Analysis of the Polymerization Initiation and Activity of *Pasteurella multocida* Heparosan Synthase PmHS2, an Enzyme with Glycosyltransferase and UDP-sugar Hydrolase Activity

Anais A. E. Chavaroche,† Lambertus A. M. van den Broek,‡ Jan Springer,§ Carmen Boeriu,§ and Gerrit Eggink*§

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Heparosan synthase catalyzes the polymerization of heparosan (1-4GlcUA1–4GlcNAcα1-), by transferring alternatively the monosaccharide units from UDP-GlcUA and UDP-GlcNAc to an acceptor molecule. Details on the heparosan chain initiation by *Pasteurella multocida* heparosan synthase PmHS2 and its influence on the polymerization process have not been reported yet. By site-directed mutagenesis of PmHS2, the single action transferases PmHS2-GlcUA+ and PmHS2-GlcNAc+ were obtained. When incubated together in the standard polymerization conditions, the PmHS2-GlcUA+/PmHS2-GlcNAc+ showed comparable polymerization properties as determined for PmHS2. We investigated the first step occurring in heparosan chain initiation by the use of the single action transferases and by studying the PmHS2 polymerization process in the presence of heparosan templates and various UDP-sugar concentrations. We observed that PmHS2 favored the initiation of the heparosan chains when incubated in the presence of an excess of UDP-GlcNAc. It resulted in a higher number of heparosan chains with a lower average molecular weight or in the synthesis of two distinct groups of heparosan chain length, in the absence or in the presence of heparosan templates, respectively. These data suggest that PmHS2 transfers GlcUA from UDP-GlcUA moiety to a UDP-GlcNAc acceptor molecule to initiate the heparosan polymerization; as a consequence, not only the UDP-sugar concentration but also the amount of each UDP-sugar is influencing the PmHS2 polymerization process. In addition, it was shown that PmHS2 hydrolyzes the UDP-sugars, UDP-GlcUA being more degraded than UDP-GlcNAc. However, PmHS2 incubated in the presence of both UDP-sugars favors the synthesis of heparosan polymers over the hydrolysis of UDP-sugars.

**Anais A. E. Chavaroche, Lambertus A. M. van den Broek, Jan Springer, Carmen Boeriu, and Gerrit Eggink**

From the *Bioprocess Engineering Group, Wageningen University and Research Center, P.O. Box 8129, 6700 EV Wageningen, The Netherlands* and *Food and Biobased Research, Wageningen University and Research Center, P.O. Box 17, 6700 AA Wageningen, The Netherlands*

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Due to its extensive use in the medical area, the world market for heparin is yearly increasing by about 15%. In 2009, it represented a turnover of ~6 billion dollars. Heparin is mainly used in surgery to prevent vein thrombosis but is also administrated in a number of settings, including kidney dialysis and acute coronary syndromes (1). In addition, recent studies have shown that heparin and derivatives, such as low molecular weight heparin, may have a larger therapeutic potential. For example, it was observed that low molecular weight heparin improved the survival of patients suffering from cancer (2). However, due to the risk of hemorrhagic complications heparin cannot be used as an anticancer therapeutic agent, and thus analogous molecules that do not exhibit anticoagulant properties should be used. The biological activity of heparin and heparin-like molecules is influenced by the disaccharide repeat composition, the chain length, and the sulfation patterns (3). The use of recombinant enzymes to synthesize heparin and analogs could enable a tight control of the polymer chain length, of the UDP-sugars incorporated, and of the sulfation patterns.

Heparin is produced in a cascade of enzymatic reactions where initiation, polymerization, N-deacetylation/N-sulfation, C5-epimerization, and O-sulfation take place in a coordinated manner. Heparosan, the unsulfated and unepimerized precursor of heparin, is constituted of the repeating disaccharide unit (1-4GlcUAβ1–4GlcNAcα1)n. In nature, apart from vertebrates, heparan polymers can be found in microorganisms. Heparosan is present in the polysaccharide capsule of certain pathogenic bacteria in order to protect them against the host immune system during infection. In *Pasteurella multocida* Type D, an animal pathogen, heparosan is synthesized by the heparosan synthase PmHS1.

PmHS1 (*P. multocida* heparosan synthase 1) and PmHS2 (the cryptic homolog of PmHS1) have been characterized, and both recombinant enzymes exhibit different polymerization properties (4–6). PmHS2 is a non-processive glycosyltransferase containing a glucuronyltransferase (GlcUA+) and an N-acetylglucosaminyltransferase (GlcNAc−) activity. Each of the catalytic transferase domains contains a DxD amino acid motif, considered to be the key residue involved in the substrate binding and the catalysis (7, 8). The inactivation of the catalytic domain by substitution of both aspartic acids by asparagines has been described for *P. multocida* hyaluronan synthase PmHAS (9, 10) and for PmHS1 (heparosan synthase 1) (11). Here we describe, based on amino acid sequence homology with PmHS1 and with *Escherichia coli* heparosan synthase KfiC and KfiA (12–14), the construction of the two PmHS2 single action transferases (PmHS2-
GlcUA$^+$ and PmHS2-GlcNAC$^+$. Both the characterization of the single action transferases and a detailed study of the polymerization process of PmHS2 allowed us to investigate the initiation process of heparosan chains. Until now, details of the first step occurring in heparosan polymerization have not been described. Here, we report how the heparosan chain initiation influences the overall polymerization process.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis of *P. multocida* pmhssB, the Gene Encoding for PmHS2**—Site-directed mutagenesis using specific primer sets was performed to introduce mutations in the DXD amino acid motif of each PmHS2 transferase domain. The positions of the point mutations in *pmhssB* (GenBank™ accession number AY292200) were based on amino acid homology with PmHS1 (11) and *E. coli* KfIC and KfIA (12–14). The primers, forward FWGlcUA (5′-CTTTAAAAATGATATGATGATGTCATCAGT-3′) and reverse RVGlcUA (5′-CATGATGACATACATCATTACTATTTGAAAG-3′), were designed to allow the substitution D215N/D217N in the glucuronyltransferase domain, leading to PmHS2-GlcNAC$^+$. To obtain the PmHS2-GlcUA$^+$, the primers forward FWGlcNAc (5′-CCCTGAATGATAACATTATCTATTCAGG-3′) and reverse RVGlcNAc (5′-CGCTTGGATAGATATGATTATACATTACAGG-3′) enabled the substitution D479N/D481N in the N-acetylglucosaminyltransferase domain. The double transferase knock-out (PmHS2-nul) was obtained by using DNA template pmhss2-GlcUA$^+$ and the primer set FWGlcUA/RVGlcUA.

To introduce the nucleotide modification into the pmhssB gene, two PCRs of 30 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min) amplified pmhssB into two distinct fragments, both of them containing the mutations. The different primer combinations are presented in Table 1. The two fragments were linked by their overlapping ends, and the DNA strands were complemented by 10 cycles of overlap PCR (94 °C for 30 s, 65 °C for 30 s, and 72 °C for 2 min). Finally the modified gene was amplified by 30 cycles of PCR (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min), and for each gene (pmhss2-GlcUA$^+$, pmhss2-GlcNAC$^+$, and pmhss2-nul), the full length was obtained using the primer set FWGlcUA (5′-GTGATCCATGAGCTCTAAAAAATAAAAAGGTAAACAGG-3′) and reverse RVGlcUA (5′-ACCTTTCAAAATAGTATGATGATGTCATCAGT-3′). Each PCR fragment was ligated into the pET101 vector and transformed into *E. coli* BL21(DE3) expression strain (Invitrogen). The nucleotide sequencing of the mutants confirmed the amino acid substitutions in both DXD motifs.

**Expression and Purification of the PmHS2 Recombinant Proteins**—The expression and the purification of the recombinant proteins (PmHS2-GlcUA$^+$, PmHS2-GlcNAC$^+$, and PmHS2-nul) was done in the same way as described previously for PmHS2 (15).

**Determination of PmHS2 Enzyme Activities**—For the standard polymerization conditions, the polymerization reaction with a final volume between 30 and 600 μl contained 40 mM Tris–HCl, pH 7.15, 4 mM MnCl₂, 4 mM MgCl₂, UDP-GlcUA, UDP-GlcNAc, and freshly purified recombinant PmHS2 enzymes (PmHS2, PmHS2-nul, PmHS2-GlcUA$^+$, and PmHS2-GlcNAC$^+$). The amount of UDP-sugar and enzymes varied with the experiments; details are given in the figure legends. The reactions were performed in the dark at 30–32 °C under mild shaking conditions for times ranging from 30 min to 24 h.

Heparosan templates (35 kDa; polydispersity index = 1.17) were synthesized using the standard polymerization condition. The polymerization reaction was quenched by heating (99 °C for 30 min) and subsequently was centrifuged (18,000 × g for 15 min) in order to discard the denaturated enzymes. The synthesized heparosan chains were used as templates and were added to a new enzymatic reaction (end concentration 0.55 ± 0.05 μM) when appropriate.

The polymerization and the hydrolysis activities of PmHS2 enzymes (PmHS2, PmHS2-nul, PmHS2-GlcUA$^+$, and PmHS2-GlcNAC$^+$) were assessed by a coupled enzyme assay, gel electrophoresis, high performance anion exchange chromatography (HPAEC), high performance size exclusion chromatography (HPSEC) analysis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The enzymatic reactions were not quenched by heat shock but were stopped by immersion in liquid nitrogen and stored at −20 °C prior to analysis. All samples were analyzed at least in duplicate.

**Coupled Enzyme Assay**—The UDP-sugars conversion was quantified by measuring the NADH reduction into NAD$^+$ at 340 nm in a coupled enzyme assay (16, 17). The assay was performed in the same way as described previously (15).

**Gel Electrophoresis**—To analyze the heparosan polymers formed during the enzymatic reactions, the quenched samples were mixed with glycerol to a final concentration of 12% (v/v). The samples were loaded on 2% agarose gel, and the gels were stained with 0.1 μg/ml ethidium bromide.
run for 2 h at 50 V in Tris acetate-EDTA (TAE) buffer and then were stained overnight in the dark in an ethanol/Stains-All buffer. The destaining was carried out in pure water as we described previously (15).

In addition, Novex 20% Tris borate-EDTA (TBE) polyacrylamide gel (Invitrogen) were used, depending on the experiment. The gels were run for 45 min at 200 V in TBE buffer and then were stained 45 min in the dark in a Stains-All buffer according to the recommendations of Sigma-Fluka. Briefly, to prepare the staining buffer, a stock solution (0.1% (w/v) Stains-All in formamide (100%)) was added in a 1:10 ratio to a dilution buffer (45 mM Tris, pH 9.2, 7.5% (v/v) formamide, 25% (v/v) isopropyl alcohol). The TBE gels were destained in pure water for 30 min in the dark.

To estimate heparan molecular mass with gel electrophoresis analysis, hyaluronan polymers of 30, 160, and 262 kDa (Hyalose) or a selected hyaluronan molecular mass marker ranging from 27 to 495 kDa (Select-HALoLadder, Hyalose) were used.

**HPAEC Analysis**—The composition of the reaction mixture (UDP-sugars and monosaccharides) was analyzed by HPAEC using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (2 × 250 mm) in combination with a CarboPac PA guard column (2 × 25 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, CA). The standards were purchased from Sigma, unless indicated: UMP (M, 368.15), UDP-GlcUA (M, 646.2), UDP-GlcNAc (M, 651.3), GlcUA (M, 194.14), GlcNac (M, 221.21; Merck), and UDP-glucuronic acid (M, 448.18; Biochemika). All samples were injected and analyzed in duplicate.

For the HPAEC analysis performed in condition 1, a flow rate of 0.3 ml/min was used with the following gradient: 0–26 min at 16 mm NaOH, 26–33 min at 16–100 mm NaOH, 33–78 min at 0–1000 mm sodium acetate in 100 mm NaOH, 78–83 min at 1000 mm sodium acetate in 100 mm NaOH. Under these conditions, N-acetylglucosamine, glucuronic acid, and UDP-GlcNac eluted at 8, 44, and 67 min, respectively, and UDP-GlcUA was not detected.

Alternatively, for combined UV and electrochemical detection, the above described HPAEC system was equipped with a VWD-3100 single wavelength detector (Dionex) and the pulsed electrochemical detector in series. In this case (condition 2), a flow rate of 0.3 ml/min was used with the following gradient: 0–2 min at 5 mm NaOH, 2–27 min at 0–875 mm sodium acetate in 5 mm NaOH, 27–32 min at 875–950 mm sodium acetate in 5 mm NaOH, 32–37 min at 950 mm sodium acetate in 5 mm NaOH. First, UV detection was performed at 260 nm, and thereafter 500 mm NaOH (0.2 ml/min) was added to the eluate in order to enable the electrochemical detection. Under these conditions, GlcUA, UMP, UDP-GlcNac, UDP, and UDP-GlcUA eluted at 11, 21, 24, 27, and 34 min, respectively.

**HPSEC Analysis**—The size distribution and the molecular weight of the polymers were analyzed by HPSEC on an Ultimate 3000 (Dionex) using three TosoH Bioscience TSK-gel columns in series (4000, 3000, and 2500 Super AW, 150 × 6.0 mm) preceded by a TSK AW-L guard column (35 × 4.6 mm; Tosoh Bioscience). Samples (20 µl) were injected and eluted at 40 °C using 0.6 ml/min 0.2 M NaNO₃. All samples were injected and analyzed in duplicate. Detection was performed using a Shodex RI 101 refractive index detector (Showa Denko K.K.). Selected hyaluronan molecular mass markers (Hyalose) of 30, 160, and 262 kDa, respectively, were used for calibration. For each HPSEC analysis, the molecular weight and the size distribution (polydispersity index) of the samples were determined by fitting the elution time of the hyaluronan molecular weight markers to a logarithmic model (15).

**MALDI-TOF MS Analysis**—An Ultraflex work station (Bruker Daltonics) equipped with a 337-nm laser was operated in the negative mode and calibrated with a mixture of peptide standards from Bruker Daltonics. Ions were accelerated with a 20-kV voltage after a delayed extraction of 180 ns. Detection was performed using the reflector mode. Samples were 10 times diluted in the matrix solution containing 10 mg/ml 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile. For analysis, 2 µl of the mixture was transferred to a MALDI sample plate and dried under a stream of warm air.

**RESULTS**

The DXD amino acid motif, present in each transferase catalytic domain of glycosyltransferases, such as PmHS2, is involved in the UDP-sugar binding through interaction with the ribose of the UDP molecule and with the divalent metal ions (7, 8). The substitution of both aspartic acids by asparagines in the DXD motif of the catalytic domain of each transferase results in a loss of charge and as a consequence inactivates the modified transferases.

In order to understand more about the polymerization of heparan chains, PmHS2-GlcUA⁺, PmHS2-GlcNac⁺, and PmHS2-nul were constructed by site-directed mutagenesis. The characterization of the single action transferases and a detailed analysis of the PmHS2 polymerization process enabled us to understand the initiation of the heparan polymerization process.

**Expression and Purification of the PmHS2 Recombinant Proteins**—Both the expression level of the recombinant proteins and the recovery level after purification were higher for PmHS2-nul, PmHS2-GlcUA⁺, and PmHS2-GlcNac⁺ than for PmHS2. PmHS2 was stable at least for 6 months at ~80 °C (15), whereas in contrast, the polymerization activity of the combined PmHS2-GlcUA⁺/PmHS2-GlcNac⁺ decreased around 50% after 1 week of storage at ~80 °C.

**Polymerization Activity of the Combined PmHS2-GlcUA⁺/PmHS2-GlcNac⁺**—PmHS2-GlcUA⁺ or PmHS2-GlcNac⁺, incubated separately in the presence of both UDP-sugars for 24 h, did not synthesize heparan polymers; neither did PmHS2-nul. However, PmHS2-GlcUA⁺ and PmHS2-GlcNac⁺ incubated together in the presence of both UDP-sugars showed polymerization activity as determined by gel electrophoresis (Fig. 1). Thus, site-directed mutagenesis of one DXD motif inactivated only the modified transferase domain and maintained the function of the other transferase.

Just as previously observed with PmHS2 (15), PmHS2-GlcUA⁺/PmHS2-GlcNac⁻ favored the formation of high molecular weight heparan polymers with a low polydispersity.
Polymerization Initiation and Activity of PmHS2

The hydrolysis of UDP-GlcUA by PmHS2, in the absence of UDP-GlcUA, took place within the first 30 min of incubation. After 24 h of incubation, about 0.03–0.05 mM UDP-GlcNAc was converted into N-acetylglucosamine and UDP. The conversion represents about 12–20% of the initial concentration (0.25 mM). In the presence of higher UDP-GlcNAc concentrations, PmHS2 did not hydrolyze a higher amount of UDP-GlcNAc.

The hydrolysis of UDP-GlcUA by PmHS2 was only observed in the reaction mixture in the presence of PmHS2 or PmHS2-GlcUA + (Fig. 3). The hydrolysis activity of the UDP-GlcUA by PmHS2 increased progressively in time. After 24 h of incubation, the hydrolysis activity of UDP-GlcUA by PmHS2 was constant for at least 8 h in the presence of 5 mM UDP-GlcUA and 5 mM UDP-GlcNAc. The specific activity of the PmHS2 polymerization activity leveled off when about 450 nmol of UDP-sugar was observed in the presence of 5 mM UDP-GlcUA and 5 mM UDP-GlcNAc. The reaction mixtures containing PmHS2 or PmHS2-GlcNAc + were not observed by HPAEC, and the analysis of the reaction mixtures by the coupled enzyme assay confirmed the absence of UDP. These results indicate that active PmHS2 is capable of hydrolyzing the UDP-sugars. The PmHS2 single action transferases (PmHS2-GlcUA + or PmHS2-GlcNAc +) are also able to hydrolyze the UDP-sugars (HPAEC condition 1) (Fig. 3).

The degradation of UDP-GlcNAc was mainly observed in the reaction mixtures containing PmHS2 or PmHS2-GlcNAc +. Unexpectedly, also a low degree of hydrolysis of UDP-GlcNAc was observed in the presence of PmHS2-GlcUA + (Fig. 3B) or PmHS2-nul (data not shown).

The hydrolysis of UDP-GlcNAc by PmHS2, in the absence of UDP-GlcUA, took place within the first 30 min of incubation. After 24 h of incubation, about 0.03–0.05 mM UDP-GlcNAc was converted into N-acetylglucosamine and UDP. The conversion represents about 12–20% of the initial concentration (0.25 mM). In the presence of higher UDP-GlcNAc concentrations, PmHS2 did not hydrolyze a higher amount of UDP-GlcNAc.
Hydrolysis of UDP-GlcUA did not exceed 0.25 mM even when higher UDP-sugars (0.25 mM) were added to the reaction mixture. As observed with UDP-GlcNAc, the hydrolysis of UDP-GlcUA did not exceed 0.25 mM even when higher UDP-GlcUA concentrations were added to the reaction mixture.

In the presence of both UDP-sugars (0.25 mM/0.25 mM), the polymerization of heparosan was favored, and the PmHS2 hydrolysis activity was reduced (Fig. 3A). The hydrolysis of UDP-GlcNAc was not detected during the first 5 h of incubation, and after 24 h, the amount of UDP-GlcNAc hydrolyzed represented less than 4% of the initial UDP-GlcNAc concentration. The hydrolysis of the UDP-GlcUA did not increase between 3 and 24 h of incubation and represented around 5% of the initial concentration.

**PmHS2-GlcNAc** or **PmHS2-GlcUA** in the Presence of Both UDP-sugars—We showed that the elongation of heparosan polymers occurred only when PmHS2-GlcUA was reconstituted with PmHS2-GlcNAc. After 24 h of incubation in the presence of a 0.5 mM concentration of each UDP-sugar, the reaction mixtures of PmHS2-GlcUA (glucuronyltransferase) incubated alone as well as PmHS2-GlcNAc (N-acetylglucosaminyltransferase) were analyzed by HPAEC by UV absorbance and by electrochemical detection. Only for PmHS2-GlcUA incubated with both UDP-sugars, the HPAEC analysis (condition 2) showed the presence of an additional product eluting at 29 min (Fig. 4A). This product was observed with UV and electrochemical detection. For the PmHS2-GlcNAc reaction mixture, no additional product was detected with the HPAEC analysis, even when non-equimolar UDP-sugar concentrations were used (Fig. 4B).

In addition, the reaction mixtures of both the PmHS2-GlcUA and the PmHS2-GlcNAc were analyzed by normal phase thin layer chromatography (TLC) with butyl alcohol/acetic acid/water (1.5:1:1) and the products were detected by shadowing with UV light. Only in the reaction mixture incubated with PmHS2-GlcUA, we observed an additional product being more polar than the UDP-sugars (UDP-GlcUA Rf 0.24 and UDP-GlcNAc Rf 0.30). This additional product (Rf 0.18) was extracted from the TLC, resuspended in pure water, and analyzed by MALDI-TOF MS (supplemental Fig. 1). It showed an m/z of 782, which corresponds to a UDP-disaccharide containing a glucuronic acid sugar unit and an N-acetylglucosamine sugar unit. In addition, the presence of mono- and disodium UDP-disaccharides was also observed by mass spectrometry (m/z 804 and 826) in the unpurified reaction mixture. Subsequently, the product was rerun by HPAEC (condition 2), and it was found to elute at 29 min; this confirmed that it is the additional product observed in the unpurified reaction mixture (data not shown).
**Polymerization Initiation and Activity of PmHS2**

**TABLE 2**

Influence of the UDP-sugar concentration on the average molecular mass and size distribution of heparosan polymers

High performance size exclusion chromatography analysis of heparosan polymers synthesized by PmHS2 (55–60 μg/ml) after 24 h of incubation in the presence of equimolar and non-equimolar UDP-sugar concentrations (mM). Mn, average molecular weight; Mw, number average molecular weight (expressed in molecular mass, kDa). Estimated polydispersity index (PDI) is calculated as Mw/Mn.

| UDP-GlcUA/UDP-GlcNAc | Mn   | Mw   | PDI (Mw/Mn) |
|-----------------------|------|------|-------------|
| 5/5                   | 40   | 65   | 1.63        |
| 0.25/0.25             | 115  | 135  | 1.17        |
| 5/0.25                | 45   | 55   | 1.22        |
| 0.25/5                | 20   | 25   | 1.25        |

**PmHS2 Polymerization Process in the Presence of Non-equimolar UDP-sugar Concentrations and Heparosan Template**—In order to determine whether one of the two UDP-sugars favors the heparosan chain initiation, the PmHS2 polymerization process was studied in the absence or in the presence of heparosan template (35 kDa) in combination with non-equimolar UDP-sugar concentration.

The influence of non-equimolar UDP-sugar concentrations on the heparosan chain length elongation and its size distribution was investigated (Table 2). In agreement with what has been observed previously in the absence of heparosan template (15), PmHS2 polymerized longer heparosan chains when incubated with low UDP-sugar concentrations. Heparosan chains with an average molecular mass of 135 and 65 kDa were synthesized in the presence of a 0.25 mM concentration of each UDP-sugar or 5 mM, respectively, after 24 h of incubation. Differences in heparosan polymer initiation and elongation were also observed when PmHS2 was incubated in the presence of non-equimolar UDP-sugar concentrations. PmHS2 incubated in the presence of 5 mM UDP-GlcUA, 0.25 mM UDP-GlcNAc elongated heparosan polymers up to 55 kDa, whereas in the presence of 0.25 mM UDP-GlcUA, 5 mM UDP-GlcNAc, the observed molecular mass was around 25 kDa. We calculated that PmHS2 initiated about 2.2-fold more heparosan chains when incubated in the presence of an excess of UDP-GlcNAc than with an excess of UDP-GlcUA. Non-equimolar UDP-sugar concentrations in the range of 0.25–20 mM for each UDP-sugar were added to PmHS2. In the presence of an excess of UDP-GlcNAc, it was observed that after 3 h of incubation, more heparosan chains were initiated, resulting after 24 h of incubation in heparosan polymers with a lower average molecular weight. The opposite was observed in the presence of an excess of UDP-GlcUA.

In addition, the influence of non-equimolar UDP-sugar concentrations on the PmHS2 polymerization process was determined using the coupled enzyme assay to quantify the UDP-sugar conversion. It is known that the amount of UDP-sugars converted during the polymerization process is determined by the availability of the limiting UDP-sugar. After 3 h of incubation in the presence of 5 mM UDP-GlcNAc and a limiting concentration of UDP-GlcUA (from 0.1 to 0.5 mM), PmHS2 fully converted the available UDP-sugars; in the presence of 1 mM UDP-GlcUA, about 63 ± 8% of the available UDP-sugars were converted (Fig. 5). In contrast, with an excess of UDP-GlcUA (5 mM) and a limiting concentration of UDP-GlcNAc (0.1–2.5 mM), only about 42 ± 6% of the UDP-sugars were converted after 3 h. After 24 h of incubation, the amount of UDP-sugar converted in order to polymerize heparosan was similar in the samples whether PmHS2 was incubated with an excess of UDP-GlcNAc or an excess of UDP-GlcUA. These results indicate that the presence of an excess of UDP-GlcNAc stimulates the PmHS2 polymerization activity during the first hours of incubation, but due to the restricted availability of UDP-GlcUA, the elongation activity levels off sooner. An excess of UDP-GlcUA slows down the polymerization activity but does not inhibit the overall heparosan synthesis process. To confirm the fact that UDP-GlcNAc favors the heparosan chain initiation, the PmHS2 polymerization process was investigated in the presence of heparosan template (35 kDa) (Fig. 6).

The HPSEC analysis of the PmHS2 reaction mixture incubated for 3 h in the presence of heparosan template and a 5 mM concentration of each UDP-sugar revealed the presence of two products (25 and 65 kDa). It was reported previously that PmHS2 initiates and elongates simultaneously heparosan polymers (15), therefore, the presence of these products suggests that PmHS2 synthesized new heparosan polymers (25 kDa) and also elongated the heparosan templates, resulting in longer polymers (65 kDa). After 6 h of incubation, the difference between the molecular masses of the two polymer groups was less visible; it resulted in an increase of the overall polydispersity. In the reaction mixture of PmHS2 incubated for 3 h with a lower UDP-sugar concentration (0.25 mM), only heparosan polymers of 130 kDa were observed. In the presence of 5 mM UDP-GlcNAc, 0.25 mM UDP-GlcUA in combination with heparosan template, PmHS2 polymerized two distinct heparosan polymer groups with an average molecular mass of 30 and 75 kDa as products of the initiation and the elongation of templates, respectively. When an excess of UDP-GlcUAc was added, only one heparosan polymer group of about 65 kDa was synthesized after 3 h of incubation. The same results were obtained when using longer heparosan templates (50 kDa).

These results indicate that PmHS2 incubated with an excess of UDP-GlcNAc can initiate and elongate heparosan...
the polymerization activity of the single action transferases with PmHS2, it is necessary to estimate the activity of each transferase catalytic domain separately. Moreover, the specific activity should be expressed in nmol of UDP-sugar converted/mol of active transferase/min because both catalytic domains cannot be active at the same time due to the stepwise addition of UDP-sugars.

A rise in the incubation temperature from 30 to 40 °C resulted in a 5-fold increase of PmHS2 polymerization activity. The leveling off of PmHS2 polymerization activity observed after the conversion of about 4.5 mM UDP-sugar is independent of the incubation temperature within the range from 30 to 39 °C. The leveling off is probably due to UDP inhibition because we have observed previously that PmHS2 polymerization activity was inhibited by UDP concentration above 4 mM (15).

**Hydrolysis Activity by PmHS2**—We have shown that PmHS2 was capable of hydrolyzing UDP-sugars into UDP and the corresponding sugar residue when incubated in the presence and in the absence of acceptor molecules. PmHS2 hydrolyzes more UDP-GlcUA than UDP-GlcNAc. Heat-inactivated PmHS2 did not hydrolyze the UDP-sugars.

Sugiuara et al. (19) reported that in the absence of enzyme, the metallic ion Mn²⁺ participates in the hydrolysis of UDP-GlcUA at the pyrophosphate bonds, resulting in the production of UMP and a monosaccharide unit. Here, we also observed in the absence of PmHS2 the hydrolysis of UDP-GlcUA into UDP-GlcUA-A and a sugar residue. However, it appeared that the presence of active or inactive PmHS2 stabilizes UDP-GlcUA. It is assumed that PmHS2 probably entraps or binds MnCl₂ and therefore prevents the role of Mn²⁺ in the hydrolysis of UDP-GlcUA. The unexpected hydrolysis of UDP-GlcNAc by PmHS2-nul and PmHS2-GlcUA⁻ was probably due to a partial inactivation of the N-acetylgalactosaminyltransferase catalytic domain. Kane et al. (11) reported for PmHS1 that the transferase catalytic domains were not completely inactivated by the amino acid substitution of the DXD motif. They observed that PmHS1 single action transferase mutants conserved about 1–2% of relative specific activity for the mutated transferase domain (11). A rise in the UDP-sugar concentration did not increase the degree of hydrolysis by PmHS2.

The addition of both UDP-GlcUA and UDP-GlcNAc to the reaction mixture results in a reduction of the hydrolysis of both UDP-sugars. It is concluded that the hydrolysis reaction is slow compared with the heparosan elongation process. Thus, in the standard incubation condition and in the presence of both UDP-sugars, the hydrolysis is not favored by PmHS2 and can be neglected.

The glycoside hydrolases or glycosidases (EC 3.2.1.-) and the glycosyltransferases (EC 2.4.x.y) are well described in the literature, and their catalytic mechanisms show similarities with the exception of the acceptor molecules, being a water molecule with the glycoside hydrolases (20–22). For both classes of enzymes, the catalytic mechanism is done according to an acid/base reaction orchestrated by two amino acid residues. One residue acts as an acid catalyst, and the other residue acts as a base catalyst. Based on the similarities, the glyco-
sidic hydrolysis of the UDP-sugars by both the inverting and the retaining transferases of PmHS2 might be due to a nucleophilic attack by a water molecule at the transferase catalytic domain. The hydrolase activity has been described for the glycosyltransferase α3GT (23) and the glycosyltransferases Toxin A and Toxin B (24). Until now, the hydrolysis of UDP-sugars by glycosyltransferases involved in the polymerization of glycosaminoglycans has not been reported, and it is unknown if other enzymes exhibit this mechanism. In view of our results, the glucuronyltransferase catalytic domain is more sensitive to the hydrolytic attack than the N-acetylgalcosaminyltransferase domain.

Despite the fact that the hydrolysis rate is rather low, PmHS2 is more versatile than expected. Thus, based on the literature, PmHS2 might be of interest to synthesize activated sugars. Indeed, it was observed that glycoside hydrolases engineered into glycosylsylthases were capable of synthesizing new and valuable compounds for therapeutic applications (25) and that versatile glycosyltransferases were found to exhibit additional activity, such as being able to synthesize rare nucleotide activated sugars (21, 26).

Initiation of the Heparosan Polymer by PmHS2-Enzymes—The reaction mixtures of PmHS2-GlcUA+ or PmHS2-GlcNAc+ were analyzed by HPAEC by electrochemical detection and UV absorbance. The absorbance in UV light is more sensitive than the electrochemical detection for UDP-sugars due to the presence of the UDP group (27). Therefore, the presence of a larger peak with UV detection, as with electrochemical detection, shows the presence of a UDP group.

HPAEC analysis showed that PmHS2-GlcUA+, incubated in the presence of both UDP-sugars, catalyzed the formation of an additional product visible with both chemical and UV detection. The UV signal was much stronger, indicating the presence of a UDP group. The presence of this additional product was also confirmed by TLC analysis. The MALDI-TOF MS analysis of the TLC purified product showed an m/z of 782, corresponding to a UDP moiety containing a glucuronic acid and a N-acetylglucosamine unit. In contrast, with PmHS2-GlcNAc+, no additional products were observed even in the presence of non-equimolar UDP-sugar concentrations.

PmHS2 elongates heparosan chains by adding sugar units at the non-reducing end of the polymer (5, 6). As a consequence, the UDP present in the chain belongs to the first acceptor molecule. Based on the literature and the results obtained, it is concluded that PmHS2-GlcUA+ catalyzes the formation of GlcUA-GlcNAc-UDP by transferring GlcUA to UDP-GlcNAc acceptor molecules and therefore initiates heparosan chains. Interestingly, Tlapak-Simmons et al. (28) reported that PmHAS catalyzed the formation of the GlcUA-GlcNAc-UDP disaccharide, but the disaccharide GlcNAc-GlcUA-UDP was not observed.

Therefore, it would be interesting to determine if these P. multocida glycosyltransferases are capable of initiating polymer chains by using one of the two UDP-sugar as acceptor or whether this result is a reflection of a competition between the two UDP-sugars for the acceptor and donor site.

The study of the PmHS2 polymerization process in the presence of different concentrations of UDP-sugars showed that an excess of UDP-GlcNAc increased the UDP-sugar conversion during the first hours of the polymerization process and favored the synthesis of a higher number of polymer chains, resulting in chains with a smaller molecular weight. With an excess of UDP-GlcUA, we observed that PmHS2 polymerization activity was reduced during the first 6 h of incubation, probably by inhibiting the initiation or by hampering the elongation; nevertheless, it did not inhibit the overall heparosan synthesis process.

In the presence of heparosan template and an excess of UDP-GlcNAc, it was observed that PmHS2 initiates and elongates heparosan polymers simultaneously, whereas in the presence of an excess of UDP-GlcUA, PmHS2 elongates exclusively the heparosan template.

Based on the results obtained in the tested conditions with the PmHS2 single action transferases and with PmHS2, it clearly appears that UDP-GlcNAc is involved in the initiation of heparosan chains. We conclude that PmHS2 initiates heparosan chains using UDP-GlcNAc as the acceptor molecule, and as a consequence, not only the UDP-sugar concentration but also the ratio of UDP-sugar concentrations is an important parameter for the PmHS2 polymerization process.

Moreover, it was concluded that UDP-GlcNAc and the heparosan template have about the same affinity for the acceptor binding site. The PmHS2 initiation and polymerization process is different from PmHS1 and PmHAS. Indeed, PmHS1 and PmHAS elongated polymers with a narrow size distribution when short oligosaccharide templates were added to the reaction mixture, whereas in the absence of oligosaccharide template, more polydisperse polymers were formed (6, 29). Williams et al. (18) reported that for PmHAS, the binding affinity at the acceptor binding site is higher for short oligosaccharides than for monosaccharides. Therefore, in the presence of oligosaccharide templates, the polymerization reaction is more efficient because the initiation step does not take place, and only the elongation of the templates occurs.

Based on our results, it appears that the heparosan chain initiation by PmHS2, in contrast with PmHS1 and PmHAS, is not controlled by the acceptor binding site affinity for short oligosaccharide but by the amount and the ratio of UDP-GlcNAc present in the polymerization reaction. According to the results obtained with polysaccharide heparosan template (35 kDa), we speculate that in the presence of short heparosan oligosaccharide templates and an excess of UDP-GlcUA, PmHS2 will only elongate the short templates, resulting in heparosan polymers with a narrower size distribution. However, more research is needed to confirm this hypothesis.

In summary, we showed that PmHS2 is an enzyme with two glycosyltransferase activities and two UDP-sugar hydrolase activities. The fact that PmHS2 is able to hydrolyze UDP-sugars could open new perspectives in the field of UDP-sugar regeneration. With the single action transferases (PmHS2-GlcUA+ and PmHS2-GlcNAc+), PmHS2, we demonstrated that the first step of the in vitro synthesis of heparosan is driven by the transfer of the GlcUA from the UDP-GlcUA moiety to a UDP-GlcNAc acceptor molecule. Knowing which
UDP-sugar initiates the polymerization reaction could enable the regulation of heparosan synthesis by the use of modified oligosaccharide templates and might result in the synthesis of heparosan analog molecules. Experimental design approaches in which the amount of each PmHS2 single action transferases and UDP-sugars are variables could enable us to have a better understanding of how the elongation of heparosan polymer is regulated. In the future, it should help us to determine the reaction conditions leading to heparosan polymers with specific molecular weight and narrow size distribution.

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