoC, which has been characterized and expressed in several microorganisms for the production of recombinant chondroitin [3–5].

Due to the involvement of chondroitin sulfates in various biological processes, chondroitin oligosaccharides are good candidate molecules that can serve as precursors for chemical or enzymatic modifications such as sulfation leading to well-defined biologically active compounds [6,7].

The bacterial production of oligosaccharides has been proven to be a good alternative to *in vitro* synthesis of oligosaccharides, because all the polysaccharide substrates, nucleotide sugars and enzymes are synthesized and act together in the recombinant host, which thus operates as a

living enzymatic reactor. This approach has been successfully applied to produce heparosan oligosaccharides [8], and more recently to produce di- and tetrasaccharides of chondroitin [5].

A key point of this approach is the good expression and activity of recombinant enzymes in bacteria. The only known enzyme allowing to generate large chondro-oligosaccharides is the bovine testicular hyaluronidase [9]. Unfortunately, this hyaluronidase contains five disulfide bonds that cannot be efficiently produced in a soluble and functional form in the reducing environment of the *E. coli* cytoplasm. *Pedobacter heparinus* synthesizes two chondroitin lyases (AC and B) that can be expressed in a recombinant form in *E. coli* [10]. However, these two enzymes have an exo-mode of action (EC 4.2.2.5 and 4.2.2.9, respectively), generally releasing unsaturated disaccharides. Endolyase P135 from *Victivallis vadensis* was recently identified and has been successfully expressed in *E. coli* [11].

In the present study, we describe a novel bacterial recombinant strain producing cytoplasmic unsulfated chondroitin able to produce a large panel of chondro-oligosaccharides via co-expression of *V. vadensis* chondroitin lyase in a recombinant *E. coli* strain. Chondroitin and chondro-oligosaccharides were purified and characterized using size-exclusion chromatography with multi-angle light scattering detection

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<https://doi.org/10.1016/j.carres.2024.109243>

Received 13 May 2024; Received in revised form 2 July 2024; Accepted 13 August 2024

Available online 14 August 2024

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Table 1
Bacterial plasmids and strains.

Gene	Description	Ref. or source
<i>tig</i>	Trigger factor (TF) from <i>Escherichia coli</i> DH1	Uniprot P0A850
<i>kfoC</i>	Chondroitin synthase from <i>E. coli</i> K4	Uniprot Q8L0V4
<i>kfoG</i>	Unknown protein from <i>E. coli</i> K4	Uniprot Q8L0V9
<i>gne</i>	UDP-GlcNAc 4-epimerase from <i>Campylobacter jejuni</i>	Uniprot A0A060NG80
<i>kfiD</i>	UDP-glucose-6-dehydrogenase from <i>E. coli</i> K5	Uniprot Q47329
<i>glcAT-P</i>	β -1,3-Glucuronyltransferase	Uniprot P59270
<i>pl35</i>	Chondroitin lyase belonging to polysaccharide lyase family 35 (CAZY classification) from <i>V. vadensis</i>	[11]
Plasmid		
pBAD33	Cloning vector, Cm, P _{BAD} promoter, P15A	[13]
pET28a	Cloning vector	Novagen
pBS-kfoCG	pBluescript derivative carrying <i>kfoCG</i>	[15]
pWKS-gne	pWKS derivative carrying <i>gne</i>	[16]
pBBR-kfiD	pBBR1MCS3 derivative carrying <i>kfiD</i>	[14]
pBBR-glcAT-P-kfiD	pBBR1MCS3 derivative carrying <i>glcAT-P</i> and <i>kfiD</i>	[15]
pBAD-tig	pBAD33 derivative carrying <i>tig</i>	[14]
pBAD-pl35	pBAD33 derivative carrying <i>pl35</i>	This work
pBAD-tig-pl35	pBAD33 derivative carrying <i>tig-pl35</i>	This work
pET28a-pl35	pET-28a derivative carrying <i>pl35</i>	This work
pET28a-tig-pl35	pET-28a derivative carrying <i>tig-pl35</i>	This work
Strain		
DJ	DH1 Δ lacZ, Δ lacA, Δ wcaJ	[12]
K-cho	DJ, pBS-kfoCG, pBBR-glcAT-P-kfiD, pWKS-wbPp	[15]
K-cho2	DJ, pBS-kfoCG, pBBR-glcAT-P-kfiD, pWKS-gne	This work
K-cho1	Strain K-cho2 with additional pBAD-tig-pl35	This work

CAZY, carbohydrate-active enzymes database.

(SEC-MALS), mass spectrometry (MS) and NMR spectroscopy.

2. Materials and methods

2.1. Materials

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* TOP 10 cells (Invitrogen) were used for plasmid propagation. *E. coli* BL21 (DE3) cells were used for protein overexpression. The *E. coli* DJ strain was used for high cell density cultures. This strain was initially designed to produce glucuronylated oligosaccharides [12].

Antibiotics were used at the following concentrations: ampicillin, 100 μ g mL⁻¹; tetracycline, 10 μ g mL⁻¹; chloramphenicol, 25 μ g mL⁻¹; kanamycin, 30 μ g mL⁻¹. Enzymes for molecular biology were obtained from Thermo Fisher Scientific.

The coding sequence of the *V. vadensis* chondroitin lyase (called “pl35”) codon optimized for expression in *E. coli* was synthesized (Genscript) flanked with *KpnI* and *Sall*, to allow insertion in those restriction enzyme sites of expression vector pBAD33 [13], resulting in the construct pBAD-pl35. This gene was also cloned into the *NdeI* and *Sall* sites of the previously prepared vector pBAD-tig [14], resulting in the construct pBAD-tig-pl35 expressing pl35 fused with trigger factor (TF) at the N-terminus. For protein overexpression and *in vitro* activity measurements, the pl35 gene and *tig-pl35* fusion gene were cloned into the *NdeI* and *Sall* sites of the pET28a vector, resulting in the constructs pET28a-pl35 and pET28a-tig-pl35, respectively. All constructs were verified by DNA sequencing (Eurofins MWG).

2.2. High cell density cultures

Cultures were carried out in 0.5 L bioreactors (Multifors, Infors) as previously described [8]. Briefly, the first growth phase was carried out

(non-induced conditions) in 200 mL of mineral medium supplemented with glucose (1.75 %) as the carbon source at 33 °C and left overnight, *i. e.* until total glucose consumption. Then (induced conditions), the culture was fed with 60 mL of feeding solution (30 g glycerol; 0.96 g MgSO₄, 7H₂O; 1.5 mL trace elements) at a flow rate of 1.4 mL h⁻¹. Temperature was lowered to 28 °C at the beginning of the feeding phase. In the meantime, L-arabinose (0.5 % w/v in feeding solution), lactose (0.25 % w/v in feeding solution) and isopropyl- β -D-thiogalactopyranoside (IPTG) (0.2 mM culture medium) were added when required. Cultures ended when the feeding solution ran out, which correspond to about 43 h of growth under induced conditions. Cell density expressed as optical density at 600 nm was about 150–170 at that time.

2.3. Extraction and purification of chondroitin and chondro-oligosaccharides

The culture was centrifuged (9000 \times g, 30 min, 20 °C) after feeding solution ran out. This step was omitted when chondroitin was extracted from the entire culture. The supernatant, composed of the extracellular medium, was removed and reserved. The cell pellet was heated for 40 min at 105 °C after adding 200 mL of deionized water; it was then mixed and centrifuged as before to recover the supernatant containing the released intracellular content. Both the extracellular and intracellular fractions were treated with Amberlite IR120 (hydrogen form) to lower the pH to about 3.5, resulting in protein precipitation. Fractions were centrifuged as before and pH was raised to 6.8 by filtration on Amberlite FPA66 anion exchange resin (free base).

Both fractions were loaded on a Dowex 1 \times 4-400 (carbonate form) column (5 \times 30 cm) at a flow rate of 3 mL min⁻¹ installed on Bio-Rad NGC system to monitor conductivity and detect unsaturated uronic acids at 235 nm. After washing with 80 mL of deionized water, 200 mL of a linear gradient of ammonium carbonate (0–1 M) was applied. The total oligosaccharide fraction (0.2–0.8 M ammonium carbonate) strongly absorbing at 235 nm due to the introduced double bonds was collected and freeze-dried.

Chondro-oligosaccharides were isolated using size exclusion chromatography with three Superdex S30 columns in series in 0.1 M ammonium carbonate (1.2 mL min⁻¹) and refractive index detection. Each separated fraction was lyophilized and kept dried for subsequent analyses.

2.4. Chondroitin quantification

Culture samples (1 mL) were centrifuged in 1.5 mL tubes (12,100 \times g, 5 min, 20 °C) to separate the extracellular medium from the pellet. The pellet was suspended in water (final volume of 1 mL) and boiled for 20 min. Uronic acid content was quantified using the H₂SO₄/m-hydroxybiphenyl assay with glucuronic acid as the standard [17].

2.5. On-line SEC-MALLS

Average molecular weight and molecular weight distributions were determined by high-performance size-exclusion chromatography (HPSEC) [LC-20AD, Shimadzu] with on-line multi-angle light scattering (MALS) [MiniDawn Treos], Wyatt Technology Corp. (Santa Barbara, CA, USA) fitted with a K5 cell and a laser wavelength of 658 nm), a refractive index detector and a viscometer. Columns [OHPAK SB-G guard column and OHPAK SB 806 M HQ column (Shodex)] were eluted with 0.1 M NaNO₃ containing 0.03 % NaN₃ at 0.5 mL/min. Solvent and samples were filtered through 0.1 μ m and 0.2 μ m filter units (Millipore), respectively.

2.6. Mass spectrometry

Mass spectra were recorded on a Bruker amaZon speed spectrometer

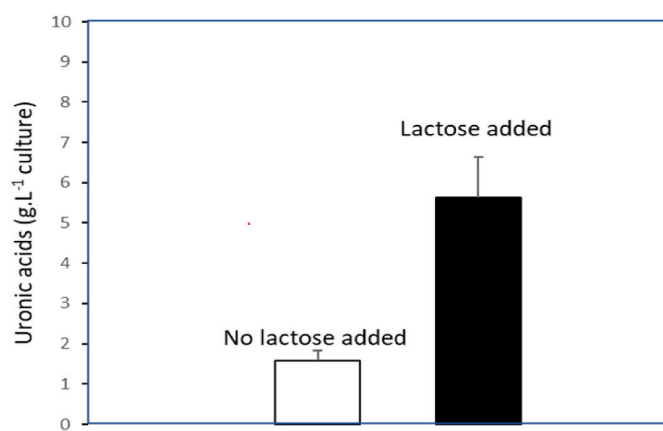


Fig. 1. Production of chondroitin in high cell density culture of *E. coli* strain K-cho2 expressing chondroitin synthesis genes after 48 h of culture.

for ESI experiments and on a Bruker autoflex® speed spectrometer for matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) spectrometry measurements.

2.7. NMR analyses

DEPTQ and proton NMR spectra were recorded with a Bruker Avance 400 spectrometer operating at a frequency of 100.618 MHz for ¹³C and 400.13 MHz for ¹H. Samples were solubilized in D₂O and spectra were acquired at a temperature of 293 K. The residual signal of the solvent was used as the internal standard: HOD at 4.85 ppm. DEPTQ spectra were recorded using 90 pulses, 20,161 Hz spectral width, 65,536 data points, 1.62 s acquisition time, 2 s relaxation delay, and 20,480 scans. Proton spectra were recorded with a 4000 Hz spectral width, 32,768 data points, 4.09 s acquisition times, 10 s relaxation delays, and 32

scans. The ¹H and ¹³C NMR assignments were based on ¹H-¹H homonuclear and ¹H-¹³C heteronuclear correlation experiments (correlation spectroscopy, COSY; heteronuclear multiple-bond correlation, HMBC; heteronuclear single quantum correlation, HSQC). They were performed with a 4000 Hz spectral width, 2048 data points, 0.255 s acquisition time, 1–1.5 s relaxation delays; 32 to 128 scans were accumulated.

2.8. Protein expression and purification

Recombinant *E. coli* BL21 (DE3) transformed with pET28a-pl35 or pET28a-tig-pl35 were grown in LB medium supplemented with 30 μg mL⁻¹ kanamycin at 37 °C until the DO₆₀₀ reached 0.6. Protein expression was then induced with 0.5 mM IPTG at 20 °C for 20 h. Bacterial pellets collected by centrifugation (4800×g, 20 min, 4 °C), resuspended in 50 mM Tris-HCl (pH 8) containing 300 mM NaCl and lysed with a cell disruption system (Constant Systems Ltd). After centrifugation (30,000×g, 30 min, 4 °C), the recombinant proteins were purified from the supernatants using nickel affinity chromatography (Ni-NTA resin; Protino, Macheray-Nagel). Based on SDS-PAGE analysis, fractions of interest were pooled together and dialyzed against 50 mM Tris-HCl (pH 8) containing 150 mM NaCl.

2.9. Enzyme assays

Unsulphated chondroitin was produced in strain K-cho [15]. Chondroitin lyase activity was assayed by incubating unsulphated chondroitin (8.3 mg mL⁻¹) with the recombinant lyase in 50 mM Tris-HCl (pH 8) at 25 °C. The change in absorbance at 232 nm due to the formation of unsaturated uronic acids was measured over 25 min.

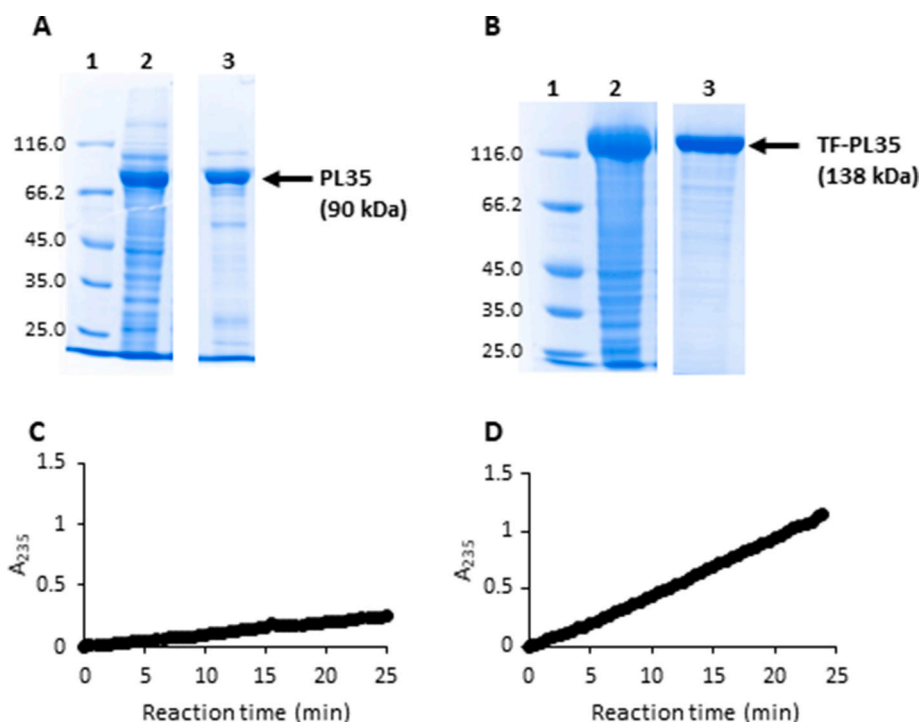


Fig. 2. SDS-PAGE analysis and activity of PL35 (A and C) and TF-PL35 (B and D) on unsulphated chondroitin. Recombinant *E. coli* BL21 (DE3) was grown until OD₆₀₀ reached 0.6 and recombinant protein expression was induced by adding IPTG to a final concentration of 0.5 mM at 20 °C for 20 h. The cells were harvested, lysed and enzymes were purified on Ni-NTA Agarose. Proteins were then analyzed on 10 % SDS-PAGE and stained with Coomassie brilliant blue R-250.1, molecular mass markers; 2, cytoplasmic fractions; 3, purified enzymes. The arrows indicate the recombinant proteins. Enzyme activity was assayed on 5 mg of unsulphated chondroitin with 200 μg of PL35 (C) and 130 μg of TF-PL35 (D) in a final volume of 600 μL of 50 mM Tris-HCl (pH 8).

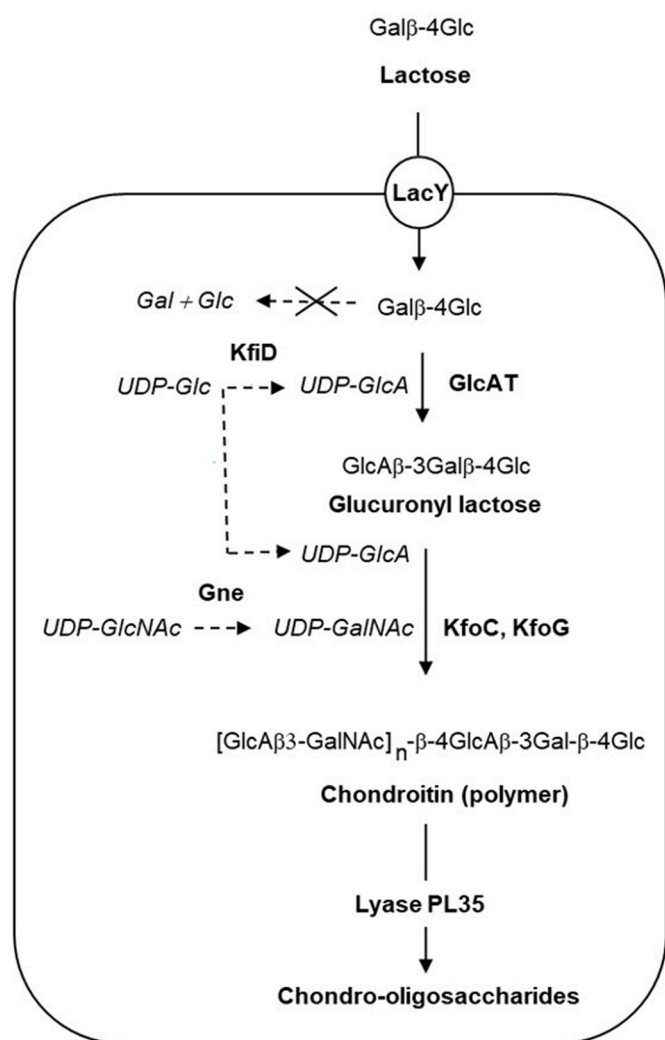


Fig. 3. Metabolic engineering pathway of the *Escherichia coli* K-cho1 strain to produce chondro-oligosaccharides. Recipient strain was an *E. coli* DJ derivative, carrying recombinant plasmids pBS-kfoCG (chondroitin synthesis), pBBR-glcATP-kfiD (priming reaction and UDP-GlcA synthesis), pWKS-gne (UDP-GalNAc synthesis), and pBAD-tig-pl35 (chondroitin lyase).

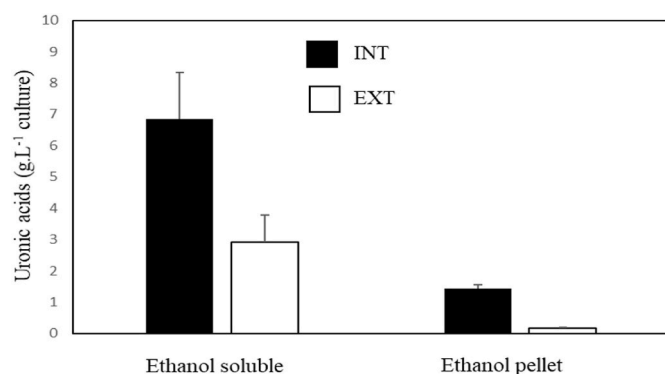


Fig. 4. Production of uronic acid carbohydrates in the intracellular (INT) and extracellular (EXT) fractions of high cell density cultures of strain K-cho1 expressing chondroitin synthase and lyase activities. Culture fractions were treated with three volumes of ethanol and centrifuged. The uronic acid assay was performed after 48 h of fed-batch cultivation. Error bars are taken from the mean of two separate cultures.

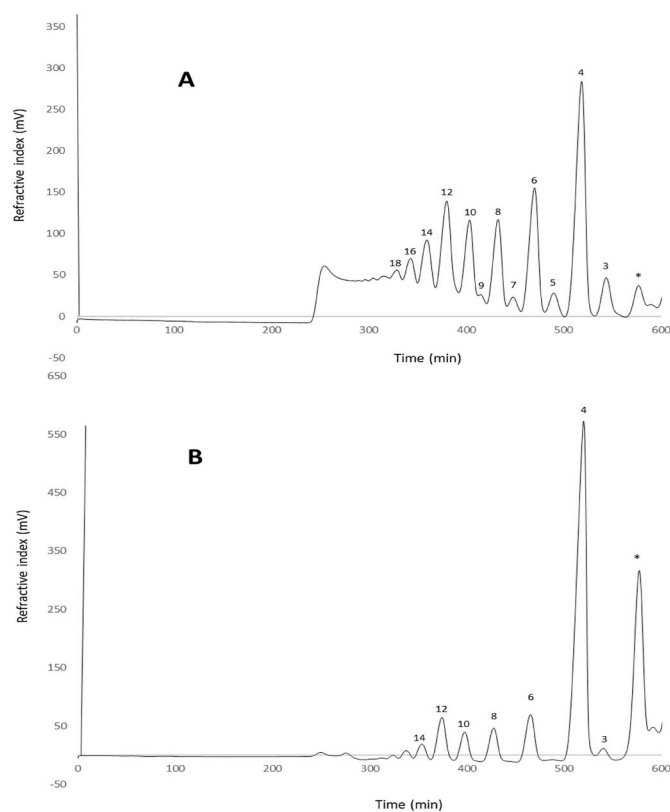


Fig. 5. Purification of chondro-oligosaccharides from the intracellular (cell pellet) fraction (A) and extracellular fraction (B). Numbers represent sugar monosaccharide units. (*) unknown compound.

3. Results

3.1. Engineering for high chondroitin production

In vivo chondroitin synthesis in engineered *E. coli* requires at least three enzymes: (1) UDP-glucose-6-dehydrogenase to convert UDP-glucose into UDP-glucuronic acid, (2) UDP-GlcNAc-4-epimerase to convert UDP-GlcNAc into UDP-GalNAc and (3) chondroitin synthase.

Generally speaking, it is best to improve the yield of production of the polymer before addressing its degradation for producing oligomers. In a previous work, we described the recombinant *E. coli* strain K-cho that produces non-secreted chondroitin by overexpressing KfoCG (chondroitin synthase of *E. coli* K4), KfiD (UDP-Glucose-6-dehydrogenase of *E. coli* K5), WbPp (UDP-GlcNAc-4-epimerase of *Pseudomonas aeruginosa*) and GlcAT-P (mouse β -1,3-glucuronyltransferase), which — upon lactose supplementation — leads to the synthesis of glucuronyllactose, an efficient priming acceptor for chondroitin synthesis [15].

We constructed a new strain, called K-cho2, having the same features as K-cho, except that we chose Gne from *Campylobacter jejuni* as the UDP-GlcNAc-4-epimerase, because Gne has better efficiency than WbPp *in vitro* for UDP-GlcNAc epimerization [18]. Moreover, the higher efficiency of Gne compared with WbPp has been demonstrated in another study carried out in our group, involving the microbial synthesis of globopentaose *in vivo* [19].

Strain K-cho2 derives from strain DH1; it therefore lacks the *kps* genes involved in group 2 capsular polysaccharide transport. Consequently, the produced chondroitin remains intracellular, as observed in recombinant heparosan synthesis [8,15].

Strain K-cho2 was cultivated in fed batch conditions. Compared with the data obtained with strain K-cho [15], the level of chondroitin obtained with K-cho2 upon lactose supplementation improved dramatically, by a factor of about 3.5 (Fig. 1). In fact, we had to add one volume

Table 2

Electrospray ionization mass spectrometry (ESI-MS) analysis of purified chondromers

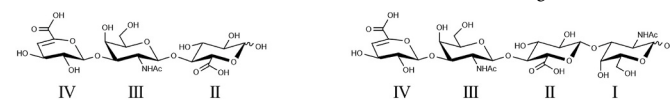
Oligomer (number of sugar units)	Composition	Exact mass (Da) (calculated)	Molecular ion m/z
3	Δ UA β 1,3GalNAc β 1,4 GlcA	555.44	(-) 554.11 (+) 578.18 (Na)
4	Δ UA β 1,3GalNAc β 1,4 GlcA β 1,3 GalNAc	758.22	(-) 756.847 (+) 781.200 (Na)
5	Δ UA β 1,3 [GalNAc β 1,4 GlcA]2	934.26	(-) 933.204 (+) 957.234 (Na)
6	Δ UA β 1,3 [GalNAc β 1,4 GlcA]2 β 1,3 GalNAc	1137.33	(-) 1136.25 (+) 1160.304 (Na)
7	Δ UA β 1,3 [GalNAc β 1,4 GlcA]3	1313.37	(-) 1312.265 (+) 1336.349 (Na)
8	Δ UA β 1,3 [GalNAc β 1,4 GlcA]3 β 1,3 GalNAc	1517.27	(-) 1515.057 (+) 1539.350 (Na)
9	Δ UA 1,4 GlcA]5	1692.48	(-) 1691.514 (+) 1715.429 (Na)
10	Δ UA β 1,3 [GalNAc β 1,4 GlcA]4 β 1,3 GalNAc	1895.56	(-) 1894.22 (+) 1918.40 (Na)
12	Δ UA β 1,3 [GalNAc β 1,4 GlcA]5 β 1,3 GalNAc	2274.90	(-) 2273.23 (+) 2297.469 (Na)
14	Δ UA β 1,3 [GalNAc β 1,4 GlcA]6 β 1,3 GalNAc	2653.76	(-) 2653.626
16	Δ UA β 1,3 [GalNAc β 1,4 GlcA]7 β 1,3 GalNAc	3032.89	(-) 3032.536
18	Δ UA β 1,3 [GalNAc β 1,4 GlcA]8 β 1,3 GalNAc	3407.97	(-) 3411.724

of water to the culture before extracting chondroitin, because it had formed a thick gel upon heating. Chondroitin extracted by ethanol precipitation had an average molecular weight of 350 ± 98 kDa according to the SEC-MALS analysis. This value is more than 10-fold higher than chondroitin produced in strain K-cho expressing the WbPp epimerase [15].

These results strongly suggest that the UDP-GalNAc concentration is a limiting factor for the kinetic incorporation and molecular weight of recombinant chondroitin in *E. coli*. Therefore, adding lactose allows a more efficient synthesis of chondroitin suggesting an initiation activity. Indeed cultivation of strain K-cho in the presence of lactose had demonstrated incorporation of the latter at the reducing end of the chondroitin produced [15], suggesting that it is also present in the chondroitin produced in this study. Nevertheless, the increase in the size of chondroitin produced in this study (20–300 kDa) makes it impossible to visualize the reducing end of the polysaccharide by ^1H NMR and therefore the lactose-specific protons.

3.2. Expression of chondroitin endo-lyase in recombinant *Escherichia coli*

A new endo-chondroitin lyase (GenBank accession no. ZP_06241351.1) from *V. vadensis* ATCC BAA-548 belonging to the polysaccharide lyase family 35 (carbohydrate-active enzymes (CAZy) database, <http://www.cazy.org/> [20], was recently discovered [11].

Table 3 ^1H and ^{13}C chemical shifts in trimer and tetramer chondro-oligosaccharides.

		α		β	
I. GalNAc	H1			5.21	4.68
	H2			4.29	4.00
	H3			4.01	3.82
	H4			4.2	4.11
	H5			4.13	3.72
	H6			3.66	3.76
	H-Me			2.01	2.01
II. GlcA	H1	5.22	4.64	4.54	
	H2	3.61	3.32	3.38	
	H3	3.8	3.6	3.60	
	H4	3.98	3.77	3.76	
	H5	4.15	3.8	3.77	
III. GalNAc	H1	4.55		4.55	
	H2	4.01		4.00	
	H3	3.90		3.91	
	H4	4.12		4.11	
	H5	3.73		3.73	
	H6	3.78		3.78	
	H-Me	2.05		2.06	
IV. GlcA Δ	H1	5.2		5.21	
	H2	3.8		3.81	
	H3	4.11		4.11	
	H4	5.95		5.96	
I. GalNAc	C1			91.2	95.02
	C2			48.8	52.29
	C3			77.25	80.31
	C4			68.58	67.73
	C5			70.27	74.87
	C6			62.72	61.19
	C-Me			22.16	21.92
II. GlcA	C1	91.92	96.06	104.23	
	C2	71	73.57	72.34	
	C3	70.95	73.90	73.71	
	C4	79.59	80	79.72	
	C5	71.41	76.08	75.77	
III. GalNAc	C1	100.82		100.82	
	C2	51.3		51.02	
	C3	79.83		79.79	
	C4	67.46		67.39	
	C5	75.03		75	
	C6	61		60.94	
	C-Me	22.41		22.35	
IV. GlcA Δ	C1	101.3		101.2	
	C2	69.4		69.32	
	C3	65.6		65.47	
	C4	107.94		108.6	
	C5	143.8		143.8	
	C6	168.8		168.6	

This enzyme, named Pl35 and described as an endo-chondroitin lyase, appeared as a good candidate for the production of chondro-oligosaccharides in engineered *E. coli*. Thus, we first overexpressed the lyase in *E. coli* BL21 (DE3) harboring plasmids pET28a-pl35 or pET28a-tig-pl35, leading to protein Pl35 or TF-Pl35, respectively. Both recombinant enzymes were expressed in frame with an N-terminal His₆Tag, amenable to purification using immobilized metal ion affinity

Table 4
Recovery of each oligomer species after purification on gel permeation.

Oligomer (number of sugar units)	mg.L ⁻¹ total culture ^a
3	157
4	1110
5	81
6	591
7	51
8	425
9	21
10	420
12	629
14	336
16	144
18	110

^a Calculated according to the recovery from both intracellular and extracellular fractions.

chromatography (Fig. 2 A and B), and exhibited activity on bacterial non-sulfated chondroitin as expected. Interestingly, TF-PI35 displayed an activity about 7.5 higher than that of PI35 (Fig. 2 C and D).

TF is a chaperone that binds to nascent polypeptide chains via ribosomal proteins, maintaining the newly synthesized proteins in an open conformation and preventing them from forming aggregates [21, 22]. The fusion of TF at the N-terminus of KfiC glucuronyltransferase sufficiently stabilizes the enzyme to allow the *in vivo* synthesis of heparosan [14]. Therefore, we cloned the fusion gene *tig-pl35* into a pBAD33 vector and the K-cho2 strain (described above) was transformed with the resulting construct to create the K-chol strain for the *in vivo* production of chondro-oligosaccharides (Fig. 3).

3.3. Engineering of *Escherichia coli* to produce chondro-oligosaccharides

The K-chol strain was prepared from strain K-cho2, adding an additional plasmid (pBAD33-*tig-pl35*) carrying a lyase sequence inducible by L-arabinose, to allow independent adjustment of lyase expression. The K-chol strain lacks β -galactosidase activity, preventing the hydrolysis of exogenously added lactose. Conversely, L-arabinose is degraded and thus continuously provided through the feeding solution.

Recombinant strain K-chol was cultivated on minimal medium in fed-batch conditions. The content of ethanol-soluble uronic acids (UA) was estimated, being indicative of the presence of low molecular weight fragments, *i.e.* oligomers. As shown in Fig. 4, most ethanol-soluble UAs were found in the intracellular fraction (see Fig. 5).

A previous report showed that intracellularly produced heparosan oligosaccharides carry six or more monosaccharide units [8]. Based on this information and based on UA content, our results indicate that a majority of relatively large oligomers were produced and retained intracellularly at a concentration greater than 10 g L⁻¹. Only a small proportion of UAs precipitated with ethanol, suggesting that most of the formed chondroitin polymers were degraded by the lyase.

3.4. Purification and characterization of chondro-oligosaccharides

Chondro-oligosaccharides from the extracellular medium and intracellular (cell pellet) fractions were purified by SEC and characterized.

The chromatogram profiles suggested the presence of two distinct series, because minor peaks were found between major peaks. According to the electrospray ionization mass spectrometry (ESI-MS) values (Table 2), the main series was composed of odd-numbered oligomers having the structure $\Delta\text{UA}\beta 1,3 [\text{GalNAc}\beta 1,4 \text{GlcA}]_n\beta 1,3 \text{GalNAc}$, whereas the minor series was apparently composed of even-numbered oligomers having the structure $\Delta\text{UA}\beta 1,3 [\text{GalNAc}\beta 1,4 \text{GlcA}]_n$.

A detailed NMR spectroscopy analysis of trimers and tetramers was performed (Table 3). In the tri-chondromer, chemical shifts of C1 from GlcA at 96.06 and 91.92 confirmed its anomeric position (mixture α/β). In the tetra-chondromer, chemical shifts of C1 from GalNAc at 91.2 and

95.02 confirmed its anomeric position (mixture α/β). Annotated NMR spectra are available in Supplemental Materials.

The production of even-numbered oligomers has already been reported in a study on the mode of action of *Streptomyces hyaluronidase* [23]. This behavior was attributed to the lack of specificity of the enzyme [24], whereby the lyase alternately cuts the $\beta 1,3$ linkage, leading to non-reducing ΔGalNAc - and reducing uronic acid ends. In this scenario, unstable ΔGalNAc is rapidly eliminated, leaving [non-reducing] ΔUA ends common to all final products. We therefore hypothesize that PI35 behaves the same way.

Larger oligomers were found particularly in the intracellular fraction, in agreement with the size/diffusion relationship previously mentioned, *i.e.*, more retention for larger degrees of polymerization. We purified oligomers with up to the 18 sugar units (Table 4). Disaccharide $\Delta\text{UA}\beta 1,3 \text{GalNAc}$ was not found, again as observed for the *S. hyaluronidase* enzyme [24], providing further support for a similar catalytic behavior of the two enzymes.

4. Conclusion

To our knowledge, this is the first report of the production of well-defined large unsulfated chondro-oligosaccharides in bacterial cultures with high structural diversity. A similar approach was recently described in *Corynebacterium*, but resulted in dimers and tetramers only [5].

The chondroitin lyase PI35 of *V. vadensis* used in the present study yielded this oligomer diversity. We found that PI35 uses a catalytic mechanism similar to that of hyaluronate lyase in *S. hyalurolyticus* [24]. This study is thus the first report of this type of enzymatic behavior on non-sulfated chondroitin.

Our objective in this study was to seek performance and we have shown that utilization of the [mouse GlcAT-P + lactose] priming system was found to considerably improve the production of chondroitin and consequently its hydrolysis products. Moreover, and although it was not a topic of this study, we kept expressing *kfoG* in the recombinant strain as we did in previous works, as we noticed a slight improvement of chondroitin synthesis when co-expressed with *kfoC* (see Fig. S4, supplemental information). *KfoG* was described as an inactive glycosyltransferase-like protein, but it might have a positive effect by interacting with *KfoC*. Such kind of promoting interaction was reported between *KfiC* and *KfiA* involved in N-acetylheparosan synthesis [25]. They are a very few reports about it, none of them addressing the putative role of *KfoG* in K4 capsule synthesis. In the paper of Krahulec [26], mutant K4 *kfoG* (-) strain was shown to produce same amount of chondroitin but size was four times lower according to the authors. Another report [27] mentions a dramatic decrease in chondroitin synthesis upon deletion of *kfoG* in recombinant *E. coli* K-12.

Another point appeared to be essential in order to increase chondroitin substrate was to express the UDP-GlcNAc 4-epimerase from *Campylobacter jejuni* which has been shown to be very active enzyme. This points out the fact the pool of UDP-GalNAc was a limiting factor of chondroitin synthesis. It correlates with a previous study where we demonstrated an increase of chondroitin production with UDP-GlcNAc 4-epimerase expression [28].

The large-scale preparation of chondro-oligosaccharides is a prerequisite for preparing derivatives such as glycoconjugates or sulfated compounds. Considering that several hundred mg of individual oligomers can be purified from 1 L of bacterial culture, the present study paves the way for the preparation of new putative biological compounds.

CRediT authorship contribution statement

Yanna André: Investigation. **Emeline Richard:** Supervision, Investigation, Conceptualization. **Mélanie Leroux:** Conceptualization. **Isabelle Jeacomine:** Investigation. **Eric Bayma:** Investigation. **Sylvie Armand:** Writing – original draft, Supervision, Investigation,

Conceptualization. **Bernard Priem**: Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors acknowledge support from the French Research National Agency (ANR AAP 2021 GAGS-nanosensor). M. Leroux completed her doctoral thesis at Université Grenoble Alpes as part of a partnership between CERMAV and HTL Biotechnology. Her fellowship was funded equally by the French National Association for Research and Technology (ANRT) and the HTL Biotechnology company. The authors thank Dr Anthony Bresin (HTL company) for his helpful comments on this paper. We acknowledge the NanoBio ICMG (UAR 2607) for providing facilities for mass spectrometry (A. Durand, L. Fort, R. Gueret) and NMR analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carres.2024.109243>.

Abbreviations

GAG	glycosaminoglycan
GlcA	glucuronic acid
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
Δ	unsaturated uronic acid at the non-reducing end
DP	degree of polymerization
KfoC	chondroitine synthase
KfiD	UDP-Glc-dehydrogenase
GlcAT-P	β-glucuronyltransferase
Gne	UDP-GlcNAc 4-epimerase
Pl35	endochondroitin lyase
TF	trigger factor
MS	mass spectrometry
MALDI-TOF	matrix-assisted laser desorption ionization - time of flight
ESI	electron spray spectrometry
NMR	nuclear magnetic resonance
DEPTQ	distortionless enhancement by polarization transfer including the detection of quaternary nuclei
SEC-MALS	size exclusion chromatography with multi-angle light scattering detection
OD	optical density
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
IPTG	isopropyl β-D-1-thiogalactopyranoside.

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